Chicken Intestinal 3D Organoids: *A Novel Method to Study Enterococci Pathogenesis and Intervention Strategies*

Enterococcus cecorum **are members of the normal microbiota in the intestinal tract of poultry, however, since the early 2000s virulent *E. cecorum* strains have emerged as a significant challenge in poultry production worldwide. Clinical outbreaks can have major economic impact through septicaemia, spondylitis, and bacterial osteomyelitis, which subsequently lead to locomotor disorders and increased mortality and condemnation rates have been reported.[2,3] Strains are considered virulent when they are recovered from typical lesions in femur and free thoracic vertebra, whereas commensal or avirulent strains are generally isolated from the intestines and cloaca of healthy birds. The colonisation of the intestine followed by translocation and bacteremia is crucial to the pathogenesis of *E. cecorum* and development of lesions. The ability of *E. cecorum* to translocate from the intestinal tract to various tissues seems linked to virulence. This process of translocation and the interaction between bacterium and intestinal epithelium has not been studied in detail due to a lack of a suitable *in vitro* chicken model.**

The aim of our study was to develop an *in vitro/ex vivo* model to investigate translocation and to differentiate between cloacal and lesion *E. cecorum* strains based on invasion and translocation. Models with increased cellular heterogeneity are more representative of the *in vivo* organ, however, typically, these more complex culture systems are more labour intensive and costly. The introduction of intestinal organoids has provided species- and organ-specific models which create more reliable intestinal responses than current *in vitro* options such as cell lines. A chicken organoid model with multiple villus-crypt structures was published in 2021, which uniquely maintains *in vivo* cellular diversity, polarity, and barrier function, incorporating cells of the lamina propria, while exhibiting an apical out orientation.[4] These 3D chicken organoids are the most physiologically-relevant *in vitro* model available for studying intestinal health with the presence of an epithelial layer and an inner core containing the lamina propria, therefore, containing all cell lineages found in the intestinal tissue *in vivo*.[5,6]

Materials & Methods

Experiments were performed using 18-day-old embryos of specific pathogen-free chicken bred at Royal GD. The generation of 3D intestinal organoids has been described previously, in brief intestinal villi are isolated by enzymatic digestion and after purification grown in a defined medium.[5] The four *E. cecorum* strains used in this study are available at GD and were previously characterised by Manders *et al.*[7] Two strains were isolated from cloacal swabs of healthy broiler reproduction animals (cloacal stains) and two strains were isolated from broilers with spondylitis and femur head necrosis (lesion strains).

After two days in culture the organoids were inoculated with 10^3–10^7 colony forming units (CFU) *E. cecorum* and after 3 or 6 hours post inoculation the number of bacteria were quantified by quantitative PCR.

Subsequently, an invasion assay was developed to determine translocation of bacteria. After inoculation with *E. cecorum* and incubation for 3 or 6 hours, the organoids were treated with a mixture of antibiotics, washed, and lysed with a detergent. The lysates were serially diluted and plated on Columbia agar supplemented with sheep blood and counted the next day.

Results

Based on microscopical analysis, the effect of *E. cecorum* on the morphology of chicken intestinal organoids was not affected after inoculation with a high dose of bacteria, independent of the bacterial strain used (Figure 1).

To investigate if *E. cecorum* lesion strains translocated at a higher rate than the cloacal strains, we first measured bacterial load by quantitative PCR. Significant differences between cloacal and lesion strains were detected after inoculation with 10^5 but not with 10^6 CFU, although the bacterial DNA levels at 6 hours were consistently higher compared to 3 hours after inoculation. At the highest inoculation dose of 10^7 CFU, a significant difference was detected between cloacal and lesions strains and, for the lesion strains, there was also a significant effect of time (Figure 2A). Because of the overlap in qPCR datapoints of cloacal and lesion strains, the usefulness of qPCR in this assay is questionable, even though the difference between cloacal and lesion strains is statistically significant. Inoculation with lower number of bacteria resulted in significant differences between cloacal and lesion strains after inoculation with 10^5 but not with 10^6 CFU.[1]

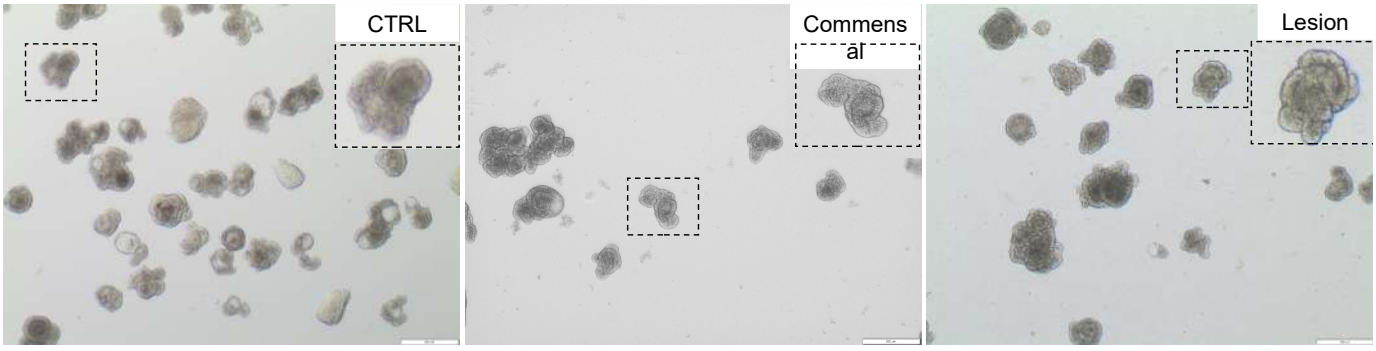

Figure 1. Representative images of organoids grown for 3 days in floating organoid medium. Organoids are inoculated with 10^7 CFU per well with a commensal or lesion strain or medium (CTRL) and imaged at 6 hours post inoculation. Enlargements of healthy organoids are given in dashed rectangles to demonstrate the morphology after treatment. Scale bars: 200 µm.

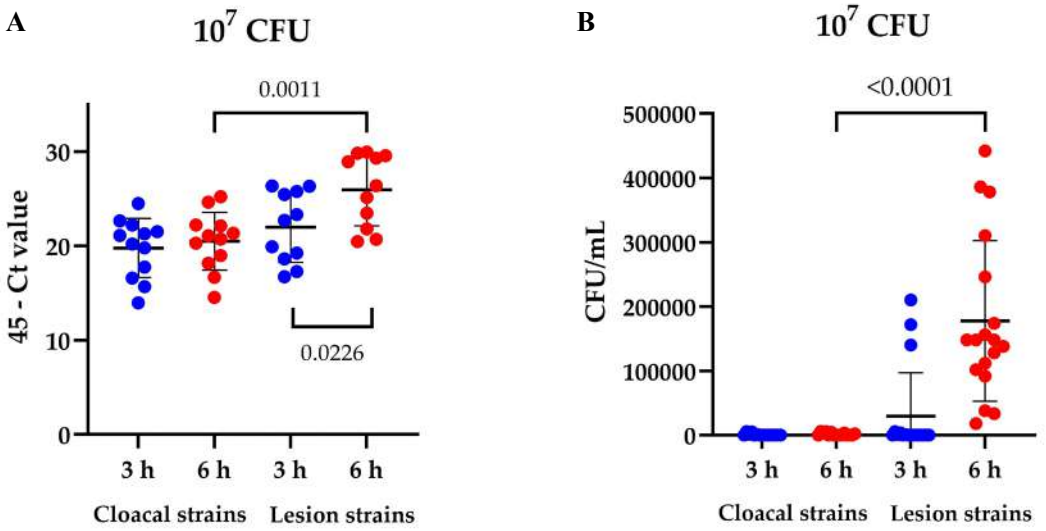

Figure 2. Quantification of E. cecorum in chicken intestinal organoids inoculated with 10^7 CFU. E. cecorum was quantified by qPCR (A) or invasion assay (B) at 3 (blue) or 6 (red) hours post inoculation. Data are presented as means with standard deviation.

Despite multiple washing steps, we cannot exclude bacteria adhering to the organoids. Since quantification by qPCR cannot distinguish between adherent bacteria and invaded bacteria, we then developed an invasion assay to quantify invaded or translocated *E. cecorum*. The number of bacteria recovered after antimicrobial treatment of the organoids differed substantially between the cloacal and lesion strains (Figure 2B). At 6 hours post inoculation, the lesion strains invaded the organoids to a significantly higher ($p < 0.0001$) extend compared to the cloacal strains. At 3 hours post inoculation, the variation within a group was too large to distinguish between the strains.

Conclusion

Although GD is renowned for it's expertise in the field of animal models, we aim to develop alternative models taking the 3Rs into account, replacement, refinement and reduction. This novel model will not only allow in depth analysis of host-pathogen interaction, but also enables us to screen intervention strategies in vitro before we perform animal trials and, therefore, an excellent route for reducing the number of animal trials needed for bringing a product to the market.[8]

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Lonneke Vervelde

Lonneke Vervelde, Prof Dr Ir graduated from Wageningen University and completed a PhD degree in 1995 on the mucosal immune responses of chickens to Eimeria tenella. She was awarded a personal chair in Veterinary Immunology and Infectious Disease at the University of Edinburgh in 2018. She recently joined Royal GD as immunologist and senior researcher and develops novel in vitro complex cellular models and expands the veterinary immunological toolbox at GD.

Jeanine Wiegel

Jeanine Wiegel, DVM completed her veterinary medicine studies at Utrecht University, with a focus on livestock and public health and graduated Cum Laude from University of Georgia, USA as a Master in Avian Health and Medicine. Her work as senior poultry veterinarian includes monitoring for the poultry industry, research projects on antimicrobial use and resistance in poultry and consultancy in the Netherlands and abroad.