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Targeted gut microbiota interventions to modulate immune responses in chickens

von

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For my mother, who would be proud

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ABBREVIATONS

16S rRNA	16S ribosomal ribonucleic acid
AID	activation induced cytidine deaminase
approx.	approximately
Aqua dest.	Aqua destillata
BLAST	basic local alignment search tool
cDNA	complementary DNA
CFU	colony forming unit
ChiBac	Chicken intestinal bacterial collection
CT	Ceacal Tonsil
DDH	DNA-DNA hybridization
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen; German Collection of Microorganisms and Cell Cultures
DTT	dithiotreitol
ELISA	enzyme-linked immunosorbent assay
EtOH	Ethanol
F	forward
FCR	feed conversion ratio
FOS	Fructooligosaccharides
GALT	gut associated lymphoid tissue
GF	Germfree
GIT	gastrointestinal tract
GO	Gene Ontology
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgJ	Immunoglobulin joining chain
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
IL6	interleukin 6
IL21	interleukin 21
IMNGS	integrated microbial next generation sequencing

INRA	Institute national de la recherche agronomique; French national institute for agricultural research
JCHAIN	Immunoglobulin joining chain (=IgJ)
MALDI-TOF	matrix assisted laser desorption ionization – time of flight
MEGA	molecular evolutionary genetics analysis
MDS	multi-dimensional scaling
MOS	Mannanoligosaccharides
MM	maternal microbiota
NCBI	National Center for Biotechnology Information
NGS	next generation sequencing
OTU	operational taxonomic unit
PBS	phosphate-buffered-saline
PCR	polymerase chain reaction
qPCR	real time quantitative polymerase chain reaction
R	reverse
RDP	ribosomal database project
Rpm	rounds per minute
rRNA	ribosomal ribonucleic acid
RT	room temperature
SPF	specific pathogen free
WCA	Wilkins-Chalgren Anaerobic broth
x g	times gravity (in terms of centrifugation)

UNITS

°C	degree Celsius
bp	base pair
g	gram
kg	kilogram
L	liter
M	molar
mg	milligram
min	minute
ml	milliliter
µg	microgram
µl	microliter
ng	nanogram
s	seconds
S	Svedberg Unit

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1 Introduction

The world of microbiomes is one of the most fascinating and most studied fields of research of the past 10 years. The term “Microbiome” describes the ecological community of commensal, pathogenic and symbiotic microorganisms in a certain habitat including bacteria, archaea, fungi, viruses and protozoans. It was first designed by Nobel prize winner Joshua Lederberg in 2001 including only the human microbiome. In 2008, the human microbiome project was founded. The microbial communities of 300 healthy human individuals were characterized for different sampling sites: Nasal and oral cavities, skin, urogenital and gastrointestinal tract (1). Host body microbiomes are not only relevant for human research. Animal, plant, soil, marine and wastewater microbiomes are analyzed all over the world. Even the microbial composition of the international space station was inspected (2). When it comes to animal microbiomes, a lot of effort is invested in understanding the gut microbiota compositions of mice, rats and fruit flies. Laboratory animals are very useful as experimental models for translational research. In contrast, minor efforts are made to study the microbiome of livestock animals. This trend is quite similar to the limited genome sequencing efforts for these animals.

Chicken meat is the main source of alimentary protein for humans worldwide. Within the past 70 years, broilers and layers were bred for high productivity traits, which means high meat and egg production within a short period of time (3). Animal wellbeing and health has often been neglected. The use of antibiotics as feed additives, so called growth promotors, led to disturbance in the gut microbiome, an underdeveloped immune system and only little resistance against pathogens. Fortunately, these growth promotors are forbidden in the European Union since 2006 (4) but the extended application of antibiotics exacerbated the crisis on antibiotic resistances (5-8). Due to the lack of alternatives, antibiotics are still the most common metaphylactic treatment for enteric infections in chickens. Thus, it is of great interest to study the chicken microbiome in detail and work towards new solutions to improve gut health.

This dissertation presents insights into the chicken gut microbiome and a possible new strategy to promote immune system development via the microbiome, potentially leading to less antibiotics usage, better animal health, and more resistance against enteric pathogens if further developed in the near future.

2 Literature review

2.1 The chicken gastrointestinal tract

2.1.1 Anatomy and Physiology

The alimentary tract of birds is different from the gastrointestinal tract (GIT) of mammals. The main function of the GIT is the mechanical, chemical and enzymatic breakdown of food to smaller components that can be used by the host. The beak is the functional counterpart to lips and teeth. It shows a great variety between different bird species and is shaped depending on the diet. For feral chickens, the beak helps picking up grains, grass, insects, worms and snails. In production facilities, the diet consists mostly of pelleted food. Food subsequently enters the crop, a bag-like dilatation of the esophagus. The crop is a storage room for ingested food, as the whole digestive system of birds is very short and thus a reservoir becomes necessary. The digesta is further transported through the esophagus to the proventriculus. This glandular stomach is capable of producing gastric acid, hydrochloric acid and pepsinogen leading to a rather low pH. The chemical and enzymatic breakdown of nutrients starts here. The ventriculus or gizzard is the following stomach, a strong muscular and lens-shaped organ. In the gizzard, the food is ground and mixed with gastric acid from the proventriculus. Small stones, the grit, help grinding down large food particles and therefore increasing the accessibility to nutrients (9). The small intestine is the place where most digestion and absorption of nutrients takes place. It consists of the duodenum, jejunum and ileum. Bile salts, bicarbonate and pancreatic enzymes are introduced to the digesta, leading to a rise in pH (9) and creating more bearable conditions for bacteria. The caeca are two reverse located blind sacs alongside the ileum. The digesta is transported into the caeca by reverse peristaltic movement and peristaltic movement empties them. Both processes lead to mixing of the digesta (10, 11).The main functions of the caeca themselves is water and electrolyte reabsorption (12). The fecal material is passing through the short colon, mixed with uric acid and drops out through the cloaca.

2.1.2 Immunological organs

The Gut-associated lymphoid tissue (GALT) is part of the mucosal immune system. It is the primary defense system where the first contact between the host and antigens takes place. In case of the GIT, antigens can be all kinds of feed components, commensal bacteria, bacterial metabolites or pathogenic microorganisms. In addition, the mucosal immune system plays a fundamental role in finding the balance between intestinal inflammations and gut homeostasis, as reviewed by Blaser et al. and Pamer (13, 14) for human host-microbiota interactions. However, corresponding data in chickens are scarce. Unlike mammals, chickens lack encapsulated lymph nodes. Instead, defined lymphoid tissues are distributed throughout the

gut: the esophageal tonsil, the pyloric tonsil, Peyer's patches, the caecal tonsil (CT) and the Bursa of Fabricius. Additionally, diffusely scattered lymphatic cells can be found throughout the gut in large numbers. The development of organized lymphoid tissues starts in the embryonic phase and is independent of antigen stimulation. Further maturation of lymphoid tissues after hatch is antigen driven. This could be shown in germfree (GF) chickens and Japanese quail, where total weights of bursa, spleen and thymus was unaffected, but only reduced lymphoid tissues could be found in GALT with no lymphoid follicles found in CT (15).

At the cellular level, the GALT consists of a variety of immunological relevant cells: T- and B-lymphocytes, immunoglobulin-producing plasma cells, intraepithelial lymphocytes, dendritic cells, macrophages, natural killer cells and heterophilic granulocytes. However, T- and B-lymphocytes make up to 90% of the cellular population under non-inflammatory conditions. Lymphatic cells can be predominantly found in mucosal epithelium and in the lamina propria that lies underneath (16). Follicles and germinal centers are formed by B-cells and permeate the lymphoid tissue. T-cells can be predominantly found in the center of villi and form interfollicular regions in the deeper regions of the lamina propria (17). γδ T-cells in particular are present between the epithelial cells, while αβ T-cells are restricted to the lamina propria and the follicular regions. The composition of immune cells in the GIT is highly interchangeably, depending on age, microbial colonization, host genetics and especially the contact to pathogenic microorganisms (18). Studies of GF animals provide insights into the impact of gut microbial colonization on immune system development. Cheled-Shoval et al. showed that goblet cells are not fully maturated in the caecum and MUC2 expression was reduced in the ileum of GF and mono-associated animals (19). By comparing GF, mono-colonized and conventional chickens, Mwangi et al. demonstrated the dependencies of microbial complexity on the αβ-T-cell repertoire (20). Another study on GF animals discovered that $CD4^+$ and $CD8^+$ T-cells are absent in gut tissues and B-cells are missing in the lamina propria. Additionally, no germinal centers were found in the CT and no intestinal or serum IgA was found until day 28 post hatch. It was possible to partially abrogate this effect by mono-association with the probiotic *E.coli* Nissle strain or a mixture of four different bacteria (21).

Three classes of immunoglobulins can be found in avian species: IgY, IgM and IgA. IgY is the functional equivalent to mammalian IgG but with a higher molecular weight and some different biochemical properties like salt precipitation conditions. IgY exists as monomer in serum and is mainly produced during the secondary antibody responses. Many publications use the term IgG instead of IgY, but IgY should be consistently used when referring to chicken (22). Chickens and other avian species lack the presence of IgD and IgE. It is assumed that their functional properties are taken over by the present antibodies (22, 23). IgM has a pentameric configuration and is the primarily expressed receptor on B-cells. It is similar to mammalian IgM and the first antibody produced after infection.

Avian IgA is similar to its mammalian counterpart. It is found in the serum and is secreted in the gut via the bile. It is the predominant antibody in body secretions. A monomeric, trimeric or tetrameric configuration of IgA were described in chickens (24). In mice and humans, it is the most abundantly produced antibody. T-cell dependent and T-cell independent mechanisms of class switch recombination to IgA are known and Transforming growth factor *beta* (TGF-β) is a critical switch factor (25). IgA-secreting plasma cells are frequently found in mucosal tissues like the gut. In mucosal fluids, the joining chain (JCHAIN, IgJ) is connected to the dimeric or trimeric IgA molecule. In serum, it is mostly present as a monomer. Mucosal epithelial cells express the polymeric-Ig-receptor (PIGR) that binds IgA. Receptor-Ig complexes can be endocytosed on the basolateral side and subsequently transported to the apical side, where secretory IgA (sIgA) is released (26). PIGR itself is partially cleaved by proteolytic enzymes with a part remaining associated with sIgA as the secretory compound (SC). This SC retains the stability of dimeric IgA in the gut lumen and supports the attachment of sIgA to mucus (27-29). Secretory IgA plays a fundamental role in microbiome research. Macpherson et al. first described in 2000 that production of sIgA is induced by the presence of bacteria in the intestine and its role in protecting the host against commensal microorganisms (30). While commensal bacteria are rapidly killed by macrophages in the lamina propria, dendritic cells under the epithelium have the capability of preserving commensals and selectively inducing bacteria-specific IgA responses (31).

2.2 The 16S rRNA gene in microbiome research

2.2.1 Analysis of cultured isolates

Ribosomes are universally present within all living cells (32). They build a complex molecular machine that translates RNA codons into amino acids which are linked together to proteins (33). The bacterial ribosome consists of two compartments, the 50S and the 30S subunits. Both subunits contain approximately 63% ribonucleic acid (rRNA) (34). The 50S subunit divides into 23S and 5S rRNA. The 30S subunit contains the 16S rRNA that is a key player in molecular microbiology as a phylogenetic clock that can be used to taxonomically classify bacteria and archaea (35-37). It contains conserved regions that all bacteria have in common but also consists of nine hypervariable regions summing up to a total of approx. 1500bp (38). These hypervariable regions are helpful to infer bacterial phylogeny (39), as they can differ from bacterial species to species. Carl Woese et al. established the method of using 16S rRNA for taxonomic classification of methanogenic bacteria (40) and archaea (41). Yet, even differences between strains of the same species can be analyzed (42). For cultured bacterial species, it is common to use Sanger sequencing with different primers to obtain the full-length sequence of 16S rRNA genes (43). The percentage of sequence identity between isolate and

known species offers first clues about their classification. In the 1960, DNA-DNA hybridization (DDH) was used to measure the overall similarity between genome sequences of different species (44, 45). A DDH threshold of 70% to delineate between two species was suggested and accepted over the last 50 years (46, 47). Stackebrandt et al. compared DDH values with 16S rRNA gene sequence similarities and found a sequence similarity threshold of 97% to be sufficient to identify new species (48). Currently, accepted thresholds are <94.5% sequence identity for new genera and <86.5% for new families. A species threshold of 98.7% is widely accepted (36). Comprehensive databases like EzBioCloud (49), SILVA (50) and the 'Ribosomal database project (RDP) (51) greatly help research on bacterial taxonomy and diversity.

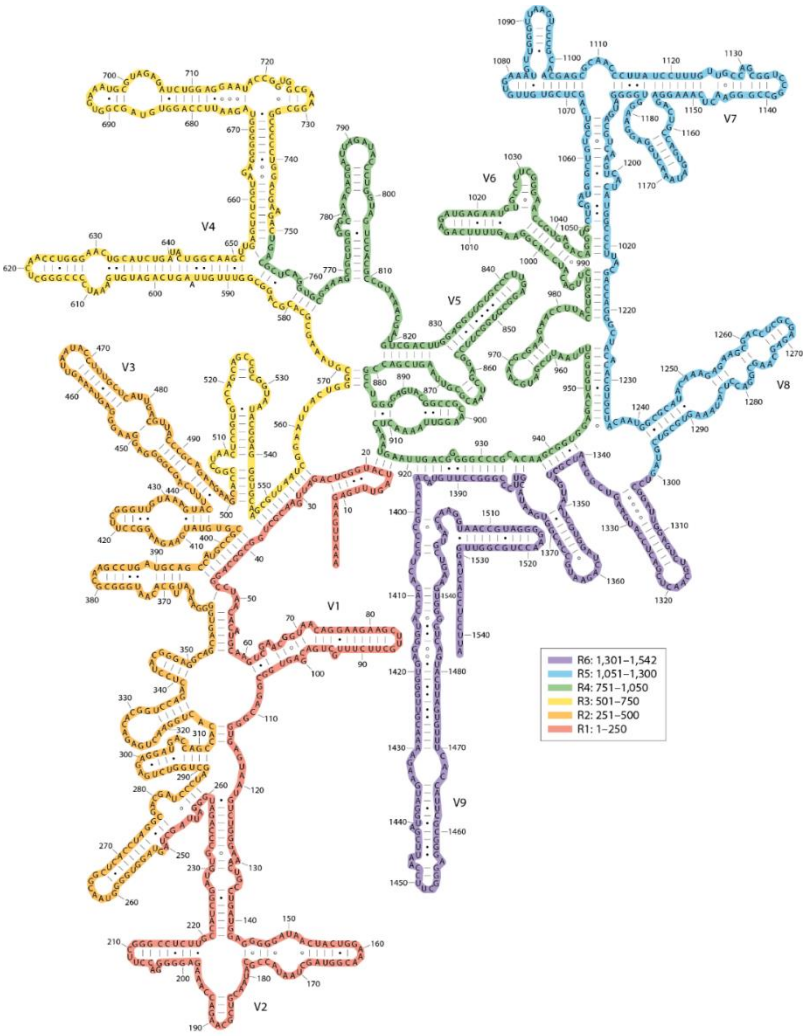

Figure 1: 16S ribosomal RNA.

Secondary structure of the 16S rRNA of Escherichia coli. In red, fragment R1 including the variable regions V1 and V2; in orange, fragment R2 including region V3; in yellow, fragment R3 including region V4; in green, fragment R4 including regions V5 and V6; in blue, fragment R5 including regions V7 and V8; in purple, fragment R6 including region V9. Adapted from Yarza et al. 2016.

2.2.2 Community analysis by sequencing

There are many potential methods that can be utilized to conduct metagenomic studies of diverse ecosystems. Microbial communities, including bacteria, archaea, fungi, viruses and protozoa can be analyzed by shotgun metagenomic sequencing. This method provides taxonomic information down to the species and potentially the strain level. Whole genomes are reassembled from sequenced reads, potentially providing novel insights into uncultured bacteria (52).

Proteomics or metaproteomics generate a vast amount of data on the protein architecture of whole communities. Therefore, data from metagenomics studies is combined with techniques of mass-spectrometry (53). Again, computing power and expensive devices, including mass spectrometer, gas chromatography, high-pressure liquid chromatography, two-dimensional liquid chromatography and surface-enhanced laser desorption-ionization (54), are needed to carry out research in this area.

We performed 16S rRNA gene amplicon sequencing to conduct our own research. It is the most common method applied in microbial ecology studies, yet allowing detailed insights into community structures (55-59). The method itself is unable to provide functional insights and is therefore limited to taxonomic diversity. Nevertheless, the cost effectiveness and the availability of downstream pipelines for data analysis are major advantages (60).

In 2010, the Knight lab introduced QUIIME (Quantitative Insights Into Microbial Ecology), the first pipeline to analyze large-scale sequencing data from raw reads to interpretation and deposition (61). Today, web-based platforms, such as the ‘Integrated microbial next generation sequencing’ (IMNGS) platform (62), made community analyses of 16S rRNA raw data available for non-expert researchers. Raw reads are demultiplexed, quality checked, filtered for chimeras and afterwards clustered together to approx. 440bp fragment reads. These clusters are ‘Operational Taxonomic Units’ (OTUs) that code for a specific bacterial taxon with a certain level of confidence by BlastN (63), EzBioCloud (49) or RDP (51). Based on the level of certainty, some OTUs can code down to species level with a sequence similarity of 100%. For others, the correct phylogenetical placement works only to the genus, the family or the order level. Newer bioinformatic tools such as DADA2 promise even higher detection rates by making use of amplicon sequence variants instead of OTUs (42).

Bioinformatics experts invest great efforts to improve and simplify their pipelines. This way, non-expert users quickly receive access to a rapidly growing field of research.

2.3 The chicken gastrointestinal microbiome

The chicken intestinal microbiome is a diverse and complex ecosystem that contains hundreds of bacterial species (64). These bacteria are responsible for breaking down nutrients (65) (66), developing immune functions (67) and excluding pathogens (68) (69) (70). Before the advent of molecular biology, microbial communities were analyzed by culture-based approaches. Since the late 19th century, scientists are cultivating bacteria from the intestine of birds (71). At that time, not much was known about anaerobic cultivation and how to find ideal culture conditions. Early cultivation studies found streptococci, lactobacilli, clostridia and coliforms in poultry carcasses (72). The quantification of different bacterial species was complicated. In 1957, Barnes et al. quantified the bacterial groups aforementioned with $<2x10^7$ to $1.2x10^{10}$ for lactobacilli, $2.2x10^8$ to $6.4x10^9$ for coliforms and $2.8x10^8$ to $1.6\ x10^9$ for streptococci per gram caecal content of 17-day old birds. *Bacteroides* was complicated to cultivate due to the presence of large numbers of different other bacteria. The Roll-Tube method by Hungate et al. revolutionized the cultivation of anaerobic bacteria (73). Salanitro et al. were one of the firsts to use this technique for the cultivation of chicken gut bacteria. They identified anaerobic Gram-positive rods (36.1%) such as *Propionibacterium acnes* and *Eubacterium* spp., gram-negative rods (18.6%) like *Bacteroides clostridiiforme, Bacteroides hypermegale* and *Bacteroides fragilis*, spore-forming rods (15.7%) such as *Clostridium* spp., pleomorphic cocci (5.2%) and *Peptostreptococcus* spp. (1.5%). Facultative anaerobes, including *Escherichia* spp. were estimated to account for 17.5% of caecal communities (74). Different culture conditions and media types were also tested to determine optimal conditions for anaerobe bacteria (75).

Cultivation approaches were the only tool to investigate microbiota compositions in different ecosystems for a long time. The development of molecular techniques including Sanger sequencing (43, 76), Polymerase Chain Reaction (77) and the use of the 16S rRNA gene as phylogenetic marker (40, 78, 79) led to a rapid evolution of microbiome research. Few studies remain culture-centric, however the combination of cultivation and molecular techniques had a renaissance in recent years (55, 80-84).

Currently most scientists use high-throughput 16S rRNA gene sequencing to gain more information about the chicken intestinal microbiome. Wei et al. made the first real bacterial census of the turkey and chicken intestinal microbiome. They state that less than 7000 sequences are necessary to gain an almost complete coverage (99%) at both species- and genus level. Poultry samples from different geographic origins, breeds and diets are needed to be studied to culture all species and reach that level of coverage (85).

2.3.1 Microbiome composition of different gut regions

Most studies focus on the caecal microbiota as the highest biomass of bacteria can be found in this organ (86, 87). However, it is important to investigate microbial communities in all gut regions to obtain a better understanding of how microbe-host interactions work throughout the GIT. An overview of the different gut compartments and their dominating microbial taxa can be seen in figure 2.

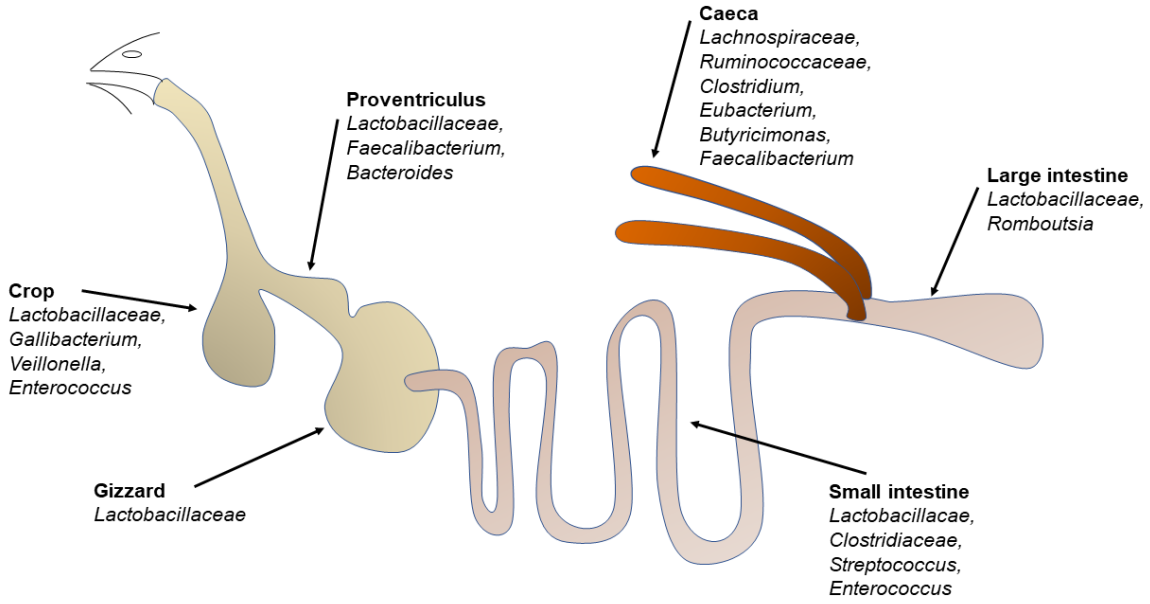

Figure 2: Spatial variance in the chicken GIT microbiota

Data was taken from the review of Pedroso et al. (88). Data for the large intestine was completed with own observations.

2.3.1.1 Crop/Proventriculus/Gizzard

The predominant bacteria in the crop are *Lactobacillaceae* with 10^8-10^9 colony forming units (CFU)/g content (89, 90). Additionally, enteric bacteria and gram-positive cocci could be isolated from the digesta and mucosa of the crop (90). At the species level, other bacterial taxa could be isolated: *Bifidobacterium* spp., *Micrococcus luteus, Staphylococcus lentus, Enterobacter aerogenes, Pseudomonas aeruginosa, Escherichia coli, Escherichia fergusonii, Klebsiella pneumoniae, Bacteroides* spp., *Eubacterium* spp., *Salmonella enterica enterica,* and *Campylobacter jejuni* (90-92). The high number of lactobacilli and low numbers of other species can be traced back to the low pH of approx. 4.5 in the crop. Some lactobacilli seem to be able to attach to host epithelial cells leading to an even stronger *Lactobacillus* dominance in the crop (89). Interestingly, adhering lactobacilli were only isolated in birds but not in mammals (93). Like in the crop, lactobacilli are the dominating bacteria in the proventriculus (94). While many studies assessed the microbial composition in other gut regions, there is only one study focusing on the proventriculus and proposing a total amount of 10^4 – 10^6 CFU/g

digesta (95). Lactobacilli and *Clostridiaceae* dominate the microbial composition of the gizzard. Interestingly, the microbial composition of the crop and the gizzard is indistinguishable (96, 97). The influx of acid from the proventriculus seems to inhibit bacterial growth and significantly lowers the levels of aerobic bacteria (91).

2.3.1.2 Small intestine

Compared to the caecum, the microbiota in the small intestine is still sparse in diversity (98). According to a study by Dumonceaux et al., *Lactobacillaceae* account for up to 90% of the composition of the small intestine (99). Another study estimated the numbers of ileal bacteria to be 70% *Lactobacillaceae*, 11% *Clostridiaceae*, 6.5% *Streptococcus* and 6.5% *Enterococcus* (100). Stanley et al. proposed that 99% of jejunal 16S rRNA gene sequences are related to *Lactobacillus* (101). A newer study found overlapping results, with *Lactobacillus* being the predominant genus in duodenum, jejunum and ileum. Moreover, the next common genus was *Romboutsia* in the ileum and *Staphylococcus* in duodenal and jejunal samples (98).

2.3.1.3 Caecum

The microbial diversity rises drastically in the caecum (98). Qu et al. found the most abundant phyla in the chicken caecum to be *Firmicutes, Bacteroidetes* and *Proteobacteria*, of which most are strict anaerobes (102). Wei et al. found the most predominant phyla to be *Firmicutes* (78%) and *Bacteroidetes* (11%), respectively (85). *Clostridia* were the predominant class within the *Firmicutes* phylum and can be further subdivided into *Lachnospiraceae* and *Ruminococcaceae* as the most abundant families (103, 104). Within these bacterial families, many strains remain uncharacterized. The cultivation of these microorganisms was previously difficult due to being strict anaerobes (74). Sergeant et al. found that the most abundant OTUs within the caecum belong to the genera *Megamonas, Veillonellaceae, Alistipes, Bacteroides* and *Pseudoflavonifractor* (105).

2.3.1.4 Large intestine

Not many studies focused on chicken colonic microbiota. Sekelja et al. investigated the origin of four dominant phylogroups (2x*Clostridia, Lactobacillaceae, Escherichia*) found in chicken feces. The *Lactobacillaceae* phylogroup had its origin most likely in the crop and the gizzard while the *Clostridia* phylogroups originated in the caecum and the colon. Caecal and colonic samples clustered together by principal component analysis (96). The microbiome of these gut compartments could certainly be similar right after the caecal content was emptied into the colon. If this is not happening, the colonic microbiome has to be more similar to the one of the small intestines. In fact, the microbial composition is not static and can be changed or influenced by external and internal factors described hereinafter.

2.3.2 Factors influencing the chicken microbiome

The development of the intestinal microbiome of farmed chickens is different from mammalian species. Mammals are directly colonized by vaginal and skin microbiota from their mothers during birth. It is well known that the microbiome of a vaginally delivered infant differs from that in infants born by caesarean section (106, 107). In contrast to mammals, birds lay eggs. The starter microbiome is thus entirely dependent on external factors. Microbial colonization starts directly after hatch with microbiota from the environment, including eggshell, the nest, the mother bird, feed, water, transport boxes, animal caretakers and litter (108).

Due to high-throughput and commercial nature of chicken hatcheries, the number of microbial species newborn chickens are exposed to, is limited. In production facilities, everything is cleaned and disinfected, the rational being to exclude common pathogens like *Clostridium* spp., *Salmonella* spp. and avian pathogenic *Escherichia coli* (APEC) from the flock, in order to generate healthy chickens and safe food for human consumption (109, 110). Nevertheless, chickens are not healthy in production facilities (111). There is a constant need for antibiotics usage to treat flocks against pathogenic bacteria (112). This leads to further accumulation of antibiotics in the food chain and therefore to a more drastic rise of antibiotic resistances (8). Looking at feral chickens or chickens living on a farm, the hatchlings are exposed to a wider range of external factors including the mother bird, other animals, fresh flora and a variety of grains. One can say that the starter microbiome is much more diverse than in facilities (113). The hypothesis is that a diverse microbiome leads to an adequate development of the immune system, more resistance against pathogens, higher animal wellbeing and safer food for consumption. The chicken GIT microbiome can be positively or negatively influenced by the main factors diet, age, antimicrobial substances, genotype, prebiotics and probiotics.

2.3.2.1 Age

The chicken intestinal microbiome develops over time (114). *Proteobacteria,* especially *Enterobacteriaceae,* is the major bacterial phylum in the first two days after hatch. The overall composition is low in diversity. After three days, there is a rapid increase in bacterial richness. The family *Ruminococcaceae* and other *Firmicutes* including *Clostridium* spp. are detected. By day 28, the *Proteobacteria* are completely outnumbered by *Firmicutes* (115). Denaturing Gradient Gel Electrophoresis (DGGE) based analysis indicated that the number of observed DGGE-bands (a proxy for species) was increasing until day 11. The number of bands was higher in the caecum, compared to other parts of the intestinal tract, but there was no further increase of bands from day 11 onwards, suggesting a plateau in diversity (116).

Glendinning et al. looked at the microbial community composition of different intestinal regions and studied its development over time. In jejunum and ileum, the increase of species richness was moderate from day 1 to day 7 (jejunum 128 to 208 species, ileum 70 to 145 species). In

the caecum, a significant increase in richness could be observed during this period (41 to 283 species). Between day 7 and day 14, species richness increased in ileum (145 to 273 species), but not in jejunum and caecum. The total species richness rose in all regions from day 15 to week 5, with the highest increase occurring within the caecum (98).

2.3.2.2 Host genotype

Another major influencing factor on the gut microbiome is the host genotype. Han et al. studied the effect of host genotype on *Campylobacter* colonization. They found that layer type birds were more affected by *Campylobacter* spp. compared to broiler type birds (117). Singh et al. compared the fecal microbial composition of two flocks; one with low feed conversion ratio (FCR) and the other with high FCR. Both groups had the same genetic background and had *ad libitum* access to feed and water. *Fusobacteriaceae* (11.4-fold), *Flavobacteriaceae* (94.3-fold), *Rhizobiaceae* (13-fold), *Vibrionaceae* (330-fold) *Xanthomonadaceae* (25.4-fold), *Comamonadaceae* (37-fold) and *Campylobacteraceae* (189.4-fold) were higher in the high FCR group. Other bacterial families including *Synergistaceae* (37.3-fold), *Prevotellaceae* (16-fold), *Rikenellaceae* (13-fold) and *Ruminococcaceae* (17.7-fold) were higher in the low FCR group. Even though the animals' genetic background was the same, due to the selection for performance traits, the microbiota composition differed significantly between the groups, possibly contributing to the observed phenotypic difference (118). Richards et al. investigated the development of caecal microbiota over time in three different broiler breeds (Cobb 500, Hubbard JA87, Ross 308). Chicks were housed together in the same room under climate-controlled conditions in a bio-secure housing unit. Hubbard and Ross broilers were mainly colonized with *Enterobacteriaceae*, while Cobb broilers were mainly colonized with *Enterococcaceae* and *Clostridiaceae* directly after hatching. More homogeneity from day 3 post hatch on was observed, but Hubbard showed more *Bifidobacteriaceae* and less *Enterobacteriaceae* than the other two breeds. From day 7 post hatch on, no significant differences were observed anymore. Although these findings suggest genotypic differences, they could come from different environmental exposure during the process of hatching (119).

2.3.2.3 Diet

Animal feed seems to have the most significant influence on the gut microbiome. Lourenco et al. compared fecal and caecal microbiomes of chickens fed on a soy-based vs. soy-free diet. They found a lower relative abundance of common foodborne pathogens such as *Campylobacter* and *Acinetobacter* combined with a significantly higher richness in the soy-free group (120). Ludvigsen et al. investigated the effect of diet combined with rearing conditions on microbial composition. They found a higher *beta*-diversity, a proxy for differences in biodiversity, within the group fed an experimental diet consisting of barley, oat and wheat compared to a commercial diet. The diet itself mainly affected highly abundant microbes (121).

Siegerstetter et al. compared microbiota of restrictive fed chickens with a group that had *ad libitum* access to food and observed a tendency towards lower diversity in the *ad libitum* fed group. Additionally, *ad libitum* fed chickens were characterized by higher relative abundance of *Enterobacteriaceae* (1.3 fold), *Turicibacteraceae* (3.9 fold) and *Peptostreptococcaceae* (3.2 fold) (122). An older study by Janczyk et al. investigated the effect of green microalga *Chlorella vulgaris* on laying hens by DGGE and found a lower species richness in the control group (123). Diet is not only affecting the microbiome in general but can also influence potentially pathogenic bacteria that can be a risk for human health. Han et al. found more CFU of *Campylobacter* sp. in a broiler feed group compared to a layer feed group. Both groups were inoculated with *Campylobacter* sp. at day 1 post hatch. This finding suggest that the layer feed is modifying the microbiome in a way that leads to more resistance against *Campylobacter* sp. (117).

2.3.2.4 Antimicrobial substances

Antibiotic growth promoters have been used in chickens since 1940. They are administered in low, sub-therapeutic dosages to prevent infections and support muscle growth in animals (124). In particular, penicillin has been linked to increased growth performance in broiler chickens (125). Singh et al. investigated the effect of penicillin on the caecal microbial composition. They supplemented 55mg/kg of penicillin to a corn-soybean diet and compared this to a control group. Not only was the body weight of antibiotic-treated chickens increased, the microbiota was significantly modified. *Firmicutes* rose from 58.15% in the control group to 91.5% in the antibiotic treatment group and *Bacteroidetes* decreased from 31.1% to 2.96% relative abundance (126). Adding antibiotics in drinking water changed the microbial community and immune parameters temporarily in the later phase of life of Ross 308 broilers. Six days after antibiotic treatment with Amoxicillin or Enrofloxacin, Shannon diversity was reduced compared to the control group. Interestingly, 16 days after antibiotic treatment, the diversity rose higher in the Enrofloxacin group compared to the control and Amoxicillin group (127).

Avilamycin was a widely used antibiotic growth promoter in poultry industry. Choi et al. investigated the role of Avilamycin as feed-additive on ileal and caecal microbiota. They observed an increase of bacterial diversity in the ileum but a decreased diversity in the caecum (128).

2.3.2.5 Prebiotics

Prebiotics are indigestible substances than can be given to the host to promote the growth or activity of potentially beneficial bacteria. The definition of the term prebiotics remains unclear due to different interests of the research community, regulating agencies, food industry and consumers (129). Prebiotics can act through different mechanisms. They provide nutrients,

prevent pathogen adhesion, interact with the immune system and affect the gut morphological structure. All these features are most likely due to gut microbiota modulation (130). Mannanoligosaccharides (MOS) are constituents of yeast cell walls (*Saccharomyces cerevisiae*). MOS show the ability to inhibit pathogens with type-1 fimbriae like *Escherichia coli* and *Salmonella* spp. (131). This effect was explained by blocking bacterial lectin that led to a reduction in enteric pathogen load (132). MOS supplementation can modulate the gut microbiota composition leading to a rise of *Bacteroidetes* and an alteration of the functional capability of the caecum (133). Moreover, Cheled-Shoval et al. described that *in ovo* administration of MOS led to an increase in goblet cells that further led to a 3-fold increase in MUC2 gene expression (134). The increased production and secretion of mucin can potentially strengthen gut barrier integrity and capture pathogenic bacteria (135). Yitbarek et al. reported that MOS supplementation in a *Clostridium perfringens* challenge experiment resulted in an upregulation of TLR2b, TLR4, interleukin-12p35 and interferon-γ in ileum and an upregulation of TLR4 in CT, thus enhancing a proinflammatory effect on *C. perfringens*-challenged chickens (136).

Intestinal digestive enzymes of poultry cannot metabolize the *beta*-glycosidic bond of fructooligosaccharides (FOS) and inulin, thus leaving them undigested before they reach the ceaca, providing nutrients for gut bacteria. FOS enhanced the growth of bifidobacteria and lactobacilli while the growth of *C. perfringens* and *E. coli* was inhibited (137-139). Saminathan et al. found *in vitro*, that lactobacilli can use FOS more efficiently than MOS (140). Most bifidobacteria can use FOS as single carbon source *in vitro,* whereas inulin was only used by some species (141). Rebolé et al. found the caecal concentration of fermentation products butyric acid and lactic acid significantly increased in inulin-fed broilers compared to a control group (142). These organic acids play a role in the inhibition of pathogenic microorganisms by altering the pH and enhancing morphology and physiology of the GIT (143).

2.3.2.6 Probiotics

There has been some confusion in the past concerning the term “Probiotics”. Lilly and Stillwell (1965) were the firsts to propose a definition: “Growth-promoting factors produced by microorganisms” (144). This definition completely lacks the fact that living prokaryotic cells are involved and was later used for feed supplements with beneficial effects on gut bacteria (Prebiotics) (145). Fuller (1989) defined a probiotic as “A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” (146). This definition has still its validity today, but the beneficial effects of many probiotics on the gut microbial ecosystem remains unclear. Recently, the international scientific association for Probiotics and Prebiotics redefined probiotics as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (147). For the poultry industry, probiotics play a crucial role in replacing antibiotics, improving feed-efficiency or excluding

pathogens. Line et al. investigated the role of yeast (*Saccharomyces boulardii*) supplementation in broiler feed in *Salmonella typhimurium* and *Campylobacter jejuni* challenge experiments. While *Salmonella* was significantly reduced by the treatment, no effects on *Campylobacter* counts could be observed (148). Saint-Cyr et al. were able to reduce *Campylobacter jejuni* loads in the caeca of broilers by using the probiotic *Lactobacillus salivarius* SMXD51 (149). In another study, *Clostridium butyricum*-fed broiler chickens had higher levels of IgA, IgY and IgM in serum compared to the control group underlining the role of gut microbiota in immunoglobulin production and acquired immunity (150). Baldwin et al. administered three different *Lactobacillus* strains directly at hatch. Even though each strain was able to colonize when given alone, only one isolate (*L. ingluviei*) was able to colonize when given in a mixture. Chickens inoculated with the probiotic mix showed higher weight by day 28 and a modified microbiome, particularly represented by less relative abundance of *Lactococcus* and *Escherichia fergusonii* and more *Clostridium*-related OTUs (151). Gao et al. compared the effect of the probiotic *Lactobacillus plantarum* with an antibiotics-treated group and a control group. Interestingly, both experimental groups showed higher average daily weight gain and a better feed conversion ratio than the control group. The probiotic-treated group elevated the relative abundance of *Lactobacillus* spp. and led to higher serum IgY and intestinal secretory IgA levels (152). In 1973, Nurmi and Rantala proposed to inhibit *Salmonella infantis* attachment in broiler gut epithelium by transplantation of gut content from adult chickens to hatched chicks. They were able to significantly reduce the total amount of *S. infantis* compared to a control group (153). The concept of giving maternal microbiota (MM) directly at hatch was further developed by mimicking fecal transfer with a bacterial cocktail of different isolates to enhance microbial colonization and stimulate immune responses. The targeted approach can therefore be seen as next-generation probiotics (154).

3 Aims

Three targets were defined for this thesis. First, it was tested whether the development of the immune system is microbiota-dependent. This was shown in many studies for mice and humans (155-160), but only in a few for chickens (67, 161, 162). Therefore, we designed an animal experiment comparing a model for conventional, facility-raised vs. farm animals. We used host RNA and 16S rRNA gene amplicon sequencing combined with immunological methods to evaluate microbiota- and host-derived effects.

The long-term goal is to utilize cocktails of cultured gut bacteria to stimulate maturation of the immune system in chickens, similar to the properties of maternal microbiota under natural conditions. Although many probiotics already exist, they do not cover many metabolic properties of simplified bacterial communities. They often consist of one or two strains of *Lactobacillus* or bifidobacteria. Competitive exclusion products aim for increasing the resistance against *Salmonella* spp., but their effects on the microbiome of the developing chicken are unclear (163-165). Hence, the second aim of this work was to create a chicken intestinal bacterial collection to work with isolates that originate directly from the target host organism.

Third, we selected nine strains according to their phylogenetic diversity, abundance and prevalence in the chicken gut, colonization properties and ability to inhibit *Campylobacter* spp.. This minimal bacterial consortium was used in an animal experiment to assess its capability of stimulating immune functions.

4 Material and Methods

Exponents for chemicals and reagents refer to the provider listed in the attachments.

4.1 Animal experiments

All animal experiments were performed according to French laws, approved by the French Animal Ethics committee and German laws, approved by the government of Oberbayern, department 54, veterinary affairs (file number: 55.2-1-54-2532.0-60-2015). Chickens were kept in aviaries throughout the experiments according to German laws, especially the law of animal wellbeing for keeping livestock animals (TierSchNutztV, §12-14). Aviaries were bedded with pellets. All animals had unlimited access to food and water. During the first three weeks, an infrared lamp was used to keep chicks warm. Temperature and humidity were documented daily. Experienced animal caretakers checked the chickens daily for the occurrence of diarrhea, ectoparasites and animal wellbeing.

4.1.1 Animal experiment 1: Proof-of-principle study

The main goal of the first animal experiment was to prove that the MM has a stimulating effect on the developing immune system. On the other hand, we wanted to show that the gut microbiota composition of a SPF facility is poor and therefore the immune system of birds under these housing conditions underdeveloped. The SPF facility is the experimental model for broiler or layer production facilities with high hygienic standards; the MM group is the model for farm animals with low hygienic standards. We analyzed caecal microbiota profiles and evaluated immunoglobulin levels in plasma, caecal content and bile. In addition, we looked at host differences in the gene expression of intestinal and spleen tissue by RNAseq.

The experiment was carried out at the ‘Institute National de la Recherche Agronomique’ (INRA, Nouzilly, France, n=17) and at the ‘Department for Veterinary Sciences’ (LMU, Munich, n=25). The INRA PA-12-layer line was used.

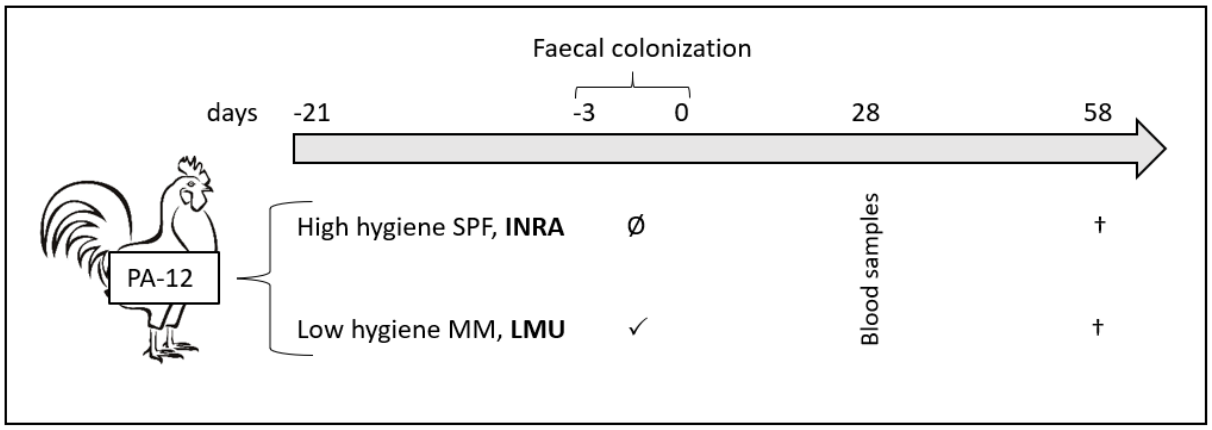

Figure 3: Experimental design of the first animal experiment

Only the low hygiene group (MM, LMU) was colonized with fecal microbiota. Therefore, five adult M-11-layer birds were allowed to put fecal droppings in the aviary three days before hatch. At hatch, the adult animals were removed and the chicks were immediately brought to the aviary for passive fecal colonization. The high hygiene group (SPF, INRA) was kept under strict hygienic measures. The room had to be entered through a disinfection mat, caretakers had to wear gloves and an overall. Blood samples for immunoglobulin measurement were taken on day 28 and day 58 in both groups. All animals were sacrificed on day 58. Caecal content was collected for microbiome analysis. Organ samples of spleen, ileum, caecum and CT were taken for RNA sequencing and qPCR analysis.

4.1.2 Animal experiment 2: Validation study

In mouse experiments, facility effects on the gut microbiome can be strong (166). Hence, we performed the second animal experiment only at the LMU, 'Department for Veterinary Sciences', with a "Lohmann's Selected Leghorn" (LSL) layer line.

The experiment was conducted with a similar experimental design. 56 hatched chicks were assigned to two groups in equal amounts (n=28). Different sampling time points were chosen to investigate the development of the caecal microbiota and plasma immunoglobulin levels over time.

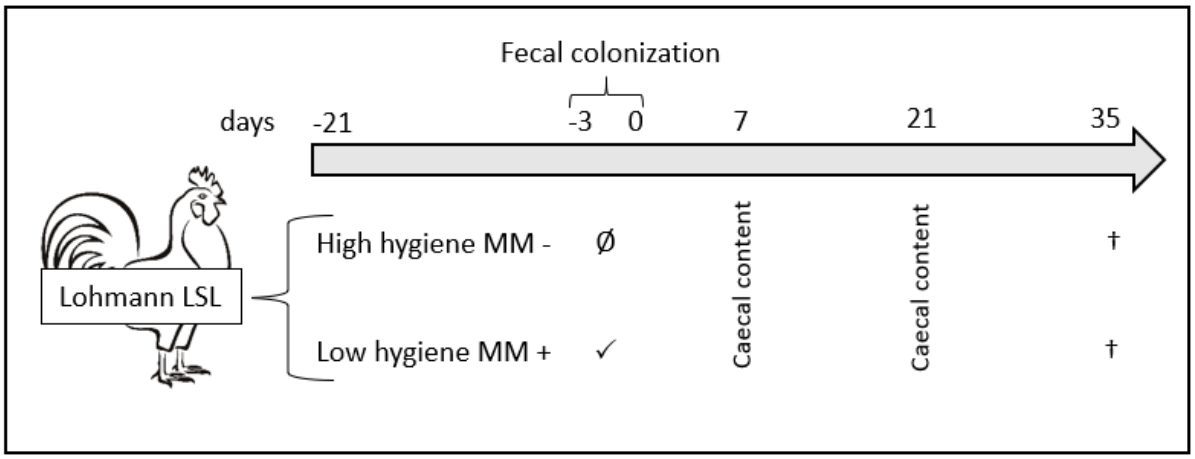

Figure 4: Experimental design of the second animal experiment

Only the low hygiene group (Maternal microbiota, MM) was passively colonized with fecal microbiota following the aforementioned approach. Blood for immunoglobulin measurement was taken on day 21 and day 35. Caecal content was collected for microbiome analysis on day 7, day 21 and day 35. We included four caecal samples of the adult donor chickens for microbiome analysis. The MM- group was kept under strict hygienic measures and was treated at first in the morning to avoid cross-contamination from the MM+ group.

4.1.3 Animal experiment 3: Colonization study

With the third animal experiment, we tested whether a minimal bacterial consortium has the potential to stimulate immune responses in the developing chicken. Three groups of the INRA PA-12-layer line were treated with either MM (=positive control), PBS (=negative control) or the minimal consortium (Cons) (=experimental group). All groups were kept at the 'Department for Veterinary Sciences', LMU Munich. 77 hatched chicks were assigned to the three groups as follows: MM (n=26), PBS (n=24) and Cons (n=27). We evaluated immunoglobulin levels in plasma and microbiome profiles in caecal content.

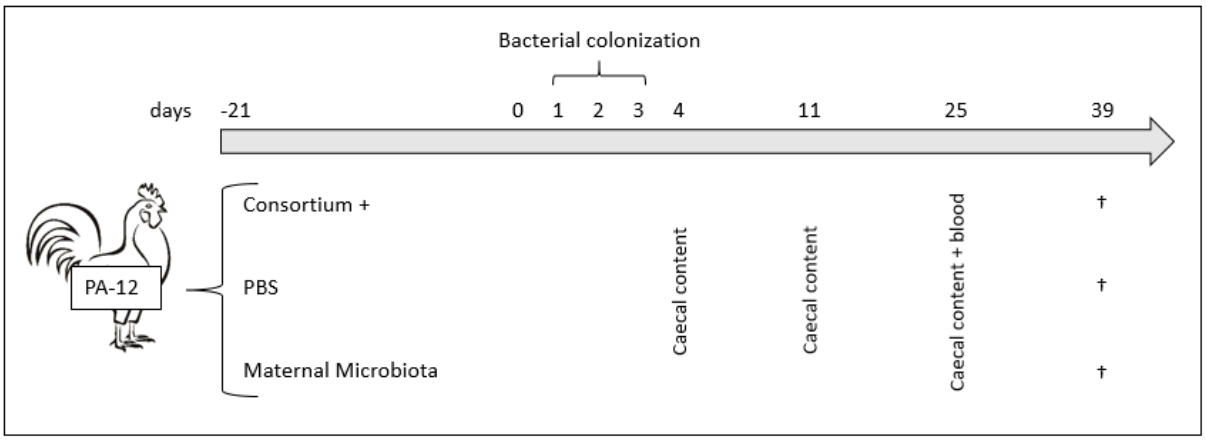

Figure 5: Experimental design of the third animal experiment

For fecal colonization, a total of 500g feces was collected from adult LSL layer chickens and the whole amount was spread throughout the bedding of the hatched chicks in the MM group. The PBS and the Cons group received 250µl of PBS or bacterial suspension orally on the day of hatch and on day one and two post hatch, respectively. The PBS and Cons group were kept under strict hygienic measures, as described above. The PBS group was treated at first in the morning, followed by the Cons group and lastly the MM group, to avoid cross-contaminations. Caecal samples for NGS sequencing were taken on day four, day 11, day 25 and day 39. Blood was collected on day 25 and day 39.

4.2 Quantitative ELISA

Material:

Coating Buffer (pH 9.6, storage 4°C)

3.1g Sodium carbonate[1] (Na_2CO_3)

6.0g Sodium bicarbonate[1] ($NaHCO_3$)

ad 1000ml Aqua dest.

Adjust pH after dissolving all reagents

Coating Antibodies: A1[17](#8330-01), G1[17](#8320-01) (storage 4°C)

PBS-T (pH 7.2, storage RT)

40g Sodium chloride[1] (NaCl)

5.75g Disodium hydrogen phosphate[1] (Na_2HPO_4)

1g Potassium chloride[1] (KCl)

1g Potassium dihydrogen phosohate[1] (KH_2PO_4)

ad 5000ml Aqua dest.

Adjust pH after dissolving all reagents

2.5ml Tween20[1] after pH adjustment

PBS (pH 7.4, storage RT)

40g Sodium chloride[1] (NaCl)

5.75g Disodium hydrogen phosphate[1] (Na_2HPO_4)

1g Potassium chloride[1] (KCl)

1g Potassium dihydrogen phosphate[1] (KH_2PO_4)

ad 5000ml Aqua dest.

Adjust pH after dissolving all reagents

TMB Buffer (pH 5.0, storage 4°C)

8.2g Sodium acetate[1]

3.15g Citric acid[1]

Ad 1000ml Aqua dest.

Casein[2] (#218682, storage RT)

FBS Superior[3] (#S0615, storage -20°C)

Round Bottom 96-well plates[15], unsterile, for dilution

Nunc Maxisorp™ 96-well plate[15]

Chicken Serum as standard for quality control

Detection Antibodies (A3-POD, 4D12-POD, manufactured in-house, storage 20°C)

Dimethylsulfoxide[1] (DMSO, storage RT)

3,3',5,5'-Tetramethylbenzidine[1] (TMB, storage RT)

Hydrogen Peroxide 33%[1] (storage RT)

Sulfuric Acid 1M[1] (storage RT)

Aqua Dest.

Biochrom Asys Atlantis Microplate Washer

Tecan Sunrise Microplate Reader

Solutions were prepared as indicated and stored at the mentioned temperature until use. All reagents, samples, sample dilutions and antibodies were applied by reverse pipetting. Incubation- and sample temperature was constantly at 22-24°C. The dilution of samples was started before blocking the ELISA plate. Sample dilutions, standard curves and quality controls were prepared on a 96-well round bottom plate and were afterwards transferred to a Maxisorp™ ELISA plate by multi-channel pipetting. For duplicates or triplicates, all approaches were prepared separately. A Nunc Maxisorp™ flat bottom 96-well plate was coated with the following coating antibodies, depending on the immunoglobulin to quantify:

IgA: 2µg/ml Mouse Anti-Chicken IgA-UNLB (SouthernBiotech, 8330-01)

IgY: 5µg/ml Mouse Anti-Chicken IgY-UNLB (SouthernBiotech, 8320-01)

All antibodies were diluted in coating buffer. Each well of the Maxisorp™ plate was coated with 100µl antibody dilution, sealed with a microplate sealing tape and stored at 4°C over night. A 1% Casein solution in PBS pH 7.4 was prepared for IgA and IgY. 4 wells were filled with 1% Casein solution in PBS pH 7.4 without antibody as a negative control.

Blocking:
The coated ELISA plate was washed in a microplate washer by siphoning the coating solution and distributing 100µl/well PBS-T. The procedure was repeated two more times by the washer. Afterwards, the plate was pounded a couple of times on a clean tissue to remove residual liquid. 200µl of the 1% Casein solution was applied to each well by multi-channel pipetting. The plate was sealed and incubated for 1h at 22-24°C.

Sample application:
The ELISA plate was washed in the microplate washer and treated as aforementioned. Samples and standard curves were applied in duplicates at the required concentrations. Quality controls were applied in triplicates. Samples, standard curves and quality controls were diluted in 1% Casein in PBS-T pH 7.4 with 10% FBS for IgA and IgY. 100µl of the antibody dilution was applied with a multi-channel pipette to each well of the Maxisorp™ ELISA plate. For the uncoated wells, the first four dilutions of the standard curves were applied. As a second negative control, the dilution liquid was applied to four coated wells. The two negative controls

helped detecting background noises that can later be subtracted from the extinction of samples. The plate was sealed and incubated for 1h at 22-24°C.

Detection antibody:

The ELISA plate was washed in the microplate washer and treated as aforementioned. 50µl of peroxidase (POD) conjugated secondary antibody was applied to each well in the following concentrations:

IgA: A3-POD (1mg/ml), 1:1000 in 1% Casein in PBS-T pH 7.4

IgY: 4D12-POD (1mg/ml), 1:2000 in 1% Casein in PBS-T pH 7.4

The secondary antibodies were manufactured in-house. The plate was sealed and incubated for 1h at 22-24°C.

Detection:

The ELISA plate was washed in the microplate washer and treated as aforementioned. A TMB Stock solution was prepared by weighing 6mg TMB to 1ml of DMSO. After thorough vortexing, the solution was stored in a dark place until usage. The TMB working solution was prepared by mixing 12.5ml TMB buffer with 415µl TMB stock solution and 3.75µl hydrogen peroxide (for one plate). 100µl of the TMB working solution was pipetted to each well. The plate was sealed and incubated for 10min in the dark at 22-24°C. Afterwards, the reaction was stopped by adding 50µl of 1M sulfuric acid to each well. The plate was read in a Tecan Sunrise plate reader with the following parameters: Absorbance, Filter 450nm, reference filter 620nm, shaking 5s outside. The raw data sheet was exported to Microsoft Excel and was further evaluated with an in-house Excel sheet for quantitative ELISA.

4.3 16S rRNA gene amplicon sequencing

16S rRNA gene amplicon sequencing of the V3-V4 region was performed at the ZIEL – Institute for Food & Health, Core Facility Microbiome, Technical University of Munich (TUM). Sample processing was divided into four main steps. DNA isolation (1), library construction by PCR (2), amplicon cleaning and dilution (3) and sequencing (4).

4.3.1 DNA isolation

Material:

Gut Content in DNA Stool Stabilizer[11] **(**#1038111100)

Polypropylene Screw-cap Tubes with 500mg Silica Beads[18] (sterile)

Guanidinethiocyanat[14] **4M** (storage 4°C)

N-Laurolylsarcosine[14] **5%** (storage 4°C)

Polyvinylpyrrolidone[12] (storage RT)

RNase[16] (storage -20°C)

Dry Ice

NucleoSpin® gDNA Clean-up Kit[19] (#740230.50)

Heraeus™Fresco™ Centrifuge

MP Biomedicals FastPrep 24® 5G Instrument

Biometra TSC Thermo Shaker

DNA isolation was carried out with a modified protocol by Godon et al. (167). 600µl of diluted gut content in DNA stool stabilizer was transferred to a 2ml screw-cap tube containing 500mg of 0.1mm silica beads. 250µl of Guanidinethiocyanat 4M for cell lysis and 500µl of 5%-N-Laurolylsarcosine as detergent were added. The tubes were vortexed thoroughly and then incubated for 60min at 70°C and 700rpm on a thermo shaker.

To lyse the cells mechanically, the tubes were shaken in an MP Biomedicals Fast Prep 24® 5G instrument three times for 40sec at 6.5m/s. Dry ice was added before each step to keep the samples cooled. 15mg of Polyvinylpolypyrrolidone was added to each sample, the tubes were vortexed and then centrifuged for 3min at 15.000xg at 4°C. The supernatant was transferred to a new 2ml tube. The tubes were again centrifuged for 3min at 15,000xg at 4°C and 500µl of the clear supernatant was again transferred to a new 2ml tube. 5µl of RNase was added and the samples were incubated for 20min at 37°C on a thermo shaker at 700rpm.

Further DNA Clean-up was performed with a NucleoSpin® gDNA kit. 500µl sample was mixed with 1500µl of Binding Buffer. The tubes were vortexed for 5sec. 680µl of the sample was put on a NucleoSpin® gDNA Clean-Up column. The columns were centrifuged for 30sec at 11,000g. The flow-through was discarded and the procedure was repeated two more times to bind a high amount of DNA to the column. The columns were washed three times by adding

600µl of Wash-Buffer followed by a centrifugation step for 30sec at 11,000xg. The flow-through was discarded each time. After the final washing step, the columns were centrifuged dry for 2min at 11,000xg. The columns were put in a new 1.5ml tube and 30-50µl warmed Elution Buffer was pipetted directly on the center of the column matrix. Samples were incubated for 1min at room temperature and then centrifuged for 1min at 11,000xg. If necessary, the elution step was repeated by pipetting the flow-through on the center of the column matrix again or by using more Elution buffer. Purified DNA was measured by NanoDrop™ 1000. 1µl of Elution Buffer was pipetted on the sample plate as blank. 1µl of sample was used for measuring the amount of DNA. 260/280 ratio was used to detect the occurrence of contaminations. DNA quantification by NanoDrop 1000 was performed to dilute the DNA to 12ng/µl for the following library construction.

4.3.2 Library construction by 2-step PCR

Material:

Phusion® HF Buffer[15] (storage -20°C)

dNTPs[20] (20µmol, #BIO-39043, storage -20°C)

Primer 341F-ovh[21] (20µM, storage -20°C)

Primer 785R-ovh[21] (20µM, storage -20°C)

Phusion® High Fidelity DNA Polymerase Hot-Start[15] (storage -20°C)

Dimethylsulfoxide[1] (DMSO, storage RT)

Nuclease-free water

Biometra Tadvanced Thermocycler

The V3-V4 regions of the 16S rRNA genes were amplified by 2-step PCR. The first step was necessary for amplification of the desired PCR product; the second step was performed to add individual barcodes to each sample for latter identification after the sequencing run. A Mastermix was prepared as follows:

Reagents	Volume µl/sample
Phusion® HF Buffer	4
dNTPs (20µmol)	0.4
341F-ovh Primer 20µM	0.125
785R-ovh Primer 20µM	0.125
Phusion® High Fidelity DNA Polymerase Hotstart	0.1
DMSO 100%	1.5
Nuclease-free water	11.75

Table 1: Mastermix of the first step PCR for library construction

DNA was diluted to 12ng/µl with nuclease-free water. 2µl of sample DNA was added to 18µl of the Mastermix. The reaction was carried out in duplicate. PCR reaction was performed in a thermocycler with settings as follows:

Step	T (°C)	Time (s)	Cycles
Initial denaturation	98	40	1
Denaturation	98	20	15
Annealing	55	40	15
Elongation	72	40	15 (go back to denaturation)
Final elongation	72	120	1
Holding	10	∞	∞

Table 2: Thermocycler conditions for amplification of the 16S rRNA gene V3-V4 region

A second Mastermix was prepared as follows:

Reagents	Volume µl/sample
Phusion® HF Buffer	10
dNTPs (20µmol)	1
e.g. 341F-ovh-HTS-SC501 (20µM)	0.313
Phusion® High Fidelity DNA Polymerase Hotstart	0.2
DMSO 100%	2.5
Nuclease-free water	31.487

Table 3: Mastermix of the second step PCR for library construction

2µl DNA of the first step PCR reaction was added to 45.5µl of the second Mastermix. 2.5µl of individual barcoded reverse primer (e.g. 785r-ovh-HTS-SA701 and subsequent) was added to each sample. The primers could be used vice versa, e.g. taking the same reverse primer for the Mastermix and individual forward primers for each sample. In the third sequencing order (colonization experiment), only one index primer was used but with 12 nucleotides instead of eight. An example table of primers used in the first and second step PCR can be found in table 5. The second PCR reaction was performed in a thermocycler with settings as follows:

Step	T (°C)	Time (s)	Cycles
Initial denaturation	98	30	1
Denaturation	98	5	10
Annealing	55	10	10
Elongation	72	10	10 (go back to denaturation)
Final elongation	72	120	1
Holding	10	∞	∞

Table 4: Thermocycler conditions for individual barcoding of samples

Step	Oligonucleotide	sequence
1	341F-ovh	5‘-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG **CCTACGGGNGGCWGCAG**-3‘
1	785r-ovh	5‘-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG **GACTACHVGGGTATCTAATCC**-3‘
2	341F-ovh-HTS-SC501	5‘-AATGATACGGCGACCACCGAGATCTACACACG **ACGTGTCG** TCGGCAGCGTC-3‘
2	785r-ovh-HTS-SA701	5‘-CAAGCAGAAGACGGCATACGAGAT **AACTCTCG** GTCTCGTGGGCTCGG-3‘

Table 5: Used primers in 2-step PCR for library construction.

Step 1: Specific primers for 16S rRNA gene in bold. Step 2: Individual barcodes in bold

The PCR products were pooled prior to cleaning, resulting in a total volume of 100µl PCR product per sample. A selection of samples was checked by gel electrophoresis as a quality control (see 4.6.5 for details).

4.3.3 Amplicon cleaning and dilution

Material:

Agencourt AMPure XP Kit[22] (storage -20°C)

Magnetic Rack[22]

Ethanol 70%[6] (storage RT)

PCR purification was performed with an Agencourt AMPure XP kit. AMPure XP beads were thawed and kept at RT for 30min. They were thoroughly vortexed to obtain a well-dispersed solution. After thermal cycling, PCR products were transferred to a 96-deep-well plate. 1.8µl of AMPure XP beads/µl PCR product was added. The entire volume was gently pipetted up and down ten times. The samples were incubated for 5min at RT. The plate was placed on a

magnetic rack at RT for 2min until the liquid appeared clear. The supernatant was removed from each well using a 200µl multi-channel pipette. 200µl of freshly prepared 70% EtOH was added to each well without disturbing the beads. After 30s incubation at RT, the supernatant was again removed and the washing step with EtOH was repeated two more times. The plate was incubated for max. 5min to let the EtOH evaporate. It was removed from the magnetic rack and the pellet was suspended in 25-33µl BE elution buffer. The entire volume was gently pipetted up and down ten times to mix it thoroughly. The plate was incubated for 2min at RT. It was again placed on the magnetic rack for 2min at RT until the liquid appeared clear. 23-31µl of the clear supernatant from each well was transferred to a new PCR-tube. Measurement of DNA concentration was performed by fluorimetry with Qubit® assay according to the manufacturers' instructions (see 4.6.3 for more details).

4.3.4 Sequencing

Material:

Illumina® MiSeq™

MiSeq™ Reagent Kits v2[23]

The 16S rRNA gene amplicon libraries were sequenced in an Illumina® MiSeq instrument in paired-end modus. The average library size for V3-V4 16S rRNA gene amplicon sequencing was therefore determined to 440bp. All sample were pooled and diluted to a final molarity of 2nM according to the following formula:

$$(\text{concentration in ng/µl}) / (660\ \text{g/mol} \times \text{average library size}) \times 10^6 = \text{concentration in nM}$$

Hereinafter the DNA pool was denatured. 10µl of a freshly prepared 0.2 N NaOH solution was mixed with 10µl of the 2nM DNA pool in a 1.5ml tube. The samples were vortexed and quickly spun in a micro centrifuge. Samples were incubated for 5min at RT followed by a 5min incubation step at 95°C and a 5min step at 4°C. All following steps were performed on ice. 980µl of cooled HT1-Buffer was added to the DNA pool to gain a concentration of 20pM. DNA was furthermore diluted to a final concentration of 4pM. 250µl of PhiX control library was added to 750µl of library pool. Samples were kept on ice until the MiSeq cartridge was ready. 600µl of the PhiX/library mix was transferred to the loading well of the MiSeq cartridge.

The sequencing run was finished after 2 days. The MiSeq instrument was washed with MilliQ + 0.5% Tween and then with only MilliQ water. The MilliQ washing step was repeated.

4.3.5 Data analysis

Programs:

Integrated Microbial Next Generation Sequencing (IMNGS)

R Version 3.5.2

R Studio 1.1.463

RHEA pipeline

Apache OpenOffice Calc 4.1.6

Adobe Acrobat Reader DC

Raw reads were assigned to their corresponding samples via demultiplexing using barcode pairs unique to each of the samples. Demultiplexing was performed by demultiplexor_v3.pl, an in-house developed Perl script of the Core Facility Microbiome (TU Munich).

4.3.5.1 IMNGS

Raw data was further loaded in the online 'integrated microbial next generation sequencing' (www.imngs.org) platform (62). Parameters were as follows:

Number of allowed mismatches in the barcode	1
Min fastq quality score for trimming of unpaired reads	20
Min length for single reads or amplicons for paired overlapping sequences	200
Max length for single reads or amplicons for paired overlapping sequences	600
Max rate of expected errors in paired sequences	3
Length of trimming at the forward side of the seqs	10
Length of trimming at the reverse side of the seqs	10
Min relative abundance of OTU cutoff (0-1)	0.005

Table 6: Selected parameters in IMNGS platform

The automated online platform IMNGS is based on UPARSE (168). Pairing, quality filtering and OTU clustering at 97% sequence identity was done by USEARCH 8.0 (169). Chimera filtering was done by UCHIME (170). Taxonomic classification was done by RDP classifier version 2.11 training set 15 (171). Sequence alignment was done by MUSCLE (172) and treeing by FastTree (173). A mapping file was provided to add metadata and to identify each sample correctly.

4.3.5.2 RHEA

The output files from IMNGS were further processed with RHEA (174), a set of R scripts to perform microbial diversity analysis. R Studio was used for convenience (175). The RHEA pipeline consists of six individual scripts that build on each other:

1. Normalization
2. *Alpha*-Diversity
3. *Beta*-Diversity
4. Taxonomic-Binning
5. Serial-Group-Comparison
6. Correlations

The normalization script is balancing different sequencing depth by calculating normalized counts. Rarefaction curves are built to help estimating the sufficiency of sequencing depth for each sample. *Alpha*-diversity parameters were calculated for each sample, including richness, an enumeration of all detectable OTUs within each sample. However, as species richness does not take into account the community structure (some OTUs could be present only once, others a hundred times), different diversity indices were also used. The most common in bacterial community analysis are the Shannon-Wiener index and the Simpson index. Both are not linear making a comparison between sample groups difficult. RHEA calculates the effective diversity of a microbial profile for a given index by estimating the number of equally abundant OTUs (176, 177). *Beta*-diversity assesses similarities between different samples. Bray-Curtis considers the similarities between samples whilst UniFrac considers the phylogenetic distances between OTUs. Weighted UniFrac is also taking the relative abundance of each OTU into account (178, 179). Unweighted UniFrac is sensitive for rare OTUs, weighted UniFrac for dominant OTUs. The generalized UniFrac is a more balanced version of both UniFrac analyses and is used by the RHEA script (180). The script generates multi-dimensional scaling plots (MDS-plots), non-metric dimensional scaling plots (NMDS-plots) and a dendrogram as output files. Permutational multivariate analysis of variance is performed to test for significant separation of the groups. The taxonomic-binning script sorts each OTU to its taxonomic level after classification by RDP (51). Some OTUs can be taxonomically assigned to genus level, e.g. Kingdom>Phylum>Class>Order>Family>Genus. For others, the taxonomic classification stops earlier, e.g. Kingdom>Phylum>Class>Order. Due to the large proportion of uncharacterized microorganisms, some OTUs cannot be classified correctly. This leads to outputs such as “unknown *Bacteroidales*”. The OTU could be assigned at the order level, but not correctly at the family level. Furthermore, misclassification on lower taxonomic levels is unavoidable. The script generates output files for each taxonomic level and a graphical overview of the microbial compositions at each level. The serial-group-comparison script uses Fisher tests and analysis of variance (ANOVA) to detect differences in composition and relative abundance between groups. Since the classical ANOVA is assuming normality of distribution and this is barely the case for OTU datasets, the script is using the non-parametric Kruskal-Wallis Rank Sum Test. Additionally, if more than two groups are compared, the script is using Mann-Whitney-U-Test to perform pairwise tests (181). Benjamini-Hochberg method is used for

multiple testing with corrected significance values (182). The user is allowed to set parameters on its own. If not mentioned otherwise, these parameters are usually set to:

- abundance_cutoff: 0.5 (values below 0.5% abundance are zeroed)
- prevalence_cutoff: 0.3 (30% of samples are positive within a given group)
- max_median_cutoff: 1 (minimum median abundance value, that must be observed in at least one group before statistical analysis)
- ReplaceZero: Yes (Zeros are replaced with NA and are not considered in statistic)
- PlotOption: 1 (graphical output without individual values as dots)
- sig.cutoff: 0.05 (significance cutoff level of 0.05%)

A reduction of tests can be applied by adjusting these values. Unnecessary tests can be avoided by increasing the prevalence cutoff. Additional tests can be conducted by removing the significance cutoff (setting it to 1). By setting the abundance cutoff to zero, low abundant communities can be evaluated. Pre-filtering datasets is important to increase the power of analysis, always depending on the addressed question (183). For the correlation script, metadata can be added and combined with the existing taxonomic variables. Their relationship is calculated by Pearson’s coefficient of correlation. The graphical output file as Corrplot gives an overview of taxa that correlate positively or negatively with the added metadata.

All tabular output files of the RHEA script can be opened in either Microsoft Excel or Apache OpenOffice Calc. All PDF output files can be opened in Abode Acrobat Reader DC.

4.4 Isolation of bacterial strains

4.4.1 Sample collection

Material:

PBS (pH 7.2, storage RT)

8g Sodium chloride[1] (NaCl)

1.15g Disodium hydrogen phosphate[1] (Na_2HPO_4)

0.2g Potassium chloride[12] (KCl)

0.2g Potassium dihydrogen phosphate[12] (KH_2PO_4)

ad 1000ml Aqua dest.

Adjust pH after dissolving all reagents

Wilkins Chalgren Anaerobic Broth[13] (#CM0643)

Cysteine Hydrochloride[14] (storage RT)

Dithiothreitol[7] (DTT, storage 4°C)

Glycerol[14] (storage RT)

Aqua Dest.

Schott Bottles with Butyl Rubber-stopper[24]

Gassing Station (custom-made)

Varioklav 400E Autoclave

Prior to the dissection of animals, Phosphate-Buffered-Saline (PBS) and Wilkins-Chalgren-Anaerobic Broth (WCA) were prepared. The WCA medium was produced by dissolving 33g of the broth powder in 1000ml Aqua dest.. Both solutions were supplemented with 0.05wt/vol L-cysteine hydrochloride and 0.02wt/vol DTT as reducing agents for the reduction of oxygen. 80ml was aliquoted into 100ml Schott bottles with butyl rubber-stopper. The solutions were gassed for approx. 10min with a gas mixture containing 94% carbon dioxide and 6% forming gas 95/5 (95% nitrogen and 5% hydrogen). All Schott bottles were autoclaved with a standard program for liquid solutions.

During sacrifice, jejunal, caecal or colonic content was first transferred in the sterile and anoxic PBS solution. It was shaken for approx. 1min to dissolve the content into the liquid. After shaking, the butyl rubber-stopper caps were soaked in 100% EtOH twice before transferring 5ml of the gut suspension in PBS into the anoxic WCA solution by using a sterile needle and syringe. This procedure helped maintaining the anoxic conditions by transferring the solution twice. The inoculated WCA solution was immediately brought to the lab for further processing. Cryo stocks were made under a laminar flow by mixing 0.5ml of the gut suspension in WCA with 0.5ml 40% glycerol leading to a final concentration of 20% glycerol. The freshly prepared

cryo stocks were immediately put on dry ice before deep-freezing them at -80°C. The samples were kept in the freezer until further processing.

Samples for bacterial isolation were only taken during the second animal experiment. To obtain more samples for bacterial isolation and to cover more diversity, intestinal samples from a free-range broiler, a free-range layer and a conventional broiler were taken following the procedure described above.

4.4.2 Isolation

Material:

Frozen Samples in Cryo Medium

Phosphate Buffered Saline[14] (PBS, anoxic, sterile)

Sterile Inoculation Loops[14]

Culture Media (see table 7 for composition)

Agar Agar[5]

Ethanol Absolute[16]

Hungate Tubes[16]

MBraun® UNIlab pro Anaerobe Workstation (custom-made)

SalvisLab Incucenter IC80

The frozen samples were brought to an MBraun® anaerobic workstation containing the same gas as described above. They were serially diluted from 10^{-2} to 10^{-5} in sterile and anoxic PBS and plated on different solid culture media (15g/l agar). The complete list of media and their composition can be found in table 7. To enrich the growth of fastidious strains, agar plates were supplemented with 5% sheep blood[10]. Plates were incubated at 37°C in an incubator and checked daily for growth and the occurrence of new colonies. After 1-14 days of initial growth, single colonies were picked and streaked at least 3 times to guarantee pure cultures. After re-streaking, single colonies were transferred into liquid medium using the Hungate technique (73) and following the instructions of the DSMZ for cultivation of strictly anaerobic bacteria (184). Inoculated Hungates were brought out of the anaerobic chamber and were incubated at 37°C, with agitation if necessary, until a dense growth was observed.

Turbid cultures were passaged for further analyses. Therefore, the butyl rubber was soaked in EtOH and then flamed twice before inoculation and once after inoculation to prevent contaminations. A 1ml syringe was flushed with the gas mixture aforementioned. 1ml of gas was injected into the Hungate to avoid a vacuum and 1ml of liquid culture was extracted and transferred into a new Hungate. The newly inoculated Hungates were again incubated at 37°C until a dense growth was observed. They were used for further identification protocols via MALDI biotyping or sequencing.

Brain Heart Infusion[13] (#CM1135)	Brain heart infusion	37g
	Cysteine Hydrochloride[14]	0.5g
	DTT[7]	0.2g
	Phenosafranine[14]	2ml (1.25mg/ml)
	Aqua dest.	1000ml
Bile Esculin Agar[14] (#48300)	Bile Esculin Agar	64.5g
	Aqua dest.	1000ml
GAM Broth[9] (#5422)	GAM broth	59g
	Cysteine Hydrochloride[14]	0.5g
	DTT[7]	0.2g
	Phenosafranine[14]	2ml (1.25mg/ml)
	Aqua dest.	1000ml
GAM Broth Modified[9] (#5433)	GAM broth modified	41.7g
	Cysteine Hydrochloride[14]	0.5g
	DTT[7]	0.2g
	Phenosafranine[14]	2ml (1.25mg/ml)
	Aqua dest.	1000ml
Wilkins Chalgren Anaerobic Broth[13] (#CM0643)	WCA broth	33g
	Cysteine Hydrochloride[14]	0.5g
	DTT[7]	0.2g
	Phenosafranine[14]	2ml (1.25mg/ml)
	Aqua dest.	1000ml
YCFA Medium (modified) (manufactured in-house)	Caseine Hydrolysate[14]	10g
	Yeast extract[5]	2.5g
	Glucose monohydrate[12]	5g
	Magnesium sulfate heptahydate[12]	45mg
	Calcium Chloride Dihydrate[12]	90mg
	Dipotassium hydrogen phosphate[12]	0.45g
	Potassium dihydrogen phosphate[12]	0.45g
	Sodium chloride[12]	0.9g
	Phenosafranine[14]	2ml (1.25mg/ml)
	Sodium carbonate[14]	4g
	Cysteine hydrochloride[14]	1g
	Hemin 90%[14]	10mg
	Aqua dest.	1000ml
	<u>Volatile fatty acids</u>	
	Acetic acid[12]	1.9ml
	Propionic acid[14]	0.7ml
	Isobutyric acid[14]	90µl
	Valeric acid[14]	100µl
	Isovaleric acid[14]	100µl
	<u>Vitamin Solution</u>	10ml
	Biotin[5]	2mg
	Folic acid[14]	2mg
	Pyridoxine hydrochloride[14]	10mg

	Thiamine hydrochloride dihydrate[14]	5mg
	Riboflavin[14]	5mg
	Nicotinic acid[14]	5mg
	Calcium pantothenate[14]	5mg
	Vitamin B12[14]	0.1mg
	p-Aminobenzoic acid[14]	5mg
	Lipoic acid[14]	5mg
	Aqua dest.	1000ml

Table 7: List of media used for cultivation of anaerobe bacteria

4.5 Strain identification by MALDI-TOF

Pure strains or single colonies were analyzed using a Bruker MALDI Biotyper® (185).

4.5.1 Liquid extraction protocol

Material:

Hungate Culture

Aqua Bidest.

Ethanol Absolute[16] (storage RT)

Formic Acid[5] (storage RT)

Acetonitrile[14] (storage RT)

Biometra TSC Thermo Shaker

Heraeus™Fresco™ Centrifuge

2ml of Hungate culture was transferred to a 2ml tube. The cups were centrifuged for 10min at 13,000rpm. The supernatant was removed and the cups were centrifuged again for 2min at 13,000rpm. The remaining supernatant was removed. 300µl of Aqua bidest. was added and the cups were put for 5min on a shaker. For washing the pellet and removing potential media residues, 900µl of ethanol (EtOH) absolute was added and the cups were again put on a shaker for 5min. They were incubated for 1min at room temperature before centrifuging them for 5min at 13,000rpm. The supernatant was removed and the cups were centrifuged again for 2min at 13,000rpm. The remaining supernatant was removed. The cups were incubated with open caps under a laminar flow for 15min to evaporate EtOH residues. To resolve the pellet, 50µl of 70% formic acid was added to the cups and they were put on a shaker for 5min. 50µl of acetonitrile was added and the cups were again put on a shaker for 5min. 5µl of the prepared sample was pipetted on three spots of the MALDI target.

4.5.2 Direct colony picking

Material:

Single Colonies of pure Culture

Sterile Inoculation Loops[14]

MALDI Target[25]

Instead of using liquid culture for MALDI analysis, it was possibly to directly pick single colonies and transfer them to the MALDI target. This direct method was less time consuming and can easily be performed from agar plates. The method was also applied for a quicker identification of new bacterial isolates without losing time for incubation in liquid cultures.

A single colony was picked with a sterile inoculation loop and was directly swapped on an empty spot of the MALDI target. It was appropriate to leave some bacterial material on the plate of the desired colony for later re-streaking and further cultivation.

4.5.3 Matrix preparation and quality control

Material:

MALDI Matrix (storage RT)

1mg α-Cyano-4-hydroxycinnamic acid[14]

100µl organic solvent

Organic Solvent

500µl Acetonitrile[14] (storage RT)

475µl Aqua dest. (storage RT)

25µl Trifluoracetic acid[14] (storage RT)

Bacterial Test Standard BTS[25] (storage -20°C)

The MALDI Matrix was prepared by dissolving 1mg of Hydroxycinnamic acid in 100µl organic solvent. This was done in a darkened room, because of the light-sensitivity of the Matrix. After the liquid or the colony had dried, the samples were overlaid by pipetting 1µl of MALDI matrix on each spot. The target was dried for 5min under a laminar flow until measurement.

To calibrate the instrument and as a performance control, a bacterial test standard (BTS, Bruker®, IVD Bacterial Test standard), containing the extracted *E. coli*-DH5-*alpha* strain spiked with two additional proteins, was used on three spots of the MALDI target and overlaid with 1µl of MALDI matrix. To prepare the test standard, 1ml of organic solvent was produced. 50µl of the organic solvent was added to the lyophilized BTS and dissolved by pipetting up and down 20 times at RT. The solution was incubated for 5min at RT and then mixed again by pipetting up and down 20 times. The tube was centrifuged briefly in a micro centrifuge. Aliquots were made and stored at -20°C until use.

4.5.4 MALDI measurement

Material:

Prepared MALDI Target with Samples

MALDI-TOF Biotyper®

The target was inserted into the MADLI-TOF Biotyper. A sample sheet was created to assign the positions of the BTS and samples. The measurement itself was self-automated and did not need further processing. The analyzed samples were divided into 3 different categories by MALDI scoring. A score from 3-2 represented a good species identification, a score from 1.7-1.9 meant that the identification was only possible at genus level and a score below 1.69 had no reliable identification (figure 6). If no peaks were found by MALDI measurement, technical issues were usually the reason. This could happen if not enough bacterial biomass was applied to the target or the matrix was not used correctly.

Result Overview

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
A1 (+++) (A)	BTS (BTS)	Escherichia coli	2.50	Escherichia coli	2.40
A2 (+) (B)	Chris1 (standard)	Lactobacillus saerimneri	1.82	Lactobacillus saerimneri	1.79
A3 (+++) (A)	Chris2 (standard)	Bacteroides vulgatus	2.06	Bacteroides vulgatus	1.88
A4 (-) (C)	Chris3 (standard)	No Organism Identification Possible	1.28	No Organism Identification Possible	1.22
A5 (+++) (A)	Chris4 (standard)	Enterococcus faecium	2.39	Enterococcus faecium	2.36
A6 (+++) (A)	Chris5 (standard)	Lactobacillus salivarius	2.21	Lactobacillus salivarius	2.18
A7 (+++) (A)	Chris6 (standard)	Lactobacillus salivarius	2.42	Lactobacillus salivarius	1.89
A8 (+++) (A)	Chris7 (standard)	Veillonella magna	2.11	No Organism Identification Possible	1.26

Figure 6: Output file of the MALDI Biotyper

Green shows a reliable identification. Yellow shows an insecure identification and red shows no possible identification.

4.6 Strain identification by 16S rRNA gene sequencing

4.6.1 DNA isolation

Material:

Hungate Culture

Polypropylene Screw-cap Tubes[14] (sterile)

DNA Stool Stabilizer[11] (storage RT)

Guanidinethiocyanat[14] **4M** (storage 4°C)

N-Laurolylsarcosine[14] **5%** (storage 4°C)

Polyvinylpyrrolidone[12] (storage RT)

RNase[16] (storage -20°C)

Dry Ice

NucleoSpin® gDNA Clean-up Kit[19] (#740230.50)

Heraeus™Fresco™ Centrifuge

MP Biomedicals FastPrep 24® 5G Instrument

Biometra TSC Thermo Shaker

To get a more precise identification, the 16S rRNA gene of bacterial isolates selected after MALDI was sequenced. A modified protocol by Godon et al. (186) was used for extraction of genomic DNA. 2ml of Hungate culture was transferred to a 2ml tube. It was centrifuged for 10min at 12,000xg. The supernatant was removed and the pellet was resolved in 600µl DNA stool stabilizer. From here on, the DNA isolation protocol is identical to the protocol for amplicon sequencing described in section 4.3.1.

4.6.2 DNA quantification by NanoDrop 1000

Material:

Isolated DNA

Elution Buffer[19] (component of the NucleoSpin® gDNA Clean-up Kit, storage RT)

NanoDrop™ 1000

Purified DNA was measured by NanoDrop™ 1000. 1µl of Elution Buffer was pipetted on the sample plate as blank. 1µl of sample was used for measuring the amount of DNA. 260/280-ratio was used to detect the occurrence of contaminations. DNA quantification by NanoDrop 1000 was performed to evaluate the volume of microliters to put in the following PCR reaction.

4.6.3 DNA quantification by Qubit dsDNA high sensitivity kit

Material:

Isolated DNA

Qubit® Buffer[15] (storage RT)

Qubit® Reagent[15] (storage RT)

Low Concentration Test Standard[15] (storage 4°C)

High Concentration Test Standard [15] (storage 4°C)

Qubit® 2.0 Fluorometer[15]

Qubit® Assay Tubes[15]

DNA quantification with Qubit® 4 Fluorometer is an indirect, fluorochrom-assisted method. The fluorochrom is intercalating with the double stranded DNA. The amount of emitted light is proportional to the concentration of DNA. A standard curve was created with a low and a high amount, calibrated DNA solutions, provided by the distributor.

1µl of sample or 10µl of standard reagent was mixed with 199µl or 190µl stock solution, respectively. The solution was incubated for 2min at RT that the fluorochrom has time to intercalate with the DNA. After measuring the two standards, the samples were measured and the DNA concentration was assessed.

4.6.4 PCR of the 16S rRNA gene

Material:

Isolated DNA

Dreamtaq Green PCR Mastermix[15] **(**#K1082, storage -20°C)

Nuclease-free Water

Primer 27F[26] (storage -20°C)

Primer 1492R[26] (storage -20°C)

PCR Tubes 0.2ml[14]

Biometra Tadvanced® Thermocycler

Dreamtaq Green Mastermix, Aqua dest., Primer 27F (AGA GTT TGA TCA TGG CTC A) and Primer 1492R (TAC GGT TAC CTT GTT ACG ACT T) were pipetted together according to the amounts in table 8. DNA was added depending on the measured concentration by NanoDrop.

Reagent	<20ng/µl	>20ng/µl
Dreamtaq Green Mastermix	20µl	20µl
Nuclease-free water	8µl	13µl
Primer 27F	1µl	1µl
Primer 1492R	1µl	1µl
=Volume each	30µl	35µl
+ DNA sample	10µl	5µl
=Total volume	40µl	40µl

Table 8: Mastermix of the PCR reaction for amplification of the 16S rRNA gene

30µl or 35µl of the Mastermix were pipetted into 0.2ml PCR tubes. 10µl or 5µl of DNA was added, respectively. Nuclease-free water was used as a negative control. The cups were brought into a thermocycler. The program parameters can be seen in table 9.

Step	T (°C)	Time (s)	Cycles
Initial denaturation	95	180	1
Denaturation	95	20	30
Annealing	55	30	30
Elongation	72	90	30 (go back to denaturation)
Final elongation	72	120	1
Holding	4	∞	∞

Table 9: Thermocycler conditions for amplification of the 16S rRNA gene

4.6.5 Gel electrophoresis

Material:

PCR Products

TAE Buffer Solution[5] (storage RT)

Agarose[14] (storage RT)

GelRed®[4] (storage 4°C)

100bp Ladder[15] (storage -20°C)

Nuclease-free Water

Gel Electrophoresis Chamber

Biometra Standard Power Pack P25T

UVP GelStudio SA

After PCR amplification, the PCR products were detected by gel electrophoresis. Therefore, a 1.5% agarose gel was poured by dissolving 1.8g agarose in 120ml TAE buffer by heating in the microwave and careful shaking. 2µl of 10,000x GelRed® was added after cooking. A gel electrophoresis chamber was filled with TAE buffer to the filling line and 4µl of a 100bp ladder or 4µl of the samples were pipetted into the wells, respectively. The gel was running for approx. 20min at 110V and 220mA. The bands were detected using an UVP GelStudio SA (Analytik Jena) with UV light.

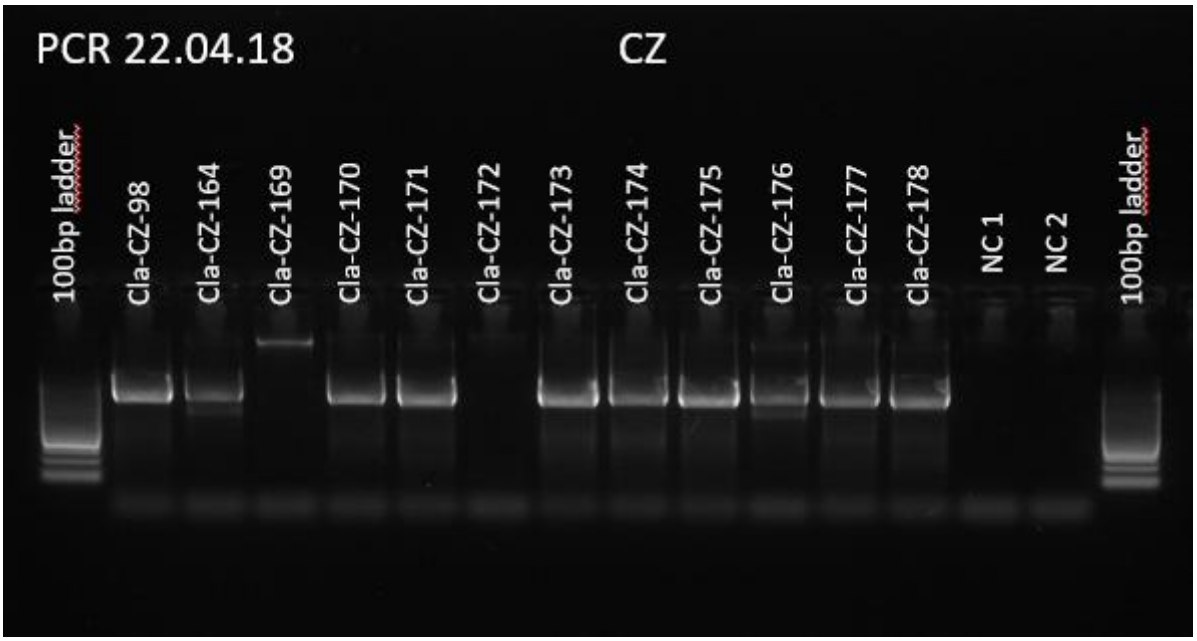

Figure 7: Gel picture with positive and negative 16S rRNA gene amplicons

Cla-CZ-169 only shows a genomic band but no 16S rRNA gene band. Cla-CZ-172 is also negative for 16S rRNA gene. All other samples are positive and are used for further processing.

4.6.6 PCR product clean-up

Material:

PCR Products

MSB® Spin PCRapace Kit[11]

Nuclease-free Water

Heraeus™Fresco™ Centrifuge

After successful detection of PCR products, the positive samples were further purified using a MSB® Spin PCRapace kit. 200µl of binding buffer was added to the PCR product and the whole solution was transferred to a silica filter column. After a 1min incubation step at room temperature, the columns were centrifuged for 4min at 15,000g. The filter columns were put into a new 1.5ml tube and 30µl of nuclease-free water was pipetted directly into the center of the silica filter. After another incubation step of 1min at room temperature, the columns were

centrifuged for 1min at 11,000g. The concentration of the purified PCR product was measured by NanoDrop described under 4.6.2 and the concentration was diluted to 50-70ng/µl with nuclease-free water. PCR products were sent to GATC sequencing service, later Eurofins Genomics sequencing service. Sanger technique was used for sequencing of the PCR amplicons with a 27F primer in forward direction.

4.6.7 Bioinformatic analysis of sequencing results

Programs:

MEGA 7

EzBioCloud

AB1 files were opened with MEGA 7 (187) trace editor and checked for noise and artefacts. The beginning and the end, where the sequencing results are usually of bad quality, were removed. This led to an evaluable average sequence length of 850bp. The fragment was entered into www.ezbiocloud.net (49). If a bacterium was new to the collection or represented a potentially new taxon, the strain was sequenced using primers 1492R, 785F and 338R. The thresholds for a new species were 98.7%, for a new genus 94.5% and for a new family 86.5% of sequence similarity to the next validly placed taxon (36).

Primer	Sequence
27F	AGAGTTTGATCATGGCTCA
1492R	TACGGTTACCTTGTTACGACTT
785F	GGATTAGATACCCTGGTAGTC
338R	GCTGCCTCCCGTAGGAGT

Table 10: Primers used for sequencing the full-length of the 16S rRNA gene

The sequences were again cut at the beginning and the end and then joined together with the MEGA 7 alignment explorer. The full-length sequence, containing approximately 1500bp, was added to the 16S-based ID pipeline of www.ezbiocloud.net to obtain a more reliable identification of the bacterial isolates.

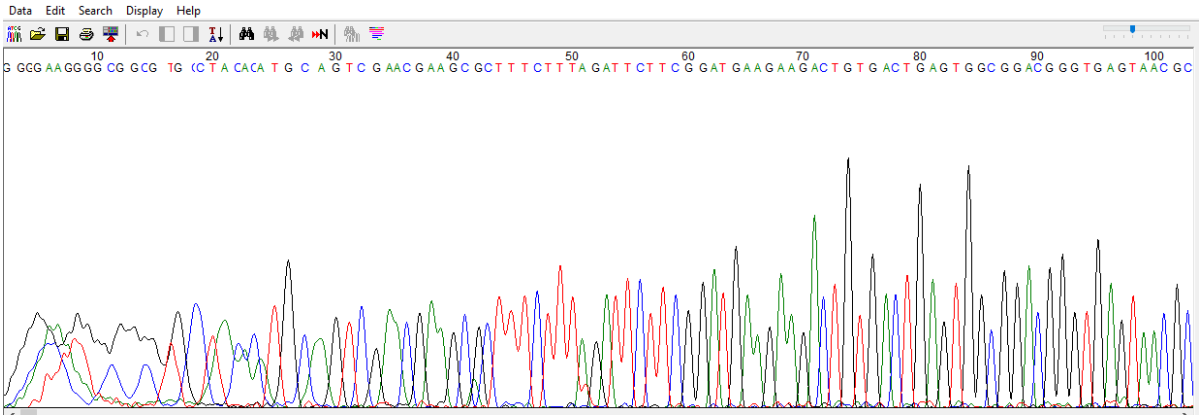

Figure 8: AB1 File in the MEGA 7 trace editor

The AB1 file visualizes results from sanger sequencing. The sequence was screened for background noises. They usually appear in case of contamination. The beginning and the end were cut out due to technical quality issues.

4.7 RNA preparation

4.7.1 Sample preparation

Material:

PeqGOLD Trifast[16] (Trizol, storage 4°C)

Screw-Cap Tubes with Ceramics Beads[16]

Petri Dishes 60mm[15]

Scalpel Blades[14]

Mettler AE 100 Analytical Scale

Precellys® Homogenizer

Screw-Cap tubes were prepared by filling them with 0.6g ceramics beads under a PCR workstation before starting the homogenization. A new petri dish was put on the scale and was tared. The sample material was removed from RNAlater, cut in pieces that weight estimated 50-100mg with a sterile scalpel blade and weighted on the analytical scale. If necessary, sample material was again cut to obtain the needed amount of material. Sample material was transferred to screw-cap tubes with ceramics beads and immediately put on ice. Spare material was put back into RNAlater.

After cutting all samples, 1ml of cold peqGOLD Trifast reagent was added to each tube. The tubes were put in a Precellys Homogenizer. Parameters were set to 1x6,500xg for 30s. After the homogenization step, samples were directly put on ice. If an insufficient homogenization occurred, the samples were again homogenized with the same parameters. Samples were directly used for RNA isolation.

4.7.2 RNA isolation

Material:

Chloroform[1] (storage RT)

Isopropanol 100%[14] (storage RT)

Ethanol Absolute[16] (storage RT)

Nuclease-free Water

Aqua Dest.

Eppendorf Centrifuge 5415R

TS-100 Thermo Shaker

A centrifuge was cooled down to 6°C. A 75% EtOH solution was prepared by mixing 30ml of EtOH absolute with 10ml Aqua dest.. 200µl Chloroform and 500µl Isopropanol per sample were aliquoted into Falcon tubes, respectively. 200µl Chloroform was added to each screw-cap tube with sample and Trizol reagent. Tubes were sealed tightly and mixed by fierce shaking for approx. 15s. After a 5min incubation step at RT, tubes were centrifuged at 12,000xg for 15min at 6°C. During that time, new 2ml tubes were prepared and filled with 500µl Isopropanol. Instantly after the centrifugation was completed, 500µl of clear supernatant was transferred to the tubes with Isopropanol. Tubes were mixed cautiously by inverting and were incubated for 10min at RT. After another centrifugation step at 12,000xg for 10min at 6°C, the supernatant was poured off, the tubes were turned around and shortly put on a clean tissue to remove residual liquid but not affecting the RNA pellet. 1ml of EtOH 75% was added and the tubes were centrifuged at 7,500xg for 5min at 6°C. The supernatant was again removed the way described above. Tubes were again centrifuged shortly and residual liquid was removed with a 100µl pipette. Open tubes were left under a laminar flow for approx. 20min to evaporate residual EtOH. Depending on the size of the pellet, 50-100µl nuclease-free water was used to completely dissolve the RNA pellet. This was obtained by carefully pipetting up and down the liquid with the RNA. Dissolved RNA was incubated for 10min at RT and subsequently for 10min at 58°C on a thermo shaker at 300rpm. Isolated RNA was stored at -80°C.

4.7.3 Contamination control by NanoDrop 1000

Isolated RNA was measured by NanoDrop 1000. 1µl of nuclease-free water was pipetted on the sample plate as blank. 1µl of sample was used for measuring the RNA. 260/280-ratio and 260/230-ratio were used to assess the occurrence of contaminations from proteins, phenols or chaotropic salts. RNA was considered uncontaminated if both ratios exceeded 1.8. In cases where these requirements were met, the RNA quality was further checked in an Agilent 2100 Bioanalyzer. Otherwise, RNA was precipitated from the contaminated solution as described under 4.7.4.

4.7.4 Clearance of RNA contaminations

Material:

Isopropanol 100%[14] (storage RT)
Sodium Acetate 3M[1] (storage RT)
Ethanol[16] **75%**
Nuclease-free Water
Eppendorf Centrifuge 5415R

All steps were performed on ice. Contaminated RNA solution was mixed with the equal volume of Isopropanol and 1/10 volume of sodium acetate 3M. Tubes were inverted and incubated for 10min at RT. After centrifugation at 12.000xg for 10min at 6°C, the supernatant was discarded and 1ml of 75% EtOH was added to each sample. Samples were centrifuged at 7.600xg for 5min at 6°C. The supernatant was discarded. Samples were again shortly centrifuged and the residual EtOH was removed with a pipette. The RNA pellet was dried for approx. 30min with open caps to let EtOH evaporate. Depending on the expected yield of RNA, the pellet was dissolved in 20-100µl nuclease-free water and the samples were again checked for contamination with NanoDrop (see 4.7.3).

4.7.5 Quality control by Agilent 2100 Bioanalyzer

Material:

Isolated RNA
Nuclease-free Water
RNAse ZAP™ [15] (storage RT)
RNA 6000 Nano Kit[27] (#5067-1511, storage 4°C)
Agilent 2100 Bioanalyzer
TS-100 Thermo Shaker
Vortex Mixer IKA MS3

All reagents were brought to RT for 30min before use. A thermo shaker was set to 70°C. 550µl of RNA Nano Gelmatrix were pipetted to a spin filter and centrifuged for 10min at 1,500xg. 65µl of filtered gel matrix were transferred to a nuclease-free 1.5ml tube. 1µl of dye was added and the gel-dye mix was centrifuged for 10min at 13,000xg. The samples were incubated for 2min at 70°C in a thermo shaker and then immediately put on ice. The Bioanalyzer electrodes were cleaned by pipetting 350µl of RNAse ZAP™ into a cleaning chip and leaving the chip in the machine for approx. 1min. Afterwards, the electrodes were cleaned two times with nuclease-free water the way described above. A new RNA Nano Chip was added to the priming station and the station was set to position C. 9µl of filtered gel-dye mix were pipetted to the well with a white G on black ground. The priming station was

closed to apply pressure for exactly 30s. After opening the station, 9µl of gel-dye mix was added to the two wells with a black G on bright ground. 5µl of RNA 6000 Nano marker was pipetted to each sample well and into the well with the ladder symbol. 1µl of each sample or 1µl of Nano ladder was added to the respective wells. The chip was vortexed in a Vortex mixer for 1min and then transferred to the Bioanalyzer. The program “Agilent 2100 Expert” was started, the samples were labeled and the Bioanalyzer run was started.

With the Agilent 2100 Bioanalyzer, it was possible to evaluate the quality of RNA samples. RNAses, shear forces, heat or contaminations with DNA, can damage the RNA and therefore lower its integrity. RNA molecules were separated by size in a chip-based capillary electrophoresis. The software calculated the RNA integrity number (RIN) from the created electropherogram, especially the 18S and 28S rRNA peaks and their relation to each other.

Usually, the RIN cutoff for sequencing experiments was set to >8. However, in our experiment, we also included three samples with a marginally lower RIN of 7.7 (1x) and 7.9 (2x). The cutoff for qPCR experiments was set to >7.

4.8 Real-time RT-PCR

4.8.1 DNAse digest

Material:

Isolated RNA

10x Reaction Buffer with MgCl[28] (component of #A5001, storage -20°C)

Nuclease-free Water

DNase, RNase-free[15] (#EN0521, 1U/µl, storage .20°C)

EDTA 50mM[15] (storage -20°C)

PCR Tubes 0.2ml[14]

BioRad T100™ Thermal Cycler

All reagents were mixed according to table 11. The volume of RNA used in the DNAse digest was calculated from the NanoDrop measurements.

Reagent	Amount for 1x
RNA	1µg
10X reaction buffer with MgCl	1µl
DNase, RNase-free (1U/µl)	1µl
Nuclease-free water	Ad 9µl

Table 11: Reagents for the DNase digest of sample RNA

Reaction mixtures were incubated for 10min at 37°C in a thermocycler and directly put on ice. 0.5µl 50mM EDTA was added to each reaction. Afterwards, samples were again incubated for 10min at 65°C in a thermocycler. The DNase digested RNA was used instantly in the reverse transcription.

4.8.2 Reverse Transcription

Material:

DNase digested RNA

GoScript™ Reverse Transcription System[28] (#A5001, storage -20°C)

Nuclease-free Water

BioRad T100™ Thermal Cycler

4.4µl of DNase digested RNA was mixed with 1µl Random Hexamer Primers (100pmol) and 4.6µl nuclease-free water. The reaction mixtures were incubated for 5min at 70°C in a thermocycler and then put on ice for 5min.

A Mastermix was prepared on ice according to the amounts in table 12.

Reagent	Amount for 1x
Nuclease-free water	2µl
GO Script 5x Reaction buffer	4µl
MgCl (25mM)	2µl
PCR nucleotide mix (0.5mM each final conc.)	0.5µl
RNasin Ribonuclease inhibitor	0.5µl
GO Script Reverse Transcriptase	1µl
Total	**10µl**

Table 12: Reagents for the reverse transcription of sample RNA

10µl of the Mastermix was added to each sample of RNA-hexamer-mix. Sample tubes were vortexed shortly and spun in a micro centrifuge. The following steps were performed in a programmed thermocycler:

5min at 25°C

60min at 42°C

15min at 70°C

After the thermocycler run, samples were directly put on ice and incubated for at least 5min. The produced complementary DNA (cDNA) was stored at -20°C or used immediately in a qPCR reaction.

4.8.3 Real-time RT PCR

Material:

cDNA

GoTaq® qPCR Master Mix[28] (#A6001, storage -20°C)

Nuclease-free Water

100X CXR Reference Dye[28] (#C5411, storage -20°C)

qPCR Primers[8 26] (storage -20°C)

96-well qPCR Plate[14]

Sealing Film for qPCR Plates[14]

Applied Biosystems 7300 Real Time PCR System

The real-time RT-PCR (qPCR) approach measures the relative amount of mRNA copies of a gene of interest. The principle is equal to an end point RT-PCR reaction: Using specific primers, the sequence of interest is copied by a polymerase throughout a number of amplification cycles. The used qPCR primers and their respective sequences can be found in table 13. For IL-21 and IgJ, Qiagen quantitect primer assays were used and therefore, no primer sequences are available. The primer assays can be ordered on the internet platform GeneGlobe provided by Qiagen under catalogue number QT00596064 for IgJ (Gg_IGJ_1_SG QuantiTect Primer Assay) and catalogue number QT00713342 for IL-21 (Gg_IL21_1_SG QuantiTect Primer Assay), respectively. cDNA was diluted 1:10 with nuclease-free water to obtain a final concentration of 40ng/µl.

Gene	Primer forward sequence	Primer reverse sequence	Annealing T (°C)
18S rRNA	CATGTCTAAGTACACACGGGCGGTA	GGCGCTCGTCGGCATGTATTA	59
IgA	CGCCCCTTCCGTCTACGT	CGAAATCGGTTGGTTTTGTTG	59
AID	CGTCTGAAACCCAGCAAGAGT	TGTCCATGTCAGCTGGGTTCT	59
IL-6	GCTTCGACGAGGAGAAATGC	GCCAGGTGCTTTGTGCTGTA	59
IgY	TGGAGGGAAGGGAAGAGTTACAG	TCCGGGCATCCCTTGAC	56
IgJ	Not available	Not available	56
IL-21	Not available	Not available	56

Table 13: Primers used in quantitative real-time PCR

A Mastermix was prepared according to the volumes in table 14.

Reagent	Amount for 1x
Primer forward	1.5µl
Primer reverse	1.5µl
Nuclease-free water	4.25µl
GoTaq Mastermix	12.5µl
100X CXR Reference Dye	0.25µl
cDNA	5µl
Total	**25µl**

Table 14: Reagents for preparing a Mastermix for qPCR reaction

Components 1-5 were mixed and 20µl was distributed on the 96-well plate. 5µl of diluted sample cDNA was added to each well in duplicate. Each well was cautiously mixed by pipetting up and down several times. The plate was sealed with a sealing film and shorty spun to remove air bubbles. The qPCR cycler was programmed as follows:

Step	Function	T	No of cycles	time
Initial activation		95°C	1x	2min
Amplification	Denaturation	95°C	40x	15s
	Annealing	56/59°C		30s
	Extension	72°C		30s
Melting curve		95°C	1x	15s
		57°C		30s
		95°C		15s

Table 15: Settings of the quantitative real-time PCR

As opposed to end point RT-PCR, the amount of amplified DNA in qPCR is not assessed at the end of the last cycle, but in every single cycle. The approach facilitates relative quantification of the original mRNA copy number in each sample. For the assessment of the amount of amplified DNA, a fluorescent dye (SYBR Green) is added to the reaction mix. SYBR Green is intercalating with DNA double strands. Intercalation induces fluorescence, which is measured by the real-time thermocycler. The intensity of the fluorescent signal is hence directly proportional to the total amount of PCR product present in the reaction mix at any time point and an amplification curve can be plotted over time. A threshold for the measured fluorescence is set within the logarithmic phase of the amplification curve. The cycle, in which this threshold is exceeded (Cycle Threshold, CT), is used for subsequent data analysis. Throughout this study, the mean CT of duplicates was calculated for each sample. Subsequently, the housekeeping gene 18S rRNA was used for normalization of raw CT values for each target

gene, eliminating errors caused by unequal original RNA input. This was done by subtracting the CT value of 18S rRNA from the CT value of the target gene for each respective sample:

$$\Delta CT = CT_{(target\ gene)} - CT_{(housekeeping\ gene)}$$

The ΔCT was further subtracted from the total amount of amplification cycles to obtain a value that is directly proportional to the expression (40-ΔCT).

4.9 RNA sequencing

RNA sequencing was performed at the Center for Translational Cancer Research, TranslaTUM, Technical University of Munich.

Library preparation for bulk 3'-sequencing of poly(A)-RNA was done as described previously by Parekh et al. (188). Briefly, barcoded cDNA of each sample was generated with a Maxima RT polymerase (Thermo Fisher) using oligo-dT primer containing barcodes, unique molecular identifiers (UMIs) and an adapter. 5' ends of the cDNAs were extended by a template switch oligo (TSO) and after pooling of all samples full-length cDNA was amplified with primers binding to the TSO-site and the adapter. cDNA was fragmented and TruSeq-Adapters ligated with the NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina® and 3'-end-fragments were finally amplified using primers with Illumina P5 and P7 overhangs. In comparison to Parekh et al. the P5 and P7 sites were exchanged to allow sequencing of the cDNA in read1 and barcodes and UMIs in read2 to achieve a better cluster recognition. The library was sequenced on a NextSeq 500 (Illumina®) with 65 cycles for the cDNA in read1 and 16 cycles for the barcodes and UMIs in read2.

Data was processed using the published Drop-seq pipeline (v1.0) to generate sample- and gene-wise UMI tables (189). Reference chicken genome GRCg6a (Gen Bank Accession: GCA_000002315.5) was used for alignment. Transcript and gene definitions were used according to the ENSEMBL annotation release 98. A "differences in gene expression" (DGE) matrix was provided for further analysis.

4.9.1 Data analysis

Programs:

Apache OpenOffice Calc 4.1.6

R Version 3.5.2

R Studio 1.1.463

R Pipeline DEBrowser

Single samples were extracted from the DGE matrix using the Apache OpenOffice Calc. OpenOffice has some advantages for bioinformatics analysis compared to Microsoft Excel. It can easily deal with tab-delimited files and does not automatically change values to dates. Single tab-delimited files were generated for each sample. They were afterwards joined together, dependent on the addressed question and which samples needed to be compared.

The DEBrowser application is designed to analyze count data from sequencing experiments. It includes all features from filtering, normalization, batch effect correction, principal component analysis, differential expression analysis, gene ontology and pathway discovery. It therefore makes use of a variety of different R packages (190). The program displays an interactive web-based graphical user interface based on R's shiny package (191).

4.9.1.1 Filtering and Normalization

Filtering helped removing genes that have low expression and low coverage. Additionally, it increased the speed and accuracy of DE algorithms. The user can choose from different filtering criteria: Minimum signal in at least one samples, minimum average signal across all samples or minimum signal in at least n samples. Filtering was set to mean with a minimum read count of 2, meaning all genes that have an average read count below 2 reads across all samples are filtered out. Batch effect correction was not performed because all samples were run in the same sequencing run. A median ratio normalization was conducted by DESeq2 package (192). The provided principal component analysis (PCA) helped detecting outliers and uncovering the variability between the samples.

4.9.1.2 Differences in gene expression

DESeq2 analyzed the counts per gene, e.g. the total number of reads that were mapped to a unique gene. Therefore, a DGE matrix with the samples in need of comparison was uploaded to DEBrowser. The respective samples were assigned to their group and the DE algorithm was started. The generated result tables expressed '*p-value'*, 'adjusted *p-value'*, 'fold change', 'log2 fold change' and 'log10 adjusted *p-value*' for each gene. For this analysis, an adjusted *p-value* <0.01 or a fold change >2 was used to detect most significantly and most differentially expressed genes. Information about how the result tables were calculated can be found in the original publication (192).

4.9.2 GO Enrichment Analysis

A list of upregulated genes in the respective organs and groups was uploaded to the web-based platform Geneontology (GO, www.geneontology.org) to perform a GO enrichment analysis with the PANTHER classification system (193). Statistical tests on the occurrence of certain genes were performed and classified to their respective GO term. The gene lists were screened against all chicken genes in the dataset and “biological process” was selected as screening criterion

5 Results

This study aimed to investigate how the gut microbiome of chickens influences the mucosal immune system development and immunological responses. First, microbial profiles were assessed in different gut regions. Second, shifts in gut microbial profiles were put in context of differences in host gene expression and differences in immunoglobulin levels. A second animal experiment validated the results from the first experiment. Third, a chicken intestinal bacterial collection (ChiBac) was established and used to design a minimal bacterial consortium. Finally, this bacterial cocktail was used for colonization of chickens.

5.1 Comparison of intestinal regions

16S rRNA gene amplicon sequencing was performed to gain a better understanding of microbial profiles in different gut regions. Eight healthy M-11-layer type chickens were sacrificed at the age of 90 days. Intestinal content of jejunum, caecum and colon was collected from each bird. Amplicon sequencing delivered a total of 201,069 high-quality reads (8,378 ± 3,645 reads/sample) representing 298 OTUs (88 ± 71 OTUs/sample).

Jejunal samples had the lowest bacterial diversity with only 30 observed species on average followed by colonic sample with an average of 61 observed species. For colonic samples, two outliers were detected that had a much higher richness with 138 and 139 observed species. Without these two samples, a lower average richness of 35 species was observed in the colon. Caecal samples had the highest richness with 174 observed species on average (figure 9, A). The distribution of Shannon effective counts, which take the evenness of populations into account, was similar to richness (figure 9, B). The phylogram and MDS plot illustrated distances between the samples. The two outliers were closer to the caecal samples and had a similar composition and richness. The MDS plot also indicated how similar the groups are to each other. Jejunum and colon were closer together and formed a mixed cluster of both groups. Caecum was far away from the other groups and formed its own cluster (figure 9, C+D).

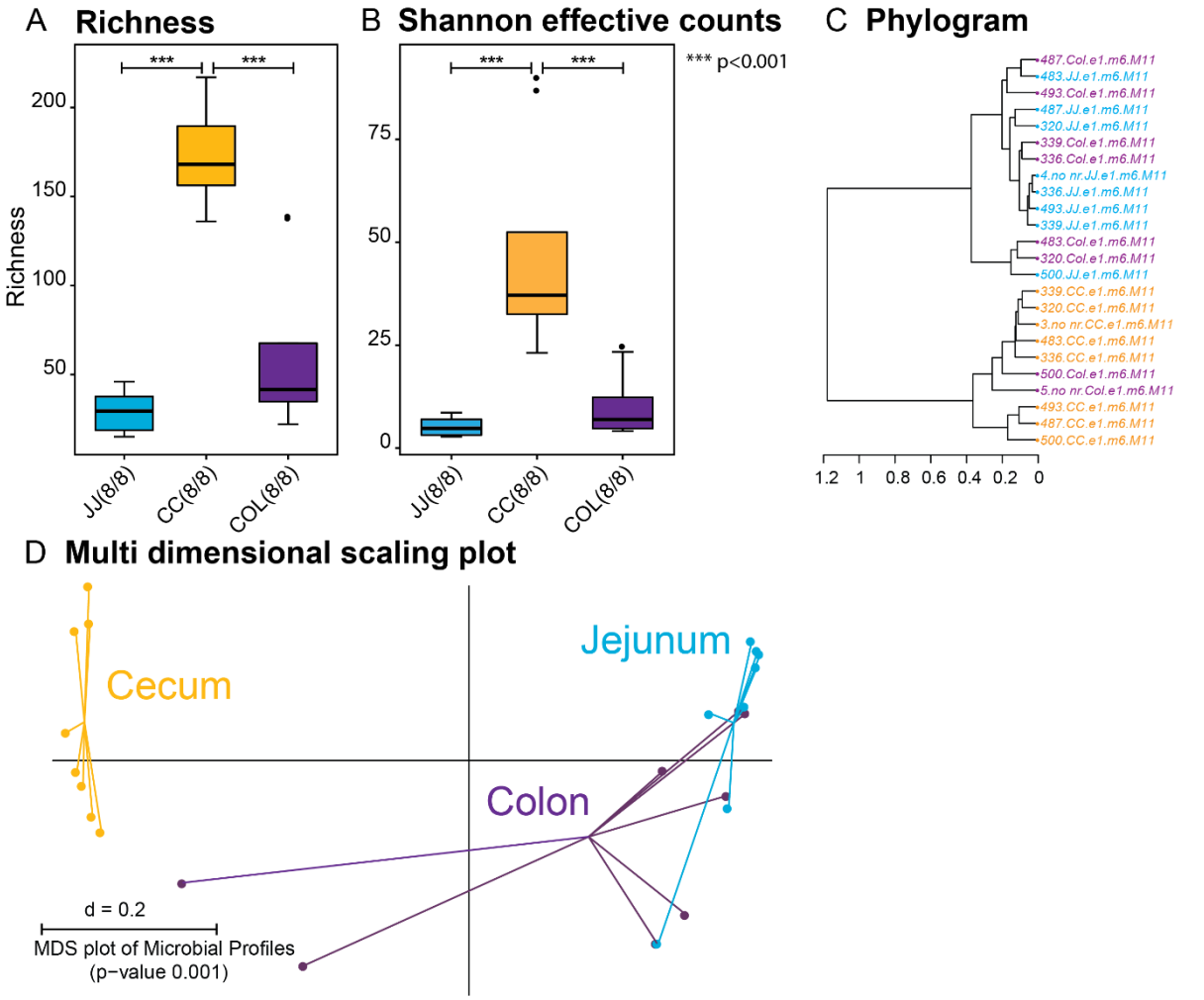

Figure 9: Microbial diversity of different gut regions

A: *Alpha*-diversity shown as Richness or B: Shannon effective counts. C: The phylogram shows each sample and their distance to each other. D: The MDS plot of microbial profiles shows the distance between groups. d=0.2 means samples are 20% distinct from each other. Each dot represents a single sample. Statistics were performed with Wilcoxon Rank Sum pairwise test. JJ: Jejunum; CC: Caecum; COL: Colon in A and B.

The evaluation of data already at the phylum level revealed interesting findings. *Firmicutes* dominated both the jejunum and the colon while *Bacteroidetes* and *Proteobacteria* occurred at higher relative abundances within the caecum (figure 10). The analysis at family level showed that mostly *Lactobacillaceae* inhabited the jejunum. Colon samples had additional *Ruminococcaceae* and *Peptostreptococcaceae* within the *Firmicutes* phylum compared to jejunum. OTU level analysis was performed to gain closer insights into the different gut regions. Most of the molecular species found in the jejunum and colon were also found in the caecum, but in lower relative abundances. The most abundant species in jejunum and colon were: *Romboutsia timoensis, Lactobacillus aviarius, Lactobacillus hayakitensis, Lactobacillus salivarius, Lactobacillus reuteri* and *Lactobacillus colehominis.* Caecum-specific molecular

species were only detected in a few jejunum and colon samples at low relative abundances (<0.8%, figure 11). The data showed that the caecum is the most diverse gut region both phylogenetically and in terms of molecular species detected.

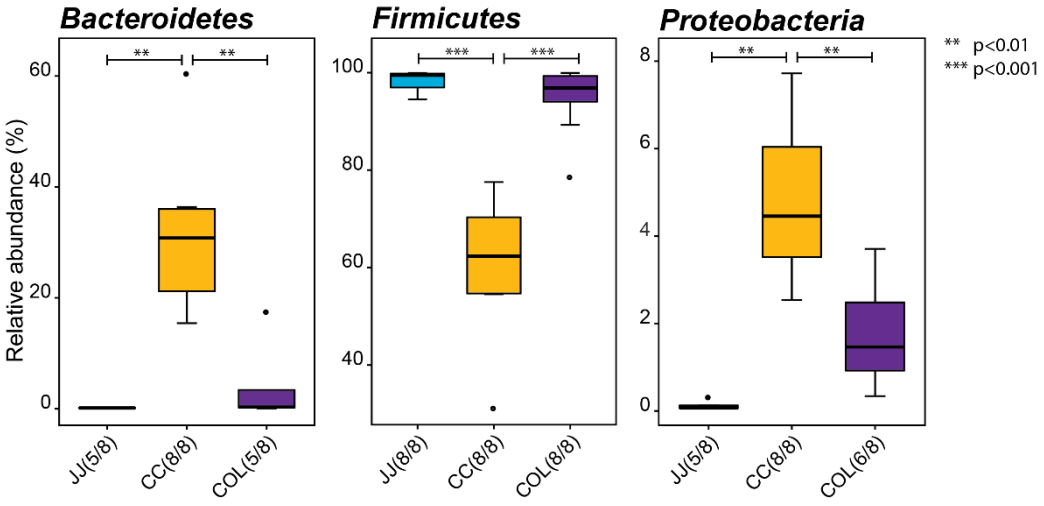

Figure 10: Bacterial composition of different gut regions at the phylum level

Only significant phyla are shown. All statistics were performed with Wilcoxon Rank Sum pairwise test. Two asterisks indicate a *p-value* <0.01. Three asterisks indicate a *p-value* <0.001.

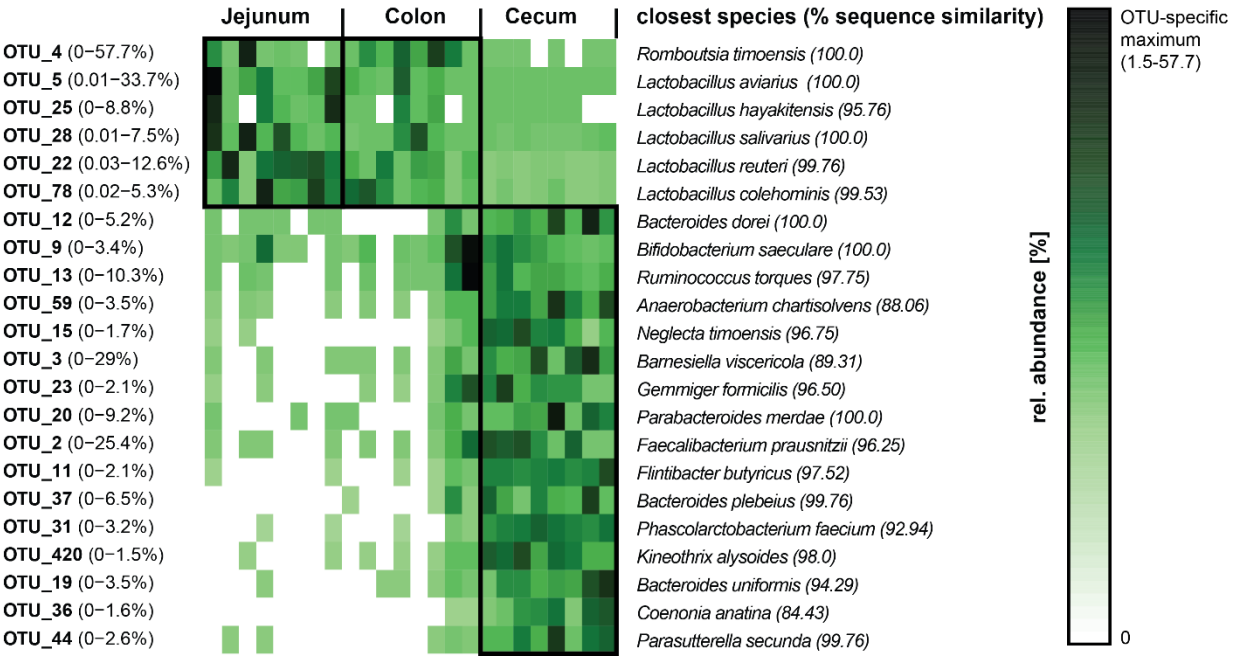

Figure 11: Heatmap of intestinal regions at the OTU level

Percentage in brackets next to OTU numbers is the relative abundance range in all samples. OTUs were identified by NCBI BlastN for 16S ribosomal RNA gene sequences. Only significant taxa are shown.

5.2 Animal experiment 1: Proof-of-principle study

The Proof-of-principle study was conducted to evaluate positive effects on the developing immune system by giving MM to hatched chickens. Therefore, immunoglobulin concentrations were measured, host gene expression differences were detected by RNAseq and qPCR and the gut microbiota composition was investigated by 16S rRNA gene amplicon sequencing.

5.2.1 Immunoglobulin levels

Plasma IgA and IgY levels were quantified by ELISA on day 28 and day 58 post hatch in plasma. IgA levels were also evaluated in bile and caecal content after sacrifice. IgA is secreted into the gut lumen and gallbladder via Poly-Ig-receptor mediated transcytosis (26) and plays an important role in the establishment and maintenance of gut microbiota (194). Significant differences between the SPF group, kept at the INRA, and a group that was colonized with MM, kept at LMU, were observed for IgA (figure 12, upper row) and IgY (figure 12, middle row) at both time points in plasma and for IgA in bile and caecal content (figure 12, lower row). IgA levels in the MM group almost doubled from day 28 (mean value 71.4 µg/ml) to day 58 (mean value 133.1 µg/ml) while the rise within SPF group was only about one quarter (mean value 32.9 µg/ml to 43.8 µg/ml, respectively). The same was observed for IgY in MM but IgY also rose drastically in SPF group from day 28 (mean value 147.8 µg/ml) to day 58 (mean value 658.1 µg/ml).

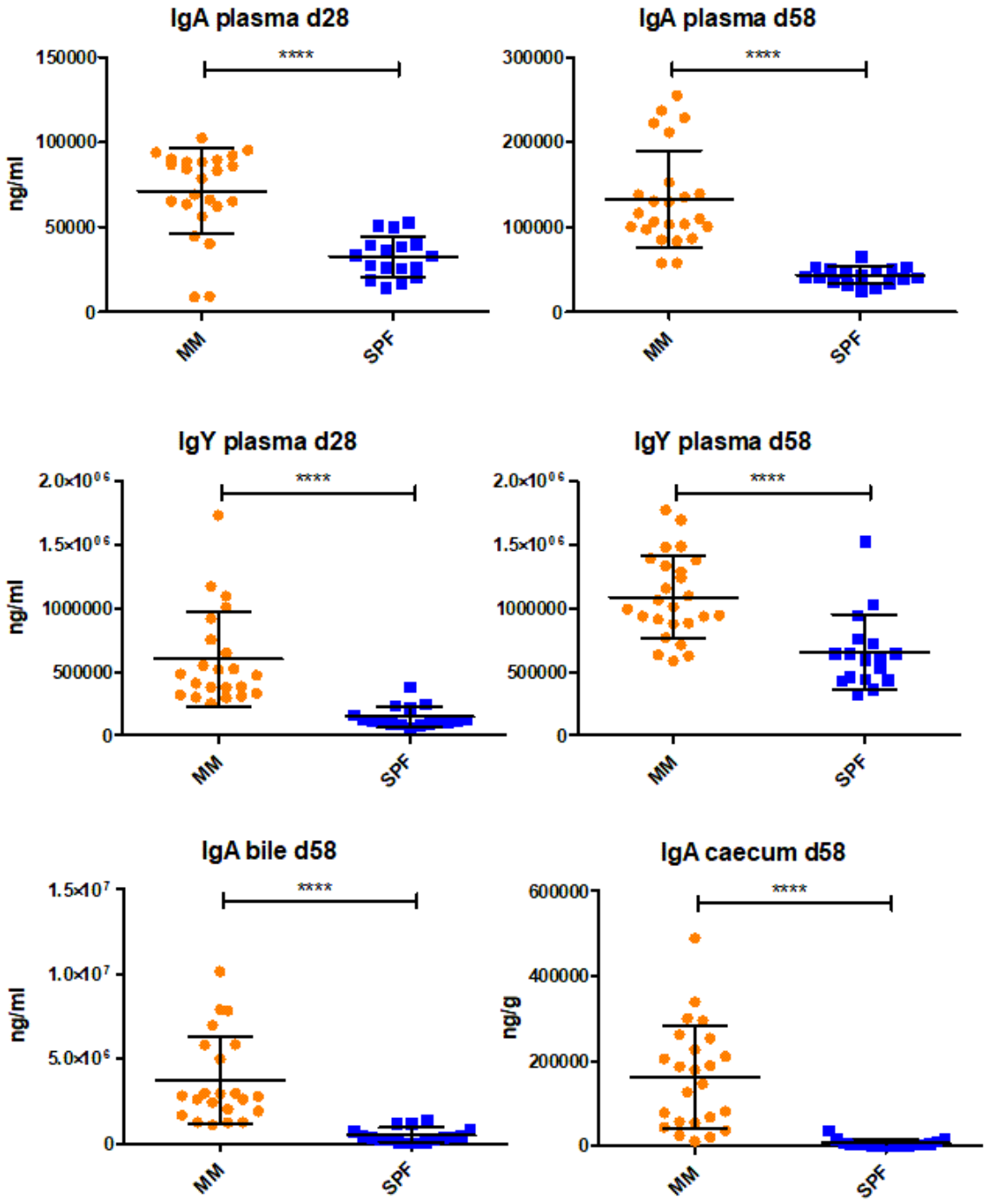

Figure 12: IgA and IgY levels on day 28 and day 58 post hatch

Upper row: IgA in plasma at day 28 and day 58. Middle row: IgY in plasma at day 28 and day 58. Lower row: IgA in bile and in caecum at day 58. MM animals were hosted at LMU; SPF animals were hosted at INRA. Each dot represents a single animal. Middle line is showing the mean value, upper and lower line the standard deviation. Statistics were performed with an unpaired t-test. All results were highly significant (*p-value* <0.0001)

5.2.2 RNAseq

RNA sequencing was performed to unravel differences in gene expression between MM group and SPF group in CT, ileum and spleen. 20,393 genes were represented in the dataset.

Caecal Tonsil (CT)

At first, the expression profile in CT was analyzed. After filtering, 10,774 genes were left for DESeq2 analysis. 6 samples of the MM group were compared with 4 samples of the SPF group. 130 genes were upregulated in the SPF group, where either an adjusted *p-value* below 0.01 was calculated or a fold change of two or higher was assessed. 47 genes were upregulated in the MM group. 10,597 genes were not differentially regulated. Principle component analysis was performed as a quality control and to detect outliers (figure 13, A). No samples were removed from the analysis. The heatmap gives an overview of the most significantly regulated genes (figure 13, B).

The immunologically relevant genes activation-induced cytidine deaminase (AID, AICDA), immunoglobulin variable region (Ig V gene), interleukin 6 receptor (IL6R), immunoglobulin joining chain (JCHAIN), interleukin 21 receptor (IL21R) and the polymeric immunoglobulin receptor (PIGR) were chosen to detect differences in immune status. Significant differences were detected for AID, Ig V gene and JCHAIN. No significant differences could be determined for IL6R, IL21R and PIGR (figure 13, C).

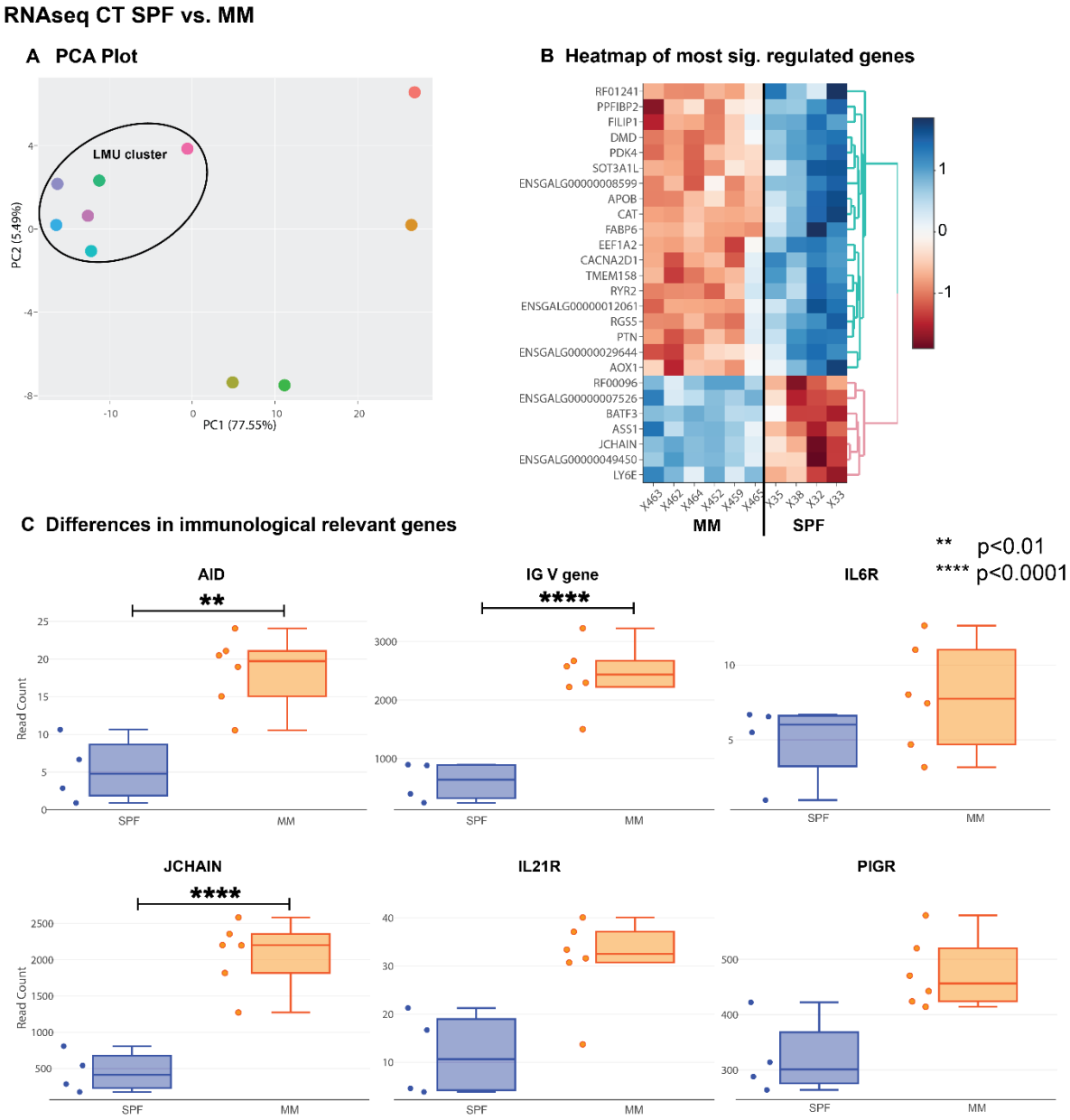

Figure 13: RNAseq in CT

A: Principal component analysis of samples in MM and SPF group. B: Heatmap of most significantly regulated genes. C: Differences in immunologically relevant genes: AID, Ig V gene, IL6R, JCHAIN, IL21R and PIGR. MM animals were hosted at LMU, SPF animals were hosted at INRA

Ileum

After filtering, 10,761 genes were left for DESeq2 analysis. 6 samples of the MM group were compared with 5 samples of the SPF group. 58 genes were upregulated in the SPF group, where either an adjusted *p-value* below 0.01 was calculated or a fold change of two or higher was assessed. 83 genes were upregulated in the MM group. 10,620 genes were not differentially regulated.

Principle component analysis was performed as a quality control and to detect outliers (figure 14, A). No samples were removed from the analysis. The heatmap gives an overview of the most significantly regulated genes (figure 14, B).

The aforementioned genes were also assessed in ileum. After filtering, the AID gene could not be detected anymore. Significant differences were detected in Ig V gene and JCHAIN. No significance was determined for IL6R, IL21R and PIGR, but a strong tendency for IL21R and PIGR is observable (figure 14, C).

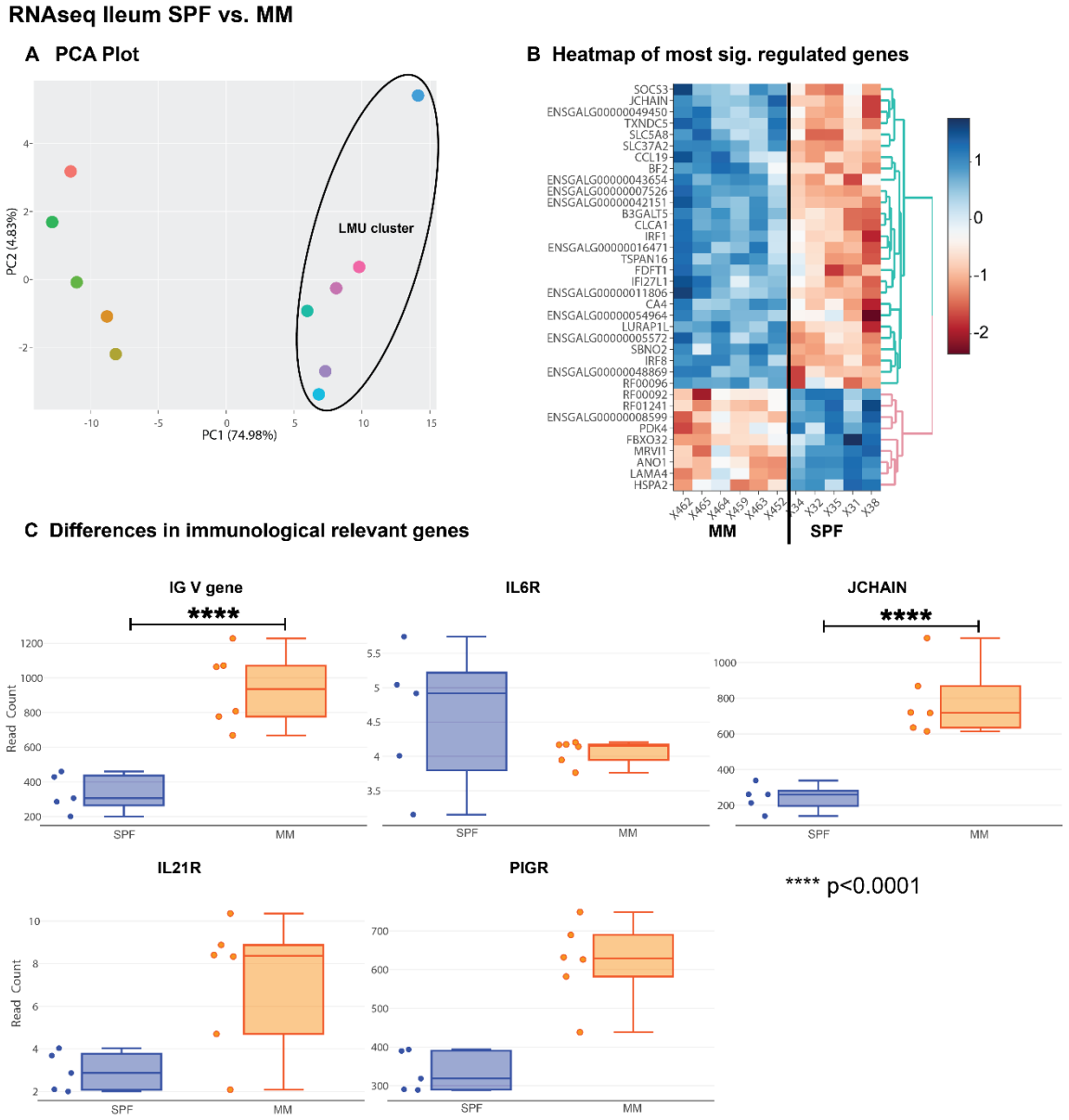

Figure 14: RNAseq in ileum

A: Principal component analysis of samples in MM and SPF group. B: Heatmap of most significantly regulated genes. C: Differences in immunologically relevant genes: Ig V gene, IL6R, JCHAIN, IL21R and PIGR. MM animals were hosted at LMU, SPF animals were hosted at INRA

Spleen

After filtering, 10,662 genes were left for DESeq2 analysis. 5 samples of the MM group were compared with 4 samples of the SPF group. 10 genes were upregulated in the SPF group, where either an adjusted *p-value* below 0.05 was calculated or a fold change of two or higher was assessed. Only one gene was upregulated in the MM group. 10,651 genes were not differentially regulated.

Principle component analysis was performed as a quality control and to detect outliers (figure 15, A). No samples were removed from the analysis. The heatmap gives an overview of all significantly regulated genes (figure 15, B). No significant differences could be detected for the assessed immunologically relevant genes (figure 15, C).

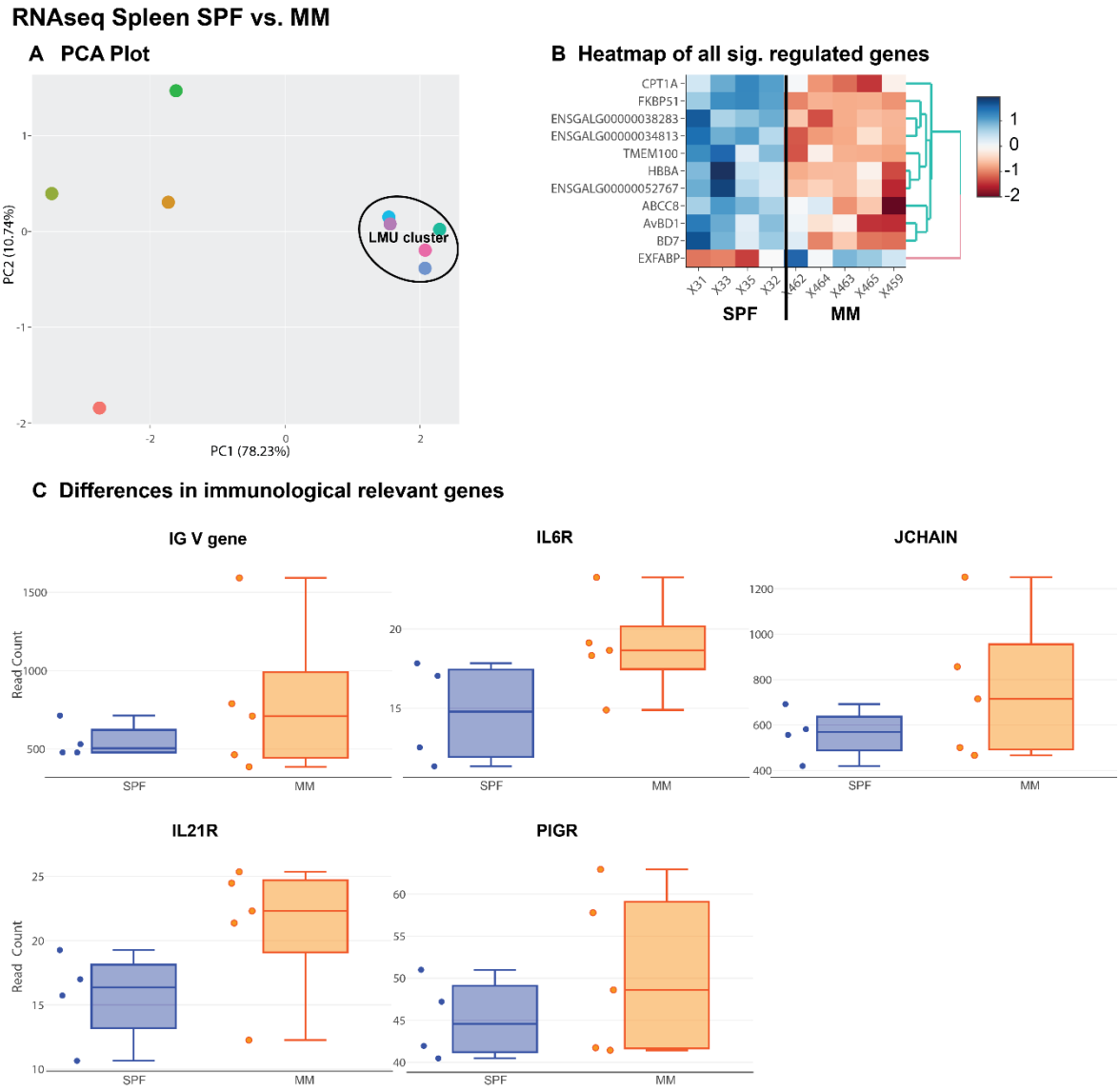

Figure 15: RNAseq in spleen

A: Principal component analysis of samples in MM and SPF group. B: Heatmap of most significantly regulated genes. C: Differences in immunologically relevant genes: Ig V gene, IL6R, JCHAIN, IL21R and PIGR. MM animals were hosted at LMU, SPF animals were hosted at INRA

5.2.3 GO enrichment analysis

GO enrichment analysis was performed to find significant gene clusters that code for the same biological process. First, the 47 upregulated genes in MM were screened. We found five GO terms to be significantly addressed by the gene list (table 16).

GO biological process	GO #	# of genes found	Raw *p-value*
Response to other organism	GO:0051707	9/47	4.09E-06
Response to external biotic stimulus	GO:0043207	9/47	4.22E-06
Response to biotic stimulus	GO:0009607	9/47	5.16E-06
Response to external stimulus	GO:0009605	12/47	1.32E-05
Response to stress	GO:0006950	15/47	1.52E-05

Table 16: Significant GO terms in CT of the MM group

Next, 130 genes that were upregulated in the SPF group were screened for their respective GO terms. Five GO terms were significant throughout the analysis (table 17).

GO biological process	GO #	# of genes found	Raw *p-value*
Fatty acid metabolic process	GO:0006631	8/130	1.18E-05
Monocarboxylic acid metabolic process	GO:0032787	10/130	1.26E-05
Cellular lipid metabolic process	GO:0044255	15/130	4.02E-06
Lipid metabolic process	GO:0006629	16/130	1.33E-05
Oxidation-reduction process	GO:0055114	16/130	1.66E-05

Table 17: Significant GO terms in CT of the SPF group

The analysis of the 83 upregulated genes in the MM group of the ileum revealed 41 significant GO terms. For overview reasons, only the most interesting for our study are shown in table 18.

GO biological process	GO #	# of genes found	Raw *p-value*
Lipid metabolic process	GO:0006629	18/83	4.74E-10
Response to cytokine	GO:0034097	9/83	9.91E-05
Immune response	GO:0006955	15/83	2.95E-10
Immune system process	GO:0002376	18/83	5.18E-08
Defense response to other organism	GO:0098542	11/83	3.18E-08
Response to external biotic stimulus	GO:0043207	16/83	1.61E-10
Defense response to bacterium	GO:0042742	5/83	8.13E-05
Response to bacterium	GO:0009617	9/83	2.67E-06

Table 18: Significant GO terms in ileum of the MM group

The analysis in the SPF group of the ileum revealed no significant GO terms. After exclusion of 7 undefined genes, that were probably not correctly assigned to a GO term, the analysis showed significantly regulated terms for “regulation of biological process”, “animal organ development”, “anatomical structure development” and “multicellular organism development”.

As there were generally only few genes differentially regulated in spleen, no significant GO terms were found in the analysis.

5.2.4 Real-time RT-PCR

Real-time RT-PCR was performed to underline the results from RNAseq analysis and to confirm the results from immunoglobulin measurement. The gene expression of IgA, AID, IL6, IgJ, IL21 and IgY was tested in CT. Significant differences in gene expression were determined for IgA, IL6, IgJ and IgY. No significant differences were detected for AID and IL21, but a tendency is observable (figure 16). The results from IgA and IgY mirror the results from immunoglobulin measurement by ELISA. The IgJ readout confirms the RNAseq results in at least one gene (JCHAIN).

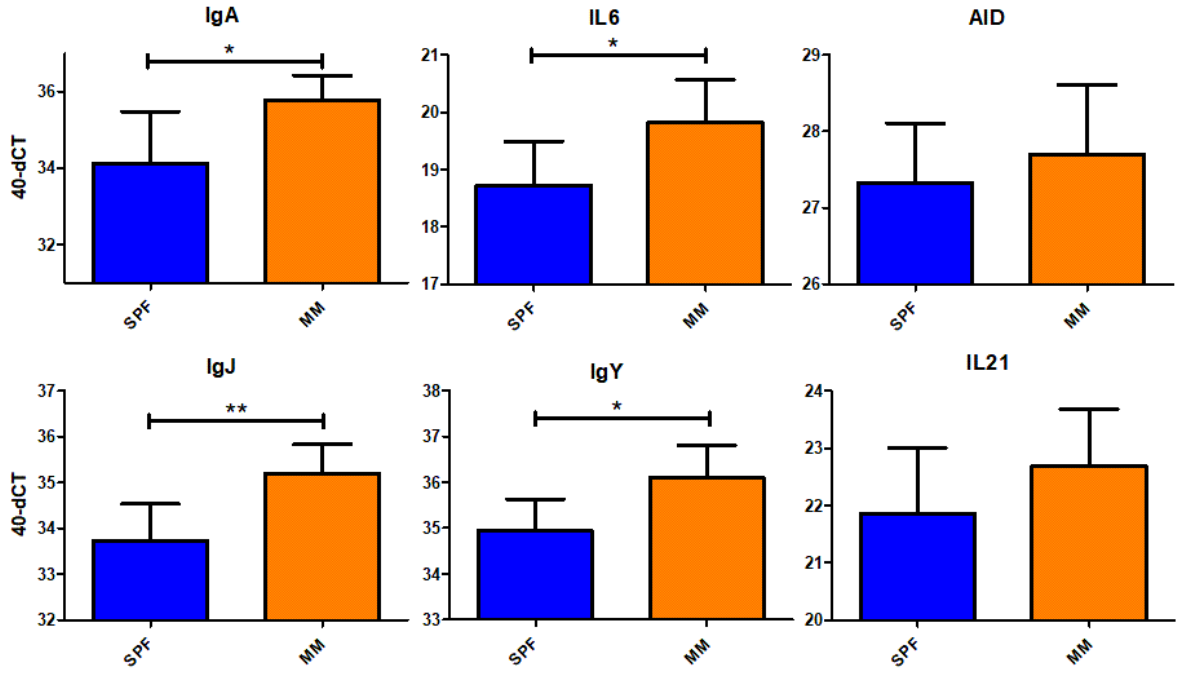

Figure 16: Gene expression in CT measured with qPCR

MM animals were hosted at LMU; SPF animals were hosted at INRA. One asterisk indicates a *p-value* <0.05, two asterisks indicate a *p-value* < 0.01. Statistics were performed by an unpaired t-test

5.2.5 Gut microbiota composition

To investigate associations between the microbiota and altered immunological phenotypes, 16S rRNA gene amplicon sequencing of caecal content was performed on day 58 from animals in the two different groups (n=8/group). Sequencing generated 138,684 high-quality reads (8,668 ± 3,081 reads/sample) representing 270 OTUs (155 ± 59 OTUs/sample). *Alpha*-diversity analysis already revealed outstanding differences:

Richness was 98 ± 6 bacterial species in the SPF group and 211 ± 13 in the MM group (figure 17, A). Shannon effective counts were 25 ± 4 in the SPF group and 71 ± 8 in the MM group (figure 17, B). The MDS plot shows significance (*p-value* = 0.001) in the *beta*-diversity of the two groups, each forming distinct clusters. The SPF group shows a strictly defined cluster while MM group shows a more diverge cluster (figure 17, C).

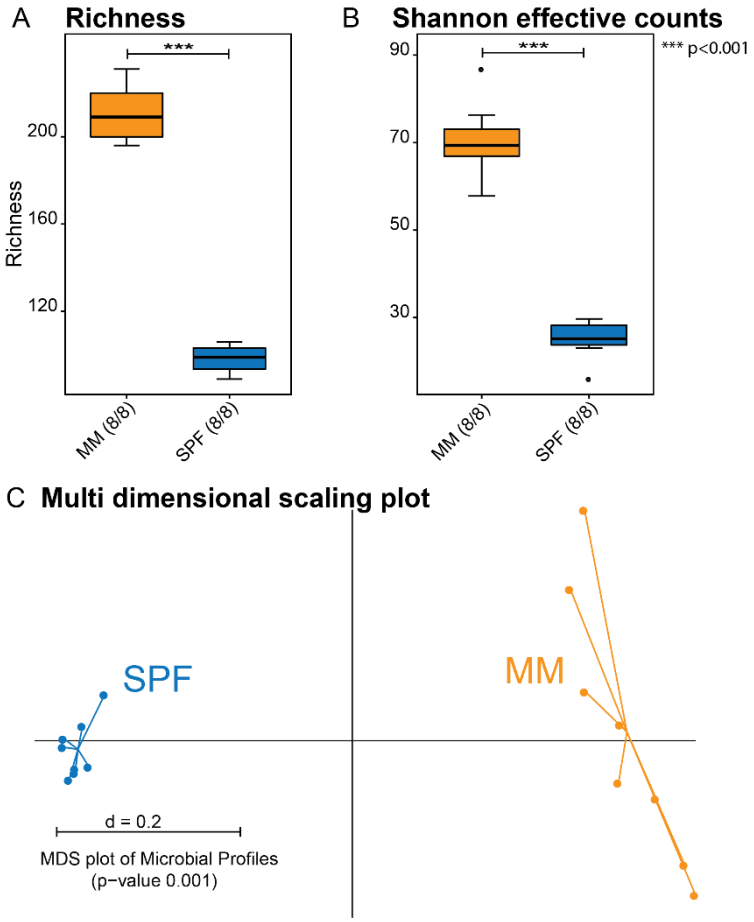

Figure 17: Microbial diversity in the first experiment

A: *Alpha*-diversity shown as richness or B: Shannon effective counts. C: The MDS plot of microbial profiles shows the distance between groups. d=0.2 means samples are 20% distinct from each other. MM animals were hosted at LMU; SPF animals were hosted at INRA Each dot represents a single sample. Statistics were performed with Wilcoxon Rank Sum pairwise test.

The bacterial composition at both the phylum and family level was further investigated. Astonishingly, only *Firmicutes* were found in the SPF group while the MM group consisted of five different phyla (figure 18, A). *Actinobacteria* were not detected in any samples. At the family level, only *Lachnospiraceae* and *Ruminococcaceae,* both belonging to the phylum *Firmicutes,* were present in high relative abundances in the SPF group. *Erysipelotrichaceae* were found in all samples of the SPF group compared to only one sample in MM group (figure 18, B). Additionally, few *Lactobacillaceae* (3/8 observations), unknown *Bacillales* (3/8 observations) and some unknown *Clostridiales* (8/8 observations) were found in the SPF group.

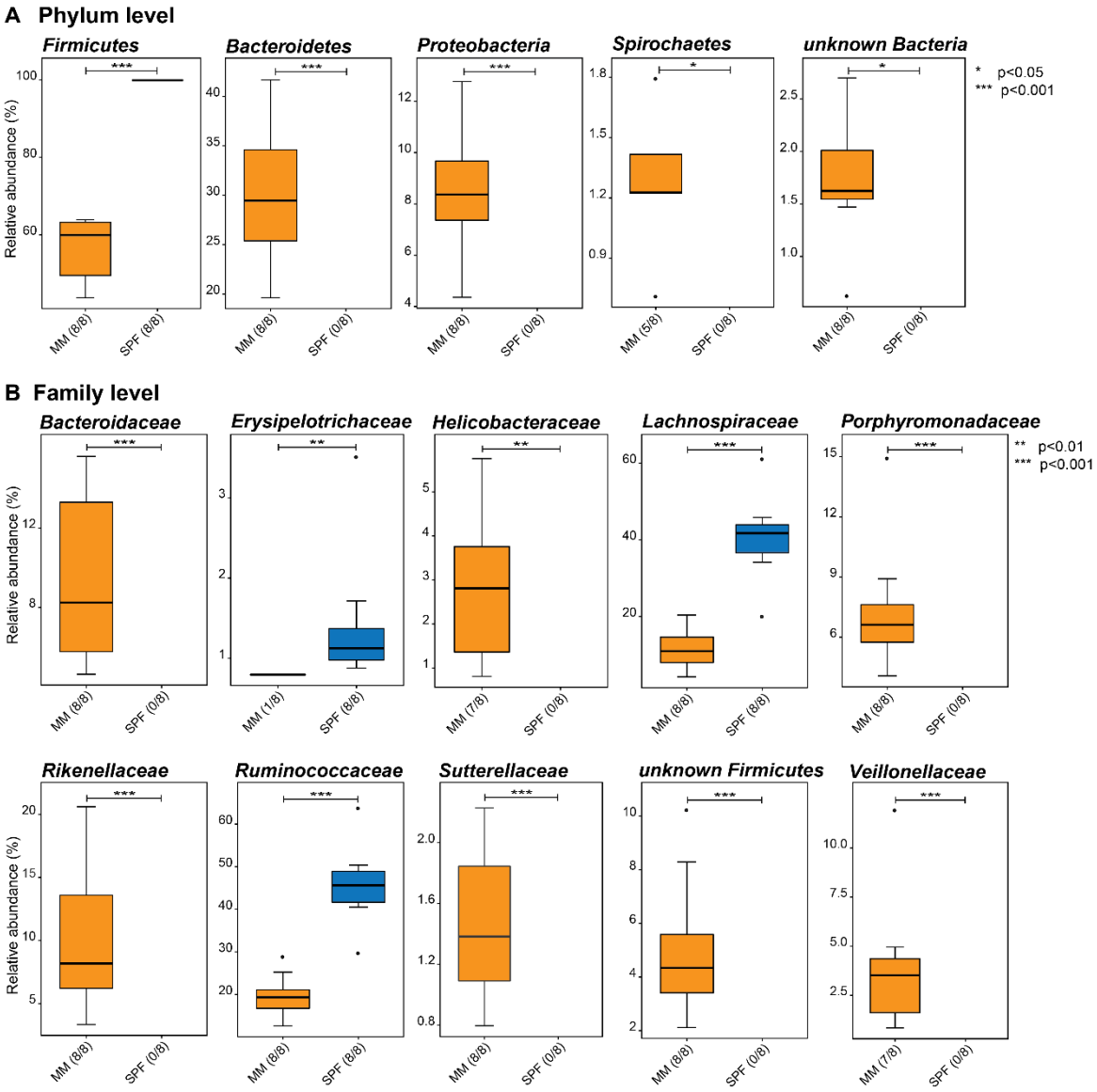

Figure 18: Bacterial composition at the phylum level

A: Bacterial composition at the phylum level or B: at the family level. Statistics for *Firmicutes, Lachnospiraceae* and *Ruminococcaceae* were performed with Wilcoxon Rank Sum pairwise test. The other statistics were performed with Fisher's exact test. Only significant results are shown. One asterisk indicates a *p-value* <0.05, two asterisks indicate a *p-value* <0.01, three asterisks indicate a *p-value* <0.001.

5.3 Animal experiment 2: Validation study

Since the Proof-of-principle study was conducted in two different facilities, the Validation study aimed to find out if the results of the first animal experiment are reproducible. Therefore, plasma immunoglobulin levels were measured and the gut microbiota composition was assessed by 16S rRNA gene amplicon sequencing.

5.3.1 Immunoglobulin levels

The plasma IgA level were quantified on day 21 and day 35 post hatch in a group with (MM+) or without (MM-) maternal microbiota, both hosted at the LMU. No significant differences were observed between the groups at both time points (figure 19). A greater increase in IgA in the MM+ group from day 21 to day 35 was observed.

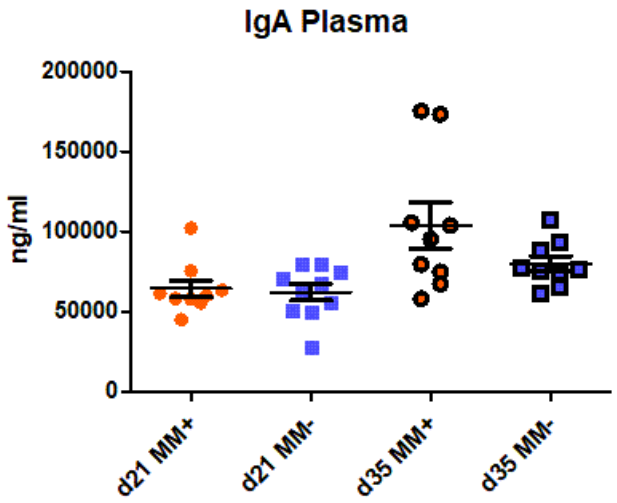

Figure 19: IgA plasma levels in the second animal experiment

Immunoglobulin levels at day 21 and at day 35. Significant differences were not detected between the groups at each time point. Statistics were performed with an unpaired t-test.

5.3.2 Gut microbiota composition

5.3.2.1 Over-time comparison of gut microbiota

First, we aimed to identify how the microbiome of the MM+ group developed over time, studying caecal samples from day 7, day 21, day 35 and the adult donor hens. In total, 268,420 high-quality reads (7,669 ± 4,722 reads/sample) were generated representing 183 OTUs (115 ± 33 OTUs/sample). The richness on day 7 had a mean value of 71 observed species and was drastically rising to 130 observed species on day 21. Richness was further rising to 142 on day 35, the adult donor animals showed a richness of 140 observed species (figure 20, A). Shannon effective counts were continuously rising from 31 to 39, 45 and 57 on day 7, 21, 35 and adult, respectively (figure 20, B). The MDS plot showed a clear separation of day 7 and

adult samples. Interestingly, day 7 samples showed a much more distinct cluster in itself, meaning each sample was at least 10% different from each other (figure 20, C). The adult samples showed a much more defined cluster. Samples on day 21 and 35 formed an overlapping cluster, meaning that these time points were very similar to each other.

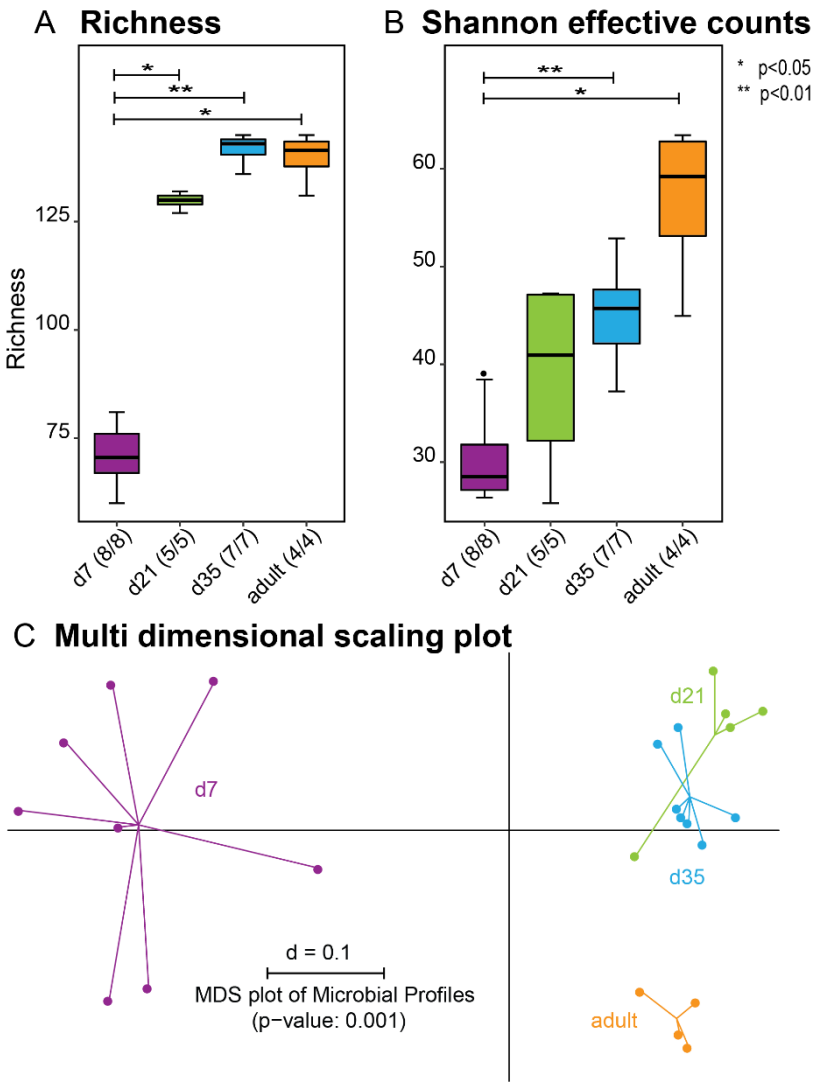

Figure 20: Over-time comparison of caecal microbiota

A: *Alpha*-diversity shown as richness or B: Shannon effective counts. C: The MDS plot of microbial profiles shows the distance between the groups. d=0.1 means samples are 10% distinct from each other. Each dot represents a single sample. Statistics were performed with Wilcoxon Rank Sum pairwise test. One asterisk indicates a *p-value* <0.05, two asterisks indicate a *p-value* <0.01.

Firmicutes was the most abundant phylum at all assessed ages. Nevertheless, the highest level of *Firmicutes* was observed at day 7. *Actinobacteria* was detected in all age groups (figure 21, A). *Elusimicrobia* were only found in samples of day 35 and in adult samples (data not shown). Further evaluation at the family level revealed that some families were completely absent at day 7: *Coriobacteriaceae, Porphyromonadaceae, Succinivibrionaceae* and *Sutterellaceae. Acidaminococcaceae, Rikenellaceae* and some unknown *Firmicutes* were only detectable in a few samples (figure 21, B).

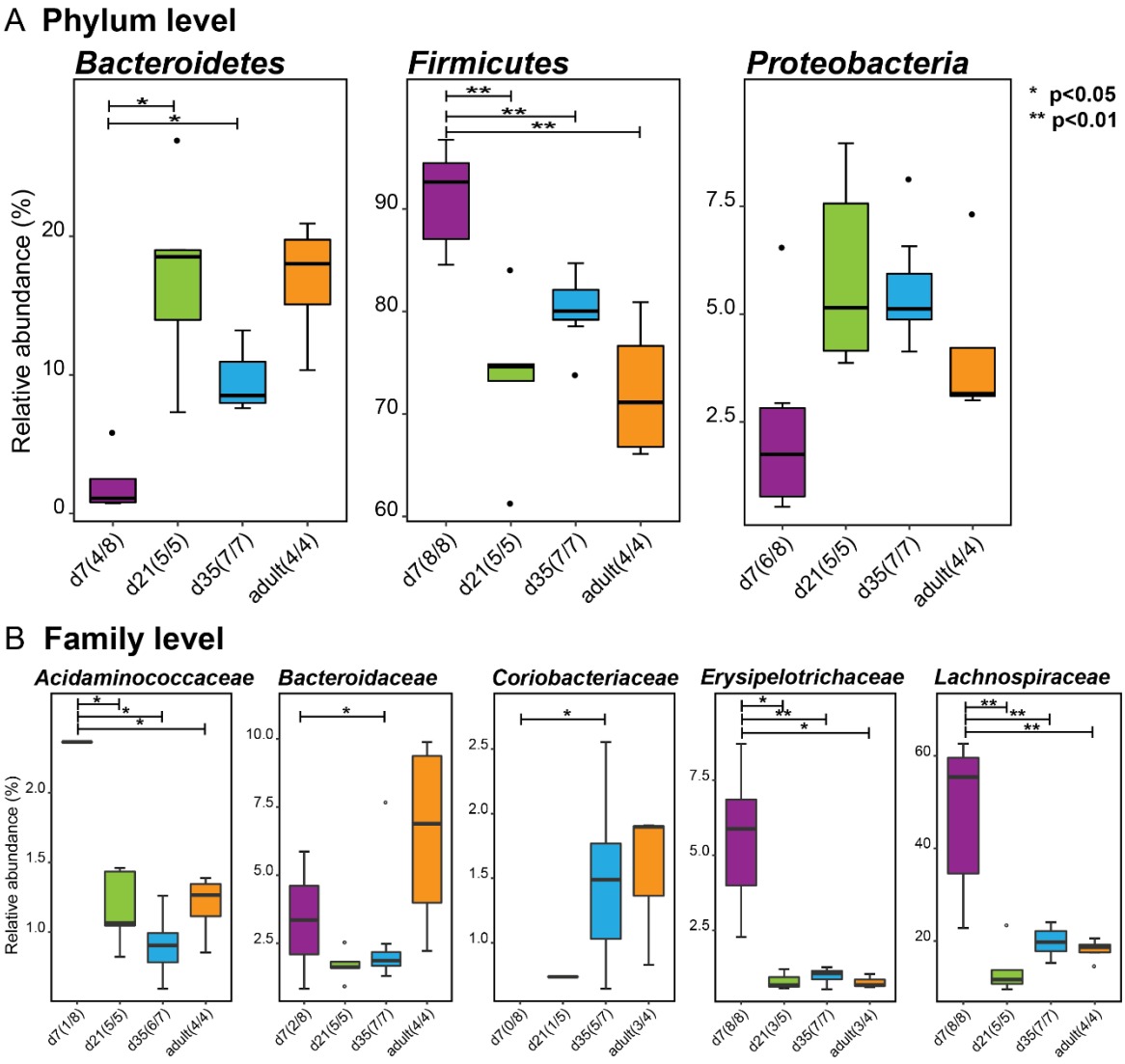

Figure 21: Over time comparison at the phylum and at the family level

A: Over time comparison at the phylum level. B: Over time comparison at the family level. Only significant families are shown. Statistics were performed with Wilcoxon Rank Sum pairwise test or Fisher's exact test. One asterisk indicates a *p-value* <0.05, two asterisks indicate a *p-value* <0.01.

5.3.2.2 Comparison of MM+ and MM- at day 35 post hatch

The next step was to assess differences between MM+ and MM- groups. Therefore, caecal samples at day 35 were analyzed and the two groups were compared. Seven samples of the MM+ group were compared with six samples of the MM- group. Sequencing generated 116,314 high-quality reads (8,947 ± 6,004 reads/sample) representing 177 OTUs (124 ± 24 OTUs/sample). Significant differences were observed in richness and in Shannon effective counts. Richness was 146 ± 5 species in MM+ compared to 100 ± 7 in MM- group (figure 22, A). Shannon effective counts were 45 ± 5 in MM+ compared to 27 ± 9 in MM- (figure 22, B). The MDS plot shows significance (*p-value* = 0.001) in the *beta*-diversity of the two groups, each forming distinct clusters (figure 22, C).

The analysis at the phylum level revealed only *Firmicutes* to be present in the MM- group. The other major bacterial phyla *Actinobacteria, Bacteroidetes* and *Proteobacteria* were not detected in MM- but in MM+. The MM- group was completed with unknown *Bacteria* but not significantly different from MM+ (figure 22, D). At the family level, only *Lachnospiraceae* and *Ruminococcaceae* were significantly higher in the MM- group. Both families belong to the phylum *Firmicutes* and make up the biggest proportion of all detected families in MM-. Additionally, low relative abundances of the families *Enterobacteriaceae* (<0.4%), *Erysipelotrichaceae* (<4%) and *Lactobacillaceae* (<4.9%) were detected in the respective samples of the MM- group. The MM+ group represented more diversity at the family level with *Bacteroidaceae, Bifidobacteriaceae, Coriobacteriaceae, Helicobacteraceae, Porphyromonadaceae, Rikenellaceae, Veillonellaceae* and unknown *Firmicutes* being only present in this group (figure 22, E).

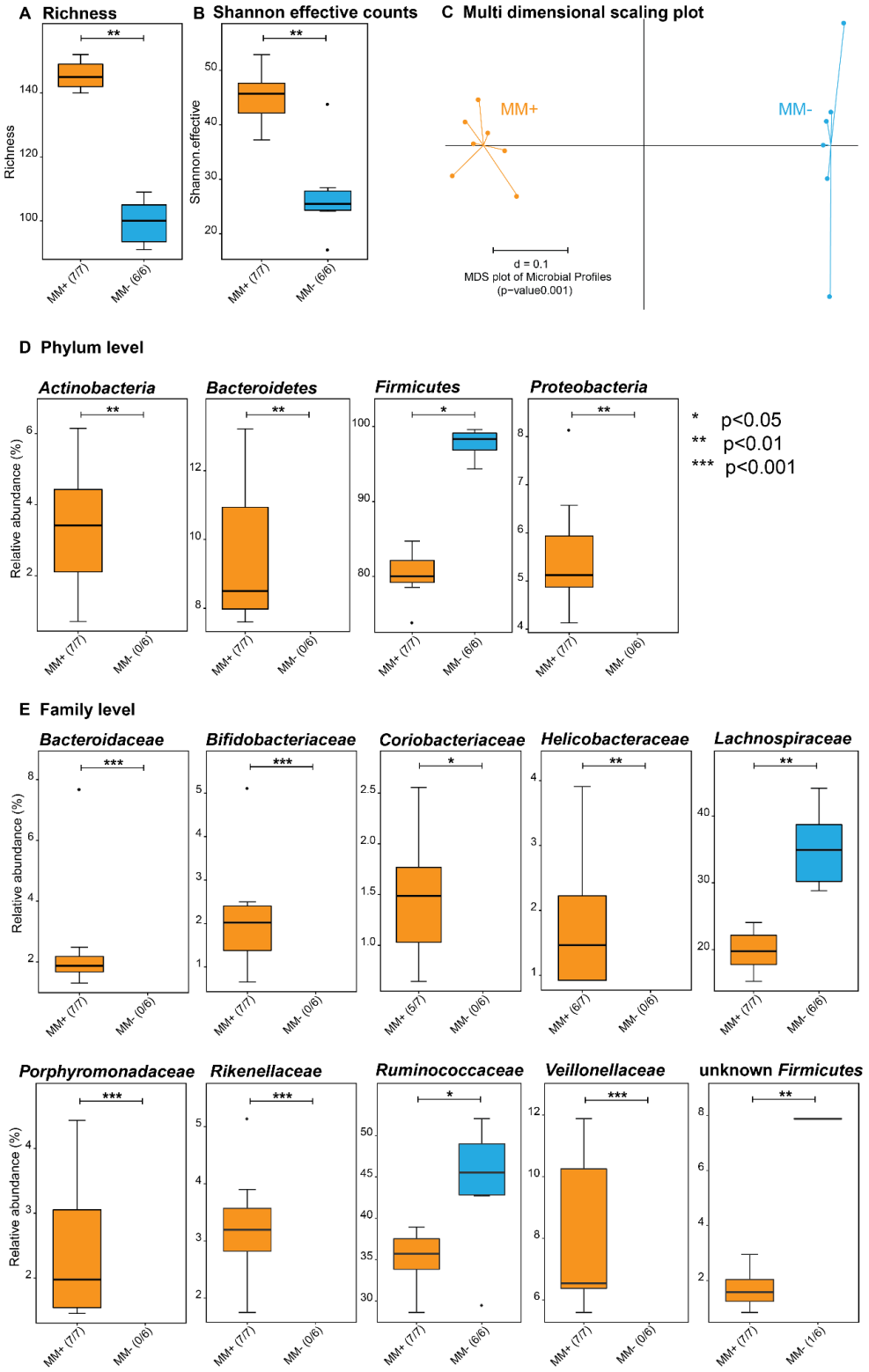

Figure 22: Microbial composition in the second animal experiment

Alpha-diversity shown as A: Richness or B: Shannon effective counts. C: MDS plot of microbial profiles. d=0.1 means samples are 10% distinct from each other. D: Bacterial composition at the phylum level. E: Bacterial composition at the family level. Statistics for Richness, Shannon effective counts, *Fimicutes, Lachnospiraceae* and *Ruminococcaceae* were performed with Wilcoxon Rank Sum pairwise test. All other statistics were performed with Fishers' exact test. One asterisk indicates a *p-value* <0.05, two asterisks indicate a *p-value* < 0.01, three asterisks indicate a *p-value* <0.001.

5.4 The Chicken intestinal bacterial collection (ChiBac)

One of the main goals of this project was to establish a bacterial collection with isolates originated from the chicken gut to design host-specific minimal bacterial consortia. The collection was deposited at the DSMZ and can be used by other scientists to perform further functional studies.

First, a total of 68 different bacterial species were isolated. Unfortunately, some strains were lost due to fastidious growth behavior. Eventually, a total of 44 strains were submitted to the DSMZ and their full-length 16S rRNA gene sequences were submitted to the nucleotide GenBank. Of these 44 isolates, seven represented potentially new taxa, including three new genera and four new species. Genome sequencing was performed for six of them so far. Work on the 7th strain is ongoing due to its fastidious nature and the incapability to produce enough biomass for high yield DNA extractions. Different media types were used to obtain the isolates. Most of the work was done in a MBraun® anaerobic chamber. Some isolates were obtained under microaerophilic conditions with an ANAEROCULT® A (Merck Millipore) test system. No isolate was obtained under aerobic conditions. Within 32 MALDI measurements, a total of 361 colonies were screened. The largest fraction of all analyzed colonies led to “no possible identification” (n=109). No peaks were found in 28 cases, probably due to technical issues. These two positions subtracted from all screened colonies led to 217 identifications at species level and 7 identifications at genus level (*Megamonas* sp.). 163 identifications were ascribed to the phylum *Firmicutes* (72.8%), 35 to *Proteobacteria* (15.6%), 15 to *Bacteroidetes* (6.7%) and 11 to *Actinobacteria* (4.9%). For identifiable strains, *Lactobacillus salivarius* (n=34), *Escherichia coli* (n=33) and *Lactobacillus johnsonii* (n=21) were most frequently identified. The genus *Lactobacillus* was identified 122 times in total followed by *Escherichia* (n=33) and *Enterococcus* (n=24) (figure 23).

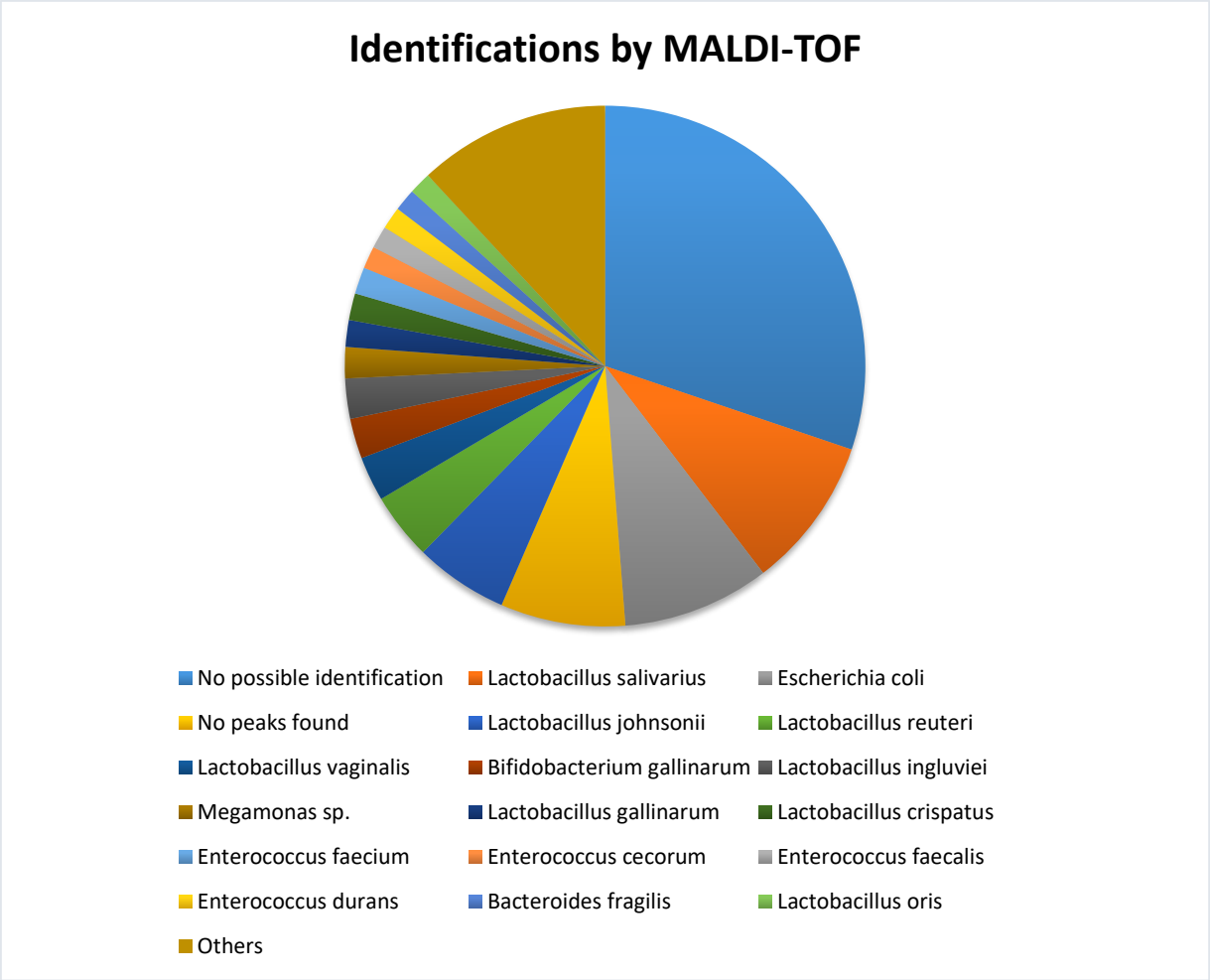

Figure 23: MALDI statistics of frequently detected strains

5.4.1 Taxonomic classification

Most of the isolates belong to the phylum *Firmicutes* (n=33), followed by *Actinobacteria* (n=5), *Bacteroidetes* (n=4) and *Proteobacteria* (n=2). The high number of Firmicutes is mostly explained by the fact, that both *Lactobacillaceae* (n=14) and *Enterococcaceae* (n=6) are comparatively easy to cultivate. At the family level, cultivation resulted in at least 15 different families: *Lactobacillaceae* (n=14), *Enterococcaceae* (n=6), *Coriobacteriaceae* (n=4) *Clostridiales* (n=4), *Clostridiaceae* (n=3), *Veillonellaceae* (n=2), *Lachnospiraceae* (n=2) *Rikenellaceae* (n=2), *Enterobacteriaceae* (n=1), *Bifidobacteriaceae* (n=1), *Desulfovibrionaceae* (n=1), *Bacteroidaceae* (n=1), *Porphyromonadaceae* (n=1), *Paenibacillaceae* (n=1) and *Ruminococcaceae* (n=1). Four isolates were not correctly assigned to a family, because they are potentially new isolates or the respective family remains undescribed. Therefore, they were assigned to the order *Clostridiales.* All isolates of the ChiBac collection are presented in figure 24.

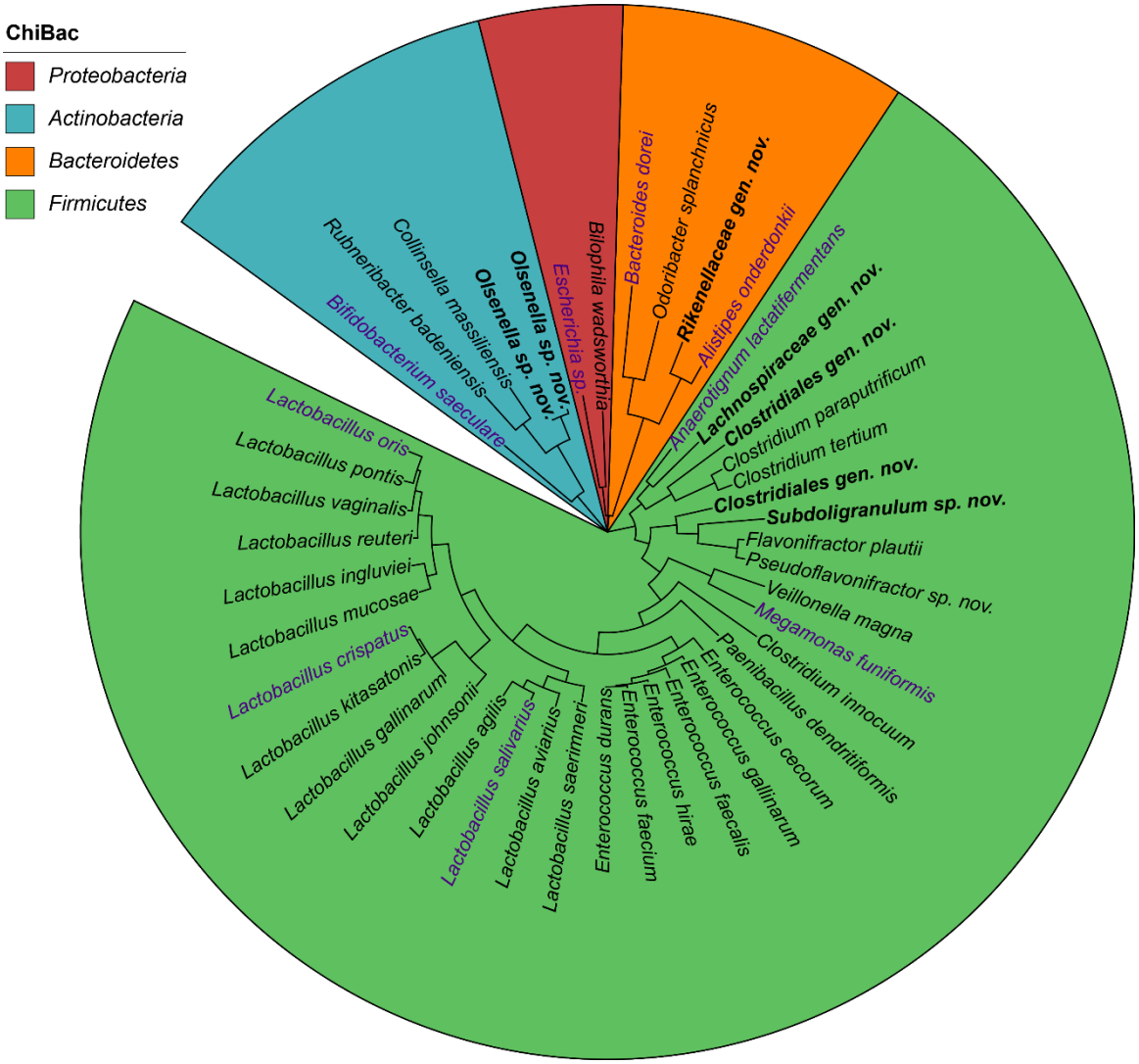

Figure 24: Phylogenetic tree of all isolates of ChiBac

Phylogenetic tree of all isolates that were deposited at the DSMZ. The tree was generated in MEGA 7 with Neighbor Joining method and 500 bootstraps. The color displays the respective phylum. Potentially new isolates are presented in bold, isolates of the minimal consortium in purple.

5.4.2 Ecological distribution

All ChiBac isolates were screened for their prevalence and relative abundance against all available chicken samples (n=1,499) in IMNGS with more than 5,000 reads. The aim was to create an overview which isolates were dominant and prevalent in chicken samples.

Prevalence

Prevalence describes how frequently an isolate can be found within the population. The isolate *Lactobacillus johnsonii* was 86.3% prevalent, i.e. it was detected in 86.3% of all screened chicken samples. The Top10 most prevalent isolates within ChiBac are presented in table 19.

Top	Species name	Prevalence (%)
1	*Lactobacillus johnsonii*	86.3
2	*Escherichia* sp.	79.0
3	***Lachnospiraceae* gen. nov.**	75.4
4	*Lactobacillus salivarius*	74.2
5	*Lactobacillus crispatus*	73.0
6	*Clostridium tertium*	58.4
7	***Pseudoflavonifractor* sp. nov.**	58.4
8	*Rubneribacter badeniensis*	52.9
9	*Lactobacillus reuteri*	50.1
10	*Anaerotignum lactatifermentans*	44.4

Table 19: Top 10 most prevalent ChiBac isolates

Average relative abundance

Average relative abundance describes the proportion of an isolate in all positive samples. For the isolate *Lactobacillus crispatus,* 11.1% relative abundance was detected meaning it was observed in a proportion of 11.1% reads within all samples. The Top10 ChiBac isolates with the highest relative abundances are presented in table 20.

Top	Species name	Average rel. abundance (%)
1	*Lactobacillus crispatus*	11.1
2	*Lactobacillus gallinarum*	8.1
3	*Escherichia* sp.	7.4
4	*Bacteroides dorei*	5.4
5	*Lactobacillus aviarius*	5.1
6	*Lactobacillus johnsonii*	4.0
7	*Lactobacillus salivarius*	3.4
8	*Lactobacillus reuteri*	3.2
9	*Enterococcus faecium*	1.8
10	*Lactobacillus vaginalis*	1.7

Table 20: Top 10 most relative abundant ChiBac isolates

5.4.3 Selection of a minimal consortium

For the development of a minimal consortium with the aim of modulating immune responses via inoculation of chickens at hatch, nine members of ChiBac were selected. Selection criteria were colonization properties, creation of an anaerobic environment, inhibitory effects on *Campylobacter* sp., reduction of pH in the gut, production of butyrate and, particularly 16S rRNA gene profiling data of own experiments. Another major criterion was to ensure the members were phylogenetically diverse, enhancing their ability to represent a healthy chicken gut microbiota. The composition of the minimal consortium, taxonomic classification and selection criteria are explained hereinafter. The final cell densities of each species included within stocks of the minimal consortium were measured by plating on WCA agar.

#	Species name	Phylum	Family	CFU/ml
1	*Escherichia* sp.	*Proteobacteria*	*Enterobacteriaceae*	$4.3x10^9$
2	*Lactobacillus salivarius*	*Firmicutes*	*Lactobacillaceae*	$1.95x10^4$
3	*Lactobacillus crispatus*	*Firmicutes*	*Lactobacillaceae*	$3.7x10^6$
4	*Lactobacillus oris*	*Firmicutes*	*Lactobacillaceae*	$4.5x10^7$
5	*Bifidobacterium saeculare*	*Actinobacteria*	*Bifidobacteriaceae*	$6.6x10^6$
6	*Bacteroides dorei*	*Bacteroidetes*	*Bacteroidaceae*	Not determined
7	*Alistipes onderdonkii*	*Bacteroidetes*	*Rikenellaceae*	$5x10^8$
8	*Megamonas funiformis*	*Firmicutes*	*Veillonellaceae*	$2.1x10^6$
9	*Anaerotignum lactatifermentans*	*Firmicutes*	*Lachnospiraceae*	Not determined

Table 21: Isolates of the minimal consortium

Escherichia sp. was selected for its potential to create an anaerobic environment in the gut (195) and its dominance in the chicken gut. It is the only member of the phylum *Proteobacteria* in the consortium. Its precise identification at the species level was not possible through the 16S rRNA gene full-length sequence and thus, genome sequencing was performed to correctly identify *Escherichia* sp., but it is still ongoing. For the species *Lactobacillus salivarius* in general, an inhibitory effect on *Campylobacter* sp. was described by Saint-Cyr et al. and by Svetoch et al. (149, 196). This inhibitory effect was confirmed against *Campylobacter jejuni* subsp. *doylei* in-house in a cross-streaking assay. *Lactobacillus crispatus* and *Lactobacillus oris* were selected for their potential to lower the pH in the gut (197). *Lactobacillaceae* in general were selected for their dominance in the chicken gut. *Bifidobacterium saeculare* was selected for its potential to produce anti-*Campylobacter* agents (198) and, more importantly, because it was not found in SPF and MM- groups within the presented experiments. *Bacteroides dorei* was selected because it was not found in SPF and MM- groups within the experiments. Additionally, the species was shown to induce Interleukin-22 production by innate lymphoid cells which has antimicrobial properties (157). *Alistipes onderdonkii* was selected because it was not detected in SPF and MM- groups. Additionally, it could be important for the presence of intraepithelial lymphocytes (199). *Megamonas funiformis* was selected because it was not observed in SPF and MM- groups. In addition, it presented properties of a good early life colonizer (200). *Anaerotignum lactatifermentans* was selected because members of the genus *Anaerotignum* are potential butyrate producers (201). Butyrate might be important for enhanced antimicrobial functions in macrophages (202).

5.5 Animal experiment 3: Colonization study

The Colonization study aimed to investigate if a minimal bacterial consortium is colonizing in chickens and if it generates comparable results to MM. Therefore, the plasma immunoglobulin levels were measured and the gut microbiota composition was assessed by 16S rRNA gene amplicon sequencing.

5.5.1 Immunoglobulin levels

Plasma immunoglobulin levels of IgA were quantified by ELISA on day 25 and day 39 and for IgY on day 25, respectively. Significant differences were detected for IgA on day 25 between Consortium (Cons) and PBS and between MM and PBS, respectively. No significant differences could be determined on day 39 for IgA. Highly significant differences were also assessed for IgY on day 25 between MM and Cons and between MM and PBS (figure 25).

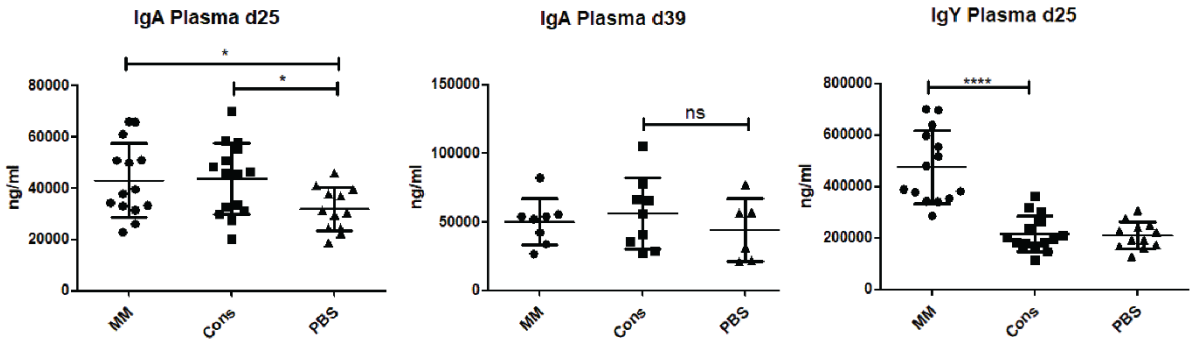

Figure 25: Plasma IgA and IgY levels in a colonization experiment

Each dot represents a single animal. Middle line is showing the mean value, upper and lower line the standard deviation. Statistics were performed with an unpaired t-test between the respective groups. One asterisk indicates a *p-value* <0.05, four asterisks indicate a *p-value* <0.0001.

5.5.2 Gut microbiota composition

The gut microbiota composition was assessed to detect microbial differences between the groups and to find out if the bacteria of the consortium colonized the animals. 16S rRNA gene amplicon sequencing was performed with samples at day 25, as significant differences in immunoglobulin quantification were detected at this time point. The caecal content of six animals of each group was analyzed. Sequencing delivered a total of 269,194 high-quality reads (14,955 ± 5,875 reads/sample) representing 156 OTUs (101 ± 24 OTUs/sample). Significantly, higher richness was detected in the MM group compared to Cons and PBS, but significance was not observed for Shannon effective counts (figure 26, A+B). The MDS plot and the phylogram showed a more defined cluster for MM and a mixed cluster of PBS and Cons (figure 26, C+D).

Significant differences were detected for *Firmicutes* between MM and Cons. A higher relative abundance of *Bacteroidetes* and a lower relative abundance of *Firmicutes* could be determined in the MM group. *Actinobacteria* were found in higher relative abundance in the PBS group, but only in 50% of samples (figure 26, E).

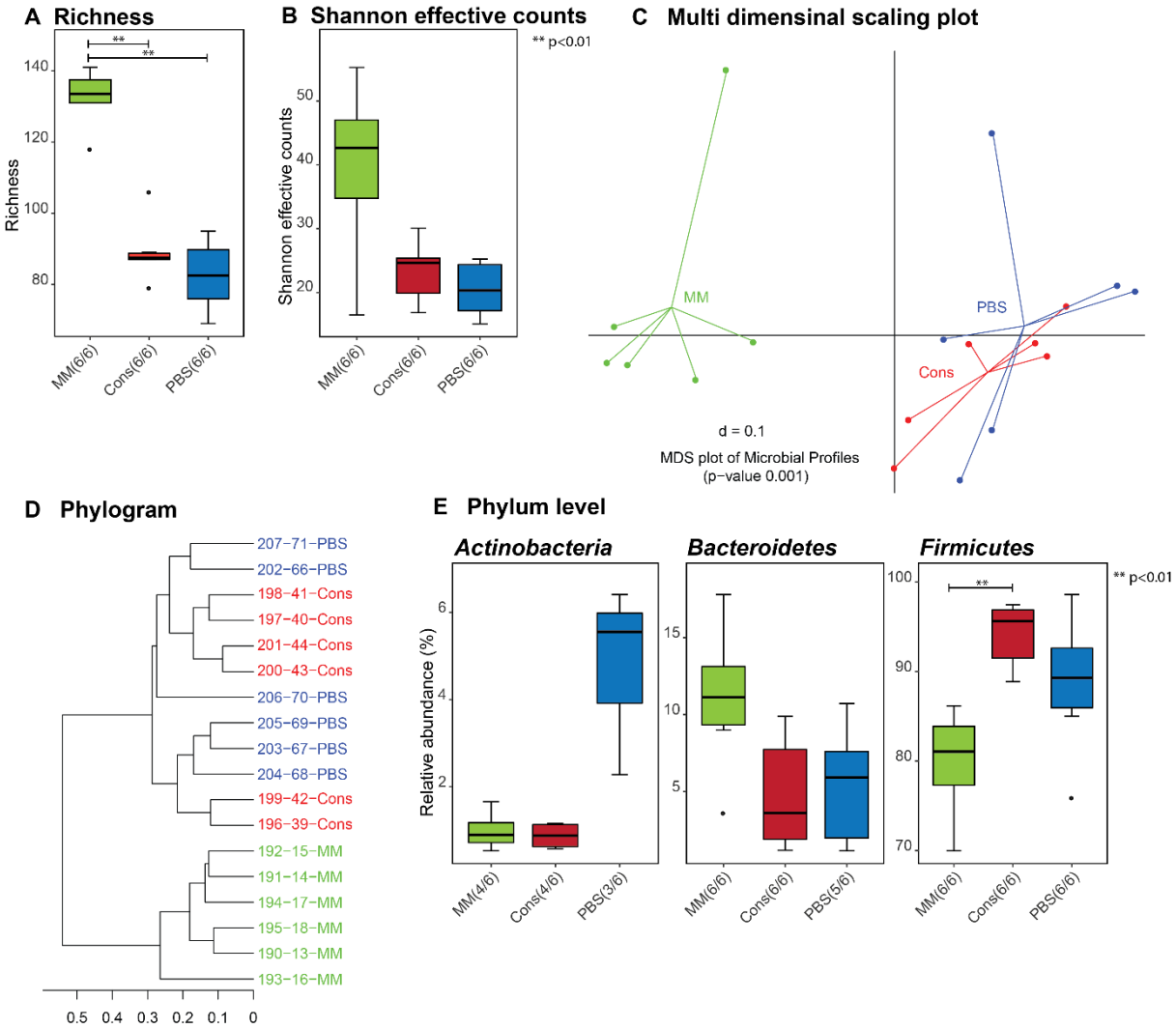

Figure 26: Microbiota composition in a colonization experiment

A: *Alpha*-diversity shown as richness or B: Shannon effective counts. C: Beta-diversity shown as MDS plot. D: Phylogram of all samples. E: Taxonomic composition at the phylum level. Statistics were performed with Wilcoxon Rank Sum pairwise test.

OTU level analysis was performed to detect single molecular species that were used for colonization of the Cons group. Therefore, the OTU table was screened with BlastN (63) and the sequence similarities were documented. Molecular species with 100% sequence similarity were found for *Lactobacillus crispatus* (OTU_42), *Lactobacillus salivarius* (OTU_79), *Bifidobacterium saeculare* (OTU_8), *Bacteroides dorei* (OTU_48), *Escherichia* sp. (OTU_76) and *Alistipes onderdonkii* (OTU_11). A slightly lower sequence similarity was found for

Megamonas funiformis (OTU_2). For *Anaerotignum lactatifermentans,* a 95.51% sequence similarity to *Anaerotignum aminivorans* (OTU_49) could be detected. A molecular species for *Lactobacillus oris* was not detected. We expected higher levels of the colonizing strains in the Cons group compared to PBS. This was only observed for *Megamonas funiformis* and *Alistipes onderdonkii. Bifidobacterium saeculare* represented equally high relative abundance in all groups, but with two outliers in the PBS group. Outliers in the PBS group were also observed for *Lactobacillus crispatus* and *Lactobacillus salivarius. Bacteroides dorei* was not detected in the PBS group and only in one observation in the Cons group in very low relative abundance (figure 27). The colonization was considered effective if the molecular species of the respective isolate was detected in minimum 80% of all observations. This was only the case for five out of nine isolates.

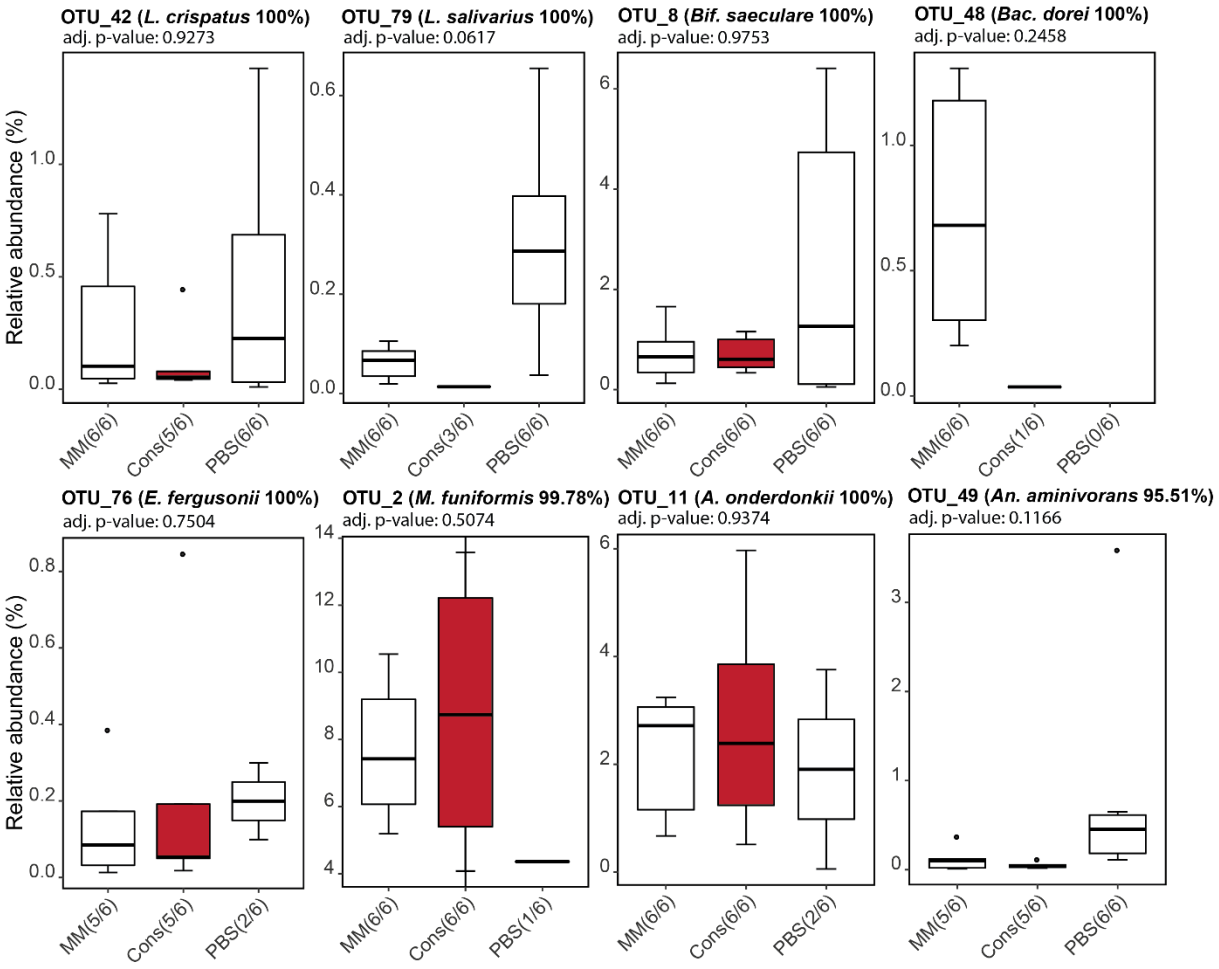

Figure 27: Molecular species of strains used for microbial colonization

6 Discussion

The chicken represents one of the most important animals for global food supply. The poultry industry tries to fulfill the high demand for animal protein with high throughput rates and high-speed poultry rearing. Animal health and wellbeing has often been neglected. Since hatched chickens grow up without any contact to parent animals, no transfer of maternal microbiota takes place. This transfer of bacteria from parents to young animals would be crucial to guarantee an adequate development of the intestinal immune system and therefore, a reduction of pathogen entry into poultry flocks. Without a trained immune system, antibiotics are often the only way to treat infections. Hence, strategies are required to strengthen the intestinal immune system. Adequate colonization by chicken microbiota will possibly increase gut health and improve animal wellbeing. This study aimed at better understanding the chicken microbiome and its impact on the development of the gut immune system. In order to assemble bacterial consortia which may help to promote proper development, anaerobic bacterial strains were isolated from chicken hosts and a first experimental consortium was developed.

6.1 Comparison of intestinal regions

Spatial variations of the microbiota are described for almost every ecosystem (98, 203-205). This is not surprising due to the fact that prokaryotes are competing for their respective niches. In this study, the gut luminal bacterial content of the jejunum, the caecum and the colon of 90-day old chickens was compared by 16S rRNA gene amplicon sequencing. Most studies, either cultivation-based or sequencing-based, discovered *Lactobacillus* to be the predominantly discovered genus within the small intestine (98-101). The next common genera were *Romboutsia* in ileum and *Staphylococcus* in duodenal and jejunal samples (98). These observations mirror our own findings with *Lactobacillus* being the most relatively abundant genus in jejunum. In contrast, *Staphylococcus* was not detected throughout the experiments, neither by sequencing, nor by cultivation. The most abundant phyla in the chicken caecum are *Firmicutes, Bacteroidetes* and *Proteobacteria* (85, 102). Our own study concords with these results, but with different relative abundances. Sergeant et al. describes the most abundant OTUs in caecum to belong to *Megamonas, Veillonellaceae, Alistipes, Bacteroides* and *Pseudoflavonifractor* (105). Similar results were observed in caecum for *Megamonas* and *Bacteroides*. *Faecalibacterium* was the most relative abundant genus in the presented study. In the colon, 75% of samples clustered closer with the jejunal samples while 25% clustered closer with the caecal samples. We hypothesized that the caecum was emptied just before sacrifice, making these samples look like caecal samples. In summary, the colon cannot be used as sampling site for gut microbiome studies as the origin could be of small intestinal or caecal nature, leading to extremely variable results. This should also be taken into account when using faecal droppings as sampling material (206). Differences between the presented study and the literature are not surprising, as every group utilizes different sequencing

techniques, different bioinformatics pipelines and targets different variable regions of the 16S rRNA gene (60, 207, 208).

6.2 Proof-of-principle

The concept of giving MM to newly hatched chicks was established by Nurmi and Rantala to prevent *Salmonella infantis* infections in chickens. Already in 1973, they stated that “broiler production is carried out under abnormally hygienic conditions” and therefore, the prevalence of *Salmonella* spp. was much higher compared to layer hens that were seemingly not kept under such strict conditions. The authors suspected the intestinal flora to normally built defense mechanisms against pathogens (153). Goren et al. copied the principle in 1988 in a large longitudinal study with more than 8 million broiler chickens that were evaluated flock-wise. The main focus was again on prevention of *Salmonella* spp. infection (209). In 2016, Varmuzova et al. colonized newly hatched chickens with caecal extracts to promote resistance against *Salmonella* enteritidis (70). Volf et al. inoculated chicken with MM to detect differences in gene expression. They identified six genes with a significantly reduced gene expression in GF compared to colonized chickens. These include AVD, IgM, Igλ, CALB1, ES1 and ISG12-2 (210).

The concept of Nurmi et al. (153) was adopted in this study, but in contrast, competitive exclusion properties were not in the focus. The aim was to evaluate host-related immunological data that is tangible and reproducible throughout the experiment with different methods. Thus, quantification of immunoglobulin levels, RNA sequencing and qPCR built on each other. The gut microbiota composition was evaluated to find major differences and possibly find the responsible microbes for immune system development. Concerning the presented experiment, one of the major biasing factors on the readouts is the facility discrepancy. The animals were of the same genetics, got the same feed and were treated equally by the caretakers. Nevertheless, each facility represents its own unique characteristics. Especially when comparing microbiome data, housing conditions are a major influencing factor (211).

The immunoglobulin levels of IgA and IgY were evaluated in plasma, bile and caecal content. Highly significant results were generated for all comparisons at each time point. The most interesting findings were therefore the results in bile and caecal content. Basically, no sIgA was detected in the SPF group (figure 12) while the MM group exhibited very high IgA levels thus, underlining the importance of a balanced gut microbiota colonization to establish competent adaptive mucosal immunity. To our knowledge, no comparative experiment on the evaluation of IgA in a SPF or germfree environment was published so far. Sarah Lettmann evaluated the IgA levels of germfree, mono-colonized, tetra-colonized and conventional (facility-raised) chickens in plasma, bile and caecal content in her doctoral thesis. Basically, no IgA was found in caecal content or bile of germfree chickens, underlining the importance of

microbial colonization for IgA induction in the gut. Tetra-colonized compared to conventional chickens secreted similar levels of IgA in bile but lower levels in caecal content (212). In summary, it seems that the SPF facility has more similarities to a germfree- than to a conventional housing environment.

RNAseq was conducted to find differently expressed genes in both groups. Heatmaps were generated to get an overview of significantly expressed genes. However, data sets were primarily analyzed for genes of the adaptive immune system in CT, ileum and spleen. No direct transcripts were found for IgA and IgY, hence we choose immunoglobulin joining chain (JCHAIN) and Ig variable region (Ig V gene) for evaluation of genes that are relevant for immunoglobulin production. The activation-induced cytidine deaminase (AID) and the polymeric immunoglobulin receptor (PIGR) were found in the DGE matrix. Transcripts for IL6 and IL21 had very low read counts and were not differentially expressed, so we choose IL6R and IL21R for our readouts. Unfortunately, the chicken genome is not fully annotated. Hence, we probably missed some genes that would be relevant for assessing the immune status. Volf et al. investigated expressional differences in chicken caecum by comparing GF with colonized chickens. The major finding was the complete absence of immunoglobulin gene transcripts in GF chickens (213). Considering that the SPF animals showed similar patterns in immunoglobulin levels, we expected immunological relevant genes to be regulated differently. CT displayed the most differently regulated set of genes. Interestingly, more genes were upregulated in the SPF group than in the MM group. Some upregulated genes in the MM group belong to immunological pathways, supporting the hypothesis that a microbial stimulus was missing in the SPF group. GO enrichment analysis found significant terms for responses to ‘external and biotic stimuli’ and ‘responses to stress’ in MM group while GO terms for lipid metabolism were found in the SPF group. Apolipoprotein B (APOB) and ‘fatty acid binding protein 6’ (FABP6) play substantial roles in fat reabsorption and transport of fatty acids (214, 215). Possibly, the lipid metabolism replaced the missing microbial fermentation of carbohydrates. In ileum, a contrary pattern was depicted, where more genes were upregulated in MM compared to SPF. GO enrichment analysis showed a similar pattern like in CT, genes for ‘immune response’, ‘immune system process’ and ‘responses to external/other organism/bacterial stimulus’ were located. Interestingly, similar small intestinal transcriptomic results were presented for nematode infected chickens (216). Additionally, the GO term ‘lipid metabolic process’ was enriched, displaying a contradictory picture to the CT. The SPF group in ileum expressed terms for ‘anatomical structure development’ and ‘multicellular organism development’, letting us assume that the structural development of this gut section is not completed and lipid metabolic pathways will be of importance at later time points. The analyzed immunological relevant genes showed a similar pattern like in CT, but IL6R was upregulated in SPF. This result was contradictory to the IL6 result from qPCR, where IL6 was significantly

upregulated in MM. IL6 plays an important role in immune stimulation but was absent in the filtered RNAseq dataset, assuming that not enough read counts were detected. An *in vitro* study in 1994 found that the treatment of a RPMI-8226 cell line with IL6 led to a lower number of expressed IL6R receptors on cell membranes (217). This could be the case in our experiment but would still be contradictory to the results in CT. However, as IL6R generally had very few read counts in the assessed organs, it was difficult to evaluate this finding. The least differentially expressed genes were found in spleen. The significance cutoff was therefore lowered to *p-value* <0.05 to find more transcriptional differences. Even after reducing the threshold, only one gene was higher expressed in MM and ten genes were higher expressed in SPF. No significant differences could be found for immunologically relevant genes. The only upregulated gene in MM was EXFABP that codes for a siderocalin with bacteriostatic properties under iron limited conditions (218). RNAseq has a high discovery rate of differentially expressed genes but their validity needs to be confirmed by qPCR (219). We used available primers for IgA, AID, IL6, IgJ, IL21 and IgY. Significant differences were detected for IgA, IgJ, IgY and IL6 but not for AID and IL21 (figure 16). The results of IgA, IgJ and IgY confirmed the findings from immunoglobulin measurement and RNAseq, where JCHAIN and Ig V gene were upregulated. The significant difference of IL6 in qPCR was not detected for IL6R by RNAseq, although a tendency was observed in CT. Differences in IL21 gene expression were not significant by qPCR and also not significant for IL21R by RNAseq in all tissues. AID gene expression was not significant by qPCR but significance in CT was detected by RNAseq. Gene expression analyses revealed differences in immunologically relevant genes, especially genes for immunoglobulin production. These differences are affiliated with a diverse gut microbiota composition discussed hereinafter.

The evaluation of 16S rRNA microbial profiles showed remarkable results. The richness and Shannon effective counts were significantly lower in the SPF group, which is not surprising for a group that is kept under strict hygienic conditions. As the animals were raised in different facilities, it would have been helpful to know if these changes were an effect of hygienic measures or an effect of MM transplantation. A comparison of the microbial profiles of animals that were kept under strict hygienic measures at both institutes, LMU and INRA, would have been useful. However, this question could only be addressed at the LMU facility in a validation study (see below). Data analysis at the phylum level showed only *Firmicutes* to be present in the SPF group, which were not sufficient to fully stimulate immune system development in comparison with the complex maternal microbiota. However, this does not exclude *Firmicutes* as immune system stimulators in general, since the MM group could have contained species of the phylum *Firmicutes*, that were not present in the SPF group, but were probably responsible for the induction of immune responses. Yang et al. showed recently that a specific isolate of the mouse commensal *Bacteroides ovatus* is a major IgA inducer, while other

Bacteroides ovatus strains did not have the same effect (220). A future perspective would therefore be to look for strong IgA inducers in the MM community.

6.3 Validation

As discussed, a second animal experiment was conducted to compensate for facility effects and to reproduce the results of the first experiment. The IgA levels were quantified at day 21 and 35 post hatch. No significant differences were detected between the groups at both time points. At day 21, the IgA levels were almost equally high. Compared to the first experiment, where IgA was quantified at day 28, the levels within the MM+ groups were similar (Exp. 1: 71.4 µg/ml, Exp. 2: 64.2 µg/ml), taking the differences in sampling time points into account. Comparing the SPF group of the first experiment with the MM- group of the second experiment, clear differences in IgA levels were observed (Exp. 1: 32.9 µg/ml, Exp. 2: 63.0 µg/ml). A tendency towards higher IgA levels within the MM+ group was noticed at day 35. Again, comparing the IgA levels of both experiments in MM+ groups (Exp. 1: 133.1 µg/ml, Exp. 2: 103.6 µg/ml), the differences were comparable by having sampling points that differed by 23 days (day 58 in Exp. 1 and day 35 in Exp. 2). Looking at the high hygiene groups, SPF birds had significantly lower levels of IgA at day 58 and were already overtaken by the MM- group at day 35 (Exp. 1: 43.8 µg/ml, Exp. 2: 79.6 µg/ml). The gut microbiome composition at day 35 was further analyzed to detected differences between the two groups. In contrast to the results of immunoglobulin quantification, the microbiome data depicted a similar picture to the first experiment. Richness and Shannon effective counts were significantly higher in MM+. Almost only *Firmicutes* were detected in the MM- group, subdividing further in *Lachnospiraceae* and *Ruminococcaceae* at the family level. These observations were nearly identical to the first experiment. However, looking closer into the data, generally more OTUs were detected in the Proof-of-Principle study (n=270) than in the validation study (n=177). We further performed OTU level analysis to detect differences and similarities between both MM+ and SPF vs. MM- groups, respectively. *Megamonas funiformis, Ruminococcus torques* and *Faecalibacterium prausnitzii* had a higher relative abundance in MM+ group in the second experiment, while *Alistipes onderdonkii, Parabacteroides merdae* and *Bacteroides uniformis* were more relatively abundant in the first experiment. By comparing MM- with SPF, *Subdoligranulum variabile* and *Anaerobium acetethylicum* were higher relatively abundant in MM- while *Roseburia faecis, Clostridium clostridioforme* and *Eubacterium coprostanoligenes* were higher in the SPF group. As the immunoglobulin levels of the MM+ groups of both experiments were comparable, the molecular species of the SPF/MM- side were more interesting. Two hypotheses can be proposed: Either the higher abundant species in the MM- group were responsible for the stimulation of an adequate immune response or the higher abundant species in SPF had an immuno-inhibitory role, leading to lesser IgA production. We assume that an initial microbial stimulus was responsible for increased immune responses in both MM+ and MM- groups.

Later, this stimulus was not sufficient enough to further increase IgA levels in MM- while the more diverse microbial population in MM+ led to even stronger IgA responses. An over-time-comparison of gut microbiota in the MM+ group was conducted to better understand the microbial shifts within the early weeks of life. The lowest richness was detected at day 7 and mostly *Firmicutes* were observed. This agrees with the first animal experiment, where *Firmicutes* were the only phylum in the SPF group. The microbiome of young chickens and chickens under high hygienic standards seems to be very similar. Between day 21 and 35, numerous changes in the microbial composition took place on the way to a stable microbial ecosystem. *Bacteroidetes* were first rising from day 7 to 21 and then falling again from day 21 to 35. *Proteobacteria* were least abundant in young chickens. They were rising on day 21 and 35 and then settling back to a lower level. The relation between microbial colonization and immune system maturation was also investigated by Volf et al., who compared the gene expression of IgA, IgM and Igλ of conventional chicken with tetra-colonized (mixture of *E. coli* Nissle1917, *Enterococcus faecium* DSM7134, *Lactobacillus rhamnosus* DSM7133 and *Clostridium butyricum* DSM10702) or mono-colonized (reconstituted with *E. coli* Nissle1917 or *Enterococcus faecium* DSM7134*)* chickens. Tetra-colonized and conventional chickens had significantly higher gene expressions of the immunoglobulins, confirming the importance of diverse microbial colonization for immune system maturation (213). The difficulty of reproducing microbiome experiments became apparent during the second study. Although the microbiome composition looked very similar to the first study, no significant differences were detected in immunoglobulin levels. Confirmation experiments are therefore required as powerful tools to make microbiome research more robust and conclusive (221).

6.4 The ChiBac collection

In 2020, Rychlik reviewed the composition and function of chicken gut microbiota and stated, that one of the most important future challenges is “to generate an extensive collection of pure cultures of chicken gut anaerobes” (108). The Rychlik group already provided 133 genomes of chicken gut anaerobes that will help to better understand the metabolic profiles of these isolates and therefore, elucidate host-microbiota interactions in chickens (82). Furthermore, Crhanova et al. suggested that half of the chicken caecal microbiota members can be grown *in-vitro* (222).

Within ChiBac (this study), 68 strains were first isolated of which only 44 were submitted to the DSMZ. This was mostly owed to contamination issues and the inability to passage liquid cultures over longer time periods. The most affected genus was *Bacteroides,* where initially five strains were isolated but only one survived for archiving in the collection. Undescribed taxa were also affected, leading from potential 13 new isolates to 7 new isolates. The ecological

distribution revealed insights into prevalence and relative abundance. Highly prevalent and highly abundant isolates were easier to cultivate, because they were present in many chicken samples in relatively high numbers. For low prevalent and low abundant strains, it was more difficult to distinguish between chicken isolates or contaminations. Three isolates had a prevalence and relative abundance below 1%. These were *Paenibacillus dendritiformis, Enterococcus durans* and *Clostridium innocuum*. These three isolates possibly originated from contaminating sources. Even though other groups are also putting great efforts into the isolation and cultivation of chicken gut anaerobes, more work is required to obtain a comprehensive library for future application research into metabolomics and studies investigating host-microbiota relations. Rychliks work and this study are the first to systematically address this challenge.

To gain insights into the role of individual bacteria or consortia, *in vivo* experiments are required, provided stringent readouts are available to quantify the effects. Based on the ChiBac library, a first minimal consortium was composed. Selection of isolates of the minimal consortium was limited by availability and the biosafety level. Thus, nine isolates were selected representing a broad phylogenetic diversity. For two isolates (*Bacteroides dorei, Anaerotignum lactatifermentans*), it was not possible to assess the amount of CFU, that was set in the consortium. This was probably due to the wrong choice of solid medium or the isolates failed to readapt to solid medium from liquid culture conditions. However, vitality was verified by native microscopy. For *Lactobacillus salivarius*, only low CFU/ml were detected, even though the culture was turbid. All other isolates ranged from 10^6 to 10^9 CFU/ml. The differences in bacterial concentrations represents a major bias in a colonization experiment. The bacteria could outcompete each other by targeting the same niche. Using the lowest common denominator would be an option to circumvent this bias but this would have the disadvantage of inoculation with very low bacterial numbers. Therefore, the decision was made to inoculate each species at the highest possible concentration to ensure colonization. The consortium was initially selected *in vitro* for potential anti-*Campylobacter* properties. However, proving the efficacy as a competitive flora against *Campylobacter* was beyond this study, but needs to be carried out in the future. Additional studies may also include further developed consortia with more and/or other species. Therefore, the ChiBac project needs to be expanded to have more bacterial isolates, which will help improving the minimal consortium.

6.5 Colonization

The concept of minimal bacterial consortia was first described by Schaedler et al. for mice, composed of *Lactobacillaceae, Streptococcus* sp., *Bacteroidaceae* and *Enterobacteriaceae* (223). Brugiroux et al. established a mouse bacterial consortium (Oligo-Mouse-Microbiota-12)

and presented good results in preventing mice from *Salmonella* colonization (224). Furthermore, whole-genome sequences of the Oligo-Mouse-Microbiota-12 consortium were provided (225) and its successful colonization in GF mice has been confirmed in different facilities (226). The purpose of the third animal experiment in this study was to establish a bacterial minimal consortium in chickens that possesses similar properties like the maternal microbiome in terms of stimulation of immune responses. Therefore, the immunoglobulin levels and microbiota composition were assessed. The results of IgA measurement at day 25 were promising. The consortium group exhibited almost equal levels of IgA as the MM group while IgA was significantly lower in the PBS group. IgY was significantly higher at day 25 in MM compared to the consortium and PBS groups. However, the total values of IgA were much lower compared to the other experiments. Only the IgA levels at day 28 in the SPF group of the first experiment were comparable to the levels of MM and consortium groups. However, the significant differences at day 25 were not reproduced at day 39, when no significant differences between the groups were detected. It can be hypothesized that the initial microbial stimulus for immune system development was stronger in MM and consortium groups, but not sufficient enough to provide a further boost at later time points. This assumption was supported by the generally low levels of IgA at day 39 in all groups, compared to the previous experiments. As significant differences in IgA levels were only detected at day 25, the gut microbiota composition was analyzed at this time point. Richness was significantly higher in the MM group, as expected. Richness was also slightly higher in the consortium group compared to PBS, which is not surprising, as we inoculated the consortium animals with nine additional species. Phylum level analysis detected more *Bacteroidetes* and less *Firmicutes* in the MM group, compared to the consortium and PBS groups. Between consortium and PBS groups, *Bacteroidetes* were equally abundant but *Actinobacteria* were higher in the PBS group and *Firmicutes* were higher in the consortium group. Compared to the other experiments, where *Firmicutes* was almost the only phylum in the SPF and MM- groups, the PBS group exhibited additional *Bacteroidetes* and *Actinobacteria.* Unfortunately, in this experiment the PBS group did not establish a limited microbiota composition as SPF birds and MM- birds in the previous studies. Introduction of more bacterial species through bedding, feed or personal in comparison with the first two studies cannot be excluded, underscoring the problems associated with microbiome research. In addition, inoculation with the consortium or PBS could have stressed the birds and thus, induced changes in the microbiome (227).

We further investigated if the minimal consortium colonized in the consortium group, because we expected higher relative abundances of the nine isolates than in the PBS group. This was only observed for *Megamonas funiformis* and *Alistipes onderdonkii*. Different reasons may have contributed that the colonization of chickens was not a complete success. First, the vitality after freezing: the consortium was processed in an anaerobe workstation and was immediately

frozen after preparation. All isolates were separately tested for their ability to survive freezing and thawing, but not in the context of a mixture. The process of freezing potentially damaged cells and the rate of survival might even be lower in isolates that were used in low concentrations (228, 229). Second, the anaerobic needs of the bacteria: the critical point was the thawing of aliquots and the oral inoculation of chickens. During that time, the bacterial cocktail was exposed to oxygen which might have killed some vital bacteria (230). Third, the establishment of the microbiome: For mammals, it is known that the microbiota develops from aerobic and facultative anaerobic to an obligate anaerobic state. Thus, newborns do not exhibit an anaerobe gut environment as such. It needs to develop over time with the establishment of the microbiome (195). Therefore, some isolates did probably not succeed in colonizing the host. In summary, the colonization of freshly hatched chickens represented some difficulties that needs to be overcome in future experiments. Freeze-drying and indirect inoculation of encapsulated bacteria mixed into the animal feed, similar to faecal microbiota transplants in humans, is imaginable (231). This procedure would reduce stress directly after hatch and ensure bacterial viability.

In summary, first steps were taken towards the development of a minimal bacterial consortium, which may provide an appropriate “starter flora” to newly hatched chickens under commercial conditions. However, establishment of an optimal combination of bacteria will require a series of *in vivo* experiments with single strains and combinations thereof. Preferably, such studies would utilize germfree birds which can be re-constituted under defined conditions in isolators (213). Since there is only one GF facility for chickens available in the EU at INRA in Tours, France, the number of studies which can be conducted is highly limited. Alternatively, and as shown here, SPF facilities provide a highly clean environment and thus a good alternative to GF isolators. However, in order to maintain the SPF status, operators of such facilities will not allow the introduction of bacterial isolates into this clean environment. This leaves researchers in the field with the option utilized in this study to raise hatchlings in a clean but not SPF environment in close proximity to ensure equal housing conditions. Such an experimental setting will come with numerous disadvantages as discussed above but may prove to be the only way to progress with understanding host-microbiota interactions.

7 Summary

A working immune system plays a crucial role in maintaining animal health, improving animal wellbeing and reinforcing defense mechanisms. Especially in intensive livestock farming, an effective immune system is needed to control pathogen colonization and therefore, minimizing the use of antibiotics. The development and maturation of the immune system is highly dependent on microbial colonization of the gastrointestinal tract. Only few studies investigated the potential of chicken gut microbiota in influencing immune system development and function. Thus, an experimental model was established to simulate natural conditions by giving maternal microbiota to chickens directly at hatch. Compared to animals kept under SPF conditions, significantly higher concentrations of immunoglobulins IgA and IgY in plasma, bile and gut content were detected by ELISA. Gene expression analyses showed particularly immunologically relevant genes, including joining chain (JCHAIN, IgJ), immunoglobulin variable region gene (Ig V gene), IgA and IgY, to be upregulated in the maternal microbiota group. Additionally, the gut microbiome of these animals represented twice as much bacterial species and it was more diverse compared to the SPF control group. This was proven by 16S rRNA gene amplicon sequencing. In conclusion, this study demonstrates that the early colonization with a complex microbiota has substantial influence on the development and function of the intestinal immune system in chickens.

Since the first experiment was conducted in two different animal facilities to enable the comparison of the impact of a clean environment (without contact to maternal microbiota) and an environment with the presence of maternal microbiota, the study was repeated with a modified concept. For this purpose, animals were kept in the same facility but were spatially separated, with one group in contact to maternal microbiota directly at hatch and a control group without microbiota contact. The 16S rRNA gene amplicon sequencing revealed a similar microbiome composition as in the previous study. Significantly fewer bacterial species were observed in the group without maternal microbiota and the phylum *Firmicutes* made up to 95% of the total composition. However, significant differences in immunoglobulin A levels were not detected, but a tendency to increased IgA production in animals in contact with maternal microbiota was observed over time.

Further, initial experiments to develop a minimal bacterial consortium with the purpose of early gut colonization were conducted. The aim of such a colonization would be to promote the development and functions of mucosal tissues and of the mucosa-associated immune system. A chicken gut bacterial collection was established to provide isolates for the use in a minimal consortium. Through anaerobic cultivation, mass-spectrometric identification and sequencing-based verification, a total of 44 isolates were obtained and deposited at the German collection for microorganisms and cell cultures. Among these, seven are potentially new and so far,

undescribed isolates. Starting from 44 isolates, nine different bacteria were selected for the minimal consortium based on their properties and their phylogenetic placement.

In a final experiment, chickens were orally inoculated with the minimal consortium, while a negative control group obtained PBS and a positive control group received maternal microbiota. First, immunoglobulin A levels were significantly reduced in the PBS group, while no differences between the maternal microbiota group and the consortium group were detected. However, no significant differences between the groups were observed at a later time point. Significantly more bacterial species were detected in the maternal microbiota group, while the consortium group exhibited only few more species compared to the PBS group. Significant differences in the microbiome composition of the different groups could not be demonstrated at the phylum level. Successful colonization of the minimal consortium was only proven for five out of nine isolates. To improve the colonization rate and to achieve stronger effects on the mucosal immune system, the consortium needs further development in the future.

The results of this work underline the importance of diverse microbial colonization for the development of the gastrointestinal immune system in chickens. The presented approach of inoculation with a minimal bacterial consortium, if further developed consequently, will contribute to the improvement of gut health and animal wellbeing.

8 Zusammenfassung

Beeinflussung der Immunantwort in Hühner durch gezielte Darmbakterienintervention

Ein funktionierendes Immunsystem spielt eine entscheidende Rolle, um die Tiergesundheit zu erhalten, das Wohlbefinden zu steigern und Abwehrmechanismen zu stärken. Besonders in der intensiven Landwirtschaft wird ein effektives Immunsystem benötigt, um die Kolonisierung mit Pathogenen zu verhindern und damit den Antibiotikaverbrauch zu reduzieren. Die Entwicklung und Reifung des Immunsystems ist stark von der mikrobiellen Besiedelung im Magen-Darm-Trakt abhängig. Nur wenige Studien beschäftigen sich mit dem Potential der Darmmikrobiota Immunantworten in Hühnern zu beeinflussen. Aus diesem Grund wurde ein Modell etabliert um die Verhältnisse von Tieren unter natürlichen Bedingungen zu simulieren, indem maternale Mikrobiota den Küken direkt nach dem Schlupf zugänglich gemacht wurde. Im Vergleich mit Tieren aus einer SPF Haltung, wurden für diese Tiere signifikant höhere Immunglobulinspiegel für IgA und IgY in Plasma, Galle und Darminhalt mittels ELISA nachgewiesen. Genexpressionsanalysen zeigten, dass insbesondere immunologisch relevante Gene, wie die J-Kette (JCHAIN, IgJ), Immunglobulin variable Region (Ig V Gen), IgA und IgY in der Gruppe mit maternaler Mikrobiota hochreguliert waren. Zusätzlich beinhaltete das Mikrobiom dieser Tiere etwa doppelt so viele Bakterienspezies und war deutlich vielfältiger im Vergleich zur SPF-Kontrollgruppe. Dies wurde mittels 16S rRNA Gen Amplicon Sequenzierung nachgewiesen. Die Untersuchung zeigt, dass auch beim Huhn eine frühe Besiedelung mit einer komplexen Mikrobiota die Entwicklung und Funktion des Darmimmunsystems erheblich beeinflusst.

Da dieser Versuch aufgrund der Versuchsanordnung (SPF Haltung vs. Haltung auf einer Einstreu mit maternaler Mikrobiota) in zwei verschiedenen Tierhaltungsanlagen durchgeführt werden musste, wurde ein modifizierter Wiederholungsversuch durchgeführt. Hierzu wurden die Tiere im gleichen Stall, aber separiert gehalten, wobei eine Gruppe unmittelbar nach dem Schlupf Kontakt zu maternaler Mikrobiota, die Kontrollgruppe aber keinen Kontakt zu dieser hatte. Die 16S rRNA Gen Amplicon Sequenzierung ergab eine sehr ähnliche Zusammensetzung des Mikrobioms wie in der vorausgegangenen Studie. Es konnten signifikant weniger Bakterienspezies in der Gruppe ohne maternale Mikrobiota nachgewiesen werden und das Phylum *Firmicutes* machte über 95% der Gesamtzusammensetzung aus. Allerdings konnten keine signifikanten Unterschiede in den IgA-Immunglobulinspiegeln festgestellt werden. Es zeigte sich jedoch eine Tendenz zu einer verstärkten IgA Bildung über die Zeit bei den Tieren mit Kontakt zu maternaler Mikrobiota.

Basierend auf diesen Daten und auf Ergebnissen aus früheren Studien wurden im Weiteren erste Untersuchungen zur Entwicklung eines bakteriellen Minimalkonsortiums zur frühen Besiedelung des Darms durchgeführt. Ziel einer derartigen Besiedelung wäre es, die

Entwicklung und Funktion des mukosalen Gewebes und des Mukosa-assoziierten Immunsystems zu fördern. Es wurde eine Sammlung von Hühner-Darm-Bakterien angelegt, um Isolate für den Einsatz in einem Minimalkonsortium verfügbar zu machen. Mittels anaerober Kultivierungstechnik, massenspektrometrischer Identifikation und Sequenzierungs-basierter Verifikation konnten insgesamt 44 Isolate bei der Deutschen Sammlung für Mikroorganismen und Zellkulturen hinterlegt werden. Darunter befinden sich auch sieben potentiell neue Isolate, die bisher noch nicht beschrieben worden sind. Ausgehend von diesen 44 Isolaten wurden neun verschiedene Bakterien anhand ihrer Eigenschaften und ihres phylogenetischen Stammbaumes für ein Minimalkonsortium ausgewählt.

In einem dritten Versuch wurde das Minimalkonsortium oral an Küken verabreicht, während eine negative Kontrollgruppe PBS erhielt und eine positive Kontrollgruppe mit maternaler Mikrobiota eingestreut wurde. Die Immunglobulin A Spiegel ergaben zunächst signifikant niedrigere Werte für die PBS-Gruppe, während die maternale Mikrobiota Gruppe und die Konsortium Gruppe keine Unterschiede aufzeigten. Zu einem späteren Zeitpunkt konnten allerdings keine Unterschiede mehr zwischen den Gruppen festgestellt werden. Eine signifikant erhöhte Anzahl an Bakterienspezies konnte in der maternalen Mikrobiota Gruppe nachgewiesen werden, während nur etwas mehr Bakterienspezies in der Konsortium Gruppe im Vergleich zur PBS-Kontrollgruppe auftraten. Bedeutende Unterschiede in der Zusammensetzung der Mikrobiota in den verschiedenen Gruppen konnten auf Phylumebene nicht gezeigt werden. Eine erfolgreiche Kolonisierung mit dem Minimalkonsortium wurde nur für fünf von neun Isolaten nachgewiesen. Um in Zukunft eine bessere Kolonisierungsrate zu erreichen und stärkere Effekte auf das mukosale Immunsystem zu erzielen, muss das Konsortium weiterentwickelt werden und die Darreichungsform muss überdacht werden.

Die Ergebnisse dieser Arbeit unterstreichen die Bedeutung einer vielfältigen mikrobiellen Besiedelung für die Entwicklung des Darmimmunsystems in Hühnern. Durch eine konsequente Weiterentwicklung des hier vorgestellten Ansatzes zur Inokulation mit einem bakteriellen Minimalkonsortium kann zukünftig ein Beitrag zur Verbesserung der Darmgesundheit und des Wohlbefindens der Tiere erreicht werden.

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10 Attachments

a. Provider of Chemicals and Reagents

The respective providers were integrated as exponents in chapter 4 (Material and Methods) according to the following list:

1. Applichem GmbH
2. Calbiochem
3. Biochrom GmbH
4. Biotium Inc.
5. Carl Roth GmbH
6. Fischar GmbH
7. Hoffmann-La-Roche AG
8. Qiagen GmbH
9. Hyserve GmbH
10. ILMED Labor- und Medizintechnik GmbH
11. Invitek Molecular GmbH
12. Merck KGaA
13. Oxoid Deutschland GmbH
14. Sigma Aldrich GmbH
15. ThermoFisher Scientific Inc.
16. VWR International LLC
17. Southern Biotech
18. MP Biomedicals
19. Machery-Nagel
20. Bioline GmbH Germany
21. Biomers.net GmbH
22. Beckman Coulter Inc.
23. Illumina Inc.
24. Dunn Labortechnik GmbH
25. Bruker Daltonik GmbH
26. Eurofins Genomics Germany GmbH
27. Agilent Technologies Inc.
28. Promega GmbH

b. Laboratory equipment

Anaerobe cultivation:

- UNILab pro anaerobe workstation (custom-made), MBraun GmbH
- Incucenter IC80, Renggli AG Salvislab

Analytical Scale:

- Mettler AE 100, Mettler Toledo GmbH

Autoclaving:

- Varioklav 400E, ThermoFisher Scientific Inc.

Bioanalyzer:

- 2100 Bioanalyzer Instrument, Agilent Technologies Inc.

Centrifugation:

- Heraeus™Fresco™ Centrifuge, ThermoFisher Scientific Inc. (Heraeus)
- Eppendorf Centrifuge 5415R, Eppendorf AG

ELISA:

- Asys Atlantis Microplate Washer, Biochrom GmbH
- Sunrise Microplate Reader, Tecan Trading AG

Gel Imaging:

- UVP GelStudio SA, Analytik Jena AG

Homogenization:

- MP Biomedicals FastPrep 24® 5G, MP Biomedicals Germany GmbH
- Precellys® Homogenizer, Bertin GmbH

MALDI:

- MALDI-TOF Biotyper®, Bruker Daltonik GmbH

qPCR:

- Applied Biosystems 7300 Real Time PCR System, ThermoFisher Scientific Inc (Applied Biosystems)

Quantification:

- NanoDrop1000™, ThermoFisher Scientific Inc
- Qubit 4 Fluorometer, ThermoFisher Scientific Inc

Power Supply:

- Biometra Standard Power Pack P25T, Analytik Jena AG

Sequencing:

- Illumina® MiSeq™, Illumina® Inc

Thermocycling:

- Biometra Tadvanced Thermocycler, Analytik Jena
- T100™ Thermal Cycler, BioRad

Thermoshaking:

- Biometra TSC Thermo Shaker, Analytik Jena AG
- TS-100 Thermo Shaker, VWR International (Peqlab)

Vortexing:

- Vortex Genie 2, Scientific Industries Inc.
- Vortex Mixer IKA MS3, IKA® GmbH

c. Used Programs and websites for data analyses

- Apache OpenOffice Calc 4.1.6
  (available from: https://www.openoffice.org/de/download/)
- DEBrowser Pipeline for R
  (available from: https://bioconductor.org/packages/release/bioc/html/debrowser.html)
- EzBioCloud: www.ezbiocloud.net
- GO Enrichment: www.geneontology.org
- GraphPad Prism 6
- IMNGS: www.imngs.org
- MEGA 7 (available from: https://www.megasoftware.net/)
- RHEA pipeline for R (available from: https://github.com/Lagkouvardos/Rhea)
- R Studio Version 1.1.463
  (available from: https://rstudio.com/products/rstudio/download/)
- R Version 3.5.2 (available from: https://www.r-project.org/)

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