Clostridium perfringens strains proliferate to high counts in the broiler small intestinal tract, in accordance with necrotic lesion severity, and sporulate in the distal intestine

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Abstract

Clostridium (C.) perfringens is the causative agent of necrotic enteritis (NE), an important enteric disease in poultry. Although a variety of virulence factors have been identified and as such the pathogenesis is well studied, data on colonization and sporulation during passage in the intestinal tract are scarce. This study, therefore, evaluated the behaviour of *C. perfringens* in the different intestinal compartments of broiler chickens during a NE trial. Necrotic enteritis-associated lesions were mostly found in the jejunum, where they were significantly more severe compared to the duodenum and ileum. Furthermore, a positive correlation between the total number of vegetative *C. perfringens* cells in the duodenum, jejunum, ileum, or distal colon and disease severity was observed. Additionally, in the caecum and distal colon, *C. perfringens* was mainly present as a spore. This observation has important consequences for NE treatment and prevention, as both the vegetative cells and *C. perfringens* spores should be targeted to avoid uptake of spores from the litter and reinfection of the birds after antibiotic treatment.

Keywords

Clostridium perfringens, necrotic enteritis, colonization, sporulation

1. Introduction

Necrotic enteritis (NE) is a devastating enteric disease, affecting different avian species, including broiler chickens, causing an estimated annual global economic loss of more than US$ 6 billion in the broiler industry (Wade and Keyburn, 2015). Although once the causative *Clostridium perfringens* strains are introduced into a flock most birds will be infected, not all will develop disease, if any (Drigo et al., 2009). Several studies mention isolation of *C. perfringens* NetB positive strains from healthy animals, clearly demonstrating a certain fraction of each flock is resistant to NE, also indicated by the lack of disease in a subset of birds in experimental models (Gholamiandekhordi et al., 2006; Drigo et al., 2009; Keyburn et al., 2010). Some of these differences in NE prevalence may be attributed to the

genetics of the birds, resulting in natural immunity. Additionally, not all birds are necessarily equally exposed to predisposing factors, such as a parasitic infection with *Eimeria* (Caly et al., 2015; Lin et al., 2017). Another reason, maybe as consequence of the above mentioned factors, could be a difference in the colonisation rate of the animals by the causative *Clostridium perfringens* strains (Moore, 2016). A higher colonisation rate could explain a more severe course of the disease.

C. perfringens is a non-invasive pathogen with ubiquitous environmental distribution in soil, food, sewage, as well as the gastrointestinal tract in both diseased and non-diseased animals (Cooper et al., 2013; Uzal et al., 2016). The observation that *C. perfringens* is highly persistent throughout nature can be attributed to another of its characteristics, which is the ability to form heat-resistant endospores in unfavourable environmental conditions (Li et al., 2016; Talukdar et al., 2017). Studies mostly mention that sporulation mainly occurs in (wet) litter since that environment is less favourable as compared to the chicken gut (Caly et al., 2015; Moore, 2016). However, questions have been raised whether that is really the case, or whether sporulation already starts in the chicken intestine and *C. perfringens* is released into the environment as spores. Previous studies have suggested that sporulation does in fact take place in the gastrointestinal (GI) tract of humans (Li et al., 2016). To our knowledge, no study which directly investigated the behaviour of *C. perfringens* in the chicken gut during NE has been conducted until today. This study therefore aimed to elucidate whether there is an association between numbers of vegetative and spore form *C. perfringens* in the different intestinal compartments of chickens and the disease severity, during a NE *in vivo* trial.

2. Materials and methods

2.1. *Strains and culture conditions*

The pathogenic *C. perfringens* type G strain CP56, isolated from necrotic lesions (Gholamiandehkordi et al., 2007), was cultured anaerobically overnight (ON) at 37°C in TGY broth (3% tryptone (Sigma

Aldrich, St. Louis, Missouri, US), 2% yeast extract (Sigma Aldrich), 0.1% glucose (Sigma Aldrich) and 0.1% L-cysteine (Sigma Aldrich)).

2.2. *Media preparation*

CHROMagar™ C. perfringens was prepared according to the manufacturer's instructions. Briefly, CHROMagar™ C. perfringens base was supplemented with CHROMagar™ C. perfringens supplement 1 to a final concentration of 2000 mg/L, as well as CHROMagar™ C. perfringens supplement 2 to a final concentration of 120 mg/L. Sterile supplements were added after sterilization of the respective media, after which they were dispersed in 120 x 120 mm petri dishes, air dried at room temperature and stored at 4 °C for maximum 30 days.

2.3. *In vivo necrotic enteritis model*

All experimental procedures involving animals were approved by the ethical committee of the Faculties of Veterinary Medicine and Bioscience Engineering of Ghent University (EC2020-045). The *in vivo* NE model used in this trial was based on a previously described study (Gholamiandehkordi et al., 2007). In short, 1-day-old unvaccinated Ross 308 broilers were randomly allocated to the treatment and control group with 12 birds/pen and 8 pens per treatment/control group. All broilers were fed a wheat/rye-based (43%/7.5%) diet supplemented with soybean meal as a protein source. From day 17 on, the diet was altered with fishmeal (30%) replacing the soybean meal as a protein source. These diets contain high levels of proteins and non-starch polysaccharides which predispose the chickens to the development of NE. On days 14 and 16, animals from the treatment group received a 10-fold dose of live attenuated *Eimeria* vaccines, respectively Hipracox (containing 5 *Eimeria* species: *E. tenella, E. acervulina, E. maxima, E. praecox and E. mitis*) (Hipra, Melle, Belgium) and Paracox-8 (containing 7 *Eimeria* species: *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox* and *E. tenella*) (MSD Animal Health, Brussels, Belgium), to induce a predisposing coccidial infection. On

day 18, 19 and 20, birds in the treatment group were challenged with an overnight culture of approximately 5 x 10^8 CFU of *netB*-positive *C. perfringens* strain CP56. On day 21, all animals were euthanized.

At necropsy, NE severity was evaluated by scoring lesions in the small intestine (duodenum, jejunum, ileum) as previously described by Keyburn et al., and the highest calculated score was allocated to the bird, regardless of the segment, as follows: score 0 = no lesions, score 1 = thin or friable wall, score 2 = focal necrosis or ulcerations (1–5 foci), score 3 = focal necrosis or ulcerations (6–15 foci), score 4 = focal necrosis or ulcerations (≥ 16 foci), score 5 = patches of necrosis of 2–3 cm long, score 6 = diffuse necrosis (Keyburn et al., 2006). Birds with a lesion score of 2 or more were considered NE positive. From birds that received each scoring class, duodenal, jejunal, ileal, caecal, and distal colon contents were collected of which a small fraction was used for plating on CHROMagar™ C. perfringens, while the rest was snap frozen and stored at -20 °C. Eleven birds were used per each scoring class.

2.4. *Assessing the numbers of relative spore levels and vegetative cells in intestinal content and faeces*

For the collection of gut content from all intestinal segments (duodenum, jejunum, ileum, caecum, and distal colon), 50-100 mg faeces or content were weighed in a 2 mL Eppendorf tube in duplicate. Each time, sterile phosphate-buffered saline (PBS) was added to the gut content and faeces (1/10 dilution). The samples were vortexed for 30 s at maximum speed followed by transferring 200 µL per sample to a 96-well plate where the samples where 10-fold serially diluted in sterile PBS. One replicate of each sample was, however, heat-shocked for 20 min at 80°C before diluting and plating 6 drops of 20 µL of each sample dilution on CHROMagar™ C. perfringens. The plates were subsequently air-dried and incubated anaerobically for 24 h at 37 °C, after which the total number of *C. perfringens* as well as the number of spores were determined by colony counting. Plates containing between 20 and 300 colonies

in 6 x 20 µl droplets were counted and numbers of CFU/g faeces were calculated. All plates with droplets consisting of more than 300 colonies were determined as uncountable.

2.5. *Statistical analysis*

Statistical analysis was performed using GraphPad Prism 8.4.3. A Kruskall-Wallis test was performed followed by Dunn's post hoc test, to identify the average score lesion in each intestinal compartment. Spearman correlation was applied to assess whether there was a link between the total *C. perfringens* numbers and disease severity, as well as relative spore numbers and disease severity, which was performed in the R statistical environment (R Core Team 2017, version 3.6.0). For all analyses, *p*-values smaller than 0.05 were considered statistically significant.

3. Results

3.1. *NE-associated lesions are more severe and prevalent in the jejunum of broiler chickens*

None of the birds from the control group, which were not challenged with *Eimeria* or *C. perfringens* strain CP56, developed NE lesions (n = 16), while a subset of birds that were *Eimeria* infected and challenged with the *netB*-positive strain CP56 developed NE. In 34.04 % (32/94) of these birds, no lesions were observed. Although lesions of NE-positive birds were observed in all segments of the small intestine, most lesions were found in the jejunum, with a prevalence of 61.70 % compared to 32.98 % and 12.76 % in the duodenum and ileum, respectively. Furthermore, the lesions in the jejunum were also significantly more severe compared to the lesions in the duodenum or ileum ($P < 0.0001$ and $P = 0.0057$, respectively), with a dominance of moderate to severe lesion scores (scores 3-4 and 5-6, respectively) present in 33.87 % (21/62) and 38.71 % (24/62) birds, respectively. The distributions of lesions scores in the small intestine are summarised in Table 1.

3.2. *NE lesion development is linked to higher C. perfringens numbers in the small intestine*

Analysis of the numbers of *C. perfringens* cells from challenged birds which developed necrotic lesions and unchallenged birds (negative control) showed significantly higher numbers of *C. perfringens* in challenged birds as compared to unchallenged birds, for all intestinal segments (all *P*-values below 0.0001, Fig. 1A). In the jejunum, caecum and distal colon, significantly higher numbers of total *C. perfringens* were found in birds that were challenged but did not develop NE lesions compared to the unchallenged birds ($P = 0.0128$ for jejunum, $P = 0.0015$ for caecum, $P = 0.0241$ for distal colon). When comparing the total *C. perfringens* numbers in challenged birds that developed NE lesions to challenged birds that did not develop lesions, significantly more *C. perfringens* was detected in the jejunum, ileum, and distal colon of the birds that developed necrotic lesions (*P*-values 0.0168, 0.0063 and 0.029, respectively). These results show that birds with higher numbers of *C. perfringens* are more likely to develop enteric NE lesions.

3.3. *Relative spore fractions are higher in challenged birds*

In addition to the total *C. perfringens* load, also the relative spore fraction in each intestinal segment was determined (Fig. 1B). No significant differences in the spore fraction were observed between challenged birds with lesions and challenged birds without lesions, in all intestinal segments. However, in the ileum, significantly more spores were observed in the challenged animals that developed lesions, compared to the negative control ($P = 0.001$, Fig. 1B). Additionally, both challenged birds with lesions, as well as challenged birds without lesions had a significantly higher fraction of spores in the caecum as compared to unchallenged birds ($P = 0.006$ for both). The same was observed in the distal colon, where challenged birds without lesions and challenged birds with lesions had significantly higher fraction of spores than the negative control birds ($P = 0.004$ and $P = 0.001$, respectively).

3.4. *Caecum and distal colon show significantly more spores as compared to other compartments*

Interestingly it was simultaneously observed that while lesions are commonly found in all compartments of the small intestine (duodenum, jejunum, ileum), the fraction of spores is overall lower in these compartments compared to the caecum and distal colon (Fig. 1B). In animals that developed NE, the fraction of spores in the caecum was significantly higher as compared to all small intestinal compartments ($P < 0.0001$). The same was observed in challenged animals that did not develop NE (duodenum vs caecum: $P = 0.039$, jejunum vs caecum: $P = 0.014$, ileum vs caecum: $P = 0.0019$) In unchallenged birds (negative control), the total *C. perfringens* load was similar between all intestinal segments. Furthermore, no differences in relative *C. perfringens* spore fraction were detected between the different intestinal segments of the unchallenged birds.

3.5. *Total C. perfringens numbers correlate with disease severity while relative spore fractions do not*

A Spearman correlation was used to assess the relationship between total *C. perfringens* numbers per intestinal segment and the disease severity (highest lesion score of each animal) (Fig. 2A). In all compartments of the small intestine, a positive correlation between the total *C. perfringens* load and the overall disease severity was observed, with the strongest correlation in the jejunum (Spearman $R = 0.67$, $P < 0.0001$). No correlation between the caecal *C. perfringens* load and NE disease severity was observed. In addition to the correlation between the total *C. perfringens* numbers and the disease severity in challenged birds, the relative spore fraction was also analysed. Nevertheless, no correlation could be observed between the % of spores and the disease severity in any of the intestinal compartments.

4. Discussion

C. perfringens is a normal inhabitant of the gut of broiler chickens, but when conditions are favourable pathogenic strains can proliferate and cause disease (Kiu and Hall, 2018). In order to successfully treat and/or prevent NE, it is necessary to understand how the causative agent, *C. perfringens*, behaves in the chicken gut. More specifically, knowing whether one intestinal compartment is more colonised by *C. perfringens* than another, and how this can affect lesion development and therefore disease severity, can be of importance when using dietary additives. During previous NE trials, our research group has observed that NE lesions are more prevalent and severe in the jejunum compared to other compartments, which is in accordance with older studies (Long et al., 1974; Damme et al., 2020). Interestingly, no lesions are found in the caecum or distal colon and the reason why the jejunum is most affected remains unknown, but a possible reason could be the colonisation level. In addition to the ileum and distal colon, the jejunum seems to be the intestinal segment with highest *C. perfringens* counts, which therefore can be linked to more lesions in that part of the chicken gut. A higher *C. perfringens* colonisation rate seems to result in more severe lesions. This, however, raises the question why no lesions whatsoever are found in the distal colon, although *C. perfringens* numbers are comparable with the jejunum and ileum. Therefore, not only the total *C. perfringens* count in each segment was assessed but also the ratio of *C. perfringens* spores vs. vegetative cells. The significantly higher number of spores in the caecum and distal colon could be one reason why no lesions are found in those parts of the intestine, since spores are metabolically dormant cells and thus not producing toxins. A possible explanation why sporulation mostly occurs in the lower parts of the intestine and not in the upper parts are bile acids. Primary bile acids are synthesised and conjugated in the liver, after which they are released into the gut lumen. Here, they are further metabolised by the gut microbiota into secondary bile acids (Ridlon et al., 2006; Bansal et al., 2020). Studies reported that in a *Clostridoides difficile* infected mouse-model,

some secondary bile acids, such as deoxycholate (DCA) or taurocholate (TCA) induce spore germination, while others, such as lithocholate (LCA) or ursodeoxycholate (UDCA), are inhibitory (Winston and Theriot, 2016). Additionally, bile acids have previously been shown to stimulate *C. perfringens* spore formation *in vitro* (Heredia et al., 1991; Park and Rafii, 2018). The small intestine most likely harbours more primary bile acids, which could induce germination of ingested spores, while secondary bile acids are more prevalent in the lower intestinal segments, such as the caecum and colon, as they might have been metabolised by the bacteria during the passage to the large intestine where they induce sporulation. Furthermore, a study showed that dietary DCA alleviated NE-induced ileal inflammation, which could mean that in chickens and for *C. perfringens*, DCA inhibited spore germination or induced sporulation (Bansal et al., 2020). Another reason for the higher spore number in the lower intestine compared to the upper intestine, could be that nutrients become scarcer in the large intestine, resulting in sporulation of *C. perfringens*, while more nutrients are available in the small intestine for *C. perfringens* growth. It has been commonly accepted and assumed that sporulation mainly takes place in the litter rather than in the animal itself (Caly et al., 2015). The high spore numbers in the caecum and distal colon of the birds, however, show that *C. perfringens* cells are mostly released as spores into the environment. This observation has important consequences for intervention and diagnosis of NE. If spores are present in high amounts in the animal, chances are high that NE might reoccur once the conditions are more favourable for *C. perfringens* to grow. Even when treated with antibiotics, spores will most likely persist in the animal and also be released into the environment, which explains why NE might reoccur although barns are thoroughly sanitised. A possible intervention could be treatments that have an inhibitory effect on *C. perfringens* spores, through for example adherence to the exosporium. This effect has been seen in *Clostridioides difficile* spores, where a vancomycin-loaded spore-targeting iron oxide nanoparticle has been developed to

successfully delay spore germination (Chiu et al., 2021). Furthermore, when developing new diagnostic tools, these findings also have to be taken into consideration, since it is likely that a diagnostic test will be based on the use of faeces, which will mostly contain *C. perfringens* spores rather than vegetative cells, and therefore no or less, active toxins.

Summarized, we demonstrated that the *C. perfringens* colonisation rate correlates with disease severity. The jejunum is the most colonized intestinal segment and the segment in which most lesions were found. No link between the fraction of *C. perfringens* cells that were present as spores and the disease severity was observed. Spores were found to be a lot more prevalent in the lower intestinal segments, where no lesions are found.

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Tables

Table 1: Score distribution of necrotic enteritis lesions in the small intestine of 21-day old broilers.

Group	Segment	Lesion score [a]						NE positive [b]	Average lesion score [c]
		0	2	3	4	5	6		
Control	Duodenum	16	0	0	0	0	0	0 % (0/16)	-
	Jejunum	16	0	0	0	0	0	0 % (0/16)	-
	Ileum	16	0	0	0	0	0	0 % (0/16)	-
	Overall							0 % (0/16)	-
CP56	Duodenum	63	17	4	7	0	3	32.98 % (31/94)	2.77 ^{A}
	Jejunum	36	13	5	16	10	14	61.70 % (58/94)	3.92 ^{B}
	Ileum	82	4	3	3	0	2	12.76 % (12/94)	3.17 ^{A}
	Overall							65.96 % (62/94)	3.91

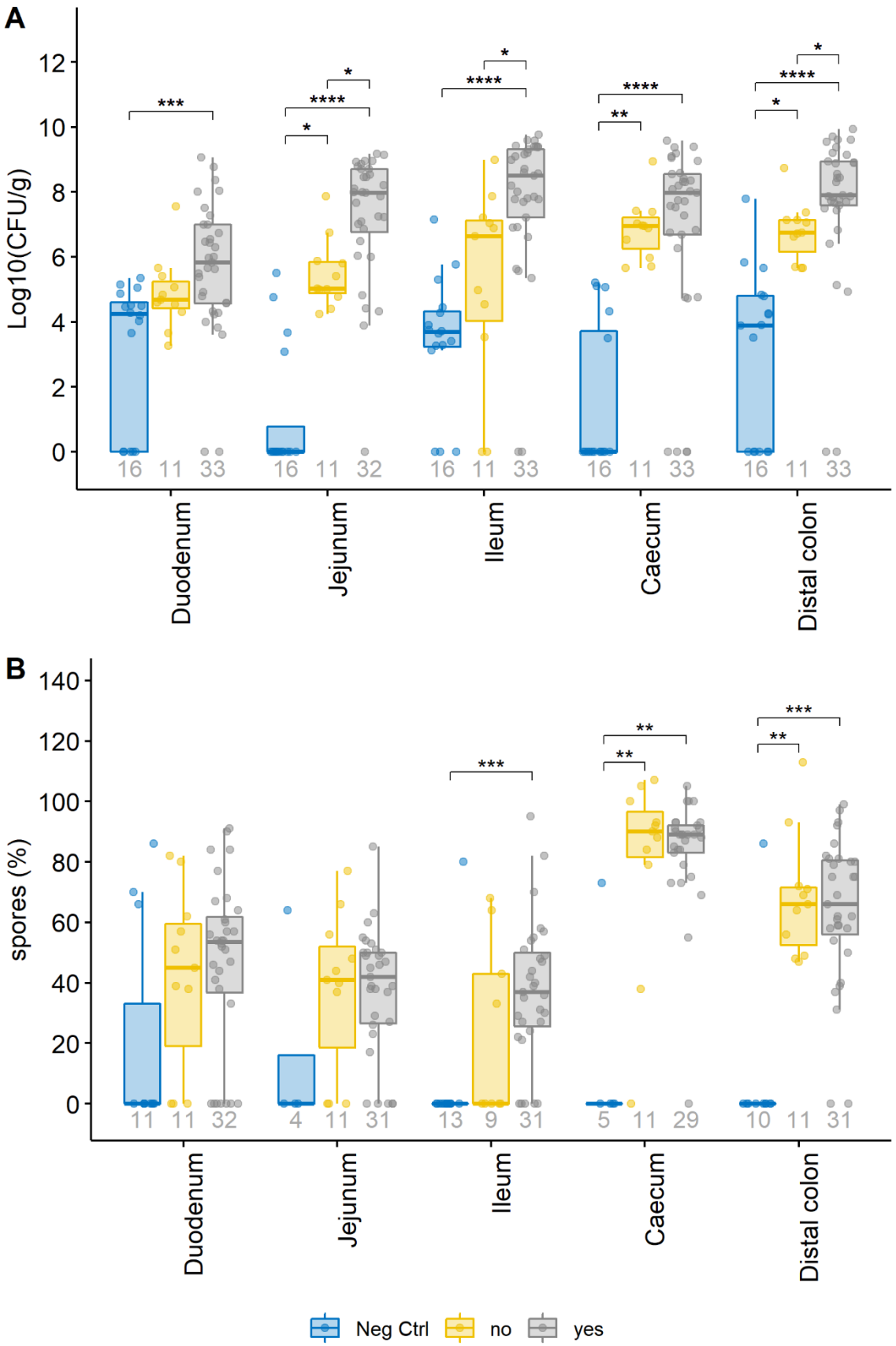
Birds were either not challenged (control group) or challenged with a ten-fold dose of a live attenuated *Eimeria* vaccine at day 14 and 16 to induce a predisposing coccidiosis infection, followed by challenge with the pathogenic *C. perfringens* strain CP56 on days 18, 19 and 20 (CP56 group). At day 21, all animals were euthanized and the small intestine was scored for necrotic enteritis (NE) lesions.

[a] Lesion scoring was performed as previously described by Keyburn et al. 2006.

[b] Birds with a score ≥ 2 are considered necrotic enteritis (NE) positive.

[c] Data representing the average lesion score per segment of NE positive birds. Means with different superscripts differ significantly ($P < 0.05$).

Figures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5 11 29
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Duodenum
Jejunum
Ileum
Caecum
Distal colon
Neg Ctrl
no
yes

Fig. 1: **Numbers of total *C. perfringens* and total spores in duodenum, jejunum, ileum, caecum, and distal colon of broiler chickens during a NE trial.** Neg Ctrl: Negative control (birds not challenged with CP56); no: birds challenged with CP56 but no intestinal lesions were observed; yes: birds challenged with CP56, intestinal lesions were observed. (A) Total number of vegetative *C. perfringens* cells in duodenum, jejunum, ileum, caecum, and distal colon. *C. perfringens* numbers are shown as $\log_{10}$ of CFU/g intestinal content/faeces. (B) Relative number of spores in duodenum, jejunum, ileum, caecum, and distal colon shown as % of spores from total number of *C. perfringens* present in birds in each segment of the intestine (spore-total *C. perfringens* ratio). The number of samples per intestinal segment are indicated at the bottom of the boxplots. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

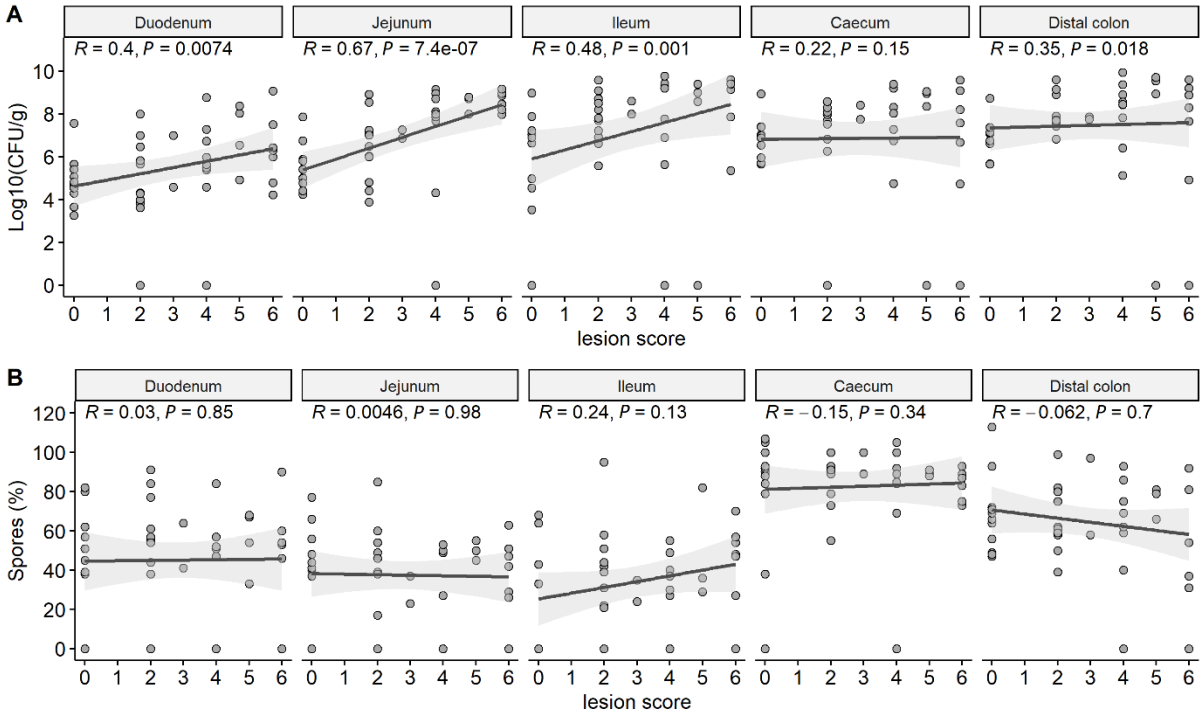

Fig 2.: Spearman correlation of *C. perfringens* numbers and disease severity. The disease severity is expressed as the highest lesion score observed in the small intestine of each bird. **(A)** $\log_{10}$ of total *C. perfringens* cell numbers (colony forming units) per gram intestinal content in all intestinal compartments. **(B)** Amount of *C. perfringens* spores in %, relative to total amount of *C. perfringens* in each intestinal compartment.