DISSERTATION

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ANALYSIS OF PHOSPHORUS UTILIZATION USING THE HOST GENOME AND MICROBIOTA VARIABILITY IN JAPANESE QUAIL

UNIVERSITY OF HOHENHEIM

Analysis of Phosphorus utilization using the host genome and microbiota variability in Japanese quail

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I dedicate this work to my beloved family,
whose persistent selfless love, support, and encouragement
helped me accomplishing everything.

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LIST OF ABBREVIATIONS

BWG	Body weight gain
Ca	Calcium
CaU	Calcium utilization
CJA	*Coturnix japonica* chromosome
cM	Centi Morgan
DNA	Desoxyribonucleic acid
F:G	Feed per gain
FA	Foot ash in mg
FA%	Foot ash in percentage
FI	Feed intake
G-BLUP	Genomic best linear unbiased prediction
GIT	Gastro intestinal tract
h^2	Heritability
m^2	Microbiability
Mb	Megabase
M-BLUP	Microbiome best linear unbiased prediction
mg	Milligram
MWAS	Microbiome wide association analysis
OTU	Operational taxonomic units
P	Phosphorus
PU	Phosphorus utilization
QTL	Quantitative trait locus
rRNA	Ribosomal ribonucleic acid
SNP	Single nucleotide polymorphism
TA	Tibia ash in mg
TA%	Tibia ash in percentage
triM	Tracing inheritance with Markov models

GENERAL SUMMARY (ENGLISH)

Phosphorus (P) is an essential element for growth and performance of avian species. It is predominantly bound as phytic acids and salts (phytate) in plant seeds. Phytases and other phosphatases can harness P by cleaving P groups. Nonruminants have low endogenous phytase activity in the gastrointestinal tract, and thus, the requirement of this element is not met from exclusive plant-based diets. Therefore, mineral P or phytase enzymes are supplemented in poultry feed. Due to the finite quantities of high quality mineral P worldwide, it is of great economic interest. P supplementation is increasingly causing environmental problems. Past studies investigated the P utilization (PU) of different poultry species. They revealed a high phenotypic variation in PU among individuals. Moderate heritabilities indicates that breeding for this trait is in principle possible.

The overall aim of this thesis was to gain a deeper understanding of the variability of P utilization in relation to host genetics, ileal microbiota composition and their interaction in the model species Japanese quail.

The objective of **chapter two** was to verify whether variation in PU in quail is a heritable trait conditioned by a few quantitative trait loci (QTL) with detectable effects. For this purpose, individuals were genome-wide genotyped with a 4k SNP chip, and a linkage map was generated. Based on this map, QTL linkage analysis was performed using multimarker regression analysis in a line-crossing model to map QTL for PU. In addition to this focal trait, genetically correlated performance traits such as feed intake, body weight gain, feed per gain and calcium utilization, as well as foot and tibia ash traits in mg and percentage, were analysed. Within the mapped QTL regions, standard single marker association analysis was considered to assess significant SNP markers for trait association. We identified a few QTL regions with significant effects. Among them was a QTL peak at *Coturnix japonica* chromosome (CJA) 3 for PU. Several genes were found in the region surrounding this peak, which requires further functional gene analysis. Based on these results, we hypothesized that these traits are polygenically determined due to several small QTL effects, which we could not detect significantly. The overlap of the QTL regions indicated linkage of the traits and confirmed their genetic correlations.

To date, animal-derived and microbiota-induced PU has only been adequately studied in quail. Therefore, with the aim of predicting microbiota-related host traits, **chapter three** examined the composition of the ileum microbiota and differential abundance analysis (DAA). Based on this study, it was shown that a sex-specific influence on microbiota composition exists. The digesta samples of all animals were dominated by five genera: Unc. *Lactobacillus*, unc. *Clostridaceae* 1, *Clostridium sensu stricto*, *Escherichia coli* and *Streptococcus alactolyticus*. These taxa contributed to more than 70% of the total ileum microbial community. In examining the microbiota composition of each of the 50 animals with the highest and lowest PU, DAA revealed genera significantly associated with PU.

In **chapter four,** we characterized the influence of performance-related gut microbiota to unravel the microbial architecture of the traits evaluated. The aim of this study was to determine whether the variation in PU is partly driven by the microbial community in the ileum. We used microbial mixed linear models to estimate microbiabilities (m^2). This determines the fraction of phenotypic variance that can be explained by the gut microbiota. The estimation of m^2 was 0.15 for PU and was highly significant. It was also highly significant for feed intake, body weight gain and feed per gain. This model was bivariately extended and showed a high microbial correlation of the traits. Based on both results, the ileum microbiota composition plays a substantial role in PU as well as in performance traits, and there is a considerable animal microbiota correlation, showing that the microbiota affects multiple traits. The microbial drivers of this microbial fraction were identified by applying microbiome-wide association studies (MWAS). By back-solving the microbial linear mixed model, we approximated the effect of single OTUs on the phenotypic traits from the microbial model solutions. An MWAS at the genus level uncovered several traits associated with bacterial genera.

Subsequently, we assessed whether the microbial community in the ileum is a heritable host trait that can be used for breeding individuals with improved PU. In **chapter five** we applied QTL analysis using specific genera to examine whether they are linked with genomic SNP markers. These QTL analyses revealed a link between some microbiota species and host genomic regions of chromosomes and SNP markers. By estimating significant heritabilities for some genera, we were able to provide evidence for the hypothesis that the microbial community and microbial features are at least partially related to host genetics. We predicted the animal microbial effects on PU and correlated performance traits by applying microbial best linear unbiased predictions (M-BLUP). In addition, genomic best linear unbiased predictions (G-BLUP) were used to predict the SNP effect for the predicted animal microbial effect. A combination of those two may help to predict genomic breeding values of the microbiota effects for future hologenomic breeding programs.

GENERAL SUMMARY (GERMAN)

Phosphor (P) ist ein essentielles Element für das Wachstum und die Leistung von Vogelspezies. Es ist in Pflanzensamen vorwiegend als Phytinsäure und dessen –salze (Phytat) gebunden. Phytasen und andere Phosphatasen können P durch Abspaltung von P-Gruppen nutzbar machen. Nichtwiederkäuer haben eine geringe endogene Phytaseaktivität im Magen-Darm-Trakt, sodass der Bedarf dieses Elements nicht ausschließlich durch pflanzliche Futtermittel gedeckt werden kann. Demzufolge werden mineralischer P oder Phytase-Enzyme im Geflügelfutter supplementiert. Aufgrund der weltweit begrenzten Menge an hochwertigem mineralischem P ist es von großem wirtschaftlichem Interesse. Die P-Supplementierung verursacht zunehmend Umweltprobleme. Vergangene Studien untersuchten die P-Verwertung (PU) verschiedener Geflügelarten. Sie zeigten eine hohe phänotypische Variation der PU zwischen Individuen. Moderate Heritabilitäten deuten darauf hin, dass eine Zucht auf dieses Merkmal möglich ist.

Das übergeordnete Ziel dieser Arbeit war es, ein tieferes Verständnis für die Variabilität der P-Verwertung in Bezug auf die Wirtsgenetik, die Zusammensetzung der ilealen Mikrobiota und deren Interaktion bei der Modellspezies japanischen Wachtel zu erlangen.

Das Ziel des **zweiten Kapitels** war es, zu überprüfen, ob die Variation der P-Verwertung bei Wachteln ein erbliches Merkmal ist, das durch einige wenige *quantitative trait loci* (QTL) mit größeren Effekten bedingt ist. Zu diesem Zweck wurden Individuen genomweit mit einem 4k SNP-Chip genotypisiert und eine Kopplungskarte erstellt. Basierend auf dieser Karte wurde eine QTL-Kopplungsanalyse unter Verwendung einer Multimarker Regressionsanalyse in einem Linienkreuzungsmodell durchführt, um QTL für PU zu kartieren. Neben dem Hauptmerkmal wurden genetisch korrelierte Leistungsmerkmale wie Futteraufnahme, Körpergewichtszunahme, Futter pro Zunahme und Kalziumverwertung, sowie die Merkmale Fuß- und Tibia Asche in mg und Prozent untersucht. Innerhalb der kartierten QTL Regionen wurde eine Standard Singlemarker Assoziationsanalyse durchgeführt, um SNPs mit signifikanter Merkmalsassoziation zu identifizieren. Wir kartierten einige QTL Regionen mit signifikanten Effekten. Darunter war für PU ein QTL Signal auf dem Wachtelchromosom (CJA) 3. In der Region um dieses Signal wurden mehrere Gene gefunden, die eine weitere funktionelle Genanalyse erfordern. Basierend auf diesen Ergebnissen stellten wir die Hypothese auf, dass diese Merkmale aufgrund mehrerer kleinerer QTL Effekte, die wir nicht signifikant nachweisen konnten, polygenetisch bestimmt sind. Die Überlappung der QTL Regionen deutet auf eine Kopplung der Merkmale hin und bestätigt deren genetische Korrelationen.

Bislang wurde die tier- und mikrobiota-induzierte PU bei Wachteln nur unzureichend untersucht. Mit dem Ziel mikrobiota-bezogene Wirtsmerkmals vorherzusagen, wurde in **Kapitel drei** die

Zusammensetzung der Ileum Mikrobiota und die differentielle Abundanzanalyse (DAA) untersucht. Anhand dieser Studie konnte gezeigt werden, dass ein geschlechtsspezifischer Einfluss auf die Mikrobiotazusammensetzung besteht. Die Digesta-Proben aller Tiere wurden von fünf Gattungen dominiert: Unc. *Lactobacillus*, unc. *Clostridiaceae* 1, *Clostridium sensu stricto*, *Escherichia coli* und *Streptococcus alactolyticus*. Diese Taxa trugen zu mehr als 70% der gesamten mikrobiellen Gemeinschaft im Ileum bei. Bei der Untersuchung der Mikrobiotazusammensetzung der 50 Tiere mit der höchsten und niedrigsten PU, konnte die DAA eine signifikante Assoziation von Gattungen mit PU zeigen.

In **Kapitel vier** haben wir den Einfluss der leistungsbezogenen Darmmikrobiota charakterisiert, um die mikrobielle Architektur der untersuchten Merkmale zu entschlüsseln. Das Ziel dieser Studie war es festzustellen, ob die Variation der PU teilweise von der mikrobiellen Gemeinschaft im Ileum angetrieben wird. Wir verwendeten mikrobielle gemischte lineare Modelle, um Microbiabilities (m^2) zu schätzen. Diese bestimmen den Anteil der phänotypischen Varianz, der durch die Darmmikrobiota erklärt werden kann. Die Schätzung von m^2 betrug 0,15 für PU und war hoch signifikant. Sie waren ebenfalls hochsignifikant für die Futteraufnahme, die Körpergewichtszunahme und Futter pro Zunahme. Dieses Modell wurde bivariat erweitert und zeigte eine hohe mikrobielle Korrelation der Merkmale. Basierend auf beiden Ergebnissen spielt die Zusammensetzung der Ileum Mikrobiota sowohl bei PU, als auch bei den Leistungsmerkmalen eine wesentliche Rolle und es besteht eine beträchtliche Tier-Mikrobiota-Korrelation, die zeigt, dass die Mikrobiota mehrere Merkmale beeinflusst. Die mikrobiellen Treiber dieser Mikrobiotafraktion identifizierten wird durch die Anwendung von mikrobiomweiten Assoziationsstudien (MWAS). Durch Auflösen des mikrobiellen gemischten linearen Modells haben wir den Effekt einzelner OTUs auf die phänotypischen Merkmale aus den mikrobiellen Modellösungen approximiert. Eine MWAS auf Gattungsebene deckte mehrere Merkmale auf, die mit Bakteriengattungen assoziierten sind.

Anschließend untersuchten wir, ob die mikrobielle Gemeinschaft im Ileum ein erbliches Wirtsmerkmal ist, das für die Zucht von Individuen mit verbesserter PU genutzt werden kann. In **Kapitel fünf** wendeten wir für spezifische Gattungen eine QTL-Analyse an, um zu untersuchten, ob sie mit genomischen SNP-Markern verknüpft sind. Diese QTL-Analysen zeigten eine Verbindung zwischen einigen Mikrobiotaspezies und genomischen Regionen von Chromosomen und SNP-Markern des Wirts. Durch die Schätzung signifikanter Heritabilitäten für einige Gattungen konnten wir Nachweise für die Hypothese liefern, dass die mikrobielle Gemeinschaft und mikrobielle Merkmale zumindest teilweise mit der Wirtsgenetik zusammenhängen. Wir schätzten die mikrobiellen Effekte der Tiere auf PU und korrelierte Leistungsmerkmale voraus, indem wir *microbial best linear unbiased predictions* (M-BLUP) anwendeten. Zusätzlich wurden *genomic best linear unbiased predictions* (G-BLUP) verwendet, um den SNP-Effekt für den vorhergesagten tierischen mikrobiellen Effekt zu schätzen. Eine Kombination

dieser beiden kann helfen, genomische Zuchtwerte der Mikrobiota-Effekte für zukünftige hologenomische Zuchtprogramme vorherzusagen.

CHAPTER I

Introduction

CHAPTER I – Introduction

1.1 Phosphorus availability in poultry production

As the global population continues to grow, the production of essential food is becoming an ever greater challenge (Thornton 2010). Phosphorus (P), among other macro- and micronutrients, is a crucial and essential mineral. Its key role as an important adjusting screw in food production is worth protecting, as it cannot be replaced by any other mineral. Phosphorus is an essential component of RNA and DNA and in carbohydrate, fatty acid, and even amino acid metabolism. When bound as adenosine mono-, di-, or triphosphate (AMP, ADP, and ATP, respectively), it provides energy in body cells. A massive proportion of minerals is stored in the bones of animals. Therefore, an adequate supply of P and Calcium (Ca) is urgently needed to ensure bone stability (Williams et al. 2000).

Phosphorus occurs naturally in an inorganic, mineral form (phosphate rock), or organically, when it is bound as phytic acids and its salt phytate (inositol compounds) in plant seeds (Eeckhout and Paepe 1994; Rodehutscord et al. 2016). These P sources are not fully usable for monogastric animals because they have a low endogenous phytase activity in the gastro intestinal tract (GIT) in the brush border membrane (Maenz and Classen 1998). These enzymatic breakdowns by phytase enzymes are necessary for hydrolyzing phytate to render contained P accessible. Such enzymatic breakdown can only take place to a small extent in poultry, and the needs of an animal are not met by purely plant-based diets. Consequently, animal feed is supplemented with mineral P or phytase enzymes.

There is a global scarcity of P from usable, high-quality sources (Cordell et al. 2009). The largest portion of the remaining high-quality, available, and mineable phosphate rock is controlled by only five countries worldwide: China, Jordan, the USA, South Africa, and Morocco (Neset and Cordell 2012). This results in the fact that P supplementation accounts for a substantial part of feed costs. In addition, the available P is not used effectively enough and is wasted at many points before it reaches the end consumer. Examples include over-fertilization of soil and eutrophication due to run-off into bodies of water (Ashley et al. 2011), which can lead to negative consequences. Additional waste results from the over-supplementation in animal feed, which occurs because of the extensive variability in the P requirements of individual animals (Punna and Roland 1999) according to the age, diet, environmental influences, or genetics of the animal. This surplus is further spread through the application of excrement as fertilizer, which incites environmental problems.

Overall, as demand for meat intensifies with population growth, the consumption of mineral P will indirectly increase in the coming years. The use of this important element must be adapted in the future to protect the long-term food security of the rising world population. There are various approaches to improve holistic P consumption and utilization. Conceivable approaches include recycling from a wide variety of sources (Leinweber et al. 2018), more accurate matching to soil needs, harnessing P from sources of poorer quality (e.g. through the removal of pollutants), and enhanced utilization by

plants and animals. Although these methods alone are unlikely to ensure more sustainable use, this work provides a starting point in the form of adapted animal breeding to achieve more efficient and sustainable use of P.

Few studies have investigated the relationship between phosphorus utilization (PU) and host genetics in poultry. They have demonstrated that, under standardized conditions, a broad phenotypic variation exists between individuals in terms of PU (Beck et al. 2014; Beck et al. 2016; Edwards 1982; Nelson 1967; Punna and Roland 1999; Zhang et al. 1998; Zhang et al. 2003). The early studies of Zhang et al. (1998) and Punna and Roland (1999) have concluded that the breed of an animal may impact PU. According these authors, the integration of PU as a trait is conceivable in breeding programs. This hypothesis has been supported by subsequent studies with different poultry species that have estimated moderate heritabilities for various traits that affect PU. For instance, Zhang et al. (2003) and Ankra-Badu et al. (2010) estimated a heritability of 0.09 for phytate P bioavailability in broilers and chickens, respectively. Furthermore, de Verdal et al. (2011) estimated a heritability of 0.22 for the ratio of P intake to P excretion in chickens, and Beck et al. (2016) estimated a heritability of 0.14 for PU in Japanese quail.

To understand the complexity of PU, genetic and phenotypic correlations with performance traits have been estimated in several poultry species. Zhang et al. (2003) observed low to moderate phenotypic and genetic correlations between phytate P bioavailability and body weight (BW), body weight gain (BWG), and feed consumption in their study with broilers. Ankra-Badu et al. (2010) also reported genetic correlations between phytate P bioavailability and BW, feed conversion ratio, and calcium (Ca) bioavailability in chickens. Beck et al. (2016) estimated negative phenotypic and genotypic correlations of feed per gain (F:G) and PU as well as positive phenotypic and genotypic correlations of BWG and PU in Japanese quail. This trend has been further evidenced by Künzel et al. (2019), who reported moderate to high phenotypic and genotypic correlations for PU and Ca utilization (CaU), BWG, feed intake (FI), and F:G. The latter was the only trait with negative correlations. Given that modern commercial layers and broilers are selected for high performance, these correlations may be crucial considerations in future breeding strategies.

1.2 Intestinal microbiota composition of quail and chickens

In addition to the phytase activity of the small intestinal brush border membrane of chickens (Maenz and Classen 1998), it is well-known that some gut microorganisms can produce phytases (Akyurek et al. 2011; Borda-Molina et al. 2019; Ptak et al. 2015). Therefore, it is worth to consider these microorganisms in PU and, ideally take them into account in breeding strategies.

In recent years, increased attention has been directed to the gut microbiota because they demonstrate a relation to performance traits, metabolism, and behavior. By definition, the term “microbiota” refers to the assemblage of specific microorganisms, such as bacteria, fungi, protozoa, and viruses, in the same environment (Marchesi and Ravel 2015). This microbiota composition can be highly

variable between animals. The metagenome encompasses all genomes and genes from the species of the microbiota. Operational taxonomic units (OTUs) describe a unit of microbial genomic sequences clustered by their sequence similarity. The concept of the microbiome represents the collection of microbiota, their metagenome, and the host as their habitat (Marchesi and Ravel 2015). Furthermore, the symbiotic relationship of the microbiota with the host can be viewed as a whole organism, namely the holobiont. The term "hologenome" refers to both genomes (Bordenstein and Theis 2015; Estellé 2019). From these multi-layered descriptions, it is apparent that the interpretation and discussion of the microbiome, or even of the hologenome, are far more complex than those of microbiota composition alone.

While some studies examined intestinal microbiota composition of Japanese quail (Borda-Molina et al. 2020; Liu et al. 2015; Su et al. 2014; Wilkinson et al. 2016; Wilkinson et al. 2020), analyses of intestinal microbiota have more commonly focused on laying hens or broilers. Poultry have a shorter gastro intestinal tract (GIT) and, accordingly, a shorter passage rate compared to mammals (Golian and Maurice 1992). The activity of the intestinal microbiota is of immense importance. Previous quail studies investigating different GIT sections reported that the intestinal microbial community of the ileum consists primarily of four phyla: *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteriodetes* (Liu et al. 2018; Su et al. 2014; Wilkinson et al. 2016). The main phyla dominating the quail ileum are also abundant in chickens (Clavijo and Flórez 2018; Oakley et al. 2014).

The colonization of individual GIT sections is always dependent on the given conditions. For instance, the proventriculus and gizzard present low colonization of microbiota compared to the small and large intestine because their pH value limits growth, and commensal microbiota species differ in their nutrient requirements (Deusch et al. 2015). In contrast, the small and large intestine have favorable conditions for the growth of many microbiota species, and these intestinal segments assume the main responsibility for nutrient digestibility and absorption. It is well-known that the digestive process is closely related to intestinal microbiota. Specialized GIT microbiota species ferment indigestible carbohydrates and polysaccharides, which are indispensable for the host (Du et al. 2020). This fermentation leads to the production of short-chain fatty acids (SCFAs) and energy (Du et al. 2020; Koh et al. 2016), which then become available to the host. These SCFAs include acetate, propionate, and butyrate, which vary in proportion depending on the microbial composition in the intestinal section. The utilization of nutrients and conversion into valuable substrates by specific microbiota species also concerns the utilization of certain essential minerals such as P. Ruminants meet a major part of their P requirements with the help of specialized intestinal microbiota species in the rumen, which harness P through their phytase enzymes (reviewed in Humer and Zebeli 2015). As noted, nonruminants have low endogenous phytase activity, which can also be supported by certain intestinal microbiota species or microbial phytases (Akyurek et al. 2011; Borda-Molina et al. 2019; Ptak et al. 2015).

1.2.1 Factors influencing the intestinal microbiota

The establishment of a healthy gut microbiota composition is relevant to animal welfare and performance. In this regard, the development and diversity of GIT microbiota are heavily influenced by numerous host-related and environmental factors. They interact among themselves, with the host and the host genetics, and with diet and feed additives. Many of these factors have been addressed in existing reviews of various poultry species (Choi et al. 2015; Diaz Carrasco et al. 2019; Kers et al. 2018; Maki et al. 2019; Rychlik 2020; Shang et al. 2018; Yadav and Jha 2019), and some are outlined below.

The microbial colonization in the GIT starts during incubation and continues immediately after hatching. The mother's cloacal microbial community determines the first contamination and condition of the egg (Apajalahti et al. 2004) and may enter the avian organism through the pores of the eggshell (Gantois et al. 2009; Lee et al. 2019; Maki et al. 2020). After hatching, the microbial colonization changes with the age of the chicken (Knarreborg et al. 2002; Lu et al. 2003; Videnska et al. 2014; Yeoman et al. 2012) and according to stress responses (Lyte et al. 2021). Besides age, both gender (Borda-Molina et al. 2020; Su et al. 2014; Torok et al. 2013; Wilkinson et al. 2016; Zhao et al. 2013), and host genetics (Kers et al. 2018; Lumpkins et al. 2010; Meng et al. 2014; Mignon-Grasteau et al. 2015; Schokker et al. 2015; Wen et al. 2019; Zhao et al. 2013) have been discussed as host-related factors that could determine the microbial community in the GIT; however, the extent to which the latter may determine the microbiome composition is controversial.

Some environmental factors that evidently affect intestinal microbiota composition in different poultry species are housing conditions (Kers et al. 2019) and, more specifically, litter (Wang et al. 2016) and stocking density (Guardia et al. 2011). Additionally, an important human-made factor is diet, which is decisive for gut health (Liu et al. 2015; Liu et al. 2018). Diet also includes the use of pre- and probiotics (Abou-Kassem et al. 2021; Fonseca et al. 2010; Parois et al. 2017; Tufan and Bolacali 2017; Vali 2009) and the maintenance of the numbers of beneficial commensal microbiota in the GIT. The diet is an energy supplier for microbiota species, and it directly impacts the development of specific microbial species that, in turn, may have a positive or negative effect on the host. For instance, the production of SCFAs can lower the pH value in GIT segments, thereby contributing to the microbial colonization of the intestinal tract through. This may lead to a reduction of pathogens (van der Wielen et al. 2000).

1.2.2 Influence of intestinal microbiota on the host and quantitative traits

In addition to the influences on the GIT microbiota, the effects of the intestinal microbiota on a wide range of host traits have been a subject of recent research. Certain stress- and behavior-related microbiota parameters, such as the fearfulness of an animal, have been examined by Kraimi et al. (2018), whose research demonstrated a reduction of fearfulness in germ-free quails compared to microbial-colonized quails. Similarly, a study on the effect of a probiotic treatment revealed diminished fearfulness and improved memory in quails (Parois et al. 2017).

Other commercial microbiota-influenced parameters are the performance parameters of an animal as well as metabolic processes, morphological changes, and the maintenance of health and immunity. The GIT microbiota are jointly responsible for breaking down and utilizing nutrient that are indigestible for the host itself. The end products of microbial fermentation are subsequently available for the host. End products, such as the aforementioned SCFAs, occupy a key role in the GIT; they provide energy for gluconeogenesis (de Vadder et al. 2014) and are partly responsible for changes in intestinal structures, such as the proliferation of intestinal epithelial cells (Pan and Yu 2014; Rinttilä and Apajalahti 2013) or an increase of villus height in the duodenum of broilers (Panda et al. 2009). Thus, microbial fermentation conditions a larger absorption surface in the GIT.

Additionally, feed efficiency (FE) and feed conversion ratio (FCR) are crucial for economic profitability of farms and achieving more sustainable production. Most studies on FCR-associated intestinal microbiota were conducted using the ratio of feed intake (FI) to BW or BWG within a defined period of time. In principle, lower FCR translates to superior and a more efficient conversion of feed into body mass or performance. In this regard, multiple studies investigated FE- or FCR-associated intestinal microbiota to specify species with positive or negative correlations with these traits and to identify many different phylotypes (Schokker et al. 2015; Singh et al. 2012; Singh et al. 2014; Stanley et al. 2013; Stanley et al. 2016; Yan et al. 2017). Among these microbiota are butyrate-producing bacteria, which are beneficial species for cellulose and starch degradation (Stanley et al. 2013). Past research proved that butyrate is a preferred energy source for epithelial cells, as it promotes cell proliferation of intestinal epithelial cells in mice (Donohoe et al. 2011). The connection with FCR is logical in view of the above-mentioned surface enlargement through an increase in villus height in broilers (Panda et al. 2009) as well as the lengthening of microvilli and increase in crypts depth in the jejunum of chickens (Leeson et al. 2005). One of the metrics for FCR is BWG. The BW of broilers was positively correlated with *Bifidobacterium* in the ileum and *Lactococcus* in the cecum and negatively correlated with the genera *Streptococcus* and *Akkermansia* in the ileum and cecum (Han et al. 2016).

Few studies estimated genetic parameters of microbial compositions in the GIT of poultry in terms of quantitative traits. Some heritable fecal microbial species were detected in studies investigating the BW of chickens (Meng et al. 2014; Zhao et al. 2013). Mignon-Grasteau et al. (2015) examined the cecal microbiota composition of chickens concerning different FE expressions. They estimated low heritabilities for individual species as well as moderate heritabilities for ratios of different species such as *Lactobacillus crispatus*, *Clostridium leptum*, *Clostridium coccoides* and *Escherichia coli*. In addition, they detected chromosome-wide significant QTL affecting the cecal microbial composition.

The reviewed studies reflect a direct connection between intestinal microbiota and quantitative traits. Since the genetic and phenotypic correlation of BWG and FCR with PU has already been illustrated (Ankra-Badu et al. 2010; Beck et al. 2016; Künzel et al. 2019; Zhang et al. 2003), and intestinal microbiota have demonstrated phytase activity (Palacios et al. 2008; Ptak et al. 2013; Selle and

Ravindran 2007), it is of critical importance to investigate the interaction of the GIT microbiota and PU. Since the mucosa of quail and chickens can provide phytase to only a limited extent (Huber et al. 2015; Maenz and Classen 1998), the host relies on specialized microbiota species, feed supplementation, or both. Because of the higher phytase requirement in the upper segments of the GIT (Huber et al. 2015; Ptak et al. 2015), most microbiota species associated with phytase activity were found in that area (Ptak et al. 2015; Witzig et al. 2015).

To date, few studies examined the effect of mineral P and phytases on the GIT microbiota. Ptak et al. (2015) indicated that changes in Ca and P supplementation have an effect on the composition and activity of GIT microbiota in broilers. This finding suggests that the gut microbiota composition adapts to and selects the supplementation of mineral P or phytases. Since GIT microbiota are presumably associated with correlated traits beyond the known associations, and they may be involved to varying degrees in the expression of such traits, it is necessary to gain a deeper understanding of host-microbiota interactions. The actual PU, with the synergy of the host and GIT symbiotic microorganisms included, is difficult to estimate. Therefore, the overall aim of this thesis is to investigate PU variability between animals without additional P supplementation while taking into account the host genetics, the ileum microbiota composition and their interaction.

1.3 Thesis outline

The research utilizes an existing large dataset of an F_2 design of Japanese quail (Beck et al. 2016). The animals were raised and maintained in a controlled environment under standardized conditions. No mineral P or phytase supplementation was applied in order to allow the quails to exhibit their full potential of PU and performance as well as the ileum digesta microbiota composition. The findings of this thesis can enhance our understanding of the link between quail genetics and PU as well as of PU-associated microbial species, which can support the development of a hologenomic selection for breeding individuals with improved PU.

In **chapter two**, we hypothesize that the variation of PU in quail is a heritable trait that is conditioned by a few detectable quail QTL. To evaluate this hypothesis, individuals were genome-wide genotyped with a 4k SNP chip, and devised a linkage map. Then, we performed QTL linkage analysis using multimarker regression analysis in a line-crossing model to map QTL for PU, FI, BWG, F:G, and CaU as well as for the bone ash traits of tibia ash (TA) and foot ash (FA) in terms of mg and percentage (TA% and FA%, respectively).

In **chapter three**, we predict microbiota-related host traits based on an examination of the ileum microbiota composition and a differential abundance analysis (DAA). In order to assess the impact of the microbiota composition in the ileum on PU, a sample of 100 trait discordant individuals were compared in the DAA. Then, **chapter four** explores whether the variation of PU is partly driven by the

microbial community in the ileum of the quail. To unravel the microbial architecture of the evaluated traits, we characterized the influence of performance-related gut microbiota on the basis of microbial mixed linear models for estimating microbiabilities (m^2) as well as bivariate extensions for determining the microbial correlation of the traits PU, FI, BWG, and F:G. Furthermore, we applied microbiome-wide association studies (MWAS) at the genera and OTU levels to identify the microbial drivers of m^2. The effects of single OTUs on the phenotypic traits from the microbial model solutions were approximated by back-solving the microbial linear mixed model. Additionally, we applied functional predictions for three levels of predictions.

For future breeding individuals with improved PU, we subsequently assess whether the microbial community in the ileum is a heritable host trait. Therefore, in **chapter five** we used a selection of 74 genera to estimate their heritabilities. These genera were additionally used as observations in a QTL analysis to investigate whether they are linked to SNP markers and regions of the genome. We predicted the animal microbial effects on PU and correlated traits by applying microbial best linear unbiased predictions (M-BLUP). In addition, genomic best linear unbiased predictions (G-BLUP) were used to predict the SNP effect for the predicted animal microbial effect. A combination of those two may help to predict genomic breeding values of the microbiota effects for future hologenomic breeding programs.

The thesis concludes with a general discussion in **chapter six**. This chapter provides additional QTL results from different analysis methods. Moreover, it debates the estimation of OTU effects and the combination of animal microbiota effects with animal genetic effects in the statistical model. Finally, the chapter considers possibilities for the integration of microbiota data sets into selection schemes and a conceivable application of the selection program.

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CHAPTER II

1st Publication

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Mapping genes for phosphorus utilization and correlated traits using a 4k SNP linkage map in Japanese quail (*Coturnix japonica*)

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Summary

A large F2 cross with 920 Japanese quail was used to map QTL for phosphorus utilization, calcium utilization, feed per gain and body weight gain. In addition, four bone ash traits were included, because it is known that they are genetically correlated with the focal trait of phosphorus utilization. Trait recording was done at the juvenile stage of the birds. The individuals were genotyped genome-wide for about 4k SNPs and a linkage map constructed, which agreed well with the reference genome. QTL linkage mapping was performed using multimarker regression analysis in a line cross model. Single marker association mapping was done within the mapped QTL regions. The results revealed several genome-wide significant QTL. For the focal trait phosphorus utilization, a QTL on chromosome CJA3 could be detected by linkage mapping, which was substantiated by the results of the SNP association mapping. Four candidate genes were identified for this QTL, which should be investigated in future functional studies. Some overlap of QTL regions for different traits was detected, which is in agreement with the corresponding genetic correlations. It seems that all traits investigated are polygenic in nature with some significant QTL and probably many other small-effect QTL that were not detectable in this study.

Keywords: feed utilization, Japanese quail, linkage map, quantitative trait loci

Introduction

Phosphorus is an essential mineral for all living organisms. It is important for energy metabolism, nucleic acid synthesis, enzyme activity and bone mineralization. Most of the phosphorus in plant seeds and feedstuffs produced thereof is present as phytic acid and its salts, called phytates (Eeckhout & Paepe 1994). Owing to low endogenous phytase activity in the digestive tract of poultry, phytate-P sources can only partially be utilized. Therefore, poultry diets are usually supplemented with mineral phosphorus, often in combination with exogenous phytase, which results in additional costs. Additionally, global mineral phosphorus resources are limited, and the phosphorus in excreta has an environmental impact. Therefore, it is desirable to minimize mineral phosphorus supplementation without compromising animal health and performance. Thus, high phosphorus utilization (PU) by animals is desirable.

Japanese quail (*Coturnix japonica*) has long been an important model species in poultry studies because of its short generation intervals, small body size, which results in a smaller space requirement (Kayang *et al.* 2004; Cheng *et al.* 2010), and similarity to other poultry species (Stock & Bunch 1982; Shibusawa *et al.* 2001). A recent study implemented an F2 experimental design with approximately 1000 Japanese quail and phenotyped the F2 individuals for PU and related traits (Beck *et al.* 2016a). The coefficient of variation for PU was 0.11, which indicated substantial variation, with a heritability of 0.14 (±0.06). By applying structural equation models some complex relationships of PU were detected with body weight gain and feed per gain ratio (Beck *et al.* 2016a). A subsequent study of the ileum microbiota composition of those birds estimated a significant microbiability for PU (Borda-Molina *et al.* 2020; Vollmar *et al.* 2020). In addition, ileal transcriptome profiles, miRNA–mRNA and gut microbiome interactions of subsets of quails with divergent PU have been studied (Oster *et al.* 2020; Ponsuksili *et al.* 2020).

Because calculation of PU involves quantitative measurement of feed intake and excretion over several days, PU is a very-hard-to-measure trait in a routine breeding enterprise. Therefore, proxy traits and genetically correlated traits are desirable and convenient to measure. Bone ash traits are features that have been used to determine the bioavailability of phosphorus in quail (Vali & Jalali 2011) and chicken (Li *et al.* 2017). Several bone ash traits were analyzed using samples from the experiment of Beck *et al.* (2016a) and the genetic correlations with PU were estimated, which were between 0.5 and 0.6 (Künzel *et al.* 2019). Thus, it might be possible to consider bone ash traits as proxy traits to breed for the improvement of PU.

Until now, it has been largely unknown whether the genetic variance of PU is caused by many QTL with small effects or if there are some large QTL that might be of special interest for breeding purposes. Therefore, the aim of this study was to map the QTL associated with the focal trait PU as well as other performance traits and bone ash traits in Japanese quail using an F2 cross. The individuals were genotyped genome-wide with 4k SNPs, and we used these data to establish a linkage map and subsequently to conduct QTL linkage and association mapping.

Material and Methods

Experimental design

The experiment was conducted in accordance with the German Animal Welfare Legislation approved by the Animal Welfare Commissioner of the University Hohenheim (approval number S371/13TE). An F2 cross of Japanese quail (*C. japonica*) was established. The details of the F2 design can be found in Beck *et al.* (2016a), and only the essential steps are described in the following. The founder lines were divergently selected for social reinstatement behavior in an earlier experiment conducted at the INRA, France (Mills & Faure 1991). The selection of these founder individuals is thus not related to the focal trait PU. Twelve males from founder line A (B) were mated to 12 females from founder line B (A) to produce the F1 generation. From this generation, 17 males and 34 females were selected, and one male was mated with two females, resulting in 920 F2 individuals. These individuals belonged to 34 full-sib families and 17 paternal half-sib families, with approximatively the same family size. A low-P-content diet was provided to allow the quails to exhibit their full PU potential. The diet did not contain mineral P supplement or phytase.

Trait records

Body weight gain (BWG) was calculated as the difference in body weight at days 10 and 15. Feed per gain ratio (F:G) was calculated as feed intake (FI) within this 5-day period divided by BWG. PU and calcium utilization (CaU) were calculated for this period based on quantitative intake and excretion of the elements as described in Beck *et al.* (2016a). The quails were slaughtered on day 15, and the right tibia and the right foot were preserved. The total amount of ash in the tibia and foot (TA and FA) as well as ash concentrations in the dry matter of the bones (TA% and FA%) were recorded as described in detail in Künzel *et al.* (2019). Descriptive statistical parameters, heritabilities and trait abbreviations are provided in Table 1. The heritabilities of the traits were estimated by Beck *et al.* (2016a) and Künzel *et al.* (2019) using mixed linear animal models.

DNA collection and SNP genotyping

One milliliter of blood was collected from each animal using EDTA-K tubes and stored at −20°C until DNA extraction was performed using the Maxwell 16 Blood DNA Purification Kit (Promega). The DNA concentration was adjusted to 50 ng/µl to ensure consistent measurements. Using a customer's Illumina iSelect chip, we genotyped 5388 SNPs. The SNP markers were mapped through the chicken genome using the method described in Recoquillay *et al.* (2015), as no quail genome was available at the time of genotyping. The following criteria were applied to filter the genotypes: one or more conflicting genotypes between parent and offspring, a MAF ≤0.03, an SNP call frequency ≤0.9 and cluster separation ≤0.4. This led to the exclusion of 842 SNPs. Furthermore, we rejected SNPs on the sex chromosomes Z or W and in the linkage group (LG) LGE22C19W28_E50C23 or E64 (information

obtained from the *C. japonica* reference genome assembly (NCBI GCA_001577835.1)). This filtering resulted in a total of 3986 SNP markers for further analysis.

Table 1 *Descriptive statistics and heritabilities of the traits.*

Trait[1,2]	abbreviation	unit	min	max	mean	h^2 (SE)
Phosphorus utilization[+]	PU	%	21.49	87.43	71.41	0.14 (0.06)
Calcium utilization*	CaU	%	19.42	84.31	60.56	0.17 (≤0.10)
Feed per gain[+]	F:G	g/g	1.21	3.92	1.78	0.12 (0.06)
Feed intake*	FI	g	16.11	62.35	42.65	0.11 (≤0.10)
Body weight gain[+]	BWG	g	5.80	37.85	24.50	0.09 (0.14)
Tibia ash (mg)*	TA	mg	19.20	83.50	45.82	0.23 (≤0.10)
Tibia ash (%)*	TA%	%	35.53	55.71	45.26	0.23 (≤0.10)
Foot ash (mg)*	FA	mg	19.60	83.60	44.76	0.34 (≤0.10)
Foot ash (%)*	FA%	%	12.10	21.91	17.30	0.31 (≤0.10)

[1] From days 10–15 of life.

[2] Measurements and heritabilities from Beck *et al.* (2016a)[†] and Künzel *et al.* (2019)* and SEs are in parentheses.

Linkage map construction

The linkage mapping software LEP-MAP2 (Rastas *et al.* 2015) was used to build a sex-averaged Japanese quail map. The software uses pedigree and marker information to assign SNP markers to LGs and computes the likelihood of the marker order within each LG using standard hidden Markov models (Rastas *et al.* 2013; Rastas *et al.* 2015). In the first step, the module *SeparateChromosomes* was used to assign markers to the LG. We used the option LOD = 1–20 to test lodLimits with a sizeLimit = 5 so that LGs with fewer than five markers were removed. A lodLimit of 5 resulted in 27 LGs with 3975 markers assigned to them. The remaining markers were assigned to LGs by using the module *JoinSingles* with lodLimit = 1–15 and lodDifference = 2. A lodLimit = 1 was selected because there was no difference compared with other lodLimits in terms of results, and an additional nine SNPs could be assigned. The module *OrderMarkers* orders the markers within each LG. This step was replicated five times to select the best order with the highest likelihood. The module was run with the options polishWindow = 30, filterWindow = 10 (both parameters are used for speeding up the computations), numThreads = 10 (maximum number of threads to use), useKosambi = 1 (using Kosambi mapping function), minError = 0.15 (because genotyping errors can lead to large map distances) and sexAveraged = 1 (to compute the sex-averaged map distances).

To compare the calculated genetic map with the reference genome *C. japonica* (NCBI GCA_001577835.1), the flanking sequences for each SNP were aligned by performing BLAST searches of the reference genome. This led to the assignment of our LGs to the chromosomes. These assignments were used throughout the rest of the study.

QTL linkage and association analysis

A line cross model was applied in this study. For this purpose, we used the package RQTL2 (Broman *et al.* 2019). This program was developed for inbred line crosses. We estimated the F_{ST} value for each SNP in the two founder populations using eq (8) in Weir & Cockerham (1984). Subsequently, we selected only those SNPs with an $F_{ST} > 0.23$, which comprised approximately half of the SNPs, and the selected SNPs were used for QTL linkage mapping. This filtering ensured that the assumptions regarding the inbred founder lines made by the software were approximatively fulfilled. In addition, we selected only those chromosomes with >40 SNPs because we applied multimarker linkage mapping. These two filter steps resulted in 1968 SNPs that were used for QTL linkage mapping on 19 chromosomes. Subsequently, we applied the RQTL2 software package and estimated QTL genotype probabilities for each F2 individual and each marker position. These probabilities were used in a regression analysis to map the QTL. We included the hatches as fixed effects in the regression model. The LOD score was used as a test statistic, and correction for multiple testing was done using the permutation test (10 000 permutations). We considered two significance criteria for each trait, i.e. 1 and 5% genome-wide significance (LOD scores 4.9–5.9 and 4.2–4.7 respectively). The QTL support intervals (SI) were approximated using the LOD drop off method with a drop of 1.5 LOD (Manichaikul *et al.* 2006). The upper and lower bounds of the SI were extended by 5 cM to be conservative. Because the assumptions of the linkage QTL mapping approach regarding the inbred founder lines were only approximated fulfilled (i.e. not every SNP with $F_{ST} > 0.23$ was divergently fixated in the two founder lines), we conducted an SNP association analyses. For this purpose, we tested all markers within the SI (i.e. also those with an $F_{ST} < 0.23$) for trait associations to support the presence of a QTL. We repeated this process for each SNP within the intervals separately by applying a mixed linear model using the software GCTA (Yang *et al.* 2011). The hatches were considered as fixed effects, and correction for putative population stratification effects was performed by including a random animal effect based on a genomic relationship matrix that was calculated using all markers except those on the chromosome under consideration (i.e. the leave-one-chromosome-out option in gcta). As only those markers in the SI were tested for associations, no correction for multiple testing was performed.

To identify positional candidate genes in the 0.5 Mbp regions up- and downstream of significant SNPs, we used Genome Data viewer from NCBI and the reference genome assembly (NCBI GCA_001577835.1).

Results

Construction of the linkage map

The summary of the linkage map is shown in Table 2, and a list of SNPs with their chromosomal position was made public available (see Data availability statement). The linkage map is plotted in Fig. S1. A total number of 3975 SNPs were assigned to 27 LGs. The map covers 1735 cM with individual LG lengths that range from approximately 3 cM [*C. japonica* chromosome (CJA) 28] to 253 cM (CJA1) (Table 2). The number of markers per chromosome varied from 5 (CJA25) to 769 SNPs (CJA1), and the average density was 0.81 markers per cM across all chromosomes. We estimated a high correlation between the genetic (cM) position of the calculated linkage map and the physical (bp) position of the reference genome assembly (NCBI GCA_001577835.1), ranging from 0.88 to 0.99 (Table 2). Overall, the order of the markers of the genetic map agreed well with the order of the physical positions of the reference genome. No LGs could be assigned to chromosome 16, because this chromosome is poorly characterized so far and no SNP could be assigned to it. Figure shows the comparison between the physical (bp) and genetic map (cM) for chromosome 2, and some outliers are visible. These outlier markers were either identified at other positions within an LG compared with the reference genome or had positions that were not yet known. The comparisons of the remaining chromosomes are shown in Fig. S1.

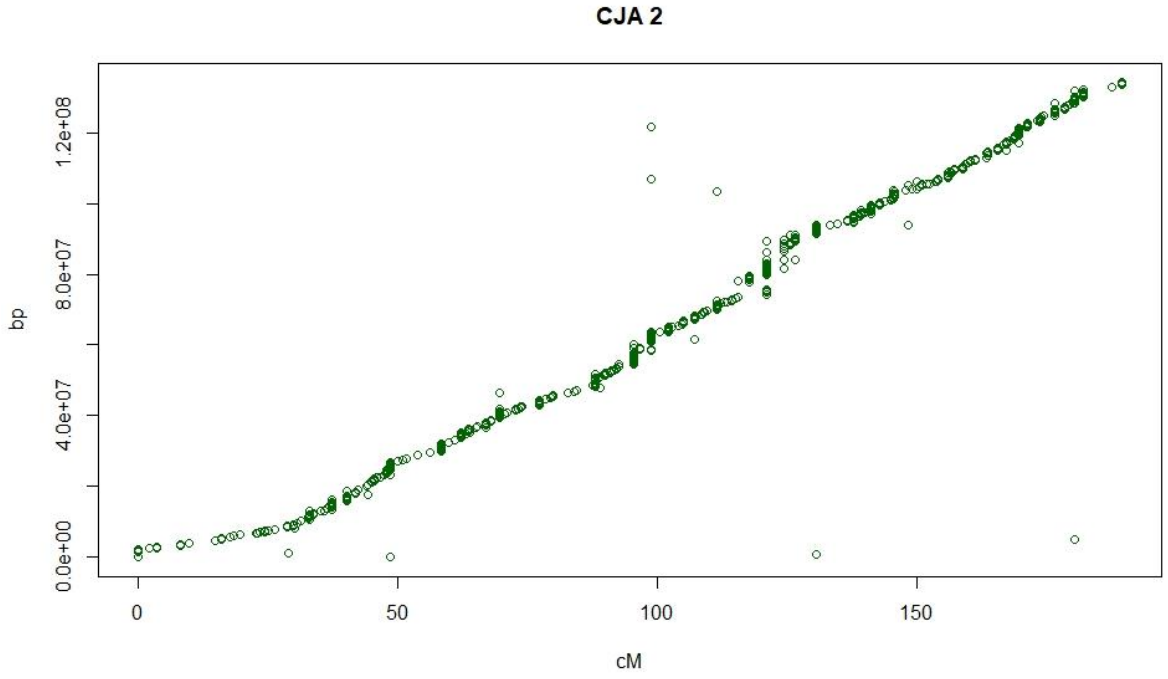

Figure 1 *Plot of SNPs that were assigned to chromosome 2. The y-axis shows the physical position (bp), which is based on the reference genome assembly (NCBI GCA_001577835.1), and the x-axis shows the genetic position (cM). Note that the SNP positions at 0 bp refer to an as yet unknown position*

Table 2 *Numbers of markers (*n *SNPs) on each chromosome (*Coturnix japonica, *CJA), length in cM and in Mb, average number of markers per cM and per Mb, and correlation between the cM and the bp positions*

CJA	n SNPs	Length		Markers		Correlation
		cM	Mb[1]	per cM	per Mb	
1	769	253.09	177	0.33	0.23	0.96
2	650	189.57	136	0.29	0.20	0.98
3	457	153.11	101	0.34	0.22	0.94
4	436	116.80	83	0.27	0.19	0.98
5	278	97.64	54	0.35	0.19	0.89
6	145	64.45	32	0.44	0.19	0.97
7	152	68.45	34	0.45	0.20	0.93
8	138	54.70	27	0.40	0.15	0.92
9	121	53.67	21	0.44	0.15	0.99
10	88	44.22	19	0.50	0.17	0.96
11	91	43.40	18	0.48	0.18	0.95
12	90	47.72	17	0.53	0.15	0.99
13	63	38.74	16	0.61	0.19	0.96
14	75	47.11	13	0.63	0.16	0.92
15	55	46.04	12	0.84	0.19	0.98
16	-	-	0.3	-	-	-
17	52	45.19	9	0.87	0.14	0.98
18	40	38.77	10	0.97	0.13	0.95
19	56	45.78	9	0.82	0.12	0.97
20	62	51.92	13	0.84	0.20	0.96
21	26	31.08	6	1.20	0.15	0.96
22	18	39.11	4	2.17	0.13	0.96
23	27	39.93	5	1.48	0.13	0.96
24	36	46.08	6	1.32	0.15	0.98
25	5	3.33	3	0.67	0.10	0.88
26	19	37.30	5	2.33	0.14	0.95
27	17	35.41	5	2.08	0.19	0.89
28	9	2.77	4	0.31	0.13	0.95
total	3975	1735.36	839.30	-	-	-
average	147	64.27	29.98	0.81	0.17	0.95

[1] Size in Mb based on the reference genome *Coturnix japonica* 2.0 (NCBI GCA_001577835.1)

Identification of QTL

The test statistic plots of the analyzed chromosomes are shown in Figs 2 & 3 for the performance and the bone ash traits respectively. A total of 21 QTL (eight QTL for 1%, 13 QTL for 5%) were mapped for all traits at a 1% (5%) genome-wide significance level. For all traits, QTL could be mapped, except for F:G. A detailed description of the QTL is given in Table 3. For PU, we identified one QTL on CJA3, and for BWG, we found one QTL on CJA3, whereas all other traits were associated with two or more QTL (Table 3, Figs. 2 & 3). Some SI overlapped for several traits. For example, the SI on CJA3 for PU and FA% and the SI on CJA4 for CaU, TA and FA overlapped (Table 3).

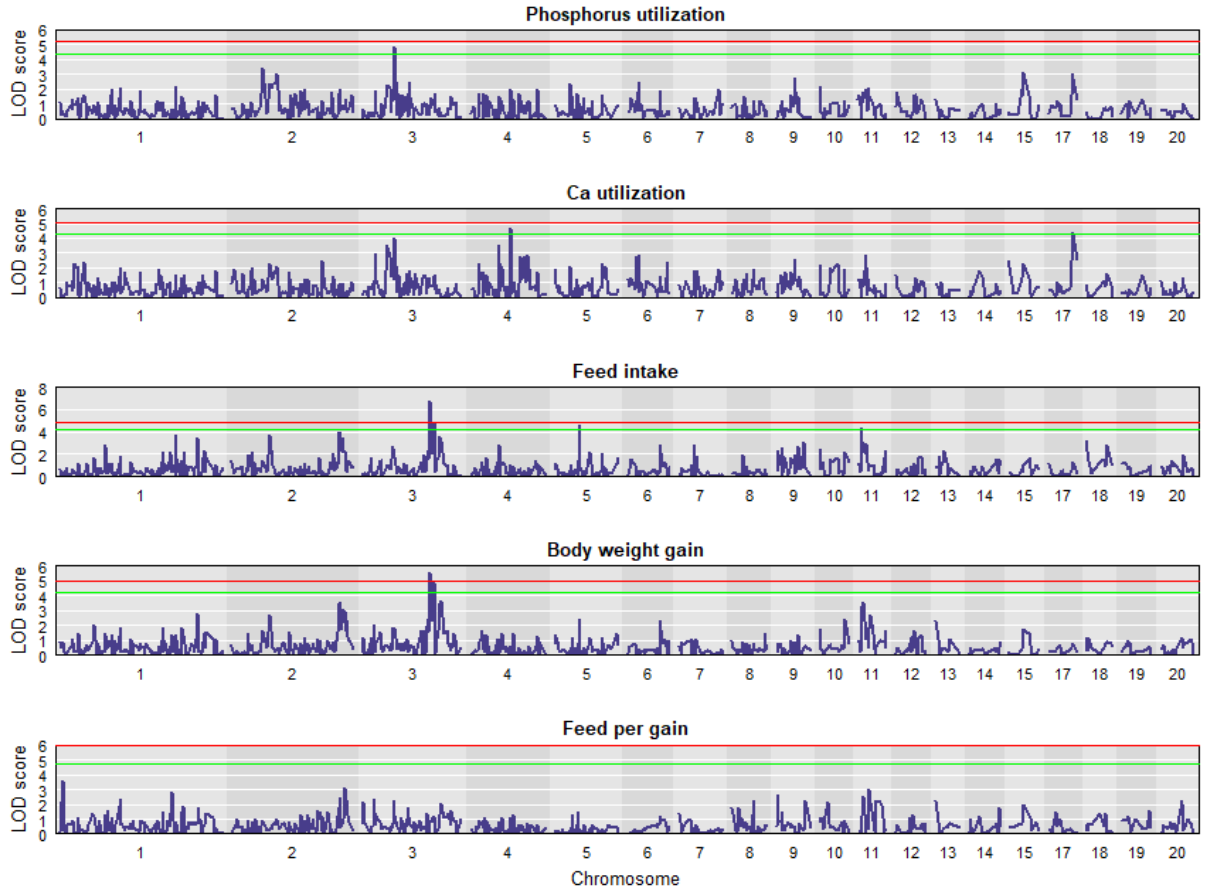

Figure 2 *Plot of the QTL linkage mapping scan of growth and efficiency traits with LOD score test statistics. The green and red lines correspond to genome-wide significance levels of 5 and 1% respectively*

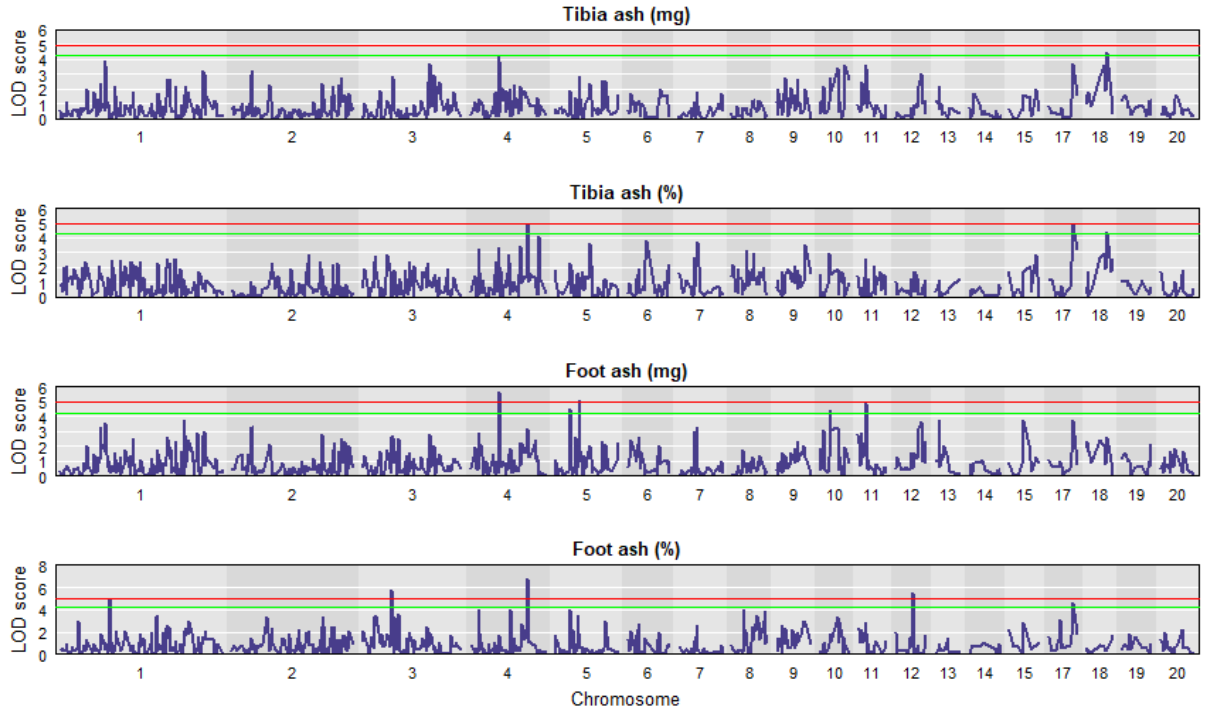

Figure 3 *Plot of the QTL linkage mapping scan of bone ash traits with LOD score test statistics. The green and red lines correspond to genome-wide significance levels of 5 and 1% respectively*

The results of the SNP association analyses are shown as the numbers of significant SNPs in the QTL regions, and the significant SNPs are listed in Table S1. A total of 127 SNPs were shown to be significant in QTL regions for all traits. Significant SNPs were found in all QTL regions for the traits PU, CaU, FI, BWG and TA (Table 3, Table S1). Although the SI on CJA3 overlapped for PU and FA%, no significant identical SNPs could be found in this region (Table S1). PU was associated with five significant SNPs, and FA% was associated with three SNPs on CJA3. Several SNPs were significantly associated with several traits. The QTL on CJA3 for FI shared five significant SNPs with the QTL for BWG (id12506 at 91 cM, id10670 at 95 cM, id10683 at 97 cM, id06748 at 101 cM and id14876 at 102 cM). Nine SNPs on CJA11 (id06872 and id32446 at 7 cM, id15452 at 11 cM, id32451, id07827, id09706, id05659 and id08551 at 13 cM, and id05029 at 16 cM) were significant within the QTL region for FI and FA (Table S1). One SNP (id08651 at 39 cM) on CJA18 was significant within the QTL region for TA and TA%. No other common significant SNP similarities could be found despite the presence of overlapping SI.

Table 3 *Trait specific positions of significant QTL (Pos) on the chromosomes (CJA), LOD score test statistics (LOD) at the 1% (**) and 5% (*) genome-wide significance level, and the corresponding support intervals (SI). SI_low and SI_high = beginning and end of the support interval respectively, with the number (*n*) of significant SNPs identified by the association analysis*

Trait[1]	CJA	Pos	LOD	SI_low	SI_high	*n* of SNPs
PU	3	48.9	4.82*	35.89	65.71	5
CaU	4	62.6	4.64*	31.71	75.64	14
	17	36.8	4.35*	20.21	57.69	6
FI	3	104.6	6.73**	90.27	119.49	7
	5	38.6	4.63*	25.99	51.10	4
	11	5.5	4.34*	0.00	27.44	10
BWG	3	104.6	5.59**	90.27	124.58	12
TA	4	44.7	4.23*	31.71	57.82	28
	18	30.6	4.43*	6.11	49.06	1
TA%	4	88.6	4.98*	72.78	120.10	0
	17	36.8	4.95*	20.21	57.69	1
	18	30.6	4.38*	6.11	49.06	1
FA	4	45.2	5.63**	32.68	57.82	0
	5	38.6	5.05**	11.00	51.10	0
	10	15.5	4.45*	0.00	42.25	6
	11	13.2	4.92*	0.00	27.44	10
FA%	1	77.1	4.99**	63.95	90.56	4
	3	44.2	5.76**	31.22	58.10	3
	4	88.6	6.74**	72.78	103.79	12
	12	27.5	5.45**	13.05	41.16	0
	17	36.8	4.62*	4.58	57.69	3

[1] For trait abbreviations, see Table 1.

Candidate genes associated with PU, performance and bone ash traits

We identified numerous genes in a 0.5 Mbp region up- and downstream of the significant SNPs in all QTL regions. For the PU QTL on CJA3 we identified 73 positional genes (see Table S2). Of these genes, 51 have known functions. No functional annotation analyses were conducted. No SNP within exon regions could be identified. Therefore, we looked for SNPs that were either intronic or obvious and were related to metabolic processes in which phosphorus might play a role. This filtering led to four genes (from the initial 73 ; Table 4), which were discussed in detail.

Table 4 *Genes and their functions[1] and positions in the reference genome within the PU QTL region of CJA3*

Official gene symbol	Gene name	Function[1]
BMP2	*Bone morphogenetic protein 2*	Ligand of TGF-beta superfamily, Induces cartilage and bone formation
PLCB1	*Phospholipase Cβ1*	Hydrolyze phospholipids into fatty acids and other lipohilic molecules, Catalyzes the formation of inositol-1,4,5-trisphosphate and diacylglycerol from phosphatidylinositol-4,5-bisphosphate, uses calcium as a cofactor, involved in intracellular transduction of many extracellular signals
PLCB4	*Phospholipase Cβ4*	Hydrolyze phospholipids into fatty acids and other lipohilic molecules, Catalyzes the formation of inositol-1,4,5-trisphosphate and diacylglycerol from phosphatidylinositol-4,5-bisphosphate, uses calcium as a cofactor, involved in intracellular transduction of many extracellular signals
TGFB2	*Transforming Growth Factor β2*	Involved in TGF-*β*-2 chains, Involved in many processes, e.g. cell differentiation, growth, or morphogenesis processes

[1] According to GeneCards and UniProt.

Discussion

In previous studies, we analyzed the impact of the quail genome (Beck *et al.* 2016a; Künzel *et al.* 2019), ileum microbiota composition (Borda-Molina *et al.* 2020; Vollmar *et al.* 2020) and transcriptomic profiles (Oster *et al.* 2020) as well as miRNA–mRNA and gut microbiota (Ponsuksili *et al.* 2020) on the focal trait PU and other related traits. Preliminary QTL mapping was done on few chromosomes and markers, without reporting any clear signals (Beck *et al.* 2016b). Hence, a thorough QTL mapping has not been done previously. This study filled this gap by conducting QTL linkage and association mapping for these traits in the same experimental design. The results clearly showed that all of the the investigated traits are polygenic in nature and are associated with several significant QTL as well as many other small-effect QTL that were not detectable.

Linkage map

Until the publication of the reference genome in 2016, only a few low-density genetic maps were available. The map calculations were based on ALFP markers (Roussot *et al.* 2003) or microsatellites (Kayang *et al.* 2004), or both types of markers (Kikuchi *et al.* 2005). Recoquillay *et al.* (2015) were the first to calculate a genome-wide linkage map based on SNP markers. Our genetic map agreed well with the map from Recoquillay *et al.* (2015). Next, based on the reference genome *C. japonica* 2.0 (NCBI GCA_001577835.1), genome assemblies for other quail species (Wu *et al.* 2018) and Japanese quail

(Morris *et al.* 2020) were developed. As our experiment with several full- and half-sib families and approximately 1000 animals across three generations can be seen as a powerful linkage mapping design, we developed a further linkage map.

The coverage and density of SNP markers were low for some LGs (Table 2, Fig. S1). This is especially noticeable for the smaller LGs (assigned to CJA25 and 28) with fewer than 10 markers. This is a result of the chosen sizeLimit = 5 in the module SeparateChromosome of the software lepmap2, as a larger sizeLimit resulted in a larger number of markers that could not be assigned to any LG. In addition, this sizeLimit was chosen to obtain the best fit based on the karyotype of Japanese quail. The genome of Japanese quail is closely related to that of the domestic chicken (*Gallus gallus domesticus*) (Wu *et al.* 2018) and shows a typical avian species karyotype that includes 10 pairs of macrochromosomes and numerous small microchromosomes (Schmid *et al.* 1989; Zlotina *et al.* 2019).

After comparison with the reference genome (e.g. Fig. 1), only a few markers could not be assigned to physical positions. However, most marker positions in the LGs were consistent with the chromosomes of the reference genome. The good fit of the map is also demonstrated by the high correlation of the linkage and physical marker positions (Table 2). Overall, the present linkage map seems to be of good quality and consistent with the reference genome. This justified the use of this map for the QTL linkage analysis.

QTL results and candidate genes

Our study adds new information for QTL in Japanese quail and provides novel QTL affecting PU, i.e. the PU QTL on CJA3 (Table 3). Owing to the use of different methods, experimental designs and trait definitions and recordings, a sophisticated comparison of QTL linkage mapping results across studies is difficult and thus was not performed in this study. QTL associated with other traits in Japanese quail have been reported by Minvielle *et al.* (2005), Esmailizadeh *et al.* (2012), Ori *et al.* (2014), Sohrabi *et al.* (2012), Recoquillay *et al.* (2015) and Knaga *et al.* (2018).

Some trait interrelationships could be identified by studying the genetic and phenotypic correlations (Beck *et al.* 2016a; Künzel *et al.* 2019) as well as the overlapping of the QTL SI (Table 3). For example, on CJA3, we detected QTL associated with PU and FA% in the same chromosomal region (Table 3). These traits are genetically correlated (0.46) (Künzel *et al.* 2019). On CJA4, we mapped QTL associated with CaU, TA and FA (Table 3), and these traits also showed substantial genetic correlations. The strong genetic correlation between BWG and FI (approximately 0.87, Künzel *et al.* 2019) can be partly explained by the QTL on CJA3, which mapped to both traits (Table 3).

Two of the four most interesting candidate genes in the PU QTL on CJA3 (Table 4) are *transforming growth factor-β 2* (*TGFB2*) and *bone morphogenetic protein 2* (*BMP2*). Both genes are members of the TGFB superfamily (Iqbal *et al.* 2018; Loozen *et al.* 2019), which is known to encode multifunctional growth factors involved in cell differentiation, growth and morphogenesis processes (Li *et al.* 2003; Darzi Niarami *et al.* 2014). *TGFB2* is also involved in the mitogen-activated protein kinase

(MAPK) signaling pathway (Kyoto Encyclopedia of Genes and Genomes, KEGG). This pathway is associated with many tissue-building and -rebuilding processes in organisms. The other two candidate genes are *phospholipase Cβ1* and *4* (*PLCB1* and *PLCB4*) (Table 4). According to KEGG analysis, they are involved in a broad spectrum of biological processes, including inositol phosphate metabolism, the calcium signaling pathway, the phosphatidylinositol signaling system, the GnRH signaling pathway and the Wnt signaling pathway. Involvement in inositol phosphate-related pathways is of specific interest, because phytate provided the main source of P in the diet. Variation in PU likewise was caused by differences in digestive phytate breakdown, thus providing a different amounts of inositol and inositol phosphates for the quail's metabolism. Also far-reaching and as an example, the Wnt signaling pathway is known to be involved in bone metabolism, which supports the connection of PU and bone ash traits (Robling 2013; Maeda *et al.* 2019; Ponsuksili *et al.* 2020). This partially explains the genetic correlation of the traits.

Conclusion

The experimental design used in this study proved to be powerful for the calculation of an SNP linkage map. Several genome-wide significant QTL could be mapped by linkage and subsequent association analyses. It seems that the focal trait PU and the other performance and bone ash traits are polygenic in nature and are associated with some significant QTL and probably many other small-effect QTL that were not detectable in this study. Some overlap of QTL regions for different traits was detected, which is in agreement with the corresponding genetic correlations. For PU, a QTL on CJA3 could be detected by linkage mapping, which was substantiated by the results of the SNP association mapping. Four candidate genes were identified for this QTL, which should be investigated in further functional studies.

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Data availability statement

Genotype and phenotype data, pedigree information and the genetic map are available through OSF and can be accessed at https://osf.io/57nty/.

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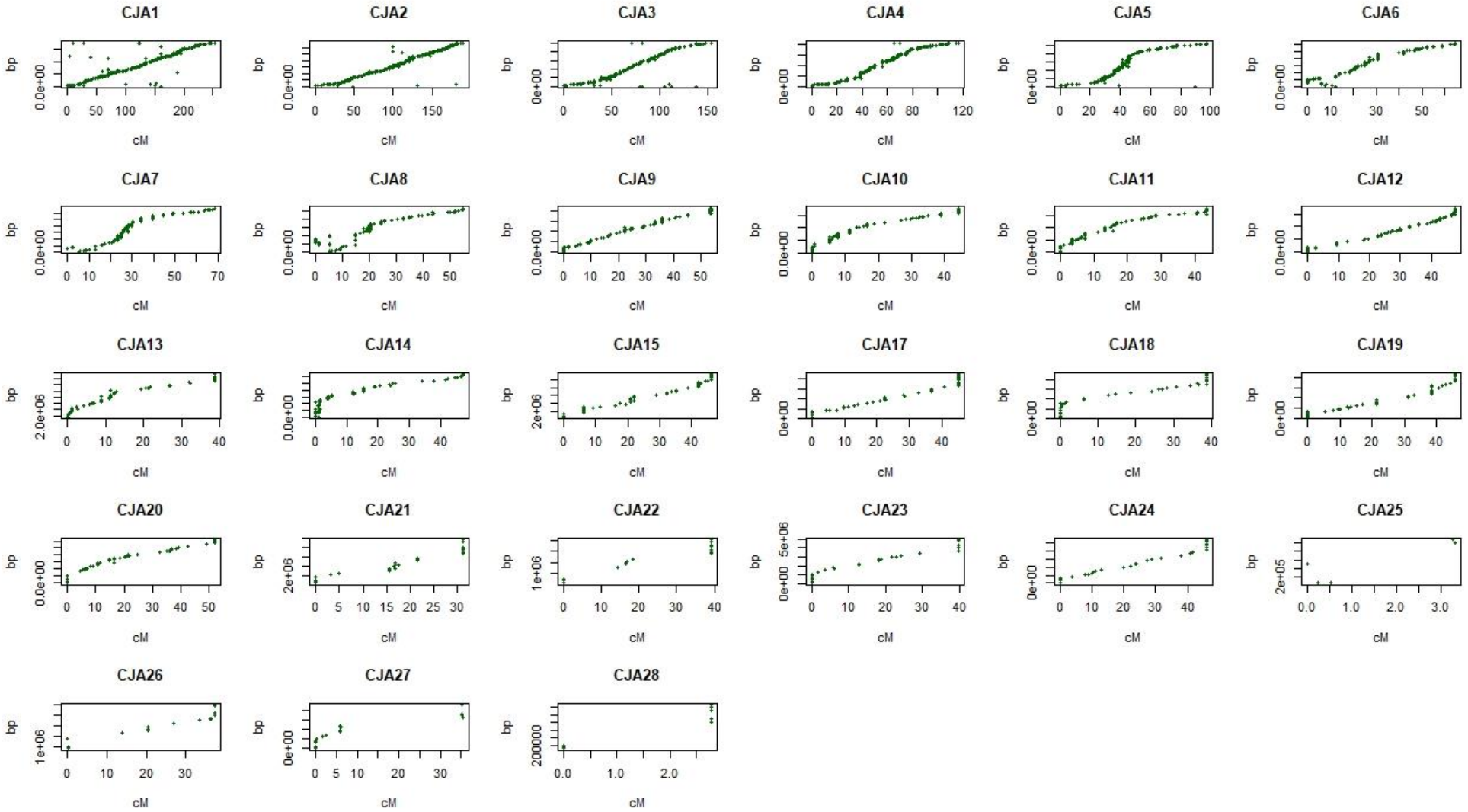
Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Comparison of genetic and physical maps

Table S1. Summary of trait-associated markers ($P \leq 0.05$) within the significant QTL regions

Table S2. Summary of 73 identified genes for PU in a 0.5 Mbp regon up- and downstream of significant SNPs



Supplemental Figure S1 *Comparison of genetic and physical maps. The y-axis shows the physical position (bp), which is based on the reference genome assembly (NCBI GCA_001577835.1), and the x-axis shows the genetic position (cM). Note that the SNP positions at 0 bp refer to an as yet unknown position.*

Supplemental Table S1 *Summary of trait-associated markers ($p \leq 0.05$) within the significant QTL regions*

Trait[a]	CJA	Marker ID	p-value	cM	Matched markers with other traits
PU	3	id10557	0.041	37.70	
	3	id01656	0.042	37.70	
	3	id14384	0.036	37.70	
	3	id14623	0.012	41.86	
	3	id10574	0.034	43.22	
CaU	4	id06563	0.027	46.69	
	4	id09235	0.048	65.59	
	4	id04477	0.048	65.59	
	4	id14317	0.025	65.59	
	4	id12663	0.012	65.59	
	4	id16422	0.042	65.59	
	4	id12664	0.042	65.59	
	4	id05313	0.043	68.33	
	4	id10860	0.034	68.33	
	4	id03398	0.042	68.33	
	4	id05311	0.042	68.33	
	4	id03657	0.040	71.08	
	4	id12682	0.017	74.72	
	4	id10870	0.048	75.27	
	17	id11675	0.002	28.28	
	17	id23952	0.019	36.76	
	17	id03867	0.044	45.19	
	17	id05727	0.013	45.19	
	17	id03250	0.013	45.19	
	17	id13349	0.036	45.19	
FI	3	id12506	0.003	90.82	BWG
	3	id10670	0.001	95.39	BWG
	3	id07565	0.038	97.35	
	3	id10683	0.019	97.35	BWG
	3	id06748	0.022	100.81	BWG
	3	id14876	0.036	101.52	BWG
	3	id29726	0.049	112.08	
	5	id21765	0.016	30.87	
	5	id10931	0.025	35.87	
	5	id32246	0.035	36.37	
	5	id32730	0.032	36.93	
	11	id06872	0.024	7.32	FA
	11	id32446	0.027	7.32	FA
	11	id15452	0.003	11.30	FA
	11	id32451	0.005	13.21	FA
	11	id07827	0.011	13.21	FA
	11	id09706	0.011	13.21	FA
	11	id05659	0.009	13.21	FA
	11	id08551	0.009	13.21	FA
	11	id05029	0.023	15.58	FA
	11	id32793	0.039	16.83	
BWG	3	id12506	0.009	90.82	FI
	3	id10670	0.002	95.39	FI

	3	id19121	0.009	97.34	
	3	id14875	0.030	97.35	
	3	id19122	0.030	97.35	
	3	id10683	0.008	97.35	FI
	3	id03367	0.028	97.35	
	3	id14232	0.046	100.81	
	3	id06748	0.039	100.81	FI
	3	id14876	0.042	101.52	FI
	3	id10530	0.028	109.31	
	3	id12548	0.013	120.70	
TA	4	id02692	0.013	38.31	
	4	id05290	0.007	38.31	
	4	id11068	0.007	38.31	
	4	id10783	0.008	38.31	
	4	id03383	0.021	38.69	
	4	id07589	0.028	38.69	
	4	id04753	0.028	38.69	
	4	id02319	0.028	38.69	
	4	id06108	0.006	38.69	
	4	id09188	0.006	38.69	
	4	id03382	0.031	38.69	
	4	id25452	0.049	38.69	
	4	id31883	0.031	38.86	
	4	id14009	0.019	39.51	
	4	id20374	0.010	39.51	
	4	id09186	0.028	39.51	
	4	id23126	0.031	39.51	
	4	id10788	0.028	39.51	
	4	id06761	0.044	39.51	
	4	id16557	0.044	39.51	
	4	id10790	0.044	39.51	
	4	id16986	0.044	39.51	
	4	id04323	0.019	39.76	
	4	id09192	0.019	40.13	
	4	id08248	0.038	44.21	
	4	id25646	0.028	46.87	
	4	id09217	0.039	47.02	
	4	id07604	0.049	49.98	
	18	id08651	0.030	38.77	TA%
TA%	17	id06643	0.001	40.83	
	18	id08651	0.029	38.77	TA
FA	10	id07281	0.049	0.88	
	10	id13413	0.026	5.22	
	10	id05642	0.011	16.49	
	10	id09667	0.043	16.49	
	10	id07288	0.027	32.10	
	10	id32850	0.004	35.04	
	11	id06872	0.023	7.32	FI
	11	id32446	0.017	7.32	FI
	11	id15452	0.012	11.30	FI
	11	id32451	0.011	13.21	FI
	11	id07827	0.021	13.21	FI

	11	id09706	0.023	13.21	FI
	11	id05659	0.022	13.21	FI
	11	id08551	0.022	13.21	FI
	11	id05029	0.041	15.58	FI
	11	id32374	0.042	16.82	
FA%	1	id33073	0.008	71.94	
	1	id03827	0.024	76.45	
	1	id09976	0.013	80.66	
	1	id11811	0.027	80.85	
	3	id10552	0.020	32.13	
	3	id02658	<0.001	32.13	
	3	id14857	0.046	48.40	
	4	id17823	0.008	74.72	
	4	id09240	0.008	74.72	
	4	id32711	0.032	74.72	
	4	id10866	0.008	74.72	
	4	id25649	0.026	77.60	
	4	id04769	0.024	79.63	
	4	id09248	0.045	83.66	
	4	id00486	0.030	88.64	
	4	id26718	0.042	90.26	
	4	id13708	0.019	91.30	
	4	id09265	0.040	103.54	
	4	id27230	0.034	103.64	
	17	id33074	0.047	9.73	
	17	id00247	0.002	17.16	
	17	id19277	0.036	36.74	

[a] For trait abbreviations, see Table 1.

Supplemental Table S2 *Summary of 73 identified genes for PU in a 0.5 Mbp region up- and downstream of significant SNPs*

Trait[a]	Gene	Gene location in bp		SNP	Location (distance to genes in bp)	SNP location	
		Start	End			cM	bp
PU	PAK5	11950702	12094008				
	LAMP5	12095782	12106823				
	PLCB4	12108448	12289519				
	PLCB1	12329777	12674893	id14384	intronic	37.703	12540995
	LOC107310957	12675334	12679908				
	TMX4	12718163	12743981				
	HAO1	12748177	12774489				
	LOC107310956	12762766	12764953				
	LOC107310980	13000416	13110455				
	BMP2	13149896	13155078				
	LOC116652411	13174497	13239523				
	LOC107310917	13273456	13326531				
	FERMT1	13326689	13344740				
	LRRN4	13349146	13357235				
	CCT4	13362618	13368311				
	FAM161A	13368497	13376521				
	DTD1	13376027	13383387				
	LOC116653205	13383435	13385218				
	SEC23B	13384133	13398061				
	POLR3F	13399683	13405682				
	DZANK1	13405772	13421996				
	BIRC5	13422245	13423482				
	KAT14	13424420	13438934				
	LOC107310984	13438522	13439387				
	OVOL2	13447675	13454039				
	MGME1	13456378	13461301				
	SNX5	13461501	13477385	id10557	upstream (13110)	37.701	13490495
	RRBP1	13509416	13529344	id10557	downstream (18920)	37.701	13490495
	DSTN	13532567	13541879				
	LOC116653173	13541962	13562948				
	LOC107310933	13564800	13567019				
	LOC107310890	13584169	13587185				
	SYNDIG1	13662551	13678715	id01656	upstream (13979)	37.703	13692694
	LOC107310995	13727528	13728659	id01656	downstream (34834)	37.703	13692694
	CST7	13770487	13778397				
	APMAP	13782846	13793306				
	ACSS1	13793652	13823150				
	TTBK1	13841207	13915358				
	SLC22A7	13918080	13930431				
	TTL	13931216	13945307				

	POLR1B	13945877	13958409				
	PTCRA	13959083	13960082				
	CNPY3	13960406	13962555				
	GNMT	13962792	13964620				
	PEX6	13965569	13977199				
	VSX1	13980174	13982882				
	ENTPD6	13996794	14007427				
	MAL	14010292	14014218				
	MRPS5	14017074	14048607				
	LOC116653272	14150276	14150648				
	SLC8A1	14175324	14272642				
	LOC116653182	14184512	14190798				
	LOC107311002	16816696	16819420				
	TGFB2	16895806	16958385				
	RRP15	16964138	16991977				
	SPATA17	17175446	17258347				
	GPATCH2	17258251	17354587	id10574	intronic	43.223	17293862
	LOC107311059	17363606	17392868				
	LOC116653249	17447035	17456715				
	ESRRG	17456684	17830994				
	LOC107311023	17471465	17474542				
	LOC116653250	17678879	17681797				
	USH2A	17848612	18204761				
	KCTD3	18205056	18228494				
	LOC107311086	18240156	18254071				
	KCNK2	18303701	18471703	id14623	intronic	41.856	18451011
	LOC107311089	18421856	18471849	id14623	intronic	41.856	18451011
	CENPF	18473857	18508763				
	PTPN14	18517965	18615002				
	SMYD2	18616279	18643814				
	LOC107311062	18650875	18657208				
	PROX1	18724302	18774251				
	LOC116653231	18899477	19022498				

[a] For trait abbreviations, see Table 1.

CHAPTER III

2nd Publication

CHAPTER III – 2nd Publication

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

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Simple Summary

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

Abstract

In this study, we aimed to investigate the ileum digesta of a large cohort of Japanese quail fed the same diet, with similar environmental conditions. We also address how P utilization (PU), Ca utilization (CaU), and bird performance (feed intake (FI), feed conversion (FC), and body weight gain (BWG)) modify intestinal microbiota of male and female quail. Despite the great number of samples analyzed (760), a core microbiome was composed of five bacteria. The Unc. *Lactobacillus,* Unc. Clostridiaceae 1, *Clostridium* sensu stricto, *Escherichia coli*, and *Streptococcus alactolyticus* were detected in all samples and contributed to more than 70% of the total community. Depending on the bird predisposition for PU, CaU, FI, BWG, and FC, those species were present in higher or lower abundances. There was a significant gender effect on the ileal microbial community. While females had higher abundances of *Lactobacillus,* males were more colonized by *Streptococcus alactolyticus.* The entire cohort was highly colonized by *Escherichia coli* (8%–15%), an enteropathogenic bacteria. It remains unclear, if microbiota composition followed the mechanisms that caused different PU, CaU, FI, FC, and BWG or if the change in microbiota composition and function caused the differences in PU, CaU, and performance traits.

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

1. Introduction

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

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

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

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

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

2. Materials and Methods

2.1. Ethical Statement

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

2.2. Sample Collection, DNA Extraction, and Illumina Library Preparation

Ileum digesta samples from 760 quail were obtained from a previous study that used an F2 design [13]. The experimental design is fully described by Beck et al. (2016). Briefly, the quails were fed with a starter diet from 1 d to 5 d (Supplementary Table S1) and then with an experimental diet (Supplementary Table S1) until the end of the experiment (15 d). Diets were designed based on the nutritional recommendations for young turkeys (Gesellschaft für Ernährungsphysiologie, 2004) [14], except for P and Ca concentration. The main feeding ingredients of the starter diet were corn, wheat, and soybean, while the experimental diet ingredients were corn, soybean, and potato protein. All information regarding phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI), body weight gain (BWG), feed conversion (FC), and gender for each animal is shown in Supplementary Table S2. On day 15 of age, birds were sacrificed [15]. The ileum was longitudinally opened and digesta collected with a sterile spoon and stored in RNA later at −80 °C until further analysis. DNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions with a preliminary step of bead beating (30 s, 5.5 m/s) in a FastPrep instrument (MP Biomedicals, Santa Ana, CA, USA).

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

2.3. Samples Grouping

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

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

2.4. Bioinformatics and Stratistical Analysis

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

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

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

OTUs between the groups were determined with the unpaired Welch's t-test with a cut-off p-value < 0.05. Shannon diversity was calculated with Primer 7 software. Correlations between OTUs and traits were estimated with the Spearman coefficient using PRISM 6 (GraphPad Software, San Diego, CA, USA) and were considered significantly different if p-value < 0.05.

3. Results and Discussion

3.1. Effect of PU, CaU, and Animal Performance on Microbial Distribution

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

The microbial community of the quail´s gastrointestinal tract has not yet been deeply analyzed, and this leads to a lack of sequencing information in the databases. As previously reported by Wilkinson et al. (2016) and other avian studies, some of the most abundant OTUs detected in the ileum could not be taxonomically classified [6,28,29]. The most abundant OTU, assigned to an unclassified Clostridiaceae1, correlated positively with PU, CaU, FI, and BWG (Supplementary Table S3). This OTU belongs to the order Clostridiales, which are known to degrade plant components, which are further fermented to short-chain fatty acids [30]. FC was negatively correlated with unclassified *Clostridium* sensu stricto (on av. 22.8%); BWG with *Streptococcus alactolyticus* (on av. 10.7%) and *Enterococcus faecium* (on av. 1.5%); PU, CaU, and FI with *Escherichia coli* (on av. 13.1%) and BWG; and FI with unclassified *Lactobacillus* (on av. 29.3%) (Supplementary Table S3). Previously positive correlations for *Lactobacillus* species with egg production and feed conversion have been reported [31]. However, in the present study, only one negative correlation was observed between a high abundant unclassified *Lactobacillus* (on av. 29.3%) and FI. The presence of *Lactobacillus* species is considered to be beneficial for the bird because they transform carbohydrates to lactic acid, inhibit pathogen adhesion to the epithelium, and decrease the pH in the ileum [12]. The pH was not measured in this study, but one

hypothesis for the high abundance of *E. coli* (on av. 13%) is the increasing presence of one member of Clostridiales (unclassified Clostridiaceae1) and the nondominance of *Lactobacillus* as indicators of a higher pH. The lower dominance of *Lactobacillus* differs from previous reports on quail [6] and broiler chicken [12]. The negative correlation between *E. faecium* and BWG contradicts the results of a previous study in broilers [32]. *E. faecium* can exert probiotic effects and enlarge the villus height in the ileum of broilers [32]. In quails, it reduced the presence of pathogens like *Salmonella* owing to the production of a bacteriocin [33].

In order to better understand the effects of P and Ca utilization and other performance parameters (BWG, FI, and FC), a priori groups based on high, low, or medium bird predisposition for each trait were established. PERMANOVA test based on those a priori groups confirmed an influence of the single factors PU, CaU, and FI on the ileal microbial community (Supplementary Table S4a), while a trend was shown for the interaction BWG × FC (p-value < 0.10) (Supplementary Table S4b). The abundance of Candidatus *Arthromitus* was higher within birds with higher PU (Figure 1). These segmented filamentous bacteria attach to the intestine and have been previously isolated from the terminal ileum of chickens [34] and turkeys [35]. Moreover, at an early age, they have been found to positively correlate to bird performance, probably owing to its immunomodulatory capabilities [35,36]. Other genera promoted in the birds with higher PU were *Bacillus* and *Leuconostoc* (Figure 1). *Bacillus* is considered as a probiotic in chickens; may improve bird performance [37]; exerts different enzymatic activities like amylase, xylanase, and pectinase [38]; and phosphatase activity can be expected from this genus, as previously reported in soils [39,40].

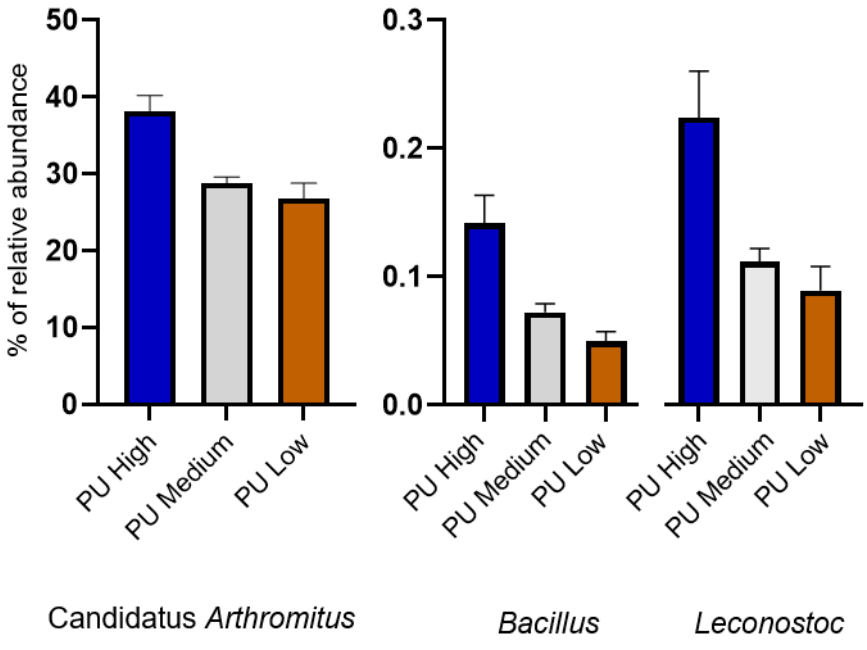

Figure 1. *Relative abundance of the genera influenced by the P utilization (PU) in the high and low groups.*

Gender had a statistically significant effect on the ileal microbial diversity of the present dataset (Supplementary Table S4c). Correspondingly, the Shannon diversity index significantly differed between males and females (Supplementary Figure S1). Previous studies demonstrated that gender differences exist in the presence of specific bacterial groups, such as *Lactobacillus* in quail [6]. In the present data set, *Lactobacillus* was more abundant in females (26% vs. 22% in males), while the abundance of *Streptococcus* tended to be the opposite (7.3% in females vs. 9.3% in males) (Supplementary Figure S2).

Considering that all birds received the same diet and were housed under the same conditions, a possible explanation for the range of performance values observed can be attributed to individual differences for diet assimilation and the presence of indigestible dietary polysaccharides [41,42]. The percentage of dissimilarity between the high, low, and medium groups for the PU, CaU, FI, BWG, and FC ranged between 52.1% and 60.9% (Supplementary Table S5). Taking into account a high individual variability not only in performance values, but also in microbial composition, it is expected that the microbial metabolic activities changed. It is possible that even bird behavior was affected as it has been demonstrated that gut microbiota affects emotional reactivity in Japanese quail [43,44].

3.2. Gender Effects on Microbiota, PU, CaU, FI, BWG, and FC

Female quail are physiologically different from males [45]; thus, it is expected to comprise different microbial resemblance. To evaluate whether gender variation exists and has an impact on PU, CaU, FI, BWG, and FC, centroids that compute the average plotting position of an a priori group of samples were calculated and ordinated using principal coordinate analysis (PCoA) (Figure 2). Gender affected the grouping of the high, medium, and low levels of PU, CaU, FI, BWG, and FC (pvalue < 0.05). A previous study using only 200 quail observed an effect of gender on PU and CaU only as a trend [42]. It is important to highlight that, in the present study, PU ranged from 21% to 86% and CaU from 11% to 84%, a higher variation compared with that observed by Beck et al. (2014). The same study did not observe any effect of gender on FI, BWG, and FC, unlike what we observed in the present study. This discrepancy might be owing to the higher number of birds used in this study originating from an F2 design and the microbiota of the GIT being used to determine these observations.

For PU, CaU, and FI, the PCoA plots depicted three clusters comprising male/female low and medium, male high, and female high (Figure 2A–C). The two principal component axes accounted for 80% (PU), 83% (CaU), and 95% (FI) of variation among groups, thus providing a good ordination of the samples. ANOSIM pair-wise comparison tests showed a significant difference between female high versus male high, female high versus female low, and male high versus male low groups for the three traits (p-value < 0.05), except for the CaU between female high versus male high where a trend was observed (p-value = 0.06) (Supplementary Table S6). The same was not observed for female low versus male low and female medium versus male medium groups. An effect of gender in the medium group was also observed (Supplementary Table S6).

Regarding FC and BWG, the PCoA plots showed separation between low, medium, and high birds (Figure 2D and E). The two principal component axes accounted for high coverage of the total microbial variation (90% for FC and 92% for BWG). ANOSIM pairwise tests showed no statistical significance between the gender for the higher and lower group, but between high and lower groups within the same gender (p-value < 0.05). Regarding BWG, the female medium group was statistically different from the male medium group, while a trend was observed between the two groups for FC (p-value = 0.1) (Supplementary Table S6).

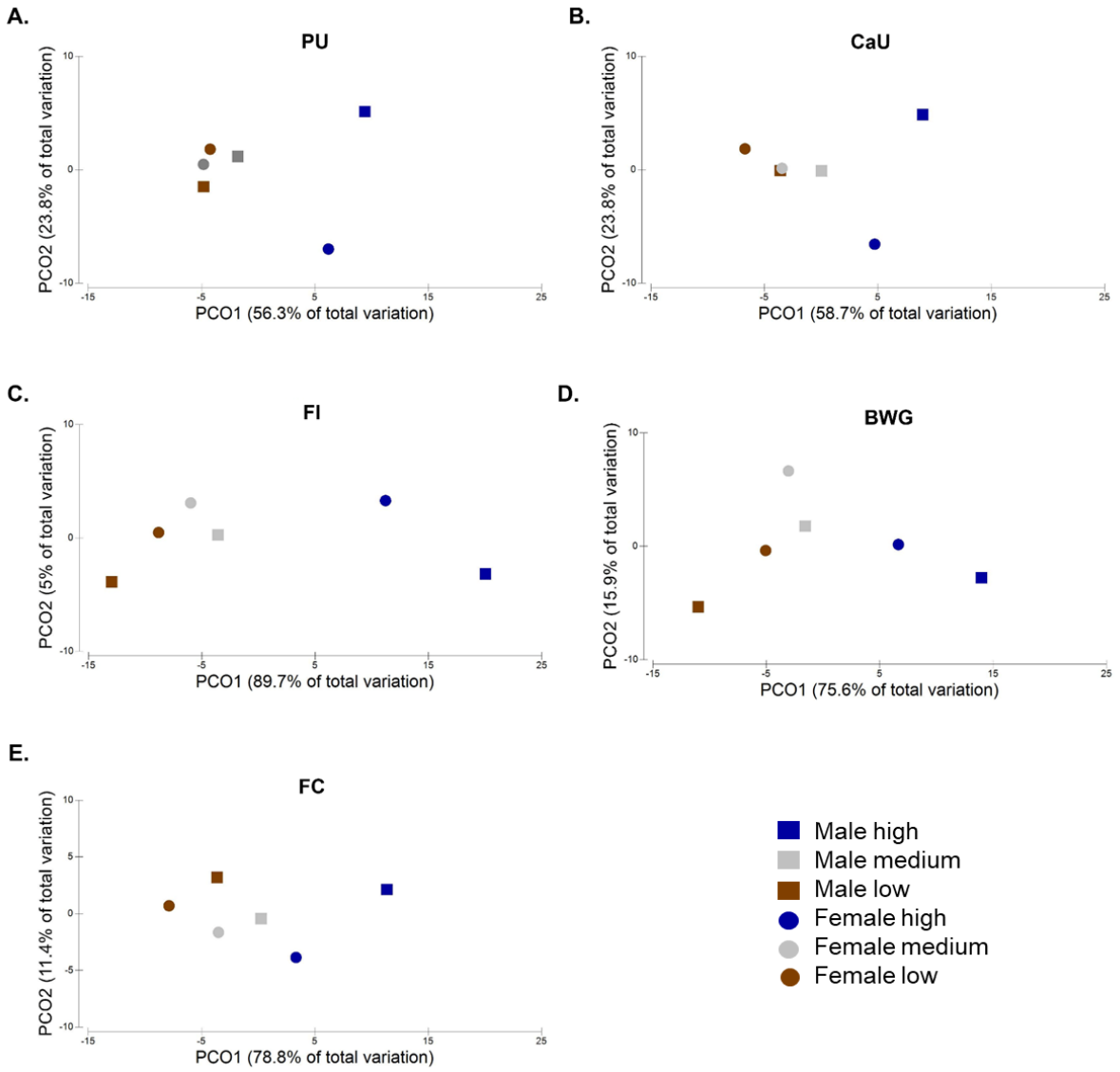

Figure 2. *Principal coordinates analysis (PCoA) plots depicting the gender effect on (A) phosphorous utilization (PU), (B) calcium utilization (CaU), (C) feed intake (FI), (D) body weight gain (BWG), and (E) feed conversion (FC) in the high, medium, and low groups.*

A group of five bacteria was responsible for the separation observed between the groups in all traits. Unclassified *Clostridiaceae1,* unclassified *Lactobacillus, Streptococcus alactolyticus,* unclassified *Clostridium* sensu stricto, and *Escherichia coli* contributed to more than 70% of the total community. Female and male groups were colonized by the same microorganisms, but relative abundances of microorganisms were different between genders. The average dissimilarity between the groups ranged from 51% to 62%, and the average similarity within the groups was between 37% and 50% (Supplementary Table S7).

Pair-wise comparisons for each of the performance measurements revealed that those five bacteria abundances significantly changed based either on gender or within the gender between the high, medium, and low groups (Supplementary Table S8). Unclassified *Clostridiaceae1* was highly abundant in the high male and female groups of all traits, with an average abundance between 32% and 49% in males and 30% and 41% in females (Figure 3 and Supplementary Table S8).

In the low female and male groups, the average abundance ranged from 20% to 28%. A significant difference in the abundance of unclassified Clostridiaceae1 was observed for PU between the groups female high versus male high (36% vs. 40%), female high versus female low (36% vs. 27%), and male high versus male low (40% vs. 26%) (p-value < 0.05) (Supplementary Table S8A). For the CaU, a trend was observed between the female high versus female low group (32% vs. 25%) (p-value < 0.06) and a statistical significance between male high and low (37% vs. 28%) (p-value < 0.05) (Supplementary Table S8B). In regards to feed intake, an effect was detected between female versus male high (41% vs. 49%), female high versus female low (41% vs. 24%), and male high versus male low (49% vs. 20%) (p-value < 0.05) (Supplementary Table S8C) and in the case of BWG between female versus male high (36% vs. 43%), female high versus female low (36% vs. 26%), and male high versus male low (43% vs. 22%) (p-value < 0.05) (Supplementary Table S8D). This microorganism belongs to the Clostridiales order, and it was previously detected in the gastrointestinal tract of broilers [12]. Clostridia are common colonizers of broiler and quail GIT [46] and are responsible for plant material degradation [30]. Generally, they are not the most dominant group, as observed in this study, but are detected in lower relative abundance [6,47]. Corn favored the abundance of clostridia in the avian GIT [48]. The quail of this study were fed with a corn-based diet [13], which might explain the higher abundance of the unclassified Clostridiaceae1 in the samples. Bird age has a remarkable impact on microbiota composition and diversity, gut modulation, and metabolic functions [46]. All previous studies characterizing quail GIT have worked with animals at the age of 4–8 weeks [6,47,49]. This impairs the comparison between those and the present study (two weeks old). In broiler chicken, bacterial changes during their lifespan are known to exist, with an establishment of more stable communities in older animals [46]. Regarding the quails' GIT, there is still no knowledge of how the GIT evolves during lifespan.

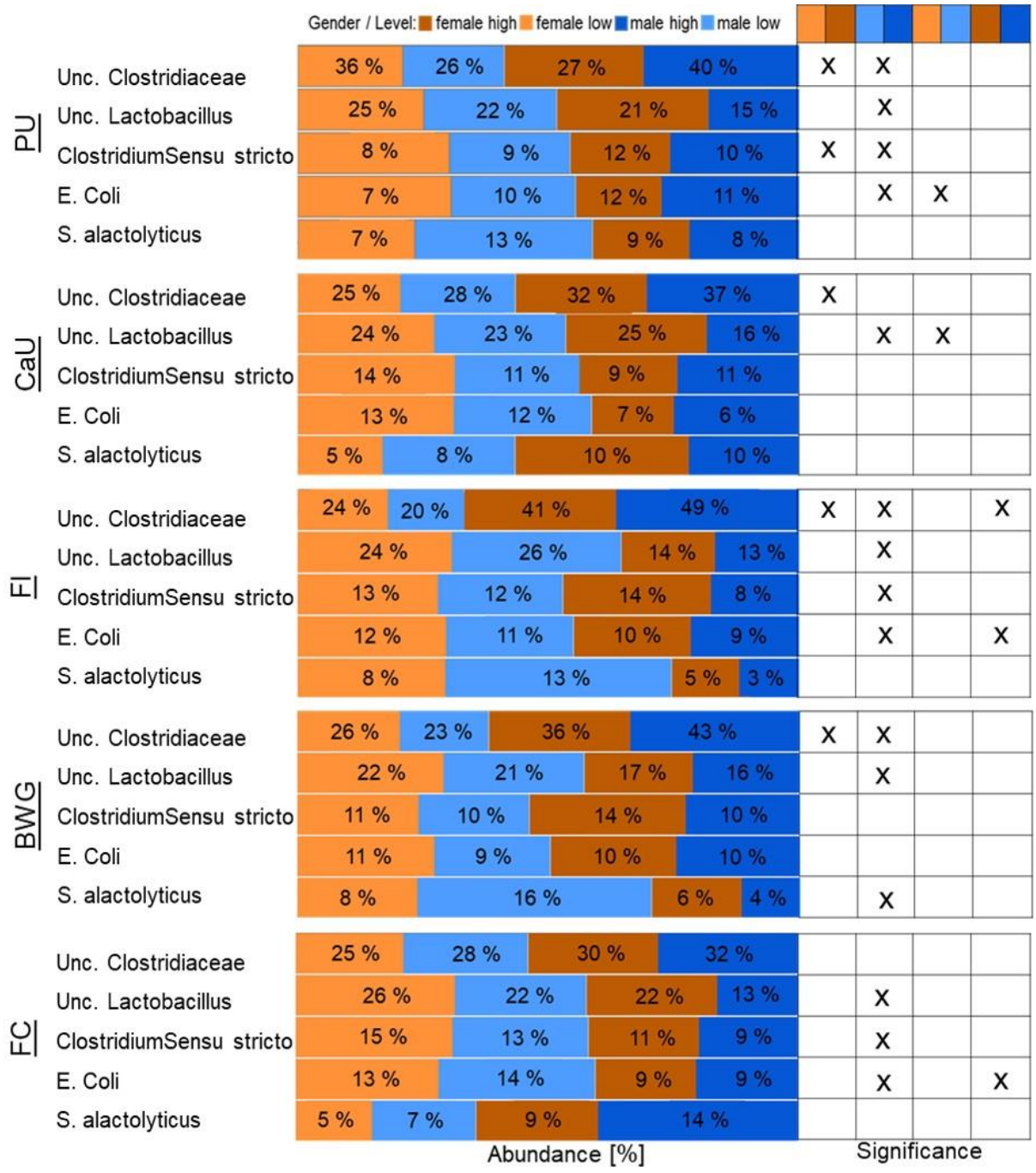

Figure 3. *Abundance variation of the five operational taxonomic units (OTUs) that contribute to 70% of total bacterial community of females and males considering phosphorous utilization (PU), calcium utilization (CaU), feed intake (FI), body weight gain (BWG), and feed conversion (FC). Statistical significances between the groups are depicted on the graph (p-value < 0.05).*

Lactobacillus are common colonizers of the ileum of broilers and quail. They are known to improve bird health, inhibit pathogen adhesion, and maintain bacterial stability [47]. They are usually considered in the literature as beneficial; however, care should be taken because they colonize the GIT together with other species and are not independent of them. They interact either positively or negatively [12,50],

and thus may have an impact on gut health. In the present study, an unclassified *Lactobacillus* was present in all traits in higher relative abundance in the low female and male groups (21%–26%) in comparison with the high groups (13%–25%) (Figure 3 and Supplementary Table S8). The female high group showed higher relative abundances (14%–25%) compared with the male group (13%–16%), while in the lower groups, the males showed higher bacterial abundance for the traits PU (22% vs. 21%) and FI (26% vs. 24%), and the females in the traits CaU (24% vs. 23%), FC (26% vs. 22%), and BWG (22% vs. 21%) (Figure 3 and Supplementary Table S8). The higher abundance of *Lactobacillus* in female birds is consistent with results by Wilkinson et al. (2016) [6], and a significant difference between gender was obtained for PU, CaU, and FCR for high and medium groups and in the medium group for FI and BWG.

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

Members of *Clostridium* sensu stricto are usually associated with pathogenesis and are indicators of imbalanced gut microbiota [52]. *Clostridium* sensu stricto was detected in higher abundance in the low female/male samples (9%–15%) in comparison with high female/male (8%–14%) (Figure 3 and Supplementary Table S8). An effect of gender on the abundance of *Clostridium* sensu stricto was observed for the medium groups of PU, CaU, and FC (Supplementary Table S8), where higher abundance was found in females. Despite the high abundance of this member of *Clostridium* sensu stricto, the birds of this experiment were healthy, and there was no effect on BWG, as previously suggested by (Apajalahti and Kettunen 2006).

Escherichia coli is an enteropathogenic bacteria that can be responsible for disease. It is a common colonizer of the avian digestive tract with no principal effect on the health status of the birds. However, it can be a potential carrier of disease to other animals and humans [53]. In this study, it was detected in a range from 10%–14% abundance in low female/male and 7%–11% in high female/male birds (Figure 3 and Supplementary Table S8). Thus, it can be hypothesized that, in comparison with chicken surveys [11,12], quail may be particularly predisposed to harbor members of the family Enterobacteriaceae, as has been reported in other studies [47]. Despite the close relative abundance between the high and low groups, statistical significance (0.05 < p-value < 0.1) was denoted between gender for PU (high group) and CaU (high group), with being males more colonized. Within gender, PU (female high vs. low), CaU (female high vs. low), FC (female high vs. low), and FC (male high vs. low) showed statistical significance (Supplementary Table S8).

4. Conclusions

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

Supplementary Materials: The following are available online at www.mdpi.com/2076-2615/10/5/885/s1, Figure S1: Shannon diversity index [H'] for the overall data, based on microbial ecology resemblance for female and male Japanese quails, Figure S2: Percentage of relative abundance of the genera detected in the ileum of female and male Japanese quails, Table S1. Ingredient composition and analyzed concentrations of the diets (Adapted from Beck et al. 2014), Table S2 (excel file): Information regarding phosphorous utilization (PU), calcium utilization (CaU), feed intake (FI), body weight gain (BWG), feed conversion (FC), and gender for each animal, Table S3: Pearson correlation and its corresponding significance value of the most abundant operational taxonomic units (OTUs) against phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI), body weight gain (BWG), and feed conversion (FC), Table S4: Multivariate statistical analysis for the overall data at OTU level. A. PERMANOVA analysis for P and Ca utilization. B. PERMANOVA analysis for BWG, FC, and FI. C. ANOSIM to test gender effect, Table S5: Average dissimilarity (%) between high, medium, and low groups for phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI) body weight gain (BWG), and feed conversion (FC) by males and females, Table S6: ANOSIM pairwise tests by groups: phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI), body weight gain (BWG), and feed conversion (FC) by males and females, Table S7 (excel file): Average similarity and dissimilarity (%) between high, medium, and low groups for phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI) body weight gain (BWG), and feed conversion (FC) by males and females, Table S8: Pairwise comparison based on t-test for phosphorus utilization, calcium utilization, feed intake, body weight gain, and feed conversion and the most abundant OTUs (unclassified Clostridiaceae1; unclassified *Lactobacillus;* unclassified *Clostridium sensu stricto* 1; *Escherichia coli; Streptococcus alactolyticus; Enterococcus faecium).* A. Phosphorus utilization. B. Calcium utilization. C. Feed intake. D. Body weight gain. E. Feed conversion.

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Supplementary Material

Figure S1: *Distance-based redundancy analysis (dbRDA) for A. Ca utilization (CaU), B. Feed intake (FI), C. Body weight gain (BWG), and D. Feed conversion (FC). Vectors indicate the direction of each performance trait and its relation to the groups high, edium, and low*

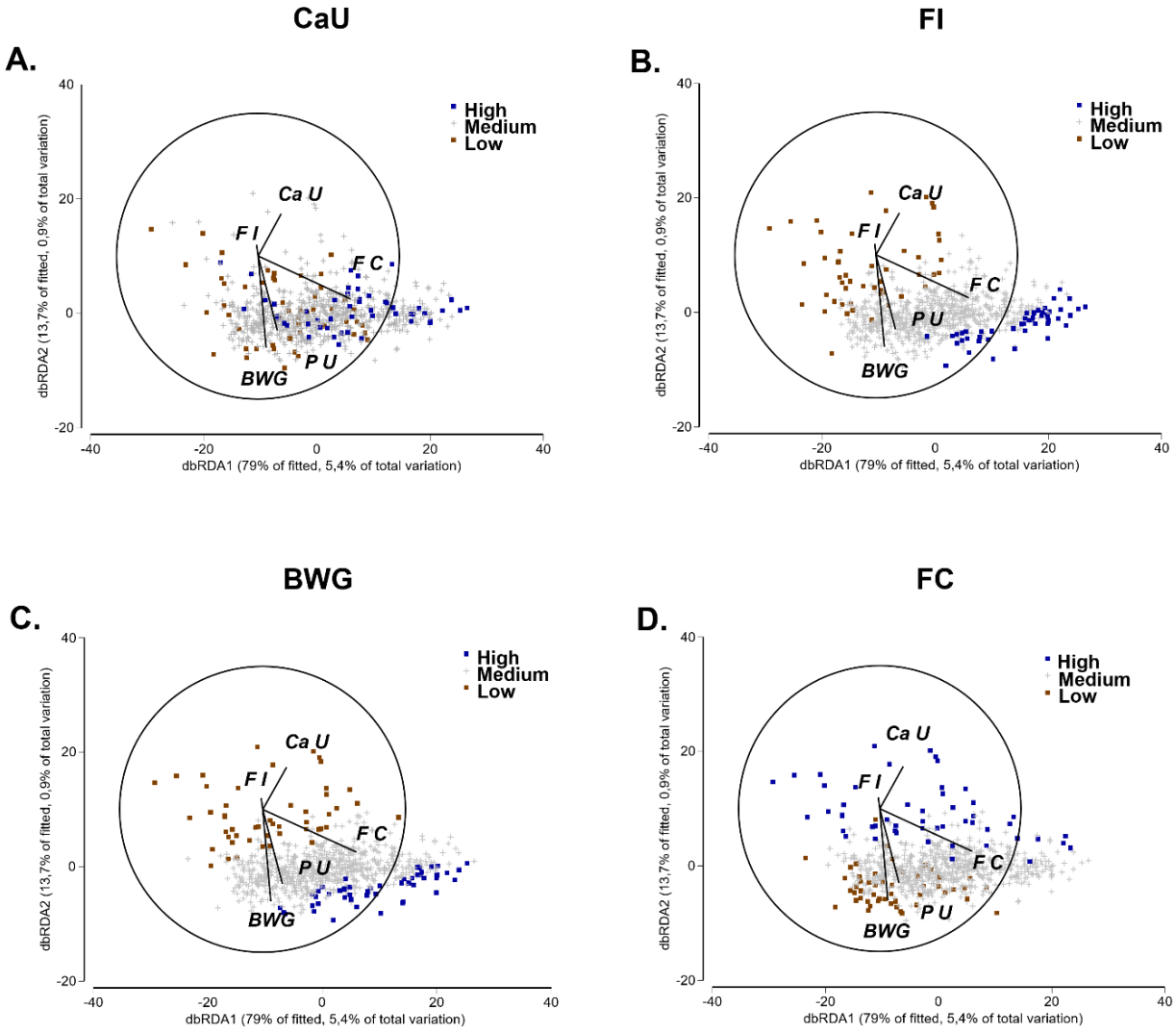
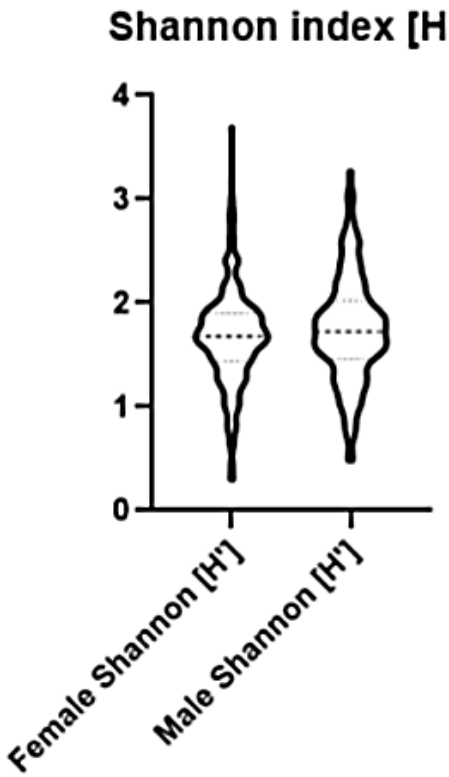


Figure S2: *Shannon diversity index [H'] for the overall data, based on microbial ecology resemblance for female and male Japanese quails*



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

Figure S3. *Percentage of relative abundance of the genera detected in the ileum of female and male Japanese quails*

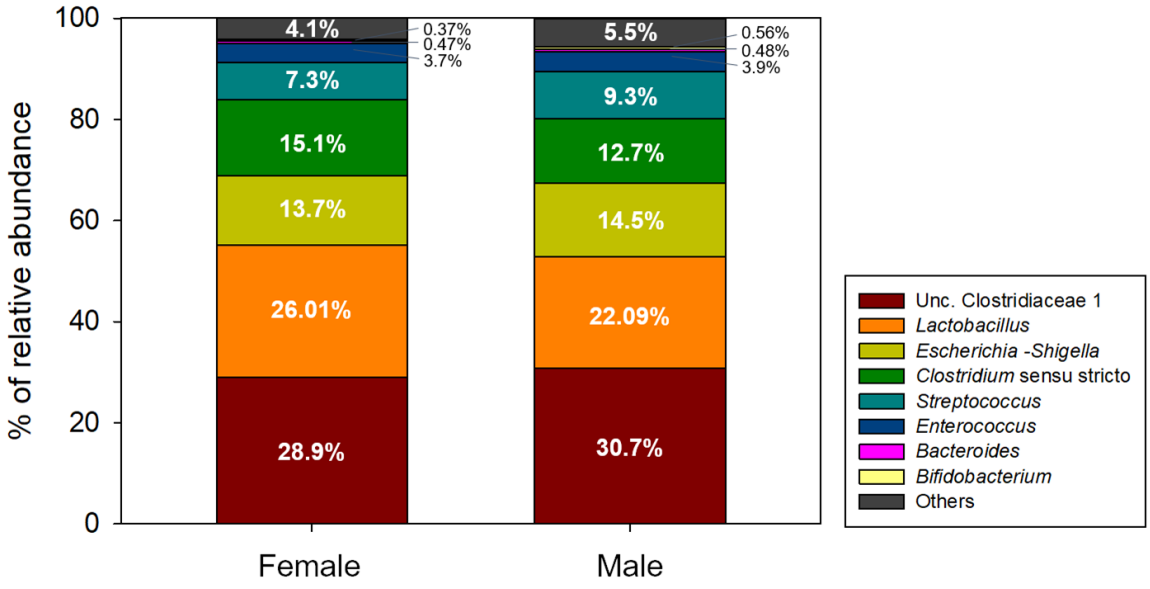


Table S1 (excel file): *Information regarding phosphorous utilization (PU), calcium utilization (CaU), feed intake (FI), body weight gain (BWG) and feed conversion (FC), and gender for each animal.*

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

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

Table S3: *Distance-based linear model (DistLM) for defined environmental data and the microbial communities of 760 samples.*

DistLM

Distance based linear models

VARIABLES

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

Total SS(trace): 1,2848E+06

MARGINAL TESTS

Variable	SS(trace)	Pseudo-F	P	Prop.
P Utilization	10822	6,4385	0,0001	0,0084226
Ca Utilization	8322,9	4,9422	0,0002	0,0064778
F I	40751	24,829	0,0001	0,031717
BWG	23757	14,28	0,0001	0,01849
FC	8172,8	4,8525	0,0003	0,006361

res.df: 758

***Table S4**: Multivariate statistical analysis for the overall data at OTU level. A. PERMANOVA analysis for P and Ca utilization. B. PERMANOVA analysis for BWG, FC and FI. C. ANOSIM to test gender effect*

A. PERMANOVA analysis for P and Ca utilization

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
P	4	15906	3976,4	2,3723	0,0013	9911
Ca	4	11154	2788,6	1,6637	0,0287	9903
P xCa**	4	11763	2940,7	1,7544	0,024	9915
Res	710	1,1901E+06	1676,2			
Total	723	1,2364E+06				

B. PERMANOVA analysis for BWG, FC and FI

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
BWG	4	7263,7	1815,9	1,1096	0,3369	9916
Feed intake	4	13694	3423,6	2,092	0,003	9912
Feed Conversion	4	6772,9	1693,2	1,0346	0,4132	9904
BWG x Feed intake	5	9381,2	1876,2	1,1465	0,2746	9893
BWG x Feed Conversion	7	15596	2228	1,3614	0,0755	9881
Feed intake x Feed Conversion	8	13081	1635,2	0,99918	0,4699	9883
BWG x Feed int. x Feed Conv.	0	0		No test		
Residuals	690	1,1292E+06		1636,5		
Total	723	1,2364E+06				

B. ANOSIM to test gender effect

Analysis of Similarities

One-Way - A

Tests for differences between unordered Gender groups

Global Test

Sample statistic (R): 0,005

Significance level of sample statistic: 1,3%

Number of permutations: 9999 (Random sample from a large number)

Number of permuted statistics greater than or equal to R: 131

Table S5: *Average dissimilarity (%) between high, medium and low groups for phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI) body weight gain (BWG) and feed conversion (FC) by males and females*

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

Table S6: *ANOSIM pairwise tests by groups: phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI), body weight gain (BWG), and feed conversion (FC)) by males and females*

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

Table S7 (excel file)*: Average- similarity and dissimilarity (%) between high, medium and low groups for phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI) body weight gain (BWG) and feed conversion (FC) by males and females*

Table S8*: Pairwise comparison based on t-test for phosphorus utilization, calcium utilization, feed intake, body weight gain, and feed conversion and the most abundant OTUs (*Unclassified *Clostridiaceae1;* Unclassified *Lactobacillus;* Unclassified *Clostridium sensu stricto 1; Escherichia coli; Streptococcus alactolyticus; Enterococcus faecium). A. Phosphorus utilization. B. Calcium utilization. C. Feed intake. D. Body weight gain. E. Feed conversion.*

CHAPTER IV

3rd Publication

CHAPTER IV – 3rd Publication

The gut microbial architecture of efficiency traits in the domestic poultry model species Japanese quail (*Coturnix japonica*) assessed by mixed linear models

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ABSTRACT

It is well known that mammals and avian gut microbiota compositions are shaped by the host genomes and affect quantitative traits. The microbial architecture describes the impact of the microbiota composition on quantitative trait variation and the number and effect distribution of microbiota features. In the present study the gut microbial architecture of feed-related traits phosphorus and calcium utilization, daily gain, feed intake and feed per gain ratio in the domestic poultry model species Japanese quail were assessed by mixed linear models. The ileum microbiota composition was characterized by 16S rRNA amplicon sequencing techniques of growing individuals. The microbiability of the traits was on a similar level as the narrow sense heritability and was highly significant except for calcium utilization. The animal microbial correlation of the traits was substantial. Microbiome-wide association analyses revealed several traits associated and highly significant microbiota features, both on the bacteria genera as well as on the operational taxonomic unit level. Most features were significant for more than one trait, which explained the high microbial correlations. It can be concluded that the traits are polymicrobial determined with some microbiota features with larger effects and many with small effects. The results are important for the development of hologenomic selection schemes for feed-related traits in avian breeding programs that are targeting the host genome and the metagenome simultaneously.

Keywords: Japanese quail, quantitative traits, microbiability, hologenomic selection

INTRODUCTION

Livestock microbiota research has received substantial attention in recent years (Estellé 2019). This is driven by the development of costeffective methods for the characterization of the microbiota composition, e.g., by the 16S rRNA amplicon sequencing approach or by sequencing the entire metagenome. The microbiota composition in the gastrointestinal tract (GIT) is strongly associated with quantitative traits such as growth and feed efficiency traits in pigs (Camarinha-Silva et al., 2017; Maltecca et al., 2019; Quan et al., 2018; Yang et al., 2017), methane emission in ruminants (Difford et al., 2018; Myer 2019; Roehe et al., 2016), and body weight gain and feed per gain ratio in poultry (Meng et al., 2014; Stanley et al., 2012). It is well known that the microbiota itself is shaped by the host genomes and, hence, it can be seen as a mediator between the individual host genome and corresponding quantitative trait records. This enables the development of hologenomic selection schemes that are targeting the host genome and the metagenome simultaneously (Estellé 2019; Weishaar et al., 2020). It was shown that especially for feed-related traits like feed or nutrient efficiency, hologenomic selection is a promising method to alleviate negative side effects of improving these traits on animal health (Weishaar et al., 2020).

Hologenomic selection requires the prediction of quantitative traits with the aid of microbiota composition (Camarinha-Silva et al., 2017; Maltecca et al., 2019; Verschuren et al., 2020) and this benefits from the knowledge of the microbial architecture of quantitative traits. The microbial architecture of a quantitative trait describes the impact of the microbiota composition in a specific GIT section, and the number and effect distribution of microbiota features affecting the trait. This can be assessed with the aid of microbial mixed linear models (Camarinha-Silva et al., 2017; Difford et al., 2018). These models contain a random animal effect with a covariance structure modeled by a microbial relationship matrix M. The elements of M are estimated from the relative microbiota operational taxonomic unit (OTU) abundances shared by pairs of animals. The microbiability (Difford et al., 2018) is the fraction of the phenotypic variance of a trait that can be explained by the microbiota composition. The marginal OTU effects can be obtained from the predicted animal effects. These models can thus be used for a multi- OTU microbiome-wide association study (MWAS), where all OTUs are fitted simultaneously. Expanding these models toward multivariate applications reveal the microbiota-driven trait correlations. Alternatively, single OTUs or bacterial genera can be used one by one in a mixed linear model to test them for trait association. The MWAS approaches can be used to identify the drives for the microbiota trait interrelation (Gilbert et al., 2016).

Japanese quail are well-established model animals in domestic poultry studies because of their short generation interval, small body size, low space requirements, and good comparability to other poultry species (Cheng et al., 2010; Kayang et al., 2004; Mills et al., 1997; Rodehutscord and Dieckmann 2005; Shibusawa et al., 2001; Stock and Bunch 1982). Only a few studies characterizing the GIT microbiota of Japanese quail were conducted (Borda-Molina et al., 2020; Liu et al., 2015; 2018; Wilkinson et al., 2016; 2020). Compared to mammals, the avian GIT is shorter in relation to body size

and digesta has a faster passage rate (Wilkinson et al., 2016). While the upper GIT segments (crop, proventriculus and gizzard) are responsible for initial feed hydrolysis, the main nutrient absorption takes place in the small intestine (duodenum, jejunum, and ileum). Thus, the ileum is a suitable location for the microbiota characterization if the interrelation between the microbiota and feed efficiency traits is to be investigated. The paired caeca are particularly important for fermentation and a high microbiota density and diversity is observed in this part of the GIT (Witzig et al., 2015; Yeoman et al., 2012).

Growing Japanese quail were used to study the variability of mineral utilization efficiency, growth, and other efficiency traits by Beck et al. (2016). A substantial phenotypic variability of these traits and a significant heritability were reported. Given the importance of the microbiota composition for efficiency traits observed in other species (Maltecca et al., 2019), it can be hypothesized that next to the host genome, feed and nutrient efficiency traits are also affected by the GIT microbiota composition. This is supported by studies on the effect of phosphorus (P) supply on the activity and composition of the microbiota in the ileum and other GIT sections in broiler chickens (Borda-Molina et al., 2016; Ptak et al., 2015; Tilocca et al., 2016; Witzig et al., 2015).

To the best of our knowledge, no studies are published so far analyzing the impact of GIT microbiota on feed-related traits in poultry using microbial mixed linear models and microbiome-wide approaches. The aim of the study was the estimation of microbial parameters for the traits phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI), feed per gain ratio (F:G), and body weight gain (BWG), as well as the application of MWAS on phylum, genera, and OTU level. The interrelation between the traits and the microbiota composition was further assessed with functional predictions.

MATERIAL AND METHODS

Experimental design

The experiment was conducted in accordance with the German Animal Welfare Legislation approved by the Animal Welfare Commissioner of the University (approval number S371/13TE) and described in detail by Beck et al. (2016). Briefly, a F2 cross of 920 individuals of Japanese quail (Coturnix japonica) was established. After plausibility testing, 888 individuals were available for further analyses. Before the quail were individually placed in metabolic units

on day five of life, they were housed in groups. After five days of acclimatization to the metabolic units, the performance testing was conducted in a strong growth period between 10th and 15th day of life, and animals were then slaughtered. Slaughtering took place at 12 different days, subsequently denoted as test-days. At slaughter the ileum was longitudinally opened and digesta was collected and stored in RNAlater at -80° until further analysis. The animals were provided with a low-phosphorus but otherwise nutrient-adequate diet. Bodyweight gain (BWG) was calculated as the difference of the body weight at day 10 and day 15. Feed per gain ratio (F:G) was calculated as feed intake (FI) within these 5 days divided by BWG. Phosphorus utilization (PU) and Calcium utilization (CaU) were calculated as the difference between total intake and total excretion of the respective element. Summary statistics are

shown in Table 1. Genetic parameters (heritability and genetic correlations) were estimated using mixed linear models and are reported by Beck et al. (2016).

Ileum microbiota characterization

Ileum microbial composition was obtained from a previous study (Borda-Molina et al., 2020). Briefly, ileum digesta samples of 760 quails were sequenced using 250bp paired-end sequencing chemistry on an Ilumina MiSeq platform (128 samples did not pass the quality filter of the sequences and were subsequently discarded). Demultiplexing and trimming of sequencing reads were done by using the default parameters from QIIME v1.9.1 pipeline (Caporaso et al., 2010), and it followed a subsampled open-reference OTU (operational taxonomic units) calling approach of the pipeline, with a maximum sequence length of 360 bp. The reads were merged into one fasta file and aligned using the SILVA Database (Release 132) (Quast et al., 2013). We used this database, because of its data are quality checked and includes more updated information. Chimeras were identified and removed using usearch (Edgar et al., 2011). Sequence reads can be accessed under the accession number PREJB37544. Sequences were clustered into operational taxonomic units (OTU) at >97% similarity and were taxonomically assigned to the closest species. OTUs were standardized by total. For further analyses, OTUs with an abundance lower than 0.0001% were removed and only phyla and genera with an average abundance higher than 0.5% are displayed in the results.

Functional predictions were carried out with the R package Tax4Fun2 (Wemheuer et al., 2020), which relied on the SILVA database (Yilmaz et al., 2014) and used the KEGG hierarchy for the assignations (Kanehisa et al., 2016). Silva database can provide more accurate information because it is regularly updated and maintained, and taxonomic assignations are manually curated (Balvočiūtė and Huson 2017). The biom table to assign this functionality was obtained from qiime pipeline (McDonald et al., 2012). Genomes from 16S rRNA gene sequences identified in this study were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/home/genomes/) in order to produce the most accurate database. Functional predictions were correlated with the quantitative traits.

Table 1 *Overview of phenotypic traits. Traits, trait abbreviations, mean, minimum (min), maximum (max) and standard deviation (SD) of the observed traits of the Japanese quail animals*

Trait[a]	abbreviation	unit	min	mean	max	SD
P utilization	PU	%	21.490	71.399	87.430	7.998
Feed intake	FI	g	16.110	42.630	62.350	7.120
Bodyweight gain	BWG	g	5.800	24.491	37.850	5.032
Feed per gain ratio	F:G	g/g	1.210	1.782	3.920	0.303
Ca utilization	CaU	%	19.420	60.554	84.310	10.018

[a] From day 10 to 15 of life.

Statistical analyses

Microbial linear mixed model: All statistical analyses were performed in R Studio (Version 3.5.2). The following microbial mixed linear model was fitted within ASReml R (Version 3.0) (Butler et al. 2009) to determine the microbial variance components:

$$y = \mu 1 + Z_{td} td + m + e, \quad (1)$$

where y is the vector with trait records (the considered traits were PU, BWG, FI, F:G, and CaU), μ is the trait mean and 1 is the vector of ones, vector $td \sim N(0, I\sigma_{td}^2)$ is the vector of random test day (i.e. the effect of the day at slaughter) effects with variance σ_{td}^2 and design matrix Z, and vector $e \sim N(0, I\sigma_e^2)$ contains the random residuals with variance σ_e^2. Vector m contains the random microbiota animal effects with distribution $m \sim N(0, M\,\sigma_m^2)$ and microbial variance σ_m^2. The microbial relationship matrix M was calculated as $M = \frac{XX^T}{N}$, where N is the number of OTUs and X is a $n \times N$ matrix, where n is the number of animals. Matrix X contains the standardized and log-transformed abundances of the OTUs (Camarinha-Silva et al. 2017). The model was applied in an univariate setting for the estimation of microbiability (m^2) as $m^2 = \frac{\sigma_m^2}{\sigma_p^2}$, with $\sigma_p^2 = \sigma_m^2 + \sigma_{td}^2 + \sigma_e^2$. The significance of microbiability was tested by conducting a likelihood-ratio test on the random animal effects. The test statistic was calculated as $D = 2[\log(L_2) - \log(L_1)]$, with L_2 being the likelihood of the full model and L_1 of the reduced model, i.e. model (1) without the random microbiota animal effect. The test statistic D under the null-hypothesis was chi-squared distributed with one degree of freedom. Next to the microbiability, the microbiota correlation between quantitative traits was of interest. For this purpose, model (1) was extended towards bivariate applications. The covariance matrix of the random microbiota animal effects became $Var\begin{bmatrix} m_1 \\ m_2 \end{bmatrix} = M \otimes \begin{bmatrix} \sigma_{m1}^2 & \sigma_{m1,m2} \\ \sigma_{m1,m2} & \sigma_{m2}^2 \end{bmatrix}$, with $\sigma_{m1,m2}$ being the covariance of the animal microbiota effects on trait 1 and 2. From the solutions of this bivariate model the animal microbiota correlations were estimated as $r_{m1,m2} = \hat{\sigma}_{m1,m2}/(\hat{\sigma}_{m1} * \hat{\sigma}_{m2})$. The significance of the correlation was tested by a likelihood ratio test as described above, with L_2 being the likelihood of the full bivariate model and L_1 of the corresponding bivariate model but with the covariance fixed at zero. In addition, phenotypic correlations between the raw trait records were calculated.

Microbiome-wide association analyses, MWAS: MWAS were conducted using two different approaches. The first approach was applied to bacterial genus level. A second filter step was applied at a minimum of 0.5% mean abundance of a bacterial genus. This reduced the number of genera down to 74, which were subject to the association analysis using the following mixed linear model

$$y = Xb + Z_{td} td + a + e, \quad (2)$$

where b is a vector with fixed effects containing the trait mean and the bacterial genus to be tested. The vector a contained the random animal effect with distribution $a \sim N(0, A\sigma_a^2)$, where A is the pedigree-based relationship matrix and σ_a^2 the additive genetic variance (Lynch and Walsh 1998). The effect of the bacterial genera was modeled as a covariate, i.e. the observation of an individual was regressed on the abundance of the bacteria genera. The regression coefficient was tested for significance using an F-Test. This model was applied for each of the 74 genera and each trait separately. The nominal p-values were corrected for multiple comparisons using the Bonferroni correction method. The correction was applied within each trait. To judge how many false-positive results were among the significant associations we calculated the false-discovery rate (FDR) (Benjamini and Hochberg 1995) using the software QVALUE (Storey and Tibshirani 2003). The FDR q-value of the significant bacterial genera with the lowest test statistic provided an estimate of the proportion of false-positive results among the significant associations.

The same approach was applied on the phylum level, with the four most abundant phyla (mean abundance > 0.5%) being tested. Because multiple testing is not a serious issue here, the nominal p-values were not corrected.

The second MWAS approach was applied at the multi-OTU level. We used model (1) for predicting the animal microbiota effects and obtained OTU effects by back-solving the effects as

$$\hat{u} = \frac{X' M^{-1} \hat{m}}{N}, \quad (3)$$

where $\hat{u}$ is the vector with estimated OTU effects, matrix X is as defined above, N is the number of OTUs, M^{-1} is the inverted microbial relationship matrix, and $\hat{m}$ is the vector with estimated animal microbiota effects (obtained from model (1)). Because all OTU effects were estimated simultaneously, they can be interpreted as marginal effects, i.e. the effect of each OTU is corrected for the effects of all other OTUs. We examined those OTU whose absolute trait association effect exceeded 0.25 σ_m more closely.

Data availability

All data generated and analyzed during this study were fully uploaded to the database of the journal. Supplemental material at figshare: https://doi.org/10.25387/g3.12123606.

RESULTS

Ileum microbiota community and functional predictions

The amplicon sequences were classified into 1188 OTUs belonging to 7 microbial phyla (Table 2). Most abundant bacterial groups at phylum level included *Firmicutes* (mean abundance in percentages 83.25), followed by *Proteobacteria* (mean abundance 14.29), *Actinobacteria* (mean abundance 1.65), and *Bacteroidetes* (mean abundance 0.70). The remaining phyla were identified as *Epsilonbacteraeota,*

Tenericutes, and *others*. The most abundant genera were *Candidatus Arthromitus* (mean abundance 29.64), *Clostridium sensu stricto* (mean abundance 14.11), *Enterococcus* (mean abundance 3.75), *Escherichia-Shigella* (mean abundance 14.17), *Lactobacillus* (mean abundance 24.33) and *Streptococcus* (mean abundance 8.25). They account for 96% of the total community. Further details regarding the microbiota characteristic are presented in (Borda-Molina et al. 2020).

The results from the functional predictions are shown in Figure 1 for three classification levels. At the broadest level of classification (level 1), the main activities were carried out for metabolism, followed by genetic information processing, and environmental information processing. In the next classification level (level 2 in Figure 1) the most abundant activities comprised carbohydrate metabolism, amino acid metabolism and nucleotide metabolism, energy metabolism, metabolism of cofactors and vitamins, and lipid metabolism (Figure 1 and Table S1).

From 352 predicted functions at the third level, a number of significant correlations with the quantitative traits were identified (Table S2). To summarize, for PU a total of 17 positive correlations with functions related to metabolism and environmental information processing were found. CaU was positively correlated with 30 functions belonging mainly to metabolism and five negative interactions. BWG showed 48 positive interactions with metabolism and 18 negative interactions. F:G registered 67 positive and 35 negative interactions. The highest number of correlations were registered with feed intake where 112 were positive and 60 were negative (Table S1). Thus, all the traits evaluated mainly interact with metabolic classified predicted functions.

Table 2 *Sample distribution at phylum level. Relative abundances at the phylum level with their minimal (min), mean, maximum (max) values, and standard deviation (SD)*

Phylum	Relative abundances			SD
	min	mean	max	
Actinobacteria	0.002	1.652	39.921	3.424
Bacteroidetes	<0.001	0.698	41.246	2.947
Epsilonbacteraeota	<0.001	<0.001	0.044	0.003
Firmicutes	16.393	83.249	99.875	12.718
others	<0.001	0.104	1.206	0.126
Proteobacteria	0.028	14.295	81.490	12.066
Tenericutes	<0.001	0.001	0.194	0.012

Table 3 *Results from the microbial linear mixed model (model 1), with microbial variance (σ_m^2), test-day variance (σ_{td}^2), residual variance (σ_e^2), and microbiability (m^2) with p values (standard errors are in in parenthesis)*

Trait[a]	$\boldsymbol{\sigma_m^2}$ (SE)	$\boldsymbol{\sigma_{td}^2}$ (SE)	$\boldsymbol{\sigma_e^2}$ (SE)	$\boldsymbol{m^2}$ (SE)	p value
PU	9.083 (3.210)	1.278 (0.997)	50.043 (3.228)	0.150 (0.050)	<0.001
FI	4.603 (1.852)	9.918 (4.618)	35.152 (2.169)	0.093 (0.037)	<0.001
BWG	4.302 (1.242)	1.504 (0.842)	17.973 (1.160)	0.181 (0.048)	<0.001
F:G	0.023 (0.005)	0.001 (0.001)	0.061 (0.004)	0.269 (0.051)	<0.001
CaU	4.463 (3.771)	5.846 (3.278)	91.457 (5.526)	0.044 (0.037)	0.235

[a] For trait abbreviations see Table 1.

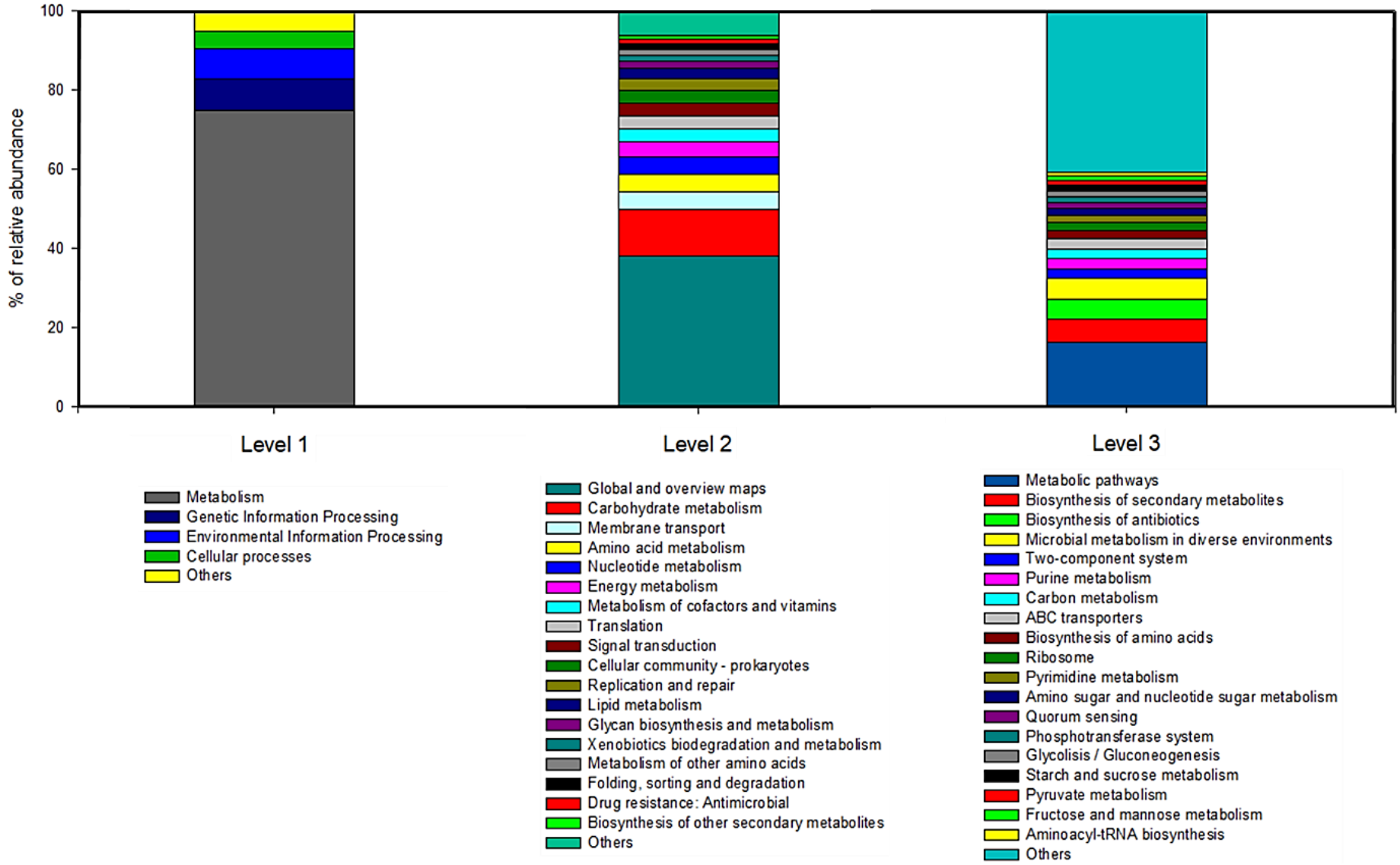

Figure 1 *Functional predictions of different levels of classification. Bar plot for the percentage of relative abundances (y-axis) of the predicted functions (x-axis) at the three levels of classification based on KEGG database.*

Microbial parameters

The estimated microbiabilities (results from model 1) were low for CaU and FI, and moderate for PU, BWG, and F:G (Table 3). They were highly significant with small standard errors, except for CaU ($P = 0.23$). Therefore, no further microbial analyses were conducted for CaU. The test-day variance component (Table 3) was small for all traits, except for FI.

The animal microbial correlations (results from the bivariate extensions of model 1, Table 4) were substantial. They were close to one for BWG-F:G and above 0.9 for PU-FI, and FI-BWG. They were highly significant and the standard errors were small in relation to the estimates. The microbial correlation coefficients were much larger than the phenotypic correlations, but the directions were the same.

Microbiome-wide association analyses

The results of the single-feature MWAS (model 2) for the four most abundant phyla revealed only weak significant associations for *Firmicutes* and *Proteobacteria* with PU. A higher abundance of *Firmicutes* increased (*P* nominal = 0.016) and a higher abundance of *Proteobacteria* decreased PU (*P* nominal = 0.048) (not shown elsewhere).

All genera and OTU effects are reported in units of σ_m. The significant associations (*P* nominal < 0.05) on the genus level are shown in Table 5. The number of microbiome-wide significant associations (p adjusted < 0.05) were 2 (3, 5, 6) for PU (FI, BWG, F:G, respectively). Remarkably, some genera showed highly significant associations for multiple traits. These were *Kurthia* (all four traits), *Candidatus Arthromitus* (PU, BWG, and FI), *Leuconostoc* (PU and BWG), *Enterococcus* and *Rothia* (both for BWG and F:G). All four PU significant genera were also significant for FI and BWG. The sign of some effects were in agreement with the signs of the microbial correlation coefficients (Table 4). The highest number of significant associations among the traits was found for F:G.

The results from the multi-OTU MWAS (model 3) are shown as Manhattan plots of marginal OTU effects in Figure 2. Several OTUs with large marginal effects ($\geq 0.025\sigma_m$) were mapped for all traits and are listed in Table 6 along with their taxonomic classifications. Among the traits, most large effect OTUs were mapped for F:G. Some large OTU affected several traits. The OTU402 showed a large effect for all four traits, OTU281 for FI, BWG, and F:G, and OTU1146 for PU and BWG. The OTU1053 affected both, PU and F:G.

Table 4 *Phenotypic vs. animal microbial correlations. Phenotypic correlations ($r_{pearson}$) and results from the bivariate microbial linear mixed model (bivariate extensions of model 1), with microbial covariance ($\sigma_{m1,m2}$), and microbial correlation ($r_{m1,m2}$) with p values (standard errors are in in parenthesis)*

Traits[a]	Phenotypic correlation		Animal microbial correlation		
	$r_{pearson}$	p value	$\sigma_{m1,m2}$ (SE)	$r_{m1,m2}$ (SE)	p value
PU – FI	0.561	<0.001	5.695 (2.085)	0.905 (0.102)	<0.001
PU – BWG	0.581	<0.001	4.671 (1.637)	0.791 (0.116)	<0.001
PU – F:G	-0.387	<0.001	-0.310 (0.097)	-0.738 (0.134)	<0.001
FI – BWG	0.849	<0.001	3.743 (1.346)	0.902 (0.059)	<0.001
FI – F:G	-0.213	<0.001	-0.282 (0.076)	-0.876 (0.117)	<0.001
BWG – F:G	-0.645	<0.001	-0.302 (0.072)	-0.982 (0.028)	<0.001

[a] For trait abbreviations see Table 1.

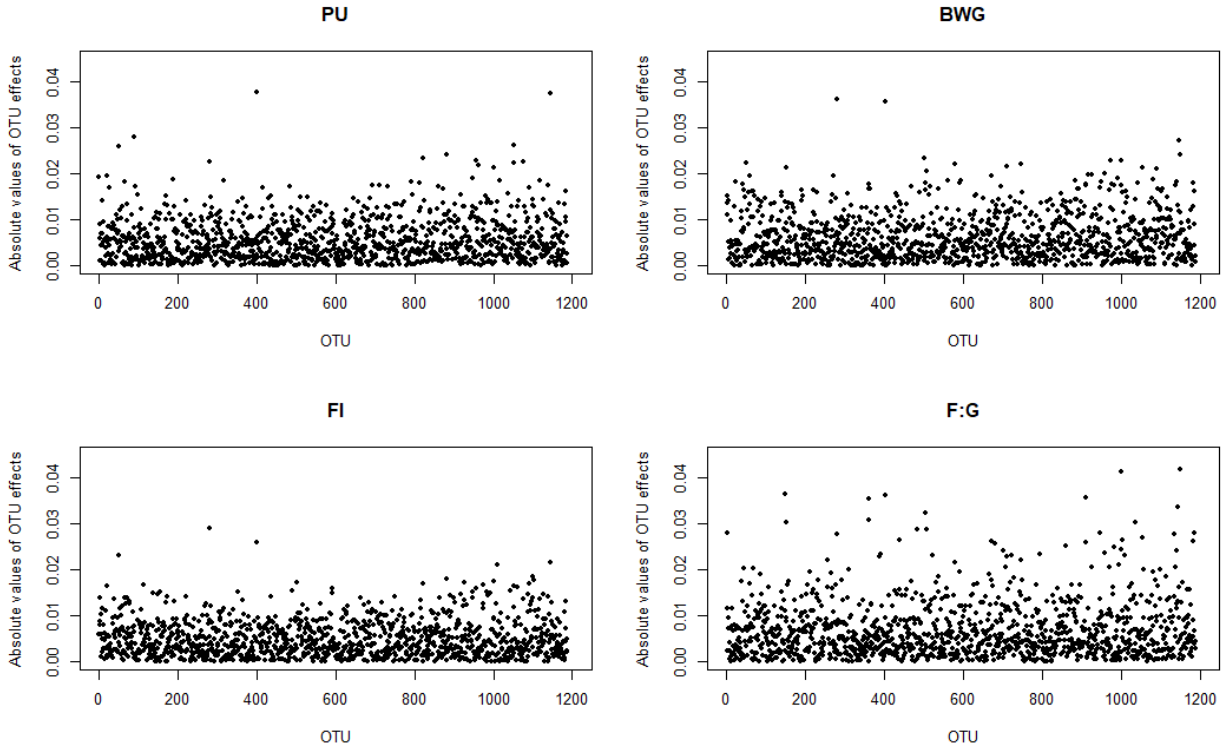

Figure 2 *Manhattan plot of results from the microbiome-wide association study conducted with model (3) for P utilization (PU), feed intake (FI), body weight gain (BWG), and feed per gain (F:G). Each dot represents absolute marginal OTU effect in units of σ_m and the corresponding OTU number.*

Table 5 *Results from the MWAS conducted with model (2) at the genus level (n = 74) with nominal p and adjusted p values, FDR q values, effect estimates $\hat{b}$ (in units σ_m, standard errors are in parenthesis)*

Trait[a]	Genus	p value	FDR q-value	p adjusted	$\hat{b}$ (SE)
PU	*Candidatus Arthromitus*	<0.001	<0.001	<0.001	0.024 (0.005)
	Kurthia	<0.001	0.011	0.022	-1.133 (0.312)
	Leuconostoc	0.005	0.089	0.291	1.083 (0.381)
	Bacillus	0.005	0.089	0.301	1.677 (0.593)
FI	*Candidatus Arthromitus*	<0.001	<0.001	<0.001	0.033 (0.006)
	Kurthia	<0.001	0.009	0.019	-1.329 (0.362)
	Leuconostoc	0.001	0.015	0.044	1.545 (0.449)
	Enterococcus	0.001	0.018	0.068	-0.040 (0.012)
	Bacillus	0.008	0.104	0.467	1.852 (0.702)
	Streptococcus	0.010	0.105	0.521	-0.018 (0.007)
BWG	*Candidatus Arthromitus*	<0.001	<0.001	<0.001	0.026 (0.005)
	Enterococcus	<0.001	<0.001	0.001	-0.040 (0.009)
	Kurthia	<0.001	0.001	0.002	-1.176 (0.281)
	Leuconostoc	<0.001	0.006	0.025	1.252 (0.348)
	Rothia	<0.001	0.006	0.028	-0.666 (0.186)
	Streptococcus	0.001	0.008	0.059	-0.018 (0.005)
	Macrococcus	0.001	0.008	0.064	-0.311 (0.093)
	Aerococcus	0.002	0.016	0.137	-0.158 (0.051)
	Unclassified Clostridiaceae1	0.002	0.016	0.145	2.266 (0.734)
	Clostridium sensu stricto	0.015	0.102	0.675	0.013 (0.005)
	Propionibacterium	0.023	0.142	0.822	2.987 (1.312)
	Clostridium XlVa	0.026	0.146	0.853	-1.022 (0.457)
	Bacillus	0.028	0.146	0.874	1.204 (0.546)
	Erysipelotrichaceae incertae sedis	0.030	0.147	0.893	-3.053 (1.402)
F:G	*Aerococcus*	<0.001	<0.001	<0.001	0.211 (0.042)
	Kurthia	<0.001	<0.001	<0.001	1.147 (0.233)
	Staphylococcus	<0.001	<0.001	0.001	0.317 (0.073)
	Enterococcus	<0.001	0.002	0.006	0.033 (0.008)
	Rothia	<0.001	0.002	0.009	0.600 (0.155)
	Macrococcus	0.001	0.009	0.050	0.264 (0.077)
	Unclassified Ruminococcaceae	0.001	0.009	0.061	0.613 (0.183)
	Cutibacterium	0.003	0.021	0.170	0.620 (0.205)
	Subdoligranulum	0.003	0.021	0.174	0.745 (0.247)
	Candidatus Arthromitus	0.004	0.026	0.230	-0.013 (0.004)
	Erysipelotrichaceae *incertae sedis*	0.004	0.028	0.265	3.350 (1.166)
	Unclassified Lachnospiraceae	0.005	0.028	0.290	0.171 (0.060)
	Lachnospiraceae *incertae sedis*	0.008	0.044	0.440	1.583 (0.594)
	Clostridium sensu stricto	0.010	0.055	0.542	-0.013 (0.004)
	Streptococcus	0.021	0.106	0.798	0.013 (0.004)
	Clostridium XlVa	0.027	0.120	0.867	0.844 (0.380)
	Sellimonas	0.028	0.120	0.875	1.385 (0.629)

[a] For trait abbreviations see Table 1.

Table 6 *Results from the MWAS conducted with model (3) with marginal absolute OTU effect estimates $\hat{b}$ (in units σ_m, standard errors are in parenthesis), and taxonomic classification. Results with $\hat{b} \geq 0.025\ \sigma_m$ are shown*

Trait[a]	OTU	$\hat{b}$	Phylum	Class	Order	Family	Genus
PU	OTU50	0.026	*Firmicutes*	*Bacilli*	*Bacillales*	*Paenibacillaceae*	*Paenibacillus*
	OTU1053	0.026	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Escherichia-Shigella*
	OTU90	0.028	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Escherichia-Shigella*
	OTU1146	0.037	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Escherichia-Shigella*
	OTU402	0.038	*Firmicutes*	*Bacilli*	*Bacillales*	*Paenibacillaceae*	*Paenibacillus*
FI	OTU402	0.026	*Firmicutes*	*Bacilli*	*Bacillales*	*Paenibacillaceae*	*Paenibacillus*
	OTU281	0.029	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Unclassified_ Ruminococcaceae*
BWG	OTU1146	0.027	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Escherichia-Shigella*
	OTU402	0.036	*Firmicutes*	*Bacilli*	*Bacillales*	*Paenibacillaceae*	*Paenibacillus*
	OTU281	0.036	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Unclassified_ Ruminococcaceae*
F:G	OTU982	0.025	*Actinobacteria*	*Actinobacteria*	*Micrococcales*	*Dermabacteraceae*	*Brachybacterium*
	OTU858	0.025	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae 1*	*Candidatus Arthromitus*
	OTU681	0.026	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae 1*	*Candidatus Arthromitus*
	OTU909	0.026	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Unclassified_ Ruminococcaceae*
	OTU1183	0.026	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Escherichia-Shigella*
	OTU672	0.026	*Actinobacteria*	*Actinobacteria*	*Micrococcales*	*Microbacteriaceae*	*Leucobacter*
	OTU1002	0.026	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Tyzzerella*
	OTU437	0.027	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
	OTU1053	0.027	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Escherichia-Shigella*
	OTU281	0.028	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Unclassified_ Ruminococcaceae*
	OTU1134	0.028	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Unclassified_ Ruminococcaceae*
	OTU1	0.028	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Ruminococcus2*

OTU1186	0.028	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*
OTU947	0.028	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*
OTU483	0.029	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Escherichia-Shigella*
OTU507	0.029	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Escherichia-Shigella*
OTU150	0.030	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Unclassified_ Ruminococcaceae*
OTU1037	0.030	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Enterococcaceae*	*Enterococcus*
OTU359	0.031	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Psychrobacter*
OTU504	0.032	*Firmicutes*	*Bacilli*	*Bacillales*	*Staphylococcaceae*	*Staphylococcus*
OTU1143	0.034	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Roseburia*
OTU361	0.035	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Lachnospiraceae_ incertae_sedis*
OTU910	0.036	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Unclassified_ Ruminococcaceae*
OTU402	0.036	*Firmicutes*	*Bacilli*	*Bacillales*	*Paenibacillaceae*	*Paenibacillus*
OTU149	0.037	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Blautia*
OTU1001	0.041	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Enterococcaceae*	*Enterococcus*
OTU1148	0.042	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae 1*	*Unclassified_ Clostridiaceae1*

[a] For trait abbreviations see Table.

DISCUSSION

This study analyzed the effect of the ileum microbiota composition on multiple quantitative traits with microbial mixed linear models. The results from functional predictions (Figure 1, Table S1, and Table S2) revealed that the ileum of quails is a highly metabolic active microbial environment. The m^2 estimates (Table 3) revealed a substantial impact of the microbiota composition on F:G and also on BWG and PU, which was also found with the functional predictions (Table S2). The m^2 estimates were on a similar level as the narrow sense heritability estimates for these traits (Beck et al. 2016a). Interestingly, the estimated animal microbiota correlations $r_{m1,m2}$ between traits were markedly high (Table 4), which is due to linkages between the traits, i.e. they were all P- related. It is known from other monogastric species that feed-related traits are affected by the GIT microbiota composition (Maltecca et al. 2020). However, the animal microbiota correlations $r_{m1,m2}$ were larger than the phenotypic correlations (Table 4) and the genetic correlations (Beck et al. 2016a). This points to the same underlying microbiota fractions affecting this class of traits. This can also be deduced from the MWAS results (Table 6), where most genera affected more than one trait. Some genera showed substantial effects with up to two or even three units of σ_m, e.g. for BWG (Table 6), even though these estimates may be biased due to multiple testing in the MWAS.

The results from the OTU level MWAS revealed some outliers with marginal effects > $0.025\sigma_m$, with many OTUs affecting more than one trait (Figure 2 and Table 6). However, no substantial peaked OTU could be identified. It might be that the large genera effect obtained from model (2) were dissected down to multiple marginal OTU effects underlying each genus. *Firmicutes* and *Proteobacteria* were also one of the most abundant phyla in other studies (Kumar et al. 2018; Shah et al. 2019; Liu et al. 2018; Wilkinson et al. 2016; Su et al. 2014). From these two phyla, four OTUs were associated with several traits (Table 6). Both OTUs of the phylum *Proteobacteria* belong to the *Escherichia-Shigella* genus, which is known as enteropathogenic microorganism. Both OTUs had negative effects on BWG and PU, while a positive effect was estimated for F:G. In broilers, abundance of *Escherichia-Shigella* in crop, ileum, and caeca samples was negatively correlated with performance traits (Rubio et al. 2015; Fonseca et al. 2010), which is consistent with our estimates for BWG and PU. One common colonizer of poultry GIT is *Candidatus Arthromitus* (Richards-Rios et al. 2020; Danzeisen et al. 2013; Gong et al. 2007) belonging to the family *Clostridiaceae* and the phylum Firmicutes. We found positive effects on several traits (Table 5), which is in agreement with other studies reporting positive correlations of this genus with animal performance traits (Danzeisen et al. 2013; Johnson et al. 2018). Both bacteria, *Bacillus* and some subspecies of *Enterococcus*, are considered as probiotic in chicken and Japanese quail (Cartman et al. 2008; Hong et al. 2005). *Bacillus* showed positive effects on several traits (Table 5). However, *Enterococcus* showed negative effects on FI and BWG, which may be due to the fact that *Enterococcus* is also known for pathogenesis and antibiotic resistance (Song et al. 2019; Quednau et al. 1998).

With regards to the trait microbial architecture it can tentative be concluded, that the traits are poly-microbial determined with some microbiota features exerting larger effects. In addition, the across-trait effects of the microbiota features point to substantial shared microbiota architecture for these traits. This is important for the development of hologenomic selection schemes that are targeting the host genome and the metagenome simultaneously (Weishaar et al. 2020).

The models applied show strong similarities with corresponding genomic models. The genomic counterpart of model (1) is a model where the microbial relationship matrix *M* is replaced by a genomic relationship matrix built by dense SNP data (Yang et al. 2011). The MWAS models (2) and (3) are closely related to genome-wide association studies (GWAS) frequently applied in livestock species, where single-marker as well as multi-marker models are used (reviewed in Schmid and Bennewitz 2017; Gilbert et al. 2016). The strength of these association models is that nuisance factors can be included straightforwardly. In this study the random test-day effects and the random genetic animal effects (with the pedigree-based genetic relationship matrix) were included. Both explained significantly a part of the variance. The inclusion of a random genetic animal effect in GWAS models is important to model the population structure and we followed this in the MWAS model (2). Alternatively, the relationships of the animals could have been modelled by the M matrix. We tested this and found in general the same significant effects, although on a somewhat lower significance level (results not shown). The latter might result from the genus under consideration being included twice in the model, i.e. as a fixed covariable and as random OTUs.

The applied models need large data sets. This is in contrast to so-called differential abundance analyses (Li 2015). These kind of studies are based on the comparison of the abundance of microbiota composition of previously selected groups of animals that differ with respect to their traits means. Naturally, also differential abundance analyses benefit from large data sets, but because group means are compared, they are applicable also to smaller data sets.

Conceptually, the main difference between the MWAS and the GWAS models is the use of relative abundances as regression variables instead of SNP genotypes. The relative abundances are compositional-type data with many zeros (Pawlowsky-Glahn et al. 2015), which are multivariate with a unit sum. It is impossible to alter the relative abundance of one feature without altering at least one of the other abundances (reviewed in Li 2015). This limits the identification of causalities from MWAS results. Methods are available to handle microbiota compositional data (Shi et al. 2016) and further research is needed to study the effect of incorporating these methods in the applied MWAS models. Thus, it is valid to conclude from the results of MWAS model (2) that the microbiota features are trait associated, but no inference of causality can be drawn. Since all features are considered simultaneously in eq (3), the problem is less evident for the results of the multi-OTU MWAS. Thus, this approach might serve as an ad hoc procedure to account for the compositional-type data structure. Further research is needed for the calculation of p values from the back-solved OTU effects as described for SNP effects obtained from genomic models by (Aguilar et al. 2019).

The multi-OTU MWAS method treated the OTU as random with normally distributed homogeneous variances. These models are convenient to apply from a computational point of view, but the downside is that large OTU effects might be regressed back too strong and thus do not peak in the Manhattan plots. Alternative models allow for a heavy-tailed distribution of OTU effects (Maltecca et al. 2019; Sanglard et al. 2020).

CONCLUSION

Except CaU, all traits were substantially influenced by the ileum microbiota composition and showed a substantial animal microbiota correlation. The latter points to the same microbiota features affecting multiple traits, which was confirmed by the results from the MWAS. The traits were poly-microbial in nature, with some microbiota features with large effects on the traits and many features with small or non-significant effects. The results might help to develop tailored breeding schemes that invoke microbial trait predictions. In this study ileum microbiota samples were used, but in practical breeding applications it is more convenient to use fecal samples. More research is needed to analyses if the microbiota composition in fecal samples are good quantitative trait predictors as well. They have to be confirmed in poultry species and lines such as laying hens or broiler chickens, which are economically more important than Japanese quail. The application of microbiome wide mixed linear models proved to be suitable to unravel the GIT microbial architecture of the traits, but have to be extended towards handling compositional type data.

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CHAPTER V

4th Publication

CHAPTER V – 4th Publication

The ileum microbiota composition is a heritable mediator between the host genome and phosphorus utilization in Japanese quail (*Coturnix japonica*)

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Keywords: Japanese quail, P utilization, intestinal microbiota, microbiability, hologenomic selection, quantitative trait loci

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in preparation

BACKGROUND

The poultry industry is a fast-growing sector of the global food supply. High efficiency meat and egg production coupled with low housing requirements makes poultry production indispensable worldwide. In order to maintain the competitiveness of poultry production, the main issues to be addressed are the utilization characteristics of essential nutrients. Phosphorus (P) is an essential nutrient needed by every living organism, with a finite global mineral occurrence and an enormous environmental impact of P excretion in the excreta (Campbell *et al.* 2017; Cordell *et al.* 2009; Pavlov *et al.* 2010). P is bound in plant seeds as phytic acid (myo-inositol hexaphosphate, $InsP_6$), the primary form of storage (Rodehutscord 2016). Better utilization of P from feed components would be a desirable goal. This can be achieved by phytases (myo-inositol hexaphosphate phosphohydrolases) and other phosphatases, a group of enzymes catalyzing the stepwise cleavage of P from $InsP_6$ or the hydrolysis of $InsP_{1\ to\ 5}$ (Sommerfeld *et al.* 2018; Zeller *et al.* 2015). It is known that non-ruminants overall have low endogenous phytase activity. Nevertheless, native InsP degradation in the gastrointestinal tract (GIT) can occur, albeit at a low level, by phytases and phosphatases derived from the endogenous mucosa of the GIT, some vegetable feed components, or from the gut microbiota (reviewed in Rodehutscord (2017)).

It is well known that the microbiota composition in avians' digestive tract is mainly associated with environmental factors, such as diet or housing condition, but numerous results in the literature point to an influence of host genetics on microbial colonization in the GIT. Significant heritabilities were found for the relative abundance of bacterial genera in the cow rumen (Difford *et al.* 2018; Saborío-Montero *et al.* 2020), for bacterial genera and operational taxonomic unit (OTU) level in the pig colon (Bergamaschi *et al.* 2020; Camarinha-Silva *et al.* 2017) as well as for cecal and fecal microbial species of chickens (Meng *et al.* 2014; Mignon-Grasteau *et al.* 2015; Zhao *et al.* 2013).

A previous study reported differences in P utilization (PU) in Japanese quail with standardized feeding and housing conditions and a heritability of 0.14 for this trait (Beck *et al.* 2016). Borda-Molina *et al.* (2020) detected differences in the relative abundance of different microbial genera between high and low PU quail from the same population. In a subsequent study, Vollmar *et al.* (2020) confirmed that PU and related traits are substantially influenced by the composition of the animals' ileal microbiota and estimated a significant microbiability, which was similar in magnitude as the heritability. This impact of the GIT microbial composition on quantitative traits was reported across livestock species, such as methane emission in ruminants (Difford *et al.* 2018; Myer 2019; Roehe *et al.* 2016; Zhang *et al.* 2020), growth and efficiency traits in pigs (Camarinha-Silva *et al.* 2017; Maltecca *et al.* 2019; Khanal *et al.* 2020) and growth traits in chickens (Wen *et al.* 2019).

In a previous study we assessed the microbial architecture of PU and related traits in Japanese quail by applying microbiome-wide association analysis and found that the traits were polymicrobial, with many traits associated with bacteria genera, but none with an exceptional large effect (Vollmar *et al.* 2020). Subsequently, we used the same data set to map QTL for PU on the genomes of the quail,

which were genotyped with 4K SNPs by applying linkage mapping (Vollmar *et al.* 2021). Several significant QTL could be mapped.

This study aimed to analyze the host genetic impact of the ileum microbiota composition in the same quail data set used in our previous studies. For this purpose, the heritabilities for the bacteria genera were estimated and QTL linkage mapping was applied. Subsequently, the hologenomic selection approach developed by Weishaar *et al.* (2020) was applied in order to assess the feasibility to breed for an improved P utilization based on the host genome and the heritable part of the ileum microbiota composition.

METHODS

Experimental Design

Details of the experimental design are described in Beck *et al.* (2016) and, thus, only the most relevant aspects are presented in the following. This animal experiment was performed according to the requirements of the German Animal Welfare Legislation and was approved by the Animal Welfare Commissioner of the University of Hohenheim (approval number S371/13TE). An F2 population of Japanese quail (*Coturnix japonica*) was established based on two divergent lines selected for social reinstatement behavior (Mills & Faure 1991). Twelve males and twelve females from each founder line were mated to generate the F1 generation. Seventeen roosters and 34 hens from the F1 generation were randomly selected and mated (one male with two females), resulting in 920 F2 individuals. These F2 birds were phenotyped between 10 and 15 days of age, while the birds were provided with a corn-soybean meal-based diet without mineral P or phytase supplements. A diet with an overall low P content was chosen to evaluate the PU potential of the quails.

Sample collection, SNP genotyping, and characterization of the ileum microbiota

The focal trait of this experiment was PU, which was calculated based on total P intake and P excretion, as well as based on FI during the experimental period. Quail BWG was quantified as the difference in body weight between days 10 and 15. The F:G ratio was computed as the FI during this 5-day period divided by the BWG. The quails were slaughtered at 15 days of age to collect ileum samples for further analysis. The birds were incubated and slaughtered on 12 different days, which were treated as test days in the statistical analysis. Estimates of the phenotypic and genetic correlations between the four recorded traits are in Beck *et al.* (2016) and Künzel *et al.* (2019).

DNA preparation, 4k SNP genotyping, and construction of a genome-wide linkage map are described in detail by Vollmar *et al.* (2021). In brief, all birds were genotyped for 5388 SNPs and the following criteria were applied to filter the genotypes: SNPs with one or more conflicting genotypes between parent and offspring, a minor allele frequency (MAF) ≤ 0.03, a SNP call frequency ≤ 0.9, and a cluster separation ≤ 0.4 were removed. We also excluded SNPs on the sex chromosomes Z and W. Finally, 3986 SNPs remained for further analysis.

Analyses of the composition of the ileum microbiota were performed by target amplicon sequencing, as described in Borda-Molina *et al.* (2020). Sequences were clustered into operational taxonomic units (OTUs) at > 97 % similarity. In total, 1188 OTU with an average relative abundance higher than 0.0001% and a sequence length greater than 250 bp were used in further analyses. Due to the use of a strict quality filter on the sequences, several samples were excluded. The final dataset included data on 758 quails with SNP genotypes, microbiota composition characteristics, and trait records (PU, FI, BWG, and F:G).

Statistical analyses

Transformation of microbial data: We used two microbial classifications for the statistical analyses, i.e., microbial genus and OTU. Genera data were filtered for a minimum of 0.5 % of the average relative abundance (RA) of each genus. This filtering step reduced the number of genera from 200 to 74. Because the distribution of the relative abundance of each microbial genus deviated remarkably from a Gaussian distribution, we applied a Box-Cox transformation with a specific lambda for each genus. The lambda was determined by a grid search to maximize the likelihood function of a normal distribution, following Box & Cox (1964):

$$\mathrm{f}(\mathbf{y}) = \begin{cases} \frac{\mathbf{y}\lambda-1}{\lambda} & (\lambda \neq 0) \\ \log \mathbf{y} & (\lambda = 0) \end{cases}, \quad (1)$$

where $\boldsymbol{y}$ is the vector of the relative abundances of each microbial genus to be transformed, and λ is the transformation parameter determined for each genus, which ranged from -2 to 0.505.

Mixed linear models for microbial composition: The following statistical analyses using a mixed linear model were performed in R Studio (Version 3.5.3) and ASReml R (Version 3.0) (Butler *et al.* 2009):

$$\boldsymbol{y} = \mu\,\mathbf{1} + \mathbf{Z_{td}}\mathbf{td} + \mathbf{Z_a}\mathbf{a} + \mathbf{e}, \quad (2)$$

where $\mathbf{y}$ is a vector of the transformed relative abundances of each genus, μ is the trait mean, and $\mathbf{1}$ is a vector of ones; $\mathbf{td}$ is a vector of the random test day effects, assumed to follow a normal distribution $\mathbf{td} \sim N(0, \mathbf{I}\sigma_{td}^2)$, where σ_{td}^2 is the variance, $\mathbf{I}$ is the identity matrix, and $\mathbf{Z_{td}}$ is the design matrix; $\mathbf{a}$ is a vector of the random animal effects, assumed to follow a normal distribution $\mathbf{a} \sim N(0, \mathbf{A}\sigma_a^2)$, where $\mathbf{A}$ is the pedigree-based relationship matrix and σ_a^2 the additive genetic variance, and $\mathbf{Z_a}$ is the design matrix. We chose to use pedigree instead of SNP genotypes here, because of the limited number of SNPs in the study. Finally, $\mathbf{e}$ is a vector of random residuals, assumed to follow a normal distribution $\mathbf{e} \sim N(0, \mathbf{I}\sigma_e^2)$, where σ_e^2 is the variance.

Using this mixed linear model, heritability (h_y^2) of each microbial genus was estimated as $h_y^2 = \frac{\sigma_a^2}{\sigma_p^2}$, with $\sigma_p^2 = \sigma_a^2 + \sigma_{td}^2 + \sigma_e^2$. Significance of the heritabilities was tested by conducting a likelihood ratio test on the random animal effects. The test statistic was computed as $D = 2[log(L_2) - log(L_1)]$, where L_2 is the likelihood of the full Model (1) and L_1 that of Model (1) without random animal effects, and is distributed as a chi-square with one degree of freedom under the null hypothesis of zero heritability. All microbial genus heritabilities with a nominal p value ≤ 0.05 were used for further analyses.

By extending model (2) to a bivariate model, we calculated the phenotypic (r_p) and genetic correlations (r_p) between the microbial genera (p ≤ 0.05) and the performance traits PU, BWG and F:G. The significance test of these correlations was done with the likelihood ratio test as described above. L_2 is the likelihood of the full bivariate model and L_1 the likelihood of the bivariate model, but with the covariances of the animal effect fixed at zero.

QTL linkage analyses of microbial genera: We used the R package R/qtl2 (Broman *et al.* 2019) for QTL linkage mapping. This program was originally set up for inbreed crosses. However, the founders in our study were not inbred, and thus this assumption was not fulfilled. Therefore, we calculated the QTL genotype probabilities for each F2 individual and each chromosomal position using the R package MAPfastR (Nelson *et al.* 2013), which was developed for outbred line crosses. Subsequently, the estimated QTL genotype probabilities were transferred to R/qtl2.

Genome scans were performed using regression of the phenotypes on two QTL genotype probability-derived regression variables, representing the QTL additive and dominant effects. The software did not allow the inclusion of random nuisance effects, other than a residual, or classification effects. Therefore, the effects of test days were included as dummy covariates in the model. The resulting logarithm of the odds (LOD) scores per cM were used as test statistics. To address the problem of multiple testing, a permutation test (10,000 permutations) was applied to derive 5 and 10% genome-wide significance thresholds for each microbial genus. Support intervals (SI) for QTL position were determined by using the 1.5 LOD drop-off method, which corresponds approximately to a 95% confidence interval (Manichaikul *et al.* 2006).

Within the SI for each identified QTL, all markers were evaluated for trait association using the single-marker association mapping approach implemented in the software package GCTA (Yang *et al.* 2011). The model regressed the phenotypes on the number of copies of the 1-allele at the SNP (i.e. 0, 1, or 2 copies) and included test days as dummy covariates and the random animal genetic effect with a SNP-derived covariance matrix, as implemented in the software using the LOCO option. During the association analysis, no correction for multiple testing was performed within the SI because the number of SNPs within a SI was usually small, reducing the problem of multiple testing in genome-wide association analysis.

Microbial linear mixed model: Microbial linear mixed models were performed using R Studio (Version 3.5.2) and ASReml R (Version 3.0) (Butler *et al.* 2009). As described above, microbiabilities estimated with this kind of models have already been described in Vollmar *et al.* (2020). However, since this model is the basis for further analyses, they will be described briefly in the following. To determine the microbial variance components and estimate $\widehat{\boldsymbol{k}}_{\boldsymbol{i}}$ of the animal microbiota effects $\boldsymbol{k}_{\boldsymbol{i}}$ on the quantitative trait we fitted the model:

$$\boldsymbol{y} = \boldsymbol{\mu}\,\mathbf{1} + \boldsymbol{Z}_{\boldsymbol{td}}\boldsymbol{td} + \boldsymbol{k} + \boldsymbol{e}, \tag{3}$$

where $\boldsymbol{y}$ is the vector of observations of the performance traits PU (BWG, FI, F:G) for n animals, $\boldsymbol{\mu}$ is the trait mean and $\mathbf{1}$ is the vector of ones, vector $\boldsymbol{td} \sim N(0, \boldsymbol{I}\sigma^2_{td})$ is the vector of random test day effects with variance σ^2_{td} and design matrix $\boldsymbol{Z}$, and vector $\boldsymbol{e} \sim N(0, I\sigma^2_e)$ contains the random residuals with variance σ^2_e. Vector $\boldsymbol{k}$ include the random microbiota animal effects with distribution $\boldsymbol{k} \sim N\left(0, \boldsymbol{M}\sigma^2_k\right)$ and microbial variance σ^2_k. The microbial relationship matrix $\boldsymbol{M}$ was calculated as described in detail in (Camarinha-Silva *et al.* 2017). The microbiability was estiamted as $m^2_y = \frac{\sigma^2_k}{\sigma^2_p}$, with $\sigma^2_p = \sigma^2_k + \sigma^2_{td} + \sigma^2_e$ (Difford *et al.* 2018). The significance of the random animal microbiota effect was tested using likelihood ratio tests as described avove.

Estimation of heritability of the microbiota effects: Estimates of the microbial animal effects $\widehat{\boldsymbol{k}}$ for each trait from model (3) were used as observations in the following genomic prediction model, as proposed by Weishaar *et al.* (2020) :

$$\widehat{\boldsymbol{k}} = \boldsymbol{\mu}\,\mathbf{1} + \boldsymbol{m} + \boldsymbol{e}, \tag{4}$$

where $\boldsymbol{\mu}$ is the overall mean, the vector $\boldsymbol{m} \sim N(0, \boldsymbol{G}\sigma^2_m)$ is the random animal genetic effect with the genomic covariance matrix $\boldsymbol{G}$, estimated using the 4k SNP genotypes following method 1 of VanRaden (2008), σ^2_m is the genomic variance of the estimated microbiota effects and $\boldsymbol{e}$ is the vectors of residuals with variance σ^2_e. Heritability of the microbiota-mediated trait $\widehat{\boldsymbol{k}}$ was calculated as $h^2_k = \frac{\sigma^2_m}{\sigma^2_m + \sigma^2_e}$. Significance tests for estimates of heritability were performed by likelihood ratio tests.

Microbial and genomic predictions: Three types of predictions were performed and evaluated using cross-validation, two genomic predictions and one microbial prediction. Model (2) was used to obtain genomic best linear unbiased predictions (GBLUP), but with the **A** matrix replaced by the **G** matrix. Model (3) was used to obtain microbial best linear unbiased predictions (MBLUP) (Camarinha-Silva *et al.* 2017). For GBLUP of the microbiota-mediated part of the trait, Model (3) was used to obtain estimates of the random microbiota effects of the animals for each of the four traits, which were subsequently used as observations in Model (4).

Microbial and genomic predictions were assessed using cross-validation with 500 repetitions, with variance components fixed at their estimated values. For each repetition, a reference population of 80 % of the animals was randomly selected to estimate the effects of OTU and/or SNP effects. The remaining 20 % of animals were used as validation population from which the animal effects were predicted. The averaged correlations, between the estimated animal effect and the observed animal phenotype $\boldsymbol{y}$, were used as accuracy of prediction. Based on these correlations, the confidence intervals were calculated from the 2.5 and 97.5 % quantile.

RESULTS

Heritabilities and correlations

Among the 74 bacterial genera examined, 27 showed a significant estimate of heritability ($p \leq 0.05$) (Table 1). The heritability ranged from 0.04 to 0.17. The highest heritabilities are estimated for *Clostridium sensu stricto*, *Lactobacillus* and *Bifidobacterium*, at 0.17, 0.12 and 0.10, respectively. All but one of the heritable genera belonged to the Firmicutes and Actinobacteria family, with the average relative abundances of the heritable genera ranging from 0.01 to 24.33%.

Table 2 shows the phenotypic correlations r_p between PU, BWG, F:G and the microbial genera, calculated with model (2). Only the phenotypic correlations are shown, as the genetic correlations showed large standard errors due to the limited number of animals. The r_p between PU and the genera are in a range of -0.086 (PU - *Streptococcus*) to 0.141 (PU - *Bacillus*). A similar range of r_p between F:G and the microbial genera were found, ranging from -0.107 (F:G – *Clostridium sensu stricto*) to 0.165 (F:G – *Enterococcus*). The r_p for BWG and the genera were slightly higher and ranged from -0.149 (BWG – *Enterococcus*) to 0.248 (BWG – *Leuconostoc*). Highest r_p of genera with our focal trait PU were found for *Bacillus* (0.141), *Leuconostoc* (0.134) and *Lactococcus* (0.129).

Table 1 *Estimations of heritable genera ($p \leq 0.05$). Genus with the corresponding phylum, their average relative abundance in percentage (RA %) and estimated heritabilities (h_y^2) with standard errors (SE) in parentheses and p values.*

Phylum	Genera	RA (%)	h_y^2	(SE)	p value
Firmicutes	*Aerococcus*	0.47	0.08	(0.04)	0.003
Firmicutes	*Anaerofilum*	0.02	0.04	(0.03)	0.040
Firmicutes	*Anaerostipes*	0.06	0.04	(0.03)	0.040
Firmicutes	*Bacillus*	0.08	0.06	(0.03)	0.006
Actinobacteria	*Bifidobacterium*	0.48	0.10	(0.05)	<0.001
Firmicutes	*Clostridium sensu stricto*	14.11	0.17	(0.07)	<0.001
Actinobacteria	*Corynebacterium*	0.15	0.05	(0.04)	0.043
Actinobacteria	*Corynebacterium*	0.47	0.06	(0.04)	0.012
Actinobacteria	*Curtobacterium*	0.01	0.06	(0.04)	0.014
Actinobacteria	*Cutibacterium*	0.06	0.08	(0.04)	0.002
Firmicutes	*Enterococcus*	3.75	0.06	(0.04)	0.011
Proteobacteria	*Escherichia / Shigella*	14.17	0.09	(0.05)	0.001
Firmicutes	*Lactobacillus*	24.33	0.12	(0.05)	<0.001
Firmicutes	*Lactococcus*	0.14	0.04	(0.03)	0.040
Firmicutes	*Leuconostoc*	0.12	0.04	(0.03)	0.029
Firmicutes	*Macrococcus*	0.23	0.06	(0.03)	<0.001
Actinobacteria	*Microbacterium*	0.02	0.05	(0.03)	0.026
Firmicutes	*Oscillibacter*	0.01	0.05	(0.04)	0.038
Firmicutes	*Ruminococcus 2*	0.28	0.05	(0.03)	0.030
Actinobacteria	*Saccharopolyspora*	0.01	0.06	(0.04)	0.021
Firmicutes	*Sellimonas*	0.03	0.05	(0.03)	0.028
Firmicutes	*Staphylococcus*	0.31	0.05	(0.03)	0.006
Firmicutes	*Streptococcus*	8.25	0.08	(0.05)	0.022
Firmicutes	*Subdoligranulum*	0.07	0.05	(0.03)	0.049
Firmicutes	*Tyzzerella*	0.08	0.07	(0.04)	0.007
Actinobacteria	Unclassified *Coriobacteriaceae*	0.01	0.05	(0.03)	0.026
Firmicutes	Unclassified *Lachnospiraceae*	0.38	0.06	(0.03)	0.010

Table 2 *Estimates of phenotypic correlations (r_p) (with standard errors (SE) in parentheses) between the considered genera and each trait.*

Genus	P utilization		Body weight gain		Feed per gain	
	r_p	(SE)	r_p	(SE)	r_p	(SE)
Aerococcus	-0.021	(0.039)	-0.119	(0.041)	0.120	(0.038)
Anaerofilum	-0.025	(0.038)	-0.044	(0.040)	0.043	(0.038)
Anaerostipes	0.075	(0.040)	0.057	(0.047)	0.072	(0.041)
Bacillus	0.141	(0.040)	0.232	(0.048)	-0.052	(0.043)
Bifidobacterium	-0.002	(0.042)	0.056	(0.050)	0.081	(0.041)
Clostridium sensu stricto	-0.025	(0.040)	0.044	(0.042)	-0.107	(0.038)
Corynebacterium	0.003	(0.039)	-0.048	(0.045)	0.024	(0.039)
Corynebacterium	-0.012	(0.041)	-0.110	(0.047)	0.053	(0.041)
Curtobacterium	0.074	(0.038)	0.185	(0.041)	-0.065	(0.039)
Cutibacterium	-0.001	(0.040)	0.043	(0.047)	0.089	(0.040)
Enterococcus	-0.085	(0.038)	-0.149	(0.038)	0.165	(0.036)
Escherichia / Shigella	-0.065	(0.040)	0.007	(0.045)	-0.086	(0.039)
Lactobacillus	-0.038	(0.047)	-0.092	(0.064)	-0.087	(0.047)
Lactococcus	0.129	(0.041)	0.236	(0.050)	-0.067	(0.045)
Leuconostoc	0.134	(0.042)	0.248	(0.051)	-0.054	(0.046)
Macrococcus	-0.006	(0.053)	-0.161	(0.072)	-0.004	(0.060)
Microbacterium	0.042	(0.039)	0.115	(0.044)	0.001	(0.039)
Oscillibacter	-0.007	(0.038)	-0.051	(0.041)	0.026	(0.038)
Ruminococcus 2	0.027	(0.040)	-0.001	(0.045)	0.106	(0.039)
Saccharopolyspora	0.039	(0.039)	0.145	(0.043)	0.020	(0.039)
Sellimonas	0.056	(0.039)	0.033	(0.045)	0.102	(0.039)
Staphylococcus	0.019	(0.045)	0.045	(0.062)	0.137	(0.043)
Streptococcus	-0.086	(0.038)	-0.109	(0.039)	0.118	(0.037)
Subdoligranulum	0.052	(0.039)	0.003	(0.041)	0.100	(0.038)
Tyzzerella	0.018	(0.040)	0.071	(0.045)	-0.011	(0.040)
Unclassified *Coriobacteriaceae*	0.039	(0.039)	0.048	(0.045)	0.063	(0.039)
Unclassified *Lachnospiraceae*	0.053	(0.040)	0.020	(0.045)	0.099	(0.039)

QTL linkage mapping results

The QTL linkage mapping results are shown as genome scan plots per heritable microbial genus with significant QTL in Figure 1. For clarity, only the first 23 *Coturnix japonica* chromosomes (CJA) are shown within the plots, since no significant peaks were observed for the other chromosomes. As described in Vollmar *et al.* (2021), none of the genotyped SNPs were located on CJA16. Six QTL with genome-wide significance thresholds of 5 and 10% were found across all genera (Table 3). Significant peaks were detected for the microbial genera *Aerococcus* on CJA3, for *Bacillus* on CJA2, for

Cutibacterium on CJA2, for *Escherichia/Shigella* on CJA24, for *Ruminococcus 2* on CJA3, and for *Streptococcus* on CJA5.

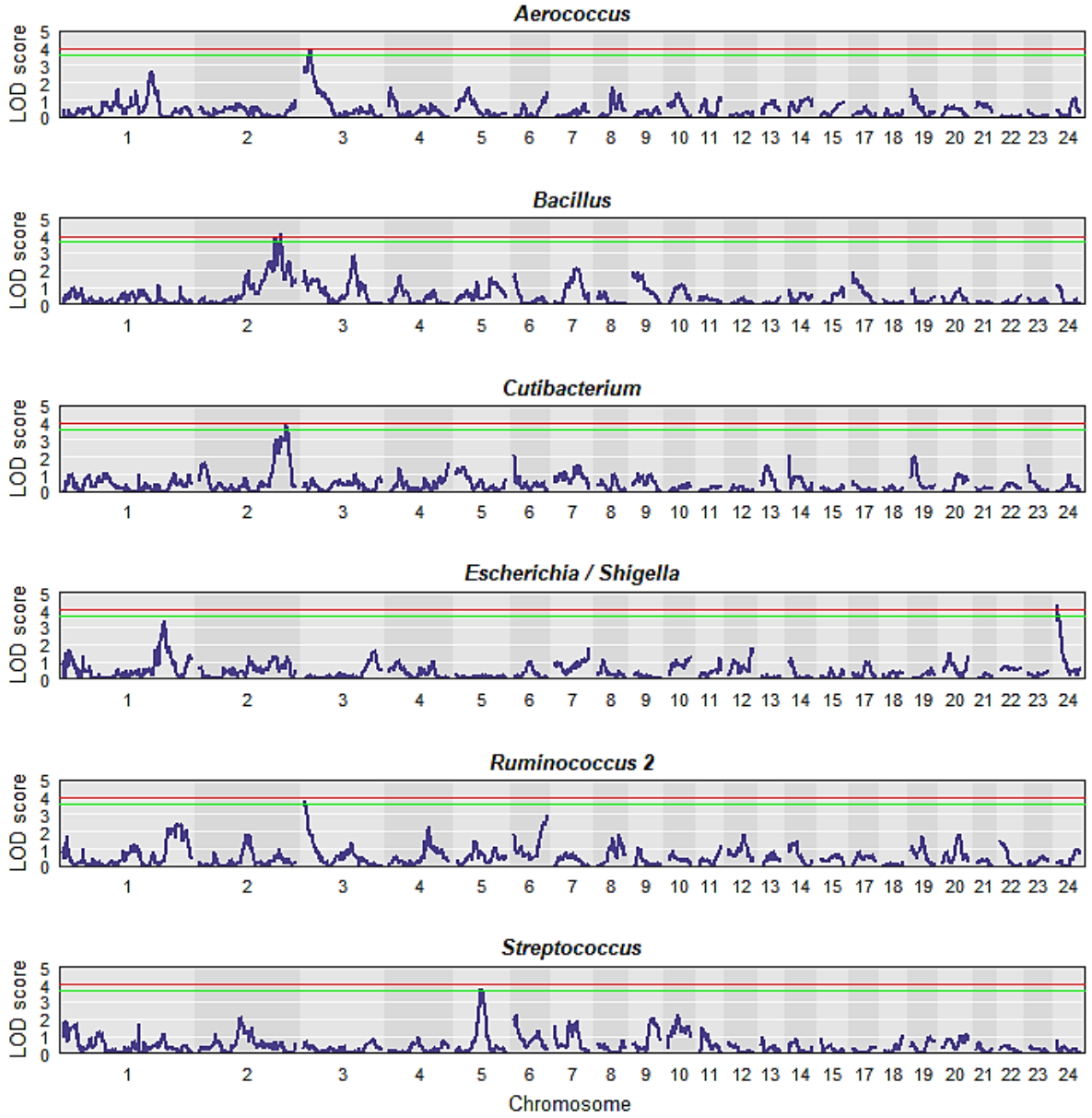

Figure 1 *Plots of the QTL linkage mapping scan of heritable genera ($p \leq 0.05$) with significant QTL. LOD score as test statistic and the red and green lines correspond to the genome-wide significance levels of 5 and 10 %, respectively.*

Results of the SNP-based association analyses for SNPs within the SI regions of the six identified QTL are in Table 3. Significant SNPs were found for each SI region, for a total of 103 significant SNPs, as listed in Supplementary Table S1. Due to overlapping SI, significant SNPs were shared between *Bacillus* and *Cutibacterium* on CJA2 and between *Aerococcus* and *Ruminococcus 2* on CJA3 (see Supplementary Table S1).

Table 3 *Results of the QTL linkage mapping. Positions in cM (Pos) of 5 % (**) and 10 % (*) genome-wide significant QTL on the Coturnix japonica chromosomes (CJA), with LOD score test statistic (LOD), and the corresponding QTL support intervals (SI). SI_low and SI_high represent the beginning and the end of the SI, respectively, and the number of significant SNPs (n of SNPs) ($p \leq 0.05$) obtained from the SNP trait association analysis.*

Trait	CJA	Pos[1]	LOD	SI_low[1]	SI_high[1]	n of SNPs
Aerococcus	3	12.0	4.00**	0.00	19.00	16
Bacillus	2	160.0	4.14**	147.00	164.00	24
Cutibacterium	2	171.0	3.83 *	147.00	178.00	34
Escherichia/Shigella	24	0.0	4.31**	0.00	8.00	2
Ruminococcus 2	3	2.0	3.74 *	0.00	10.00	10
Streptococcus	5	50.0	3.78 *	44.00	57.00	17

[1] Specification in cM, the corresponding genetic linkage map can be found in Vollmar *et al.* (2021).

Genomic and microbial trait predictions

Estimates of heritability for the animal microbiota effects for the four traits based on Model (4) were 0.07 (SE = 0.04, p value = 0.020) for PU, 0.14 (SE = 0.05, p value ≤ 0.001) for FI, 0.06 (SE = 0.04, p value = 0.020) for BWG, and 0.03 (SE = 0.03, p value = 0.267) for F:G. For all traits, except F:G, the estimate of heritability of animal microbiota effects was significant ($p \leq 0.05$). The results of the cross-validation of microbial and genomic predictions are in Table 4. Genomic predictions, $\hat{\mathbf{g}}$, and genomic predictions of the microbiota-mediated part of the traits, $\hat{\mathbf{m}}$, had similar correlations with the trait phenotypes. Average correlations between the microbial predictions, $\hat{\mathbf{k}}$, and the trait phenotypes were slightly higher than GBLUP accuracies for PU and FI, and markedly higher for BWG and F:G.

Table 4 *Estimated mean accuracy and confidence intervals (CI) of the genomic and microbial trait predictions. Estimated accuracies of the MBLUP and GBLUP of the trait observations and GBLUP of the microbiota-mediated part of the trait observations.*

Trait[1]	MBLUP		GBLUP		Microbiota mediated GBLUP	
	Accuracy	95 % CI	Accuracy	95 % CI	Accuracy	95 % CI
PU	0.22	0.09:0.35	0.18	0.05:0.32	0.16	0.01:0.31
FI	0.31	0.17:0.43	0.24	0.10:0.35	0.22	0.07:0.38
BWG	0.34	0.20:0.46	0.13	-0.01:0.25	0.14	-0.03:0.29
F:G	0.31	0.10:0.47	0.10	-0.05:0.23	0.07	-0.07:0.23

[1] PU = P utilization, FI = Feed intake, BWG = Body weight gain, F:G = Feed per gain.

DISCUSSION

In previous studies, we investigated the impact of host genetics (Beck *et al.* 2016; Künzel *et al.* 2019; Vollmar *et al.* 2021), the ileum microbiota composition (Borda-Molina *et al.* 2020; Vollmar *et al.* 2020), the interactions between the host miRNA-mRNA and the microbiome (Ponsuksili *et al.* 2020; Ponsuksili *et al.* 2021) and the ileal transcriptomic profiles (Oster *et al.* 2020) on our focal trait PU and related traits in Japanese quail. To complement those studies, we modeled the microbiota composition as a host trait and investigated how the microbiota composition and the host genome can be used together to predict the traits considered in the study. This fills an important gap about the feasibility assessment of hologenomic selection methods.

Mixed linear models, heritabilities and correlations

It is well known that gut microbial colonization is determined by the environmental and genetic background of animals. External factors, such as diet, husbandry, photoperiod and litter effects, can overlay or mask the effects of genetics (Hieke *et al.* 2019; Hubert *et al.* 2019; Shang *et al.* 2020; Wang *et al.* 2016). To reduce external influences on gut microbiota and to ensure comparability of animals, standardized housing and management conditions were used for all animals in this study. The microbiota composition DNA samples used in this study originated from an experiment that took place several years ago (Beck *et al.* 2016), and at that time, the importance of having control samples of feed, water, litter, DNA extraction, etc. was underestimated.

Heritabilities were estimated for cecal and fecal microbiota species in chickens (Meng *et al.* 2014; Mignon-Grasteau *et al.* 2015; Zhao *et al.* 2013), but no heritabilities of the ileal microbial genera have been studied in Japanese quail so far. Therefore, our results are not comparable to these studies. The three genera with the highest heritabilities were *Clostridium sensu stricto* (h_y^2 = 0.17), *Lactobacillus* (h_y^2 = 0.12) and *Bifidobacterium* (h_y^2 = 0.10). These heritabilities are lower than those calculated by Camarinha-Silva *et al.* (2017) and Estellé *et al.* (2014) for ileal bacterial genera in pigs and Org *et al.* (2015) in mice, but a solid comparison across species is questionable. Mignon-Grasteau *et al.* (2015) estimated moderate heritabilities for relative abundances of members of the genera *Lactobacillus* and *Clostridium* in the ceca of chickens.

The phenotypic correlations between the bacterial genera and the performance traits were within a low to medium range (Table 2). Because of the limited number of animals in our study, estimates of genetic correlations had large standard errors. Some studies (Han *et al.* 2016; Johnson *et al.* 2018; Siegerstetter *et al.* 2017) identified higher correlations between the performance traits and the ileal microbial genera in chickens compared to our results. The authors classified some of the same microbial genera, with the identical signs of phenotypic correlations for the recorded traits. Han *et al.* (2016) found a similar correlation between body weight and *Lactococcus* in broiler chickens, as we calculated between BWG and this genus in Japanese quail. In our results, the three bacterial genera *Bacillus*, *Lactococcus* and *Leuconostoc* dispose of the highest significant correlations with PU und BWG. Our

results are in agreement with those of Vollmar *et al.* (2020), who reported an association of *Bacillus* and *Leuconostoc* with PU using a MWAS, and also with those of Borda-Molina *et al.* (2020), who confirmed a positive phenotypic association of the relative abundance of *Bacillus* and *Leuconostoc* in the ileum with PU. Lactic acid bacteria are known to be phytase degraders and some species of the genus *Bacillus* showed extracellular phytase activity that might improve PU efficiency (Valente *et al.* 2013, Künzel *et al.* 2021).

The performance trait F:G displays the needed feed per body weigth gain. A good feed conversion rate indicates low feed intake for high performance. These explanations are consistent with the negative correlations of F:G with the traits PU and BWG (Beck *et al.* 2016; Künzel *et al.* 2019; Vollmar *et al.* 2020), as well as the negative correlations with the genera *Bacillus* (r_p = -0.052), *Lactococcus* (r_p = -0.067) and *Leuconostoc* (r_p = -0.054). The microbial genus *Clostridium sensu stricto* correlated negatively with the trait F:G (r_p = -0.107) and the genus with the highest positive correlation between the microbial genera and F:G is *Enterococcus* (r_p = 0.165). Surprisingly, negative correlations were found between *Lactobacillus* and the observed performance traits, although some *Lactobacillus* strains are considered probiotics (Gao *et al.* 2017; Wang *et al.* 2017; Wu *et al.* 2019). In summary the bacterial genera B*acillus*, *Lactococcus, Leuconostoc, Clostridium sensu stricto* affect PU in a positive, *Enterococcus* and *Streptococcus* in a negative way.

QTL linkage analyses of the microbial genera

To date, only a few QTL studies have been conducted in quail. In addition to Vollmar *et al.* (2021), Knaga *et al.* (2018), Ori *et al.* (2014) and Recoquillay *et al.* (2015) mapped QTL for different behavioral and performance traits on the quail genome. However, several authors have investigated host QTL for microbial colonization of the GIT in other species. For instance, Mignon-Grasteau *et al.* (2015) performed QTL analyses of microbial genera in the ceca of chickens. The consideration of the microbial colonization of the ileum at the genus level, with the inclusion of the quail genome, to perform a QTL analysis is unique to date.

In our study, six significant host QTL for microbial composition in quail were detected (Figure 1). One of these significant QTL was for *Bacillus*, on CJA2. Relative abundance of *Bacillus* was most highly correlated and showed the highest recursive relationships with PU, FI, and BWG. Interestingly, the SI of a previously identified QTL for growth rate on this chromosome (Knaga *et al.* 2018) overlaps with the SI of the QTL on CJA2 for *Bacillus*. Similarly, Essa *et al.* (2021) identified a QTL for BWG on chicken chromosome 2. The SI for he QTL on CJA2 for *Bacillus* also overlapped with that of the QTL for *Cutibacterium* (Table 3) and several common significant SNPs were detected (see Additional Table S1). The SI of the QTL identified for *Aerococcus* on CJA3 overlapped with that of *Ruminococcus 2*, and several common SNPs were also detected for this overlapping region. On CJA5, the SI of a QTL for *Streptococcus* overlapped with the SI for QTL for FI and foot ash reported in Vollmar *et al.* (2021).

Previously, Vollmar *et al.* (2020) found a trait association between *Bacillus* and PU using a MWAS and Borda-Molina *et al.* (2020) confirmed a phenotypic association between the increased relative abundance of *Bacillus* in the ileum with increased utilization of P. The involvement of the microbiota in the expression of PU, BWG and F:G is evident based on the results and can also be argued from a biological point of view. If we look at the genus Bacillus as an example, we can assume from the significant phenotypic correlation and identified QTL on CJA2 that *Bacillus* may directly influences the microbial colonization of the ileum with *Bacillus*, as well as indirectly influences the performance traits PU, FI and BWG. In future studies, structural equation models may help to examine the relationship between the quantitative traits and composition of the ileal microbiota.

The genus *Bacillus* is already used as a probiotic in chicken birds and is known to improve performance traits (Li *et al.* 2018; Abdel-Moneim *et al.* 2020), positively affecting the immune system (Bai *et al.* 2017; Li Gong *et al.* 2018; Fazelnia *et al.* 2021), increasing digestive enzyme activity (Li Gong *et al.* 2018; Wang *et al.* 2020; Fazelnia *et al.* 2021) and synthesizing phytases (Latorre *et al.* 2016). Some subspecies of *Lactococcus* supplied in the diet of broilers resulted in lower F:G, increased body weight, reduced mortality, and positive effects on the immune system (Fajardo *et al.* 2012; Zhang *et al.* 2016) and the carcass quality (Mujnisa *et al.* 2018). For some strains of *Leuconostoc* studies found weak enzymatic activities, including the formation of acid phosphatase (Paula *et al.* 2015) and the immunomodulatory activity due to induced cytokine production has been demonstrated (Seo *et al.* 2012). As noted above, the genus *Enterococcus* has a negative impact on PU and BWG. In humans, some members of this genus are considered opportunistic pathogens due to their antibiotic resistance (Hollenbeck & Rice 2012; Maasjost *et al.* 2015). In chickens, these bacteria can lead to increased one-day mortality (Gregersen *et al.* 2010) and the formation of toxic metabolites by bacterial metabolisation of protein has also been reported (King *et al.* 2009). Relative abundance of the genera *Staphylococcus* and *Streptococcus* also negatively influenced the traits analyzed in our study. There are different references to these bacterial genera, which can cause different diseases and health restrictions in poultry. Depending on the bacterial species, several clinical observations that range from drowsiness and poor feed intake to increased mortality (reviewed in Logue *et al.* (2020)).

Microbial and genomic predictions

The results from the microbial and genomic predictions (Table 4) illustrated a strong effect of the ileum's microbial composition on the phenotypic expression of the traits. The two-step procedure proposed by Weishaar *et al.* (2020) to estimate breeding values for the microbiota-mediated part of a trait was also successful in our study, in particular for PU and FI. The GBLUP accuracy for the microbiota-mediated part of the host phenotype was only slightly lower than the prediction accuracy of the conventional GBLUP. This might be because the genetic effect of the host on the trait mediated by the microbiota is much stronger than the direct genetic effect of the host. To substantiate this hypothesis, we fitted Model (1) with an additional random animal effect with the microbiota-based covariance matrix **M** for PU.

Compared to a model with only the microbiota effect, the estimate of microbiability for PU remained at almost the same level (0.15) but the estimate of heritability dropped from 0.12 to 0.07 (results not shown). A similar pattern was observed by Difford *et al.* (2018) in a study on dairy cattle rumen microbiota composition and methane production. This clearly shows that fitting both random effects simultaneous is beneficial but that assuming a zero covariance between the two random effects is too simplistic. How to model both effects simultaneously and how to interpret the results from such models biologically is an ongoing research topic (Christensen *et al.* 2021, Pérez-Enciso *et al.* 2021, Saborío-Montero *et al.* 2021) but this is outside the scope of our study.

Conclusion

A significant host genetic effect on the ileum microbiota composition in quail was detected. From the 74 bacteria genera, 27 showed a significant heritability. QTL linkage mapping for these 27 genera revealed several significant QTL, but no major one. The application of microbial and genomic mixed linear models allowed accurate prediction of PU and related traits. In particular, applying these models made it possible to predict the microbiota-mediated part of the traits, demonstrating the feasibility of hologenomic selection.

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SUPPLEMENTARY INFORMATION

Supplementary Table S1 *Summary of the trait-associated markers from GCTA ($p \leq 0.05$), within the significant QTL regions. All markers that are significantly associated with another characteristic are listed in the last column.*

Trait	CJA	Marker ID	p value	Pos[1]	matched markers with other genus
Aerococcus	3	id00986	< 0.001	0.000	*Ruminococcus2*
	3	id29156	0.027	0.000	
	3	id00575	< 0.001	0.000	*Ruminococcus2*
	3	id13672	0.007	0.360	*Ruminococcus2*
	3	id15191	0.028	1.830	
	3	id07523	0.016	3.082	*Ruminococcus2*
	3	id10549	0.033	3.082	
	3	id02839	0.030	3.082	*Ruminococcus2*
	3	id14154	0.028	8.013	*Ruminococcus2*
	3	id12388	0.019	8.024	*Ruminococcus2*
	3	id24815	0.003	9.433	
	3	id18519	0.009	11.689	
	3	id03758	0.021	16.649	
	3	id13768	0.014	17.290	
	3	id05996	0.008	17.290	
	3	id12382	0.008	17.290	
Bacillus	2	id09016	0.003	147.885	
	2	id05956	0.023	148.328	
	2	id32858	0.006	148.948	
	2	id12291	0.013	150.053	
	2	id33001	0.014	150.574	*Cutibacterium*
	2	id25447	0.008	150.911	
	2	id30476	0.021	151.894	
	2	id03102	0.002	154.051	
	2	id00923	0.003	154.051	
	2	id04053	0.002	157.198	*Cutibacterium*
	2	id33819	0.008	157.198	
	2	id10445	0.002	157.198	*Cutibacterium*
	2	id13471	0.001	158.582	
	2	id06720	0.001	158.587	*Cutibacterium*
	2	id02198	< 0.001	158.750	*Cutibacterium*

	2	id04692	0.041	159.867	
	2	id01497	0.002	160.198	*Cutibacterium*
	2	id10454	< 0.001	160.198	*Cutibacterium*
	2	id02833	0.003	160.198	*Cutibacterium*
	2	id02131	0.002	160.317	*Cutibacterium*
	2	id12311	0.028	161.185	
	2	id08123	0.028	161.185	
	2	id09029	0.011	163.378	
	2	id09032	0.032	163.648	*Cutibacterium*
Cutibacterium	2	id33001	0.032	150.574	*Bacillus*
	2	id04053	0.015	157.198	*Bacillus*
	2	id10445	0.049	157.198	*Bacillus*
	2	id18905	0.023	157.199	
	2	id06720	0.043	158.587	*Bacillus*
	2	id02198	0.039	158.750	*Bacillus*
	2	id01497	0.045	160.198	*Bacillus*
	2	id10454	0.009	160.198	*Bacillus*
	2	id02833	0.011	160.198	*Bacillus*
	2	id02131	0.039	160.317	*Bacillus*
	2	id09032	0.035	163.648	*Bacillus*
	2	id01918	0.037	165.518	
	2	id13472	0.037	165.518	
	2	id32230	0.045	168.860	
	2	id15179	0.032	168.860	
	2	id32700	0.041	168.871	
	2	id17401	0.014	169.669	
	2	id23477	0.013	169.669	
	2	id28643	0.013	169.669	
	2	id08129	0.011	169.669	
	2	id10474	0.007	170.978	
	2	id31089	0.001	171.280	
	2	id12342	0.003	171.280	
	2	id08131	0.001	171.280	
	2	id15180	0.020	171.280	
	2	id03809	0.002	171.280	
	2	id01498	0.002	171.280	
	2	id09043	0.017	173.562	

	2	id32704	0.044	173.563	
	2	id10482	0.021	173.563	
	2	id17403	0.044	173.567	
	2	id13854	0.003	174.374	
	2	id00622	0.001	176.564	
	2	id17758	0.014	176.564	
Escherichia / Shigella	24	id10195	0.042	0.000	
	24	id13533	0.041	0.000	
Ruminococcus2	3	id00986	< 0.001	0.000	*Aerococcus*
	3	id18286	0.039	0.000	
	3	id00575	0.001	0.000	*Aerococcus*
	3	id13672	0.009	0.360	*Aerococcus*
	3	id06525	0.001	0.360	
	3	id07523	0.009	3.082	*Aerococcus*
	3	id02839	0.014	3.082	*Aerococcus*
	3	id34021	0.002	3.082	
	3	id14154	0.008	8.013	*Aerococcus*
	3	id12388	0.009	8.024	*Aerococcus*
Streptococcus	5	id14925	< 0.001	44.417	
	5	id08312	0.035	44.614	
	5	id15246	0.026	45.341	
	5	id03766	0.005	45.342	
	5	id17506	0.015	45.342	
	5	id01172	0.038	45.342	
	5	id04785	0.007	45.342	

CHAPTER VI

General Discussion

CHAPTER VI – General Discussion

Food of animal origin has to become more effective at meeting the needs of the growing world population (Thornton 2010). Therefore, this work was carried out to provide an approach to possibly reduce supplementation of P based on the individual genetic variability of an animal and its gut microbiota. For this purpose, quantitative trait locus (QTL) analyses were performed (chapter two), and further analyses of the ileum microbiota were applied (chapters four, five and six). **Chapter two** includes the study of the calculation of a linkage map, with subsequent QTL analysis. **Chapter three** contains a study that considers of the ileum microbiota composition of Japanese quail, followed by **chapter four**, which involves a study using microbial mixed linear models to test the effects of the microbiota on our quantitative target traits. This is followed by **chapter five**, where the microbial mixed linear model was extended to genomic parameters, a hologenomic selection approach was investigated, and QTL mapping was applied to microbiota data.

In this chapter, additional results of QTL analyses are shown, the methods of ileum microbiota composition analysis, the practical application of results, and some remaining open questions are discussed.

6.1 QTL Analysis

To fundamentally discuss QTL analysis methods, some fundamentals must be considered. The choice of the analysis method is of great importance. Suitable for our F_2 design, the basic idea was to apply association mapping (AM) methods. When applying this technique, mainly unrelated or related animals without an experimental design are used. This seemed fitting because our founder lines were not related to each other. Compared to linkage analysis (LA), AM is considered to be simpler because many statistical methods can be used, whereas LA is computationally complex because it requires adapted probability calculations. However, AM was not the most appropriate method, as our animals were genotyped using a 4k SNP chip. Therefore, the marker density was too low for AM methods. LA was originally designed for low-density microsatellite maps. Applying this technique always requires an informative design and is additionally based on recombination events between markers within the generations of this design. This enabled us to apply a LA with a low marker density for QTL analysis and our informative F_2 design in chapter two, with good results.

This led to an adjustment, a decisive factor for QTL mapping: marker density. It was shown that distances between markers can differ slightly between populations (Alonso-Blanco et al. 2006), but if the distance between markers is less than 10 cM, the accuracy of QTL mapping does not automatically increase (Darvasi et al. 1993; Piepho 2000). Hence, the calculation of our own linkage map specifically for our population was of high interest. After calculating the linkage map, the average marker distance was 0.81 markers/cM with a total length of 1735.36 cM for all calculated chromosomes, and the chosen

4000 markers were evenly distributed over the genome (chapter two). Nevertheless, a lower mapping resolution may result due to the lower number of markers and thus fewer recombination events (Crooks et al. 2011). Consequently, wide QTL confidence intervals (CIs) are expected, which was not the case in our study (chapter two). Our CIs were short, with fast, sharp drops, which may have been due to possible inaccurate estimation of genotype probabilities. Neighboring genotype probabilities of loci may have dropped sharply and appeared in short CIs. However, the density of markers is not the most decisive factor for the accuracy of QTL mapping; more important is the number of individuals used (Ledur et al. 2010). Applying the LA method, our dataset with approximately 900 informative F_2 animals was a sufficiently large population (chapter two). A larger test population results in additional costs for genotyping and phenotyping.

It is possible that a higher accuracy may be achieved by using more markers and, in particular, more individuals. Larger gaps between markers might be reduced to illuminate all loci of the genome, detecting QTL with medium or low effects by linkage disequilibrium with very close markers or estimating more precise genotype probabilities and thus creating smoother QTL peaks and QTL detection.

In addition to the fundamentals already mentioned, the choice of experimental design is important for the QTL mapping strategy. Our experimental founder lines were selected for a behavioral trait (chapter two), and the aim of our first study was to map QTL for the target trait P utilization for animals of the F_2 generation. Cross-breeding experiments usually produce individuals with divergently segregating alleles. The differentiation of our two founder lines were indicated by investigating an F_{ST} value of 0.31 (Beck et al. 2016). Therefore, we presumed to obtain sufficient divergently segregating alleles in the F_2 generation. Assuming, that in inbred line crosses the genotypes can be traced to their origin, genotype probabilities can be calculated for each marker position. These markers may be linked to a QTL of interest in inbred lines. Taking this into account, using an F_2 design in our experiment was advantageous.

Because of our results (chapter two), we assumed that the QTL CI could not be reliably estimated because the genotype probabilities were too imprecise and incorrectly estimated. Thus, methods of outbred lines provided an alternative method and were further investigated in two approaches. In outbred populations, animals may have the same genotypes by chance, which is to be expected in inbred populations, as they have kinship relationships within and between families (Gonzales and Palmer 2014). It is assumed that markers are not fixedly linked to QTL of interest, and flanking markers do not provide a reliable statement about the origin of the desired allele (Crooks et al. 2011). Accordingly, the genotypes are traced using a pedigree. Their genotype probabilities can then be calculated with a possible higher accuracy.

Given this evidence for uncertain estimated QTL CIs, we tested other QTL mapping methods. The results of additional QTL analyses are shown below.

When using the MAPfastR module (Nelson et al. 2013), a software program for outbred crosses, a triM (tracing inheritance with Markov models) algorithm is used to calculate QTL genotype probabilities instead of a hidden Markov model as used in R/qtl2 (Broman et al. 2019). The QTL genotype probability method underlying our QTL analysis using R/qtl2 (chapter two) is based on an algorithm (Haley et al. 1994) that was not originally designed for denser marker maps or outbred crosses (Crooks et al. 2011). The QTL genotype probabilities of MAPfastR were applied using the triM algorithm, which more accurately estimates the line origin probability and thus QTL genotype probabilities. Good adaptation of the algorithm to our data set was expected. In chapter two, this adaptation is best adjusted by marker selection via F_{ST} values. We noted the calculated QTL genotype probabilities and then implemented R/qtl2 software because both modules use least square estimation for the QTL genome scan (Haley-Knott regression) (Haley et al. 1994). This was carried out for two reasons: better QTL genotype probabilities were expected, and both modules used the same basic assumptions. Hence, R/qtl2 was applied in the following steps, as the interface of the module R/qtl2 is easier to handle than MAPfastR. All following analysis steps were performed as described in chapter two in 'QTL linkage and association analysis', except for marker selection.

Figure 1 shows the results for performance traits. No significant QTL peak was identified for the performance traits. These results indicate no advantage in using MAPfastR software for the given data sets. In fact, the plots depict sharp peaks as in chapter two, which may indicate a rapid decline in genotype probabilities of flanking markers.

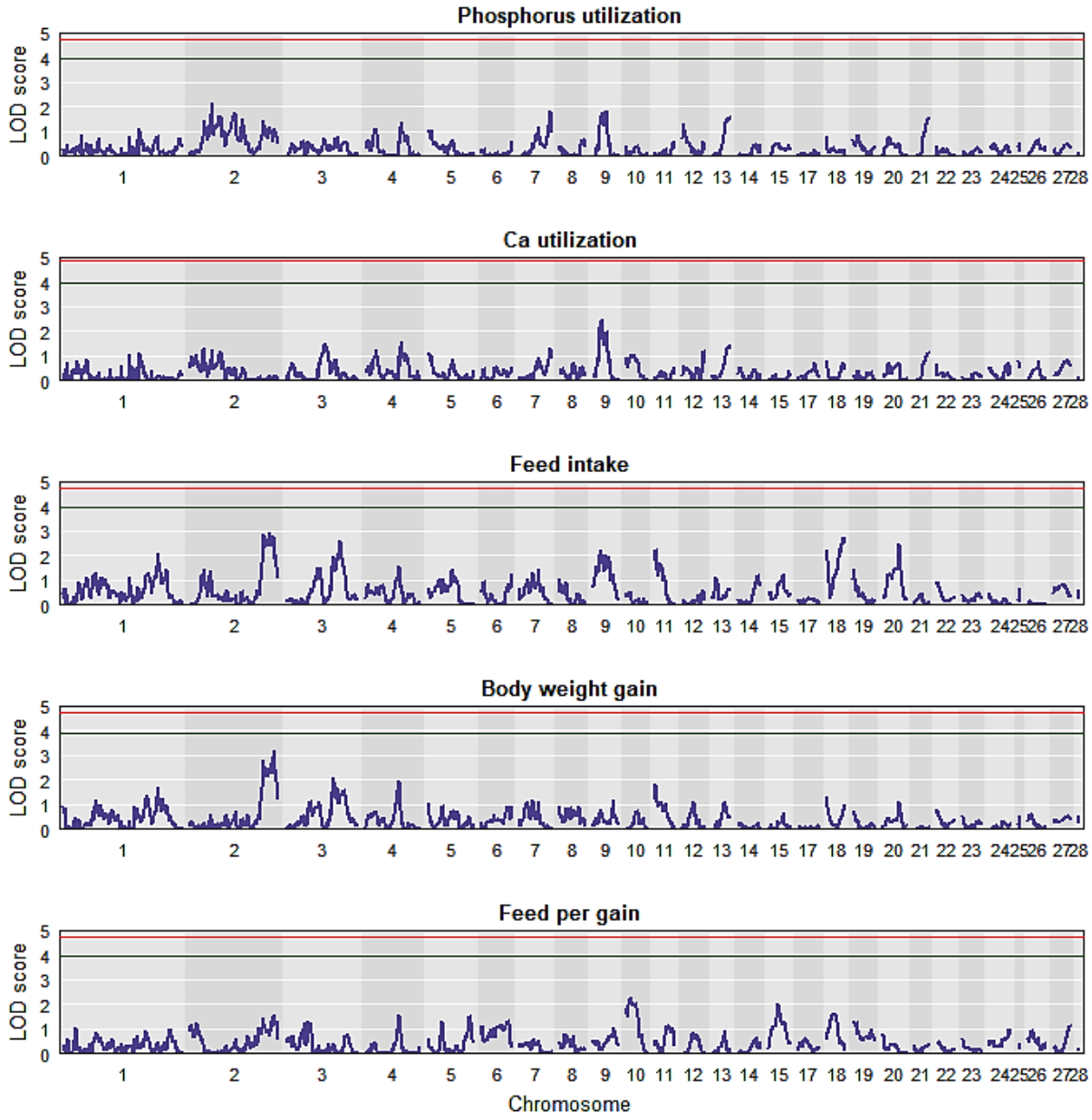

Figure 1 *Plot of a QTL linkage mapping scan of performance traits with LOD score test statistics using QTL genotype probabilities of MAPfastR, a software package for outbred line-crosses, and the interface of the module R/qtl2. The red and green lines correspond to genome-wide significance levels of 1 and 5%, respectively.*

Therefore, we tested another software program, QTLMap (Filangi et al. 2010), which uses a half-sib design and interval mapping methods. The test statistic is based on a likelihood ratio test (LRT) and corresponds to the Haley and Knott (1992) regression as well, scanning for QTL every 1 cM in the quail genome. A QTL peak with a *p*-value <0.05 and <0.001 at the genome-wide level was retained as significant, and highly significant, respectively. To obtain the 95% confidence intervals of the QTL peak, we used the LOD drop-off method.

The test statistic plots of QTLMap results are shown in Figure 2. We identified seven QTL peaks (two QTL for 1% and five QTL for 5%) for all traits at a 1% and 5% genome-wide significance level. We mapped QTL for all traits, except for PU. For FI, we mapped one QTL at CJA11 ($1\%_{\text{genome-wide}}$) and one QTL peak at CJA2 ($5\%_{\text{genome-wide}}$), and one QTL at CJA2 for BWG ($5\%_{\text{genome-wide}}$). For F:G, we identified one peak at CJA2 ($1\%_{\text{genome-wide}}$); for CaU, we identified two QTL peaks at CJA1 and 20 ($5\%_{\text{genome-wide}}$) (Figure 2). The QTL peaks overlapped for BWG and F:G at CJA2.

Although we used the same animals for the analyses, a comparison cannot be reliably made due to different methods. To fully judge which method is most appropriate for this dataset, a simulation study with known QTL should be conducted. This was, however, outside the scope of this thesis. In addition, our study (chapter two) offered initial results regarding the trait P utilization using a large number of experimental animals and SNP markers. Under the given conditions, the application of R/qtl2 was the best possible QTL mapping software because of its easy computational application and results. Taking into account the statistical results, we can assume that a more refined algorithm and genotype probabilities calculated differently will not necessarily lead to improved results.

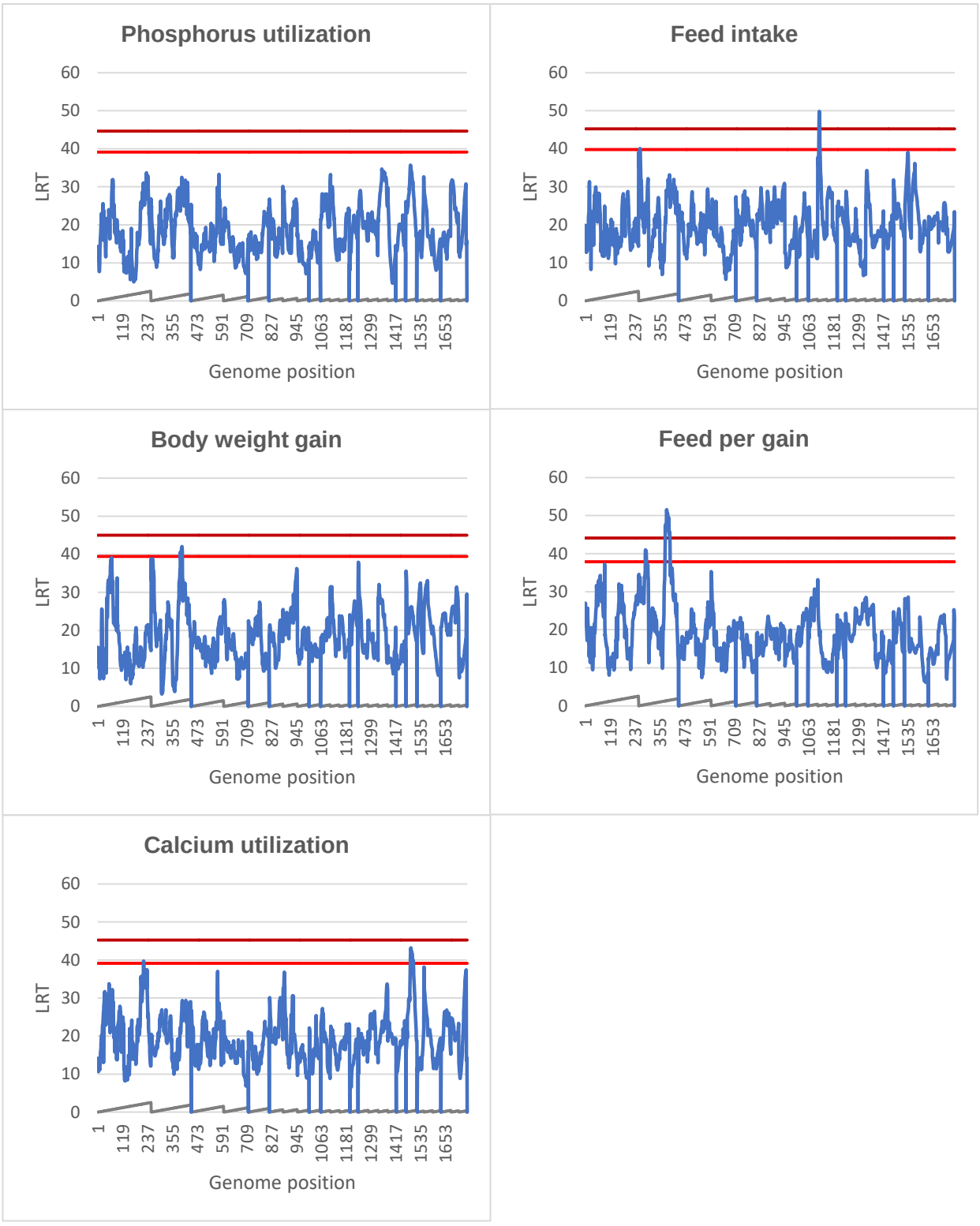

Figure 2 *Plot of a QTL linkage mapping scan of performance traits with likelihood ratio test (LRT) statistics using QTLMap, a software package for outbred half-sib models. The light red and dark red lines correspond to genome-wide significance levels of 5 and 1%, respectively. The gray line at the bottom declines the genetic distances in cM, whereby an increase in the line reflects a chromosome.*

6.2 Intestinal microbiota composition

Understanding the various factors that alter microbiota-driven traits or the microbiota composition of the quail gastrointestinal tract (GIT) may help to improve individualized performance strategies and pre- or probiotic therapeutic feed supplementation. The GIT of Japanese quail harbors a complex and diverse assemblage of microbiota, which varies in each section of the intestine (Su et al. 2014; Wilkinson et al. 2016). In addition to host-related and environmental factors influencing microbiota composition, the choice of analysis method is of great importance for microbial studies.

6.2.1 Technologies for assaying the intestinal microbiota composition

One main consideration is what exactly should be collected from which part of the intestine or feces using which method of analysis. Studies have shown that intestinal sections differ in their microbial community in both quail (Su et al. 2014; Wilkinson et al. 2016) and chicken (Shang et al. 2018; Xiao et al. 2017). This can be explained by the various tasks of the GIT sections. The small intestine is responsible for food digestion and absorption of nutrients and therefore harbors microorganisms that enable enzymatic breakdown of nutrients (Deusch et al. 2015; Gong et al. 2002). We observed this intestinal section, as the phytase activity of feed supplements and bacteria with phytase activity is expected to be high in this section (Dersjant-Li et al. 2015; Palacios et al. 2008). In addition, differences within a section of the intestine can be detected in luminal or mucosal samples of poultry (Awad et al. 2016; Borda-Molina et al. 2016; Stanley et al. 2013a) because the intestinal microbiota near epithelial cells can have slightly different functionality than those in the lumen. These differences in luminal and mucosal content are difficult to detect and are often summarized as digesta since postmortem GIT movement causes mixing of the intestinal contents. To date, microbiota analyses based on digesta samples are common in experimental approaches, even though they have to be collected postmortem. Digesta samples of living animals can be collected from cattle or sheep using intestinal fistulae (Henderson et al. 2013; Martinez-Fernandez et al. 2019), but there is no alternative for experiments with poultry. Another intestinal content sampling method is the collection of fecal samples. Sampling feces is conceivable for practical application and breeding companies as a practical, noninvasive, repeatable, and cost-effective method. Although some microbial taxa living in the small intestine can also be detected in the feces, only a few members are well represented in fecal samples (Yan et al. 2019). Moreover, some studies have shown that fecal samples do not represent the intestinal microbiota of the different sections, such as the mainly observed ceca (Oakley and Kogut 2016; Stanley et al. 2015). Therefore, analyzing fecal samples instead of GIT digesta samples may distort the results, with low reliability. Hence, the most trustworthy analyses are not applicable in practice, and this data collection step needs further research.

A question remains open regarding which method should be used to analyze samples. In recent years, high-throughput next-generation sequencing has been developed and provides large-scale analysis. These technologies enable sequencing of RNA and DNA in a rapid and cost-effective manner.

Basically, four approaches of microbiota analyses are used: DNA, RNA, protein or metabolite analyses. Through DNA analyses, the relative abundances of microbiota species and taxonomic compositions can be collected and identified. Further analyses of microbial proteins through metaproteomics help to understand interactions of microbiota with the host and microbial metabolite analyses approaches through metabolomics to investigate which chemical reactions are responsible for microbial activities. Evaluation on a DNA basis provides an overview of all microorganisms in the intestine, but it does not show which species are active. For this purpose, analysis of 16S ribosomal RNA (rRNA) is a good choice and is therefore one of the most popular gene sequence analyses used to identify associations of microbiota and the host. The 16S rRNA gene of the 30S small subunit of a prokaryotic ribosome contains nine hypervariable regions (V regions), each surrounded by highly conserved regions. By determining the V regions and the sequence variations contained therein, it is possible to determine and assign bacterial taxonomy through comparison (Choi et al. 2015). Different V regions of the 16S rRNA gene have been used to determine the intestinal microbiota composition in poultry studies (Mohd Shaufi et al. 2015; Singh et al. 2012; Stanley et al. 2013a). In particular, because of all the different research methods, comparison of studies is difficult. When recording the microbiota composition, care must be taken to select a representative intestinal segment and microbiota species for the trait of interest; in our case, the small intestine and P utilization.

6.2.2 Factors influencing the intestinal microbiota

The intestinal microbiota is influenced by various factors and some of them are already described in chapter one (1.2.1 Factors influencing the intestinal microbiota). Indeed, the microbiota composition is not static and changes due to host-related and environmental influences, as well as the interaction of microbiota themselves. The host genetics (e.g. Schokker et al. 2015; Wen et al. 2019; Zhao et al. 2013), the age of an animal (e.g. Knarreborg et al. 2002; Lu et al. 2003; Videnska et al. 2014), gender (e.g. Zhao et al. 2013) or metabolic components, including changes in physiological conditions of the intestine, are discussed to have an impact on the intestinal microbiota. Some parts of the host-related influences are shown by genetic-microbial relationships in our studies (chapters five and six), as well as an effect of gender in chapter three. Certain conditions (e.g., pH value, anaerobic) prevail in the GIT, exerting selective pressure on the intestinal microbiota the requirement of specialized microbiota. In essence, microorganisms always compete for nutrients and habitat. The microbial community may also be determined by the type of poultry because layer or meat-type chickens may have physiological differences and needs.

Environmental influence arises from housing or feeding conditions. In order to meet hygiene standards of hatcheries, hatching eggs are often disinfected to eliminate microorganisms of the surface of the eggshell. The disinfection is also used in experimental avian studies, e.g., investigating the influence of gut microbiota on behavior in quail (Kraimi et al. 2018) or to assess the influence of disinfection of

hatching eggs of broilers (Olsen et al. 2017). In addition to the direct vertically transmission of cloacal microflora on the eggshell surface and through the pores, the mother's antibodies may protect against harmful microorganisms already in the egg yolk and thus indirectly influence the microbial composition (Gantois et al. 2009; Grindstaff et al. 2003; Hamal et al. 2006). Pedroso et al. (2005) showed that the small intestine of young chicks already carries a complex microbial community. The surrounding environment of the animals immediately after hatching influences their microbial composition, such as the incubator (Cason et al. 1994). Since chicks have been artificially incubated for commercial benefit, many natural influences such as brooding, nesting or feeding behavior by older animals have been eliminated. Gut microbiota composition is formed predominantly from environmental and human-made influences (Apajalahti et al. 2004; Stanley et al. 2013b). By investigating the housing system, it was shown that among animals living together, pen partners ingest excrement or feather components of others (Meyer et al. 2012), and thus their gut microbiota may adjust to certain degree to that of the others. This aspect was not considered in our study or other studies because the animals were housed individually during the experimental period (chapters four, five and six, e.g., Zhao et al. 2013). Therefore, some alignment of the gut microbiota of individuals is to be expected in practical application and requires practical validation studies. In particular, different studies are difficult to compare due to these variable environmental influences. Well-controlled conditions are needed to capture small effects. All these influencing factors were standardized in our experiment, and cannot be optimized at this magnitude in practice. This also limits transferability to practical implementation. Both host-related and environmental effects of the animal must be considered.

It should be emphasized that microorganisms have constant influence on themselves. The microbiota structure is described by compositional data (discussed in chapter four). The GIT microbiota is in constant competition for resources, e.g. nutrients and habitat (Douglas and Werren 2016). This competition also determines the genetic diversity of the gastrointestinal tract microbiota and thus describes a nongenetic portion of the microbial variance, which is also determined by environmental influences. If the microbial composition changes such that a gap is created, it is immediately occupied and repopulated by competitors. If one OTU is changed, others change automatically as well (Li 2015).

6.2.3 Intestinal microbiota influencing the host and quantitative traits

In addition to the factors influencing the intestinal microbiota, the microbiota may influence the host and its quantitative traits. It is widely known that there is a symbiotic and close interaction between the intestinal microbiota and host (Pan and Yu 2014). Because of the presence of different microbiota species, indigestible feed components can be broken down by microbial enzymes and converted into usable components. The influence of the intestinal microbiota on quantitative traits such as BWG (Meng et al. 2014) or feed conversion (Stanley et al. 2012) in chickens has already been shown and is described more in detail in chapter one (1.2.2 Influence of intestinal microbiota on the host and quantitative traits).

To quantify the effects of the gut microbial community on the desired performance traits, the estimated microbiabilities (m_y^2) describe the proportion of traits that can be explained by the ileum microbiota composition (chapter four). To understand the architecture of a quantitative trait, both the animal's genetics and GIT microbiota, as well as their interaction, are of great interest. The effects of host genetics on these traits can be shown by estimated narrow sense heritabilities (h_y^2) (chapter two). The slightly higher estimates of m_y^2 (except for FI and CaU) compared to h_y^2 suggest that the microbiota composition influences traits in approximately the same way as host genetics. These results confirm the influence of the gut microbiota on quantitative traits. Estimating the heritability of animal microbiota effects (h_k^2) and microorganisms (chapter five) is one step further in terms of a hologenomic breeding scheme. According to the results in chapter five, specific microbiota species can be considered for breeding for a particular trait.

6.2.4 Estimation of OTU effects

Chapter four already addresses the linear estimation of OTU effects. A multi microbiome-wide association study (MWAS) at the OTU level verified that many OTUs had small effects, with some outliers having larger effects. We assumed that trait microbial architectures were poly-microbial determined (chapter four). However, this might also be the case because of the model assumptions. The OTU frequencies were transformed by log transformation and standardized to mean zero, and we assumed homogeneous variance. The normal distribution of OTU effects regressed large OTU effects towards the mean. This linear regression of OTU effects of complex traits has been used in some studies of broilers and pigs (Han et al. 2016; Weishaar et al. 2020, respectively). It is possible that this estimation of OTU effects using linear regression may violate the assumptions of the model. Thus, genera with large effects could not be detected because they were split into many small OTU effects (chapter four).

To reliably estimate OTU effects on quantitative traits, a representative identification of the intestinal microbiota composition is required. Therefore, a higher sequencing depth may increase the probability of identifying rare OTUs (Schloss and Handelsman 2005). At the same time, including false positive OTUs in the analysis might lead to bias, as they will increase as well. We excluded very rare OTUs with relative abundances of <0.001% (chapter four). They were assumed to be rather insignificant, to avoid distortions and to take into account those that occur frequently and thus might be more easily modifiable in breeding terms. The functional importance of rare OTUs may also be high, but whether they have similar functionality to more common OTUs needs to be investigated. Nevertheless, it is always important that all OTUs, even if they occur only in small numbers, be considered to act as a whole community and affect each other.

6.3 Combination of animal microbiota effects and animal genetic effects

This subchapter derives an approach for formulating a mixed linear model with two random effects that are not independent of each other. The following equation is conceivable:

$$\boldsymbol{y} = \boldsymbol{Xb} + \boldsymbol{T_A} + \boldsymbol{T_M} + \boldsymbol{e}\ ,$$

where $\boldsymbol{y}$ is a vector of observations for *n* animals (PU, FI, BWG, F:G, or CaU) and $\boldsymbol{b}$ is the vector of fixed effects and the corresponding design matrix $\boldsymbol{X}$. $\boldsymbol{T_A}$ and $\boldsymbol{T_M}$ denote the animal genetic effects and animal microbiota effects, respectively. Thus far, the following covariance structure has been adopted:

$$\boldsymbol{Cov}\begin{bmatrix}\boldsymbol{T_A}\\ \boldsymbol{T_M}\end{bmatrix} = \begin{bmatrix}\boldsymbol{A\sigma_A^2} & - \\ - & \boldsymbol{M\sigma_M^2}\end{bmatrix}.$$

$\boldsymbol{A}$ describes the genomic relationship matrix following VanRaden (2008), and $\boldsymbol{M}$ the microbial relationship matrix following Camarinha-Silva et al. (2017) in the dimension *n* animal x *n* animal. $\boldsymbol{\sigma_A^2}$ and $\boldsymbol{\sigma_M^2}$ are the genomic and microbial variances, respectively. This incorrectly assumes that they are independent of each other.

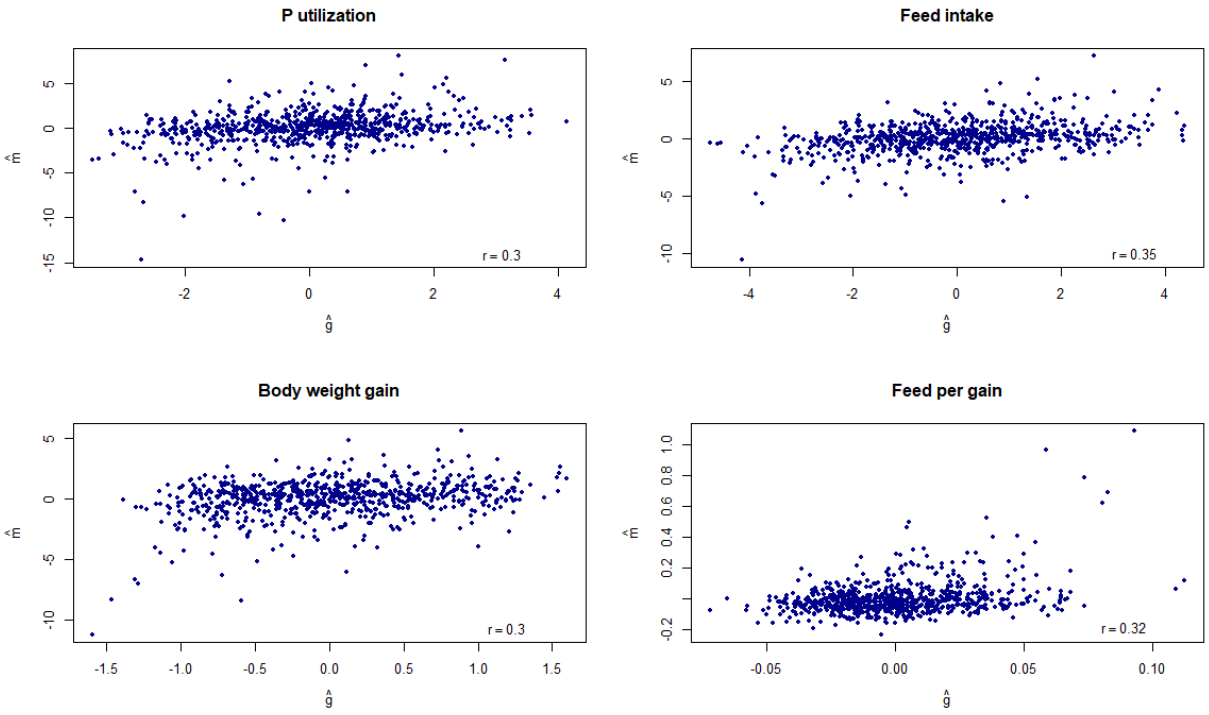

Figure 3 *Scatterplots of animal microbial effects ($\hat{m}$) and animal genetic effects ($\hat{g}$) and their Pearson correlation coefficients (r).*

The animal microbial correlations in chapter four and Figure 3 clearly show that independence cannot be assumed but is rather dependent on both sides. This could lead to the conceivable covariance structure.

$$\boldsymbol{Cov}\begin{bmatrix}\boldsymbol{T_A}\\ \boldsymbol{T_M}\end{bmatrix} = \begin{bmatrix}\boldsymbol{A\sigma_A^2} & \boldsymbol{V\sigma_{A,M}}\\ \boldsymbol{V\sigma_{A,M}} & \boldsymbol{M\sigma_M^2}\end{bmatrix}.$$

$\boldsymbol{\sigma_{A,M}}$ denotes the covariance of both random effects. Here, it is assumed that $\boldsymbol{T_A}$ and $\boldsymbol{T_M}$ have a common scattering of variance. To date, no previous study has taken this into account. However, these random animal effects are not independent (regarding the mentioned correlations), and because it is known that the genetics of an animal also influences the gastrointestinal microbiota composition, $\boldsymbol{\sigma_{A,M}}$ should be considered. Regardless, there is no solution for $\boldsymbol{V}$ in the literature, and we have yet to resolve this issue.

6.4 Transferring results in practical breeding programs

After many theoretical and technical discussions, one question remains unanswered: How can the findings be translated into practical animal breeding? To breed quail or poultry for efficient and sustainable P utilization, sophisticated breeding programs are necessary and sensible. Breeding for a new trait requires comprehensive knowledge about it. PU is an extremely hard-to-measure trait, and QTL mapping in chapter two revealed that PU and related traits are polygenic in nature and thus represent typical quantitative traits. It is conceivable that the microbiota composition can be used as an explanatory variable to predict PU and production traits, which is confirmed by our findings in chapters four and five. We found some significant QTL and many QTL with very small effects that were not significant (chapter two). The overlapping confidence intervals of these QTL peaks between the traits confirmed the genetic correlations between the same (Künzel et al. 2019) and the MWAS in chapter four. Many microbiota species significantly influenced the same multiple traits (chapter four and five). Thus, we have heritable, microbiota-mediated traits that can possibly be used as explanatory variables. That possibility of innovative breeding schemes for PU is shown by the heritabilities of Beck et al. (2014) (shown in chapter two). In addition, it can be concluded from the results that both host genetics and the ileum microbiota composition have approximately equal influences on PU.

6.4.1 Challenging standardization of phenotyping

Targeted breeding for trait P utilization requires phenotyping of animals and is a great challenge in the processing of a trait. A larger number of phenotyped animals is desirable to improve the estimation of genetic and microbial parameters. For practical application, it must be possible to collect data for many animals in a manner as stress-free and noninvasive as possible (Brito et al. 2020). According to this, single-cage housing, as used in our experiment, or digesta and mucosa samples from euthanized animals is not appropriate. Such approaches are not in conformity with animal protection and welfare and are be

acceptable to the consumer. This leads to the fact that even auxiliary traits, such as bone ash traits (Künzel et al. 2019), are not a useful tool to measure the P content and thus draw conclusions on P utilization.

Stanley et al. (2015) showed that a large proportion of microbiota species from cecal and fecal samples are consistent. One main advantage of fecal collection in practical use, as opposed to digesta sampling, is its repeatability. Digesta samples can only be raised once. Despite the fact that fecal samples may be a useful tool for research questions concerning ceca or large intestine segments, they do not represent the intestinal microbiota composition of the upper GIT segments (see also chapter 6.2.1), such as the small intestine (Sekelja et al. 2012; Stanley et al. 2014). Therefore, traits should be recorded by individual feed intake (Inra et al. 2019), feed samples, growth observations and body analyses, or microbiota profiling in feces if possible. In addition, large numbers of animals will be needed to reliably identify the host genome-microbiota interactions.

The age of the animal at phenotyping may also be important. Our quails were tested at a very young growth stage (day 10 to day 15 of life) when they were not yet ready to lay eggs. Young avian animals need a high level of minerals, such as P and Ca, because of bone formation and growth (Nelson 1967; Shim et al. 2012). Older animals mobilize minerals stored in the body for the formation of eggshells. The developmental stage of the individual GIT and the microbiota contained therein is also of interest because it has been shown that both change with age (Stanley et al. 2013a; Tilocca et al. 2016; Yeoman et al. 2012). Therefore, to obtain representative data, young animals should be examined.

6.4.2 Integration of microbiota data in selection schemes

Some studies have shown a conceivable selection of a hologenomic approach for ruminants or pigs (Ross et al. 2013; Weishaar et al. 2020) to consider both the host genome and GIT microbiota metagenome, namely the hologenome (Bordenstein and Theis 2015). For the selection approach, the relationship between the host genome and microbiota composition is crucial. At the same time, the development of a breeding strategy that follows the hologenomic approach is controversially discussed and has potential weaknesses (Douglas and Werren 2016; Moran and Sloan 2015; Stencel and Wloch-Salamon 2018). Estellé (2019) described the acquisition of the microbiota through vertical (between generations; directly from the parent animal) and horizontal (within populations; uptake of new microbiota from the environment at each host generation) transmission. Vertical transmission was confirmed by the results of our studies through the heritabilities of microbiota composition in chapter five. In contrast, Douglas and Werren (2016) argue that vertically transferred organisms can be distorted on the maternal side (e.g., by the birth process, as already described in 6.2.3) and that possible genetic interpretations can be biased by this. Horizontal transfer within populations is discussed to reduce the impact of heritable microbiota (van Vliet and Doebeli 2019). Moreover, environmental microbiota influences and horizontal transfer are reduced by standardized test conditions but may play a larger role in practical breeding and negatively affect hologenomic selection (van Vliet and Doebeli 2019). The

current idea of selection pursues the goal of increasing biological performance, improving efficiency, and considering health aspects. The intestinal microbiota composition is not yet considered in this selection and requires further research.

In addition to the critically discussed aspects, the hologenomic approach is intended to improve animal husbandry and food production. It seems to be beneficial and conceivable for traits with strong interactions with microorganisms, such as efficiency traits. This especially includes feed-related traits (Maltecca et al. 2020; Weishaar et al. 2020), as well as our chosen trait PU and related traits in our studies (chapters four, five, and six). According to the results of chapter four, a hologenomic approach is particularly useful because these traits are poly-microbial determined and share the same microbial architecture. With this approach several traits can be influenced simultaneously. To date, only correlations between phenotype and intestinal microbiota data have often been determined to show these relationships.

Some technical and sampling extraction issues still require research. For practical applications, better considerations of the environment should be applied. Our studies showed an effect of the test day (chapters three, four and five). In addition, animals to be tested should be compared to a reference population from the same housing, to avoid bias. The same applies to the survey of OTU frequencies, as already discussed in chapter 6.2.4., as well as the choice of a representative intestinal segment and type of sample (chapter 6.2.1.). For more accurate results, larger data sets with more complex procedures might be helpful (Shi et al. 2016).

6.4.3 Conceivable selection program

As described above, a hologenomic selection scheme is a conceivable approach. Other approaches, such as genome editing, may not be the solution for complex traits such as PU because the complexity of the traits is rarely understood with their interactions with genes, physiology and environmental influences (Tizard et al. 2019).

A first two-step hologenomic breeding selection approach was published by Weishaar et al. (2020). It can be applied based on the genomic selection model (Meuwissen et al. 2001; Meuwissen et al. 2016). The animals of the reference population need to be phenotyped for the trait of interest, genotyped using SNP chips, and characterized for their microbial composition for the GIT section of interest. First, the estimation of OTU and microbial animal effects has to be performed using a microbial mixed linear model (as model one in chapter four). Second, the estimation of genetic effects (SNP effects) for estimated animal microbiota effects (as model four in chapter five) has to be performed using a genomic mixed linear model. These results combined with genomic estimated breeding values (EBVs) can lead to an estimation of genomic-microbial breeding values for potential selection candidates. Consequently, a classical EBV and a microbial EBV are obtained for one trait. A hologenomic selection index can be created by combining these breeding values with some weighting factors (Weishaar et al.

2020). The selection candidates can be used as parents for the next generation. As a result of selection, these candidates have a GIT microbiome composition optimized for the trait of interest.

Before applying this method, important aspects still need to be considered and clarified in further studies. Applications such as MWAS (chapter four) are analogous to genomic models and require large data sets. Few methods provide the possibility for smaller data sets, such as differential abundance analyses (Li 2015), and the size of the reference population should be clarified to estimate reliable OTU effects.

6.5 Conclusion

The results provided in this thesis prove that the hypotheses can be confirmed. The outcome of this research suggests that, in addition to host genetics, the ileum microbiota composition have an approximately equal contribution to PU variability. Both the trait PU itself and the microbial composition are heritable traits that can be altered through breeding, which leads to the conclusion that the knowledge presented in this work may be the beginning of a hologenomic selection approach. It is conceivable that, in the future, we may be able to effectively capture genetic and microbial variability through breeding. Although this selection idea alone is unlikely to ensure more sustainable use of resources, this research provides a starting point by analyzing adapted animal breeding for more efficient and sustainable use of P and for more sustainable resource utilization.

This thesis also provides practical implications; however, before the hologenomic selection idea may be applied in practice, some questions must be clarified through further studies. The results of this thesis and further studies must be transferred from Japanese quail to poultry species in order to verify and apply this knowledge to the actual poultry species used in food production. Some studies have shown that the genomes of these two species are quite similar (Recoquillay et al. 2015; Sasazaki et al. 2006; Shibusawa et al. 2001); therefore, upon transferring our results, the similarities should be examined. Other questions include: How will the phenotypic traits and GIT microbiota samples be standardized in practice? Should other environmental interactions be considered, in addition to the genotype-microbiota interaction, as envisioned by Estellé (2019)? How will breeding companies be able to deal with a future increase in data? Will breeding and production companies agree on different ways to save resources?

I opened this thesis by noting that, as the global population continues to grow, production of essential food will become an ever greater challenge. Overall, one aspect of food production should always be on sustainable use of resources. If something cannot be continued forever as it is currently used, it is not sustainable. We will need to improve recycling and the use of P in any form in order to continue to produce food and live in the future.

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