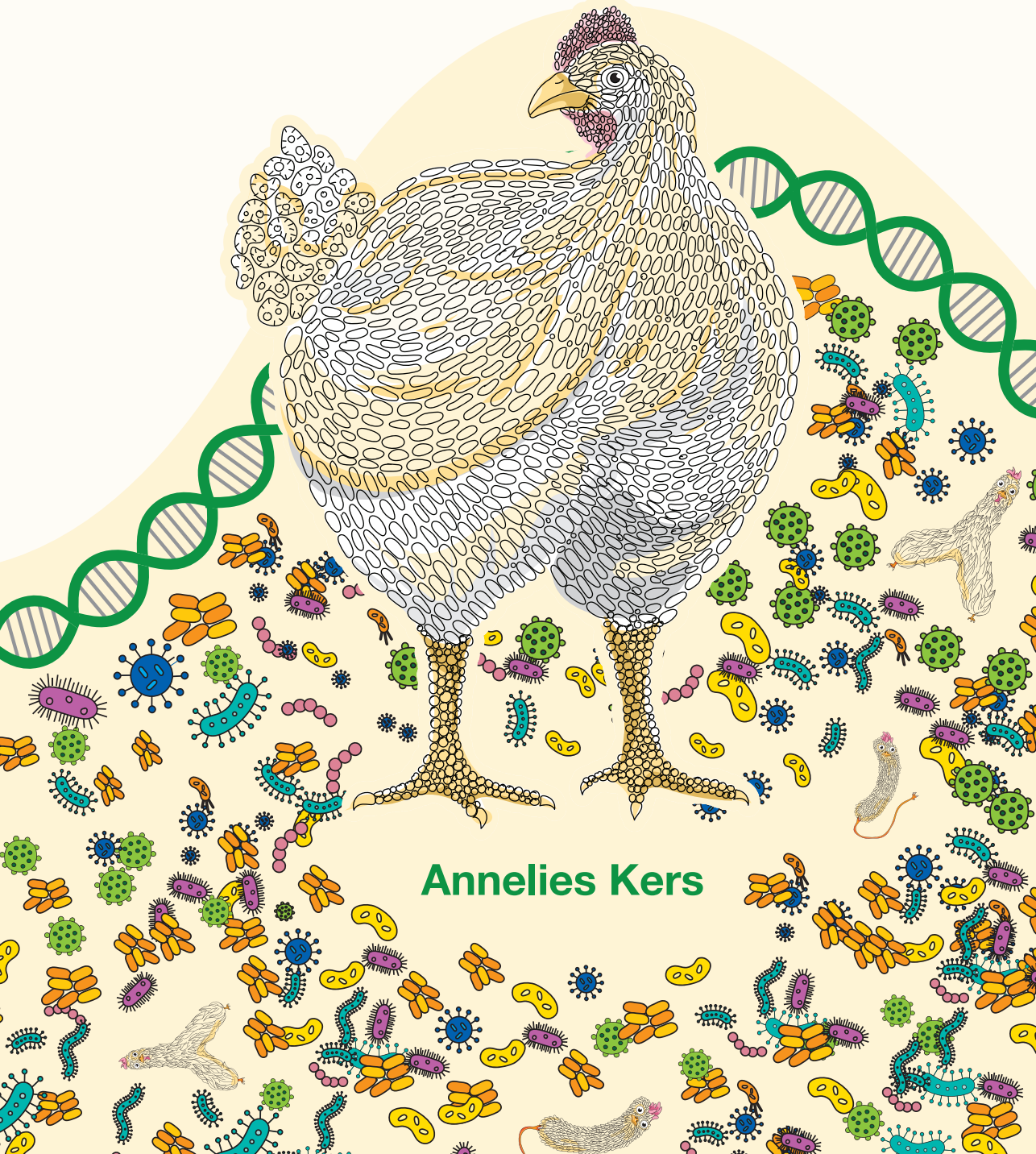


# Intestinal microbiota and broiler health: hype or hope?



Annelies Kers



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## **Colofon**

Intestinal microbiota and broiler health: hype or hope?

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# Intestinal microbiota and broiler health: hype or hope?

Intestinale microbiota en vleeskuiken gezondheid: hype of hoop?

(met een samenvatting in het Nederlands)

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*After nourishment, shelter and companionship,  
stories are the thing we need most in the world  
- Philip Pullman*

# CHAPTER 1

## General introduction

## **Why would we study broiler chickens?**

We, humans, have created through genetic selection, improved feed quality, and management a fast-growing, highly efficient broiler chicken. The growth rate and feed conversion ratio of broiler chickens have vastly improved over the last decades (Zuidhof et al., 2014). For example, the 28 day body weight of a 1957 Ross broiler strain was 316 gram, while the 2005 Ross broiler strain had a body weight of 1396 gram and currently Ross 308 standards indicate a body weight of 1573 gram (Aviagen, 2019, Zuidhof et al., 2014). Worldwide the poultry sector is the largest category of meat production and is expected to continue to grow in the coming decade (OECD, 2019). Broilers are the champions in meat production, and the conversion ratio of dietary feed ingredients into chicken meat is 1.6 after 42 days (Aviagen, 2019). In addition to their contribution to human food production, chickens are also used to perform groundbreaking research, for example, for the discovery of vitamin K (Nobel Price, 1943, Henrik Dam, Edward Doisy), and the discovery of the cellular origin of retroviral oncogenes (Nobel price, 1989, J. Michael Bishop, Harold E. Varmus). Thus, chickens are of great value for society and therefore it is of great importance to gain further scientific knowledge to improve broiler health.

## **The challenges of rearing broiler chickens**

Unfortunately, the high productivity of broilers also has some drawbacks. One of the drawbacks is the preventive use of antibiotics to achieve high production performances. Early in the 1950s, it was observed that livestock with antibiotic supplemented to the feed gained weight more efficiently. Antibiotic resistance, however, has become one of the biggest threats for global health, and therefore Europe banned in-feed antibiotics growth promoters (AGPs) from livestock feed in 2006. After the ban, intestinal health problems in broilers significantly increased, which also increased the risk of foodborne diseases in humans and the therapeutic use of antibiotics (Van Immerseel et al., 2004, Onrust et al., 2015, McEwen et al., 2017). Intestinal problems reported in broilers are often associated with a microbial imbalance, defined as a deviation from the composition observed in a healthy state, regularly referred to with the ill-defined terms 'dysbiosis' or 'dysbacteriosis' (Teirlinck et al., 2011, M'Sadeq et al., 2015, Ducatelle et al., 2018). Those problems result in reduced production performance, reduction of feed efficiency and animal welfare problems such as wet litter (Collett, 2012, Dunlop et al., 2016). Several studies have revealed that those intestinal health problems are correlated with the gut microbiota composition (Hofshagen and Kaldhusdal, 1992, Han et al., 2016a, Johnson et al., 2018). Therefore, knowledge of the development and variation of intestinal microbiota composition would be of great value for optimizing the resilience of broiler flocks and reduce the need for preventive and therapeutic antibiotics.



Increased knowledge of the working mechanisms underlying associations between intestinal microbiota and chicken health can thus have a large worldwide impact on public health and the sustainability of chicken production.

## Why should we study the intestinal microbiota?

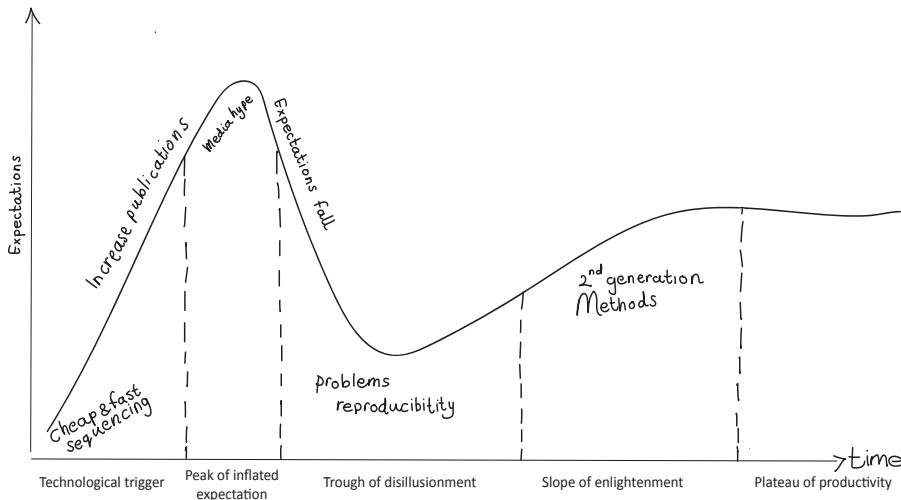
The intestinal microbiota in humans and animals has an important role in health and disease. For example, animals raised without microbes showed an underdeveloped immune system and are more susceptible to diseases (Williams, 2014). It has been shown that the intestinal development in high hygiene environments, such as isolators, negatively influences the development of the intestinal microbiota composition (Mulder et al., 2011, Inman et al., 2010). It also has been suggested that healthy microbiota may protect against colonization of pathogenic bacteria (Rolhion and Chassaing, 2016).

## What are microbes, microbiome and microbiota?

The word microbe is derived from the Greek words μικρός (mikrós, “small”) and βίος (bíos, “life”), small life (Berg et al., 2020). But what is the definition of a microbiome or microbiota? Before I was born the microbiome was defined “as a *characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties*” (Whipps et al., 1988). The term thus not only refers to the microorganism involved but also encompasses their theatre of activity (Whipps et al., 1988). More than a decade later the microbiome is described within a certain environment: “A *microbiome is an ecological community of commensal, symbiotic and pathogenic microorganisms within a body space or the environment*” (Lederberg J, 2001). In 2015 it was defined in much more detail: “*The term microbiome refers to the entire habitat, including the microorganisms (bacteria, archaea, lower and higher eukaryotes, and viruses), their genomes (i.e., genes), and the surrounding environmental conditions. The microbiome is characterized by the applications of one or combinations of metagenomics, metabolomics, metatranscriptomics, and metaproteomic combined with clinical or environmental metadata*” (Marchesi and Ravel, 2015). This is quite a comprehensive definition while in general, most recent publications on the microbiome are based on 16S Svedberg ribosomal RNA (16S rRNA) gene amplicon sequencing, and only look at bacteria and archaea, and not at fungi, protists, viruses, and phages, like in this thesis. In published microbiota research, the term microbiome is often used synonymously with the term microbiota, however, there are important differences. Often, if only 16S rRNA sequencing is used, and when the microorganisms are found within a specific environment, the term microbiota should be used. The term microbiome, in turn, is used to describe the collection of genomes from all the microorganisms and their “theatre of activity” in the environment (Berg et al., 2020).

### What is the current state of microbiota research?

A new technology or research field follows a certain track of development. The Gartner hype cycle is a model that explains the process of introducing a new technology in the market and can also be applied to the scientific world. This hype cycle describes the maturity of emerging technologies through five phases (Fenn and Raskino, 2008). Figure 1 shows how the phases of the hype cycle can be applied to microbiota research.



**Figure 1:** The Gartner hype cycle, adapted from Fenn and Raskino, 2008.

It all started with a *Technological Trigger*: In the past, microscopes and cell culture techniques were used to study microbes. However, many microbes cannot be culture-grown under laboratory conditions and this limits research in microbiology. By using DNA-based technologies the non-culturable microbes can be studied as well. Those molecular tools of modern microbial ecology are currently mainly based on 16S rRNA gene amplicon sequencing and are used to identify bacterial DNA within a sample. Therefore, molecular genetics-based approaches, such as sequencing, have resulted in new research opportunities to study microbes. As the cost of sequencing has decreased substantially, it has become more and more affordable to study the phylogeny and taxonomy from complex microbiomes. Also, the sequencing time has dramatically dropped, as in 2000 the draft of the human genome was finished after 13 years, whereas currently, it will just take an hour to sequence it (NIH, 2019). This resulted in the possibility to study a large number of samples and changed the focus of microbiota research towards population research as ecology and epidemiology.

This led to the second stage, *the Peak of Inflated Expectations*: In 2009 there were

just 260 publications with microbiome or microbiota in the title, while ten years later this number is 5777\* (\*NCBI, PubMed, microbiota [ti] OR microbiome [ti]). Microbiota research is a rising star. This is the stage during which several success stories regarding microbiota research are shared, but not many researchers or companies are involved in the research field. This is a period of heightened expectation, as in high impact journals it was published that patients with e.g. an inflammatory bowel disease as Crohn's disease or ulcerative colitis, have a different intestinal microbiota compared to healthy controls (Manichanh et al., 2006, Qin et al., 2010). In this stage, also the media show interest, although it might be unrealistic and overly optimistic. This period may last for several months or even several years until the expectations change. Between 2009 and 2019 The New York Times published 53 papers with microbes, microbiota, microbiome or bacteria-I in the title (Table 1), with just one publication in 2009 and the largest number of publications in 2018.

Then the third phase starts, *Trough of Disillusionment*: the expectations begin to fall sharply because of unpromising experimental results or the failure of commercialization efforts. Often media's interest wanes, and the media become skeptical of technology's market value. In the case of microbiota research, the interest of the media seems to have slightly decreased in 2019 but has not become negative or skeptical (Table 1). However, as contradicting observations have been reported in scientific publications, there is a critical discussion within the scientific world about the utility of microbiota research (Pollock et al., 2018). For example, in some studies, a reduced ratio of the bacterial phyla Bacteroidetes and Firmicutes was associated with obesity (Bervoets et al., 2013, Kasai et al., 2015), whereas other studies reported an opposite association (Xu et al., 2012, Armougom et al., 2009) or no association at all (Hu et al., 2015, Mbakwa et al., 2018). Also, in chickens a highly variable intestinal microbiota development has been observed between repeated experiments (Stanley et al., 2013). Another problem is the fallacy that correlation is not equivalent to causality, e.g. that patients with inflammatory bowel disease have a different intestinal microbiota compared to healthy persons. Does the microbiota cause inflammatory bowel disease or do those patients have a different microbiota because they have a disease and therefore a different diet? Still, the resulting differences in microbiota could contribute to perpetuating the disease, even if it is not causally responsible for its onset. Particularly questions addressing causal relationships are not that easy to answer in humans. In addition, because of the hype, many people want to perform microbiota research, but some may not have the experience yet to accurately perform this kind of research, let alone correctly interpret what they find. This may result in semi-optimal experimental designs, misleading or incorrect conclusions, and has led to problems with reproducibility of scientific research. The hype has also led to a wide range of data analysis methods, which have a great impact on study outcomes and the interpretation (Schloss, 2009, Salter et al., 2014,

Caruso et al., 2019). These method-linked differences in microbiota research make it difficult to disentangle biological and technical variation when comparing results across studies. Currently, it is not clear which factors are the most important drivers that influence microbiota research results.

**Table 1:** Overview of publications in The New York Times between 2009 and 2019, with microbes, microbiota, microbiome or bacteria-I in the title. More articles related to microbiota research have been published in this newspaper within this time frame, but titles only including terms as 'germs', 'fecal transplants', 'diet', 'probiotics', 'antibiotics' or 'stool', have been excluded from this summary.

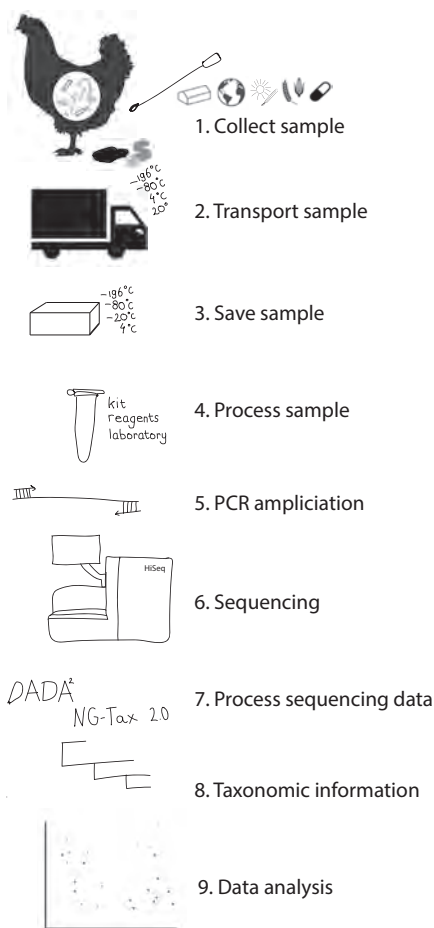
New York Times – headings	Date
When the Menu Turns Raw, Your Gut Microbes Know What to Do	Oct. 23, 2019
Postpartum Peeing, The Microbiome!? and Daylight Saving Strategies	Oct. 19, 2019
Seeking an Obesity Cure, Researchers Turn to the Gut Microbiome	Sept. 10, 2019
Could a Gut Bacteria Supplement Make Us Run Faster?	June 26, 2019
Breast Milk Is Teeming With Bacteria — That's Good for the Baby	June 12, 2019
Germs in Your Gut Are Talking to Your Brain. Scientists Want to Know What They're Saying.	Jan. 28, 2019
Does the Gut Microbiome Ever Fully Recover From Antibiotics?	Dec. 21, 2018
The Ecosystem in Immigrants' Guts Is Shaped by the Place They Call Home	Nov. 8, 2018
Scientists Are Retooling Bacteria to Cure Disease	Sept. 4, 2018
Keep Your Gut Healthy and Your Skin May Follow	Aug. 27, 2018
The Bacteria Babies Need	June 17, 2018
Your Cute Rubber Duck May Be a Haven for Bacteria	March 28, 2018
C-Sections and Gut Bacteria May Contribute to Overweight Kids	Feb. 28, 2018
The Importance of Infants' Exposure to Micro-Organisms	Feb. 5, 2018
Gut Microbes Combine to Cause Colon Cancer, Study Suggests	Feb. 1, 2018
The Weekly Health Quiz: Calories, Exercise and Gut Bacteria	Jan. 5, 2018
Exercise Alters Our Microbiome. Is That One Reason It's So Good for Us?	Jan. 3, 2018
Unlocking the Secrets of the Microbiome	Nov. 6, 2017
Gut Bacteria May Play a Role in Weight Loss	Sept. 15, 2017
Gut Bacteria Can Fluctuate With the Seasons	Aug. 24, 2017
Does Colon Cleansing Wipe Out Our Gut Microbiome?	Aug. 11, 2017
What Does Your Microbiome Say About You?	July 31, 2017
Lovers Share Colonies of Skin Microbes, Study Finds	July 31, 2017
Can I Test the Health of My Gut Microbiota?	July 7, 2017
A Baffling Brain Defect Is Linked to Gut Bacteria, Scientists Say	May 10, 2017
Exciting Microbe Research? Temper That Giddy Feeling in Your Gut	March 6, 2017
Microbes, a Love Story	Feb. 10, 2017

A Gut Makeover for the New Year	Dec. 29, 2016
Your A.T.M. Is Covered in Microbes, but Mostly Harmless	Nov. 18, 2016
Gut Bacteria Are Different in People With Chronic Fatigue Syndrome	July 7, 2016
Federal Microbiome Project Aims to Solve Tiny Riddles of Science	May 12, 2016
40 Trillion Bacteria on and in Us? Fewer Than We Thought.	Feb. 15, 2016
Scientists Urge National Initiative on Microbiomes	Oct. 28, 2015
Can the Bacteria in Your Gut Explain Your Mood?	June 23, 2015
Hidden Life Forms: Investigating Microbial Diversity on Our Bodies and in Our Homes	March 25, 2015
Among New York Subway's Millions of Riders, a Study Finds Many Mystery Microbes	Feb. 5, 2015
There Is No 'Healthy' Microbiome	Nov. 1, 2014
Fighting Poisons With Bacteria	Sept. 15, 2014
Rice Has a Microbiome	Sept 15, 2014
Our Microbiome May Be Looking Out for Itself	Aug. 14, 2014
We Are Our Bacteria	July 14, 2014
My No-Soap, No-Shampoo, Bacteria-Rich Hygiene Experiment	May 22, 2014
Gut Bacteria From Thin Humans Can Slim Mice Down	Sept. 5, 2013
Human Microbiome May Be Seeded Before Birth	Aug. 29, 2013
Bacteria in the Intestines May Help Tip the Bathroom Scale, Studies	March 27, 2013
Better Eating, Thanks to Bacteria	Sept. 17, 2012
Tending the Body's Microbial Garden	June 18, 2012
In Good Health? Thank Your 100 Trillion Bacteria	June 13, 2012
Our Microbiomes, Ourselves	Dec. 3, 2011
In Some Cases, Even Bad Bacteria May Be Good	Oct. 31, 2011
Bacterial Ecosystems Divide People Into 3 Groups, Scientists Say	April 20, 2011
How Microbes Defend and Define Us	July 12, 2010
Microbes 'R' Us - <i>The human body is home to a vast array of bacteria. So what are they up to?</i>	July 21, 2009

After the trough of disillusionment phase, the *Slope of Enlightenment* starts: This is the stage in which there is a comprehensive understanding of how a new technology can be of value e.g. for public health. Often second-generation and third-generation products or methods begin to emerge with enhanced features. This is also the case in microbiota research as second-generation bioinformatics tools have been developed. In 2010 an open-source bioinformatics pipeline was developed for performing microbiota analysis from raw DNA sequencing data, Quantitative Insights Into Microbial Ecology (QIIME) 1 (Caporaso et al., 2010). Currently QIIME 1 is no longer supported because development and support are entirely focused on its successor, QIIME 2. Also, other analysis methods for high-throughput community sequencing

data were further developed, such as Mothur (Schloss et al., 2009), an *open-source, platform-independent, community-supported software for describing and comparing microbial communities*. And new methods, such as NG-tax: *a highly accurate and validated pipeline for analysis of 16S rRNA amplicons from complex biomes* (Ramiro-Garcia et al., 2016), and DADA2: *High resolution sample inference from Illumina amplicon data* (Callahan et al., 2016), and many others were developed (Edgar, 2013, Allali et al., 2017). More insight on how to conduct robust microbiota research has been developed over the past years (Laukens et al., 2016, Knight et al., 2018). Although many factors that influence the outcome of these analyses are currently still unknown, some factors are known to influence the outcome of the microbiota composition analysis. Figure 2 shows a schematic view of a routine 16S rRNA gene sequencing workflow, with important factors that warrant careful attention before the start of research. The choices to make depends on the research questions but also on the feasibility of the sample collection.

1. First, the sample collection: it is important to define potential confounders like age, sex/gender, sample type, housing conditions, location, season or temperature, feed and medication use (Kers et al., 2018, Knight et al., 2018)
2. Sample transport: the composition of a fresh sample can be affected by the growth of certain micro-organisms during transport but freezing samples might change the ratio between microbes (Choo et al., 2015, Vandeputte et al., 2017)
3. Sample storage: in glycerol, 95% ethanol or a commercial product, the duration and condition of storage can influence the observed microbiota composition (Lauber et al., 2010, Vandeputte et al., 2017, Pollock et al., 2018)



**Figure 2:** Schematic illustration of the basic workflow of 16S rRNA amplicon sequencing

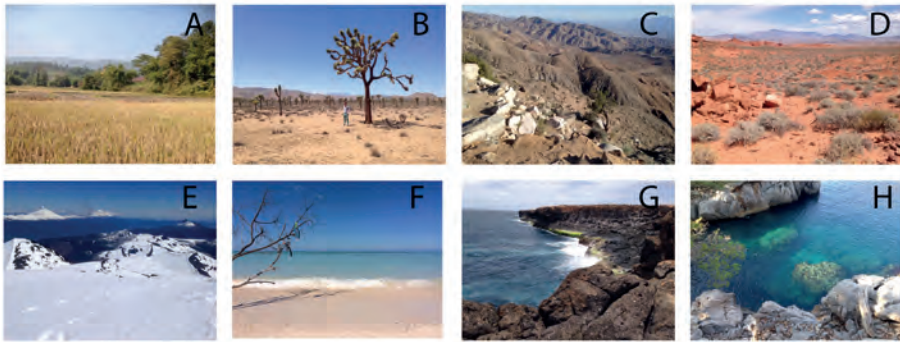
4. Standardizing technical factors and sample processing as e.g. DNA isolation, to control for variation introduced by kits, reagents, bead-beating steps or contamination of the lab environment
5. Different primers used to amplify bacterial 16S rRNA gene are more sensitive to observe certain microbes, also the number of PCR cycles is important to standardize
6. Sequencing 16S rRNA genes on different types of platforms often limits the possibilities to compare data between studies, as it is difficult to compare new to older platforms (D'Amore et al., 2016)
7. The raw sequence data needs to be aligned to a reference database for taxonomic classification. Different databases result in different research results (Schloss, 2009)
8. Then, community and phylogenetic analysis of sequences can be started
9. Finally, multivariate data analysis is needed to answer research questions

The Plateau of Productivity starts: This is the stage in which the product is widely recognized as useful and marketable. One can argue whether this stage has been reached for the use of fecal microbiota transplantation as treatment of recurring and refractory infection with *Clostridioides difficile* (Quraishi et al., 2017). It has shown to be an effective method, but fecal microbiota transplantation is still not widely applied in clinical practice. One of the reasons is that screening for a healthy donor requires extensive and expensive testing. Therefore, the first stool-transplant bank has been opened in 2016 at Leiden University Medical Center, in the hope to improve the efficacy of fecal microbiota transplantation. In poultry, however, stool-transplant-like products are commercially available, defined as competitive exclusion products, to help establish or restore a healthy microbiota in chickens and to compete with colonization by pathogenic bacteria (Nurmi et al., 1992, Mead, 2000). In part III of this thesis, a competitive exclusion product is used as tool. In sum, defining the current state of microbiota research is not that easy, but presumably we are not yet at the plateau of productivity yet.

### Comparing ecosystems to characterizing the microbiota

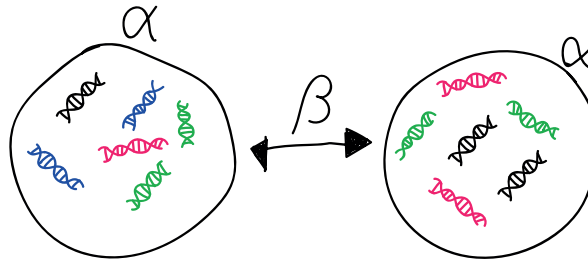
After obtaining the taxonomic data, the question often is whether there are differences between the microbiota of different experimental groups, e.g. chickens fed different diets or kept in different environments. There are many ways to compare an ecosystem. Figure 3 shows eight different photos of an ecosystem. A common question is which ecosystem has the lowest diversity? This is probably photo E, snowy mountains, but which ecosystem has the highest diversity? Photo A is a forest and photos F, G and H are different seas. Probably the absolute number of individuals is highest in a sea due to the larger area, but the number of

different species is probably highest in the forest. And which of these ecosystem photos are most similar, those of the seas or the mountains? This example indicates



**Figure 3:** Eight different ecosystems, credits G. Koop.

it is not easy to compare ecosystems. Two commonly used measurements to define diversity are the alpha diversity and beta diversity. The alpha diversity measurement is used to define how ‘complex’ an ecosystem is. If many taxa are present this results in a higher alpha diversity and this means a more complex ecosystem. The presence of taxa can be defined on e.g. the richness, evenness or phylogenetic diversity of the taxa. The beta diversity is used to define how similar two ecosystems are, to indicate which taxa are shared, including the abundance of each of these taxa. Identical ecosystems have a beta diversity of 0, and ecosystems that are completely different have a beta-diversity of 1 (Figure 4).

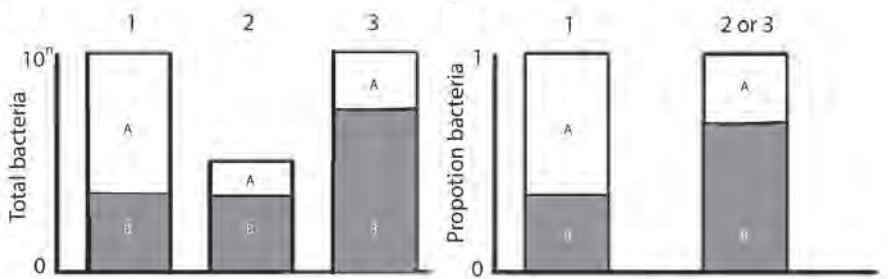


**Figure 4:** Systematic overview of beta- and alpha-diversity

In addition to different diversity measures, statistical methods are often used to answer research questions, and with new techniques like 16S rRNA amplicon sequencing, new statistical methods are needed as well. This type of data is preprocessed into relative abundance of bacteria (Figure 5). Many statistical methods assume that sequencing data are equivalent to ecological data where the counts of reads assigned to organisms are often normalized to a constant area or volume (McMurdie and Holmes, 2013, Weiss et al., 2017, Gloor et al., 2017).



Nevertheless, the relative abundance of 16S rRNA data is not truly independent, because the abundance of the taxa is calculated using all other taxa, which is an issue for many statistical tests (Gloor et al., 2017). Statistical testing is based on rejecting the null hypothesis of ‘no association’. However, if also many associations are tested, which is the case within microbiota research, the probability of incorrectly rejecting a null hypothesis is increased, with potentially many ‘false positive’ associations (type 1 errors) as a result of chance. Therefore, it is important to correct for multiple testing. An example where it is often applied is in genome-wide associations studies (GWAS), as in this field Bonferroni correction is commonly  $5 \times 10^{-8}$ , obtained by dividing 0.05 by 1,000,000 assessed single-nucleotide polymorphism (SNPs) (Risch and Merikangas, 1996). This is based on an estimate of the number of independent SNPs, contrary to the total number of SNPs. Bonferroni’s correction, however, is too strict for microbiota data, and similar strategies as in GWAS are needed (Jiang et al., 2017). In microbiota research, clearly, there is a need for rigorous statistical methods (Willis, 2019).

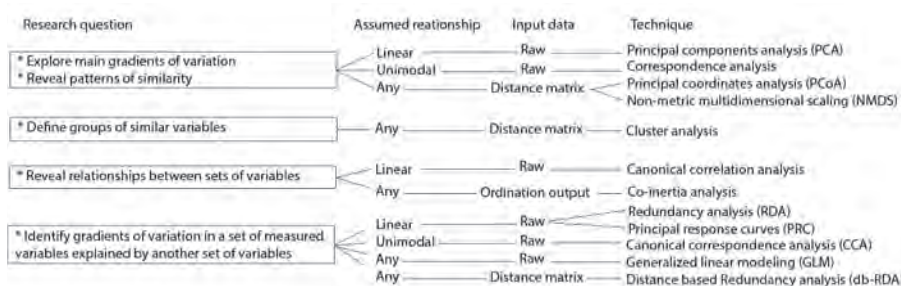


**Figure 5:** Credits Gloor et al., 2017. “The bar plots show the difference between the total count of and the proportion of bacteria for two different taxa, A (white) and B (gray) in three samples. The left bar graphs show the total counts for three samples, and the height of the color illustrates the total count of the taxa. When the three samples are sequenced, we lose the absolute count information and only have relative abundances, proportions, or “normalized counts” as shown in the right bar graph. The taxa A and B in samples 2 and 3 appear with the same relative abundances, even though the counts in the environment are different.”

Nevertheless, one test that is often used is the Permutational analysis of variance (PERMANOVA) test. This tests location effect (‘means’), defined as if the centroid of a group of samples for one group differs from the centroids of the samples of the other group and assumes equal dispersions (‘variance’). The limitation is that if groups have different dispersions,  $p$ -values are not adequate. This is not a problem if differences in dispersion matter as much as differences in location. Another limitation is that this test is only for categorical data. A continuous variable as body weight cannot be included in the test. Also, the available statistical tools for longitudinal data analysis are far away from meeting the needs of modeling the dynamic microbiota data (Xia and Sun, 2017).

Moreover, to observe an association between groups, the sample size needs to be large enough. Power and sample size determination are important for decent

study designs to reduce the probability of concluding that groups are not different when they are ('false negative' as a result of underpowered design). There is not that much known about multivariate statistics and sample size calculations for this type of data. To develop power calculation tools for microbiota studies, two questions need to be answered. First, how much of a difference between the microbiota compositions does one need to be able to differentiate two groups? Second, how much of an effect size is biologically relevant (Hanson and Weinstock, 2016)? Another challenge comes from the often relatively small sample sizes of the current microbiota studies which is usually approximately 100-200 individuals or less. The sample sizes necessary to detect significant associations in many GWAS of common diseases are thousands to tens of thousands of individuals (Davenport et al., 2015, Dabrowska and Witkiewicz, 2016). Although limited, there are specific statistical tools for microbial ecology data. Paliy and Shankar (2016) reviewed applications of multivariate statistical techniques. Figure 6 was adapted from this review, and describes the potential multivariate techniques based on the research goal, assumed relationship among variables, and input data structure. Across this thesis different multivariate techniques have been used to answer the research questions.



**Figure 6:** Credits Paliy and Shankar 2016, diagram of potential choices of multivariate techniques based on the research goal, assumed relationship among variables, and input data structure.

## Broiler chickens and intestinal microbiota

From an ecological perspective, the intestines of poultry are a rather selective habitat (Cressman et al., 2019). It is important to be aware of the structural differences of the intestinal tract of poultry compared to that of mammals. For example, the intestinal tract of poultry is shorter (relative to the body length) than that of most mammals. Consequently, the digesta passes the intestinal tract faster, resulting in shorter retention time in poultry (5–6 h in total) (Shires et al., 1987). Also, in poultry the pair of ceca harbor the highest diversity of the intestinal microbes (Salanitro et al., 1974, Gong et al., 2007a, Owens et al., 2008, Stanley et al., 2014a). Much has been learned about the effect of diet and antimicrobial growth promoters (AGP), but much remains to be learned about how factors modulate the development and eventual assemblage of the intestinal microbiota.

## Objectives and research questions

The overall objective driving the research described in this thesis is to improve the understanding of the interaction between the intestinal microbiota and broiler health. The main three research questions that will be addressed in this thesis are:

1. How should we design intestinal microbiota poultry research?
2. Which phenotypes are related to the intestinal microbiota composition and variation?
3. Can the intestinal microbiota be manipulated to improve broiler health and reduce the chance of colonization of pathogens?

## Aim and outline of this thesis

The first part of the thesis is about how to design intestinal microbiota research in poultry. In Chapter 2 we review the available literature with respect to the potential host and environmental factors affecting the intestinal microbiota in chickens and we show that microbiota research has to deal with many hidden host and environmental variables, which are not all known. Chapter 3 describes investigations on how one of those variables, i.e. housing conditions, affects the interplay of a nutritional intervention and the intestinal microbiota of broiler chickens. In this chapter a feed with and without medium-chain fatty acids (MCFAs) is used as a tool to generate differences in cecal microbiota composition between the chickens in different pens within a housing condition. Chapter 4 evaluates different invasive and non-invasive sampling methods to characterize gut microbiota composition throughout a production cycle of broiler chickens.

The second part of this thesis focuses on the development and the variation of the cecal microbiota in individual broilers, flocks and farms. We conducted a field survey and collected phenotypic characteristics of broilers raised across different broiler farms and poultry houses.

Chapter 5 describes a detailed study on two broilers farms and used clinical scoring systems, such as a morphometric evaluation of 'dysbacteriosis', i.e. a 'gut score' (Teirlynck et al., 2011) and the *Eimeria* lesion score (Johnson and Reid, 1970) for intestinal lesions associated with the disease coccidiosis, to observe if there is a correlation between microbiota composition and clinical scoring systems. In Chapter 6, we explore characteristic microbial community types (clusters) in the cecal microbiota of broiler chickens. In addition, we investigate factors that explain the variation in microbiota composition within and between broiler flocks.

In the final part of this thesis, part III, we move from observational studies to intervention studies to manipulate the intestinal microbiota in broilers. In Chapter 7, we manipulate the intestinal microbiota with a competitive exclusion product under experimental circumstances to reduce the spread of an Extended Spectrum

Beta-Lactamase (ESBL)-producing *Escherichia coli* at flock level. This product is also used as a tool to test the effect of differences in initial colonization on the development of the intestinal microbiota and the activation of the innate immune system (Chapter 8). In Chapter 9, the same competitive exclusion product and nutritional intervention with MCFA are used to alter the intestinal microbiota and explore its effect under an intestinal challenge with *Eimeria maxima* and *Clostridium perfringens*.

Chapter 10 concludes with a general discussion of this thesis in a broader context, and offers directions for future research and topics of debate.



# PART I

How to design intestinal  
microbiota poultry  
studies?







# CHAPTER 2

## Host and environmental factors affecting the intestinal microbiota in chickens

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## Abstract

The initial development of intestinal microbiota in poultry plays an important role in production performance, overall health and resistance against microbial infections. Multiplexed sequencing of 16S ribosomal RNA gene amplicons is often used in studies, such as feed intervention or antimicrobial drug trials, to determine corresponding effects on the composition of intestinal microbiota. However, considerable variation of intestinal microbiota composition has been observed both within and across studies. Such variation may in part be attributed to technical factors, such as sampling procedures, sample storage, DNA extraction, the choice of PCR primers and corresponding region to be sequenced, and the sequencing platforms used. Furthermore, part of this variation in microbiota composition may also be explained by different host characteristics and environmental factors. To facilitate the improvement of design, reproducibility and interpretation of poultry microbiota studies, we have reviewed the literature on confounding factors influencing the observed intestinal microbiota in chickens. First, it has been identified that host-related factors, such as age, sex, and breed, have a large effect on intestinal microbiota. The diversity of chicken intestinal microbiota tends to increase most during the first weeks of life, and corresponding colonization patterns seem to differ between layer- and meat-type chickens. Second, it has been found that environmental factors, such as biosecurity level, housing, litter, feed access and climate also have an effect on the composition of the intestinal microbiota. As microbiota studies have to deal with many of these unknown or hidden host and environmental variables, the choice of study designs can have a great impact on study outcomes and interpretation of the data. Providing details on a broad range of host and environmental factors in articles and sequence data repositories is highly recommended. This creates opportunities to combine data from different studies for meta-analysis, which will facilitate scientific breakthroughs towards nutritional and husbandry associated strategies to improve animal health and performance.

**Keywords:** gut microbiota, poultry, confounding factors, microbiome, gut health, 16S rRNA

## Introduction

In recent years several articles have been published on the intestinal microbiota composition of chickens and its associations with production and health (Nava et al., 2007, Brisbin et al., 2008, Kohl, 2012, Stanley et al., 2012a, Yeoman et al., 2012). For instance, some studies have described differences in bacterial species abundance for broilers with high versus low growth and feed efficiency (Stanley et al., 2012a, Singh et al., 2014). Another important topic in microbiota research is *Clostridium perfringens*-associated necrotic enteritis that can cause severe production losses and disease in broilers, and can cause foodborne illness in humans (Hook et al., 1996, Van Immerseel et al., 2004). Necrotic enteritis is associated with perturbations in microbiota composition, but whether these are cause or effect of *C. perfringens* proliferation remains unclear (Stanley et al., 2012b, Antonissen et al., 2016, Moore, 2016). Also, the ban on antibiotic growth promoters in the European Union has prompted research into developing alternative nutritional strategies aiming at stimulation of beneficial microbiota in chickens (Stanley et al., 2014a). These examples illustrate that it is essential to increase our biological understanding of the host-microbe interactions, which may eventually result in effective strategies to promote sustainable poultry production.

Although much progress has been made in this rapidly expanding research field, researchers using next generation sequencing (NGS) tools have reported large differences in microbiota composition across and within studies (Stanley et al., 2013, Brooks et al., 2015, Reardon, 2016). A meta-analysis of gut microbiota studies across different avian species showed that a large factor contributing to the observed variation in avian intestinal microbiota composition was the study itself (Waite and Taylor, 2014). Within the same study, differences in intestinal microbiota composition may also occur across independent poultry trials, even when the research conditions are carefully controlled and intended to be similar across trials (Stanley et al., 2013, Thibodeau et al., 2017). Comparison of the outcomes of microbiota studies might be hampered by differences in technical aspects, biological variation within and between hosts, and environmental factors (Lozupone et al., 2013, Brooks et al., 2015, Laukens et al., 2016). Multiplex sequencing of 16S ribosomal RNA (rRNA) gene amplicons, which is often used to profile the composition of the intestinal microbiota, is associated with technical variation. Differences in the sequencing platforms used, the choice of PCR primers and corresponding region to be sequenced, the number of PCR cycles, DNA extraction protocols, and the storage of samples can create variation in outcomes between studies. These technical factors have been reviewed previously (Pissavin et al., 2012, Lozupone et al., 2013, Brooks et al., 2015, Hermes et al., 2015, Laukens et al., 2016, Allali et al., 2017), and are therefore beyond the scope of this review. The aim of this review is to provide an overview of poultry-specific host and environmental factors that

affect the composition of the intestinal microbiota of poultry, and to create awareness of confounding factors in poultry microbiota studies. Knowledge of these factors will enable the improvement of design and reproducibility of outcomes of poultry microbiota studies. An overview of biological and environmental factors potentially influencing chicken microbiota composition described in the literature is shown in Figure 1. In the following sections, known effects of host characteristics and environmental factors on intestinal microbiota will be described, followed by a discussion of the potential implications of these confounding factors for microbiota research in poultry.

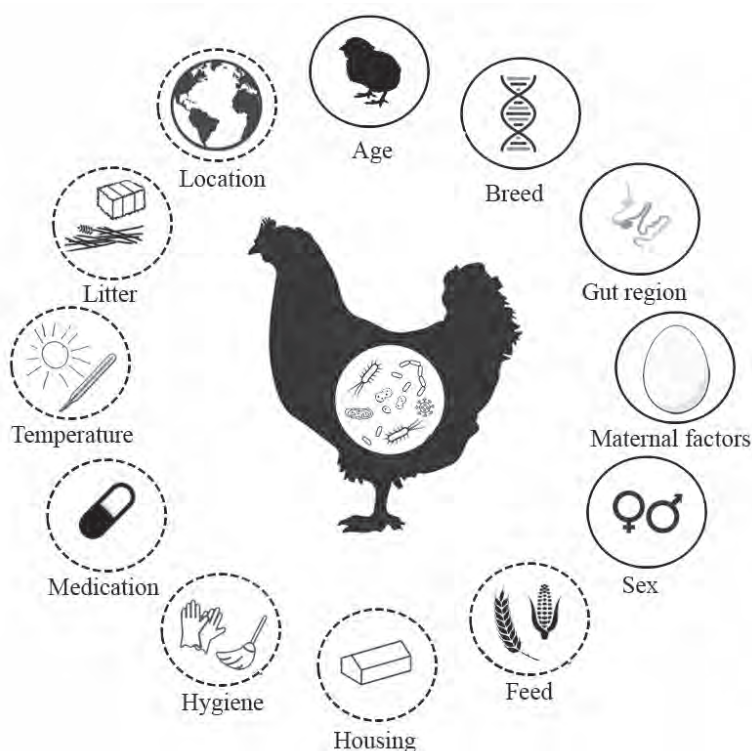
## **Host characteristics influencing intestinal microbiota in poultry**

### ***Development of the chickens***

One day-old broiler chicks already carry a community of microorganisms in their intestinal tract (Ballou et al., 2016). Microorganisms can be acquired in the pre-hatching phase, directly from the mother in the oviduct of the hen (Gantois et al., 2009) or from the environment through the pores in the eggshell (Cason et al., 1994, Roto et al., 2016). In a recent publication it was shown that broiler eggs contaminated with cecal microbiota on the egg surface of other birds reduced the bird-to-bird variation in the cecal microbiota composition after hatch but not the composition itself (Donaldson et al., 2017). This means that the cecal microbiota on the egg surface resulted in more similarity between the microbiota samples of the individual broilers, but the microbiota of the donors associated with high or low performance was not actually transferred to the newly hatched broilers. After hatch, the young chicks might be colonized before arriving at the farm by microbiota from the environment at the hatchery or during transport (Shapiro et al., 1949, Pedroso et al., 2005).

The microbiota composition may also be influenced by maternal antibodies supplied through the yolk. Maternal antibodies can provide protection against colonization by certain pathogens generally until two weeks post-hatch (Grindstaff et al., 2003, Hamal et al., 2006), and this may affect the chicks' intestinal microbiota. In mammals it is known that maternal antibodies can affect the interaction between intestinal bacteria and the immune system (Cebra, 1999). Although the mechanism behind the interaction of bacteria and the immune system is not exactly clear, the altered development of the immune system in germ-free animals suggests that it is at least partly shaped by the microbiota (Williams, 2014).

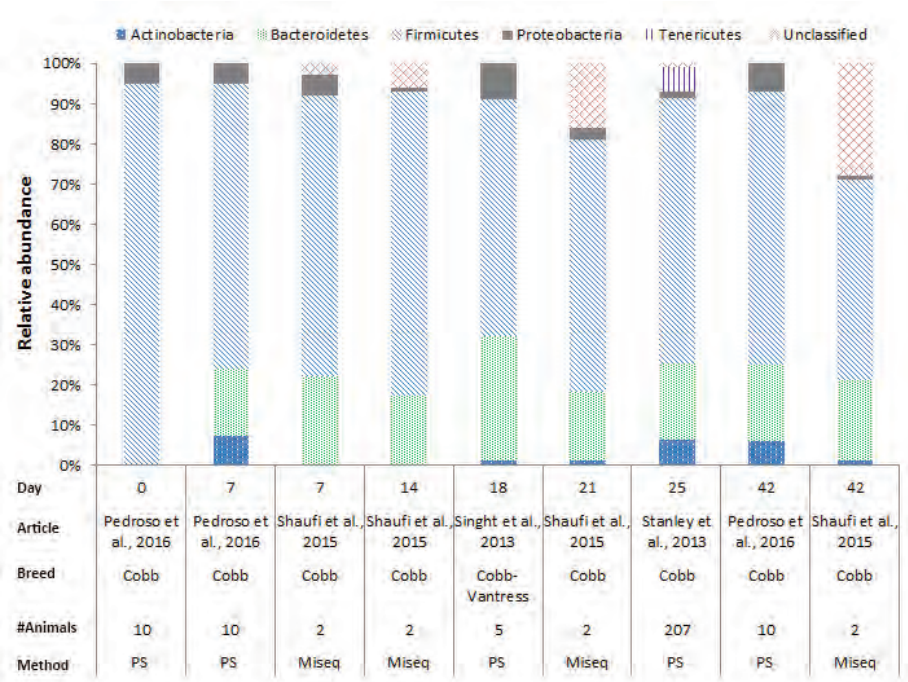
In chickens the intestinal microbiota richness, i.e. the number of different microbial taxa, increases during the first weeks of life (Gerard et al., 2008, Danzeisen et al., 2011, Ballou et al., 2016), while the individual variation in microbiota composition decreases as the chickens age (Crhanova et al., 2011). A compilation of 16S rRNA gene amplicon sequencing data from cecal samples of two different broiler breeds (meat



**Figure 1:** Factors that affect the intestinal microbiota composition of chickens. Factors found in the literature that determine the development of the intestinal microbiota in broiler chickens. Solid line indicates host characteristics, dashed line indicates environmental factors. The gut regions comprise the crop, proventriculus, gizzard, duodenum, jejunum, ileum, ceca, large intestine and cloaca. Maternal factors include horizontal transmission, vertical transmission and maternal antibodies.

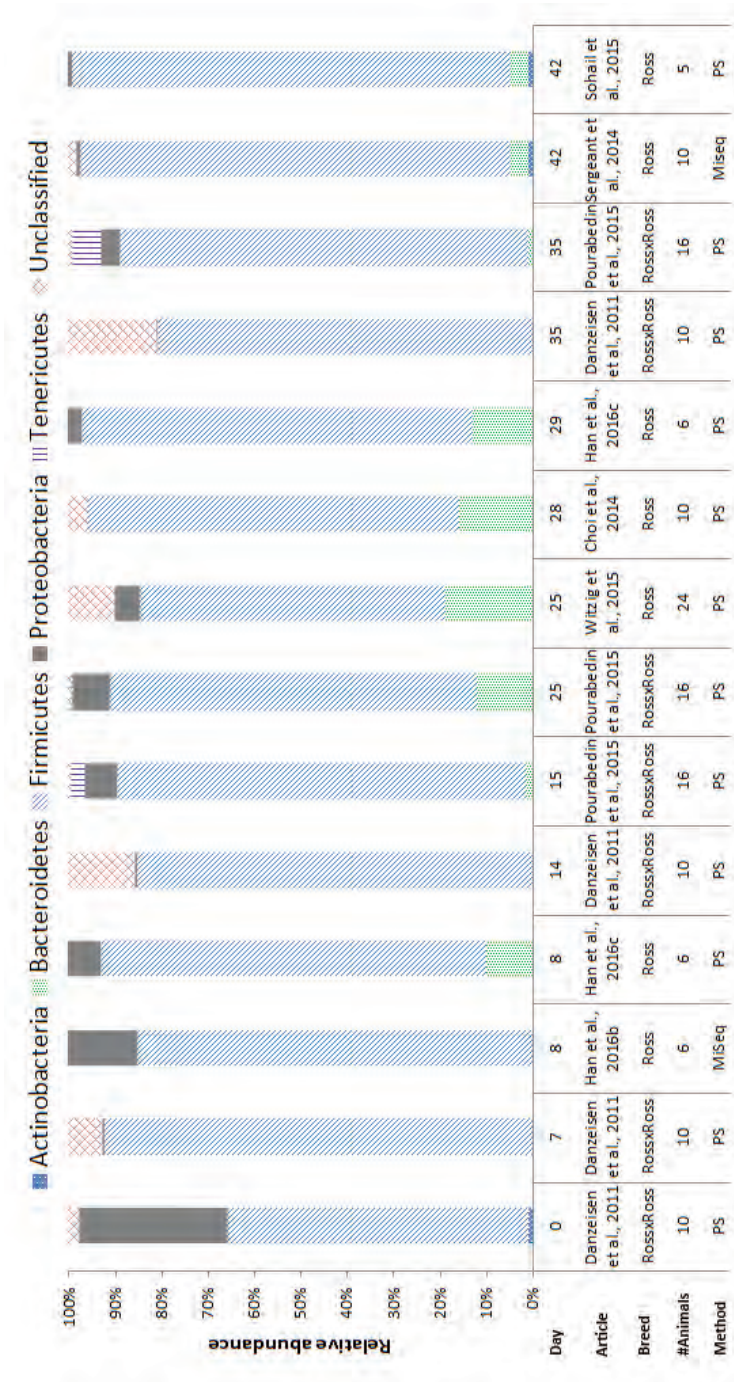
production) and layer-type chickens (egg production) shows variation at the phylum level across studies, and at different time points (Figures 2, 3, 4). This compilation is based on a systematic literature search. However, the limited number of articles with 16S rRNA gene amplicon sequencing data, and the large methodological differences between poultry studies did not allow for accurate re-analyses of original raw data to provide figures that would represent a true meta-analysis of studies. Therefore only the relative abundance at phylum level of the chickens in groups not exposed to specific treatments (control groups) is summarized to illustrate some general differences in microbiota development with regard to breed and age. *Firmicutes* were the most abundant phylum across the two broiler breeds throughout the production period from 0 to 42 days of age (Figures 2, 3). It is striking that *Firmicutes* were found to be the most abundant phylum on day 0 in meat-type chickens whereas *Proteobacteria* were most abundant in layer-type chickens (Figure 4). On day 0 the relative abundance of *Proteobacteria* in layer-type chickens was above 85% (Figure

4) (Ballou et al., 2016), whereas in meat-type chickens this phylum only accounted for approximately 30% (Danzeisen et al., 2011)(Figure 3), and 5% (Pedroso et al., 2016) (Figure 2). *Firmicutes* become the most abundant phylum also in layer-type chickens from day 7 onwards (Figure 4). For humans it has been shown that facultative anaerobic *Proteobacteria* are the most abundant phylum in the first period of life (Reinhardt et al., 2009, De Filippo et al., 2010, Schwartz et al., 2012), which is also seen in laying hens (Videnska et al., 2014a, Ballou et al., 2016) but not in broilers. The variation in colonization pattern between layer-type and meat-type chickens might be explained by the differences in exposure to microbiota, husbandry factors, and feed composition, but biological differences between these chicken types are most likely to play an important role as well and will be discussed in the next section.



**Figure 2:** The composition of the cecal microbiota in Cobb broilers at phylum level. General composition of the cecal microbiota in Cobb broilers across different ages, from control groups, not exposed to specific treatments. The data is from four different studies, based on 16S rRNA 454 pyrosequencing (PS), n = 3 and MiSeq sequencing n = 1. Pedroso et al. (2016), Figure 7, stacked bar chart A was used. Mohd Shaui et al. (2015), Figure 4, the last four bars were used. Singh et al. (2013), the data from Figure 1 was used. Stanley et al. (2013), the data from MG-RAST was used to combine the data from Figure 6.





**Figure 3:** The composition of the cecal microbiota in Ross broilers at phylum level. General composition of the cecal microbiota in Ross broilers across different ages, from control groups, not exposed to specific treatments. The data is from eight different studies, based on 16S rRNA 454 pyrosequencing (PS), n = 6 and MiSeq sequencing n = 2. Danzeisen et al. (2011), the data from the supporting information Supplementary Table S1 was used. Han et al. (2016b), the data from Figure 6, the first bar of the histogram was used. Han et al. (2016c), the data from Figure 7, pie chart A and C was used. Pourabedin et al. (2015), the data from the supplementary data, Supplementary Figure S1A was used. Witzig et al. (2015), the data from the Supplementary Data, Supplementary Table S4 was used. Choi et al. (2014), data from Figure 1 was used. Sergeant et al. (2014), the data from the Supplementary Data 1 was used. Sohail et al. (2015), the data from Supplementary Table 2 was used.



**Figure 4:** The composition of the cecal microbiota in layer-type chickens at phylum level. General composition of the cecal microbiota in laying hens across different ages, from control groups, not exposed to specific treatments. The data is from four different studies, based on 16S rRNA 454 pyrosequencing (PS), n = 1 and MISeq sequencing n = 3. Bailou et al. (2016), data from Figure 2B was used. Videnska et al. (2014b), data from the Supplementary Data S1 for ages 7, 14, 21, 28, and 56, data from the Supplementary Data S2 for ages 4, 7, 13, 16, and 19, and data from the Supplementary S3 data for age 21 was used. Polansky et al. (2016), data from Figure 1, bar 1 and 3 from the histogram was used. Han et al. (2016b) the data from Figure 6, the second bar of the histogram was used.



### **Chicken type and breed**

The genetic background of the host has been recognized as a factor that might influence intestinal microbiota composition (Benson et al., 2010, Org et al., 2015, Schokker et al., 2015, Han et al., 2016b). Considerable physiological differences exist between layer-type and meat-type chickens. Over decades, breeding programs have selected laying hens for maximal egg production and broilers for maximal meat production. This has resulted in large differences in growth, with an average body weight of laying hens of 450 g compared to 2800 g at 6 weeks of age in broilers<sup>1</sup>. These chicken breeding programs seem to have affected intestinal physiology (Uni et al., 1996) and immune function (Simon et al., 2014). Morphological differences in the intestinal tissue between laying hens and broiler chickens with respect to villus height, villus width, and crypt depth influence the intestinal absorptive area and have been associated with the higher body weight of broilers (Uni et al., 1996). Moreover, it has been shown that the expression of IgA, IgM and IgY in the ileum is higher in broilers compared to laying hens (Simon et al., 2014). These and other differences in intestinal physiology and immune system development between laying hens and broiler chickens are likely to influence microbiota composition and vice versa. Studies on differences between broilers and laying hens with regard to microbiota composition are scarce. To our knowledge, only two studies compared the microbiota composition between broilers and laying hens. It should be noted, however, that the first study was done with three week old broilers and 62 week old laying hens (Videnska et al., 2014a). This large age difference, as well as the difference in exposure to microbes in the housing environment and substantial differences in the composition of the diet for broilers and laying hens, may also have influenced microbiota composition, which hampers conclusions on the effect of chicken type (Videnska et al., 2014a). In the second study, as expected, differences in the development of local immunity and the colonization pattern of commensal bacteria between chicken types were found, and these differences were also shown to significantly alter the response to inoculation with *Campylobacter* (Han et al., 2016b). This indicates that differences in chicken breeds or genetic lines can impact important study outcomes.

In addition to biological differences between layer-type and meat-type chickens, there are also differences within chicken breeds of the same chicken type. As a previous observational study has shown, broiler breed was a factor associated with colonization by antibiotic resistant strains of *E. coli* (Persoons et al., 2011), and in an experimental study it was shown that different broiler breeds significantly differed in disease susceptibility to necrotic enteritis (Jang et al., 2013). In a study with different broiler breeds, hatched in the same hatchery, it was shown that each breed also had its own unique ileum microbiota composition at the age of 20 days (Kim et al., 2015). In 20-day-old Cobb broilers, *Bacteroidetes* were found in the ileum content, but were absent in Ross broilers. In turn, in Ross broilers, *Actinobacteria* were found

<sup>1</sup> [http://en.aviagen.com/assets/Tech\\_Center/Ross\\_Broiler/Ross-Broiler-Handbook-2014i-EN.pdf](http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-Broiler-Handbook-2014i-EN.pdf),  
[http://www.hyline.com/userdocs/pages/weekly\\_performance\\_standards\\_tables\\_-\\_Commercial\\_12-30-15.pdf](http://www.hyline.com/userdocs/pages/weekly_performance_standards_tables_-_Commercial_12-30-15.pdf),  
<http://www.lohmannngb.co.uk/files/Classic-Colony-Manual-28-Mar-2011.pdf>.

in the ileum content, but not in Cobb broilers (Kim et al., 2015). Similar results were found in other studies, i.e. absence of *Bacteroidetes* and presence of *Actinobacteria* in Ross broilers at 21 days of age (Nakphaichit et al., 2011) and 25 days of age (Pourabedin et al., 2015) and Cobb broilers without *Actinobacteria* but with *Bacteroidetes* at 23 days of age (Mohd Shaufi et al., 2015). In contrast, in another recent study a relative abundance of 22% of *Bacteroidetes* was reported in the ileum of 18-day-old Ross broilers (Han et al., 2016a). The presence of *Bacteroidetes* in the latter study and absence in the other studies may be caused by inevitable differences in diet or other experimental conditions, the younger age at sampling, differences in sequencing technology; as pyrosequencing versus Illumina MiSeq, or the differences in the primers used.

We compiled the data of studies for which 16S rRNA gene amplicon sequencing data of cecal samples was available for two broiler breeds (Figures 2, 3). This compilation shows that in cecal samples *Actinobacteria* are present in all four Cobb studies (100%) and in three out of eight Ross studies (38%), and that *Bacteroidetes* are present in all four Cobb studies (100%) and in six out of eight Ross studies (75%) (Figures 2, 3). These figures might suggest that breed influences the microbiota composition, but it is more likely that Cobb and Ross broilers had a different exposure to microbiota due to differences in parent flock or due to differences in the immune responses caused by differences in the genetic background (Emam et al., 2014, Schokker et al., 2015). Furthermore, it should be noted that, unfortunately, many articles on chicken microbiota data do not contain information about the breed (Qu et al., 2008, Corrigan et al., 2015, Lim et al., 2015, Schokker et al., 2015, Li et al., 2016, Oakley and Kogut, 2016, Lin et al., 2017).

Within certain broiler breeds, there is also a distinction between low and high body weight lines. As several studies have revealed, broilers from lines with low and high feed conversion ratio (FCR) show differences in their bacterial communities. In fecal samples, broiler lines with low FCR, indicating a more efficient use of feed for growth, showed higher counts for *Lactobacillus* compared to broilers lines with high FCR (Zhao et al., 2013, Meng et al., 2014, Mignon-Grasteau et al., 2015). Broiler line comparison in another study showed that the composition of the microbiota differed while microbial diversity did not, which might suggest that different chicken lines harbor different microorganisms for the same intestinal function (Schokker et al., 2015). The mechanisms behind the variation in intestinal microbiota between different broiler lines remain unclear, but it has been suggested that genetic background and the immune system influence establishment of gut microbiota after hatch (Schokker et al., 2015). Commercial selection programs for high production may result in co-microevolution of the microbiota and immune system of the host (Yang et al., 2017), although other factors, such as differences in exposure to microbial communities, cannot be excluded.

## Sex

In poultry, sex difference is part of the disparate production system, because layer-type chicken flocks predominantly consist of hens, whereas in broiler flocks males and females are often raised together. Broiler males generally have a higher growth rate and lower FCR than broiler females. Differences in bacterial communities between male and female broilers are also influenced by non-growth related factors, because no differences in growth rate were observed until day 21, whereas already at day 3 differences were observed in the intestinal microbiota composition (Lumpkins et al., 2008). In this study, the intestinal microbiota communities, determined by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments, showed less than 30% similarity between male and females (Lumpkins et al., 2008). Another study, where female and male broilers (age 22 and 42 days) were compared using quantitative PCR (qPCR), showed differences in abundance of *Lactobacillus salivarius*, *L. crispatus*, *L. aviarius* and *Escherichia coli* in their ceca (Torok et al., 2013). These are four out of the five potential performance-related bacteria of the qPCR format used (Torok et al., 2013). In a study on intestinal microbiota composition in chickens of 245 days of age and different broiler lines, i.e. a high (HW) and low body weight (LW) line, the relative abundance of 48 microbial species was significantly different between sexes (Zhao et al., 2013). Furthermore, there was a significant interaction between genotype and sex. In HW lines, males and females had 30 species of bacteria that were different between them, and LW lines 17 species (Zhao et al., 2013).

In animal studies, often only males are used to create a stable baseline model that is not affected by cyclical reproductive hormone levels (Zucker and Beery, 2010). An interaction between probiotic treatment and sex for *Bifidobacterium* was found in 42-day-old broilers (Mountzouris et al., 2015). These results reinforce that the sex of a chicken might be a confounding factor. Many broiler and microbiota studies contain only data from males (Dumonceaux et al., 2006, Burkholder et al., 2008, Guardia et al., 2011, Akyurek and Yel, 2011, La-ongkhum et al., 2011, Stanley et al., 2012a, Akbarian et al., 2014, Goodarzi Boroojeni et al., 2014, Huff et al., 2015, Ruiz et al., 2015) or the sex of the broilers is unknown (Stanley et al., 2012b, Corrigan et al., 2015, Oakley and Kogut, 2016). This sex bias in literature might influence our understanding of the microbiota development in chickens and therefore the sex of the chicken should always be reported.

## Sampling the gastrointestinal tract of chickens

The gastrointestinal tract (GIT) regions consists of the crop, proventriculus, gizzard, duodenum, jejunum, ileum, ceca, large intestine and cloaca. The GIT regions have different functions that impact microbiota dynamics and should be considered when determining the sampling protocol and study design. Differences in composition and

abundances of bacteria between the different GIT regions have been reviewed in detail previously (Yeoman et al., 2012, Stanley et al., 2014a, Deusch et al., 2015). Each GIT region has its own specific function in the digestion of feed, suggesting that there are differences in requirements for the types of microbiota that need to be present in each part. The crop primarily stores and pre-processes feed for further digestion (Richardson, 1970). For example, crop samples have been observed to show large differences in microbiota composition between individual broilers on the same diet (Sekelja et al., 2012, Choi et al., 2014). To illustrate, in one study with three individual 28-day-old broilers, the relative abundances of *Firmicutes* amounted to 95%, 40% and 32%, of *Proteobacteria* 5%, 55% and 19%, of *Bacteroidetes* 0%, 3% and 36%, and of *Actinobacteria* 0%, 2% and 13% for the three broiler chickens (Choi et al., 2014). The large individual variation in this study may have been related to the time between feeding and sampling. This variable will be discussed in more detail in the section about feed access. The gizzard mechanically grinds feed and acts as a microbial barrier due to its low pH (Stanley et al., 2014), the duodenum receives digestive enzymes from the bile- and pancreatic ducts, and the main function of the ileum is the absorption of nutrients. Those three regions, however, are all dominated by *Lactobacillus* species (Konsak et al., 2013, Stanley et al., 2014a).

The main function of the cecum is fermentation of nutrients (Clench, 1995). From microbiota data of individual broilers it is known that there is more variability between individual ileum and cloaca samples than between ceca samples (Pissavin et al., 2012, Choi et al., 2014). The cecum is the part of the GIT with the highest microbial richness and is mainly colonized by anaerobic microorganisms (Salanitro et al., 1974, Videnska et al., 2013). The cecal microbiota is more diverse, has a greater richness, and is more stable compared with microbiota residing in the ileum (Gong et al., 2007, Owens et al., 2008, Stanley et al., 2014). In addition, an adequate sample size for a study depends on the type of samples as well. The high individual variation in crop samples compared to cecal samples will result in a lower number of cecal samples needed to find a potential difference.

Intestinal samples of chickens can only be acquired post-mortem, and therefore, in many studies a less invasive method of sampling is preferred. Fecal samples and collection of cecal droppings have been used to determine intestinal microbiota composition. Cecal droppings reflect broilers' cecal microbiota, whereas fecal droppings do not (Pauwels et al., 2015, Stanley et al., 2015). Cecal droppings are difficult to collect because they are usually more easily trampled by the chickens and are produced less frequently than fecal droppings, with one cecal dropping for every seven to eight fecal droppings (Williams, 1995). Consequently, for comparisons between groups or studies, the location from which the intestinal samples originate should be taken into account to avoid misinterpretation of results.

## Environmental factors influencing intestinal microbiota in poultry

### *Biosecurity level*

In poultry production, it has been suggested that compared to the situation where a chicken is hatched by the mother hen, the relatively high hygiene levels of hatcheries have an effect on the development of the GIT and immune system. It is suggested that this is due to a delayed exposure to a 'healthy' microbiota (Bailey, 2010), which is comparable to the 'hygiene hypothesis' postulated for humans (Lashner and Loftus, 2006). Moreover, the high hygiene levels within hatcheries may also result in variable intestinal microbiota between batches of newly hatched chickens. It has been hypothesized that their intestinal bacterial community is shaped rather randomly and is quite heterogeneous due to exposure to bacteria from a variety of environmental sources after hatch, rather than colonization by maternally derived bacteria (Stanley et al., 2013). These environmental sources include people handling the chicks, transport crates, the first feed and the litter in the poultry house. In broilers raised in isolators, it was shown that the intestinal morphology was altered with shorter villi, more shallow crypts and a reduced production of acidic mucin compared with conventionally raised broilers (Forder et al., 2007), which might result in a different microbiota composition. In studies with other animals, for example in pig studies it has been shown that the intestinal development in high hygiene environments, such as isolators, negatively impacts a normal succession of the intestinal microbiota because it influences the expression of large numbers of immune-related genes (Mulder et al., 2011), and reduces the microbiota diversity compared to piglet siblings raised on a farm (Inman et al., 2010).

### *Housing*

Studies in humans have reported that individuals who live together show less variation of the intestinal microbiota compared to a group of random individuals (Yatsunenکو et al., 2012, Schloss et al., 2014). In animal studies, a living-together effect, also referred to as a cage effect, is well-known, especially for animals that are coprophagic such as mice. Since chickens are coprophagic as well, a cage effect is likely to occur in chicken studies (McCafferty et al., 2013, Laukens et al., 2016). To avoid cage effects and to prevent uncontrolled intake of particles and feathers containing potentially influencing intestinal microbiota (Meyer et al., 2012), some researchers house the birds individually (Zhao et al., 2013, Org et al., 2015). Cage was also a factor in a study on *C. perfringens*, which showed that the variation in *C. perfringens* count tended to be smaller between birds from the same pen (Hofshagen and Kaldhusdal, 1992). Furthermore, as researchers recently proposed, different experimental units may differentially shape especially the non-dominant microbiota in broilers (Ludvigsen et al., 2016).

Also, the type of production system can influence microbiota composition. In a study comparing organic farms to conventional farms, a higher number of *C. perfringens* was found in ileum and cecum samples of broilers from organic farms (Bjerrum et al., 2006). In this case, the researchers suggested that this difference might be due to the antimicrobial drug salinomycin, applied as coccidiostat in the conventional feed, which has antibiotic properties that can affect the intestinal microbial composition (Bjerrum et al., 2006). Moreover, they found lower counts of *Enterobacteriaceae* and higher lactobacilli numbers in the ileal content of the birds raised on the organic farms (Bjerrum et al., 2006). Access to an outdoor range was demonstrated to enrich *Bifidobacterium* in ceca and ileum in Ross broilers (Gong et al., 2008), and resulted in a higher proportion of *Bacteroidetes* in the cecum and a lower *Firmicutes/Bacteroidetes* ratio in Dagu chickens (Xu et al., 2016). In the ceca of Dagu chickens housed in free-range systems, a higher abundance of bacteria associated with functions involved in amino acids and glycan metabolic pathways was observed (Xu et al., 2016). In the previous example, access to range may have altered the composition of the microbiota, but the timing of access to the range may be important as well. When access to range occurred during the last four weeks only, instead of from the beginning of the production period onwards, no change in the richness of the broiler intestinal microbiota was found (Gong et al., 2008). Furthermore, the broiler density in a flock was also shown to affect the performance and the intestinal bacterial community (Guardia et al., 2011). In a flock with a stocking density of 17 birds per m<sup>2</sup> a decrease in growth performance and bacterial composition in the cecal samples was found, compared to a stocking density of 12 birds per m<sup>2</sup> (Guardia et al., 2011). This effect was more pronounced in the first half of the broiler production period. However, whether this was a direct effect of the alterations in microbiota or due to other health and management problems associated with increased stocking densities remained unclear.

### **Litter**

In poultry farming, litter is a mix of fecal and composted bedding material. Litter is an important environmental factor since chickens peck and forage in the litter. Litter is also used to collect samples to determine the intestinal composition of a flock. It has been demonstrated that the microbiota composition of litter samples collected from different production systems clustered with the corresponding microbiota composition of cecal samples (Mancabelli et al., 2016), suggesting that microbiota is exchanged between the chickens and the litter.

Depending on the litter type, litter quality and litter management the bacterial composition of chickens varies (Torok et al., 2009, Pan and Yu, 2014). It has been shown that litter type can affect the intestinal microbiota composition, for example birds raised on softwood sawdust versus chopped straw showed significant

differences in cecal microbial communities at 28 days of age (Torok et al., 2009). Also, it has been shown that female broilers grow slower on paper litter than on wood litter (Torok et al., 2011c). This stresses the importance of the choice of litter material for microbiota studies, as it might affect interventions.

The quality of litter has, in several studies, been associated with the performance of the chickens (Welfare Quality, 2009, de Jong et al., 2014). Litter quality has been observed to vary also within the same poultry house, with for example, higher moisture content of litter underneath nipple drinkers (Dumas et al., 2011). Wet litter was found to have greater microbiota (alpha) diversity than dry litter, and this might influence the intestinal microbiota as well (Dumas et al., 2011, Oakley et al., 2013). Although in general litter samples of the same flock do not share many taxa with fecal samples, wet litter was more similar to fecal samples than dry litter (Oakley et al., 2013).

Reused litter may harbor pathogens from the previous flock (Stanley et al., 2004). In broilers reared on 7-day-old fresh litter the ileal microbiota was dominated by *Lactobacillus* spp., whereas in broilers reared on reused litter a group of unclassified *Clostridiales* were the dominating bacteria in the ileal microbiota (Cressman et al., 2010). In the litter the microbial (alpha) diversity between fresh litter and reused litter became similar at day 42 (Cressman et al., 2010). Another study showed that as litter aged, litter microbial diversity decreased (Pedroso et al., 2013), whereas the opposite tendency was observed for chicken intestinal microbiota.

### **Feed access**

After the first ingestion of feed after hatch a large increase in bacterial numbers in the chicken intestine can be observed (Shapiro and Sarles, 1949, Mountzouris et al., 2015). Access to feed stimulates villus heightening and increased generation of cells in the crypt in young chicks (Gonzalez-Moran et al., 1985). In young chicks, delay in access to feed affects the development of the intestinal surface area (Uni et al., 1998, Lamot et al., 2014), and therefore potentially also the microbiota composition (Flint et al., 2012). Feed withdrawal later in life has also been associated with changes in microbiota composition (Burkholder et al., 2008, Vossen et al., 2009). Temporary feed withdrawal can result in an increased intestinal pathogen colonization (Thompson et al., 2008), for instance with *Salmonella* (Burkholder et al., 2008). After six hours of feed deprivation, large changes in the bacterial community were observed in the proximal part of the GIT (Vossen et al., 2009). Daily cycles of light and darkness, feeding rhythm, or temperature affect eating behavior of animal hosts which creates a daily rhythm of the digestive system. As a consequence, many bacteria experience substantial environmental changes during the day, due to eating behavior of animal hosts, which is referred to as a bacterial circadian clock (Johnson et al., 2017). In a mouse study, cyclical changes in the intestinal microbiota from feeding/fasting rhythms added to the intra-individual variation (alpha diversity) of intestinal



microbiota (Zarrinpar et al., 2014). Therefore, it is important that the time of feeding and/or feed deprivation and the moment of sampling are kept similar between birds or groups and are documented in scientific articles. Unfortunately, details on the duration of fasting before sampling are often not described.

### ***Climate and geographical location***

The local climate in a poultry house is an important factor that is well known to influence the performance of chickens. The number of studies describing the effects of climate on microbiota, using 16S rRNA gene amplicon sequencing, are however limited. For heat stress, however, some studies are available that describe both the large effects on performance and alterations of the microbiota composition of broilers (Lan et al., 2004, Sohail et al., 2015). These alterations can lead to susceptibility to by *E. coli* (Laudadio et al., 2012) and can contribute to increased intestinal colonization by *Salmonella* (Soliman et al., 2009, Burkholder et al., 2008). When birds experienced stress due to exposure to higher temperatures for 24 hours, greater changes were shown to occur in the ileal content compared to cecal samples, indicating that the microbiota in the ileum may be more sensitive to changes than the cecal microbiota (Burkholder et al., 2008).

The geographic location may affect the climate in the poultry house and as a consequence may influence the intestinal microbiota of chickens (Videnska et al., 2014a, Zhou et al., 2016). Although temperature in poultry houses is often controlled, broiler production may decrease because of the unfavorable influence of a hot environment (Laudadio et al., 2012). Current guidelines define this as an environment above 29°C and with 70% relative humidity, according to management guidelines<sup>2</sup>. This may be the reason why in one flock in Austria in the years 2003 to 2006 and 2013 no seasonal effect was identified (Sofka et al., 2015). Recently, a between-sample (beta) diversity analysis did not show specific clustering based on the different geographical locations. However, effects of the geographical location were detectable when comparing species richness and intra-individual diversity (Siegerstetter et al., 2017). For many studies, geographical location and its effects on the climate the birds are exposed to are unknown. It is therefore often difficult to evaluate to what extent these factors may influence the research results.

### ***Implications of confounding factors affecting the intestinal microbiota in chickens***

The aim of this review was to provide an overview of host and environmental factors that affect the composition of the intestinal microbiota of poultry, to create awareness of confounding factors in poultry microbiota studies. We summarized the currently available knowledge regarding potential confounding factors separately, but of course, many of those factors cannot be seen independently. This review emphasizes

<sup>2</sup> [http://en.aviagen.com/assets/Tech\\_Center/Ross\\_Broiler/Ross-Broiler-Handbook-2014i-EN.pdf](http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-Broiler-Handbook-2014i-EN.pdf)



the relevance of comprehensive documentation and reporting, as well as control of relevant host and environmental factors and molecular approaches in poultry microbiota studies, as previously suggested for studies with humans and mammals (Kilkenny et al., 2010, Laukens et al., 2016).

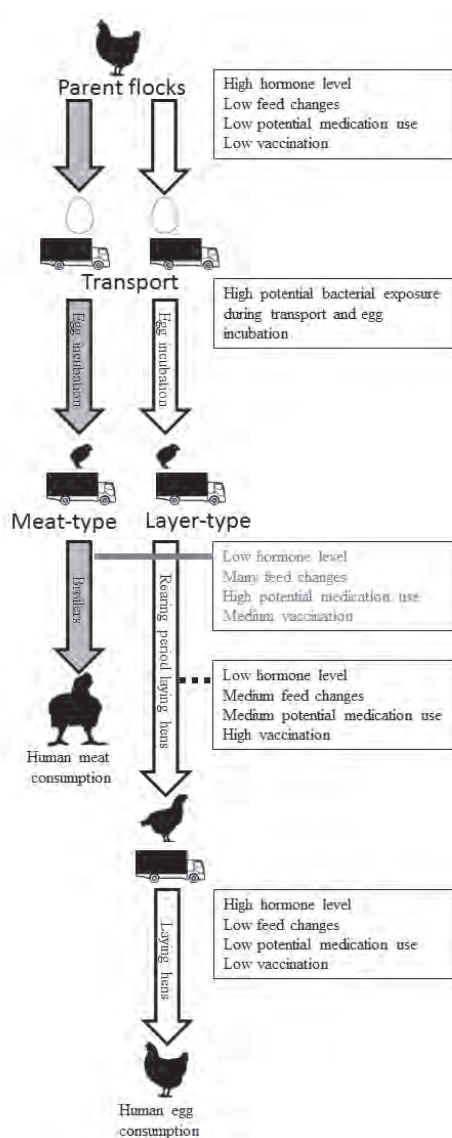
Of the factors that influence poultry microbiota composition (Figure 1), antibiotics and feed composition are well-known for their effects on performance and the intestinal microbiota. Antibiotics and feed composition are often the main interventions that are the focus of a given study and were *not* discussed in this review, as our aim was to show which host and environmental factors that are not under investigation act as confounders, and may unintentionally have a large impact on the study outcome. For example, rather than the antibiotic treatment, a stronger effect on the composition of the microbiota was attributed to the environment in which chickens were raised, i.e. battery cages versus floor pens (Pedroso et al., 2006). Furthermore, there are examples of studies that indicated an unexpected lack of differences in intestinal microbiota composition between diet interventions (van der Hoeven-Hangoor et al., 2013, Park et al., 2015), but did show clustering for different GIT regions, age groups and cages (Park et al., 2015, Ludvigsen et al., 2016). Consequently, not taking confounding factors properly into account with study design and data analysis might explain why antibiotics or a feed intervention does not show effects or an unrepeatable effect on the intestinal microbiota composition. Thus, host characteristics and environmental factors can have a large impact on conclusions that can be drawn from experiments and field studies.

Using knowledge of relevant confounding factors to improve study designs is an essential prerequisite to being able to generate data that will facilitate thorough understanding of the phylogeny and composition of the microbiota, and functionality of host-microbiota interactions. Although controlling for confounders is not always possible, detailed recording and reporting of these factors should be considered as an integral and essential part of each study. Providing details on study variables such as feed composition, feed access, feed changes, feed deprivation, medications (preventive and therapeutic), vaccines, age, sex, hygiene protocols, housing systems, litter type, flock density, housing temperature and location in the methodology part of publications, will improve the repeatability, the reproducibility, and the interpretation of chicken microbiota studies.

During the life of a chicken, many of the host and environmental factors discussed in this review can exert their respective effects on chickens. Figure 5 provides a compilation of known and potential effects on the intestinal microbiota composition of chickens, illustrating that many factors can have both short- and long-term effects and may even originate from the hatching stage or a previous generation. Layer-type and meat-type chickens have different production systems and are therefore displayed separately. The influences of hormonal changes

due to the reproduction cycle are limited in broilers, due to their short lifespan, and also limited in the first part of the rearing period of layers. Near the end of the rearing period and the first part of the laying period, development of the reproductive organs and the start of egg production may affect GIT microbiota composition, as shown in mice (Org et al., 2016). Exposure to bacteria early in life, before, during and shortly after hatching, during transport or at the start of the rearing period (for layers) or production period (for broilers) has a potentially large impact on microbiota composition and immune development for both the short- and the long-term (Maynard et al., 2012). No or a delayed feed access, for example during transport to a farm, also can influence intestinal microbiota composition (Simon, 2016). Nevertheless, if this delayed feed access effect is biologically relevant in terms of stimulation and functionality of the immune system is still unclear. It is clear that perturbation of early life microbial colonization has long-term effects on immune development (Simon et al., 2016; Schokker et al., 2017).

The rearing period is associated with more feed changes, more preventive and therapeutic treatments and vaccinations than the laying period. In broilers, many feed changes, treatments, and vaccinations occur in a very short lifespan of approximately 6 weeks. It is known that different *Salmonella* vaccines used in broilers can change cecal microbiota composition (Park et al., 2017). This most likely also happens in the rearing



**Figure 5:** Known and potential factors that affect the intestinal microbiota composition of chickens during life. These factors can have short- and long-term effects and may even originate from the hatching stage or a previous generation. Grandparent flocks and the rearing period of the parent flock are not included in the figure. Layer- (white arrow) and broiler-type (gray arrow) have different production systems and are therefore displayed separately.

period of laying hens. Since it is currently unknown how all those potential factors influence intestinal microbiota composition of chickens, further investigation is needed.

In Figure 2-4, 16S rRNA gene amplicon sequencing data is combined from different studies, although it should be noted that there is still limited 16S rRNA gene amplicon sequencing data available for day 0 to 7-day-old broilers. In addition, the sample size of most studies is also limited, especially in the laying hen studies where the sample size is 3-6 birds. Another important observation that follows from these data is that in some studies, 30% (Figure 2) to 20% (Figure 3) of 16S rRNA multiplex amplification data has remained unclassified. To increase our biological understanding of host-microbe interactions these unknown microbes need to be identified. Consequently, there is still limited evidence that the colonization pattern of layer- and meat-types is different. Despite the limited availability of data and methodological differences between studies, it seems safe to conclude that layer- and meat-type chickens follow a different colonization pattern compared to mammals. For example, in human babies, a period has been identified where members of the phylum *Actinobacteria* are present in a high proportion (Reinhardt et al., 2009, De Filippo et al., 2010, Ottman et al., 2012, Schwartz et al., 2012). This period with a high proportion of *Actinobacteria* is neither observed in laying hens (Videnska et al., 2014b, Polansky et al., 2016, Ballou et al., 2016), nor in broilers (Figures 2, 3). In contrast with mammals that drink milk during the first weeks of life, chickens ingest solid feed from the day of hatch onwards, which might explain the variation in colonization pattern between mammals and birds. Another possible explanation for the observed large differences between data of mammalian studies and poultry studies, is that in mammalian studies often fecal samples are used, whereas in chicken studies cecal samples are most often collected.

## Concluding remarks

Comprehensive analyses of intestinal microbiota will lead to better understanding of dynamics in microbial community structure and function, which will increase our understanding of intestinal health in poultry. Hence, research aimed at identifying biologically relevant characteristics of a healthy poultry microbiota, for instance as a foundation for nutritional and husbandry associated strategies to replace antimicrobial drugs, is both promising and challenging. It has been shown that microbiota studies have to deal with many hidden host and environmental variables, which are not all known. Therefore, it is essential to be aware of the large impact the choice of study designs has on the results and thus on the interpretation of the outcomes of studies into the intestinal microbiota. Furthermore, providing details on study variables and sequence data repositories creates opportunities to combine data from different studies for meta-analysis, which will facilitate scientific breakthroughs towards innovative microbiota-inspired intervention strategies.

### ***Conflict of Interest***

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

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### ***Author Contributions***

JGK, FCV, EAJF, JAS and HS initiated the project. JGK searched the databases for potentially eligible articles based on their titles and abstracts and wrote the paper. FCV, EAJF, GDAH, JAS and HS contributed to the development of the manuscript as a whole by giving constructive feedback on the manuscript during its preparation. All authors gave approval of the manuscript for publication.





# CHAPTER 3

## Take care of the environment: housing conditions affect the interplay of nutritional interventions and intestinal microbiota in broiler chickens

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## Abstract

**Background:** The intestinal microbiota is shaped by many interactions between microorganisms, host, diet, and the environment. Exposure to microorganisms present in the environment, and exchange of microorganisms between hosts sharing the same environment, can influence intestinal microbiota of individuals, but how this affects microbiota studies is poorly understood. We investigated the effects of experimental housing circumstances on intestinal microbiota composition in broiler chickens, and how these effects may influence the capacity to determine diet related effects in a nutrition experiment. A cross-sectional experiment was conducted simultaneously in a feed research facility with mesh panels between pens (Housing condition 1, H1), in an extensively cleaned stable with floor pens with solid wooden panels (H2), and in isolators (H3). In H1 and H2 different distances between pens were created to assess gut microbiota exchange between pens. Feed with and without a blend of medium-chain fatty acids (MCFA) was used to create differences in cecal microbiota between pens or isolators within the same housing condition. Male one-day-old Ross broiler chickens (n=370) were randomly distributed across H1, H2, and H3. After 35 days cecal microbiota composition was assessed by 16S ribosomal RNA gene amplicon sequencing. Metabolic functioning of cecal content was assessed based on high-performance liquid chromatography. Results: Microbial alpha diversity was not affected in broilers fed +MCFA in H1 but was increased in H2 and H3. Based on weighted UniFrac distances, the nutritional intervention explained 10%, whereas housing condition explained 28% of cecal microbiota variation between all broilers. The effect size of the nutritional intervention varied within housing conditions between 11%, 27%, and 13% for H1, H2, and H3. Furthermore, performance and metabolic output were significantly different between housing conditions. The distance between pens within H1 and H2 did not influence the percentage of shared genera or operational taxonomic units (OTUs). Conclusions: The cecal microbiota of broilers was modifiable by a nutritional intervention, but the housing condition affected microbiota composition and functionality stronger than the diet intervention. Consequently, for interpretation of intestinal microbiota studies in poultry it is essential to be aware of the potentially large impact of housing conditions on the obtained results.

**Keywords:** Transmission, Microbiome, 16S rRNA, Fatty acids, Poultry



## Background

Knowledge of the factors that affect the diversity and functioning of intestinal microbes is essential to facilitate the development of new strategies to improve health, to reduce the use of antibiotics and to improve production performance of broiler chickens (Lan et al., 2005, Johnson et al., 2018). Numerous interactions between micro-organisms, diet, host and environmental factors affect the composition of the chicken intestinal microbiota (Wei et al., 2013, Stanley et al., 2014a, Kers et al., 2018). Knowledge about how those factors and their interactions shape the intestinal microbiota in broilers is limited but is important for the design and interpretation of experiments, especially for nutritional research. One of those poorly understood factors is the transmission of bacteria present in the living environment to the hosts (environment-to-host transmission) and exchange of microbiota between hosts sharing the same environment (host-to-host transmission), and how this shapes the intestinal microbial communities within hosts (Browne et al., 2017).

Previous studies have indicated that housing conditions have a major effect on the health of a host and the composition of its intestinal microbiota (Muller et al., 2016, Lundberg et al., 2017, Combes et al., 2017, Ridaura et al., 2013). Broilers raised in isolators showed alteration of the intestinal morphology, with shorter villi, shallower crypts and reduced production of acidic mucin, compared to conventionally raised broilers. This alteration of the intestinal morphology was suggested to be instigated by differences in the bacterial colonization (Forder et al., 2007). Piglets raised in an isolator had a different succession of species during the development of the intestinal microbiota than piglets raised under conventional circumstances (Schmidt et al., 2011, Mulder et al., 2009). The alteration in the intestinal microbiota was associated with an altered expression of immune-related genes (Mulder et al., 2009). The intestinal microbiota is not only altered in extreme environments, such as isolators, but also in other experimental environments differences in cecal microbiota in broiler chickens were observed (Stanley et al., 2013, Ludvigsen et al., 2016). For example, in an experiment with broiler chickens it was observed that both feed intervention as well as housing conditions (i.e. two different experimental rooms that were presumed identical) affected cecal microbiota, with OTUs associated with room being on average approximately 3-fold less predominant than those associated with diet (Ludvigsen et al., 2016).

Another factor related to housing conditions that may shape the intestinal microbiota community is the transmission of microbes between hosts (Browne et al., 2017). It is roughly estimated that 50% to 60% of the bacterial genera from the intestinal microbiota of healthy humans produce resilient spores, which are specialized for host-to-host transmission (Browne et al., 2016). In humans, it was found that the intestinal microbiota of individuals who live together show less variation between individuals compared to the variation in a group of randomly selected individuals

(Yatsunenkeno et al., 2012, Schloss et al., 2014). This has been observed in chickens as well, as the variation between birds within the same pen tended to be smaller than between birds within the same diet group (Nordentoft et al., 2011, Hofshagen and Kaldhusdal, 1992). A study on *Campylobacter jejuni* and *Escherichia coli* showed that spatial distance between pens delayed its transmission from infected to naïve chickens (van Bunnik et al., 2014). Consequently, transmission of microbes between spatially separated chickens within a research environment might be an unknown confounding factor. Hence, it is difficult to determine the potential effects of these processes on the reproducibility and outcomes of broiler nutritional interventions. There is a lack of knowledge on the sizes and mechanisms of effects of this transmission of microbes and the exposure to microbes from the environment on broiler intestinal microbiota composition and functioning.

Therefore, the aim of this study was to compare the effect of different experimental housing conditions for broiler chickens on cecal microbiota composition and the concomitant interpretation of a nutritional intervention. The same nutritional intervention was performed simultaneously in three different housing conditions; housing condition 1 (H1), a standard grow-out feed trial facility; housing condition 2 (H2), a facility with floor pens for small-scale experiments; and housing condition 3 (H3), isolators. In H1 and in H2 different distances between pens were created, to observe if distance between pens could influence the intestinal microbiota. Previous studies have shown that the addition of medium-chain fatty acids (MCFA) to feed can significantly change intestinal microbiota composition (van der Hoeven-Hangoor et al., 2013, Zentek et al., 2012). Therefore, a diet with and without MCFA was used as a tool to generate differences in cecal microbiota composition between the chickens in different pens within a housing condition. At 35 days of age, we determined cecal microbiota composition based on 16S ribosomal RNA (rRNA) gene amplicon sequence analysis. The metabolic output of the microbes was determined by measuring the production of acetate, butyrate, isobutyrate, lactate, and propionate, and effects on production performance were determined based on body weight on day 35. This research provides insights into the potential effects of interactions between hosts, and hosts and their environment, on the composition and functioning of intestinal microbiota in broiler chickens and the interpretation of nutritional interventions.

## Results

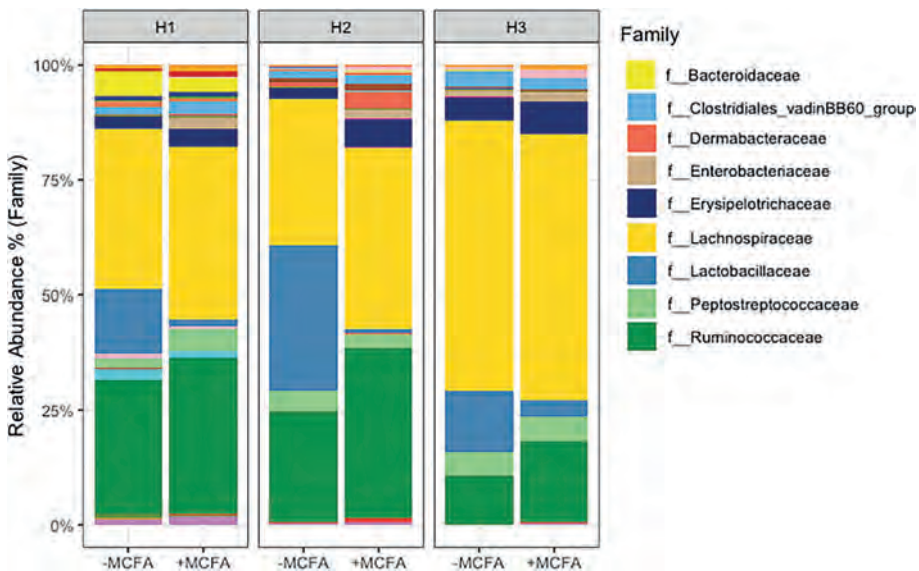
### ***Biosecurity level among three housing systems***

The bacterial loads, as determined using Rodac plates, were different between the housing conditions before the broilers arrived in the experimental facilities (Additional file: Figure S1). CFU per Rodac plate were highest in H1 and lower in H2 and H3

(H1-H2,  $F = 12.1$ ,  $p < 0.001$  and H1-H3,  $F = 10$ ,  $p < 0.001$ ). Based on the Rodac plate results, three out of ten isolators were disinfected again with vaporized hydrogen peroxide before the broilers arrived.

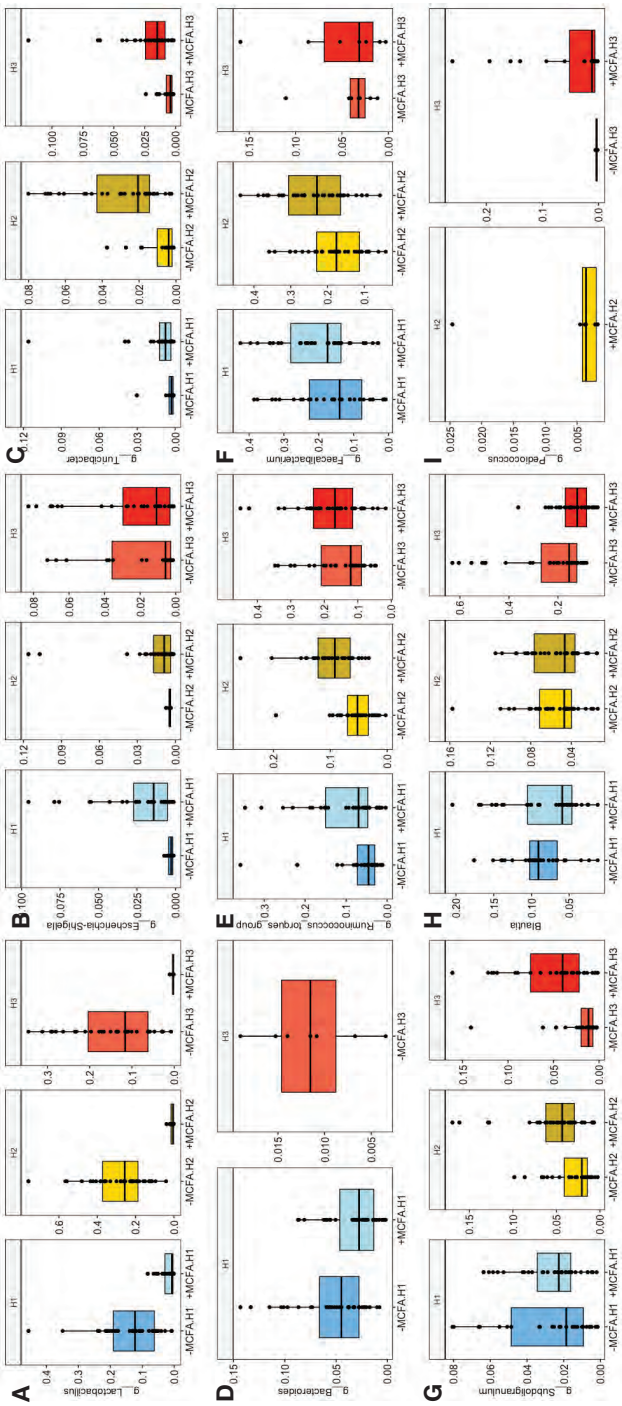
### ***Dietary effect of MCFA on cecal microbiota in different housing systems***

On day 35 the cecal content of 210 male Ross 308 broiler chickens from the three different housing conditions were analyzed, with a total of seven cecal samples (i.e. broilers) per pen. Figure 1 provides an overview of the nine most abundant microbial families in the cecal microbiota of the broilers across the three housing conditions and for -MCFA and +MCFA feed (for a complete overview of the relative abundance of all families, see Additional file: Table S1).



**Figure 1:** Bar chart with the cumulative relative abundance (%). The legend only contains the most abundant bacterial families. Per housing condition and diet intervention, with an average of 35 birds per bar. For a complete overview of the relative abundance of all 46 families, see Additional file: Table S1.

In the ceca of broilers fed +MCFA, the relative abundance of *Lactobacillus* was significantly lower in all three housing conditions compared to broilers fed -MCFA (Figure 2a). The reduction in relative abundance for the +MCFA broilers of *Lactobacillus* varied per housing condition, and was 12, 28 and 14 percent points in H1, H2 and H3 (see Additional file: Table S2 for  $p$ -values). There was a concomitant increase in the relative abundance of *Escherichia-Shigella* and *Turicibacter* in +MCFA broilers in all three housing conditions (Figure 2b, c).



**Figure 2:** Box plots of nine genera that were significantly different in relative abundance between the broilers on the diet -MCFA or +MCFA. The results are based on differences of relative abundance (Wilcoxon rank-sum test, adjusted  $p$ -values for multiple testing, BH,  $<0.05$ ). The genera *Lactobacillus*, *Escherichia-Shigella* and *Turicibacter* show the same trend across housing conditions (a-c), while some effects are unique in a subset of one housing condition (d-i).

In addition to the differences that were observed in all housing conditions, some differences were only found in certain housing environments. In H1 the relative abundances of an unknown member of the *Peptostreptococcaceae* family, and the genus *Bacteroides* were lower in +MCFA broilers (Figure 2d). In H2 the genera *Ruminococcus torques* group, *Faecalibacterium* and *Subdoligranulum* were higher in +MCFA broilers (Figure 2e-g). In H3 the relative abundances of the genus *Blautia* (Figure 2h) and *Clostridium innocuum* group were lower, whereas the relative abundance of an uncultured group within the *Lachnospiraceae* and the genera *Subdoligranulum*, *Pediococcus* (Figure 2g, i) and *Erysipelatoclostridium* were higher in broilers fed +MCFA. In total 46 genera differed (adjusted  $p < 0.05$ ) in relative abundance between the feed interventions within one housing condition (Supplementary materials: Table S2 and Figure S2).

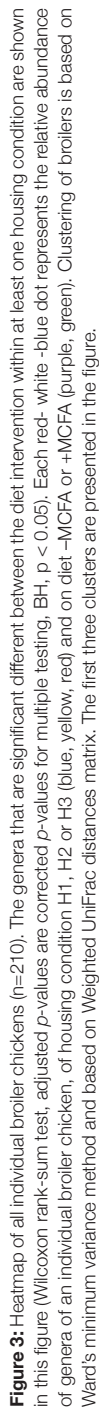
The heatmap in Figure 3 shows all genera that significantly differed in relative abundance between the feed interventions for each housing condition. Hierarchical clustering of broilers revealed three clusters; cluster one coinciding with housing condition H3, cluster two containing most broilers of H1, and the third cluster contained all broilers of H2 and 10 broilers in H3 and 14 broilers in H1 (Figure 3). Pen effects can be identified, for example, the first seven birds in the first cluster, with a higher relative abundance of *Blautia*, were all raised in the same isolator in H3. Cluster three also contained one isolator of H3 (see \*), while in this cluster also eight broilers of H1 cluster together, although these were housed in different pens (Figure 3). All aforementioned results show that the housing conditions had a larger effect on the microbiota composition than the MCFA feed intervention.

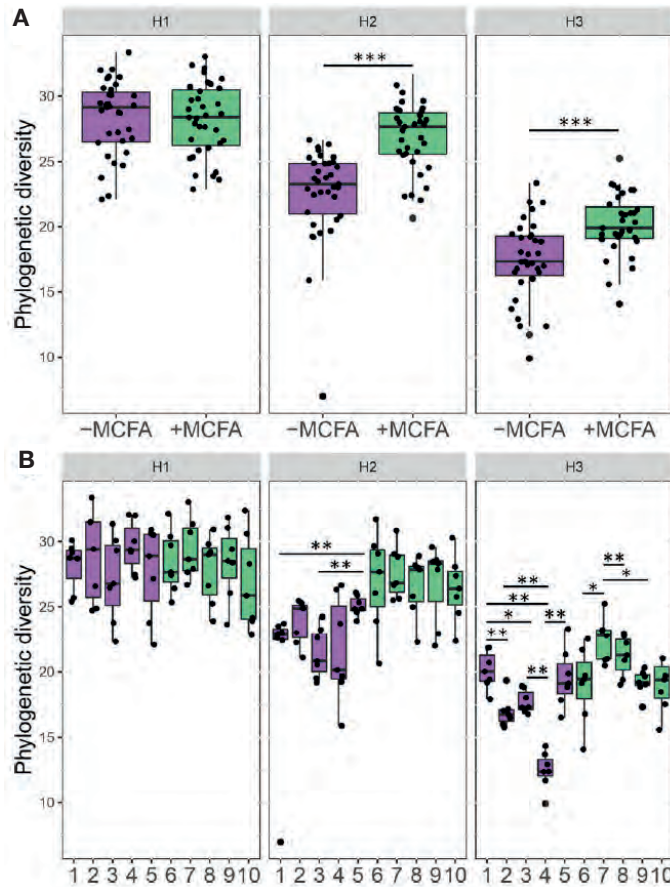
For both -MCFA and +MCFA broilers together, significant differences in relative abundance of genera were also found between housing conditions (Supplementary materials: Table S2). In H1 a higher relative abundance of the genus *Bacteroides* were found compared to H2 (Figure 2d). In H1 and H2 we also observed a higher relative abundance of *Faecalibacterium* than in H3, and a lower relative abundance for the *Ruminococcus torques* group and *Blautia* (Figure 2f, e, h). Overall, a large number ( $n = 103$ ) of differences between the housing conditions were observed (total overview in Supplementary materials: Table S2 and Figure S2).

### **Housing and dietary effect on microbial alpha diversity**

When comparing cecal microbiota alpha diversity of broilers within the same housing condition, different effects were observed in response to the MCFA intervention. In H1 no effect was observed, whereas in H2 and in H3 the +MCFA resulted in a higher phylogenetic diversity (Figure 4a). Overall, the phylogenetic diversity was highest in broilers housed in the feed trial facility (H1) and lowest in the isolators, H3 (Figure 4a). Other alpha diversity metrics were in agreement (Additional file: Figure S3). The effect size of the +MCFA was highest in H2 ( $H1, \chi^2 = 0.03, p = 0.846$ ;  $H2, \chi^2 = 29, p < 0.001$ ;  $H3, \chi^2 = 11, p < 0.001$ ). In addition, in housing conditions H2 and H3, but not H1, diversity was different between a subset of the pens within the same intervention (Figure 4b, Supplementary materials: Table S3).







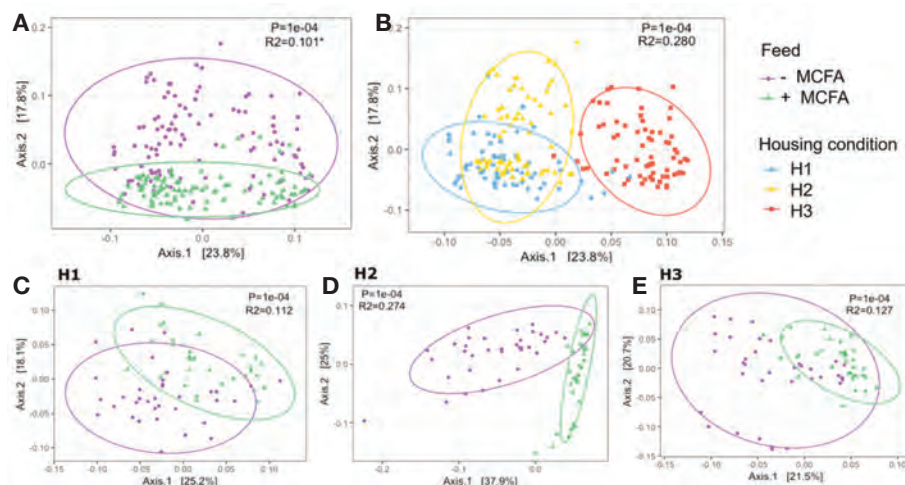
**Figure 4:** Phylogenetic diversity of the cecal microbiota of the six different experimental groups. A. The phylogenetic diversity (OTU level) was higher in the ceca of broilers fed +MCFA in H2 and H3 but not in H1. The phylogenetic diversity was highest in H1 and lowest in H3. (n=35 broilers per group, Kruskal-Wallis, \* =  $p < 0.05-0.01$ , \*\* =  $p < 0.01-0.001$ , \*\*\* =  $p < 0.001$ ). B. Phylogenetic diversity was different between a subset of pens within the same diet intervention (n= 7 broilers per pen).

### Housing and dietary effect on microbial beta diversity

Weighted UniFrac based analysis of cecal microbiota showed that the +MCFA feed intervention explained 10% ( $R^2$ ) of microbiota variation independent of the housing condition (Figure 5a, PERMANOVA, pseudo-F = 23,  $p < 0.001$ ). It should be noted that also the beta dispersion was significantly higher in the -MCFA groups (Figure 5a,  $p = 0.021$ ). In the total dataset the housing condition explained 28% of microbiota variation (Figure 5b, pseudo-F = 40,  $p < 0.001$ , beta dispersion  $p = 0.295$ ).

In H1, diet explained 11% of the total microbiota variation (Figure 5c, pseudo-F = 9,  $p < 0.001$ , beta dispersion  $p = 0.968$ ), while in H2, it explained 27% (Figure 5d, pseudo-F = 26,  $p < 0.001$ , beta dispersion  $p = 0.450$ ). In H3 the feed intervention

explained 13% but beta dispersion was also significantly different between feed groups (Figure 5e, pseudo-F = 9,  $p < 0.001$ , beta dispersion  $p = 0.007$ ). Pen explained 26%, 53% and 55% of microbiota variation in housing unit H1, H2 and H3. Including pen in the analysis increased the explained variation by definition because we cannot disentangle pen and feed as in each pen broilers were exposed to the same feed intervention. Nevertheless, this suggests the strongest pen effect in H3, since in H3 an increase from 13% (feed only) to 55% (feed and pen) explained microbiota variation was observed.



**Figure 5:** Weighted UniFrac based PCoA analysis across the six different experimental groups. A. Diet effect across all three housing units ( $n=210$ ). B. Housing condition effect. C. Diet effect in housing condition 1 (PERMANOVA, OTU level), Diet:  $R_2 = 11\%$ ,  $p < 0.001$ , Pen:  $R_2 = 26\%$ ,  $p < 0.001$ ,  $n=70$ ). D. Diet effect in housing condition 2 (PERMANOVA, Diet:  $R_2 = 27\%$ ,  $p < 0.001$ , Pen:  $R_2 = 53\%$ ,  $p < 0.001$ ,  $n=70$ ). E. Diet effect in housing condition 3 (PERMANOVA, Diet:  $R_2 = 13\%$ ,  $p < 0.001$ , Pen:  $R_2 = 55\%$ ,  $p < 0.001$ ,  $n=70$ ). \* beta dispersion  $< 0.05$ .

The above described results were confirmed with Bray-Curtis and Jaccard metrics, which showed that the feed explained most of the variation in H2 and the cage explained most of the variation in H3 (Supplementary materials: Figure S4). In contrast, Unweighted UniFrac, which only considers presence or absence of OTUs showed that feed explained most variation in H3 instead of H2 (Supplementary materials: Figure S4). Across housing conditions, the effect sizes based on unweighted UniFrac were slightly higher than for weighted UniFrac, whereas the opposite trend was observed for corresponding Jaccard and Bray-Curtis dissimilarity (Supplementary materials: Figure S4). This suggests that the most abundant taxa were more phylogenetically related (i.e. more similar) compared to the low abundant taxa and that not all of those low abundant taxa were shared between the housing conditions.

### **Percentage of microbial taxa shared between pens**

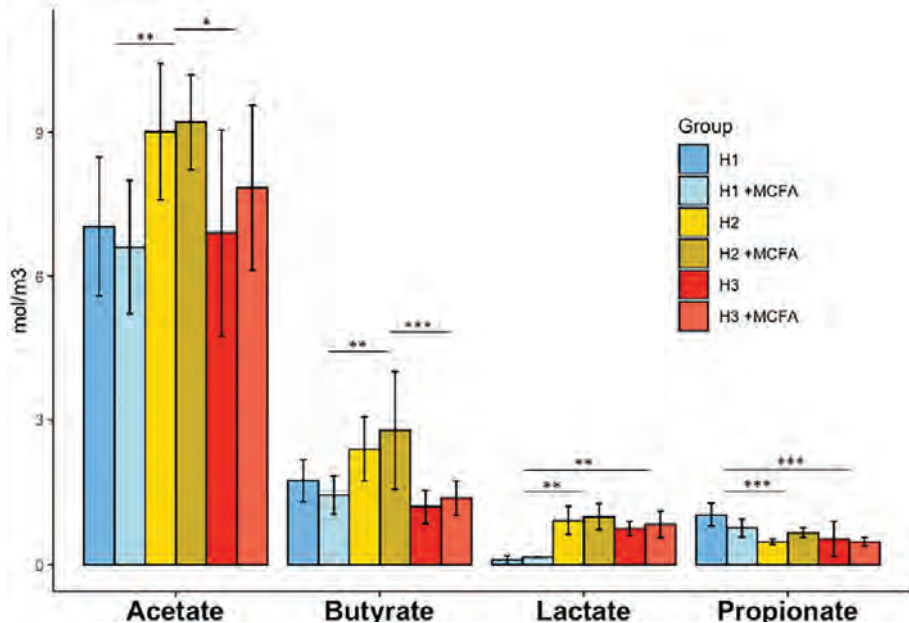
We used the percentage of shared genera or shared OTUs as a proxy for



putative host-to-host and environment-to-host transmission. The total number of genera identified in each housing condition was 125, 98 and 102, and the average percentage of genera shared between pens was 74%, 74% and 55% in H1, H2 and H3. In the pens of H1 and H2 the feed intervention had no effect on percentage of shared genera, i.e. 75% versus 73%, and 78% versus 74% (+MCFA and -MCFA, H1:  $p = 0.926$ , H2:  $p = 0.078$ ). However, in H3 there was a significant difference in shared genera between the two feeds where pens fed +MCFA shared 66% and pens -MCFA 49% ( $p = 0.006$ ). The percentage of shared OTUs was lower compared to percentage of genera, but the trend was the same. Broilers with MCFA in their diet shared more OTUs compared to -MCFA fed broilers (Additional file: Table S4). Strikingly, physical distance between pens was not correlated with the percentage of shared OTUs or genera (Additional file: Figure S5).

### ***Metabolic output of the cecum in broiler chickens within and between housing conditions***

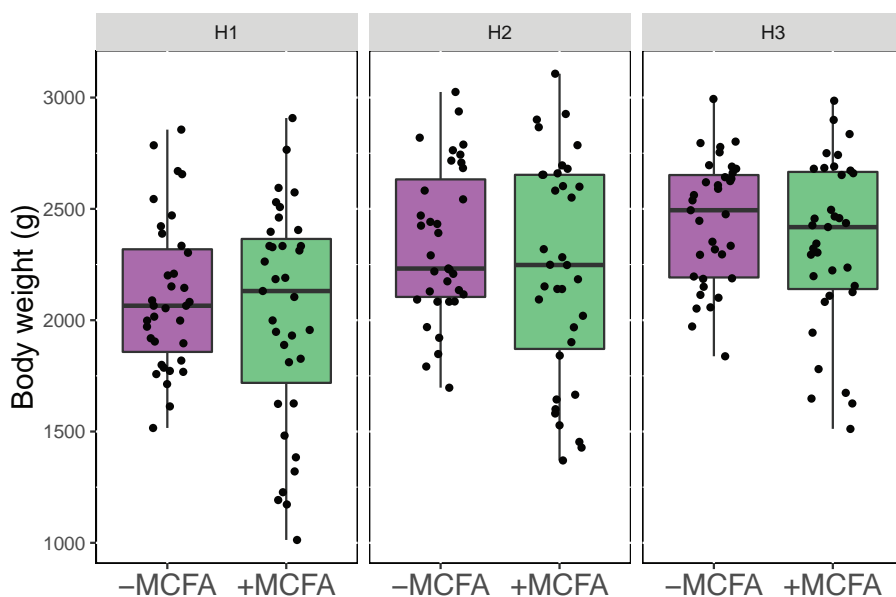
Within housing conditions no significant difference between dietary treatment groups was observed for acetate, butyrate, lactate or propionate levels in the cecum (Figure 6). Isobutyrate was not detected in the cecal samples. However, in contrast, butyrate, acetate, lactate and propionate concentrations were significantly different between housing conditions (Figure 6), with acetate and butyrate highest in H2 and propionate highest in H1. The concentration of lactate was lowest in H1 (Additional file: Table S5).



**Figure 6:** Acetate, butyrate, lactate and propionate concentrations in the cecal content of the six different experimental groups. (n=35 broilers per group, \*  $p < 0.05$ -0.01, \*\*  $p < 0.01$ -0.001, \*\*\*  $p < 0.001$ )

### Growth performance

After 35 days the average body weight was not different between the dietary treatments but differed per housing condition. Body weights of the broilers in H1 were significantly lower than those of broilers in H2 or H3 on the same dietary treatment (Figure 7, Supplementary materials: Table S6). Only in the period 14-35 the average daily gain and the average daily feed intake were lower in +MCFA broilers (Supplementary materials: Table S6). In all other measured growth performance data, only housing conditions resulted in different performance (Supplementary materials: Table S6).



**Figure 7:** Body weight of broilers on day 35 of age. (n=35 broilers per group)

### Discussion

The aim of this study was to compare the effect of different experimental housing conditions for broiler chickens on cecal microbiota composition and the concomitant interpretation of a nutritional intervention. The variation and composition in the cecal microbiota differed strongly across housing conditions, even with similar management, genetic background, and feed in 35 day old broilers. These findings are in line with previous studies, where housing conditions were shown to influence intestinal microbial composition (Muller et al., 2016, Lundberg et al., 2017, Combes et al., 2017, Ridaura et al., 2013, Parker et al., 2018). In addition to the cecal microbiota composition, also metabolic output and growth performance were different between the housing conditions. We also varied the physical distances between pens, to

determine the potential effects of exchange of microbes on the outcomes of the experiment. In this study, the distances between pens did not show any significant correlation with the percentages of genera or OTUs shared between pens. However, our results are based on a single observation after 35 days, and hence, we cannot determine whether early in life the distance between pens could have influenced microbiota composition temporarily.

Few studies have been performed on effects of distances between cages on microbial spread (van Bunnik et al., 2014, Warfel et al., 2012). As previously described, small distances could delay the spread of *Campylobacter jejuni* and *Escherichia coli* for days but ultimately did not prevent transmission (van Bunnik et al., 2014). How and to what extent an environment influences the composition of the intestinal microbiota, is still unclear, although in humans, it is proposed that spore-forming bacteria influence host-to-host and environment-to-host transmission (Browne et al., 2017). Although it is difficult to accurately quantify microbiota transmission, the results of this study strongly suggest differences in the level of transmission of microbes between broilers for the different housing conditions. We found no difference in the alpha diversity between +MCFA and -MCFA fed broilers in H1, while in H2 and H3 the +MCFA broilers displayed a higher cecal microbiota alpha diversity. In addition, the effect of diet or pen on the microbiota composition was lowest in H1. This suggests transmission of microbes may have occurred between the diet groups in H1, and therefore differences between groups due to the diet were leveled out. Additionally, the initial diversity and richness of H1 was higher before the start of the experiment, which might have worked in tandem with the transmission to cause less variation overall in this environment.

In addition to host-to-host transmission, spore-forming bacteria can also influence research environments (Ludvigsen et al., 2016, Browne et al., 2016) and this might also be an important factor that influences the intestinal microbiota in broilers. Though, the genera that were either present or absent in the different housing conditions (*Bacteroidetes* and *Pediococcus*), are surprisingly both non-spore-formers. In H3, the isolators, the relative abundances of genera *Faecalibacterium*, *Blautia*, and *Ruminococcus torques* group were found to be different compared to H1 and H2. These genera are also not known to produce spores. The genus *Bacillus*, known to include endospore-forming bacteria, was present in all housing conditions, while the endospore-forming genus *Clostridium* was only present in H3. Therefore, it is not possible to explain the observed differences between housing conditions by spore-forming bacteria only.

In the H3 there was a low risk of introducing microbes from the shared environment, i.e. surrounding facility or animal technicians, and this might have resulted in a stronger pen effect in H3 compared to H1 and H2. In addition, large differences in exposure to microbes due to differences in levels of biosecurity are likely to have

been caused by the different cleaning approaches and downtime between experiments within each housing condition, as suggested by the Rodac plate results. To support this observation, a more extensive characterization of microbial exposure in the different housing conditions should also be included in future studies in order to allow assessing the presence of those microorganisms in the host itself. In one isolator from H3 the relative abundance of the genus *Blautia* was much lower than in the other isolators, and therefore this single isolator had a large impact on the identification of differentially abundant genera independent of the diet intervention. This difference between pens and isolators within the same housing condition might be a result of stochastic variation in the early life microbial colonization of the birds' intestines (Zhou and Ning, 2017).

Our observations are in line with a piglet study, that showed that piglets raised in isolators showed a lower microbiota diversity compared to siblings raised at a farm (Inman et al., 2010). In addition, also unique genera were observed per housing condition. We succeeded in keeping the temperature and humidity the same across the three housing conditions as well as the light schedule, however, small differences in the local climate of the three units may have occurred. The light intensity in H3 was higher than in H1 and H2. It is not likely that this has had large effects, as light intensity has not been associated with an altered performance (Newberry et al., 1988, Blatchford et al., 2009, Deep et al., 2010), nor is it known to influence the intestinal microbiota. Despite that we tried to keep all conditions that potentially might influence the intestinal microbiota as consistent as possible, the three housing conditions seem to have their own facility-specific effect on the intestinal microbiota.

Despite the difference in alpha and beta (intra- and inter-individual) diversity across the housing conditions, the +MCFA diet lowered the relative abundance of the genus *Lactobacillus* and heightened the relative abundance of *Escherichia-Shigella* and *Turicibacter* in all three housing conditions. This is in line with other studies where MCFA reduced lactobacilli (van der Hoeven-Hangoor et al., 2013, Zentek et al., 2012). The observation that *Escherichia-Shigella* (family *Enterobacteriaceae*) was higher in relative abundance in broilers +MCFA for all three housing conditions, is in line with another study that showed that MCFA promoted members of the family *Enterobacteriaceae* in the ileum of broilers (van der Hoeven-Hangoor et al., 2013). MCFA are also known to control and decrease the spread of pathogens in poultry (Van Immerseel et al., 2006, van Gerwe et al., 2010) and improve feed efficiency (van der Hoeven-Hangoor et al., 2013). However, contrasting effects of *Lactobacillus* spp. on the performance of chickens have been observed earlier (Ríos-Covián et al., 2016), possibly because of the different relative abundance of *Lactobacillus* or the presence of different species of *Lactobacillus*. In H1 the genus *Bacteroides* and family *Peptostreptococcaceae* were higher in +MCFA fed broilers, which are both associated with a healthy intestine (Browne et al., 2017, Macdonald et al., 2017,

Wlodarska et al., 2015). In H2 the relative abundances of *Ruminococcus torques* group, *Subdoligranulum*, and *Faecalibacterium* were heightened in +MCFA fed broilers. Of these, the *Ruminococcus torques* group has been associated with better performance (Torok et al., 2011b). It has been observed to be more abundant in broilers treated with zinc bacitracin, and those broilers also showed a reduced feed conversion ratio (Crisol-Martinez et al., 2017). This correlation of the relative abundance of the *Ruminococcus torques* group with reduced feed conversion ratio was not observed in our data.

The observed lower mean body weight in H1 compared to H2 and H3, may have been caused in part by an infection with the intestinal protozoal parasite *Eimeria* in H1. This is a common infection in commercial broiler chickens, which was prevented by the high biosecurity level and long downtimes between experiments for the other two housing conditions. For instance, *Eimeria* has been described to decrease the richness and diversity of the intestinal microbiota (Hauck, 2017), while we found the highest diversity in H1. Also, we found no genera that were previously associated with an *Eimeria* spp. infection (Hauck, 2017, Stanley et al., 2014b, Perez et al., 2011). Lastly, post mortem and clinical findings suggested that *Eimeria tenella*, the species affecting the ceca, was not present. Thus, a limited effect of the *Eimeria* spp. infection on the microbiota can be expected. In addition, the limited number of birds (n=35) and pens (n=5) to measure differences in performance might also be the reason for not finding clear effects on performance data between +MCFA and -MCFA fed broilers. It is known that substantial variation in performance between individual broilers requires large bird numbers to detect significant differences. For instance, in similar studies 108 or 96 broilers were used to observed potential differences between diet interventions (van der Hoeven-Hangoor et al., 2013, Lamot et al., 2016).

To characterize the carbohydrate catabolism of the cecal microbiota, lactate and the short chain fatty acids (SCFAs) acetate, butyrate and propionate and were measured. The metabolite analyses supported the observation that the microbiota varied per housing condition. Not only with respect to composition, but also regarding activity. Although, within each housing condition, no difference in metabolic activity was observed while the composition of the microbiota did vary. Thus, similar functions can be exerted by different species and genera. It is therefore important to assess the impact that microbiota has on e.g. intestinal metabolism, rather than only describing which microbial taxa are present.

This study contributes to understanding of the complex underlying mechanisms leading to differences in intestinal microbiota composition and activity in diet intervention experiments and factors confounding these observations. Differences in housing conditions can act as substantial confounding factors in microbiota studies. Although we have not elucidated the exact mechanisms explaining these differences, we have shown that differences with regard to biosecurity level at the start of an

experiment, but also with regard to contact with the environment and between pens, may be part of the underlying mechanisms explaining these differences. More knowledge on how to modulate the function of the intestinal microbiota will help to improve the resilience of broiler chickens against pathogens and may reduce the need to use antimicrobial drugs. It is important to realize that in addition to pathogens, also commensals can spread which can impact the reproducibility of microbiota studies (van Bunnik et al., 2014). In addition to other known and unknown host- and environmental factors contributing to these observed differences, exposure to microbes present in the living environment is an important factor that can shape the intestinal microbiota community of broiler chickens.

### **Conclusion**

The same nutritional intervention can modify the intestinal microbiota in the same direction under different housing conditions, however, in this study housing condition affected the microbiota composition and functionality stronger than the nutritional intervention. The unique differences found per housing condition resulted in a different interpretation of the dietary MCFA intervention on the microbial changes. Therefore, it is essential to be aware of the potentially large impact of housing conditions on the interpretation of intestinal microbiota experiments. A challenging task for further nutritional microbiota research is to discover the mechanisms to distinguish transmission between hosts, and between hosts and the experimental environment, to improve the repeatability of microbiota research. To improve understanding of the working mechanisms of diet and the interaction with the intestinal microbiota, nutritional experiments should be repeated and also performed under field conditions, to elucidate the mode of action and assess its efficacy.

## **Methods**

### ***Experimental design***

A total of 370 one-day-old male broiler chickens (Ross 308) were purchased from a commercial hatchery (Lagerwey Hatchery, the Netherlands). All chicks were derived from the same 42 week old broiler breeder flock. At the hatchery the chicks were randomly allocated to two different experimental facilities (H1 and H2 + H3). The chicks were transported to these two facilities in the same truck. After a 30 minutes (H2 & H3) and 50 minutes' (H1) drive, the day-old broilers arrived (day 0 of the experiment) and were placed in three different housing conditions (Figure 8), i.e. H1, a grow-out feed trial facility, H2, a floor stable, and H3, isolators (Supplementary materials: Figure S6).

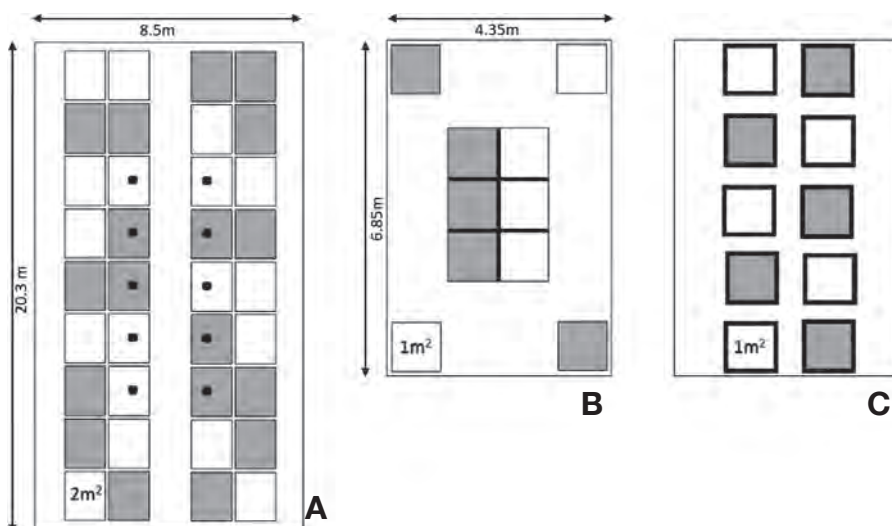
H1 is a research facility at Cargill Animal Nutrition Innovation Center (Velddriel, the Netherlands), and consisted of standard grow-out pens used for broiler feed

experiments. A total of 170 chicks were randomly allocated to 10 pens (2.26 m (b) x 0.90 m (w), 2.03 m<sup>2</sup>) (Figure 8a). The distance between the two blocks separated by an aisle was 1.96 m. In each pen, 10 out of 17 broilers were followed individually throughout the grow-out period. Between the pens steel mesh panels were used as dividers, and the raised metal floor was covered with paper and a 2 cm layer of wood shavings. In this facility a downtime period of two week between experimental rounds was used, and between rounds the facility was cleaned and disinfected with a product with quaternary ammonium compounds and glutaraldehyde (MS Megades, Schippers, the Netherlands).

H2 and H3 were located at the Faculty of Veterinary Medicine of Utrecht University (Utrecht, the Netherlands). Broilers were randomly allocated to H2 or H3. In H2, 100 broilers were randomly distributed over 10 floor pens (1.00 m x 1.00 m, 1 m<sup>2</sup>), in one research unit (Figure 8b). Adjacent pens were separated by solid wooden panels with only a mesh panel at the front of the pen. A single pen was present in four corners, and in the middle six pens were connected with each other (Figure 8b). The floor of each pen was covered with a 2 cm layer of wood shavings. Before placement of the chicks, H2 was extensively cleaned and disinfected with vaporized hydrogen peroxide. In H3, 100 broilers were randomly distributed over 10 negative pressure HEPA filtered isolators (0.65 m x 1.5 m, 1 m<sup>2</sup>; Figure 8c). All materials entering or leaving the isolators were passed through a chlorine tank sealed off with a removable lid. The floor consisted of a box (0.65 m x 0.65 m, 0.42 m<sup>2</sup>) filled with wood shavings to the same amount and from the same batch as in H1 and H2. The other 0.58 m<sup>2</sup> consisted of a plastic mesh floor. All 10 isolators in H3 were extensively cleaned and disinfected with vaporized hydrogen peroxide before the experiment. There was a downtime period of six weeks with the previous experimental flocks for H2 and H3.

Between H1, H2 and H3 bird management conditions were kept as equal as possible. The wood shavings were transported from H1 to H2 and H3 three weeks before the start of the experiment and were stored under comparable conditions. Although the sizes of the pens were slightly different, the chick densities were the same. After 5 days, the number of chicks was reduced to 15 chicks per pen in H1 and 8 chicks per pen in H2 and H3 (Figure 8). This resulted in a stocking density of 7.5 birds per m<sup>2</sup> for H1, and 8 birds per m<sup>2</sup> for H2 and H3. In the pens of H1 and H2 artificial lighting was set at 100 lux for 23 hours/day (h/d) from day 0-3, 20 h/d from day 4-6 and 18 h/d from day 7-35. In the isolators artificial lighting was set with the same schedule, but with a light intensity of 200-400 lux. Temperature gradually decreased from 34 °C at day 0 with 2.5 °C per week to 20°C at day 35. Temperature was monitored twice a day and corrected when needed. The birds were observed twice a day, and presence of clinical signs, abnormal behavior and mortality were recorded. At day 7 all birds in all facilities were vaccinated against Newcastle Disease virus (Avinew® Neo, Boehringer Ingelheim, Germany) with the same battery-operated backpack sprayer (H1 and H2) or handheld garden sprayer (H3).





**Figure 8:** Schematic overview of the three experimental housing conditions. Grey pens are +MCFA, white pens are -MCFA. A. Housing condition 1 (H1) is a grow-out feed trial facility, with mesh panels separating pens. Only the 10 pens with a dot were individually followed and sampled for 16S rRNA gene amplicon sequencing. B. Housing condition 2 (H2), an extensively cleaned floor stable with different distances between the pens and adjacent pens separated by solid wooden panels, with only a mesh panel at the front of the pen. C. Housing condition 3 (H3), isolators, high biosecurity level and protected from environmental contamination.

### Experimental feeds

All broilers had *ad libitum* access to feed and water throughout the experimental period. To establish differences in intestinal microbiota, feeds with and without MCFA (+MCFA and -MCFA) were used (van der Hoeven-Hangoor et al., 2013). The two different feeds were formulated to meet the nutrient requirements of broilers and based on digestibility and nutrient data provided by the Feed Tables from the Dutch Central Bureau of Livestock Feeding (CVB, 2016). For each feeding phase, a starter and grower basal feeds were produced. Starter and grower feeds contained 2,850 and 2,925 kcal of apparent metabolizable energy (AME)·kg<sup>-1</sup> and 10.48 and 9.87 g·kg<sup>-1</sup> apparent fecal digestible lysine. The feed was wheat-corn soybean meal based and in the +MCFA feed, a blend of 0.3% C10:0 capric acid and 2.7% C12:0 lauric acid (Sigma-Aldrich, the Netherlands) was added. Diets were kept isocaloric by exchanging the MCFA blend with soybean oil and animal fat based on the ingredient energy values. Diets were produced at Research Diet Services (the Netherlands) and pelleted using steam addition (approximately 80°C) at 2.5 mm (starter feeds; 0 to 14 days of age) and at 3.0 mm (grower feeds; 14 to 35 days of age). Diets did not contain antimicrobial additives.



### **Data collection**

After cleaning and disinfection of the three housing units (H1-3), a hygienogram was made with Replicate Organism Detection And Counting (Rodac) plates. The Rodac plates (55 mm diameter) contained medium with 16 g/l agar, 1 mL/l tween 80, 1 g/l ammonium carbonate, 2 g/l lecithin, 1 g/l l-histidine, 5 g/l sodium chloride, 10 g/l meat extract, 10 g/l peptone (tryptone + meat peptone) and 0.5 g/l sodium thiosulphate (5H<sub>2</sub>O) (GD Animal Health, the Netherlands). In every pen at least one Rodac plate was pressed gently on a surface for 30 seconds. After incubation for 24h at 38°C the number of colonies was counted to determine the number of colony forming units (CFU).

In all three housing conditions, individual broiler weights were recorded at the start of the experiment (day 0) and at days 14 and 35. In addition to body weights, also feed consumption was recorded for each pen. The gain to feed ratio was calculated as kg of weight gain/kg of feed consumed for each time period (0 to 14, 14 to 35 and 0 to 35 days of age). On day 35, all broilers in the different housing units were euthanized, using carbon dioxide (H1) or electrocution followed by cervical dislocation (H2 and H3). A pen from H2 and H3 were selected alternately for euthanasia, to avoid a sampling effect due to time differences between housing conditions. Cecal content of each broiler was gently squeezed into sterile cryotubes and snap frozen on dry ice and stored at -80°C for microbial genomic DNA extraction. Between sampling of each broiler sterile gloves were changed, and the table, scissors and tweezers were cleaned with 70% ethanol to prevent cross contamination between samples.

### **DNA extraction**

DNA was extracted, from 0.25 g cecal content, using 700 µl Stool Transport and Recovery (STAR) buffer (Roche Diagnostics Nederland BV, the Netherlands). The cecal sample was transferred to a sterile screw-capped 2 mL tube (BIOplastics BV, the Netherlands) containing 0.5 g of zirconium beads (0.1 mm; BioSpec Products Inc., USA) and 5 glass beads (2.5 mm; BioSpec Products Inc., USA). The samples were treated in a bead beater (Precellys 24, Bertin technologies, France) at a speed of 5.5 ms<sup>-1</sup> for 3 × 1 min, followed by incubation at 95 °C with agitation (15 min and 300 rpm). The lysis tube was centrifuged (13,000 g for 5 min at 4 °C), and the supernatant was transferred to a 2 mL microcentrifuge tube. Thereafter, the above described process was repeated with 300 µl of STAR buffer. An aliquot (250 µL) of the combined supernatants from the sample lysis was then transferred into the custom Maxwell® 16 Tissue LEV Total RNA Purification Kit cartridge. The remainder of the extraction protocol was then carried out in the Maxwell® 16 Instrument (Promega, the Netherlands) according to the manufacturer's instructions. DNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, USA), and DNA was stored at -20 °C until further use.

***Microbiota composition***

Extracted DNA was diluted to 20 ng/ $\mu$ L in nuclease free H<sub>2</sub>O. All PCR plastics were UV irradiated for 15 min before use. For 16S rRNA gene-based microbial composition profiling, barcoded amplicons covering the variable regions V5-V6 of the 16S rRNA gene were generated by PCR using the 784F and 1064R primers (Ramiro-Garcia et al., 2016).

Each sample was amplified in duplicate using Phusion hot start II high fidelity polymerase (Finnzymes, Finland), checked for correct size and concentration on a 1% agarose gel and subsequently combined and purified using CleanNA magnetic beads (CleanNA the Netherlands). The 50  $\mu$ L PCR reactions contained 36.5  $\mu$ L nucleotide free water (Promega, USA), 0.5  $\mu$ L of 2 U/ $\mu$ L polymerase, 10  $\mu$ L of 5  $\times$  HF buffer, 1  $\mu$ L of 10  $\mu$ M stock solutions of each of the forward (784F) and reverse (1064R) primers, 1  $\mu$ L 10 mM dNTPs (Promega) and 1  $\mu$ L template DNA.

Reactions were held at 98°C for 30 s and amplification proceeded for 25 cycles at 98°C for 10 s, 42°C for 10 s, 72°C for 10 s and a final extension of 7 min at 72°C. Synthetic communities of known composition were added as positive controls (Ramiro-Garcia et al., 2016), and samples with nuclease free water were added as no-template negative controls to ensure high quality sequencing data. A composite sample for sequencing was created by combining equimolar amounts of amplicons from the individual samples, followed by a final purification step with magnetic beads to remove any remaining contaminants. The resulting libraries were sent to GATC Biotech (Germany; now part of Eurofins Genomics Germany GmbH) for sequencing on an Illumina HiSeq2500 instrument.

Data was analyzed using NG-Tax (Ramiro-Garcia et al., 2016). In short, paired-end libraries were filtered to contain only read pairs with a perfect match to the primers and perfectly matching barcodes, to demultiplex reads by sample. OTU were defined as unique sequences. The OTU picking strategy was based on an open reference approach. First, reads were sorted by abundance per sample and OTUs with an abundance of < 0.1% were discarded. In a second step the remaining reads were matched to the first set of OTUs allowing for one mismatch. Taxonomy was assigned using SILVA 128 16S rRNA gene reference database (Quast et al., 2013).

***High-performance liquid chromatography (HPLC)***

After DNA extraction, from the same 2 mL cryo tubes, 100 mg of cecal content was diluted in 900  $\mu$ L Milli Q, and centrifuged (13,000 g for 15 min at 4°C). Supernatant was stored at -20°C until HPLC analysis. Crotonate was used as internal standard, and the external standards were acetate, butyrate, isobutyrate, lactate and propionate. Substrate conversion and product formation were measured with a Spectrasystem HPLC (Thermo Scientific, the Netherlands) equipped with a Shodex SUGAR SH1011 column with guard column SUGAR KS-G 6B (Agilent, the Netherlands) for the separation of organic acids and carbohydrates. Measurements

were conducted at a column temperature of 45 °C with an eluent flow of 0.8 mL min<sup>-1</sup> flow and the detector RID 20a.

### **Statistical analysis**

All statistical analyses were performed in R version 3.4.2 (R Foundation for Statistical Computing, Austria (Team, 2008)), using the packages: Phyloseq, Microbiome, and Vegan (McMurdie and Holmes, 2013, Lahti et al., 2017, Oksanen, 2010). To test for differences in relative abundance of genera between two groups, we used a Wilcoxon rank-sum test and corrected for multiple testing with Benjamini-Hochberg (BH). Alpha diversity (within sample) was determined using phylogenetic diversity (Faith, 2006), Shannon, Inverse Simpson and Fisher. Faith's phylogenetic diversity not only takes into account the numbers of bacteria, but also the phylogenetic relatedness of those bacteria (Faith, 2006). Beta diversity (between samples) was determined using Jaccard, Bray-Curtis, weighted and unweighted UniFrac metrics (Bray and Curtis, 1957, Jaccard, 1912, Lozupone et al., 2007). Differences in alpha diversity between treatment groups were tested with a Kruskal-Wallis test and pairwise comparisons were tested using a Wilcoxon rank-sum test. Multivariate microbiota data were visualized using principal coordinates analysis (PCoA), and non-parametric permutational analysis of variance (PERMANOVA) tests were used to analyze group differences within multivariate community data (Anderson, 2001). Growth performance data (body weight, average daily gain, average daily feed intake and gain to feed ratio) and concentrations of butyrate, acetate, propionate and lactate concentrations were analyzed with ANOVA test with Tukey's post-hoc test, using pen as experimental unit.

### **Supplementary Materials**

<https://link.springer.com/article/10.1186/s42523-019-0009-z#Sec20>

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### **Availability of data and materials**

Raw sequence data were submitted into the Sequence Read Archive (SRA) at the NCBI under accession number PRJNA553870.

### ***Authors' contributions***

FCV, EAJF, JGK, JAS and HS initiated this project. DML, FCV and JGK performed sample collection. JGK did data processing, analysis and manuscript writing. FCV, EAJF, DML, JAS and HS contributed to the design of this experiment. FCV, DML, EAJF, GH, JAS and HS contributed to the development of the manuscript by giving constructive feedback on the manuscript during its preparation. All authors gave approval of the manuscript for publication.

### ***Ethics approval***

The animal experiment was approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee and was in compliance with all relevant legislation.

### ***Competing interests***

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





# CHAPTER 4

## Comparison of different invasive and non-invasive methods to characterize intestinal microbiota throughout a production cycle of broiler chickens

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## Abstract

In the short life of broiler chickens, their intestinal microbiota undergoes many changes. To study underlying biological mechanisms and factors that influence the intestinal microbiota development, longitudinal data from flocks and individual birds is needed. However, post-mortem collection of samples hampers longitudinal data collection. In this study, invasively collected cecal and ileal content, cloacal swabs collected from the same bird, and boot sock samples and cecal droppings from the litter of the broilers' poultry house, were collected on days 0, 2, 7, 14 and 35 post-hatch. The different sample types were evaluated on their applicability and reliability to characterize the broiler intestinal microbiota. The microbiota of 247 samples was assessed by 16S ribosomal RNA gene amplicon sequencing. Analyses of  $\alpha$  and  $\beta$  measures showed a similar development of microbiota composition of cecal droppings compared to cecal content. Furthermore, the composition of cecal content samples was comparable to that of the boot socks until day 14 post-hatch. This study shows that the value of non-invasive sample types varies at different ages and depends on the goal of the microbiota characterization. Specifically, cecal droppings and boot socks may be useful alternatives for cecal samples to determine intestinal microbiota composition longitudinally.

**Keywords:** microbiome; 16S rRNA, methods, gut, avian, poultry



## Introduction

In the six- to eight-week life span of commercial broilers the intestinal microbiota composition changes rapidly (Awad et al., 2016, Ijaz et al., 2018). Several studies have revealed associations between intestinal microbiota composition and health, and the production performance of broiler chickens (Han et al., 2016b, Johnson et al., 2018, Stanley et al., 2012a, Yeoman et al., 2012, Rinttilä and Apajalahti, 2013, Mohammed et al., 2019), but underlying mechanisms have remained unclear. To elucidate how intestinal microbiota composition interacts with parameters related with health and performance, e.g., maturation of the immune system, growth and feed conversion, longitudinal data is of great value. To this end, monitoring of intestinal microbial development from the first week of life onwards in individual birds would be especially important. This would help to improve the research of underlying mechanisms of intestinal microbiota succession and may facilitate new interventions to increase health and performance of broiler chickens (Goodrich et al., 2014, Corrigan et al., 2015, Ballou et al., 2016). The added value of longitudinal data has also recently been demonstrated in humans, where daily sampling revealed that similar foods had different effects on the intestinal microbiota in different individuals and that the variation of the microbiota depends on at least two days of dietary history (Johnson et al., 2019).

The sample type has a large impact on the observed intestinal microbiota, because the diversity and functionality of the intestinal microbiota substantially differs per intestinal region in mammals and poultry (Pereira and Berry, 2017, Choi et al., 2014, Stanley et al., 2014a). In the ceca of chickens, the microbiota is quantitatively and functionally most developed compared to other parts of the chickens' intestinal tract (Svihus, 2014, Ducatelle et al., 2018). The microbiota in the cecal content has been widely investigated because its functionality, mostly fermentation, is affected by diet (Stanley et al., 2014a, Svihus, 2014, Clench and Mathias, 1995, Józefiak et al., 2004, Yegani and Korver, 2008). The ileum plays an important role for digestion and absorption of nutrients (Noy and Sklan, 1995, Svihus, 2014), and its microbial composition is more sensitive to transient shifts, and shows lower diversity and richness compared to cecal content (Stanley et al., 2014a, Thaiss et al., 2015, Gong et al., 2007a, Owens et al., 2008, Burkholder et al., 2008). Therefore, in broiler intestinal microbiota research cecal content samples are often of main interest, both in experimental settings and field studies. The collection of ileal and cecal content samples has to be done post-mortem, which is not only undesirable because it requires euthanasia of broilers, but also makes longitudinal sampling of individual birds impossible. Fecal droppings of chickens would facilitate longitudinal sampling, however, several studies in chickens have shown that those samples do not reflect the cecal microbiota (Stanley et al., 2015, Pauwels et al., 2015, Videvall et al., 2018). Also, in literature about humans the use of fecal (stool) samples as a reference for the

intestinal microbiota is under debate (Pereira and Berry, 2017, Lavelle et al., 2015, Vandeputte et al., 2016, Wang et al., 2016a). Those samples are easy to collect longitudinally but might be less useful to unravel functionalities of the intestinal microbiota (Pereira and Berry, 2017, Lavelle et al., 2015, Gevers et al., 2014). In addition, there is individual variation in the broilers' intestinal microbiota composition, both between as well as within studies and flocks (Stanley et al., 2013, Thibodeau et al., 2015). Hence, re-sampling of the same bird throughout an entire study could be advantageous in many types of microbiota research (Pauwels et al., 2015, Knight et al., 2018).

A non-invasive sample type used in several studies is the cloacal swab (Stanley et al., 2016, Donaldson et al., 2017, Pedroso et al., 2005). The intestinal microbiota from cloacal swabs was distinct from cecal content samples in broilers of 25 days of age but showed some overlap with ileal content samples (Stanley et al., 2015). A recent study showed that microbiota composition assessed using cloacal swabs was not reflecting the cecal content or ileal content samples but most closely represented the microbiota found in litter samples (Johnson et al., 2018). In four- to six-week-old ostriches, cloacal swabs showed similar results as fecal samples and also differed from cecal content and ileal content samples (Videvall et al., 2018). Another non-invasive sample type is cecal droppings, as in addition to fecal droppings, chickens also excrete cecal droppings (Williams, 1995). The similarity of the cecal content and cecal droppings has been described before for broilers at the end of the production cycle (Pauwels et al., 2015), but not in broilers during the production cycle. A third non-invasive sample type are boot socks samples, which are commonly used in poultry houses to monitor the presence of *Salmonella* (Skov et al., 1999, Berghaus et al., 2013). Theoretically, boot socks might also be applicable as a method to measure the microbiota of a poultry house but have not been applied in microbiota research so far. To our knowledge, there are no published reports where the potential usability of boot socks or cecal droppings was evaluated for microbiota research from shortly after hatch throughout a broiler production cycle.

Therefore, the aim of this study is to evaluate invasively collected samples and non-invasively collected samples with regard to characterization of the intestinal microbiota composition. On two different broiler farms cloacal swabs were collected intravitally and from the same birds cecal and ileal content was collected at post-mortem at 0, 2, 7, 14 and 35 days of age. At the same time points, cecal droppings and boot socks samples were obtained from the broiler house. We compared the microbiota composition of cecal and ileal content to cloacal swabs, and compared cecal content to cecal droppings, and cecal content with boot socks samples. In addition to the microbiota composition, also the discriminative properties of the sample types were assessed, by evaluating its ability to distinguish farms or the age of the broilers. The microbiota composition of 247 samples was assessed by 16S

ribosomal RNA (rRNA) gene amplicon sequencing, to determine intestinal microbiota composition longitudinally in broiler flocks and in an experimental setting. Results from this study show that cecal droppings represented the cecal microbiota of the five individual birds across different time points quite well, even though we collected those samples in pools from the litter in the poultry house. Early in the production cycle, the composition measured with the boot socks was close to that of cecal content samples. Cecal and ileal content samples are, however, distinct from cloacal swabs. Therefore, cecal droppings and boot socks may be useful alternatives for cecal samples to determine intestinal microbiota composition longitudinally. This study contributes to expanding the toolbox for the collection of valuable data under field and experimental circumstances. With these tools, novel strategies can be developed to steer towards more resilient gut microbiota.

## Materials and Methods

### *Ethical Statement*

The Dutch Central Authority approved the animal experiment for Scientific Procedures on Animals and the Animal Experiments Committee (registration number AVD108002016442, 7 April 2016) of Utrecht University (Utrecht, the Netherlands), and all procedures were done in full compliance with all relevant legislation.

### *Data Collection*

Data for this study were obtained from two broiler farms in The Netherlands, with Ross 308 broilers, low historic use of antibiotics, and good production performance. No antibiotics were used. Coccidiostatic drugs were applied as standard in the feed of both flocks. Both farms used a combination of the coccidiostatic drugs nicarbazin and narasin (Maxiban® G160, Elanco, Houten, The Netherlands). Farm 1 used nicarbazin and narasin from day 0 until day 28 followed by narasin (Monteban® G100, Elanco, Houten, The Netherlands) until the end of the production period. Farm 2 used nicarbazin and narasin from day 0 until the end of the production period. At both farms water and feed were supplied *ad libitum* but the farms had a different feed supplier and received broilers from different commercial hatcheries. Diets on both farms were wheat based, combined with feeding of whole wheat at later ages. In addition to soybean meal, sunflower seed meal and rapeseed meal were added at maximum 6.5% inclusion.

The age of the parent stock was 55 weeks for Farm 1, and 35 weeks for Farm 2. The artificial lighting was set for 23 h/day (h/d) from days 0–3, 20 h/d for days 4–6 and 18 h/d for days 7–35. Temperature was set to gradually decrease from 34°C at day 0 to 20°C at day 35. Farm 1 used wood shavings and Farm 2 peat as litter material.

During one production cycle both farms were visited on day 0 (day of arrival of the

day-old chicks on the farm), 2, 7, 14 and 35. One farm was visited in August 2016, and the second farm was visited in June 2017. From each poultry house, five broilers were randomly selected, one from each corner and in the middle of the poultry house. The start of the sampling of broilers took place at least 30 min after the end of a dark period, to reduce potential effects of fasting on the amount of content in the intestinal tract and microbiota composition.

### ***Sampling Methods***

During each visit samples were taken from the five broilers per house before, i.e., cloacal swabs, and after euthanization, i.e., cecal content and, ileal content, and from the poultry house, i.e., cecal droppings from special feces collection boxes, and boot socks. The broilers were collected from the broiler house and immediately a cloacal swab (Rayon swab tip, Copan 155C, Copan Italia, Brescia, Italy) was taken. The swab was placed in a 2 mL sterile cryotube (Brand™ 114832, Fisher Scientific, Wertheim, Germany) and cut with a sterile scissor to fit in the tube. Each tube with swab was placed on dry ice before being stored in a freezer (−80°C) for future processing. Subsequently, the broilers were euthanized by cervical dislocation, immediately followed by removal of the gastrointestinal tract. This procedure was carried out as sterile as possible, and between broilers sterile gloves were changed and the table, scissors, and tweezers were cleaned with 70% ethanol to prevent cross contamination between broilers. The distal end of the cecum was cut to collect cecal content. Ileal content was collected distal and close to the Meckel's diverticulum. The intestinal content was gently squeezed into a 2 mL sterile cryotube and snap frozen on dry ice and stored at −80°C for microbial genomic DNA extraction. The time between euthanization and placing the samples on dry ice was between 3–5 min.

In each poultry house five fecal collection boxes of wood with iron netting (70 cm × 50 cm × 5 cm) were placed on the litter, with one in each corner and one in the middle of the poultry house (Figure S1). At the start of each sample collection day, these fecal collection boxes were lined with clean oilpaper (soda paper treated with paraffin, Vendrig packaging, Woerden, The Netherlands) to collect cecal droppings. The cecal droppings were collected in the poultry house 4–6 h after placement of the boxes in the poultry house, using a sterile spoon (SteriPlast Bio, 2.5 mL, Bad Belling, Germany) or a sterile surgical disposable scalpel (Carbon Steel scalpel, #10, Braun, Tuttlingen, Germany). At least three different cecal droppings per box were put in one 2 mL cryotube and put on dry ice and stored at −80°C until DNA extraction.

Per broiler house, at each sampling day, five boot socks (bioTRADING Benelux B.V., Mijdrecht, The Netherlands) were used to collect litter samples at the same locations where the fecal collection boxes were placed (Figure S1). Boot socks were worn over the foot while walking through a poultry house to collect adhering fecal and litter material. The poultry house boots were covered with clean plastic boot socks to

avoid contamination with feces already present on the boots. Sterile gloves were used to apply the boot socks for sampling and after walking the surface of the designated location, the boot socks were placed in a plastic bag and put on dry ice and stored at  $-80^{\circ}\text{C}$  until DNA extraction.

### **DNA Extraction**

In total, 50 cecal and 50 ileal content samples, 47 cecal droppings, 50 cloacal swabs and 50 boot socks were analyzed. DNA was extracted from 0.25 g cecal and ileal content and cecal droppings using 700  $\mu\text{L}$  of Stool Transport and Recovery (STAR) buffer (Roche Diagnostics Nederland BV, Almere, The Netherlands). All samples were transferred to a sterile screw-capped 2 mL tube (BIOplastics BV, Landgraaf, The Netherlands) containing 0.5 g of zirconium beads (0.1 mm; BioSpec Products, Inc., Bartlesville, Oklahoma, USA) and 5 glass beads (2.5 mm; BioSpec Products) was used.

The cloacal swab samples were directly transferred to a sterile screw-capped 2 mL tube (BIOplastics BV) containing also 0.5 g of zirconium beads (0.1 mm; BioSpec Products, Inc) and 5 glass beads (2.5 mm; BioSpec Products) but 300  $\mu\text{L}$  of Stool Transport and Recovery STAR buffer.

Before DNA was extracted from a boot sock sample, the boot sock was cut in half with a sterile scalpel and put into two different 50 mL Falcon tubes. Then 35 mL phosphate-buffered saline (PBS) was added to both 50 mL tubes and the tubes were vortexed for 20 s (s) at maximum speed. Thereafter the tubes were placed for 30 min in a carousel (Shake 'n' Stack Thermo Hybaid, Thermo Fisher Scientific, Landsmeer, The Netherlands) to soak the feces from the boot sock (room temperature). From both tubes, 5 mL was pipetted in a 10 mL sterile tube. Next the tubes were vortexed for 5 s at maximum speed to mix and 1.5 mL of boot sock content was transferred to a sterile 2 mL microcentrifuge tube and centrifuged (13,000 g for 5 min at  $4^{\circ}\text{C}$ ). The supernatant was removed and of the 10 mL tube again 1.5 mL boot sock content was added and centrifuged (13,000 g for 5 min at  $4^{\circ}\text{C}$ ). The pellet was resuspended in STAR buffer. All samples were treated in a bead beater (Precellys 24, Bertin technologies, Montigny-le-Bretonneux, France) at a speed of 5.5 m/s for  $3 \times 1$  min, followed by incubation at  $95^{\circ}\text{C}$  with agitation (15 min and 300 rpm). The lysis tube was centrifuged (13,000 g for 5 min at  $4^{\circ}\text{C}$ ), and the supernatant was transferred to a 2 mL microcentrifuge tube. Thereafter, the aforementioned process was repeated with 300  $\mu\text{L}$  STAR buffer for the cecal, cecal droppings and ileal content samples, and with 200  $\mu\text{L}$  STAR buffer for the swab and boot sock samples.

An aliquot (250  $\mu\text{L}$ ) of the combined supernatants from the sample lysis was then transferred into the custom Maxwell® 16 Tissue LEV Total RNA Purification Kit cartridge (Promega, Leiden, The Netherlands). The remainder of the extraction protocol was then carried out in the Maxwell® 16 Instrument (Promega, Leiden, The

Netherlands) according to the manufacturer's instructions. The DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, Delaware, USA), and the DNA samples were stored at  $-20^{\circ}\text{C}$  until further use.

### **16S rRNA Gene Amplification Analysis**

Extracted DNA was diluted to  $20\text{ ng }\mu\text{L}^{-1}$  in nuclease free  $\text{H}_2\text{O}$ . All polymerase chain reaction (PCR) plastics were ultraviolet (UV) radiated for 15 min before use. For 16S rRNA gene-based microbial composition profiling, barcoded amplicons covering the variable regions V5-V6 of the bacterial 16S rRNA gene were generated by PCR using the 784F and 1064R primers as described in Ramiro-Garcia et al. (2016).

Each sample was amplified in duplicate using Phusion hot start II high fidelity polymerase (Finnzymes, Espoo, Finland) checked for correct size and concentration on a 1% agarose gel and subsequently combined and purified using CleanNA magnetic beads (CleanN, Waddinxveen, The Netherlands). A detailed description of the PCR reactions was described previously (Kers et al., 2019b).

Positive and negative controls were added to the data set to ensure high quality sequencing data. As positive controls we used synthetic communities of known composition (Ramiro-Garcia et al. 2016), and as negative controls we used nuclease-free water. The resulting libraries were sent to GATC Biotech (Konstanz, Germany; now part of Eurofins Genomics Germany GmbH) for sequencing on an Illumina HiSeq2500 instrument. The 16S rRNA data was analyzed using the NG-tax pipeline (Ramiro-Garcia et al. 2016). In short, paired-end libraries were filtered to contain only read pairs with a perfect match to the primers and perfectly matching barcodes, to demultiplex reads by sample. Amplicon sequence variants (ASV) were defined as unique sequences. The ASV picking strategy was based on an open reference approach. First, reads were sorted by abundance per sample and ASVs with an abundance of  $<0.1\%$  were discarded. In a second step the remaining reads were matched to the first set of ASVs with one mismatch. Taxonomy was assigned using the SILVA 128 16S rRNA gene reference database (Quast et al., 2013). Raw sequence data were deposited into the Sequence Read Archive (SRA) at the NCBI under accession number PRJNA574842.

### **Statistical Data Analysis**

To compare sample types we applied widely used diversity measures and univariate and multivariate statistical analyses. Alpha diversity (within sample) was determined using Faith's phylogenetic diversity (Faith, 2006) which not only takes into account the numbers of bacteria, but also the phylogenetic relatedness of those bacteria (Faith, 2006). Differences in  $\alpha$  diversity between measured microbiota by the different sample types were tested with a Kruskal–Wallis test and pairwise comparisons were tested

using a Wilcoxon rank-sum test. Beta diversity (between samples) was determined using Jaccard, Bray–Curtis, weighted and unweighted UniFrac measures (Bray and Curtis, 1957, Jaccard, 1912, Lozupone et al., 2007). Multivariate microbiota data were visualized using principal coordinates analysis (PCoA), and non-parametric permutational analysis of variance (PERMANOVA) tests were used to analyze differences within multivariate community data (Anderson et al., 2001). To test for differences in relative abundance of genera between two sample types, we used a Wilcoxon rank-sum test and corrected for multiple testing with Benjamini–Hochberg (BH). We compared the cecal and ileal content to cloacal swabs, and cecal content with cecal droppings, and cecal content with boot sock samples. The comparisons between ileal content and boot socks and ileal content and cecal droppings were excluded from the data analysis because this was considered as not biologically relevant. All statistical analyses were performed in R version 3.4.2, using the packages: Phyloseq, Microbiome and Vegan (McMurdie and Holmes, 2013, Lahti et al., 2017, Oksanen, 2010).

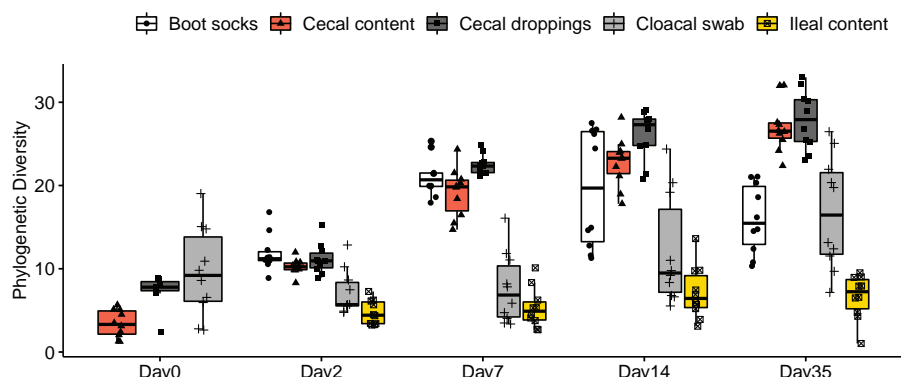
## Results

### **Technical Results of 16S rRNA Gene Sequencing**

In total, 50 cecal content, 40 ileal content, 50 cloacal swabs, 47 cecal droppings and 40 boot socks samples passed our quality control standards. The amplicon sequence variants (ASV) associated with an unknown domain or the order Mitochondria were removed from all sequenced samples. About 90% of the families in the negative controls were associated with five families: *Bacillaceae*, *Burkholderiaceae*, *Halomonadaceae*, *Micrococcaceae*, or *Shewanellaceae*. The ileal content and boot sock samples of day 0 were excluded because these contained a large number of families associated with the negative control samples, and therefore did not pass our quality control standards (Figure S2).

### **Alpha Diversity Analysis across Different Sample Types**

When the data from all ages and farms were analyzed, the phylogenetic diversity was significantly higher in cecal content compared to the cloacal swab samples and the phylogenetic diversity was lower in ileal content compared to the cloacal swab samples (Figure 1, Table 1:  $\chi^2 = 10.6$ ,  $p = 0.001$  and  $\chi^2 = 18.5$ ,  $p < 0.001$ ). The cecal content was not significantly different from cecal droppings and boot socks based on phylogenetic diversity (Table 1:  $\chi^2 = 3.6$ ,  $p = 0.058$ ;  $\chi^2 = 2.6$ ,  $p = 0.108$ ). Alpha diversities Chao 1 and Shannon, that do not take into account the phylogenetic relatedness, showed one different result of the comparisons. According to Chao 1 and Shannon measures, the ileal content samples were not significantly different when compared to cloacal swab samples (Table 1).



**Figure 1:** Phylogenetic diversity (ASV level) of different sample types for both sampled farms across 0, 2, 7, 14 and 35 days of age. Whiskers show 95% interval, box 50% interval,  $n = 10$ , circles are boot socks, triangles are cecal content, squares are cecal droppings, pluses are cloacal swabs and squares with cross are ileal content samples.

### Beta Diversity Analysis across Different Sample Types

Sample type explained 6.6%, 4.7%, 10.3% and 14.6% of the variation between the cecal content samples and cloacal swabs for Bray–Curtis, Jaccard, unweighted and weighted UniFrac distances based analysis, respectively (Table 2). For ileal content versus cloacal swabs sample, type explained 3.2%, 2.4%, 6.5%, 10.7% of the variation depending on the distance metric (Table 2). Based on unweighted UniFrac distances, no significant difference between cecal content and cecal droppings were measured, whereas on based Bray–Curtis, Jaccard and weighted

**Table 1:** Different diversity measures (ASV level) across different samples types. Number of broilers tested is specified in the table row (Kruskal–Wallis).

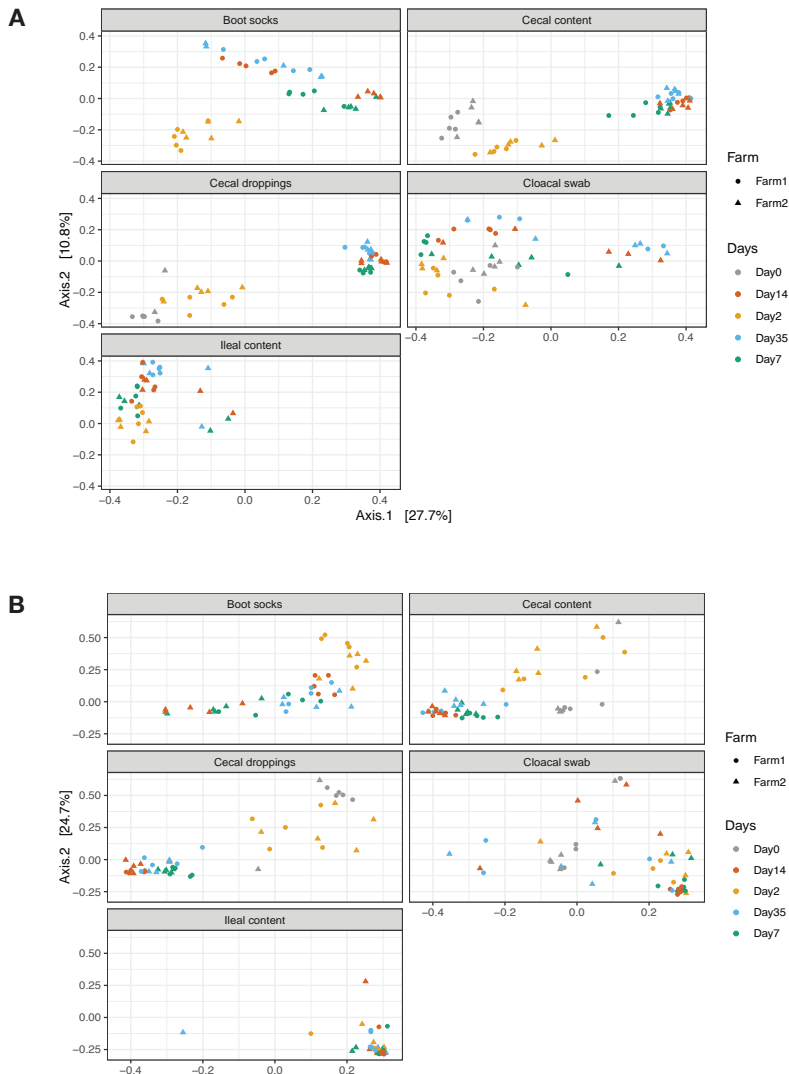
Sample type	Phylogenetic diversity		Chao 1		Shannon	
	$\chi^2$	p-value	$\chi^2$	p-value	$\chi^2$	p-value
Cecal content versus cloacal swab ( $n=100$ )	10.633	0.001	15.076	$1.0 \times 10^{-4}$	26.947	$2.1 \times 10^{-7}$
Ileal content versus cloacal swab ( $n=80$ )	18.501	$1.7 \times 10^{-5}$	0.245	0.620	3.379	0.066
Cecal content versus cecal droppings ( $n=97$ )	3.604	0.058	6.623	0.010	6.116	0.013
Cecal content versus boot socks ( $n=80$ )	2.582	0.108	0.241	0.624	1.447	0.229
Cecal content	$\chi^2$	p-value	$\chi^2$	p-value	$\chi^2$	p-value
n=50						
Age	43.848	$6.9 \times 10^{-9}$	38.147	$1.7 \times 10^{-7}$	37.252	$1.6 \times 10^{-7}$
Farm	0.350	0.554	0.001	0.938	0.115	0.734
Ileal content						
n=40						
Age	3.902	0.272	3.547	0.315	11.545	0.009
Farm	0.026	0.871	0.751	0.386	1.230	0.267
Cloacal swab						
n=50						
Age	14.888	0.005	13.255	0.010	13.917	0.007
Farm	2.688	0.101	2.765	0.096	0.886	0.347
Cecal dropping						
n=47						
Age	38.679	$8.1 \times 10^{-8}$	33.46	$9.4 \times 10^{-7}$	32.865	$1.3 \times 10^{-6}$
Farm	0.007	0.932	0.009	0.924	0.016	0.898
Boot socks						
n=40						
Age	17.729	$5.7 \times 10^{-4}$	24.527	$1.9 \times 10^{-5}$	22.945	$4.1 \times 10^{-5}$
Farm	4.801	0.029	4.120	0.042	3.484	0.062



analysis showed that sample type explained 3.4%, 2.6% and 2.9% of the variation UniFrac distances between the cecal content and cecal droppings. This suggests that the composition of the cecal content and cecal droppings were phylogenetically not different, but that the abundance of genera were different. The principal coordinate analysis (PCoA), in Figure 2 shows that cecal content samples and cecal droppings samples are near each other (and Figure S3 with different sample types combined in single plots). Between the cecal content and boot sock samples 8.5%, 6.2%, 10.7% and 22.7% of the variation was explained by the sample type (Table 2).

**Table 2:** Beta diversity analysis across different samples types. Overview of different distance measures that determine the microbiota interindividual diversity, R<sup>2</sup> = Percentage of the variation between broilers explained,  $p$  =  $p$ -value permutational analysis of variance (PERMANOVA) test, FDis =  $p$ -value multivariate dispersions test.

Samples		Bray-Curtis				Jaccard				unweighted UniFrac				weighted UniFrac			
		R <sup>2</sup>	$p$	FDis	R <sup>2</sup>	$p$	FDis	R <sup>2</sup>	$p$	FDis	R <sup>2</sup>	$p$	FDis	R <sup>2</sup>	$p$	FDis	$p$
Sample type		<i>n</i>															
Cecal content vs cloacal swab	100	6.6	1 x 10 <sup>-4</sup>	0.042	4.7	1 x 10 <sup>-4</sup>	0.034	10.3	1 x 10 <sup>-4</sup>	0.318	14.6	1 x 10 <sup>-4</sup>	0.627				
Ileal content vs cloacal swab	80	3.2	0.0031	0.685	2.4	0.0137	0.672	6.5	2 x 10 <sup>-4</sup>	0.569	10.7	1 x 10 <sup>-4</sup>	0.002				
Cecal content vs cecal droppings	97	3.4	3 x 10 <sup>-4</sup>	0.001	2.6	2 x 10 <sup>-4</sup>	0.001	1.6	0.155	0.169	2.9	0.028	0.370				
Cecal content vs boot socks	80	8.5	1 x 10 <sup>-4</sup>	0.001	6.2	1 x 10 <sup>-4</sup>	0.001	10.7	1 x 10 <sup>-4</sup>	0.626	22.7	1 x 10 <sup>-4</sup>	0.682				
Cecal content	50	3.5	0.039	0.135	3.1	0.029	0.188	2.9	0.165	0.825	1.6	0.519	0.990				
Cecal content	50	37.8	1 x 10 <sup>-4</sup>	0.041	26.9	1 x 10 <sup>-4</sup>	0.058	55.7	1 x 10 <sup>-4</sup>	0.001	62.0	1 x 10 <sup>-4</sup>	0.564				
Ileal content	40	6.5	0.006	0.884	5.5	0.005	0.887	3.4	0.174	0.012	5.1	0.019	0.970				
Ileal content	40	23.0	1 x 10 <sup>-4</sup>	0.005	18.8	1 x 10 <sup>-4</sup>	0.007	29.6	1 x 10 <sup>-4</sup>	0.959	18.3	1 x 10 <sup>-4</sup>	0.245				
Cloacal swab	50	4.1	0.01	0.866	3.6	0.014	0.871	3.4	0.065	0.678	3.4	0.131	0.497				
Cloacal swab	50	30.3	1 x 10 <sup>-4</sup>	0.009	23.4	1 x 10 <sup>-4</sup>	0.010	34.4	1 x 10 <sup>-4</sup>	0.755	38.7	1 x 10 <sup>-4</sup>	0.027				
Cecal droppings	47	3.8	0.061	0.827	3.5	0.033	0.841	3.9	0.110	0.782	2.0	0.364	0.674				
Cecal droppings	47	42.7	1e-04	0.937	30.5	1 x 10 <sup>-4</sup>	0.882	64.5	1 x 10 <sup>-4</sup>	0.006	68.9	1 x 10 <sup>-4</sup>	0.206				
Boot socks	40	10.0	0.002	0.932	8.9	6 x 10 <sup>-4</sup>	0.939	9.2	0.004	0.966	11.4	0.004	0.850				
Boot socks	40	50.6	1e-04	0.005	38.6	0.387	0.041	51.0	1 x 10 <sup>-4</sup>	0.034	55.9	1 x 10 <sup>-4</sup>	0.001				



**Figure 2:** Principal coordinate plots (PCoA) based on A. unweighted UniFrac and B. weighted UniFrac distances between cecal and ileal content, cloacal swabs, cecal droppings and boot socks. Different sample types are shown in separate plots for clarity (Combined Figure S3). Different colors indicate different sampling days, circles are samples of Farm 1 and triangles are samples of Farm 2.

### ***The Effect of Age on the Bacterial Microbiota across Different Sample Types***

The phylogenetic diversity of the cecal content and cecal droppings increased with the age of the broilers (Figure 1,  $\chi^2 = 43.8$ ,  $p < 0.001$ ;  $\chi^2 = 38.7$ ,  $p < 0.001$ , Table 1). The cloacal swabs and boot sock samples also showed a significant increase in phylogenetic diversity across time but with a smaller effect size ( $\chi^2 = 14.9$ ,  $p = 0.005$ ;  $\chi^2 =$

17.7,  $p < 0.001$ ). No age effect was found for ileal content samples (Table 1). For the samples of day 0, cecal content showed a lower phylogenetic diversity compared to cecal droppings and cloacal swabs (Figure 1, Table S1). On days 2, 7 and 35 no difference in the phylogenetic diversity between cecal content and cecal droppings was observed (Figure 1, Table S1). On day 7 no differences in phylogenetic diversity were detected between cecal content and the boot socks, and between ileal content samples and cloacal swabs (Figure 1, Table S1). Also on day 14 cecal content and boot socks were not different, but the cecal content showed a higher phylogenetic diversity compared to the cecal droppings (Figure 1, Table S1). All other comparisons between sample types across age were significantly different (Table S1).

For the cecal content samples the PCoA, based on unweighted UniFrac and weighted UniFrac distances, showed that the age of the broilers explained 56% and 62% of the variation (Figure 2, Table 2). When using Bray–Curtis and Jaccard distances, that do not incorporate phylogenetic distances, age explained 37.8% and 26.9% of the variation (Table 2, Figure S3). Taken together, this suggests that the taxa found in the cecal content samples were phylogenetically distinct across ages. For the ileal content samples, weighted UniFrac distances showed that age explained just 18.3% of the variation (Figure 2, Table 2).

In the cloacal swab samples age explained 38.7% of the variation based on weighted UniFrac distances (Figure 2, Table 2), however, also  $\beta$  dispersion was found to be different across different ages within cloacal swabs (Table 2). Based on weighted UniFrac distances was shown that in cecal droppings age explained 68.9% of the variation (Figure 2, Table 2). In addition, the results based on weighted UniFrac distances showed that also in boot socks broiler age explained most of the variation (55.9%, Figure 2, Table 2).

### ***The Effect of Farm on the Bacterial Microbiota across Different Sample Types***

No differences in phylogenetic diversity between the two farms were observed in cecal content, cecal droppings, cloacal swabs and ileal content, but differences were observed between boot sock samples from the two farms ( $\chi^2 = 4.8$ ,  $p = 0.029$ ; Table 1 and Figure S4). When comparing the different sample types on the two farms, similar results were found; the only difference was that in Farm 1 the phylogenetic diversity in cecal content was higher compared to that of cloacal swabs, while in Farm 2 this difference was not observed ( $p = 0.012$  versus  $p = 0.058$ ; Table S1 and Figure S4). The larger individual variation between cloacal swabs samples in Farm 2 might be the reason why we observed this difference between farms (Figure S4).

Weighted UniFrac distances between cecal content microbiota showed that farm did not explain any of the variation (Figure 2, Table 2). When using Bray–Curtis and Jaccard distances that do not incorporate phylogenetic distances, farm explained 3% of the variation (Table 2). Taken together, this suggests that the taxa

found in the cecal content samples were phylogenetically related across farms. For the ileal content samples, based on weighted UniFrac distances, results showed that 5.1% of the variation was explained by farm (Figure 2, Table 2). The Bray–Curtis and Jaccard distances also showed that farm explained 5–6% of the variation (Table 2).

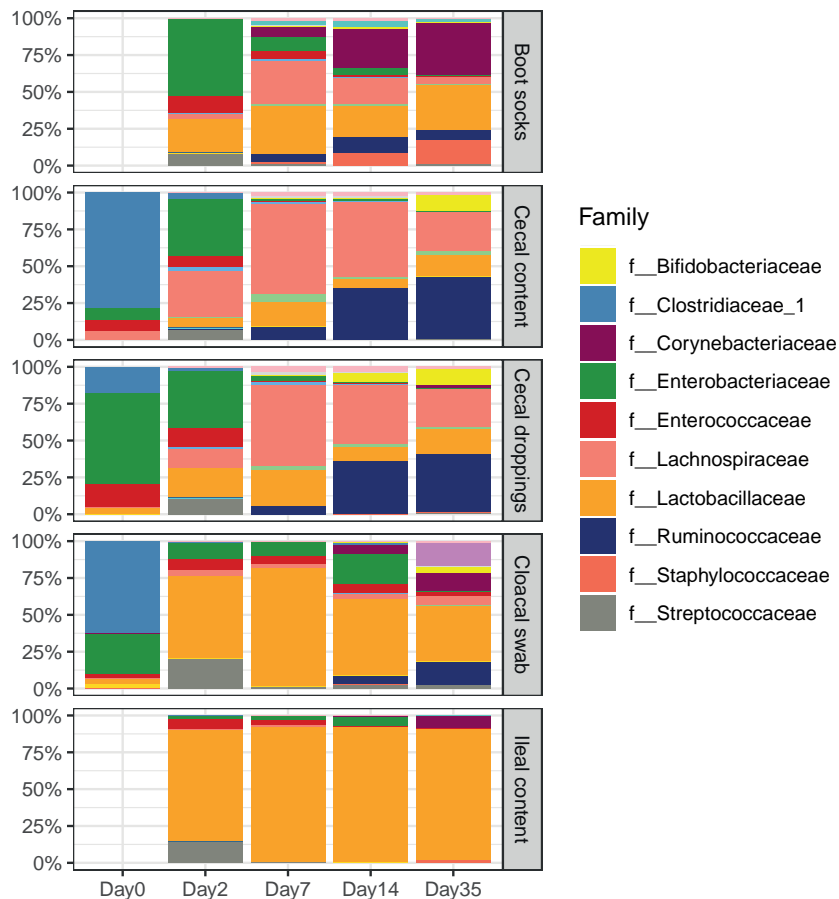
For the cloacal swab samples the results showed that farm did not explain a significant part of the variation based on unweighted and weighted UniFrac, but it did explain part of the variation based on Bray–Curtis (4.1%) and Jaccard (3.6%) (Figure 2, Table 2). For cecal droppings based on weighted UniFrac distances, showed that the farm did not explain the variation (Figure 2, Table 2). Only the Jaccard distance, taking into account the presence or absence of an ASV, showed that farm explained 3.5% of the variation (Table 2). The results based on weighted UniFrac distances showed that for boot socks, the farm explained 11.4% of the variation (Table 1). This was mainly an effect of the large difference between farms and boot socks on day 14 (Figure 2).

### ***Microbial Taxa that Differ across the Invasive and Non-Invasive Sample Types***

Figure 3 shows the relative abundance across age and different sample types at family level. On day 0 both the cecal content samples and cloacal swabs were found to be predominated by members of the family *Clostridiaceae* (Figure 3). From day 2 onwards the same microbial families were highly abundant within the cecal content and cecal dropping samples (Figure 3, Table S2).

On day 2 *Enterobacteriaceae* and *Lachnospiraceae* were predominant in the cecal content, and *Lachnospiraceae* were predominant on day 7. On day 14 and day 35 *Ruminococcaceae* and *Lachnospiraceae* were the most predominant families in the cecal content samples.

The only difference in the abundance of genera found between cecal content and cecal droppings was the increased relative abundance of the genus *Lactobacillus* in the cecal droppings compared to the cecal content on day 2 (Figure S5a). The genus *Lactobacillus* was also more abundant in the cloacal swabs compared to the cecal content but also many other genera were different (Table S3). Only on day 0, no differences between genera were observed between the cecal content and cloacal swab samples. On day 2 only the genus *Escherichia-Shigella* was higher in relative abundance in the cloacal swab samples compared to the cecal content (Figure S5b, Table S3). In addition, on day 2 no differences were observed between cecal content and boot socks and between ileal content and cloacal swabs.



**Figure 3:** Relative microbial abundance across age and different sample types at family level. Only the labels of the 10 most abundant families are shown, the other families are not labeled for clarity. All families are presented in Table S2.

The differences in relative abundance of genera observed in cecal content and boot socks varied across time (Table S3). The genera *Lactobacillus* (13–17% points), *Corynebacterium* (14–28% points) and *Staphylococcus* (11–18% points) showed a higher relative abundance in the boot socks compared to cecal content samples (Figure S5a, c, d, and Table S3). Only on day 35 did the cloacal swabs and the ileal content show differences in five genera, with a main increase in the genus *Faecalibacterium* (11% points) in the cloacal swab samples (Figure S5e, Table S3).

Concerning differences between farms, within the cecal content samples the only difference observed was a higher relative abundance of the genus *Bacteroides* in Farm 2 compared to Farm 1 (Figure S5f,  $p = 0.005$ ). Only in the boot socks was it also possible to observe a higher relative abundance in *Bacteroides* in Farm 2

(Figure S5f,  $p = 0.029$ ). No difference in genera between farms could be detected in cecal droppings, cloacal droppings, or ileal content samples.

## Discussion

The aim of this study was to evaluate different types of samples with regard to characterization of the intestinal microbiota of individual broilers and flocks from the first week of life onwards until the end of the production cycle. In particular, we compared invasively collected cecal and ileal content samples to non-invasive samples, such as cloacal swabs, cecal droppings and boot sock samples collected from the litter. In addition, the invasive and non-invasive sample types were evaluated on the ability to distinguish between ages and among farms. The outcomes of this study suggest that cecal droppings and boot socks are useful non-invasive alternatives for cecal content samples to determine intestinal microbiota longitudinally in broilers and broiler flocks.

In our study, the cecal microbiota had the highest diversity and showed less variation over time compared to the microbiota of the ileal content, which is consistent with other studies (Stanley et al., 2014a, Gong et al., 2007, Owens et al., 2008). Age had the most profound effect on the cecal microbiota as demonstrated by other authors (van der Wielen et al., 2002, Gong et al., 2008). Although the sample size in this study was limited to five samples per farm and time point, clear differences in microbiota across sample types were observed. It should be noted, however, that the evaluation of different sample types was based on finding no statistically significant differences between sample types. As we used non-parametric tests corrected for multiple testing, that have a low chance of false-positive results, we may have underestimated the microbiota composition differences between sample types. Therefore, we compared different methods, different  $\alpha$  and  $\beta$  diversity measures and applied univariate and multivariate statistical analyses together, to determine which differences in the measured intestinal microbiota between different sample types were considered relevant.

No differences between the farms were observed in cecal content based on  $\alpha$  diversity and  $\beta$  diversity taking phylogenetic relatedness into account. This is in contrast with another study where differences in microbiota composition of cecal content were found between broiler flocks (Johnson et al., 2018). Our results indicate a comparable development of the microbiota across age for both farms, despite differences between farms, such as feed supplier, bedding materials, parent stock and management. Except for these differences, both farms were similar with respect to good production performance and health status of the flock, including not using antibiotics. A comparable regimen of in-feed coccidiostat drugs in starter and grower feed, and the geographical proximity of the two farms might in part explain the absence of detectable differences in cecal microbiota composition. However, when

phylogenetic relatedness was not taken into account, the farm explained a small, but significant amount of variation between the cecal content samples, indicating that the development was comparable but there were also differences in the microbiota composition between the farms. This result ties in well with a previous study wherein the choice of litter material influenced the cecal microbiota (Torok et al., 2009), which might also be the case in this study.

When comparing non-invasive sample types to the cecal content, the cecal droppings represented the cecal microbiota of the five individual birds across different time points quite well, even though we collected those samples in pools from the litter in the poultry house. Only on day 2 of age did cecal droppings show a higher relative abundance of the genus *Lactobacillus* compared to cecal content. These results add new information to previous results described in literature, which only showed that cecal droppings reflected cecal content microbiota of broilers at the end of the production cycle (Pauwels et al., 2015). Cecal droppings might be a challenge to collect, since chickens produce just one cecal dropping for every seven to eight fecal droppings (Williams, 1995). Nevertheless, this sample type may be of value to monitor the cecal microbiota composition in broilers non-invasively and longitudinally.

Another surprising potentially useful non-invasive sample type to monitor the cecal microbiota at flock level are boot socks samples. This method is commonly used in poultry houses to monitor the presence of *Salmonella* (Skov et al., 1999, Berghaus et al., 2013), and has recently been described to evaluate human-pathogen interactions for *Campylobacter* in the environment (Jones et al., 2017). To our knowledge, we are the first to describe a microbiota analysis using 16S rRNA gene amplicon sequencing with boot sock samples collected in a poultry house. The composition measured with the boot socks was close to that of cecal content samples early in the production cycle. On day 2, no difference between the boot socks and cecal content samples was observed, and on day 7 only the genera *Lactobacillus*, *Escherichia-Shigella* and *Enterococcus* were lower in relative abundance in the boot socks. However, on day 35, 20 genera were different between the boot socks and the cecal content, which indicated that boot socks were less useful to reflect the cecal content microbiota near the end of the production cycle. When comparing our results to the literature, it must be pointed out that *Corynebacterium*, *Sphingobacterium*, and *Lactobacillus* were observed to be highly abundant in litter samples on day 35 (Wang et al., 2016b), which indicates that the boot socks collected at the end of the production cycle may mostly represent the litter rather than the cecal or ileal content. This may suggest that the litter has developed its own specific microbiota. Within Farm 1 the composition of the boot socks at day 14 was closely related to the cecal content composition on day 35, while in Farm 2 the composition of day 14 was closely related to the cecal composition on day 7. Based on the differences we observed at day 14 between farms, we can speculate that this process

of developing a litter microbiota may have occurred faster in Farm 1 than in Farm 2, potentially influenced by the difference in bedding materials with wood shavings in Farm 1 and peat in Farm 2. This is in line with the literature that showed that litter type could affect the intestinal microbiota composition (Torok et al., 2009, De Cesare et al., 2019). It was expected that cecal and ileal content samples would be distinct from cloacal swabs, because that has been described before (Johnson et al., 2018, Stanley et al., 2015, Videvall et al., 2018). The ileal content is described to harbor a relatively simple microbial composition, with mainly the genera *Lactobacillus*, *Streptococcus* and *Clostridium* (van der Wielen et al., 2002, Lu et al., 2003a, Knarreborg et al., 2002), compared to the rich microbial composition in the ceca (Stanley et al., 2014a, Gong et al., 2007b, Owens et al., 2008, Videnska et al., 2013). Only on day 2 did we not observe any significant difference in the  $\alpha$  diversity between the ileal content and cloacal swabs. Therefore, cloacal swab samples might be a useful tool early in the production round depending on the goal of the microbiota characterization.

Consistent with our present findings, previous research also showed that age explained part of the individual variation in ileal content microbiota of broilers (Knarreborg et al., 2002). Nevertheless, we observed that age explained only 18% of the ileal microbiota variation, whereas age explained 39% and 62% of the intestinal microbiota variation in cloacal swabs and cecal content. In contrast to the  $\beta$  diversity, the  $\alpha$  diversity was not affected by age in the ileal content samples. This difference might be because ileal and cloacal microbiota are more sensitive to fluctuations of fasting or partial emptying of parts of the intestinal tract compared to cecal microbiota. This might also be the reason why the homogeneity in phylogenetic diversity between the cloacal swab samples is lower compared to homogeneity between the cecal content samples, explaining why in farm 2 the phylogenetic diversity was not significantly higher in cecal content compared to the cloacal swab samples, while all other comparisons showed significant differences. This decreases the usability of cloacal swabs for characterization of cecal or ileal microbiota based on 16S rRNA amplicon sequencing. However, major differences in the intestinal microbiota can potentially be observed with a large sample size of cloacal swabs.

Currently, it is unclear how the intestinal microbiota can be manipulated to optimize the robustness of broiler flocks, for example, to improve the alertness of the immune system while maintaining production efficiency (Broom, 2019). Knowledge about the age-related development of microbiota may contribute to future research focusing on the timing of nutritional interventions used to steer the microbiota in broiler chickens to more resilient and healthier chickens. Therefore, non-invasive longitudinal sampling methods are required in order to collect baseline samples before and after the onset of disease or a nutritional intervention. This will improve our understanding of the dynamics of intestinal microbiota in healthy individuals and those with diseases and facilitates evaluation of efficacy of interventions at the



individual level. Although the current high cost and time-consuming process of sequencing hampers the use of microbiota data as an applicable diagnostic tool of interventions in the field, this may change in the future. Furthermore, in experimental settings non-invasive longitudinal collection of data can already be of great value to gain more understanding of changes in intestinal microbiota composition and its associations with pathological and physiological processes in broilers.

## Conclusions

In conclusion, this study shows that the value of non-invasive sample types varies at different ages and depends on the goal of the microbiota characterization. We have shown that cecal droppings and boot socks, collected from a broiler house, are useful alternatives for cecal samples collected during post-mortem, to determine intestinal microbiota composition longitudinally in broiler flocks and in an experimental setting. These sample types may be a useful expansion of the current toolbox for microbiota studies. Further studies should be done to validate the use of those microbiota sample types as a diagnostic tool early in the production cycle, e.g., by studying broiler flocks with differences in health and productivity status in further detail. Non-invasive longitudinal sampling to monitor the development of the intestinal microbiota will facilitate the development of new and better interventions to improve the health and performance of broiler chickens.

## Supplementary Materials

The following are available online at

<https://www.mdpi.com/2076-2607/7/10/431#supplementary>

## Author Contributions

Conceptualization, F.C.V., J.A.S. and H.S.; Methodology, F.C.V., J.A.S., E.A.J.F., J.G.K. and H.S.; Investigation, J.G.K. and F.C.V.; Writing—Original Draft, J.G.K.; Writing—Review and Editing, F.C.V., J.A.S., E.A.J.F. and H.S.; Funding Acquisition, F.C.V.; Supervision, F.C.V., J.A.S., E.A.J.F. and H.S.

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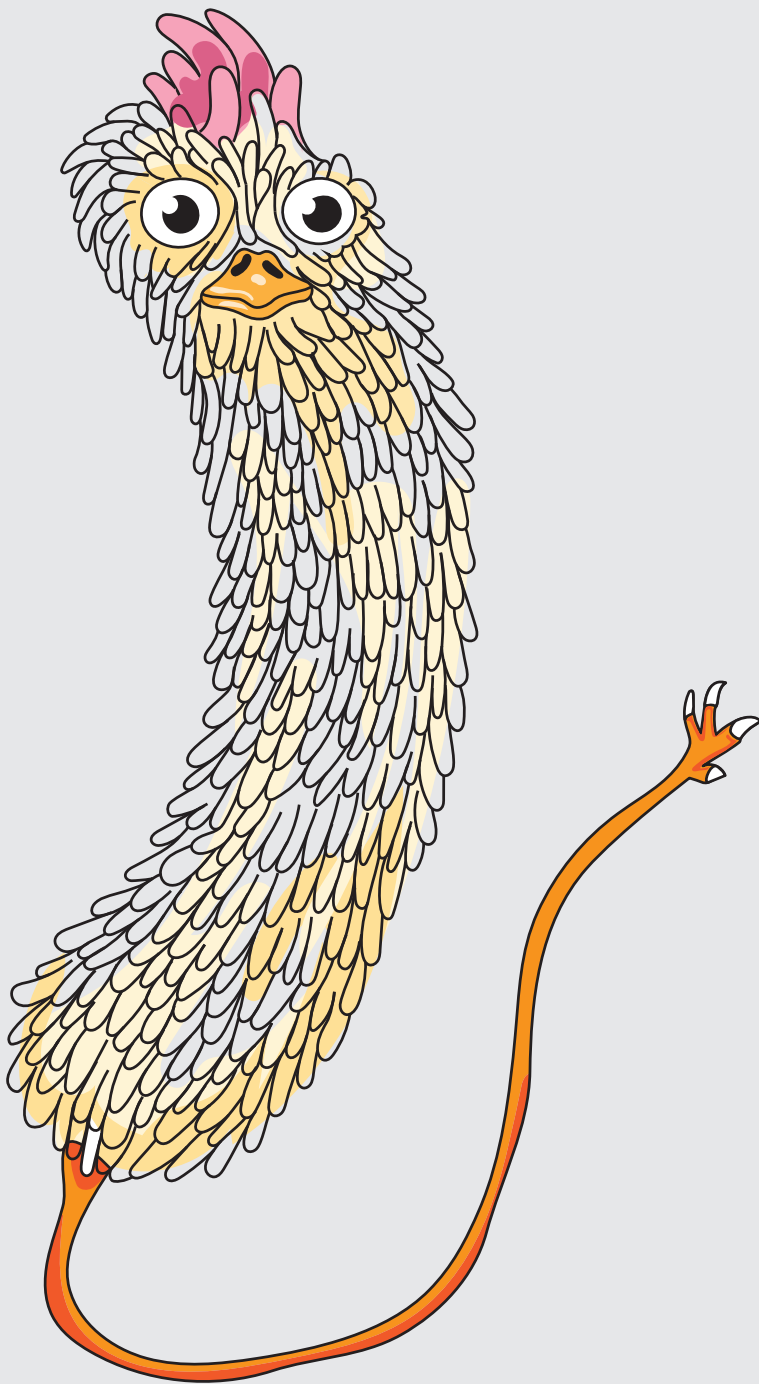
### ***Conflicts of Interest***

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.



## PART II

Do phenotypic characteristics explain the intestinal microbiota composition and its variation in poultry farms?





# CHAPTER 5

## Associations between phenotypic characteristics and clinical parameters of broilers and intestinal microbial development throughout a production cycle: a field study

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## Abstract

Disturbances in intestinal health are a common problem affecting commercial broiler chickens worldwide. Several studies have revealed associations between health, production performance and intestinal microbiota. The aim of this study was to describe the development of the intestinal microbiota of broilers within a production cycle to evaluate to what extent clinical parameters and phenotypic characteristics can explain the intestinal microbiota variation. Of four well-performing flocks within two farms the cecal content was collected of nine broilers at 0, 2, 4 or 5, 7, 11 or 12, 14, 21, 28, 35 and 40 days of the production cycle. In total 342 samples were analyzed using 16S ribosomal RNA gene amplicon sequencing. Variables as macroscopic gut abnormalities, gut lesions, age, individual body weight, sex, footpad integrity, color of ceca and foam in cecal content were determined. Ileum tissue was collected for histological quantification of villus length and crypt depth. Flock infection levels of the intestinal disease coccidiosis were measured in pooled feces from the poultry house. Increases in phylogenetic diversity were observed from hatch until day 21 of age. Constrained multivariate analysis indicated that age, farm, body weight, ileum crypt depth, cecal color, and the coccidiosis lesion score were important variables to describe the variation in cecal microbiota. These results contribute to determining relevant variables in flocks that may be indicative of the intestinal microbiota composition. Moreover, this knowledge increases the awareness of interactions between the intestinal microbiota and broiler health as well as their relative importance.

**Keywords:** cecal microbiota, field study, poultry, 16S rRNA gene, phenotypes



## Introduction

Disturbances in intestinal health in broiler chickens are a common worldwide challenge (M'Sadeq et al., 2015). Intestinal problems reported in broilers are often associated with a microbial imbalance, defined as a deviation from the composition observed in a healthy state, regularly referred to with the ill-defined terms 'dysbiosis' or 'dysbacteriosis' (Teirlynck et al., 2011, Ducatelle et al., 2018). Intestinal health problems have been associated with a loss in production performance, and several studies have correlated this with intestinal microbiota composition (Hofshagen and Kaldhusdal, 1992, Han et al., 2016a, Johnson et al., 2018). Therefore, knowledge of the development and variation of intestinal microbiota composition is pivotal for the design of strategies towards optimizing the intestinal health of broiler flocks. In poultry, it has been observed that the gut microbiota composition varies between flocks, flock cycles, breeds and, housing conditions and that many other unknown host- and environmental factors exist (Johnson et al., 2018, Stanley et al., 2013, Cuperus et al., 2018, Kim et al., 2015, Kers et al., 2018, Kers et al., 2019b).

Currently, it is unclear which phenotypic characteristics and clinical parameters are important to be able to differentiate and describe the intestinal microbiota composition associated with either healthy or unhealthy humans and animals. In humans, for example, the self-assessed Bristol stool scale score to assess stool consistency, was identified as an important phenotypic factor that showed the largest effect size to explain the variation in fecal microbiota composition (Falony et al., 2016). Food is also known to have an impact on microbiota composition, but similar foods can have different effects on people's microbiota (Johnson et al., 2019). In chickens, the use of antibiotics, feed composition and feed additives are well-known factors that influence intestinal microbiota composition (Burel and Valat, 2009, Torok et al., 2011a, Wei et al., 2013, Gao et al., 2017), however, knowledge with respect to factors related to intestinal health is limited.

For the evaluation of intestinal health, two macroscopic scoring systems are commonly used in broiler flocks. The first one, the coccidiosis lesion score (CLS), is used to determine the presence and severity of infections with *Eimeria* species, causing coccidiosis (Johnson and Reid, 1970). The second is a macroscopic score to assess the severity of intestinal health problems, ranging from a subclinical microbial imbalance to enteritis. This score was developed and introduced as a morphometric evaluation of 'dysbacteriosis', further referred to as the gut score (GS) (Teirlynck et al., 2011). It has also been described that a normal cecal lobe contains dark free-flowing fecal material, and that pale ceca with dense non free-flowing content have been associated with inflammation (Atterbury et al., 2011). These observations have, however, not yet been studied in association with cecal microbiota composition.

Knowledge of the dynamics in the development of the cecal microbiota is important, as it will help to optimize the timing of interventions. However, in literature,

there is still a lack of consensus on the age of the maturation or stabilization of the cecal microbiota of broiler chickens. One study showed that the cecal content microbial community resulted in no differences between 14 to 28 days of age (Lu et al., 2003a). This is in contrast with a study that showed higher individual variability in cecal microbiota of broilers of 14 days versus 28 days of age, suggesting that the cecal microbiota is not yet stable in 14-day-old broilers (Torok et al., 2009). Another study showed no differences in cecal microbiota composition in broilers between day 22 and day 36 of age (Ranjitkar et al., 2016).

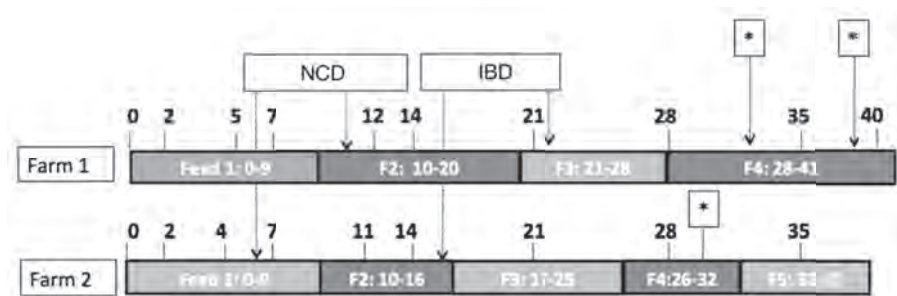
The purpose of the present study was therefore to describe the development of the intestinal microbiota composition of broiler chickens within a production cycle from different flocks, and to evaluate whether and to what extent phenotypic characteristics and clinical parameters can explain the observed microbial variation. Two commonly used clinical scoring systems, the CLS and GS (Teirlynck et al., 2011, Johnson and Reid, 1970), were used to assess possible correlations between health status and cecal microbiota composition. Furthermore, phenotypic characteristics, such as broiler age, individual body weight, sex, footpad integrity and color of the ceca and foam in cecal content were measured. At poultry house level, the number of *Eimeria* oocysts per gram was measured in pooled feces at days 14, 21, 28 and 35 to determine flock infection levels of the intestinal disease coccidiosis. The microbiota composition of cecal content was assessed by 16S ribosomal RNA (rRNA) gene amplicon sequencing. In addition, ileum tissue was collected to measure villus length and crypt depth. In total, the data of 342 broilers, and 14 phenotypic characteristics and clinical parameters as explanatory variables, were included in multivariate distance-based redundancy analysis to study associations with intestinal microbial development.

## Materials and Methods

### ***Farm selection***

Data for this study were obtained from two broiler farms in the Netherlands, both with Ross 308 broilers. The farms were selected for good production performance, as we were interested in a healthy intestinal microbiota. Also, to reduce the chance of including flocks treated with antibiotics, only farms with an antimicrobial use in the previous months below 15 DDDA<sub>f</sub> (defined daily dose per animal year on farm level) were recruited for the study. Two poultry houses were selected within a farm. They were equal with regard to size, year of construction, heating system, cleaning protocols and, feed and water system. The farms received chickens from different commercial hatcheries. The age of the breeder stock was 55 weeks for Farm 1 and 35 weeks for Farm 2. At both farms, water and feed were supplied *ad libitum*.

The two farms obtained feed from different suppliers, with small differences in feed composition. The diets on both farms were wheat-based and were combined with the addition of whole wheat at the farm at later ages. In addition to soybean meal, sunflower seed meal and rapeseed meal were added at a maximum of 6.5% inclusion. Farm 1 started with a bird density of 21 birds/m<sup>2</sup> and Farm 2 started with 24 birds/m<sup>2</sup>. Figure 1 provides an overview of the different feed changes, vaccination and flock thinning moments during the production cycle. Farm 1 used wood shavings as litter material, and Farm 2 peat. In both farms artificial lighting was set to 23 hours/day (h/d) for days 0- 3, 20 h/d for days 4-6 and 18 h/d for days 7-35. The temperature was set to gradually decrease from 34°C at day 0 to 20°C from day 35 onwards. Both farms used a combination of the coccidiostatic drugs nicarbazin and narasin (Maxiban® G160, Elanco, Houten, The Netherlands). Farm 1 used nicarbazin and narasin from day 0 until day 28 followed by narasin (Monteban® G100, Elanco, Houten, The Netherlands) until the end of the production period. Farm 2 used nicarbazin and narasin from day 0 until the end of the production period. No other antimicrobial treatments were applied during the study.

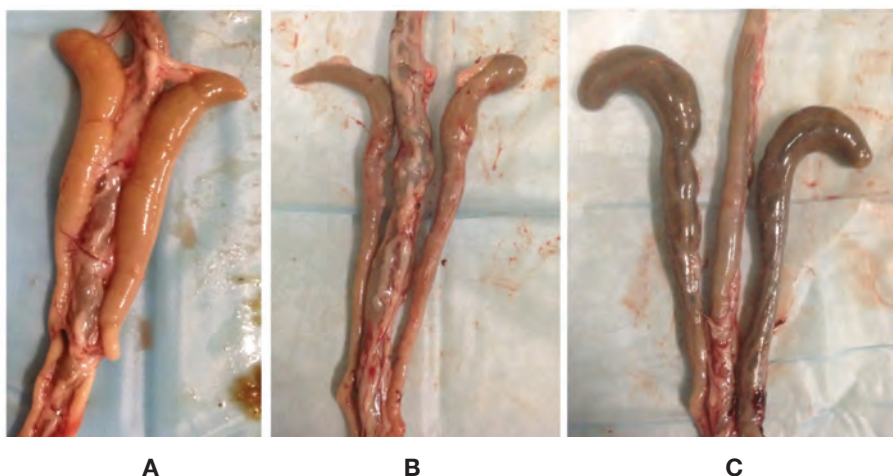


**Figure 1:** Schematic outline of sampling days and the different feed (F) and vaccination schedules at the two farms included in this study. Both farms have their own feed change schedule. This overview shows the different vaccine and sampling moments at the farms. NCD = vaccine against Newcastle disease, IBD = vaccine against Infectious Bursal Disease. \* = thinning, i.e. 15-30% of broilers are transported to the slaughterhouse.

### Data collection

During the production cycle Farm 1 was visited on days 0 (day of chick placement), 2, 5, 7, 12, 14, 21, 28, 35 and 40 (August 2016), and Farm 2 on days 0, 2, 4, 7, 11, 14, 21, 28 and 35 (June 2017). From each poultry house, nine broilers were randomly selected for sampling at each of the visits. Between the sampling of the two poultry houses on the same farm, coveralls, footwear and all sampling materials were changed. The start of the sampling of broilers took place at least 30 min after the end of a dark-period, to avoid low amounts of content in the intestinal tract at sampling. The broilers were individually weighed and checked for external abnormalities, including evaluation of absence or presence of footpad abnormalities. Footpad integrity, such as discolorations or lesions, were scored according to

methods developed for broiler welfare legislation (Broiler Directive 2007/43/EC), with a score of 2 or above considered as presence of footpad abnormalities (Welfare Quality, 2009). The broilers were euthanized by cervical dislocation. The gastrointestinal tract was quickly but carefully removed, and this procedure was carried out as much as possible in a sterile fashion, as previously described in detail (Kers et al., 2019a). The cecal lobes were classified into three classes based on external visual inspection: light color of the cecal content (A), dark content without stripes (B), and dark content with stripes (C) (Figure 2). Also, the presence of foam in the cecal content was recorded. The distal dead end of the cecum was cut to collect cecal content. The cecal content was gently squeezed into a 2 mL sterile cryotube, snap frozen on dry ice and stored at -80 °C until further use for DNA extraction. The time between euthanization and placing the cecal samples on dry ice ranged between 3-5 min. After the collection of cecal content, the gonads were inspected to determine the sex of the broiler. Between broilers, sterile gloves were changed, and the table, scissors, and tweezers were cleaned with 70% ethanol to prevent cross contamination between broilers.



**Figure 2:** Color classification of cecal lobes. Three different colors of ceca. A. light content, with foam B. Dark content, no stripes and no foam C. Dark content, with stripes and no foam.

On days 14, 21, 28 and 35, the intestinal tract was scored macroscopically using two methods. We quantified intestinal mucosa lesions indicative for *Eimeria* spp. infections, CLS (Johnson et al., 1970), and we applied the macroscopic ‘dysbacteriosis’ score system, GS (Teirlynck et al., 2011). The CLS was quantified on a scale from 0-4, with score 0 when lesions were absent and score 4 to indicate the most severe lesions. This score was determined for three separate parts of the intestinal tract, corresponding with the multiplication sites of the three most common

*Eimeria* spp. in broilers: the duodenum (*E. acervulina*), jejunum (*E. maxima*) and ceca (*E. tenella*). The total CLS score ranged from 0, no lesions, to 12, severe lesions in multiple sites in the intestinal tract. The GS consisted of ten parameters that were assessed using a binary system, with or without the presence of: (1) ballooning of the gut; (2) significant redness or dilated blood vessels cranial or (3) caudal to Meckel's diverticulum; (4) reduced gut wall thickness or increased fragility of the gut cranial or (5) caudal to Meckel's diverticulum; (6) reduced tonus (flaccidness) of the gut cranial or (7) caudal to Meckel's diverticulum; (8) abnormal appearance of the contents in the lumen of the gut cranial or (9) caudal to Meckel's diverticulum; and (10) undigested feed particles caudal to the ileocecal junction (2). The GS score was 0, when no gut abnormalities were found, and ranged to 10, indicating the presence of all ten gut abnormalities included in the scoring system. Per timepoint CLS and GS data were collected from nine broilers from Farm 1, and eighteen broilers from Farm 2.

On days 14, 21, 28 and 35 the number of oocysts per gram of feces (OPG) was quantified in a pooled fecal sample collected from the litter. A modification of a McMaster oocyst counting chamber technique was used as previously described (Velkers et al., 2010). As the McMaster technique is not suitable for reliable species identification, and each *Eimeria* species might have a specific impact on microbiota composition, a quantitative polymerase chain reaction (qPCR) for seven chicken *Eimeria* spp. (*E. acervulina*, *E. maxima*, *E. tenella*, *E. mitis*, *E. brunetti*, *E. necatrix*, and *E. praecox*) was performed on day 35 of the production cycle for species identification (Peek et al., 2017).

### **Immunohistochemistry**

To study the development of the gut, villus length and crypt depth were measured. Jejunum segments were taken near Meckel's diverticulum from nine broilers per farm on each sampling day. Samples were fixed in formalin, dehydrated in xylene, embedded in paraffin and sectioned in 5 µm thick slices. The tissue was then stained with hematoxylin-eosin and examined under a light microscope (Olympus, Tokyo, Japan). Representative images were taken, and crypt and villus length were measured employing image software (CellSence®). For each broiler, measurements were done blinded in 30 well-oriented villus and crypts. Villus height was measured from the tip of the villus to the base of an adjacent crypt. Crypt depth was measured from the villus-crypt axis to the base of the specific crypt.

### **16S rRNA gene amplicon sequence analysis**

DNA extraction, 16S rRNA gene-targeted PCR and analysis of microbiota composition was done as previously described Kers et al., 2019b. Briefly, DNA was extracted from 0.25 g cecal content, using 700 µl of Stool Transport and Recovery (STAR) buffer (Roche Diagnostics Nederland BV, the Netherlands). All 342 cecal samples were

transferred to sterile screw-capped 2 mL tubes (BIOplastics BV, the Netherlands), used for bead beating. The DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, USA), and the DNA samples were stored at  $-20^{\circ}\text{C}$  until further use. Barcoded primers covering the variable regions V5-V6, amplified with 784F and 1064R of the 16S rRNA gene, were used for microbial composition profiling (Ramiro-Garcia et al., 2016). To ensure high quality sequencing data, synthetic Mock communities of known composition were used as positive controls (Ramiro-Garcia et al., 2016), and nuclease free water as negative controls. Sequencing of resulting libraries was performed by GATC Biotech (now part of Eurofins Genomics Germany GmbH, Konstanz, Germany) on an Illumina HiSeq2500 instrument. The 16S rRNA gene amplicon data was analyzed using NG-tax 2.0 (Ramiro-Garcia et al., 2016, Poncheewin et al., 2020). In short, to generate Amplicon Sequence Variants (ASVs), NG-Tax 2.0 employs a fast de novo ASV-picking algorithm. To assign taxonomy the SILVA 128 16S rRNA gene reference database was used (Quast et al., 2013).

### ***Statistical analysis***

All statistical analyses were performed in R version 3.4.2 (R Foundation for Statistical Computing, Austria), using the packages: Phyloseq, Microbiome, Vegan and nlme (McMurdie et al., 2013, Lahti et al., 2017, Oksanen et al., 2019, Pinheiro et al., 2018). To test for differences in the relative abundance of genera between two groups, we used a Wilcoxon rank-sum test and corrected for multiple testing with Benjamini Hochberg (BH) ( $q$ -value) and values of  $q < 0.05$  were considered significant. Alpha diversity (within sample) was determined using phylogenetic diversity (Faith, 2006). Beta diversity (between samples) was determined using Bray-Curtis, Jaccard, and weighted and unweighted UniFrac distance metrics (Bray and Curtis, 1957, Jaccard, 1912, Lozupone et al., 2007). Differences in alpha diversity between treatment groups were tested with a Kruskal-Wallis test, and pairwise comparisons were tested using a Wilcoxon rank-sum test. Multivariate microbiota data were visualized using Principal Coordinate Analysis (PCoA), and non-parametric permutational analysis of variance (PERMANOVA) tests, to analyze group differences within multivariate community data (Anderson et al., 2001). PERMANOVA was performed with 9999 permutations. To examine differences in total microbiota composition between poultry houses over time, a principal response curve (PRC) analysis was performed. PRC was originally developed to analyze time-series data and carries out partial redundancy analysis (RDA) ordination to obtain estimates of community changes using time as a predictor variable (Van den Brink and Braak, 1999). In addition, distance-based unweighted UniFrac (UF) and weighted UniFrac redundancy analysis (WUF-db-RDA), a multivariate canonical ordination analysis method that takes the phylogenetic makeup of microbial communities into consideration, was performed

using ASV level data (Shankar et al., 2017). To determine the most parsimonious constrained ordination model, a stepwise selection (both directions) was used based on Akaike information criterion (AIC) selection, and a variance inflation factor (VIF) was used to verify the absence of multicollinearity.

## Results

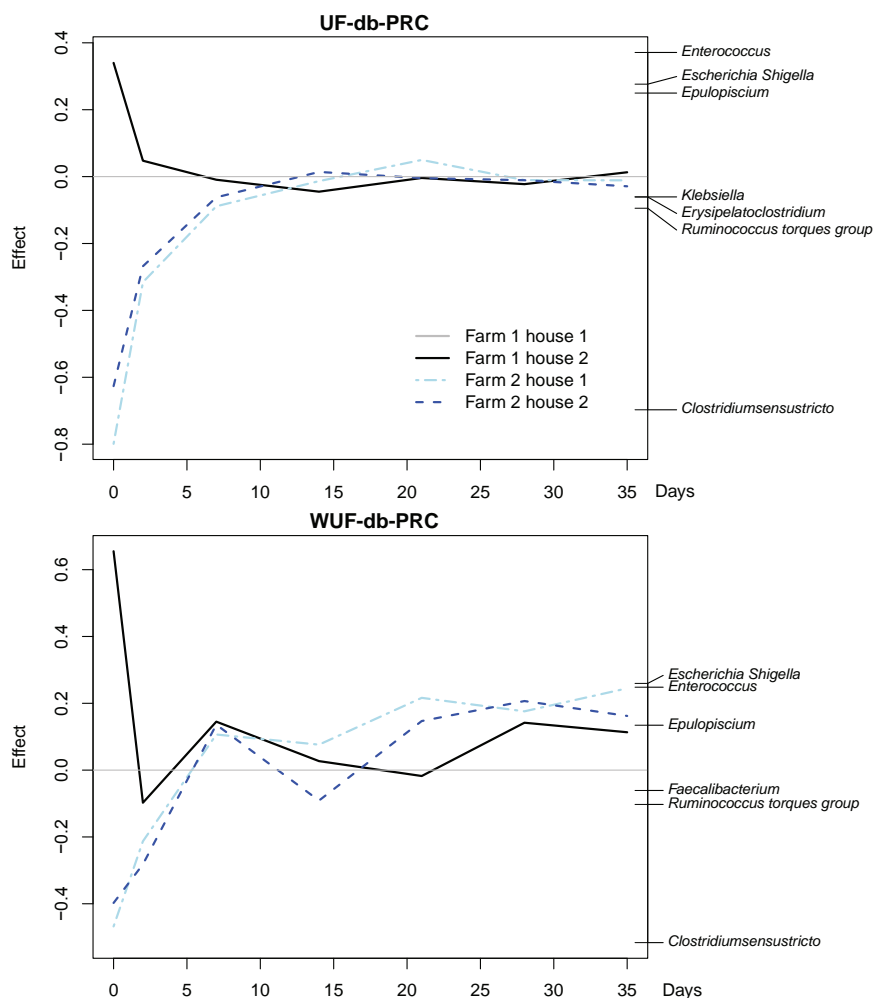
### *Performance of the flocks and health status*

Based on the body weight and feed conversion ratios, all flocks were considered as well-performing compared to Ross 308 broiler production standards (Table S1). Body weight of the broilers was comparable between farms and poultry houses on the same farm (Figure S1). The oocyst counts indicated some differences in *Eimeria* flock infection dynamics between flocks on days 21, 28 and 35, but in all cases, counts were indicative of rather standard levels of infection on broiler farms (Table S1). On both farms, the *Eimeria* qPCR at day 35 revealed only the presence of *E. acervulina*. The condemnation rate at slaughter was slightly above (Farm 2) or considerably lower (Farm 1) than average for European broiler farms. All these findings were in line with the good production performance of both farms and clinical observations during farm visits.

### *Intestinal microbiota development*

Potential differences in the dynamic development of cecal microbiota composition between farms and houses were first globally assessed using PRC analysis based on UF and WUF distances between individual samples and using Farm 1, poultry house 1, as a reference poultry house (Figure 3). Differences in microbial composition between poultry houses were highest on day 0 (day of chick placement), and decreased over time. Based on UF-db-PRC, the main difference between Farm 1 and Farm 2 was the higher relative abundance of *Clostridium sensu stricto* in Farm 2, and the lower relative abundance of the genera *Escherichia*, *Shigella*, *Enterococcus*, and *Epulopiscium* in Farm 1 (Figure 3a). After day 7 and until the end of the study, the variation between the poultry houses was limited. Observed differences in temporal development in the different farms were more extensive when using WUF distance data but similar in terms of the genera associated with these differences (Figure 3b).





**Figure 3:** Differences in temporal dynamics of cecal microbiota composition between farms and houses. Principle Response Curve (PRC) analysis based on unweighted A. and weighted B. UniFrac distances between samples at genus level. Genus weights contributing with an effect above 0.1 to each statistical model are shown on the right side of each panel. Each curve corresponds to one of the poultry houses, with Farm 1, poultry house 1, set as reference baseline. For each house and timepoint, data from nine individual broilers is included.

### Diversity analysis of cecal microbiota

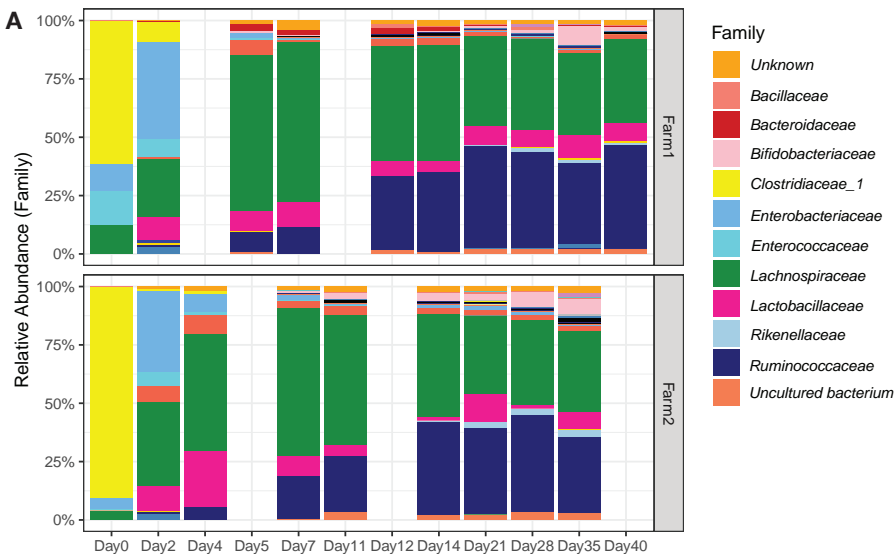
Overall, in both farms, members of the family *Clostridiaceae* predominated the cecal microbial community on day 0 (Figure 4a). On day 2, *Enterobacteriaceae* and *Lachnospiraceae* were most predominant, whereas members of the *Lachnospiraceae* were predominant on day 4 until day 7. From day 14 until day 40, *Ruminococcaceae* and *Lachnospiraceae* were the most predominant families.

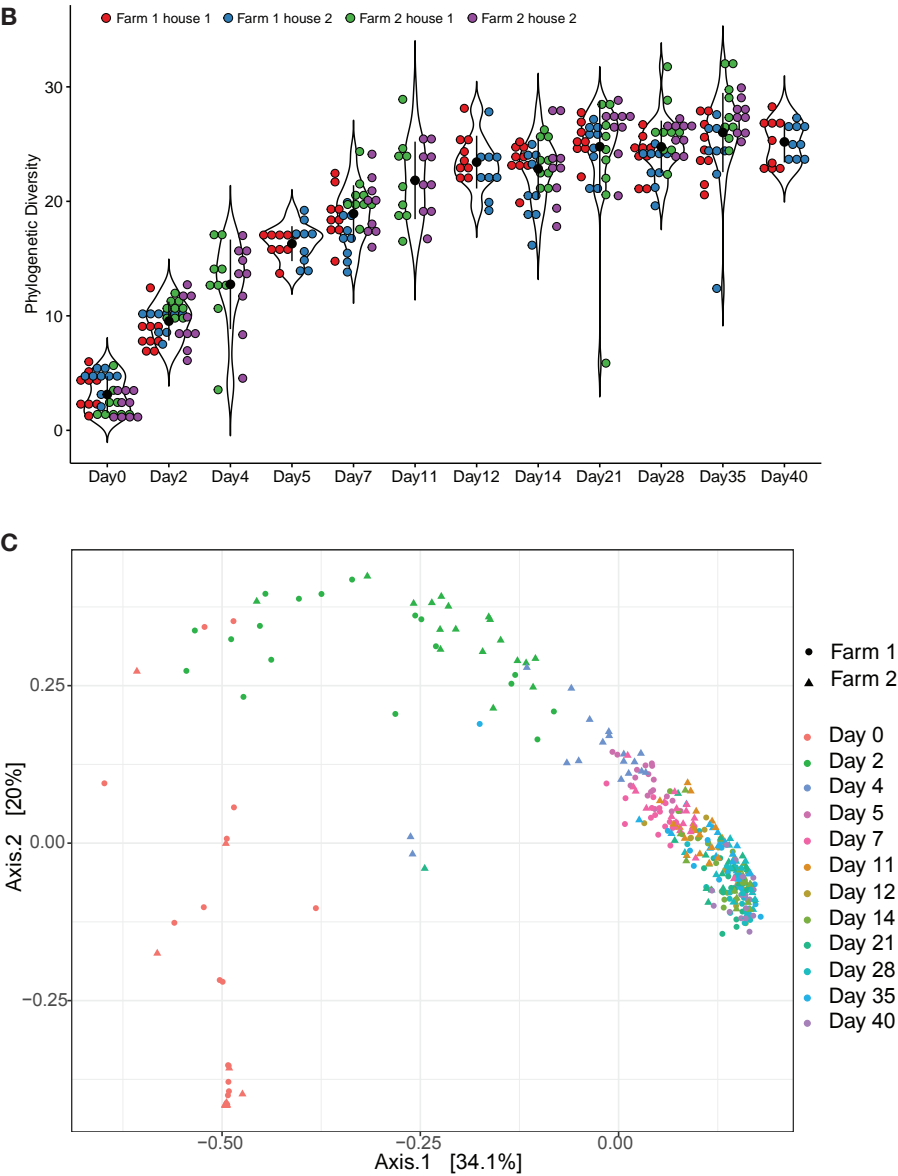


The phylogenetic diversity (alpha) increased with broiler age in both farms until day 21 (Figure 4b). After day 21, however, no significant difference in phylogenetic diversity was found between days, indicating stabilization of microbiota complexity. Although both farms showed the same trend, the phylogenetic diversity was higher in Farm 2 compared to Farm 1 (chi-squared = 4.62, df = 1,  $p = 0.032$ ). When stratified per day, however, significant differences between farms were only found for days 0, 2, 7, 28 and 35, but not for days 14 and 21 (Table S2).

PERMANOVA of cecal microbiota compositional data using WUF showed that age explained 18.6%, whereas farm explained only 1.2% of the observed microbiota variation between broilers, in line with the grouping observed in the PCoA (Figure 4C, Table S3). Depending on whether the analysis was based on UF- or WUF distance measures, the difference between farms was highest at day 0 or day 2, in line with the corresponding UF- and WUF-db-PRC results (Figure 3). In contrast, poultry house did not significantly contribute to explaining the observed variation, and neither did sex, whereas cecal color and foam explained 5.5% and 3.4% of the variation, respectively (WUF, Table S3).

Only on days 21, 28 and 35 the clinical parameters to assess intestinal health, i.e. GS and total CLS scores, showed values above zero. The highest average total CLS was found on day 28 and for GS on day 35, but both scores were rather low with a highest value of 5 (Table S4). Therefore, the effect of the GS and total CLS on the microbiota variation between broilers was only tested in a subset of the data for days 21, 28 and 35. The GS did not significantly contribute to explaining the variation but the total CLS explained 12.4% of the variation (WUF, Table S3).





**Figure 4:** Development of cecal microbiota composition. Development of cecal microbiota in Farm 1 (top panel) and Farm 2 (bottom panel) at the family level. For each timepoint, data from 18 animals was included (9 animals per house). B. Phylogenetic diversity per flock increased with broiler age ( $n=342$ ) until day 21, (pairwise Wilcoxon-rank sum test, adjusted  $p$  value of  $q > 0.05$ ). C. Analysis of beta diversity using Principle Coordinate Analysis based on weighted UniFrac distances. Farm and sampling time are indicated as variables.

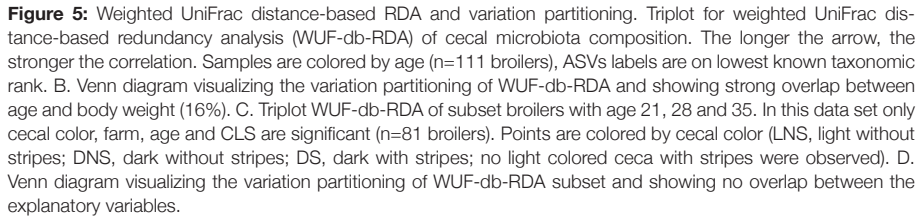
### **Factors that explain the intestinal microbiota variation of broilers**

To further disentangle the different effects of phenotypic characteristics and clinical parameters on cecal microbiota composition, WUF-db-RDA was carried out using data at ASV level. The first model contained 148 broilers of both farms of which all 11 phenotypic characteristics were measured (Table S5). The most parsimonious model to explain the variation of the cecal microbiota included five significant explanatory variables, i.e. crypt depth, age, body weight, cecal foam, and farm, and explained 34.8% of the cecal microbiota variation. The variation in the relative abundance of members of the genus *Clostridium sensu stricto*, strongly predominant on days 0 and 2, was most accurately accounted for by the model (Figure S2). Therefore, those days were removed in the next model. The most parsimonious WUF-db-RDA model based on 111 broilers, contained the four significant explanatory variables age, farm, body weight, and crypt depth, and explained 28.9% of the variation (Figure 5a). The relative abundance of a member of the genus *Faecalibacterium* was associated with the ordination in broilers with older age, higher body weight, and crypt depth (Figure 5a). Venn diagrams, visualizing the partitioning of variation of WUF-db-RDA and shared variance explained by the significant explanatory variables, showed strong overlap between age and body weight among the explanatory variables tested, accounting for the largest explained variance (16%, VIF 17) (Figure 5b). In addition, the four significant explanatory variables also explained part of the variation independently, i.e. age (5%), farm (2%), body weight (1%) and crypt depth (1%) (Figure 5b).

The final model contained only data of days 21, 28 and 35 and included the clinical parameters ( $\log_{10}$  OPG, CLS, and GS), and the previously described significant explanatory phenotypic variables age, farm, body weight, and cecal color (no crypt depth data was available for these broilers). The most parsimonious model to explain the variation in this dataset included cecal color, farm, age, and CLS, and explained 28.5% of the cecal microbiota variation (Figure 5c). The analysis of variance partitioning among the explanatory variables tested showed only 1% overlap between farm and cecal color. The explanatory variables that individually explained part of the variation were CLS (8%), farm (4%), cecal color (4%) and age (3%) (Figure 5d). CLS was associated with a higher relative abundance of a member of the genus *Bifidobacterium* (Figure 5c).

## **Discussion**

This comprehensive study described the dynamics of the development of the cecal microbiota for four different broiler flocks that were frequently sampled throughout a production cycle. Furthermore, the variation in cecal microbiota composition was associated with phenotypic characteristics and clinical parameters of broilers chickens.



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al., 2015). *Lachnospiraceae* became predominant from day 8 onwards (Mon et al., 2015), and *Lactobacillaceae* were not observed in day-old chicks but were observed in chicks from 3 days of age onwards (Gong et al., 2008), which is in line with our observations. Our study showed no difference in genera between days 21 and 28, and phylogenetic diversity seemed to stabilize after 21 days, despite feed changes that occurred on both farms after 21 days. These results indicate maturation of the cecal microbiota, with no large developmental changes afterwards.

Studies on the dynamics of the development of the microbiota of children have shown that in the first months of life *Proteobacteria* are predominant. At the age of one year the genus *Bifidobacterium* is highly abundant and at the age of two years members of the class *Clostridia* are among the predominant taxa (Korpela and de Vos, 2018). Although this succession in early life has been based on the five most predominant bacterial taxa of healthy vaginally born children, they showed overlap with the development observed in this broiler chicken study. On day 2, *Enterobacteriaceae* (*Proteobacteria*) were predominant in the cecal content of both farms. After day 7 *Ruminococcaceae* and *Lachnospiraceae* (both *Clostridia*) showed the highest relative abundance in the cecal content. However, in this study, on day 0 the cecal content was predominated by *Clostridiaceae*, a family of mainly obligate anaerobes. This is notable because in human research it is suggested that the first colonizers of the gut are predominantly facultative anaerobes and that later in life obligately anaerobic bacteria take over (Bäckhed et al., 2015, Nagpal et al., 2016). In children born through cesarean section, also a higher relative abundance of obligately anaerobic taxa and lower abundance of facultatively anaerobic taxa has been observed (Nagpal et al., 2016). As commercial chickens hatch under strict hygiene practices in hatcheries, they are exposed to a diverse range of microorganisms from environmental sources rather than from their parents, which might explain the predominance of an obligately anaerobic genus as *Clostridium sensu stricto* (*Clostridiaceae*). This predominance of the genus *Clostridium* has also been reported in day-old turkeys on a commercial farm (Smith and Rehberger, 2018). Nevertheless, probably not all members of the genus *Clostridium* are truly obligately anaerobic (Wiegel et al., 2006). Although they cannot use oxygen as the terminal electron acceptor, many members of this genus can tolerate oxygen, especially under non-growth conditions, i.e., in the absence of utilizable substrates and energy sources (Wiegel et al., 2006). This suggests that members of the genus *Clostridium sensu stricto* are among the first colonizers of the avian ceca in the field setting studied here, and that after a few hours/days members of the phylum *Proteobacteria* colonize the ceca, followed later in life by other members of the *Clostridiaceae*. Members of the family *Clostridiaceae* are known as spore-forming bacteria (Wiegel et al., 2006). This might be how they find their way into the egg of day-old-broilers (Richards-Rios et al., 2020b).

Explanatory variables as CLS, cecal color, farm, and age were associated with

the microbiota variation in broilers of 21 days and older. In the total dataset, age was found to be the most important factor, whereas within the last three timepoints (21, 28, 35 days), age was significant but not the most important explanatory variable as the microbiota composition had become more mature and thus stable. Despite the fact that only a limited number of lesions were observed, it is striking that it was still the most important explanatory variable in the dataset comprising days 21, 28, 35, suggesting that on farms with more severe coccidiosis problems even larger effects may be expected. The CLS was associated with a higher relative abundance of *Bifidobacterium*. This is in line with an experimental study where broilers with an *E. tenella* infection, a species multiplying in the ceca, also showed a higher relative abundance of *Bifidobacterium* compared to broilers without infection (Macdonald et al., 2017). Nevertheless, *E. tenella* was not observed in the pooled fecal samples by qPCR nor were lesions in the ceca observed at post-mortem. The species *E. acervulina* that multiplies in the duodenum was the only species observed on both farms, which may have caused shifts in the microbiota composition downstream as described previously (Perez et al., 2011, Hauck, 2017).

In humans, medication, stool consistency and transit time, were variables that explained part of the stool microbiota variation (Falony et al., 2016, Müller et al., 2020). In our study, also the color of the ceca was associated with cecal microbiota composition, although no overlap with any of the clinical parameters was observed. In literature pale ceca, due to white blood cell infiltration, have been associated with an infection by e.g. *Salmonella* (Atterbury et al., 2011). We can speculate that the lack of overlap between cecal color with any of the clinical parameters suggests that in our study the color was not associated with infection but rather by fermentation phase or transit time.

Though the farms included in this study were both well-performing, farm explained part of the variation, in contrast to the flock. As every flock lived in a different house, the variable flock was expected to influence the microbiota composition as previous research has shown (Kers et al., 2019b). Because the flocks in the two poultry houses on each of the farms started with a similar exposure to microbes, we can speculate that this similar early life exposure to microbes affected and shaped microbiota composition then, and later in life.

Previous research using the GS system showed correlations of gut scores measured at days 10, 17 and 20, indicating that the prevalence of gut abnormalities at day 10 can be predictive for scores later (Caekebeke et al., 2020). It is also likely that disruptions in microbiota composition occur before clinical signals, such as production losses, become apparent. In this study, however, the gut score was not associated with cecal microbiota composition. All flocks in this study were well-performing, and only low CLS and GS values were observed. This may have limited the chance of observing associations between clinical parameters and the intesti-

nal microbiota. This limitation in the current analysis also addresses an important general challenge for these types of studies. A lot of different host-related factors and infectious and non-infectious challenges may directly or indirectly affect the broiler microbiota composition, and can consequently influence broiler health and performance (Kers et al., 2018). In humans, it has been described that over 500 individuals per group are needed to study shifts in microbiota composition in e.g. obese versus lean people (Falony et al., 2016). In this study, we analyzed nine birds per timepoint per flock. Although the total sample size is larger than in most other poultry studies published to date, this might still not be enough, especially considering that within the same flock, the sampled broilers are not completely independent of each other. Only a limited number of phenotypic or clinical characteristics were found to be associated with cecal microbiota variation in this study. A larger sample size per flock and a larger number of farms, with more distinct differences in health and performance status, might have revealed more associations between microbiota composition and phenotypic and clinical characteristics.

This study was performed using the V5-V6 region and within the used pipeline it was shown to outperform the V4-region e.g. allowing for higher differentiation within the *Enterobacteriaceae* family and showing great consistency with full-length analyses (Ramiro-Garcia et al., 2016). This limited the comparison of our results across literature because the hypervariable regions give specific taxonomic signatures. However, there is no clear consensus on the preferred region within poultry microbiota studies and a wide variation of regions is used.

To further understand the mechanisms that drive the development and to identify important causes of disruptions in the microbiota associated with reduced health, species- or even strain-level information and knowledge of the actual functioning of the microbes will be needed. Species-level classification for 16S rRNA gene sequences remains a serious challenge, which limits the interpretation of the results. For example, *Clostridium perfringens* has been associated with dysbacteriosis and bacterial enteritis (Teirlynck et al., 2011, Roberts et al., 2015), but based on the used SILVA reference database the species *Clostridium perfringens* is a member of the genus *Clostridium sensu stricto* 1 that is predominant in day-old broiler chickens without any clinical signs. Approaches providing a higher level of taxonomic and functional resolution will be needed, such as shotgun metagenomics, that allows for differentiation of functionally distinct, but phylogenetically similar populations. However, even with metagenomics, the functional insights are limited to functional predictions and no information is obtained for the metabolic activity. Thus, multi-omics approaches such as combination of metagenomics with metabolomics are necessary in order to identify not only the bacteria but also their metabolites that are important for broiler physiology, production and health. Furthermore, more observational longitudinal studies including farms with poorer production performance and health

status are needed. Such studies may, for instance, validate our associations of the higher CLS with a higher relative abundance of *Bifidobacterium* in the microbiota composition and may reveal other associations with clinical data as well.

In conclusion, in this study age, farm, body weight, crypt depth, cecal color and CLS were important explanatory variables to explain the variation of the cecal microbiota composition in broiler chickens under field conditions. Our results suggest that the temporal development is major in the first week of life and that after 21 days of age the cecal microbiota composition can be considered as mature and stable in a well-performing broiler flock. This knowledge contributes to the understanding of the development and the interaction between the intestinal microbiota and broiler health.

### ***Ethical statement***

The animal experiment was approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee (registration number AVD108002016442) and was in compliance with all relevant legislation.

### ***Funding***

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### ***Author Contributions***

Jannigje G. Kers: Conceptualization (supporting); Formal analysis (lead); Data Curation (lead); Investigation (lead); Writing original draft; Writing review and editing (equal); Visualization (lead). Jean E. de Oliveira: Conceptualization (supporting); Writing review and editing (supporting). Egil A.J. Fischer: Conceptualization (supporting); Writing review and editing (supporting). Monique H.G. Tersteeg-Zijderveld: Resources (supporting); Investigation (supporting). Prokopis Konstanti: Formal analysis (supporting); Investigation (supporting); Writing – review and editing (supporting). J. Arjan Stegeman: Conceptualization (supporting); Funding Acquisition (supporting); Writing review and editing (supporting). Hauke Smidt: Conceptualization (supporting); Funding Acquisition (supporting); Writing review and editing (supporting). Francisca C. Velkers: Conceptualization (supporting); Data Curation (lead); Funding Acquisition (lead); Investigation (supporting); Writing – review and editing (equal)



### Data Availability statements

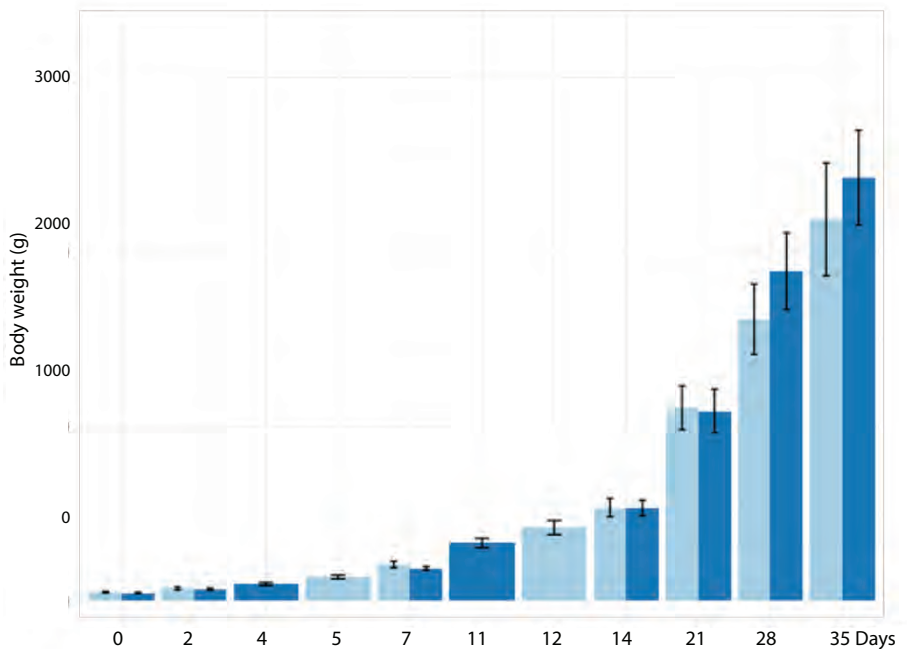
The raw sequence data generated during the current study are available in the Sequence Read Archive (SRA) repository at the NCBI under accession PRJNA644193.

### Acknowledgments

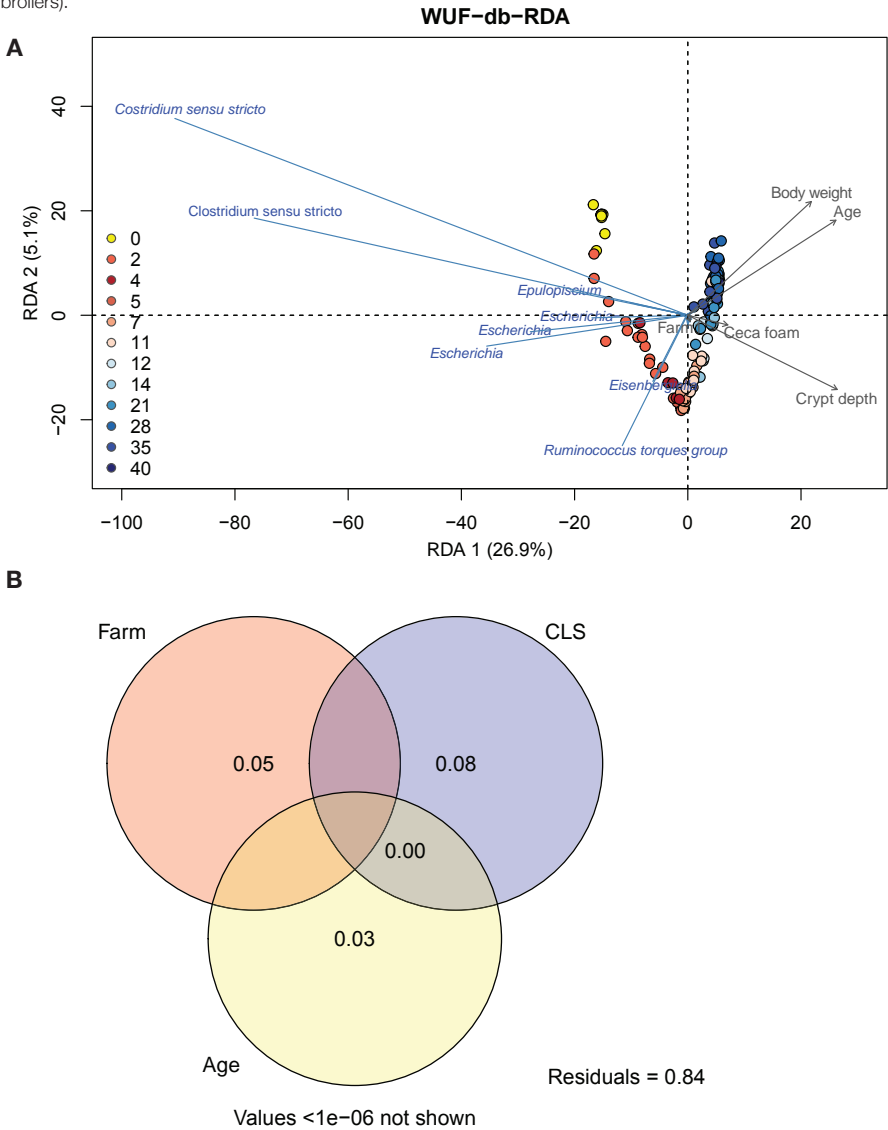
The authors wish to thank the farmers and veterinarian Sible Westendorp of the commercial farms participating in this study, the animal technicians from the Department of Farm Animal Health of Utrecht University and Cargill Animal Nutrition and Thijs Manders and Amerins Mulder for assisting with the collection of the samples at the farms, and Ineke Heikamp-de Jong and Mirin Spaninks for all the lab work to process the samples. Furthermore, we are grateful to Edoardo Saccenti for his input on the sample size.

### Supplementary Materials

**Figure S1:** Body weight of broilers across time between farms. No statistical difference in performance was observed, between the farms (Farm 1 dark blue, Farm 2 light blue). In both farms, the body weight was above the Ross 308 performance standard (Aviagen, 2019).



**Figure S2:** Weighted UniFrac-db-RDA of cecal microbiota composition. Triplot for weighted UniFrac-db redundancy analysis (WUF-db-RDA) of cecal microbiota composition. The longer the arrow, the stronger the correlation. A. All timepoints included in model. B. The Venn diagram displaying the variation partitioning of the WUF-db-RDA subset showed no overlap between the explanatory variables farm, coccidiosis lesion scores (CLS) and age (n=148 broilers).



**Table S1:** Performance across farms.

	<b>Farm 1 Flock 1</b>	<b>Farm 1 Flock 2</b>	<b>Farm 2 Flock 1</b>	<b>Farm 2 Flock 2</b>	<b>Ross 308 standards</b>
<b>Body weight at slaughter (g)</b>	2819 (D41)	2819 (D41)	2613 (D37)	2643 (D37)	2334 (D37) 2715 (D41)
<b>Feed conversion ratio (FCR)</b>	1.580	1.580	1.505	1.489	1.587 (D37) 1.667 (D41)
<b>Oocyst output (Log<sub>10</sub> OPG)</b>	D21 – 2.64 D28 – 4.05 D35 – 5.05	D21 – 3.00 D28 – 4.65 D35 – 4.08	D21 – 3.48 D28 – 4.63 D35 – 4.89	D21 – 4.23 D28 – 4.76 D35 – 3.19	n.a. #1
<b>Condemnation rate at slaughter</b>	0.20%	0.22 %	1.38%	1.38%	n.a. #2

#1 Rather standard levels of infection on broiler farms (26). #2 For Farm 2 the condemnation rates were slightly above (Farm 2) and below average (Farm 1) compared to average rates for European broiler farms and the reasons for condemnation did not indicate specific abnormalities related to poor health (Salines et al., 2017, Löhren, 2012), D = day, (Aviagen, 2019).

**Table S2:** Comparison of phylogenetic diversity between farms at different broiler ages. Differences in phylogenetic diversity (alpha diversity) between treatment farms, stratified per age, tested with a Kruskal-Wallis test.

	<b>chi-squared</b>	<b>p-value</b>
<b>All days</b>	4.62	0.032
<b>Age 0</b>	9.61	0.002
<b>Age 2</b>	4.10	0.042
<b>Age 7</b>	6.57	0.010
<b>Age 14</b>	0.40	0.527
<b>Age 21</b>	0.57	0.448
<b>Age 28</b>	13.47	2.42e-04
<b>Age 35</b>	15.64	7.66e-05

**Table S3:** Beta diversity based on different distance metrics. R<sup>2</sup> = Percentage of the variation between chickens explained, p-value = based on PERMANOVA test, GS = gut score (2), CLS = coccidiosis lesion score (18), \*\*\* =  $p < 0.001$ , \*\* =  $p < 0.01$ , \* =  $p < 0.05$ . The age of the broilers explains 18.6% of the variation between all the cecal samples, while farm just explained 1.2% (Weighted UniFrac, WUF). When we stratified the data per age, around 10% (7%-12%) of the variation was explained by farm based on weighted UniFrac. Furthermore, unweighted UniFrac distance was slightly higher than the weighted UniFrac distance between the two farms, whereas the opposite trend was observed for corresponding Jaccard and Bray-Curtis distances that are not phylogenetically weighted. Taken together, this indicated that abundant taxa are more phylogenetically related compared to the less abundant taxa between the two farms.

Table S3:

		Bray-Curtis			Jaccard			UF			WUF		
		n	R <sup>2</sup>	p-value	R <sup>2</sup>	p-value	R <sup>2</sup>	R <sup>2</sup>	p-value	R <sup>2</sup>	R <sup>2</sup>	p-value	p-value
Age	Farm 1 + 2	341	12.1%	1.00e-04 ***	7.5%	1.00e-04 ***	19.1%	1.00e-04 ***	1.00e-04 ***	18.6%	1.00e-04 ***	1.00e-04 ***	1.00e-04 ***
	Farm 1	179	14.4%	1.00e-04 ***	9.1%	1.00e-04 ***	21.4%	1.00e-04 ***	1.00e-04 ***	20.6%	1.00e-04 ***	1.00e-04 ***	1.00e-04 ***
	Farm 2	162	12.8%	1.00e-04 ***	8.3%	1.00e-04 ***	20.1%	1.00e-04 ***	1.00e-04 ***	18.7%	1.00e-04 ***	1.00e-04 ***	1.00e-04 ***
	21 + 28 + 35	107	2.7%	1.00e-04 ***	2.1%	1.00e-04 ***	4.5%	1.00e-04 ***	1.00e-04 ***	4.2%	1.00e-04 ***	1.00e-04 ***	1.00e-04 ***
F1 vs F2	All ages	341	2.0%	1.00e-04 ***	1.5%	1.00e-04 ***	2.0%	1.00e-04 ***	1.00e-04 ***	1.2%	1.00e-04 ***	0.002	**
	Age 0	36	24.9%	1.00e-04 ***	23.3%	1.00e-04 ***	19.6%	1.00e-04 ***	1.00e-04 ***	11.5%	1.00e-04 ***	0.013	*
	Age 2	36	22.8%	1.00e-04 ***	14.9%	1.00e-04 ***	17.1%	1.00e-04 ***	1.00e-04 ***	12.3%	1.00e-04 ***	0.002	**
	Age 7	36	11.1%	1.00e-04 ***	7.7%	1.00e-04 ***	18.4%	1.00e-04 ***	1.00e-04 ***	9.7%	1.00e-04 ***	8.00e-04	***
	Age 14	36	6.1%	3.00e-04 ***	4.9%	1.00e-04 ***	13.6%	1.00e-04 ***	1.00e-04 ***	7.1%	1.00e-04 ***	0.026	*
	Age 21	35	9.3%	1.00e-04 ***	7.1%	1.00e-04 ***	10.7%	1.00e-04 ***	1.00e-04 ***	8.6%	1.00e-04 ***	3.00e-04	***
	Age 28	36	8.3%	1.00e-04 ***	6.3%	1.00e-04 ***	10.9%	1.00e-04 ***	1.00e-04 ***	10.7%	1.00e-04 ***	1.00e-04	***
	Age 35	36	10.7%	1.00e-04 ***	7.9%	1.00e-04 ***	13.9%	1.00e-04 ***	1.00e-04 ***	8.4%	1.00e-04 ***	0.003	**
	21 + 28 + 35	107	5.2%	1.00e-04 ***	3.7%	1.00e-04 ***	7.0%	1.00e-04 ***	1.00e-04 ***	6.2%	1.00e-04 ***	1.00e-04	***
	All ages	179	1.1%	0.018	*	1.0%	0.009	**	0.299	0.6%	0.385		
F1: poultry house	Age 21 + 28 + 35	53	5.7%	1.00e-04 ***	4.3%	1.00e-04 ***	3.9%	1.00e-04 ***	0.006	**	2.9%	0.109	
	Age 35	18	8.8%	0.048	*	7.9%	0.040	*	0.376	4.2%	0.649		
	All ages	162	0.6%	0.387	0.6%	0.376	0.4%	0.376	0.646	0.2%	0.931		
	Age 21 + 28 + 35	54	2.3%	0.170	2.2%	0.128	2.2%	0.128	0.231	1.6%	0.597		
F2: poultry house	Age 35	18	6.1%	0.375	6.1%	0.338	6.9%	0.338	0.238	5.2%	0.491		
	All ages	341	0.2%	0.924	0.2%	0.920	0.1%	0.942	0.946	0.1%	0.946		
	All ages	340	1.9%	1.00e-04 ***	1.3%	1.00e-04 ***	3.8%	1.00e-04 ***	1.00e-04 ***	3.4%	1.00e-04 ***		
	Age 21 + 28 + 35	107	1.8%	0.0033	**	1.5%	0.002	**	0.010	*	0.009	**	
Cecal color	All ages	340	3.7%	1.00e-04 ***	2.5%	1.00e-04 ***	5.4%	1.00e-04 ***	1.00e-04 ***	5.5%	1.00e-04 ***		
	Age 21 + 28 + 35	107	4.0%	1.00e-04 ***	3.1%	1.00e-04 ***	4.8%	1.00e-04 ***	1.00e-04 ***	5.6%	1.00e-04 ***		
	Age 21 + 28 + 35	81	4.7%	0.639	4.8%	0.665	5.1%	0.348	0.348	5.1%	0.365		
	Age 21 + 28 + 35	81	6.2%	0.060	.	5.6%	0.076	.	0.048	*	12.4%	0.045	*
Footpad integrity	Age 21 + 28 + 35	107	1.3%	0.064	.	1.2%	0.048	*	0.015	*	1.1%	0.290	

**Table S4:** Overview of total CLS and GS scores. The coccidiosis lesion score (CLS), is used to determine the presence and severity of infections with *Eimeria* species (18) and can range from 0-4 with higher scores indicating more severe lesions. CLS was determined for duodenum, jejunum and ceca. The total CLS is the sum for all three intestinal parts and can range from 0-12. The morphometric evaluation of 'dysbacteriosis' referred to as the gut score can range from 0-10 (GS) (2). Average values and standard deviation (SD) for total CLS and GS, and the percentage of broilers for three categories of scores are provided for day 21, 28 and 35 of the study.

		Day 21		Day 28		Day 35	
Total CLS	Average (SD)	0.19 (0.48)		0.81 (0.78)		0.56 (1.08)	
(0-12)	0	85%	0	37%	0	67%	
	1 or 2	15%	1, 2 or 3	63%	1,2 or 5	33%	
	> 3	0%	>3	0%	>6	0%	
GS	Average (SD)	2.11 (1.01)		2.37 (1.01)		2.48 (1.09)	
(0-10)	1 or 2	59%	1 or 2	63%	1 or 2	48%	
	3, 4 or 5	41%	3, 4 or 5	37%	3, 4 or 5	52%	
	>6	0%	>6	0%	>6	0%	

**Table S5:** Phenotypic characteristics and clinical parameters at flock and individual level

Phenotypic characteristics	Flock level	Individual level
All days	Farm (1 or 2) Flock (1 of 2)	Age (integer)
		Body weight (numeric)
		Sex (male or female)
		Footpad integrity (yes/no)
		Cecal color (A, B, C)
		Cecal foam (yes/no)
		Villus length (numeric)
		Crypt depth (numeric)
		Villus/crypt ratio (numeric)
Clinical parameters	Flock level	Individual level
Days 14, 21, 28 and 35	Number of <i>Eimeria</i> oocysts in pooled feces ( $\log_{10}$ OPG)	Coccidiosis Lesion Scores (0-12) Gut Score (1-10)



# CHAPTER 6

## Conserved developmental trajectories of the cecal microbiota of broiler chickens: a field study

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In preparation

## Abstract

There is great interest in identifying gut microbiota development patterns and underlying assembly rules to help identify strategies to improve broiler health and performance. Population stratification by using community types (clusters) helps to simplify complex ecosystem principles of the intestinal microbiota. This study aimed to identify community types to increase insight in intestinal microbiota variation between broilers and to reveal factors that explain this variation. Ten well-performing poultry flocks on four different farms were followed. In each flock, the cecal content of nine broilers was collected at 7, 14 and 35 days post-hatch. The cecal microbiota composition of 270 broilers was analyzed using 16S ribosomal RNA gene amplicon sequencing. Two robust community types were observed, one of which was dominated by 7-day-old broilers, and one by 35-day-old broilers. Fourteen-day-old broilers were divided across both community types. In broilers of the same age, the cecal microbiota could not be robustly separated into different community types. This is the first study that showed conserved cecal microbiota development trajectories based on different clustering methods between different commercial broiler flocks. In addition to the observed development with age, cecal microbiota variation between broilers was explained by the poultry flock, body weight, and the different feed components. However, those variables could not be disentangled from the influence of the variables farm. These data support the notion of a conserved development of cecal microbiota, but further investigation of mechanisms underlying microbiota development and function and effects on broiler performance is needed.

**Keywords:** microbiome, field study, poultry, 16S rRNA, microbial community, gut, intestinal



## Introduction

The intestinal microbiota is associated with the health and production performance of broiler chickens (Stanley et al., 2014, Stanley et al., 2016, Han et al., 2016, Johnson et al., 2018). Therefore, there is great interest in identifying the biological principles that underlie the structure and function of these microbiological ecosystems. This knowledge can contribute to the development of beneficial nutritional, management or diagnostic tools, to improve broiler health. However, several studies in broilers have described the intestinal microbiota as highly variable within and between repeated experiments (Stanley et al., 2013, Thibodeau et al., 2017, Cuperus et al., 2018). Feed, antimicrobial products, host and environmental factors have all been shown to attribute to the variation in intestinal microbiota (Apajalahti et al., 2001, Borda-Molina et al., 2018, Kers et al., 2019b). Recently it has been showed that the effect of a feed intervention on the composition of broiler microbiota was highly dependent on the rearing environment (Kers et al., 2019b). Furthermore, in humans, it has also been shown that similar foods can have different effects on the microbiota (Johnson et al., 2019). Therefore, it is important to identify which factors influence the microbiota composition of broilers, and to which extent.

It has been proposed that microbial communities coevolve with their hosts (Backhed et al., 2005, Filippo et al., 2010, Dethlefsen et al. 2008). Although it is generally accepted that the composition of the intestinal microbiota is unique per individual, conserved patterns, termed enterotypes, were discovered across adult human individuals, independent of age, gender, cultural background, and geography (Arumugam et al., 2011). Recently, this enterotype concept was further refined, acknowledging that statistical support for robust clusters was variable and a range of confounding factors could affect the initially defined discrete clusters. Nevertheless, stratification using cluster structures, can still serve as a powerful tool to reduce the complexity of the microbiota community landscape (Costea et al., 2018). In human infants and adults, clusters, defined as microbiota community types have been associated with differences in microbial functionality, diseases and with differences in diet (Arumugam et al., 2011, Costea et al., 2018, Borewicz et al., 2019, Zhong et al., 2019). There have also been attempts to define microbiota community types in poultry. In 31 broilers of 56 days of age, the fecal microbiota was classified into four potential community types based on principal component analysis (Kaakoush et al., 2014). These community types were defined as being dominated by *Firmicutes* alone, or in combination with *Proteobacteria*, *Actinobacteria* or *Bacteroidetes*, respectively (Kaakoush et al., 2014). Another study observed three community types in duodenum content of broilers of 77 days of age. One cluster was predominated by *Bacteroides* and *Escherichia-Shigella*, one by *Ochrobactrum* and *Rhodococcus*, and the third by *Bacillus* and *Akkermansia* (Yuan et al., 2020).

Although the cecal microbiota has been widely investigated because of its functionality, which in broilers is especially related to the fermentation of feed (Stanley et al., 2014, Svihus 2014), the factors that contribute to the normal cecal microbiota variation in healthy broiler populations has remained under-investigated. Therefore, the actual main sources of cecal microbiota variation remain unknown. In humans, factors such as stool consistency and medication have been shown to be important in explaining adult fecal microbiota variation (Falony et al., 2016, Müller et al., 2020). However, broilers have a short life span, and this increases the difficulty to disentangle the factors that influence their microbiota because the microbiota is still developing at the end of their lives. In human infants aged 3 to 46 months, ten community types were observed and described in a transition model consisting of three phases: a developmental, transitional and stable phase (Stewart et al., 2018). Another study showed that infants belonging to different community types also had different degradation patterns of human milk oligosaccharides (Borewicz et al., 2019a). Thus, probing the cecal microbiota of broilers for community types can provide useful insights in the cecal microbiota development, as well as the factors that can affect this development and their importance.

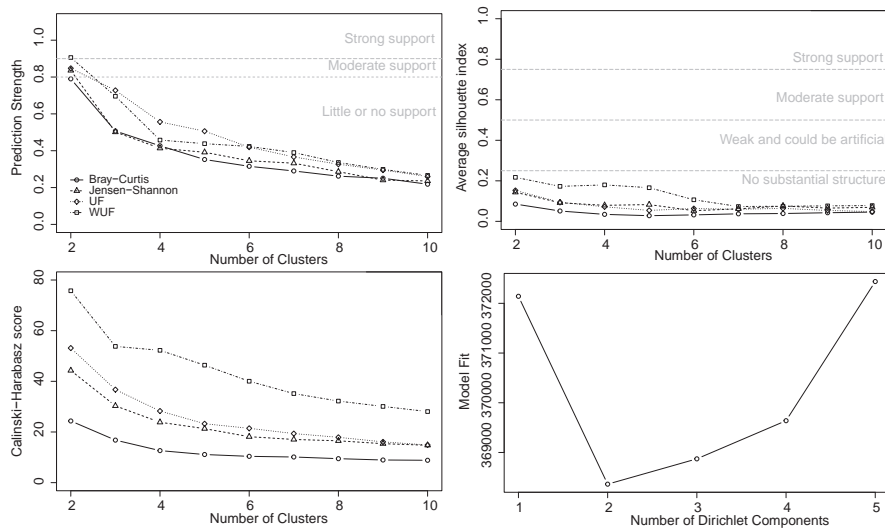
The aim of this study was therefore to explore whether stratification of the cecal microbiota into community types helps to provide insight into ecosystem principles and define the variables impacting the cecal microbiota within and between broiler flocks across age. A longitudinal study in four well-performing broiler farms with a total of ten flocks was performed. From each flock, nine individual broilers were sampled on days 7, 14 and 35 days old. In total, the cecal microbiota of 270 broilers was determined by sequencing of 16S ribosomal RNA (rRNA) gene amplicons. The outcomes of community types analyses have been shown to be highly dependent on the clustering algorithms used (Koren et al., 2013, Costea et al., 2018). Therefore, to robustly define community types within the cecal microbiota, two clustering methods were used; Dirichlet Multinomial Mixtures (DMM) clustering and partitioning around medoid (PAM) clustering with four beta diversity distance metrics. In addition to the community types, host characteristics, environmental factors, and feed components were included in multivariate distance-based redundancy analysis, to study the relative impact of these factors on the variation in microbiota composition between broilers.

## Results

### ***Microbiota stratification into community types***

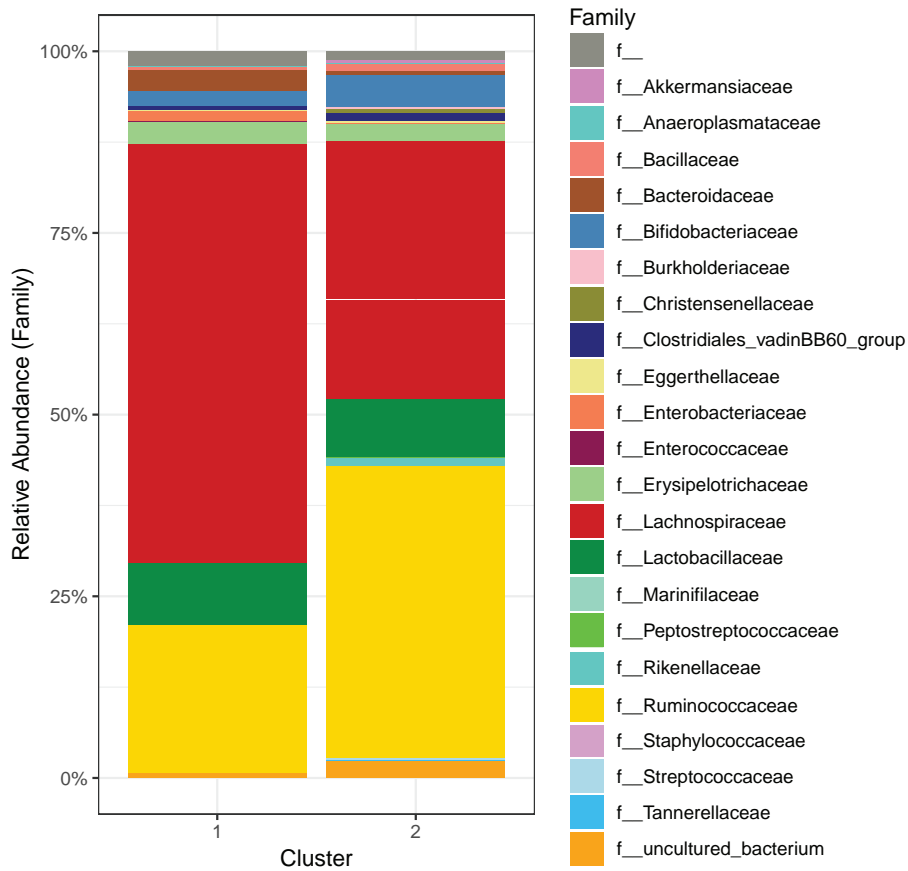
Identifying community types in compositional datasets depends not only on the structure of the data but can also be sensitive to the methods applied. Nonetheless,

all applied clustering methods were in concordance, indicating an optimum of two clusters within the 270 broiler chickens of 7, 14 and 35 days old (Figure 1).



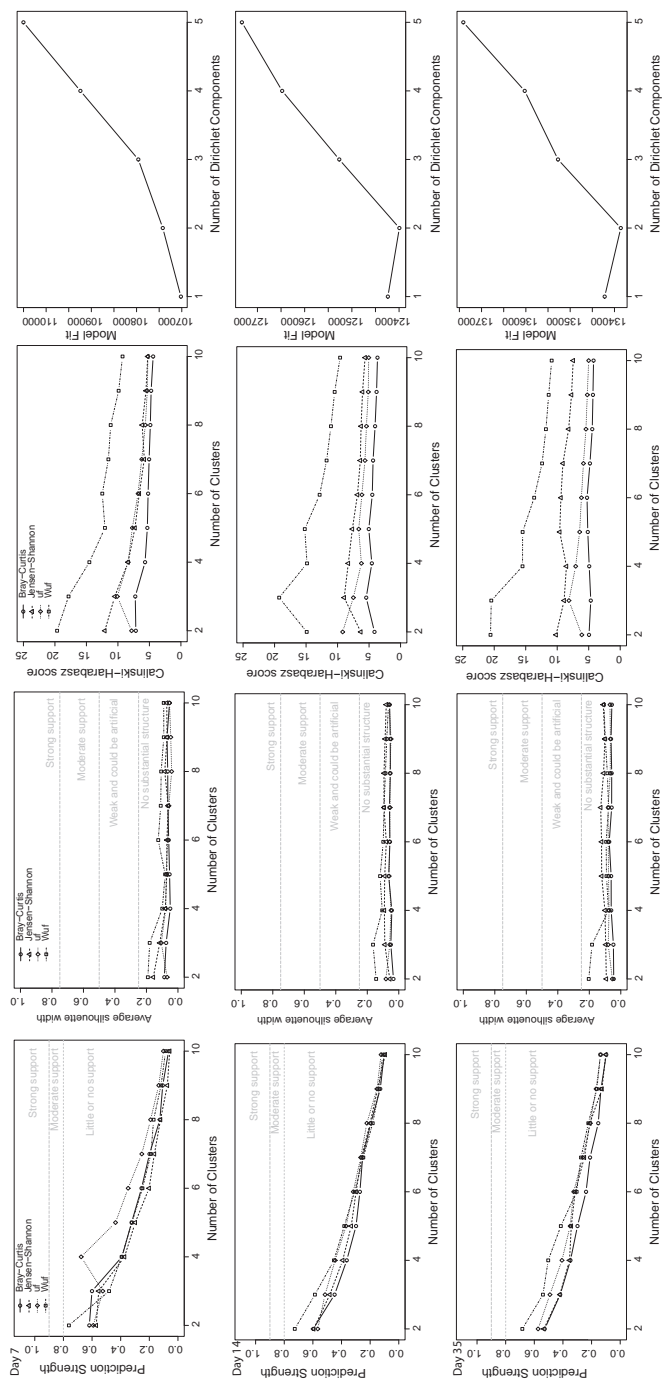
**Figure 1:** Clustering scores for structures in broiler cecal samples based on partitioning around medoid (PAM) and dirichlet multinomial mixtures (DMM) clustering. The dataset contained 270 broilers of age 7, 14 and 35 days old. All four figures show most support for two clusters. The thresholds for significance of clustering scores are indicated as dashed lines on the plots. A. Based on the prediction strength, strong support was observed for the PAM weighted UniFrac (PAM-WUF), and moderate support when using other distance metrics (Bray-Curtis (BC), Jensen-Shannon (JS), and unweighted UniFrac (UF). B. No support for two clusters was observed based on the silhouette index, although the highest score was also observed for two clusters. C. All distance metrics showed the highest score for two clusters based on the Calinski-Harabasz score. The PAM-WUF methods showed again the highest score for two clusters. D. The Dirichlet Multinomial Mixture cluster score showed also highest evidence for two clusters.

Across all methods, the two clusters were associated with age, as almost all 7-day-old broilers were in cluster 1, and all 35-day-old broilers were in cluster 2, while the 14-day-old broilers were distributed across both clusters (Figure S1). However, individual broilers were classified in different clusters depending on the applied method (Figure S1). For example, on day 14 in the data of Farm 1, PAM clustering based on unweighted UniFrac (PAM-UF) resulted in 11/18 broilers in cluster 1, PAM clustering based on Weighted UniFrac (PAM-WUF) resulted in 6/18 broilers in cluster 1, and DDM clustering resulted in 9/18 broilers in cluster 1 (Figure S1). The predominant families were *Lachnospiraceae* in cluster 1 and *Ruminococcaceae* in cluster 2 (Figure 2). The top 25 amplicon sequence variants (ASVs), that differed in relative abundance between the two clusters, were largely independent of clustering method (Table S1).



**Figure 2:** Mean relative abundance of microbial taxa at family level for two clusters based on DMM clustering. Relative microbial abundance (%) of the two robust community types at family level. In total 270 broilers of 7, 14 or 35 days of age were included.

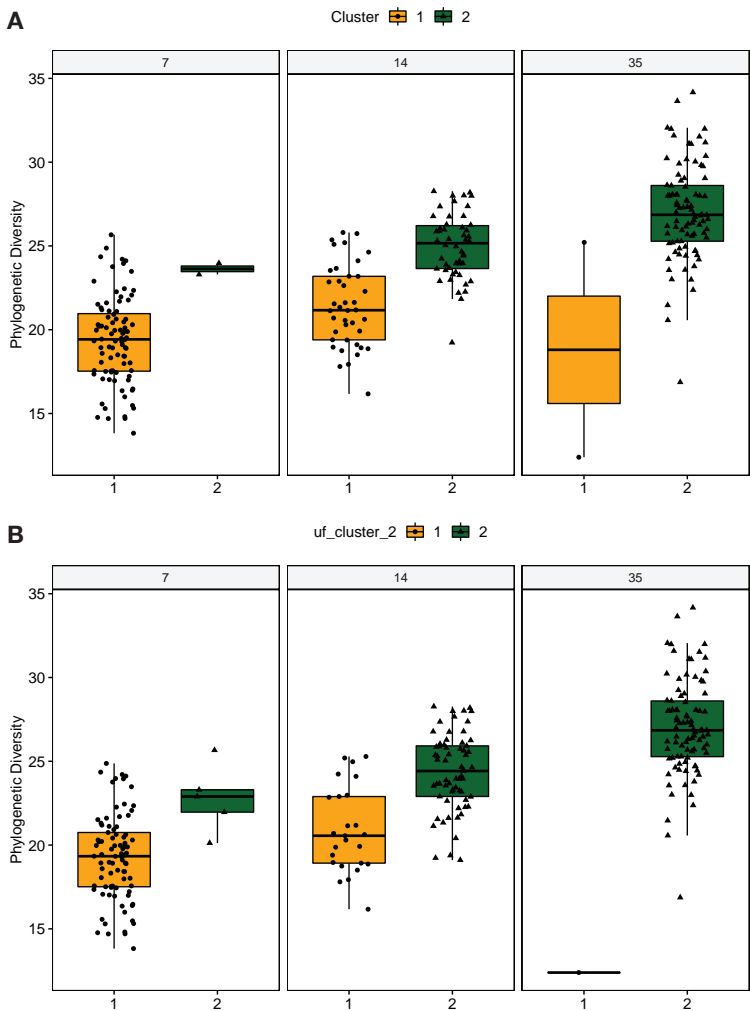
Commercial broilers have a short life span between hatch and slaughter of approximately five to eight weeks, and during this life phase their intestinal microbiota composition changes rapidly. Therefore, the cluster analyses were also performed stratified by age, to identify potential age-specific clusters. These analyses showed that different clustering algorithms resulted in differences in optimal cluster structures, which suggests that there were no robust age-specified clusters within 7-, 14- or 35-day-old broilers with the number of samples included in this study (Figure 3, Figure S2).

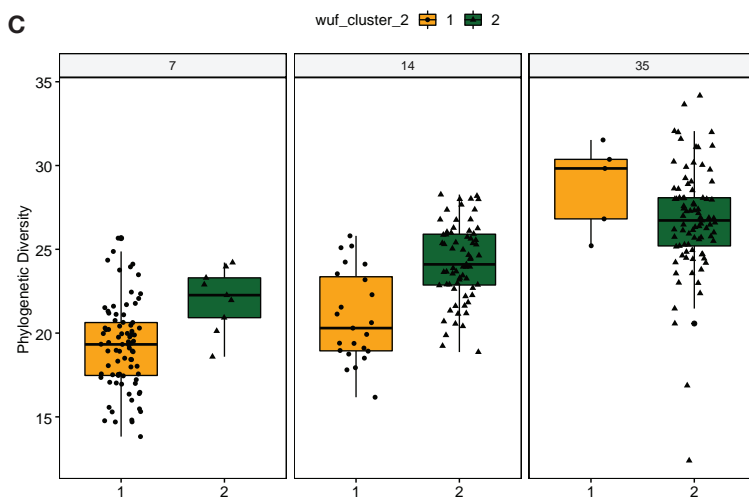


**Figure 3:** Cluster analysis stratified by sampling day. Prediction Strength, Average Silhouette Width, Calinski-Harabasz index and Laplace approximation. In the cecal content of 7-day-old broilers the PAM-WUF method showed near-moderate support (0.78, threshold at 0.80) for two clusters, however, the other methods showed little or no support for significance based on the prediction strength. Although there was no support for clusters, the prediction strength was higher for four clusters instead of two clusters. B,C. Based on the silhouette index and the Calinski-Harabasz score, the PAM-UF distance metrics resulted in three clusters, and all other distance metrics in two clusters. D. Based on DMM, no clusters were observed. This indicated that depending on the clustering method, the number of clusters varied in 7-day-old broilers. E. On day 14 little to no support was observed for all distance metrics based on the prediction strength, however, the highest support was for two clusters independent of distance metrics. F. No support for clusters was observed based on the silhouette index as well. G. PAM-BC, -JS and -WUF showed the highest Calinski-Harabasz score for three clusters, and PAM-UF the highest Calinski-Harabasz score for two clusters. H. The DMM method resulted in two clusters. I,J. In the broilers of 35 days old, also little to no support was observed based on all distance metrics, although again the highest support was for two clusters (prediction strength, J). K. The Calinski-Harabasz score showed that distance metrics BC and JS resulted in two clusters, and distance metrics UF and WUF resulted in three clusters. L. The DMM method resulted in two clusters.

**Diversity and developmental trajectories of cecal microbiota**

To assess whether the two identified community types differed in alpha and beta diversity, DMM-, PAM-UF- and PAM-WUF-derived clusters were compared. Within sample (alpha) diversity defined as phylogenetic diversity was higher in cluster 2 compared to cluster 1, independent of clustering method (Figure 4). Alternative alpha diversity metrics such as ASV richness and Shannon diversity confirmed these results (Table S2). As the 14-day-old broilers were distributed across both clusters we also tested whether, within this age category, a difference between clusters could be identified. The phylogenetic diversity of 14-day-old broilers in DMM, PAM-UF or PAM-WUF cluster 1 was lower compared to 14-day-old broilers in cluster 2 (Figure 4).





**Figure 4:** Phylogenetic diversity of the cecal microbiota across different cluster methods and stratified per age. Phylogenetic diversity (ASV level) of different clustering methods the individual sample types are shown in separate age plots for clarity (n=270). Whiskers show 95% interval, box 50% interval. A. DMM cluster (Kruskal-Wallis  $\chi^2 = 161.49$ ,  $p$ -value  $< 2.2e-16$ ) B. PAM-UF cluster, ( $\chi^2 = 151.73$ ,  $p < 2.2e-16$ ). C. PAM-WUF cluster ( $\chi^2 = 117.35$ ,  $p < 2.2e-16$ ). Within broilers of 14 days old, DMM,  $\chi^2 = 36.60$ ,  $p$ -value  $< 1.5e-09$ , PAM-UF,  $\chi^2 = 25.04$ ,  $p$ -value  $< 5.6e-07$ , PAM-WUF,  $\chi^2 = 19.84$   $p$ -value  $< 8.4e-06$ ).

PERMANOVA based on UF and WUF distances of cecal microbiota compositional data allowed to assess to which extent the different community types accounted for the observed total microbiota variation. The variation explained by the two clusters for the different methods was for DMM clustering 16.4% and 18.1%, for UF-PAM clustering 16.5% and 17.6% and for WUF-PAM clustering 14.1% and 22.1%, respectively.

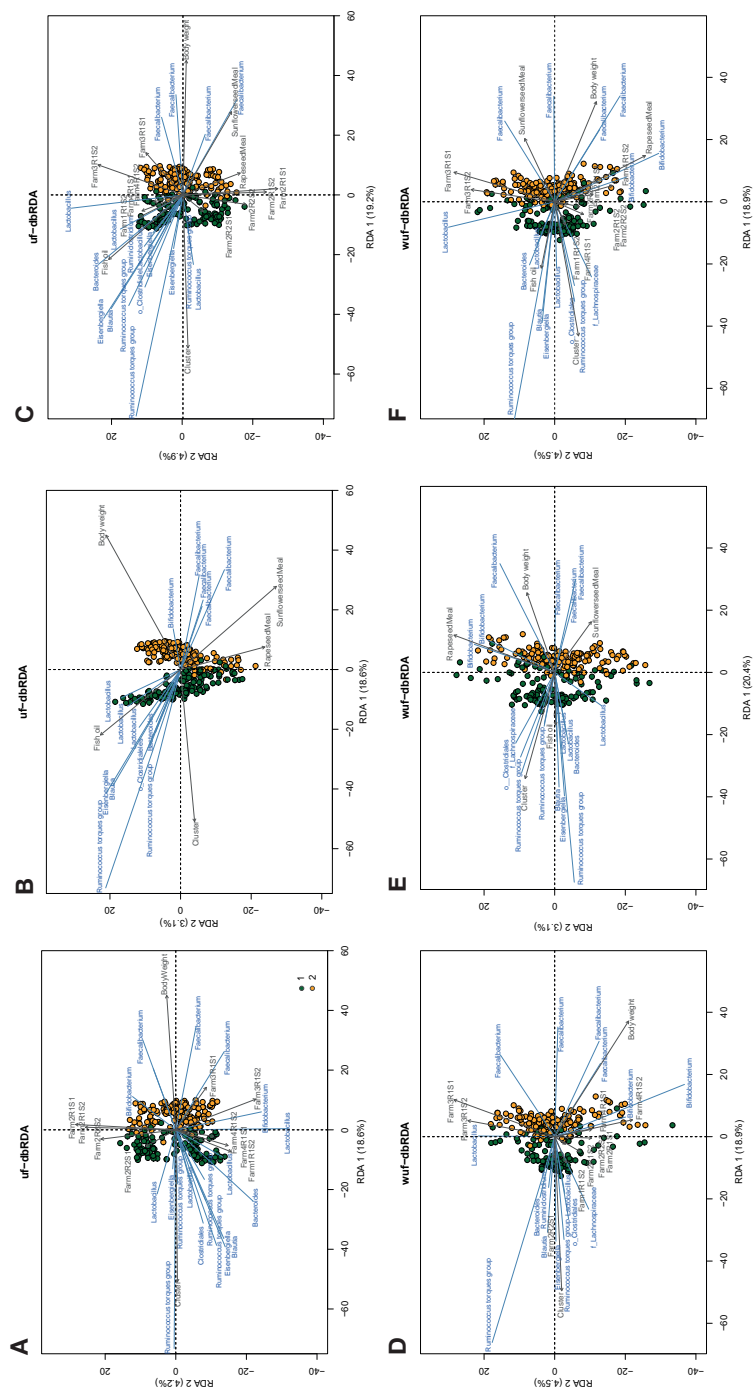
In addition to the two community types, 12 host and environmental characteristics (categorical and continuous), were added as explanatory variables in a UF and WUF distance-based redundancy analysis (db-RDA) (Table S3). These included environmental characteristics at flock level, such as flock size, surface of the poultry house, bird density per m<sup>2</sup>, litter type, age of the parent stock, hatchery, feed producer, antibiotic use, but also individual host data, such as individual body weight and sex, and factors such as farm and flock (Table S3). The db-RDA analysis allowed to determine the relative impact of these variables on modulation of microbiota composition (microbiota covariates) during the development of cecal microbiota. The most parsimonious UF-db-RDA model, verified on collinearity, contained only three explanatory variables which could be related to microbiota composition: the DMM based community types, flock and body weight. This model explained 31.7% of the cecal microbiota variation, which was very similar to the analysis based on WUF (30.6%) with the same variables (Figure 5a, d). Only minor differences were observed

with the PAM clusters (33.3 % and 31.0%, data not shown). It should be noted, however, that the two community types are related to the age of the animals, and in turn, age and body weight are also highly related in these fast growing chickens. Therefore, we included age in the model, which resulted in a variance inflation factor (VIF) value of 27, suggesting that the variable age contains little or no unique information (collinearity) in the model with community types, flock, and body weight, and therefore can be considered redundant in this set of explanatory variables (UF and WUF). Venn diagrams visualizing the variation partitioning, also showed strong overlap (collinearity) between cluster, body weight and age (Figure 3S, 11 % and 9%). Because of the overlap between these variables, we cannot unequivocally conclude that the relative abundance of members of the genus *Faecalibacterium* was only associated with higher body weight (Figure 5a, d), and whether the relative abundance of a member of the genus *Ruminococcus torques* group was strongly associated with the ordination in broilers within cluster (Figure 5a, d), because both are also associated with the age of the broiler.

In order to assess the impact of differences in feed components on the cecal microbiota, 13 feed components were added to the explanatory model (Table S3). Body weight, sunflower seed meal %, rapeseed meal % and fish oil % were related to microbiota composition and together with the community types explained 26.4% (UF-db-RDA) and 27.9% (WUF-db-RDA) of cecal microbiota variation (Figure 5b, e). This most parsimonious model did not contain flock anymore, however, when flock was included in the model again, this increased the explanatory power of the model to 37.0% and 36.7% (UF, WUF, Figure 5c, f). Flock resulted in a VIF value of 4.3, suggesting that the variable flock contains little or no unique information. This collinearity is also visualized in db-RDA triplots, showing that e.g. fish oil % was associated with Farm 1 and rapeseed meal % with Farm 2 (Figure 5c, d). This indicates that it is difficult to disentangle the contribution of the different feed components from the inevitable effect of flock and farm within this dataset.

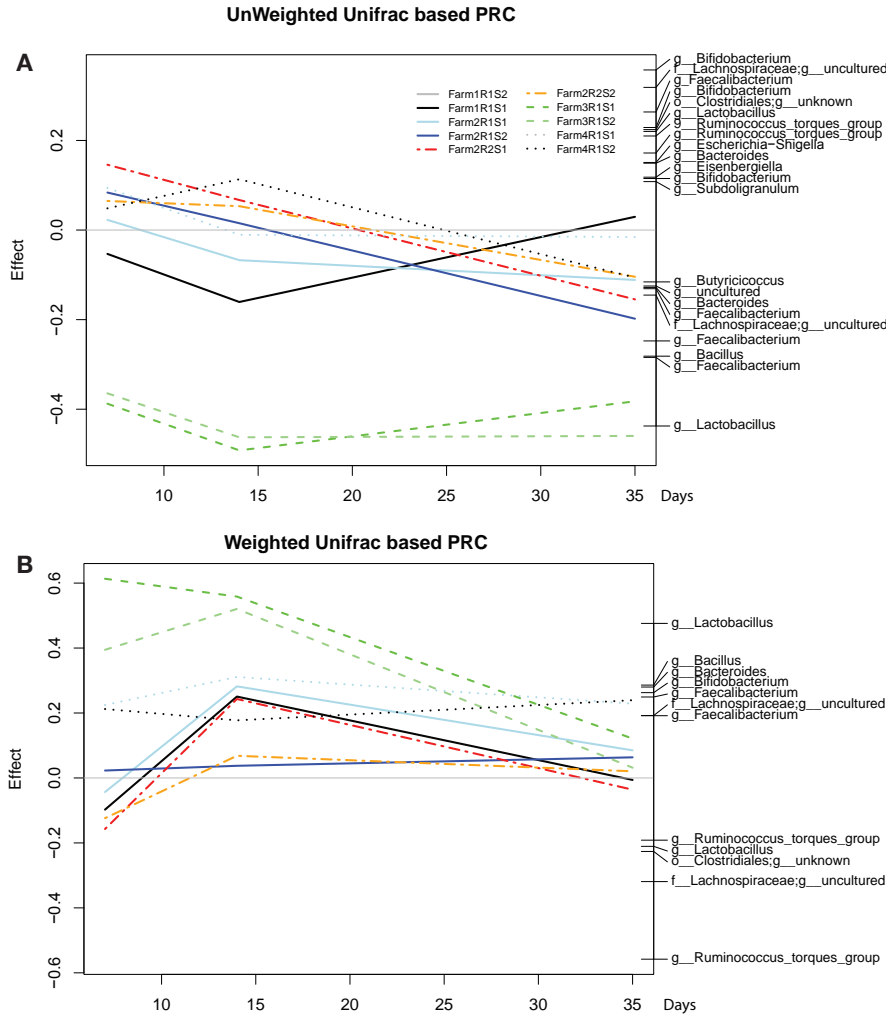
Potential differences in the development of cecal microbiota composition between poultry flocks over time were visualized using Principle Response Curve (PRC) analysis based on UF and WUF distances between individual samples and using flock 2 from Farm 1 as a reference flock (Figure 6). With UF, all farms were relatively similar in composition on day 7, except for Farm 3 (Figure 6a). Farm 3 remained distinct from the rest, due to presences of a member of the genus *Lactobacillus* (Figure 6a), this is also shown in Figure 5d, f. The WUF based PRC showed that Farm 3 and 4 started with a deviant composition, but through time the composition at all farms converged to a similar composition except for Farm 4 (Figure 6b). Thus, taken together, we observed that feed components, flock, and body weight had a substantial impact on the cecal microbiota composition, but the identified





**Figure 5:** Triplots of the different distance based redundancy analyses to explain the variation observed in cecal microbiota composition on ASV level. The longer the arrow length, the stronger the correlation. Samples are colored by DMs-based cluster and ASVs labels are on lowest known taxonomic rank. A. UF-db-RDA with explanatory variables cluster, flock, and body weight. B. UF-db-RDA with cluster, body weight, sunflower seed meal %, rapeseed meal % and fish oil %. C. UF-db-RDA with cluster, flock, body weight, sunflower seed meal %, rapeseed meal %, and fish oil %. D. WUF-db-RDA with cluster, flock, and body weight. E. WUF-db-RDA with cluster, body weight, sunflower seed meal %, rapeseed meal %, and fish oil %. F. WUF-db-RDA with cluster, flock, body weight, sunflower seed meal %, rapeseed meal % and fish oil %.

clusters were independent of these important microbiota covariates. This substantiating the existence of conserved developmental patterns exemplified by the identified clusters. However, because especially feed associated variables were associated with the farm, the independent effects of the variables on microbiota composition could not be disentangled further.



**Figure 6:** Differences in temporal dynamics of cecal microbiota composition between farms and houses. Principle Response Curve (PRC) analysis based on unweighted A. and weighted B. UniFrac distances between samples at ASV level. ASV weights contributing to each statistical model are shown on the right side of each panel. Microbial community changes over time are depicted on Y axis as the difference between microbiota composition, Farm 1, poultry flock 2 has been used as reference (gray line). A. unweighted UniFrac distance-based dbPRC (effect size below 0.1 are not shown for clarity) B. weighted UniFrac distance-based dbPRC (effect size below 0.2 are not shown for clarity).

## Discussion

The aim of this study was to explore whether stratification of the cecal microbiota, collected from broilers in commercial broiler farms, into community types would provide insight into factors that explain the cecal microbiota variation within and between broiler flocks across age. This study showed a conserved cecal microbiota development based on different clustering methods between different commercial broiler flocks. Two robust community types were found, one dominated by broilers of seven days old, and one dominated by broilers of 35 days old. The broilers of 14 days old were divided across both community types. This indicates that the development of the cecal microbiota followed a general trajectory across farms, and an important transition occurred around the second week of life.

The temporal development of the intestinal microbiota towards a developed and matured intestinal microbiota in chickens is not fully understood (Yin et al., 2010, Ballou et al., 2016, Donaldson et al., 2017, Kers et al., 2018, Jurburg et al., 2019). Early life colonization, feed additives, and antimicrobial drugs play a large role in microbiota composition and affect the development (Ballou et al., 2016, Gao et al., 2017). Our results indicate that the temporal cecal microbiota development of broiler chickens undergo two distinct phases, independent of factors such as farm or a flock. In human infants, ten clusters, and three phases were observed (Stewart et al., 2018). The fact that the broilers of 14 days old were divided across both clusters suggests that the period around the second week of life is an important transitional phase. In contrast to other studies that suggest stabilization of the community richness (alpha diversity) by day 14 in cecal or fecal content (Lu et al., 2003b, Jurburg et al., 2019), our data showed an increase in community richness around this age. This may suggest that in the other experimental studies the transition phase was earlier, compared to our field study. Also, with the large time interval between day 14 and 35 the exact moment of stabilization could not be determined in this study and may have occurred shortly after day 14.

In general, identifying community types is sensitive to the methods applied (Koren et al., 2013). The highest prediction strength, silhouette index, and Calinski-Harabasz score were observed with the PAM-WUF method. This is in line with previous suggestions that the WUF distance metric is the best choice for cluster or enterotyping structures (Koren et al., 2013). Our results showed high to moderate support for two community types within the total data set based on the prediction strength, but the silhouette index did not support this observation. This trend of high to moderate support based on the prediction strength and a limited support based on the silhouette index has been observed before. It has been noticed that prediction strength is better able to recover community types (Koren et al., 2013, Yuan et al., 2020). In contrast with previous studies, where four fecal community types and three duodenum community types were identified in broiler chickens of 57 days and 77

days old (Kaakoush et al., 2014, Yuan et al., 2020), no robust community types were observed in our age-stratified analysis of 7-, 14- or 35-day-old broilers. This might be because the broilers in our study were much younger. In humans, the establishment of mature cluster structures has been estimated to occur between the age of 9 and 36 months (Bergström et al., 2014, Zhong et al., 2019). Furthermore, the limited number of individual samples in the stratified data analysis (n= 90) might also be an explanation for why we did not observe robust clusters within age groups.

Previous research in broilers also showed that continuous supply of in-feed antibiotics decreased the maturation of the intestinal microbiota, while feed with the probiotic bacterium *Lactobacillus plantarum* accelerated the development of intestinal microbiota (Gao et al., 2017). On days 3 until 6 of the production cycle of Farm 3, the flock in poultry house 2 received a three-day antibiotic treatment (trimethoprim + sulfamethoxazol). However, this early life antibiotic treatment did not result in different clustering according to our analysis of the community types. Although the PRC analysis showed that Farm 3 (poultry houses 1 and 2), was distinct from the other farms on day 7, this was due to the higher relative abundance of a member of the genus *Lactobacillus*. This higher relative abundance of *Lactobacillus* was also observed on day 14 and 35, and in house 2 as well in house 1, where no flock treatment with antibiotics was applied. This suggests that the difference between Farm 3 and the other farms could not be attributed to the early life treatment with antibiotics, or that the houses were not distinct due to transmission of microbes between poultry houses. Alternatively, it can also be argued that this deviance from other farms was more related to poor chick health on Farm 3, and not to whether or not antibiotic treatment was applied. Both houses on the farm received day-old chicks from the same hatchery. Early life mortality was high in both houses, due to omphalitis, but was slightly higher in house 2, which prompted the antibiotic use. Therefore, we cannot rule out that the effects on microbiota were mostly caused by a lower chick quality and disease in both houses than by the use of antibiotics.

In addition to the observed community types, the body weight, and the feed components sunflower seed meal %, rapeseed meal %, and fish oil % explained 26-27% of the cecal microbiota variation between broilers. However, when including flock in the analysis, the explained variation increased with around 10% but also the collinearity increased between the variables. This indicates that it is very difficult to disentangle the contribution of the different feed components from the inevitable effect of flock and farm, unless a much larger number of farms would be included in the analysis.

In one study, four fecal community types were found in two farms, suggesting that these groupings did not occur by chance because a considerable microbiota variation between farms was observed (Kaakoush et al., 2014). In our study, the two community types were also observed across the different poultry

flocks. However, all flocks were located in the Netherlands, where similar feed compounds are available for different feed suppliers, and all farms used wheat-based feeds. Therefore, the variation between feed and flock is small, which may limit the generalizability of the relevance of the identified feed compounds to other countries.

In humans, diet can provoke a shift in the community types (Wu et al., 2011, Kovatcheva-Datchary et al., 2015), and although the feed in different farms was obtained from different feed suppliers, this was not reflected in our clusters. During the production cycle feed shifts occurred on days 9 (Farm 1 & 2), 10 (Farm 2 & 4) and 12 (Farm 3), however, with a retention time of the digestive tract of less than 12 hours (McWhorter et al., 2009, Sundu, 2009), we assume that the microbiota was already adjusted to the feed shift before day 14. Also, if feed or farm would largely influence the temporal development, then the optimal number of clusters would have been three, associated with the age of the broilers, or four associated with the farm.

Only a few studies have focused on providing insight into the factors that influence the temporal development and microbiota configurations of broiler chicken intestinal microbiota (Johnson et al., 2018). This research remains challenging, because of the wide array of available approaches, each with their advantages and disadvantages (Costea et al., 2018). No single method is perfect and although the db-RDA resulted in the same important factors, a less stringent VIF threshold influences the results and increases explained variation (Zuur et al., 2010). However, increasing the explained variation does not mean that we better understand the interactions between the microbiota and the factors that are important to explain the variation.

In summary, stratification of microbiota composition into community types helps to provide insight into the factors that explain the microbiota variation. Two robust community types were identified in the cecal microbiota of broiler chickens, indicating a conserved developmental trajectory of the cecal microbiota in ten different commercial broiler flocks on four different farms. This emphasizes the importance for further investigation of mechanisms underlying microbiota development and functions that affect broiler performance. This mechanistic knowledge can contribute to the development of new nutritional interventions, improved management as well as better diagnostic tools to improve broiler health.

## Materials and Methods

### *Ethical statement*

The field study was approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee (registration number AVD108002016442) and was carried out in compliance with all relevant legislation.

### Farm selection and data collection

Data for this study were obtained from four broiler farms in the Netherlands, each with two similar houses. All farms had conventional Ross 308 broilers, both male and female. The farms were selected for good production performance, as we were interested in a healthy intestinal microbiota. Also, to reduce the chance of including flocks treated with antibiotics, only farms with an antimicrobial use in the previous months below 15 DDAAf (defined daily dose per animal year on farm level) were recruited for the study. All selected farms were within the target zone for antimicrobial use according to national benchmark thresholds for poultry farms (Netherlands Veterinary Medicines Authority, SDa, 2017). Notwithstanding, two flocks were treated with antibiotics. The flock in poultry house 2 of Farm 3 received a three-day antibiotic treatment (trimethoprim + sulfamethoxazol) between day 3 and 6 of the production cycle, and the flock in poultry house 2 of Farm 4 received a three-day antibiotic treatment (amoxicillin) from day 22 onwards of the production cycle (Table 1). The farms were visited on days 7, 14 and 35 of the production cycle. On one of the farms (Farm 2) data was collected from two consecutive production cycles. The farms received chicks from different commercial hatcheries. An overview of the different farm characteristics of the poultry flocks is described in Table 1. The diets on all farms were provided *ad libitum* and were mostly wheat-based, but there were differences in composition of the feed and feed suppliers between farms. Table S4 provides an overview of the feed details per time point. Coccidiostatic drugs were standardly applied in all flocks (Table S4).

**Table 1:** Farm characteristics. The broilers of Farm 2 cycle 1 and 2 are of the same parent flock. Each poultry house contains one broiler flock.

	Farm 1		Farm 2 Cycle 1		Farm 2 Cycle 2		Farm 3		Farm 4	
<b>Start production cycle (visit)</b>	August 2016		June 2017		June 2017		August 2017		August 2017	
<b>Hatchery</b>	Hatchery A		Hatchery B		Hatchery B		Hatchery C		Hatchery D	
<b>Age of the parent flock (weeks)</b>	55		35		42		49		54	
<b>Type of litter</b>	Woodshavings		Peat		Peat		Strawpellets		Strawpellets	
<b>Feed supplier</b>	Supplier A		Supplier B		Supplier B		Supplier C		Supplier B	
<b>Poultry house</b>	1	2	1	2	1	2	1	2	1	2
<b>Size poultry house (m<sup>2</sup>)</b>	1313	1313	972	968	972	968	1600	1850	1350	1730
<b>Flock size</b>	28000	28000	23500	23500	23500	23500	32200	38400	30500	39000
<b>Antibiotic treatment</b>	No	No	No	No	No	No	No	Yes (day 3)	No	Yes (day 22)

### ***Cecal content collection and 16S rRNA gene amplicon sequencing***

From each poultry house, nine broilers were randomly selected for sampling at each of the visits. Between the sampling of the two poultry flocks on the same farm, coveralls, footwear and all sampling materials were changed. The start of the sampling of broilers took place at least 30 min after the end of a dark-period, to avoid low amounts of content in the intestinal tract at sampling. Broilers were individually weighed, checked for abnormalities and euthanized by cervical dislocation. The gastrointestinal tract was quickly but carefully removed, using a procedure that was as sterile as possible, as previously described in detail (Kers et al., 2019a). The cecal content was stored at -80°C before extraction of DNA as previously described (Kers et al., 2019b). Briefly, DNA was extracted from 0.25 g cecal content, using 700 µL of Stool Transport and Recovery (STAR) buffer (Roche Diagnostics Nederland BV, the Netherlands) and repeated bead beating. DNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, USA), and samples were stored at -20°C until further use. Barcoded amplicons covering the variable regions V5-V6, amplified with primers 784F and 1064R were used for amplification of the bacterial 16S rRNA gene as described before (Ramiro-Garcia et al., 2016). To ensure high quality sequencing data, synthetic communities of known composition were used as positive controls (Ramiro-Garcia et al., 2016), and nuclease free water as negative controls. Sequencing of resulting libraries was performed by GATC GmbH on an Illumina HiSeq2500 instrument (now part of Eurofins Genomics Germany GmbH, Konstanz, Germany). The 16S rRNA gene sequence data was analyzed using NG-tax 2.0 (Poncheewin et al., 2020). In short, NG-Tax 2.0 employs a fast de novo ASV-picking algorithm. To assign taxonomy the SILVA 128 16S rRNA gene reference database was used (Quast et al., 2013).

### ***Statistical analysis***

Statistical analyses were performed in R, version 3.4.3 (RStudio Team, 2017), using packages: Phyloseq, Vegan, Microbiome, DirichletMultinomial, and RVAideMemoire (McMurdie and Holmes 2013, Lahti et al., 2017, Oksanen et al., 2010).

Clustering was performed according to the partitioning around medoid (PAM)-based clustering protocols using Jensen-Shannon divergence (PAM-JSD) on ASV-level (Arumugan et al., 2011), Bray-Curtis (PAM-BC) (Bray and Curtis 1957), unweighted UniFrac (PAM-UF), and weighted UniFrac (PAM-WUF) (Lozupone et al., 2010). The optimal number of clusters was calculated using prediction strength, average silhouette width (silhouette index), Calinski-Harabasz index and Laplace approximation (Koren et al., 2013, Holmes et al., 2012). Also, Dirichlet Multinomial Mixtures (DMM), a probabilistic model, was applied to cluster the 16S rRNA gene sequence data at ASV level (Holmes et al., 2012). To test for differences in relative abundance of genera between clusters, Wilcoxon rank-sum test corrected for multiple comparisons using Benjamini-Hochberg (BH)

was used. A  $p$ -value of  $<0.05$  was considered statistically significant.

Shannon diversity, ASV richness and Faith's phylogenetic diversity (Faith, 2006) were calculated to define microbial alpha diversity for each sample. Differences in alpha diversity were tested with a Kruskal-Wallis test, and pairwise comparisons were tested using Wilcoxon rank-sum test and corrected for multiple testing with BH. Beta diversity (between samples) was determined using Bray-Curtis, Jaccard, weighted and unweighted UniFrac metrics (Bray and Curtis, 1957, Jaccard, 1912, Lozupone et al., 2007). Multivariate microbiota data were visualized using principal coordinates analysis (PCoA), and non-parametric permutational analysis of variance (PERMANOVA) tests were used to analyze group differences within multivariate community data (Anderson, 2001).

To examine the multivariate differences between microbiota compositions of poultry houses over time, principal response curve (PRC) analysis was used. PRC was originally developed to analyze time-series data and carries out partial redundancy analysis (RDA) ordination to obtain estimates of community changes using time as a predictor variable (Van den Brink and Braak, 1999).

In addition, to provide insight in which explanatory variables impact broiler cecal microbiota, distance-based redundancy analysis (db-RDA), based on weighted (WUF) and unweighted Unifrac (UF) distances were performed, with the capscale function from the vegan package (Oksanen et al., 2010). This is a multivariate canonical ordination analysis method that takes the phylogenetic makeup of microbial communities into consideration. The analysis was performed using ASV level data (Shankar et al., 2017). To determine the most parsimonious model, the model that explained the microbiota variation with the least number of explanatory variables, a stepwise selection (both directions) was used based on the Akaike information criterion (AIC), using the ordistep function from vegan. The most parsimonious model was verified on collinearity, and variance inflation factors (VIF) higher than 3 were removed from the model one by one (Zuur et al., 2010). High VIF values suggest that the variable contains little or no unique information, and therefore is redundant in the set of explanatory variables.

### ***Acknowledgements***

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Conflicts of Interest

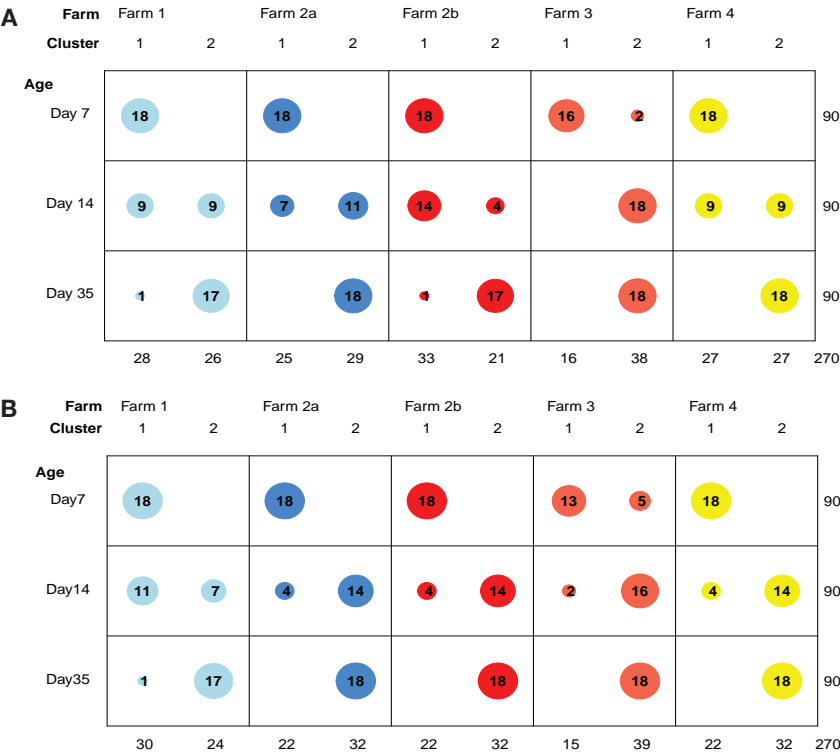
The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

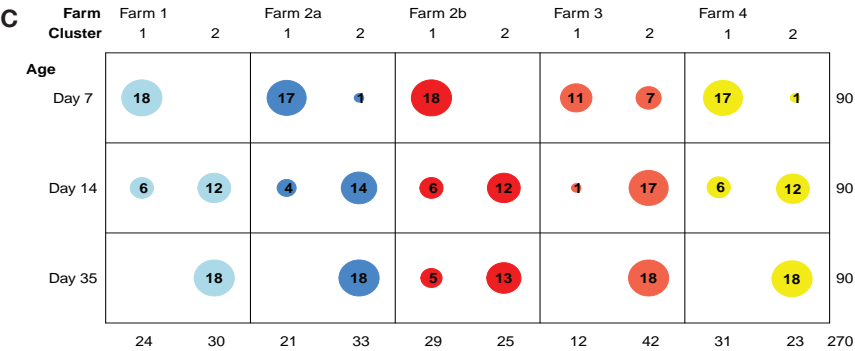
Author Contributions

Conceptualization, F.C.V., J.A.S. and H.S.; Methodology, F.C.V., J.A.S., E.A.J.F., J.G.K. and H.S.; Investigation, J.G.K. and F.C.V.; Data analysis, J.G.K and G.D.A.H; Writing Original Draft, J.G.K.; Writing Review and Editing, G.D.A.H, F.C.V., J.A.S., E.A.J.F. and H.S.; Funding Acquisition, F.C.V.; Supervision, F.C.V., J.A.S., E.A.J.F. and H.S.

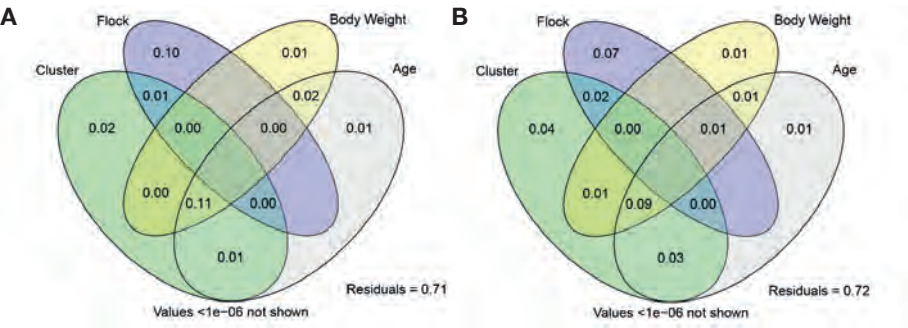
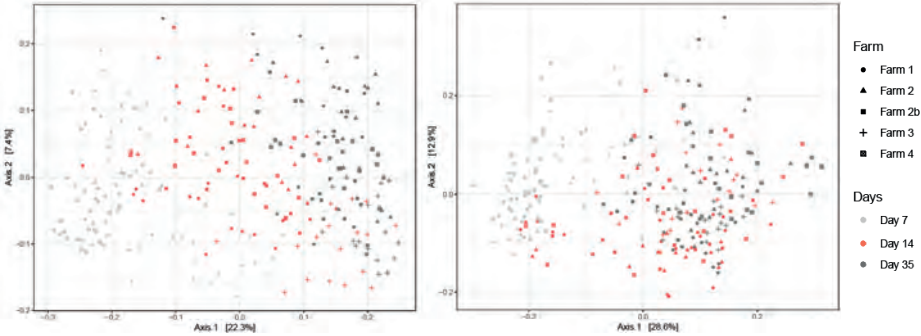
Supplemental Materials

**Figure S1:** Distribution of clusters across farms and animal age. Clusters were assigned using PAM clustering based on unweighted (A) or weighted (B) UniFrac distances, and DMM clustering (C). The variation of individual samples through different cluster methods stratified per farm. Farm 1 is light blue, Farm 2 is dark blue and red (a and b are different production cycles), Farm 3 is light red, Farm 4 is yellow.





**Figure S2:** Principal coordinate plots (PCoA) based on (A) unweighted UniFrac and (B) weighted UniFrac distances. Different colors indicate different sampling days, and different shapes indicated different farms.



**Table S1:** Top 25 ASVs differing in relative abundance between two clusters identified by different clustering methods. The difference in ASVs between cluster 1 and cluster 2, across different methods. Results are based on differences of relative abundance tested with Wilcoxon rank-sum test. Adjusted P-values (0.05) are corrected for multiple testing with BH. Bold names indicated the variation between methods. % Indicated increase (+) or decrease (-) between cluster 1 versus cluster 2.

Top	DMM	%	PAR-UF	PAM-WUF	PAM-BC	PAM-JS
1	g_Faecalibacterium	+	g_Faecalibacterium	g_Faecalibacterium	g_Faecalibacterium	g_Faecalibacterium
2	g_Ruminococcus torques	-	g_Ruminococcus torques	g_Ruminococcus torques	g_Ruminococcus torques	g_Ruminococcus torques
3	g_Eisenbergiella	-	g_Eisenbergiella	g_Eisenbergiella	g_Eisenbergiella	g_Eisenbergiella
4	o_Clostridiales	-	o_Clostridiales	o_Clostridiales	g_Bifidobacterium	o_Clostridiales
5	f_Lachnospiraceae	-	f_Lachnospiraceae	f_Lachnospiraceae	o_Clostridiales	f_Lachnospiraceae
6	f_Lachnospiraceae	-	g_Bifidobacterium	g_Bifidobacterium	f_Lachnospiraceae	g_Bifidobacterium
7	g_Bifidobacterium	+	g_Lactobacillus	g_Lactobacillus	g_Lactobacillus	g_Lactobacillus
8	g_Bacteroides	-	g_Subdoligranulum	f_Lachnospiraceae	g_Bacteroides	f_Lachnospiraceae
9	g_Subdoligranulum	+	g_Bacteroides	g_Bacteroides	f_Lachnospiraceae	g_Bacteroides
10	g_GCA-900066575	-	f_Lachnospiraceae	g_Subdoligranulum	g_Subdoligranulum	g_Subdoligranulum
11	g_Alistipes	+	g_Alistipes	g_GCA-900066575	<b>g_Blaulia</b>	g_CA-900066575
12	g_Escherichia-Shigella	-	g_GCA-900066575	g_Alistipes	g_GCA-900066575	g_Alistipes
13	g_Fuscatenibacter	+	g_Escherichia-Shigella	g_Lachnoclostridium	g_Alistipes	g_Lachnoclostridium
14	o_Clostridiales	-	g_Lachnoclostridium	o_Clostridiales	g_Lachnoclostridium	g_Escherichia-Shigella
15	g_Lachnoclostridium	-	o_Clostridiales	g_Escherichia-Shigella	g_Escherichia-Shigella	o_Clostridiales
16	g_Erysipelatoclostridium	-	g_Sellimonas	g_Sellimonas	o_Clostridiales	g_Erysipelatoclostridium
17	g_Christensenellaceae	+	g_Ruminococcaceae_UCG-14	g_Bacillus	g_Sellimonas	g_Sellimonas
18	g_Bacillus	+	g_Ruminococcaceae_UCG-005	g_Erysipelatoclostridium	g_Fuscatenibacter	g_Fuscatenibacter
19	g_Ruminococcaceae_UCG-14	+	g_Erysipelatoclostridium	g_Ruminococcaceae_UCG-14	g_Bacillus	g_Ruminococcaceae_UCG-14
20	g_Ruminococcaceae_UCG-005	+	g_Fuscatenibacter	g_Ruminococcaceae_UCG-005	g_Ruminococcaceae_UCG-14	g_Ruminococcaceae_UCG-005
21	g_Sellimonas	-	g_Christensenellaceae	g_Christensenellaceae	g_Ruminococcaceae_UCG-005	g_Christensenellaceae
22	<b>g_Ruminiclostridium</b>	-	g_Bacillus	<b>g_Ruminiclostridium</b>	g_Erysipelatoclostridium	g_Bacillus
23	<b>g_Akkermansia</b>	+	g_uncultured_bacterium	g_Fuscatenibacter	g_Christensenellaceae	g_Butyricoccus
24	<b>g_Ruminiclostridium</b>	+	<b>g_Ruminiclostridium</b>	<b>g_Akkermansia</b>	g_uncultured_bacterium	g_uncultured_bacterium
25	g_uncultured_bacterium	+	<b>g_Akkermansia</b>	g_uncultured_bacterium	<b>g_Ruminiclostridium_</b>	<b>g_Akkermansia</b>

**Table S2:** Alpha diversity metrics. Differences in alpha diversity were tested with a Kruskal-Wallis test.

<b>Community types 1 vs 2</b>				
<b>ASV richness</b>	<b><math>\chi^2</math></b>	<b><math>p</math> – value</b>		
DMM cluster	109.67	$p < 2.2\text{e-}16$		
PAM-UF	88.06	$p < 2.2\text{e-}16$		
PAM-WUF	74.33	$p < 2.2\text{e-}16$		
Age	85.86	$p < 2.2\text{e-}16$		
<b>Shannon diversity</b>	<b><math>\chi^2</math></b>	<b><math>p</math> – value</b>		
DMM cluster	47.19	$p = 6.5\text{e-}12$		
PAM-UF	39.85	$p = 2.7\text{e-}10$		
PAM-WUF	32.82	$p = 1.0\text{e-}8$		
Age	36.81	$p = 1.0\text{e-}8$		
<b>Age 7 vs 14 &amp; 14 &amp; 35</b>	<b>Day 7 vs 14</b>		<b>Day 14 vs 35</b>	
<b>Phylogenetic diversity</b>	<b><math>\chi^2</math></b>	<b><math>p</math> – value</b>	<b><math>\chi^2</math></b>	<b><math>p</math> – value</b>
DMM cluster 1	<b>14.43</b>	<b>1.5e-4</b>	0.03	0.859
DMM cluster 2	1.10	0.295	<b>20.65</b>	<b>5.5e-6</b>
PAM-UF cluster 1	<b>6.01</b>	<b>0.014</b>	2.78	0.096
PAM-UF cluster 2	2.44	0.118	<b>33.64</b>	<b>6.6e-9</b>
PAM-WUF cluster 1	<b>5.76</b>	<b>0.016</b>	<b>11.49</b>	<b>7.0e-4</b>
PAM-WUF cluster 2	<b>6.33</b>	<b>0.012</b>	<b>31.63</b>	<b>1.8e-8</b>
<b>ASV richness</b>	<b><math>\chi^2</math></b>	<b><math>p</math> – value</b>	<b><math>\chi^2</math></b>	<b><math>p</math> – value</b>
DMM cluster 1	0.96	0.326	0.03	0.859
DMM cluster 2	2.47	0.116	2.66	0.103
PAM-UF cluster 1	0.71	0.400	2.78	0.095
PAM-UF cluster 2	3.21	0.073	<b>11.03</b>	<b>8.9e-4</b>
PAM-WUF cluster 1	0.02	0.885	<b>11.09</b>	<b>8.7e-4</b>
PAM-WUF cluster 2	<b>4.89</b>	<b>0.027</b>	<b>7.63</b>	<b>5.8e-3</b>
<b>Shannon diversity</b>	<b><math>\chi^2</math></b>	<b><math>p</math> – value</b>	<b><math>\chi^2</math></b>	<b><math>p</math> – value</b>
DMM cluster 1	0.02	0.902	0.01	0.906
DMM cluster 2	0.44	0.505	2.18	0.140
PAM-UF cluster 1	0.03	0.856	2.78	0.096
PAM-UF cluster 2	1.3e-4	0.991	<b>6.99</b>	<b>8.2e-3</b>
PAM-WUF cluster 1	0.43	0.513	<b>11.08</b>	<b>8.7e-4</b>
PAM-WUF cluster 2	0.13	0.718	3.25	0.072

**Table S3:** Explanatory variables host and environmental characteristic and feed components**Host and environmental characteristic (n=12)**

Farm (4 farms), age (7, 14, 35), body weight, flock (n=10), sex, age parent stock, hatchery, litter type, flock size, density (number of animals per m<sup>2</sup>), Surface (poultry house in m<sup>2</sup>), Feed producer, antibiotic use (yes/no)

**Feed compounds (n=13)**

Wheat %, Farmers wheat %, Maize %, Oats %, Soybean meal %, Potato protein %, Rapeseed meal%, Sunflower seed meal %, Fish oil %, Metabolizable energy (AME)·kg<sup>-1</sup>, Phosphorous, Fecal digestible lysine g·kg<sup>-1</sup>, Methionine + Cysteine

**Table S4:** Feed characteristics and coccidiostats

	Farm 1	Farm 2 Cycle 1	Farm 2 Cycle 2	Farm 3	Farm 4
<b>Day 7</b>					
Wheat %	34	42	42	40	47
Farmers wheat %	0	0	0	0	0
Maize %	22.2	20	20	25	17
Oats %	9.5	1.5	1.5	5.0	1.5
Soybean meal %	23.1	28.0	28.0	19	24
Potato protein %	1.0	0	0	0.5	0
Rapeseed meal %	1.5	0	0	0	0
Sunflower seed meal %	0	0	0	2	0
Fish oil %	0.4	0	0	0	0
Metabolizable energy (AME)·kg <sup>-1</sup>	2970 kcal	2925 kcal	2925 kcal	2945 kcal	2925 kcal
Phosphorous	3.9	5.4	5.4	3.48	4.5
Fecal digestible lysine g·kg <sup>-1</sup>	12.6	11.8	11.8	10.3	11.4
Methionine + Cysteine	8.5	8.7	8.7	7.82	8.4
Coccidiostatic drugs	Narasin and nicarbazin	Narasin and nicarbazin	Narasin and nicarbazin	Narasin and nicarbazin	Narasin and nicarbazin
<b>Day 14</b>					
Wheat %	43	55	55	43	42
Farmers wheat %	12	11	11	16	10   12
Maize %	20.4	7.5	7.5	20	20
Oats %	4.0	2.0	2.0	5.0	3.0
Soybean meal %	22.9	20.0	20.0	19	22.0
Potato protein %	0.3	0	0	0	0
Rapeseed meal %	1.0	3.0	3.0	0	2.0
Sunflower seed meal %	0	3.5	3.5	2.5	0
Fish oil %	0	0	0	0	0

Metabolizable energy (AME)-kg- 1	3000 kcal	2935 kcal	2935 kcal	3020 kcal	2925 kcal
Phosphorous	3.7	3.8	3.8	3.12	3.3
Fecal digestible lysine g·kg- 1	12.1	11.1	11.1	10.2	10.4
Methionine + Cysteine	8	8.3	8.3	7.9	7.8
Coccidiostatic drugs	Narasin and nicarbazin	Narasin and nicarbazin	Narasin and nicarbazin	Narasin and nicarbazin	Salinomycin

**Day 35**

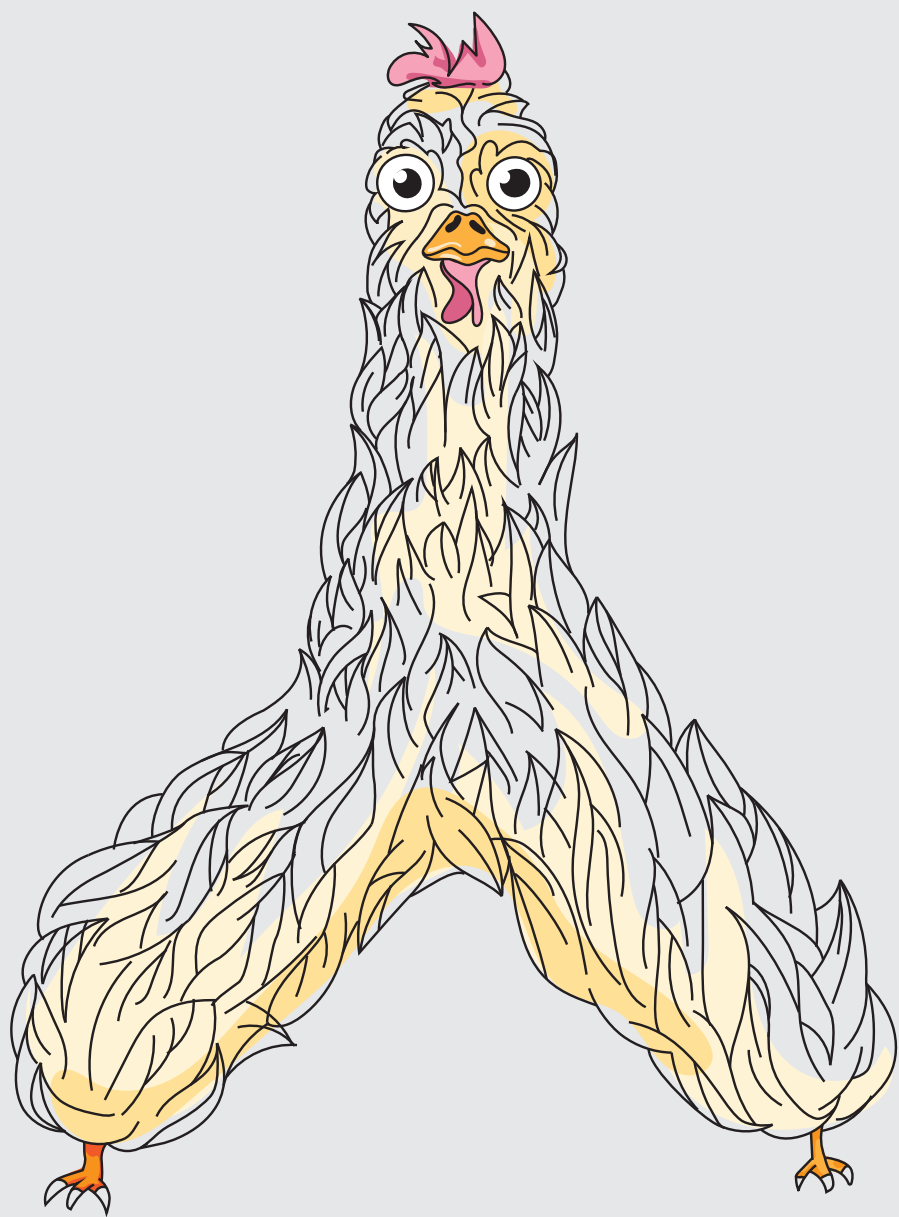
Wheat %	48	62	62	53	55
Farmers wheat %	37	25	25	35	34
Maize %	18.5	8.0	8.0	15	10
Oats %	0	0	0	2.5	0
Soybean meal %	19.9	17.5	17.5	18	21.0
Potato protein %	0	0	0	0	0
Rapeseed meal %	4.5	0	0	0	2
Sunflower seed meal %	0	2.8	2.8	2.5	0
Fish oil %	0	0	0	0	0
Metabolizable energy (AME)-kg- 1	3050 kcal	3025 kcal	3025 kcal	3070 kcal	3025 kcal
Phosphorous	3.2	2.9	2.9	2.8	2.9
Fecal digestible lysine g·kg- 1	10.7	9.4	9.4	10.0	10.0
Methionine + Cysteine	7	7.2	7.2	7.8	7.6
Coccidiostatic drugs	Narasin	none	none	None	none



## PART III

Can we manipulate the intestinal microbiota to improve broiler health and reduce the risk of colonization with pathogens?







# CHAPTER 7

## Competitive exclusion prevents colonization and compartmentalization reduces transmission of ESBL-producing *Escherichia coli* in broilers

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Submitted

## Abstract

Extended spectrum  $\beta$ -lactamase (ESBL)-producing bacteria are resistant to extended-spectrum cephalosporins and are common in broilers. Interventions are needed to reduce the prevalence of ESBL-producing bacteria in the broiler production pyramid. This study investigated two different interventions. The effect of a prolonged supply of competitive exclusion (CE) product and compartmentalization on colonization and transmission, after challenge with a low dose of ESBL-producing *Escherichia coli*, in broilers kept under semi-field conditions, were examined. One-day-old broilers (Ross 308) (n=400) were housed in four experimental rooms, subdivided in one seeder (S/C1)-pen and eight contact (C2)-pens. In two rooms, CE product was supplied from day 0 to 7. At day 5, seeder-broilers were inoculated with *E. coli* strain carrying bla<sub>CTX-M-1</sub> on plasmid Inc11 (CTX-M-1-*E. coli*). Presence of CTX-M-1-*E. coli* was determined using cloacal swabs (day 5-21 daily) and cecal samples (day 21). Time until colonization and cecal excretion (log<sub>10</sub> CFU/g) were analyzed using survival analysis and linear regression. Transmission coefficients within and between pens were estimated using maximum likelihood. The microbiota composition was assessed by 16S ribosomal RNA gene amplicon sequencing in cecal content of broilers on days 5 and 21. None of the CE broilers was CTX-M-1-*E. coli* positive. In contrast, in the untreated rooms 187/200 of the broilers were CTX-M-1-*E. coli* positive at day 21. Broilers in C2-pens were colonized later than seeder-broilers (Time to event Ratio 3.53, 95% CI 3.14 – 3.93). The transmission coefficient between pens was lower than within pens ( $3.28 \times 10^{-4} \text{ day}^{-2}$ , 95% CI  $2.41 \times 10^{-4} - 4.32 \times 10^{-4}$  versus  $6.12 \times 10^{-2} \text{ day}^{-2}$ , 95% CI  $4.78 \times 10^{-2} - 7.64 \times 10^{-2}$ ). The alpha diversity of the cecal microbiota content was higher in CE broilers than in control broilers at days 5 and 21. The supply of a CE product from day 0 to 7 prevented colonization of CTX-M-1-*E. coli* after challenge at day 5, likely as a result of CE induced effects on the microbiota composition. Furthermore, compartmentalization reduced transmission rate between broilers. Therefore, a combination of compartmentalization and supply of a CE product may be a useful intervention to reduce transmission and prevent colonization of ESBL/pAmpC-producing bacteria in the broiler production pyramid.

**Keywords:** poultry, compartments, intervention, antimicrobial resistance, ESBL, *Escherichia coli*, colonization.

## Introduction

Extended Spectrum Beta-Lactamase and plasmid AmpC Beta-Lactamase (ESBL/pAmpC)-producing bacteria are resistant to extended-spectrum cephalosporins (ESC). ESBL/pAmpC-producing bacteria are present in humans, animals and the environment (Blaak et al., 2015). Poultry is known as a source of ESBL/pAmpC-producing bacteria and high prevalence in poultry and poultry products have been reported in several European countries, as reviewed by (Saliu et al., 2017). ESBL/pAmpC-producing bacteria are present at all levels of the broiler production pyramid (Dierikx et al., 2013, Agersø et al., 2014, Nilsson et al., 2014, Zurfluh et al., 2014, Projahn et al., 2018). Different routes of transmission within the broiler production pyramid have been described, for example between generations, via the hatcheries, and on and between farms (Dame-Korevaar et al., 2019a). Introduction of ESBL/pAmpC-producing bacteria can occur at several levels of the broiler production pyramid, for example at the farm or at the hatchery. A recent study estimated that, based on the proportional similarity index (PSI), the average transfer of ESBL/pAmpC genes between subsequent generations in the broiler production pyramid is almost 50% (Apostolakos et al., 2019). However, for most of the routes it is unknown to what extent they contribute to the presence of ESBL/pAmpC-producing bacteria in the broiler production pyramid. In the Netherlands, antimicrobial resistance in broilers has decreased significantly since 2010 (Hesp et al., 2019), following the trend of reduced antimicrobial usage. However, additional interventions are needed to further reduce this prevalence in the broiler production pyramid.

Interventions can aim to reduce exposure of broilers to ESBL/pAmpC-producing *Escherichia coli*. This can be done by improving biosecurity. For example hygiene barriers can help reduce exposure to bacteria from the farm environment, or by cleaning and disinfection between production rounds. However, even after cleaning and disinfection, ESBL/pAmpC-producing bacteria might remain in the poultry house and result in colonization of the new flock (Daehre et al., 2018). In addition, housing measures may reduce the prevalence of ESBL/pAmpC-producing *E. coli* in poultry flocks. In turkeys, subdividing the flock was associated with a reduced risk for the presence of resistant *E. coli* in the farm (Jones et al., 2013). Experimental studies showed that spatial separation between infectious and susceptible animals reduced the transmission rate of *Campylobacter* in broilers (Van Bunnik et al., 2012) and *Streptococcus suis* in pigs (Dekker et al., 2013). Further, interventions aiming at preventing colonization by ESBL/pAmpC-producing *E. coli* in broilers have been described, such as acid-based feed additives (Roth et al., 2017) or competitive exclusion (CE) products (Nuotio et al., 2013, Ceccarelli et al., 2017, Methner et al., 2019, Dame-Korevaar, 2020). CE products are aimed at establishing a natural community of intestinal bacteria to protect broilers from colonization by invaders (Nurmi et al., 1992). In modern broiler production, due to strict hygiene practices in commercial

hatcheries, the initial bacterial load to colonize the chicken intestinal tract shortly after hatch is low (Donaldson et al., 2017, Varmuzova et al., 2016). Eggs are usually disinfected to remove bacterial contamination before placement in the hatcher. Consequently, the chicks are exposed mostly to bacteria from environmental sources rather than parental sources upon hatching. Microbial treatment supplied after hatch has been shown to affect the development of bacterial taxa found in growing chickens (Schokker et al., 2017, Ballou et al., 2016). This suggests that early supply of CE products might influence microbiota composition and act as a possible intervention to prevent colonization by ESBL/pAmpC-producing *E. coli* in young broilers. A single supply of CE product before challenge with a high dose of ESBL-producing *E. coli* has already showed to reduce colonization, cecal and fecal excretion (CFU/g), as well as transmission of ESBL-producing *E. coli* (Nuotio et al., 2013, Ceccarelli et al., 2017, Methner et al., 2019). Additionally, CE products resulted in a reduced intestinal and cecal excretion (CFU/g) after challenge with pathogenic *E. coli* (Hofacre et al., 2002). A prolonged supply of CE product via the drinking water to broilers kept in isolators, from day of hatch until day 14 resulted in a delay and even prevention of colonization after challenge with a in the field realistic low dose of ESBL-producing *E. coli* (Dame-Korevaar 2020).

The aim of this study was to determine the effect of interventions on colonization and transmission of ESBL-producing *E. coli* in young broiler chicks kept under semi-field circumstances. Two interventions were included: 1) prolonged supply of CE product from day of hatch until day 7, and 2) compartmentalization of a broiler flock. To investigate the effect of CE product on microbial composition, microbiota in cecal content was assessed before and after challenge by 16S ribosomal RNA (rRNA) gene amplicon sequencing.

## **Material and Methods**

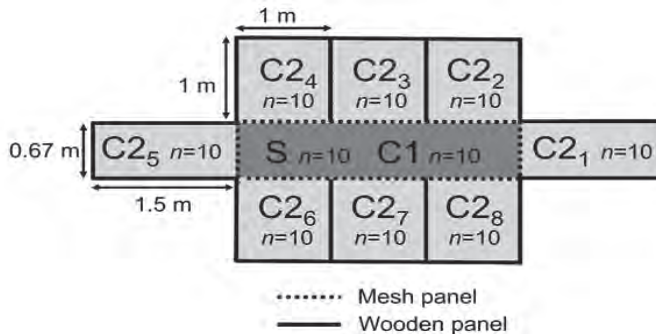
### ***Ethics of experimentation***

Broilers were observed daily and the presence of clinical signs, abnormal behavior and mortality was recorded. The study protocol was approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee of Utrecht University (Utrecht, the Netherlands) under registration number AVD108002015314; all procedures were done in full compliance with all legislations.

### ***Birds, housing and management***

Conventional broiler chicks (Ross 308, n=416), from a parent stock flock of 37 weeks of age, were transported directly after hatch to the animal facilities (Utrecht University, Utrecht, the Netherlands). Upon arrival, the broilers were individually tagged,

weighed, and randomly divided over four experimental rooms ( $n=104$  broilers per room). Each room was subdivided into nine pens, with one seeder (S/C1)-pen in the middle ( $2 \text{ m}^2$ ,  $n=24$  broilers), surrounded by eight contact (C2)-pens ( $1 \text{ m}^2$ ,  $n=10$  broilers per pen) (Figure 1). The S/C1-pen was separated from the C2-pens by a mesh panel (30 cm solid panel at the bottom, 40 cm mesh panel, 10 cm solid panel on top). Feed and water systems were also separated, and strict hygiene measures between pens were taken. No direct contact between the broilers was possible, but small particles (e.g. litter, dust) could be transferred between pens potentially. The C2-pens were separated from each other with wooden panels of 80 cm height, assuming no contact and no spread of particles was possible. At day 5, just before challenge with ESBL-producing *E. coli*, the number of broilers in the S/C1-pen was reduced to 20, by removing the surplus broilers. Ten of the remaining 20 broilers in each S/C1-pen were randomly selected and transported to four separate isolators. In these isolators, the broilers (seeder (S) broilers) were inoculated with CTX-M-1-*E. coli* and after one hour moved back to the original S/C1-pens. Before the start of the experiment the parent flock, hatchery and research facilities were tested for the absence of ESBL/pAmpC-producing bacteria.



**Figure 1.** Schematic representation of the experimental set up of one of four broiler rooms (1 – 4). Each room was subdivided in nine pens, with one seeder (S/C1) pen in the middle ( $2 \text{ m}^2$ ) ( $n=10$  S-broilers, 10 C1-broilers), surrounded by eight contact (C2) pens ( $1 \text{ m}^2$ ) ( $n=10$  broilers per pen). The S/C1-pen was separated from the C2-pens by 80 cm high mesh panels. The C2-pens were separated from each other by 80 cm high wooden panels.

Broilers were housed on fine wood shavings. A standard broiler diet without any antibiotics or coccidiostats, radiated with 9 Gy, was available *ad libitum*. The intervention was supplied in the drinking water; therefore, drinking water was not available *ad libitum* during the first seven days of the experiment in both intervention and control groups. Five broilers died or were euthanized before the end of the experiment due to causes unrelated to the experiment.

**Intervention competitive exclusion**

In two of four rooms a competitive exclusion (CE) product was supplied, containing natural, live intestinal microbiota derived from specific pathogen free (SPF) chickens and manufactured by fermentation (Aviguard®, MSD Animal Health, the Netherlands). From the moment of arrival in the rooms (day 0, 10:00 a.m.) until day 7, (4:00 p.m.), CE product was supplied in the drinking water, twice per day. Water solutions containing the CE product were prepared in predilution, with a dose level according to recommendations of the manufacturer, i.e. 0.125 gram CE product per 10 broilers. The amount of drinking water was restricted between day 0 and 7, based on the expected water consumption of 10 (C2-pen) and 20 (S/C1-pen) broilers in a pen to ensure that all supplied CE product would be consumed.

***E. coli* challenge**

Broilers were challenged with *E. coli* strain E38.27, which carries the ESBL gene bla<sub>CTX-M-1</sub> on an IncI1 plasmid (CTX-M-1-*E. coli*), isolated from conventional healthy broilers at slaughter age and resistant to cefotaxime (Dierikx et al., 2010). Oral inoculation of seeder (S) birds was performed on day 5 at 8:00 a.m. using a 1 mL syringe without a needle with 0.5 mL of 10<sup>2</sup> CFU/mL. The bacterial dilution was measured with the McFarland reader and retrospective colony counting. From one hour after inoculation onwards, 10 contact (C1) birds were exposed to 10 seeder birds, by moving the inoculated seeder birds to the corresponding S/C1-pens containing the contact birds.

***Cloacal and cecal samples***

Samples were taken using sterile dry cotton swabs (Copan 155C, Copan Diagnostics, USA). Broilers were sampled at day 5 at 4:00 a.m., just before inoculation to confirm absence of ESBL/pAmpC-producing bacteria, and from day 6 until day 21 daily at 8:00 a.m. At day 21, after the last sampling, post mortem examination was done within 30 minutes after euthanasia for each broiler. Broilers were weighed and sex was determined, exterior and interior abnormalities were assessed, and ceca were collected and stored on dry ice for further analysis.

***Microbiota sample collection and analysis***

Cecal content samples were collected from five surplus broilers of the control group and from five surplus broilers of the CE intervention group (n=10) at day 5. At day 21, cecal content of all broilers in the S/C1-pen in all four rooms (n=80) was collected. The closed side of one of the two ceca was cut and cecal content was gently squeezed into a 2 mL sterile cryotube and snap frozen on dry ice and stored at -80°C for genomic DNA extraction. To determine the microbial composition of the CE product, Aviguard® was suspended in PBS according to the manufacturer's



instructions and four aliquots of 2 mL were stored at -80°C for bacterial genomic DNA extraction. The full protocol for DNA extraction and determining microbiota composition was previously described (Kers et al., 2019b). Briefly, DNA was extracted from 0.25 g cecal content or frozen CE product, using 700 µL of Stool Transport and Recovery (STAR) buffer (Roche Diagnostics Nederland BV, the Netherlands). All 94 samples were transferred to a sterile screw-capped 2 mL tube (BIOplastics BV, the Netherlands), used for bead beating. The DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, USA), and the DNA samples were stored at -20°C until further use. Barcoded amplicons covering the variable regions V5-V6 and primers 784F and 1064R were used for 16S rRNA gene-based microbial composition profiling as previously described (Ramiro-Garcia et al., 2016). To ensure high quality sequencing data, synthetic communities of known composition were used as positive controls (Ramiro-Garcia et al., 2016) and nuclease free water as negative controls. Sequencing of resulting libraries was performed on Illumina Hiseq2500 (Eurofins Genomics Germany GmbH). The 16S rRNA data was analyzed using NG-tax 2.0 (Ramiro-Garcia et al., 2016). In short, to generate amplicon sequence variants (ASVs), NG-Tax 2.0 employed a fast de novo ASV-picking algorithm. To assign taxonomy the SILVA 128 16S rRNA gene reference database was used (Quast et al., 2013). Raw sequence data were deposited into the Sequence Read Archive (SRA) at the NCBI, under accession number PRJNA647260.

### ***ESBL-producing E. coli detection***

All cloacal samples were enriched in 3 mL Luria Bertani (LB) broth. After overnight incubation at 37°C, 10 µL broth were inoculated on MacConkey plates supplemented with 1 mg/L cefotaxime and incubated overnight at 37°C. *E. coli* colonies growing on MacConkey plates supplemented with cefotaxime were referred to as CTX-M-1-*E. coli*. If visual assessment was not conclusive on the presence of *E. coli*, colonies were selected for further analyses using MALDI-TOF MS (Bruker Daltonik, Germany).

### ***ESBL-producing E. coli and total E. coli quantification***

At day 21, content from one of two ceca of 80 selected broilers from rooms 1 and 2 was collected. For both rooms, selection included all broilers (n=20) from the S/C1-pen and additionally 20 broilers from the C2-pens which were excreting CTX-M-1-*E. coli*. Samples were processed as previously described (Dame-Korevaar et al., 2019b). Concentrations of ESBL-producing *E. coli* and total *E. coli* were determined semi-quantitatively. CFU/gram feces was calculated based on the highest dilution showing growth of typical *E. coli* colonies (Jett et al., 1997) and the weight of the feces on the swabs or the amount of cecal content collected (Ceccarelli et al., 2017). *E. coli* colonies growing on MacConkey plates supplemented with cefotaxime were referred to as CTX-M-1-*E. coli*. If visual assessment was not conclusive on the presence of *E. coli*, colonies were selected for further analyses using MALDI-TOF MS.

**Statistical analysis**

Statistical analyses were performed in R, version 3.4.3, using packages survival, phyloseq, microbiome and vegan.

**Time until colonization**

Time until colonization was analyzed using parametric survival regression with an accelerated failure time model using a Weibull distribution (Kalbfleisch and Prentice, 2011). The hazard ratio was expected to be non-proportional during the experiment, because of the compartmentalization. This accelerated failure time model models the effect of the variables on the acceleration or deceleration of the time until colonization with CTX-M-1-*E. coli*. Colonization of individual broilers was measured as excretion of CTX-M-1-*E. coli* and time until colonization was defined as the time point of the first cloacal swab of two consecutive cloacal swabs tested positive for CTX-M-1-*E. coli*. If the last swab (day 21) and the ceca tested positive, broilers were assumed to be colonized at day 21. If only the ceca tested positive, broilers were not included as colonized birds within the time span of the experiment.

**Microbiota composition**

Alpha and beta diversity metrics were calculated and univariate and multivariate statistical analyses were applied to determine differences in the cecal microbiota. Alpha diversity (within sample richness) was determined using Faith's phylogenetic diversity, taking into account the phylogenetic relatedness (Faith, 2006). Differences in alpha diversity were tested using a non-parametric Kruskal-Wallis test. Beta diversity (between sample differences) was determined using weighted and unweighted UniFrac metrics (Lozupone et al., 2007). Principal coordinates analysis (PCoA) was used to visualize the data. To test differences within multivariate community data, non-parametric permutational analysis of variance (PERMANOVA) were used (Anderson, 2001).

**Transmission coefficient**

The transmission coefficients for within and between pen transmission ( $\beta_{\text{within}}$  and  $\beta_{\text{between}}$ ) were estimated based on a stochastic multi-pen SI model (Velthuis et al., 2007, Klinkenberg et al., 2002) in which the number of new cases is determined by transmission from excreting (I) birds to susceptible (S) birds for a total population of (N) birds, using maximum likelihood estimation.

The probability ( $p_k$ ) for a susceptible animal in pen  $k$  to become colonized during time interval  $\Delta t$  was calculated based on the force of infection (**foi**) within the pen and between pens (S/C1-pen to C2-pen):

$$p_k = 1 - e^{-(foi_{within} + foi_{between})\Delta t} \quad \text{Eq. 1}$$

Two models were used in which the **foi** was based in model 1 on direct transmission or in model 2 on indirect transmission with a build-up of infectivity in the environment. In model 1 **foi** was determined by the proportion of excreting birds in the same pen ( $\frac{I_k}{N_k}$ ) and the proportions of excreting birds in the adjacent pen connected through a mesh panel ( $\frac{I_{adj}}{N_{adj}}$ ) during a time interval  $\Delta t$ :

$$p_k = 1 - e^{-(\beta_{within} \frac{I_k}{N_k} + \beta_{between} \frac{I_{adj}}{N_{adj}})\Delta t} \quad \text{Eq. 2}$$

The unit of  $\beta_{within}$  and  $\beta_{between}$  in model 1 is 1/day, and is interpreted as the number of new colonized broilers per day, due to one positive broiler.

In model 2 the **foi** in pen  $k$  was assumed to be a result of a build-up of infectivity in the environment. The cumulative sum of hours that all excreting birds were excreting in a pen (cumexcrhours<sub>k</sub>) and in the adjacent pen connected with a mesh panel (cumexcrhours<sub>adj</sub>) up to the beginning of the interval was used as a measure for environmental accumulation:

$$p_k = 1 - e^{-(\beta_{within} \text{cumexcrhours}_k + \beta_{between} \text{cumexcrhours}_{adj})\Delta t} \quad \text{Eq. 3}$$

The unit of  $\beta_{within}$  and  $\beta_{between}$  in model 2 is 1/day<sup>2</sup> and is interpreted as the number of new colonized broilers per day, caused by each day that one positive broiler has been excreting CTX-M-1-*E. coli* (Dekker et al., 2013, Dame-Korevaar, 2020).

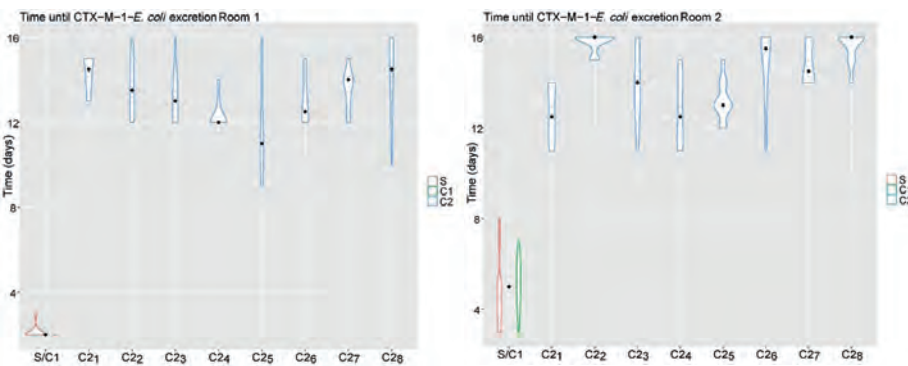
### **Cecal excretion levels**

The differences in cecal content of total *E. coli* and CTX-M-1-*E. coli* (CFU/g) were tested using a linear regression model including the variables room, pen, sex, weight at day of hatch, weight at day 21, type of bird (S, C1, C2) and time until colonization. The best fitting model was obtained by backward selection based on lowest AIC value. The correlation between cecal content of CTX-M-1-*E. coli* and time until colonization was tested using Pearson's correlation coefficient.

# Results

## Time until colonization

Broilers in the CE groups (room 3 and 4) were not colonized with CTX-M-1-*E. coli*. In the control groups all broilers in room 1 (n=100), and 87/100 broilers in room 2 were colonized at the end of the experiment (Figure 2). Time until colonization was delayed for broilers in room 2 compared to broilers in room 1 (Time Ratio (TR) 3.00, 95% CI 1.82 – 4.95), and for C2 broilers compared to seeder broilers (TR 3.53, 95% CI 3.14 – 3.93). No difference in time until colonization was observed between seeder and C1 broilers (TR 1.14, 95% CI 1.00 – 1.30). Weight at day of hatch, weight at day 21 and sex did not influence time until colonization (Table 1).



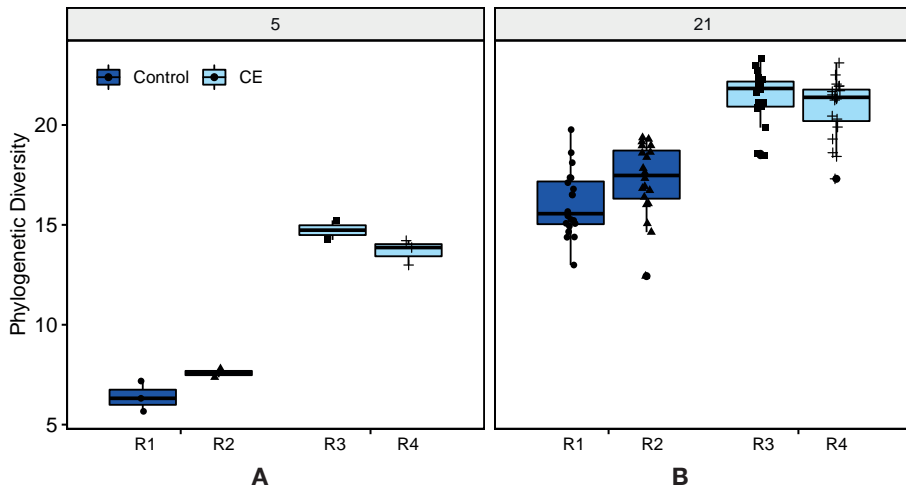
**Figure 2.** Time until colonization (days) of CTX-M-1-*E. coli* per pen (S/C1, C2<sub>1</sub>, C2<sub>2</sub>, C2<sub>3</sub>, C2<sub>4</sub>, C2<sub>5</sub>, C2<sub>6</sub>, C2<sub>7</sub>, C2<sub>8</sub>) and type of bird [seeder (S), contact 1 (C1), contact 2 (C2)] for room 1 (left) and room 2 (right). The violin plot indicates the total range of observations; the black dot indicates the median.

**Table 1.** Regression coefficients of time until colonization (95% CI) of CTX-M-1-*E. coli* for an accelerated failure time model.

Variable	Accelerated failure time (days, 95% CI)
Baseline survival (Room 1, Seeder, Male)	3.00 (1.82 – 4.95)
Room 2	1.24 (1.15 – 1.33)
Animal type	Contact 1 1.14 (1.00 – 1.30)
	Contact 2 3.53 (3.14 – 3.93)
Sex	Female 0.97 (0.91 – 1.03)
Body weight at day 0	1.00 (0.99 – 1.02)
Body weight at day 21	1.00 (1.00 – 1.00)

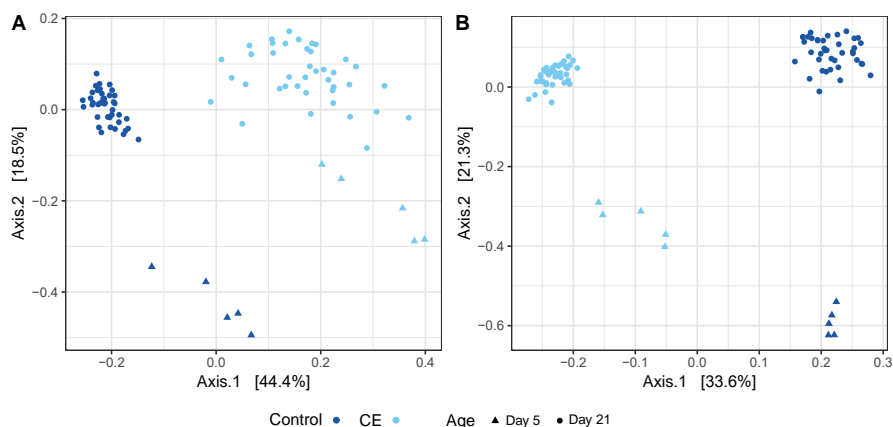
### Microbiota composition in cecal content

The alpha diversity (phylogenetic diversity) was higher in cecal content samples of the broilers supplied with CE product (CE broilers) compared to the control broilers on day 5 and day 21 (Figure 3). On day 21 no differences in alpha diversity between the two intervention rooms were observed ( $\chi^2 = 1.90$ ,  $p = 0.17$ ), but the control broilers in room 1 had a lower alpha diversity than control broilers from room 2 ( $\chi^2 = 4.92$ ,  $p = 0.03$ ). Within rooms, no differences between seeder and contact (C1) broilers were found. Weight at day of hatch, weight at day 21 and sex did not influence the alpha diversity (data not shown).



**Figure 3.** Alpha (phylogenetic) diversity of cecal microbiota at day 5 ( $n=5$  broilers per intervention) and day 21 ( $n=40$  broilers per intervention), for the control (rooms (R) 1, 2) and intervention groups (rooms (R) 3, 4).

In weighted and unweighted UniFrac (WUF and UF) distance based analysis, the supply of the CE product explained 60% (WUF) and 69% (UF) of the variation between the cecal content samples on day 5 (Figure 4, principal coordinate analysis (PCoA), PERMANOVA, WUF:  $R^2 = 0.598$ ,  $p = 0.009$ , uf:  $R^2 = 0.688$ ,  $p = 0.008$ ). On day 21, application of the CE product explained 46% (WUF) and 51% (UF) of the variation between the cecal content samples (Figure 4, PERMANOVA, uf:  $R^2 = 0.461$ ,  $p = 1.0 \times 10^{-4}$ , WUF:  $R^2 = 0.510$ ,  $p = 1.0 \times 10^{-4}$ ). Within rooms, being a seeder or contact broiler did not explain any of the variation between the cecal content samples. The variation between the two control groups was larger than between the two intervention rooms (WUF:  $R^2 = 0.351$  versus  $R^2 = 0.210$ ).



**Figure 4.** Principal coordinate analysis (PCoA) of microbiota composition based on weighted UniFrac A. and un-weighted UniFrac B. distances between control (dark blue) and CE (light blue) groups. Different symbols indicate different sampling days: triangles are samples of day 5, and circles are samples of day 21.

The heatmap (Figure 5) shows all genera that significantly differed in relative abundance between CE broilers and control broilers at day 5 and 21. Selection of the first four clusters reveal two clusters with control broilers: one for the broilers of 5 days of age, and one for the broilers of 21 days of age. The other two clusters consist of CE broilers, one cluster contains broilers of both 5 and 21 days of age, while the second cluster contains only CE broilers of 21 days of age.

In the CE product, 22 different genera were identified (Table 2). Of these genera, five were more abundant in CE broilers than in control broilers at day 5: *Collinsella*, *Eubacterium*, *Flavonifractor*, *Lachnoclostridium* and *Lactobacillus*. At day 21, genera *Eubacterium coprostanoligenes*, *Bacteroides*, *Collinsella*, *Enterococcus*, *Eubacterium*, *Megamonas*, *Megasphaera*, *Slackia* and *Sutterella* were more abundant in CE than in control broilers (Table 2).

### Transmission

Broilers in the CE groups (room 3 and 4) were not colonized with CTX-M-1-*E. coli*, and transmission was thus not observed. In the control groups, the transmission coefficient between pens ( $\beta_{\text{between}}$ ) was lower than the transmission coefficient within pens ( $\beta_{\text{within}}$ ) for both models. Model 2, with accumulated environmental transmission, was preferred over model 1, assuming direct transmission (AIC 402.1 vs. 438.1, Table 3). For model 2,  $\beta_{\text{between}}$  was  $3.28 \times 10^{-4} \text{ day}^{-2}$  (95% CI  $2.41 \times 10^{-4} - 4.32 \times 10^{-4}$ ) and  $\beta_{\text{within}}$  was  $6.12 \times 10^{-2} \text{ day}^{-2}$  (95% CI  $4.78 \times 10^{-2} - 7.64 \times 10^{-2}$ ) (Table 3).

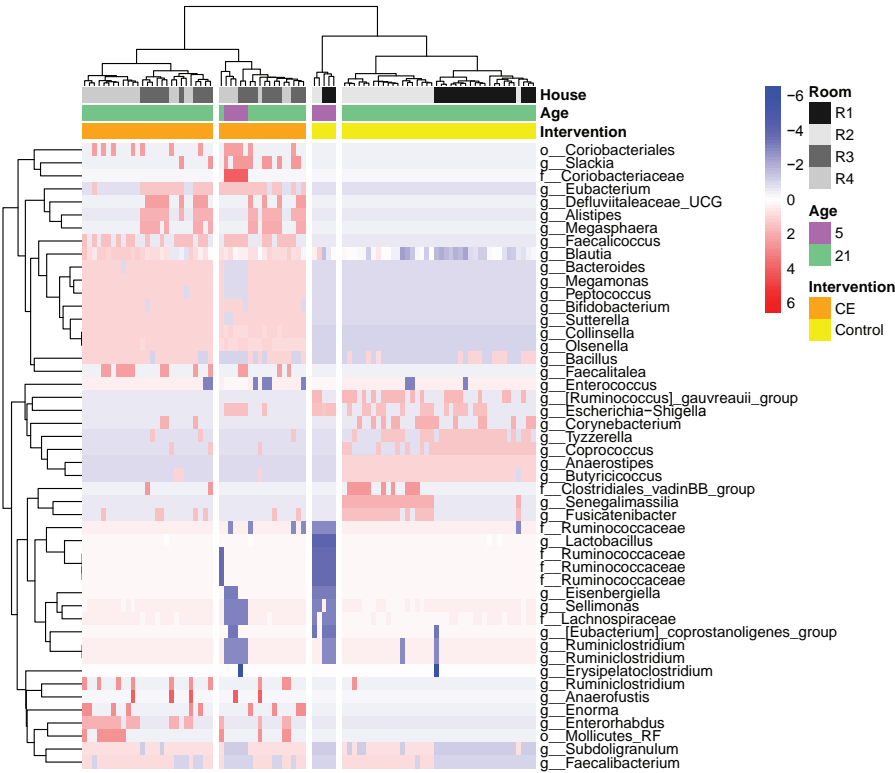
**Table 2.** Relative abundance and standard deviation (SD) of genera that were present in the CE product, and the significantly different relative abundance in cecal content of CE broilers versus control broilers at day 5 (n=10) and 21 (n=80). Results are based on differences of relative abundance tested with Wilcoxon rank-sum test. Adjusted *p*-values (<0.05) are corrected for multiple testing with Benjamini-Hochberg (BH). - = not detected.

Relative abundance CE product			Change in relative abundance CE vs control broilers					
Genera	Relative abundance (%)	SD (%)	Day 5 Relative abundance (%)			Day 21 Relative abundance (%)		
			Control	CE	<i>p</i> -value	Control	CE	<i>p</i> -value
[Eubacterium] coprostanoligenes group	0.65	0.22				0.70	1.11	8.05×10 <sup>-07</sup>
Bacteroides	0.47	0.06				-	1.12	9.06×10 <sup>-15</sup>
Blautia	0.30	0.09				18.48	6.64	2.75×10 <sup>-12</sup>
Candidatus_Soleaferrea	0.39	0.06						
Clostridium sensu stricto 1	2.77	0.45	14.67	0.72	0.03	0.04	-	0.04
Clostridium sensu stricto 2	0.72	0.12						
Collinsella	0.53	0.07	-	12.98	0.03	-	4.28	3.34×10 <sup>-15</sup>
Enterococcus	10.80	1.07	31.76	13.40	0.03	0.46	0.94	2.67×10 <sup>-03</sup>
Erysipelatoclostridium	2.53	0.09			0.03	1.84	0.99	0.03
Escherichia-Shigella	0.57	0.02	15.09	0.99	0.03	0.09	3.72×10 <sup>-03</sup>	2.04×10 <sup>-04</sup>
Eubacterium	0.66	0.04	-	3.31	0.03	-	0.20	2.22×10 <sup>-07</sup>
Flavonifractor	1.02	0.14	-	0.79	0.03			
Lachnoclostridium	9.78	0.93	-	1.77	0.03			
Lactobacillus	14.96	1.33	-	10.77	0.03			
Megamonas	1.55	0.56				-	10.36	3.34×10 <sup>-15</sup>
Megasphaera	3.30	0.74				-	0.27	3.82×10 <sup>-05</sup>
Negativicoccus	3.62	0.66						
Oscillibacter	1.94	0.18						
Peptostreptococcus	30.97	4.04						
Sellimonas	1.31	0.38				1.14	0.64	2.60×10 <sup>-04</sup>
Slackia	0.34	0.09				-	0.03	7.57×10 <sup>-03</sup>
Sutterella	1.76	0.21				-	0.99	3.34×10 <sup>-15</sup>
uncultured	4.45	3.56						
unknown	0.08	0.09						

**Table 3.** Transmission coefficients ( $\beta_{\text{within}}$  and  $\beta_{\text{between}}$ , 95% CI) using an SI-model for transmission based on the proportion of excreting birds (model 1) and the accumulative excretion time (model 2).

Transmission coefficient ( $\beta$ , 95% CI)				
	Unit*	$\beta_{\text{within}}$ (95% CI)	$\beta_{\text{between}}$ (95% CI)	AIC
<b>Model 1</b>	day <sup>-1</sup>	1.31 (1.07 – 1.59)	0.03 (0.02 – 0.04)	438.1
proportion excreting birds				
<b>Model 2</b> accumulative excretion time	day <sup>-2</sup>	$6.12 \times 10^{-2}$ ( $4.78 \times 10^{-2}$ – $7.64 \times 10^{-2}$ )	$3.28 \times 10^{-4}$ ( $2.41 \times 10^{-4}$ – $4.32 \times 10^{-4}$ )	402.1

\* The unit in model 1 is 1/day, and is interpreted as the number of new colonized broilers due to one positive broiler per day. The unit in model 2 is 1/day<sup>2</sup> and is interpreted as the number of new colonized broilers caused by each day that one positive broiler has been excreting.



**Figure 5.** Heatmap representing the abundance of amplicon sequence variants (ASVs) in all individual broiler chickens analyzed (n=90). Only ASVs that are significantly different at day 5 and day 21 between CE and control are shown (Wilcoxon rank-sum test, adjusted p-values are corrected p-values for multiple testing, Benjamini-Hochberg,  $p < 0.05$ ). Each red, white, or blue rectangle represents the relative abundance of a genus in an individual broiler. Clustering of broilers is based on Ward's minimum variance method and based on weighted UniFrac distances matrix.



### Cecal excretion levels

Mean CTX-M-1-*E. coli* ( $\log_{10}$  CFU/g) was lower in cecal samples from broilers from C2-pens than from the S/C1-pen, except for pen C2<sub>6</sub> and C2<sub>7</sub> (Table 4). CTX-M-1-*E. coli* ( $\log_{10}$  CFU/g) was lower in cecal samples from broilers kept in room 2 than broilers kept in room 1 (estimate -0.52, 95% CI -0.91 – -0.13  $\log_{10}$  CFU/g). Broilers with a higher body weight at day of hatch had slightly higher cecal CTX-M-1-*E. coli* levels (estimate 0.08, 95% CI 0.01 – 0.15  $\log_{10}$  CFU/g). Cecal CTX-M-1-*E. coli* levels were correlated with time until colonization, the shorter the time until colonization, the higher the cecal level ( $r = -0.60$ , 95% CI -0.73 – -0.43). Mean *E. coli* levels in cecal content did not differ between rooms or pens.

**Table 4.** Parameter estimates for cecal content levels at day 21 ( $\log_{10}$  CFU/g cecal content, 95% CI) of CTX-M-1-*E. coli* (n=75) using a linear regression model.

Variable	Estimate CTX-M-1- <i>E. coli</i> (95% CI)
Room 1, pen Seeder/C1 (intercept)	3.95 (0.93 – 6.98)
Room 2	-0.52 (-0.91 – -0.13)
Pen C2 <sub>1</sub>	-1.17 (-1.91 – -0.43)
Pen C2 <sub>2</sub>	-2.28 (-3.23 – -1.33)
Pen C2 <sub>3</sub>	-1.85 (-2.95 – -0.75)
Pen C2 <sub>4</sub>	-2.01 (-2.64 – -1.39)
Pen C2 <sub>5</sub>	-0.86 (-1.40 – -0.33)
Pen C2 <sub>6</sub>	-0.59 (-1.52 – 0.33)
Pen C2 <sub>7</sub>	0.34 (-0.77 – 1.46)
Pen C2 <sub>8</sub>	-2.69 (-3.49 – -1.88)
Body weight day of hatch (day 0)	0.08 (0.01 – 0.15)

## Discussion

The supply of CE product via drinking water from day of hatch until day 7 prevented colonization of broilers with ESBL-producing *E. coli* after challenge of seeder birds. In the control group, 93.5% of the broilers were colonized at the end of the experiment. These results are in line with earlier experiments within isolators, in which a continuous supply of CE product during the first 14 days was able to prevent colonization (Dame-Korevaar, 2020). In the isolators in which at least one bird was colonized with ESBL-producing *E. coli*, application of CE products reduced the rate of colonization, decreased excretion (CFU/g) and reduced transmission, as previously shown in studies applying a single supply of CE (Hofacre et al., 2002, Nuotio et al., 2013, Ceccarelli et al., 2017, Methner et al., 2019). The enhanced effect of CE product found in this study compared to these earlier studies could have resulted from the prolonged supply, the longer period between start of CE product and exposure to ESBL-producing *E. coli*, or the moment of challenge with ESBL-producing *E. coli* and the low challenge dose used in our study.

The microbiota composition was more diverse in the CE broilers than in the control broilers on day 5 and 21. This supports the hypothesis that microbial diversity plays a role in preventing colonization. Successful competitive exclusion of ESBL-producing *E. coli* by specific genera being present in the CE broilers could also have prevented colonization. Intestinal colonization with microbiota of adult donor hens is associated with increased resistance against colonization, e.g. with *Salmonella* (Varmuzova et al., 2016). In a study where newly hatched layer chicks were exposed to an adult hen, transfer of microbiota occurred within 24 hours of contact and a 1-3 days longer contact period resulted in an even more developed chick microbiota (Kubasova et al., 2019). In our study, supplying a CE product derived from intestinal bacteria of adult chickens possibly increased resistance and prevented colonization with ESBL-producing *E. coli*. The higher diversity observed in broilers at day 5 was maintained during the experiment. At day 21, two weeks after the last supply of the CE product, the intestinal microbiota composition was still more diverse in the CE broilers. Next to genera identified in the CE product, also other genera were found to be different between CE and control groups, indicating that the intestinal microbiota of CE broilers was early and persistently different compared to the composition of the microbiota as observed in the control broilers.

Direct competition between specific bacteria and inoculated *E. coli* in CE broilers might have played a role in preventing colonization, including competition for binding sites or limiting nutrients (Nurmi et al., 1992, Callaway et al., 2008). This could be related also with the production of antimicrobial compounds, including volatile fatty acids, inhibiting or eliminating species that compete for the same niche (Callaway et al., 2008). Some genera were present exclusively in the CE product and in CE broilers, but not in control broilers (Table 2). Due to competition, these genera might have prevented

colonization. Next to preventing colonization with *E. coli*, CE products have shown to prevent or reduce colonization of different bacteria, e.g. *Salmonella* (Nakamura et al., 2002, Ferreira et al., 2003, Markazi et al., 2018, Luoma et al., 2017) and *Campylobacter* (Schneitz and Hakkinen, 2016).

In the control groups compartmentalization resulted in a significantly lower transmission between pens than within pens. Transmission between pens shows that environmental transmission can occur and presence of ESBL/pAmpC-producing bacteria in litter, air or dust plays a role in transmission (Blaak et al., 2015, Daehre et al., 2018, Friesse et al., 2013, Laube et al., 2013, Blaak et al., 2014). Delayed transmission as result of compartmentalization has been described for other pathogens (Videnska et al., 2014b, Jurburg et al., 2019). The effect of compartmentalization can be two-fold: the physical barrier prevents direct contact between broilers, and next to that, during the time needed for transmission between pens to occur, the microbiota of the susceptible broilers might develop further, making it more difficult for ESBL-producing *E. coli* to colonize. In chickens, microbiota in the first week of life contains *Enterobacteriaceae* (Ballou et al., 2016, Videnska et al., 2014, Jurburg et al., 2019) suggesting that *E. coli* can easily colonize during the first week. Older bird might get less susceptible for colonization (Dame-Korevaar, 2020), however in our study colonization with ESBL-producing *E. coli* still occurred at 21 days of life, maybe as a result of accumulation of excreted ESBL-producing *E. coli* in the environment. Once transmission between pens occurred, transmission within pens followed rapidly. In room 1, within S/C1-pen transmission occurred very fast: all except one bird were positive at the first sampling after challenge. Therefore, this pen could not be included in the estimation of within pen transmission.

Estimation of the transmission coefficients was done using the proportion of excreting birds (model 1) and the accumulative excretion time (model 2). The model including excretion time fitted better to the observed data, indicating that accumulation of ESBL-producing *E. coli* in the environment most likely plays a role in the transmission within a flock. This increased infectivity by accumulation of the bacteria has been modelled also for other pathogens in pigs and chicken (Dekker et al., 2013, Lurette et al., 2008, van Bunnik et al., 2014). In our model, environmental accumulation is assumed to be unlimited, whereas in practice it is likely that there is a certain maximum, as postulated by Van Bunnik and colleagues that assumed the force of infection to be limited by a maximum exposure capacity for recipient animals (van Bunnik et al., 2014). However, in our study models including a maximum exposure capacity rendered a model that did not converge, which might indicate that the maximum exposure capacity was not yet reached at the end of the experiment.

In poultry practice, the interventions studied in this experiment could be used to control the spread of ESBL-producing *E. coli*. A competitive exclusion product could be supplied on the farm via the drinking water system. Supply should be done as soon as possible after hatching, before exposure to ESBL-producing *E. coli* occurs. In this

study compartmentalization, including separation of feed- and water systems and strict hygiene measures, reduced transmission of ESBL-producing *E. coli* but could not prevent it. In practice, with less strict hygiene measures, the effects might be smaller.

## **Conclusions**

Overall, our study shows that competitive exclusion is a useful intervention tool to prevent colonization of ESBL-producing bacteria after challenge with a low dose in the first week in a broiler flock. Transmission within a flock could be delayed by compartmentalization, however as soon as ESBL-producing bacteria are excreted and accumulate in the environment spread to other birds seems inevitable. Therefore, compartmentalization of large flocks into smaller groups of birds, which is for instance more common in breeding flocks at higher levels of the broiler production chain, could be combined to enhance the efficacy of other interventions. Competitive exclusion products could be supplied to young chicks after hatching at different levels of the broiler production pyramid to prevent colonization of birds. The insights provided by this study may provide a basis for further developments towards practically applicable measures to further reduce antimicrobial resistance in poultry.

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## **Authors Contributions**

Experiment design: AD, EF, JG, FV, DC, DM, AS. Experiment execution: AD, FV. Data analysis: AD, JK, EF. Manuscript writing: AD, JK. All authors discussed, read, contributed to, and approved the final manuscript.

## **Conflict of Interest**

We have no conflict of interest to declare.





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# CHAPTER 8

## Early life inoculation with a competitive exclusion product accelerates maturation of intestinal microbiota and enhances NK cell activation in broiler chickens

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## Abstract

Studies in mammals and chickens have shown that the development of the immune system is affected by interactions with intestinal microbiota. Early life modulation of microbial colonization may affect the innate and adaptive immune development and may have long lasting effects on health of broiler chickens. This study investigated the effects of inoculation of broiler chickens with a competitive exclusion product on intestinal microbiota composition and development of natural killer (NK) cells. We hypothesized that inoculation directly upon hatch (day 0) with an adult-derived microbiota (AM) product would induce an alteration in microbiota composition shortly after hatch, and subsequently affect intestinal NK cell subsets and activation. Microbiota composition of cecal and ileal content of chickens of 1, 3, 7, 14, 21 and 35 days of age was assessed by sequencing of 16S ribosomal RNA gene amplicons. In parallel, subsets and activation of intestinal NK cells were analyzed by flow cytometry. In the cecal content of 1- and 3-day-old AM chickens, a higher alpha diversity was observed compared to control chickens, whereas ileal microbiota was unaffected. Regarding beta diversity, cecal microbiota could be clustered into three distinct community types. Cluster A represented cecal microbiota of 1-day-old AM chickens and 1- and 3-day-old control chickens. Cluster B included microbiota of seven of eight 3- and 7-day-old AM and 7-day-old control chickens, and cluster C comprised microbiota of all chickens of 14 days and older, independent of inoculation. In 3-day-old AM chickens an increase in the percentage of intestinal IL-2R $\alpha$ <sup>+</sup> NK cells and activated NK cells were observed compared to control chickens of the same age. In addition, an increase in relative numbers of intestinal cytotoxic CD8 $\alpha$  T cells was observed in 14- and 21-day-old AM chickens. Taken together, these results indicate that early exposure to AM shapes and accelerates the maturation of the cecal microbiota, which is paralleled by an increase in IL-2R $\alpha$ <sup>+</sup> NK cells and enhanced NK cell activation. The observed association between early life development of intestinal microbiota and immune system indicates possibilities in developing microbiota-targeted strategies that strengthen the immune system and thereby improve health and resilience of broiler chickens.

**Keywords:** poultry, avian immunology, gut microbiome, innate immunity, intraepithelial lymphocytes, natural killer cells, succession, inoculation.



## Introduction

Health and production efficiency of broiler chickens is of major importance as chicken meat is an important and sustainable source of animal protein for the growing human population (OECD., 2019, Skunca et al., 2018). Restrictions of the use of antimicrobials in poultry production have made other strategies to maintain or improve health of broiler chickens, such as enhanced immune responsiveness, increasingly important.

The intestinal microbiota plays a crucial role in broiler health and production performance, as it is involved in many physiological processes, including nutrient digestion and absorption, metabolism, intestinal barrier function, and development of intestinal immunity (Kogut, 2019, Pedroso and Lee, 2015). The maturation of the intestinal microbiota of chickens entails rapid successional changes, developing from a simple, to a more complex and diverse composition due to gradual colonization with microbiota (Cressman et al., 2010, Oakley and Kogut, 2016, Jurburg et al., 2019). Early life exposure to microbiota is an important driver of intestinal microbiota development, which can also affect health later in life. This has been shown in human infants (Dominguez-Bello et al., 2010, Borewicz et al., 2019b, Borewicz et al., 2020), and other mammals and hatchlings treated with antibiotics early in life or raised under extreme hygienic conditions, e.g. germ-free or SPF environments (Coloe et al., 1984, Inman et al., 2010, Mulder et al., 2011, Schokker et al., 2017, Xi et al., 2019). Also, in laying-hens, early transiently colonizing bacteria have been shown to have a large effect on intestinal microbiota composition later in life (Ballou et al., 2016, Polansky et al., 2016, Volf et al., 2016). However, due to hatching in the clean hatchery environment, colonization in commercial chickens starts with microbiota from environmental, rather than parental sources. As these environmental microorganisms may include pathogenic bacteria, competitive exclusion products such as Broilact and Aviguard, derived from intestinal microbiota of healthy adult chickens, have been developed to compete with colonization by pathogenic bacteria (Mead, 2000). When supplied *in ovo* or to hatchlings, Aviguard has been shown to accelerate bacterial colonization (Crhanova et al., 2011, Pedroso et al., 2016, Yin et al., 2010) and to decrease the occurrence of undesirable bacteria such as *Salmonella* and *Escherichia coli* (Ceccarelli et al., 2017, Kerr et al., 2014, Mead, 2000). The intestinal immune system plays an important role in the defence against pathogens that enter a host via the gut. Underneath the mucus layer a layer of epithelial cells including immune cells such as the intraepithelial lymphocytes (IEL) is observed. The population of IEL consists of high numbers of  $\gamma\delta$  T cells, adaptive CD8<sup>+</sup> T cells and innate natural killer (NK) cells (Göbel et al., 2001). During embryonic development and early life, when resistance against pathogens relies on innate immune responses since the adaptive immune system is not yet fully developed, NK cells are important players (Sharma and Tizard, 1984). Chicken NK cells have also been reported in multiple organs in-

cluding the intestine, lung, spleen and blood (Göbel et al., 2001, Jansen et al., 2010, Jansen et al., 2013). Previously, it has been showed that a high percentage of intestinal NK cells in chickens are recognized by the marker 28-4, which was identified as CD25 or IL-2R $\alpha$  (Göbel et al., 2001). In mammals, the IL-2R $\alpha$  chain is expressed on NK cells early upon activation, and this is followed by enhanced NK cell mediated killing and IFN $\gamma$  production (Leong et al., 2014). Another marker found to be expressed on intestinal NK cells was 20E5 (Meijerink in prep). It is also expressed on cells that show NK cell activation (Jansen et al., 2010). Furthermore, elsewhere in the body, increased surface expression of CD107 indicative of NK cell activation was observed on primary chicken NK cells in lung, spleen and blood upon infections with avian viruses (Bertzbach et al., 2019, Jansen et al., 2013, Vervelde et al., 2013).

In the intestinal tract many interactions occur between the microbiota and immune cells (Hooper et al., 2012, Round and Mazmanian, 2009). These interactions are important for the development of the immune system, as was shown in mammals (Macpherson and Harris, 2004, Rooks and Garrett, 2016) and chickens (Yin et al., 2010, Broom and Kogut, 2018, Schokker et al., 2017). For example, early life transplantation of adult microbiota has resulted in increased natural antibody titers in laying chickens paralleled by long lasting effects on mRNA levels of pro-inflammatory cytokines (van der Eijk et al., 2020, Metzler-Zebeli et al., 2019). Disturbing the early life microbiota in 1-day-old broiler chickens by antibiotics resulted in reduced numbers of macrophage-like cells in the jejunum (Schokker et al., 2017). Differences in rearing environment, e.g. a reduction in environmental microbial exposure, resulted in lower expression levels of  $\beta$ -defensins (Butler et al., 2016).

Studies in rodents and humans have shown that specific probiotic microorganisms enhance intestinal NK cell activity and cytokine production (Aziz and Bonavida, 2016) either directly via their interaction with receptors expressed on NK cells (Carrillo-Bustamante et al., 2016, Temperley et al., 2008), or indirectly via cytokine production of resident myeloid or epithelial cells (Sonnenberg and Artis, 2012). Also the adaptive immune system can be modulated via interactions with the microbiota (Brisbin et al., 2012, Gao et al., 2017, Siwek et al., 2018, Yitbarek et al., 2019), or indirectly through innate immune cell activities. As other studies in rodents and humans have shown, the microbiota affects activation of  $\gamma\delta$  T cells (Nielsen et al., 2017, Yang et al., 2018) and CD8 $^{+}$  T cells (Tanoue et al., 2019). Taken together, this indicates that the composition and activity of the microbiota and its effects on the immune system in early life may have long term consequences on the health of individuals.

In chickens, previous studies addressed the effect of microbiota on innate immune responses in the intestine, spleen and blood by studying mRNA levels of immune related genes (Butler et al., 2016, Metzler-Zebeli et al., 2019) by immunohistochemistry (Schokker et al., 2017) and by analysis of natural antibody titers (van der

Eijk et al., 2020). In this study, we used tools that we developed previously for the analysis of the phenotype and the function of chicken innate immune cells (Jansen et al., 2010, De Geus et al., 2012) to assess whether and to what extent differences in early life microbial colonization would affect the development of NK cells locally (in the intestine) and systemically (in spleen and blood).

We hypothesized that inoculation with a competitive exclusion product derived from adult microbiota (AM) upon hatch would induce an alteration in microbiota development and affect the presence and activation of intestinal NK cells. Indeed, AM inoculation resulted in an accelerated maturation of the intestinal microbiota, an increase of IL-2R $\alpha$ <sup>+</sup> NK cells and enhanced activation of NK cells. The observed association between early life development of intestinal microbiota and the immune system indicates possibilities to apply microbiota-targeted strategies that can accelerate maturation of intestinal microbiota and strengthen the immune system to improve the health and resilience of broiler chickens.

## Materials and Methods

### *Birds and husbandry*

Ross 308 17- and 18-day old embryonated eggs were obtained from the same parent flock of a commercial hatchery (Lagerwey, the Netherlands). The 17- day old eggs (hatch group A) and 18- day old eggs (hatch group B) were disinfected with 3% hydrogen peroxide and placed in disinfected egg hatchers. All eggs hatched at day 21. Directly upon hatch, chickens (day 0 in age) were randomly divided into two treatment groups, weighed, labelled and inoculated. Next, the chickens of the two treatment groups were placed in separate floor pens of 2 m x 1.5 m (pens 1 and 2), with a solid wall separating the pens. Each pen was divided in two equal parts of 1 m x 1.5 m for chickens from hatch group A and B. The pens were lined with wood shavings (2 kg/m<sup>2</sup>, sterilized by autoclavation). Non-sterilized standard commercial starter and grower feeds (Research Diet Services, the Netherlands) and water was provided *ad libitum*. No antibiotics, coccidiostatic drugs or commercial vaccines were applied during the experiment. A standard lighting and temperature scheme for Ross broiler chickens was used, and conditions were kept the same for all compartments.

### *Experimental design*

Chickens were inoculated immediately after hatch to reduce opportunities for prior exposure to microbiota. First, the control group received an oral inoculation with 0.5 mL PBS (Lonza, Basel, Switzerland). The other group, henceforth referred to as the AM group, was inoculated with 0.5 mL of PBS containing 0.05 g/mL of Aviguard®

(MSD Animal Health, the Netherlands). This is a freeze-dried product consisting of fermented, undefined cultures from intestinal microbiota of healthy specific-pathogen-free birds. To determine the microbial composition of the AM inoculum, four aliquots of 2 mL were stored at -80°C for DNA extraction.

### ***Sample collection***

At day 0 (upon hatch), four non-inoculated chickens per hatch group were randomly selected and sacrificed, to collect cecal and ileal content for microbiota analyses, as has been described in Kers et al. (2019a). Ileal content was collected distal and close to the Meckel's diverticulum. The intestinal content was gently squeezed into a 2 mL sterile cryotube, snap frozen on dry ice and stored at -80°C for DNA extraction. The time between sacrificing and placing the intestinal samples on dry ice was between 3-5 min. To avoid cross contamination, all management and biotechnical procedures were completed first with the control group and for each compartment at the same time. At days 1 (24 hours after inoculation), 3, 7, 14, 21 and 35, eight chickens (four from the control and four from the AM group) were randomly selected per hatch group (A/B) and sacrificed to collect cecal and ileal content as described above. At day 0 and day 1, the chickens were too small to collect sufficient cells for immunological analyses. Therefore, ileum tissue, spleen and blood were collected from day 3 onwards from six of these eight chickens (n = 3 per hatch group). All chickens were weighed prior to post-mortem analyses.

### ***DNA extraction***

In total, 56 cecal and 56 ileal content samples, consisting of 28 samples per treatment group, and four samples of the AM inoculum were analyzed for microbiota composition. DNA was extracted from 0.25 g content, using 700 µl of Stool Transport and Recovery (STAR) buffer (Roche Diagnostics Nederland BV, the Netherlands). All samples were transferred to sterile screw-capped 2 mL tubes (BIOplastics BV, the Netherlands) containing 0.5 g of zirconium beads (0.1 mm; BioSpec Products, Inc., USA) and 5 glass beads (2.5 mm; BioSpec Products). All samples were treated in a bead beater (Precellys 24, Bertin technologies, France) at a speed of 5.5 m/s for 3 × 1 min, followed by incubation at 95°C with agitation (15 min and 300 rpm). The lysis tube was centrifuged (13,000 g for 5 min at 4°C), and the supernatant was transferred to a 2 mL microcentrifuge tube. Thereafter, the above-described process was repeated with 300 µl STAR buffer. An aliquot (250 µl) of the combined supernatants from the sample lysis was then transferred into the custom Maxwell® 16 Tissue LEV Total RNA Purification Kit cartridge. The remainder of the extraction protocol was then carried out in the Maxwell® 16 Instrument (Promega, the Netherlands) according to the manufacturer's instructions. DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, DE, USA), and

the DNA samples were stored at  $-20^{\circ}\text{C}$  until further use.

### ***qPCR, 16S rRNA gene amplification, sequencing and data processing***

Extracted DNA was diluted to  $20\text{ ng}\mu\text{l}^{-1}$  in nuclease free  $\text{H}_2\text{O}$ . All PCR plastics were UV irradiated for 15 min before use. To validate the AM inoculation, absolute quantification of the bacterial 16S rRNA genes by real-time PCR amplification was performed for the cecal content samples of day-old chickens. For ileal content samples the amount of DNA was too low to reliably determine gene copy numbers. All qPCR assays (CFX384™ real-time PCR detection system, Bio-Rad, Hercules, CA, USA) were performed in triplicate with  $25\text{ }\mu\text{l}$  reactions and was described previously (Verlaet et al., 2019). For 16S ribosomal RNA (rRNA) gene-based microbial composition profiling, barcoded amplicons covering the variable regions V5-V6 of the bacterial 16S rRNA gene were generated by PCR using the 784F and 1064R primers as described before (Ramiro-Garcia et al., 2016). Each sample was amplified in duplicate using Phusion hot start II high fidelity polymerase (Finnzymes, Finland), checked for correct size and concentration on a 1% agarose gel and subsequently combined and purified using CleanNA magnetic beads (CleanNA, the Netherlands). A detailed description of the PCR conditions is given elsewhere (Kers et al., 2019b). Positive and negative controls were added to the data set to ensure high quality sequencing data. As positive controls we used synthetic mock communities of known composition (Ramiro-Garcia et al., 2016), and as negative controls we used nuclease free water. The resulting libraries were sent to Eurofins Genomics GmbH (Germany) for sequencing on an Illumina HiSeq2500 instrument. The 16S rRNA data was analyzed using NG-tax 2.0 (Poncheewin et al., 2020). In short, paired-end libraries were filtered to contain only read pairs with a perfect match to the primers and perfectly matching barcodes, to demultiplex reads by sample. Amplicon sequence variants (ASVs) were defined as unique sequences. The ASV picking strategy was based on a de novo reference approach. Taxonomy was assigned using the SILVA 128 16S rRNA gene reference database (Quast et al., 2013). Cecal content samples of day 0 and ileal content samples of day 0 and 1 were excluded from the analysis, because these contained a large number of families associated with the negative control samples, and therefore did not pass our quality control standards.

### ***Isolation of tissues and cells***

Ileum segments ( $\pm 10\text{ cm}$  distal from Meckel's diverticulum), spleens and blood ( $5\text{ mL}$ ) were collected. Ileum segments were washed with PBS to remove contents and cut in sections of  $1\text{ cm}^2$  and washed again. Subsequently, IELs were collected by incubating three times in EDTA-medium (HBSS 1x (Gibco BRL) supplemented with 10% heat-inactivated FCS (Lonza); 1% 0.5M EDTA (Sigma-Aldrich) at 200 rpm for 15 min at  $37^{\circ}\text{C}$ . Supernatants were collected and centrifuged 5 min at 1200 rpm

at 20°C. Cells were then resuspended in PBS, lymphocytes were isolated using Ficoll-Paque Plus (GE Healthcare, the Netherlands) density gradient centrifugation for 12 min at 1700 rpm, washed in PBS using centrifugation for 5 min at 1300 rpm and resuspended at  $4.0 \times 10^6$  cells/mL in NK medium (IMDM medium supplemented with 8% heat-inactivated FCS (Lonza); 2% heat-inactivated chicken serum, 100 U/mL penicillin/ streptomycin and 2 mM glutamax I; Gibco BRL, United Kingdom). Spleens were homogenized using a 70  $\mu$ m cell strainer (Beckton Dickinson (BD) Biosciences, NJ, USA) to obtain a single cell suspension. Next, lymphocytes in spleen and blood were isolated by Ficoll-Paque density gradient centrifugation (20 min at 2200 rpm), washed in PBS and resuspended at  $4.0 \times 10^6$  cells/mL in NK medium as described for ileum.

### ***Flow cytometry***

Presence and activation of NK and T cell subsets were determined in IEL, spleen and blood. Unless described otherwise, all antibodies were obtained from Southern Biotech (AL, USA). Markers known to be expressed on chicken NK cells (hybridomas provided by Göbel, T.W., Ludwig Maximilians University, Germany), such as mouse-anti-chicken-28-4 (IL-2R $\alpha$ ; IgG3) and -20E5-BIOT (IgG1) were co-stained with mouse-anti-chicken-CD45-FITC (IgM) and -CD3-APC (CT3; IgG1) mAb to exclude T cells. The T cell panel included the following markers: mouse-anti-chicken-CD3-PE (CT3; IgG1), -CD4-APC (CT4; IgG1), -TCR $\gamma\delta$ -FITC (TCR-1, IgG1), -CD8 $\alpha$  (EP72, IgG2b) and -CD8 $\beta$ -BIOT (EP42, IgG2a). Secondary antibody staining was performed using goat-anti-mouse-IgG3-PE and streptavidin-PercP (BD Biosciences) in the NK cell panel, and goat-anti-mouse-IgG2b-APC/Cy7 and streptavidin-PercP in the T cell panel. To assess CD107 expression on NK cells, lymphocytes were washed in (polyclonal B-cell activator) PBA and stained with mouse-anti-chicken-CD3-PE, -TCR $\gamma\delta$ -BIOT (TCR-1, IgG1), -28-4 and -CD41/61-FITC (11C3, IgG1, Serotec) to exclude thrombocytes from analysis. Secondary antibody staining was performed using streptavidin-PercP and goat-anti-mouse-IgG3-APC/Cy7. All staining procedures were incubated for 20 min at 4°C in the dark, washed in PBA and subsequently stained with a live/dead marker (Zombie Aqua™ Fixable Viability Kit, Biolegend) for 15 min at RT in the dark to exclude dead cells. Finally, lymphocytes were fixed using 2% paraformaldehyde (Merck, Germany) for 10 min at RT, washed and resuspended in PBA. Flow cytometry (FACSCANTO II Flowcytometer, BD Biosciences) was used to measure 150  $\mu$ l or 50,000 lymphocytes cells in the live gate, and data was analyzed with software program FlowJo (Tree star Inc, OR, USA).

### ***NK cell activation assay***

NK cell activation was determined using the CD107-assay, which measures increased surface expression of CD107 as a result of degranulation of perforin and

granzymes (Jansen et al., 2010). Briefly, lymphocytes isolated from IEL, spleen and blood were resuspended in NK medium, and  $1 \times 10^6$  lymphocytes per sample were used. Lymphocytes were cultured in presence of 1  $\mu$ l/mL Golgistop (BD Biosciences) and mouse-anti-chicken-CD107-APC mAb (5G10, IgG1, hybridomas provided by Göbel) during 4 hours at 37°C, 5% CO<sub>2</sub>. Next, cells were washed, stained with monoclonal antibodies and analyzed by flow cytometry.

### **Data analysis**

Statistical analyses for microbiota and the relation between microbiota and the immune system were performed in R (R Foundation for Statistical Computing, Austria), using the packages Phyloseq, Microbiome, Vegan and DirichletMultinomial (McMurdie and Holmes, 2013, Lahti et al., 2017, Oksanen et al., 2010, Morgan, 2019). A Kruskal-Wallis test was used to test for difference in 16S rRNA gene counts in cecal content of day-old chickens between treatment groups.

Alpha and beta diversity metrics and multivariate statistical analyses were applied to determine differences in the measured intestinal microbiota between the two treatment groups and with age. The alpha diversity (within sample) data was determined using Faith's phylogenetic diversity. Faith's phylogenetic diversity not only takes the number of different taxa (ASVs) into account, but also the phylogenetic relatedness of these taxa (Faith, 2006). To test for differences in relative abundance of genera between treatment groups, we used a Wilcoxon rank-sum test and corrected for multiple comparisons using the Benjamini-Hochberg (BH) procedure. The beta diversity (between samples) was determined using weighted and unweighted UniFrac metrics (Lozupone et al., 2007). Multivariate microbiota data were visualized using principal coordinates analysis (PCoA, multidimensional scaling method), and non-parametric permutational analysis of variance (PERMANOVA) tests were used to analyze group differences within multivariate community data (Anderson, 2001).

To assess whether the development of the microbiota proceeded through different stages of maturation in the two treatment groups, Dirichlet Multinomial Mixtures (DMM) modelling was applied, using a probabilistic model, to identify possible clusters (types) of microbial composition 16S rRNA gene sequence data (Holmes et al., 2012) based on the relative abundance of the microbial groups at genus level. Two separate DMM models were used to study clustering of the microbiota data of the cecal content and ileal content separately. Next, to test whether the observed differences in the microbial development between treatments were associated with differences in immune development, Wilcoxon rank-sum test, corrected for multiple comparisons using BH was used to test for associations between the identified DMM clusters of microbial composition and immunological parameters. As ileal microbiota clustering did not indicate differences in microbial development between treatments, only the clusters identified for the cecal microbiota were used. Associations were



tested for a subset of immunological parameters that showed differences between AM and control chickens of the same age. Furthermore, parameters with fewer than four observations per treatment group and day of age were omitted. The final selection of parameters included percentages and absolute numbers of intestinal IL-2R $\alpha$ <sup>+</sup>, 20E5<sup>+</sup> and CD107<sup>+</sup> NK cells, and CD8 $\alpha\alpha$ <sup>+</sup> T cells.

Statistical analyses for the immunological parameters were done with GraphPad Prism 7.0 software (GraphPad Software Inc., USA), using the Mann-Whitney U test to test differences between treatment groups at a specific day of age. A  $p$  value of  $<0.05$  was considered statistically significant.

## Results

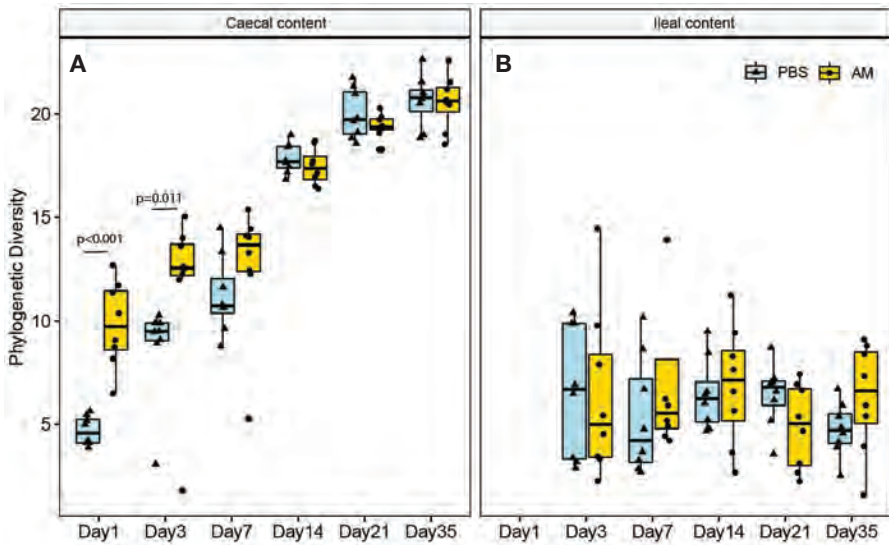
### ***AM treatment influences the composition and development of the intestinal microbiota in newly hatched chickens.***

The total bacterial 16S rRNA gene copy numbers 24 hours after inoculation were significantly higher in cecal content samples at day 1 in AM inoculated compared to control chickens, indicating the presence of a higher quantity of bacteria after inoculation with AM (Fig. S1).

To investigate the effect of AM inoculation on the microbiota composition at different ages in the broiler chickens, alpha and beta diversities, as well as differences in relative abundance of individual microbial taxa, were assessed. The phylogenetic diversity metric, providing information on the number as well as phylogenetic relatedness of observed microbial taxa at the ASV level, was used as an alpha diversity measure to determine differences between AM and control chickens. The phylogenetic diversity of the cecal content was higher in 1- and 3-day-old AM chickens compared to controls, but not for any of the other ages (Fig. 1A). In contrast, the phylogenetic diversity of ileal content microbiota did not differ between treatment groups at any age (Fig. 1B).

Beta diversity, i.e. the similarity in composition between samples, was determined using the weighted and unweighted UniFrac distance metrics to determine the influence of age and treatment on the composition. Two dimensional visualization of the cecal microbiota in PCoA plots placed 3-day-old and 7-day-old AM inoculated chickens closely together, indicating high similarity in microbiota composition between these age groups (Fig. 2). PERMANOVA of cecal content microbiota showed that treatment explained 6-9% of the variation in cecal microbiota composition between samples ( $p < 5e-04$ ; unweighted UniFrac,  $p < 2e-04$ , weighted UniFrac), whereas age explained 49-41% of the variation between samples ( $p < 5e-04$ ; unweighted UniFrac,  $p < 2e-04$ , weighted UniFrac).





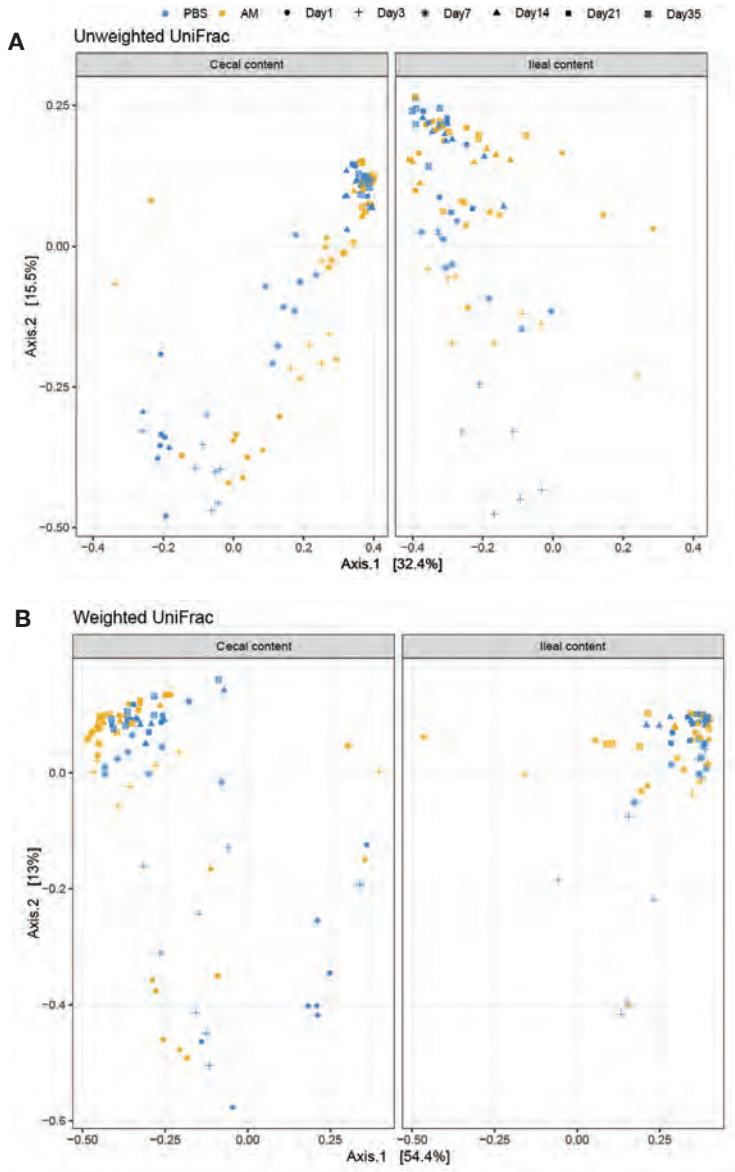
**Figure 1:** Phylogenetic diversity of the ileal and cecal content microbiota at different ages. A. The phylogenetic diversity (alpha diversity, at ASV level) was only significantly higher in the cecal content of AM chickens compared to controls on day 1 and day 3. B. In the ileal content microbiota no differences were observed at any of the ages.  $n = 8$  chickens per treatment per day of age, whiskers show 95% interval, box 50% interval.

PERMANOVA of ileal content samples showed that treatment explained 4% of the variation in ileal microbiota composition based on unweighted UniFrac, whereas treatment did not significantly contribute to explaining the observed variation using the weighted UniFrac distance metrics ( $p = 0.038$ ; unweighted UniFrac,  $p = 0.355$ , weighted UniFrac, Fig. 2B). This indicates that differences in microbiota of ileal samples between treatment groups concerned mostly in presence/absence of taxa occurring at low relative abundance. Age explained 29–24% of the variation between the ileal content samples ( $p < 1e-04$ ; unweighted UniFrac,  $p < 1e-04$ , weighted UniFrac).

In the AM inoculum 24 different genera were detected, for which the relative abundances in cecal and ileal samples were compared between AM and control chickens. A higher relative abundance in cecal content of AM chickens compared to controls was found for ten of these 24 genera at day 1, five on day 3, four at day 7 and two at day 14 and 21. At day 35 none of these genera differed in relative abundance between AM and control chickens (Table 1). This indicates that AM inoculation had an impact on the relative abundance of genera at an early age but did not permanently influence the relative abundance of these genera in the cecal content samples. For ileal content, no differences in the relative abundances of the 24 genera of the AM inoculum were observed at any of the different ages (data not shown).

**Table 1:** Relative abundance of genera present in the AM inoculum and differences in relative abundance in cecal content for AM compared to control chickens. The AM inoculum contained 24 different genera. The genera for which significant differences in relative abundance of AM inoculated chickens (AM) versus controls (PBS) were found for day 1, 3, 7, 14 and 21 are indicated in bold. No differences were observed between treatments on day 35 (data not shown). The analyses were based on 8 chickens per group. Results are based on differences of relative abundance tested with Wilcoxon rank-sum test. Adjusted  $p$ -values ( $<0.05$ ) were corrected for multiple testing with Benjamini-Hochberg (BH). - = not detected). RA = Relative abundance Aviguard® (%), AM / PBS = Relative abundance in AM / control group (%),  $P$  =  $p$ -value, only values above 0.05 are shown.

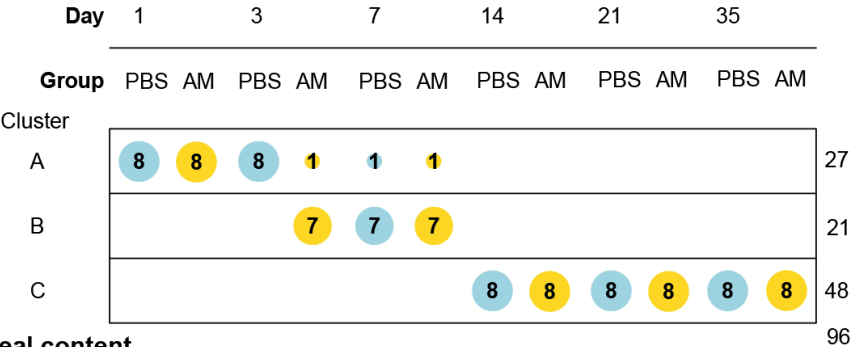
Genera in AM inoculum	RA %	Day 1			Day 3			Day 7			Day 14			Day 21		
		SD %	AM%	PBS%	P	AM%	PBS%	P	AM%	PBS%	P	AM%	PBS%	P	AM%	PBS%
<b>Eubacterium coprostanoligenes group</b>	0.65	0.22	-	-	-	0.06	-	-	-	-	-	0.84	1.12	-	0.71	0.65
<b>Bacteroides</b>	0.47	0.06	-	-	-	3.91	-	0.045	2.57	-	0.018	2.01	1.12	-	3.01	3.57
<b>Blautia</b>	0.30	0.09	9.17	-	0.006	5.86	3.92	-	4.67	16.63	-	4.35	13.14	-	6.10	10.68
<b>Candidatus Soleaferrea</b>	0.39	0.06	0.56	-	0.006	-	-	-	-	-	-	-	-	-	-	-
<b>Clostridium sensu stricto 1</b>	2.77	0.45	24.32	53.93	0.033	1.96	22.30	-	-	0.19	-	-	-	-	0.03	-
<b>Clostridium sensu stricto 2</b>	0.72	0.12	0.77	-	0.006	-	-	-	-	-	-	-	-	-	-	-
<b>Collinsella</b>	0.53	0.07	0.68	-	0.034	4.65	-	0.018	3.64	1.04	-	1.54	4.30	-	3.60	2.02
<b>Enterococcus</b>	10.80	1.07	10.12	17.64	-	16.19	18.55	-	0.86	0.87	-	0.32	0.36	-	0.43	0.48
<b>Erysipelatoclostridium</b>	2.53	0.09	0.26	0.00	-	2.56	-	0.027	0.05	2.07	0.018	0.81	2.01	-	0.44	1.06
<b>Escherichia-Shigella</b>	0.57	0.02	32.73	3.36	0.006	0.72	11.07	0.044	0.16	0.73	-	-	0.03	-	-	-
<b>Eubacterium</b>	0.66	0.04	0.30	-	0.016	0.46	0.11	-	0.92	0.28	-	0.15	0.19	-	0.07	0.12
<b>Flavonifractor</b>	1.02	0.14	1.23	-	0.006	0.90	0.66	-	0.13	0.44	-	0.05	0.43	0.010	-	-
<b>Lachnospirillum</b>	9.78	0.93	2.30	-	0.006	3.28	-	0.018	0.77	0.85	-	0.66	0.72	-	0.41	0.16
<b>Lactobacillus</b>	14.96	1.33	8.46	-	6.83	1.12	-	-	8.05	3.44	-	5.34	12.34	-	13.85	10.14
<b>Megamonas</b>	1.55	0.56	0.02	-	4.05	-	-	-	30.21	-	0.018	27.46	-	-	0.009	7.46
<b>Megasphaera</b>	3.30	0.74	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<b>Negativicoccus</b>	3.62	0.66	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<b>Oscillibacter</b>	1.94	0.18	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<b>Peptostreptococcus</b>	30.97	4.04	0.19	-	0.034	-	-	-	-	-	-	-	-	-	-	-
<b>Sellimonas</b>	1.31	0.38	-	-	-	-	-	-	0.31	0.75	-	0.60	0.84	-	0.50	0.88
<b>Slackia</b>	0.34	0.09	0.02	-	0.03	-	-	-	0.43	-	0.037	0.01	0.05	-	0.08	0.10
<b>Sutterella</b>	1.76	0.21	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<b>Uncultured</b>	4.45	3.56	0.00	0.00	-	1.40	0.27	-	1.07	2.15	-	1.37	1.96	-	1.11	1.48
<b>unknown</b>	0.08	0.09	0.11	0.24	-	0.16	0.21	-	0.53	0.13	-	0.42	0.47	-	0.59	0.63



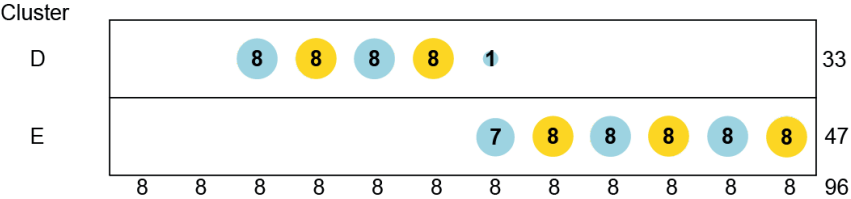
**Figure 2:** PCoA plot visualizing cecal and ileal microbiota. A. Unweighted UniFrac distance based PCoA on cecal (left) and ileal (right) content. B. Weighted UniFrac distance based PCoA on cecal (left) and ileal (right) content.  $n = 8$  chickens per treatment per day of age. Cecal microbiota showed that treatment explained 6-9% of the variation between samples (PERMANOVA, A.  $p < 5e-04$ ; unweighted UniFrac, B.  $p < 2e-04$ , weighted UniFrac), whereas age explained 49-41% of the variation between samples ( $p < 5e-04$ ; unweighted UniFrac,  $p < 2e-04$ , weighted UniFrac). Ileal microbiota showed that treatment explained 4% of the variation between samples, based on unweighted UniFrac, whereas treatment did not significantly contribute to explaining the observed variation using weighted UniFrac distance metrics (A.  $p = 0.038$ ; unweighted UniFrac, B.  $p = 0.355$ , weighted UniFrac).

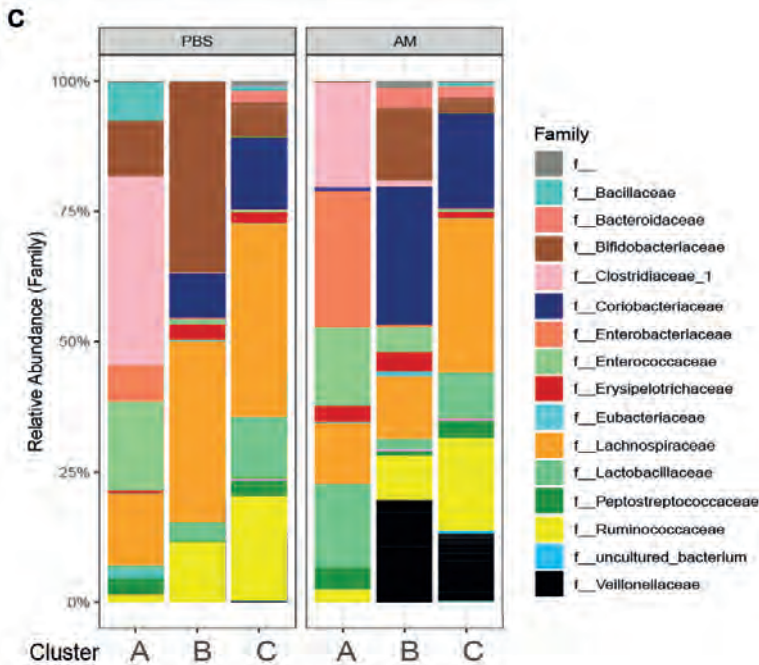
To assess if AM inoculation affected the development of the microbial composition from hatch towards a mature microbiota, microbiota compositions were subjected to DMM clustering of 16S rRNA gene sequencing data based on the relative abundance of microbial taxa at genus level. The DDM method showed the best model fit, based on lowest Laplace approximation, for three clusters in the cecal content samples (Fig. 3A). Cluster A contained 27 samples, with all 1-day-old AM and control chickens and all 3-day-old controls. Cluster B consisted of 21 samples, containing seven of the eight 3-day-old AM chickens and 7-day-old AM and control chickens. The remaining 48 samples were in cluster C, which contained all AM and control chickens of 14, 21 and 35 days old. This difference in distribution of AM and control chickens over cluster A and B in the first week of life indicates an accelerated maturation of cecal microbiota for AM chickens. In contrast, clustering for the ileal microbiota only showed an effect of age, with cluster D dominated by 3- and 7-day-old chickens of both treatments, and cluster E by chickens of 14, 21 and 35 days old of both treatments (Fig. 3B).

**A cecal content**



**B ileal content**

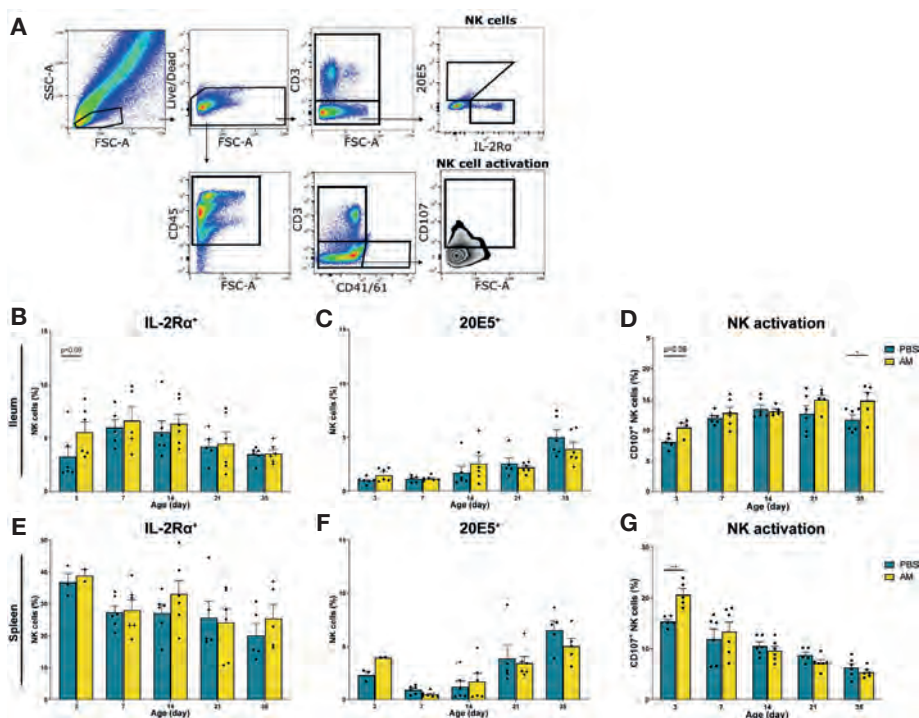




**Figure 3:** Dirichlet multinomial mixtures (DMM) clustering of 16S rRNA gene sequencing data for cecal and ileal microbial. A. DMM clustering showed the best model fit for three clusters in the cecal content (lowest Laplace approximation). Cluster A contains 27 samples, Cluster B 21 samples, and the remaining 48 samples are in cluster C. Cluster B contains 3-day old AM chickens, and almost all AM and control chickens of day 7, indicating acceleration of microbiota maturation in the cecal content. B. In the ileal content samples two distinct clusters were observed. Nodes are colored according to intervention (AM or PBS) and ordered according to age. C. Relative microbial abundance of the clusters observed in the cecal content stratified by the intervention at family level.

### **AM treatment affects presence of NK cell subsets and their activation**

Possible differences in subsets and activation of intestinal NK cells from AM and control chickens were determined. Local effects of AM inoculation on intestinal NK cells were compared to systemic effects measured in spleen and blood. Within the live lymphocytes, the CD3 negative IL-2R $\alpha$ <sup>+</sup> or 20E5<sup>+</sup> NK cells were quantified (Fig. 4A). In parallel, NK cell activation was determined by analysis of enhanced CD107 surface expression on CD3 negative and CD41/61 negative cells. At day 3, the percentage of intestinal IL-2R $\alpha$ <sup>+</sup> NK cells tended to be higher in AM chickens ( $5.61 \pm 0.95\%$ ) compared to controls ( $3.25 \pm 0.93\%$ ,  $p = 0.09$ , Fig. 4B). No differences between treatment groups were observed in intestinal 20E5<sup>+</sup> NK cells (Fig. 4C). Increased CD107 expression on intestinal NK cells was observed at day 3 in AM chickens ( $10.52 \pm 0.70\%$ ), when compared to controls ( $8.07 \pm 0.47\%$ ,  $p = 0.06$ , Fig. 4D). At day 35, an increase in activation of intestinal NK cells was observed in AM chickens ( $14.86 \pm 1.27\%$ ) compared to the controls ( $11.71 \pm 0.75\%$ ,  $p = 0.04$ , Fig. 4D). No differences between treatment groups were observed in CD107 expression of intestinal NK cells at other ages (Fig. 4D).



**Figure 4:** Effect of adult microbiota (AM) on NK cells in broiler chickens. A. Gating strategy after isolation of lymphocytes from IEL to analyze NK cell subsets and activation. B-E. Frequencies of NK cell subsets by characterization of surface markers IL-2R $\alpha$ <sup>+</sup>, C, F. 20E5<sup>+</sup> during aging in B-D. IEL and E-G spleen. D, G. Frequencies of NK cell activation during aging as assessed by measuring the surface marker CD107. Mean + SEM of chickens is shown (n = 6), however, chickens were excluded from analysis when number of events in the gate of interest was < 100. Statistical significance is indicated as \* p < 0.05 and \*\* p < 0.01.

Relative numbers of IL-2R $\alpha$ <sup>+</sup> and 20E5<sup>+</sup> NK cells in spleen and blood were similar in both treatment groups (Fig. 4E-F and S3A-B). However, NK cell activation was significantly increased in splenic NK cells in 3-day-old AM chickens ( $20.74 \pm 1.10\%$ ) compared to controls ( $15.35 \pm 0.40\%$ ,  $p = 0.004$ , Fig. 4G). No difference in CD107 surface expression on blood-derived NK cells was found between treatment groups (Fig. S2C). Furthermore, AM inoculation did not affect total lymphocyte numbers in the intestine, spleen and blood (Fig. S3 A, E, I). In addition to the percentages of the different NK subsets, absolute numbers were determined. Similar trends were observed in absolute number of IL-2R $\alpha$ <sup>+</sup>, 20E5<sup>+</sup> and CD107<sup>+</sup> NK cells although the differences between treatments were less pronounced (Fig. S3).

### **AM treatment affects intestinal cytotoxic CD8 $\alpha\alpha$ T cells in 14- and 21-day-old chickens**

In addition to NK cell subsets and NK cell activation, effects of AM inoculation on

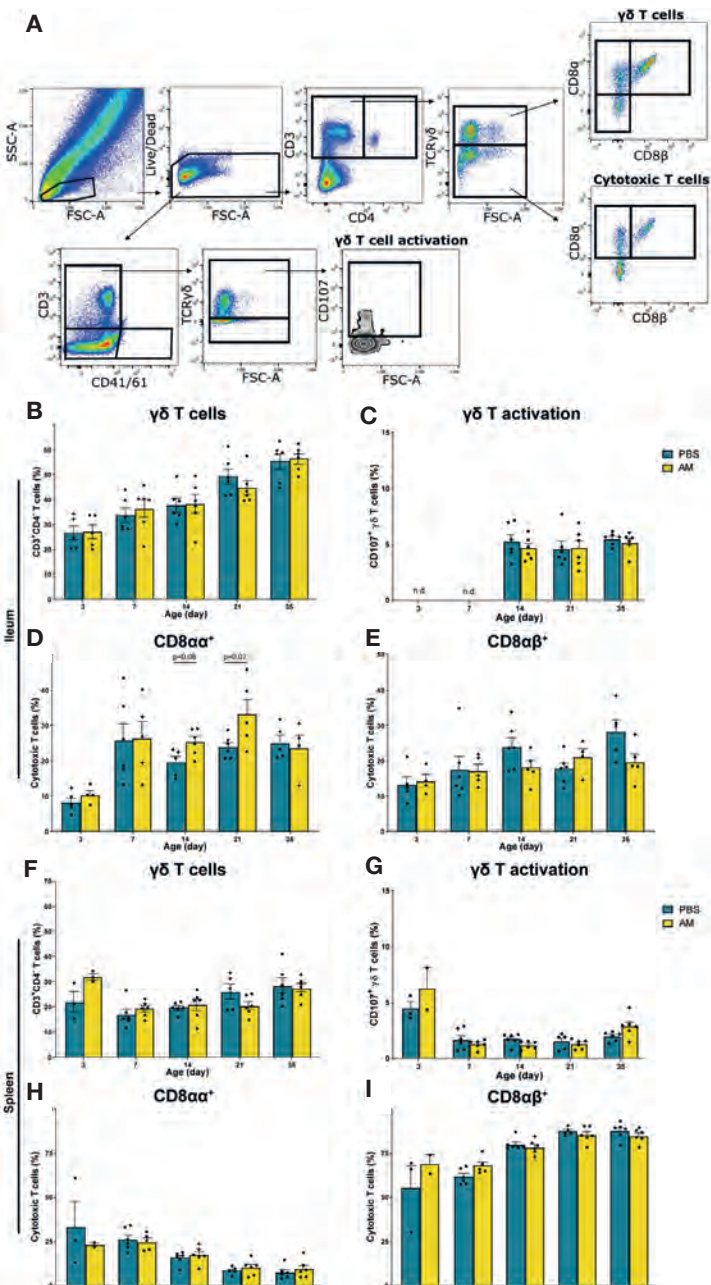
presence and function of  $\gamma\delta$  T cells and presence of cytotoxic  $CD8^+$  T cells were studied. Within the  $CD3^+$  and  $CD4^+$  lymphocytes, both  $TCR\gamma\delta^+$  and  $TCR\gamma\delta^-$  cell populations were analyzed for  $CD8\alpha\alpha$  and  $CD8\alpha\beta$  expression (Fig. 5A). In parallel, activation of  $\gamma\delta$  T cells was determined at day 7, 14 and 21 by analyzing increased surface expression of CD107 on  $CD3^+CD41/61^+ TCR\gamma\delta^+$  cells (Fig. 5A). No differences between AM and control chickens were observed in the percentage of intestinal  $\gamma\delta$  T cells (Fig. 5B),  $CD8^+$ ,  $CD8\alpha\alpha^+$  and  $CD8\alpha\beta^+$  gamma delta subsets (data not shown) and activation of  $\gamma\delta$  T cells (Fig. 5C). The percentage of intestinal  $CD8\alpha\alpha^+$  T cells tended to be higher in 14-day-old ( $25.3 \pm 1.5\%$ ) and 21-day-old AM chickens ( $33.2 \pm 4.2\%$ ) compared to controls ( $19.5 \pm 1.7\%$ ,  $p = 0.08$  and  $24.0 \pm 1.3\%$ ,  $p = 0.07$ , respectively, Fig. 5D). No differences between groups were found at any age in the percentages of intestinal  $CD8\alpha\beta^+$  T cells (Fig. 5E). Furthermore, no differences between AM and control chickens were observed in the percentage of  $\gamma\delta$  T cells (Fig. 5F and S3D), subsets (data not shown),  $\gamma\delta$  T cell activation (Fig. 5G and S3E) and cytotoxic T cells in spleen and blood (Fig. 5H, I and S3 F, G). Absolute numbers of these parameters were investigated and did not show any differences between AM and control chickens, although an increase in numbers of both treatments was observed with age (Fig. S4).

### ***Association between cecal microbiota clusters and immune cells***

Clustering of the cecal microbiota samples suggests that AM chickens showed an earlier maturation of cecal microbiota compared to controls. Also, differences in  $IL-2R\alpha^+$  NK cells, NK cell activation and  $CD8\alpha\alpha$  T cells were observed between AM chickens compared to the controls. To assess a possible relationship between the observed differences in the microbial development between treatments and the detected differences in immune parameters, we used the previously identified DMM clusters to test for correlations between the cecal microbiota (i.e. stages of successive microbiota maturation) and immune parameters.

Clusters A, B and C were based on relative abundance of genera present in the cecal microbiota of chickens and represent different stages during the early life development of cecal microbiota. Correlations to relative and absolute numbers of  $IL-2R\alpha^+$ ,  $20E5^+$ ,  $CD107^+$  NK cells and cytotoxic  $CD8\alpha\alpha^+$  T cells in the ileum were investigated. The percentage of intestinal  $IL-2R\alpha^+$  NK cells was higher in cluster B compared to cluster A ( $p = 0.026$ , Table 2), and compared to cluster C ( $p = 0.044$ , Table 2) regardless of treatment (Fig. 6A). The percentage of  $IL-2R\alpha^+$  NK cells in cluster C tended to be higher compared to cluster A, but this was not significant ( $p = 0.068$ , Table 2, Figure 6A).





**Figure 5:** Effect of adult microbiota (AM) on T cells in broiler chickens. A. Gating strategy after isolation of lymphocytes from IEL to analyze T cell subsets and  $\gamma\delta$  T cell activation. B,F. Frequencies of total  $\gamma\delta$  T cells, subsets (data not shown) and C,G.  $\gamma\delta$  T cell activation by characterization of surface markers TCR $\gamma\delta$  and CD107 during aging in B-E. IEL and F-I. spleen. D,H. Frequencies of cytotoxic T cell subsets using the surface markers CD8 $\alpha\alpha$  and E,I. CD8 $\alpha\beta$  during aging. Mean + SEM of chickens is shown ( $n = 6$ ), however, chickens were excluded from analysis when number of events in the gate of interest was < 100.



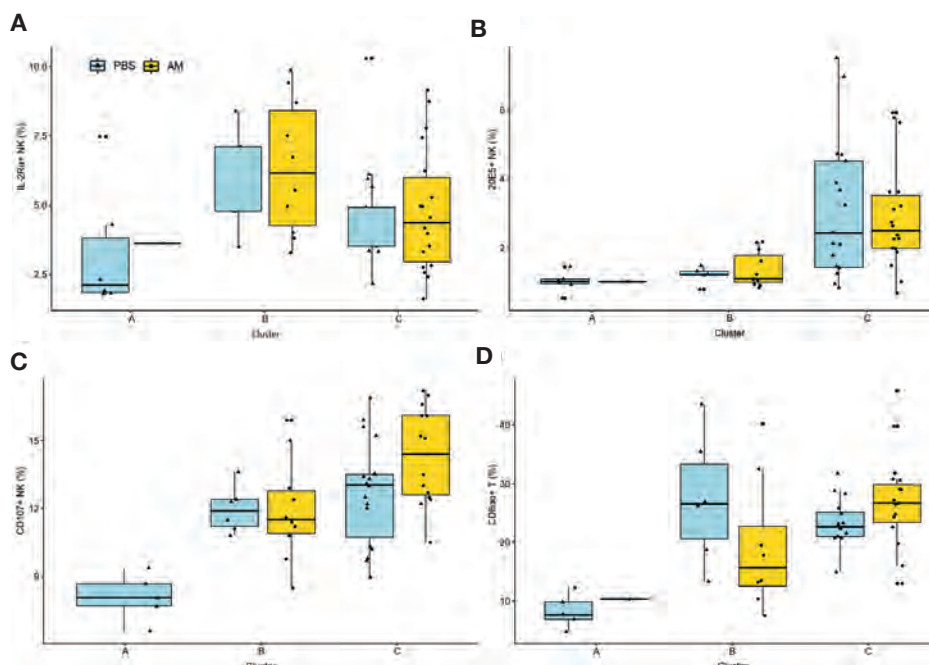
Relative numbers of intestinal 20E5<sup>+</sup> NK cells were similar between clusters A and B and highest in cluster C (Fig. 6B, Table 2). Relative numbers of intestinal CD107<sup>+</sup> NK cells were highest in cluster C and lowest in cluster A (Table 2, Fig. 6C). Within cluster C, the percentage of CD107<sup>+</sup> NK cells tended to be higher in AM chickens (Fig. 6C). Relative numbers for intestinal cytotoxic CD8αα<sup>+</sup> T cells were higher in cluster B and C compared to cluster A and did not differ between cluster B and C (Fig. 6D, Table 2). Similar correlations were observed between clusters and absolute numbers of intestinal 20E5<sup>+</sup>, CD107<sup>+</sup> NK cells and cytotoxic CD8αα<sup>+</sup> T cells (Table 2). These results indicate significant associations between cecal microbiota clusters and subsets of intestinal immune cells.

**Table 2.** Statistical differences in relative (%) and absolute (cells/mg) numbers of intestinal immune cells between caecal microbiota clusters.

Immune cells	Cluster A vs B	Cluster B vs C	Cluster A vs C
IL-2Rα <sup>+</sup> NK (%)	<b>0.026</b>	<b>0.044</b>	<b>0.068</b>
20E5 <sup>+</sup> NK (%)	0.124	<b>3.0e-4</b>	<b>0.001</b>
CD107 <sup>+</sup> NK (%)	<b>0.003</b>	<b>0.020</b>	<b>0.001</b>
CD8αα <sup>+</sup> T (%)	<b>0.001</b>	0.254	<b>4.1e-4</b>
IL-2Rα <sup>+</sup> NK (cells/mg)	<b>0.011</b>	0.051	<b>2.7e-4</b>
20E5 <sup>+</sup> NK (cells/mg)	<b>0.039</b>	<b>5.3e-7</b>	<b>2.1e-6</b>
CD107 <sup>+</sup> NK (cells/mg)	0.398	<b>4.0e-6</b>	<b>1.1e-4</b>
CD8αα <sup>+</sup> T (cells/mg)	<b>0.008</b>	<b>9.5e-6</b>	<b>6.4e-4</b>

## Discussion

In this study, we aimed to induce an alteration in the intestinal microbiota shortly after hatch by administration of adult-derived microbiota, and compared presence and function of NK cells, as representatives of developing innate immunity, to those of non-inoculated controls. We hypothesized that early exposure to adult-derived microbiota would accelerate intestinal microbiota colonization and affect subsets and activation of intestinal NK cells. Our results indicate that the inoculation with the adult-derived microbes mostly affected the early development of the cecal microbiota and induced an earlier maturation of cecal microbiota compared to control broiler chickens. This development was paralleled by an increase in intestinal IL-2Rα<sup>+</sup> NK cells and enhanced activation of NK cells early in life and CD8αα<sup>+</sup> T cells later in life.



**Figure 6:** Associations between cecal microbiota clusters and immune cells. Associations between the identified DMM clusters of cecal microbiota composition and relative numbers of intestinal NK cell subsets. A. IL-2R $\alpha$ +, B. 20E5+, C. CD107+ and D. cytotoxic CD8 $\alpha\alpha$ + T cells were analyzed using Wilcoxon rank-sum test. Adjusted p-values ( $< 0.05$ ) were corrected for multiple testing with BH.

The AM inoculation delivered immediately after hatch successfully altered intestinal microbiota composition, especially in the first week of life, but did not permanently influence the diversity of cecal microbiota. In addition, with respect to the genera found in the AM product, a higher relative abundance was only found shortly after inoculation. More specifically, a higher relative abundance in AM chickens was found for ten of the 24 genera in the inoculum on day 1, but this quickly declined to two genera by the end of the first week. These findings are in line with previous studies with the same product: inoculation with Aviguard *in ovo* enhanced development of intestinal microbiota of broiler chickens and increased diversity and reduced the abundance of *Enterobacteriaceae* (Pedroso et al., 2016). Similar to our study, not all genera present in the inoculum permanently colonized the intestine; they were assumed to have been transient colonizers facilitating the development of a complex microbiota by temporarily altering the microenvironment (Pedroso et al., 2016). Similar observations have been reported for 1-day-old laying hens inoculated with Aviguard. Not all bacteria of the product, nor of the mother hen, were effectively transferred to the chickens' gut, but compared to controls, cecal microbiota enriched for the phyla *Bacteroidetes* and *Actinobacteria* was observed within a week

in both Aviguard treated chickens and in chickens naturally exposed to a mother hen (Kubasova et al., 2019). Like chickens hatched in commercial hatcheries, the control chickens in our study were gradually exposed to microbiota in the hours and days after hatch from different sources, such as the housing environment, litter, feed and water. This colonization was delayed compared to the chickens inoculated with AM directly after hatch, as indicated by the clustering of cecal microbiota samples of 3-day-old controls with 1-day-old AM inoculated chickens, and of 7-day-old controls with 3-day-old AM chickens. This accelerated maturation of cecal microbiota has also been observed in a study where a topical spray treatment of eggs with adult cecal content significantly altered broiler chicken microbiota immediately after hatch (Richards-Rios et al., 2020b). As in our study, the effect on the cecal microbiota was highest at 3 days of age, and diminished over time (Richards-Rios et al., 2020b). In contrast, swabbing of the egg surface once during incubation with diluted adult cecal content did not lead to significant differences in alpha diversity nor in the pattern of bacterial colonization between treated and control broiler chickens (Donaldson et al., 2017). This difference may be a result of the egg inoculation technique, suggesting that perhaps a lower number of spores and vegetative cells was applied to the eggshell in the latter study.

Although many of the available poultry microbiota studies have focused on broiler chickens, its relation with the innate immune system has not previously been elaborately investigated. We observed an increase in IL-2R $\alpha$ <sup>+</sup> NK cells and activation of NK cells within the first days of life, together with an increase in relative numbers of cytotoxic CD8 $\alpha\alpha$ <sup>+</sup> T cells from day 14 onwards in chickens that were inoculated with AM.

The increased NK cell activation observed in AM chickens may suggest a mildly increased cytotoxic capacity against potential pathogens, as the CD107 expression can increase up to 30% upon viral infections (Vervelde et al., 2013), which is more than two fold higher than the NK cell activation observed in this study. This result is in line with the observed increase in IL-2R $\alpha$ <sup>+</sup> NK cells in this study. Studies in humans have shown that increased IL-2R $\alpha$  expression is associated with an early stage of NK cell activation (Leong et al., 2014), and this was also observed in chickens (Abdolmaleki et al., 2018, Jahromi et al., 2018). In addition to the local effect on NK cell activation, our observation of increased splenic NK cell activation in 3-day-old AM chickens also indicates there is a systemic effect. No effects of AM inoculation on immune cells in the blood were observed.

The observed differences between AM and control chickens with respect to immune parameters suggest an interaction between microbial and immune development. This was further substantiated by the significant associations between IL-2R $\alpha$ <sup>+</sup> NK cells, CD107<sup>+</sup> NK and CD8 $\alpha\alpha$ <sup>+</sup> T cells and cecal microbiota clusters: cluster A includes chickens with a starting microbiota, cluster B chickens in the middle of the

maturation process and cluster C chickens with a more matured successive microbiota composition from day 14 onwards. These clusters follow the successional patterns of microbiota development as previously described for broiler chickens, with bacterial community richness increasing rapidly over time and stabilizing from day 14 onwards (Cressman et al., 2010, Jurburg et al., 2019, Oakley and Kogut, 2016, Chapter 5). Our analyses showed that cluster B was associated with an increase in IL-2R $\alpha$ <sup>+</sup> NK cells and an enhanced NK cell activation regardless of treatment. This suggests that the accelerated microbiota colonization due to AM inoculation affected the development of NK cells locally and systemically. Interestingly, the IL-2R $\alpha$ <sup>+</sup> NK cell subset was higher in relative numbers in cluster B compared to the starting microbiota cluster A, but subsequently decreased in relative numbers in the more mature microbiota cluster C. The 20E5<sup>+</sup> NK cell subset and NK cells that express CD107 further increased in relative numbers between cluster B and cluster C. This fits with the observation in mammals that an increase in IL-2R $\alpha$  expression is associated with an early stage of NK cell activation, which is followed by enhanced NK cell mediated killing. Cluster C was associated with an increased relative number of intestinal cytotoxic CD8 $\alpha\alpha$ <sup>+</sup> T cells. As the cecal microbiota in this cluster shows a matured composition similar in AM and control chickens of the same age, this suggests that early life inoculation with AM also affected the adaptive immune development in the intestine.

Although these results indicate associations between early life microbiota colonization and immune system development, the data from this study cannot elucidate exactly how these processes are related. As has been shown in humans and mice, microbiota can signal to immune cells in various ways either locally or systemically (Ganal et al., 2012, Sonnenberg and Artis, 2012). Locally, microorganisms interact directly with NK cells via TLRs and NCRs resulting in cytokine production by NK cells, and indirectly via cytokine production of resident myeloid or epithelial cells that consequently affect NK cell responses (Sonnenberg and Artis, 2012, Poggi et al., 2019). Systemically, microbiota can induce instructive signals to non-mucosal antigen-presenting cells and by producing among others IL-15, TNF $\alpha$  and IFN, subsequently prime optimal splenic NK cell responses (Ganal et al., 2012). Since chicken NK cells have been shown to express TLRs (Kannaki et al., 2010) and NCRs (Straub et al., 2013, Jansen et al., 2016), the interactions between microbiota and NK cells probably follow similar routes to those in humans and mice.

In mammals, specific commensal bacterial strains have been linked to modulation of NK cells. Several reports established that bacteria within the *Lactobacillus* genus can induce IFN $\gamma$  and cytotoxicity responses in intestinal NK cells as a result of IL-12 production by dendritic cells after TLR engagement with bacteria (Fink et al., 2007, Koizumi et al., 2008, Aziz and Bonavida, 2016). Furthermore, *Bacteroides fragilis* can stimulate innate and adaptive immune pathways directly through TLR

signaling and indirectly by inducing cytokine production (Troy and Kasper, 2010). Although we did observe significant differences in the relative abundance of genera between AM and control chickens at day 1 and 3, we cannot pinpoint a specific genus responsible for the observed effect on NK cells. Interestingly, the genus *Bacteroides* showed a significantly higher prevalence and relative abundance in 3- and 7-day-old AM chickens and the genus was absent in control chickens of similar age. This could suggest that the observed effects on NK cells in 3-day-old AM chickens may be linked to a higher presence of *Bacteroides* bacteria. We did not find differences in the prevalence of *Lactobacillus* bacteria due to AM inoculation. Other genera that showed significant differences in their prevalence and/or relative abundance between AM and control chickens at 1 and 3 days of age have not been described as specifically interacting with NK cells.

In addition, microbiota has been shown in mice and humans to interact directly with  $\gamma\delta$  T cells, and increased frequencies of CD8<sup>+</sup>  $\gamma\delta$  T cells and  $\gamma\delta$  T cell activation were observed during intestinal inflammation (Bhagat et al., 2008, Nielsen et al., 2017). Under non-inflammatory conditions similar to those of our study, application of adult cecal content on eggs altered and accelerated the microbiota of 3-day-old chickens but did not affect  $\gamma\delta$  T cells in cecal tonsils (Richards-Rios et al., 2020). Furthermore, AM inoculated chickens in, our study showed an increased presence of intestinal CD8 $\alpha\alpha$ <sup>+</sup> T cells at two and three weeks of age. Although in previous studies with mice no CD8 $\alpha\alpha$  and CD8 $\alpha\beta$  subsets were investigated, microbiota was shown to have a direct (Tanoue et al., 2019) and indirect (Luu et al., 2018) effect on cytotoxic T cells, as a result of induction of IFN $\gamma$  production.

In conclusion, our study showed a relation between an accelerated maturation of intestinal microbiota and the enhanced NK cell response early in life. This interaction between microbiota and the developing innate immune system indicates possibilities in developing strategies to improve health and resilience of broiler chickens. One such possibility is through feed interventions or the use of products with adult-derived microbiota directly after hatch, both of which can affect microbiota composition and may accelerate microbiota maturation and consequently can strengthen the innate immune system, conferring direct protective effects early in life as well as influencing adaptive immunity later in life. The combination of a well-developed microbiota and immune system will result in more robust broiler chickens with higher resilience against health challenges, such as disturbances in gut health and invading pathogens.

### ***Ethical statement***

The animal experiment was approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee (registration number AVD1080020174425) of Utrecht University (the Netherlands) and all procedures were done in full compliance with all relevant legislation.

### ***Funding***

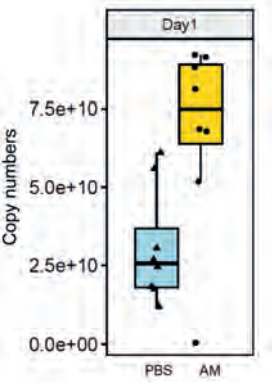
This work is part of the research programme of NWO Earth and Life Sciences (ALW) with project number 868.15.020, which is financed by the Dutch Research Council (NWO) and by Cargill Animal Nutrition and Health.

### ***Conflict of Interest Statement***

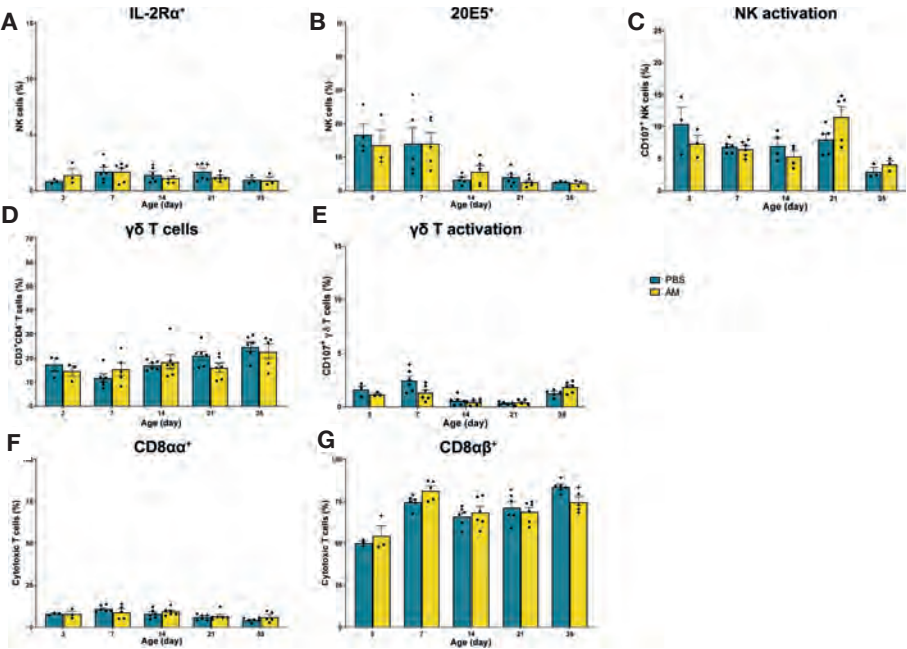
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Materials

**Figure S1.** Visualization of 16S rRNA gene count data in 1-day-old chickens as determined by qPCR. AM chickens (yellow) and controls (PBS, blue). The gene copy numbers in cecal content samples of AM chickens were higher compared to those found in control chickens ( $p = 0.021$ ).

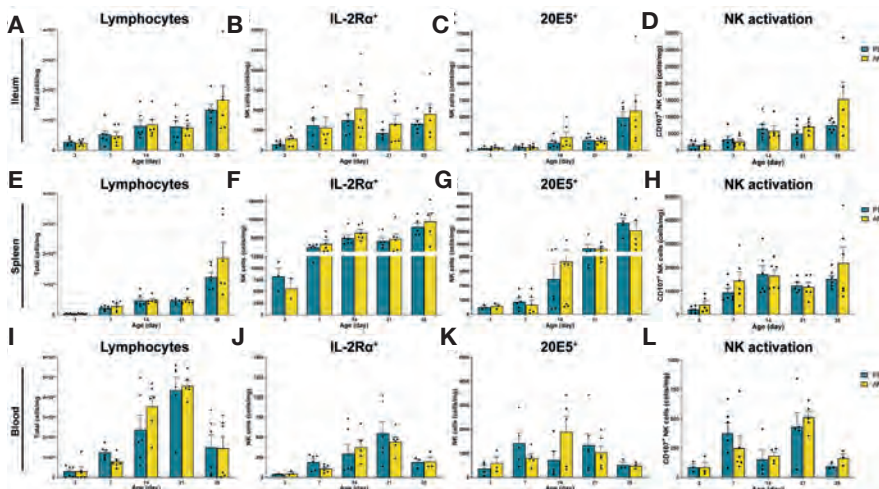


**Figure S2.** Effect of adult microbiota (AM) on NK and T cells in blood of broiler chickens. (A) Frequencies of NK cell subsets by characterization of surface markers IL-2R $\alpha$  and (B) 20E5 during aging in blood. (C) Frequencies of NK cell activity during aging as assessed by measuring the surface marker CD107. (D) Frequencies of total  $\gamma\delta$  T cells and (E)  $\gamma\delta$  T cell activation by characterization of surface markers TCR $\gamma\delta$  and CD107, respectively. (F) Frequencies of cytotoxic T cell subsets using the surface markers CD8 $\alpha\alpha$  and (G) CD8 $\alpha\beta$  during aging in blood. Mean + SEM of chickens is shown ( $n = 6$ ), however, chickens were excluded from analysis when number of events in the gate of interest was  $< 100$ .

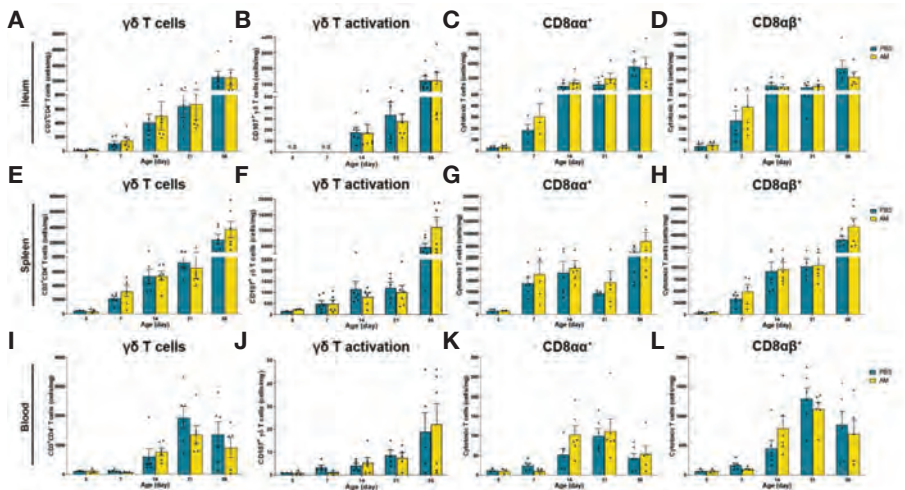




**Figure S3.** Effect of adult microbiota (AM) on NK cell numbers in broiler chickens. (A) Total cell numbers per mg organ isolated from IEL, (E) spleen and (I) blood. (B,F,J) Absolute NK cell numbers for subsets by characterization of surface markers IL-2R $\alpha$  and (C,G,K) 20E5 during aging in (A-D) IEL, (E-H) spleen and (I-L) blood. (D,H,L) Cell numbers for NK cell activation during aging as assessed by measuring the surface marker CD107. Mean + SEM of chickens is shown (n = 6), however, chickens were excluded from analysis when number of events in the gate of interest was < 100.



**Figure S4:** Effect of adult microbiota (AM) on T cell numbers in broiler chickens. (A,E,I) Absolute cell numbers for total  $\gamma\delta$  T cells and (B,F,J)  $\gamma\delta$  T cell activation by characterization of surface markers TCR $\gamma\delta$  and CD107, respectively, during aging in (A-D) IEL, (E-H) spleen and (I-L) blood. (C,G,K) Absolute cell numbers for cytotoxic T cell subsets by characterization of surface markers CD8 $\alpha\alpha$  and (D,H,L) CD8 $\alpha\beta$  during aging. Mean + SEM of chickens is shown (n = 6), however, chickens were excluded from analysis when number of events in the gate of interest was < 100.









# CHAPTER 9

## Effects of a competitive exclusion or dietary medium-chain fatty acids intervention to alter intestinal microbiota on broiler health and performance after subclinical intestinal challenge

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In preparation

## Abstract

Necrotic Enteritis (NE) is an intestinal disease causing large economic losses and severe health and welfare problems in commercial broilers. NE is caused by proliferation of pathogenic strains of *Clostridium perfringens* in combination with pre-disposing factors, including the disruption of the gut microbiota. It is still unknown whether the aberrant microbiota of diseased birds is a cause or effect in NE disease development. To study associations between microbiota and NE susceptibility, the intestinal microbiota of broilers was altered using a competitive exclusion (CE) product at day of hatch (D0) or medium-chain fatty acids (MCFA) fed from D0 onwards. A subclinical NE challenge was induced by oral inoculation with *Eimeria maxima* at D7 and *C. perfringens* at D14. Differences in the response to the NE challenge between CE and MCFA interventions, and negative (non-challenged, non-treated) and positive (challenged but non-treated) controls, were evaluated based on production performance, intestinal microbiota, intestinal lesions and microbial metabolites. The study included 720 broilers of which the intestinal microbiota of 240 broilers was assessed by 16S ribosomal RNA gene amplicon sequencing. Cecal concentrations of the microbial metabolites acetate, butyrate, formate, and propionate were determined using high-performance liquid chromatography. Subclinical NE challenge reduced growth performance between D7-D16 for challenged controls compared to non-challenged controls, indicating successful challenge. In CE broilers no effects on growth performance and metabolic output were observed directly after NE challenge compared to positive controls. Broilers fed MCFA showed reduced growth performance due to the NE challenge compared to negative controls, but less compared to positive controls and CE broilers. Microbial composition was different for CE and MCFA broilers compared to controls at D15. Also, the propionate concentration was lower in MCFA compared to CE broilers and controls. These results suggest that MCFA-induced alterations in the intestinal microbiota can improve the resistance of broilers against a subclinical NE challenge. Future research is needed to better understand underlying mechanisms and interactions between intestinal microbiota, nutrition, the immune system and pathogens.

**Keywords:** gut microbiota, poultry, microbiome, gut health, 16S rRNA

## Introduction

Several studies have revealed associations between intestinal microbiota composition, health status, and the production performance of broiler chickens (Stanley et al., 2012a, Yeoman et al., 2012, Rinttilä and Apajalahti, 2013, Johnson et al., 2018). The intestinal microbiota plays a key role in intestinal health and disturbances in intestinal health in broiler chickens are a common worldwide challenge (M'Sadeq et al., 2015). Necrotic Enteritis (NE) is an intestinal disease that is estimated to result in a substantial global annual economic loss of over US\$ 6 billion (Wade, 2015, Kaldhusdal et al., 2016). NE can present as an acute clinical disease, causing high broiler mortality, and as a subclinical (or chronic) condition. In subclinical form, NE is associated with poor digestion and absorption of nutrients, reduced weight gain and increased feed conversion ratio causing significant economic loss (Cooper and Songer, 2016). Both types are typically caused by the proliferation of *Clostridium perfringens* type A that expresses NetB toxins (Songer and Meer, 1996, Allaart et al., 2013, Moore, 2016).

In healthy poultry flocks, non-pathogenic *C. perfringens* strains are circulating at low levels (Timbermont et al., 2009, Lacey et al., 2016). For *C. perfringens* to cause NE, colonization and proliferation of pathogenic isolates and predisposing factors are needed (Moore, 2016). Such predisposing factors include: i) physical changes to the gut, ii) changes to bird immune status, and iii) disruption of the gut microbiota (Moore, 2016). It has been reported that broilers that developed NE showed differences in gut microbiota compared to broilers that remained healthy (Feng et al., 2010, Stanley et al., 2012b). Several studies have shown associations between the abundance of *Lactobacillus* and the development of NE (Antonissen et al., 2016, Li et al., 2017, Yang et al., 2019). For example, broilers with a co-infection of *C. perfringens* in conjunction with several species of *Eimeria* had a significantly reduced species diversity and relative abundance of *Lactobacillus* in jejunal microbiota compared to non-infected birds (Yang et al., 2019).

After the ban on the use of in-feed antimicrobial growth promoters (AGPs) in many countries, NE prevalence has increased (Van Immerseel et al., 2009, Gaucher et al., 2015) and has created a need for new interventions. With mounting evidence for associations between intestinal microbiota and NE disease development, an improved understanding of the interaction of the intestinal microbiota and the development of NE may provide useful tools to reduce NE disease impact (Antonissen et al., 2016). It has not been demonstrated whether the differences in the microbiota of diseased birds result from the disease process, or whether some underlying types of microbiota make birds more susceptible to disease and that within the birds with “susceptible microbiota”, disease is more likely to develop (Moore et al., 2016, Antonissen et al., 2016).

In this study we used two commercially available products known to be able to alter the intestinal microbiota, but with different modes of action. One of these

products is a competitive exclusion (CE) product. Previous studies have shown that CE products were effective in reducing *C. perfringens* associated NE gross lesions (Hofacre et al., 1998) and colonization (Abudabos, 2013). It is used commercially to reduce susceptibility for the colonization of pathogens and has also shown to be able to accelerate the maturation of intestinal microbiota (Hofacre et al., 1998, Chapter 7 and 8 of this thesis). The second intervention to alter microbiota is a feed containing medium-chain fatty acids (MCFA). MCFA blends have shown to induce a significant shift in intestinal microbiota composition (Van der Hoeven et al., 2013, Kers et al., 2019b). Furthermore, MCFA and their esters are known to have antimicrobial properties and inhibit the proliferation of *C. perfringens* (Kabara and Marshall, 2005, Lensing et al., 2010, Yang et al., 2019).

The objective of this study was to determine if an altered intestinal microbiota, via the CE principle or a nutritional intervention with MCFA, can affect disease resistance of broilers to a subclinical NE challenge. NE was induced using a previously proven model with oral inoculation of *Eimeria maxima* at 7 days of age and *C. perfringens* at 14 days of age. Differences in the response to the intestinal challenge between interventions and negative (non-challenged, non-treated) and positive (challenged but non-treated) controls were assessed based on production performance, intestinal microbiota composition, intestinal lesions and microbial fermentation metabolites.

## **Materials and Methods**

### ***Ethics approval***

The study protocol was approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee (registration number AVD220002016387) and all procedures were done in full compliance with all relevant legislation. The broilers were observed twice a day, and the presence of clinical signs, abnormal behavior and mortality was recorded.

### ***Experimental design***

At the start of the experiment (day 0) 720 one-day-old male Ross 308 broiler chickens, originating from a parent flock of 54 weeks of age, were obtained from a commercial hatchery. Broilers were divided into four groups and randomly assigned to 12 replicate pens, each with 15 broilers, using a randomized complete block design. The two control groups included non-challenged, non-treated broilers (negative control group, NC) or challenged and non-treated broilers (positive control group, PC). Broilers in the intervention groups received either a CE product at day of hatch or were fed a diet containing MCFAs from day 0 until the end of the experiment at day 34.

### ***Necrotic enteritis challenge***

Broilers in the NC group were inoculated with 1 mL of sterile saline on day 7, and 1 mL of sterile liver broth (Difco, Detroit, MI) on day 14. Broilers in the PC group and intervention groups were orally inoculated with 1 mL tap water containing 4500 sporulated *E. maxima* oocysts on day 7 (Weybridge strain, GD Animal Health, Deventer, the Netherlands), followed by an oral inoculation with 1 mL liver broth containing a dose of  $10^8$  colony forming units of *C. perfringens* type A (Strain GD 5.11.53, GD Animal Health, Deventer, the Netherlands) on day 14. The alpha, beta2 and NetB toxin positive *C. perfringens* strain, isolated from chickens with NE, was used in combination with the same *E. maxima* strain previously used in similar trials to successfully induce a subclinical NE challenge (Lensing et al., 2010).

### ***Competitive exclusion and nutritional intervention***

Broilers of the CE intervention group received the CE product, Aviguard (MSD Animal Health, Boxmeer, the Netherlands) a freeze-dried product derived from intestinal microbiota of healthy, specified pathogen-free birds and manufactured by fermentation. At the start of the experiment, the product was suspended in water (dosed according to the manufacturer's instructions) and sprayed on the day-old-chicks before placement in the pens. To avoid cross contamination of the CE product to broilers in other groups, CE broilers were sprayed in a separate room. Another group of broilers received a diet containing 2% of MCFA (Grolux Synergy, Cargill, Rotterdam-Botlek, the Netherlands). All broiler chickens received a wheat-based starter feed from day 0 to 16 (1.05 dLys and 2825 kcal ME Broiler; pelleted 2.5 mm) and grower feed from day 16 until day 34 (1.02 dLys and 2900 kcal ME Broiler; pelleted 3.0 mm). Diets were formulated based on digestibility and nutrient data provided by Feed Tables from the Dutch Central Bureau of Livestock Feeding (CVB, 2017). No antimicrobial additives or coccidiostatic drugs were applied.

### ***Housing and management***

Pens (2.26 m (l) × 0.90 m (w), 2.03 m<sup>2</sup>) were lined with 2 cm of wood shavings and separated from each other using steel mesh panels that did not allow direct contact between birds from adjacent pens. The 48 pens were divided across two rooms. Each pen was equipped with two nipple drinkers adjustable in height. Artificial lighting was set at 23 h/day (h/d) from day 0 to 3, 20 h/d from day 4 to 6 and 18 h/d from day 7 to 34. Temperature was gradually decreased from 34 °C at day 0 with 2.5 °C per week to 20 °C at day 34. On day 3 and day 21 all broilers were vaccinated against Newcastle Disease virus (Avinew Neo, Boehringer Ingelheim, Alkmaar, the Netherlands) with a battery-operated backpack sprayer. In advance of the study, the facility was cleaned and disinfected with quaternary ammonium compounds and glutaraldehyde (MS Megades, Schippers, Bladel, the Netherlands).

### **Data collection**

Feed intake at pen level and body weight (BW) of individual birds were measured on days 7, 14, 16, 21, 28 and 34. Average daily gain (ADG), average daily feed intake (ADFI) and gain to feed ratio (G:F) were calculated based on the BW gain and feed intake per pen. The intestinal content was collected according to procedures described by Kers et al. (2019b). On days 7 (before challenge), 21, 28 and 34 one broiler was sacrificed per pen and on day 15, the day after *C. perfringens* inoculation, two broilers were sacrificed per pen. In-between individual bird samplings, sterile gloves were changed and the table, scissors, and tweezers were cleaned with ethanol 70% to prevent cross-contamination between intervention groups. Three different intestinal scores were measured to assess intestinal health on days 15, 21, 28 and 34.

The first clinical intestinal scoring was the *C. perfringens* lesions score (Lensing et al., 2010), categorizing the severity of NE on a scale from 0 to 4 with 0 = no lesions, 1 = 1 to 5 small white lesions (spots of less than 1 mm in diameter), 2 = more than 5 small white lesions (spots of less than 1 mm in diameter) or 1 to 5 larger lesions (spots of 1 to 2 mm in diameter), 3 = more than 5 larger lesions (1 to 2 mm in diameter) or erosive zones and 4 = dead birds with positive NE diagnosis at post mortem. Scoring was done in the duodenal and jejunal segment of the small intestine. The second clinical lesion scoring used was the coccidiosis lesion score (CLS) to quantify lesions in the jejunum caused by *E. maxima* (Johnson and Reid, 1970). CLS was quantified on a scale from 0 to 4, with 0 score for absence of lesions, 1 and 2 for few to more numerous petechiae and score 3 and 4 with intestinal wall thickening and ballooning, and mucous and blood clots in the lumen of parts or most of the length of the jejunum. The third clinical lesion scoring was based on a macroscopic scoring system referred to as gut score (GS, Teirlynck et al., 2011). The GS score system consists of ten parameters, assessed using a binary system, with or without the presence of: (1) ballooning of the gut; (2) significant redness or dilated blood vessels cranial or (3) caudal to Meckel's diverticulum; (4) reduced gut wall thickness or increased fragility of the gut cranial or (5) caudal to Meckel's diverticulum; (6) reduced tonus (flaccidness) of the gut cranial or (7) caudal to Meckel's diverticulum; (8) abnormal appearance of the contents in the lumen of the gut cranial or (9) caudal to Meckel's diverticulum; and (10) undigested feed particles caudal to the ileocecal junction (2). The GS score was 0, when no gut abnormalities were found, and ranged to 10, indicating the presence of all ten gut abnormalities included in the scoring system.

On days 7, 10, 15, 21, 28 and 34, pooled fecal droppings were collected from the litter of each pen to measure the number of *Eimeria* oocysts per gram of feces (OPG) using a modification of a McMaster oocyst counting chamber technique as previously described (Velkers et al., 2010).

On day 15 one cecal content sample of each pen was used to measure the concentrations of acetate, butyrate, isobutyrate, lactate, propionate and formate us-



ing high-performance liquid chromatography (HPLC) as described previously (Kers et al., 2019b).

### ***DNA isolation and microbiota profiling***

In total, 240 cecal content and 72 ileal content samples were analyzed. A detailed description of the DNA isolation and determining the microbiota composition has been described in detail elsewhere (Kers et al., 2019b). In short, DNA was extracted from 0.25 g content, using 700 µL of Stool Transport and Recovery (STAR) buffer (Roche Diagnostics Nederland BV, the Netherlands) and repeated bead beating. The DNA concentrations were measured, and extracted DNA was stored at -20 °C until further use. Barcoded amplicons covering the variable regions V5-V6 and primers 784F and 1064R were used for 16S rRNA amplicon sequencing as described previously (Ramiro-Garcia et al., 2016). To ensure high quality sequencing data, synthetic communities of known composition were used as positive controls (Ramiro-Garcia et al., 2016), and nuclease free water as negative controls. Sequencing of resulting libraries was performed by Eurofins (Genomics Germany GmbH) on Illumina HiSeq2500. The 16S rRNA data was analyzed using NG-tax 2.0 (Poncheewin et al., 2020). Briefly, to generate amplicon sequence variants (ASVs), NG-Tax 2.0 employs a fast de novo ASV-picking algorithm. To assign taxonomy the SILVA 128 16S rRNA gene reference database was used (Quast et al., 2013).

### ***Statistical analysis***

All statistical analyses were performed in R, using the packages: Phyloseq, Microbiome, Vegan and RVAideMemoire (McMurdie and Holmes, 2013, Lahti et al., 2017, Oksanen, 2010, Pinheiro, 2018).

Growth performance data (BW, ADG, ADFI and G:F) were analyzed using a mixed model:  $Y_{ij} = \mu + \alpha_i + B_j + E_{ij}$ , where  $Y_{ij}$  is the dependent variable,  $\mu$  the overall mean,  $\alpha_i$  the fixed effect of treatment ( $i = \text{PC, NC, CE or MCFA}$ ),  $B_j$  the random block effect ( $j = 1, 2, 3, \dots, 12$ ) and  $E_{ij}$  the residual error term.

Microbial alpha diversity (within sample) was determined using Faith's phylogenetic diversity, observed number of ASV and Shannon index. Beta diversity (divergence in microbiota composition between samples) was determined using Bray-Curtis, Jaccard, weighted (WUF) and unweighted UniFrac (UF) distance metrics (Bray and Curtis, 1957, Jaccard, 1912, Lozupone et al., 2007). Differences in alpha diversity between groups were tested with a Kruskal-Wallis test and pairwise comparisons were tested using a Wilcoxon rank-sum test and corrected for multiple testing with Benjamini Hochberg (BH). Differences in beta diversity between groups were tested using Tukey honest significant differences. The variation between cecal microbiota samples was visualized using principal coordinates analysis (PCoA), and non-parametric permutational analysis of variance (PERMANOVA) tests were used to analyze

group differences within multivariate community data (Anderson, 2001). To test for differences in relative abundance of genera between two interventions, we used a Wilcoxon rank-sum test and corrected for multiple testing with BH.

In addition, WUF and UF distance-based redundancy analysis (db-RDA), a multivariate canonical ordination analysis method was performed (Shankar et al., 2017). The models were performed based on microbiota information of the individual broiler, and the explanatory variables: individual body weight, group, individual intestinal health score (NE, CLS and GS score) and the OPG per pen (after  $\log_{10}$  transformation to normalize the data,  $\log_{10}(\text{OPG}+1)$ ). The concentrations of acetate, butyrate, isobutyrate, lactate and propionate were included in the model on day 15. To determine the most parsimonious constrained ordination model, a stepwise selection (both directions) was used based on Akaike information criterion (AIC) selection.

## Results

### *Growth performance*

Performance did not differ among groups from day 0 to 7 prior to NE challenge (Table 1). Inoculation with *E. maxima* at day 7 reduced ADG and ADFI and worsened G:F from day 7 to 14 for all NE challenged groups compared to the NC group ( $p < 0.001$ ; Table 1). A similar response was observed for BW at day 14. However, within the challenged intervention groups the ADG and ADFI of the MCFA group were higher ( $p < 0.001$ ; Table 1) than for the PC, with the CE group in-between. Also, BW at day 14 was higher ( $p < 0.001$ ; Table 1) for the MCFA group compared to both the PC and CE groups.

Inoculation with *C. perfringens* at day 14 lowered ADG and ADFI between day 14 to 16 for the PC and CE groups compared to the NC, with the MCFA group in-between (Table 1). Nevertheless, BW at day 16 was lower for all NE challenged groups compared to the NC group, but higher for the MCFA group compared to both the PC and CE groups ( $p < 0.001$ ; Table 1).

Between day 16 to 34, ADG was higher ( $p = 0.034$ ) for the MCFA group compared to the NC group, with the PC and CE groups in-between (Table 1). In the same age period, G:F ratio was higher for all NE challenged groups compared to the NC control group ( $p = 0.001$ ; Table 1). During this age period, BW was lowered at day 21 for the PC and CE groups compared to the NC group, with the MCFA group in-between ( $p = 0.001$ ; Table 1).

Between day 0 to 34, G:F ratio was higher for the MCFA group compared to all other groups ( $p < 0.001$ ; Table 1), whereas BW at day 34, nor ADG and ADFI from day 0 to 34, were affected. Challenge with *E. maxima* or *C. perfringens* did not affect mortality in any of the groups (data not reported).

**Table 1:** Effect of dietary treatment on mean body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), and gain to feed ratio (G:F) of broiler chickens from 0 to 34 days of age across different interventions groups.

Variable	NC - No NE	PC - NE	CE	MCFA	SEM	P-Value
BW 0d, g	41.8	41.9	42.3	42.2	0.2	0.304
BW 7d, g	161	162	160	166	2	0.083
BW 14d, g	406 <sup>c</sup>	357 <sup>a</sup>	362 <sup>a</sup>	380 <sup>b</sup>	5	<b>&lt;.001</b>
BW 16d, g	505 <sup>c</sup>	442 <sup>a</sup>	448 <sup>a</sup>	475 <sup>b</sup>	6	<b>&lt;.001</b>
BW 21d, g	796 <sup>b</sup>	748 <sup>a</sup>	746 <sup>a</sup>	783 <sup>ab</sup>	10	<b>0.001</b>
BW 28d, g	1322	1315	1305	1347	20	0.358
BW 34d, g	1931	1940	1939	1987	29	0.420
ADG 0-7d, g	16.9	17.0	16.7	17.5	0.3	0.227
ADFI 0-7d, g	17.7	17.7	17.8	18.1	0.3	0.601
G:F 0-7d	0.963	0.966	0.958	0.982	0.007	0.082
ADG 7-14d, g	35.5 <sup>c</sup>	27.8 <sup>a</sup>	28.8 <sup>ab</sup>	30.6 <sup>b</sup>	0.6	<b>&lt;.001</b>
ADFI 7-14d, g	43.9 <sup>c</sup>	37.6 <sup>a</sup>	39.4 <sup>ab</sup>	39.8 <sup>b</sup>	0.6	<b>&lt;.001</b>
G:F 7-14d	0.808 <sup>b</sup>	0.739 <sup>a</sup>	0.745 <sup>a</sup>	0.770 <sup>ab</sup>	0.012	<b>&lt;.001</b>
ADG 14-16d, g	50.7 <sup>b</sup>	43.7 <sup>a</sup>	44.3 <sup>a</sup>	47.6 <sup>ab</sup>	1.2	<b>&lt;.001</b>
ADFI 14-16d, g	63.7 <sup>b</sup>	57.9 <sup>a</sup>	57.9 <sup>a</sup>	60.6 <sup>ab</sup>	1.4	<b>0.012</b>
G:F 14-16d	0.799	0.758	0.795	0.774	0.020	0.420
ADG 16-34d, g	79.1 <sup>a</sup>	83.1 <sup>ab</sup>	82.4 <sup>ab</sup>	83.8 <sup>b</sup>	1.3	<b>0.034</b>
ADFI 16-34d, g	114	117	114	117	2	0.475
G:F 16-34d	0.696 <sup>a</sup>	0.711 <sup>b</sup>	0.712 <sup>b</sup>	0.714 <sup>b</sup>	0.004	<b>0.001</b>
ADG 0-34d, g	52.0	51.2	51.4	52.4	0.7	0.616
ADFI 0-34d, g	70.2	69.9	70.1	70.5	1.0	0.974
G:F 0-34d	0.732 <sup>a</sup>	0.732 <sup>a</sup>	0.732 <sup>a</sup>	0.743 <sup>b</sup>	0.002	<b>&lt;.001</b>

<sup>a,b,c</sup>Means within the same row with different superscripts differ ( $p$ -value <0.05). Each group consisted of 12 pens with 15 male broilers at the start of the experiment. Body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), and gain to feed ratio (G:F). Interventions: not challenged with Necrotic Enteritis (NE) nor treated (NC), challenged with NE but not treated (PC), challenged with NE and receiving either a competitive exclusion product at day of hatch (CE) or broilers challenged with NE and fed a diet containing medium chain fatty acids (MCFA).

### **Alpha and beta diversity of the intestinal microbiot**

To determine effects of the NE challenge on cecal microbial diversity, the NC and PC groups were compared with each other. To assess the effect of the interventions on microbiota diversity upon challenge, the two intervention groups (CE, MCFA) were compared to PC, as these broilers also received the NE challenge.

On days 7, 21, 28 and 34 no differences were observed in alpha diversity among

the groups between the cecal or ileal content (Table 2). On day 15 the observed number of ASVs and the Shannon index were higher in the cecal content of broilers in the NC group compared to the PC group (Table 2). In broilers of the CE group the observed number of ASVs was higher compared to PC broilers. In the broilers fed the MCFA diet the Shannon index was higher compared to PC.

Beta diversity was determined using Jaccard, Bray-Curtis and unweighted (UF) and weighted UniFrac (WUF) measures. On day 7 no differences in cecal microbial composition were observed between the NC and PC groups and the MCFA intervention (Table 3). There was, however, a difference between the PC and the CE broilers based on UF distances (Table 3,  $R^2 = 7.8\%$ ,  $p = 0.023$ ). This indicated the presence of different phylogenetically distinct ASVs between these groups. On day 15, the cecal microbiota composition was different between the PC and the MCFA broilers. When taking the phylogenetic information into account, the microbiota composition of PC and CE broilers was different across all days (Table 3). The microbiota composition of the PC versus CE broilers remained phylogenetically different until the end of the experiment (UF, Table 3). Only on day 15, a significantly lower dissimilarity in microbiota composition between broilers was found within the PC group compared to the NC group based on Bray-Curtis and Jaccard distances (Fig. 1,  $p$ -adjusted 0.025, and 0.018; data from other days are not shown). Ileal content samples showed no difference in beta diversity across interventions on day 21 (Table 3).

### **Difference in relative abundance of microbial taxa across all groups**

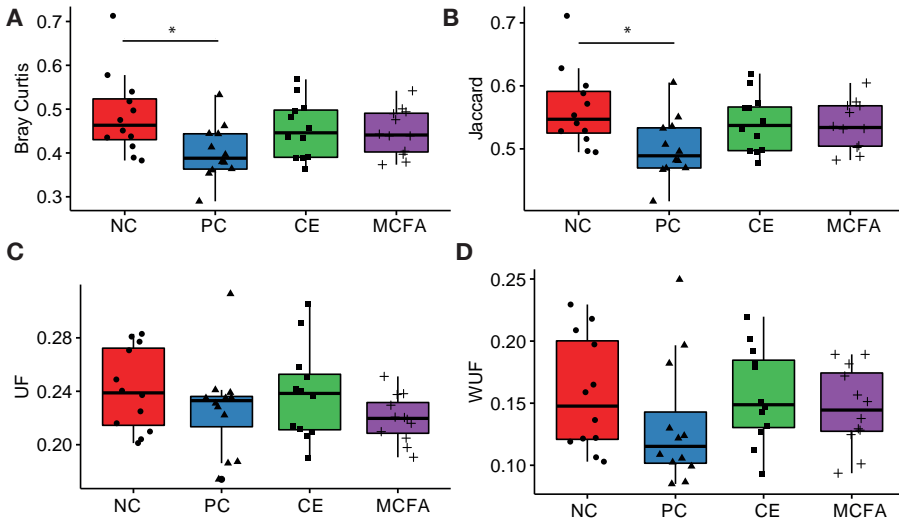
Across intervention and age group, cecal microbiota of the broilers was dominated by the families *Lactobacillaceae*, *Lachnospiraceae* and *Ruminococcaceae* (Sup. Figure S1). On day 7 no significant differences in the relative abundance of any taxa at genus level in the cecal microbiota were observed among the NC, PC, CE or MCFA groups. On day 15, the relative abundance of the genus *Lactobacillus* was higher and of *Ruminiclostridium\_9* and *Defluviitaleaceae\_UCG-011* lower in PC compared to NC broilers (Fig. 2a). In the MCFA broilers the relative abundance of *Lactobacillus* was lower compared to PC broilers on day 15 (Fig. 2a). In CE broilers the relative abundance of the genus *Collinsella* was higher compared to the other groups on days 15, 21 and 28. In CE broilers compared to NC, *Bacteroides* was higher in relative abundance (Fig. 2a). On day 28 in CE broilers, *Faecalicoccus* and *Senegalimassilia* were higher in relative abundance compared to NC and PC broilers (Fig. 2b). In broilers with the CE intervention, *Olsenella* (day 28) was higher abundant compared to NC (Fig. 2b). No other significant differences based on relative abundance of individual genera were observed between the four groups.

**Table 2:** Alpha diversity measurements of the cecal microbiota of broilers at day 7, 15, 21, 28, and 34. Each group consisted of 12 male broilers. Means in the same row with different superscripts across intervention differ ( $P_{\text{adj}} < 0.05$ ), SD between brackets. Pairwise comparisons using Wilcoxon rank sum test corrected with BH. Interventions: not challenged with Necrotic Enteritis (NE) nor treated (NC), challenged with NE but not treated (PC), challenged with NE and receiving either a competitive exclusion product at day of hatch (CE) or broilers challenged with NE and fed a diet containing medium chain fatty acids (MCFA). ASV = amplicon sequence variance.

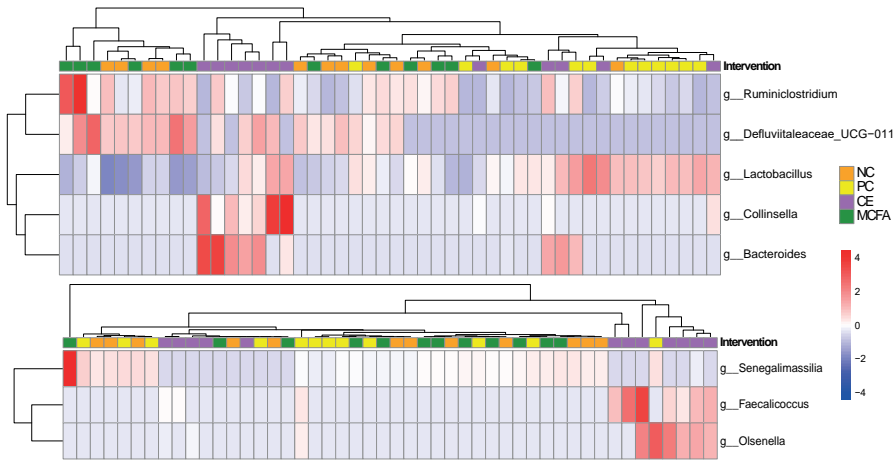
<b>Intervention</b>	<b>Phylogenetic diversity</b>		<b>Observed number ASV</b>		<b>Shannon</b>	
<b>Day 7</b>	<b>Mean</b>	<b>SD</b>	<b>Mean</b>	<b>SD</b>	<b>Mean</b>	<b>SD</b>
NC	17.86	(3.25)	100.00	(22.86)	3.36	(0.61)
PC	17.88	(2.12)	98.67	(20.28)	3.53	(0.30)
CE	18.70	(2.07)	104.50	(18.96)	3.57	(0.44)
MCFA	16.73	(2.09)	94.58	(20.48)	3.52	(0.43)
<b>Day 15</b>						
NC	24.63	(2.13)	146.17 <sup>b</sup>	(26.53)	4.00 <sup>b</sup>	(0.30)
PC	22.53	(2.27)	116.41 <sup>ac</sup>	(13.95)	3.43 <sup>a</sup>	(0.26)
CE	23.66	(1.69)	133.17 <sup>b</sup>	(17.11)	3.69 <sup>ab</sup>	(0.46)
MCFA	23.54	(1.08)	130.42 <sup>bc</sup>	(15.12)	3.97 <sup>b</sup>	(0.17)
<b>Day 21 cecal content</b>						
NC	23.96	(2.11)	138.42	(19.04)	4.02	(0.17)
PC	25.32	(1.46)	150.33	(17.57)	4.22	(0.26)
CE	24.87	(2.54)	135.42	(26.10)	4.03	(0.35)
MCFA	23.84	(1.79)	131.83	(17.08)	3.97	(0.26)
<b>Day 21 ileal content</b>						
NC	4.42	(3.17)	48.25	(16.27)	1.93	(0.38)
PC	3.23	(1.36)	53.17	(29.95)	2.05	(0.68)
CE	3.26	(1.74)	43.01	(18.18)	1.81	(0.37)
MCFA	3.72	(1.18)	40.58	(19.93)	1.62	(0.46)
<b>Day 28</b>						
NC	26.21	(1.21)	157.83	(20.11)	4.30	(0.21)
PC	25.59	(1.53)	145.00	(13.46)	4.10	(0.15)
CE	25.03	(2.77)	142.33	(25.17)	4.10	(0.23)
MCFA	25.31	(2.24)	144.33	(17.85)	4.16	(0.17)
<b>Day 34</b>						
NC	25.60	(2.37)	144.42	(21.00)	4.09	(0.29)
PC	25.51	(2.49)	142.33	(22.04)	4.11	(0.23)
CE	25.12	(1.88)	138.50	(13.50)	3.94	(0.27)
MCFA	25.46	(2.26)	140.50	(21.05)	4.13	(0.30)

**Table 3:** Beta diversity stratified per different timepoints and interventions.  $R^2$  = Percentage of the variation between chickens explained,  $p$  = value PERMANOVA test. UF unweighted unfrac. WUF weighted UniFrac

	Bray-Curtis			Jaccard			UF			WUF	
NC vs PC	R <sup>2</sup>	p-val		R <sup>2</sup>	p-val		R <sup>2</sup>	p-val		R <sup>2</sup>	p-val
Day 7	0.033	0.876		0.038	0.833		0.043	0.453		0.022	0.897
Day 15	0.084	0.010	*	0.068	0.012	*	0.090	0.004	**	0.166	0.001 ***
Day 21	0.041	0.504		0.043	0.469		0.039	0.664		0.048	0.343
Day 21 ileal	0.037	0.439		0.043	0.397		0.048	0.316		0.048	0.313
Day 28	0.030	0.833		0.036	0.803		0.030	0.960		0.056	0.211
Day 34	0.029	0.889		0.033	0.930		0.043	0.427		0.036	0.588
NC vs CE											
Day 7	0.029	0.934		0.034	0.933		0.049	0.254		0.032	0.705
Day 15	0.053	0.174		0.049	0.178		0.081	0.002	**	0.142	0.002 **
Day 21	0.052	0.156		0.048	0.175		0.064	0.035	*	0.132	0.002 **
Day 21 ileal	0.024	0.703		0.037	0.759		0.079	0.070	.	0.029	0.534
Day 28	0.045	0.329		0.046	0.287		0.098	1e-4	***	0.178	1e-4 ***
Day 34	0.044	0.338		0.045	0.307		0.072	0.006	**	0.119	0.008 **
NC vs MCFA											
Day 7	0.051	0.197		0.049	0.186		0.058	0.108		0.054	0.237
Day 15	0.050	0.228		0.047	0.264		0.106	3e-4	***	0.065	0.136
Day 21	0.038	0.702		0.040	0.745		0.040	0.594		0.037	0.556
Day 21 ileal	0.043	0.387		0.418	0.443		0.020	0.884		0.029	0.498
Day 28	0.028	0.914		0.033	0.916		0.033	0.987		0.023	0.914
Day 34	0.041	0.471		0.041	0.511		0.061	0.064	.	0.071	0.092 .
PC vs CE											
Day 7	0.034	0.837		0.038	0.833		0.078	0.023	*	0.016	0.983
Day 15	0.056	0.153		0.051	0.153		0.092	0.001	***	0.085	0.047 *
Day 21	0.045	0.339		0.044	0.366		0.066	0.007	**	0.134	0.001 **
Day 21 ileal	0.036	0.496		0.040	0.467		0.044	0.393		0.070	0.189
Day 28	0.037	0.584		0.040	0.526		0.067	0.024	*	0.070	0.109
Day 34	0.044	0.368		0.046	0.288		0.085	0.001	**	0.128	0.002 **
PC vs MCFA											
Day 7	0.044	0.431		0.044	0.427		0.038	0.621		0.041	0.478
Day 15	0.103	0.003	**	0.079	0.004	**	0.096	1e-4	***	0.234	1e-4 ***
Day 21	0.050	0.217		0.047	0.226		0.053	0.149		0.087	0.019 *
Day 21 ileal	0.070	0.135		0.064	0.127		0.061	0.191		0.070	0.194
Day 28	0.032	0.758		0.038	0.680		0.041	0.569		0.072	0.093
Day 34	0.038	0.533		0.040	0.539		0.072	0.019	*	0.051	0.286



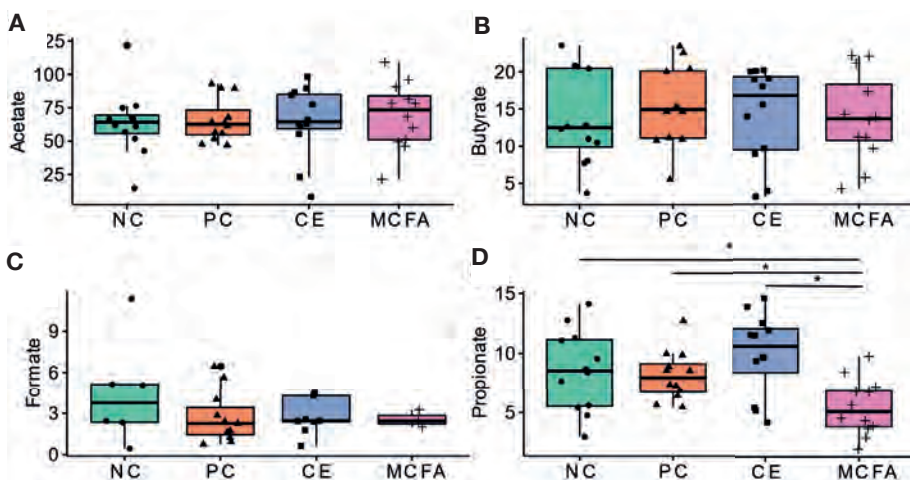
**Figure 1:** Divergence in microbial composition between broilers from the same group on day 15. Higher distance measures indicate higher dissimilarity, where 1 represents a completely dissimilar bacterial community. A. Bray–Curtis distances between the microbiota in cecal samples across the four groups. The PC broilers showed a significant lower dissimilarity compared to NC broilers ( $p$ -adjusted 0.025). B. Jaccard distances, PC showed a significant lower dissimilarity compared to NC ( $p$ -adjusted 0.018). C. Unweighted UniFrac (UF) D. Weighted UniFrac (WUF).



**Figure 2:** Heatmap of the genera that were significantly different in relative abundance between broilers. Heatmap of all individual broiler chickens ( $n = 48$ , 15 days old). The genera that are significant different between the intervention are shown A. Day 15 B. Day 28 (Wilcoxon rank-sum test, adjusted  $p$ -values are corrected for multiple testing, BH,  $p < 0.05$ ). Each red- white -blue dot represents the relative abundance of genera of an individual broiler chicken, NC, PC, CE, MCFA (orange, yellow, purple, green). Clustering of broilers is based on Ward's minimum variance method and based on weighted UniFrac distances matrix.

### Metabolic output of the ceca across groups on day 15

Concentrations of acetate, butyrate, and formate were not different across groups (Fig. 3a-c). A low concentration of formate was measured in 28 out of the 48 samples; in six of the NC broilers, in all 12 PC broilers, in seven of the CE broilers and in three of the MCFA broilers (Fig. 3c). The concentration of propionate was lower in MCFA broilers compared to all other groups (Fig. 3d). Isobutyrate and lactate were only measured in a small subset of the samples (5/48 and 15/48) and therefore not included in the results.



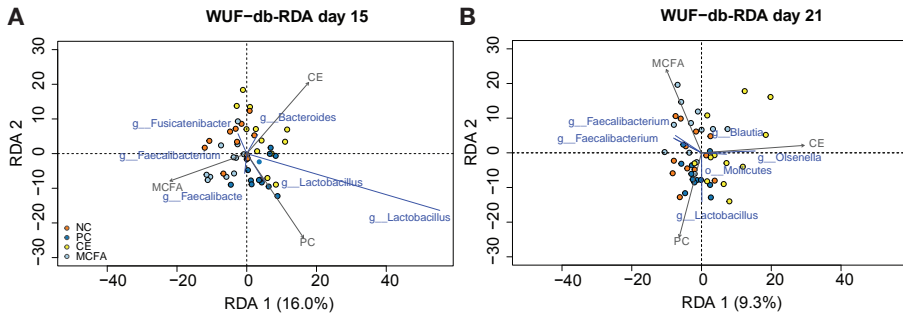
**Figure 3:** Acetate, butyrate, formate and propionate concentrations in the cecal content across groups on day 15. Each group contained 12 broilers of 15 days old. All concentration in mM. A. Acetate B. Butyrate C. Formate and D. Propionate. Formate was detected in 6 NC broilers, in all 12 broilers of the PC group and in 7 CE and 3 MCFA broilers. Propionate was lower in MCFA broilers compared to all other groups ( $p = 0.048$ ,  $p = 0.025$ ,  $p = 0.022$ , based on pairwise comparisons using Wilcoxon rank sum test corrected for multiple testing with BH  $< 0.05$ ).

### Clinical intestinal parameters and the cecal microbiota

In order to assess whether clinical parameters are associated with variation in cecal microbiota composition, UF and WUF db-RDA were performed. These analyses allowed to determine the relative impact of the intervention, BW, NE score, CLS score, GS score and OPG on microbiota composition of day 15 and 21. At day 15, the most parsimonious model to explain the variation of the cecal microbiota included only group ( $F = 3.82$ ,  $p = 0.005$ ) and explained 22.0% of the cecal microbiota variation (Fig. 4). BW ( $F = 2.06$ ,  $p = 0.055$ ), NE score ( $F = 0.74$ ,  $p = 0.635$ ), CLS score ( $F = 0.89$ ,  $p = 0.530$ ), GS ( $F = 1.02$ ,  $p = 0.335$ ) and OPG ( $F = 2.08$ ,  $p = 0.065$ ) were not included in the WUF-db-RDA model. Important ASVs (classified at genus level) for the ordination were members of *Fusicatenibacter* in NC broilers, *Lactobacillus* in PC broilers, *Bacteroides* in CE and *Faecalibacterium* in MCFA broilers (Fig. 4a). The



UF-db-RDA resulted in the same model, with only group included as variable, but only explained 14.1% of the cecal microbiota variation. An overview of the NE, CLS and GS scores shows that only low numbers of broilers had a positive NE score, i.e. a score above 0, in both control groups (8%) and that generally low mean CLS and GS scores were found in all groups (Sup. Table S1). Results at day 21 were comparable to the results of day 15, with the most parsimonious model to explain the variation of the cecal microbiota also including only the variable group and none of the clinical parameters (WUF-db-RDA, Fig. 4b) and explained 14% of the variation.



**Figure 4:** Weighted UniFrac distance based redundancy analysis. Triplot for weighted UniFrac distance based redundancy analysis (WUF-db-RDA) of cecal microbiota composition. The longer the arrow length, the stronger the correlation. Points are colored by intervention, ASVs labels are on lowest known taxonomic rank. A. day 15 B. day 21.

## Discussion

In this study we used two different interventions aimed at altering the broilers intestinal microbiota, one following the competitive exclusion principle at day 0, whereas the second included a MCFA nutritional intervention that was fed from day 0 onwards. Next, broilers were inoculated with *E. maxima* and *C. perfringens* at day 7 and 14 respectively to induce a subclinical NE challenge. The NE challenge had the largest effect on production performance, microbiota composition and clinical parameters directly after *C. perfringens* inoculation.

The lowered production performance in the NE challenged broilers of the PC group compared to the non-challenged broilers in the NC group indicates that the challenge successfully induced subclinical NE. Moreover, a lower dissimilarity in microbiota was observed in the PC compared to the NC group, indicating that the NE challenge affected the microbiota towards a more homogeneous composition. A lower dissimilarity has been associated with an unhealthy microbiota composition (Manichanh et al., 2006, Bäckhed et al., 2012). However, this effect was only observed based on Bray-Curtis and Jaccard distances and not when the phylogenetic information of the microbiota composition was taken into account. This indicates that the NE challenge model affected the microbiota composition but

that the homogenization of the microbiota concerned mostly phylogenetically similar microorganisms.

Especially on day 15, when the NE challenge showed its highest impact, differences in microbiota composition between CE and MCFA broilers compared to PC broilers could be observed. Also, differences in relative abundance of specific genera for the unchallenged and challenged controls were different than those observed for CE and MCFA compared to PC broilers. This suggests that, although a clear effect on microbiota composition remained undetectable before day 15, the interventions successfully induced alterations in intestinal microbiota and affected the response to the NE challenge. An explanation for this may be the use of cecal content to evaluate microbiota composition, as in previous similar studies more clear effects were detected in ileal and jejunal content, where *Eimeria* and *C. perfringens* induce the most severe lesions (Lin et al., 2017, Yang et al, 2019).

The CE product did not result in any positive or negative effect on production performance compared to PC broilers. Reduced losses in production performance in NE challenged broilers were observed in a previous study with CE (Hofacre et al., 1998), but in a more recent study no effects on production performance were found, whereas mortality and NE lesion scores were reduced (Hofacre et al., 2019). In this experiment, an effect of CE on mortality and NE lesion scores was not found, but as the PC and NC groups were also not different for these parameters, this may have been a result of the subclinical character of the NE challenge. A higher relative abundance of the genus *Enterococcus* has also been described in broilers that received CE (Pedroso et al., 2014). Although we did not observe this difference, we did observe differences in *Collinsella*, *Bacteroides*, *Faecalicoccus*, *Senegalimassilia*, and *Olsenella* compared to the NC group, and *Collinsella*, *Faecalicoccus*, and *Senegalimassilia* compared to the PC group. The genus *Collinsella* is associated with altering the host's metabolism (Gomez-Arango et al., 2018, Candela et al., 2016). However, beside the lower number of broilers where formate was detected in cecal content, i.e. in 7 of 12 CE broilers, instead of in all 12 broilers in the PC group, no differences in metabolic output (concentrations of acetate, butyrate, formate, and propionate) were observed in the cecal content of CE broilers, which again may have been as a result of the subclinical character of the NE challenge.

In contrast, in broilers fed MCFA the loss in production performance due to the subclinical NE intestinal challenge was clearly reduced when compared to the PC and CE groups. Improved production performance due to feeding MCFA or MCFA esters has been shown before (Lamot et al., 2016), also in studies with NE challenge (Lensing et al 2010). The improved production performance by MCFA could be explained by the antimicrobial properties of MCFA and their esters that may reduce the intestinal damage and microbiota perturbations due to *C. perfringens* inoculation (Kabara and Mashall 2005, Lensing et al 2010, Yang et al., 2019). However, feeding

of MCFA may also have resulted in more rapid recovery of the intestinal tract during the recovery phase (day 16 to 34) of the NE challenge, as suggested before by Lamot et al. (2016). Also, the concentration of propionate was lower in MCFA broilers compared to all other interventions. In pigs fed with MCFA, lower concentrations of propionic acid (the conjugate base of propionic acid) and propionate were observed (Dierick et al., 2004, Zentek et al., 2012). Increased concentrations of propionate have been associated with improved health (Reichardt et al., 2014), however, in our study we observed a reduced concentration. Nevertheless, as the broilers fed with MCFA showed the best production performance, apparently the intestinal metabolism was beneficially altered, which suggests that the interpretation of the metabolic output is not unambiguous. We observed a reduced relative abundance of the genus *Lactobacillus* in the cecal content of broilers fed MCFA on day 15. This is in line with previous studies where a reduced relative abundance of the genus *Lactobacillus* was found in the jejunal microbiota of NE challenged broilers also fed MCFA (Yang et al., 2019). However, also in broilers without a NE challenge but fed with MCFA, a reduced relative abundance of the genus *Lactobacillus* has been observed (Van der Hoeven-Hangoor, et al., 2013, Kers et al., 2019b).

In addition, the presence of formate in the cecal content of all 12 tested PC broilers was striking, although the concentration was low, as in the other groups it was only measured in half of the broilers or even less. Formate, a product of fermentation of carbohydrate substrates, can be produced by different bacteria, e.g. members of *Enterococcus*, *Bacteroides*, and *Bifidobacterium* (Duncan et al., 2004, Tremaroli and Bäckhed, 2012). Acids such as formate normally behave as intermediates in microbial metabolism in the gut and are often onwards converted into other metabolic products such as acetate (Clench and Mathias 1995, Flint et al., 2012). Recently, however, elevated concentrations of formate have been associated with inflammation (Hughes et al., 2017) and suggested as a potential biomarker (Ducatelle et al., 2018). In a mouse model, it has been shown that increased levels of formate contribute to the bloom of the family *Enterobacteriaceae* in an inflamed gut (Hughes et al., 2017). In our data we did not observe differences in members of this family between the groups. In another study, however, a decrease was observed in fecal *Enterobacteriaceae* and *C. perfringens* counts in NE challenged broilers fed with MCFA (Abdelli et al., 2020). In our study, formate was only detectable in three out of the 12 broilers fed MCFA, which suggests that the intestinal inflammation in the MCFA broilers may have been reduced compared to the other groups. These results indicate that identification of formate as potential biomarker may be of added value in experiments or in the field, and merits further investigation.

For elucidation of underlying mechanisms of the ameliorated negative effects of the subclinical intestinal NE challenge, studying interactions between intestinal microbiota, metabolites and clinical parameters, combined with interactions with

immune responses, both locally in the intestinal tract and systemically is needed.

In summary, our study revealed that alterations in intestinal microbiota composition, induced by MCFA, improved the disease resistance of broilers against a subclinical intestinal NE-challenge, resulting in a better maintained growth performance during the NE challenge and improved recovery afterwards. Future research is needed to better understand underlying mechanisms and interactions between intestinal microbiota, nutrition, the immune system and pathogens to help identify strategies to reduce the impact of NE in broilers.

**Acknowledgement**

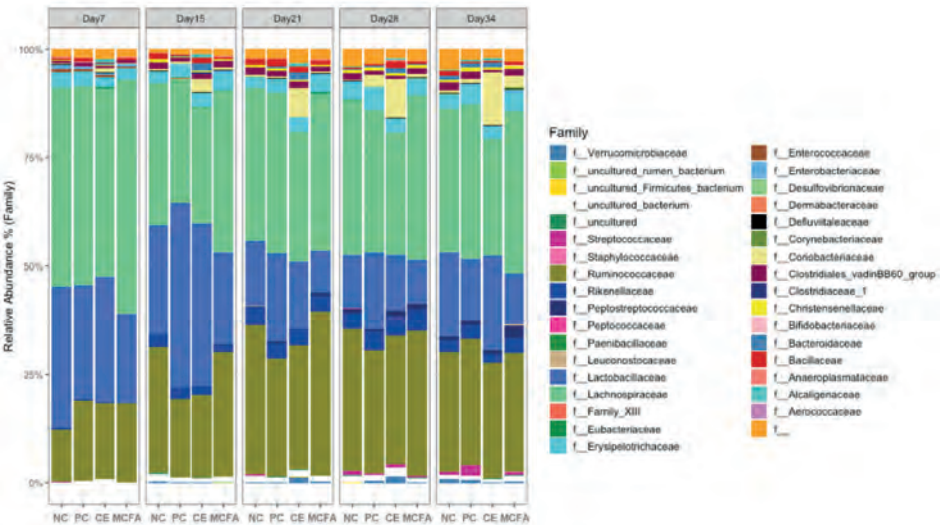
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**Supplementary Materials**

**Figure S1:** Barplot of relative abundance of all families.

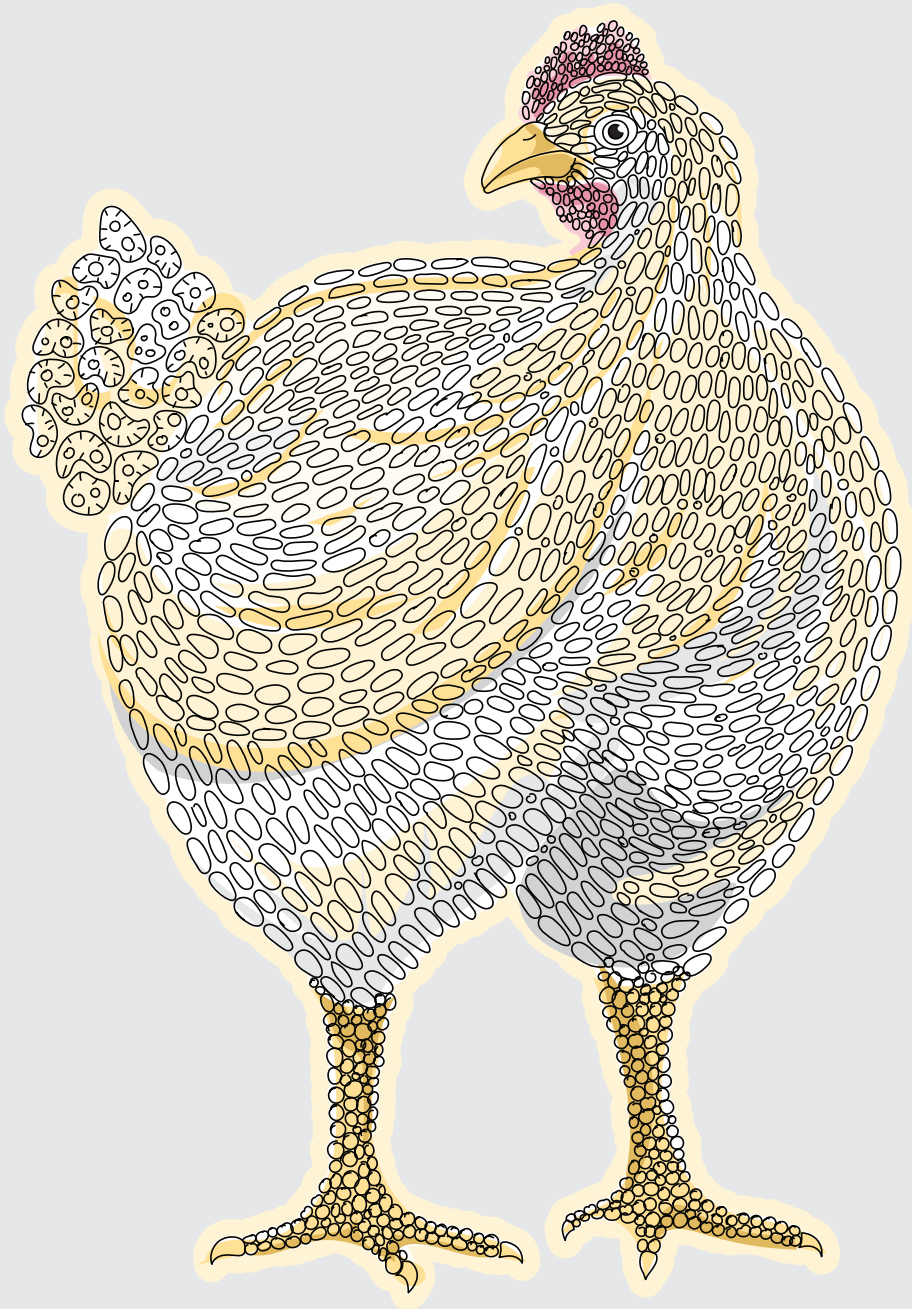


**Table S1:** Clinical parameters on day 15. The percentage of broilers scored positive (>0) for *Clostridium perfringens* lesions (NE score), coccidiosis lesion score (CLS score, *Eimeria maxima*), and gut score (GS) and the severity of the score presented as mean values. Interventions: not challenged with Necrotic Enteritis (NE) nor treated (NC), challenged with NE but not treated (PC), challenged with NE and receiving either a competitive exclusion product at day of hatch (CE) or broilers challenged with NE and fed a diet containing medium chain fatty acids (MCFA). (1 day post infection, n = 24 per intervention, only of 12 broiler cecal content).

Intervention	NE score		CLS score		GS score	
	Positive birds (%)	Mean score (0 to 4)	Positive birds (%)	Mean score (0-4)	Positive birds (%)	Mean score (scale 0-10)
NC	8%	1.00	33%	1.75	92%	2.33
PC	0%	0	50%	1.17	100%	2.58
CE	25%	1.33	17%	1.50	100%	1.67
MCFA	17%	1.00	17%	1.00	100%	2.08
<i>n=12 broilers per intervention clinical parameters measured and cecal content collected</i>						
	Positive birds (%)	Mean score (0 to 4)	Positive birds (%)	Mean score (0-4)	Positive birds (%)	Mean score (scale 0-10)
NC	4%	1.00	33%	1.75	96%	2.47
PC	4%	1.00	38%	1.11	100%	2.48
CE	33%	1.38	21%	1.20	96%	2.13
MCFA	17%	1.00	21%	1.20	96%	2.13
<i>n=24 per intervention only clinical parameters measured</i>						

## PART IV

General discussion  
and summary







# CHAPTER 10

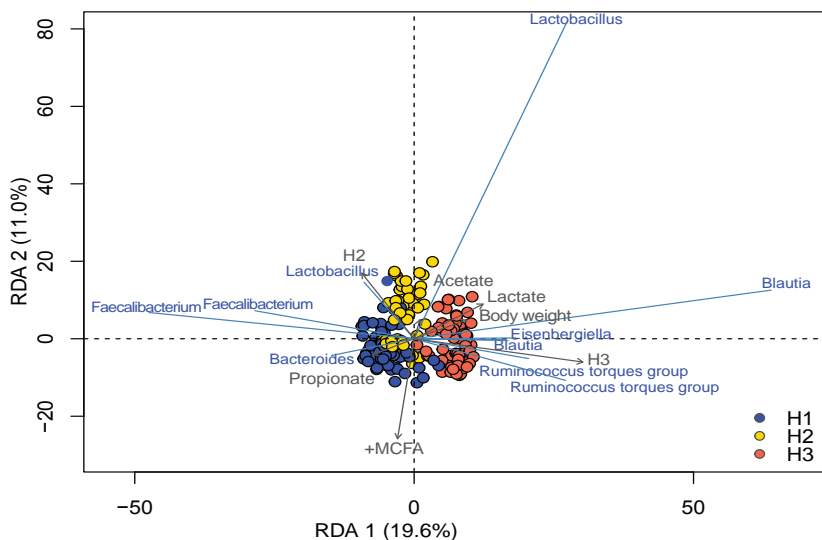
## General discussion

In recent years, large-scale studies have increased our understanding of the interactions between microbes and health in humans and animals. The goal of this thesis was to improve the understanding of the interaction between the intestinal microbiota and broiler health. Various pieces of the puzzle have been obtained in this thesis and will be discussed here, together with those pieces that still need to be found and correctly placed in the bigger picture in future research. Finally, the conclusions that can be drawn based on the research described in this thesis are summarized.

## **Part 1 - Study design is everything**

### ***Be aware of the environment***

In general, meta-analyses provide insight into the available level of evidence of research outcomes by locating, appraising, and summarizing similar studies. Similar studies, however, are difficult to find within the research field of poultry intestinal microbiota (Chapter 2). This is partly due to the technical variation in analytical methods used, including for example 16S rRNA sequencing, and partly because of the broad range of host- and environmental factors that can affect the outcome of chicken research. One of the factors that have been investigated in more detail in this thesis was how important housing conditions are with respect to the interplay of nutritional interventions and intestinal microbiota in broiler chickens (Chapter 3). The concept of the relevance of the environment is not new, as the Dutch botanist and microbiologist Baas-Becking proposed in 1934 already: *“Everything is everywhere, but the environment selects”*. In Chapter 3 we described in detail that the environment affected microbiota composition and functionality stronger than the diet intervention. In Chapter 3 of this thesis, the microbiota composition and the metabolic output were discussed as separate parts, but these data can also be combined within one analysis. This additional comprehensive analysis included the variables environment (three different housing conditions; a feed research facility close to the practical poultry situation, extensively cleaned floor pens, and isolators with a very high hygiene status), a feed intervention (feed with or without supplementation of medium chain fatty acids, MCFA), individual body weight of the 35-day-old broilers, and measured metabolic output of the cecal content (propionate, butyrate, acetate, and lactate). To analyze the contribution of these variables to cecal microbiota composition, distance-based redundancy analysis (db-RDA) was performed. This analysis showed that the housing environment of the broilers still explained most of the variation (first axis), before the actual feed intervention (second axis). In total 42.9% of the variation of the cecal microbiota could be explained with six explanatory variables: the housing condition, the feed intervention, body weight, and the concentration of the measured metabolic output propionate, acetate, and lactate (Figure 1).



**Figure 1:** Triplot for weighted UniFrac distance-based redundancy analysis (WUF-db-RDA) of the cecal microbiota composition of 210 broilers of 35 days old (data Chapter 3) of amplicon sequence variance (ASV) level. The longer the arrow length, the stronger the correlation. ASVs labels are on lowest known taxonomic rank. Samples are colored by housing condition (H1 = feed research facility, H2 = extensively cleaned floor pens, H3 = isolators).

In this reanalysis, broilers without MCFA in their feed showed a strong correlation with a member of the genus *Lactobacillus* in cecal content compared to broilers with MCFA (Figure 1). A higher concentration of lactate was correlated with a higher body weight, and with a member of the genus *Blautia* (Figure 1). The relative abundance of *Lactobacillus* was significantly lower in all the broilers fed MCFA, and the relative abundance of *Blautia* was higher in the broilers that were raised in the isolators (Chapter 3). Through which mechanism the presence of higher concentrations of lactate in the cecal content is associated with body weight cannot be concluded unequivocally from this dataset, due to the dynamic process of production and utilization by gut bacteria. This means that a higher lactate concentration can indicate that less lactate could have been converted into butyrate or more lactate could have been produced in the gut (Duncan et al., 2004, Meimandipour et al., 2009, De Maesschalck et al., 2015, Onrust et al., 2015). Having said that, it is interesting to note that members of the genus *Blautia* are lactate-utilizing butyrate-producing bacteria (Duncan et al., 2004, De Maesschalck et al., 2015), which suggests that the cecal microbiota of broilers with a higher body weight most likely produced more lactate. Broilers raised in the isolators (H3) had a higher body weight compared to the broilers raised in the feed research facility (H1), but no difference in the relative abun-

dance of *Lactobacillus* was observed between those housing conditions (Chapter 3). Therefore, it is not likely that in this case the abundance of the genus *Lactobacillus* explains the higher production of lactate. Members of the genus *Ruminococcus* torques group might also produce lactate (Crost et al., 2018), and this might result in the higher body weight observed in the isolators. In humans, a higher concentration of lactate has been associated with severe inflammatory bowel disease and with an inflamed mucosa (Vernia et al., 1988, Hove et al., 1995, Duncan et al., 2004). During the post-mortem examination of the broilers, no evidence of inflammation of the mucosa was observed. The post-mortem examination was performed at day 35 of age, hence we cannot rule out the presence of inflammatory processes in the gut earlier in life. However, if that had been the case, a negative impact on body weight would have been expected, instead of a higher body weight. With only one time point available, we can only speculate on what has caused an elevated concentration of lactate in the cecal content of broilers with a higher body weight.

When exactly the microbiota composition and functionality has changed, at the start of the experiment, or during the experiment, is unknown and might have been caused by stochastic changes. Such stochastic changes over time can drive environmental effects, as for example shown in a mice study, where an inoculation with a specific microbial community was insufficient to eliminate this so-called cage effect (McCafferty et al., 2013). Thus, when and how the difference between the broilers across housing conditions started remains unclear. This stresses the importance to move from cross-sectional studies to longitudinal studies to further understand the mechanisms underlying the colonization and development of the intestinal microbiota and broiler health. Therefore, non-invasive sampling methods are needed to measure the intestinal microbiota within the same broiler flock and preferably even within the same broilers across time.

### ***Non-invasive sampling methods***

One challenge frequently encountered within in vivo poultry research is that intestinal content for microbiota analyses is often collected post-mortem, hampering longitudinal data collection. Suitability of fecal droppings of chickens for that purpose would facilitate longitudinal sampling. However, in humans, the usability of fecal (stool) samples is under debate because they might not be representative with respect to in situ functionalities of the intestinal microbiota, especially at locations higher upstream in the intestinal tract (Lavelle et al., 2015, Vandeputte et al., 2016, Pereira and Berry, 2017). Therefore, we compared different invasive and non-invasive methods to characterize the cecal microbiota throughout a production cycle of broiler chickens (Chapter 4). Based on our study, cecal droppings collected from the poultry house litter, and boot sock samples, collected by walking over the litter, were found to be useful alternatives for cecal samples to determine cecal microbiota composition lon-

gitudinally. In infection and transmission experiments where the dynamic process of infection of a host upon exposure to a pathogen of interest is studied, one should be aware that removing cecal and fecal materials might influence the research results. In that case, cloacal swabs might be a better alternative. The limitation of cloacal swabs, though, is that they generally show a large variation in microbiota composition between individuals (Chapter 4). Thus, a larger number of samples should be taken to be able to detect differences in microbiota composition between groups. Recently, it was shown that cloacal swabs are an unreliable source for estimating the microbiota of the lower gastrointestinal tract in broilers (Williams and Athrey, 2020). Nevertheless, further studies should be performed to validate our observations and further study the applicability of cecal droppings, boot socks and cloacal swabs under different field and experimental conditions. However, the current cost and time-consuming process of sequencing still hampers the use of microbiota data as an applicable diagnostic tool in practice, but this might change in the future.

### ***Perform field experiments as soon as possible***

Like in any other research field, it is very important to think carefully about the most important research question and the future application prior to collecting the data. In addition, due to the large impact of the study design on the outcome of microbiota research (Laukens et al., 2016, Knight et al., 2018), it is important for poultry researchers to perform field experiments as soon as possible in order to facilitate the translation from experimental research to commercial poultry production. However, it has been shown that microbiota studies have to deal with many hidden host and environmental variables, that are not all known (Chapter 2), and that limits the reproducibility of research outcomes. It has been proposed for rodent studies that the use of natural or “wild” gut microbiota can help to improve the reproducibility of research outcomes (Rosshart et al., 2017, Rosshart et al., 2019). Microbial communities encountered under field circumstances are not the same as those in a lab setting, and this can substantially affect how e.g. the immune system develops and functions, which can lead to incorrect assumptions of how the immune system works under natural circumstances (Rosshart et al., 2017, Rosshart et al., 2019). This reasoning applies also to poultry intestinal microbiota research. However, not all types of research can be done under field circumstances. Therefore, we need tools to minimize or compensate for these differences when studying the intestinal microbiota. Such tools are, for example, intestinal organoids or a ‘microbiome-on-a-chip’ (Rubert et al., 2020, Stanley and van der Heijden, 2017). Those tools will improve knowledge on interactions between diet, microbiota and the intestinal epithelium to further decipher the difference between a healthy and unhealthy intestinal microbiota (Rubert et al., 2020). Still, the “normal” intestinal microbiota is important as it can strongly affect research outcomes, therefore experiments to test interventions should also be

repeatedly performed in commercial poultry farms. Although this type of research is sometimes referred to as a 'microbial black box', as it might not provide much insight into what is taking place at the cellular level and on how different microbes interact, it contains the complex and variable growing environment of broilers which is lacking under lab settings.

## **How our picture of biology changes over time – technical hurdles**

In the last fifteen years, the discussion on limited reproducibility and prevailing biases affecting research outcomes has received increasing attention (Ioannidis, 2005, Begley and Ioannidis, 2015, Baker, 2016). The actual biology does not change, but rather the ways how we interpret, or measure our observations have been and continue to be adjusted and optimized. For example, in 1923, 48 human chromosomes were counted through the microscope by Theophilus Painter. Until 1956 his 'error' was copied by others until Joe Hin Tjio coincidentally determined the true number of 46 human chromosomes (Matthews, 2013). In the microbiota research field, it is a combination of new insights and technical hurdles that influence how we are able to observe and understand biological mechanisms. New species of bacteria are constantly discovered, and this results in reclassification of the microbial taxonomy (Parks et al., 2018). This information is used in reference databases that are used to align and classify raw sequence data. Thus, the interpretation of 16S rRNA gene amplicon sequencing data depends on the used reference databases and on the version of the database, e.g. GreenGenes (last updated in 2013) or SILVA 138 (updated in 2019). This also limits the comparability of studies and therefore also the reproducibility, unless raw data from previous studies is available and can be re-analyzed.

In addition, a wide variety of sample processing protocols exists as well. We used a validated protocol for human fecal (stool) samples for a variety of sample types for chicken intestinal content. This means that our protocols were not optimized for the sample types collected for this thesis, and this might explain an unexpected result observed in ileal content samples, which we found to be predominated (>90-100 %) by members of the genus *Lactobacillus* (Chapter 4). Although it was expected that the ileal content harbored a relatively simple microbial composition, other genera e.g. within the family *Clostridiaceae*, *Streptococcus*, and *Enterococcus* were also expected (van der Wielen et al., 2002, Lu et al., 2003b, Richards-Rios et al., 2020a). Ileal content samples are, however, substantially different compared to fecal samples. Ileal content samples may be relevant to study effects of nutritional interventions at this specific site of the intestinal tract, but as they can still contain an undigested feed fraction, such as wheat grains, those samples might need a different processing protocol to extract DNA. Another approach to measure the ileal microbiota, for instance

for studies on interactions between microbiota and intestinal cells, could be the use of mucosal scrapings of the ileum, as they are expected to contain a different microbiota, both with respect to members as well as their relative abundances (Yasuda et al., 2015, Schroeder, 2019).

Another technical challenge is the appropriate use of statistical methods. Errors in analyses are sometimes even caused by trivial reasons, e.g. incorrect use of R functions to analyze the data. For example, the Jaccard distance measure (Jaccard index) can be interpreted in different ways, and is often defined as the presence/absence of taxa, but can also be computed based on the Bray-Curtis dissimilarity, which would result in a different Jaccard index<sup>1</sup>. Those technical issues can be easily resolved by sharing analysis codes between researchers on online platforms. Another important prerequisite for a comprehensive accumulation of data for poultry microbiome research is to apply the Findable, Accessible, Interoperable, Reusable (FAIR) guiding principles (Wilkinson et al., 2016). Providing details in a machine-readable way (referred to as accompanying ‘metadata’ and ‘data ontology’) for a broad range of experimental methods, host and environmental factors and data analysis in articles, and depositing raw sequence data in repositories, is needed to efficiently reuse data. Fortunately, there are current initiatives to apply this to the microbiome research field<sup>2,3</sup> and, also funders of research often support, stimulate or demand the reuse of data. For poultry microbiota research, including the work presented in this thesis, important metadata to share is for example the feed composition, egg incubation and hatching conditions, parent flocks, poultry type (for egg production or meat) or breeds, housing and husbandry conditions, transport during life or medication and vaccination history (Chapter 2).

### ***Power and sample size calculations***

Another well-known phenomenon that can hamper progress in every research field concerns publication biases in reporting mainly positive findings (Ioannidis, 2005). In microbiota research this might even occur rather unintentionally, by using certain alpha and beta diversity metrics. To illustrate this, we used our housing experimental data (Chapter 3) to perform power calculations afterwards. The power was calculated across different diversity measures as the fraction of times the null hypothesis was rejected over 100 or 1000 repetitions of the test (threshold  $p < 0.01$ ; Figure 2, 3). Based on this dataset we observed that the Shannon and Bray-Curtis metrics are most sensitive to observe differences between groups, resulting in a lower sample size. Based on this retrospective power calculation we can draw two conclusions about our study design. First, we did not sample enough broilers to observe a difference in the alpha

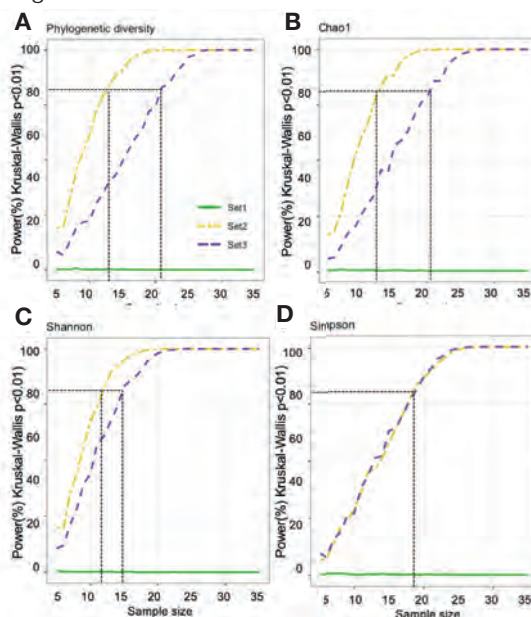
<sup>1</sup> <https://github.com/vegandevs/vegan/issues/153>

<sup>2</sup> Manifesto of the FAIR Microbiome Implementation Network, <https://www.go-fair.org/implementation-networks/overview/fair-microbiome/>

<sup>3</sup> Unlock, microbial potential, FAIR data platform, <https://m-unlock.nl/fair-data-platform>

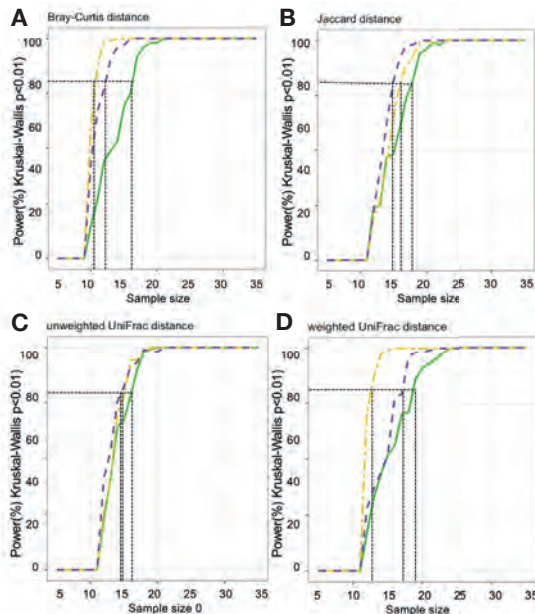
diversity between broilers fed with or without MCFA raised in housing condition 1, the feed research facility with a more natural field-type environmental microbiota (Chapter 3, Figure 2). Second, with 15 broilers less we would also have observed a higher alpha and beta diversity in broilers fed with MCFA in housing condition 2 and 3, the floor pens and isolators under more controlled and hygienic lab settings (Figure 2, 3).

As shortly explained in the introduction (Chapter 1), to develop power calculation tools for microbiota studies, we need to know 1) how much of a difference in the microbiota composition is needed to be able to differentiate between two groups, and 2) how much of an effect size is biologically relevant (Hanson and Weinstock 2016). The difference in microbiota composition needed to differentiate two groups depends on the measurements used, and the question to answer. In our data, for example, alpha diversity measures were less sensitive to observe differences between the broilers compared to the beta diversity (Chapter 3, Figure 2, 3). Although no difference in alpha diversity between broilers fed with or without MCFA raised in housing condition 1 was observed, the average daily gain and the average daily feed intake were lower in MCFA broilers (Chapter 3). Therefore, the difference only observed based on the beta diversity might already be biologically relevant and hence sufficient to draw conclusions in this case. Future research will have to investigate different datasets, e.g. based on simulated data and at different taxonomic levels, to observe if those trends observed in our data can be generalized.



**Figure 2:** The cecal microbiota composition of 35-day-old broilers raised in three different housing conditions, fed with or without MCFA, H1 = feed research facility, H2 = extensively cleaned floor pens, H3 = isolators, (six different experimental groups, n=35 per group, data Chapter 3). Across different alpha diversity metrics H2 is most sensitive to observe the difference between the feed groups based on their microbial communities (n=1000 repetitions, power 0.80). A, B. Phylogenetic diversity and Chao1 showed comparable sensitivity, and a sample size of 13 or 20 individuals per group. C. Shannon index showed the smallest sample size needed to observe the difference between the microbial communities. D. Simpson index showed no difference in samples size between data sets.





**Figure 3:** Different beta diversity metrics to estimate different sample sizes ( $n=100$  repetitions). A. Bray-Curtis distance metrics is most sensitive to observe difference between groups (16 samples needed). B. Jaccard distance is the only metrics that showed that dataset 3 needed the smallest sample size, indicating that between the groups in dataset 3 specific ASV are absent between the groups. C, D. Weighted UniFrac is more sensitive compared to unweighted UniFrac to observe a difference between the groups based on their microbial communities.

## Part 2 – Stochastic and deterministic processes in the establishment of the broiler cecal microbiota

### *Conserved development of the cecal microbiota*

The goal of this part of the thesis was to increase insight into the dynamics of intestinal microbiota composition in broilers and broiler flocks. In ecology, different processes are described that can shape a habitat. This includes stochastic processes such as dispersal, diversification, and ecological drift, and deterministic processes such as interactions between species, individuals, and environmental conditions (Burke et al., 2011, Stegen et al., 2012, Pereira and Berry, 2017, Stegen et al., 2016, Zhou and Ning, 2017). The intestine is also described as a habitat, or defined as a dynamic ecosystem, where those processes occur (Pereira and Berry, 2017). We performed a field study to collect cecal microbiota samples across different healthy broiler flocks. The data was used to answer two questions. First, if stratification of the cecal content samples into clusters would help to provide insight into ecological processes, and second, which factors are important to explain the variation in microbial composition observed between broilers. To analyze the datasets presented in Chapter 5 and 6 further, for the purpose of this discussion, we merged these two field study datasets. Combined, the cecal microbiota of 557 individual broilers was determined, from four different farms, across ten flocks. The data collected on days



The merged analysis is in agreement with the conclusions obtained from the separate analyses (Chapter 5, 6). Similar to those results, the phylogenetic diversity of the cecal microbiota increased until day 21 (Chapter 5). Thereafter, no significant difference was found, indicating stabilization of microbiota complexity (Chapter 5). The principal response curves also showed high variation between flocks within the first weeks of life, whereas thereafter the variation between the flocks was limited (Chapter 5, 6). This merged analysis showed two extra clusters for the broilers before 7 days of age (Figure 4). Clusters 3 and 4 in the new analyses were comparable to the clusters 1 and 2 described in Chapter 6. In human infants of 3 to 46 months of life, ten clusters were observed and described in a transition model, with three phases: the developmental phase, the transitional phase and the stable phase (Stewart et al., 2018). As commercial broilers have a short life span, those different phases are difficult to observe. A constraint of this analysis is that we did not measure the same individual broiler over time like in the human infants. However, the results indicate a conserved developmental trajectory of cecal microbiota composition that follows the same general trend across farms and that the variation in maturity across individuals is especially large around the second week of life. This is in line with other research that suggests that the assembly of the gut microbiota is largely deterministic (Pereira and Berry, 2016). Other researchers, however, suggested that stochastic factors might also play a significant role in shaping the structure of the microbial communities within the first days of life (Burke et al., 2011, Schloss et al., 2012). This is based on the 'lottery' theory that whoever gets there first in the gut 'wins' the space (Munday, 2004, Burke et al., 2011). Our results, however, show that independent of flock/poultry house, all individual cecal microbiota compositions end up within one cluster.

***The world is essentially a stochastic and highly nonlinear system - (Heesterbeek et al., 2015)***

Although there is a conserved developmental trajectory of cecal microbiota, further investigation is needed to understand which factors are important to explain the intestinal microbiota composition and variation. In addition to the observed developmental clusters, the factors body weight, the poultry house and feed ingredients (sunflower seed meal, rapeseed meal, and fish oil inclusion level), explained around 37% of the cecal microbiota variation between broilers (Chapter 6). In the field study described in Chapter 5, it was also shown that parameters such as intestinal lesions, explained around 29% of the variation in combination with factors such as the color of the ceca, farm, and age (Chapter 5). This means that approximately 63 to 70% of the variation is not explained by the factors we have measured. This indicates that either unmeasured (unknown) factors contribute, but also that stochastic effects can have a profound implication for the formation of the microbiota (Faust and Raes, 2012, Hildebrand et al., 2013). Most likely, both play an important role. Our data

provides also evidence for the importance of factors such as poultry house, and feed composition in shaping cecal microbiota. This is a key step towards the awareness of interactions between management factors and the cecal microbiota and broiler health.

## **Biological hurdles within broiler field studies**

As discussed in the first part of this General discussion, the study design is important, but the optimal design is not always possible. Broilers within the same poultry house eat the same feed and peck in the same litter that contains feces of other broilers. Therefore, it remains difficult to separate biological interactions within the microbiota of an individual from those that occur in other animals of the flock and in the housing environment. Individual housing of broilers in experiments is far from the commercial situation and therefore less interesting to study as it would limit the real dynamics of the intestinal microbiota. Merging the microbiota data or pooling the microbiota samples would create “*Frankenstein*” compositions, i.e. compositions that are artificially composed and not observed in individuals. This also limits possibilities to further investigate underlying mechanistic functions of the cecal microbiota as the variation between individuals is lost. One way to combat this problem is to study microbe-to-microbe interactions within the microbiota outside the host (Foster et al., 2017). Another way to combat this challenge is to enlarge datasets, with broilers raised in different areas of the world, with different feed compositions, management conditions and different health status. This will increase the evidence and understanding of which factors are most important to explain the variation between broiler flocks. Nevertheless, even in large datasets it will be a challenge to disentangle the biological interactions due to the rearing conditions of commercial broiler flocks.

## **Part 3 – Reproducible manipulation of the intestinal microbiota**

### ***Competition has pro and cons***

For the third and final part of the research described in this thesis, manipulations of the intestinal microbiota were performed to improve broiler health and reduce the risk of colonization with pathogens. Across studies, with different research questions, we used the same competitive exclusion product (Chapter 7, 8, 9). The concept of competitive exclusion was originally developed to combat *Salmonella* colonization and is defined as “*the establishment of adult-type resistance to salmonellas in newly hatched chickens by administering adult intestinal microorganisms*” (Nurmi et al., 1992). The product we used was a freeze-dried product, containing intestinal microbiota, derived from healthy, specified pathogen-free birds and manufactured

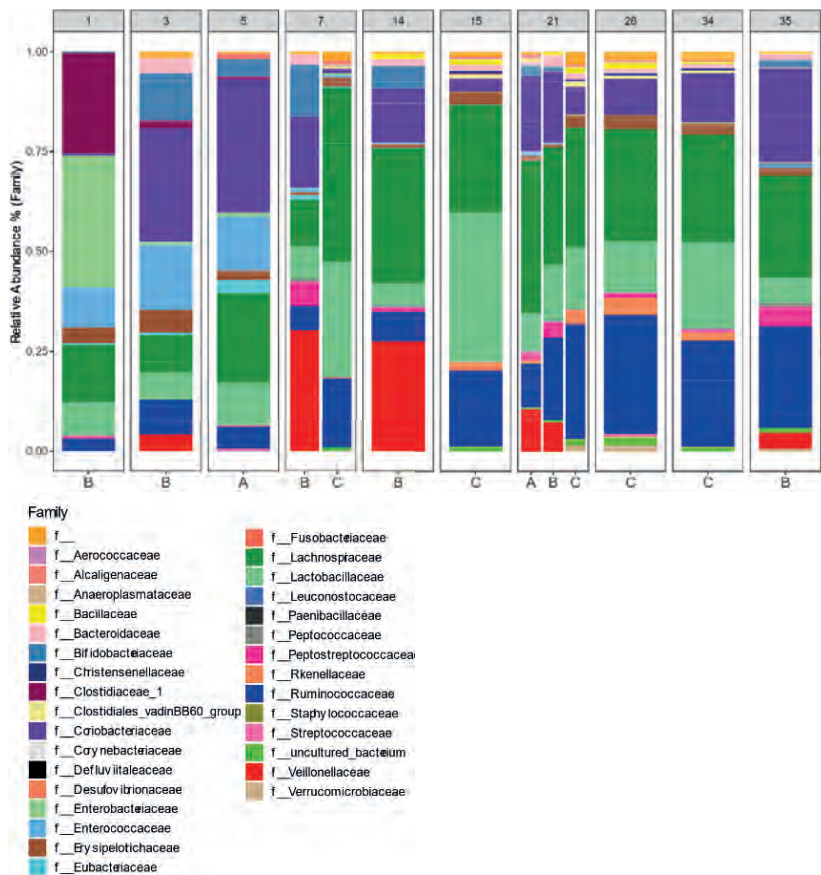
by fermentation (Aviguard®, MSD Animal Health, the Netherlands). Overall, our studies show that this competitive exclusion product (CE product) was shown to be a useful intervention to prevent colonization of Extended Spectrum Beta-Lactamase (ESBL)-producing *Escherichia coli* after challenge with a low dose in the first week in a broiler flock (Chapter 7), that it accelerated the maturation of the cecal microbiota (Chapter 8, also referred to as adult-derived microbiota (AM)), but that it did not ameliorate the loss of performance induced by a subclinical intestinal challenge (Chapter 9). Despite the different ways of administration of the product, via drinking water for 5 days (Chapter 7), oral inoculation directly upon hatch (Chapter 8) and spray on day-old broilers (Chapter 9), the three chapters showed the same trend with a higher relative abundance of the genus *Collinsella* and *Bacteroides* both present in the CE product and in the treated broilers. The genus *Collinsella* is associated with a reduced expression of tight junction proteins (Chen et al., 2016) and altering the hosts' metabolism (Gomez-Arango et al., 2018, Candela et al., 2016). Although *Collinsella* is part of the normal intestinal microbiota in humans (Tremaroli and Bäckhed, 2012), it is also suggested to be pro-inflammatory in humans (Candela et al., 2016). In addition, there is a patent that designates the use of *Collinsella* for the treatment of inflammatory bowel disease in mammals (Robert Saalman, 2018). This indicates that there is no consensus on the functionality of *Collinsella*. Nevertheless, we did not observe any positive or negative effect on growth performance, nor a difference in the metabolic output (concentrations of acetate, butyrate, formate, and propionate) in the cecal content of CE treated broilers (Chapter 9).

To explore the effect of the CE product in more detail, the three datasets were combined. Although we observed a conserved developmental trajectory of cecal microbiota with age (Chapter 6), different ages are difficult to compare, and therefore only broilers of the same age were compared. In dataset A (Chapter 7), the broilers received the CE product from 0 until 5 days of age to reduce the spread of an Extended Spectrum Beta-Lactamase (ESBL)-producing *E. coli* at flock level. In dataset B the CE product was used as a tool to test the effect of differences in initial colonization on the development of the intestinal microbiota due to oral inoculation direct upon hatch (Chapter 8), and in dataset C the CE product was sprayed on day-old broilers to alter the intestinal microbiota and explore its effect under an intestinal challenge with *Eimeria maxima* and *Clostridium perfringens* (Chapter 9). One of the observations that stands out is that dataset B (Chapter 8) was characterized by a higher relative abundance of the family Veillonellaceae compared to dataset C (Chapter 9) on day 7 (Figure 5). This was also observed on day 21, where in datasets A and B the family *Veillonellaceae* is present but not in dataset C (Figure 5). One of the major differences between the three experiment is, besides the method of administration of the CE product, the facility where the experiment was performed. Experiments A and B were performed at the research facilities of Utrecht University

whereas experiment C was performed at a research facility of Cargill Animal Nutrition. In our field study data, the family *Veillonellaceae* was also not observed (Chapter 5, 6) and it was only present at low relative abundance in broiler raised in the isolators (Chapter 3). This indicated that this family might be present as a result of environmental factors rather than due to the administration of the CE product, although the family *Veillonellaceae* were also present in the CE product (Chapter 7). The observed number of ASVs (alpha diversity) in the cecal content of broilers that received the CE product indicated a significantly lower alpha diversity in datasets A and B compared to dataset C on day 21 (Figure 6a). Nevertheless, the difference can still be caused by the different ways of the administration of the product. The two-dimensional visualization of the cecal content microbiota in the principal coordinate plot (PCoA) placed the broilers of the three chapters far from each other (Figure 6b). In contrast, the broilers of experiment A of day 5 (gold circles) are placed relatively close to the 1- and 3-day-old broilers of experiment B, which suggest that the administration of the CE product had a similar effect on the microbiota composition at a young age, but that this effect vanished over time (Figure 6b).

## Moving towards functionally

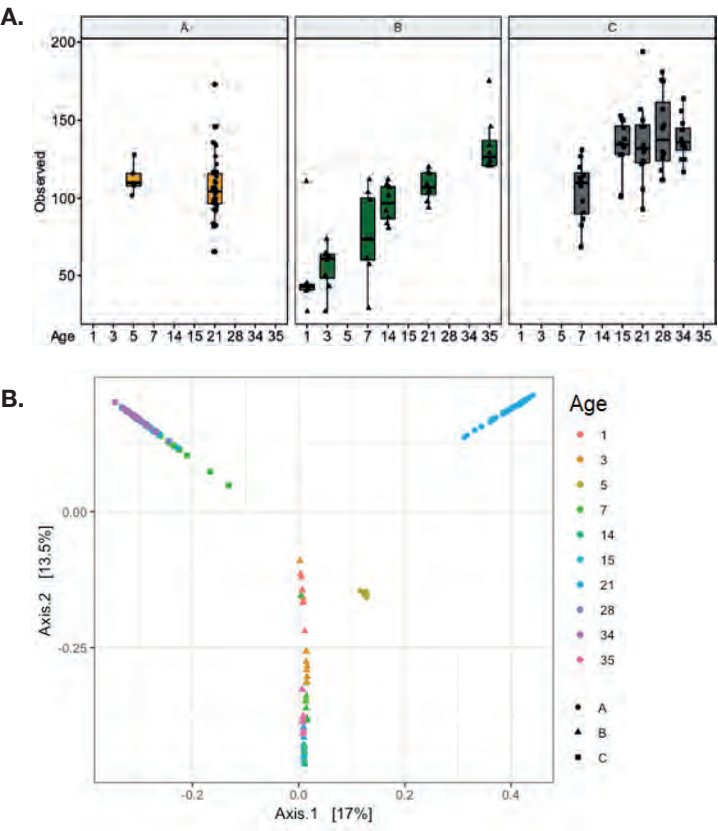
In two of the studies described in this thesis, an MCFA feed intervention was used (Chapter 3, 9), and both chapters showed a reduced relative abundance of the genus *Lactobacillus* in the cecal content of those broilers. This feed intervention improved the growth performance (Chapter 3) and reduced the loss of growth performance induced by a subclinical intestinal challenge (Chapter 9). In Chapter 3 the metabolic output could not be correlated with the feed intervention, but the concentration of propionate was lower in MCFA fed broilers compared to all other interventions in Chapter 9. Propionate has been suggested to have an anti-inflammatory effect in inflammatory bowel diseases (Tedelind et al., 2007). In the first MCFA study, we only used healthy broilers and therefore we might not have observed any difference in metabolic output, whereas in Chapter 9 we challenged the broilers with *Eimeria maxima* and *Clostridium perfringens*. This challenge may have affected different digestive and inflammatory processes in the small intestine, which may have affected metabolic processes in the ceca as well. In addition, the microbiota composition of the broilers fed MCFA, defined as the relative abundance of genera, showed differences between those two chapters (day 35 Chapter 3, day 34 Chapter 9). This might be because of the slight differences in the MCFA product between the chapters, as in Chapter 3 we used a blend of 0.3% C10 and 2.7% C12 and in Chapter 9 a blend of 6% C10 and 46.5% C12.



**Figure 5:** Relative microbial abundance at family level across broiler age and of different experiments described in different chapters of this thesis. Only the families that have a relative abundance of more than 0.1% in the cecal content of broilers are shown. Data shown are taken from Chapter 7 (A, broilers received the CE product via drinking water for 5 days), Chapter 8 (B, broilers received the CE product via oral inoculation directly upon hatch), and Chapter 9 (C, broilers received the CE product via spray).

Also, the inclusion was slightly different: 3% (Chapter 3) vs. 2% (Chapter 9). However, in both chapters broilers fed with MCFA showed an improved production performance although the microbiota composition was different. Thus, similar functions of the microbiota can be exerted by different species and genera, and therefore it is important to assess the impact of the cecal microbiota on e.g. intestinal metabolism, rather than only describing which microbial taxa are present. Other techniques might be able to answer those questions e.g. metagenomics, metatranscriptomics, or metabolomics.





**Figure 6:** A. The observed number of ASVs (alpha diversity) in the cecal content of broilers that received the CE product. B. Principal coordinate plot (PCoA) based on Jaccard distances between cecal content samples of broilers across chapters. Different colors indicate a different sampling age of the broilers, the shapes indicate the different chapters from which the data is derived (A=Chapter 7 (circle), B= Chapter 8 (triangles), C= Chapter 9 (square)).

## Conclusions

This thesis presents new insights into the intestinal microbiota in broiler chickens. We showed that it is essential to be aware of the large impact of the study design on the results and thus on the interpretation of the outcomes with respect to the intestinal microbiota and its correlation with host-associated measurements such as health, growth performance and immune development. In particular, we showed that the same nutritional intervention can modify the intestinal microbiota in the same direction under different housing conditions, but that housing condition affected the microbiota composition and functionality stronger than the nutritional intervention.



We have also shown that the temporal development and dynamics are major in the first weeks of life and that after three weeks of age the cecal microbiota composition can be considered as stable in a well-performing broiler flock. Our results also suggest a conserved development of the cecal microbiota, but non-invasive longitudinal sampling to monitor the development of the intestinal microbiota is needed to improve our understanding of the development of the intestinal microbiota and the interaction with broiler health.

Future studies should provide details on study variables and sequence data repositories to create opportunities to combine data from different studies for meta-analysis. This will facilitate scientific breakthroughs toward innovative microbiota-inspired intervention strategies. More collaboration among research groups, nationally, internationally, and globally, to maximize the quality and validity of research results is therefore also needed. Another challenging task for further intestinal microbiota research is to discover the mechanisms to distinguish transmission between hosts, and between hosts and the experimental environment, to improve the repeatability of microbiota research. Then questions addressing for example the best timing and type of intervention may be answered. In addition, further research is needed to better understand the function of the intestinal microbiota and the interaction with nutrition and the immune system. This will contribute to the development of new nutritional interventions, improved management as well as better diagnostic tools to improve broiler health.



**This book may be finished, but the story continues.**

Within years, the complex interplay between host and microbiota within the intestinal ecosystem, will be discovered - piece by piece. So probably not tomorrow, but in the foreseeable future, microbes will improve broiler health. This gives me hope.

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## Summary

Broiler chickens are created through genetic selection and are fast-growing, highly efficient chickens for human meat consumption. In the past, antibiotics have been used preventively to achieve high production performances. Antibiotic resistance, however, has become one of the biggest threats for global health, and therefore Europe banned in-feed antibiotic growth promoters from livestock feed in 2006. After the ban, intestinal health problems in broilers significantly increased. Those intestinal health problems are often associated with an imbalance in the microbiota. Microbiota is defined as the assemblage of living microorganisms present in a defined environment. Knowledge on the development and variation of the intestinal microbiota will be of great value to optimize the health of broilers and to prevent the need for therapeutic antibiotics. The objective described in this thesis is to improve the understanding of the interaction between the intestinal microbiota and broiler health.

The first part of the thesis is about how to design intestinal microbiota research in poultry. Considerable variation of intestinal microbiota composition has been observed both within and across studies. Such variation may in part be attributed to technical factors, such as sampling procedures, sample storage, DNA extraction, the choice of PCR primers and corresponding region to be sequenced, and the sequencing platforms used. **Chapter 2** is a literature study on host and environmental factors affecting the intestinal microbiota in chickens. Host-related factors, such as age, sex, and breed, have a large effect on intestinal microbiota. The diversity of chicken intestinal microbiota tends to increase most during the first weeks of life, and corresponding colonization patterns seem to differ between layer- and meat-type chickens. Environmental factors, such as biosecurity level, housing, litter, feed access and climate influence the composition of the intestinal microbiota as well. In **Chapter 3** a cross-sectional feed experiment was simultaneously conducted in three different experimental housing environments. Feed with and without a blend of medium-chain fatty acids (MCFA) was used as a tool to create differences in cecal microbiota between pens, within the same housing environments. The cecal microbiota was modifiable by this feed intervention, but the housing environment affected microbiota composition and functionality stronger than the diet intervention. Consequently, for interpretation of intestinal microbiota studies in poultry it is essential to be aware of the potentially large impact of housing conditions on the obtained results. Longitudinal research would improve our knowledge on how e.g. factors such as environment influence the intestinal microbiota. Therefore **Chapter 4** evaluates different invasive and non-invasive sampling methods to characterize intestinal microbiota throughout a production cycle of broiler chickens. This study addresses that the value of non-invasive sample types varies at different ages and



depends on the goal of the microbiota characterization. Instead of cecal content collected post-mortem, cecal droppings and boot socks can be useful alternatives to determine intestinal microbiota composition longitudinally.

The second part of this thesis focuses on if phenotypic characteristics can explain the intestinal microbiota composition and its variation in poultry farms. **Chapter 5** gives the results of a field study that described the development of the intestinal microbiota of broilers within a production cycle and evaluates to what extent clinical parameters and phenotypic characteristics can explain the intestinal microbiota variation. An increase in phylogenetic diversity was observed from hatch until day 21, and factors such as age, farm, body weight, ileum crypt depth, cecal color, and the coccidiosis lesion score were important variables to describe the variation in cecal microbiota. In **Chapter 6**, we explored population stratification by using clusters (community types) to simplify the complex ecosystem of the intestinal microbiota. This study showed conserved cecal microbiota development trajectories based on different clustering methods across different commercial broiler flocks. In addition, the cecal microbiota variation between broilers was explained by the poultry flock, body weight, and the different feed components. Those variables, however, could not be disentangled from the influence of the farm. This emphasizes the importance for further investigation of mechanisms underlying microbiota development and functions that affect broiler performance.

In the third part, the focus shifts from observational studies to intervention studies. In **Chapter 7**, we manipulate the intestinal microbiota development with a competitive exclusion (CE) product, supplied via drinking water directly upon hatch until 7 days of age. All broilers were challenged with Extended Spectrum Beta-Lactamase (ESBL)-producing *Escherichia*, but those which received the CE product were not colonized. The cecal content of those broilers had a higher phylogenetic diversity even when 21 days old. In **Chapter 8** this CE product is used as a tool as well, to test the effect of differences in initial colonization on the development of the intestinal microbiota and the activation of the innate immune system. Stratification by using clusters showed that early exposure to CE product accelerates the maturation of the cecal microbiota, which is paralleled by an increase in IL-2R $\alpha$ <sup>+</sup> NK cells and enhanced NK cell activation. The observed association between early life development of intestinal microbiota and immune system indicates possibilities in developing microbiota-targeted strategies that strengthen the immune system and thereby improve health and resilience of broiler chickens. In **Chapter 9**, again the CE product and a comparable MCFA intervention as in **Chapter 3** was used to alter the intestinal microbiota. In addition, the broilers were challenged with Necrotic Enteritis (NE), an intestinal disease causing large economic losses and severe health and welfare problems. Broilers fed MCFA showed reduced growth performance due to the NE challenge compared to negative controls, but less

reduced growth performance compared to positive controls and CE broilers. These results suggest that MCFA-induced alterations in the intestinal microbiota can improve the resistance of broilers against a subclinical NE challenge.

In **Chapter 10**, the general discussion, the main results and further research are discussed. First, study design is everything, and in poultry the rearing environment does influence the microbiota. Therefore, it is highly recommended to provide details on a broad range of host and environmental factors in articles and sequence data repositories. This creates opportunities to combine data from different studies for meta-analysis, which will facilitate scientific breakthroughs towards nutritional and husbandry associated strategies, and eventually improve broiler health. Second, our picture of biology changes over time. Microbiota research has technical hurdles but has to deal with stochastic and deterministic processes that establish broiler cecal microbiota as well. Nevertheless, a conserved development of the cecal microbiota has been observed. Finally, we were able to reproducibly manipulate the intestinal microbiota of broilers. To improve broiler health, it is needed to gather more knowledge on intestinal metabolism, rather than only describing which microbial taxa are present. Other techniques might be able to answer those questions e.g. metagenomics, metatranscriptomics, or metabolomics.

## Samenvatting

Vleeskuikens zijn door genetische selectie een zeer efficiënte bron van vleesproductie geworden. In het verleden werden antibiotica preventief gebruikt om vleeskuikens te kunnen laten groeien met weinig gezondheidsproblemen. Antibioticaresistentie is echter een van de grootste bedreigingen voor de wereldwijde gezondheid. Daarom is sinds 2006 het preventief gebruik van antibiotica niet meer toegestaan binnen de veehouderij in Europa. Na het verbod namen de darmgezondheidsproblemen bij vleeskuikens aanzienlijk toe. Deze darmgezondheidsproblemen worden vaak geassocieerd met een disbalans in de microbiota. Microbiota wordt gedefinieerd als de verzameling van levende micro-organismen die aanwezig zijn in een bepaalde omgeving. Kennis over de ontwikkeling en variatie van de darmmicrobiota is van grote waarde om de gezondheid van vleeskuikens te optimaliseren en de noodzaak van therapeutische antibiotica te verminderen. Het doel van het onderzoek in dit proefschrift is om meer inzicht te krijgen in de interactie tussen de darmmicrobiota en de gezondheid van vleeskuikens.

Het eerste deel van dit proefschrift gaat over hoe darmmicrobiota-onderzoek bij pluimvee ontworpen zou moeten worden. Zowel binnen als tussen studies is namelijk een aanzienlijke variatie in de samenstelling van de darmmicrobiota waargenomen. Een dergelijke variatie kan gedeeltelijk worden toegeschreven aan technische factoren zoals bemonsteringsprocedures, monsteropslag, DNA-extractie, de keuze van PCR-primers en het corresponderende gebied waarvan de sequentie moet worden bepaald, en de gebruikte sequencingplatforms. **Hoofdstuk 2** is een literatuurstudie naar gastheer- en omgevingsfactoren die de darmmicrobiota bij kippen beïnvloeden. Gastheer-gerelateerde factoren, zoals leeftijd, geslacht en ras, hebben een groot effect op de microbiota. De diversiteit van de microbiota van kippen neemt in de eerste levensweken het meest toe en de overeenkomstige kolonisatiepatronen lijken te verschillen tussen kippenrassen. Omgevingsfactoren, zoals het bioveiligheidsniveau, huisvesting, strooisel, toegang tot voer en klimaat beïnvloeden de samenstelling van de darmmicrobiota eveneens. In **Hoofdstuk 3** wordt een parallel uitgevoerd voedingsexperiment in drie verschillende omgevingen beschreven. Voyer met en zonder een mengsel van middellange ketenvetzuren (MCFA) werd gebruikt als hulpmiddel om verschillen in cecale microbiota tussen hokken te creëren binnen dezelfde huisvestingsomgevingen. De cecale microbiota was aanpasbaar door deze voerinterventie, maar de huisvestingsomgeving beïnvloedde de samenstelling en functionaliteit van de microbiota sterker dan de dieetinterventie. Bijgevolg is het voor interpretatie van intestinale microbiota-onderzoeken bij pluimvee essentieel om op de hoogte te zijn van de potentieel grote impact van huisvestingsomstandigheden op de verkregen resultaten. Longitudinaal onderzoek is nodig om kennis over hoe factoren zoals de omgeving de darmmicrobiota beïnvloeden. Daarom evalueert **Hoofdstuk 4** verschillende invasieve en niet-invasieve bemonsteringsmethoden om de

darmmicrobiota te karakteriseren gedurende een productiecycclus van vleeskuikens. Deze studie laat zien dat de waarde van niet-invasieve monstertypes varieert per leeftijden en afhankelijk is van het doel van de microbiota-karakterisering. In plaats van cecale inhoud die post-mortem wordt verzameld, kunnen cecale uitwerpselen en overschoenen nuttige alternatieven zijn om longitudinaal de samenstelling van de darmmicrobiota te bepalen.

Het tweede deel van dit proefschrift gaat in op de vraag of fenotypische kenmerken de samenstelling van de darmmicrobiota en de variatie in pluimvee-bedrijven kunnen verklaren. **Hoofdstuk 5** geeft de resultaten van een veldonderzoek dat de ontwikkeling van de darmmicrobiota van vleeskuikens binnen een productiecycclus beschrijft en evalueert in hoeverre klinische parameters en fenotypische kenmerken de variatie in de darmmicrobiota kunnen verklaren. Een toename in fylogenetische diversiteit werd waargenomen vanaf dag 1 tot dag 21. Factoren zoals leeftijd, boerderij, lichaamsgewicht, ileum vlokken diepte, cecale kleur en de coccidiose laesiescore waren belangrijke variabelen om de variatie in cecale microbiota te beschrijven. In **Hoofdstuk 6** is door middel van stratificatie het complexe ecosysteem van de darmmicrobiota vereenvoudigd. Deze studie toonde een geconserveerd ontwikkelingstraject van cecale microbiota aan. Daarnaast werd de variatie in de cecale microbiota tussen vleeskuikens verklaard door de pluimveestal, het lichaamsgewicht en verschillende voercomponenten. Deze variabelen kunnen echter niet worden gescheiden van de invloed van de boerderij. Dit benadrukt het belang voor verder onderzoek van mechanismen die ten grondslag liggen aan de ontwikkeling van microbiota en functies die de gezondheid van vleeskuikens beïnvloeden.

In het derde deel verschuift de focus van observationele studies naar interventiestudies. In **Hoofdstuk 7** manipuleren we de ontwikkeling van de darmmicrobiota met een competitief uitsluitingsproduct (CE) dat vanaf uitkomst tot 7 dagen oud via het drinkwater wordt toegediend. Alle vleeskuikens werden geïnoculeerd met een *Escherichia* die een uitgebreide spectrum Beta-Lactamase (ESBL) produceerden. De vleeskuikens die het CE-product kregen, werden niet gekoloniseerd met de ESBL. De cecale darminhoud van deze vleeskuikens had een hogere fylogenetische diversiteit, zelfs als ze 21 dagen oud waren. In **Hoofdstuk 8** wordt dit CE-product ook als hulpmiddel gebruikt om het effect van verschillen in initiële kolonisatie op de ontwikkeling van de darmmicrobiota en de activering van het aangeboren immuunsysteem te testen. Stratificatie door middel van clusters toonde aan dat vroege blootstelling aan CE-product de ontwikkeling van de cecale microbiota versnelt, wat gepaard gaat met een toename van en verbeterde natuurlijke celactivering. De waargenomen associatie tussen het immuunsysteem en de darmmicrobiota biedt mogelijkheden om strategieën te ontwikkelen om het immuunsysteem versterken en daardoor de gezondheid en veerkracht van vleeskuikens te

verbeteren. In **Hoofdstuk 9** werden opnieuw het CE-product en een vergelijkbare MCFA-interventie als in Hoofdstuk 3 gebruikt om de darmmicrobiota te manipuleren. Bovendien werden de vleeskuikens blootgesteld aan darmziekte Necrotische Enteritis (NE), die grote economische schade en ernstige gezondheids- en welzijnsproblemen veroorzaakt. Vleeskuikens die voer met MCFA kregen, vertoonden verminderde groeiprestaties als gevolg van de NE-darmziekte in vergelijking met kuikens zonder ziekte, maar minder in vergelijking met kuikens op voer zonder MCFA of met CE-product en met NE darmziekte. Deze resultaten suggereren dat door MCFA veroorzaakte veranderingen in de darmmicrobiota de weerstand van vleeskuikens tegen een subklinische NE-uitdaging kunnen verbeteren.

In **Hoofdstuk 10**, de algemene discussie, worden de belangrijkste resultaten en verder onderzoek besproken. Ten eerste is studiedesign erg belangrijk omdat bij pluimvee de opfokomgeving de microbiota beïnvloedt. Het wordt daarom ten zeerste aanbevolen om details over een breed scala aan gastheer- en omgevingsfactoren te beschrijven in artikelen en data-opslagomgevingen van sequentiegegevens. Dit biedt mogelijkheden om gegevens uit verschillende studies te combineren voor meta-analyse, wat wetenschappelijke doorbraken zal vergemakkelijken in de richting van voedings- en houderij-gerelateerde strategieën om de gezondheid van vleeskuikens te verbeteren. Ten tweede, verandert ons beeld van biologie in de loop van de tijd. Microbiota-onderzoek kent technische hindernissen, maar de ontwikkeling van de cecale microbiota van vleeskuikens heeft ook te maken met stochastische en deterministische processen die de samenstelling beïnvloeden. Desalniettemin werd een geconserveerde ontwikkeling van de cecale microbiota waargenomen. Ten slotte was het mogelijk om de darmmicrobiota van vleeskuikens reproduceerbaar te manipuleren. Om de gezondheid van vleeskuikens te verbeteren, is het nodig om meer kennis te verzamelen over het darmmetabolisme, in plaats van alleen te beschrijven welke microbiota aanwezig zijn. Andere technieken kunnen die vragen misschien beantwoorden, bijvoorbeeld metagenomics, metatranscriptomics of metabolomics.

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## Curriculum Vitae

Jannigje Gerdien (Annelies) Kers was born in Acquoy, The Netherlands on April 12<sup>th</sup>, 1989. After attending high school at the Heerenlanden College in Leerdam, she started the bachelor Biomedical Sciences at the VU University in Amsterdam in 2007. During her studies, she attended a variety of extracurricular courses. After a gap year, she started the Master Biomedical Sciences and Master Business Administration in 2011. She wrote a thesis on 'Deoxyribonucleic acid Methylation in Alzheimer's Disease' under the supervision of prof. dr. JC van Swieten and a thesis on 'Commercialisation of Academic Intellectual Property Disease' under the supervision of dr. ir. JC van Burg and prof. dr. E Masurel. In 2014 she started as junior researcher at the VU University Medical Center Amsterdam, Department of Community Genetics & Public Health Genomics, and was a Visiting Scholar at Emory University, Rollins School of Public Health, Epidemiology, Atlanta, USA (Oct 2014 - Sep 2015). In November 2015 Annelies started as a PhD student at the Department of Farm Animal Health of the faculty of Veterinary Medicine of Utrecht University in collaboration with Laboratory of Microbiology (MIB), Wageningen University & Research. She will continue in science as a postdoc at MIB. Annelies currently lives in Utrecht with her husband, baby son and three cats.

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A handwritten signature in black ink that reads "Annelies". The script is cursive and fluid, with the first letter 'A' being particularly large and stylized.

