

Morphology, microbiota, and metabolome along the intestinal tract of female turkeys

Janina Rzeznitzek ^{*}, Frederic J. Hoerr [†], Ivan Rychlik,[‡] Karen Methling,[§] Michael Lalk,[§] Alexandra Rath,[#] Alexandra von Altrock,[#] and Silke Rautenschlein ^{*},¹

^{*}*Clinic for Poultry, University of Veterinary Medicine Hannover, Foundation, 30559 Hannover, Germany;* [†]*Veterinary Diagnostic Pathology, Fort Valley, VA 22652, USA;* [‡]*Veterinary Research Institute, 621 00 Brno, Czech Republic;* [§]*Institute of Biochemistry, University of Greifswald, 17487 Greifswald, Germany;* and [#]*Clinic for Swine, Small Ruminants and Forensic Medicine, University of Veterinary Medicine Hannover, Foundation, 30173 Hannover, Germany*

ABSTRACT The global turkey industry is confronted with emerging challenges regarding health and welfare. Performance and disease resilience are directly linked to gut health. A clear definition of a healthy gut is a prerequisite to developing new strategies for improved gut health and, thus, general health, welfare and productivity. To date, detailed knowledge about gut health characteristics, especially during the critical fattening period, is still lacking for turkeys. Therefore, the goal of this study was to describe the morphology, microbiota, and metabolome along the intestinal tract of clinically healthy *Salmonella*- and *Campylobacter*-free commercial turkey hens throughout the fattening period from 7 to 10 wk posthatch, and obtain information on the stability of the investigated values over time. Feed changes were avoided directly preceding and during the investigation

period. Investigation methods included histomorphometric measurement of intestinal villi and crypts, Illumina-sequencing for microbiota analysis, and proton nuclear magnetic resonance spectroscopy for metabolite identification and quantification. Overall, the study demonstrated a high repeatability across all 3 experiments and gut section differences observed coincided with their functions. It was demonstrated that gut maturation, defined by gut microbiota stability, is reached earlier in the ceca than any other intestinal section where morphological changes are ongoing throughout the fattening period. Therefore, the present study provides valuable information necessary to advise future studies on the development and implementation of measures to support gut maturation and establish a protective microbiota in commercial turkeys.

Key words: turkey, age, fattening, gut microbiota, intestinal metabolome

2022 Poultry Science 101:102046

<https://doi.org/10.1016/j.psj.2022.102046>

INTRODUCTION

To meet increasing global demands, the commercial poultry industry is currently one of the fastest growing sectors of animal agriculture (Carrasco et al., 2019). A challenge faced by the industry is optimizing production efficiency whilst preserving animal health and welfare standards (Hafez and Attia, 2020). Performance and health have been directly linked to gut health (Scupham, 2007; Awad et al., 2018). Whilst enabling nutrient and fluid absorption, the intestinal barrier also prevents invasion of pathogens and harmful toxins (Awad et al.,

2018). The gut epithelium is continuously renewed by enterocyte proliferation in crypts and migration to villi tips (Iji et al., 2001). Infectious and noninfectious causes may lead to mucosal damage with villus shortening, followed by crypt hyperplasia where enterocyte turnover is increased (Iji et al., 2001; Adji et al., 2019). Villus stunting can negatively influence absorption processes in the gut, impairing weight gain in affected animals (Awad et al., 2008).

Recently, the complex symbiotic network between microbiota and their host as part of the intestinal barrier has received much attention (Wilkinson et al., 2017; Carrasco et al., 2019; Bindari and Gerber, 2022). Indigenous microbes use an extensive signaling system among each other and with their host to maintain a stable microenvironment (Carrasco et al., 2019). They constantly compete for attachment sites and available nutrients derived from ingesta, mucin, or bile (Sekirov et al., 2010). In turn, commensals produce metabolites,

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Received April 5, 2022.

Accepted June 29, 2022.

¹Corresponding author: silke.rautenschlein@tiho-hannover.de

which provide the host with nutrients, energy, bioactive compounds, and structural components (Ndagijimana et al., 2009). By changing the local pH and oxygen levels, microbial metabolites can modify the microenvironment, influencing microorganism colonization (Sekirov et al., 2010; Awad et al., 2018). Any imbalance (dysbiosis) to the ecosystem decreases disease resistance and risks bacterial or fungal overgrowth, affecting production efficiency (Sekirov et al., 2010).

Host factors, such as breed, age, and sex, as well as differences in climate, season, feed composition, and management style are key aspects affecting intestinal health (Kogut, 2017; Kers et al., 2018). Nowadays, phase feeding is common practice in commercial turkey production (Gous et al., 2019a). During the brooding and rearing phase, (pre-)starter crumb or small pellet protein-rich diets supplemented with vitamins and minerals ensure improved nutrient digestibility for maximum growth and development of bones, muscles, and internal organs (Grimes, 2015; Gous et al., 2019a). At 6 wk of age, the start of fattening, toms and hens are separated, allowing matching of feeding phases with sex-specific growth stages and requirements (Gous et al., 2019a). Because of moderately reduced protein content and increased metabolizable energy, pelleted grower diets encourage intestinal development whilst retarding overall growth (Laudadio et al., 2012). Protein-restricted diets fed at this stage promote intestinal stability and disease resilience (Auckland and Morris, 1971; Plavnik and Hurwitz, 1990). Prior to slaughter, high-energy finishing diets promote compensatory gain to overcome the deficit (Lemme et al., 2006).

Therefore, the early fattening period, which begins at 6 wk of age, is especially crucial for intestinal development and gut health (Grimes, 2015). Optimizing diets and feeding programs to influence and stabilize the gut microenvironment in this critical period, not only improves gut health but also animal health, welfare, and production long-term (Grimes, 2015). However, a basic understanding of the individual components of the gut itself is essential to develop new strategies (Awad et al., 2008). Most studies describing the poultry intestinal tract focus on chickens (Kogut, 2017; Awad et al., 2018) whereas turkeys are largely understudied. Therefore, the goal of this study was to advance knowledge about the gut of clinically healthy *Salmonella*- and *Campylobacter*-free commercial turkey hens, particularly focusing on the intestinal structure, microbiota composition, and metabolome at different time points throughout the fattening period and comparing these components across repeat experiments.

MATERIALS AND METHODS

Ethical Approval

All work was approved by the Ethics Department of the Lower Saxony State Office for Consumer Protection and Food Safety in Oldenburg, Germany, and

conducted under the project license number 33.8-42502-04-19/3207.

Experimental Design

Experimental Animals Per experiment, we obtained 18 nonvaccinated female day-old poultts from a commercial hatchery (Moorgut Kartzfehn Turkey Breeder GmbH, Bösel, Germany) ($n = 54$ animals in total). We worked with the hybrid line British United Turkey 6 (**B. U.T. 6**), which represent the most common commercial meat-type turkey line in Europe (Grashorn and Bessei, 2004). The study only included female turkeys to minimize the influence of sex on investigated gut parameters and because hens and toms are separated before fattening in commercial settings due to sex-specific nutritional and management requirements (Kers et al., 2018; Gous et al., 2019b). Upon arrival, the poultts received individual wing tags. They were all housed in a single temperature- and light-controlled littered floor pen measuring 7.36 m² and isolated from other birds at the housing facility of the Clinic for Poultry, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany. The same pen was used in all experiments and was cleaned and disinfected in between. Wet litter and excreta were removed, and fresh wood shavings provided on a weekly basis. Thoroughly cleaned and disinfected perches as well as fresh autoclaved straw were supplied as enrichment. The turkey poultts had access to water via automatic bell drinkers at all times. Throughout the experiments, the birds received commercial turkey feed (Deuka, Deutsche Tiernahrung Cremer GmbH & Co. KG, Düsseldorf, Germany) ad libitum. For the first 3 wk of life, they were fed a small-pelleted protein-rich starter diet (Table 1). Then, protein-restricted grower feed was gradually introduced over the course of 1 wk and fed exclusively until termination of the experiment at 10 wk (Table 1).

General Study Procedure and Sample Acquisition

The animal trial was conducted 3 times (**EXP 1-3**). Turkey poultts were checked daily and received individual clinical scores based on their general wellbeing, range of movement, wounds or injuries, respiratory symptoms, and fecal consistency. Cloacal swabs were taken on a weekly basis to confirm their *Campylobacter*-negative status by initial enrichment in Preston broth (Thermo Scientific Inc., Waltham, MA) in a microaerobic environment (5% O₂, 10% CO₂, and 85% N₂) at 37.5°C for 48 h and subsequent incubation on *Campylobacter*-selective charcoal cefoperazone deoxycholate agar (CCDA) plates (Thermo Scientific Inc., Waltham, MA) under the same conditions. *Salmonella* detection was performed according to international standard DIN EN ISO 6579 based on EU regulations 517/2011 and 1190/2012. In short, fresh droppings collected from the turkey pen were mixed with buffered peptone water (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) in a 1 to 10 ratio (wt/vol) and incubated in an aerobic environment at 37°

Table 1. Commercial turkey starter and grower diets.

Feeding regime and dietary composition	Starter diet*	Grower diet*
Age when fed (weeks)	1–3	4–10
Metabolizable energy (kcal/kg)	2,796.4	2,915.8
Composition (%)		
Crude protein	26.0	22.0
Crude fat	5.4	6.3
Crude fiber	3.2	3.3
Crude ash	7.0	5.9
Calcium	1.3	1.1
Phosphorus	0.9	0.7
Sodium	0.14	0.14
Lysine	1.60	1.45
Methionine	0.60	0.56
Main ingredients	Soybean meal, wheat, corn, barley, potato protein, plant-based fatty acids, vegetable oils	Wheat, soybean meal, corn, plant-based fatty acids, potato protein, barley, vegetable oil, soybeans
Nutritional additives		
Vitamin A (IU/kg)	10,000.0	10,000.0
Vitamin D ₃ (IU/kg)	5,000.0	5,000.0
Vitamin E (mg/kg)	80.0	80.0
Iron (mg/kg)	35.0	35.0
Iodine (mg/kg)	0.8	0.8
Copper (mg/kg)	11.0	11.0
Manganese (mg/kg)	112.0	112.0
Zinc (mg/kg)	84.0	84.0
Selenium (mg/kg)	0.3	0.3
Coccidiostat		
Monensin sodium (mg/kg)	100.0	100.0

*Deuka, Deutsche Tiernahrung Cremer GmbH & Co. KG, Düsseldorf, Germany.

C for 18 h. After incubation, the suspension was applied to Modified Semi-Solid Rappaport-Vassiliadis agar (Thermo Scientific Inc., Waltham, MA) and incubated aerobically at 41.5°C for 24 h. If no growth was observed, agar plates were incubated for another 24 h.

On study d 49, 56, and 70 (from here on referred to as 7, 8, and 10 wk of age), 6 birds were humanely stunned with an electrical impulse to their head, and then instantly exsanguinated, complying with EU law for euthanasia of research animals (Directive 2010/63/EU). Thereafter, birds were weighed and samples obtained from the middle of the duodenum, adjacent to Meckel's diverticulum (jejunum), 5 cm proximal to the ileocecal junction (ileum), and mid-cecum. Moving distally from specified sampling locations, material was collected for histological, microbiota, and metabolome analysis in a sequential order. New utensils were used for each gut section to prevent cross-contamination. For histology, gut content was gently squeezed from 1.5 cm gut sections before fixing the tissue in 4% (wt/vol) phosphate-buffered formalin. From each sampling location, approximately 3.0 mL luminal content were stored in 2 sterile 1.5 mL microcentrifuge tubes (Sigma-Aldrich, St. Louis, MO) and frozen at -20°C for microbiota and metabolome analysis.

Histomorphometric Analysis and Heterophil Counts

After fixation for a minimum of 48 h, tissue samples were trimmed prior to dehydration in a graded series of ethanol and subsequent embedding in paraffin. A HistoCore MULTICUT microtome from Leica (Nussloch,

Germany) was used to cut the tissue samples into 4 μ m sections, which were stained with hematoxylin and eosin and mounted onto glass slides according to standard protocols. The preparations were viewed at 25x and 100x total magnification with a DMLB binocular light microscope equipped with a DFC320 camera from Leica (Nussloch, Germany) to capture the images. Morphometric measurements were carried out using ImageJ1 software (version 1.53e, National Institute of Health, Bethesda, MD) (Schneider et al., 2012). Only epithelial sections with an intact lamina propria were selected. For each specimen, 10 villi and 10 crypts were measured to determine average villus height (VH), villus width (VW), and crypt depth (CD) (Awad et al., 2015). Additionally, the villus height to crypt depth ratio (VH:CD) and the villus surface area (VSA) were calculated as previously described (Awad et al., 2015). Finally, heterophils were counted in 10 randomly selected cecal epithelial sections at 400x total magnification.

Microbiota Analysis

To investigate the microbiota composition, intestinal content was initially homogenized with zirconia silica beads (BioSpec Products, Bartlesville, OK) in a MAGNALyzer (Roche Diagnostics, Basel, Switzerland) prior to DNA extraction using a QIAamp DNA stool minikit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Subsequently, the DNA quality and concentration were evaluated spectrophotometrically and adjusted to 5.0 ng/ μ L. The PCR and Illumina-sequencing (Illumina Inc., San Diego, CA) of the 16S rRNA gene V3/V4 regions was carried out as described previously without modifications (Polansky et al.,

2016). Afterward, QIIME 2 software package was used to match the Illumina-generated sequences with operational taxonomic units (OTU) to identify and classify bacterial species present. A clustering threshold of 97% was applied (Caporaso et al., 2010; Bolyen et al., 2019). Because of low numbers of reads (<1,000), 29% of duodenum and 6% of jejunum samples were excluded from data analysis. For this reason, sample analysis in EXP 3 was limited to ileal and cecal samples. QIIME 2 was further used to analyze within-sample (α -diversity) metrics. OTU richness was determined by the total number of phylotypes identified. We also analyzed diversity using the nonphylogenetic Chao-1 estimator, which accounts for unobserved species and provides variance estimates based on counted individuals within samples (Willis, 2019). Further, we included the Shannon diversity index, which takes evenness and relative species abundances into account, providing information about the entropy of a community of microorganisms (Chao and Shen, 2003). To examine how taxonomic abundance profiles compared between different samples (β -diversity), we used weighted UniFrac distance matrix-based principal coordinate analysis (PCoA) ordination, which incorporates phylogenetic distances and abundance information between 2 samples (Lozupone and Knight, 2005).

Metabolome Analysis

Only samples from EXP 1 and 2 were included in the analysis due to experimental limitations. Samples were thawed on ice. To determine the DM content, 1 g gut content was weighed out, dried at 104°C for 24 h, weighed again, and discarded thereafter (Gous et al., 2019a). For the metabolome analysis, 300 mg intestinal content was homogenized with 1 mL distilled water by vigorous vortex-mixing. Next, the mixtures were centrifuged at 10,000 $\times g$ and 0°C for 10 min. The supernatant was subsequently passed through a Filtropur S 0.2 μm pore size syringe filter with a polyethersulfone membrane (Sarstedt AG & Co. KG, Nuembrecht, Germany). Proton nuclear magnetic resonance ($^1\text{H-NMR}$) spectroscopic analysis of the sterile supernatant was performed for metabolite identification and quantification as previously described by Dorries and Lalk (2013) and Troitzsch et al. (2021) with few modifications summarized below. In short, 400 μL sample were combined with 200 μL 0.2 M sodium hydrogen phosphate buffer containing 30% deuterium oxide (Eurisotop, St-Aubin Cedex, France) and 1.74 mM 3-trimethylsilyl-[2,2,3,3-D₄]-1-propionic acid (TSP) (Sigma-Aldrich, St. Louis, MO). After centrifugation (13,000 $\times g$, 4°C), samples were analyzed in 5 mm glass tubes (Bruker Biospin GmbH, Rheinstetten, Germany) using a Bruker AVANCE-NEO 600 NMR spectrometer with a SampleJet autosampler and 5 mm QCI cryo-probe, run with TOPSPIN 4.0.6 software (Bruker Biospin GmbH, Rheinstetten, Germany). Spectra were attained at 600.27 MHz and 300 K before being analyzed using

AMIX Viewer 3.9.15 software (Bruker Biospin GmbH, Rheinstetten, Germany). The spectral width of the internal reference TSP was adjusted to 0 ppm to align the analytical sample spectra for metabolite identification. Obtained spectra were then matched with signals acquired from known reference compounds to identify metabolites within analyzed samples. Before metabolite quantification, the AMIX underground removal tool was applied to reduce background noise. An external reference signal was then generated using the ERETIC (Electronic Reference To access In vivo Concentrations) method based on the PULCON (Pulse Length-Based Concentration) determination principle (Wider and Dreier, 2006). Successively, signal intensity determined by peak integration was compared between the external control and analytical sample spectra to determine the absolute metabolite concentration. Resulting metabolite concentrations were normalized against the corresponding DM content.

Statistical Analysis

Statistical analysis was performed using SAS Enterprise Guide software (version 7.15, SAS Institute Inc. Cary, NC). Normally distributed data were analyzed using a two-way ANOVA and Tukey-Kramer test for pairwise comparisons. If a normal distribution could not be assumed, data were analyzed using a non-parametric Kruskal-Wallis test initially, followed by individual pairwise comparisons with Wilcoxon's two-sample tests and post hoc Bonferroni-Holm correction method ($\alpha = 0.05$). Statistical significance was assumed when $P \leq 0.05$. Graphs were created using GraphPad Software Prism 9 (version 9.2.0, San Diego, CA).

RESULTS

Bird Health

The turkeys in this study appeared clinically healthy and exhibited no macroscopic or microscopic lesions on post-mortem or histological examination, respectively. Further, they maintained a *Salmonella*- and *Campylobacter*-negative status as confirmed by culture and 16S rRNA sequencing. At 10 wk, body weights between 5.43 kg and 6.87 kg were recorded.

Histomorphometric Analysis and Heterophil Counts

VH, VW, VSA, CD, and VH:CD differed between gut sections (Suppl. Table 1). VH decreased steadily along the intestinal tract, being greatest in the duodenum ($P < 0.001$) and smallest in the cecum ($P < 0.001$), averaging 2.79 mm and 0.20 mm, respectively (Figure 1A). With a mean width of 0.14 mm, villi in the ileum were 1.4 to 1.75 times wider than those in the jejunum ($P < 0.001$) and cecum ($P < 0.001$) (Figure 1B). VSA was the largest in the duodenum (1.06 mm²) ($P < 0.001$) and the

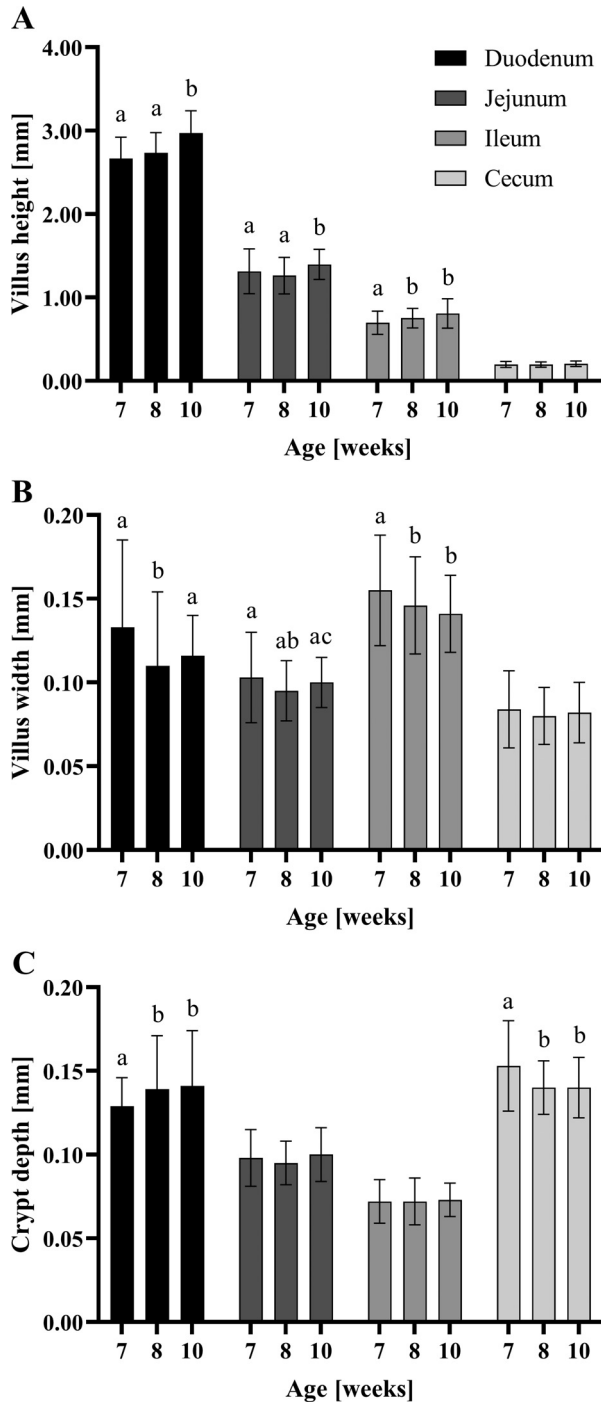


Figure 1. Histomorphometric measurements of gut villi and crypts. Histograms depict (A) villus height, (B) villus width, and (C) crypt depth of the duodenum, jejunum, ileum, and cecum of female turkeys at 7, 8, and 10 wk of age, $n = 18$. Data were summarized for all 3 experiments. Per specimen, 10 villi and 10 crypts were measured microscopically at 25x and 100x magnification. Vertical error bars depict standard deviation. Different letters indicate statistically significant differences between age groups ($P \leq 0.05$). Wilcoxon's two-sample test, post hoc Bonferroni-Holm correction ($\alpha = 0.05$).

smallest in the cecum (0.05 mm^2) ($P < 0.001$) but did not differ between the jejunum and ileum ($P > 0.05$) (data not shown). On average, crypts were the shallowest in the ileum (0.07 mm) followed by the jejunum (0.10 mm) and the deepest in the duodenum and cecum (0.13 mm and 0.14 mm , respectively) ($P < 0.05$) (Figure 1C). Calculated VH:CD was smallest in the

cecum (1.39) ($P < 0.001$) and largest in the duodenum (21.06) ($P < 0.001$) (data not shown). Further, average cecal heterophil counts ranged from 10.6 to 29.2 (median: 2.8) (data not shown).

We recorded an age effect on histomorphometric measurements. On average, villi in the duodenum were 0.17 to 0.20 mm longer at 10 compared to 7 and 8 wk of age ($P < 0.001$) (Figure 1A). This trend was also observed in the jejunum and ileum ($P < 0.001$) but not the cecum ($P > 0.05$) (Figure 1A). Additionally, in the duodenum and ileum, we observed a reduction of mean VW by 0.01 to 0.03 mm over time ($P < 0.001$ and $P = 0.004$, respectively) (Figure 1B). There was no difference between VSA at the beginning and the end of the fattening period in any gut section ($P > 0.05$) (data not shown). CD increased in the duodenum and decreased in the cecum between 7 and 10 wk of age ($P = 0.002$ and $P < 0.001$) but remained unchanged in the jejunum and ileum ($P > 0.05$) (Figure 1C). VH:CD increased in all gut sections over time ($P < 0.05$) (data not shown).

Morphometric measurements varied very little between experiments. The sole difference was that duodenal villi were slightly wider and, subsequently, calculated VSA and VH:CD mildly elevated in EXP 3 compared to EXP 1 and 2 ($P < 0.05$) (data not shown). This allowed us to summarize data from all 3 experiments from here on forth (Figure 1).

Microbiota Analysis

Across all intestinal sections, investigated time points, and experiments, the largest number of organisms identified belonged to the phylum *Firmicutes*, ranging from 76.00% in the proximal to 85.07% in the distal tract (data not shown). *Lactobacillaceae* (30.73%) and *Peptostreptococcaceae* (15.39%) were the most common families found in the jejunum but were hardly present in the cecum (1.52% and 1.97%, respectively) (Figure 2). There the dominating microorganisms belonged to the families *Lachnospiraceae* (32.50%) and *Ruminococcaceae* (18.44%), which only contributed to 11.05% and 2.35% of microbes residing in the jejunum (Figure 2). Bacteria belonging to the *Clostridia* families *UCG-014* (8.59%) and *vadinBB60 group* (6.07%) as well as *Oscillospiraceae* (8.43%) were more prominent in the distal than in the proximal tract where there were more *Erysipelotrichaceae* (4.12%), *Aerococcaceae* (2.43%), and *Staphylococcaceae* (2.38%) (Figure 2). Microorganisms belonging to the phylum *Bacteroidota* only made up a small percentage of the overall composition compared to *Firmicutes* (data not shown). In the jejunum, they mainly consisted of *Bacteroidaceae* (1.93%) whereas in the cecum, they were predominated by *Rikenellaceae* (8.88%) (Figure 2). Bacteria of the phylum *Proteobacteria* were mainly exemplified by the family *Enterobacteriaceae*, constituting 5.60% of the total microbiota in the proximal intestinal tract vs. 2.33%

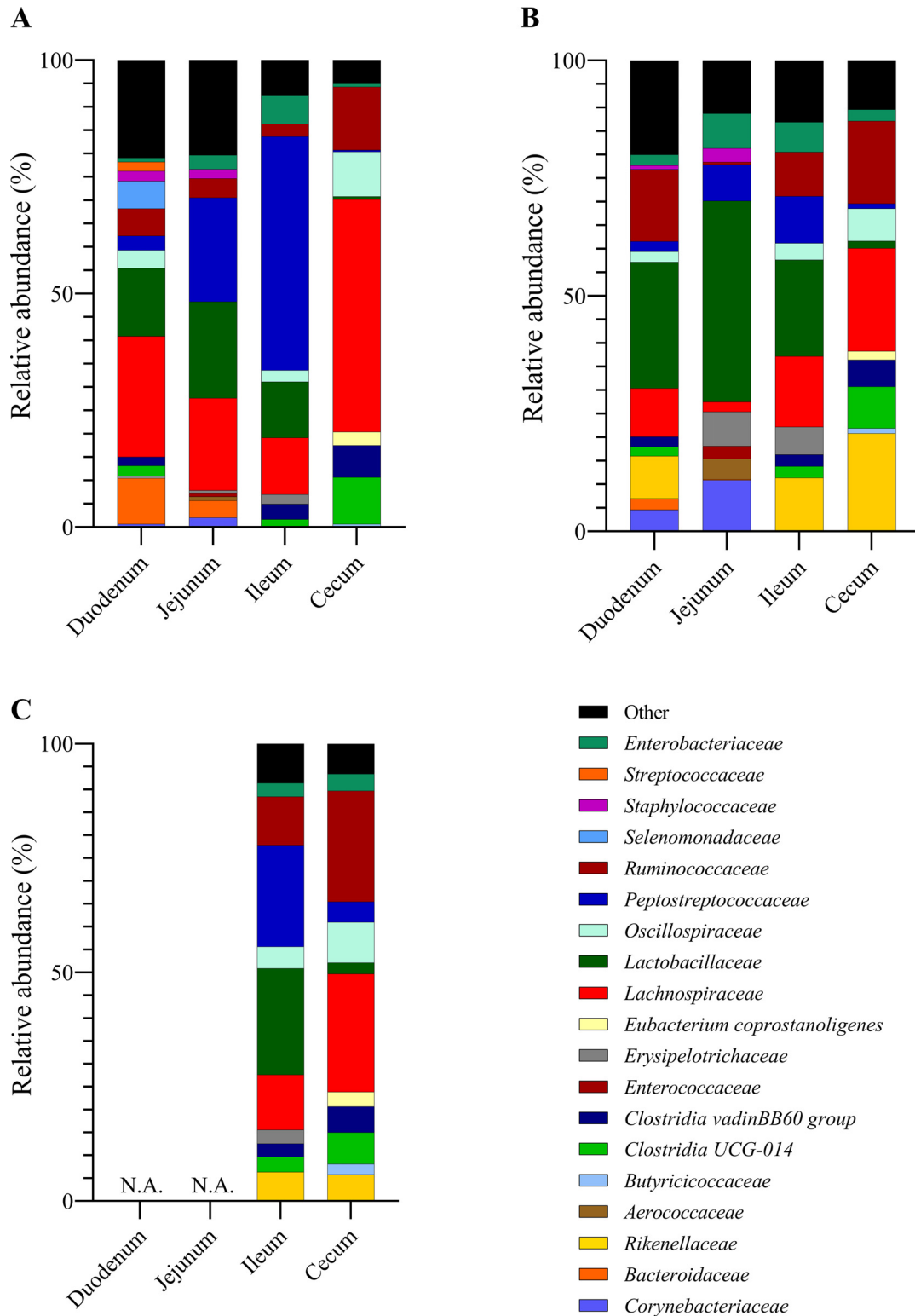


Figure 2. Microbiota composition at bacterial family level. Histograms depict relative abundances (%) of bacterial families in the duodenum, jejunum, ileum, and cecum of female turkeys in (A) Experiment 1, (B) Experiment 2, and (C) Experiment 3, $n = 18$. In Experiment 3, duodenal and jejunal samples were not analyzed (N.A.). Data were summarized for turkeys aged 7 to 10 wk. Samples were analyzed using Illumina-sequencing and matched with operational taxonomic units, applying a clustering threshold of 97%. Families were summarized as “other” if the average abundance was below 1.0%.

in the distal (Figure 2). *Actinobacteriota* mainly represented by *Corynebacteriaceae*, were found in the jejunum at significantly higher levels (6.18%) than in the cecum ($P = 0.014$) (Figure 2).

Phylotype richness generally increased along the intestinal tract. In the duodenum, the total number of

distinguishable taxa ranged from 1,560 to 69,780, averaging 15,057, and was therefore lowest of all intestinal samples ($P < 0.001$). In contrast, species richness was highest in the cecum ($P < 0.001$), averaging 58,424 OTUs (data not shown). The Chao-1 estimator also increased along the intestinal tract in EXP 2 and 3 ($P <$

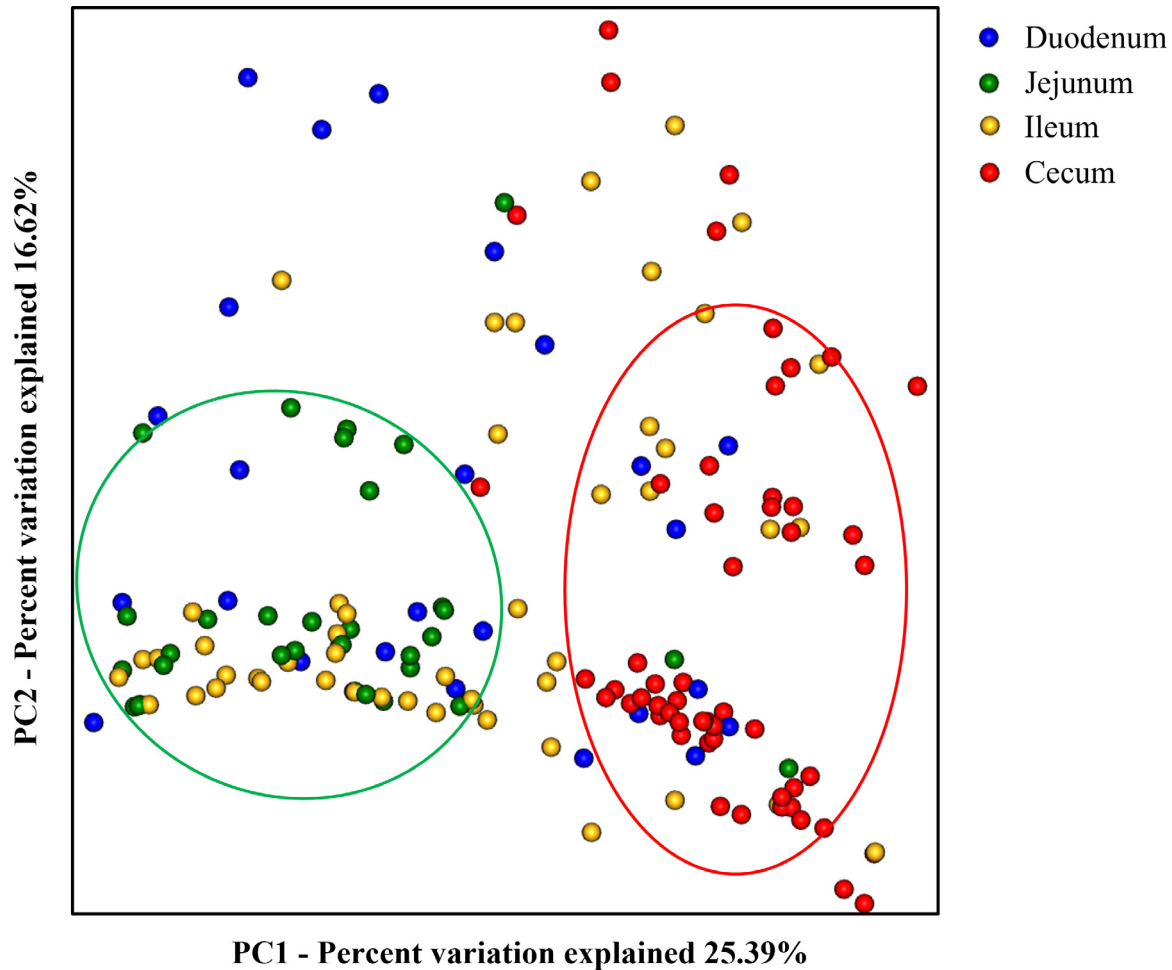


Figure 3. Beta-diversity of gut microbiota. Principal coordinate analysis ordination based on weighted UniFrac distance matrix depicts the similarity of bacterial communities illustrated by proximity between dots in the ordination. Dots represent individual duodenum, jejunum, ileum, and cecum samples from female turkeys, $n = 54$. Data were summarized for 3 repeat experiments and ages 7 to 10 wk.

0.01). However, the index did not differ between the gut sections in EXP 1 ($P = 0.935$) (data not shown). Though not significant statistically ($P > 0.05$), the Shannon diversity index tended to be highest in the cecum, followed by the duodenum, and lowest in the ileum (data not shown). The PCoA plot depicts separate clusters of samples derived from the jejunum and cecum but not for the duodenum and ileum (Figure 3).

In this study, neither the microbiota composition ($P > 0.05$) nor the richness and diversity determined by the total number of OTUs identified and the Chao-1 estimator were affected by age ($P = 0.972$ and 0.281 , respectively) (data not shown). Only the Shannon index was slightly higher at 8 wk compared to 10 wk in the ileum ($P = 0.041$) (data not shown). No cluster formation on age groups was identified on the PCoA plot, indicating that between-sample diversity was not affected by age either (data not shown).

The microbiota composition varied between experiments. Although *Firmicutes* members remained the most common phylum, their levels were overall lower in EXP 2 compared to EXP 1 and 3 ($P = 0.025$ and 0.028 , respectively) (data not shown). This was mainly due to a relative decrease of *Peptostreptococcaceae* in the ileum and *Lachnospiraceae* in the cecum (Suppl. Table 2). In

the jejunum, both families of microorganisms were considerably reduced (Suppl. Table 2). In turn, there was a higher percentage of *Rikenellaceae* (*Bacteroidota*) in ileum and cecum samples ($P = 0.005$ and 0.015) and *Corynebacteriaceae* (*Actinobacteriota*) in the jejunum ($P = 0.003$) in EXP 2 compared to the other experiments (Suppl. Table 2). Richness and diversity barely differed between experiments, except for OTU richness being higher and the Chao-1 estimator lower in cecal samples of EXP 1 compared to the other 2 ($P = 0.001$ for both) (data not shown). Neither the Shannon diversity index ($P = 0.867$) nor β -diversity varied between experiments.

Metabolome Analysis

In total, 43 metabolites were identified and quantified in investigated gut samples (Figure 4, Suppl. Table 3). Most metabolites identified in the duodenum consisted of different proteinogenic amino acids (AA). Except for glutamate, mean concentrations of proteinogenic AAs ranged from 30.22 mmol/kg DM in the proximal to 6.74 mmol/kg DM in the distal tract. Figure 5A depicts alanine levels at different sampling locations,

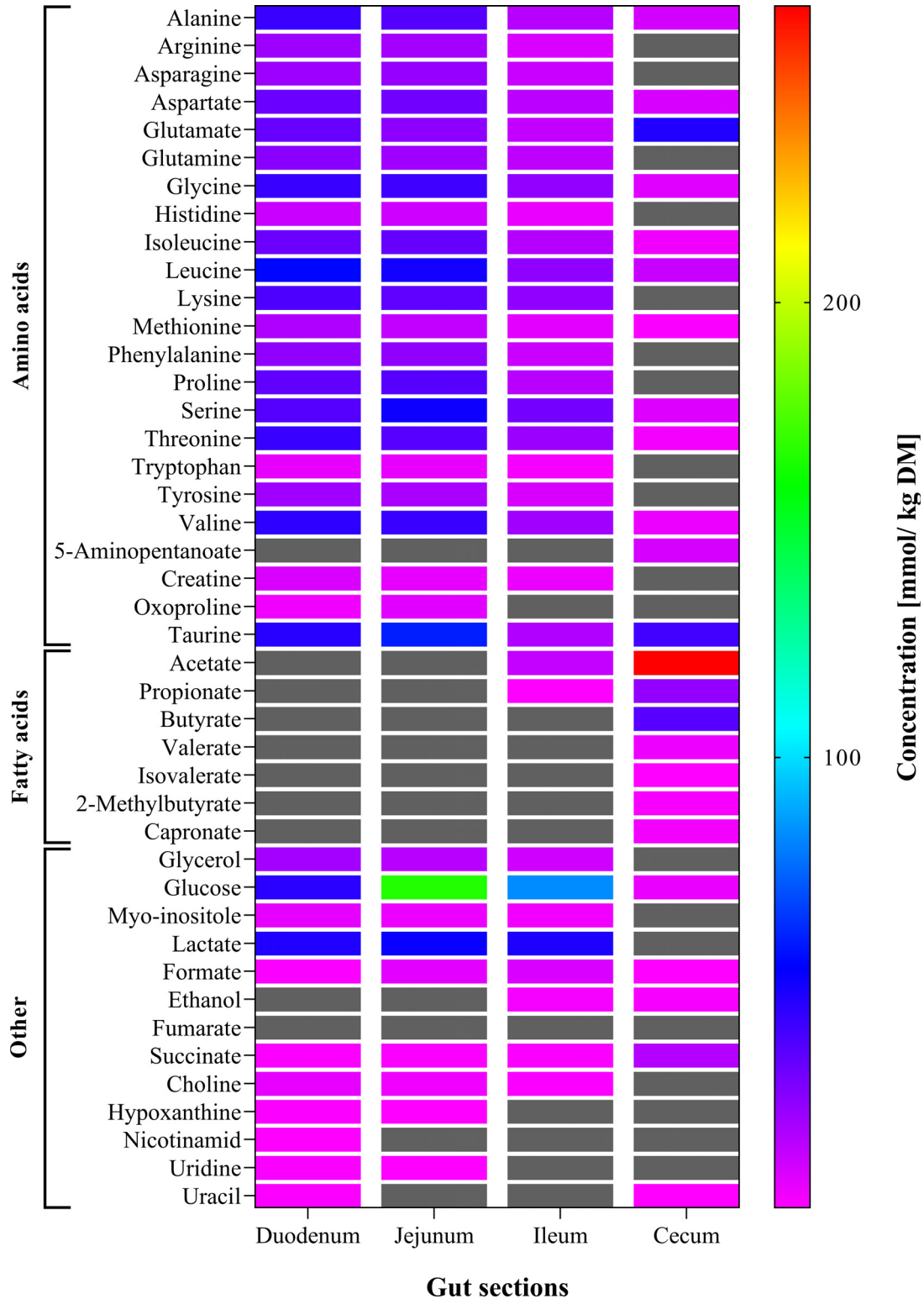


Figure 4. Gut metabolite identification and quantification. Heat map depicts metabolites identified and quantified in the duodenum, jejunum, ileum, and cecum of female turkeys, $n = 36$. Data were summarized for 2 experiments (EXP 1 and EXP 2) and turkeys aged 7 to 10 wk. Metabolites were identified and quantified using proton nuclear magnetic resonance spectroscopy. Results were normalized against DM content of gut ingesta. Gray represents quantities below detection limit. The pink and purple colors are low, the red high concentrations in relation to all metabolites identified.

representing typical proteinogenic AA metabolism. In contrast, glutamate initially decreased from the duodenum to the ileum before increasing more than 3-fold in the cecum ($P < 0.001$) (Figure 5B). However, creatine and oxoproline followed patterns similar to the other proteinogenic AAs, starting with 8.71 and 7.17 mmol/kg

DM in the proximal tract, respectively, and reaching non-detectable levels in the distal sections (Figure 4). 5-aminopentanoate was only detected in the cecum in concentrations varying from 2.00 to 48.99 mmol/kg DM (Figure 4). The level of taurine varied most along the intestinal tract, reaching its highest level in the jejunum

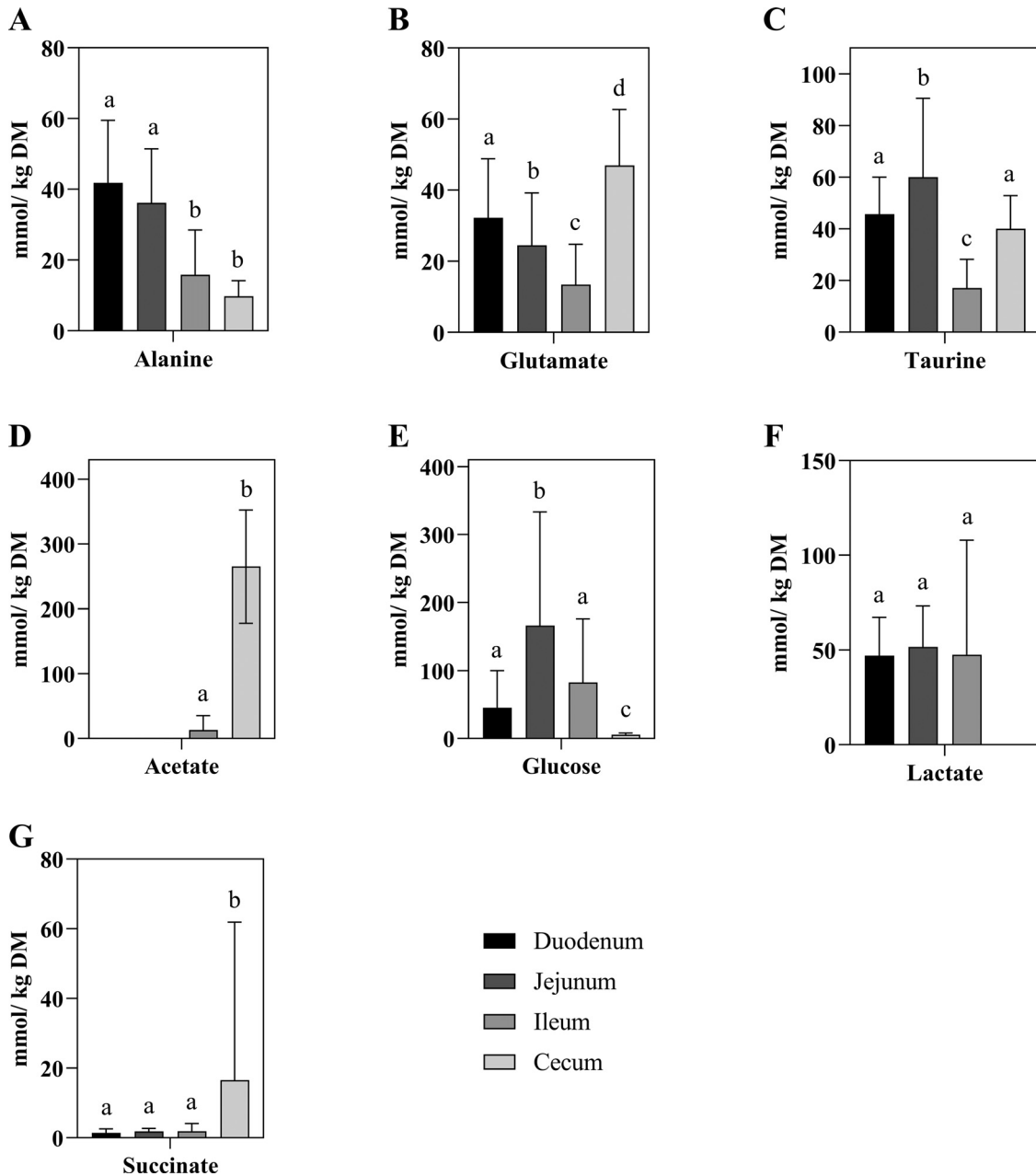


Figure 5. Concentrations of selected metabolites. Histograms depict concentrations of (A) alanine, (B) glutamate, (C) taurine, (D) acetate, (E) glucose, (F) lactate, and (G) succinate in the duodenum, jejunum, ileum, and cecum of female turkeys, $n = 36$. Data were summarized for 2 experiments (EXP 1 and EXP 2) and turkeys aged 7 to 10 wk. Metabolites were identified and quantified using proton nuclear magnetic resonance spectroscopy and normalized against ingesta DM content. Vertical error bars represent standard deviation. Different letters indicate statistical significances in metabolite concentrations between gut sections ($P \leq 0.05$). Wilcoxon's two-sample test, post hoc Bonferroni-Holm correction ($\alpha = 0.05$).

and its lowest in the ileum ($P < 0.05$) before increasing again in the cecum (Figure 5C).

No short-chain fatty acids (SCFA) were identified in the proximal intestinal tract but comprised most metabolites identified in the cecum (Figure 4). By far, the SCFA with the highest concentration was acetate, reaching levels over 300 mmol/kg DM in the cecum (Figure 5D). Butyrate and propionate were also identified at concentrations up to 35.40 and 22.97 mmol/kg DM in the distal tract, respectively (Figure 4). Valerate, isovalerate, 2-methylbutyrate, and medium-chain fatty acid caproate were only present in small amounts (< 5.00 mmol/kg DM) in cecal samples (Figure 4).

Other identified metabolites included glycerol, glucose (Figure 5E), myo-inositol, and lactate (Figure 5F), which all decreased steadily along the intestinal tract ($P < 0.05$) (Figure 4). Glucose had a very large individual variability, ranging from 1.68 to 698.27 mmol/kg DM in the jejunum and reaching its lowest concentration in the cecum ($P < 0.05$) (Figure 5E). Further, formate initially increased from the duodenum to the ileum (8.54 mmol/kg DM) and then decreased close to the detection limit in the cecum ($P < 0.05$) (Figure 4). Ethanol and succinate followed opposite patterns. Ethanol was only detected in the ileum and cecum, at similar levels ($P = 0.388$) and with large variations from 0.89 to 8.52 mmol/kg DM (Figure 4). Succinate levels were

initially low but increased distally ($P < 0.05$) with great individual variability, ranging from 0.30 to 194.81 mmol/kg DM (Figure 5G). The remaining metabolites fumarate, choline, hypoxanthine, nicotinamide, uridine, and uracil all had average levels below 6.00 mmol/kg DM in the duodenum and decreased further along the intestinal tract ($P > 0.05$), reaching concentrations below 1.00 mmol/kg DM in the cecum (Figure 4).

Age barely influenced metabolite concentrations in our study. For instance, uracil concentrations were minimally lower in the ileum at 10 compared to 7 wk of age ($P = 0.008$). Meanwhile, cecal butyrate levels were approximately 30% higher at the end of the fattening period compared to the middle ($P = 0.007$) but indifferent from the beginning at 7 wk of age ($P > 0.05$) (data not shown).

Apart from one exception (5-aminopentanoate), identified metabolite quantities were all slightly higher in EXP 2 compared to EXP 1 ($P = 0.0231$) (data not shown).

DISCUSSION

To date, there is no clear definition of turkey gut health, which is necessary to develop new strategies for improving gut health and, thus, animal health, welfare, and productivity. Therefore, the goal of the present study was to provide this fundamental knowledge by giving a comprehensive overview of the morphology, microbiota composition, and metabolome along the intestinal tract of clinically healthy *Salmonella*- and *Campylobacter*-free commercial B.U.T. 6 turkey hens at 7, 8, and 10 wk of age. We selected different time points during the fattening period, a production phase critical for intestinal development, to investigate a potential change of gut health characteristics over time. In total, 3 experiments were carried out with comparable results.

Gut Section Differences

Histomorphometric differences observed between intestinal sections of the investigated turkeys were consistent with their digestive function and reports from previous studies (Svihus, 2014; Bindari and Gerber, 2022). For instance, the present study confirmed the large surface area in the small intestine for rapid water and nutrient absorption (Svihus, 2014; Bindari and Gerber, 2022) and relatively shallow crypts associated with a low epithelial turnover (Iji et al., 2001). Cecae were confirmed to possess a smaller surface area, associated with a slower ingesta transit time and time-consuming microbial fermentation processes (Adji et al., 2019). Additionally, their deeper crypts indicate a higher enterocyte replacement rate (Iji et al., 2001).

As previous research on the microbiota composition of chickens and turkeys has suggested, *Firmicutes*, followed by *Bacteroidota*, *Proteobacteria*, and *Actinobacteriota*, were also the primary taxonomic phyla

identified in the present study (Wilkinson et al., 2017; Clavijo and Florez, 2018). At family level, however, the findings diverge from one another due to a lack of standardized approaches concerning potential influences on gut microbiota (Kohl, 2012). However, prior studies agree that microbiota composition varies between different intestinal sections once initial diversification during the brooding phase is completed (Yeoman et al., 2012; Xiao et al., 2021; Bindari and Gerber, 2022). *Lactobacilli* were often named common inhabitants of the proximal intestinal tract whereas *Lachnospiraceae*, *Ruminococcaceae*, and *Clostridia* were frequently found colonizing the ceca of humans and chickens (Biddle et al., 2013; Vacca et al., 2020; Yang et al., 2020; Xiao et al., 2021), which agrees with our results. To the best of our knowledge, the present study is the first to describe the microbiota composition of *Campylobacter*-free turkeys. Over the last decade, especially *Campylobacter jejuni* has increasingly been implicated in causing structural, functional, as well as microbiota changes in the gut of colonized chickens (Awad et al., 2018). In general, few bacteria identified in the gut of the turkey poult belonged to the families *Erysipelotrichaceae*, *Staphylococcaceae*, *Corynebacteriaceae*, *Enterobacteriaceae*, and *Rikenellaceae*. These bacterial families include some opportunistic pathogens, which have occasionally been associated with inflammatory processes, reduced SCFA production, enterocyte invasion, and disease during dysbiosis in humans and chickens but their role in turkeys remains unclear (Sannasiddappa et al., 2011; Shi et al., 2014; Kaakoush, 2015). Nevertheless, the number of beneficial bacteria predominated in this study. For example, *Lactobacilli* produce lactate, which lowers the local pH in the gut lumen, therefore strengthening the epithelial mucus layer and modulating the microbiota composition (Zdunczyk et al., 2015; Yang et al., 2020). *Lachnospiraceae*, *Ruminococcaceae*, *Clostridia*, and *Oscillobacter*, commensal anaerobes we identified in the hindgut, produce SCFAs (Biddle et al., 2013; Vacca et al., 2020), which significantly contribute to gut health by enhancing immune tolerance of the gut, preventing apoptosis, increasing mucus production, and improving transepithelial resistance (Yang et al., 2020).

As expected, within-sample richness and diversity were highest in the ceca (Asare et al., 2021). The number of identified OTUs increased along the gut. However, this was only partially true for the Chao-1 and Shannon indices where diversity was indifferent or decreased from the duodenum to the ileum in some experiments, which was in agreement with previous findings in broilers (Bjerrum et al., 2006; Lv et al., 2021). Moreover, research on layer hens also showed that Chao-1 is better suited than the Shannon index in revealing differences between gut sections (Xiao et al., 2021). The β -diversity was expected to be greatest between proximal and distal gut sections (Xiao et al., 2021), which was largely confirmed by this study. Illustrated by 2 separate clusters in the PCoA plot, the microbiota of jejunum and cecum samples were most dissimilar. Meanwhile, samples from the duodenum and ileum were scattered, sharing

phylogenetic similarities with both jejunal and cecal samples. Turkeys commonly exhibit coprophagic behavior which could explain the similarity between the duodenal and cecal content we found (Kers et al., 2018).

The concentration of identified metabolites varied between gut segments mostly as expected. Because the digestion of AAs, fats, simple carbohydrates, nucleotides, vitamins, and minerals occurs in the small intestine (Svihus, 2014), we anticipated their concentration to decrease from the duodenum to the ileum as they are absorbed from the lumen, which was confirmed in our metabolome analysis. In the ceca, microbial fermentation products, such as short- and medium-chain fatty acids, ethanol, succinate, lactate, and formate were all projected to increase (Den Besten et al., 2013), which coincided with most of our findings. Surprisingly, lactate and formate levels were reduced in cecal content. In this study, we predominantly found lactate- and formate-producing facultative anaerobes, such as *Lactobacilli* and *Romboutsia* species, in the proximal gut (Gerritsen et al., 2014; Ganzle, 2015; Xiao et al., 2021). Therefore, their fermentation products were likely absorbed by the host or utilized by other bacteria before reaching the ceca (Yeoman et al., 2012; Svihus, 2014). Overall, those metabolites largely produced by microbial fermentation, had the greatest variability in concentration, coinciding with the high fluctuations of microbiota composition between individuals observed in this study.

The remaining metabolites in cecal content may also be traced back to microbial activity. Glutamic acid is produced by *Lactobacilli*, especially when soy products are added to feed, which was the case in the present study (Zareian et al., 2012). Further, *Escherichia coli* were shown to produce uracil (Ha, 2016). Belonging to *Ruminococcaceae*, *Flavonifractor plautii* are involved in lysine catabolism, resulting in 5-aminopentanoate production (Medvecky et al., 2018). These bacteria were part of the cecal microbiota in our study but composed less than 1.00% of the total microbiota. Taurine is produced by yeasts and fungi (Cook and Denger, 2006), which are expected to colonize the cecum, explaining the spike of taurine in cecal content, but were not part of the microbiota investigation in this study. However, deconjugation of taurine-conjugated bile acids may also explain the increase of taurine in the cecal lumen (Volf et al., 2021). As bile resorption mainly occurs in ileum (Volf et al., 2021), taurine may proceed to the cecum. Additionally, taurine is common in feed, especially in plants, which is released during microbial fermentation of complex carbohydrates in the cecal lumen (Cook and Denger, 2006).

Age Effect

During the first week post-hatch, the turkey gut is populated with microorganisms. Initially the microbial community is comprised of facultative anaerobe *Proteobacteria*, which are gradually replaced by anaerobic *Firmicutes* as the oxygen-depleted distal tract grows and develops (Xiao et al., 2021). This diversification during

the early rearing phase is accompanied by structural and functional changes, including increases in VH and CD to maximize absorptive surface area as well as expression of nutrient transporters and brush border enzymes (Iji et al., 2001; Awad et al., 2008). Gut maturation is usually completed during the fattening period, between 7 and 9 wk of age (Grimes, 2015; Gous et al., 2019a; Gous et al., 2019b; Xiao et al., 2021).

Despite a consistent diet during the fattening period, this study revealed structural changes, especially in the small intestine, between 7 and 10 wk. Explicitly, villi generally became slightly longer and narrower, leaving VSA unaffected by age. Previous research on broilers reported similar age-related morphological changes of small-intestinal villi without increased absorptive area or nutrient requirement (Iji et al., 2001). We further observed deeper crypts in the duodenum toward the end of the fattening period compared to the beginning, indicating a higher turnover rate of enterocytes (Iji et al., 2001). However, this was different from former research on mice and pigs describing a decrease in proliferative activity of crypts and lower enterocyte turnover rate postweaning as the gut matures (Morita et al., 1994; Brunsgaard, 1997). Though this was not true for the small intestine in the present study, we recorded that cecal crypts became shallower over time, which coincided with our observation that cecal villi themselves did not undergo morphological changes over time, unlike their small intestinal counterparts. Our findings suggest that the ceca reach maturity earlier than the proximal gut.

The stability in diet along with high zoohygienic standards and strict biosecurity measures were reflected in gut microbiota composition and diversity as well as metabolite concentrations, which varied little over time in the present study. Only few microorganisms and metabolites were minimally affected by age, which, presumably, was biologically insignificant. Studies in broilers also revealed stability of the microbiota composition if feed is kept constant and once maturation is reached 30 d posthatch, the end of the grower phase (Gao et al., 2017). Similar to our study, these birds were fed single base diets to avoid phase feeding during the maturation process (Gao et al., 2017). Therefore, it supports our findings that the microbiota community changes very little in healthy birds once gut maturation is completed and strict biosecurity prevents the contact with other animals, such as wild birds, rodents, and insects.

Variations Between Experiments

Overall, our study produced results with a high repeatability across experiments. To keep potential influencing host-factors as constant as possible, we chose animals of the same breed, sex, and age (Kers et al., 2018). In general, a repetition of these experiments with male turkeys and other breeds is recommended. We employed the same feeding and management regime in each experiment. Additionally, our study is unique in

such manner that dietary changes were avoided during the investigation period to exclude any direct effects on gut components (Bindari and Gerber, 2022). In the field, the fattening period is normally accompanied by multiple feeding phases, which may directly affect gut morphology, microbiota, and metabolites. Despite tightly controlled experimental circumstances, we expected external influences, such as differences in season, the environment, feed composition, and parent flocks to cause some variation of the investigated factors between experiments (Kers et al., 2018; Bindari and Gerber, 2022).

Season has a direct impact on climactic conditions, such as the temperature and humidity, within poultry houses. In our study, EXP 1 was conducted in fall, EXP 2 in summer, and EXP 3 in winter months. Even though the housing facility in this study is temperature-controlled, extreme weather conditions occasionally lead to temperature fluctuations as high as 4°C. EXP 3 was affected by an unusual cold-period in the third and fourth week, during which the birds had a reduced weekly body weight gain. Both cold and heat stress can alter intestinal morphology by causing villus stunting and reduced VSA (Adji et al., 2019). In our study, histomorphometric samples were only collected after the birds had regained their weight in wk 7–10. Surprisingly, we found wider villi with an increased surface area compared to the other 2 experiments. To meet the high nutritional demand for compensatory growth, VSA was likely increased by inducing enterocyte proliferation (Iji et al., 2001). Unfortunately, due to the experimental setup, it was impossible for us to measure feed intake.

In EXP 2, the microbiota composition was significantly different from the other 2 experiments. *Lachnospiraceae* and *Peptostreptococcaceae* were partially displaced by *Rikenella* and *Corynebacteria* species. The biological significance of this microbial shift remains unknown and it was not reflected in the metabolite composition. It remained unchanged with the exception that the concentrations of all but one metabolite were increased. We calculated metabolite concentrations per kg DM content, which we determined from pooled luminal content of each intestinal section. It is possible that intestinal content collected during the summer months had a higher DM content, artificially increasing metabolite concentrations during EXP 2. However, the fact that general metabolite profiles were comparable between EXP 1 and 2 despite the shift in microbiota, it is likely that the effect occurred locally.

Implications and Limitations

Poultry performance has a direct link to intestinal health (Scupham, 2007; Awad et al., 2018). Especially in commercial turkeys, the poultry industry still lacks a clear definition of a healthy gut which goes beyond the mere absence of disease (Kogut, 2017). In a world where antimicrobial resistance has emerged as a silent pandemic (Hafez and Attia, 2020), there is a pressing need to define “gut health” and use this information to guide

the development of new strategies to improve gut health and, thus, achieve maximum productivity and performance (Bindari and Gerber, 2022). To the best of our knowledge, the present study was the first to describe the morphology, microbiota, and metabolome of *Salmonella*- and *Campylobacter*-free commercial turkey hens during the fattening period.

The study expanded on past research of the avian gut, which predominantly focused on chickens, and confirmed differences in intestinal structure, microflora, and metabolites, depending on the sampling location in turkeys. In addition, we demonstrated that gut maturation, signified by a stable microbial community, intestinal metabolites composition, and intestinal structure, is reached earlier in the ceca than in the small intestine where the epithelium still undergoes morphological changes throughout the fattening period. As the ceca are the predominant colonization location for most gut microorganisms, this is an important finding to inform future studies investigating gut health improvement strategies in turkeys. There is circumstantial evidence in humans, swine, and poultry that a balanced gut microbiota community is not easily modified once microbial maturity is reached, highlighting the necessity to define the age of gut maturation (Barba-Vidal et al., 2018; Wieers et al., 2020; Xiao et al., 2021). Probiotics microorganisms, such as *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacillus*, *Pediococcus*, *Streptococcus*, and *Saccharomyces spp.*, and prebiotics, such as inulin, have been utilized to fuel the growth of beneficial microorganisms and modify gut structure in the field (Zdunczyk et al., 2015; Dobrowolski et al., 2019). However, data has been inconsistent and difficult to reproduce (Barba-Vidal et al., 2018). In the present study, we reported variability in microbiota composition between experiments despite relatively consistent experimental conditions, offering a potential explanation for inconsistent results concerning probiotic use in the field. Therefore, the impact of the environment, in which an animal is raised, should not be underestimated when investigating gut microorganisms. How an animal is raised, largely affects the rate of initial gut colonization, being slowest in a high biosecurity setting with limited environmental microorganisms and quickest when contact with mother hens facilitates natural microbiota transfer (Kubasova et al., 2021). In the present study, we demonstrated that cecal maturity in female B.U.T.6 turkeys is reached before 7 wk of age if they are clinically healthy, *Campylobacter*- and *Salmonella*-free, management regimes are kept stable, biosecurity measures are high, and a single diet is fed. Field studies have to be conducted on a larger scale to elaborate on these findings. Even though these results are based on a relatively small number of animals and the investigation period was restricted to 4 wk, these findings suggest that gut maturity can be reached under these particular circumstances. Antibiotics and disease delay gut maturation whereas probiotics can be used to accelerate gut microbiota stability by several wk, increasing disease resilience (Gao et al., 2017). Overall, results from the present study suggest that pre-

and probiotic preparations may be more effective when implemented earlier, before the fattening period, when gut maturation has not been completed or when it is disturbed due to disease, stress, or antibiotic use, but further aspects need to be addressed in future studies with fattening turkeys (Gao et al., 2017).

ACKNOWLEDGMENTS

This study was kindly funded by the German Research Foundation, Bonn, Germany (grant numbers SR-767/8-1, AL-2182/2-1, 398967434, SFB/TR261). Work carried out at the University of Greifswald was part of the Mecklenburg-Western Pomerania State Excellence Initiative, funded by the European Social Fund grant KoInfekt (grant number ESF_14-BM-A55-0005_16) and Leibnitz WissenschaftsCampus ComBio-Cat (grant number W10/2018). This Open Access publication was funded by the German Research Foundation, Bonn, Germany, (grant number 491094227) “Open Access Publication Costs” and the University of Veterinary Medicine Hannover, Foundation. The authors would also like to thank Mrs. Hilke Bartels and Mrs. Rebecca Lindenwald from the Clinic for Poultry, University of Veterinary Medicine Hannover, for her help with laboratory work and advice on statistical analysis, respectively.

DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102046.

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