Porcine proliferative enteropathy:

transmission dynamics and immune response

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Dedication

To my daughter, Emilia

General abstract

Lawsonia intracellularis is a gram negative, intracellular bacterium that affects pigs and other species. In pigs, it produces porcine proliferative enteropathy (PPE), a costly problem in the swine industry worldwide. This gastrointestinal disease mainly affects the small intestinal tract. The symptoms can be subclinical, with loss of average daily gain (ADG), which can easily become chronic with soft, watery diarrhea, producing uneven growth rates. The less common acute form of the disease, porcine hemorrhagic enteropathy (PHE), occurs in finishing pigs and replacement gilts, producing bloody diarrhea, weakness, depression and sometimes sudden death.

Vaccination against *Lawsonia intracellularis*, with an oral live attenuated isolate of the bacterium or more recently with a killed intramuscular bacterin, is available to help control the infection. However, a comprehensive comparison of the impact that vaccination has on the transmission and induced immunity in vaccinated animals is needed in order to choose the right intervention in farms that may result in reducing transmission rates and generating immunological response. In this thesis, I aimed to 1) compare the impact of vaccination on the transmission of *L. intracellularis* and 2) compare the impact of vaccination on the local and systemic immune responses of vaccinated and non-vaccinated pigs.

The results from my studies highlight the importance of vaccination in the control of *L. intracellularis*. The length of *L. intracellularis* shedding as well as the transmission rate were reduced after both oral and intramuscular vaccination. The systemic immune response represented by serological IgG antibodies were stronger in the intramuscularly vaccinated group, while local immune responses represented by secretory IgA in the intestinal lumen and the cell mediated immunity were similarly induced from both vaccination protocols.

The results demonstrated in this thesis contribute to the better understanding of the epidemiology, transmission and immune responses to *L. intracellularis* in vaccinated pigs, using the two commercially available vaccines.

Chapter 2: Impact of vaccination on transmission of *Lawsonia intracellularis* in pigs

Porcine proliferative enteropathy (PPE) is a highly prevalent enteric disease in pigs caused by *Lawsonia intracellularis*. PPE is commonly controlled using antibiotics in sub-therapeutic doses or by vaccination. Nevertheless, the regulations for use of antimicrobials have directly affected the use of this approach to control PPE. Therefore, the use of vaccination has become an effective alternative. Effect of vaccination has been largely studied focusing on immune response or reduction of clinical disease, but these studies do not elucidate the impact of vaccination on transmission at the population level. Using a seeder-pig sentinel model, this study evaluated differences in transmission patterns and spread of *L. intracellularis* in naïve and vaccinated pigs. A total of 90 21-day-old pigs were distributed into three groups (27 pigs/group) and allocated into nine pens (3 pens/group). Nine seeder pigs were similarly allocated in a separate barn. Pigs in treatment 1(T1) were orally vaccinated with a live vaccine (Enterisol® Ileitis, Boehringer Ingelheim). In the treatment 2 (T2), pigs received intramuscular vaccine (Porcilis™ Ileitis, Merck Animal Health) and pigs in treatment 3 (T3) remained non-vaccinated. On day 21 after vaccination, nine seeder pigs were orally inoculated with a 10^8 dose of *L. intracellularis*. Seven days after inoculation, which was 28 days after vaccination, the nine seeder pigs were commingled (one seeder pig per pen) with pigs in treatment groups T1, T2, and T3 for transmission assessment. *L. intracellularis* shedding was monitored by quantitative PCR of fecal samples. The serological IgG response was monitored using the immuno peroxidase monolayer assay (IPMA) technique. Transmission patterns were assessed according to the susceptible–infectious (SI) model, without

the possibility of recovery during the study period. Expected probabilities of a non-vaccinated and vaccinated animal becoming positive were estimated using a mixed-effect logistic regression model considering the room as a random effect. Pigs were considered infectious when the fecal PCR was equal to or more than 10^3 *L. intracellularis*/gram of feces. Using the method described by Velthuis et al. (2003) (1). The transmission rate was 2.6 new cases/pig-week for non-vaccinated animals. The transmission rate was significantly reduced in both vaccinated groups when in contact with infectious (seeder) pigs ($P<0.05$). The transmission rate for the orally vaccinated group was 1.86 new cases/pig-week and the transmission rate for the intramuscularly vaccinated group was 1.75 new cases/pig-week. Pigs that received the oral vaccine shed *L. intracelullaris* for a median time 43.3% shorter than pigs that received no vaccine. Pigs that received the intramuscular vaccine shed *L. intracelullaris* for a median time 25.5% shorter than that of the pigs that received no vaccine. The serology results showed a significantly higher humoral response in the group vaccinated with the killed, intramuscular vaccine. However, that humoral response did not reflect a significant reduction in transmission rate or length of shedding compared with the oral vaccination. The results indicate that vaccination reduces the transmission rate and the length of *L. intracellularis* shedding after contact with infectious animals.

Chapter 3: Characterization of the local and systemic immune response to *Lawsonia intracellularis* infection in vaccinated pigs

Porcine proliferative enteropathy (PPE), caused by the Gram negative, intracellular bacterium *L. intracellularis*, is an important disease in the swine industry. It is currently controlled largely by vaccination and the use of subtherapeutic doses of antibiotics. The purpose of this study was to characterize the local and systemic immune responses to *L. intracellularis* infection after

vaccination with an oral modified live vaccine (Enterisol® Ileitis, Boehringer Ingelheim) and a killed intramuscular vaccine (Porcilis™ Ileitis, Merck Animal Health). A total of 90 three-week-old animals were divided into four groups: Oral vaccinated challenged (OV); Intramuscular vaccinated challenged (IM); Non-vaccinated non-challenged (NN); and Non-vaccinated challenged (NC). Twenty-one days after vaccination, pigs received gut homogenate via intragastric gavage containing 10^8 *L. intracellularis* organisms per pig. On days 0, 7, 21 and 28 post-infection, 6 pigs per group were humanely euthanized for sample collection. Sera and ileal lavages were collected to evaluate the systemic and local humoral immune response, respectively, using IPMA. Lymphocytes were purified from the peripheral blood (peripheral blood mononuclear cells; PBMC), ileum mucosa and ileocecal lymph nodes for ELISPOT testing. One animal (1/6) from the OV group showed the earliest detection of intestinal IgA secretion, at 7 days after challenge. Twenty-one days after challenge, all pigs showed *L. intracellularis*-specific IgA secretion in both vaccinated groups, with a higher titer for OV pigs. Systemic humoral IgG response was significantly higher in the IM group, where 79% seroconverted 21 days post-vaccination, while 4% seroconverted in the OV group. Pigs from both vaccinated groups showed no differences in number of interferon gamma (IFN-γ) producing lymphocytes from the PBMCs and intraepithelial lymphocytes, with no statistical differences among treatments. In summary, IM vaccinated pigs had stronger humoral systemic IgG responses than the OV group of pigs. An earlier local IgA response was detected in the OV group, with a greater number of IFN-γ producing T cells in ileocecal lymph nodes after vaccination and before the challenge.

General Introduction

Porcine proliferative enteropathy (PPE) is among the most important enteric diseases in swine production worldwide. The serological prevalence can reach 100% in growing-finishing stages of pig production. The non-hemorrhagic presentation, porcine intestinal adenomatosis (PIA), is more common among young pigs (40- to 80-lb). This form is characterized by pasty to watery diarrhea and reduction in the production parameters. Affected animals may recover spontaneously or develop chronic necrotic enteritis. The hemorrhagic clinical form, proliferative hemorrhagic enteropathy (PHE), mainly affects older animals. Historically, the negative effects of the disease have been mitigated through the use of antimicrobials and vaccination. However, the new trend is to reduce the use of antibiotics in the production of animals for human consumption, leaving vaccination and farm management as the only options to control bacterial diseases. There are two commercially available vaccines against *L. intracellularis*. The live attenuated oral vaccine (Enterisol® Ileitis, Boehringer Ingelheim) has been available for over a decade and the use of this vaccine has helped the swine industry to reduce the negative effects of the disease. More recently, a killed intramuscular vaccine (Porcilis™ Ileitis, Merck Animal Health) was commercially introduced in the market. However, there is lack of information about the impact of any form of vaccination on the transmission of PPE. Assessing the transmission rates among immunized populations is key to determine measures to control the disease. Moreover, there is a lack of information comparing the immune responses through the use of oral and intramuscular vaccines. The objective of this thesis is to determine the impact of vaccination on the epidemiology and immunity of vaccinated pigs that have been vaccinated with oral and intramuscular vaccines against *L. intracellularis*. More specifically, the goals are: 1) Evaluate differences in transmission patterns and spread of *L. intracellularis* in naïve and vaccinated pigs after contact with an infected animals,

and 2) characterize the local and systemic immune responses to *L. intracellularis* infection after oral and intramuscular injectable vaccination.

My thesis proposes a series of two studies to determine the transmission patterns and the immune responses mediated using commercial vaccines against *L. intracellularis*. The conclusions may help guide us to a better understanding of the epidemiology and immunology concepts around the use of vaccination to control PPE in swine production. Finally, this thesis also identifies opportunities for future research studies addressing epidemiology, control, and prevention of PPE in swine farms.

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List of abbreviations

PPE: Porcine proliferative enteropathy

PIA: Porcine intestinal adenomatosis

PHE: Proliferative hemorrhagic enteropathy

EPE: Equine proliferative enteropathy

L. intracellularis: Lawsonia intracellularis

IPMA: Immunoperoxidase monolayer assay

IHC: Immunohistochemistry

PCR: Polymerase chain reaction

ISH: *in situ* hybridization

ELISPOT: Enzyme-linked immune absorbent spot

CHAPTER 1: Literature Review

Porcine proliferative enteropathy (PPE), is a disease caused by *Lawsonia intracellularis* (*L. intracellularis*), a gram-negative, obligate intracellular bacterium, that is widely distributed worldwide among swine production systems (2). The most common macroscopic finding in affected animals is the thickening of the mucosa of the small intestine, especially the ileum, and sometimes the large intestine. This is caused by the cellular proliferation and significant reduction of goblet cells in the intestinal mucosa (3). Clinical syndromes of *L. intracellularis* infection can vary, however, there are two main clinical presentations. The first one is porcine intestinal adenomatosis (PIA), which mainly affects growing pigs under four months of age. PIA is usually chronic and often subclinical with nonspecific symptoms including diarrhea and reduced weight gain with consequent poor performance. The second one, occurring in naïve older pigs, is an acute syndrome known as proliferative hemorrhagic enteropathy (PHE), which can lead to sudden death (2). The common lesion in both syndromes is hyperplasia of intestinal epithelial cells, occurring anywhere from the jejunum through the colon, but most often in the ileum. Even though the term is misleading, PPE is commonly known as ileitis among producers and field veterinarians. The negative economic impact that the disease causes is mainly due to the reduction in daily weight gain, greater feed consumption, increased feed waste in affected herds as well as the increased cost in treatment after an outbreak.

Etiology:

The disease was first reported as porcine intestinal adenoma in 1931 (4), described as "degeneration of the epithelium, formation of adenomatous growths and transition from goblet cells to undifferentiated non-mucin containing cells in the ileum and colon". But it was not until 1993

when the bacterium was isolated in cell culture from infected pigs and the disease could be reproduced demonstrating that an intracellular organism is the causal agent of PPE (5) (6). The bacterium was described as an obligate intracellular, gram-negative curved rod. It was also established that the bacteria cannot be cultivated on conventional media and needed mitotic cells to be cultivated *in vitro* (7). In 1995, the bacteriological characteristics based on 16s rDNA sequence and protein electrophoresis were described as clearly different from other intracellular bacteria previously described (2). The bacterium was named in honor of Dr. Gordon Lawson, who was the first to associate intracellular bacteria with the lesion, as *Lawsonia intracellularis*. The laborious isolation requirements and the need for *in vitro* maintenance of *L. intracellularis* in cell culture have complicated and delayed the research on the bacteria. *L. intracellularis* is 1.25 to 1.75 μm in length, is 0.25 to 0.43 μm in width, and has a trilaminar outer envelope and unipolar flagellum that has been demonstrated in the extracellular stage (2). The bacteria also require mitotic cells and a microaerophilic environment (83.2% nitrogen, 8.8% carbon dioxide, and 8% oxygen) at 37°C to grow *in vitro*. As an alternative, a system employing a 10% hydrogen, 10% carbon dioxide, and 80% nitrogen gas tank with an evacuation-replacement system to create a microaerophilic atmosphere has been used as well (8). The peak of infection in cell culture monolayers occurs between 5 and7 days post-infection (9). Several mammalian cell lines support *L. intracellularis* growth *in vitro*. There are few *L. intracellularis* isolates reported worldwide, and among them, only two European isolates (10) and one U.S. isolate () have successfully reproduced clinical signs and lesions consistent with PPE. *L. intracellularis* infection has been reported in a variety of mammalian and non-mammalian species including hamsters (11), rabbits (12), rats (13), guinea pigs (14), ferrets (15), foxes (16), dogs (17), sheep (18), deer (19), emus (20), ostriches (21), horses (22) and non-human primates (23). However, to this date, it has not been shown that the bacterium has a zoonotic potential (24). In 2012, Vannucci and colleagues were able to achieve PPE infection

in pigs using the *L. intracellularis* isolate PHE/MN1-00 after up to 20 cell passages *in vitro*. Conversely, they demonstrated the lack of clinical signs, serological IgG response, macroscopic or histologic lesions in pigs using the isolate after 40 cell passages, suggesting the attenuation of virulence of *L. intracellularis* after serial *in vitro* passages (25). Host adaptation of the bacteria was demonstrated with an experiment where horses and pigs were challenged with their correspondent strain showing typical clinical signs, longer periods of bacterial shedding and stronger serologic immune responses (25) compared with non-species specific strains. Nevertheless, no genotypic differences in homologous isolates or strains at low or high passages were found based on 16S ribosomal DNA sequences (26).

Pathogenesis:

L. intracellularis has been shown to be inside of enterocytes as soon as 12 hours after oral challenge of pigs (27). The bacterium seems to persist in the intestinal crypts from day 5 to 28 postinfection (27) (28). The polar flagellum in extracellular bacteria is thought to play an important role in the early stages of pathogenesis to enable the bacteria to enter the crypts (29). *L. intracellularis* has been found close to the cell surface after 10 minutes and internalized after 3 hours post exposure of enterocytes *in vitro* (30). The bacterium divides by binary fission after escaping the vacuole from 2 to 6 days in cell culture and at 5 days after infection in hamsters while macroscopically changes were only observed from 10 to 12 days post infection (31).

Clinical presentation of lesions:

There are two common clinical manifestations of proliferative enteropathy in pigs: Porcine intestinal adenomatosis (PIA) is common in pigs younger than 20 weeks of age, and proliferative hemorrhagic enteropathy (PHE) is common in naïve young adults 4 to 12 weeks of age (2). PIA is a chronic disease characterized by poor and uneven weight gain sometimes with presence of watery diarrhea. Most affected pigs recover spontaneously, but a significant number develop chronic necrotic enteritis with progressive emaciation that decreases average daily gain and increases feed conversion rates (32). Conversely, PHE is the acute presentation characterized by bloody diarrhea due to the extravasation of blood from the intestinal mucosa (especially ileum), anemia, anorexia and mortality that usually affects naïve older pigs (33). Macroscopic lesions are commonly observed in and generally limited to the ileum. However, lesions can be also be identified in the distal jejunum and proximal colon (5). In PIA, the characteristic gross lesion is thickening of the mucosa with an irregular nodular or folded appearance. In PHE, the intestine is dilated with edema and mucosal proliferation. The lumen of the intestine usually contains one or more formed blood clots and fibrin necrotic debris (34).

Epidemiology:

PPE is endemic in swine populations worldwide (35). Prevalence studies performed in Europe demonstrated that 99% of pigs on fattening farms are seropositive for the etiologic agent (36). Evaluations of gross lesions from slaughter plant have shown that between 5 and 20% of the pigs are affected with PPE (37). However, these evaluations may be underestimating the incidence of PPE because gross lesions usually resolve before slaughter age (38). *L. intracellularis* infection

has been described in other domestic and wild species. Nevertheless, the role of other species as a source of *L. intracellularis* in domestic pigs is not clear (39). Genomic analysis has confirmed a difference in genotypes of *L. intracellularis* isolated from pigs and equine species (40). This suggests that the bacterium may have adapted to different hosts (25). Transmission among pigs has been demonstrated when mixing animals or by the introduction of non-immune animals to infected herds (34). Clinical disease may vary according to different farm managements, pig flows, individual immunity and use of antimicrobials. In continuous flows, *L. intracellularis* infection occurs after weaning which is thought to be due to the reduction of maternal immunity. The main transmission route of *L. intracellularis* is the oral route, with the ingestion of contaminated feces (41), or material contaminated by feces. Pigs that are recovered from infection do not shed detectable *L. intracellularis* to transmit the infection to other pigs. *L intracellularis* can be viable in feces up to 2 weeks at 5-15 degrees Celsius (41). Fomites contaminated with feces or insects and rodents can be routes of transmission as well (42). Therefore, rodent control in farms is important to reduce the transmission of *L. intracellularis*. Sows may also be a source of infection for suckling piglets (43). However, piglets often have detectable levels of antibodies during the first six weeks of age and this suggests that piglets may be protected against *L. intracellularis* infection (44). Therefore, the protection of the piglets against *L. intracellularis* infection may depend on the immunity of the dams. Even though there is basic knowledge about transmission of *L. intracellularis*, there is a lack of information on transmission of the bacterium comparing vaccinated and non-vaccinated populations.

Diagnosis:

PPE is often subclinical in presentation, so diagnosis based on clinical signs is difficult. However, there are many laboratory techniques to confirm a clinical diagnosis at the field level. The most common samples to confirm a diagnosis are feces, ileum mucosa and portions of the ileum for PCR and serum for serology (45, 48). Given that bacteria isolation by culture is difficult and limited to only some laboratories in the world, antemortem diagnosis is commonly performed by PCR and serology. Immunohistochemistry (IHC) of intestinal tissues remains the best method to confirm a diagnosis of PPE (51).

Fecal samples and intestinal mucosal are generally used to diagnose *L. intracellularis* by PCR. However, many inhibitory factors in feces may interfere with PCR amplification reactions (45). The pooling of fecal samples from two to three animals is one way to reduce PCR costs but there is an inherent reduction in the sensitivity of the test (46). The sensitivity of PCR is high and many PCR assays can detect a low number of organisms per gram of feces (47). Therefore, the interpretation of results by PCR should be taken with caution, given that positivity does not imply economic implications due to the pathogen.

Detection of *L. intracellularis*-specific IgG in serum is also a way to diagnose *L. intracellularis* infection that provides evidence of historical exposure to the bacteria. The sensitivity of the test is approximately 90% and the specificity is approximately 100% (48). To develop IPMA, the anti-species secondary antibody is labeled with peroxidase, which leads to brown staining by a substrate that can be evaluated by a skilled technician using a light microscope. Even though serology tests such as IPMA are convenient in terms of cost and number of samples, the disadvantage of these

tests, as with any other serology test, is that detectable antibodies are not generally seen until 2 weeks after the infection (49). Further, the test is not commercially available worldwide.

The identification of *L. intracellularis* is possible with the use of *L. intracellularis*-specific monoclonal or polyclonal antibodies in immunohistochemical staining of formalin-fixed, paraffin embedded tissue sections (50). In addition, *in situ* hybridization (ISH) can be done with the use of specific probes. The important advantage of IHC and ISH is the ability to demonstrate the relationship between the development of microscopic lesions and the presence of bacteria within these lesions. The specificity of IHC for *L. intracellularis* identification is 100% and sensitivity 90.9%, and are comparable to the ISH specificity and sensitivity (51).

Prevention and control:

In vitro studies have demonstrated that *L. intracellularis* isolates are sensitive to numerous disinfectants. Cleaning and disinfection of pens, facilities, and fomites are important to prevent transmission between pig groups (52). Quaternary ammonium compounds inactivated the bacteria after contact for 10 minutes at 400 ppm of concentration in water. The same results were obtained when quaternary ammonium compounds were combined with aldehydes or using oxidizing agents, biguanide, phenol, and iodine solutions (52). A control program for rodents and insects is also important for the effective control of *L. intracellularis*. Prevention and control of *L. intracellularis* are commonly done by use of subtherapeutic dosage of antimicrobials in feed or water. *In vitro* studies by use of cell culture demonstrated that penicillin, erythromycin, difloxacin, virginiamycin, and chlortetracycline are the most effective compounds. Tiamulin and tilmicosin were the next most effective and lincomycin and tylosin were relatively inactive against *L. intracellularis* (53).

On the other hand, acute or hemorrhagic presentations of the disease should be treated aggressively by medicating the clinically affected pigs using tiamulin (120 ppm), tylosin (100 ppm), or tylvalosin (100 ppm) for 14 days (53) . Other chemical treatments have also been used, such as the use of copper, zinc or feed acidifiers, but are not effective for *L. intracellularis* control (8). Natural infection confers solid immunity against *L. intracellularis* (41). Use of vaccination is another method to prevent infection. However, vaccine immunity is not always capable of totally blocking infection and shedding (54). A live, attenuated *L. intracellularis* vaccine is commercially available. Live, attenuated vaccines are administered orally, and an antibiotic-free window of three days is required prior to and after vaccine administration. Most recently, an inactivated bacterin-based vaccine has been marketed, which is administrated individually by the parenteral route (33). Serologic studies have shown that a period of three to four weeks is necessary to create protective immunity after vaccination with live attenuated vaccines (55).

Immunity:

The difficulties regarding the growth of *L. intracellularis* have delayed research and understanding of the immune responses following a natural or experimental infection. Most of the research has focused on the humoral immune responses (IgG) following a natural infection or vaccination against *L. intracellularis*. In general IgG titers from experimentally infected animals challenged either with intestinal homogenate or *L. intracellularis* pure culture, start at 2 weeks and peak at 3 weeks post inoculation and can last up to 2 to3 months (10). However, given that *L. intracellularis* is an enteric organism, specific secretory IgA response in the intestine is thought to play a more important role in preventing infection since these antibodies bind the bacterial surface in the intestinal lumen. Thus, IgA may prevent the attachment of the bacteria to the mucosal cells (56).

Previous studies have demonstrated that *L. intracellularis*-specific mucosal IgA can be detected 3 weeks after challenge (8).

Immune responses induced by *L. intracellularis* are not fully elucidated. The absence of signs of inflammatory reaction throughout early *L. intracellularis* colonization (until 11 days post infection) suggests that both the innate and the adaptive immune responses are mild or delayed. This could help the bacteria to survive commensal competition and evade cytokines (57) (58). Limited infiltration of CD8+, CD25+, and B cells have been reported in the lamina propria of the ileum, jejunum and colon of pigs affected by PHE or PIA (58). Cytokine serum profiles after *L. intracellularis* infection in pigs showed an increase in IL-10 from 5 to 10 days post infection and increased TGFβ from 0 to 5 days post infection as well as an increase in TNF-α, IFNγ, and IL-6 titers from 5 to 40 days post infection (59). Down-regulation of the gene coding for signaling lymphocytic activation molecule (SLAM7; also known as CD2) was demonstrated, which is required for T-cell activation as well as the up-regulation of the genes encoding MHC-I in infected enterocytes (60).

Concluding remarks:

Lawsonia intracellularis is a fastidious bacterium that causes porcine proliferative enteropathy (PPE) in pigs. This disease is characterized by soft, watery, pasty or bloody diarrhea according to its presentation. Conventional production practices to control PPE have included the use of therapeutic or sub-therapeutic antibiotic doses along with biosecurity measures. However, recent regulations that limit the use of antibiotics have resulted in an increased prevalence and occurrence of PPE with the consequent impact on the economy of swine production. Vaccination has emerged

as an alternative to successfully prevent and control PPE in swine farms. Production of immunity through vaccination aims to reduce the clinical manifestation of *L. intracellularis* but there is a lack of information about the impact of vaccination on transmission and the production of local and systemic immunity.

Assessing transmission of *L. intracellularis* in vaccinated populations would allow the identification of measures to control the infection. It would also help to establish biosecurity procedures in swine production such as gilt acclimation that could reduce PPE incidence. Furthermore, understanding the differences in the immune responses that are produced by the different commercial vaccines will enable the producers and veterinarians to select the best alternative for the control of this important pathogen in a new non-antibiotic scenario.

Chapter 2: Impact of Vaccination on Transmission of *Lawsonia intracellularis* in Pigs

This chapter is in preparation to be submitted to a peer reviewed journal.

Introduction:

Lawsonia intracellularis is a gram-negative, flagellated and non-spore-forming obligate intracellular bacterium that colonizes intestinal mucosa cells of equines (61), rodents (13), dogs (17), pigs (2) and other species. In pigs, *L. intracellularis* causes porcine proliferative enteropathy (PPE), characterized by clinical or subclinical infections with the presence of different grades of diarrhea that impacts growth performance and mortality (62). Even though PPE is a highly prevalent enteric disease, little is known about the epidemiology and transmission of the disease (63).

Pigs shed *L. intracellularis* through feces, and fecal shedding in pigs as young as three weeks of age can occur (63). Infection commonly occurs a few weeks after weaning on endemic farms. The most important routes for PPE transmission for pigs include the fecal-oral route and direct contact with contaminated fomites (39). Previous reports indicate lateral transmission as a route of transmission, but not all pigs within a population are necessarily infected simultaneously (52). Previous studies also show that fecal shedding can happen up to 12 weeks after a *L. intracellularis* challenge (10). Production practices, farm design, use of antibiotics and different levels of immunity within populations modify the transmission rates and dynamics of infection with *L. intracellularis*. Transmission rates and the probability of infection in naïve and vaccinated animals after exposure to such pathogens as swine influenza and *Mycoplasma hyopneumoniae* are better understood (64) (65), while the transmission rates for *L. intracellularis* infection have not been previously assessed.

The common strategies to control PPE in the United States and worldwide are antibiotics in sub-therapeutic doses and the use of commercially available vaccines. Recent sporadic outbreaks of PPE have occurred, and these outbreaks include acute (hemorrhagic) and chronic forms, the latter

often presented subclinically. The regulations on the use of antimicrobials as growth promoters have directly impacted the use of antibiotics as a prevention measure (66). As a result of increased regulations on antibiotic use, the efficacy of vaccination to control PPE should be studied more intensively. Vaccination studies have focused on the evaluation of immune response or reduction of clinical disease as critical parameters for assessment of vaccine efficacy at the individual level (67); however, these studies have not addressed the impact of vaccination on transmission at the population level. Understanding vaccination effect on transmission at the population level is needed in order to develop options for PPE control in integrated swine systems. The objective of this study was to evaluate differences in transmission patterns and spread of *L. intracellularis* in naïve and vaccinated pigs after contact with an infectious animal.

Materials and Methods

Ethics statement

Protocols and procedures followed during the study were approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC protocol 1712-35393A), and the Institutional Biosafety Committee (IBC).

Animal allocation

Ninety crossbred, 21-day-old pigs were obtained from a source known to be *L. intracellularis* negative. Negative status for *L. intracellularis* was confirmed by serology using the immunoperoxidase monolayer assay (IPMA) and fecal shedding using a quantitative polymerase

chain reaction (qPCR) assay. The sows were non-vaccinated in the source farm for *L. intracellularis* prior to starting the study.

At entry, each pig was individually weighed and identified by an ear tag. Pigs were ranked by weight and gender and randomly allocated into three different pens. Three Swiffer samples were collected from each room, pooled by room and tested for *L. intracellularis* using qPCR. Twenty-seven pigs were allocated into each of three different treatments (treatments 1, 2 and 3) and segregated into three pens (three treatments, three pens per treatment, nine pigs per pen). Nine seeder pigs were housed in a different barn. Pigs in treatment 1 were orally vaccinated with Enterisol® Ileitis (Boehringer Ingelheim). Pigs in treatment 2 received 2 mL of Porcilis™ Ileitis (Merck Animal Health) intramuscularly in the right side of the neck and pigs in treatment 3 remained non-vaccinated. Pigs were fed a commercial diet that was formulated by a nutritionist and was deemed appropriate for the age and weight of the pigs. Feed samples were taken directly from the manufacturer and from the feeders and sent for analysis on the day of vaccination to confirm that antibiotic residues were not detected in the feed used. Feeder weigh backs were done at the time of all pen weights to determine the amount of feed present at weighing.

Challenge material preparation and seeders inoculation

Pigs clinically affected with PE were necropsied and affected sections of ileum mucosa were scraped and stored at -80 ºC until use. Selected material was mixed with a solution of 10% Fetal Bovine Serum – Sucrose Phosphate Glutamate (FBS-SPG) and mixed until it reached a concentration of about $1x10^8$ *L. intracellularis* organisms per ml. One ml of the solution was then diluted into 40 ml of sterile carrier buffer. On study day 14, nine seeder pigs were inoculated by

gastric lavage with the 40 ml of mucosal homogenate resulting in about 1 x10^8 *L. intracellularis* organisms per pig. The nine seeder pigs were comingled with the non-vaccinated and vaccinated groups for seven days after inoculation. The experimental design is summarized in Figure 1.

Euthanasia

All pigs were humanely euthanized on day 49 (21 days after contact with seeders) with a lethal injection, consisting of an overdose of Fatal-Plus (Fatal-Plus® Vortech Pharmaceuticals, Dearborn, MI), a barbiturate solution containing 390 mg pentobarbital per mL, which was dosed and administered to the pigs as 1 mL per 10 pounds body weight.

Data recording and sample collection

Animal weight and feed intake were collected to calculate the average daily gain (ADG) and feed conversion rate (FCR). Data collection took place at entry, before vaccination, 21 days post vaccination, before challenge, and at necropsy. Clinical diarrhea was scored once a week, following the procedures described by Pedersen et al (68). The serological response was monitored prior to vaccination and every 2 weeks until necropsy using the immuno peroxidase monolayer assay (IPMA) technique (69). *L. intracellularis* shedding was monitored by collecting 2 grams of feces directly from the rectum by digital palpation from each animal. The feces were tested by qPCR using a commercial kit (LSI VetMAX™ *Lawsonia intracellularis*, Thermo Fisher Scientific) at arrival, prior to vaccination, every other day after vaccination until challenge, daily for 7 days post challenge (seeder pigs) and 1 time per week after the first 7 days until necropsy. After necropsy, intestinal tissues (ileum and cecum) were evaluated by immunohistochemistry, using the

following categories: 0 = No infected crypts, 1 = 1-25% of crypts infected, 2 = 26-50% of crypts infected, 3 = 51-75% of crypts infected, 4 = 76-100% of crypts infected (8). Values determined by microscopic evaluation represented an estimate of the percent of colonized crypts in a section of intestine.

Laboratory testing and procedures

Immunoperoxidase monolayer assay (IPMA)

All of the collected sera were tested for antibodies against *L. intracellularis* using the IPMA (69). An acetone-methanol-fixed monolayer of McCoy cells highly infected with *L. intracellularis* were rehydrated with a PBS solution for 10 minutes at 37ºC in 96-well plates (Nunc, Rochester, NY). Sera samples were diluted in PBS with 5% skim milk serially in 2-fold dilutions starting at 1:30. Fifty microliters of the diluted samples were added to the test wells and incubated at 37ºC for 45 minutes. After incubation, the plate was washed three times with PBS. Fifty microliters of Goat anti-porcine IgG-peroxidase conjugate was diluted to 1:4000 in Phosphate Buffered Saline with Tween (PBST) and added to the 96-well plates. The plates were incubated for 45 minutes at 37ºC, and then washed three times with PBS. After washing, 100 microliters of chromogen (3-amino-9-ethyl-carbazole) was added to each well and the plate was incubated at room temperature for 20 minutes. The wells were washed three times with PBS and allowed to dry. The 96-well plates were analyzed with an inverted microscope. The data was recorded as endpoint titers for *L. intracellularis* IgG, with a cutoff titer of 1:30 dilution as positive.

Quantitative PCR analysis

qPCR. The purified DNA was analyzed by qPCR for presence of *L. intracellularis* aspA gene copies (70). For each target gene, two primers and an internal, fluorescent-labeled TaqMan probe [5′end, reporter dye FAM (6-carboxyflourescein), 3′end, quencher dye TAMRA (6-carboxytetramethylrhodamine)] was designed using Primer Express software (Applied Biosystems, Foster City, California, USA). TaqMan PCR systems were validated using 2-fold dilutions of genomic DNA (gDNA), testing positive for the target genes. Dilutions were analyzed in triplicate and a standard curve was plotted against the dilutions. The slope of the standard curve was used to calculate amplification efficiencies using the formula E = 10 1/−s minus;1 (71).

Statistical analysis

Transmission patterns were assessed according to the susceptible–infectious (SI) model, without possibility of recovery during the study period. Pigs were considered infectious when fecal PCR was equal or more than 10^3 *L. intracellularis/*g of feces (72). Transmission rate was estimated from the data using a GLM model (binomial distribution with cloglog link function), experimental group and block as predictors, the proportion of infectious at each sampling point as offset, and with standard errors adjusted for clustering at the pen level. The duration of shedding ≥10^3 *L. intracelullaris*/g of feces was analyzed with a parametric survival regression model (Weibull regression) (73) with standard errors adjusted for clustering at the pen level. The probability of shedding ≥1 x 10^3 *L. intracelullaris*/g of feces over time was analyzed using a logistic regression model. The predictors for the logistic regression model were the experimental group, time, block, and the interaction between the experimental group and time. The estimation was carried out using

generalized estimating equations (GEE) with an independent working correlation and robust standard errors. All analyses were done in Stata 15.1 and a significant level of 0.05 was established *a priori*.

Scores for diarrhea, macroscopic lesions, histopathology, and immunohistochemistry were compared among groups using Kruskal-Wallis equality of populations rank test.

Results

Serology

All pigs were negative for *L. intracellularis*-specific antibodies at the beginning of the study. On day 28 post vaccination, the intramuscular vaccine induced *L. intracellularis*-specific antibodies in 62% (17/27) of pigs. Pigs from the intramuscular vaccine treatment showed significantly higher levels of serum IgG than the other two treatments ($P<0.01$), with a mean reciprocal titer between 1:30 to 1:480 and the dilution mean *L. intracellularis* IPMA titer was 91.15 ±115.56. The oral vaccine induced *L. intracellularis*-specific antibodies in 33% (1/27) of the pigs, with a mean reciprocal titer of 1:30. In the non-vaccinated group, all pigs remained negative for *L. intracellularis* before challenge. At necropsy (28 days post-contact with seeder pigs), the intramuscular vaccinated group showed a robust amount of *L. intracellularis* antibodies, with 100% (27/27) of the pigs demonstrating seroconversion. The intramuscular vaccine produced a significantly higher level of antibodies than the other two treatments ($P<0.01$) with a mean reciprocal titer between 1:30 to 1:7680 and the mean *L. intracellularis* IPMA titer was 931.11±1803.46. At necropsy, the orally vaccinated group showed *L. intracellularis* specific serological IgG in 50% (13/26) of the pigs, with a mean reciprocal titer between 1:30 to 1:480 and

the mean *L. intracellularis* IPMA titer was 88.84±123. The nonvaccinated group showed *L. intracellularis* specific antibodies in 25% (7/27) of the pigs, with a mean reciprocal titer between 1:30 to 1:480 and the mean *L. intracellularis* IPMA titer was 52.22±115.

Clinical signs

During the study, the abdominal appearance and demeanor of the pigs remained normal (score 0) for all three treatment groups. One animal from the oral vaccinated group died at week 2 of the transmission assessment period. On the second week of contact, 22% (6/27) of the intramuscular vaccinated pigs, 11% (3/27) of the orally and non-vaccinated pigs showed fecal scores between 1 and 2, with no statistical difference among the groups (Table 1). On the third week of contact, 40% (11/27) of the intramuscularly vaccinated, 34% (9/26) of the orally vaccinated, and 29% (8/27) of the non-vaccinated pigs had fecal scores between 1 and 4, with no statistical difference among the groups. In the last week of contact, 55% (15/27) of the intramuscularly vaccinated pigs, 26% (7/26) of the orally vaccinated, and 40% (11/27) of the non-vaccinated pigs had fecal scores between 1 and 4, with no statistical difference among the groups. On week 4 of contact, 37% (10/27) of the intramuscularly vaccinated, 11% (3/26) of the orally vaccinated, and 25% (7/27) of the non-vaccinated pigs had fecal scores between 1 and 4, with no statistical difference among the groups.

Immunohistochemistry (IHC)

At necropsy (28 days post-contact with seeder pigs), 33% (9/27) of the intramuscularly vaccinated animals were IHC positive, while 66% (18/26) of the orally vaccinated and non-vaccinated animals

had IHC positive results. There was a statistical difference between the intramuscularly vaccinated group and the other two treatment groups (P=0.012), as shown in Table 2.

Average daily gain (ADG) and feed conversion efficacy (FCE)

The mean average daily gain was 0.61Kg in the nonvaccinated animals, while in the orally and intramuscular vaccinated pigs the mean ADG was 0.57Kg. The data showed a statistical difference between the intramuscular and non-vaccinated treatments (P=0.029) and no statistical difference between the two vaccinated treatments. The mean feed conversion efficiency in the intramuscularly vaccinated group was 2.33±0.39, in the orally vaccinated group it was 2.36±0.31, and in the non-vaccinated group it was 2.27±0.21; no statistical difference among the groups was found.

qPCR

Only week 3 of the transmission assessment period demonstrated a statistical difference among the treatment groups for quantitative *L. intracellularis* shedding determined by qPCR. The average number of bacteria in the IM, OV, and the non-vaccinated groups were table 3

, respectively. Comparing the orally vaccinated group and two other treatment groups, a statistically significant difference was found (P<0.01), while there was no statistical difference among the intramuscularly vaccinated and the non-vaccinated treatment groups. When analyzing weeks 1, 2 and 4, there was no statistical difference among the various treatments.

Transmission model

The transmission rate was 2.6 new cases/pig-week (95% CI: 2.35 new cases/pig-week, 2.85 new cases/pig-week) for non-vaccinated animals. The transmission rate was significantly reduced in both vaccinated groups when in contact with infectious (seeder) pigs ($p<0.05$). The transmission rate for the orally and intramuscularly vaccinated groups was 1.86 new cases/pig-week (95% CI: 1.3 new cases/pig-week, 2.42 new cases/pig-week) and 1.75 new cases/pig-week (95% CI: 1.31 new cases/pig-week, 2.2 new cases/pig-week), respectively.

Pigs that received the oral vaccine shed *L. intracelullaris* for a median time that was 43.3% shorter (95% CI: 81.8% shorter, 77.2% longer; P=0.329) than that of the pigs that received no vaccine. Pigs with the intramuscular vaccine shed *L. intracelullaris* for a median time that was 25.5% shorter (95% CI: 81.7% shorter, 204% longer; P=0.682) than that of the pigs that received no vaccine.

Using a GEE logistic regression model, the analysis reported a significant simple effect of vaccine ($P<0.0001$) and of time (P value=0.0001), as well as a significant interaction between vaccine and time (P=0.0044) was observed. The adjusted predicted probability of shedding $\geq 10^3$ *L. intracellularis*/g of feces was higher in pigs receiving the oral vaccine compared with those receiving the intramuscular vaccine and those receiving no vaccine at the beginning of the commingling. The probability of shedding increased with time for all of the groups, but the increment was significantly higher in pigs that did not receive any vaccine (Figure 2).

Discussion

In field studies, vaccination against *L. intracellularis* has demonstrated enhanced daily weight gain and reduction of antibiotic use compared to non-vaccinated pigs (74). Therefore, *L. intracellularis* vaccination has become a common practice in swine production to prevent PPE and its impact.

However, there was a lack of information around the impact of vaccination on length of shedding and transmission patterns in vaccinated and non-vaccinated populations. The present study showed adequate efficacy of both vaccination protocols, oral and intramuscular, by reducing the length of *L. intracellularis* shedding after natural exposure through the contact with infectious animals. Both vaccinated groups were able to significantly decrease the transmission rate of *L. intracellularis* when unvaccinated shedder pigs were introduced into a vaccinated population.

Intramuscular vaccination against *L intracellularis* has been shown to induce significant serological IgG titers compared to non-vaccinated pigs (75). This finding was also observed in the present study. Additionally, we demonstrated a significant serological response from the intramuscular killed vaccination compared with the live modified oral vaccination. The differences of serological IgG titters between vaccinated groups did not reflect significant differences in the transmission rates between the vaccination groups. However, the intramuscular vaccination resulted in the higher proportion of negative animals (19/27) by IHC compared with the oral vaccination (8/26). Experimental studies to determine disease transmission after vaccination for other diseases in pigs, such as influenza (64), and*Mycoplasma hyopneumoniae* (76), have shown an association between immunity generated by vaccination and reduction of transmission rates.

No statistical differences were observed for the presence of clinical signs, feed conversion and average daily gain between both vaccinated and control groups. However, other studies have shown a reduction in the incidence of microscopic lesions of PPE in vaccinated groups compared to controls (75). More studies are necessary to assess the impact of vaccination against *L. intracellularis* in production parameters under field conditions.

From a field perspective, these results highlight the importance of implementing vaccination programs at the system level rather than site-specific interventions. Vaccination strategies against

L. intracellularis decrease the risk of commingling batches of pigs from vaccinated and non-vaccinated sources and therefore may reduce the impact of PPE in downstream flows. Under the conditions on this study, there was a significant reduction in transmission rate and length of *L. intracellularis* shedding after contact with infectious animals when comparing vaccinated with non-vaccinated groups. However, differences were not observed when comparing transmission rates of orally and intramuscularly vaccinated pigs. Therefore, we encourage the use of vaccination in general to reduce transmission and clinical disease related to *Lawsonia intracellularis*.

Chapter 3: Characterization of local and systemic immune response to *Lawsonia intracellularis* infection after oral and injectable vaccination

Introduction

Porcine proliferative enteritis (PPE), caused by the Gram-negative, intracellular bacterium *Lawsonia intracellularis* (*L. intracellularis*), negatively affects the swine industry worldwide (2). Antibiotics in subtherapeutic doses and vaccination have been the prophylactic measures that the producers and veterinarians have used to prevent the disease (3). For over a decade, oral vaccination (Enterisol® Ileitis, Boehringer Ingelheim) has significantly reduced the economic impact of PPE in the swine industry (77). However, specific immune response mechanisms induced through the oral vaccination have not been fully characterized. In 2016, an injectable killed vaccine (Porcilis™ Ileitis, Merck Animal Health) became commercially available in the United States. A preliminary study conducted by the manufacturer showed specific serological IgG response in sera after vaccination (75) but cell-mediated or local humoral immune responses have not been assessed or compared to the oral vaccination.

Cell-mediated immune response (CMI) is considered the most important mechanism of immunity induced by intracellular pathogen infection (78). CMI response results in production of interferon gamma (IFN-γ), a pro-inflammatory cytokine produced after an infection by immune cells (79) and which activates other immune cells such as macrophages, T-helper 1 (Th1), monocytes as well as up-regulates antigen presentation molecules (80). Characterizing the IFN-γ response as a result of *L. intracellularis* challenge can help to better understand the pathogenesis of the disease. The enzyme-linked immunosorbent spot (ELISPOT) assay measures antigen-specific T cells (81), and it detects the secretion of IFN-γ produced by activated T-lymphocytes used to evaluate the T-helper 1 (Th 1) response on the single-cell level (80). The limited inflammatory response associated with *L. intracellularis* infection after exposure, suggest that both the innate and the adaptive immune responses are mild or delayed which can help the bacteria to survive commensal

competition and evade cytokines (57) (58).

Recently, an inactivated intramuscular bacterin against *L. intracellularis* became commercially available (Porcilis® Ileitis, Merck Animal Health, Medison, NJ, USA), consisting of whole-cell *L. intracelullaris* in mineral oil and alpha-tocopherol as coadjutant (75). The vaccine does not require the antibiotic-free window. The manufacturer recommends vaccinating pigs older than 21 days. The intramuscular vaccine has been shown to induce a robust IgG response in serum as well as reduce *L. intracellularis* shedding up to 15-fold, while lowering the microscopic lesions, clinical signs, and bacterial burden (75). Our hypothesis is that the oral immunization using a modified-live vaccine (Enterisol® Ileitis) induces a more robust and stronger gut-associated humoral and cellular immune response against *L. intracellularis* infection. The aim of the present study was to comprehensively investigate and compare the local and systemic immune response to *L. intracellularis* infection after oral and injectable vaccination, followed by experimental infection. The objective of this study is to understand the magnitude of the local and systemic immune responses induced by oral (Enterisol®) and injectable (Porcilis™) vaccines that are commercially available.

Materials and Methods

Experimental design

A total of 90 healthy crossbred 3-week-old pigs were obtained from a known *L. intracellularis* negative source. Sows were non-vaccinated for PPE (no prior use of Enterisol Ileitis or Porcilis Ileitis). On study day -28, each pig was individually weighed and identified by ear tag at entry into the research barn. Animals were then ranked by weight, gender, and randomly assigned treatment

in a systematic permutation, ensuring balance for starting weight. There were 4 treatments: 1) Oral vaccinated (OV) n=24. 2) Intramuscular vaccinated (IV) n=24. 3) Non-vaccinated non-challenged (NN) n= 24. 4) Non-vaccinated challenged (NC) n=18. Twenty-four pigs from the OV and IV treatments were allocated and divided into threepens (8 pigs/pen). Twenty-four NN pigs were housed in a separate pen. The 18 NC pigs were allocated in a different barn. On study day -21, after 7 days of acclimation, the 24 pigs from the OV treatment group were orally drenched with one ml of life modified vaccine (Enterisol® Ileitis, Boehringer Ingelheim). The 24 pigs from the IV treatment received two mL on the right side of the neck (Porcilis™ Ileitis, Merck Animal Health). The remaining pigs were not vaccinated. Solid partitions were placed between vaccinated animal pens and other pens to avoid potential shedding of the live oral vaccine. On study day 0 (21 days after vaccination), six pigs from the treatment groups OV, IV and NN were humanely euthanized for sample collection. There was not sampling from the NC group at this time. Pigs from the OV, IV and NC treatments were orally challenged via intragastric gavage using an intestinal homogenate containing a 10^8 dose of *L. intracellularis* homogenate diluted into 40 ml of sterile SPG (Sodium-phosphate glutamate) buffer. Pigs from the NN treatment remained non-challenged. Pigs were fed a commercial diet that was formulated by a nutritionist and deemed appropriate for the age and weight of the pigs. On study days 7-, 21- and 28-days post infection, six pigs per group were humanely euthanized for sample collection (Figure 3).

Experimental infection and monitoring

On study day 0 (21 days after vaccination) the pigs from the treatments IV, OV and NC were challenged. The challenge material from gut homogenate was screened for known swine pathogens such as PCV2, *Salmonella, Brachyspira, E. coli*, PRRSV, PED, TGE, Rotavirus and Deltacoronavirus. For challenge, pigs were restrained briefly by hand, a speculum was placed

between their incisors, and a size 18-French red rubber feeding tube was passed into the esophagus. The inocula was administered using a 60 mL catheter tip syringe. *L. intracellularis* homogenate inocula was prepared by Dr. Dana Beckler at the Gut Bugs, Inc. laboratory. Animals were fed a commercial feed that met the recommended nutritional requirements. Water was provided at libitum throughout the study. No antibiotics were offered to the pigs through feed before or after vaccination or challenge.

The general health of the pigs was observed and recorded daily. Pigs were especially monitored after vaccination and challenge for any side effects. Clinical diarrhea was monitored based on a scoring system as follows: Diarrhea score: 0 (none); 1(Soft cow-pie consistency); 2 (Moderately loose, falls through the slats); 3(Watery, possibly blood-tinged, possible necrotic debris). Body weights were taken on study days -28, 7, 21 and 28.

The study was blinded to laboratory scientists performing the sampling, laboratory testing, personal performing as animal caretakers and veterinarians performing gross and histological evaluations.

Laboratory testing and procedures

IgG antibodies

Sera were collected on study days -21, 0, 7, 21 and 28. All sera were tested for seroconversion against *L. intracellularis* using the immunoperoxidase monolayer assay IPMA (69). Briefly, 96-well plates (Nunc, Rochester, NY) containing an acetone-methanol-fixed monolayer of McCoy cells highly infected with *L. intracellularis* were rehydrated with a PBS solution for 10 minutes at 37°C. Sera samples were diluted in PBS with 5% skim milk in serial two-fold dilutions starting at 1:30. Fifty microliters of the diluted sample was added to the test wells and incubated at 37°C for 45 minutes. After incubation, the plate was washed 3 times with PBS. Fifty microliters of goat

anti-porcine IgG-peroxidase conjugate diluted 1:4000 in PBST were added to the wells and incubated for 45 minutes at 37°C. The wells were washed three times with PBS. After washing, 100 ul of chromogen (3-amino-9-ethyl-carbazole) were added to the wells and incubated at room temperature for 20 minutes. The wells were washed three times with PBS and allowed to dry. After they were dried, the wells were analyzed with an inverted microscope. Results were provided as endpoint titers for *L. intracellularis* IgG, with a cutoff value of 1:30 dilution as positive.

IgA antibodies

Intestinal lavages were collected on study days 0, 7, 21 and 28. All lavages were tested for presence of anti-*L. intracellularis* IgA using the immunoperoxidase monolayer assay IPMA (69). Briefly, 96-well plates (Nunc, Rochester, NY) containing an acetone-methanol-fixed monolayer of McCoy cells highly infected with *L. intracellularis* were rehydrated with a PBS/5% skim milk solution for 10 minutes at 37°C to block non-specific reactions. Intestinal lavages were diluted in PBS with 5% skim milk in serial two-fold dilutions starting at 1:8. Lavages were centrifuged after collection for 7 minutes at 1000 revolutions per minute, and the resultant supernatant was collected. Fifty microliters of the diluted sample was added to the test wells and incubated at 37°C for 30 minutes. After incubation, the plate was washed five times with PBS containing 0.05 % tween 20 (PBST). Fifty ul of goat anti-porcine IgA-peroxidase conjugate, diluted 1:1000 in PBST, were added to the wells and incubated for 45 minutes at 37°C. The wells were washed five times with PBST. After washing, 100 ul of chromogen (3-amino-9-ethyl-carbazole) were added to the wells and incubated at room temperature for 20 minutes. The wells were washed three times with PBS and allowed to dry. After they were dried, the wells were analyzed with an inverted microscope. Results were provided as endpoint titers for *L. intracellularis* IgA, with a cutoff value of 1:8 dilution as positive.

Immunohistochemistry (IHC) for heavily infected cells (HIC)

Uniform sections of the ileum, 1 inch proximal to the ileo-cecal junction, were excised and fixed in enough quantity of 10% buffered formalin to supply a 10:1 formalin to tissue ratio on study days 0, 7, 21 and 28. All ileum samples were evaluated for percent of heavily infected cells (HIC) by blinded personal. Results were provided on a numerical point system. Values represent an estimate of the percent of HIC in a section.

The level of infection was assessed by IHC based on the amount of positive labeled antigen present in the intestinal sections under this scoring system: score 0 = Negative, score 1 = 1- 25%, score 2 = 26-50%, score 3 = 51-75%, score 4 = 76-100%. IHC procedures were conducted in the Immunohistochemistry Section of the Veterinary Diagnostic Laboratory of the University of Minnesota

Fecal shedding:

Fecal samples were collected on study days -21, 0, 7, 21 and 28 to be evaluated by qPCR. Briefly, the purified DNA was analyzed by qPCR for presence of *L. intracellularis* aspA gene copies (71). For each target gene, two primers and an internal, fluorescent-labeled TaqMan probe [5′end, reporter dye FAM (6-carboxyflourescein), 3′end, quencher dye TAMRA (6-carboxytetramethylrhodamine)] was designed using Primer Express software (Applied Biosystems, Foster City, California, USA). TaqMan PCR systems were validated using two-fold dilutions of genomic DNA (gDNA), testing positive for the target genes. Dilutions were analyzed in triplicate and a standard curve was plotted against the dilutions. The slope of the standard curve was used to calculate amplification efficiencies using the formula E = 10 1/−s minus;1 (72).

Results were reported as the number of *L. intracellularis* organisms/ml by converting the Ct value using a standard curve (82).

Immune response assays:

Elispot

Whole blood (>5ml), ileocecal lymph nodes (n=2) and ileum samples were taken on study days 0, 7, 21 and 28 for detection of IFN-γ producing cells.

Peripheral blood mononuclear cells (PBMC):

A volume of >5 ml of whole blood was collected in EDTA vacutainer tubes (BD Vacutainer® EDTA). PBMC were isolated by gradient centrifugation. Briefly, individual EDTA-collected blood samples were diluted 1:1 in sterile PBS, overlaid onto lymphocyte separation medium (Corning® 500 mL Lymphocyte Separation Medium) and centrifuged at 2000 rpm for 30 minutes at 20℃. After centrifugation, PBMC's were withdrawn from the Lymphocyte Separation MediumL/plasma interface and immediately washed two times with sterile PBS. After washing, the PBMC's were counted. Cells were stimulated by *L. intracellularis* antigen (10 ug/ml), McCoy cells (10 ug/ml as a negative control), and artificial IFN-γ stimulant mitogen Concanavalin A from *Canavalia ensiformis* (Sigma-Aldrich) (10 ug/ml as a positive control), diluted in complete medium (Roswell Park Memorial Institute + l-glutamine + MEM Non-Essential Amino Acids Solution + Sodium Pyruvate + 10% FBS), and were added to four different wells of a 96-well assay plate for ELISpot (MilliporeSigma&trade MultiScreen™) for each PBMC sample. Then, 100 ul of the PBMC suspension in complete medium were added to each well to a density of 10^6 for each treatment well (*L. intracellularis* antigen and McCoy negative control), and 50 ul were added to the Concanavalin A treatment wells (positive control) to have a final concentration of 10^5 cells per well. The cells were incubated for 18 hours in a 5% CO_2 incubator. After incubation, the plates were washed six times with sterile PBS. The samples were analyzed with the ELISPOT porcine

INF- γ kit (R&D Systems) and ELISPOT color development (R&D Systems) following the manufacturer's instructions. The plates were read by the ELISPOT reader (ImmunoSpot® S6), which counts the number of IFN-γ producing cells. The results were reported as the resulting counts averaged for each *L. intracellularis* stimulated sample and calibrated by subtracting the averaged counts for the McCoy cells. The artificial Concanavalin A stimulated wells were counted to ensure that the PBMC were secreting INF-y. All the treatment and control samples were tested in duplicate.

Ileocecal lymph nodes:

Two pieces of the ileocecal lymph nodes per pig were collected in sterile Ziploc bags containing 20 ml of RPMI with 2% FBS and antibiotics (100 IU/ml penicillin, 100, ug/ml streptomycin, 0 5, ug/ml amphotericin). The lymph nodes were smashed in the bags and the smashed content was filtered through a 70-um cell strainer (Falcon™). The filtered cells were suspended in 25 ml of Hanks' balanced salt solution (HBSS) and centrifuged at 1500 rpm at 4°C degrees for 10 minutes. After centrifugation, the supernatant was discarded and one ml of Ammonium-Chloride-Potassium (ACK) red cells lysis buffer (Lonza™ BioWhittaker™) was added for five minutes. Then, the lymphocytes were washed with HBSS twice. After washing, the lymphocytes were counted and the protocol for ELISpot was followed (see PBMC procedure). The results were reported as the resulting counts averaged for each *L. intracellularis* stimulated sample and calibrated by subtracting the averaged counts for the McCoy cells.

Intraepithelial lymphocytes (IEL):

Five to eight cm of ileum (without the payer's patches) closest to the ileocecal junction were collected in Ziploc bags containing 20 ml of RPMI with 2% FBS and antibiotics (100 IU/ml

penicillin, 100, ug/ml streptomycin, 0,5 ug/ml amphotericin). The intestine samples were cut into small pieces (less than 0.5 cm) and digested for 30 minutes in Hank's Balanced Salt Solution (HBSS) supplemented with 1.26 mmol EDTA and 0.94 mmol dithiothreitol (Sigma, Deisenhofen, Germany) and antibiotics (100 IU/ml penicillin, 100 ug/ml streptomycin, 0,5 ug/ml amphotericin, Seromed) to remove the epithelium and mucosa and isolate the lymphocytes. After digestion, the cells in the supernatant were filtered through a 70-um cell strainer (Falcon™), collected in 50 ml centrifugation tubes, and washed twice with HBSS. A third wash was done with HBSS supplemented with 0.1 mg/ml DNAse. After washing, the lymphocytes were counted and the protocol for ELISpot was followed (see PBMC procedure). The results were reported as the resulting counts averaged for each *L. intracellularis* stimulated sample and calibrated by subtracting the averaged counts for the McCoy cells.

Data analysis:

Pairwise Wilcoxon tests, corrected for multiple comparisons with Bonferroni-Holm correction, for each variable separately was necessary because the data was not normally distributed and the number of individuals for each time point were low.

Results:

Non-vaccinated pigs developed clinical disease consistent with *L. intracellularis* infection after challenge with presence of diarrhea and specific antibodies in 100% of the pigs, validating the infection model used in the study.

IgG:

After 3 weeks post-vaccination, the intramuscular vaccine induced *L. intracellularis* specific serological IgG in 79% (19/24) of the pigs, significantly higher than the other three treatments (*P=<0.01*), with a mean reciprocal titer between 1:30 to 1:480. The oral vaccine induced serological *L. intracellularis* antibodies (IgG) in 4% (1/24) of the pigs, with a mean reciprocal titer of 1:240. On study day 7 after challenge, the intramuscularly vaccinated group showed *L intracellularis* IgG antibodies in 94% (17/18) of the pigs, significantly higher than the other three treatments (*P=<0.01*) with a mean reciprocal titer between 1:60 to 1:1920. The oral vaccinated group showed *L. intracellularis* specific antibodies in 23% (4/17) of the pigs, with a mean reciprocal titer between 1:30 to 1:60. One pig died and was diagnosed with Mulberry heart disease and was not included for analysis. The NC group showed *L. intracellularis* specific antibodies in 27% (5/18) of the pigs, with a mean reciprocal titer between 1:30 to 1:240. On study day 21, (3 weeks after challenging) the intramuscular vaccine induced *L. intracellularis* specific antibodies in 100% (11/11) of the pigs, but not significantly higher than the other three treatments, with a mean reciprocal titer between 1:60 to 1:1920. One pig from this group died from a cause non-related to *L. intracellularis* and was not included for analysis. The oral vaccinated group showed *L. intracellularis* IgG specific antibodies in 90% (10/11) of the pigs, with a mean reciprocal titer between 1:60 to 1:960 (Figure 4). The NC group showed *L. intracellularis* specific IgG in 63% (7/11) of the pigs, with a mean reciprocal titer between 1:30 to 1:960. On study day 28, the intramuscular vaccine group had100% (5/5) of the pigs positive, but this was not statistically higher than the other three treatments, with a mean reciprocal titer between 1:120 to 1:3840. The oral vaccinated group showed *L. intracellularis* IgG in 83% (5/6) of the pigs, with a mean reciprocal titer between 1:60 to 1:960. The NC group showed *L. intracellularis* specific antibodies in 100% (6/6) of the pigs, with a mean reciprocal titer between 1:60 to 1:960. The NN group remained

negative in all time points. See Table 3.

Immunohistochemistry:

Animals in all treatment groups were negative by IHC on study day 0 (challenge day). On study day 7, *L. intracellularis* antigen was detected in 16% of the pigs (1/6) in the OV and NC treatment groups with a score of 3 and 1, respectively. The IV group remained negative at this time point. On study day 21, *L. intracellularis* antigen was detected in 66 % of the pigs (4/6) in the IV treatment group, with scores between 1 and 4. *L. intracellularis* was detected in 100% of the pigs (5/5) in the OV treatment group with scores between 1and 4. In the NC treatment group, *L .intracellularis* was detected by IHC in 83% (5/6) of the pigs with scores between 3 and 4. On study day 28, *L. intracellularis* antigen was detected by IHC in 40% (2/5) of the pigs from the IV treatment, with scores between 1 and 3. *L. intracellularis* was detected in 50% (3/6) of the pigs from the OV treatment with scores between 1 and 4. In the NC group, *L. intracellularis* was detected in 83% of the pigs (5/6) with scores between 1 and 4. The NN treatment group remained negative in all time points (Figure 5, Table 4).

IgA:

All the pigs from the NN, IV and OV treatments were negative for *L. intracellularis* specific IgA on study day 0 (challenge day). Seven days after challenge, one animal from the oral vaccine group showed *L. intracellularis* specific IgA, with the titer of 1:32. Pigs from the NN, NC and IV treatments were still negative at this time point. On study day 21, the OV, IV and NC groups showed *L. intracellularis* specific IgA in 100% of the pigs with mean reciprocal titers between 32 and 1024, 16 and 256, and 16 and 2048, respectively. On study day 28, all pigs from treatment groups IV and NC remained positive with mean reciprocal titers between 8 and 960 and between

16 and 1024, respectively. Pigs from the NN treatment group remained negative in all time points (Figure 6, Table 5).

Peripheral blood mononuclear cells (PBMC's)

The proportion of pigs positivefor *L. intracellularis* specific INF- γ produced by peripheral blood mononuclear cells was (66%, 4/6) for NN and IV groups at day 0 of the study compared to 50% (3/6) for the OV group. However, at day 7 of the study, the proportion of positive pigs was higher in OV group (83%, 5/6) compared to both the NN and IV groups (33%, 2/6) and NC group (50%, 3/6). This pattern was maintained at 21 and 28 days with a higher proportion of positive pigs in vaccinated groups compared to NN and NC groups (Table 6).

Ileocecal Lymph Node

Cells producing INF-γ were detected in all pigs throughout the study. At day 0, 33% (2/6) of the pigs were positive for *L. intracellularis* specific INF-γ in the NN and IV groups compared to 83% (5/6) in the OV group. However, the proportion of positive pigs increased at 21 and 28 days of the study for NN, NC and IV groups. However, this positivity decreased for OV pigs (Figure 7, Table 7).

Intraepithelial Lymphocytes (IEL):

The proportion of positive pigs with *L. intracellularis* specific INF-γ produced by intraepithelial lymphocytes was (50%, 3/6) in the IV and OV groups at day 0 and 16% (1/6) of the NN pigs. This proportion changed at 21 days with 83% (5/6) of positivity for NN, 100% (6/6) for NC, 83% (5/6) for IV and 80% (4/5) for the OV group. At day 28 of the study, the NN group was negative for production of *L. intracellularis* specific INF-γ by intraepithelial lymphocytes, compared to 50%

(3/6) of NC, 60% (3/5) of IV and 33% (2/6) of OV groups (Table 8).

Average daily gain

The average daily gain in the OV and IV groups was similar at day 7 of study (0.245 and 0.232 kg, respectively) while in the NN group the ADG was 0.313 kg. At day 21 of the study, the average daily gain was 0.360 kg in the OV group, 0.386 kg in the IV group, 0.512 kg in the NC group and was 0.736 kg in the NN group. Finally, at day 28 of the study, the average daily gain was 0.437, 0.436, 0.570 and 0.967 kg for the OV, IV, NC and NN groups, respectively (Table 9).

Fecal Shedding

Fecal shedding of *L. intracellularis* was negative at the beginning of the study for all treatment groups. On day 0 (21 days after vaccination), four animals in the oral vaccinated group showed positive fecal shedding with an average of 8.31E+02 organisms per gram of feces. The other groups were still negative at this time point. At seven days post challenge, the oral vaccinated group showed a numerically higher value of 2.77E+05 compared to the NC and IV groups. At 21- and 28-days post challenge, the NC group shed the highest number of *L. intracellularis* organisms, with an average of 4.63E+08 and 7.36E+07, respectively. (Table 10).

Attachments area

Discussion

Vaccination, as a tool to protect naïve pigs against infectious disease, is becoming crucial in the current optimal antibiotic-free scenario. The avirulent modified-live oral vaccine (Enterisol®, Boehringer Ingelheim), commercially available for over 15 years, has been largely used in the swine industry to protect naïve animals in the nursery. The manufacturer recommended the use of the vaccine in piglets older than 3 weeks-of-age with the need of a non-antibiotic window of 3 days before the vaccine (87) (55). Oral vaccination has been reported to prevent clinical signs and increase average daily gain, but does not totally prevent shedding (87). The effect of *L. intracellularis* vaccination in experimental conditions have been previously investigated. Production of cytokines in serum profiles after *L. intracellularis* infection in pigs showed an increase in IL-10 from 5 to 10 dpi and increased TGFβ from 0 to 5 dpi as well as increased TNF-α, IFNγ, and IL-6 titers from 5 to 40 dpi (59). Down-regulation of the gene coding for signaling lymphocytic activation molecule (SLAM7; also known as CD2), which is required for T-cell activation as well as the up-regulation of the genes encoding MHC-I in infected enterocytes, was also demonstrated (60). However, field studies in pigs affected by PPE did not demonstrate clear changes in gene expression and cytokine secretion (63). In this study, pigs from both vaccinated groups showed no differences in number or proportion of positive pigs or in the production of humoral and local cellular immune response after vaccination.

In a previous study, it was also reported that an elevated dose (10-times) of the avirulent oral vaccine induced a greater expression of TGFβ and TNFα measured in intestinal mucosal scrapings that protected the animals from infection after a subsequent challenge (83). This finding suggests that these cytokines may play an important role balancing inflammatory and immunosuppressive local reactions. By day 14 after infection, an elevated number of activated macrophages next to

the *L. intracellularis* infected crypts was shown, which may explain the pro-inflammatory cytokines produced at this time point in pigs (84) (59). At the same time, IgA titers in intestinal lavages were also elevated (57) (81), which has been interpreted as activation of the adaptive immune system clearing the bacteria. In our study, an earlier local IgA response was detected in the OV group, with a greater number of IFN-γ producing T cells in ileocecal lymph nodes after vaccination and before the challenge compared to the IV and NC groups. This finding is expected given the direct stimulation of the intestinal mucosa through the oral vaccine.

Measurements of the levels of IFN-γ in serum in another study showed that it was maintained elevated until 40 days after infection (59), with increased numbers of IFN-γ-producing cells until 91 days after infection (10). The importance of IFN-γ against *L. intracellularis* infection was demonstrated by a study that showed that IFN-γ receptor knock-out mice were unable to resolve the infection after 35 days while in wild type mice the infection was resolved after 21 days (85). In our study, the production of IFN-γ producing lymphocytes from the PBMCs and intraepithelial lymphocytes did not have statistical differences among treatments.

On the other hand, pigs challenged with virulent bacteria were reported to have increased levels of antigen-specific IgG and IFN-γ in serum when compared to pigs vaccinated with the oral vaccine (54). In a study published by Guedes and Gebhart in 2003, it was reported that IgG seroconversion and a higher IFN-γ concentration occurred 35 days post-vaccination (10). In our study, the IV and OV groups had an earlier production of IgG compared to the NN and NC groups. However, the proportion of positive pigs with circulating IgG antibodies in serum was higher in the IV group compared to the OV group. These results demonstrate that an intramuscular vaccination produces a faster and higher production of humoral response compared to an oral vaccination.

Production of local immunity is likely important for *L. intracellularis* protection because of the bacteria tropism and the fact that mucosal cells are the target for infection. Therefore, any local response induced by vaccination would have a critical role for the protective immune response. However, the findings of this study suggest that there is not a difference in the impact of *L. intracellularis* infection after oral or intramuscular vaccination. Interestingly, the present study demonstrates limited activation of local cell-meditated immune response using an intramuscular killed vaccine. Future studies will be crucial to further investigate the immunological mechanisms involved in this process.

In summary, even though there were numerical or statistical differences (humoral response for the IV group), no differences in average daily gain and fecal shedding were observed. Therefore, this study provides important information for producers and veterinarians at the time to choose an alternative to immunize pigs against PPE.

Chapter 4: General discussion and conclusions

Lawsonia intracellularis (*L. intracellularis*) is the causative agent of proliferative enteropathy (PPE), an enteric disease with great economic importance in theswine industry worldwide (14). Vaccination for is available to mitigate the negative economic impact that the disease causes. An oral vaccine, consisting of a live attenuated *L. intracellularis* strain, was the first commercially available (83). An killed intramuscular vaccine consisting of whole-cell bacteria, was recently released to the market (75) . Comparison of transmission and immune responses between the two commercially available vaccines has not been studied and it would add novelty information for swine producers worldwide. Previous studies that aimed to evaluate shedding after experimental infection were demonstrated shedding of the bacteria up to 12 weeks (10) (43). Understanding the transmission patterns in vaccinated and non-vaccinated populations and the immunity generated after vaccination and subsequent exposure would allow a better understanding of measures to control *L. intracellularis* shedding and prevent PPE. Vaccination against *L. intracellularis* contributes to improve herd immunity to reduce PPE infection and transmission. A reduction in transmission and disease prevalence translates into better production performance with the consequent economic benefit for producers.

In the second chapter of my thesis, we compared the transmission rates and fecal shedding in vaccinated (orally or intramuscular) and non-vaccinated animals after contact with infected pigs using a seeder pig model as a experimental setting to mimic infection spread in field conditions. Transmission was significantly reduced after vaccination, from a rate of 2.6 new cases/week in non-vaccinated animals to 1.86 and 1.75 cases/week after oral and intramuscular vaccination, respectively. We also identified a reduction in the length of *L. intracellularis* shedding after exposure to infected pigs of 43.3% and 25.5% for the oral and intramuscular vaccines, respectively,

as compared to non-vaccinated animals. Therefore, under the conditions of this study, vaccination with either oral or intramuscular vaccine was effective at reducing transmission rate and length of shedding for *L. intracellularis*. This information is relevant for producers in order to design PPE control programs, such as gilt acclimation. Basic reproduction number (R0), defined as the number of secondary cases after the introduction of one infectious animal, is used in transmission studies to measure the spread of an infectious disease. Previous studies has determined reduction of the R0 for influenza from 9.8 in non-vaccinated pigs to 1.2 in vaccinated pigs (64). We attempted to measure R0 in our study, however, the nature of *L. intracellularis* infection (shedding may occur 6-9 weeks after infection and even intermittently up to24 weeks after infection) did allow us to estimate theR0 (57). The period of time we comingled infectious animals with vaccinated or naïve pigs lasted 4 weeks for this study.

Intramuscular vaccination against *L intracellularis* has produced significant serological humoral response compared to non-vaccinated pigs (75). It has been demonstrated that management practices such as use of all-in-all-out can be associated with a lower seroprevalence to *L. intracellularis* in breeding units, and seropositivity in grower-finisher animals is a risk factor for replacement animals (84). Therefore, the use of vaccination can contribute to improved herd immunity with the consequent reduction of risk of transmission and disease prevalence. In our study, pigs vaccinated with intramuscularly had significantly higher levels of antibodies in the serum than oral vaccinated and non-vaccinated pigs. However, there was no correlation between serum antibody levels and transmission rates. This finding indicates that serological diagnostic tools (i.e. IPMA) used to assess *L. intracellularis* exposure through the detection of IgG in the serum should to be interpreted with caution when trying to associate serum response with protective immunity.

From a practical perspective, these results highlight the importance of implementing vaccination programs at the system level rather than site-specific interventions. Vaccination strategies against *L. intracellularis* decrease the risk of disease as a result of commingling batches of pigs from vaccinated and non-vaccinated sources and therefore reducing the impact of the PPE in downstream flows.

Tables

Table 1. Fecal scores in study by group and time (weeks).

Treatments	Weeks of contact with seeder pigs				
	0	1	2	3	4
Oral vaccinated	11%(3/27)	11%(3/27)	34%(9/26)	26%(7/26)	11%(3/26)
Intra-muscular vaccinated	0%	22%(6/27)	40%911/27)	55%(15/27)	37%(10/27)
Non-vaccinated challenged	0%	0%	29%(8/27)	40%(11/27)	25%(7/27)
Non-vaccinated non-challenged	0%	0%	0%	0%	0%

Table1. Fecal scores per group during the 4 weeks of contact with the seeders. Score 1 = firm and shaped, score 2 = soft and shaped, score 3 = loose, and score 4 = watery (69).

Table 2. IHC scores for all treatments

	Score				
Treatment	**Neg**	**1**	**2**	**3**	**4**
Oral (N= 26)	8	1	5	7	5
IM (N= 27)	19	4	1	3	1
Non-vacc (N= 26)	8	8	3	1	6

Immunohistochemistry results by group. Number of pigs with each score.

Table 3. Proportion of pigs positive by IPMA for serum IgG.

Treatment	Study day				
	-21	0	7	21	28
NN	NEG	NEG	NEG	NEG	NEG
NC	NEG	NEG	27% (5/18)	63% (7/11)	100% (6/6)
IV	NEG	79% (19/24)	94% (17/18)	100% (11/11)	100% (5/5)
OV	NEG	4% (1/24)	23% (4/17)	90% (10/11)	83% (5/6)

Table 4: Proportion of positive animals by immunohistochemistry by group

Treatment	Study Day			
	0	7	21	28
NN	NEG	NEG	NEG	NEG
NC	no sampling	16% (1/6)	83% (5/6)	83% (5/6)
IV	NEG	NEG	66% (4/6)	40% (2/5)
OV	NEG	16% (1/6)	100% (5/5)	50% (3/6)

Proportion of positive animals by immunohistochemistry by group IV (Intramuscular vaccine), NC (Non vaccinated challenged), NN (Non vaccinated non challenged), OV (Oral vaccine) and time (0, 7, 21 and 28 days).

Table 5: Proportion of IgA positive animals

Treatment	Study Day			
	0	7	21	28
NN	NEG	NEG	NEG	NEG
NC	no sampling	NEG	100% (6/6) 16-2048	100% (6/6) 16-1024
IV	NEG	NEG	100% (6/6) 16-256	100% (5/5) 8-960
OV	NEG	16% (1/6) 32	100% (5/5) 32-1024	83% (5/6) 8-512

Proportion of positive animals for IgA by group IV (Intramuscular vaccine), NC (Non vaccinated challenged), NN (Non vaccinated non challenged), OV (Oral vaccine) and time (0, 7, 21 and 28 days).

Table 6: Proportion of positive pigs for IFN-gamma produced by peripheral blood mononuclear cells and average of spots

Treatment	0		7		21		28	
	Proportion	Average Spots per 10^6	Proportion	Average	Proportion	Average	Proportion	Average
NN	66% (4/6)	9.5	33% (2/6)	7.5	33% (2/6)	13.5	20% (1/5)	1
NC	no sampling	--	50% (3/6)	3.3	83% (5/6)	3.6	66% (4/6)	14.5
IV	66% (4/6)	10.5	33% (2/6)	3	100% (6/6)	4	100% (5/5)	6.6
OV	50% 3/6)	11.6	83% (5/6)	4.5	80% (4/5)	6.5	66% (4/6)	1

Proportion of positive pigs for peripheral blood mononuclear cells and Average spots by group IV (Intramuscular vaccine), NC (Non vaccinated challenged), NN (Non vaccinated non challenged), OV (Oral vaccine) and time (0, 7, 21 and 28 days).

Table 7. Proportion of positive pigs with of INF-γ producing cells in lymph nodes and average of cells.

Treatment	0		7		21		28	
	Proportion	Average	Proportion	Average	Proportion	Average	Proportion	Average
NN	33% (2/6)	20.5	83% (5/6)	4.8	50% (3/6)	3.3	40% (2/5)	2.5
NC	no sampling	--	16% (1/6)	5	50% (3/6)	7.66	66% (4/6)	8.25
IV	33% (2/6)	2.5	50% (3/6)	7.33	83% (5/6)	5	60% (3/5)	6.6
OV	83% (5/6)	16.5	83% (5/6)	4.5	40% (2/5)	3.5	50% (3/6)	2

Proportion of positive pigs with INF-γ producing cells in lymph nodes and average of cells by group IV (Intramuscular vaccine), NC (Non vaccinated challenged), NN (Non vaccinated non challenged), OV (Oral vaccine) and time (0, 7, 21 and 28 days).

Table 8. Proportion of positive pigs with intraepithelial lymphocytes and average of cells

Tnt	0		7		21		28	
	Proportion	Average	Proportion	Average	Proportion	Average	Proportion	Average
NN	16% (1/6)	190	33% (2/6)	13	83% (5/6)	25.6	NEG	--
NC	no sampling	--	66% (4/6)	3.25	100% (6/6)	37.16	50% (3/6)	12.3
IV	50% (3/6)	80.6	100% (6/6)	5.16	83%	41.8	60% (3/5)	5.33
OV	50% (3/6)	102	16% (1/6)	6	80% (4/5)	40	33% (2/6)	8

Proportion of positive pigs with intraepithelial lymphocytes and average of cells by group IV (Intramuscular vaccine), NC (Non vaccinated challenged), NN (Non vaccinated non challenged), OV (Oral vaccine) and time (0, 7, 21 and 28 days).

Table 9. Average daily gain in study groups (Kg)

Treatment	Study Day			
	0	7	21	28
NN	0.171	0.313	0.736	0.967
NC	NI	NI	0.512	0.570
IV	0.147	0.232	0.386	0.436
OV	0.139	0.245	0.368	0.437

Average daily gain (Kg) by group. IV (Intramuscular vaccine), NC (Non vaccinated challenged), NN (Non vaccinated non challenged), OV (Oral vaccine) and time (0, 7, 21 and 28 days).

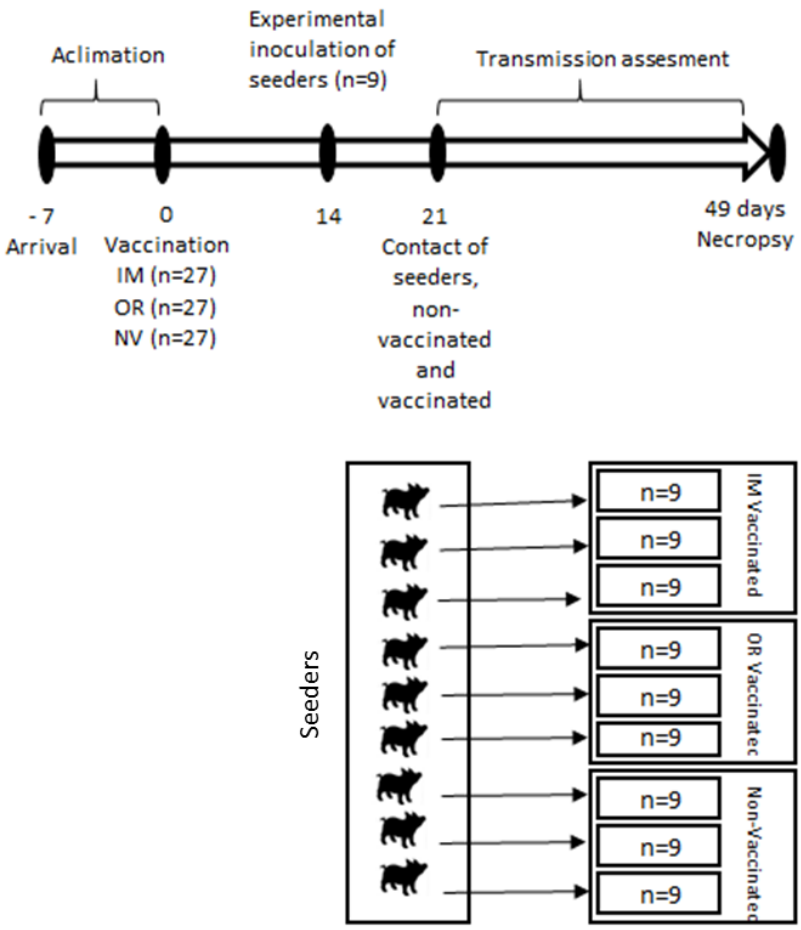
Table 10. Average number of *L. intracellularis* organisms quantified by q-PCR

	Study day			
Treatment	0	7	21	28
NN	Neg	Neg	Neg	Neg
NC	Neg	2.60E+05 (1.86E+02 - 3.21E+06)	4.63E+08 (1.47E+05 - 2.42E+09)	7.36E+07 (6.04E+04 - 3.51E+08)
OV	8.31E+02 (1.86E+02 - 7.60E+2)	2.77E+05 (2.34E+03 - 7.55E+07)	1.38E+08 (5.71E02 - 1.18E+08)	2.71 E+04 (1.87E+02 - 1.24E+05)
IV	Neg	1.32E+05 (9.08E+01 - 1.56E+06)	1.46E+06 (2.56E+04 - 3.33E+06)	9.40E+05 (3.85E+01 - 9.40E+05)

Average number of *L. intracellularis* organisms by group. IV (Intramuscular vaccine), NC (Non vaccinated challenged), NN (Non vaccinated non challenged), OV (Oral vaccine) and time (0, 7, 21 and 28 days). Minimum and maximum values are shown in parenthesis

Figures

Figure 1. Experimental design



Study timeline and treatment allocation.

Figure 2. Adjusted predicted probabilities of shedding ≥10^3 *L. intracelullaris*/g of feces by experimental group and time (in weeks)

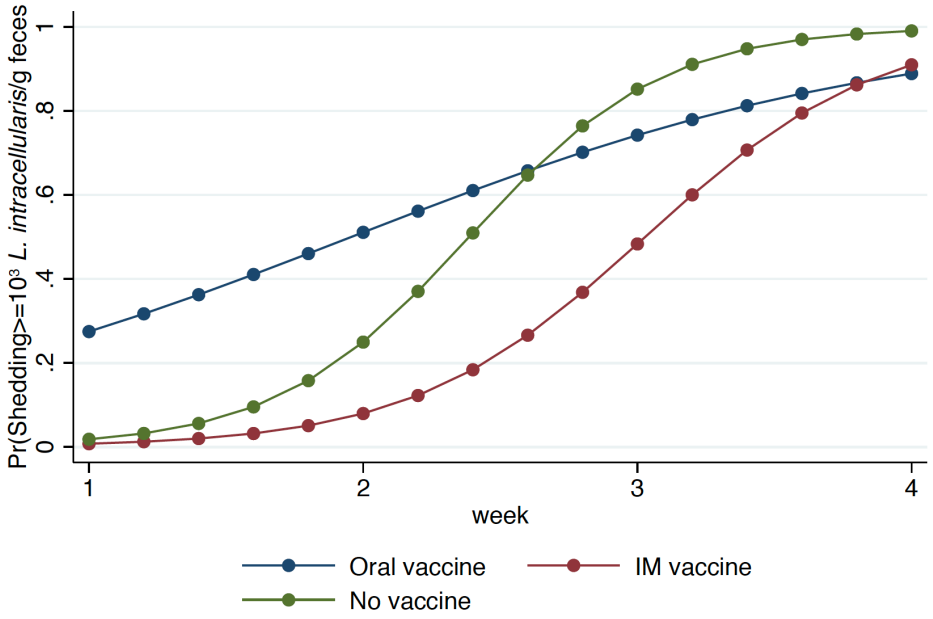


Figure 3. Representation of the experimental design and timeline (days)

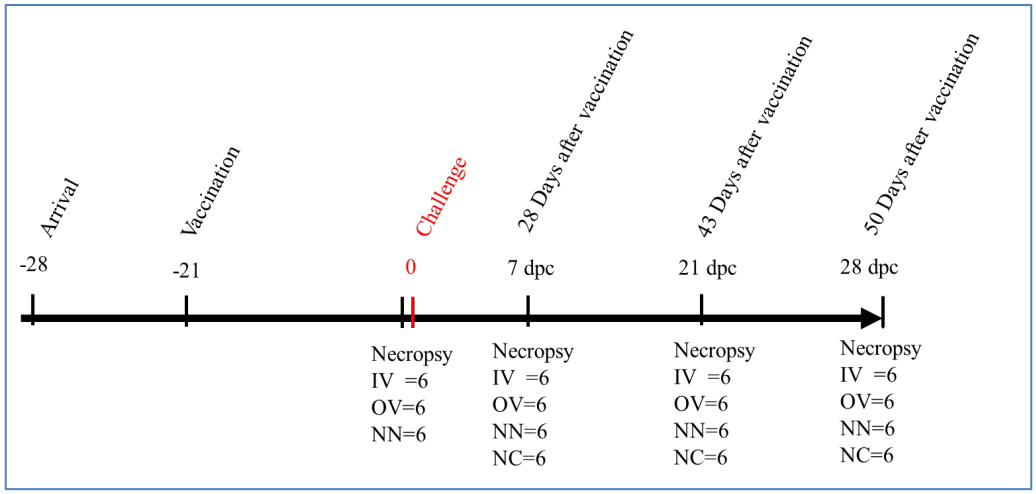


Figure 4. IgG serum

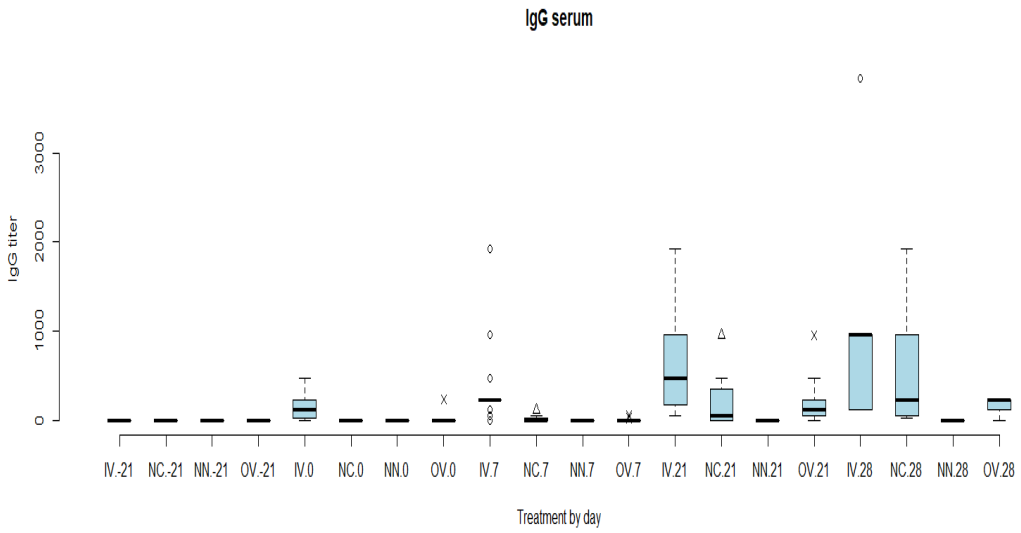


Figure 4. Distribution of IgG titers in pigs by group IV (Intramuscular vaccine), NC (Non vaccinated challenged), NN (Non vaccinated non challenged), OV (Oral vaccine) and time (-21, 0, 7, 21 and 28 days).

Figure 5. Immunohistochemistry Distribution

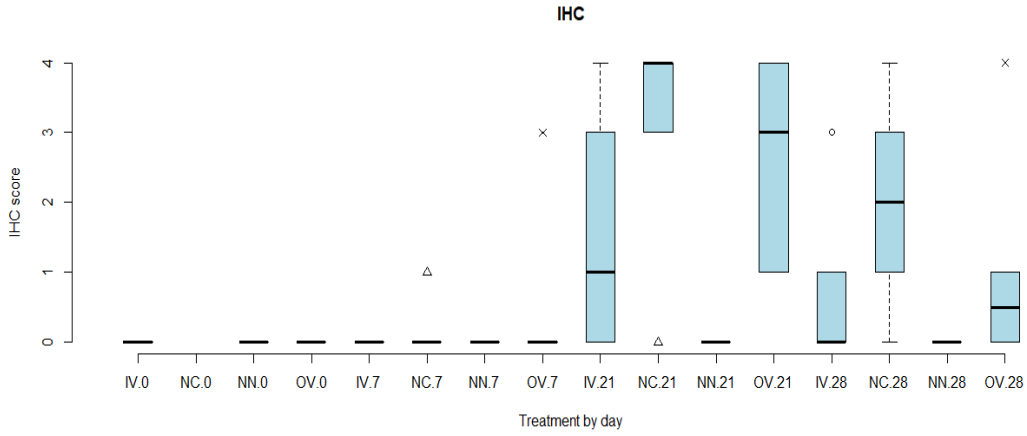


Figure 5. Distribution of immunohistochemistry scores (0 = no presence of bacteria, 1 = 1 – 25%, 2= 26 – 50%, 3= 51 – 75% and 4= 76 – 100%) by group IV (Intramuscular vaccine), NC (Non vaccinated challenged), NN (Non vaccinated non challenged), OV (Oral vaccine) and time (0, 7, 21 and 28 days).

Figure 6. Distribution of IgA titers

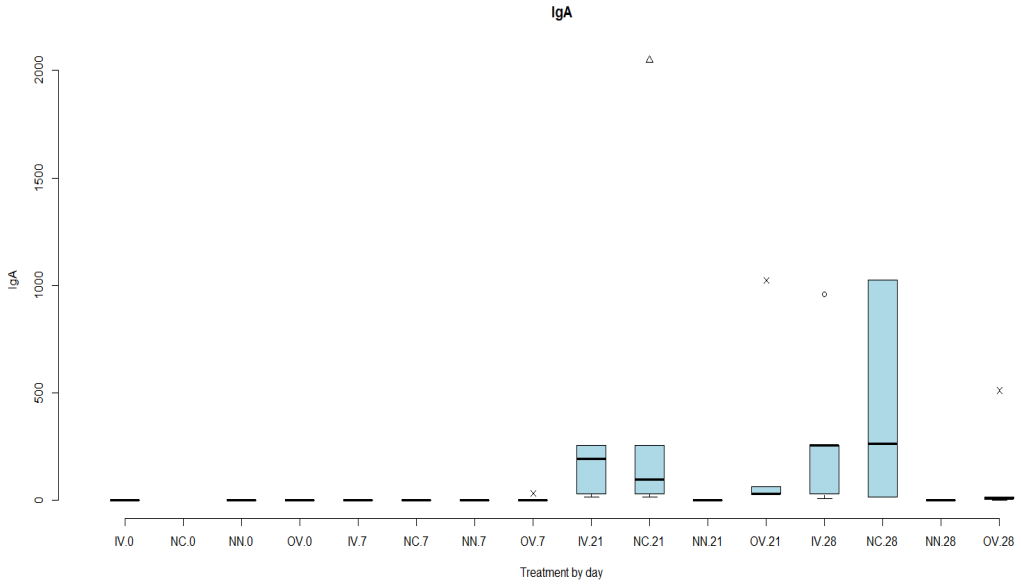


Figure 6. Distribution of IgA titers in pigs by group IV (Intramuscular vaccine), NC (Non vaccinated challenged), NN (Non vaccinated non challenged), OV (Oral vaccine) and time (0, 7, 21 and 28 days).

Figure 7. Number of cells producing INF-γ in lymph nodes

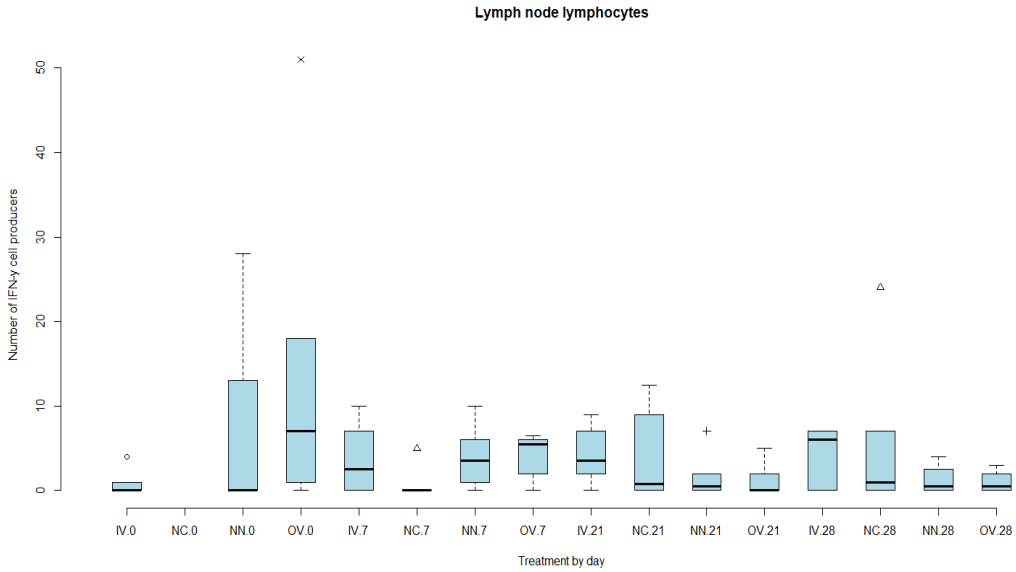


Figure 7. Number of cells producers of INF-γ in lymph nodes in pigs by group IV (Intramuscular vaccine), NC (Non vaccinated challenged), NN (Non vaccinated non challenged), OV (Oral vaccine) and time (-21, 0, 7, 21 and 28 days).

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