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## Varying starch to fat ratios in pelleted diets: II. Effects on intestinal histomorphometry, *Clostridium perfringens* and short-chain fatty acids in *Eimeria*-challenged broiler chickens

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### ABSTRACT

1. The hypothesis behind the study was that a high dietary starch level (HS) would lead to impaired gut health compared to a low-starch diet (LS) in *Eimeria*-challenged broilers. The effects of two diets with different starch to fat ratios on intestinal histomorphometry, *Clostridium perfringens* counts and toxin profile, necrotic enteritis prevalence and abundance of short-chain fatty acids (SCFAs) were examined.

2. A total of 1,920 one-day-old Ross 308 broiler chickens were fed one of two isocaloric diets formulated either with high (32:1) or low (2:1) starch to fat ratios from d 10 to 29 of age. Each treatment group had 12 pen replicates containing 80 broilers each. On d 17, the chickens were challenged with *Eimeria* vaccine strains. Samples were collected on d 16, 21–23 and 29.

3. Whereas villus length increased gradually throughout the study in the HS group, a peak level was reached on d 21–23 in the LS group. On d 29, the HS group had significantly longer villi than the LS group.

4. Caecal SCFA concentrations were higher in the HS group compared to the LS group on d 16. In both groups, the SCFA level peaked on d 21–23, with the most pronounced increase seen in the LS group.

5. The *C. perfringens* *netB:cpa* ratio increased from d 16 to 29 in the HS group. *C. perfringens* counts and necrotic enteritis prevalence were similar between the two groups.

6. Diet affected the dynamics of small intestinal villus length and caecal SCFA abundance. These findings suggest that structural remodelling of the small intestine is an adaptation to different dietary starch levels, and that caecal SCFA abundance is associated with the availability of substrate for the microbiota in the posterior intestinal segments. Chickens adapted to higher levels of dietary starch might be more robust against *Eimeria* infections due to increased mucosal surface area. Studies with other dietary starch sources are required to clarify the impact of dietary starch levels on intestinal health in *Eimeria*-challenged broilers.

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### Introduction

Energy in broiler diets is mainly provided by carbohydrate and fat sources. Starch is, due to availability and cost-efficiency, often considered the primary source of energy, and is typically provided from cereals which, quantitatively, are the most important components in modern poultry diets (Svihus 2014; Zaefarian et al. 2015). Hence, the proportion of carbohydrates in the diet is much higher for modern broilers than for their wild ancestors. It has been estimated that insects can comprise more than 50% of the diet in wild jungle fowl chicks (Klasing 2005), and fat is, therefore, a more important energy-providing nutrient in an omnivore versus a granivore diet (Barker et al. 1998).

Intensive artificial selection through breeding for efficiency and growth have resulted in modern broiler breeds with a larger appetite and increased voluntary feed intake per day (Tallentire et al. 2016). The ability of broilers to digest nutrients does not necessarily always match their feed intake, and excessive undigested nutrients may reach the lower intestines (Svihus 2011). Substrate preferences differ between microorganisms, and the intestinal microbiota composition is largely determined by nutrient availability and chemical composition of the digesta (Apajalahti

et al. 2004). As an example, the bacterium *Clostridium perfringens* (associated with necrotic enteritis in broilers) produces enzymes necessary to break down and utilise starch and sugar (Groves and Grounland 1969; Shih and Labbe 1996), and the risk of necrotic enteritis has been linked to specific dietary carbohydrate sources (Annett et al. 2002; Kaldhusdal and Hofshagen 1992; Kaldhusdal and Skjerve 1996; Riddell and Kong 1992). Dietary fat sources may influence the growth of *C. perfringens*, and it has been shown that animal fat can increase *C. perfringens* counts compared to vegetable oil (Knarreborg et al. 2002). Consequently, it is possible to influence the growth of potentially pathogenic bacteria through dietary manipulation.

Furthermore, it is well established that coccidiosis caused by *Eimeria* spp. impairs intestinal health in its own right, and is an important predisposing factor for intestinal *C. perfringens* proliferation and the development of necrotic enteritis in broilers (Hermans and Morgan 2007). *Eimeria* spp. infections are common in commercial flocks, and display a dynamic pattern characterised by low infection levels in the youngest chicks and a peak infection level at an age interval that varies with *Eimeria* spp., farming system and health management. Short-chain fatty acids (SCFAs) are produced in the posterior intestine mainly

from microbial fermentation of carbohydrates which escape absorption in the small intestine (Morrison and Preston 2016). There are numerous effects associated with SCFAs, and some of them are involved in host energy metabolism, energy supply, intestinal function and epithelial cell morphology (den Besten et al. 2013; Scheppach 1994). The amount and type of fermentable substrates reaching the lower intestinal segments affects the composition of the intestinal microbiota and, hence, the SCFA concentration and profile (Cummings and Macfarlane 1991). Undigested dietary carbohydrates primarily lead to the formation of volatile fatty acids; acetate, propionate and butyrate (Wong et al. 2006). A specific diet that leads to increased production of SCFAs lowers the pH in the intestine, which could create a favourable environment for some microbes and disadvantageous conditions for others (den Besten et al. 2013).

The ability to digest and absorb carbohydrates is believed to be highly adaptable in poultry (Murugesan et al. 2014; Suvarna et al. 2005), and the digestive capacity of the intestine can be increased through the expansion of surface area which occurs with lengthening of villi (Moran 1985). The turnover rate of small intestinal epithelium in chickens allows for rapid adjustments of crypts and villi, ensuring that chickens can adapt quickly to altered nutrient availability (Imondi and Bird 1966).

The following study was based on the hypothesis that a HS diet would result in excessive amounts of undigested starch reaching the distal part of ileum and caecum, which could affect the count and toxin gene profile of *C. perfringens* and the prevalence of necrotic enteritis. Based on the considerations above, it was expected that the different macronutrient contents in the HS and the LS diet would influence small intestinal histomorphometry and production of SCFAs. The objective of this study was to investigate the effects of two diets with different starch to fat ratios on intestinal histomorphometry, *C. perfringens* counts and toxin profile, occurrence of necrotic enteritis and abundance of short-chain fatty acids (SCFAs) in *Eimeria*-challenged broiler chickens. Effects on nutrient digestibility and production performance have been discussed in an accompanying paper (Itani et al. *in press*).

## Materials and methods

### Animals and housing

The experiment was approved by the national animal research authority (Norwegian Food Safety Authority, approval ID 8824), and performed in accordance with national and international guidelines for the care and use of experimental animals. The housing, management and environment are described in detail in an accompanying paper (Itani et al. *in press*). Briefly, 1,920, one-day-old Ross 308 broiler chickens were obtained from a commercial hatchery (Nortura Samvirkekylling, Våler, Hedmark, Norway) and housed in floor pens on new wood shavings in a climate-controlled poultry research facility (Scandinavian Poultry Research, Våler, Hedmark, Norway). Water and feed were provided *ad libitum*.

### Diets & experimental design

From d 0 to d 9, all chickens were fed a common commercial starter diet containing 366 g/kg starch (mostly derived from wheat) and 61 g/kg fat (mostly derived from soybean oil and

animal fats), providing a starch to fat ratio of 6:1. On d 10, the chickens were randomly divided into two treatment groups, both comprising 12 pens with 80 chickens each with a balanced male to female distribution per pen. During the experimental period from day 10 to day 29, chickens in the two treatment groups were fed either a high starch to fat ratio (HS; ratio of 32:1) or a low starch to fat ratio (LS; ratio of 2:1) diet. The diets were formulated to be isocaloric by replacing wheat starch in the HS diet by rapeseed oil and silica sand in the LS diet. Both diets were pelleted and formulated to meet or exceed the Ross 308 nutrition specifications (Aviagen 2014). All diets were free from in-feed antimicrobials, including antibacterial growth promoters and anticoccidials. More details on diet composition and feed processing are described in the accompanying paper (Itani et al. *in press*).

On d 17, chickens in both treatment groups were challenged orally with a 10-fold dose of the vaccine Paracox-5 vet. (MSD Animal Health, Boxmeer, the Netherlands) containing live, sporulated oocysts from five attenuated strains of *Eimeria* spp. (one precocious line each of *Eimeria acervulina* [approximately 5750 oocysts per broiler], *Eimeria mitis* [approximately 11,500 oocysts], and *Eimeria tenella* [approximately 5750 oocysts], and two precocious lines of *Eimeria maxima* [approximately 3450 oocysts]) administered through the drinking water. On d 16, 21–23 and 29, 12 chickens per treatment group (one chicken from each replicate pen) were randomly selected and euthanised by a cranial blow immediately followed by cervical dislocation before necropsy and sample collection.

### Morphometric analysis

Unopened intestinal segments of approximately 1 cm taken from the transition between duodenum and jejunum at the end of the duodenal loop were collected during necropsy on d 16, 21–23 and 29. The samples were fixed in 10% neutral-buffered formalin solution. Formalin-fixed intestinal tissue samples were embedded in paraffin and sectioned at 5 µm. Standard protocols for haematoxylin and eosin staining of histological paraffin sections were followed. Villus length and crypt depth were measured using a microscope (Nikon Eclipse 80i, Nikon Instruments Europe B.V., Amsterdam, the Netherlands) fitted with a digital camera (Nikon DS-Ri1, Nikon Instruments Europe B.V.) using the image software NIS-Elements D v4.40 (Nikon Instruments Europe B.V.). Villus length was measured from the tip of the villus to the crypt-villus junction. Crypt depth was measured from the base to the crypt-villus junction. The mean of ~10 measured villi and crypts per chicken was calculated. Only clearly defined and fully finger-shaped villi were included and histological sections of suboptimal quality not suitable for morphometric analysis were excluded. Mean villus length, crypt depth and villus:crypt ratio in each treatment group were calculated.

### *Clostridium perfringens* quantification and toxin gene analysis

On d 21, 22 and 23 (d 4, 5 and 6 after *Eimeria* challenge, respectively) caecal samples (1.0–2.0 g) were collected in sterile stomacher bags and immediately subjected to cultivation in order to quantify *C. perfringens*. Briefly, caecal samples were diluted 1:100 in peptone saline water (0.1% peptone, Difco Laboratories Inc., Detroit, MI, USA, and 0.85% NaCl) and homogenised for 30 seconds (Bagmixer

400 CC, Interscience, Saint Nom, France). Serial dilutions were made with non-buffered peptone water until a dilution of  $10^{-6}$  was reached. Aliquots of 100  $\mu$ l from the dilutions  $10^{-2}$ ,  $10^{-4}$  and  $10^{-6}$  were plated onto sheep blood agar plates and incubated anaerobically at 37°C for 24 hours (Genbox anaer, Biomérieux, Marcy-l'Étoile, France). Single colonies with double haemolysis were counted, and colony-forming units per gram (cfu/g) of caecal content was calculated. Typical colonies were selected for pure cultivation and confirmed as *C. perfringens* by a matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometer (Bruker Daltonics, Bruker Corp., Billerica, MA, USA).

Caecal contents were collected in 1.5 ml Eppendorf tubes and immediately put on dry ice during necropsy on d 16, 21–23 and 29. Samples were stored at  $-80^{\circ}\text{C}$  until further processing. DNA was extracted from 200 mg of caecal content using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with some modifications. After adding 1.4 ml of ASL lysis buffer, the samples were homogenised on a vortexer, followed by heat treatment at 70°C for 5 min. The total suspension from each sample was transferred into Precellys 2.0 ml homogenisation tubes pre-filled with 0.1 mm ceramic beads (Bertin Technologies, Montigny-le-Bretonneux, France). Mechanical lysis was done with a Precellys 24 homogeniser (Bertin Technologies) for  $2 \times 30$  seconds at 6800 rpm. Samples were centrifuged for 1 min at 14,000 rpm in order to pellet both the beads and large particles. The supernatant was transferred into new 2 ml tubes and heated at 70°C for 5 min. One InhibitEX tablet was added to each sample, and, for the rest of the procedure, the protocol provided by the manufacturer was followed. DNA yields were measured with the Qubit double-stranded DNA Broad-Range Assay Kit on a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The purity of the DNA extracts was determined by measuring the ratios of absorbance at 260/280 and 260/230 using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). DNA was stored at  $-20^{\circ}\text{C}$  until use.

The relative abundances of the *C. perfringens* pathogenicity-associated necrotic enteritis toxin B-like gene (*netB*) and the omnipresent alpha toxin gene (*cpa*) were analysed using a multiplex real-time qPCR in order to determine the ratio of presumptively pathogenic to total *C. perfringens* (*netB:cpa* ratio) in intestinal samples. The qPCR assay used primers and probes as described previously (Albini et al. 2008; Schlegel et al. 2012) with some modifications (Table 1). *C. perfringens* strain 56 (CP56) was used as positive control, and nuclease-free water was used as negative control. The CP56 was originally isolated from the gut of a broiler chicken with severe necrotic enteritis lesions, and the strain has previously been classified as a *netB* toxin positive strain (Gholamiandekordi et al. 2006). The *cpa* gene served as an internal positive control for each PCR reaction due to its presence on the chromosome of all *C. perfringens* isolates

(Songer 1996). Each reaction was performed in duplicate in a 25  $\mu$ l total reaction mixture containing 12.5  $\mu$ l Brilliant III Ultra-Fast qPCR Master Mix (Agilent Technologies, Santa Clara, California, US), 20  $\mu$ M of primers and probes, and 3  $\mu$ l of the DNA sample. A Bio-Rad CFX-96 real-time PCR instrument (Bio-Rad Laboratories Inc., Hercules, California, US) was used. The qPCR conditions were 1 cycle at 50°C for 10 min and 1 cycle at 95°C for 5 min, followed by 48 cycles at 95°C for 30 s and 60°C for 1 min. Data analysis was performed using Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories Inc.) and Microsoft Office Excel 2016 (Microsoft, Redmond, Washington, US). The standard curve was based on data from serial 10-fold dilutions ( $10^0$ – $10^{-4}$ ) of DNA from CP56. Amplification efficiency for both PCRs were calculated from these titrations to confirm that it was within the range of 90–110%. The slope and shape of amplification curves were inspected and quantification cycle (Cq) thresholds above 40 were excluded from the analysis. The threshold lines were set at the same level for both PCRs, at a point above the background fluorescence and in the beginning of the exponential phase. The amplification signals from the target genes in the undiluted positive control CP56 overlapped at this point, indicating that the ratio of *netB* to *cpa*, based on Cq values for this positive control, was 1:1. The percentage of *netB*-positive samples was calculated based on the presence or absence of the *netB* gene signal. The relative abundances of the *cpa* and the *netB* genes were determined from the standard curve, and the *netB:cpa* ratios in caecal samples were calculated by dividing the relative *netB* abundance by the relative *cpa* abundance.

### Short chain fatty acids

Caecal samples were collected in Eppendorf tubes and immediately put on dry ice during necropsy on d 16, 21–23 and 29. Samples were stored at  $-80^{\circ}\text{C}$  until further analysis. The SCFA concentrations in the caecal samples were determined according to a previously described method (De Weirdt et al. 2010). In short, SCFAs were extracted using diethyl ether, and 2-methylhexanoic acid (99%) was added to each sample as internal standard. The extracts were analysed using a GC-2014 gas chromatograph (Shimadzu, BB 's-Hertogenbosch, the Netherlands), equipped with a capillary fatty-acid free EC-1000 EconoCap column (Alltech, Laarne, Belgium).

### Necrotic enteritis

The small intestine of 12 chickens (one per replicate pen) per treatment were opened longitudinally and inspected for pathological changes indicating necrotic enteritis on d 16, 21, 22, 23 and 29. A modified necrotic enteritis scoring system was used (Lovland et al. 2004). If no macroscopic mucosal ulcers, depressions or pseudomembranes were present, the chickens were classified as 'necrotic enteritis

**Table 1.** Primers and probes used for qPCR analysis.

Target	Description	Nucleic acid sequences 5' to 3' with probe dyes (Tm)	Product length (bp)	Reference
<i>cpa</i>	Forward primer	AAG AAC TAG TAG CTT ACA TAT CAA CTA GTG GTG (57.3°C)	124	(Albini et al. 2008)
	Reverse primer	TTT CCT GGG TTG TCC ATT TCC (55.4°C)		
	Probe	HEX-TTG GAA TCA -ZEN- AAA CAA AGG ATG GAA AAA CTC AAG-IBFQ (58.4°C)		
<i>netB</i>	Forward primer	GGC GGT AAT ATA TCT GTT GAA GG (53.1°C)	168	(Schlegel et al. 2012)
	Reverse primer	ACC GTC CTT AGT CTC AAC (51.0°C)		
	Probe	FAM-ACT GCT GGT -ZEN- GCT GGA ATA AAT GCT TCA-IBFQ (60.8°C)		



negative'. Identification of minimum one mucosal ulcer, depression or pseudomembrane resulted in classification as a 'necrotic enteritis positive' chicken.

### Gizzard scores

Gizzards from 12 chickens per treatment (one per pen replicate) on d 16, 21, 22, 23 and 29 were opened, emptied and carefully washed in order to inspect the gizzard linings. A score for each gizzard was given on the basis of score 0 for no visible erosions (negative), 1 for definite erosions with restricted or focal distribution (mild), 2 for multiple erosions with widespread distribution but more than 50% normal mucosa remaining (moderate) and 3 for erosions covering more than 50% of the gizzard surface area (severe).

### Statistical analysis

Samples were collected from one chicken from each replicate pen (12 chickens per treatment group) on each sampling occasion. Initially, data from all variables were checked for normality and homogeneity of variance using Shapiro-Wilk test and Variance-comparison test, respectively. If the criteria for using parametric testing were fulfilled (normal distribution and homogeneity of variance), an independent two-sample t-test was used. If the assumptions of normality and/or homogeneity of variance were violated, the non-parametric Mann-Whitney U test (Wilcoxon rank-sum test) was used. For categorical data, Fisher's exact test was used. Choice of statistical tests for each outcome variable are given in the results sections and/or in tables and figures. Differences between means were considered significant at  $P < 0.05$ . Statistical analyses were performed using Stata version 14.2 (StataCorp LLC, College Station, TX, USA) and graphics were made using R version 3.5.3 (R Foundation for Statistical Computing, Vienna, Austria).

## Results

### Morphometric analysis

There was no significant difference in mean villus length, crypt depth or villus:crypt ratio in the duodenojejunal junction between the two groups on d 16 and 21–23 (Table 2, t-test). On d 29, chickens fed the HS diet had longer villi compared to chickens fed the LS diet ( $P < 0.001$ ). There was no difference in mean crypt depth on d 29. As a result of the difference in villus length, the mean villus:crypt ratio on d 29 was higher in chickens fed the HS diet compared to chickens fed the LS diet ( $P = 0.037$ ).

The length of villi in the HS group increased gradually from d 16 to day 29 (Table 2). This increase was most pronounced between d 16 and d 21–23 (25% increase,  $P = 0.001$ , t-test). In the LS group, villus length increased strongly from d 16 to d 21–23 (39% increase,  $P < 0.001$ , t-test), but decreased in the period from d 21–23 to 29 (13% decrease,  $P = 0.045$ , t-test).

### *Clostridium perfringens* quantification and toxin gene analysis

There was no significant difference in caecal *C. perfringens* counts (cfu/g) between the two treatment groups between d 21–23 ( $P = 0.098$ , Mann-WhitneyU test) (Figure 1).

**Table 2.** Effect of dietary starch to fat ratios on intestinal histomorphometry on days 16, 21–23 and 29.<sup>1</sup>

Age	Variable	Diet <sup>2</sup>		P-value <sup>3</sup>
		HS	LS	
16 days	Villus length (µm)	1255 ± 33	1187 ± 30	0.154
	Crypt depth (µm)	175 ± 5	179 ± 4	0.493
	Villus:crypt ratio	7.23 ± 0.2	6.67 ± 0.3	0.153
21–23 days	Villus length (µm)	1570 ± 78	1650 ± 83	0.497
	Crypt depth (µm)	175 ± 7	192 ± 7	0.111
	Villus:crypt ratio	9.09 ± 0.6	8.74 ± 0.6	0.669
29 days	Villus length (µm)	1689 ± 48	1440 ± 33	<0.001
	Crypt depth (µm)	174 ± 5	176 ± 8	0.875
	Villus:crypt ratio	9.83 ± 0.5	8.32 ± 0.4	0.037

<sup>1</sup>Values are means ± SEM.

<sup>2</sup>HS: high starch to fat ratio diet; LS: low starch to fat ratio diet.

<sup>3</sup>Independent two-sample t-test.

The qPCR analysis revealed that all caecal samples from both treatment groups were *cpa*-positive. The frequency of samples with presence of the *netB* gene was calculated (Table 3). From d 16 to d 29, the overall percentage of *netB*-positive caecal samples in both treatment groups increased from 79% to 100% ( $P = 0.05$ , Fisher's exact test). There was no statistically significant effect of diet on *netB* prevalence at any time point or in the overall experimental period.

The *netB:cpa* ratio was similar (Figure 2) in the two treatment groups on d 16 ( $P = 0.354$ ), d 21–23 ( $P = 0.624$ ) and d 29 ( $P = 0.299$ ). In the HS group, the *netB:cpa* ratio increased from d 16 to 29 ( $P = 0.018$ , Mann-Whitney U test), indicating that the relative abundance of *netB* genes present in caecal samples from chickens fed the HS diet increased to a greater extent than the relative abundance of *cpa* genes in this period. The HS group on d 29 was the only subgroup with mean and median *netB:cpa* ratio above 1.0, indicating that the relative abundance of *netB* was higher than the relative abundance of *cpa* in this group at this time point.

### Short chain fatty acids

On d 16, caecal samples from chickens fed the HS diet had higher levels of acetic acid ( $P = 0.016$ ) and propionic acid ( $P = 0.006$ ) compared to caecal samples from chickens fed the LS diet (Table 4). This resulted in higher total SCFA concentration ( $P = 0.016$ ) in the caecum from chickens fed HS diet at this time point.

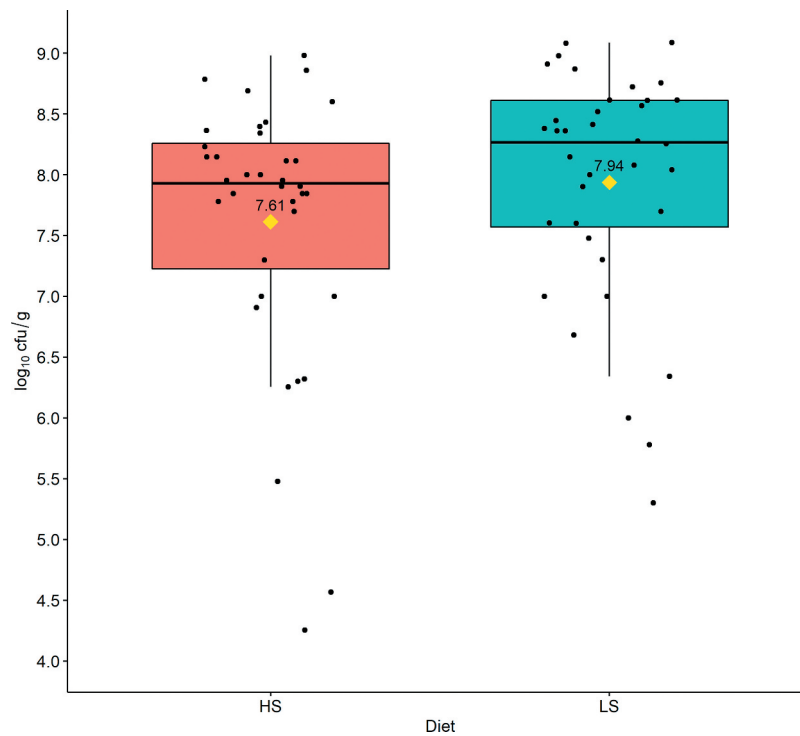
The dynamic development of total caecal SCFA concentration was similar in the two diet groups, with a maximum level reached on d 21–23. The increase from d 16 to d 21–23 was significant in the LS group ( $P < 0.001$ , t-test).

### Necrotic enteritis

The overall prevalence of necrotic enteritis on d 16 was 8.3% (2/24 chickens). In the period following *Eimeria* challenge (d 21–23) the prevalence was 16.7% (6/36 chickens) in the HS group and 13.9% (5/36 chickens) in the LS group. This difference in necrotic enteritis prevalence was non-significant ( $P > 0.05$ , Fisher's exact test). On d 29, no mucosal ulcers, depressions or pseudomembranes indicating necrotic enteritis were detected in the small intestine of any of the chickens examined.

### Gizzard scores

On d 16, the mean gizzard score was 2.0 and 1.75 in the HS and LS group, respectively ( $P = 0.508$ , t-test). During the days



**Figure 1.** Effect of dietary starch to fat ratios on caecal *Clostridium perfringens* counts ( $\log_{10}$  cfu/g) during days 21–23. Boxplots with medians indicated by horizontal lines and means indicated by diamonds. Based on samples from 36 chickens per diet. HS: high starch to fat ratio diet; LS: low starch to fat ratio diet. cfu/g = colony forming units per gram.

**Table 3.** The percentage of *netB*-positive and *netB*-negative caecal samples.<sup>1</sup>

Age	Diet	<i>netB</i> -negative		<i>netB</i> -positive	
16 days	HS	25%	21%	75%	79%
	LS	17%		83%	
21–23 days	HS	8%	8%	92%	92%
	LS	8%		92%	
29 days	HS	0%	0%	100%	100%
	LS	0%		100%	
Total			10%		90%

<sup>1</sup>Based on samples from 12 chickens per diet per time point. HS: high starch to fat ratio diet; LS: low starch to fat ratio diet.

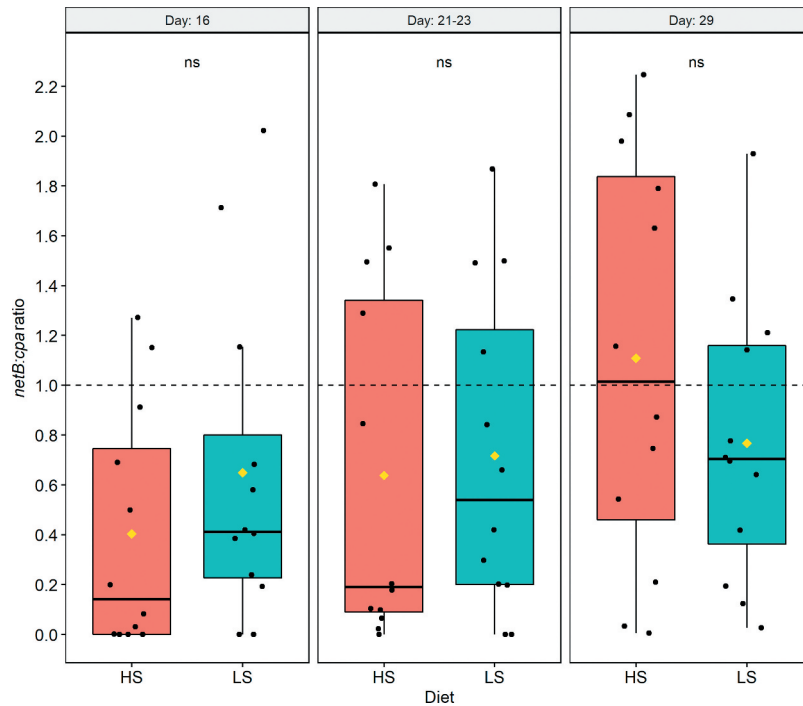
following *Eimeria* challenge (d 21, 22 and 23), the overall median gizzard score was 3 in both treatment groups ( $P = 0.489$ , Mann-Whitney U test). At the end of the experiment on d 29, the mean gizzard score was 2.7 in chickens fed the HS diet and 2.8 in chickens fed the LS diet ( $P = 0.368$ , t-test). There was no effect of diet on gizzard scores in the experiment.

## Discussion

The use of intestinal *C. perfringens* counts as an indicator of gastrointestinal health in this study is based on this bacterium's association with gizzard erosions (Novoa-Garrido et al. 2006), necrotic enteritis (Kaldhusdal and Hofshagen 1992) and growth depression (Stutz and Lawton 1984) in broiler chickens. *C. perfringens* produces amylases and has the capacity to break down and utilise starch (Shih and Labbe 1996). Any undigested starch reaching lower gut regions could thus encourage overgrowth of this opportunistic pathogen. In spite of the higher starch content in the HS diet, this diet did not lead to higher caecal *C. perfringens* counts than the LS diet at d 21–23. As shown in the accompanying paper, the concentration of starch in ileal digesta tended to be higher on d 16 (28% more starch,  $P = 0.067$ ), and was clearly higher on d 29 (31% more starch,  $P = 0.015$ ) in the HS group compared to the LS group (Itani et al. *in press*). These findings

indicated that more undigested starch reached the lower intestinal regions in chickens fed the HS diet, but the difference in ileal substrate availability at these time points did not affect caecal *C. perfringens* counts significantly during the most critical time interval after *Eimeria spp.* challenge (d 21–23). This result was in agreement with the lack of difference in overall gizzard scores and necrotic enteritis frequency between chickens fed the two diets. Neither of the treatment groups in this study had very high levels of starch in the ileum on the day before *Eimeria spp.* challenge (80 and 58 g/kg in the HS and LS group, respectively) unlike chickens in another study (222 g/kg) which had poor starch digestibility (Svihus and Hetland 2001). The relatively low levels of starch, and limited substrate availability in the posterior gut regions, could explain the lack of difference in important gut health variables between the treatment groups in this study. The presence of other microbes outcompeting *C. perfringens* in their use of starch as fermentation substrate is an additional or alternative possible explanation for the lack of impact of diet on caecal *C. perfringens* counts and necrotic enteritis prevalence.

In chickens, *C. perfringens* type G (previously designated type A) has been reported as the predominant toxinotype causing necrotic enteritis (Rood et al. 2018, Van Immerseel et al. 2004). The alpha-toxin-encoding gene *cpa* is present on the chromosome of all types of *C. perfringens* strains (Petit et al. 1999), and was used as an indicator of total *C. perfringens* abundance in caecal samples in a quantitative PCR (qPCR) assay in this study. Another toxin, the pore-forming plasmid-encoded toxin designated NetB, is believed to be a key virulence factor associated with necrotic enteritis in chickens (Keyburn et al. 2008). The current trial showed a relatively high percentage of *netB*-positive caecal samples on d 16 prior to *Eimeria spp.* challenge in (Table 3). In a previous study, *netB*-positive *C. perfringens* strains were present in a commercial broiler



**Figure 2.** Effect of dietary starch to fat ratios on the *Clostridium perfringens netB:cpa* ratio in caecal samples on days 16, 21–23 and 29. Boxplots with medians indicated by horizontal lines and means indicated by diamonds. Based on samples from 12 chickens per diet per time point. Statistical analysis carried out by Mann-Whitney U test. HS: high starch:fat ratio diet; LS: low starch:fat ratio diet; ns = not significant.

**Table 4.** Effect of dietary starch to fat ratios on short chain fatty acids (SCFAs;  $\mu\text{mol/g}$ ) in caecum.<sup>1</sup>

	Day 16			Day 21–23			Day 29		
	HS	LS	P-value	HS	LS	P-value	HS	LS	P-value
Acetic acid	58.24 $\pm$ 4.17	43.43 $\pm$ 2.95	0.016	66.75 $\pm$ 2.76	67.28 $\pm$ 4.99	0.729	62.34 $\pm$ 3.80	55.79 $\pm$ 4.37	0.184
Propionic acid	3.29 $\pm$ 0.33	1.85 $\pm$ 0.35	0.006	4.32 $\pm$ 0.49	4.39 $\pm$ 0.70	0.624	4.33 $\pm$ 0.24	4.09 $\pm$ 0.47	0.356
Butyric acid	10.21 $\pm$ 0.85	9.57 $\pm$ 0.95	0.580	12.96 $\pm$ 1.27	13.72 $\pm$ 0.86	0.419	11.24 $\pm$ 1.37	11.67 $\pm$ 1.31	0.773
Total SCFAs	73.94 $\pm$ 4.99	55.96 $\pm$ 3.78	0.016	86.89 $\pm$ 4.01	88.23 $\pm$ 5.65	0.356	80.64 $\pm$ 5.19	73.59 $\pm$ 5.82	0.299

<sup>1</sup>Values are means ( $\mu\text{mol/g}$ )  $\pm$  SEM. Statistical analysis carried out by Mann-Whitney U test. HS: high starch to fat ratio diet; LS: low starch to fat ratio diet.

house prior to placement of one-day-old broilers (Engstrom et al. 2012). From this background, it seems reasonable to suggest that *netB*-positive *C. perfringens* strains could have been present in the environment from placement of the one-day-old broilers in the current study. The frequency of *netB*-positive caecal samples increased from 16 to 29 d of age. Although no necrotic enteritis was detected on d 29, all caecal samples were *netB*-positive at this age. A possible explanation was that *netB*-positive strains were transmitted from broilers with subclinical necrotic enteritis to healthy birds, and eventually colonised the intestine of all broilers independent of health status. The interpretation of the presence of the *netB* gene with regard to pathogenicity of *C. perfringens* can be complex, and previous studies showed that *netB*-positive *C. perfringens* strains alone are apparently not enough to cause disease without predisposing factors being present (Keyburn et al. 2010; Yang et al. 2019).

The significant increase in *netB:cpa* ratio from d 16 to day 29 in the HS group implied that the relative abundance of *netB* increased to a greater extent than the relative abundance of *cpa* in this period. Since a significant increase in *netB:cpa* ratio was not present in the LS group, it was possible that diet and starch level played a role in this development. As mentioned before, *C. perfringens* can utilise starch, which was available in larger amounts in ileal contents in the HS group compared to the LS group (Itani et al. in press). Thus, starch may have predisposed birds towards intestinal

colonisation by *netB*-positive *C. perfringens* in this study. The *netB:cpa* ratio has been suggested as a faecal marker to monitor subclinical necrotic enteritis in broilers (Goossens et al. 2019). The current study was not designed to compare *netB:cpa* ratios in groups with and without necrotic enteritis, but the results demonstrated that the *netB:cpa* ratio was not directly associated with prevalence of necrotic enteritis at the time of sampling. It is, however, possible that this ratio can be useful as an indicator of previous necrotic enteritis occurrence in the examined flock. More work is required to determine the use of this marker.

In addition to age-related lengthening of intestinal villi in broilers (Alshamy et al. 2018), villus length and development are influenced by feed composition and nutrient availability (Moran 1985). Elongation of villi increases the absorptive surface of the intestine and augments the amount of brush border enzymes available to break down oligosaccharides originating from the activity of amylase on dietary starch in the anterior small intestine. Hence, increased villus length and consequently expansion of the mucosal surface area is considered an important mechanism in adaptation to higher starch levels in the diet (Moran 1985). The difference in villus length between the HS and LS group on d 29 supported this view.

The development of villus length was similar in chickens fed the LS and HS diets during the time interval between d 16 and d 21–23. This similarity might have been associated with

the *Eimeria spp.* challenge on d 17, considering that lengthening of villi as a compensatory mechanism following *Eimeria acervulina* infection has been described previously (Fernando and McCraw 1973). However, the two diet groups showed clearly distinct developments in villus length from d 21–23 to d 29. The decrease in villus length in the LS group was in contrast to the continued increase of villus length in the HS group. Both groups were challenged with *Eimeria spp.* and samples were taken at the same age. The difference in villus length development between d 21–23 and d 29 was thus most likely related to the impact of nutrients and diet. Digestion of fat and fatty acids is believed to occur mainly in the jejunum and the upper ileum (Tancharoenrat et al. 2014), while the greatest part of starch digestion occurs in the duodenum and jejunum (Osman 1982; Riesenfeld et al. 1980). Intestinal adaptation to increased levels of fat in the diet, such as raised expression of fatty acid binding proteins in epithelial cells (Krogdahl 1985), is thus likely to primarily take place in more posterior regions of the small intestine than adaptation to increased starch levels. The results from this study suggested that a diet containing a higher level of starch stimulates villus elongation in the duodenojejunal junction to a larger extent than a diet containing less starch and more vegetable fat.

The higher total caecal SCFA concentration in chickens fed the HS diet compared to chickens fed the LS diet on d 16 implied a larger number of SCFA-producing bacteria in the intestines of this group (den Besten et al. 2013). The dynamic development of total caecal SCFA concentration showed a similar trend in both the HS and the LS group, with an initial increase and a maximum level reached during d 21–23, followed by a moderate and non-significant decrease towards the end of the experiment (Table 4). The increase in total SCFA concentration from d 16 to d 21–23 was, on average, 18% in the HS group and 58% in the LS group, which suggested a more considerable increase in activity of SCFA-producing bacteria in the LS group. A possible explanation for this may be that the infection process following exposure of both groups to the same dose of *Eimeria spp.* on d 17 affected a smaller area of available digestive surface in the HS group than in the LS group, since the HS group (from d 10) adjusted to the higher level of dietary starch by the relative increase of intestinal surface area and villus length in the anterior small intestine (Moran 1985). A reduction in starch digestibility associated with *Eimeria spp.* challenge (Amerah and Ravindran 2015) could have affected the HS group to a lesser extent compared to the LS group, due to the larger remaining functional intestinal surface area. Consequently, more starch potentially reached posterior gut regions in the LS group in this period (data not shown), resulting in larger amounts of substrates accessible for SCFA-producing bacteria and a more pronounced increase in total SCFA concentration. This reasoning was supported by the fact that starch digestibility was improved from d 16 to 29 in the HS group but not in the LS group (data presented in the accompanying paper Itani et al. *in press*).

There was no clear-cut difference in necrotic enteritis prevalence, caecal *C. perfringens* counts or gizzard scores between chickens fed the two diets in this study. The hypothesis that a HS diet would lead to unfavourable effects on intestinal health in broiler chickens compared to a LS diet was not confirmed in this study. As discussed in the accompanying paper, feed processing and the physical form of starch sources were potentially confounding factors (Itani et al. *in press*). The unintentionally higher extent of

starch gelatinisation and the use of isolated wheat starch in the HS diet could have contributed to the relatively small difference in ileal starch level between the two groups, in spite of the marked difference in dietary starch content (45% in HS and 23% in LS). Taking this into account, the current data cannot be used to reject the hypothesis that high levels of dietary starch may impair intestinal health in broiler chickens.

In conclusion, there was an impact of diet on the length of anterior small intestinal villi and abundance of SCFAs in the caecum. A possible explanation for the difference in villus morphology was that the two groups had to adapt to distinct dietary macro-nutrient ratios, which required different ways of extracting energy from the feed. Due to ongoing adaptation to higher levels of dietary starch through villus elongation and increased mucosal surface (Moran 1985), chickens fed the HS diet were conceivably less vulnerable to the loss of absorptive capacity caused by the *Eimeria spp.* challenge. Chickens fed the LS diet had to rapidly adjust to loss of absorptive function in a larger proportion of the duodenal-jejunal mucosal surface in the period following challenge, and apparently accelerated the lengthening of villi in order to maintain nutrient absorption capacity. This structural remodelling of the intestine was energy-demanding, and this cost was reflected in reduced weight gain during d 15 to 24 and poorer accumulated feed conversion in the LS group (Itani et al. *in press*). The reduction in absorptive capacity of chickens fed the LS diet was underpinned by the more pronounced increase in total caecal SCFA concentration from d 16 to d 21–23 in this group, which strongly suggested that more undigested starch reached lower gut regions and were available for bacterial fermentation in this period. Collectively, these results suggested that chickens which were adapted to a diet with a higher level of starch were better prepared to cope with intestinal damage caused by a mild to moderate *Eimeria spp.* challenge affecting the anterior small intestine, compared to chickens fed a diet with a lower level of starch. This hypothesis was supported by the fact that the LS group had clearly poorer production performance during d 15–24 (Itani et al. *in press*), an age interval that included peak oocyst excretion following *Eimeria spp.* challenge. However, because the study was not designed to investigate the relationship between dietary starch level and *Eimeria* infections, this hypothesis needs to be tested experimentally.

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