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Effects of *Lawsonia intracellularis* infection in the proliferation of different mammalian cell lines

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ABSTRACT

Lawsonia intracellularis is an obligate intracellular bacterium that causes proliferative enteropathy in various animal species. While cellular proliferation of intestinal cells is recognized as the hallmark of *L. intracellularis* infection *in vivo*, it has not been demonstrated in *in vitro* models. In order to assay the effect of *L. intracellularis*, various cell lines were infected with pathogenic and non-pathogenic passages of the bacterium. Because of the high proliferative rate of these cell lines, serum deprivation, which is known to reduce proliferation, was applied to each of the cell lines to allow the observation of proliferation induced by *L. intracellularis*. Using antibodies for Ki-67 and *L. intracellularis* in dual immunofluorescence staining, we observed that *L. intracellularis* was more frequently observed in proliferating cells. Based on wound closure assays and on the amount of eukaryotic DNA content measured over time, we found no indication that cell lines infected with *L. intracellularis* increased proliferation and migration when compared to non-infected cells ($p > 0.05$). Cell arrest due to decreased serum in the culture media was cell-line dependent. Taken together, our findings provide data to support and expand previous subjective observations of the absence of *in vitro* proliferation caused by *L. intracellularis* in cell cultures and confirm that cell lines infected by *L. intracellularis* fail to serve as adequate models for understanding the cellular changes observed in proliferative enteropathy-affected intestines.

1. Introduction

Proliferative enteropathy (PE), caused by *Lawsonia intracellularis*, has been recognized in pig herds since the early 1990's (Lawson et al., 1993) and is endemic in pig herds worldwide (Holyoake et al., 2010; Resende et al., 2015; van der Heijden et al., 2004; Wu et al., 2014). In addition to pigs, PE also affects birds, horses, non-human primates and other animal species (Vannucci and Gebhart, 2014). In weaned foals, PE can be responsible for severe clinical signs and often death (Gabardo et al., 2015; McGurrin et al., 2007; Pusterla and Gebhart, 2013). The hallmark lesion of PE is the thickening of intestinal mucosa due to an increased number of epithelial cells in the affected crypts. Despite the clinical relevance of PE, its pathogenesis is not well understood. The lack of a suitable *in vitro* model that replicates the cellular proliferation observed *in vivo* has limited the understanding of the mechanisms used by *L. intracellularis* to cause the characteristic intestinal lesions. Therefore, the advancement of alternatives for prevention and control of the disease has been hindered.

L. intracellularis is a Gram-negative, obligate intracellular bacterium that requires a specific microaerophilic environment and permissive cell cultures to be cultivated *in vitro* (Vannucci and Gebhart, 2014). For *L. intracellularis* isolation and propagation, the following cell lines have been used: IPEC-J2 (porcine jejunum cell), IEC-18 (rat small intestinal cells), INT-407 (human fetal intestine), CRL 1677 (rat colonic adenocarcinoma), PK-15 (pig kidney) and McCoy (mouse fibroblasts) (Lawson et al., 1993; McOrist et al., 2006; Oh et al., 2010; Vannucci et al., 2012). To date, there is no evidence that *L. intracellularis* infection induces cellular proliferation *in vitro*. However, this absence of evidence for cellular proliferation caused by *L. intracellularis* is based only on qualitative analysis (McOrist et al., 2006) or on a single publication that evaluated a mouse fibroblast-derived cell line (Vannucci et al., 2012). Considering that cellular proliferation is the expected outcome of *L.*

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intracellularis infection in cell lines, deprivation of growth factors by the reduction of fetal bovine serum (FBS) concentration in the culture media may allow us to distinguish any proliferation induced by the bacterium from the intrinsic proliferation characteristics of each cell line. In addition, it is known that *L. intracellularis* loses its capability to cause disease in pigs at high *in vitro* passages (> 40), while it is pathogenic at low passage (≤20) (Vannucci et al., 2013). Therefore, we also hypothesized that the effects on cellular proliferation could be different, depending on the passage of *L. intracellularis* used.

The objectives of this study were to evaluate whether intestinal epithelial cell lines proliferate when infected by *L. intracellularis* at high or low passages, and to determine whether culture of various cell lines under conditions of reduced growth factors would facilitate detection of cellular proliferation *in vitro* induced by *L. intracellularis*.

2. Materials and methods

2.1. L. intracellularis propagation

L. *intracellularis* isolates (PHE/MN1-00, ATCC PTA-3457) at low (≤20) and high (> 40) passages (Vannucci et al., 2013) were propagated in a mouse fibroblast-derived cell monolayer (McCoy cells; ATCC® CRL-1696™). Dulbecco's Modified Eagle Medium (DMEM; Gibco Invitrogen Corporation) and 7% fetal bovine serum (FBS; Corning™ 35011CV, heat inactivated) were used for cell culture as described elsewhere (Guedes and Gebhart, 2003a, b). McCoy cell monolayers at about 30% confluence were infected with *L. intracellularis* within 24 h after cell passage, and then incubated at 37 °C under a controlled microaerophilic atmosphere (Vannucci et al., 2012). Seven days after infection, the McCoy cell monolayers were lysed by immersion in sterile 0.1% potassium chloride, mechanical lysing, centrifuged, and suspended in sterile sucrose phosphate glutamate (SPG) solution (Guedes and Gebhart, 2003a, b). The resultant suspensions were then filtered through 0.80 µm sterile filters to remove any remaining McCoy cells and nuclei, and then used to infect the cell lines evaluated.

2.2. Cell lines and culture conditions

The following intestinal epithelial cell lines were tested: IPEC-J2 (courtesy of Dr. David Brown, University of Minnesota), IEC-18 (ATCC® CRL-1589™) and Caco-2 (colorectal adenocarcinoma cells; ATCC® HTB-37™). In all experiments, McCoy cells were used as base-line to compare growth and proliferation of the other cell lines tested. L. intracellularis growth in McCoy cells in these experiments was also compared to a previous report (Vannucci et al., 2012).

Each cell line was grown with two different FBS (Heat Inactivated Premium FBS, Corning) concentrations, the standard (ST), according to the ATCC® recommendation for each cell line, and a deprived (D) condition. McCoy and IEC-18 cells were cultured in DMEM (Gibco, Thermo-Fisher Scientific) supplemented with 7% FBS (standard - ST) and 1% FBS (deprived – D); IPEC-J2 cells were grown in DMEM/F12 (Gibco, Thermo-Fisher Scientific) supplemented with insulin, transferrin and sodium selenite (5 ng/ml each; ITS, BD Biosciences) and 5 ng/ml epidermal growth factor (EGF; Sigma-Aldrich) with 5% FBS (ST) or 0.7% FBS (D); Caco-2 cells were grown in Eagle's Minimum Essential Medium (EMEM; ATCC® 30–2003) supplemented with 20% FBS (ST) or 3% FBS (D). Cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2, and subcultured when confluency reached 70–90% using 0.25% trypsin- ethylenediaminetetraacetic acid (EDTA, Gibco, Thermo-Fisher Scientific).

Cell lines were infected with *L. intracellularis* at low (≤20) and high (> 40) passages (Vannucci et al., 2013). Uninfected cell lines served as negative control groups and were subjected to similar manipulations and ST or D FBS concentrations in the culture media to control for variables not related to the experimental design. Cells were infected with *L. intracellularis* inocula containing between 10^5 and 10^6 microorganisms/ml as quantified by qPCR.

2.3. L. intracellularis growth over time

Cell lines were seeded into 96-well plates (Nuclon Delta Surface, ThermoScientific) and each plate contained all 4 cell lines, with all treatment groups. After infection, plates were stained for *L. intracellularis* antigen by a modified immunocytochemistry assay (Guedes et al., 2002), using rabbit polyclonal antibody (Guedes and Gebhart, 2003a, b). Evidence of bacterial intracellular propagation was based on the amount of *L. intracellularis* in the cell cytoplasm. Cells containing > 30 *L. intracellularis* organisms were considered highly infected cells (HIC) (McOrist et al., 1995; Wattanaphansak et al., 2009). The numbers of HICs were counted in an inverted microscope (Nykon, TMS) with a 200x objective lens by a trained person, blinded for the treatments. Counts were done at 1, 4 and 7 days post infection (dpi) by determining the proportion HICs in the whole well and were presented as percentages of total eukaryotic cells. The experiment was repeated twice, with four replicates for each treatment group, in each cell line, and in each experiment.

2.4. Cellular proliferation assay

The amount of DNA content of each cell line with or without *L. intracellularis* infection was used to estimate cellular proliferation over time. The CyQuant NF Cell Proliferation Assay Kit (Molecular Probes; Invitrogen Corp.) was used according to the manufacturer's instructions. Briefly, cell lines were seeded into four different 96-well plates, each plate containing four repetitions of each treatment. At various time points (0, 1, 4 and 7 dpi), one plate was selected for cellular DNA content measurement using the CyQuant kit. The fluorescence signal generated by the reaction of the binding of the fluorescent probe with DNA was measured in a plate reader (BioTek Plate Reader, Synergy H1), with excitation of 485 nm and emission of 530 nm and gain of 50%. The experiment was performed in three independent repetitions, each one containing four replicates for each treatment group.

2.5. Wound closure assay

Cell lines were seeded into 8-well chamber slides (Millicell EZ SLIDE, MilliporeSigma), in treatment groups that included ST or D FBS concentrations, and non-infected or infected with *L. intracellularis* at low (≤20) and high (> 40) passages. This resulted in six treatment groups for each of the four cell lines tested. When about 80% confluence was achieved (around 24 h post seeding), a scratch was made down the center of each well using a 200 µl sterile pipette tip (Li et al., 2013; Ying et al., 2014).

The scratched areas were photographed with a digital camera at 400x in an inverted microscope (Visual Dynamix, EXI-300) every 24 h. Pictures were then analyzed using NIH ImageJ software 1.46 r (National Institute of Health; downloaded from http://rsbweb.nih.gov/ij/). Wound widths, characterized by the distance between the scratch borders, were measured at 0, 24 and 48 h post-scratching. The wound closure effect was determined by measuring the width of the wound area at each time point compared with the wound width of the previous day. The following formula was then applied to the measurements:

wound closure = [(wound width day X – wound width day X + 1)/ wound width day X] x 100

The experiment was performed in three independent repetitions, each one containing two replicates for each treatment group.

2.6. Dual immunofluorescence: Ki-67 and L. intracellularis

The chamber slides used for the wound healing assay, fixed 48 h

post infection, were subsequently used to verify the presence of Ki-67 (a proliferation marker expressed by eukaryotic cells during the division process) and its association with *L. intracellularis* antigen. After removal of culture supernatant, cell monolayers were fixed with cold acetone and then re-hydrated with Tris buffered saline (TBS) containing 0.1% triton-x (TBS-T) for 10 min at room temperature. The cells were then incubated with 10% normal goat serum (ZC1213, Vector Laboratories) in TBS-T, for 30 min at room temperature. Afterwards, *L. intracellularis* mouse monoclonal antibody (Guedes and Gebhart, 2003a, b) was diluted 1:10 in TBS-T and incubated for 1 h at room temperature. After 3 washes with TBS-T, the rabbit Ki-67 antibody (1:200 in TBS-T, CRM325B, Biocare Medical) was added and incubated for 2 h at room temperature. Following another series of 3 washes, secondary Alexa-Fluor 488-labelled goat anti-mouse antibody (1:250, ab150113, Abcam) was added and incubated for 30 min at room temperature. After another series of 3 washes, secondary Cy3-labelled goat anti-rabbit antibody (1:250, ab97075, Abcam) was added and incubated for 30 min. Slides were washed and mounted with Prolongold (Diamond Antifade Mountant - P36971, ThermoFisher) and glass coverslips. Fluorescent signals were detected by a fluorescence microscope (Olympus, BX53) coupled with a digital camera. Immunofluorescent images were taken of ten random fields per sample at 600x, and then the numbers of *L. intracellularis* infected cells, Ki-67 positive cells and double positive cells (*L. intracellularis* and Ki-67) were counted with NIH ImageJ software 1.46 r.

2.7. Statistical analysis

Quantitative results are shown as mean ± standard error of the mean (SEM). Two-way analysis of variance (ANOVA) was used to assess differences within treatment groups and among treatment groups. Tukey's post hoc test was used to verify differences between two groups, with $p < 0.05$ considered statistically significant. T-student test was used to compare results for the dual-immunofluorescence assay. GraphPad 7.0 software for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) was used to perform the analysis.

3. Results

3.1. L. intracellularis growth over time

The growth of L. *intracellularis* in each treatment and each cell line was monitored by the estimation of the percentage of highly infected cells (HIC) (McOrist et al., 1995; Wattanaphansak et al., 2009) over time. All cell lines tested were susceptible to *L. intracellularis* infection with typical intracellular bacterial growth of about 30–100 per cell in the cytoplasm of infected cells (Fig. 1). Cell monolayers were 100% confluent after 6 days of incubation regardless of treatment group. Non-infected groups remained negative throughout the experiment and none of the infected groups, independent of the cell line, had detectable *L. intracellularis* growth as evidenced by HIC at 1 dpi (data not shown). IPEC-J2 and McCoy cells had increasing amounts of HIC over time from 1 dpi through the end of this study at 7 dpi and reached 100% HIC for all treatment groups. The number of HIC in the IEC-18 cell line plateaued about 50 to 30% HIC 4 dpi for all the treatment groups. In Caco-2 cells, the amount of HICs increased over time reaching 50 to 75% HIC, except for the treatment using high passage *L. intracellularis* under serum deprived conditions, which decreased from about 50 to 30% HIC from 4 to 7dpi (Fig. 2).

3.2. Cellular proliferation assay

Cellular proliferation was quantified at different time points (1, 4 and 7 dpi) in all cell lines and treatment groups (Fig. 3). Two-way ANOVA was used to assess differences within treatment groups and among treatment groups. Comparison within groups indicates time effect: and any significant differences between a given day and the previous day are indicated by an asterisk. Comparisons among treatment groups were evaluated at the same time point and the fluorescence intensity result for a treatment group in relation to the other groups on a given dpi. The outcomes of the deprived FBS concentration (D) varied over time and were cell line-dependent. The common findings for all cell lines tested were the lack of statistical difference within or among groups at 0 and 1 dpi and the absence of increased proliferation in any cell line infected by *L. intracellularis*, regardless of the bacterial passage status.

In IPEC-J2 cells, while the cells in the standard FBS treatment group (ST) had significant proliferation between 4 and 7 dpi ($p = 0.004$), difference was not observed in cells under the deprived FBS concentration (D). Therefore, deprivation of growth factors by decreasing FBS concentration in culture media resulted in cell arrest between 4 to 7 dpi. However, while the cells under ST FBS concentration that were infected with *L. intracellularis* at low passage had proliferation from 4 to 7 dpi ($p < 0.05$), infection in the D treatment group did not result in recovery of the proliferation rate that was reduced by the FBS deprivation. This means that *L. intracellularis* infection did not lead to cell proliferation in that condition. In the groups infected with *L. intracellularis* at high passage no statistical differences were observed within or between treatment groups from 4 to 7dpi. When comparing the respective fluorescence intensity at the same time point among groups, it was observed that at 7 dpi, the ST treatment group had higher fluorescence intensity than D treatment groups, regardless of the infection status (non-infected, infected with *L.intracelluaris* at low passage or at high passage). In general, it was observed that IPEC-J2 cells that where infected by *L. intracellularis* and maintained at D FBS had lower proliferation rate than the ST non-infected group (Fig. 3A).

In IEC-18 cells, comparison within groups resulted in increased/decreased? proliferation between 1 and 4 dpi in all treatment groups, regardless of the FBS status. For the cellular growth between 4 and 7 dpi, differences were observed in all treatment groups, with exception of the ST group. When comparing the fluorescence intensities among groups at a given dpi, it was observed that at 4 dpi the treatment groups with deprived FBS had lower fluorescence intensities than the ST treatment group, meaning that infection with *L. intracellularis*, at low or high passage, did not result in compensation of the decreased proliferation rate due to FBS deprivation. At 7 dpi, the only group that was different from the ST treatment group was the group with deprived FBS and infected with *L. intracellularis* at high passage (ST > D High passage, $p = 0.01$), which showed lesser proliferation (Fig. 3B).

In Caco-2 cells, none of the groups had any differences in cell proliferation rate from 0 to 1 dpi or from 1 to 4 dpi. Differences were observed from 4 to 7 dpi; however, these differences were observed in all of the treatment groups at ST FBS concentration, regardless of their infection state. There was no statistical difference between any treatment groups and the non-infected Caco-2 cells under ST conditions at either 4 or 7dpi. In fact, Caco-2 cells had a very similar pattern of proliferation rate regardless the FBS concentration or the *L. intracellularis*-infection status (Fig. 3C).

In McCoy cells, the deprivation of FBS did not result in cell arrest of the D group at any time point. In fact, the only treatment group in which the cell proliferation was not higher at 7dpi in relation to 4dpi was the group with ST FBS concentration, infected with *L. intracellularis* at high passage. When comparing the cell proliferation at 4 dpi among groups, only from ST treatment group was statistically different compared to the treatment group with D FBS infected with *L. intracellularis* at high passage, and that had lower proliferation ($p = 0.03$). At 7dpi, all treatment groups had similar fluorescence intensity (Fig. 3D).

3.3. Wound closure assay

To verify whether cells infected by *L. intracellularis* would

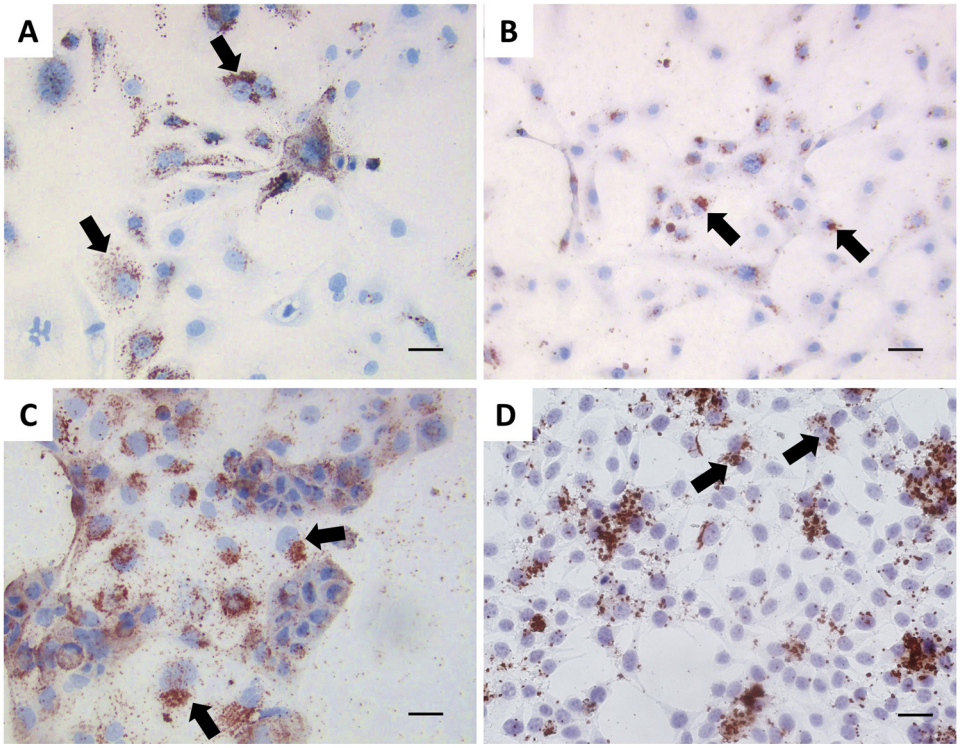


Fig. 1. *L. intracellularis* infection in intestinal epithelial cells and in mouse fibroblasts. Highly infected cells (HIC, arrows) were observed in all cell lines tested at 4 days post infection. Cells were stained by a modified immunocytochemistry for *Lawsonia intracellularis*. A) IPEC-J2 cells; B) IEC-18 cells; C) Caco-2 cells; D) McCoy cells. Scale bar: 25 µm.

proliferate and migrate faster than non-infected cells through a scratched monolayer, a wound closure assay was executed. The percent of wound closure was measured from 0 to 24 h and from 24 h to 48 h. There were no differences among treatment groups for wound closure at any time point (Fig. 4). The percent of wound closure from 0 to 24 h was below 30% for all cell lines tested except McCoy cells, and for all treatment groups. The percent of wound closure ranged from 43.5% to 93.5% for IPEC-J2 cells from 24 to 48 h. With IEC-18 cells, the percent

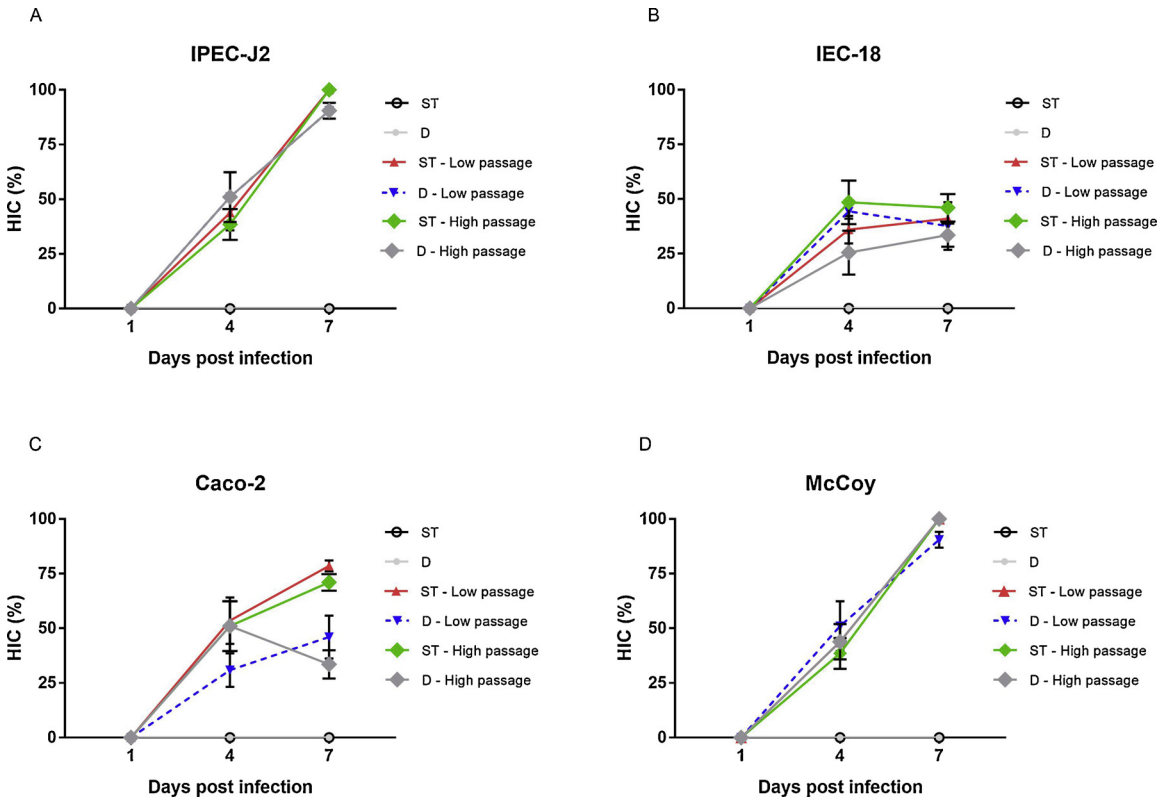


Fig. 2. Percentage of highly infected cells (HIC) over time. The percentage of HIC was measured for each treatment group at different days post infection as compared to total cells in the cell culture monolayer by direct observation of infected cells stained by a modified immunocytochemistry assay. A) IPEC-J2 cells; B) IEC-18 cells; C) Caco-2 cells; D) McCoy cells. Bars represent means ± standard error of the mean.

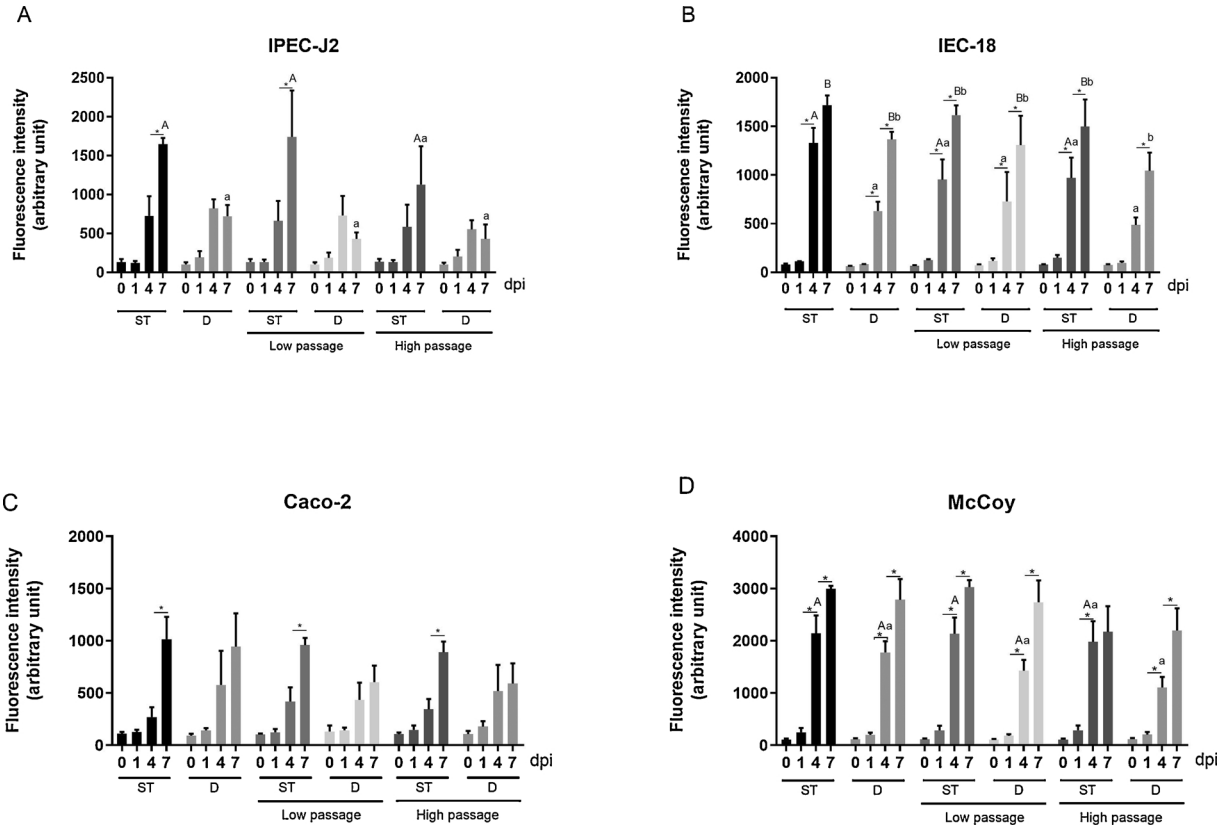


Fig. 3. Cellular proliferation of cell cultures after *L. intracellularis* infection. Eukaryotic DNA content, measured by fluorescence intensity, was quantified at different time points (0,1, 4 and 7 dpi) in all cell lines and treatment groups. Two-way ANOVA was used to detect differences within treatment groups and among treatment groups ($p < 0.05$ was considered statistically significant). Comparison within groups indicate a time effect and significant differences between a given day and the previous day are indicated by an asterisk. Comparisons among treatment groups were used to verify treatment effect at the same time point and the fluorescence result for a treatment group in relation to the other groups in a given dpi. The same letters indicate the comparison in the same dpi and capitalized and non-capitalized letters indicate significative differences between groups ("A" and "a" for 4dpi and "B" and "b" for 7dpi). Bars represent means ± standard deviation of the mean.

of wound closure ranged from 20.8% to 69.6% from 24 to 48 h. With Caco-2-cells, the non-infected and *L. intracecllularis* high passaged-infected groups at D FBS conditions the width of the wound was wider at 48 h than at 24 h, which resulted in the percent of wound closure varying from -4.8% to 27.5%. On the other hand, wound closure for McCoy cells tended to be faster, independent of the treatment, with the percent of wound closure higher than 60% for all of the treatment groups from 24 to 48 h, with the non-infected group with ST FBS achieving 100% of wound closure in 48 h.

3.4. Dual immunofluorescence: Ki-67 and L. intracellularis

It is suggested that *L. intracellularis* preferentially infects actively proliferating cells in intestinal crypts, where *L. intracellularis* infection would lead to a higher mitotic index (Vannucci and Gebhart, 2014). To explore this hypothesis *in vitro*, we investigated whether *L. intracellularis* would co-localize with the proliferation marker Ki-67 in infected cell lines at 48 h post infection. In IPEC-J2 cells, only the high passage *L. intracellularis* group grown in ST conditions showed higher numbers of infected Ki-67 positive cells compared with Ki-67 negative cells ($p = 0.008$, Fig. 5-IA). For IEC-18 cells ($p \leq 0.0001$, Fig. 5-IB), Caco-2 ($p < 0.05$, Fig. 5-IC) cells and McCoy cells ($p < 0.05$, Fig. 5-ID), the number of Ki-67 positive cells infected by *L. intracellularis* was higher than the number of Ki-67 negative cells, independent of the FBS concentration (ST or D) and of the *L. intracellularis* passage (low or high). Overall, we observed that, in the majority of treatment groups and with the exception of the IPEC-J2 cell line, the proportion of cells that were double positive (*L. intracellularis* was co-localized with Ki-67) was higher than cells that were *L. intracellularis* infected, but negative for Ki-67.

4. Discussion

Many advances in understanding the pathogenesis of proliferative enteropathy have been achieved through studies conducted *in vivo* using animal models. Although extremely valuable, *in vivo* animal trials are expensive, time demanding, of ethical concern and do not allow for the study of mechanisms of pathogenesis. In addition, *in vivo* studies often have confounding factors, such as uncontrolled environment, microbiome modulation, immune responses, and individual variation which may complicate the interpretation of the outcomes. As an alternative, two-dimensional *in vitro* cell cultures have been used over the years both to propagate *L. intracellularis* and to study *L. intracellularis* pathogenesis (Lawson et al., 1993; McOrist et al., 2006; Oh et al., 2010; Vannucci et al., 2012). Despite the advances provided by the *in vitro* models, there has been no observations of increased proliferation in these cell lines when infected by *L. intracellularis* (Vannucci et al., 2012). However, only McCoy cells have been methodically evaluated for *L. intracellularis*-induced proliferation (Vannucci et al., 2012) to date. It is important to highlight, however, that McCoy cells are mouse fibroblastic cells and do not model the intestinal epithelium which is the site where *L. intracellularis* normally infects and causes lesions. Intestinal epithelial cells would, therefore, be the most representative model to study *L. intracellularis* infection *in vitro*.

The mechanisms of pathogenesis involved in *L. intracellularis* infection and consequent intestinal lesions *in vivo* are not known. Since *L.*

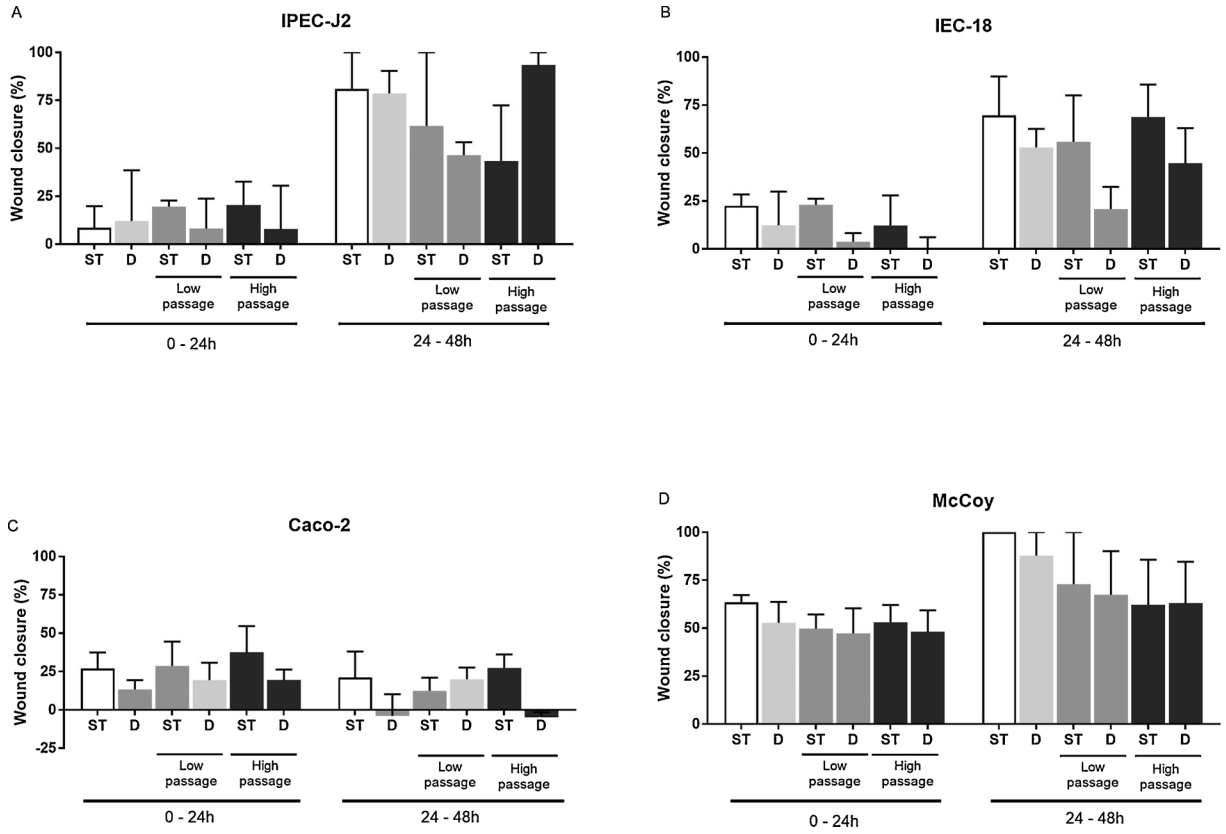


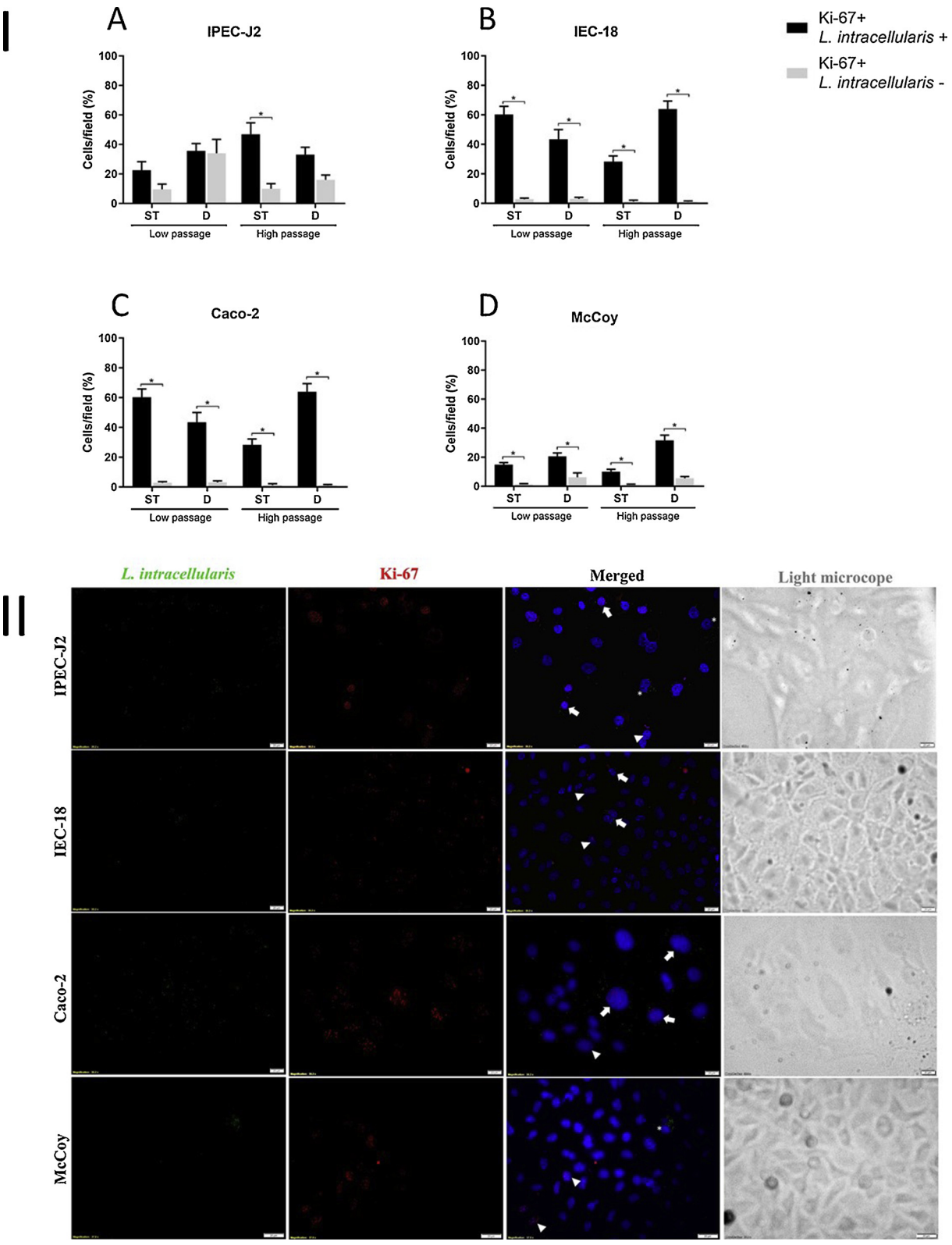
Fig. 4. Wound closure assay. Wound closure results for scratched cell lines cultured with standard (ST) and deprived (D) cocentrations of fetal bovine serum (FBS) and infected with *L. intracellularis* at low and high passages. Bars represent means ± standard error of the mean.

intracellularis is observed mainly in the intestinal crypt cellular compartments of affected animals, it is proposed that *L. intracellularis* preferably infects immature cells with proliferative potential (Lawson et al., 1993; Smith and Lawson, 2001; Gebhart and Guedes, 2010). These immature cells would thus provide an optimum environment for bacterial propagation. However, since cell lines are derived from cancer, immortalized or maintained in undifferentiated states to allow for continuous culture, it is plausible that changes in proliferation induced by *L. intracellularis* infection in cell line cultures may be masked by the already high proliferative rate of such cell lines. Cell lines grown *in vitro* have the capacity to quickly proliferate in optimum culture conditions until they achieve confluence. Manipulation of media is one strategy to detect changes in the proliferation rate (Yao and Asayama, 2017). Since fetal bovine serum (FBS) is the most common supplement for cell culture media, it is one of the medium components that can be manipulated in cellular proliferation studies (Khammanit et al., 2008; Mengual Gómez et al., 2010; Yao and Asayama, 2017). Nevertheless, deprivation of growth factors by decreased concentration of FBS did not significantly affect the cellular proliferation of any of the *L. intracellularis*-infected cell lines studied here.

We also performed the CyQuant assay to quantify cellular proliferation by assessing the amount of DNA present over time in cell cultures. Even though the CyQuant assay does not specifically target eukaryotic DNA, the amount of *L. intracellularis* DNA is negligible in comparison to the amount eukaryotic cell DNA over the 7-day growth period in our *in vitro* model. The doubling time for *L. intracellularis* is not known, but considering that at 1 dpi there are some infected cells (McOrist et al., 1995) and that it takes about 7 days for a cell to become a HIC (> 30 *L. intracellularis* organisms in the cytoplasm), the approximate doubling time for *L. intracellularis* is calculated at about1 day. The *L. intracellularis* genome comprises 1.46 Mb, whereas mouse eukaryotic cells carry approximately 2654 Mb. Since the approximate doubling time for IPEC-J2 in *in vitro* culture, for example, is approximately 40 h, it is not likely that the amount of bacterial DNA would approach even 0.01% of the amount of eukaryotic DNA within that period of 7 days. Furthermore, although some statistical differences were detected, they did not indicate a higher proliferation rate of intestinal epithelial cells infected with pathogenic *L. intracellularis* in relation to non-infected cells, highlighting the negligible influence of *L. intracellularis* DNA on the CyQuant results. This lack of cell proliferation was also observed with McCoy cells (non-epithelial, non-intestinal cells) in this study, which is in agreement with a previous publication (Vannucci et al., 2012) that also reported no increased proliferation of *L. intracellularis*-infected McCoy cells.

The wound closure assay failed to demonstrate higher migration/proliferation in any of the treatment groups infected with *L. intracellularis*, independently of the bacterial virulence (passage). Interestingly, some treatment groups cultured in Caco-2 cells had an increase in the wound area instead of a closure effect. This finding can be explained by the cancerous nature of Caco-2 cells. Caco-2 cells were derived from colorectal cancer, a malignant epithelial tumor and, therefore, have higher migration capacity. Also, cell-cell contact is no longer an inhibitor for proliferation of cancer cells. With that, Caco-2 cells lose the stimulus to grow towards the wound, which may help to explain why the wound width increased in some of the treatment groups of Caco-2 cells, but not in the other cell lines (Hanahan and Weinberg, 2011).

Although our results did not detect cellular proliferation induced by *L. intracellularis* in the various tested conditions, we did observe through the Ki-67/*L. intracellularis* dual immunofluorescence staining analysis that the number of *L. intracellularis*-infected cells that were actively proliferating was higher than the number of *L. intracellularis*-infected cells that were not proliferating ($p < 0.05$). This finding supports the hypothesis that actively proliferating cells offer more optimal



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conditions for *L. intracellularis* infection. The confluence of the cellular monolayer impacts the proliferative index of the cultured cells. Cells lose the mitotic stimulus when they start to contact each other, and since the doubling time of each cell is variable, each cell line had a different confluence at 48 h after infection. This may be the reason why the number of Ki-67 positive cells tended to be lower in McCoy cells and IPEC-2 cells compared with the other cell lines, since their confluence was higher at the time when cells were fixed. Confluent monolayers are not a suitable environment for *L. intracellularis* propagation (Lawson et al., 1993; McOrist et al., 2006, 1995), and this may be due to the monolayers' low mitotic indices at this point in culture. Therefore, the evaluation of the dual immunofluorescence staining at a single time

Fig. 5. Proportion of *L. intracellularis* infected cells at proliferative and non-proliferative stage. The mean of the total number of *L. intracellularis*-infected cells per field was determined by counting the total number of cells, the number of *L. intracellularis*-infected cells there were also Ki-67 positive, and the number of *L. intracellularis*-infected cells that were Ki-67 negative in 10 random fields, photographed with an inverted microscope using a 600x objective coupled with a digital camera. From the total number of cells, the percent of *L. intracellularis* positive cells that were Ki-67 positive cells and the percent of *L. intracellularis* positive cells that were Ki-67 negative cells were computed. **Panel I:** Bars represent mean ± standard error of the mean. Black bars represent *L. intracellularis* infected cells that were also Ki-67 positive; gray bars represent *L. intracellularis* infected cells that were Ki-67 negative. A) IPEC-J2 cells; B) IEC-18 cells; C) Caco-2 cells; D) McCoy cells. T-Student test was used to check significant differences ($p < 0.05$) that are indicated by (*). Overall, we observed that in the majority of the treatment groups the proportion of cells that were double positive (*L. intracellularis* was co-localized with Ki-67) was higher than cells that were *L. intracellularis*-infected, but negative for Ki-67. **Panel II:** immunofluorescence images of cell lines infected with *L. intracellularis* at low passage and under FBS deprived conditions at 2 dpi (D – Low passage). *L. intracellularis* antigen (green) was revealed by mouse monoclonal antibody followed by AlexaFluor 488- labelled goat anti-mouse secondary antibody. Ki-67 protein antigen (red) was revealed by rabbit polyclonal antibody followed by a Cy3-labelled goat anti-rabbit secondary antibody. Nuclear staining was performed with DAPI (blue). Arrows indicate cell that were double positive (*L. intracellularis* and Ki-67 positive); arrow heads indicate cells that were Ki-67 positive and *L. intracellularis* negative; asterisks indicate *L. intracellularis*-infected cells that were Ki-67 negative. Last column shows the same fields in bright field. Scale bar: 20 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

point constitutes a limitation of this study. Ideally, this evaluation would be done at different time points, allowing the measurements of the dual immunofluorescence staining at different time points after infection and in a variety of cell monolayer confluence levels.

Taken together, our findings have decisively shown that two-dimensional intestinal epithelial *in vitro* cultures do not reproduce the characteristic proliferative effect of *L. intracellularis* infection *in vivo*. Our work reinforces the need for the development of more appropriate models for the study of *L. intracellularis* pathogenesis *in vitro*.

Authors' contributions

TPR contributed to the acquisition, analysis and interpretation of data and to the drafting of manuscript. CJG, FAV and MSS contributed to the conception, design and data analysis and interpretation of the study. CERP, AGSD and EV contributed to the acquisition, analysis, and interpretation of data. All authors critically reviewed the manuscript and gave their final approval.

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Declaration of conflicting interests

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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