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Original Article

Changes in intestine integrity and caecal microbiome of Cobb-500 meat bird chickens during 24-hour feed withdrawal

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ABSTRACT

Poultry meat is one of the main sources of animal protein globally and this category is associated with human entero-pathogens *Salmonella* and *Campylobacter*. The management and/or prevention of these bacteria is multi-facet from farm to fork. Feed withdrawal (FW) time of meat chickens is an on-farm intervention to reduce gross and entero-pathogen contamination. The current practice of 8-12 hours FW is to ensure caecal emptying and intestinal integrity thus minimising intestinal rupture during processing. A controlled seeder bird challenge (10^5 CFU/mL) study investigated the effect of FW on the modern Cobb 500 meat bird at 2-hour intervals for 24 h. Anatomical gross gut (intestine integrity score, tensile strength) morphology showed no real change. The significance of a transient improvement in intestine tonicity at 14 and 16 hours is unclear but interesting. Histologically, the ileum observed a subtle change in gross diameter and increases of villi height and crypt depth with FW. Caecal microbiome was investigated by classical enumerations of entero-pathogens and 16SrRNA analysis. Populations of caecal *Campylobacter* remained stable (10^8CFU/g at 100% prevalence), throughout FW, whereas, *Salmonella* populations were at low levels (10^2 CFU/g) in caeca and did not change with FW. There was a higher prevalence of *Salmonella* seen in 16SrRNA as compared to cultural methods. The microbiota changes seen by 16SrRNA analysis are subtle but do demonstrate increases in the proportion of two families of bacteria *Bacteroidaceae* and *Enterobacteriaceae* and the decrease in *Lachnospiraceae* and *Lactobacillaceae* over FW time.

KEYWORDS: enteropathogen, *Salmonella*, *Campylobacter*, feed holding, macro and microscopic changes, 16SrRNA.

INTRODUCTION

Poultry meat consumption is increasing throughout the developed world [1] and this category has been associated and attributed to the two largest causes of human bacterial gastroenteritis, *Campylobacter* and *Salmonella*, with reported case rates of *Salmonella* and *Campylobacter* (per 100,000 people) of 14.4 and 13.3 in USA [2], 64.8 and 19.7 in EU [3] and 146.9 and 74.7 in Australia [4]. Even though many bacterial enteric outbreaks in recent years have been linked to fresh produce and fruit, enteric disease is still often attributed to poultry meat [5-7].

On-farm measures to control enteropathogens in commercial meat chicken flocks, aim to reduce the enteropathogen colonisation of the bird. Control methods include animal husbandry, biosecurity, probiotics and vaccinations [6, 8]. Preslaughter methods are required to reduce the probability of carcass contamination during evisceration and dressed carcase processing [9, 10].

Feed withdrawal (FW) is a common preslaughter method used by industry. FW is the process of

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removing access to feed for a set period of time prior to processing. This method was first described by Smidt *et al*. (1964) who measured the effects of FW on carcass yields [11]. Wabeck (1972) observed that FW reduced gross faecal contamination during transport [12]. Previous research suggests 8 to 12 hours of FW period as optimum in decreasing the incidence of contamination without affecting carcass yield [9, 13]. When FW is <8 h the gastrointestinal tract contains digesta and may have a high probability of contamination, while at >12 h, the integrity of the gut degrades, resulting in rupturing of the intestines during evisceration, increasing faecal contamination of the carcass [12, 14]. While a period of FW reduces the amount of ingesta in the intestines, both *Salmonella* and *Campylobacter* remain in the crop and caeca [13, 15]. The increased retention in the digestive tract of these key pathogens and subsequent rupturing of the intestine during evisceration is a common source of carcass contamination in a processing plant [16].

Currently, implemented FW times vary between poultry producing regions, Australia 8 to 12 [17], or to 18 hrs [18], USA 8 to 14 [19] while the EU sets the limit "subject to welfare issues", with no specific time frame guidelines [20, 21]. These industry guidelines have been produced by considering published research that investigated changes to intestine morphology and microbiological content of broilers in response to FW [13, 22-25].

The objective of this study was to measure the effects of FW on macro and microscopic physiological gut changes and their corresponding bacteria flora population changes, using both classical cultural methods and 16s RNA typing. The trial was designed to mimic real-world conditions: commercial meat birds (Cobb-500) were grown under commercial conditions in pens to 42 days of age, and challenged with field isolates of *Salmonella* serovar Typhimurium and *Campylobacter jejuni* at 3 days of age.

MATERIALS AND METHODS

Animal ethics

All experimental work was approved by the Birling Animal Ethics Committee, in accordance with the Animal Research Act of NSW (1985).

Birds and husbandry

Commercial broilers (Cobb Valance 500 line birds) were raised with a standard commercial feed program [26], in miniaturised floor pens (3.5 m^2) to Australian commercial stocking density with *ad libitum* access to feed and water for 43 days. A total of 350-day old chicks were placed in 10 pens (35 birds per pen), monitored daily, pen weight measured weekly and any mortalities were autopsied.

Salmonella and *Campylobacter* challenge

At placement 10 seeder birds from each pen were tagged and orally inoculated with field-isolated strains of *Salmonella enterica, enterica* serovar Typhimurium 135a and *Campylobacter jejuni* at 7.6 x 10^5 CFU/mL and 4.8 x 10^5 CFU/mL, respectively. The birds were monitored for the presence of both *Salmonella* and *Campylobacter* throughout their grow out period using weekly drag swabs of the pen floor and cloacal swabs (2 swabs per pen from wing tagged seeders and 4 from randomly selected birds).

The presence of both *Salmonella* and *Campylobacter* in drag swabs and cloacal swabs collected during bird grow out were culture confirmed using modified Australian standard culture methods [27, 28]. For *Salmonella* the drag or cloacal swabs were covered in buffered peptone water (BPW) and incubated at 37 °C overnight. The non-selective BPW culture was then transferred into two selective enrichment media, Rappaport-Vassiliadis (RVS) (Edwards Group) and Tetrathionate Hajna (TT) (Edwards Group). For RVS enrichment 100 µl of BPW was transferred into in 9.9 ml of RVS and incubated overnight at 42 °C. For TT enrichment 1 ml of BPW was transferred into 9 ml of TT broth and incubated overnight at 37 °C. The selective broths were plated onto Hektoen and XLD plates (Edwards Group), and incubated overnight at 37 °C. Typical colonies were transferred onto ChromID *Salmonella* agar plates (Edwards Group), and incubated overnight at 37 °C. Typical colonies were then transferred to Nutrient Agar and incubated overnight at 37 °C. The isolates were then confirmed as *Salmonella* Typhimurium by serological grouping with O:5 and H:i antisera (Cell Biosciences). For *Campylobacter* the swabs were incubated micro-aerophilically at 42 °C for

48 hr in Bolton broth, then 10 µl of the enrichment broth was streaked on *Campylobacter* Food Agar (CFA) plates. After microaerophilic incubation at 42 °C for 48 hours typical colonies were confirmed as *Campylobacte*r by mass spectrometry (VITEK-MS, Biomerieux).

Feed withdrawal

A total of 12 time points of FW were investigated, from 2 to 24 hours off feed, at 2-hour intervals. Each time point sampled 10 individual birds, with 2 birds randomly collected from 5 different pens. FW was divided into groups so that sampling time was reduced to 12 h. The feed from the first group (pens 1-5) for time points 14-24 hours was removed the evening before, and the second group (pens 6-10) for the 2-12 hour time points the morning of the experiment. After FW the birds remained in the pens for 2 more hours with access to water. Birds to be used for the experiment were then transferred in poultry transport crates (5 birds per crate). With the exception of the 2 hour time point birds (that were crated and transported at 1 hour and 45 min), the crated birds were then transported in a trailer to the laboratory scale processing site. Birds were processed every hour in the following order: 2, 14, 4, 16, 6, 18, 8, 20, 10, 22, 12 and 24 hours off feed. Before processing the birds were held in an air-conditioned room (22 °C) out of sight of the processing line.

The birds were processed *via* gas stunning 20% CO_2, 20% N_2 and 60% O_2 (premixed gas cylinder, BOC Australia) for 2 minutes in a dark chamber (as is standard practise in commercial gas stunners). Once stunned the birds were slaughtered by cervical incision and bleed for 2 min. Qualified veterinarians then dissected and scored the gastrointestinal tract. Each gastrointestinal tract was laid out, photographed and gut integrity scores were determined; samples for tensile strength, histology, and microbial analysis were collected.

Gut integrity scores

The gut integrity for each carcass was scored out of 10, using methods modified from Teirlynck *et al.* (2011) [29]. The final accumulative gut score included observations of crop content (empty=0, scarce=1, full=2), intestinal tonicity (present/has tone=0, absence/no tone=1), translucency (absence=0 presence=1) and the content (empty=0, scarce=1, full=2). The intestinal tonicity, translucency and content were measured in both cranial and caudal regions and were designated a cumulative maximum score of 10. Descriptions of any gut content and the presence of mucus were also recorded.

Tensile strength of the ileum

The intestine of each bird was tested using a texture analyser (Model TA-XT2, Key Diagnostics) fitted with a noodle rig, based on previously described methods [14]. The content of the gut was not removed as it's an integral part of gut integrity/strength. The assayed section (20 cm) was dissected from the ileum from 5 cm (cranially) from the end of the cecum tips. The rig setup had the two arms set at 5 cm apart, the intestinal ends were wrapped around the two arms, using a gauze strip (to prevent slipping), any slack was taken out of the sections without stretching them, and then they were pulled apart at 100 mm/min. The maximum force and the distance to break point were all measured. If the sample slipped or did not break it was excluded from analysis. All experiments were performed immediately after dissection at room temperature.

Histology

A 2 cm section of the ileum was dissected cranially adjacent to the ileum section used in the tensile strength experiments. The sections were fixed in 10% formalin (Merck) within 10 min of bird death and stored at room temperature. Histological analysis of haematoxylin and eosin stained sections was performed by the Elizabeth Macarthur Agricultural Institute (NSW, Australia). To ensure accurate measurements care was taken to orientate the sections in the embedding cassettes so that sections were uniform cross-sections. The fixed sections were measured for their diameter, circular muscularis, total muscularis, villus height and crypt depth by qualified histopathogists.

Microbiological analysis of caecal content

The caeca contents were used to enumerate the total viable count (TVC), *Salmonella* and *Campylobacter*

present using a miniature most probable number (mMPN) system [30, 31]. The contents of one of the caeca was homogenised in 1:10 (w/v) of BPW (Edwards Group). This emulsion was then used to set up 2 separate plates of mMPN dilutions. The first mMPN plate used BPW as the diluent and was used to enumerate the TVC and *Salmonella*. The second plate used modified Boltons broth (Edwards Group), containing 25 ug/mL sulfamethoxazole (Sigma-Aldrich) and 150 ug/mL 2,3,5-triphenyltertrazolium (Sigma-Aldrich) as the diluent and was used to enumerate *Campylobacter*. The dilutions for each mMNP used a plate of racked 2 ml tubes, the first row containing the neat caecal emulsion, the next 11 rows 900 µl of the diluent, and a 1:10 serial dilution was performed 11 times allowing for accurate enumeration from 10^0 to 10^{11} CFU/g.

The BPW mMPN was incubated at 37 °C for 18 hrs, and the TVC results were read by the presence or absence of turbidity. The BPW plate was then used for the enumeration of *Salmonella*. A *MSRV selective* enrichment was set up by transferring 100 ul of the BPW enrichment to a duplicate plate of racked 2 ml tubes containing 500 ul of *MSRV*; this plate was incubated at 42 °C for 48 hours. The *MSRV* enrichment was then struck onto ChromID *Salmonella* (Edwards Group) plates (divided into 8 divisions). After incubation for 18 hrs at 37 °C the plates were observed and any typical colonies were considered positive for *Salmonella*.

Campylobacter mMPN was incubated in microaerophilic conditions at 42 °C for 48 hrs; a red colour is an indication of the presence of *Campylobacter*. Confirmation of *Campylobacter* was made by streaking 1 µl of the Bolton's (ThermoFisher) enrichment onto CFA (BioMerieux) plates. After microaerophilic incubation at 42 °C for 48 hours the presence of *Campylobacter* was confirmed based upon the growth of typical colonies.

Metagenomics

10 individual birds from each time point (except 20 hrs where one of the libraries failed) had a ceaca (which were stored at -80 °C) designated for metagenomic analysis using 16SrDNA. DNA was extracted from 200 mg of caecal content using the QIAamp Fast DNA Stool Kit (Qiagen). The Ion 16S™ Metagenomics Kit (ThermoFisher) was then used to make libraries that were sequenced on the Ion Chef/S5 system (ThermoFisher)) using a templating size of 200 bp and sequencing with 500 flows. Bam files from the sequencing were then analysed using Ion reporter software (https://ionreporter.thermofisher.com/ir/secure/home.html), using the default Metagenomics workflow and both the Curated Greengenes v13.5 and Curate MicroSEQ®16S Reference Library v2013.1 databases. The default settings were used; the minimum alignment coverage for a read to be included was 90%. To make a genus ID the percentage identity was 97% and the species ID 99.0%. A minimum of 10 reads were required for an operational taxonomic unit (OTU) to be included in the analysis.

Statistical analysis

Results for the gut integrity scores, tensile strength, histology, bacterial enumeration and metagenomics were analysed with Stata 14. The level of significance (α) was set to 0.05.

RESULTS AND DISCUSSION

Bird grow out

At 41 days the birds reached a mean weight of 3.17 Kg±0.079 Kg which meets the Cobb-500 growth target of 3.17 Kg at 41 days [26]. A comparison of the weekly weight gain to the Cobb-500 standard and a previously published broiler performance trial [32] demonstrate the birds gaining weight as expected for commercial Cobb 500 broilers. For the first 4 weeks the birds weigh less that the optimal weight of the Cobb - 500 standard, indicating that the starter feed was not optimal (Supplementary Table 1). The birds raised can be considered to be a good representation of birds produced by a larger scale commercial broiler operation. There was a slight non-significant (Student t test = 0.24) pen effect with pens 1-5 having lighter birds at 3.1 Kg (0.04) compared to pens 6-10 weighing 3.2 Kg (0.12).

The use of the seeder bird method to infect the flock with *Salmonella* and *Campylobacter* had the

Table 1. *Salmonella* and *Campylobacter* flock prevalence during grow out.

	Day 7				Day 14				Day 21				Day 28				Day 35				Day 41			
Pen	***Salmonella* Drag Swab (+/-)**	***Salmonella* Cloacal Swab (n/N)**	***Campylobacter* Drag Swab (+/-)**	***Campylobacter* Cloacal Swab (n/N)**	***Salmonella* Drag Swab (+/-)**	***Salmonella* Cloacal Swab (n/N)**	***Campylobacter* Drag Swab (+/-)**	***Campylobacter* Cloacal Swab (n/N)**	***Salmonella* Drag Swab (+/-)**	***Salmonella* Cloacal Swab (n/N)**	***Campylobacter* Drag Swab (+/-)**		***Salmonella* Drag Swab (+/-)**	***Salmonella* Cloacal Swab (n/N)**	***Campylobacter* Drag Swab (+/-)**		***Salmonella* Drag Swab (+/-)**	***Salmonella* Cloacal Swab (n/N)**	***Campylobacter* Drag Swab (+/-)**		***Salmonella* Drag Swab (+/-)**	***Salmonella* Cloacal Swab (n/N)**	***Campylobacter* Drag Swab (+/-)**	
1	+	2/6	+	6/6	+	2/6	+	6/6	+	1/6	+	6/6	+	2/6	+	6/6	+	2/6	+	6/6	+	1/6	+	6/6
2	+	1/6	+	4/6	+	1/6	+	6/6	+	0/6	+	6/6	+	0/6	+	6/6	+	1/6	+	6/6	+	1/6	+	6/6
3	-	0/6	+	6/6	+	0/6	+	6/6	+	0/6	+	6/6	+	1/6	+	6/6	+	2/6	+	6/6	+	1/6	+	6/6
4	+	2/6	+	6/6	+	1/6	+	6/6	+	0/6	+	6/6	+	0/6	+	6/6	+	2/6	+	6/6	+	1/6	+	6/6
5	-	1/6	+	5/6	+	0/6	+	6/6	+	2/6	+	6/6	+	2/6	+	6/6	+	2/6	+	6/6	+	0/6	+	6/6
6	+	0/6	+	3/6	+	0/6	+	6/6	+	1/6	+	6/6	+	0/6	+	6/6	-	1/6	+	6/6	+	0/6	+	6/6
7	+	1/6	+	4/6	+	2/6	+	6/6	+	2/6	+	6/6	+	2/6	+	6/6	+	2/6	+	6/6	+	2/6	+	6/6
8	+	1/6	+	6/6	+	2/6	+	6/6	+	3/6	+	6/6	+	2/6	+	6/6	+	0/6	+	6/6	+	2/6	+	6/6
9	+	0/6	+	5/6	+	2/6	+	6/6	+	2/6	+	6/6	+	2/6	+	6/6	+	2/6	+	6/6	+	0/6	+	6/6
10	+	1/6	+	5/6	+	1/6	+	6/6	+	4/6	+	6/6	+	4/6	+	6/6	+	2/6	+	6/6	+	3/6	+	6/6
Total		9/60		50/60		11/60		60/60		15/60				15/60				16/60				11/60		

Drag swab results are presented as *Salmonella* or *Campylobacter* detected (+) or not detected (-).
Cloacal swabs are presented as the number of *Salmonella* or *Campylobacter* positive swabs (n) detected out of the total 6 (N) collected per pen.
Cloacal swabs were not taken for *Campylobacter* detection from day 21 onwards.

aim of mimicking natural field colonisation rates that would be observed in commercial flocks. Other FW studies of *Salmonella* in birds use high 10^{8-9} CFU challenges at 5-7 days before bird sacrifice, with antibiotic-resistant laboratory strains paired with subsequent antibiotic treatment of the bird resulting in high prevalence of *Salmonella* [13, 33, 34]. While this makes it easier to detect *Salmonella* in experimental birds, these models are not a true representation of the naturally infected flocks, with antibiotic treatment modifying the other background microbiota. In this study the use of field isolates of both *Salmonella* and *Campylobacter* that had been isolated from the poultry food chain ensured a colonisation of the birds as would happen in commercially raised birds. The introduction *Salmonella* at placement in 30% of birds (*via* the seeder birds) also allowed horizontal colonisation of the flock.

At 1-week post challenge with *Salmonella* Typhimurium, the in-flock prevalence for *Salmonella* was 15% (9/60 cloacal swabs) that increased to18% (11/60) the following week and then stabilised around the 20-25% for the duration of the grow out phase (Table 1). Globally, commercial flocks positive for *Salmonella* have 18-26% individual birds positive for *Salmonella* (USA (18-21.7%), Canada (23%) and Australia (26.5%)) [35-38]. In contrast, *Campylobacter* once having entered a flock quickly colonises all birds [39-41]. Such a colonisation pattern was observed in this study. Prevalence at week 1 was 83.3% (50/60 cloacal swabs) that reached 100% by week 2. *Campylobacter* drag swabs showed that all pens were positive throughout the bird grow out period. The seeder bird method used in this study provides a good model for natural flock infection of both *Salmonella* and *Campylobacter*.

Macro and microscopic analysis of ileum

Examination of the images of the intestinal layouts did not show any obvious gross macroscopic changes over time (Figure1, Supplementary Figure 1); in general the intestines were heterogenous at each time point. This is contrast to a previous study that describes the shape of the intestine changing from round (0-3 hours), to flat (9-14 hrs) back to round (but gas filled) from 14-24 hrs post FW [23]. These observed differences to previous studies could be caused by a range of factors such as bird breed, stress during transport; however due to extreme environmental conditions on the day of FW the birds were held in a temperature-controlled

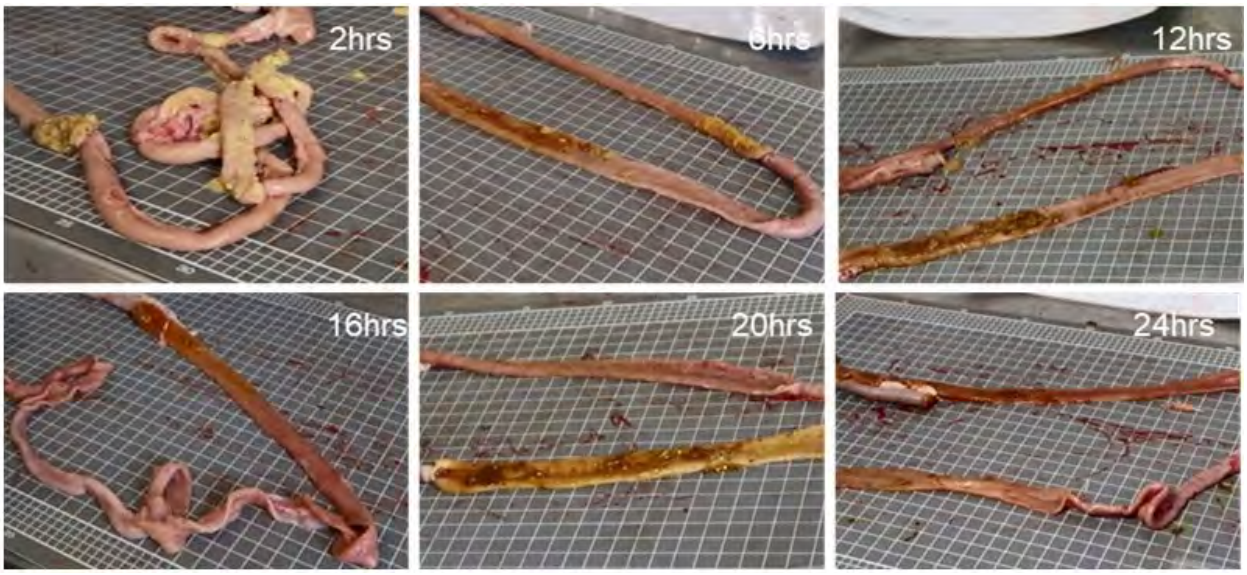

Figure 1. Macroscopic changes to intestine in response to feed withdrawal. Examples of the intestine content at 2, 6, 12, 16, 20, and 24 hours.

laboratory (25 ºC) resulting in potentially less stress, dehydration and weight loss than would normally have occurred during holding in a commercial slaughtering environment.

The gut integrity score (GIS) was used to quantify the general condition of the intestine in response to FW. GIS ranged from 0 to 6 with no statistically significant difference over time (Table 2), but a decrease (improvement) in the gut integrity score at the 14 h and 16 h time points were observed. The crop and the cranial section of intestine were full of feed and/or water at the earlier time points of 2 and 4 h post FW in 20% of birds and then remained scarce or empty from 6 h FW. The caudal section was full in 50% of birds at the 2 h FW, and then, with the exception of a single bird at 8 h FW, remained scarce or empty for the remaining time points. Previous studies have shown that FW clears feed from the crop within 3-9 hrs [23] and ingesta from the intestine within 8-12 hrs [42]. The data in this trial was able to reproduce the previous findings of feed clearance from the digestive system with FW.

The tonicity of the intestine was good (there was tone) in 100% of birds cranially for the first 6 h FW and caudally at the 2 h FW (Table 2). The percentage of birds with poor tonicity (there was no observable tone) increased in both the cranial and caudal sections up until the 12 h FW point when at least 60% of the birds had poor tonicity (Table 2). A transient improvement in tonicity was observed at the 14 and 16 h FW with 90-100% of birds having good tonicity. After this time point the intestines lost tone with the majority of birds in the 18-24 time points having no observable tone. The translucency scoring did not vary significantly with FW, with only 0 to 20% of birds at any time point having observable translucency in either the cranial or caudal section. The use of the GIS scoring system was adapted from a method used to quantify bird intestinal health [29] with the view to identify diseased birds. In retrospect this system was not suitable for this study, as the birds were healthy. The qualitive description of the presence/absence of intestinal content is a more useful indication of appropriate FW for commercial processing than a complex GIS scoring system.

Tensile strength experiments (Table 3) showed large amounts of variability within each time point. The study mean (SD) for maximum force was 345.1±81.22 g and the break distance was 63.37±27.0429 mm. The tensile strength significantly changed with FW. Maximum force had significantly decreased over time when analysed as a linear, quadratic and Log transformed data. The break distance significantly changed when analysed quadratically. These changes were similar to what was seen in a previous study of 52-day old broilers, where a reduced tensile strength was observed with FW [14]. In this previous study the force required to break the intestine of 42-day old birds was 244 g and the break distance was 20 mm, both these measurements were much less than this study. While the variation between studies could have been due to a number of reasons such as age or bird breed, the most likely factor, in this study, was not removing the content from the ilium before measurement. In this study there was on average SD of 20% for the maximum force measurements and 30.7% for the break distance, demonstrating a large variability in the data. This can be in part attributed to the variation in the preparation of the samples. Trimming connective tissue without damaging the intestine was difficult, incomplete removal gave higher values and accidental damage to intestine caused lower values. In general, using this method to measure tensile strength was difficult and not a reliable quantitative measure and would not be recommended for future studies of the tensile strength of broiler intestines.

The histological analysis (Supplementary Figure 2) of the ileum section allowed for observation of the microscopic changes during FW. The histological measurements (circular muscularis, total muscularis, villus height, crypt depth and ratio of villus height to crypt depth) were investigated for any linear or quadratic relationship to FW. A quadratic relationship with FW was observed for the gross diameter, villus height and crypt depth (Table 4). When compared to the 2 hrs FW there were some significant differences in the 6 histological measurements examined at individual time points (Table 4). The gross diameter of the ileum was significantly smaller for all but the 4 and 14 hr FW points, and the circular muscularis was

Table 2. Feed withdrawal time effect on intestine gut integrity score (GIS).

		GIS	Crop Fill Score (%)			Cranial					Caudal				
						Gut Fill Score (%)					Gut Fill Score (%)				
Time off Feed (h)	N		Empty	Scarce	Full	Empty	Scarce	Full	Translucency (%)	Tonicity (%)	Empty	Scarce	Full	Translucency (%)	Tonicity (%)
2	10	2.3 (2.11)	60	20	20	80	.	20	90	100	40	10	50	90	100
4	10	1.6 (1.84)	90	.	10	50	30	20	90	100	80	20	.	70	90
6	10	1.1 (1.45)	80	20	.	60	40	.	100	100	80	20	.	90	80
8	10	1.4 (0.70)	100	.	.	70	30	.	90	50	80	10	10	100	80
10	10_a	1.6 (1.13)	100	.	.	90	10	.	100	40	78	22	.	100	33
12	10	1.8 (0.63)	100	.	.	90	10	.	90	20	90	10		100	30
14	10_b	0.6 (1.06)	100	.	.	90	10	.	80	100	75	25	.	88	100
16	10_c	1 (0.87)	100	.	.	70	30	.	80	90	89	11	.	78	100
18	10	2.5 (0.97)	100	.	.	60	40	.	100	20	80	20	.	80	10
20	10	1.6 (1.27)	100	.	.	90	10	.	80	50	90	10	.	100	30
22	10_d	2.2 (1.09)	100	.	.	100	.	.	90	20	90	10	.	67	22
24	10	1.9 (0.99)	100	.	.	80	20	.	100	10	100	.	.	90	30

GIS is out of max score of 10, represented as mean (SD).
N_a = 9 for GIS and caudal measurements at 10 hours off feed, N_b = 8 for GIS and caudal measurements at 14 hours off feed, N_c = 9 for GIS and caudal measurements at 16 hours off feed, N_d = 9 for GIS and caudal measurements at 22 hours off feed.
Translucency and tonicity are presented as the percentage (%) of "good" translucency or tonicity samples out of the total (N) number of samples at each time point off feed. Percentages are rounded to the nearest integer.

Table 3. Feed withdrawal time effect on intestine tensile strength.

Time off Feed (h)	N	Max force (g)	Break distance (mm)
2	10	358.6 (97.72)	64.4 (22.78)
4	10	348.3 (76.81)	74.1 (16.96)
6	10	377.4 (66.73)	83.8 (33.67)
8	10	384.7 (66.14)	101.5 (32.80)*
10	10	353.3 (99.83)	87.2 (28.73)
12	10	323.1 (95.88)	49.5 (24.52)
14	10	306.7 (50.61)	71.1 (7.15)
16	10	344.7 (68.31)	81.6 (11.59)
18	10	334.8 (56.01)	100.1 (27.94)*
20	10	320.4 (73.62)	105 (34.04)*
22	10	305.4 (31.84)	46.2 (20.87)
24	10	316.3 (39.64)	58.9 (15.05)
P values			
Linear		0.024	0.382
Log transformed		0.091	0.223
Quadratic		<0.001	0.034
Log transformed		<0.001	0.054

Max force, break distance and gut integrity score presented as mean (SD).
*P < 0.05 when regressed against what is measured at 2 hours off feed.

significantly larger at the 4, 8, 12 and 22 hr FW points. The total muscularis measurement was significantly larger at the 4, 12 and 22 hr FW points. The villus height was significantly larger at all but the 24 hr FW, the crypt depth significantly larger at the 8, 20 and 22 hr FW and the V/C measurement was larger at the 10, 18 and 24 hr FW.

These results are different from a previous study of intestinal morphology changes in broilers with FW of 8, 12 and 24 hrs [25]. This previous study observed ileal villi heights to be unaffected by FW, but villus width and crypt depth decreased. The jejunal villus height increased, while crypt depths increased until 12 h of FW and then declined at 24 h which correlates well to the observations of the ilium section in this study. Villi sloughing has previously been described to occur from as early as 9 hours after FW, as determined by a fairly subjective assay 'the relying on feeling villi vibrations with the technician's fingers' [23]. The histological examination in this study did not identify any generalised cellular sloughing even at 24 hours of FW. Thompson and Applegate (2006) also observed a significant reduction in mucus content with FW. They concluded that mucus reduction was an important factor in increasing the risk of *Salmonella* colonisation of the intestine; therefore FW had a negative effect on the risk of carcass microbial contamination during processing [25]. Neither the macro nor microscopic observation in this study identified changes in mucus with FW.

Neither the macro nor micro examination of the gut over FW times trialled demonstrated appreciable differences which could be used in a

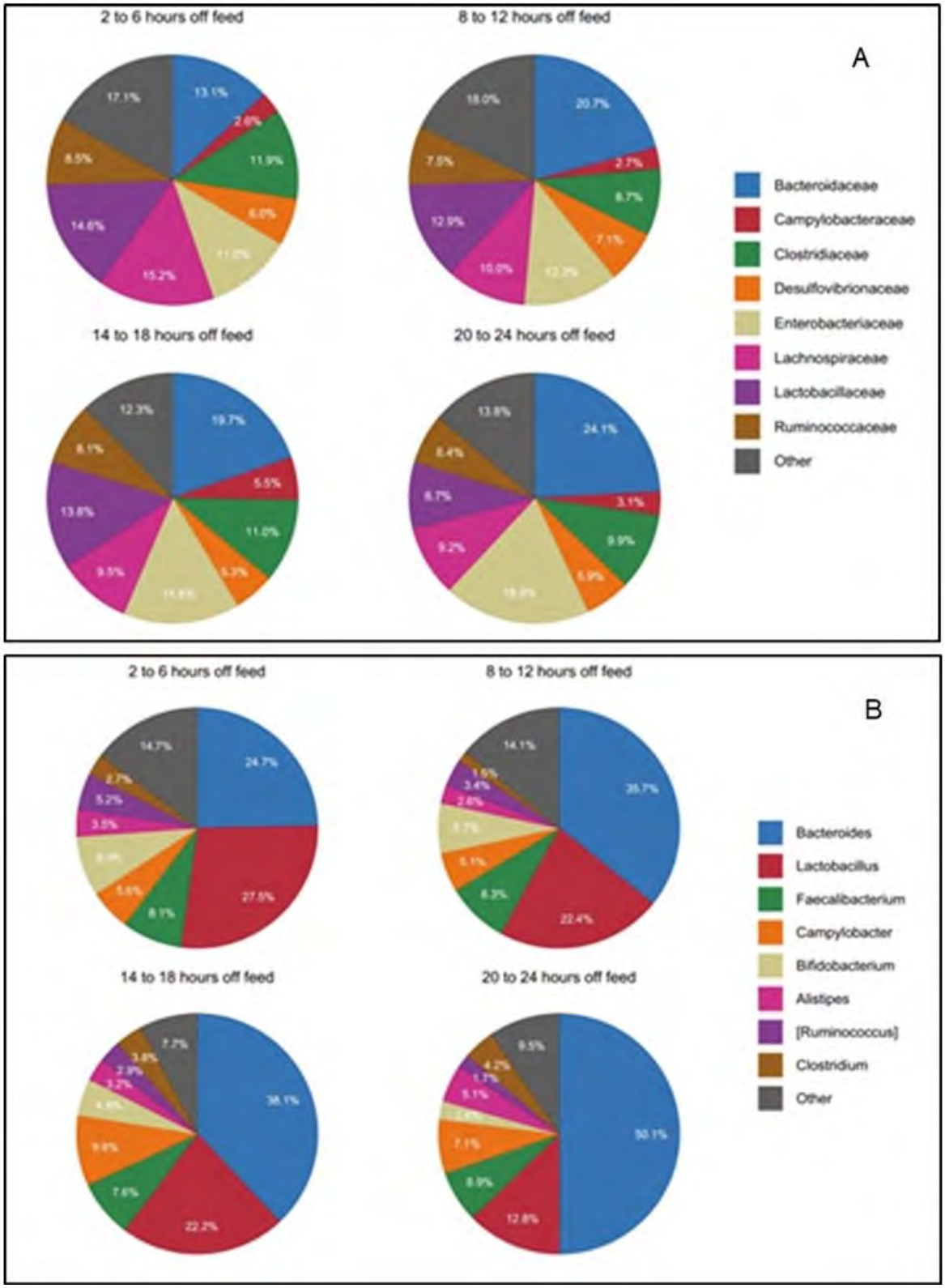

Figure 2. Relative abundance of family and genus with feed withdrawal. Changes in relative abundance of the family (A) and genus (B) with feed withdrawal represented as average of 2-6, 8-12, 14-18 and 19-24 hour time points.

Table 4. Histological measurements of the ileum at the feed withdrawal time points.

Ileal histomorphology (µm)						
Time off feed (h)	**Gross diameter**	**Circular muscularis**	**Total muscularis**	**Villus height†**	**Crypt depth**	**V/C (ratio)**
2	81.3 (4.29)	128.4 (16.74)	212.9 (27.87)	1044.1 (153.35)	218.4 (46.13)	4.7 (1.49)
4	71.7 (4.22)	191.3 (73.75)*	316.9 (41.95)*	1310.0 (147.48)*	243.8 (36.25)	5.6 (0.75)
6	62.8 (4.54)*	155.2 (37.26)	244.6 (60.53)	1350.0 (145.77)*	235.0 (39.55)	5.9 (0.93)
8	67.9 (5.44)*	175.9 (30.04)*	273.5 (60.13)	1430.0 (120.42)*	273.4 (44.31)*	5.4 (0.68)
10	65.7 (10.49)*	145.6 (14.73)	243.7 (49.39)	1410.0 (114.02)*	233.0 (34.10)	6.2 (0.91)*
12	61.6 (1.95)*	199.5 (56.43)*	308.5 (77.43)*	1450.0 (100.00)*	260.6 (23.52)	5.67 (0.79)
14	77.1 (11.21)	163.6 (11.98)	254.8 (22.31)	1420.0 (90.83)*	265.9 (27.72)	5.4 (0.70)
16	69.4 (12.38)*	166.4 (34.02)	254.0 (57.35)	1480.0 (90.83)*	266.1 (40.60)	5.7 (0.72)
18	65.1 (4.90)*	169.5 (28.94)	260.5 (46.56)	1530.0 (168.08)*	257.0 (30.07)	6.1 (1.30)*
20	67.6 (5.58)*	169.8 (26.68)	273.2 (57.49)	1260.8 (154.06)*	288.1 (47.87)*	4.5 (0.52)
22	64.9 (11.20)*	178.9 (23.43)*	277.9 (22.23)*	1290.0 (108.40)*	284.7 (36.13)*	4.6 (0.73)
24	69.7 (7.01)*	160.8 (9.99)	246.8 (8.37)	1203.4 (218.88)	203.6 (68.68)	6.2 (1.20)*
P values						
Linear	0.134	0.39	0.810	0.614	0.214	0.981
Log transformed	0.134	0.16	0.589	0.615	0.370	0.950
Quadratic	0.053	0.344	0.591	0	0.033	0.488
Log transformed	0.049	0.149	0.555	0	0.370	0.313

Values presented as means (standard deviation) with n=5 replicate birds per time point.
P values for linear and quadratic regression are presented for each measurement against time off feed for untransformed and log transformed measurements.
*P < 0.05 when regressed against what is measured at 2 hours off feed.
† N=4 for villus height at 2 hours off feed due to outlier removal.

primary processing plant to gauge adherence to FW. However, it must be noted that within this trial bird holding temperature was controlled at 25 °C and the effects of dehydration were not observed. Future work should repeat the above trial conditions but change the holding temperatures to include 20, 25, 30 and 35 °C to show whether holding temperature affects the gut morphology and strength.

Microbiome of Caecal with feed-withdrawal

The mean(±SD) study wide enumeration by mMPN for the aerobic count, *Campylobacter* and *Salmonella* of the caecal content collected during FW was $\log_{10}$ 7.8±1.07, $\log_{10}$ 7.2±1.52 and $\log_{10}$ 0.6±1.24 CFU/g, respectively. A linear regression analysis of the aerobic count (AC), *Campylobacter* and *Salmonella* enumeration with respect to FW did not identify a relationship to any of the microbes' enumeration and FW (Table 5). These enumeration values are what is expected, and reflect the different host/pathogen relationships that occur with *Salmonella* and *Campylobacte*r colonisation in the chicken caeca.

Salmonella is an intra cellular organism and thus the enumeration of populations may be only those that are extra cellular or have left the host enterocyle [43]. The pathogensis of *Salmonella* within poultry host has been reviewed by Foley *et al*. [44] showing that *Salmonella* populations within the caecal are in low numbers and transit in

Table 5. Caecal microbial enumeration and prevalence (%) in response to feed withdrawal.

Enumeration						**Prevalence**			
Time off feed (h)	**Percentage of male birds**	**N (mMNP)**	**Aerobic bacteria MPN (log10 cfu/g)**	***Campylobacter* MPN (log10 cfu/g)**	***Salmonella* MPN (log10 cfu/g))**	***Salmonella* detected MPN (%)**	**N (16SRNA profiling)**	**16SRNA Genus profiling**	**16SRNA Species profiling**
2	80	10	7.3 (0.40)	7.4 (1.28)	0.5 (0.77)	30	10	60	20
4	100	10	7.9 (0.58)	7.4 (1.07)	<0.1	ND	10	60	50
6	80	10	7.8 (0.51)	7.4 (1.71)	0.8 (1.03)	30	10	80	40
8	80	10	7.6 (0.80)	7.4 (0.58)	0.7 (0.87)	20	10	70	60
10	90	10	8.0 (0.71)	7.8 (0.98)	0.4 (0.80)	10	10	80	60
12	90	10	7.4 (0.37)	7.1 (1.36)	0.2 (0.40)	10	10	70	40
14	100	10	8.1 (0.73)	7.5 (0.84)	1.0 (1.30)	50	10	60	30
16	100	10	8.1 (0.66)	7.6 (0.37)	0.9 (1.11)	40	10	80	60
18	80	10	7.6 (0.80)	7.2 (0.75)	0.9 (0.92)	30	10	70	50
20	90	10	8.1 (0.49)	7.6 (0.73)	<0.1	ND	9	89	55
22	60	10	8.0 (0.95)	7.8 (1.17)	0.4 (0.58)	20	10	100	100
24	60	10	8.3 (0.51)	8.3 (0.40)	0.9 (1.11)	40	10	50	50
P values									
Linear regression			0.051	0.233	0.567				

nature [45]. Individual birds periodically shed *Salmonella* over time at low populations in their respective faeces [45]. The caeca enumeration for this trail was also observed in previous pen trial [31, 33].

In contrast to *Salmonella*, *Campylobacter* has a pseudo-symbiotic relationship with poultry and is seen at maximum populations [46]. This is partially because the chicken homeostatic temperature is 42 ºC, which is the optimum *Campylobacte*r growing temperature [46]. This temperature (42 ºC) relationship between organism and host shows why colonisation of the entire flock was achieved very quickly with *Campylobacte*r populations of Log_{10} 8.0 CFU/g. The caeca provides a microaerophilic environment where *Campylobacte*r predominates [47].

This microaerophilic requirement and fastidious nutrient requirement for *Campylobacter* makes it very difficult to grow in the laboratory. The ISO (International Standard Organisation) *Campylobacter* culture method [48] recommends direct plating for the enumeration of *Campylobacter*. In practice, direct plating on solid media, with selective supplements, may reduce the recovery of injured or stress cells leading to under-reporting and missing of viable but non-cultural strains [49, 50]. In liquid media 'the enrichment step is inherently better, supporting the recovery and growth of sub-lethally injured cells than direct plating on selective agar.' Based upon Richardson's (2009) observations [50] the mMPN assay used in this study [30] was designed to reduce oxygen stress and enumerated injured cells in a broth solution resulting in more accurate *Campylobacter* enumeration.

Traditionally, the ability to look at the changing poultry microbiome (due to age, feed and antibiotic use) was limited by the technology available. The identification and enumeration of bacterial populations was biased towards microbes which were easy to culture [51, 52]. A review by Shang *et al*. (2018) outlines the technologies used to characterise the microbiome [53]. The chicken gut microbiome has been characterised using Next Generation Sequencing of 16SRNA, and has identified complex changes of microbiota that occur with the addition of antibiotics to feed [54, 55]. There has only been one study reviewing the changes in the chicken microbiome with respect to FW [56]. The conclusion was that the diversity of bacteria present decreases with increasing FW time, although the method used could not identify the type of bacteria present [56].

A detailed investigation of changes to the total caecal microbiome was undertaken by 16SRNA analysis. The total number of valid reads identified by Ion reporter software was 177,288±87,429 per sample, and 104,258±59,195 could be mapped to the Greengenes database. At the phylum level seven different phylum were identified in the caecal contents (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Tenericutes, Synergistetes* and *Verrucomicrobia*). *Firmictes*, *Proteobacteria* and *Bacteroidetes* were the most predominant phylum, study wide accounting for 48.7±6.81, 25.3±5.61 and 23.2±5.17% respectively. At each time point *Firmictes* were the most predominant phylum, while *Probacteria* and *Bacteroidetes* varied as the 2nd and 3rd most frequent. *Actinobacteria* was found consistently and accounted for an average 2.7±1.32% of mapped reads, while the other phyla are minor components (<0.05%) of the microbiome of the caeca.

Study wide there were 94 individual families identified (Supplementary Table 2). The 7 most common families (>5%) were *Bacteroidaceae* (17.2±5.87%), *Enterobacteriaceae* (13.2±5.70%), *Lactobacillaceae* (11.4±5.05%), *Lachnospiraceae* (10.5±4.05%), *Clostridiaceae* (9.73±3.13%), *Ruminococcaceae* (7.2±2.27%) and *Desulfovibrionaceae* (5.6±1.92%). The next 19 most prevalent families have a study wide average of <5-0.1%, while the remaining families identified had study wide average prevalence of <0.1% with many not being present at all time points. At the genus level 63 genus were identified (Supplementary Table 2). The 10 most common (>1%) were, *Bacteroides* (32.4±17.13%), *Lactobacillus* (17.5±10.37%), *Faecalibacterium* (6.4±3.29%), *Campylobacter* (5.8±4.01%), *Bifidobacterium* (4.2±2.84%), *Alistipes* (3.2 ±2.78%), *Ruminococcus* (2.8±1.91%), *Clostridium* (2.8±1.60%), *Parabacteroides* (2.0±4.18%) and *Escherichia* (1.0±0.88%). The profile of bacteria

at the phylum, family and genus levels in the cecum throughout FW correlates very well to other published data of high throughput 16SRNA sequencing of mature broiler chicken microbiomes [57, 58]. The three most abundant phyla found in the broiler chickens are *Bacteroidetes*, *Firmicutes* and then *Proteobacteria*; the relative abundance varies with the location in the gastrointestinal tract [58] and age [57] of the birds. The data in this study was able to reproduce the previous findings [57, 58] on phylum, family and genus bacterial compositions in mature chickens as cited.

The direct comparisons of the percentage of 8 most common family and genus at the 2 h time point to all other time points demonstrated some significant changes. At the family level *Bacteroidaceae* increased at 20 and 22 hrs, *Clostridiaceae* significantly decreased at 12 hrs, *Enterobacteriaceae* increased at 14 and 16 hrs, *Lachnospiraceae* significantly decreased at 12- 20 hrs and *Lactobacillaceae* significantly decreased at 8 hrs. At the genus level, *Alistipes* significantly decreased at 16 and 22 hrs, *Bacteroides* increased at 16-24 hrs, *Bifidobacterium* significantly decreased at 18, 20 and 24 hrs, *Campylobacter* increased at 22 hrs, *Clostridium* decreased at 10 and 12 hrs, *Faecalibacterium* increased at 6,10 and 24 hrs, *Lactobacillus* decreased at 8,16, 22 and 24 hrs, and Ruminococcus decreased at 12,14, 18-24 hrs. These changes reflect the variability between individual caeca rather than having any true significance.

Negative binomial regression analysis identified significant changes in the abundance with increasing time of FW at the family and genus level (Supplementary Table 3 and 4). At the family level *Bacteroidaceae* and *Enterobacteriaceae* abundance increased by 3% for every hour of FW (P value <0.001 and 0.022), while *Lachnospiraceae* and *Lactobacillaceae* decreased by 3% (P=0.001 and <0.001). At the genus level, for every hour off feed the abundance of *Bacteroides* and *Clostridium* increased by 4 and 3%, (P <0.001 and 0.001). In contrast the abundance of *Bifidobacterium* and *Lactobacillus* decreased by 6 and 4% (P <0.001 and 0.001). A graphical representation of these changes in microbiome is shown in Figure 2.

Previous studies have shown changes in the microbiome of the crop and caeca in response to FW; however the changes reported are not consistent even within studies [33, 34]. In general, the aerobic bacteria in the crop or caeca either have no change or increase with FW. *Enterobacteriaceae* sometimes increase in the caeca while in the crop the changes are inconsistent. Lactic acid bacteria decrease with FW, which is consistent with the decrease observed for *Lactobacillaceae* in this study. Studies on the effect of FW on the presence of *Salmonella* have been inconclusive with both increases and decreases observed in the crop [13, 34]. In the caeca if birds have been challenged with *Salmonella* relatively soon before processing there is an observable increase; however examination of the incidence in commercial flocks demonstrates no changes with FW [13, 33].

Salmonella is present in the caeca at relatively low levels as seen by enumeration by mMPN. It is therefore more appropriate to examine any changes using prevalence rather than enumeration. The *Salmonella* prevalence data using mMPN growth showed a steady (non-significant) decline in prevalence from 30% at 2 h to a trial low of 10% for the time points 10 and 12 h. However, at time point 14 the cultural *Salmonella* prevalence increased to 50% and stayed relatively high for the remaining time points >14 h. The 10 and 12 hrs prevalence decrease was not seen when the prevalence is considered by 16SRNA analysis, with 80 and 70% of caeca containing detectable *Salmonella* at the genus level (Table 5).

The sensitivity of *Salmonella* detection varied between the mMPN enumeration and 16SRNA analysis (Table 5). *Salmonella* was not detected by mMPN at the 4 and 20 hour time points but was detected at all time points by 16SRNA analysis. Analysis at the genus versus species level was also more sensitive. Of note was the observation that for many samples the number of sequence counts for *Salmonella* were very close to the cut off value for inclusion of 10 reads. It is therefore likely that the true prevalence of *Salmonella* in the caeca is higher than recorded. These results demonstrate a common issue with the microbiological detection/enumeration of

Salmonella. As *Salmonella* is present in small numbers in the bird's digestive tract [31, 33] the true prevalence is difficult to accurately determine. This may be due to the fact that *Salmonella* populations are lower than the limit of detection for classical cultural methods due to Poisson distribution. Subsequent conclusions need to be treated with caution even if there is statistical significance. Even though results for the effect of FW *Salmonella* contamination risk are not reliable, *Salmonella* does belong to the *Enterobacteriaceae* family which is the second most abundant family in the caeca and was demonstrated in this study to increase in abundance with FW.

At the family level 16SRNA analysis ranked *Campylobacteraceae* as the 8th most abundant family, while at the genus level *Campylobacter* was ranked the 4th most abundant genus. Even though *Campylobacter* could be cultured from every individual bird in the study, for the mMPN only 94% of birds had *Campylobacter* identified at the family level by 16SRNA analysis. At the species level 16SRNA analysis identified *C. coli* (18% of birds tested) and not the challenged *C. Jenjuni*. While it is possible that the flock was subsequently colonised by *C. coli* it is also likely that the 16SRNA analysis has difficultly identifying reads as *Campylobacte*r and can't accurately split the *Campylobacter* at the species level. 16SRNA analysis can have difficulties identifying sequences if the organism is not well represented in the reference database or if there are multiple very similar sequences assigned to different but closely related organisms. *Campylobacter* has previously been shown to increase in the crop but not change in the caeca of birds in response to FW [15], while the levels of *Campylobacter* on carcase with FW of up to 12 hours has conflicting results with both no changes and an increase being reported [16, 59]. The observations that the caeca remains full of digesta during FW [34] and that the relative population of *Campylobacter* remains high throughout FW, may indicate that the risk of carcase contamination from caecal sources from *Campylobacter* doesn't change with FW.

The primary purpose of FW practice on farm was to aid the modern processor using automated equipment to reduce gross contamination of the chicken during transport to primary processing. This gross contamination is seen in two forms, feed in crop and faecal contamination of live birds during transport. The data presented above shows that the recommended time period of 8-12 hours results in no feed in crop nor any faecal matter in the intestinal tract of the bird but limited gross morphological changes to the intestinal tract.

CONCLUSION

The data presented in this study showed no detectable loss of gut integrity or gross morphological changes, even up to 24 hrs, during FW measurement, which is in contrast to previous studies. This may be due to decreased bird stress, due to reduced travel time and controlled holding temperature (25 ºC) prior to slaughter. Future research should be around repeating this trial conditions but with different holding temperatures of 20, 25, 30 and 35 ºC to determine if external temperature stress affects the intestinal and microbial loads differently.

The most practical method of determining the appropriate FW window to reduce the risk of carcase contamination during slaughter is the examination of the clearance of the intestinal content. The ingesta clears from intestines in the majority of birds from 10 hours, which is within the 8-12-hour FW window. The data shows that the current guidelines for FW are appropriate for the poultry industry to ensure empty gut content. The food borne pathogens of concern *Salmonella* and *Campylobacter* do not change significantly in the caeca with FW of up to 24 hours. In retrospect investigation *Salmonella* and *Campylobacter* prevalence in crop or other sections of the digestive tract may have been more informative.

The microbiota changes seen by 16SRNA analysis are subtle but do demonstrate increases in the proportion of two families of bacteria *Bacteroidaceae* and *Enterobacteriaceae* and the decrease in *Lachnospiraceae* and *Lactobacillaceae* over FW time. As *Salmonella* is a member of the *Enterobacteriacaea* family this increase requires further investigation.

ACKNOWLEDGEMENTS

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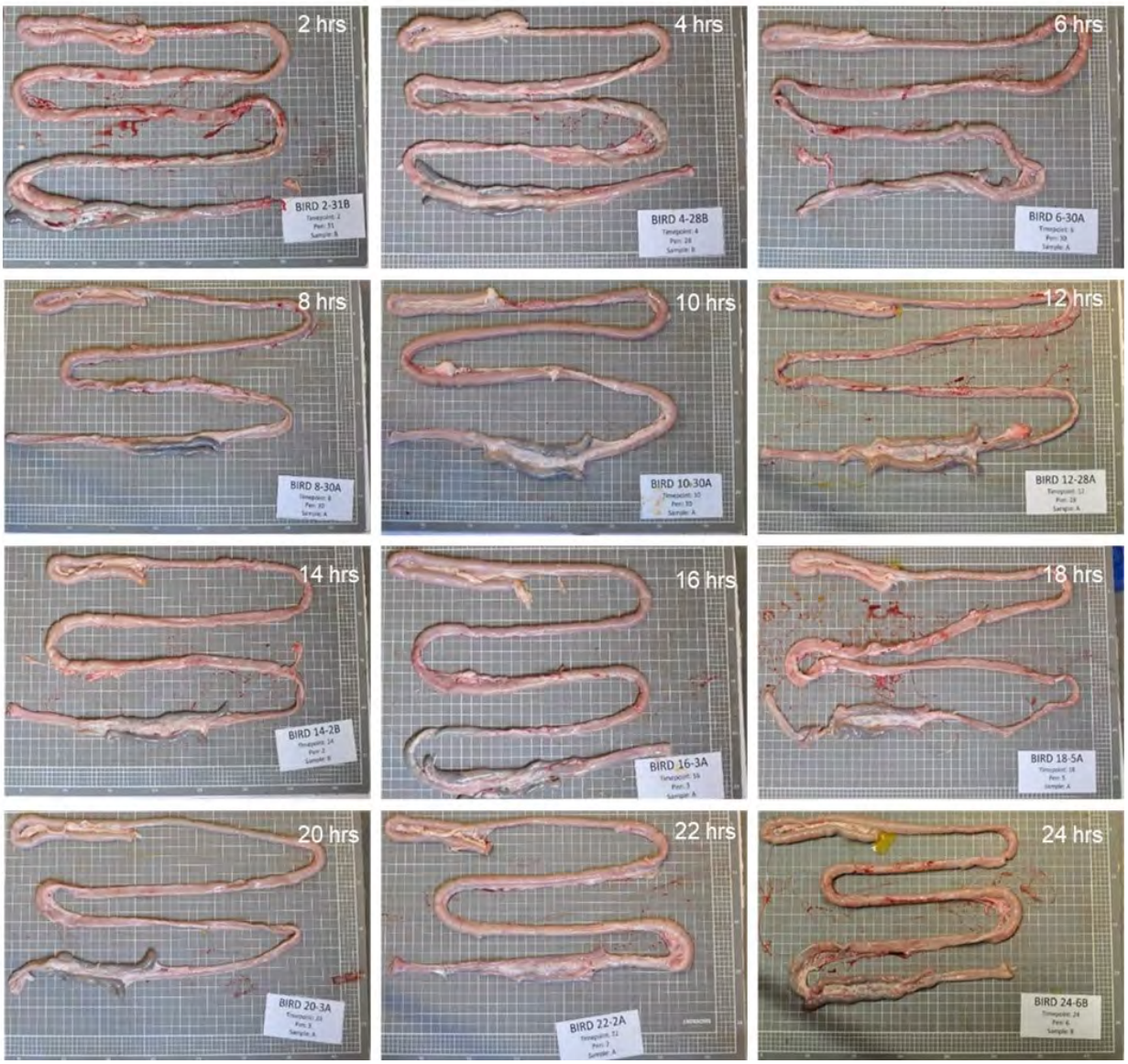
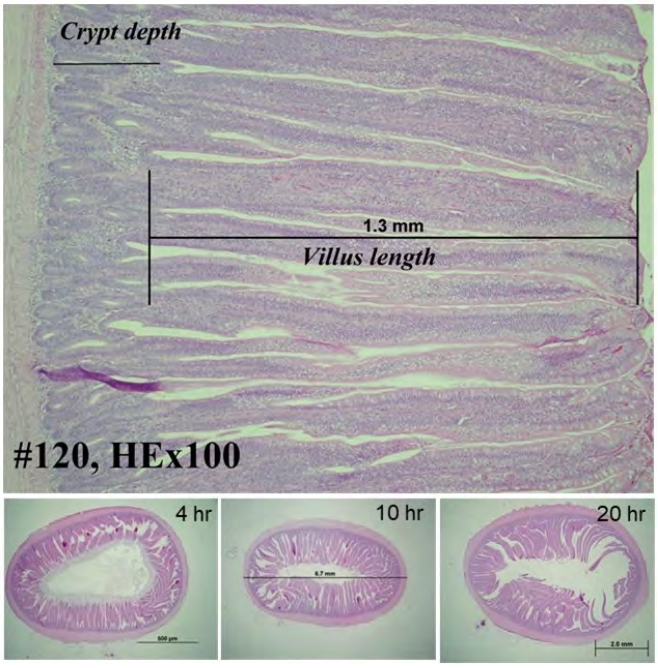
CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Representative intestine layouts from each time point.



Supplementary Figure 2. Representative histology images.

Supplementary Table 1. Pen grow out data.

Pen	Day 7	Day 14	Day 21	Day 28	Day 35	Day 41
	Weight (kg)					
1	0.183	0.491	1.006	1.794	2.503	3.123
2	0.171	0.471	1.011	1.769	2.526	3.132
3	0.160	0.427	0.952	1.652	2.413	3.139
4	0.171	0.459	0.976	1.713	2.532	3.122
5	0.165	0.447	0.993	1.748	2.528	3.236
6	0.181	0.494	1.056	1.806	2.571	3.020
7	0.174	0.471	1.006	1.788	2.600	3.261
8	0.176	0.464	1.005	1.771	2.542	3.217
9	0.179	0.487	1.050	1.810	2.607	3.269
10	0.169	0.471	0.991	1.823	2.537	3.217
Average	0.173±0.0072	0.468±0.0206	1.005±0.0310	1.767±0.0519	2.536±0.0546	3.174±0.079
Collins *et al*., (2014)	0.175±0.0020	0.470±.0.074	1.151±0.0256	1.548±0.0433	2.149±0.0611	2.734±0.0778*
Cobb Valance. (2022)	0.202	0.570	1.116	1.783	2.521	3.170
Mortality per interval (n/N)	10/350	9/340	3/331	13/328	5/315	10/310

A total of 35 birds were assigned to each pen at the beginning of the trial (day 0). On day 42, 12 birds were selected from each pen to proceed onto the feed withdrawal study.
Weight (kg) presented as mean per pen calculated as pen weight/number of birds per pen.
*Data from this study was from 42, not 41-day old birds.

Supplementary Table 2. Study wide relative abundance of family, genus and species identified by 16SRNA analysis (%).

Family		Genus		Species	
Bacteroidaceae	17.22326 (5.869531)	*Bacteroides*	32.4369 (17.13906)	*Bacteroides fragilis*	15.0949 (13.93219)
Enterobacteriaceae	13.22432 (5.701752)			*Bacteroides ovatus*	0.0876 (0.06123)
Lactobacillaceae	11.41194 (5.059253)			*Bacteroides sp.*	1.9967 (1.47381)
Lachnospiraceae	10.46402 (4.053779)			*Bacteroides thetaiotaomicron*	0.0124 (0.00747)
Clostridiaceae	9.73799 (3.132322)			*Bacteroides uniformis*	0.5843 (0.77833)
Ruminococcaceae	7.24400 (2.274989)			*Bacteroides caccae*	0.1253 (0.08327)
Desulfovibrionaceae	5.61291 (1.926741)			*Bacteroides faecichinchillae*	0.0403 (0.02278)
Campylobacteraceae	3.13830 (1.890515)			*Bacteroides vulgatus*	14.6695 (12.80167)
Bifidobacteriaceae	2.58749 (1.403116)	*Lactobacillus*	17.5210 (10.37492)	*Lactobacillus acidipiscis*	0.0007 (*)
Porphyromonadaceae	2.08823 (2.843001)			*Lactobacillus agilis*	0.7459 (0.92277)
Rikenellaceae	1.70104 (1.281211)			*Lactobacillus crispatus*	0.0098 (*)
Erysipelotrichaceae	1.33717 (0.547694)			*Lactobacillus gasseri*	0.0046 (*)
unclassified Clostridiales	1.12212 (0.654346)			*Lactobacillus panis*	0.0687 (0.06619)
Eubacteriaceae	1.00439 (0.517310)			*Lactobacillus reuteri*	0.1122 (0.11976)
Bacillaceae	0.72025 (0.376509)			*Lactobacillus salivarius*	5.2663 (3.74364)
Paenibacillaceae	0.55247 (0.308934)			*Lactobacillus sp.*	0.0206 (*)
Streptococcaceae	0.40780 (0.308463)			*Lactobacillus vaginalis*	0.7508 (0.63096)
Hyphomicrobiaceae	0.32853 (0.182472)	*Faecalibacterium*	6.3574 (3.29197)	*Faecalibacterium prausnitzii*	22.3095 (7.49924)
Peptococcaceae	0.29336 (0.122176)	*Campylobacter*	5.8193 (4.01137)	*Campylobacter coli*	0.0547 (0.07300)
Peptostreptococcaceae	0.28287 (0.391759)			*Campylobacter upsaliensis*	0.0172 (0.00889)
Oscillospiraceae	0.25779 (0.175937)	*Bifidobacterium*	4.2697 (2.84058)		
Rhodospirillaceae	0.18963 (0.387028)	*Alistipes*	3.1796 (2.77866)	*Alistipes putredinis*	8.9380 (5.58847)
unclassified Rhizobiales	0.18284 (0.213853)	*[Ruminococcus]*	2.7859 (1.90846)	*[Ruminococcus] gnavus*	3.2635 (1.87540)
Christensenellaceae	0.15104 (0.095425)			*[Ruminococcus] torques*	5.0850 (2.63936)
Clostridiales Family XI. Incertae Sedis	0.12742 (0.0978742)	*Clostridium*	2.7561 (1.59855)	*Clostridium hiranonis*	0.0671 (0.05282)
Veillonellaceae	0.11850 (0.0847191)			*Clostridium sp.*	1.7305 (1.47229)

Supplementary Table 2 continued..

Sphingomonadaceae	0.09830 (0.1483667)	*Parabacteroides*	1.9944 (4.18239)	*Parabacteroides distasonis*	5.7898 (4.81395)
Staphylococcaceae	0.09022 (0.0830814)	*Escherichia*	1.0517 (0.88009)	*Escherichia coli*	3.7082 (1.54174)
Peptoniphilaceae	0.08402 (0.0610118)			*Escherichia sp.*	0.0355 (0.01826)
Aerococcaceae	0.06708 (0.0647677)	*Blautia*	0.7716 (0.42471)	*Blautia glucerasea*	0.4988 (0.76812)
Kineosporiaceae	0.06256 (0.0325446)			*Blautia hydrogenotrophica*	0.6137 (0.36070)
Halomonadaceae	0.06004 (0.0641909)			*Blautia obeum*	0.0039 (*)
Enterococcaceae	0.05480 (0.065294)			*Blautia producta*	0.0444 (0.07391)
Neisseriaceae	0.05146 (0.061908)			*Blautia sp.*	0.4437 (0.23105)
Acetobacteraceae	0.05107 (0.066935)	*Roseburia*	0.7697 (0.49952)	*Roseburia faecis*	0.2987 (0.27761)
Catabacteriaceae	0.04858 (0.035254)			*Roseburia intestinalis*	0.0268 (0.03121)
Coriobacteriaceae	0.04461 (0.030401)	*Butyricicoccus*	0.5697 (0.77943)	*Butyricicoccus pullicaecorum*	0.7461 (1.31819)
Planococcaceae	0.03697 (0.036023)	*Flavonifractor*	0.4906 (0.53443)		
Oxalobacteraceae	0.03213 (0.036096)	*Pseudoflavonifractor*	0.4659 (0.49683)	*Pseudoflavonifractor capillosus*	0.4745 (0.57328)
Brevibacteriaceae	0.02712 (0.026581)	*Gemmiger*	0.4309 (0.34905)		
Alteromonadaceae	0.01838 (0.018871)	*Streptococcus*	0.4245 (0.29281)	*Streptococcus alactolyticus*	0.2140 (0.32788)
Prevotellaceae	0.01496 (0.009925)	*Ruminococcus*	0.3098 (0.16311)	*Ruminococcus sp.*	0.0769 (0.07237)
Brucellaceae	0.01442 (0.043312)	*Stomatobaculum*	0.2377 (0.15193)	*Stomatobaculum longum*	0.6806 (0.40551)
Dermabacteraceae	0.01393 (0.01203)	*Tyzzerella*	0.2040 (0.17964)	*Tyzzerella lactatifermentans*	0.7266 (0.63699)
Victivallaceae	0.01248 (*)	*Megamonas*	0.1871 (0.17701)	*Megamonas hypermegale*	0.2967 (0.25152)
Thermoanaerobacterales Family IV. Incertae Sedis	0.01225 (0.020187)	*Erysipelatoclostridium*	0.1811 (0.33824)	*Erysipelatoclostridium spiroforme*	0.6495 (1.02263)
Geobacteraceae	0.01031 (0.021857)	*Anaerostipes*	0.1161 (0.09844)	*Anaerostipes butyraticus*	0.4906 (0.38379)
Moraxellaceae	0.01021 (0.012824)	*Anaerotruncus*	0.1158 (0.12034)	*Anaerotruncus colihominis*	0.4798 (0.49436)
Oceanospirillaceae	0.00828 (0.010618)	*Eubacterium*	0.1149 (0.11541)	*Eubacterium ventriosum*	0.0069 (*)
Microbacteriaceae	0.00695 (0.025029)	*Salmonella*	0.0987 (0.05929)	*Salmonella enterica*	0.1094 (0.03572)
Acholeplasmataceae	0.00624 (0.008428)	*Lachnospira*	0.0823 (0.07200)		
Nocardioidaceae	0.00526 (0.006207)	*Acetanaerobacterium*	0.0768 (0.06784)		

Supplementary Table 2 continued..

Clostridiales Family XIII. Incertae Sedis	0.00506 (0.004224)	*Subdoligranulum*	0.0766 (0.08238)	*Subdoligranulum variabile*	0.1691 (0.19864)
Carnobacteriaceae	0.00493 (0.013008)	*Desulfovibrio*	0.0639 (0.0500)		
Spiroplasmataceae	0.00482 (0.003928)	*Shigella*	0.0576 (0.08341)	*Shigella dysenteriae*	0.0129 (0.00608)
Micrococcaceae	0.00473 (0.002869)			*Shigella flexneri*	0.1341 (0.08523)
Synergistaceae	0.00356 (0.004561)			*Shigella sonnei*	0.0244 (0.01302)
Verrucomicrobiaceae	0.00351 (0.005663)			*Shigella sp.*	0.0512 (0.04812)
Syntrophomonadaceae	0.00326 (0.002584)	*Ethanoligenens*	0.0415 (0.13096)		
unclassified Gammaproteobacteria	0.00290 (*)	*Enterorhabdus*	0.0404 (0.03311)		
Cohaesibacteraceae	0.00237 (0.005638)	*Staphylococcus*	0.0275 (0.03552)		
Gracilibacteraceae	0.00229 (0.002545)	*Jeotgalicoccus*	0.0173 (0.03070)		
Pasteurellaceae	0.00165 (0.001398)	*Oscillibacter*	0.0113 (0.01582)		
Sphingobacteriaceae	0.00148 (0.003198)	*[Eubacterium]*	0.0112 (0.02113)		
Mycoplasmataceae	0.00086 (0.001270)	*Hydrogenoanaerobacterium*	0.0089 (0.01887)		
Coxiellaceae	0.00084 (*)	*Brevibacterium*	0.0082 (0.01486)	*Brevibacterium aureum*	0.0178 (0.01347)
Leuconostocaceae	0.00067 (*)	*Akkermansia*	0.0080 (0.01104)	*Akkermansia muciniphila*	0.0505 (0.05332)
Xanthomonadaceae	0.00067 (0.000894)	*Gordonibacter*	0.0075 (0.01122)	*Gordonibacter pamelaeae*	0.0508 (0.05803)
Francisellaceae	0.00061 (0.001449)	*Trabulsiella*	0.0053 (0.00486)	*Trabulsiella farmeri*	0.0229 (0.01872)
Shewanellaceae	0.00060 (0.000723)	*Denitrobacterium*	0.0052 (0.00798)	*Denitrobacterium sp.*	0.0116 (0.00287)
Corynebacteriaceae	0.00058 (0.000592)	*Brachybacterium*	0.0046 (0.01072)	*Brachybacterium faecium*	0.0166 (0.01540)
Flavobacteriaceae	0.00053 (0.003462)	*Lachnoclostridium*	0.0039 (0.00530)		
Parvularculaceae	0.00045 (0.001954)	*Proteus*	0.0037 (0.01298)		
Piscirickettsiaceae	0.00033 (0.000223)	*Enterobacter*	0.0035 (0.00563)	*Enterobacter hormaechei*	0.0042 (0.00281)
Clostridiales Family XII. Incertae Sedis	0.00027 (*)			*Enterobacter sp.*	0.0075 (0.00526)
Caulobacteraceae	0.00027 (*)	*Haemophilus*	0.0033 (0.00367)	*Haemophilus parainfluenzae*	0.0167 (0.01111)

Supplementary Table 2 continued..

Rhizobiaceae	0.00023 (0.000053)	*Acetatifactor*	0.0028 (0.01865)		
Clostridiales Family XIV. Incertae Sedis	0.00021 (0.000212)	*Aerococcus*	0.0027 (0.00718)		
Bacillales incertae sedis	0.00021 (0.000568)	*Moryella*	0.0024 (0.00506)		
Acidaminococcaceae	0.00021 (0.000386)	*Senegalimassilia*	0.0024 (0.00336)		
Clostridiales Family XIX. Incertae Sedis	0.00018 (0.001045)	*Paraeggerthella*	0.0022 (0.00432)		
Rhodobacteraceae	0.00018 (*)	*Rickettsiella*	0.0021 (*)		
Puniceicoccaceae	0.00017 (*)	*Cronobacter*	0.0016 (0.00323)	*Cronobacter sakazakii*	0.0113 (0.01445)
Thiotrichaceae	0.00016 (*)	*Acetivibrio*	0.0012 (0.00506)	*Acetivibrio ethanolgignens*	0.0190 (0.01581)
Anaeroplasmataceae	0.00012 (*)	*Corynebacterium*	0.0011 (0.00251)	*Corynebacterium ammoniagenes*	0.0135 (0.01307)
Alcaligenaceae	0.00011 (*)			*Corynebacterium stationis*	0.0146 (*)
Ktedonobacteraceae	0.00007 (*)				
Halobacteroidaceae	0.00006 (*)	*Dorea*	0.0010 (0.00422)		
Desulfobacteraceae	0.00005 (0.00024)	*Sodalis*	0.0007 (0.00066)	*Sodalis glossinidius*	0.0067 (0.00372)
Caldicoprobacteraceae	0.00005 (*)	*Anaerofustis*	0.0007 (0.00066)	*Anaerofustis stercorihominis*	0.0072 (*)
Thermoanaerobacterales Family III. Incertae Sedis	0.00001 (*)	*Edwardsiella*	0.0007 (0.00127)	*Edwardsiella ictaluri*	0.0054 (0.00391)
Bradyrhizobiaceae	0.00001 (*)	*Enterococcus*	0.0003 (0.00211)	*Enterococcus cecorum*	0.0124 (*)
Methylobacteriaceae	0.00001 (*)	*Citrobacter*	0.0002 (0.00019)	*Citrobacter farmeri*	0.0027 (*)
Holosporaceae	0.00001 (*)			*Citrobacter sp.*	0.0036 (0.00188)
				Ruminiclostridium viride	0.0094 (*)

Average percentage of each operational taxonomic unit (OTU) over all data analysed, ()=SD, * indicates that only one timepoint identified this OTU.

Supplementary Table 3. The Relative Abundance of the 8 Most Common Bacterial Families Found in the Ceaca During Different Feed Withdrawal Times.

Time point (h)	*Bacteroidaceae*	*Campylobacteraceae*	*Clostridiaceae*	*Desulfovibrionaceae*	*Enterobacteriaceae*	*Lachnospiraceae*	*Lactobacillaceae*	*Ruminococcaceae*
2	10.5 (8.24, 16.31)	0.5 (0.17, 3.43)	11.8 (10.41, 16.74)	6.8 (1.49, 9.98)	4.1 (2.06, 10.28)	15 (9.7, 16.75)	15 (12.59, 18.27)	6.9 (5.2, 8.89)
4	11.1 (8.64, 13.64)	0.3 (0.09, 1.83)	12 (7.41, 17.49)	4.4 (3.09, 9.23)	9.3 (0.95, 14.71)	15.3 (9.55, 18.81)	17.7 (9.64, 26.51)	7.7 (4.88, 15.5)
6	13.4 (8.34, 15.19)	0.5 (0.05, 4.1)	12.1 (7.96, 13.15)	5.6 (2.18, 7.43)	14.2 (8.95, 20.25)	13.6 (10.25, 19.46)	10 (3.27, 17.17)	7.1 (6.28, 9.08)
8	20.2 (15.88, 34.85)	0.4 (0.12, 0.5)	8.1 (7.66, 12.63)	7.6 (4.26, 11.36)	7.3 (4.38, 18.22)	9.8 (8.46, 14.01)	9.2 (7.32, 10.24)*	7.4 (5.38, 9.67)
10	17.1 (12.1, 23.96)	2.5 (0.09, 4.65)	9.5 (6.69, 12.14)	7.1 (4.02, 11.1)	13.6 (8.29, 17.67)	11.7 (8.04, 14.01)	11.2 (9.28, 14.46)	7.6 (6.43, 9.56)
12	21.9 (10.59, 27.96)	1.1 (0.09, 6.49)	7.2 (6.46, 8.98)*	5.3 (2.52, 7.85)	7 (5.31, 12.45)	8.3 (6.21, 8.76)*	10.1 (6.54, 19.13)	5.2 (3.47, 9.02)
14	17.5 (6.86, 21.61)	2.3 (1, 16.59)	9.3 (6.96, 10.34)	3.4 (1.94, 5.54)	13.5 (6.15, 22.11)*	7.4 (5.63, 9.52)*	16.1 (8.28, 22.87)	5.9 (3.82, 9.77)
16	21.7 (12.62, 32.53)	1.4 (0.96, 2.3)	9 (5.86, 13.77)	4 (2.65, 7.52)	17.4 (7.04, 25.47)*	10.7 (6.72, 14.13)*	9.9 (3.07, 10.07)	6.1 (4.23, 11.3)
18	20.4 (14.88, 24.55)	3.5 (0.87, 8.21)	10.3 (7.8, 16.73)	5 (1, 8.52)	8.2 (5.55, 16.35)	8.4 (4.79, 12.18)*	8.8 (6.02, 24.52)	5.3 (4.77, 7.67)
20	25.6 (23.86, 30.1)*	0.5 (0.22, 5.54)	6.6 (4.77, 12.03)	6.7 (3.79, 7.49)	9.9 (6.83, 12.81)	9.1 (6.15, 10.33)*	13.5 (3.62, 23.25)	4.6 (3.34, 7.95)
22	20.4 (16.69, 27.4)*	3 (1.25, 6.46)	10.8 (8.75, 13.02)	6.2 (1.47, 7.49)	8.9 (8.35, 32.96)	11.2 (8.79, 11.46)	9.9 (4.53, 14.07)	5.9 (3.43, 6.92)
24	23 (17.79, 26.5)*	0.4 (0.25, 5.66)	10.8 (7.05, 12.55)	5.2 (1.19, 10.57)	21 (11.15, 30.68)*	8.2 (7.63, 9.63)*	1.3 (1.07, 7.13)*	8.2 (5.34, 9.64)

Supplementary Table 3 continued..

Negative binomial regression								
Incidence-rate ratio	1.03 (95% CI: .1.013, 1.041)	1.02 (95% CI: 0.989, 1.060)	1.00 (95% CI 0.986, 1.004)	0.99 (95% CI: 0.977, 1.010)	1.03 (95% CI: 1.014, 1.053)	0.97 (95% CI: 0.964, 0.985)	0.97 (95% CI: 0.951, 0.996)	0.99 (95% CI: 0.977, 1.004)
P value	<0.001	0.185	0.249	0.439	0.001	<0.001	0.022	0.172

Median OTU percentage (Interquartile range).
N=10 with the exception of 20 hr where N=9.
*Percentage of the family count observed at this time point is significantly different (P<0.05) to the percentage observed at two hours off feed, as determined by the Two-sample Wilcoxon rank-sum (Mann-Whitney) test.

Supplementary Table 4. The relative abundance of the 8 most common bacterial genus found in the ceaca during different feed withdrawal times.

Time point (h)	***Alistipes***	***Bacteroides***	***Bifidobacterium***	***Campylobacter***	***Clostridium***	***Faecalibacterium***	***Lactobacillus***	***[Ruminococcus]***
2	1.1 (0.6, 1.48)	21 (16.08, 31.09)	7 (3.72, 8.41)	1 (0.34, 7.41)	1.9 (1.44, 3.31)	3.8 (2.64, 4.49)	29.5 (19.91, 34.52)	4.7 (3.22, 8.64)
4	1.2 (0.86, 3.03)	23.2 (12.69, 28.12)	8 (2.64, 16.01)	0.9 (0.19, 4.11)	3.2 (1.33, 4.53)	8.5 (4.19, 14.86)	31.1 (18.85, 45.54)	3.8 (2.51, 5.58)
6	2.4 (0.46, 5.29)	26 (17.1, 32.89)	7.7 (3.35, 9.72)	1.2 (0.13, 6.77)	2.4 (1.94, 3.37)	7.7 (4.91, 11.21)*	16.2 (4.92, 36.26)	4.1 (2.79, 5.68)
8	1 (0.32, 1.3)	42.3 (26.4, 61.99)	6 (3.55, 6.57)	0.8 (0.24, 1.05)	1.8 (1.37, 2.53)	7.8 (3.98, 10.19)	16.1 (14.47, 23.57)*	2.7 (1.87, 7.61)
10	1.1 (0.43, 3.83)	36.3 (24.32, 42.81)	4.8 (3.9, 6.67)	5 (0.21, 10.83)	1 (0.69, 1.91)*	9.2 (5.13, 12.04)*	22.6 (16.75, 27.73)	4.5 (2.2, 6.55)
12	0.5 (0.39, 1.19)	37.1 (15.28, 40.08)	6.1 (2.17, 16.67)	2 (0.13, 9.02)	1.2 (0.71, 1.65)*	7.2 (1.99, 12.54)	14 (6.97, 27.4)	1.6 (1.12, 2.45)*
14	0 (0, 1.5)	27.2 (13.39, 46.62)	4.8 (1.01, 7.45)	4 (2.08, 25.28)	2.4 (1.09, 5.91)	5.4 (1.2, 12.82)	21.8 (11.8, 32.59)	1.3 (1.18, 1.81)*
16	0 (0, 0.27)*	47 (32.07, 58.1)*	3 (0.55, 6.3)	3.7 (2.13, 6.63)	3.4 (1.83, 4.1)	5.9 (2.22, 8.35)	15.3 (7.89, 23.08)*	2.5 (1.14, 3.42)

Supplementary Table 4 continued..

18	0.4 (0, 1.84)	39.7 (34.18, 44.17)*	2 (1.28, 4.02)*	7.4 (1.7, 19.8)	2.3 (1.29, 6.11)	5.6 (2.18, 8.76)	18 (10.22, 35.34)	2.1 (1.16, 3.42)*
20	0.6 (0, 3.47)	45.6 (41.71, 52.07)*	1.2 (0.63, 3.29)*	0.8 (0.38, 10.61)	1.7 (1.11, 2.82)	3.3 (1.37, 6.68)	19.9 (5.08, 35.78)	1.5 (1.23, 2.24)*
22	0 (0, 0.57)*	49.7 (40.34, 53.83)*	3.2 (1.23, 6.62)	10.3 (4.61, 15.69)*	2.1 (1.14, 5.61)	4.1 (3.24, 6.75)	13.4 (6.16, 19.56)*	2.1 (0.87, 2.86)*
24	7.9 (0, 17.04)	48.7 (45.64, 63.89)*	1 (0.82, 1.5)*	1.1 (0.48, 14.53)	3.2 (2.86, 4.05)	9.8 (6.14, 13.39)*	1.6 (0.68, 12.31)*	1.6 (0.84, 2.15)*
Negative binomial regression								
Incidence-rate ratio	1.03 (95% CI: .0.979, 1.079)	1.04 (95% CI: 1.023, 1.047)	0.94 (95% CI: .0.909, 0.973)	1.03 (95% CI: 0.993, 1.061)	1.03 (95% CI: 1.014, 1.053)	1.00 (95% CI: 0.974, 1.017)	0.96 (95% CI: 0.937, 0.984)	1.00 (95% CI: 0.974, 1.017)
P value	0.271	<0.001	<0.001	0.124	0.001	0.686	0.001	0.686

Median OTU percentage (Interquartile range).
N=10 with the exception of 20 hr where N=9.
*Percentage of the family count observed at this time point is significantly different (P<0.05) to the percentage observed at two hours off feed, as determined by the Two-sample Wilcoxon rank-sum (Mann-Whitney) test.

REFERENCES

1. OECD, 2021, OECD-FAO Agricultural Outlook 2021-2030.
2. Ray, L. C., Collins, J. P., Griffin, P. M., Shah, H. J., Boyle, M. M., Cieslak, P. R., Dunn, J., Lathrop, S., McGuire, S., Rissman, T., Scallan Walter, E. J., Smith, K., Tobin-D'Angelo, M., Wymore, K., Kufel, J. Z., Wolpert, B. J., Tauxe, R. and Payne, D. C. 2021, Morb. Mortal Wkly. Rep., 70, 1332.
3. EFSA and ECDC, 2018, EFSA Journal, 16.
4. Bell, R., Draper, A., Fearnley, E., Franklin, N., Glasgow, K., Gregory, J., Harlock, M., Hope, K., Kane, S., Miller, M., Pingault, N., Sloan-Gardner, T., Stafford, R., Ward, K. and Wright, R. 2021, Commun Dis Intell., 45.
5. Codex, 2011, Codex Alimentarius: Guidelines for the Control of Campylobacter and Salmonella in Chicken Meat, CAC/GL 78-2011, Joint FAO/WHO Codex Alimentarius Commission. World Health Organization, Rome.
6. Cox, J. M. and Pavic, A. 2010, J. Appl. Microbiol., 108, 745.
7. EFSA, 2019, EFSA Journal, 17, e05926.
8. Doyle, M. P. and Erickson, M. C. 2006, Poult. Sci., 85, 960.
9. Barreiro, F. R., Baraldi-Artoni, S. M., Pinto, F. R., Barbosa, M. M. C., Barbosa, J. C. and Amaral, L. A. 2012, Poult. Sci., 91, 2778.
10. Stern, N. J., Fedorka-Cray, P., Bailey, J. S., Cox, N. A., Craven, S. E., Hiett, K. L., Musgrove, M. T., Ladely, S., Cosby, D. and Mead, G. C. 2001, J. Food Prot., 64, 1705.
11. Smidt, M. J., Formica, S. D. and Fritz, J. C. 1964, Poult. Sci., 43, 931.
12. Wabeck, C. J. 1972, Poultry Science, 51, 1119.
13. Ramirez, G. A., Sarlin, L. L., Caldwell, D. J., Yezak, C. R. Jr., Hume, M. E., Corrier, D. E., Deloach, J. R. and Hargis, B. M., 1997, Poult Sci, 76, 654.
14. Bilgili, S. and Hess, J. 1997, Appl. Poult. Sci., 279.
15. Byrd, J. A., Corrier, D. E., Hume, M. E., Bailey, R. H., Stanker, L. H. and Hargis, B. M. 1998, Avian Dis, 42, 802.
16. Northcutt, J. K., Buhr, R. J., Berrang, M. E. and Fletcher, D. L. 2003, Poult Sci, 82, 1820.
17. FSANZ, 2018, Compendium of Microbiological Criteria for Food.
18. RSPCA, 2020, RSPCA APPROVED FARMING SCHEME STANDARD: Meat Chickens (v1.1). RSPCA Australia, Australian Capital Territory.
19. USDA-FSIS, 2008, Estimating human illnesses from Salmonella on young chickens. FSIS Risk Assessment for Guiding Public Health-Based Poultry Slaughter Inspection. USDA.
20. EFSA, 2007, Council Directive 2007/43/EC of 28 June 2007 laying down minimum rules for the protection of chickens kept for meat production. OJL. Vol. 182. 19.
21. Nielsen, S. S., Alvarez, J., Bicout, D. J., Calistri, P., Depner, K., Drewe, J. A., Garin-Bastuji, B., Gonzales Rojas, J. L., Schmidt, C. G., Miranda Chueca, M. Á., Roberts, H. C., Sihvonen, L. H., Spoolder, H., Stahl, K., Calvo, A. V., Viltrop, A., Winckler, C., Candiani, D., Fabris, C. and Michel, V. 2019, EFSA Journal, 17, e05849.
22. Corrier, D. E., Byrd, J. A., Hargis, B. M., Hume, M. E., Bailey, R. H. and Stanker, L. H. 1999, Avian Dis, 43, 453.
23. Northcutt, J. K., Savage, S. I. and Vest, L. R. 1997, Poult Sci, 76, 410.
24. Thompson, K. and Applegate, T. J. 2007, Purdue Unverisity extention unit notes,
25. Thompson, K. L. and Applegate, T. J. 2006, Poult. Sci., 85, 1535.
26. Cobb, 2022, Cobb 500 Broilers Preformance and Nutrition Supplement.
27. AS 5013.6, 2015 Food Microbiology - Examination For Specific Organisms - Campylobacter AS 5013.6.
28. AS 5013.10, 2009 Microbiology of food and animal feeding stuffs: Horizontal method for the detection of Salmonella spp. AS 5013.10-2009.
29. Teirlynck, E., Gussem, M. D. E., Dewulf, J., Haesebrouck, F., Ducatelle, R. and Van-Immerseel, F. 2011, Avian Pathol., 40, 139.
30. Chenu, J. W., Pavic, A. and Cox, J. M. 2013, J. Microbiol. Methods, 93, 12.
31. Pavic, A., Groves, P. J., Bailey, G. and Cox, J. M. 2010, J. Appl. Microbiol., 109, 25.
32. Collins, K. E., Kiepper, B. H., Ritz, C. W., McLendon, B. L. and Wilson, J. L. 2014, Poult. Sci., 93, 2953.

33. Hinton, A. Jr., Buhr, R. J. and Ingram, K. D. 2000, Poult. Sci., 79, 483.
34. Hinton, A., Buhr, R. J. and Ingram, K. D. 2000, Poult. Sci., 79, 212.
35. Bailey, J. S., Cox, N. A., Craven, S. E., Cosby, D. E. 2002, J. Food Prot., 65, 742.
36. Collineau, L., Phillips, C., Chapman, B., Agunos, A., Carson, C., Fazil, A., Reid-Smith, R. and Smith, B., 2020, Prev. Vet. Med., 174, 104823.
37. Siemon, C. E., Bahnson, P. B. and Gebreyes, W. A. 2007, Avian Dis., 51, 112.
38. Abraham, S., O'Dea, M., Sahibzada, S., Hewson, K., Pavic, A., Veltman, T., Abraham, R., Harris, T., Trott, D. and Jordan, D. 2019, PLoS ONE, 14, e0224281.
39. Newell, D. G. and Fearnley, C. 2003, Appl. Environ. Microbiol., 69, 4343.
40. Prachantasena, S., Charununtakorn, P., Muangnoicharoen, S., Hankla, L., Natthaporn Techawal, N., Chaveerach, P., Tuitemwong, P., Chokesajjawatee, N., Williams, N., Humphrey, T. and Luangtongkum, T. 2016, Plos One, 11, e0149585.
41. Pumtang-on, P., Mahony, T. J., Hill, R. H., Pavic, A. and Vanniasinkam, T. 2020, Poult. Sci., 100, 100891.
42. Papa, C. M. 1991, Poult. Sci., 70, 375.
43. Jajere, S. M. 2019, Vet World, 12, 504.
44. Foley, S. L., Johnson, T. J., Ricke, S. C., Nayak, R. and Danzeisen, J. 2013, Microbiol. Mol. Biol. Rev., 77, 582.
45. Ishola, O. O. 2009, Afr. J. Biotechnol., 8.
46. Hakeem, M. J. and Lu, X. 2021, Front. Cell. Infect. Microbiol., 10, 615049.
47. Bhunia, A. K. 2018, Campylobacter and Arcobacter. Foodborne Microbial Pathogens. Food Science. Foodborne Microbial Pathogens. Food Science Springer, New York.
48. ISO:10272-1:2017, Microbiology of the food chain — Horizontal method for detection and enumeration of Campylobacter spp. — Part 1: Detection method.
49. Gharst, G., Oyarzabal, O. A. and Hussain, S. K. 2013, J. Microbiol. Methods., 95, 84.
50. Richardson, L. J., Cox, N. A., Bailey, J. S., Berrang, M. E., Cox, J. M., Buhr, R. J., Fedorka-Cray, P. J. and Harrison, M. A. 209, J. Food Prot., 75, 972.
51. Blaut, M., Collins, M. D., Welling, G. W., Dore, J., Van Loo, J. and De Vos, W. 2002, Br. J. Nutr., 87, S203.
52. Fleet, G. H. 1999, Int. J. Food Microbiol, 50, 101.
53. Shang, Y., Kumar, S., Oakley, B. and Kim, W. K. 2018, Front Vet. Sci., 5, 254.
54. Johnson, T. J., Youmans, B. P., Noll, S., Cardona, C., Evans, N. P., Karnezos, T. P., Ngunjiri, J. M., Abundo, M. C. and Lee, C. W. 2018, Appl. Environ. Microbiol., 84, e00362.
55. Kumar, S., Chen, C., Indugu, N., Werlang, G. O., Singh, M., Kim, W. K. and Thippareddi, H. 2018, PloS one, 13, e0192450.
56. Thompson, K., Burkholder, K., Patterson, J. and Applegate, T. J. 2008, Poult. Sci., 87, 1624-32.
57. Ocejo, M., Oporto, B. and Hurtado, A. 2019, Sci. Rep., 9, 2506.
58. Xiao, Y., Xiang, Y., Zhou, W., Chen, J., Li, K. and Yang, H. 2017, Poult. Sci., 96, 1387.
59. Northcutt, J. K., Berrang, M. E., Dickens, J. A., Fletcher, D. L. and Cox, N. A. 2003, Poult. Sci., 82, 169.