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Fecal microbiota transplant from highly feed efficient donors shows little effects on age-related changes in feed efficiency-associated fecal microbiota in chickens

Running title: Fecal transplant and feed efficiency

Sina-Catherine Siegerstetter[a], Renée M. Petri[a], Elizabeth Magowan[b], Peadar G. Lawlor[c], Qendrim Zebeli[a], Niamh E. O´Connell[d], Barbara U. Metzler-Zebeli[a]

[a]Institute of Animal Nutrition and Functional Plant Compounds, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine Vienna, Vienna, Austria.

[b]Agri-Food and Biosciences Institute, Agriculture Branch, Hillsborough, Northern Ireland, UK.

[c]Teagasc, Pig Development Department, Animal & Grassland Research & Innovation Centre, Moorepark, Ireland.

[d]Institute for Global Food Security, Queen´s University Belfast, Northern Ireland, UK.

Address correspondence to Barbara U. Metzler-Zebeli, barbara.metzler@vetmeduni.ac.

ABSTRACT

Chickens of good and poor feed efficiency (FE) have been shown to differ in their intestinal microbiota composition. This study investigated differences in the fecal bacterial community of good and poor feed efficient chickens at 16 and 29 days post-hatch (dph) and evaluated whether a fecal microbiota transplant (FMT) from feed efficient donors early in life can affect the fecal microbiota in chickens at 16 and 29 dph, chicken's FE and nutrient retention at four weeks of age. One-hundred-ten chickens were inoculated with a FMT or a control transplant (CT) on 1, 6 and 9 dph and ranked according to residual feed intake (RFI; metric for FE) on 30 dph. Fifty-six chickens across both inoculation groups were selected as the extremes in RFI (29 low, 27 high). RFI-related fecal bacterial profiles were discernible at 16 and 29 dph. Particularly, *Lactobacillus salivarius*-, *Lactobacillus crispatus*- and *Anaerobacterium*-operational taxonomic units were associated with low RFI (good FE). Multiple administration of the FMT only slightly changed the fecal bacterial composition, which was supported by weighted UniFrac analysis, showing similar bacterial communities in feces of both inoculation groups at 16 and 29 dph. Moreover, the FMT did not change RFI and nutrient retention of good and poor feed efficient recipients, whereas it tended to increase feed intake and body weight gain in female chickens. This may suggest that host- and environment-related factors may more strongly affect chicken's fecal microbiota and FE than the FMT.

IMPORTANCE

Modulating the chicken's early microbial colonization using a FMT from highly feed efficient donor chickens may be a promising tool to establish a more desirable bacterial profile in recipient chickens, thereby improving host FE. Although FE-associated fecal bacterial profiles at 16 and 29 dph could be established, the microbiota composition of a FMT, when administrated early in life, may not be a strong factor modulating the fecal microbiota at two

to four weeks of life and reducing the variation in chicken's FE. Nevertheless, the present FMT may have potential benefits on growth performance in female chickens.

KEYWORDS: fecal microbiota transplant, chicken, fecal microbiota, nutrient retention, feed efficiency

INTRODUCTION

The gastrointestinal tract of chickens harbors complex microbial communities, with the highest bacterial diversity found in the ceca (1). The ceca are dominated by strictly-anaerobic bacteria, mainly *Ruminococcaceae* and *Lachnospiraceae* (1, 2), while in the crop, small intestine and feces *Lactobacillus* is highly abundant (1, 3). The establishment of the chicken's intestinal microbiota begins immediately post-hatch and is influenced by internal (e.g., host genetics) and external factors (e.g., diet and encountered environmental microbes) (4). The development of a mature microbial community mostly occurs until three weeks of age (1). Maturational changes within the gastrointestinal microbiota significantly impact host's phenotype by modulating the development of the digestive tract, secretion of bile acids and digestive enzymes, which influences nutrient digestion and absorption and consequently may affect growth performance and feed efficiency (FE) (5). Moreover, the commensal intestinal microbiota play important roles in stimulating intestinal immune functions and in colonization resistance against pathogens (4, 5).

Although different FE-associated intestinal microbiota have been reported for chickens, the identification of target bacteria proves to be challenging, as bacteria associated with good FE vary greatly both within and between studies (6, 7, 8). Moreover, many of the FE-associated phylotypes failed to be classified at the genus or species level, rendering it difficult to cultivate or nutritionally target those bacteria (7, 8). Also, the complex interactions with the microbial communities cannot be considered by administrating single-strain inocula. In human medicine, complex fecal microbiota transplants (FMT) have been effectively used to treat severe intestinal dysfunctions such as recurrent *Clostridium difficile* infections (9, 10). Similarly, in chickens, administration of the fecal microbiota from healthy adults has been used to transfer colonization resistance against *Salmonella* to newly-hatched chickens (11). Inoculating the surface of incubating eggs with cecal contents from high or low feed efficient donor chickens has been shown to reduce bird-to-bird variation in microbiota composition,

but did not impact FE in the growing chicks (12). By contrast, previous work in mice colonized with human microbiota indicated that a FMT can induce obesity (13) and hence may modify the FE of the host. Whether a FMT may be more efficacious to improve chicken's FE has not been sufficiently investigated so far. As differences in the fecal microbiota between low and high feed efficient chickens exist (6, 14), we hypothesized that excreta collected from highly feed efficient chickens may influence chicken's early microbial colonization and subsequently improve FE.

Because improvements in chicken FE can reduce nutrient excretion (15), we further hypothesized that, if the FMT can improve chicken FE, this should be associated with enhanced nutrient retention and reduced excretion of environmental pollutants. The objectives of the present study were to investigate 1) the FE-associated bacterial profiles in feces of chickens at 16 and 29 days post-hatch (dph), 2) the effect of administrating a FMT from highly feed efficient donors early in life on the fecal microbiota of good and poor FE chickens at 16 and 29 dph, and 3) whether the FMT could modify chicken's FE and nutrient retention at slaughter age. To assess whether the fecal microbiota generally impacted host FE and physiological traits, correlations between fecal bacterial abundances and performance traits and excreta characteristics were calculated.

RESULTS

Performance, excreta characteristics and nutrient retention. The effects of residual feed intake (RFI) rank and inoculation treatment on FE and performance traits between 9 and 30 dph, combined for both sexes and separately for females and males, are summarized in Table 1. Across sexes, the RFI was 289g lower in low (good feed efficient) compared to high (poor feed efficient) RFI chickens ($P < 0.001$). Furthermore, chickens with a low RFI showed a 278.5g lower total feed intake (TFI) compared to their high RFI counterparts ($P < 0.001$). Male chickens ate more feed, were heavier ($P < 0.001$) and showed a better FE ($P < 0.05$)

than female chickens. The FMT had no effect on RFI in both sexes and, in males, on TFI and total body weight gain (TBWG) ($P > 0.10$). However, in females, the FMT tended to increase TFI and TBWG by 147 and 82g, respectively, compared to the control transplant (CT) ($P < 0.10$).

Excreta characteristics and nutrient retention data across sexes are presented according to RFI rank and inoculation treatment in Table 2. Chickens with a low RFI had a 4.15 and 7.05% higher protein ($P < 0.05$) and P ($P < 0.10$) retention, respectively, compared to their high RFI counterparts. The FMT did not affect nutrient retention and excreta characteristics across RFI ranks ($P > 0.10$). However, a FMT × RFI interaction ($P < 0.05$) indicated that the FMT compared to the CT increased the dry matter (DM) content in excreta of low RFI chickens, but not in high RFI chickens.

16S rRNA sequencing metrics. After quality control and chimera check, a total of 3,266,165 sequencing reads with a mean of 25,516 (SD ± 7,616) sequences per sample were obtained for the 112 fecal (FMT chickens, $n = 28$/time point; and CT chickens, $n = 28$/time point), 8 FMT inoculum, 6 diet and 2 water samples. Rarefaction curves, using a maximum rarefaction depth of 10,000 sequences and the Observed operational taxonomic units (OTUs) index, are presented in Fig. S1.

Bacterial composition of the FMT, diet and water samples. The FMT comprised mainly the phyla *Proteobacteria* (77.7%) and *Firmicutes* (22.1%, Fig. 1a), whereby an *Escherichia*/*Shigella*-OTU (OTU1; 71.7%) and a *Turicibacter*-OTU (OTU3; 16.2%) dominated (Fig. 1b). The bacteria found in diet and water samples are presented at the phylum and OTU level in Tables S1 and S2. Bacterial communities of diets and feces formed separate clusters as indicated by weighted UniFrac β-diversity analysis (see Fig. S2).

Structure and time-related shifts in the fecal bacterial community. The overall bacterial community structure was similar between sexes as indicated by β-diversity analysis (Fig. 2a). At the phylum level, *Proteobacteria* (50.8 %) and *Firmicutes* (48.6%) clearly

129 dominated in chicken's feces across inoculation treatment groups, RFI ranks and time points,
130 with all other phyla showing much lower abundance (<0.5%; Fig. 3a). At the genus level, an
131 unclassified *Enterobacteriaceae* genus was dominant (48.8%), followed by *Lactobacillus*
132 (18.4%), an unclassified *Clostridiales* genus (12.2%), *Turicibacter* (6.5%), an unclassified
133 *Ruminococcaceae* genus (5.7%), *Acinetobacter* (1.1%) and *Ruminococcus* (1.0%), while all
134 other genera showed abundances <1.0% (Fig. 3b). Comparisons of α-diversity and the
135 microbiota composition at all taxonomic levels (i.e., phylum, family, genus and OTU level)
136 indicated maturational changes in the fecal community from 16 to 29 dph (Fig. 3, Tables 3
137 and 4, Table S3). Beta-diversity analysis using weighted UniFrac distances, in turn, did not
138 show differences in the overall bacterial community structure between the two sampling time
139 points (Fig. 2b). Alpha-diversity indices based on species richness and evenness (Shannon
140 and Simpson) tended to decrease from 16 to 29 dph ($P < 0.10$; Table 3). This was
141 accompanied by a decrease in the fecal abundance of highly abundant *Firmicutes* and low
142 abundant *Actinobacteria*, while highly abundant *Proteobacteria* increased from 16 to 29 dph
143 ($P < 0.05$; Fig. 3a). Moreover, time point-related effects existed within the high abundant
144 *Lactobacillus* genus and for two *Eubacterium*-OTUs (OTU4 and OTU15), which were 2.7-,
145 3.2- and 3.1-fold more abundant at 16 dph than at 29 dph ($P < 0.05$; Fig. 3b, Table S3). In
146 contrast, highly abundant *Escherichia*/*Shigella*-OTUs (OTU1 and OTU6) and *Turicibacter*-
147 OTUs (OTU3 and OTU22) increased by 1.3-, 1.4-, 11.5- and 13.1-fold from 16 to 29 dph,
148 respectively ($P < 0.05$).

149 **RFI-associated differences in the fecal microbiota.** Weighted UniFrac-based distances
150 showed no difference in the fecal microbiota composition between RFI ranks (Fig. 2c).
151 Compositional differences in the fecal bacterial abundances between low and high RFI
152 chickens at the phylum, family and genus level were hardly detectable (Fig. 3, Table 4).
153 However, *Enterobacteriaceae* and within this family an unclassified genus tended to be 1.2-
154 fold less abundant in low compared to high RFI chickens ($P < 0.10$). At the OTU level, low

RFI was significantly associated with increased abundance of four *Lactobacillus*-OTUs, with the closest reference strains being *Lactobacillus salivarius* (OTU47 and OTU43) and *Lactobacillus crispatus* (OTU51 and OTU67), and *Anaerobacterium*-OTU81 ($P \leq 0.05$; see Tables S3 and S4). Furthermore, six other *Lactobacillus crispatus*-OTUs (OTU5, OTU8, OTU21, OTU30, OTU42 and OTU98) tended to be increased, while *Klebsiella*-OTU18 tended to be decreased in low compared to high RFI chickens ($P < 0.10$).

FMT-related microbiota shifts. Weighted UniFrac distances indicated high similarities in the structure of fecal bacterial communities between treatment groups and the FMT (Fig. 2d). The high-abundant *Escherichia*/*Shigella*-OTU1 in the FMT also dominated in chicken's feces at 16 (38.6%) and 29 dph (50.1%), but did not differ between the two inoculation groups ($P > 0.10$; see Table S3). Likewise, the second most abundant OTU in the FMT (*Turicibacter*-OTU3) was predominant in feces across time points (0.83% at 16 dph and 9.6% at 29 dph), but again was equally abundant between both inoculation groups. Administration of the FMT decreased the abundance of one *Anaerobacterium*-OTU (OTU56) and *Klebsiella*-OTU18 by 2.8- and 2.6-fold ($P < 0.05$). Moreover, the FMT tended ($P < 0.10$) to decrease the abundances of five *Anerobacterium*-OTUs (OTU16, OTU28, OTU58, OTU119 and OTU130) and one *Acetivibrio*-OTU (OTU80) compared to the CT, while one *Comamonas*-OTU (OTU113) tended ($P < 0.10$) to be more abundant in the FMT compared to the CT chicken group. Likewise, the FMT tended ($P < 0.10$) to increase *Comamonas* at the genus level (Fig. 3b). At the phylum level, low abundant *Actinobacteria* tended to be less abundant in the FMT compared to the CT chicken group ($P < 0.10$; Fig. 3a).

Correlations between fecal bacterial abundances at 29 dph and RFI, TBWG, TFI and excreta characteristics. Multiple fecal bacterial associations with FE, growth performance and excreta characteristics were observed across treatment groups (Fig. 4). Only positive correlations between OTU abundances at 29 dph and RFI existed ($P < 0.05$). Accordingly, three OTUs positively correlated with high RFI; two *Gracilibacter*-OTUs

(OTU50; r = 0.33 and OTU88; r = 0.34) and one *Clostridium*-OTU (OTU39; r = 0.38). One *Anaerobacterium*-OTU (OTU29) negatively correlated with TFI (r = -0.37; $P < 0.05$). *Anaerobacterium*-OTU29 further negatively correlated with TBWG (r = -0.36) as well as *Hespellia*-OTU64 (r = -0.33; $P < 0.05$). Furthermore, two *Lactobacillus*-OTUs were negatively correlated with fecal pH (OTU7; r = -0.36 and OTU46; r = -0.35; $P < 0.05$). *Negativibacillus*-OTU70 positively correlated with fecal DM (r = 0.33; $P < 0.05$). Furthermore, three *Turicibacter*-OTUs (OTU3; r = 0.36, OTU22; r = 0.36 and OTU44; r = 0.38) positively correlated with fecal NH_3 ($P < 0.05$).

DISCUSSION

The present study investigated differences in the fecal bacterial community in chickens divergent for FE and evaluated whether the application of a FMT in the first week post-hatch would modify the fecal microbiota and promote FE and nutrient retention in recipient chickens. Differences in the fecal bacterial abundances at 16 and 29 dph and Pearson's correlations supported that especially *Lactobacillus* and *Anaerobacterium* species could be used as indicators for low RFI in the present chicken population. Moreover, results support that low RFI chickens more efficiently used the dietary protein than high RFI chickens (15). However, the FMT from highly feed efficient donor chickens led only to few compositional changes in the fecal microbiota of recipient chickens and had little effects on RFI-associated bacterial abundances at 16 and 29 dph. Also, the current microbiota profile used as FMT did not improve chicken's RFI or nutrient utilization at four weeks of age, indicating, together with the bacterial data, that other probably host- and environment (e.g., diet)-related factors more strongly affected chicken's microbiota maturation and FE than the application of an external microbial inoculum within the first days of life (16). By contrast, the FMT effectively reduced the time taken to reach slaughter weight in female chickens, as it tended to stimulate feed intake and as a consequence BW gain in females, which may be

associated with differences in microbe-host signaling between females receiving the FMT and those receiving the CT.

In contrast to our previous study using the same chicken line, housing and dietary formulation (17) different FE-associated bacterial taxa were found in the present study. This may be partly ascribed to maturational changes in the intestinal microbiota, as the age of the chickens at fecal sampling between the two studies differed, with the current chickens being six days younger than in our previous experiment (17). Maturational changes also tended to decrease the diversity of the fecal bacterial community from 16 to 29 dph in the present study, which was in contrast to previous findings for the fecal bacterial community in chickens 7, 21 and 42 dph (18). Similar to the maturational changes reported by Ranjitkar et al. (1) for the chicken cecum at 15 and 29 dph, *Lactobacillus* was more abundant in feces at 16 dph compared to 29 dph in the present study. As the chicken aged, we observed an increase in *Turicibacter* and *Escherichia/Shigella* phylotypes in feces, which was contrary to previous findings for the cecum (1, 19) and may be related to the intestinal site and differences in the succession of diets fed to the chickens during the respective experiment.

Despite the age-related changes in the fecal bacterial composition, similar RFI-associated bacteria could be identified at 16 and 29 dph. In particular, mainly *L. salivarius*-OTUs and *L. crispatus*-OTUs were associated with low RFI and thus improved FE. Both *L. salivarius* and *L. crispatus* are resistant to bile salts and acidic pH, inhibit the growth of potential pathogens such as *Escherichia coli* and *Salmonella enteritidis* and have immunomodulatory properties (20, 21). Moreover, *L. salivarius* and *Lactobacillus agilis* may enhance intestinal butyrate production, which may be beneficial for intestinal health via cross-feeding, as shown for an *in vitro* simulated chicken cecum (22). Nevertheless, inconsistent findings were reported for the association between lactobacilli and FE in chickens (7, 8, 23, 24), which may be due to particularities in experimental design, microbiota composition, diet and the intestinal site investigated. When correlating individual RFI values with bacterial

abundances in feces at 29 dph, results indicated that RFI-associations existed with other low abundant phylotypes (relative abundance 0.09 to 0.18% at 29 dph). These phylotypes were classified as one *Clostridium saccharolyticum*- and two *Gracilibacter*-OTUs using the NCBI database. *Clostridium saccharolyticium* has been shown to possess saccharolytic activities (25). An association of this taxa with poor RFI might be therefore indicative for enhanced intestinal fermentation (4) in high RFI chickens, which may reduce the nutrient availability for the host. This may also explain the positive relationships between RFI and the fecal abundance of proteolytic *Gracilibacter* (26). However, the fecal bacterial community is determined by the microbiota originating from different intestinal sections (27); therefore, caution should be taken when using the microbiota composition in feces to infer the microbiota in other intestinal segments (28). It was probably the complex interplay between all these taxa together that influenced chicken's intestinal homeostasis and FE. However, it cannot be ignored that the driving force behind these differences in taxa abundances may have been the higher feed intake in high RFI chickens, thereby altering the intestinal substrate availability with consequences for host-microbiota interactions and chicken's FE.

The current FMT represented the fecal microbial community of highly feed efficient chickens, with *Escherichia*/*Shigella* and *Turicibacter* being the two most abundant OTUs in the FMT. These phylotypes were previously associated with improved FE in chickens (14, 17). Therefore, the present FMT may have been an appropriate inoculum to influence chicken's early microbial colonization and concurrently bird's FE. Although some bacterial losses due to the FMT preparation steps likely occurred and low pH in the gizzard may have further decreased bacterial numbers of particular taxa (1), weighted UniFrac distance supported that the bacterial community in the feces of FMT chickens at 16 and 29 dph closely resembled the bacterial community in the FMT inoculum. Nevertheless, the current microbiota profile in the FMT was not efficacious to strongly modify the fecal bacterial abundances at 16 and 29 dph, chicken's RFI or nutrient retention in low and high RFI

recipient chickens. Accordingly, inoculating incubating eggs with cecal contents from good feed efficient donors did not transfer the dominant bacterial population from donors to the ceca of recipient birds as well as did not improve chicken's FE (12).

Considering that the FMT was prepared from feces of 30 dph-old chickens, it is conceivable that not all bacteria from the more mature FMT inoculum may have been able to successfully colonize the intestinal environment during the first week of life, thereby indicating a potential mismatch between donor and recipient birds. Due to the high dynamics in the re-organization of the intestinal bacterial community with time, normal maturational processes within the chicken microbiota may have been therefore more influential in shaping host's intestinal microbial community than the FMT (12). Especially, the diet is a major factor shaping the intestinal microbiota (29) and all chickens received the same starter, grower and finisher diets. Therefore, the equal fecal abundance of the predominant bacterial taxa in the FMT, *Escherichia/Shigella* and *Turicibacter,* between chickens of the FMT and CT groups may be explained by the fact that the diet and possibly chicken's feed intake more strongly affected their fecal abundances in the grower-finisher period than the administration of the FMT early in life. Moreover, as fecal samples were first collected at 16 dph, while the FMT was administered only during the first days post-hatch, it might be possible that effects of the FMT on early bacterial colonization were missed, as they may have disappeared until 16 dph. This may be supported by the trend of the higher abundance of the early colonizer *Comamonas* in FMT chickens compared to CT chickens, which was evident at 16 dph, but not any longer at 29 dph. However, we cannot draw any conclusion whether the influence of the FMT on bacterial colonization may have been more apparent and more permanent in other intestinal segments. Nonetheless, the FMT application consistently decreased the fecal abundance of several cellulolytic *Anaerobacterium* species within the *Ruminococcaceae* family (30) over the two sampling time points, thereby supporting that a certain effect of the

FMT application on the intestinal community was measurable in feces at two and four weeks post-application.

Although sex-related differences in fecal microbiota profiles (17, 31) were small in the present study, differences in intestinal microbe-host signaling between males and females associated with the FMT may have mediated the higher TFI and TBWG of females in the present study. The intestinal microbiota has been reported to alter the feeding behavior of the host via fermentation metabolites (e.g., short chain fatty acids), production of toxins, receptor recognition and stimulation of the vagus nerve, thereby affecting the secretion of satiety-regulating hormones and controlling satiety and feed intake (32, 33).

In conclusion, this study demonstrated RFI-related differences in the fecal bacterial community in chickens at 16 and 29 dph, with mainly *L. salivarius* and *L. crispatus*-OTUs being indicative for good FE. However, multiple applications of a FMT within the first 9 dph only slightly modified the fecal bacterial community in recipient chickens, and were not successful to improve the RFI and nutrient retention in chickens. This indicates that other probably host- and environment-related factors were more important for chicken's fecal microbiota composition at 16 and 29 dph and variation in RFI than the administration of a FMT early in chicken's life

MATERIALS AND METHODS

Ethical approval. This study was conducted at the Institute of Animal Nutrition and Functional Plant Compounds (University of Veterinary Medicine Vienna, Austria). The animal procedures were approved by the institutional ethics committee of the University of Veterinary Medicine Vienna and the Austrian national authority according to paragraph 26 of Law for Animal Experiments, Tierversuchsgesetz 2012 – TVG 2012 (GZ 68.205/0131-II/3b/2013).

Animals and diets. A total of 110 day-old Cobb 500 broiler chicks of both sexes were used in two replicate batches (batch 1, n = 54; and batch 2, n = 56). One more female and one more male were used in batch 2 compared to batch 1. From 1 to 8 dph, chicks of the same sex were group-housed (n = 5-6 chicks/cage). On 9 dph until the end of the experiment (30 dph), chickens were randomly allocated to individual cages to determine their individual feed intake. Housing and environmental conditions were previously described (15). Chickens were housed in stainless steel metabolic cages throughout the study, with flooring made of wire mash (10 mm × 10 mm) and padded with rubber tubing. A tray was put under each cage and was laid out with parchment paper to facilitate excreta collection. Each cage was equipped with one manual feeder and drinker. All chickens had *ad libitum* access to starter (1 to 8 dph), grower (9 to 20 dph) and finisher (21 to 30 dph) corn-soybean meal based diets and demineralized water. The detailed dietary ingredient and chemical composition can be found in Table S5. Diets were free of antimicrobials and coccidiostats. Fresh feed was provided at 9:00h every morning and feeders were re-filled with feed at 15:00h to ensure *ad libitum* access to feed.

Inoculation and preparation of the FMT. Immediately upon arrival before having access to feed and water and on 6 and 9 dph, chickens were either inoculated with 100µl of the FMT [10^4 colony forming units (CFU)] or a CT [(sterile phosphate buffered saline (PBS)]. Chickens housed together received the same transplant. The transplant was orally administrated at the back of the tongue and chicks were supervised that they swallowed. On 6 and 9 dph, feed was withheld for 15min before and after the administration.

For preparation of the FMT, freshly dropped excreta from low RFI (good FE) chickens (females, n = 4; and males, n = 2) were aseptically collected on 30 dph in a previous chicken experiment. Fecal droppings were immediately processed per bird under anaerobic conditions and were kept on ice throughout the procedure. The white portion of the excreta mainly comprising uric acid was removed. Twice the amount of PBS was added and the mixture was

thoroughly homogenized. To separate undigested feed and particulate material from the microbial fraction, the slurry was centrifuged at low speed (800 × g for 3min at 4°C; Eppendorf Centrifuge 5810 R, Eppendorf, Hamburg, Germany). To ensure microbial survival during storage (-80°C), the supernatant from each chicken was mixed with sterile glycerol (10% vol) and kept on ice for 60min to allow the glycerol to penetrate the bacterial cells. The fecal suspension from each chicken was then aliquoted to avoid multiple thawing and freezing cycles for the single inoculations. On the inoculation days, one aliquot of the fecal suspension from each low RFI female and male chicken was thawed on ice and equal volumes of the single suspensions were combined and homogenized to form the FMT stock.

Anaerobic and aerobic culturing and quantitative PCR (qPCR) was used to estimate the bacterial numbers in the prepared FMT stock before the start of the chicken experiment. Analysis with qPCR was also used to verify the administrated bacterial gene copies on each inoculation day. For aerobic and anaerobic cultivation, a 1:10 dilution series of the FMT stock using Ringer's solution (Fresenius Kabi, Graz, Austria) was prepared and plated onto Tryptic soy agar plates (TSA). Plates were either aerobically or anaerobically incubated in an anaerobic jar (Oxoid, Wesel, Germany) containing one sachet of anaerobic atmosphere generator (bioMérieux, Marcy l'Etoile, France) for 48h at 37°C. The DNA extraction and qPCR amplification protocol are described below. The FMT stock contained 7.28×10^7 CFU of culturable aerobic and anaerobic bacteria and 8.4 $\log_{10}$ total bacterial 16S rRNA gene copies per milliliter. For administration, a 1:100 dilution of the FMT stock was prepared using PBS. All female and male chickens in the FMT group were inoculated with the same FMT dilution.

Determination of FE. For the calculation of chicken's TFI, the individual feed intake of each chicken was determined weekly. For this, the amount of feed provided and feed refusals and spills were recorded. Feed refusals were collected before feeding at 9:00h daily and spills were collected before recording feed intake on 9, 14, 21, 28 and 30 dph. Body weight was

361 measured upon arrival and on 6, 9, 14, 21, 28 and 30 dph. The RFI was determined for the
362 experimental period from 9 to 30 dph. A nonlinear mixed model (SAS Stat Inc., version 9.4;
363 Cary, NC, USA) based on data for TFI, metabolic mid-test body-weight and TBWG from 9 to
364 30 dph was used to estimate chicken's RFI as the residuals over the test period (15).
365 Regression analysis was performed for each batch individually. In order to investigate
366 whether the FMT could improve the RFI of poorly feed efficient (high RFI) chickens without
367 impairing the RFI of good feed efficient (low RFI) chickens, chickens with the most extreme
368 RFI values were selected. A total of 15 low (females, n = 8; and males, n = 7) and 13 high
369 (females, n = 7; and males, n = 6) RFI chickens receiving the FMT and 14 low (n = 7/sex) and
370 14 high (n = 7/sex) RFI chickens receiving the CT were selected. Only fecal samples from
371 these selected chickens were analyzed for nutrient content and microbiota composition.
372 **Sample collection.** The gastrointestinal origin of the chicken feces determines the fecal
373 bacterial composition (27). Therefore, for the microbiota analysis, freshly dropped excreta of
374 paste-like texture without the uric acid-containing white part were predominantly collected on
375 16 and 29 dph. Within 5 to 10 min after defecation, feces were aseptically collected, placed
376 into sterile 2-ml cryotubes (Sarstedt, Nümbrecht, Germany), snap frozen in liquid N_2 and
377 stored at -80°C until DNA extraction. Moreover, water and diet samples (n = 2 per starter,
378 grower and finisher diet) were collected for microbial analysis. On 28 dph, freshly dropped
379 excreta samples were collected and stored at -20°C until analysis for NH_3 and pH. To
380 determine fecal DM concentration and retention of nutrients, excreta were collected on 29 and
381 30 dph and stored at -20°C.
382 **DNA extraction.** Total DNA was extracted from 300µl of the prepared FMT stocks,
383 250mg fecal and water samples and 150mg of diet samples using the PowerSoil DNA
384 isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA) as described previously (34). The
385 Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) with the Qubit dsDNA HS
386 Assay Kit (Life Technologies) was used to quantify DNA concentration.

16S rRNA sequencing and bioinformatic analysis. An aliquot of each of the extracted DNA sample (fecal samples, *n* = 112; FMT inoculum, *n* = 8; water samples, *n* = 2; diet samples, *n* = 6) was sent for PCR amplification of the 16S rRNA gene, library preparation and DNA sequencing to a commercial provider (Microsynth AG, Balgach, Switzerland). Primers 357F-HMP (CCT ACG GGA GGC AGC AG) and 926R-HMP (CCG TCA ATT CMT TTR AGT) targeting the V3-5 region of the 16S rRNA gene were used for amplification to generate an approximate amplicon size of 570bp (35). The Nextera XT sample preparation kit (Illumina Inc., San Diego, CA, USA) was used according to the manufacturer's recommendations. For each library, equimolar amounts were pooled and sequenced on an Illumina MiSeq Personal Sequencer using a 300bp read length paired-end protocol. The resultant overlapping paired-end reads were stitched and quality-filtered by Microsynth.

The pre-filtered and stitched reads were processed using the Quantitative Insights Into Microbial Ecology (QIIME) package (version 1.9.1) (36). Fastq files were quality trimmed using the "multiple_split_libraries_fastq" script for demultiplexed Illumina fastq data using a quality threshold of q<15. Chimeric sequences were removed with the UCHIME method using the 64-bit version of USEARCH (37, 38) and the GOLD database (drive5.com). Sequences were clustered into OTUs (97% similarity) using open-reference OTU picking and UCLUST (37). Taxonomy was assigned against the 13_8 Greengenes default database in QIIME (version 1.9.1) (http://qiime.org/home_static/dataFiles.html) (39). The OTUs with less than 10 sequences were removed. The most abundant OTUs in the FMT as well as OTUs differently affected by time point, FMT and RFI were additionally classified against the National Center for Biotechnology Information (NCBI) nucleotide database using Blastn for taxonomic classification and the database limited to the 16S rRNA target (https://blast.ncbi.nlm.nih.gov/). A rarefaction depth of 10,000 sequences was used for α- and ß-diversity analyses of diet, fecal and FMT samples, thereby excluding one fecal sample with fewer reads (low RFI, male, CT, 16 dph). Beta-diversity was determined using unweighted

and weighted UniFrac distance (40, 41). Additionally, rarefaction curves for all diet, fecal and FMT samples were calculated using a maximum rarefaction depth of 10,000 sequences and the Observed OTUs index.

qPCR. The DNA concentrations of the FMT stocks were adjusted. QPCR was performed on a Stratagene Mx3000P qPCR System (Agilent Technologies, Santa Clara, CA) in 20µl reaction volume using 10µl of the Fast-Plus EvaGreen Master Mix with Low ROX (Biotium, Hayward, CA, USA Technologies), forward and reverse primers 341-357F and 518-534R (62.5nmol each) and 0.3ng DNA extract as previously described (34). The amplification specificity was determined by melting curve analysis. Standard curves were generated using 10-fold serial dilutions (10^7 to 10^3 molecules/µl) of the purified and quantified 16S rRNA gene PCR product generated by standard PCR (PCR efficiencies: 95-102%; R^2=0.999) (39).

Chemical analyses. To determine NH_3 in excreta, the indophenol method was used (42). The pH in fresh excreta was measured in a 1:9 (vol/vol) dilution and the DM content was determined by oven-drying at 105°C overnight (43). Prior to proximate analysis [DM, crude protein (protein), crude ash (ash) and P)] as described previously (15), total excreta samples were pooled per chicken, freeze-dried and ground through a 0.5-mm screen. Acid insoluble ash, analyzed in feed and feces (43), was used as inert marker for calculation of nutrient retention.

Statistical analyses. Descriptive statistics on bacterial composition of the FMT inoculum, diet and water samples at the phylum and OTU level were performed using the MEANS procedure in SAS. To test for normality, FE, performance traits, excreta parameter, nutrient retention and microbiota data were first analyzed using Shapiro-Wilk test with the UNIVARIATE procedure in SAS (SAS Stat Inc., version 9.4; Cary, NC, USA). After establishing normal distribution of our parameters, data were analyzed by ANOVA using the MIXED procedure in SAS. To analyze FE, performance traits, excreta parameter and nutrient retention data, the fixed effects of batch, sex, FMT, RFI and the two-way-interaction FMT ×

RFI were considered in the main model. Because batch affected some of the performance traits, batch was considered as random effect in the final model. Chicken was the experimental unit. As sex was significant for the FE and performance data, a second model for these parameters was adjusted and data were additionally separately analyzed for females and males. The second model included the fixed effects of FMT, RFI and the two-way-interaction FMT × RFI. For the microbiota data, fixed effects also included the time point of excreta collection and the three-way-interaction time point × FMT × RFI. Measurements taken on the same chicken at different time points were considered as repeated measures in the model. The experimental unit was chicken nested within batch. Degrees of freedom were approximated by the method of Kenward-Roger. Least squares means were computed using the pdiff statement. A *P* value of 0.05 or less was considered significant, whereas a *P* value between 0.05 and 0.10 was considered as trend. Bacterial families, genera and OTUs comprising a relative abundance >0.05% across both sampling time points and sexes were statistically analyzed.

Pearson's correlation analysis (CORR procedure of SAS) was used to establish and quantify the relationships between fecal abundances of OTUs at 29 dph and individual RFI, TFI, TBWG, fecal pH, fecal DM and fecal NH_3. Correlations were visualized using the R packages corrplot and RColorBrewer (version 3.4).

Sequence data accession number. Raw sequencing data are available in NCBI's BioProject SRA database under accession no. PRJNA392215.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at

SUPPLEMENTAL FILE 1, PDF file, 254 kB.

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B.M.Z., E.M., P.L. and N.O. conceived and designed the study. S.C.S. and B.M.Z. conducted the animal study and together with R.P. collected samples. S.C.S. and B.M.Z. laboratory analysis. S.C.S., B.M.Z. and R.P. performed bioinformatic analysis. Q.Z. provided resources. B.M.Z. wrote the codes for statistical analysis and S.C.S. statistically analysed all data. S.C.S. and B.M.Z. collated and interpreted the data, wrote and edited the manuscript. R.P., P.L. and Q.Z. revised the manuscript. All authors read and approved the final version of this manuscript.

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FIGURE LEGEND

FIG 1 Bacterial composition of the fecal microbiota transplant: (a) phyla and (b) operational taxonomic units (OTUs). FMT inoculum, *n* = 8 (FMT inoculum of the three individual inoculation days of the two batches and pooled samples of the FMT inocula across the three inoculation days per batch).

FIG 2 Principal coordinate analysis (PCoA) plot of weighted UniFrac analysis: (a) fecal samples of females and males; (b) fecal samples at 16 and 29 days post-hatch (dph); (c) fecal samples of low and high residual feed intake (RFI) broiler chickens and (d) broiler chickens receiving either a fecal microbiota transplant (FMT chickens) or a control transplant (CT chickens) and the FMT inoculum. Yellow circles in (d) represent the FMT inoculum of the different inoculation days of the two batches and pooled samples of the FMT inocula across the three inoculation days per batch. Low RFI FMT females, *n* = 8/time point; low RFI FMT males, *n* = 7/time point; high RFI FMT females, *n* = 7/time point; high RFI FMT males, *n* = 6/time point; low RFI CT females, *n* = 7/time point; low RFI CT males, *n* = 6 at 16 dph and *n* = 7 at 29 dph; high RFI CT females, *n* = 7/time point; high RFI CT males, *n* = 7/time point; FMT inoculum, *n* = 8. Rarefaction depth of 10,000 sequences per sample removed 1 sample from the dataset (male, low RFI, CT, 16 dph).

FIG 3 Relative abundances (%): (a) bacterial phyla and (b) most abundant bacterial genera (relative abundance >0.5%) in feces at 16 and 29 days post-hatch (dph) in low and high residual feed intake (RFI) broiler chickens receiving either a fecal microbiota transplant (FMT) or a control transplant (CT). * $P \leq 0.05$, effect of time point; ** $P \leq 0.10$, trend for time point effect; † $P \leq 0.10$, trend for RFI rank effect; and †† $P \leq 0.10$, trend for FMT effect. Low RFI FMT females, *n* = 8/time point; low RFI FMT males, *n* = 7/time point; high RFI FMT females, *n* = 7/time point; high RFI FMT males, *n* = 6/time point; low RFI CT females,

650 *n* = 7/time point; low RFI CT males, *n* = 7/time point; high RFI CT females, *n* = 7/time point;
651 high RFI CT males, *n* = 7/time point. Uncl., Unclassified.
652
653 **FIG 4** Correlations between operational taxonomic units (OTUs) at 29 day post-hatch and
654 feed efficiency, performance traits and excreta characteristics. Correlations were
655 significant ($P \leq 0.05$) if correlation coefficients were ≤-0.33 or ≥0.33. The OTUs were
656 included in the matrix if they occurred in at least half of the chickens and if they were
657 significant for at least one of the parameters. Low RFI FMT females, *n* = 8; low RFI FMT
658 males, *n* = 7; high RFI FMT females, *n* = 7; high RFI FMT males, *n* = 6; low RFI CT females,
659 *n* = 7; low RFI CT males, *n* = 7; high RFI CT females, *n* = 7; high RFI CT males, *n* = 7. RFI,
660 residual feed intake; TFI, total feed intake; TBWG, total body weight gain; DM, dry
661 matter; NH3, ammonia; FM, fresh matter .

TABLE 1 Total feed intake (TFI), total body weight gain (TBWG) and residual feed intake (RFI) values of low and high RFI broiler chickens receiving either a fecal microbiota transplant (FMT) or a control transplant (CT)

	FMT		CT			*P* value		
Item[a]	Low RFI	High RFI	Low RFI	High RFI	SEM	FMT	RFI	FMT×RFI
Both sexes								
TFI (g)	2315	2631	2327	2568	57.3	0.665	<0.001	0.517
TBWG (g)	1641	1654	1656	1640	39.8	0.980	0.964	0.724
RFI (g)	-108	198	-117	155	25.2	0.310	<0.001	0.506
Females								
TFI (g)	2233	2544	2117	2366	82.2	0.087	0.002	0.707
TBWG (g)	1564	1516	1472	1444	45.0	0.081	0.407	0.837
RFI (g)	-104	259	-103	192	38.8	0.405	<0.001	0.390
Males								
TFI (g)	2398	2694	2537	2770	85.7	0.221	0.005	0.713
TBWG (g)	1710	1789	1841	1837	63.2	0.163	0.551	0.517
RFI (g)	-105	128	-130	118	31.7	0.591	<0.001	0.814

Data are presented as least-square means and pooled SEM.

Low RFI FMT females, $n = 8$; low RFI FMT males, $n = 7$; high RFI FMT females, $n = 7$; high RFI FMT males, $n = 6$; low RFI CT females, $n = 7$; low RFI CT males, $n = 7$; high RFI CT females, $n = 7$; high RFI CT males, $n = 7$.

[a]TFI, TBWG and RFI were calculated for the experimental period from 9 to 30 days post-hatch.

Sex affected TFI, TBWG ($P < 0.001$) and RFI ($P < 0.05$).

TABLE 2 Excreta characteristics and retention of nutrients in low and high residual feed intake (RFI) broiler chickens receiving either a fecal microbiota transplant (FMT) or a control transplant (CT)

	FMT		CT			*P* value		
Item	Low RFI	High RFI	Low RFI	High RFI	SEM	FMT	RFI	FMT×RFI
Dry matter content (%)	18.4^{a}	17.0^{ab}	16.6^{bB}	18.2^{abA}	0.63	0.592	0.926	0.024
pH	6.8	6.7	7.0	6.7	0.15	0.486	0.243	0.629
Ammonia (µmol/g fresh sample)	51.0	41.9	42.1	47.6	3.68	0.666	0.631	0.052
Retention (%) of								
Dry matter	82.3	78.7	81.8	80.9	1.68	0.625	0.181	0.434
Crude ash	48.9	42.6	50.1	45.1	4.56	0.682	0.220	0.885
Crude protein	79.5	74.7	79.5	76.0	1.94	0.727	0.037	0.739
Phosphorus	58.0	50.4	56.7	50.2	3.93	0.858	0.081	0.880

Data are presented as least-square means and pooled SEM.

Low RFI FMT females, $n = 8$; low RFI FMT males, $n = 7$; high RFI FMT females, $n = 7$; high RFI FMT males, $n = 6$; low RFI CT females, $n = 7$; low RFI CT males, $n = 7$; high RFI CT females, $n = 7$; high RFI CT males, $n = 7$.

^{a,b}Different superscripts within a row indicate significant difference ($P \leq 0.05$).

^{A,B}Different superscripts within a row indicate a tendency ($P \leq 0.10$).

680 **TABLE 3** Differences in α-diversity indices in feces at 16 and 29 days post-hatch (dph) in low and high residual feed intake (RFI) broiler chickens

681 receiving either a fecal microbiota transplant (FMT) or a control transplant (CT)

	16 dph				29 dph									
	FMT		CT		FMT		CT			*P* value				
Item[a]	Low RFI	High RFI	Low RFI	High RFI	Low RFI	High RFI	Low RFI	High RFI	SEM	T[b]	FMT	RFI	FMT×RFI	T×FMT×RFI
Shannon	4.0	2.8	3.4	3.6	2.8	2.8	3.0	3.2	0.42	0.076	0.568	0.489	0.167	0.475
Simpson	0.77	0.61	0.69	0.68	0.61	0.58	0.62	0.62	0.060	0.053	0.811	0.248	0.306	0.692
Chao1	972	521	707	842	577	578	652	792	143.1	0.248	0.425	0.686	0.096	0.355

682 Data are presented as least-square means and pooled SEM.

683 Low RFI FMT females, *n* = 8/time point; low RFI FMT males, *n* = 7/time point; high RFI FMT females, *n* = 7/time point; high RFI FMT males, *n* = 6/time point; low RFI CT

684 females, *n* = 7/time point; low RFI CT males, *n* = 6 at 16 dph and *n* = 7 at 29 dph; high RFI CT females, *n* = 7/time point; high RFI CT males, *n* = 7/time point.

685 [a]Rarefaction depth of 10,000 sequences per sample removed 1 sample from the dataset (male, low RFI, CT, 16 dph).

686 [b]T, time point.

TABLE 4 Differences in relative abundance (%) of most abundant bacterial families in feces at 16 and 29 days post-hatch (dph) in low and high residual feed intake (RFI) chickens receiving either a fecal microbiota transplant (FMT) or a control transplant (CT)

	16 dph				29 dph									
	FMT		CT		FMT		CT			*P* value				
Item	Low RFI	High RFI	Low RFI	High RFI	Low RFI	High RFI	Low RFI	High RFI	SEM	T[a]	FMT	RFI	FMT×RFI	T×FMT×RFI
Enterobacteriaceae	32.80	56.79	33.19	46.05	57.02	54.92	52.38	58.07	8.186	0.021	0.621	0.095	0.889	0.403
Lactobacillaceae	30.53	24.41	32.87	20.54	12.83	9.73	11.60	4.99	6.419	0.001	0.663	0.107	0.572	0.964
Ruminococcaceae	13.24	4.84	9.14	12.39	3.41	4.91	4.77	7.91	3.476	0.030	0.486	0.964	0.238	0.424
Turicibacteraceae	1.24	2.14	0.45	0.26	15.17	14.56	10.71	7.59	3.619	<0.001	0.176	0.769	0.727	0.809
Lachnospiraceae	2.36	0.64	1.42	2.31	0.44	0.77	0.55	0.79	0.519	0.004	0.583	0.867	0.109	0.196
Moraxellaceae	0.27	0.29	0.05	0.24	1.10	2.86	1.43	2.43	1.027	0.018	0.902	0.319	0.843	0.821
Unclassified *RF39*	0.28	0.01	0.11	0.07	0.04	0.05	0.05	0.47	0.127	0.692	0.386	0.743	0.084	0.095
Comamonadaceae	0.19	0.40	0.21	0.14	0.005	0.007	0.006	0.01	0.100	0.002	0.421	0.611	0.332	0.589

Data are presented as least-square means and pooled SEM.

Low RFI FMT females, n = 8/time point; low RFI FMT males, n = 7/time point; high RFI FMT females, n = 7/time point; high RFI FMT males, n = 6/time point; low RFI CT females, n = 7/time point; low RFI CT males, n = 7/time point; high RFI CT females, n = 7/time point; high RFI CT males, n = 7/time point.

[a]T, time point.

Sex affected *Ruminococcaceae* ($P < 0.10$).