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# Evaluation of mouse enteroids as a model for *Lawsonia intracellularis* infection

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## Abstract

*Lawsonia intracellularis*, an obligate intracellular bacterium, is an important enteric pathogen in pig herds and horse farms worldwide. The hallmark feature of *L. intracellularis* infection is the proliferation of epithelial cells in intestinal crypts. A major limitation to the study of *L. intracellularis* infection is the lack of an in vitro model that reproduces the changes observed in proliferative enteropathy. Here we investigated the suitability of mouse enteroids as a model to study *L. intracellularis* infection. Mouse enteroids were microinjected with *L. intracellularis*, filter-sterilized *L. intracellularis* culture supernatant, or sterile cell culture media (DMEM). *L. intracellularis* antigen was detected in mouse enteroids by immunohistochemistry and was located mostly in the basal region of the epithelium. There was no differential growth of enteroids among treatment groups, and cellular proliferation was not increased in *L. intracellularis*-infected enteroids in relation to non-infected enteroids based on immunofluorescence staining. *L. intracellularis* infection did not induce changes in gene expression of *Ki-67* (proliferation marker), *Sox9* (marker for transit amplifying cells) and *Muc2* (marker for goblet cells). These results indicate that although *L. intracellularis* antigen is detectable in mouse enteroids, indicating susceptibility to infection, mouse enteroids fail to replicate the cellular proliferation and gene expression changes observed in proliferative enteropathy. Nevertheless, we have successfully demonstrated that mouse enteroids can be used to model days-long intracellular pathogen infection, serving as potential models for the study of other pathogens of interest in veterinary medicine.

## Introduction

Pig herds and horse farms worldwide are regularly challenged by proliferative enteropathy (PE). This disease is caused by *Lawsonia intracellularis* and is characterized by the thickening of the small intestinal mucosa due to proliferation of intestinal epithelial cells. PE causes diarrhea and compromised weight gain in pigs [1, 2]. In weaned foals, PE causes diarrhea and hypoproteinemia, occasionally resulting in death [3]. Other species are also affected by PE, including ratite birds, rabbits, non-human primates, rats and mice [4].

*Lawsonia intracellularis* is a Gram-negative bacterium that requires an intracellular culture system and a specific atmosphere to be propagated in vitro [5].

Traditional single cell cultures (cell lines) are permissive to *L. intracellularis* propagation, but they have failed to reproduce the increased cellular proliferation observed in PE-affected animals [6]. The lack of an in vitro model that represents the in vivo progression of PE has limited advancement in knowledge of the pathogenesis mechanisms utilized by *L. intracellularis*. Hence, identification of new alternatives for control and prevention of PE has been difficult, and pig producers and horse owners have been relying on available commercial vaccines (whole cell vaccines) and antimicrobials to prevent and control PE [7–10]. The costs associated with producing whole cell vaccines is reflected on the commercial price of the vaccines, hampering the access to some producers to the commercial vaccines, and, therefore preventing PE. In addition, the use of antimicrobials as growth promoters in swine production has raised concerns about the selection of antibiotic-resistant organisms in treated herds [11]. Therefore, a deeper understanding

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of PE pathogenesis is necessary for the development of novel non-antimicrobial based prevention and treatment methods such as recombinant vaccines or antimicrobial alternative strategies [11–14].

The currently held hypothesis is that *L. intracellularis* infects intestinal epithelial cells, especially in the crypt compartment, leading to cellular proliferation and decreased differentiation in goblet cells along with increased apoptotic events [4, 15, 16]. The mechanisms involved in epithelial changes during the course of PE are still unclear. Thus, knowledge about interactions between *L. intracellularis* and intestinal epithelial cells, determined using a controlled environment, could generate a better understanding of how *L. intracellularis* induces cellular proliferation.

A promising alternative to single cell cultures are tridimensional multi-cell type cultures, also known as organoids. One of the most relevant advantages of organoids in relation to the traditional in vitro single cell culture is the similarity of the organoids to their respective tissue of origin [17, 18]. Enteroids, small intestinal organoids, for instance, possess not only enterocytes, but also enteroendocrine cells, transit amplifying cells, goblet cells and stem cells. All these cells form structures with cell distributions that are similar to crypts and villi found in the small intestine, and possess a lumen where cell debris and secretion are shed continuously [17]. Enteroids have been recently used to study host–pathogen interactions of important enteric bacteria in human medicine [19–23], but they have not been investigated as a model for bacterial infections in the field of veterinary medicine [24, 25].

The objective of this study was to evaluate mouse enteroids as an in vitro model for *L. intracellularis* infection. Infected enteroids were monitored over time by size along with changes in expression of genes that have been reported to change during PE. We found evidence of infection with *L. intracellularis* in enteroids followed for up to 7 days post-infection (dpi). The genetic profile of infected mouse enteroids, however, diverged from the gene expression changes observed in PE.

## Materials and methods

### Growth and passaging of mouse enteroids

Mouse enteroid preparation and maintenance were performed as described elsewhere [26] (IACUC approval number 1606-33871A). The formulation of the enteroid culture medium was as described previously [27].

### *L. intracellularis* propagation

A *L. intracellularis* isolate (PHE/MN1-00, ATCC PTA-3457, Manassas, VA, USA) at low ( $\leq 20$ ) passage [28] was propagated in McCoy mouse fibroblast-derived monolayers (ATCC® CRL-1696™). McCoy cells were

cultured as described elsewhere [29]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco ThermoFisher, Waltham, MA, USA) with 7% fetal bovine serum (FBS; heat inactivated, Corning™ 35011CV, Corning, NY, USA). McCoy cells at 30% confluency were infected with about  $10^7$  *L. intracellularis* and incubated at 37 °C under a controlled microaerophilic atmosphere created with a mixture of gas containing 10% hydrogen, 10% carbon dioxide, and 80% nitrogen [30]. Seven days after infection, cell monolayers were mechanically lysed by passage through a 23-gauge needle coupled to a syringe after immersion in sterile 0.1% potassium chloride and then centrifuged at  $200 \times g$  to separate cell debris from *L. intracellularis* organisms. The pellets were discarded and the remaining supernatant was then filtered through 0.80  $\mu\text{m}$  sterile filters (Millipore Sigma, Burlington, MA, USA) to remove any remaining McCoy cells and nuclei. After a final centrifugation at  $8000 \times g$  at 4 °C the bacterial pellet was either used to re-infect McCoy cells, or to prepare the inoculum suspended in approximately 500  $\mu\text{L}$  of its own supernatant and used as inoculum.

### Enteroid acclimation in microaerophilic and antibiotic-free conditions

As *L. intracellularis* growth requires special atmospheric conditions [30], we first tested whether the atmosphere conditions required for *L. intracellularis* growth would impact growth and development of mouse enteroids. One plate with enteroids was maintained under atmospheric conditions specific for *L. intracellularis* i.e., 10% hydrogen, 10% carbon dioxide, and 80% nitrogen [30] while another plate was maintained at 5%  $\text{CO}_2$  as a control. Both plates were incubated at 37 °C. Since *L. intracellularis* manipulation in vitro requires an antibiotic-free sterile system, antibiotics (Penicillin/Streptomycin) were excluded from both plates to assess sterility of the cultures. The plates were observed every 48 h for 3 weeks, with media replacement and enteroid passage as described previously.

### Pilot study—incubation and seed

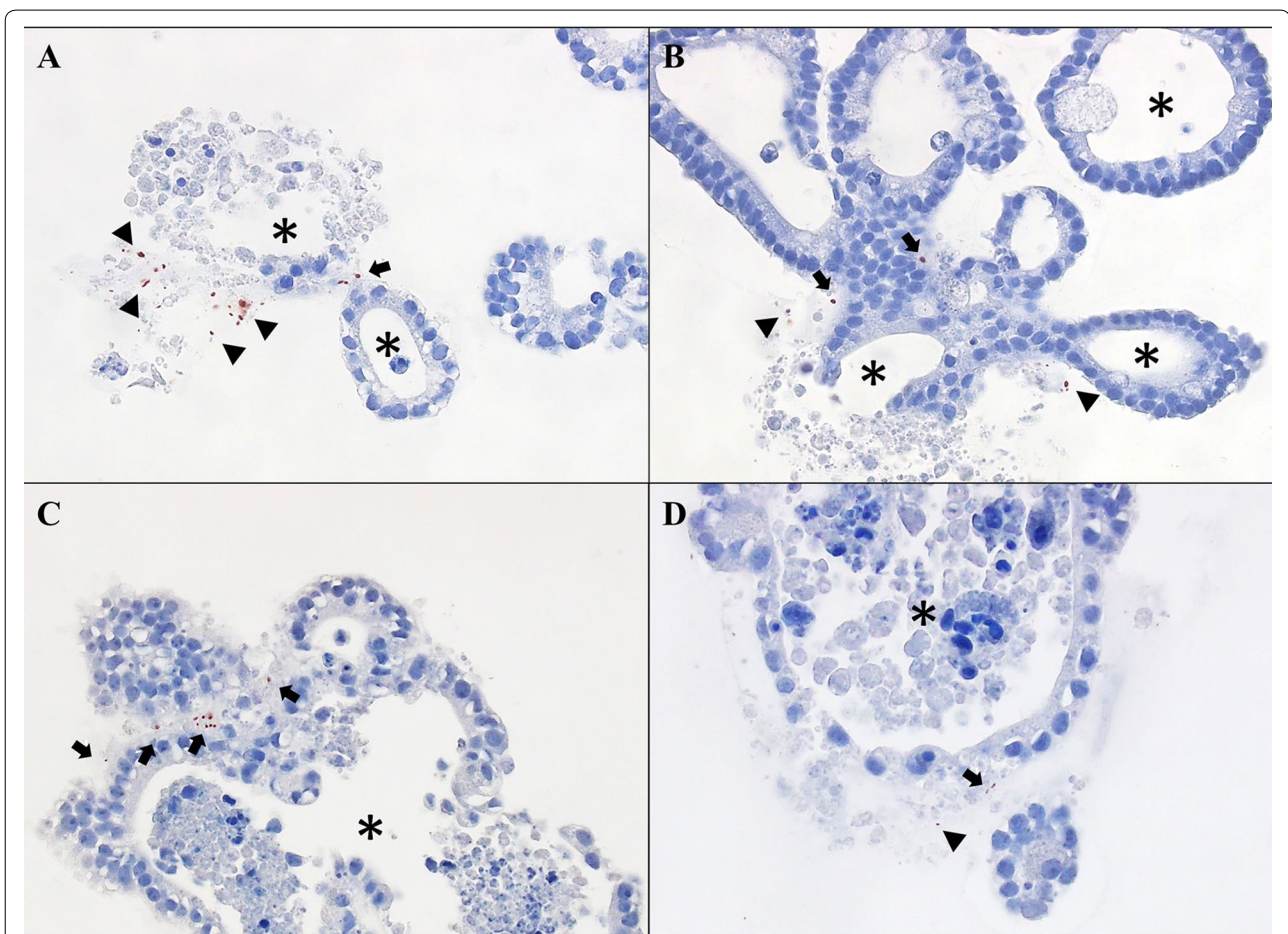
To standardize conditions for infection of mouse enteroids with *L. intracellularis*, we adopted the “incubation and seed” method [31]. Briefly, immediately after enteroid passage and before plating, enteroids were directly mixed with *L. intracellularis* suspended in 100  $\mu\text{L}$  of enteroid culture medium (resulting in an inoculum with  $\sim 10^9$  *L. intracellularis* organisms/mL), with occasional gentle agitation, and incubated at 37 °C under special atmospheric conditions generated with a gas tank of 10% hydrogen, 10% carbon dioxide, and 80% nitrogen for 30 min. The infected enteroids were briefly centrifuged

at  $200 \times g$  for 1 min, mixed with 50–100  $\mu\text{L}$  ice-chilled Matrigel (Corning, USA), and then seeded into culture plates. Enteroids were harvested 15 days post-infection (dpi) by removing culture media and suspending the Matrigel in phosphate buffered saline (PBS). After centrifugation for 1 min at  $200 \times g$ , the pellet was suspended in 4% formaldehyde and incubated for 2 h at room temperature. Enteroids were then washed in PBS, centrifuged at  $200 \times g$  for 2 min, suspended in Histogel (ThermoFischer, USA) and placed into a biopsy mold. Histogel blocks were inserted into a cassette and maintained in 70% alcohol until they were processed for histological evaluation. Four-micrometer sections were placed onto glass slides and used for hematoxylin and eosin (H&E) staining and immunohistochemistry [32]. Although *L. intracellularis* antigen was detected by IHC in the infected enteroids by

this method (Figure 1), we opted to employ the microinjection approach described below to facilitate contact of *L. intracellularis* with the cellular apical membrane in an effort to improve the level of infection.

#### Enteroid infection with *L. intracellularis*

Mouse enteroids were cultured and passaged until a sufficient number of enteroids (average of 20 enteroids per treatment group, per time point) with 100  $\mu\text{m}$  diameter were obtained for the trial. Wnt3a protein, which regulates the signaling pathways related to the cellular proliferation in the intestinal crypt compartment [33], was removed from the enteroid culture media at least 3 days before infection to enable cells to better differentiate. Twenty-four hours prior to infection, enteroids were divided into three treatment groups: DMEM: sterile



**Figure 1** *L. intracellularis* infection in epithelial cells of mouse enteroids infected by the “incubation and seed” method. **A** *L. intracellularis* antigen is associated mostly with cell debris (arrow heads),  $\times 200$ . **B** *L. intracellularis* antigen is associated with cell debris (arrow heads) and in close proximity to enteroid cells (black arrows),  $\times 200$ . **C** *L. intracellularis* antigen is observed in close association with mouse enteroid cells (black arrows),  $\times 200$ . **D** A few *L. intracellularis* antigens are observed in this image, some close to mouse enteroid cells (black arrow), and some associated with cell debris (arrow head),  $\times 200$ . Enteroid lumen is indicated by an asterisk (\*).

culture media; SN: sterile-filtered *L. intracellularis*-culture supernatant; *L. intracellularis*: suspension with about  $10^6$  *L. intracellularis* organisms/mL. Each enteroid received approximately 100 nL of each respective treatment. Treatments were placed directly into the lumen of the enteroids by microinjection using a microinjector (Nanoject II, Drumond Scientific, Broomall, PA, USA) operated by a trained technician. Experiments were performed in four independent replicates.

#### Enteroid area monitoring

Microinjected enteroids were monitored for 1 week, from the day prior to infection until 7 dpi. Images were captured at  $200\times$  magnification using an inverted microscope coupled with a digital camera immediately after injection (0 dpi), and at 1, 3 and 7 dpi. The enteroid area was measured using an image software (NIH Image) software 1.46r, National Institute of Health, Bethesda, MD, USA). The relative enteroid area (enteroid growth) was measured by subtracting the enteroid area from the area recorded at its previous measurement.

#### Enteroid harvesting and fixation

Enteroids from each treatment group were harvested at 1, 3 and 7 dpi and prepared for immunofluorescence. Matrigel was disrupted with a sterile pipet tip and the contents in each well were transferred to a 1.5 mL tube and centrifuged at  $200 \times g$  for 4 min. The supernatant was discarded, and the pellet suspended in 1 mL of PBS. This step was repeated two times to remove Matrigel. The resulting enteroid pellet was suspended and fixed in 4% paraformaldehyde for 1 h at room temperature. After fixation, enteroids were washed three times in PBS and centrifuged at  $200 \times g$  for 4 min.

Fixed enteroids were embedded in Histogel (Richard-Allan Scientific HistoGel, ThermoFischer, USA) and then either placed in optimum cutting temperature (OCT, Tissue-Tek; Sakura Finetek, Beaver Creek, CO, USA) for preparing cryosections or dehydrated in 70% ethanol and embedded in paraffin.

#### Immunohistochemistry

Paraffin-embedded sections ( $4 \mu\text{m}$ ) on charged slides were used for IHC for detection of *L. intracellularis* using specific rabbit polyclonal antibodies and a previously described method [32]. Stained sections were evaluated by bright field microscopy.

#### Immunofluorescence

OCT-embedded blocks were sectioned ( $4 \mu\text{m}$ ), placed on charged glass slides and stored at  $-20^\circ\text{C}$  until immunofluorescence staining. Anti-Ki-67 immunofluorescence was performed as described elsewhere [6]. Briefly, slides

were hydrated with Tris buffered saline (TBS) containing 0.1% triton-x (TBS-T) for 10 min and then blocked with 10% normal goat serum (ZC1213, Vector Laboratories, Burlingame, CA, USA) for 30 min to prevent non-specific binding. Sections were then incubated with anti-Ki-67 antibody (1:200 in TBS-T, CRM325B, Biocare Medical, Pacheco, CA, USA) for 2 h, followed by a 5 min wash in TBS-T and incubation with Cy3-labeled goat anti-rabbit antibody (1:250, ab97075, Abcam, Burlingame, CA, USA) for 30 min. All incubations were carried out at room temperature. Slides were washed and mounted with Prolonggold (ThermoFisher, USA) and covered with glass coverslips. The number of Ki-67-positive cells and the total number of cells were counted in at least 5 random fields at  $400\times$  using an Olympus BX53 fluorescence microscope.

#### Real-time quantitative PCR

Enteroids were harvested at 0, 1, 3 and 7 dpi, released from Matrigel as described above, lysed with 1.5 mL of TRIzol (ThermoFisher, USA) and stored at  $-80^\circ\text{C}$  until RNA extraction. RNA extraction was performed using chloroform and isopropanol precipitation following TRIzol protocols and then cleaning with the RNeasy mini kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. Total RNA was quantified using a Nanodrop 2000 (Thermo Scientific, USA) and 400 ng of RNA were transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) with random hexamer primers.

Quantitative PCR reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Amplification was performed with the following conditions: initial activation at  $95^\circ\text{C}$  for 10 min, followed by 40 cycles of denaturation at  $95^\circ\text{C}$  for 15 s and annealing at  $60^\circ\text{C}$  for 60 s. Expression of the housekeeping gene *GAPDH* was monitored as reference. Relative gene expressions were normalized to *GAPDH* using the primer efficiencies. The expression levels of *Ki-67* (marker for cellular proliferation), *Sox9* (marker for transit amplifying cells) and *Muc2* (marker for goblet cells) in enteroids of all treatment groups were measured at 1, 3 and 7 dpi. Fold change in each case was calculated in relation to expression in enteroids from day 0 (control). All primers used are listed in Table 1.

#### Statistical analysis

Results are presented as a mean  $\pm$  standard deviation of the mean (SEM) for each group. Two-way ANOVA followed by Geisser-Greenhouse correction was used to verify statistical differences with  $p < 0.05$  considered

**Table 1 Primers used for gene expression in mouse enteroids**

Target	Sequence of primers—forward	Sequence of primers—reverse
GAPDH	TCA AGA AGG TGG TGA AGC AGG	TAT TAT GGG GGT CTG GGA TGG
Sox9	CTG GAG GCT GCT GAA CGA GAG	CGG CGG ACC CTG AGA TTG C
Muc2	AGA ACG ATG CCT ACA CCA AG	CAT TGA AGT CCC CGC AGA G
Ki-67	TTT CAG GTC TCT GGA AGC AGT CA	ATC TCC ATA ATT GCT TTG ATT GCA

statistically significant. GraphPad Prism 8.1 software for Windows (GraphPad Software, La Jolla, CA, USA) was used to perform the analysis.

## Results

### Enteroid monitoring in microaerophilic conditions

There was no difference in growth or morphology between enteroids maintained for 1 week in the microaerophilic atmosphere relative to those maintained under regular conditions, i.e., 5% CO<sub>2</sub> incubator (data not shown), indicating that growth conditions required for *L. intracellularis* do not impact enteroid viability or growth.

### Detection of *L. intracellularis* in mouse enteroids

Mouse enteroids were infected with *L. intracellularis* by microinjection. Enteroids were harvested and analyzed by IHC for *L. intracellularis* antigen at 1, 3, and 7 dpi. *L. intracellularis* antigen presence was observed at all time points, indicating successful infection with *L. intracellularis*. Unexpectedly, although *L. intracellularis* antigen was observed in the cytoplasm of enteroid cells, most of the antigen, especially at 7 dpi, was found in the basal region of epithelial cells (Figure 2). This is in contrast to the location of *L. intracellularis* in vivo, which is in the apical region of the epithelial cells [16, 32].

### Enteroid area was not affected by infection

To determine whether *L. intracellularis* infection would lead to increased size or secretory activity of infected enteroids, the area of enteroids in each treatment group was measured at 0, 1, 3 and 7 dpi. *L. intracellularis*-infected enteroids did not have higher area compared with SN or DMEM treatment groups (Figure 3A). Representative images of enteroids from each treatment group are shown in Figure 3B.

### Cellular differentiation

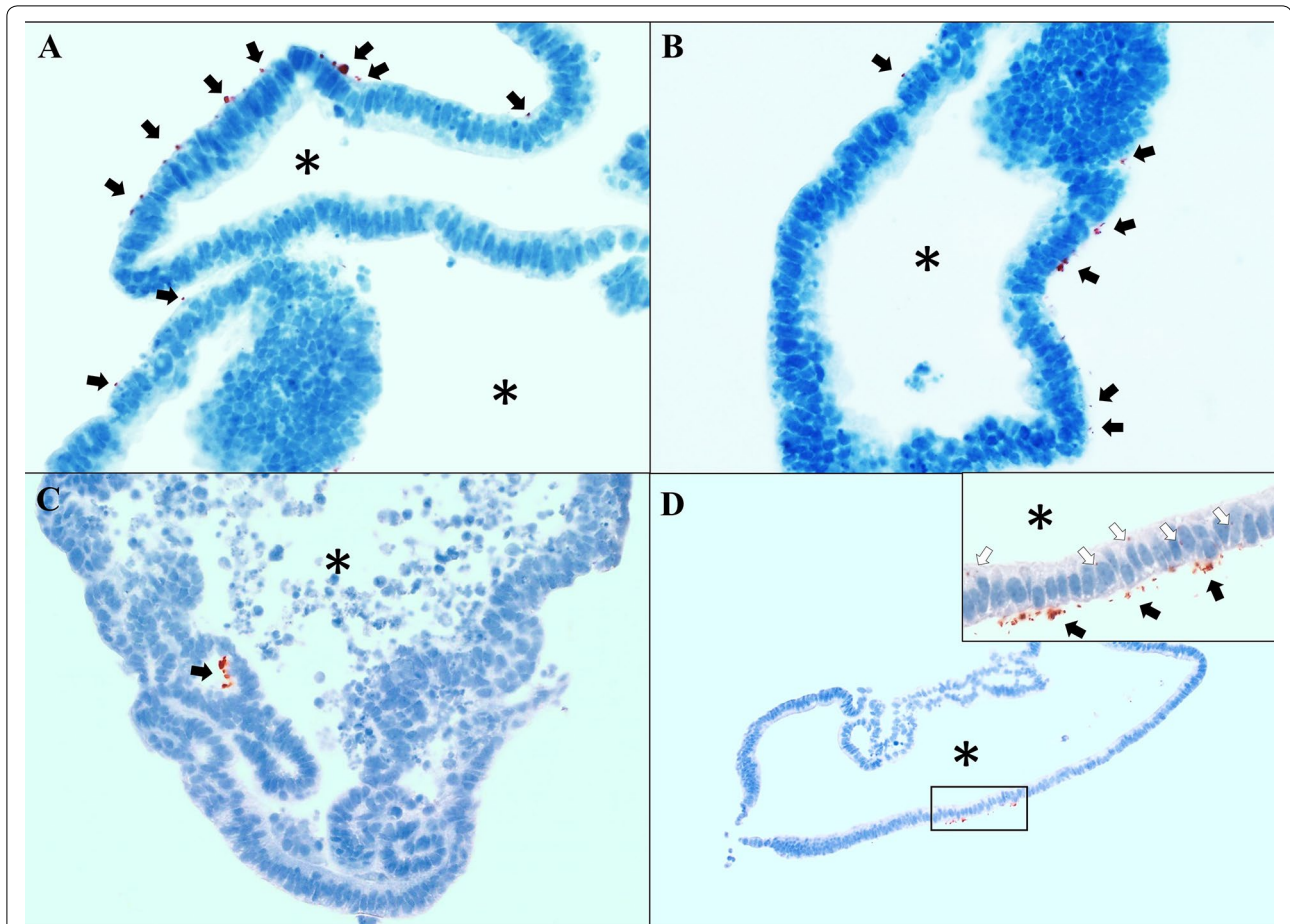
To determine whether *L. intracellularis* infection induces changes in the proliferation and differentiation of enteroid epithelial cells, as observed in the swine intestine, we evaluated expression of *Ki-67*, *Sox9* and *Muc2* in enteroids harvested at 1, 3 and 7 dpi relative to expression in enteroids at 0 dpi. There were no significant differences in expressions of any of the genes analyzed in

the treatment groups independent of the time point (Figure 4). Immunofluorescence staining confirmed that none of the treatment groups showed increased Ki-67 expression overtime, although in the *L. intracellularis* infected group considerable variability in the in Ki-67 expression throughout was noted (Figure 5).

## Discussion

Classical single cell culture systems have been extensively used in studies to understand host–pathogen interactions. Although they provide valuable information, these models are limited by their inability to represent the tissue organization observed in vivo since most of the single cell systems are either cancer-derived or transformed immortalized cell lines [19, 34]. These limitations make it difficult to assess the proliferative effects of *L. intracellularis* infection [6]. The mechanism by which *L. intracellularis* causes proliferation of intestinal epithelial cells remains unclear. Recently, the effects of *L. intracellularis* infection on the proliferation of monocultures of non-intestinal, non-epithelial cells, as well as intestinal epithelial cells were investigated under various culture conditions [6] and the findings supported previous observations that cell monocultures infected with *L. intracellularis* do not accurately represent proliferation observed in lesions in the intestine of affected animals. In vivo, *L. intracellularis* accesses the cell cytoplasm via the apical membrane and the organisms are observed in the cytoplasm throughout the course of PE. *L. intracellularis* is more frequently observed in crypt cells than cells of the villus [16, 35, 36]. Therefore, we hypothesized that enteroids would be a suitable model to further investigate *L. intracellularis* pathogenesis in vitro by providing polarized epithelial cell culture with all the crypt and villus epithelial cell types found in the mammalian intestine and similar cell exchange ratio to the in vivo intestine [37].

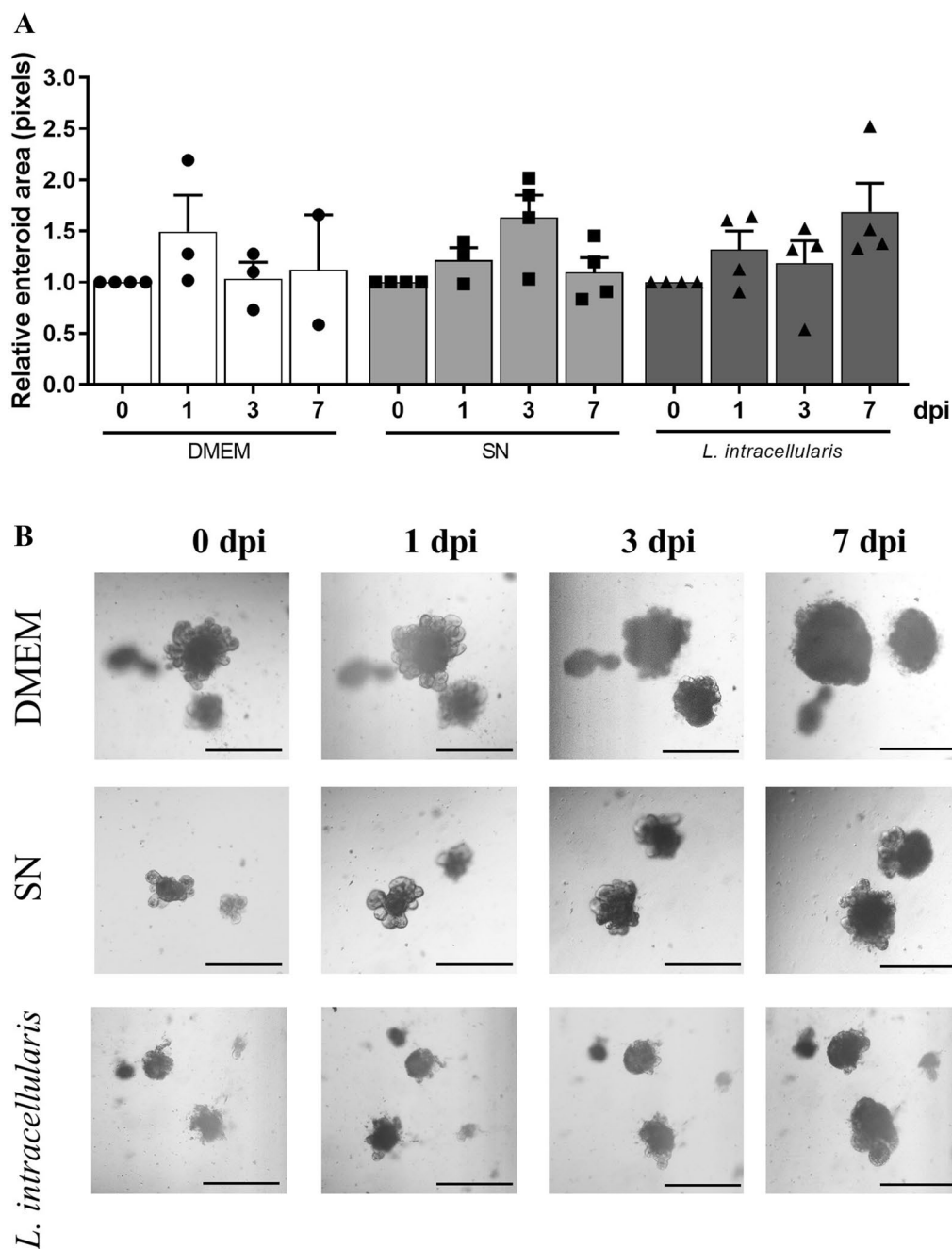
Enteroids were firstly developed in 2009 [38] and have been used to study intestinal morphophysiology as well as pathogenesis of some enteric diseases [21, 23, 39, 40]. Human enteroids can be obtained by culture of intestinal crypts isolated from biopsies. Mouse enteroids are more widely used in research as the ethical and biosafety regulations for human enteroids are more restrictive.



**Figure 2** *L. intracellularis* infection in epithelial cells of mouse enteroids infected by the microinjection method. **A–C** *L. intracellularis* antigen is observed on the basal region of mouse enteroids cells (black arrows). Most of the antigen is observed as amorphous clusters, instead of the typical rod-shaped morphology seen in vivo.  $\times 400$ . **D** *L. intracellularis* antigen is observed on the basal region of mouse enteroids cells (black arrows) and in cellular cytoplasm (white arrows on the insert). Main image:  $\times 100$ , insert:  $\times 400$ . Enteroid lumens are indicated by asterisks (\*).

Additionally, mouse intestinal physiology is well-characterized and molecular tools to study intestinal cell proliferation and differentiation are available. The presence of *L. intracellularis* DNA has been detected in feces of mice trapped in pig farms and in the surroundings of horse farms [41, 42], suggesting that mice are susceptible to infection. Furthermore, mice experimentally infected with *L. intracellularis* develop intestinal lesions that resemble lesions in affected pigs and horses, although the lesions in mice are less extensive, less severe and mostly localized in the large intestine when compared to the lesions in pigs and horses [42–44]. In addition, *L. intracellularis* has been successfully propagated in vitro using McCoy mouse fibroblast cells [6, 30, 45]. Based on this knowledge, we hypothesized that mouse enteroids may serve as a feasible model to investigate the progression of *L. intracellularis* infection and the mechanisms involved during cell proliferation.

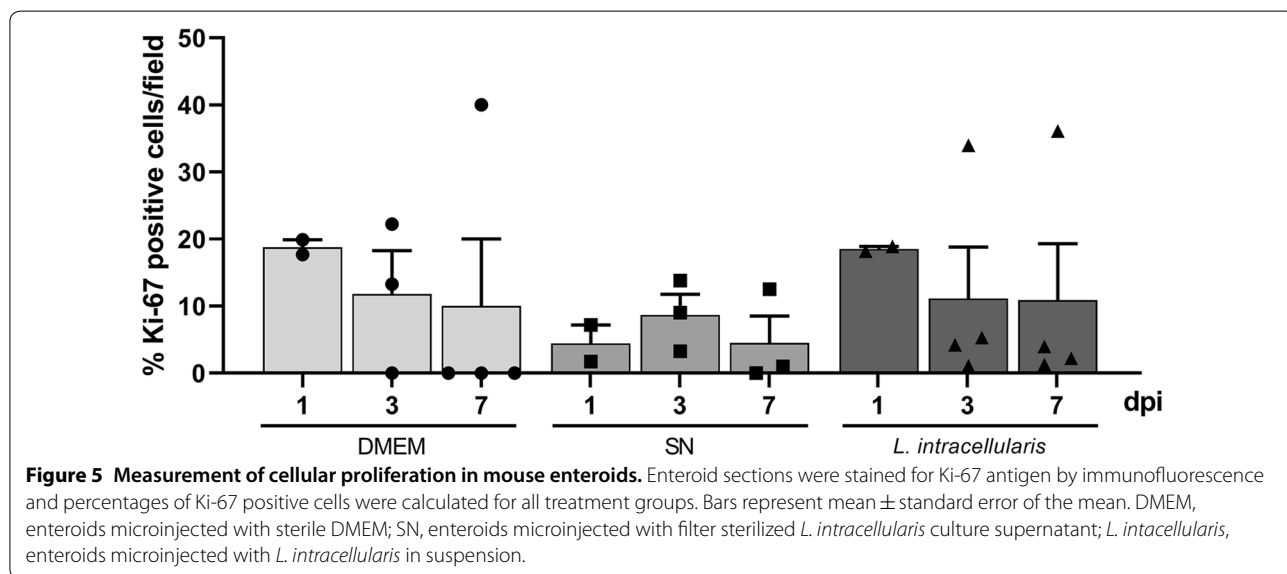
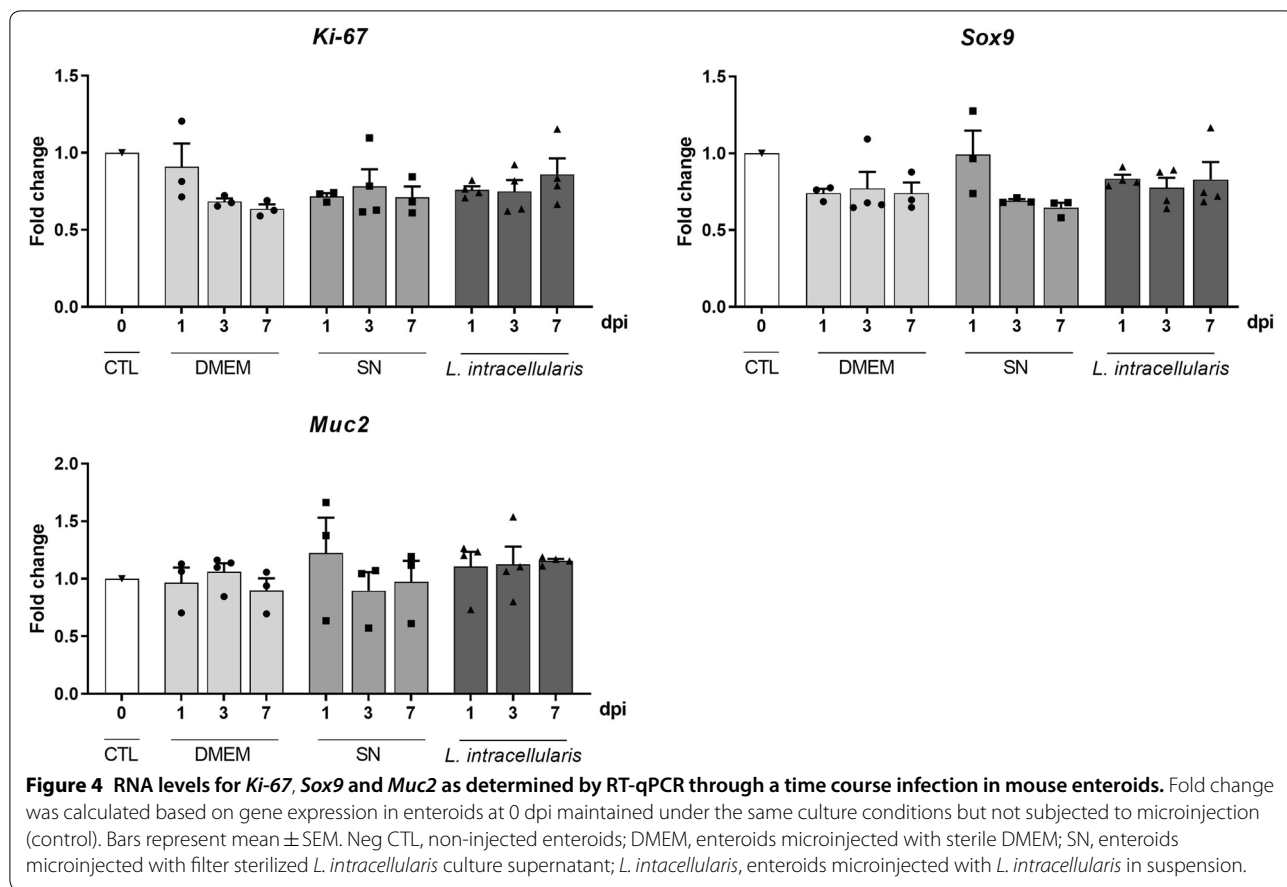
Mouse enteroids in the tridimensional culture conditions, as spheres composed of polarized epithelium, have their cellular apical side oriented to the center of the enteroid. Hence, *L. intracellularis* infection in this study was performed by microinjecting the bacterial suspension directly into the lumen of the enteroid. Microinjection is a technique that has inherent limitations in controlling the bacterial inoculum, i.e., the exact number of bacteria each enteroid was exposed to. Although we plated enteroids to obtain an average of 10 enteroids per well, the actual number of enteroids per well and their size at the time of injection was found to be variable. These technical limitations may have contributed to the high variability observed in our results and constitute one of the limitations of using tridimensional enteroids as models for the study of luminal pathogens. Others have used mouse enteroids [20, 21] and human enteroids [22, 23] to study bacterial pathogenesis using microinjection. However, none of these



**Figure 3 Relative enteroid area over time. A** The relative enteroid area was calculated by the subtraction of the area of a given enteroid from the area of the same enteroid on the previous day. Bars represent mean  $\pm$  SEM. **B** Bright-field images of each treatment group at 0, 1, 3 and 7 dpi. Scale bar 500  $\mu$ m. Neg CTL, non-injected enteroids; DMEM, enteroids microinjected with sterile DMEM; SN, enteroids microinjected with filter sterilized *L. intracellularis* culture supernatant; *L. intracellularis*, enteroids microinjected with *L. intracellularis* in suspension.

studies evaluated infection times longer than 20 h [22, 23]. Because of the nature of *L. intracellularis* infection, longer durations, i.e., several days, are needed to observe the expected changes. To our knowledge, this is the first enteroid infection experiment reporting

successful bacterial infection of enteroids for up to 7 dpi. Whether the length of the infection times had a significant effect on the number of bacteria present and variability on the evaluated parameters remains to be defined.



One of the most intriguing findings in the present experiment was the unexpected location of *L. intracellularis* in the infected enteroids. While *L. intracellularis*

antigen was observed at all time points tested (1, 3 and 7 dpi), the antigen was mostly observed localized in the extracellular space proximal to the basal region of the



epithelium of the mouse enteroids. In vivo, *L. intracellularis* organisms are consistently observed on the apical side of the cytoplasm at similar infection time points to those used in this study [16, 36]. A possible explanation for this difference in localization is that, in contrast to conditions in vivo, nutrients necessary for enteroid growth and maintenance are placed over the Matrigel, in a region that corresponds to the basal lamina in vivo and not directly in the enteroid lumen. We speculate that the gradient of nutrients on the exterior of the enteroid could be causing the *L. intracellularis* to move towards the nutrients to support their growth. It is also possible that conditions of the enteroid culture environment could result in less permissible conditions for *L. intracellularis* closer to the lumen. Another possibility is that the fibroblasts and immune cells in the basal region of the intestinal epithelium in vivo may have a regulatory effect on *L. intracellularis* traffic in the intestine, and the lack of those cells in the mouse enteroid culture would modify *L. intracellularis* traffic in vitro.

Immunohistochemistry for *L. intracellularis* is considered the gold standard method for diagnosis of proliferative enteropathy [32, 46]. In the present study, we used immunohistochemistry to verify whether the mouse enteroids, microinjected with *L. intracellularis* suspension ( $\sim 10^6$  *L. intracellularis* organisms/mL), were infected and to visually demonstrate the level of infection in these microinjected enteroids. Our results were not only surprising in regards to the localization of *L. intracellularis* antigen, as discussed before, but also in regards to the amount and morphology of the stained antigen. Generally, *L. intracellularis* antigen stained by immunohistochemistry is observed as small bacilli in the cytoplasm of cell cultures [5, 47, 48] and in the apical cytoplasm of intestinal epithelial cells of naturally and experimentally-infected pigs. In the present study, instead of observing similar morphology of *L. intracellularis* antigen, we observed mostly the antigen as a “bundle” accumulated on the basal region of the enteroid cells. Nevertheless, occasional well-shaped *L. intracellularis* antigens were observed in the cytoplasm of some enteroid cells (Figure 2D), which is an indication that after the microinjection in the lumen of the enteroid, *L. intracellularis* organisms gained entrance into the cell cytoplasm. The events involving the changes in the morphology of the antigen and its final location in the basal side of enteroid cells remains unclear and may deserve more investigation. The homeostasis of the intestinal epithelium involves various signaling pathways of which canonical Wnt signaling is responsible for maintaining a balance between the pool of undifferentiated cells with proliferation capacity, named transit amplifying cells, and differentiated cells [33, 49]. In vitro, when

enteroids are cultured in the presence of Wnt3a in the media, the transit amplifying cells stay undifferentiated for a longer period of time in relation to in vivo conditions [17], resulting in enteroids in a pro-proliferative state. In the present study, one of the main objectives was to verify the effects of *L. intracellularis* on cell proliferation, therefore we removed Wnt3a from the culture media 3 days before microinjection and during the experiment to allow the epithelial cells to differentiate, and better reproduce the proportion of proliferative cells of the normal intestinal epithelium. However, we failed to detect increased proliferation in *L. intracellularis*-infected enteroids by RT-qPCR or immunofluorescence. It is possible that the removal of Wnt3a 3 days before microinjection was not sufficient to reduce proliferation to a level where we could detect changes induced by *L. intracellularis*. Another possible explanation for this lack of difference in proliferation between treatment groups was the small amount of *L. intracellularis* delivered to the enteroid lumen, thus requiring a longer time for the bacteria to replicate in numbers to produce a detectable change in proliferation markers. The low amount of antigen detected in the microinjected enteroids indicate that *L. intracellularis* did not encounter an ideal environment for propagation as it does in McCoy cells and in the intestines of mice and other rodents [6, 30, 41, 43, 50]. The infection efficacy of enteroids derived from the intestines of more *L. intracellularis* susceptible species, such as pigs and horses, would help to confirm this hypothesis.

Changes in the area of enteroids can indicate an expansion on the number of cells or an increase in secretory activity. Secretory activity of enteroids has been demonstrated by swollen enteroids as a result of accumulation of luminal secretions after stimulation with forskolin [51]. In the present study, we expected to observe increased enteroid areas of infected enteroids that could be due to increased numbers of cells (proliferation) or secretory activity. In contrary to our expectations, we observed an increased volume from 1 dpi to 3 dpi on the SN group without changes in proliferation, suggesting the possibility of a secreted product with capacity to induce intestinal secretion. Further confirmation of this phenomenon is required.

*L. intracellularis* is a unique bacterium, requiring intracellular conditions and special atmospheric conditions for propagation in vitro. Little is known about *L. intracellularis* survival in the cellular cytosol, or the host-cellular pathways that are disrupted during *L. intracellularis* infection. By using mouse enteroids as an in vitro model for *L. intracellularis* infection, our aim was to develop a tool to further define several aspects of *L. intracellularis* pathogenesis. The localization of *L. intracellularis* more abundantly in outside

of the basal side of the enteroid epithelial cells may indicate nutrients or a microenvironment preferred by *L. intracellularis* and merits further investigation. Likewise, little is known about mouse enteroids maintained with a pathogenic enteric bacterium for a period longer than 24 h [20, 21]. More information is needed to determine if long-term infection of enteroids mirrors the dynamics of epithelium in vivo in terms of secretion, differentiation and apoptosis observed in chronic enteric diseases. Finally, yet importantly, although we were able to demonstrate infection of mouse enteroids, we did not observe the changes associated with *L. intracellularis* infection that have been noted in vivo in more susceptible species. Testing *L. intracellularis* infection in enteroids from other more susceptible species will provide further insight to species susceptibility to pathogenesis.

#### Abbreviations

cDNA: complementary deoxyribonucleic acid; DMEM: Dulbecco's Modified Eagle Medium; FBS: fetal bovine serum; H&E: hematoxylin and eosin; IHC: immunohistochemistry; OCT: optimum cutting temperature; PBS: phosphate buffered saline; PE: proliferative enteropathy; RNA: ribonucleic acid; RT-qPCR: reverse transcriptase real time quantitative PCR; SN: filter sterilized *L. intracellularis* culture supernatant; TBS: triphosphate buffered saline; TBS-T: triphosphate buffered saline with triton X.

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#### Authors' contributions

TPR contributed to the acquisition, analysis and interpretation of data and preparation of the manuscript. RLM contributed to execution of the experiments, data acquisition and interpretation. YG contributed in the execution of the experiments. CG, FAV and MSS contributed to the conception, design, data analysis and interpretation of the study. All authors read and approved the final manuscript.

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#### Availability of data and materials

The data collected in this project is shown in our results section. Further information can be available upon request.

#### Ethics approval and consent to participate

Mouse manipulation was approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC Approval Number 1606-33871A).

#### Competing interests

The authors declare that they have no competing interests.

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#### References

- Collins AM, Barchia IM (2014) The critical threshold of *Lawsonia intracellularis* in pig faeces that causes reduced average daily weight gains in experimentally challenged pigs. *Vet Microbiol* 168:455–458
- Lawson GHK, Gebhart CJ (2000) Proliferative enteropathy. *J Comp Pathol* 122:77–100
- Pusterla N, Gebhart C (2013) *Lawsonia intracellularis* infection and proliferative enteropathy in foals. *Vet Microbiol* 167:34–41
- Vannucci FA, Gebhart CJ (2014) Recent advances in understanding the pathogenesis of *Lawsonia intracellularis* infections. *Vet Pathol* 51:465–477
- Lawson GHK, McOrist S, Jasni S, Mackie RA (1993) Intracellular bacteria of porcine proliferative enteropathy: cultivation and maintenance in vitro. *J Clin Microbiol* 31:1136–1142
- Resende TP, Pereira CE, de Souza Daniel AG, Vasquez E, Saqui-Salces M, Vannucci FA, Gebhart CJ (2019) Effects of *Lawsonia intracellularis* infection in the proliferation of different mammalian cell lines. *Vet Microbiol* 228:157–164
- Roerink F, Morgan CL, Knetter SM, Knetter SM, Passat M-H, Archibald AL, Ait-Ali T, Strait EL (2018) A novel inactivated vaccine against *Lawsonia intracellularis* induces rapid induction of humoral immunity, reduction of bacterial shedding and provides robust gut barrier function. *Vaccine* 36:1500–1508
- McOrist S, Smits RJ (2007) Field evaluation of an oral attenuated *Lawsonia intracellularis* vaccine for porcine proliferative enteropathy (ileitis). *Vet Rec* 161:26–28
- Holyoake PK, Collins A, Donahoo M, Lising R, Emery D (2009) Identifying obstacles to reducing the use of antibiotics to control porcine proliferative enteropathy. *Aust Vet J* 87:33–34
- Karupannan AK, Opriessnig T (2018) *Lawsonia intracellularis*: revisiting the disease ecology and control of this fastidious pathogen in pigs. 5:181
- Lloyd DH, Page SW (2018) Antimicrobial stewardship in veterinary medicine. *Microbiol Spectr*. <https://doi.org/10.1128/microbiolspec.ARBA-0023-2017>
- Kathayat D, Helmy YA, Deblais L, Rajashekara G (2018) Novel small molecules affecting cell membrane as potential therapeutics for avian pathogenic *Escherichia coli*. *Sci Rep* 8:555–565
- Johnson BK, Abramovitch RB (2017) Small molecules that sabotage bacterial virulence. *Trends Pharmacol Sci* 38:339–362
- Yin Y, Bijvelds M, Dang W, van der Eijk AA, Knipping K, Tuysuz N, Dekkers JF, Wang Y, de Jonge J, Sprengers D, van der Laan LJ, Beekman JM, Ten Berge D, Metselaar HJ, de Jonge H, Koopmans MP, Peppelenbosch MP, Pan Q (2015) Modeling rotavirus infection and antiviral therapy using primary intestinal organoids. *Antiviral Res* 123:120–131
- Huan YW, Bengtsson RJ, Macintyre N, Guthrie J, Finlayson H, Smith SH, Archibald AL, Ait-Ali T (2017) *Lawsonia intracellularis* exploits  $\beta$ -catenin/Wnt and Notch signalling pathways during infection of intestinal crypt to alter cell homeostasis and promote cell proliferation. *PLoS One* 12:e0173782
- Guedes RMC, Machuca MA, Quiroga MA, Pereira CER, Resende TP, Gebhart C (2017) *Lawsonia intracellularis* in pigs: progression of lesions and involvement of apoptosis. *Vet Pathol* 54:620–628
- Sato T, Clevers H (2013) Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications. *Science* 340:241–260
- Clevers H (2016) Modeling development and disease with organoids. *Cell* 165:1586–1597
- Ranganathan S, Doucet M, Grassel CL, Delaine-Elias B, Zachos NC, Barry EM (2019) Evaluating *Shigella flexneri* pathogenesis in the human enteroid model. *Infect Immun* 87:e00740
- Wilson S, Tocchi A, Holly M, Parks WC, Smith JG (2014) A small intestinal organoid model of non-invasive enteric pathogen-epithelial cell interactions. *Mucosal Immunol* 8:352–361

21. Zhang Y-G, Wu S, Xia Y, Sun J (2014) Salmonella-infected crypt-derived intestinal organoid culture system for host–bacterial interactions. *Physiol Rep* 2:e12147
22. Forbester JL, Goulding D, Vallier L, Hannan N, Hale C, Pickard D, Mukhopadhyay S, Dougan G (2015) Interaction of *Salmonella enterica* serovar Typhimurium with intestinal organoids derived from human induced pluripotent stem cells. *Infect Immun* 83:2926–2934
23. Karve SS, Pradhan S, Ward DV, Weiss AA (2017) Intestinal organoids model human responses to infection by commensal and Shiga toxin producing *Escherichia coli*. *PLoS One* 12:e0178966
24. Koltes DA, Gabler NK (2016) Characterization of porcine intestinal enteroid cultures under a lipopolysaccharide challenge 1. *J Anim Sci* 94:335–339
25. Li L, Fu F, Guo S, Wang H, He X, Xue M, Yin L, Feng L, Liu P (2019) Porcine Intestinal Enteroids: a new model for studying enteric coronavirus Porcine Epidemic Diarrhea Virus infection and the host innate response. *J Virol* 93:e01682
26. Mahe MM, Sundaram N, Watson CL, Shroyer NF, Helmuth MA (2015) Establishment of human epithelial enteroids and colonoids from whole tissue and biopsy. *J Vis Exp* 97:e52483
27. Vila MF, Trudeau MP, Hung YT, Zeng Z, Urriola PE, Shurson GC, Saqui-Salces M (2018) Dietary fiber sources and non-starch polysaccharide-degrading enzymes modify mucin expression and the immune profile of the swine ileum. *PLoS One* 13:e0207196
28. Vannucci FA, Beckler D, Pusterla N, Mapes SM, Gebhart CJ (2013) Attenuation of virulence of *Lawsonia intracellularis* after in vitro passages and its effects on the experimental reproduction of porcine proliferative enteropathy. *Vet Microbiol* 162:265–269
29. Guedes RM, Gebhart CJ (2003) Comparison of intestinal mucosa homogenate and pure culture of the homologous *Lawsonia intracellularis* isolate in reproducing proliferative enteropathy in swine. *Vet Microbiol* 93:155–166
30. Vannucci FA, Wattanaphansak S, Gebhart CJ (2011) An alternative method for cultivation of *Lawsonia intracellularis*. *J Clin Microbiol* 50:1070–1072
31. Wang N, Zhang H, Zhang BQ, Liu W, Zhang Z, Qiao M, Zhang H, Deng F, Wu N, Chen X, Wen S, Zhang J, Liao Z, Zhang Q, Yan Z, Yin L, Ye J, Deng Y, Luu HH, Haydon RC, Liang H, He TC (2014) Adenovirus-mediated efficient gene transfer into cultured three-dimensional organoids. *PLoS One* 9:e93608
32. Guedes RMC, Gebhart CJ (2003) Preparation and characterization of polyclonal and monoclonal antibodies against *Lawsonia intracellularis*. *J Vet Diagnostic Invest* 15:438–446
33. Koch S (2017) Extrinsic control of Wnt signaling in the intestine. *Differentiation* 97:1–8
34. Pampaloni F, Reynaud EG, Stelzer EHK (2007) The third dimension bridges the gap between cell culture and live tissue. *Nat Rev Mol Cell Biol* 8:839–845
35. McOrist S, Gebhart CJ, Brad TB (2006) Evaluation of porcine ileum models of enterocyte infection by *Lawsonia intracellularis*. *Can J Vet Res* 70:155–159
36. Johnson EA, Jacoby RO (1978) Transmissible ileal hyperplasia of hamsters. II. Ultrastructure. *Am J Pathol* 91:451–468
37. Barker N (2014) Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration. *Nat Rev Mol Cell Biol* 15:19–33
38. Sato T, Vries RG, Snippert HJ, Van De Wetering M, Barker N, Stange DE, Van Es JH, Abo A, Kujala P, Peters PJ, Clevers H (2009) Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 459:262–265
39. Foulke-Abel J, In J, Yin J, Zachos NC, Kovbasnjuk O, Estes MK, De Jonge H, Donowitz M (2016) Human enteroids as a model of upper small intestinal ion transport physiology and pathophysiology. *Gastroenterology* 150:638–649
40. Holly MK, Smith JG (2018) Adenovirus infection of human enteroids reveals interferon sensitivity and preferential infection of goblet cells. *J Virol* 92:e00250
41. Hwang JM, Seo MJ, Yeh JY (2017) *Lawsonia intracellularis* in the feces of wild rodents and stray cats captured around equine farms. *BMC Vet Res* 13:233
42. Collins AM, Fell S, Pearson H, Toribio JA (2011) Colonisation and shedding of *Lawsonia intracellularis* in experimentally inoculated rodents and in wild rodents on pig farms. *Vet Microbiol* 150:384–388
43. Viott AD, França SA, Vannucci FA, Cruz EC Jr, Costa MC, Gebhart CJ, Guedes R (2013) Infection of sparrows (*Passer domesticus*) and different mice strains with *Lawsonia intracellularis*. *Pesq Vet Bras* 33:372–378
44. Gabardo MP, Sato JPH, Daniel AGS, Andrade MR, Pereira CE, Rezende TP, Otoni LV, Rezende LA, Guedes RM (2017) Evaluation of the involvement of mice (*Mus musculus*) in the epidemiology of porcine proliferative enteropathy. *Vet Microbiol* 205:75–79
45. Guedes RMC, Gebhart CJ, Deen J, Winkelman NL (2002) Validation of an immunoperoxidase monolayer assay as a serologic test for porcine proliferative enteropathy. *J Vet Diagnostic Invest* 14:528–530
46. Guedes RMC, Gebhart CJ, Winkelman NL, Mackie-Nuss RA, Marsteller TA, Deen J (2002) Comparison of different methods for diagnosis of porcine proliferative enteropathy. *Can J Vet Res* 66:99–107
47. McOrist S, Jasni S, Mackie RA, Berschneider HM, Rowland AC, Lawson GH (1995) Entry of the bacterium ileal symbiont *intracellularis* into cultured enterocytes and its subsequent release. *Res Vet Sci* 59:255–260
48. McOrist S, Gebhart CJ, Boid R, Barns SM (1995) Characterization of *Lawsonia intracellularis* gen. nov., sp. nov., the obligately intracellular bacterium of porcine proliferative enteropathy. *Int J Syst Bacteriol* 45:820–825
49. Clevers H (2013) The intestinal crypt, a prototype stem cell compartment. *Cell* 154:274–284
50. Murakata K, Sato A, Yoshiya M, Kim S, Watarai M, Omata Y, Furuoka H (2008) Infection of different strains of mice with *Lawsonia intracellularis* derived from rabbit or porcine proliferative enteropathy. *J Comp Pathol* 139:8–15
51. Dekkers JF, Wiegerinck CL, de Jonge HR, Bronsveld I, Janssens HM, De Winter-de Groot KM, Brandsma AM, De Jong NW, Bijvelds MJ, Scholte BJ, Nieuwenhuis EE (2013) A functional CFTR assay using primary cystic fibrosis intestinal organoids. *Nat Med* 19:939–945

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