PROLIFERATIVE ENTEROPATHY: PATHOGENESIS AND HOST ADAPTATION

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Dedication

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Abstract

Proliferative enteropathy (PE) is an infectious disease caused by an obligate intracellular bacterium, *Lawsonia intracellularis*, and characterized by thickening of the intestinal epithelium due to enterocyte proliferation. The overall goals of this thesis were to improve the understanding of the pathogenesis of PE by evaluating phenotypic traits, genome variations and transcriptome patterns of *L. intracellularis* infection and to evaluate the adaptation of the bacterium to porcine and equine hosts.

In the first section, the susceptibility of pigs to a homologous porcine *L. intracellularis* isolate continuously grown *in vitro* was assessed. A loss of virulence after 40 passages of the bacteria in culture was established. The comparative whole genome analysis of the pathogenic (low passage) and non-pathogenic (high passage) isolates identified the loss of a prophage-associated genomic island in the non-pathogenic variant. This chromosomal island proved not to be essential for the virulent phenotype, since it was not identified in horses clinically affected with PE. However, this genetic element was associated with host-adapted *L. intracellularis* variants. While pathogenic porcine isolates harbor this genetic element, it was absent in equine isolates and PE-affected horses. Gene expression profiling of a porcine pathogenic isolate showed a wider transcriptional landscape compared with the non-pathogenic variant. In addition, genes highly activated *in vitro* by the pathogenic variant also were significantly expressed *in vivo*. However, genes identified in the genomic island were not expressed by intracellular bacteria either *in vitro* or *in vivo*. The proliferative changes exhibited by the infected enterocytes *in vivo* were associated with deregulation of the G_1 phase of the host cell

cycle and repression of membrane transporters related to nutrient acquisition, characterizing a malabsorptive syndrome as the major mechanism involved in the poor performance and growth of affected animals.

Finally, an alternative method for cultivation of *L. intracellularis* was developed in order to perform a cross-species experimental study evaluating the susceptibility of pigs and horses to a porcine and an equine *L. intracellularis* isolate. Evident clinical signs, longer periods of bacterial shedding and stronger serologic immune responses were observed in animals infected with species-specific isolates indicating that host susceptibility is driven by the origin of the *L. intracellularis* strain.

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SECTION I

GENERAL INTRODUCTION AND LITERATURE REVIEW

GENERAL INTRODUCTION

Proliferative enteropathy (PE) is an infectious intestinal disease characterized by thickening of the intestinal epithelium due to enterocyte proliferation. *Lawsonia intracellularis* is the etiologic agent of PE (McOrist et al., 1995a). Cell proliferation is directly associated with bacterial infection and replication in the intestinal epithelium. As a result, mild to severe diarrhea is the major clinical sign described in infected animals (Lawson and Gebhart, 2000). The dynamics of *L. intracellularis* infection *in vitro* and *in vivo* have been well-characterized. Nevertheless, little is known about the genetic basis for the pathogenesis or ecology of this organism. In addition, the molecular mechanisms for infection and virulence remain undetermined.

Since the 1990s, PE has been endemic in swine herds and has been occasionally reported in various other species, including wild mammalians and ratite birds (Cooper et al., 1997a; Pusterla et al., 2008b). Outbreaks among foals began to be reported on breeding farms worldwide within the last four years (Guimarães-Ladeira et al., 2009; McGurrin et al., 2007; Pusterla et al., 2008a). Therefore, PE is now considered an emerging disease of the horse population (Lavoie and Drolet, 2009). Intestinal hyperplastic lesions are present in every case of PE; however, there are some differences between clinical and pathological presentations among affected species. Despite these observations of host-specificty, no genotypic differences have been demonstrated based

on 16S ribosomal DNA sequences among isolates from different affected species (Cooper et al., 1997a). Susceptibility of pigs to the horse isolate or vice-versa has not been reported and may provide relevant information about fitness and adaptation of different bacterial isolates in these species. Phenotypic and genotypic differences have potential to reveal novel bacterial subspecies or subtypes.

The present thesis focused on the study of the pathogenesis and host adaptation of *L. intracellularis* infections. The overall goals were to characterize the genomic and transcriptome features associated with the virulent phenotype of *L. intracellularis* in pigs and to evaluate the susceptibilities of horses and pigs to *L. intracellularis* infection using porcine and equine isolates. Following a literature review (Chapter 1), incorporating the recent findings into previous knowledge on the pathogenesis of PE (Section I), this thesis comprises sections dedicated to study the pathogenesis (Section II) and the host adaptation (Section III), of *L. intracellularis* infections, followed by a general conclusion (Section IV) integrating the results from the previous sections.

Section II is divided into four chapters. In Chapter 2, the susceptibility of weaning pigs to a porcine *L. intracellularis* isolate continuously grown *in vitro* was evaluated. Studies presented in Chapter 3 compared the whole genome sequence of two homologous porcine isolates that showed pathogenic (low passage) and non-pathogenic (high passage) phenotypes, respectively, in the previous study. Chapter 4 describes the gene expression profiles of pathogenic and non-pathogenic porcine *L. intracellularis* variants during the infection *in vitro*. Finally, Chapter 5 comprehensively evaluates the transcriptional

response of *Lawsonia*-infected enterocytes *in vivo* and presents a bacterial interactome analysis based on the genes highly expressed by intracellular *Lawsonia*.

Section III contains two chapters. Chapter 6 was directed to develop an alternative method for cultivation of *L. intracellularis* in order to amplify the bacterial inoculum to be used for the experiment described in Chapter 7. This last chapter describes a cross-species experimental study evaluating the susceptibility of pigs and horses to a porcine and an equine *L. intracellularis* isolate.

CHAPTER 1

Recent advances in the pathogenesis of *Lawsonia intracellularis* infections – Literature review

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1.1 Introduction

Lawsonia intracellularis is the sole species in the bacterial genus *Lawsonia* and is the etiologic agent of proliferative enteropathy (PE) (McOrist et al., 1995a). The disease is characterized macroscopically by thickening of the intestinal epithelium due to enterocyte proliferation. As a result, mild to severe diarrhea represent the major clinical signs described in affected animals (Lawson and Gebhart, 2000). Since the 1990s, PE has been endemic in swine herds and occasionally reported in various other species (Cooper et al., 1997b; Pusterla et al., 2008b). There have been numerous reports of outbreaks among foals on breeding farms worldwide within the last five years (Guimarães-Ladeira et al., 2009; McGurrin et al., 2007; Pusterla et al., 2008a). Therefore, PE is now considered an emerging disease of horse populations (Lavoie and Drolet, 2009; Pusterla and Gebhart, 2009). Among the other species in which PE has been diagnosed are hamsters (Jonas et al., 1965), rabbits (Umemura et al., 1982), ferrets (Fox et al., 1994), foxes (Landsverk, 1981), dogs (Collins et al., 1983), rats (Vandenberghe and Marsboom, 1982), mice (Abshier et al., 2001), sheep (Cross et al., 1973), deer (Drolet et al., 1996), emus (Lemarchand et al., 1997), ostriches (Cooper et al., 1997b), non-human primates (Klein et al., 1999) and guinea pigs (Elwell et al., 1981). Since *L. intracellularis* has never been identified in humans with enteric disorders, PE is not considered to be a zoonotic disease (Michalski et al., 2006). Difficulties associated with *in vitro* cultivation of this organism, linked with its ubiquitous presence and ability to cause disease in a variety of animal species, highlight the needs for higher resolution diagnostics to provide

a better understanding of the dynamic of the inter-species transmission and the realistic importance of the disease in different species.

PE was first reported in 1931 as porcine intestinal adenoma (Biester and Schwarte, 1931). Afterward, published reports were limited to laboratory hamsters in which PE was known as "wet-tail disease" based on the appearance of the perianal region of affected animals (Sheffiled and Beveridge, 1962). Bacteria and viruses were speculated to be its causative agents due to the enzootic characteristic involved in the PE outbreaks (Jonas et al., 1965). In the early 1970s Dr. G. H. K. Lawson's group in the United Kingdom started to investigate field outbreaks of PE in pigs. The presence of intracellular bacteria within proliferative lesions was then described in affected pigs (Rowland et al., 1973). It was not until 1993 that this intracellular bacterium was isolated in cell culture from infected pigs using a rat enterocyte cell line by Lawson et al (1993) and the disease was reproduced, fulfilling Koch's postulates (Lawson et al., 1993; McOrist et al., 1993). After the successful growth and maintenance *in vitro*, a new genus and species was established and the organism was named *Lawsonia intracellularis* in honor of Dr. Lawson (McOrist et al., 1995a).

The main phenotypic characteristic of the *L. intracellularis* infection characterized by cellular proliferation has not been reproduced *in vitro* (Lawson et al., 1993; Vannucci et al., 2012e). As a result, comprehensive studies on the progression of lesions have been conducted using experimental infection models in pigs and hamsters (Guedes and Gebhart, 2003a; Jasni et al., 1994b; Vannucci et al., 2010). The cell proliferation in the intestinal epithelium described as enterocyte hyperplasia is directly

associated with bacterial infection and replication in the intestinal epithelium (McOrist et al., 1996a). However, little is known about the genetic basis for the pathogenesis or physiology of this organism. In addition, the molecular mechanisms for infection and virulence remain undetermined (Jacobson et al., 2010).

Since the first report of PE, *L. intracellularis* has been found to have unique properties such as inducing unusual pathological changes in infected animals. Limited knowledge of the pathogenesis of this infection suggests that this organism has acquired mechanisms of survival and pathogenesis that are unique among bacterial pathogens (Gebhart and Guedes, 2010). The present article reviewed and discussed the current knowledge of PE focusing on the recent advances regarding the pathogenesis of *L. intracellularis* infections.

1.2 Characteristics of *L. intracellularis*

After the first report showing the presence of intracellular bacteria in association with enterocyte proliferation (Rowland et al., 1973), a few designations were attributed to the organism (*Campylobacter-like*, *ileal symbiont intracellularis* and *ileobacter intracellularis*) (McOrist et al., 1995a). In 1995, *Lawsonia intracellularis* was classified as a member of the delta subdivision of Proteobacteria belonging to the *Desulfovibrionaceae* family (Gebhart et al., 1993). Based on the DNA sequence of the 16S ribosomal gene, *L. intracellularis* was taxonomically distinct from other intracellular pathogens such as *Rickettsiae* and *Chlamydiae* (Dale et al., 1998; McOrist et al., 1995a). A free-living anaerobic human pathogen, *Bilophila wadsworthia* was the closest

organism genetically related to *L. intracellularis* exhibiting 92% similarity of the 16S rDNA sequence (Sapico et al., 1994). *Desulfovibrio desulfuricans* a sulfate-reducing bacterium was 91% similar (Gebhart et al., 1993).

Lawsonia intracellularis is a gram negative, non-spore-forming, microaerophilic, curved or vibroid-shaped rod and obligately intracellular. The bacterium is 1.25-1.75μm in length and 0.25-0.43μm in width and exhibits a trilaminar outer envelope (McOrist et al., 1995a). A unipolar flagellum was demonstrated extracellularly in cultivated organisms as well as darting motility *in vitro* upon escape from infected enterocytes (Lawson and Gebhart, 2000). The intracellular bacteria locate in the apical membrane of infected enterocytes and are commonly associated with free ribosomes and scattered mitochondria (Johnson and Jacoby, 1978).

Since the first reports of cultivation and maintenance of *L. intracellularis in vitro* (Lawson et al., 1993), various cell lines have supported the bacteria's growth, including insect and avian cells (Evans et al., 2011; Guedes et al., 2002; McOrist et al., 1995b). To date, growth of the bacteria in axenic (cell-free) media has not been reported. Regardless of the cell type, the bacteria require dividing cells in a specific microaerophilic atmosphere for cultivation (Lawson et al., 1993; Vannucci et al., 2012e). *L. intracellularis* has been conventionally cultivated in static adherent monolayer in a humidified tri-gas incubator with 83.2% nitrogen, 8.8% carbon dioxide, and 8% oxygen at 37°C (Collins et al., 1996; Lawson et al., 1993; McOrist et al., 1995b). Alternative chambers have also supported the bacterial growth by providing similar microaerophilic atmospheres using a cylinder containing 10% hydrogen, 10% carbon dioxide, and 80%

nitrogen gas (Vannucci et al., 2012e). Suspension cultures have also been used for cultivation in large-scale and production of vaccine (Kroll et al., 2004). These cultures are propagated in spinner flasks or automatized bioreactors with automatized agitation and monitoring of temperature and gas mix (Knittel and Roof, 1999). The peak of the infection occurs between five and seven days post-inoculation reaching 80-90% of infected cells (Gebhart and Guedes, 2010).

Clinical and subclinical disease has been previously reproduced using pure cultures of *L. intracellularis* at low passages (4 to 20) in cell culture (Guedes and Gebhart, 2003b; McOrist et al., 1993; McOrist et al., 1996b; Vannucci et al., 2013). Lack of clinical and pathological changes was demonstrated in pigs experimentally infected with a porcine isolate passed 40 times *in vitro* indicating that the attenuation process occurs between passage 20 and 40 (Vannucci et al., 2013). The loss of pathophysiological properties of *L. intracellularis* associated with the adaptation to *in vitro* conditions due to mutational events at DNA level has been speculated. However, standard DNA-based typing techniques, such as pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and variable number tandem repeat (VNTR) have shown identical genotypes in homologous isolates at low passage and high passage (Beckler et al., 2004; Kelley et al., 2010; Oliveira and Gebhart, 2008).

The whole genome of a porcine *L. intracellularis* isolate (PHE/MN1-00) was sequenced and annotated using Sanger-based sequencing and revealed a total of 1,719,014 base pairs distributed into one small chromosome and three plasmids. This information followed by the recent advances in sequencing technology has been crucial

for studies focused on the differences regarding genome composition and gene expression between isolates at low and high passages (Vannucci et al., 2012a; Vannucci et al., 2012c). The identification of high expression of numerous bacterial genes encoding hypothetical proteins during *L. intracellularis* infection *in vitro* and *in vivo* suggests that this organism has adopted mechanisms of survival and pathogenesis that are unique among bacterial pathogens (Vannucci et al., 2012b; Vannucci et al., 2012c).

1.3 Differences among host species

Intestinal hyperplastic lesions are present in every case of PE; however, there are some differences between clinical and pathological presentations among affected species. Pyogranulomatous enteritis has been specifically reported in hamsters in the late stage of the disease (Jacoby et al., 1975; Vannucci et al., 2010). Two main clinical presentations have been recognized in pigs, porcine intestinal adenomatosis (PIA) and proliferative hemorrhagic enteropathy (PHE). While PIA is most commonly observed in post-weaned pigs between 6 and 20 weeks of age, PHE mainly affects young adults 4–12 months of age which are represented by finishing pigs, gilts and boars (McOrist and Gebhart, 2012). In severe cases, the end-stage of PIA may result in necrotizing enteritis characterized by coagulative necrosis of the adenomatous mucosa (Rowland and Hutchings, 1978). An aggravation of the adenomatous chronic form of PE has also been reported in horses, but the clinical presentation is characterized by rapid deterioration, hypoproteinemia, anorexia, fever, lethargy and secondary bacteremia presumably caused by opportunistic organisms (Deprez et al., 2005; Duhamel and Wheeldon, 1982; Page et al., 2012). In

some cases, hypoproteinemia due to hypoalbuminemia may represent the only clinicopathological abnormality of *L. intracellularis* infections, specifically in horses without evidence of diarrhea (Frazer, 2008).

Despite these host-specific features, no genotypic differences have been demonstrated based on 16S ribosomal DNA sequences among isolates from different affected species (Cooper et al., 1997a). Experimental infections have demonstrated the reproduction of typical PE in animals infected with species-specific isolates (Jacoby, 1978; McOrist et al., 1993; Pusterla et al., 2010). Hamsters experimentally infected with intestinal homogenate from affected hamsters showed cannibalism, aggressiveness, dehydration and profuse diarrhea (Jacoby, 1978; Jacoby et al., 1975). A cross-species experimental study using pure culture of *L. intracellularis* showed marked clinical signs, longer periods of bacterial shedding and stronger serologic immune responses in foals and pigs experimentally infected with their species-specific isolates (Vannucci et al., 2012d). This study also showed lack of clinical signs, low levels of bacterial shedding and low levels or absence of serologic immune responses in foals infected with the porcine isolate and pigs infected with the equine isolate. These results suggest that host susceptibilities to PE are driven by the origin of the *L. intracellularis* isolate. Additionally, based the uncommon opportunity of pigs and foals to share the same environment in the modern pig production system or horse breeding farms, any direct cross-species transmission in the field between these two species is unlikely (Vannucci et al., 2012d). Sampieri et al (2013) showed that the host adaptation previously demonstrated in pigs and horses seems to extend to hamsters and rabbits, respectively,

which are used as experimental infection models. Rabbits were more susceptible to equine-derived isolates than to porcine variants and hamsters were more susceptible to porcine-derived isolates than to equine variants (Sampieri et al., 2013). Taken together, this evidence suggests that *L. intracellularis* variants may have evolved to be adapted to more than one host species. However, the evolutionary ecology responsible for driving host-adapted variants remains to be determined.

The fastidious properties related to the cultivation of *L. intracellularis in vitro* has limited studies associating the phenotypic observations described above with genotypic analysis of bacterial isolates derived from different species. So far, there are approximately 17 isolates worldwide. A recent study identified the presence of a prophage-associated genomic island in the genome of pathogenic porcine *L. intracellularis* isolates and in pigs naturally infected with PE by fecal PCR (Vannucci et al., 2012a). Conversely, neither a pathogenic equine isolate nor positive fecal samples by PCR from PE infected horses and rabbits exhibited the presence of this genomic island. This region comprises 15 genes downstream from the prophage DLP12 integrase gene, but it does not appear to be associated with virulence (Vannucci et al., 2012a). However, this genetic element represents the major genomic variation reported among *L. intracellularis* isolates since, to date, the bacterium has been considered a monotypic organism (Kroll et al., 2005). The genetic diversity characterized by the absence of this chromosomal island in horse isolates may drive the characterization of novel *L. intracellularis* subspecies or species-adapted genotypes. Additionally, it is possible that this genetic element contributed to an ecological specialization of pig-adapted variants.

Although there are many challenges to studying the diversity of *L. intracellularis* isolates, more comprehensive studies are required in order to define whether this prophage-associated genomic island is exclusively harbored by porcine-specific variants.

1.4 Diagnosis

The diagnosis of PE has been historically based on clinical and pathological characteristics of the *L. intracellularis* infection (McOrist and Gebhart, 2012). The hemorrhagic form, proliferative hemorrhagic enteropathy (PHE), is commonly observed in replacement gilts and boars from high health herds that were introduced into a new farm site (Kroll et al., 2005). PHE-affected pigs show acute red-tinged loose to watery diarrhea and often are found dead, with no prior clinical signs detected, and with residual fecal contents in the perianal region. Differential diagnosis includes acute brachyspiral colitis (caused by *Brachyspira hyodysenteriae* and "*B. hampsonii*") salmonellosis, gastric ulcers and hemorrhagic bowel syndrome (HBS).

Pigs affected with the chronic form, porcine intestinal adenomatosis (PIA), predominantly exhibit diarrhea, anorexia and poor growth. Subclinical cases have often been recognized especially in endemic herds by the intermittent shedding of *L. intracellularis* in the feces of infected pigs which is associated with poor growth (Jacobson et al., 2003). Significant variation on the growth performance among animals of the same age is commonly the first indicator to pursue the diagnosis in subclinically affected herds. Based on the high prevalence of the *L. intracellularis* in swine herds worldwide, the single detection of the bacterial DNA in the feces may not represent the

final diagnosis at herd level. As a result, a more comprehensive diagnostic investigation is required in order to establish the health status of the herd by also considering other enteric diseases that mimic the clinical presentation of PE, such as brachyspiral colitis, salmonellosis, colibacillosis, transmissible gastroenteritis (TGE), diarrhea caused by porcine circovirus 2 (PCV2) and hypersensitivity to soybean (McOrist and Gebhart, 2012).

In horses, PE is referred to as equine proliferative enteropathy (EPE) and is primarily observed in post weaning foals (2-8 months) and occasionally in adult animals (Frazer, 2008). Clinically affected horses commonly show lethargy, anorexia, fever, peripheral edema (ventrum, sheath, throatlatch and distal limbs), weight loss, colic and diarrhea. Other common gastrointestinal disorders in horses considered in the diagnostic investigation of EPE include parasitism, bacterial infections (*Clostridium* spp., *Salmonella* spp., *Rhodococcus equi*), rotavirus, coronavirus, ulcerations and intestinal obstructions (Pusterla and Gebhart, 2009). The most consistent finding in EPE-affected horses is hypoproteinemia due to hypoalbuminemia. Furthermore, abdominal ultrasonography has been used to demonstrate increased thickness of the intestinal wall in affected horses (Deprez et al., 2005; Pusterla and Gebhart, 2009).

Regardless of the affected species, the *ante mortem* diagnosis of PE is based on the detection of *L. intracellularis*-specific IgG by serology and the molecular identification of *L. intracellularis* DNA in feces by PCR (Guedes et al., 2002; Pusterla et al., 2009b). While positive serology characterizes exposure to infection rather than disease, the positive results by PCR indicate shedding of the bacteria and active infection.

Horses clinically affected exhibit higher serological titers than pigs when tested for *L. intracellularis*-specific antibodies by immunoperoxidase monolayer assay (IPMA) (Vannucci et al., 2012d). On the other hand, affected horses shed bacteria for shorter periods of time compared with pigs (Guedes and Gebhart, 2003b; Pusterla et al., 2010). This host-specific pattern of shedding may represent one of the reasons the subclinical form has been well-described in pigs by the intermittent shedding throughout the growing stage, but in horses has been challenging to diagnose. Additionally, the association of subclinical cases and chronic growth retardation or unthriftiness in horses remains unclear (Pusterla and Gebhart, 2009).

The primary lesion observed in all PE-affected species is the cellular proliferation described as hyperplasia of the intestinal crypt cells (Gebhart and Guedes, 2010). The *post mortem* diagnosis is based on the indicative gross lesions associated with histological changes characterized by the adenomatous proliferation of enterocytes predominantly in the crypts of Lieberkühn (Gebhart and Guedes, 2010). Macroscopic lesions are typically found in the aboral portion of the small intestine but also are observed in the upper and mid jejunum, cecum and proximal colon. The intestine of pigs affected by PHE shows dilatation, thickening and corrugation of the serosal surfaces. Blood and fibrin clots are commonly found in the intestinal lumen (Rowland and Lawson, 1973). Chronic cases in all susceptible species are recognized by the thickening of the intestinal mucosa. The progression of the chronic proliferative lesions can lead to rapid clinical decline and death worsening due to mucosal destruction (Ward and

Winkelman, 1990). Hypertrophy and thickening of the muscularis mucosa may occur at later stages of necrotic enteritis.

The adenomatous aspect of the intestinal mucosa is histologically characterized by the proliferation of enterocytes in the intestinal crypts which are elongated, enlarged and crowded with immature epithelial cells (Rowland and Lawson, 1974). Marked reduction of goblet cells in affected areas is another important component associated with typical PE lesions. Infiltration of inflammatory cells is minimal or absent and only evident in later stage lesions (McOrist et al., 2006). Therefore, inflammation is not a primary characteristic of the *L. intracellularis* infections. The presence of vibrio-shaped intracellular bacteria in the apical cytoplasm of these proliferative enterocytes can be demonstrated by Warthin-Starry silver staining. However, the technique does not specifically identify *L. intracellularis* organisms and has limitations in autolyzed and necrotic samples (Jensen et al., 1997). Immunohistochemistry staining (IHC) using *L. intracellularis*-specific antibody has been applied as the gold standard for *post mortem* diagnosis of PE. In addition, this procedure is also used to determine the level of infection based on the amount of labeled *L. intracellularis*-specific antigen present in the intestinal mucosa (Guedes and Gebhart, 2003a; Vannucci et al., 2013; Vannucci et al., 2010).

1.5 Pathogenesis

1.5.1 Exposure

Comprehensive studies describing the progression of the PE lesions have been well-described in hamsters (Jacoby et al., 1975; Vannucci et al., 2010) and pigs (Guedes and Gebhart, 2003a; Roberts et al., 1977). However, limited studies have explored the early events involved in *L. intracellularis* infection. Boutrup et al (2010) showed the presence of intracellular bacterium in mature enterocytes at the tips of villi 12 hours after oral inoculation in pigs (Boutrup et al., 2010a). Although the study showed the ability of *L. intracellularis* to infect differentiated mature enterocytes, the organism seems to persist in the crypt cells once heavily infected crypts have been described, from day 5 to 28 days post-infection (Boutrup et al., 2010a; Vannucci et al., 2013).

During the estimated 12 hours until the bacteria reach the enterocyte following oral exposure; it is assumed that *L. intracellularis* should be able to cope with stressful and competitive microenvironments, such as gastric pH and oral and intestinal microbiota. The functional analysis of the *L. intracellularis* genome showed the presence of two systems that play a crucial role in the maintenance of pH homeostasis, glutamate decarboxylase (GAD) system and the F_0F_1-ATPase operon. Interestingly, the GAD system has been primarily identified in bacteria which transit the low pH of the gastric environment prior to causing foodborne disease, such as *Listeria monocytogenes*, *Shigella flexneri* and *Escherichia coli* (Smith, 2003). The intracellular decarboxylase enzyme in the bacterial cell uses glutamate to neutralize protons (H^+) producing the neutral compound γ-aminobutyrate (GABA). As a result, intracellular H^+ is consumed in the reaction, contributing to increased cytoplasmic pH. The F_0F_1-ATPase operon is essential for pH homeostasis in bacterial cells by encoding a transmembrane protein that

generates a proton motive force capable of removing intracellular H^+ when it hydrolyzes ATP through its cytoplasmic domain (Ryan et al., 2008). Although there is no mechanistic evidence demonstrating the activity of these acid tolerant systems in *L. intracellularis*, their role in responding to acid stress has been well-described among various other enteric pathogens (Bearson et al., 1997).

The presence of a single polar flagellum in *L. intracellularis* demonstrated in extracellular bacteria present in the supernatant of infected cells *in vitro* also represents an important phenotypic component that potentially is involved in the early stages of the infection (Smith and Lawson, 2001). Enteric pathogens exhibiting flagellar activity are able to exist and penetrate the mucous layer and thus reach the apical membrane of enterocytes. Although the flagellum has not been visualized either on bacteria at the surface or in the intracellular location *in vivo*, the whole genome sequence of a porcine *L. intracellularis* isolate shows various genes involved in the flagellar assembly. As a result, this element is likely to contribute to survival or colonization on extracellular stages.

Experimental observations have suggested an important influence of the intestinal microbiota on the ability of *L. intracellularis* to colonize and survive in the intestinal tract and, consequently, cause proliferative lesions. While germ-free pigs are not susceptible to infection by a pure culture of *L. intracellularis* (McOrist et al., 1994), pigs exposed to intestinal material from infected animals develop the disease (McOrist and Lawson, 1989). In addition, the influence of diet on the infection has been reported in hamsters and pigs (Boesen et al., 2004; Jacoby and Johnson, 1981). Molbak et al (2008) described a relative reduction in the amount of *L. intracellularis* in the total ileal microbiota of pigs

fed with a non-pelleted diet (Mølbak et al., 2008). However, the specific role of either diet or intestinal microbiota in facilitating *L. intracellularis* infection remains unclear.

1.5.2 Intracellular entry

Following the inoculation of *L. intracellularis* in cultured enterocytes, bacteria were found in close association with cell surfaces after 10 min and internalized within membrane-bound vacuoles after three hours (McOrist et al., 1995b). Virulence factors associated with adhesion and entry into enterocytes have not been experimentally characterized. While the attachment mechanism appears to require specific host-bacterium interaction, the process of invasion does not depend on the viability of the *L. intracellularis* cells (Lawson et al., 1995; McOrist et al., 1995b). Lawson et al. (1995) demonstrated internalization of formalin-killed organisms and reduced bacterial invasion after blocking the cytoskeleton rearrangement using cytochalasin D *in vitro*. This evidence suggests that bacterial entry is dependent on the host cell activity and actin polymerization (Lawson et al., 1995). Nevertheless, the refereed study also described intracellular bacteria in cytochalasin D-treated cells suggesting that other mechanisms are involved in the entry process.

The analysis of the genome sequence of a porcine *L. intracellularis* isolate revealed the presence of genes encoding the entire apparatus of the type 3 secretion system (T3SS) divided into two distinct chromosomal regions. The expression of three proteins representing the energizing, core element and structural component of the T3SS

were demonstrated in three other porcine isolates (Alberdi et al., 2009). This study indicates that the T3SS element is conserved among *L. intracellularis* isolates and potentially contributes to the pathogenicity during the entry process, regardless of the host cell activity.

1.5.3 Survival and replication

Ultrastructural studies *in vitro* and *in vivo* have shown that *L. intracellularis* is typically found living free within the enterocyte cytoplasm during the active stage of the infection (Johnson and Jacoby, 1978; McOrist et al., 1995b). The dynamic of the infection after internalization has only been well-characterized using an *in vitro* model (McOrist et al., 1995b). The majority of the intracellular bacteria were observed lying free in the cell cytoplasm after three hours of exposure, indicating that the breakdown of the membrane-bound vacuoles occurs shortly after bacterial entry. A recent study identified high expression of *L. intracellularis* genes included in an operon genetically related to the *Salmonella* pathogenicity island 2 (SPI2) (Vannucci et al., 2012b). Since the activation of all of the three genes comprising this SPI2-related operon was observed to be expressed by *L. intracellularis* in the enterocyte cytoplasm *in vivo*, this operon appears to play a similar role from the SPI2 during the intracellular stage of the *Salmonella* infection (Vannucci et al., 2012b). The proteins identified in the *L. intracellularis* SPI2-related operon are expressed by *S. enterica* Typhimurium in the cell endosome and translocate virulence factors into the host cytoplasm (Hensel et al., 1998). These secreted proteins interfere with the host intracellular trafficking and have a central role for survival of the *Salmonella* in infected macrophages (Uchiya et al., 1999). In other

enteric pathogens, such as *Shigella*, *Listeria* and *Rickettsia* spp, the secretion of lytic toxins (cytolysins or hemolysis) has been reported as an important mechanism for escape into the host cytoplasm (Huang and Brumell, 2009). Although the expression of a hemolytic protein and the cytolytic activity of *L. intracellularis in vitro* have been reported (McCluskey et al., 2002), more recent studies did not identify the expression of the gene encoding hemolysin by intracellular bacteria during infection *in vitro* or *in vivo* (Vannucci et al., 2012b; Vannucci et al., 2012c). Therefore, the specific function of this protein remains to be defined.

Following vacuolar escape, *L. intracellularis* divides by binary fission in the apical portion of the cell cytoplasm beginning from two to six days after infection *in vitro* (McOrist et al., 1995b). Inhibitors of cell growth reduced the bacteria multiplication *in vitro*, suggesting that actively dividing cells are required for bacterial replication (Lawson et al., 1995). An ultrastructural study *in vivo* showed replication of bacteria free in the cytoplasm of villus and crypt cells at day 5 post-infection, but the hyperplastic changes characterized by proliferation and elongation of crypt cells were histologically and macroscopically observed from 10 and 12 days post-infection, respectively (Jacoby, 1978; Johnson and Jacoby, 1978). During the active stage of the infection, numerous dividing bacteria are located in the apical cytoplasm which is otherwise composed almost entirely of free ribosomes and scattered mitochondria (Johnson and Jacoby, 1978). The microaerophilic properties of *L. intracellularis* and its proximity to the host mitochondria creates a paradox for intracellular survival of the bacterium, mainly due to the oxidative

phosphorylation supported by the continuous transport of oxygen through the mitochondrial membrane (Koyama et al., 1989).

Evaluation of the transcriptional profiling of *L. intracellularis in vitro* and *in vivo* revealed high levels of expression of a sophisticated oxidative protection mechanism involving Cu-Zn superoxide dismutase (*sodC*), rubrerythrin-rubredoxin operon (*rubY-rubA*) operon and dioxygenases (Vannucci et al., 2012b; Vannucci et al., 2012c). Surprisingly, the gene *sodC* which plays a key role in bacteria's ability to cope with the oxidative stress is located immediately upstream of the gene encoding ATP/ADP translocase. This enzyme has been shown to be critical for metabolism of *Lawsonia* and two other groups of obligate intracellular bacteria (*Chlamydiales* and *Rickettsiales*) (Schmitz-Esser et al., 2008; Schmitz-Esser et al., 2004). In these organisms, ATP/ADP translocase catalyzes the exchange of bacterial ADP for host ATP and thus allows bacteria to exploit their hosts' energy pool, a process referred to as energy parasitism. Taken together, this evidence suggests that *L. intracellularis* is able to cope with the oxidative stress while utilizing the host energy pool.

1.5.4 Persistence and spread

The consistent presence of *L. intracellularis* in intestinal crypt cells observed during the active stage of the infection reveals a clear tropism of this organism for immature dividing enterocytes. Additionally, rapidly dividing cells in culture promote the growth of the bacteria better than differentiated non-dividing cells (McOrist et al., 1995b). The continuous proliferation and vertical migration of crypt cells through the crypt-villus axis mediates the spread of bacteria in the intestinal epithelium (Jacoby,

1978). This point highlights the particular adaptation of *L. intracellularis* to a specific intracellular microenvironment.

Highly infected enterocytes *in vitro* release numerous *L. intracellularis* organisms within balloon-like protrusions in the host cell cytoplasm, indicating that these released organisms are capable of infecting neighboring enterocytes (McOrist et al., 1995b). Cell-to-cell movement has also been speculated for *L. intracellularis* spread, the benefit of which would be to spread without exposing the bacteria to the extracellular milieu (Smith and Lawson, 2001). The propulsion of *Shigella* and *Listeria* into adjacent cells by polymerized actin has been well-described. However, these enteroinvasive bacteria are randomly distributed in the cell cytoplasm, while *L. intracellularis* is mostly present in the apical cytoplasm of infected enterocytes (Carlsson and Brown, 2006). Therefore, the potential mechanism of actin-based motility by which *L. intracellularis* utilizes to spread among infected cells may be different from those used by other intracellular pathogens.

PE lesions are restricted to the intestinal epithelium and the dissemination of active infection to other organs has not been reported. The detection of bacterial antigens in mesenteric lymph nodes and tonsilar crypt cells has been demonstrated, but attributed to the carriage of *L. intracellularis* antigens by infected macrophages (Gebhart and Guedes, 2010). Bacterial antigens persist within the site of lesions at later stages of the disease (Gebhart and Guedes, 2010).

The movement of *L. intracellularis* through the lamina propria has been recently discussed as an alternative route for spreading the infection from crypt-to-crypt in the intestinal tract (Boutrup et al., 2010a; Jensen et al., 2006). Intact and degraded organisms

in the cytoplasm of macrophages present in the lamina propria were first described by Jacoby and Johnson (1978) using transmission electron microscopy (Johnson and Jacoby, 1978). Recently, indirect evaluation of the bacterial integrity in the lamina propria was reported based on fluorescence in-situ hybridization (FISH) (Boutrup et al., 2010a). The authors demonstrated the presence FISH-positive *L. intracellularis* at early stages of the infection suggesting that the bacteria were intact and viable. Although these studies suggest the viability of *L. intracellularis* within phagocytic cells or in the extracellular matrix, it is also possible to speculate that these organisms will soon be degraded by phagocytosis and then replaced by other viable organisms coming from the epithelial layer. A chronological study evaluating the ability of *L. intracellularis* to persist within the cytoplasm of macrophages may be crucial to elucidate this route of spreading.

1.5.5 Inducing enterocyte proliferation

Intracellular replication of *L. intracellularis* is directly associated with enterocyte proliferation (McOrist et al., 1996a). But the means by which the bacterium induces proliferative changes is unknown. Infections caused by *Citrobacter rodentium*, the etiologic agent of transmissible murine colonic hyperplasia, produce morphological changes in the intestinal epithelium that most resemble the lesions seen in PE. However, *C. rodentium* induces cell proliferation when attached to the apical membrane (Luperchio and Schauer, 2001) revealing the potentially unique mechanism adopted by *L. intracellularis* to induce proliferation.

The dynamics of *L. intracellularis* infections have been well-described *in vitro* and *in vivo* (Johnson and Jacoby, 1978; McOrist et al., 1995b). While proliferative

changes *in vivo* follow an increase in the number of intracellular bacteria, the intracellular organisms are not able to induce proliferation in *in vitro* models of infection (Vannucci et al., 2012e). This interesting observation brings up the question of whether *L. intracellularis* expresses its virulence factors *in vitro* or whether the infected cells in culture are not able to exhibit the proliferative response due to the lack of factors present during the infection *in vivo*. Recent studies have shown that most of the *L. intracellularis* genes highly expressed *in vitro* are also significantly activated *in vivo* (Vannucci et al., 2012b; Vannucci et al., 2012c). These findings suggest that the lack of proliferation *in vitro* likely is related to the inability of the host cells to mimic the proliferative response observed *in vivo*. Further studies testing the proliferative responses of different cell types *in vitro*, including undifferentiated lineages, would be valuable to establish an *in vitro* model displaying the proliferative phenotype and which can then overcome this limitation to evaluating the host response. The chronic course of the disease in experimentally-infected animals is an additional complicating feature in studying the pathogenesis of *L. intracellularis* infections. A ligated intestinal loop model was applied to study early stages of PE, but it is not feasible for investigating the molecular pathogenesis involved in the cell proliferation (Boutrup et al., 2010b).

A microarray-based study of fibroblastic-like cells *in vitro* identified altered transcription of genes related to cell cycle and cell differentiation (Oh et al., 2010). However, the main phenotypic changes characterized by cell proliferation were not reproduced and the results need to be confirmed *in vivo*. Additionally, the referred study was based on the response of mesenchymal tissue-derived cells which may not

sufficiently mimic the response of enterocytes in affected animals. Another microarray analysis of field cases of pigs with diarrhea focused more on the host immune response and demonstrated changes on the expression of genes related to cell differentiation in pigs affected with the hemorrhagic form of PE (Jacobson et al., 2011). The authors did not identify any significant changes in the expression of cell cycle-associated genes regardless of the clinical form of PE. Although this study provided an interesting snapshot in time of the host response, porcine circovirus type 2 was present in some samples and confounded the evaluation of the gene expression (Jacobson et al., 2011). Additionally, this use of the entire intestinal tissues for microarray analysis needs to be taken into consideration when interpreting any changes in gene expression. The heterogeneity of the cell population in the intestine may compromise conclusions regarding the specific host response of *Lawsonia*-infected enterocytes.

Recently the host-bacterial interaction was studied *in vivo* by integrating laser microdissection and RNA-seq technology. *Lawsonia*-infected enterocytes were isolated and the gene expression profile was characterized (Vannucci et al., 2012b). Proliferative infected enterocytes showed significant activation of DNA transcription, protein biosynthesis and genes acting on the G_1 phase of the host cell cycle (RhoA, RhoB and Rho GTPase). While an increase in protein synthesis and transcription is required during the G_1 phase for preparing the host cell for subsequent division (Schafer, 1998), the aberrant activation of Rho-genes has been well-described in oncogenesis by causing deregulation of cell cycle progression and promoting cell proliferation (Pruitt and Der, 2001). Rho proteins specifically act on the G_1-checkpoint when the transition to commit

the cell to the proliferative stage occurs. If the signals responsible for promoting this transition are not present, then the cells enter into the non-proliferative phase (G_0) (Oswald et al., 2005). Bringing these findings into the context of bacterial infections, Rho proteins have been shown to be pathologically activated by bacterial toxins, known as cyclomodulins (Nougayrède et al., 2005). Cytotoxic necrotizing factor (CNF) found in uropathogenic *Escherichia coli*, *Pasteurella multocida* toxin (PMT) and dermonecrotic toxin of *Bordetella* spp. act directly on Rho family proteins promoting their irreversible activation (Lemonnier et al., 2007). Although there is no information regarding the production of toxins by *L. intracellularis*, various genes highly expressed during the infection *in vitro* and *in vivo* encode hypothetical proteins that were not recognized so far (Vannucci et al., 2012b; Vannucci et al., 2012c). It is possible to speculate that the presence of an unrecognized cyclomodulin is encoded by these highly expressed genes, but their specific properties and functions need to be determined. Some bacterial toxins have been shown to specifically activate Rho GTPase protein (Lemonnier et al., 2007). In addition to the study *in vivo* mentioned above, Rho GTPase gene was highly up-regulated during *L. intracellularis* infection *in vitro*, but no proliferative phenotype was observed in cultured cells (Oh et al., 2010). The co-activation of other Rho-genes (RhoA and RhoB) observed *in vivo* but not *in vitro* might be essential to induce the proliferative signaling in infected cells. Significant expression of the gene encoding the insulin-like growth factor binding protein IGFBP-3 was described *in vitro* and in field cases of diarrhea; however, the role of this growth factor in the *L. intracellularis* infection has not been elucidated.

Uropathogenic *E. coli* expressing CNF toxin establish a persistent intracellular infection in the urogenital tract and suppress apoptosis by affecting the transcription levels of the Bcl-2 family genes (Lemonnier et al., 2007). It is thought that apoptosis inhibition in target cells may favor bacterial persistence at the epithelium surface, thereby favoring bacterial replication and spread inside the host cell (Mulvey et al., 1998). A predominant activation of anti-apoptotic-related genes was observed during *L. intracellularis* infection *in vivo*, but the specific activation of Bcl-2 was not statistically significant (Vannucci et al., 2012b). The role of apoptosis in *L. intracellularis* infections has been speculated over the years and still needs to be elucidated. Initially, a temporary reduction of apoptosis was thought to be an important mechanism involved in cell proliferation (McOrist et al., 1996a). Later studies suggested an increase in apoptosis based on the Caspase-3 immunohistochemical staining (Boutrup, 2008; Gebhart and Guedes, 2010). Boutrup (2008) studied the dynamic of the Caspase-3 staining through the chronological evaluation of experimentally-infected pigs and showed variations on the pattern of Caspase-3 staining within different parts of the intestinal mucosa (Boutrup, 2008). The gene-encoding Caspase-3 was found to activate infected enterocytes *in vivo*, but it did not reach the parameters for statistical significance (Vannucci et al., 2012b). These inconsistent findings associated with the complexity of the apoptosis process suggest that the stage of the infection may directly influence the dynamic of apoptotic events throughout the course of the infection. A comprehensive study focusing on the regulatory events specifically related to the apoptotic networks might elucidate these questions.

1.5.6 Preventing enterocyte differentiation

Along with the cell proliferation potentially induced by deregulation of the G_1 phase of the host cell cycle, the lack of differentiation in infected enterocytes was demonstrated recently by the repression of membrane transporters related to digestion and nutrient acquisition (Jacobson et al., 2011; Vannucci et al., 2012b). The transporters anchored on the apical membrane of *Lawsonia*-infected enterocytes that are most affected are those involved in the absorption of carbohydrates (sodium/glucose transporter), amino acids (cationic amino acid transporter), bile acid (ileal sodium/bile acid transporter), lipids (lipid phosphate phosphohydrolase) and Vitamin B_{12} (cubilin receptor) (Vannucci et al., 2012b). This reduction in nutrient absorption indicates that *L. intracellularis* is able to prevent the differentiation of infected enterocytes and promote a malabsorptive syndrome resulting in poor performance and growth of affected animals (Bihr, 2003; Vannucci et al., 2010).

Another potential component involved in the pathophysiology of the diarrhea in PE affected animals is an exacerbated increase of electrolyte secretion due to the large presence of proliferating crypt cells that physiologically secrete electrolytes into the intestinal lumen through a chloride channel (Moeser and Blikslager, 2007; Welsh et al., 1982). However, the gene encoding this chloride channel was significantly down-regulated in *Lawsonia*-infected enterocytes, suggesting that the inhibition of cell differentiation occurs at early stages in undifferentiated crypt cells (Vannucci et al., 2012b). As a result, disruption of the secretory process does not appear to be involved in the pathogenesis of PE. Based on this evidence, the excessive presence of non-absorbed

solutes in the intestinal lumen osmotically retains water resulting in osmotic/malabsorptive diarrhea.

1.5.7 Immune response

The primary observation regarding the immune response in *L. intracellularis* infection is the very limited inflammatory infiltration reported in animals severely affected with PE and while exhibiting fully developed proliferative lesions (Rowland and Rowntree, 1972). The indication of an immunosuppressive mechanism induced by *L. intracellularis* has been discussed and evidenced over the years. MacIntyre et al (2003) demonstrated reduction in the number of T cells and B cells in heavily infected pigs (MacIntyre et al., 2003). Gene expression studies *in vivo* have consistently shown down-regulation of genes related to the immune response including those acting against intracellular pathogens (e. g. tumor necrosis factors) (Jacobson et al., 2011; Vannucci et al., 2012b). Interestingly, the significant up-regulation of the MHC class I gene in infected enterocytes *in vivo* indicates that *Lawsonia*-derived antigen is presented on the basolateral membrane to cytotoxic T cells located in the lamina propria (Vannucci et al., 2012b). However, the same study showed significant down-regulation of the signaling lymphocytic activation molecule SLAM7 (also known as CD2) which acts as a co-receptor (accessory molecule) required for T cell activation (Bierer and Hahn, 1993). We hypothesize that this limited activation of cytotoxic T cells associated with the reduction in the overall population of lymphocytes within the infection site plays a major role in modulating the immune response and favoring persistence of the bacterial infection.

Humoral immune responses were first detected 2 weeks after challenge of pigs with pure culture of *L. intracellularis* and persisted in some pigs for 13 weeks after exposure (Guedes and Gebhart, 2003b). This study also demonstrated a cell-meditated response through detection of *Lawsonia*-specific interferon gamma produced by peripheral blood mononuclear cells (Guedes and Gebhart, 2003b). Serological IgG responses peaked near the end of the third week and then tended to drop (Knittel et al., 1998). Naturally infected pigs showed a large accumulation of IgA in the apical cytoplasm of proliferating enterocytes (McOrist et al., 1992). Although this study did not evaluate the specificity of this IgA to *L. intracellularis*, Guedes and Gebhart (2010) detected *Lawsonia*-specific IgA in intestinal lavage of experimentally-infected pigs three weeks after infection (Guedes and Gebhart, 2010). Pigs recovering from the clinical disease and exhibiting detectable IgG titers re-inoculated with *L. intracellularis* were protected from re-colonization and clinical disease (Collins and Love, 2007). As a result, naturally infected animals have a fair degree of immunity to a second exposure.

1.6 Conclusions

Proliferative enteropathy caused by *L. intracellularis* has been well-established as an important pathogen in the swine industry. While important advances have contributed to the recognition and control of the disease in pigs, studies focusing on the pathogenesis of PE have not been largely explored. Recently, the increasing reports of PE in horses brought new challenges and opportunities in understanding the pathogenesis and source of the infection. The question whether porcine *L. intracellularis* evolved into a horse-

adapted variant or whether the equine infection was underdiagnosed in the past encourages the development of new diagnostic tools to monitor the disease and potentially find unrecognized host species involved in the epidemiology of PE. The complete genome sequencing of *L. intracellularis* was essential for characterizing the transcriptional landscape of the bacterium *in vitro* and *in vivo* and for comparative genomic analysis. These studies have integrated genomic properties of *L. intracellularis* into phenotypic traits observed in PE-affected animals. Based on the comprehensive analysis of the bacterial transcriptome, future studies will be able to focus on specific properties of proteins involved in the organism's virulence and their potential usefulness for diagnosis and control of PE. Finally, new sequencing technologies will allow expansion of the comparison of bacterial genomes derived from different host species.

SECTION II

PROLIFERATIVE ENTEROPATHY – PATHOGENESIS

CHAPTER 2

Attenuation of virulence of *Lawsonia intracellularis* after *in vitro* passages and its effects on the experimental reproduction of porcine proliferative enteropathy

Vannucci F.A.; Beckler D.; Pusterla N.; Mapes S.M.; Gebhart C.J. (2013) Attenuation of virulence of *Lawsonia intracellularis* after *in vitro* passages and its effects on the experimental reproduction of porcine proliferative enteropathy. *Vet Microbiol* 162(1): 265-269.

Summary

Non-pathogenic *Lawsonia intracellularis* variants have been obtained through multiple passages in cell culture but there is no information regarding the number of passages necessary to attenuate a pathogenic isolate. The present study evaluated the susceptibility of pigs to *L. intracellularis* after 10, 20 and 40 passages *in vitro*. Three groups (six animals/group) were inoculated with pure culture of *L. intracellularis* from passage 10, 20 or 40 and one group with placebo. The animals were monitored for clinical signs, fecal shedding and serological IgG response during 28 days post-inoculation. Gross and histologic lesions and the level of infection based on the amount of *L. intracellularis*-specific antigen in the intestinal mucosa identified by immunohistochemistry were evaluated in two animals from each group on days 14, 21 and 28. Animals inoculated with passages 10 and 20 demonstrated proliferative lesions typical of porcine proliferative enteropathy associated with the presence of *Lawsonia*-specific antigen in the intestinal mucosa. Passage 40-inoculated pigs did not show proliferative lesions or presence of *Lawsonia* antigen at any time point throughout the study. Similar patterns of fecal shedding were observed in passage 10 and 20-infected pigs but those infected with passage 40 shed for a short period. Serological IgG responses in passage 10 and 20-inoculated pigs were detected from day 14 post-infection but not at all in passage 40-inoculated animals. These results demonstrate attenuation of the virulence properties of *L. intracellularis* between 20 and 40 cell passages *in vitro*. This information will be valuable for design of future experimental models and for studying the mechanisms involved in the attenuation of *L. intracellularis* virulence.

2.1. Introduction

L. intracellularis is an obligate intracellular bacterium and causative agent of porcine proliferative enteropathy (PPE). Experimental reproduction of PPE was initially described using homogenates of intestinal mucosa derived from infected pigs (Roberts et al., 1977). Despite the relative ease in obtaining and preparing the inoculum from infected intestinal mucosa, it is difficult to control for the presence of other potential pathogenic organisms in the inoculum. This may introduce bias in controlled studies to investigate the dynamics of *L. intracellularis* infection and host-pathogen interaction. Following the first isolation of *L. intracellularis* (Lawson et al., 1993), the disease was experimentally reproduced using pure culture, fulfilling Koch's postulates (McOrist et al., 1993). In contrast to the mucosal homogenate infection model, the pure culture model allows more rigid control of the inoculum and the accurate titration of the challenging dose of bacteria (Guedes and Gebhart, 2003a). The drawback for using this model is the difficulty of isolating, growing and maintaining virulence of the bacterium *in vitro*.

Clinical and subclinical disease has been previously reproduced using pure cultures of *L. intracellularis* harvested after 4 to 13 passages in cell culture (Guedes and Gebhart, 2003b; McOrist et al., 1993; McOrist et al., 1996b; Smith and McOrist, 1997). In addition, a non-pathogenic variant obtained through multiple passages in culture has been the basis for producing the commercial modified-live vaccine (Enterisol® Ileitis, Boehringer Ingelheim) (Kroll et al., 2004). Although our lab has observed a consistent loss of pathogenicity in various *L. intracellularis* isolates at high passage, there is no information regarding the number of passages necessary to attenuate a pathogenic *L.*

intracellularis isolate and the effects of serial passages on the clinical disease, bacterial shedding and immune response throughout the course of the infection. The objective of the present study was to evaluate the susceptibility of pigs to a homologous *L. intracellularis* isolate after 10, 20 and 40 passages *in vitro*. This data will be valuable for developing future experimental models and for studying the mechanisms involved in the attenuation of *L. intracellularis* virulence *in vitro*.

2.2 Materials and Methods

Animals

Twenty-four 3-week-old pigs were obtained from a herd with no history of PPE and divided into four groups (n=6/group). Blood and fecal samples were collected and tested for *L. intracellularis*-specific antibodies by immunoperoxidase monolayer assay (IPMA) and qPCR, respectively, in order to document the negative status for each animal. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Minnesota. On the challenge day, three groups were orally inoculated with pure culture of *L. intracellularis* isolate PHE/MN1-00 at passage 10, 20 or 40 and one group with sucrose-potassium glutamate solution (negative control group).

Experimental infection

A porcine *L. intracellularis* isolate, PHE/MN1-00 (ATCC PTA-3457), was previously obtained from the intestines of a 230-day-old gilt (Guedes and Gebhart, 2003a). The isolate was grown in continuous passages in cell culture using murine

fibroblast-like McCoy cells (ATCC CRL 1696) as described by Guedes and Gebhart, 2003a. At each of passages 10, 20 and 40 the bacteria were pelleted, suspended in sucrose-potassium glutamate (pH 7.0; 0.218 M sucrose, 0.0038 M KH_2PO_4, 0.0072 M K_2HPO_4 and 0.0049 M potassium glutamate) solution with 10% fetal bovine serum and stored at -80^{o}C until the day of infection. The molecular identity of the isolate was confirmed on each of the three different passages using variable number tandem repeat (VNTR) analysis (Beckler et al., 2004). The number of *L. intracellularis* organisms in each inoculum was assessed by direct counting after immunoperoxidase staining of serial 10-fold dilutions prepared in sterile phosphate-buffered saline using polyclonal *L. intracellularis*-specific antibody (Guedes and Gebhart, 2003c). Quantitative PCR (qPCR) was also performed to validate the direct counting and standardize the challenge doses for each of the passages.

Monitoring and sample collection

The animals were monitored daily for general attitude and diarrhea during 28 days post-inoculation (PI). Once weekly, the weight was individually recorded and blood was collected to determine average daily weight gains and humoral serologic responses, respectively. IPMA was performed to measure anti-*L. intracellularis* specific IgG responses, as previously described (Guedes et al., 2002). Positive serum samples (titer ≥ 30) were tested to endpoint dilution and titers were reported as the reciprocal of the dilution. Additionally, feces were collected every other day and analyzed by qPCR, as previously described (Pusterla et al., 2008b). Absolute quantification was calculated

using a standard curve for *L. intracellularis* and expressed as copy numbers of the *aspA* gene of *L. intracellularis* per gram of feces.

Two pigs from each group were randomly selected and euthanized on days 14, 21 and 28 PI and evaluated for typical PPE lesions. Intestinal samples from terminal jejunum, terminal ileum and proximal cecum were collected. Two histologic sections were prepared: one section was stained by hematoxylin and eosin and the other by immunohistochemistry (IHC) using the streptavidin method with polyclonal antibodies specific for *L. intracellularis* (Guedes and Gebhart, 2003c). The level of infection was assessed based on the amount of positively labeled antigen present in the intestinal sections: Grade 0 (-) = no positive antigen labeled; Grade 1 (+) = one isolated focal area of antigen labeled; Grade 2 (++) = multi-focal areas of antigen labeled; Grade 3 (+++) = majority of the mucosa has positive antigen labeled; and Grade 4 (++++) = all of the mucosa has positive antigen labeled (Guedes and Gebhart, 2003a). The pathologist was blinded to the sample identity for evaluation of the tissue sections.

Statistical analysis

The serologic response and pathological findings were reported in a descriptive way due to the small number of animals. The area under the curve (AUC) based on the amount and the duration of fecal shedding was calculated for each animal and the Kruskal-Wallis test (at $p < 0.05$) was performed using SAS software to assess differences between the infected groups (passages 10, 20 and 40).

2.3 Results

Based on the direct counting of *L. intracellularis* organisms and qPCR analyses, the challenge doses were standardized to 10^9 bacterial organisms per pig in all four study groups. Clinical disease based on watery consistency of the feces throughout the study period was observed intermittently in groups inoculated with passages 10 and 20 from days 10 and 12 PI, respectively. In addition, reduced average daily gains were observed in the passage 10 (0.32 $\pm$ 0.07 kg/day) and 20 (0.39 $\pm$ 0.08) group compared to the passage 40 (0.61 $\pm$ 0.10) and negative control (0.59 $\pm$ 0.09) groups.

Macroscopic lesions typical of PPE characterized by thickening of the ileal mucosa were observed at necropsy in all pigs inoculated with passages 10 and 20. On day 14 PI, necropsied animals showed focal corrugation of the ileal mucosa and mild to moderate hyperemia of the mesentery. The lesions were more severe and extensive in the passage 10 and 20-inoculated pigs which were necropsied 21 and 28 days PI. In one animal inoculated with bacteria after 10 passages *in vitro*, typical PE lesions extended to the mid-jejunum on day 21 PI. One animal from the same group showed focal macroscopic lesions in the cecum 28 days PI. No lesions were observed in the ceca of pigs inoculated with passage 20 at any time points. Interestingly, one passage 20-inoculated animal exhibited macroscopic lesions in the jejunum on day 28 PI.

Multifocal to coalescing hyperplasia of immature enterocytes typical of PPE and associated with absence of goblet cells were histologically observed in all pigs inoculated with the passages 10 and 20 of *L. intracellularis*. The severity of the proliferative lesions was more evident on days 21 and 28 PI and lesions were consistently associated with the

presence of *Lawsonia*-specific antigen in the intestinal mucosa demonstrated by IHC. Passage 40-inoculated pigs and the negative control group did not have proliferative lesions or the presence of *Lawsonia*-specific antigen at any time point throughout the study. The level of infection was evaluated based on the amount of *Lawsonia*-specific antigen determined by IHC in the intestinal mucosa of the jejunum, ileum and cecum (Table 2.1). The lack of positive samples in the group inoculated with passage 40 suggests complete loss of the pathogenic properties of this *L. intracellularis* isolate.

The magnitude and duration of fecal shedding in all three *L. intracellularis*-inoculated groups are summarized in Figure 2.1. Higher and longer shedding were observed in pigs inoculated with passages 10 ($p < 0.01$) and 20 ($p < 0.05$) compared with passage 40-infected animal. There was no statistical difference between passage 10 and 20-infected pigs, in which *L. intracellularis* DNA was observed intermittently from 2 to 8 days PI and then it was consistently detected from days 10 PI until the end of the study period (day 28 PI). Three out of six animals inoculated with the passage 40 showed fecal shedding of the bacteria on days 8, 10 and 14 PI. The peaks of bacterial shedding were 10^7 (days 18 and 20 PI) and 10^5 (days 16, 18 and 20 PI) in pigs infected with passage 10 and 20, respectively. Additionally, the earlier detection of the *L. intracellularis* DNA in the feces (day 2 PI) may be due to the higher molecular sensitivity of the qPCR compared to the conventional PCR (Nathues et al., 2009).

Serological IgG responses were observed in all pigs infected with passages 10 and 20 of *L. intracellularis* (Figure 2.2). Passage 10-infected pigs showed detectable titer ($\geq$ 30) earlier (day 14 PI) compared with passage 20-infected pigs (day 21 PI). Additionally,

IgG titers persisted or increased from days 21 to 28 in both passage 10 and 20-infected pigs. Passage 40-infected animals demonstrated no detectable titers at any time points throughout the study period.

Table 2.1. Level of infection based on the amount of *Lawsonia*-specific antigen determined by immunohistochemistry in the intestinal mucosa of two pigs per group per time point at necropsy.

Days PI	Passage 10			Passage 20			Passage 40		
	Jejunum	Ileum	Cecum	Jejunum	Ileum	Cecum	Jejunum	Ileum	Cecum
14	++	++	+	+	+	-	-	-	-
	+	+	+	+	+	+	-	-	-
21	+++	+++	++	++	++	+	-	-	-
	++++	+++	++	+	+++	+	-	-	-
28	++	+++	+	+++	++	+	-	-	-
	+	+++	++	++	+++	+	-	-	-

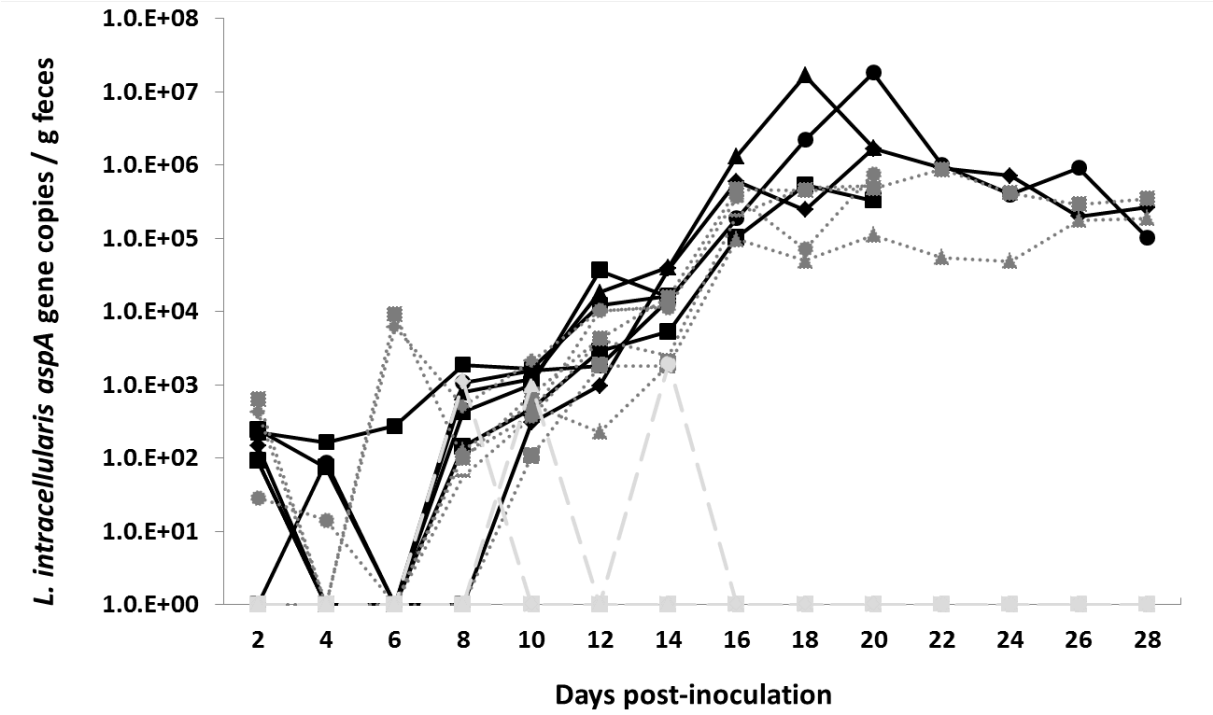

Figure 2.1. Quantities of *L. intracellularis* DNA in the feces performed by quantitative PCR. The results are expressed as copy numbers of *aspA* gene copies of *L. intracellularis* per gram of feces in animals infected with *L. intracellularis* isolate PHE/MN1-00 after 10, 20 and 40 passages in cell culture. (Black-solid lines: passage 10 group, dark gray dotted lines: passage 20 group and gray dashed lines: passage 40 group).

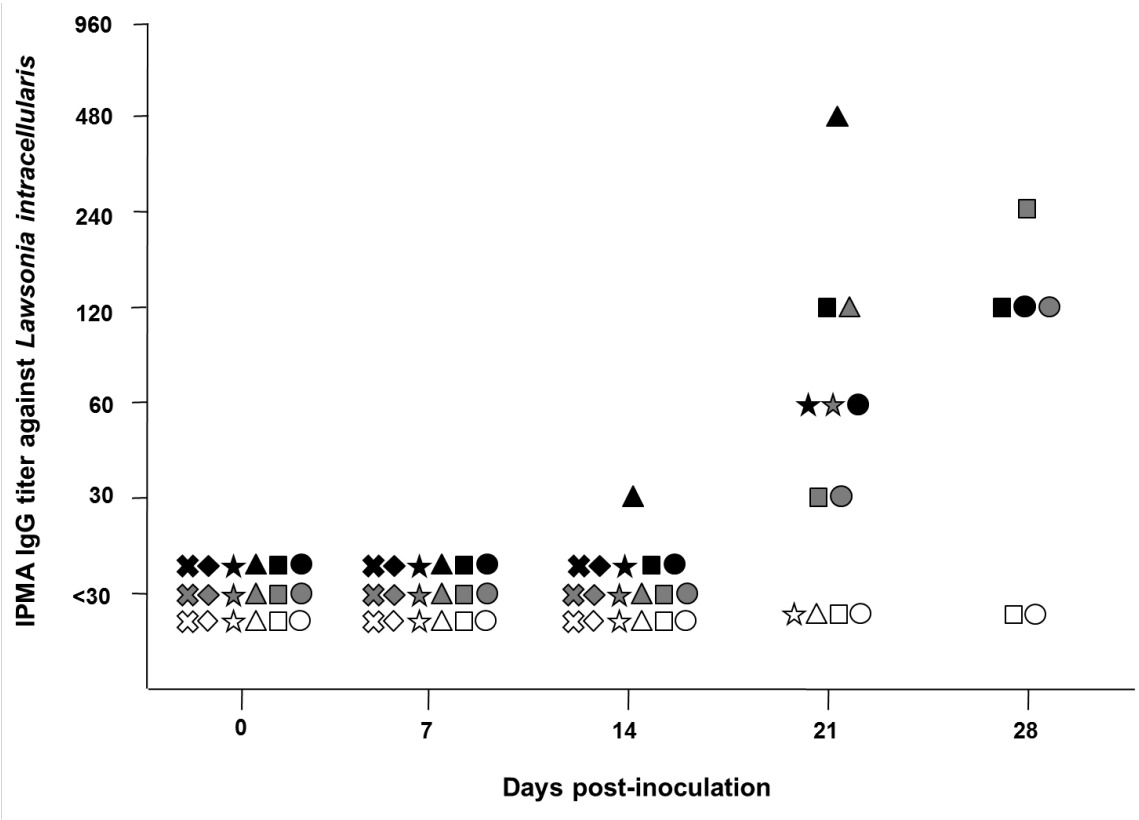

Figure 2.2 Serologic IgG response against *L. intracellularis* measured by IPMA throughout the study period (Black points: passage 10 group, gray points: passage 20 group and white points: passage 40 group).

2.4 Discussion

Experimental reproduction of clinical PPE characterized by diarrhea and poor growth performance has been reported using pure cultures after four to 12 passages in cell culture (Guedes and Gebhart, 2003a, b; McOrist et al., 1996b; Smith and McOrist, 1997). Similarly to these previous reports, in the present study diarrhea was more often observed between two and three weeks PI in passage 10 and 20-infected pigs. Using the same *L. intracellularis* isolate (PHE/MN1-00) at passage 9 containing 10^9 bacterial organisms, Guedes and Gebhart (2003a) described macroscopic lesions in the jejunum and ileum 21 days PI. McOrist et al. (1993) reported thickening of ileal and colonic mucosa 22 days PI in pigs inoculated with 10^6 *L. intracellularis* using a different bacterial isolate (916/91) at passage 6. However, this study detected no lesions in animals inoculated with 10^5 bacteria at passage 13, suggesting that either a minimal dose or minimal number of passages of *L. intracellularis* is required to reproduce lesions of PPE. In other experimental trials, Smith and McOrist (1997) challenged pigs with the *L. intracellularis* isolate 916/91 at passage 12 containing 10^8 bacterial organisms and described typical lesions of PE 21 days PI. Despite the fact that these two independent studies demonstrated gross lesions in pigs inoculated with the same bacterial isolate (916/91) at passage 12 but not at passage 13, the difference of 10^3 bacteria in the challenge doses may bias any conclusion regarding the loss of virulence from 12 to 13 passages *in vitro*. In addition, occasional microscopic lesions in the ileum were identified in passage 13-inoculated pigs (McOrist et al., 1993).

Previous studies using the pure culture infection model have monitored fecal shedding weekly by conventional PCR. In these studies, *L. intracellularis* DNA was first detected seven (Guedes and Gebhart, 2003a, b; Schwartz et al., 1999) or 14 (Smith and McOrist, 1997) days PI. The peak of bacterial shedding has varied between 14 to 28 days PI (Guedes and Gebhart, 2003a; Schwartz et al., 1999) but intermittent shedding has lasted 10 to 12 weeks in approximately 10% of infected animals after inoculation (Guedes and Gebhart, 2003b; Smith and McOrist, 1997).

Humoral immune responses in pure culture infection models have been measured using IPMA and indirect fluorescence antibody tests (IFAT) (Guedes and Gebhart, 2003b; Schwartz et al., 1999). Schwartz et al. (1999) used the N343 isolate between passages 8 and 12 and detected IFAT titers from day 21 PI. Guedes and Gebhart (2003) used the PHE/MN1-00 isolate at passage 9 and identified animals serologically positive by IPMA from day 14 PI until the end of the study period (13 weeks PI). In the same study, pigs exposed to the modified-live *L. intracellularis* vaccine (Enterisol® Ileitis, Boehringer Ingelheim) through the drinking water demonstrated IPMA titers from day 35 PI. This finding suggests that animals infected with passage 40 could show detectable IgG titers in the serum by IPMA at some point after 28 days PI.

Although our study phenotypically characterized the attenuation of virulence of *L. intracellularis* after serial *in vitro* passages, the molecular mechanisms responsible for this phenotype remain unclear. The loss of important pathophysiological characteristics associated with adaptation to *in vitro* conditions has been mainly due to mutational events at the DNA level and/or regulatory changes in gene expression (Fux et al., 2005). The

genetic adaptation to one environment may lead to loss of fitness in others. This hypothesis has been studied in laboratory-adapted *E. coli* in which repeated mutations are beneficial in one environment and detrimental in others (Cooper and Lenski, 2000). Therefore, genetic alterations during several generations *in vitro* are not random and have evolved bacterial genomes to an ecological specialization driven by the specific growth conditions (Cooper et al., 2003). Deletion, single-nucleotide polymorphism and tandem duplication have been reported in laboratory-adapted strains in other bacterial species and may account for their attenuated phenotypes (Fux et al., 2005). Additionally, these genomic variations also differently affect the immune response following the exposure to an attenuated strain (Behr, 2002). As a result, attenuation of virulence cannot be the only phenotypic characteristic required for development of live attenuated vaccines against intracellular bacteria. Ability to induce appropriate repertoire of immune responses including $CD4^+$ and $CD8^+$ T cell responses, to grow and maintain the bacterium in large scale conditions and to demonstrate safety, especially regarding the lack of reversion to virulence, are important requirements for an attenuated strain to be suitable as a vaccine. In the present study, the phenotypic observation in the attenuation of *L. intracellularis* virulence may be a starting point for mechanistic studies of this unexplored area involving the genetic basis for the attenuated phenotype and its potential association with the immune response.

In conclusion, the experimental reproduction of PPE was achieved using the *L. intracellularis* isolate PHE/MN1-00 up to 20 cell passages *in vitro*. The lack of clinical signs, serological IgG response, macroscopic or histologic lesions and *Lawsonia*-specific

antigen in passage 40-infected pigs suggests that the attenuation process occurs between 20 and 40 cell culture passages *in vitro*. Although this study represents the first published report regarding the attenuation of the virulence of *L. intracellularis* through multiple passages *in vitro*, the vaccine isolate also have exhibited this characteristic, which suggests a common property among different isolates of *L. intracellularis*. We believe this information will be valuable for design of future experimental challenge trials and for studying the mechanisms involved in the attenuation of *L. intracellularis* virulence *in vitro*.

CHAPTER 3

Comparative genome sequencing identifies a prophage-associated genomic island linked to host adaptation of *Lawsonia intracellularis* infections

Vannucci F.A.; Kelley M.R.; Gebhart C.J. (2013) Comparative genome sequencing identifies a prophage-associated genomic island linked to host adaptation of *Lawsonia intracellularis* infections. *Vet Res* (In review)

Summary

Lawsonia intracellularis is an obligate intracellular bacterium and the causative agent of proliferative enteropathy (PE). The disease is endemic in pigs, emerging in horses and has also been reported in a variety of other animal species, including nonhuman primates. Comparing the whole genome sequences of a homologous porcine *L. intracellularis* isolate cultivated for 10 and 60 passages *in vitro*, we identified a 18-kb prophage-associated genomic island in the passage 10 (pathogenic variant) that was lost in the passage 60 (non-pathogenic variant). This chromosomal island comprises 15 genes downstream from the prophage DLP12 integrase gene. The prevalence of this genetic element was evaluated in 12 other *L. intracellularis* isolates and in 53 infected animals and was found to be conserved in all porcine isolates cultivated for up to 20 passages and was lost in isolates cultivated for more than 40 passages. Furthermore, the prophage region was also present in 26 fecal samples derived from pigs clinically affected with both acute and chronic forms of the disease. Nevertheless, equine *L. intracellularis* isolates evaluated did not harbor this genomic island regardless of the passage *in vitro*. Additionally, fecal samples from 21 clinically affected horses and four wild rabbits trapped in horse farms experiencing PE outbreaks did not show this prophage-associated island. Although the presence of this prophage-associated island was not essential for a virulent *L. intracellularis* phenotype, this genetic element was porcine isolate-specific and potentially contributed to the ecological specialization of this organism for the swine host.

3.1. Introduction

Lawsonia intracellularis is a gram-negative obligate intracellular bacterium and the etiologic agent of proliferative enteropathy. The disease is characterized by thickening of the intestinal epithelium due to enterocyte proliferation which is directly associated with the presence of intracytoplasmic bacteria (Gebhart and Guedes, 2010). PE is endemic in pigs, an emerging disease in horses and is also described in various other species, including nonhuman primates, wild mammalians and ratite birds (Cooper et al., 1997a; Klein et al., 1999; Pusterla et al., 2008b).

PE was first reported in 1931 (Biester and Schwarte, 1931), but the causative bacterium was isolated in 1991 from hamsters and in 1993 from pigs using cell culture under strict microaerophilic environmental conditions (Lawson et al., 1993; Stills, 1991). To date, growth of the bacteria in axenic (cell-free) media has not been reported. The fastidious properties related to the isolation and cultivation of *L. intracellularis* has limited molecular studies comparing bacterial isolates and so far, there are approximately 15 isolates worldwide. The whole genome of a porcine *L. intracellularis* isolate (PHE/MN1-00) has been sequenced and annotated (accession: PRJNA183) using Sanger-based sequencing and a total of 1,719,014 base pairs are distributed into one small chromosome (1.46Mb) and three plasmids. The presence of few potential virulence factor-encoding genes identified by comparative sequence analysis and many hypothetical proteins suggest that this organism has adopted mechanisms of survival and pathogenesis that are unique among bacterial pathogens (Gebhart and Guedes, 2010).

Non-pathogenic isolates obtained through multiple passages in cell culture have not been successful at inducing typical PE lesions or reversing virulence in experimentally-infected pigs (Knittel and Roof, 1999). Conversely, clinical and subclinical disease has been previously reproduced using cultivated *L. intracellularis* at low passages (4 to 20) in cell culture (Guedes and Gebhart, 2003b; McOrist et al., 1993; McOrist et al., 1997b; McOrist et al., 1996b). Vannucci et al (2013) recently described attenuation of the virulence properties between 20 and 40 cell passages *in vitro*, but the molecular mechanisms responsible for this phenotype remain unclear. The loss of pathophysiological properties of *L. intracellularis* associated with its adaptation to *in vitro* conditions due to mutational events at the DNA level has been speculated. However, standard DNA-based typing techniques, such as pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and variable number tandem repeat (VNTR) have shown identical genotypes in both pathogenic (low passage) and non-pathogenic (high passage) variants (Beckler et al., 2004; Kelley et al., 2010; Oliveira and Gebhart, 2008). A more comprehensive genomic analysis of these two phenotypic variants is crucial to determine genomic variations which might potentially be associated with virulence properties. In addition, the characterization of genomic variations may reveal DNA sequence markers useful for studying genetic variations among clinical samples of *L. intracellularis* without requiring bacterial isolation.

The present study used high-throughput DNA sequencing technology to compare and characterize genomic variations between homologous pathogenic (passage 10) and non-pathogenic (passage 60) *L. intracellularis* variants. The prevalence of a prophage-

associated genomic island which was present in the pathogenic but not in the non-pathogenic variant was evaluated in porcine and equine isolates, as well as in in fecal samples positive for the presence of *L. intracellularis* DNA derived from infected animals.

3.2 Materials and Methods

L. intracellularis isolate and growth conditions

The porcine *L. intracellularis* isolate PHE/MN1-00 previously obtained from a pig with the hemorrhagic form of PE was used (Guedes and Gebhart, 2003b). A previous study using experimentally-infected pigs confirmed the pathogenic and non-pathogenic properties of these two variants (Vannucci et al., 2013). Murine fibroblast-like McCoy cells were grown in Dulbecco's Modified Eagles Medium with 1% L-glutamine, 7% fetal bovine serum and 0.5% amphotericin B without antibiotics (Guedes and Gebhart, 2003c). A pure culture of the bacteria at passage 6 (pathogenic variant) and 56 (non-pathogenic variant) was thawed and grown in McCoy cells (ATCC CRL 1696) for three continuous passages in order to allow the bacteria to recover from frozen storage. T_{75} cell culture flasks containing one-day-old McCoy cells (30% confluence) were infected with bacterial suspensions containing approximately 10^4 *L. intracellularis* organisms.

After a recovery period, pure cultures of *L. intracellularis* at passages 10 and 60 were prepared and used for DNA sequencing. Inoculated cultures were placed in an anaerobic chamber that was evacuated to 500 mmHg and refilled with hydrogen gas. Infected cultures were then incubated for seven days in a Tri-gas incubator with 83.2%

nitrogen gas, 8.8% carbon dioxide, 8% oxygen gas and a temperature of 37^{o}C, as previously described (Lawson et al., 1993). During each passage, the cell culture infection was monitored by counting the number of heavily infected cells (HIC) using immunoperoxidase staining with polyclonal antibody specific for *L. intracellularis* (Guedes et al., 2002). All procedures used in the present study were approved by the Institutional Sponsored Projects Administration of the University of Minnesota.

DNA preparation and genome sequencing

Bacterial cultures were prepared for extraction and sequencing using the supernatants of infected cell cultures in order to minimize the presence of eukaryotic DNA and enhance the purity of the bacterial suspension for DNA extraction. Briefly, cell culture supernatants were passed through 0.8µm sterile filters to remove potential detached McCoy cells present in the cell monolayer supernatant. Filtered bacterial suspensions were then pelleted by centrifugation at 8,000g for 20 minutes. The pellets were used for DNA extraction using DNeasy Blood & Tissue Kit® (Qiagen), according to the manufacturer's instructions.

The sequencing procedures were conducted through the core facility of the Biomedical Genomics Center at the University of Minnesota. Following quantification using the PicoGreen Assay of the DNA generated in the library preparation, the samples were loaded on the Illumina® Genome Analyzer GA IIx platform with paired reads of 76 bp. Base calling and quality filtering were performed following the manufacturer's instructions (Illumina® GA Pipeline).

Genome assembly and comparison

The sequence contigs were generated using Velvet assembly software (Zerbino and Birney, 2008) followed by alignment onto the reference genome using the Sequencer® 5.0 (Gene Codes Corporation). The assembly visualization was performed using Tablet software which allowed comparative genome analysis, evaluation of the genome coverage and the identification of single nucleotide substitutions (SNPs), insertions and deletions (indels) (Milne et al., 2010). Based on the sequence visualization, manual annotation, manipulation and comparison were performed between both variants. Indels and SNPs previously identified in the comparative visualization analysis were confirmed by Sanger-based sequencing of directed PCR amplicons generated from the corresponding regions.

Prevalence of the prophage-associated genomic island L. intracellularis isolates

The presence of the prophage-associated genomic island identified by whole-genome sequencing in the pathogenic variant (passage 10) of the porcine *L. intracellularis* isolate PHE/MN1-00 but not in the non-pathogenic variant (passage 60) was evaluated in 12 other isolates from our collection at low and high passages (Table 3.1). Additionally, a total of 53 fecal samples submitted to the Minnesota Veterinary Diagnostic Laboratory from different states of the United States, which had previously tested positive for the presence of *L. intracellularis* DNA by PCR, were evaluated. A total of 26 fecal samples were collected from naturally infected pigs in seven states (Iowa, Illinois, Minnesota, Missouri, Nebraska, Oklahoma and North Carolina), along with samples from 21 naturally infected horses from eight states (Florida, California,

Iowa, Kentucky, Minnesota, Tennessee, Texas and Virginia) and four wild rabbits captured in a California horse breeding farm experiencing clinical cases of *L. intracellularis* infections.

Specific primers targeting all 15 genes included in the prophage-associated genomic island and the two flanking genes present immediately before and after the prophage region were designed (Table 3.2). PCR reactions were performed in a 25µl volume using HotStar®Taq DNA polymerase (Qiagen) and 0.6µM of each primer under the following conditions: 95°C for 15 min, 35 cycles (94°C/30 sec, 52°C/60 sec, 72°C/30 sec) and final extension at 72°C for 10 min.

3.3 Results

Genome comparison between porcine L. intracellularis isolate (PHE/MN1-00) at low and high passages

The present study resequenced the isolate PHE/MN1-00 which was used to generate the reference genome of *L. intracellularis* available at the National Center for Biotechnology Information (NCBI accession: PRJNA61575) using Sanger-based sequencing. Although there is no information available regarding the number of passages *in vitro* used in this first sequencing project, our results showed identical DNA contents using the passage 10. This strongly indicates that the first genome project used low passage-cultivated bacteria.

The whole genome sequencing generated 83,999,466 and 128,258,573 millions of bp for *L. intracellularis* cultivated at 10 and 60 passages *in vitro*, respectively. For

passage 10, the average coverage of 42X was assembled with mean contig length of 11,845 bp (minimum: 2,175 and maximum: 112,744) and 14.1% of unused data that represented eukaryotic-derived DNA. The data from the passage 60 generated an average coverage of 66X assembled with mean contig length of 10,674 bp (minimum: 1,189 and maximum: 119,534) and 11.3% of eukaryotic -derived DNA.

The comparative analysis between homologous pathogenic (passage 10) and non-pathogenic (passage 60) *L. intracellularis* variants reveals a major deletion of 18,086 base pairs (bp) in the passage 60 which includes 15 genes starting from the prophage DLP12 integrase gene (LI0173). General features of the genome comparison are summarized in Table 3.3. Additionally, four single nucleotide polymorphisms (SNPs) were identified in the chromosome and one in the plasmid C of the passage 60 variant (Table 3.4). DNA sequences from plasmids A and B were identical in both the passage 10 and 60 variants.

Characterization of the L. intracellularis prophage-associated genomic island

A prophage-associated genomic island located in the chromosome represented the major genomic deletion identified in the porcine *L. intracellularis* isolate PHE/MN1-00 at passage 60. This deletion is located at the position 218,578 to 236,664 and comprises 15 genes starting from the prophage DLP12 integrase gene (Figure 3.1). Besides this prophage integrase, four more genes with known function have been annotated within the region: ribosomal protection tetracycline resistance protein (LI0179); endonuclease (LI0181); recombination protein-phage associated (LI0182) and MoxR-like ATPases (LI0185). The other ten genes were predicted to encode hypothetical proteins. Sequence

analyses of these genes using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database reveals the presence of six motif sequences: three proteins (LI0177, LI0184 and LI0186) with unknown functions but previously reported in other bacterial organisms; ferric reductase NAD binding domain (LI0174); RNA polymerase III subunit RPC82 helix-turn-helix domain (LI0175) and cobalamin biosynthesis protein CobT (LI0187).

The presence of all 15 genes included in the prophage-associated genomic island was confirmed by PCR and Sanger-based sequencing (Figure 3.2A). The loss of this genomic island in the passage 60 was confirmed by the absence of amplified products from all 15 genes and the presence of the amplified product targeting the flanking regions immediately before and after the prophage region (Figure 3.2B). The prevalence of the prophage-associated island in *L. intracellularis* isolates and infected animals was evaluated based on the PCR panel targeting all 15 genes within the genomic region described in Figure 3.2. In those bacterial isolates or infected animals that harbor this genomic island all 15 genes were found. Its absence was represented by an absence of the entire region. This observation characterizes the chromosomal island as a mobile genetic element.

Prevalence of the prophage-associated genomic island in L. intracellularis isolates

The presence of the prophage-associated genomic island was evaluated in a total of 13 *L. intracellularis* isolates, ten from pigs, two from horses and one from a hamster (Table 3.1). Regardless of the origin of the bacterial isolate (North America, South America or Europe), porcine isolates at low passages *in vitro* (up to 20) show the

presence of the prophage-associated island. However, this genetic element was entirely lost in all porcine isolates passed more than 40 times in cell culture (Table 3.1).

A distinct scenario was observed in the two equine isolates that do not harbor this genomic island regardless of the number of passages *in vitro*. Although the pathogenicity of the equine isolate at low passage (E40504) has been demonstrated in previous studies (Pusterla et al., 2009a; Pusterla et al., 2010; Vannucci et al., 2012d), the prophage island was not found even in lower passages (four, six and eight) of this isolate (data not shown).

Prevalence of the prophage-associated genomic island in infected animals

A total of 26 porcine samples that previously tested positive for the presence of *L. intracellularis* DNA in the feces were evaluated, derived from seven states of the United States (Iowa, Illinois, Minnesota, Missouri, Nebraska, Oklahoma and North Carolina). Twenty-two samples were derived from pigs affected with the chronic form of PE and four from pigs affected with the acute form of the disease, known as proliferative hemorrhagic enteropathy (PHE). Regardless of the clinical presentation of the disease or the state of origin, all the porcine-derived samples consistently exhibited the prophage-associated island characterized by the presence of all 15 genes within the chromosomal region.

Twenty-one horse samples that were positive for *L. intracellularis* by fecal PCR from eight states (Florida, California, Iowa, Kentucky, Minnesota, Tennessee, Texas and Virginia) were evaluated. None of the genomic island genes were present in any equine-derived samples, confirming the observations from the two equine isolates previously

described in this study. The absence of the prophage island also was observed in samples obtained from four wild rabbits captured in a horse breeding farm experiencing clinical cases of PE.

Table 3.1 Summary of the *L. intracellularis* isolates used in the study to evaluate the presence of the prophage-associated genomic island.

Strain ID	Origin of the strains		Number of passage	Presence of prophage
	Host species	Country*		
PHE/MN1-00	Swine	USA	10	+
			20	+
			40	-
NWumn05	Swine	USA	20	+
			40	-
VPB4	Swine	USA	10	+
			>150	-
GBI06	Swine	USA	10	+
DBumn06	Swine	USA	10	+
D15540	Swine	DK	20	+
PHE-BR	Swine	BR	10	+
963/3	Swine	UK	80	-
916/91	Swine	UK	60	-
LR189/5/83	Swine	UK	90	-
Foal96	Equine	USA	> 100	-
E40504	Equine	USA	10	-
Ham1	Hamster	USA	> 100	-

* USA: United States of America, DK: Denmark, BR: Brazil, UK: United Kindom

Table 3.2 Primers targeting the prophage-associated genomic island of porcine *L. intracellularis* isolates and the two flanking genes.

Gene	Gene product	Entrez Gene ID	Primers (5' → 3')
LI0172*	Hypothetical protein	4059866	ATTGATGCTCCTGTCCCACG ACCACATGGTGGATTCGTCC
LI0173	Prophage DLP12 integrase	4059867	CGTCGTATTCTGCGCTTTGG ATCATCAGCTACACGAGCGG
LI0174	Hypothetical protein	4059868	CAGGAAGATGCTGTGTGGCT ATTCGCTTTCGCAATACGGC
LI0175	Hypothetical protein	4059869	CCCACGGACGAAGACTTTGA TCAGCTTTCGGGCATGGATT
LI0176	Hypothetical protein	4059870	ACAGACCTCTATGCTCCCGT TCAGCGTCTTGGGGCTTTAG
LI0177	Hypothetical protein	4059871	ACACCACCATTACCACTGCT ACACCACCATTACCACTGCT
LI0178	Hypothetical protein	4059872	TTCCTCCTGCGTGTCGTAAC ATTTCTCCCTGGCTCTGCAC
LI0179	Ribosomal protection tetracycline resistance protein	4059813	CGTCAGCAAAGCGGAAACAA AAACGGCCTTGGCATTCAAC
LI0180	Hypothetical protein	4059873	GAACCGGTGAGCCAAGTGTA TTCCTTCGGGAGTCGAGGAT
LI0181	Endonuclease I	4059874	AGGCTAAGCGCATACTGCAA CAGCATTGACAGACCCGACT
LI0182	Recombination protein-phage associated	4059875	TGGATTTCCAGCACAGCCAT TGAACCGTCCTGAAGCTCAC
LI0183	Hypothetical protein	4060170	CTCTCGACGCATCTTCCCTC TCCATTCCGCCGTCATGAAA
LI0184	Hypothetical protein	4060171	TTGGACTGGCTCTTACGCAG GATGAAGCCCACGTCAGGAA
LI0185	MoxR-like ATPases	4060172	GTCATGCGTCAGAACATCGC CCTGTTTGGAGAGAGGCTGG
LI0186	Hypothetical protein	4060173	CCTTCCTGGGCCAACATCAT CACGCTTGGGCATATTTCCG
LI0187	Hypothetical protein	4060174	ATCGGTTCTTCGGATACCGC GAATCCTGCGTAGATCGGCA
LI0188*	ATP-dependent Zn proteases (*ftsH*)	4059755	GAGCTGTAGCTGGTGAAGCA ACGTGCAACAAGTGCATGTC

* Flanking genes located before and after the prophage-associated genomic island

Table 3.3 General characteristics of the two *Lawsonia intracellularis* homologous variants.

L. intracellularis PHE/MN1-00	Passage 10	Passage 60
Chromosome size	1,457,619	1,439,533
G+C content (%)	33.3	33.1
Total number of protein-coding genes	1,183	1,168
Prophage element	1 (18.1Kb)	0
Plasmids	3	3
Variant-specific genes	15	0

Table 3.4 Single nucleotide substitutions in passage 10 and passage 60 of the porcine *L. intracellularis* isolate PHE/MN1-00.

Gene locus (Position)	Description	Amino acid change		Substitution
		Passage 10	Passage 60	
Chromosome				
LI0064 (86,203)	Transcriptional regulator	Glutamic acid (**G**AA)	Lysine (**A**AA)	Non-synonymous
LI0473 (577,207)	DNA methylase	Lysine (**A**AA)	Glutamic acid (**G**AA)	Non-synonymous
LI0532-LI0533 (651,651)	Intergenic region	Isoleucine (AT**C**)	Isoleucine (AT**T**)	Synonymous
LI0905 (1,129,621)	DNA-directed RNA polymerase	Lysine (**A**AG)	Glutamic acid (**G**AG)	Non-synonymous
Plasmid C				
LIC103 (190,854)	Methyl-accepting chemotaxis	Cysteine (TG**C**)	Cysteine (TG**T**)	Synonymous

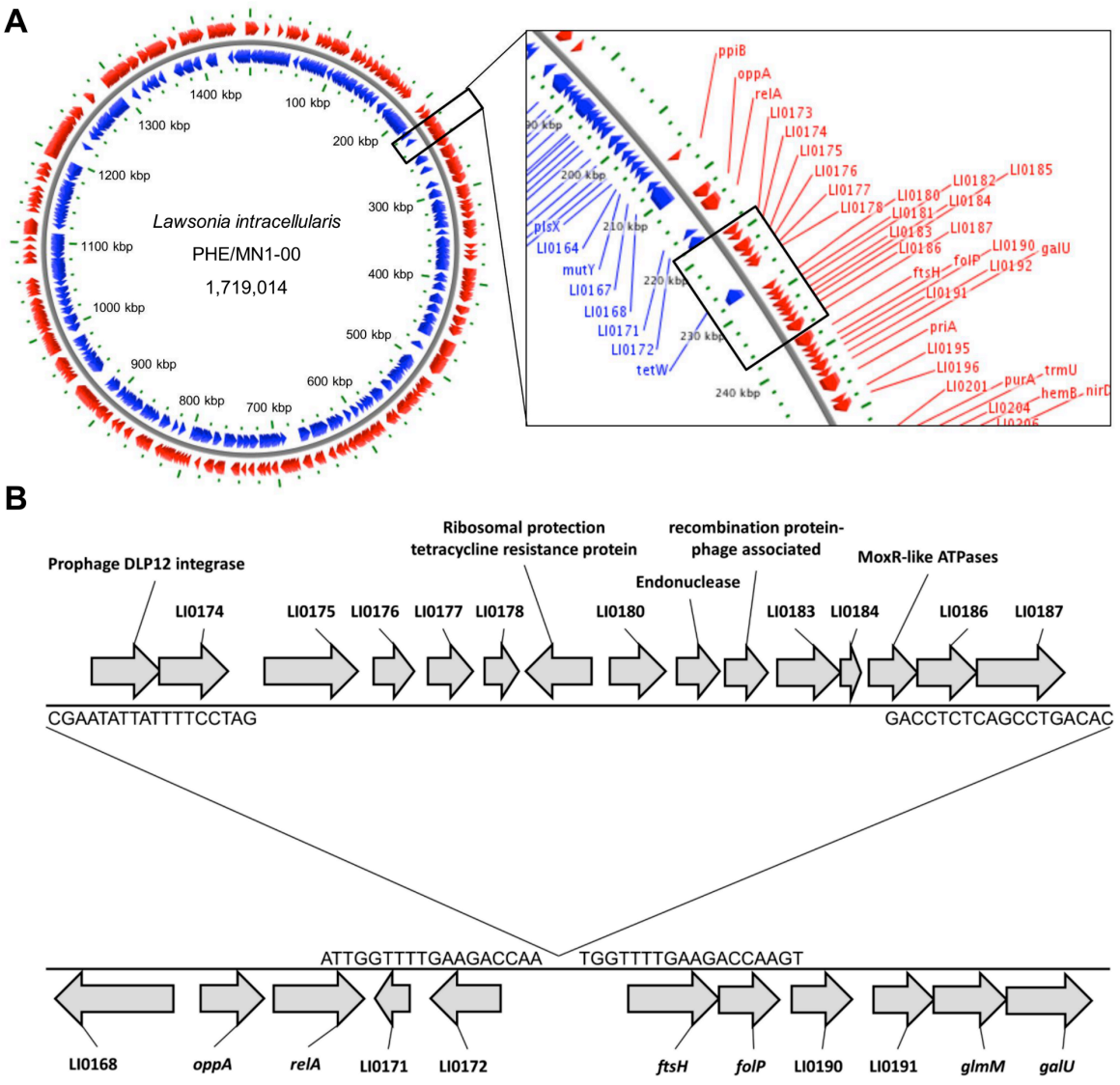

Figure 3.1 Prophage DLP12-associated genomic island. (A) Chromosome map of the porcine *L. intracellularis* isolate PHE/MN1-00 at passage 10 showing the genomic location of the prophage element. (B) Genetic organization of the prophage region (from prophage DLP12 integrase to LI0187 gene). Loci between LI0172 and *ftsH* gene from where the prophage excised and is no longer present in the equine and rabbit-derived isolates and in the porcine *L. intracellularis* isolate PHE/MN1-00 at passage 60.

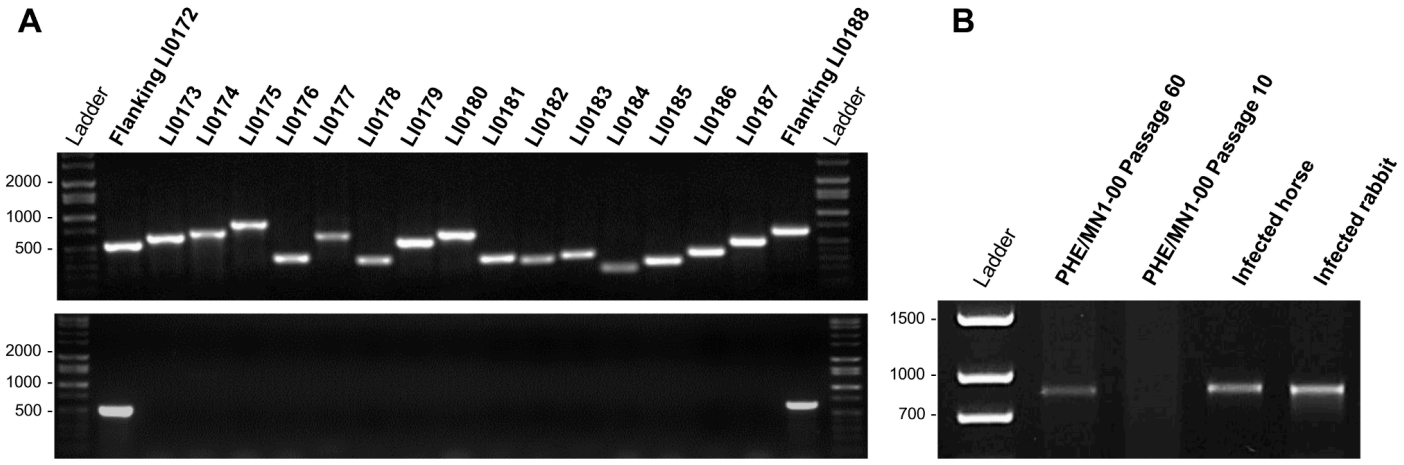

Figure 3.2 PCR detection of the prophage DLP12-associated genomic island. (A) PCR panel for detection of all 15 genes included in the prophage region and the two flanking genes. Porcine *L. intracellularis* isolate PHE/MN1-00 at passage 10 showing the presence of all prophage-associated genes (above). Loss of the prophage element at passage 60 showing the presence of flanking genes (below). (B) Absence of the entire prophage-associated island confirmed in the porcine isolate PHE/MN1-00 at passage 60 and infected horse and rabbit isolates by obtaining PCR products from the amplification of a primer pair targeting the regions immediately before and after the prophage region (forward: 5'-TCGTGAGAAACTTGTATCAATCCA-3' and reverse: 5'-TGACAATGTTAGAGCAATGACTTTTTA3-3').

3.4 Discussion

Comparing the whole genome sequences of a porcine *L. intracellularis* isolate cultivated for 10 and 60 passages *in vitro*, we identified the loss of a prophage-associated genomic island in the passage 60. This chromosomal deletion comprises a total of 15 genes (18,086 bp) starting in the defective prophage DLP12 (a defective lambdoid prophage at the 12th minute of the chromosome) previously described in *E. coli* (Lindsey et al., 1989). The full length of the prophage DLP12 in *E. coli* K-12 contains 22 genes included in a region of 21,302 bp (Wang et al., 2010). From an evolutionary perspective, this evidence suggests that the element was partially acquired by *L. intracellularis* or underwent a partial deletion once integrated into the *L. intracellularis* genome. While DLP12 genes in *E. coli* encoding a two-component lysis cassette (holing-endolysin) are responsible for inducing lysis of the host bacterial cell, this cassette was not found in the prophage DLP12-associated island of *L. intracellularis*. As a result, this defective prophage integrated into *L. intracellularis* may have lost a function essential for lytic growth and can no longer liberate infectious particles.

Although the impact of the prophage DLP12 in cell physiology and virulence remains unclear, studies have associated the presence of this integrated prophage with resistance to environmental stress and biofilm formation (Toba et al., 2011; Wang et al., 2010). Rhodius et al (2006) showed activation of DLP12 genes mediated by the transcription regulatory factor σ^E during the stress response (Rhodius et al., 2006). A recent study demonstrated the role of the DLP12 genes in maintenance of the bacterial cell wall and in biofilm development by curli-producing *E. coli* (Toba et al., 2011). To

date, no information has been presented regarding the ability of *L. intracellularis* to produce biofilm. However, empiric observations of recurrent clinical infections in certain swine production systems may support future investigations regarding biofilm production, based on the potential persistence of *L. intracellularis* in swine installations and/or fomites. Previous studies did not identify expression of the 15 genes included in the prophage-associated island by *L. intracellularis* in the host cytoplasm *in vitro* or *in vivo* (Vannucci et al., 2012b; Vannucci et al., 2012c). Therefore, similar to curli-producing *E. coli*, the expression of the DLP12 genes in *L. intracellularis* is likely to occur during the stationary phase of the extracellular life cycle.

The authors identified the persistence of the prophage DLP12-associated element for at least 20 passages *in vitro* in two porcine isolates and its loss after 40 passages in cell monolayer (Table 3.1). The low excision frequency reported for this prophage in *E. coli* K-12 (<1 per 100,000 cells) may explain the gradual loss of this genetic element during *L. intracellularis* cultivation in cell monolayer (Wang et al., 2010). During the cultivation of *L. intracellularis in vitro* the infection is passed weekly (Lawson et al., 1993), which gives the prophage DLP12 a stability of at least 20 weeks. The excision rate of integrated phages is directly related to the adverse conditions encountered by the host bacterial cells followed by the induction of the SOS response in the bacteria and conversion of the phage from its lysogenic to lytic form (Brüssow et al., 2004). However, prophage DLP12 in *E. coli* lost the ability to excise and form phage particles on SOS response challenge (Wang et al., 2010). This defective prophage may now suffer the same fate as its host. The beneficial impact of defective prophages on cell physiology

includes enhancing nutrient utilization and increasing the host's tolerance to general environmental stress and antibiotics. Therefore, the loss of this element does not compromise essential host physiological functions and it becomes dispensable under standard culturing conditions (Wang et al., 2010). Additionally, the role of the DLP12 genes in cell wall maintenance and composition suggests an important means for bacteria to improve their fitness. It has been theorized that introduction of novel genes by phages can confer beneficial phenotypes that allow the exploitation of competitive environments (Canchaya et al., 2003).

A gene encoding ribosomal protection protein associated with tetracycline resistance was found in this prophage-associated island. Although the widely use of antimicrobials in the swine industry over the years has been speculated to select resistant and/or virulent bacterial strains (Chee-Sanford et al., 2001; McEwen, 2012), the phenotypic consequence of this tetracycline resistance gene in *L. intracellularis* infections is unclear. Wattanaphansak et al (2008) evaluated the antimicrobial activity *in vitro* of chlortetracycline against ten porcine *L. intracellularis* isolates that originated from North America and Europe. Regardless of the geographical origin and the number of passages in culture, which ranged from 7 to 170, the authors found a large variation among these bacterial isolates in regard to their susceptibilities to chlortetracycline to *in vitro* (Wattanaphansak et al., 2009). This study also showed a higher antimicrobial activity of chlortetracycline against intracellular *L. intracellularis* compared with extracellular organisms. Associating this information with the lack of expression of this tetracycline resistance gene by *Lawsonia,* especially by intracellular organisms, and the

properties of DLP12 prophage to cope with environmental stress (previously discussed), it is possible to speculate that this gene may be expressed extracellularly and contribute to a higher antimicrobial tolerance during the extracellular stage. However, this hypothesis needs to be specifically addressed in a future study.

Despite the limited number of *L. intracellularis* isolates available worldwide, our results showed the presence of the entire prophage region in ten porcine isolates at low passage *in vitro* indicating the consistent adaptation and specificity of this genomic island to porcine-derived isolates, regardless of the clinical form (acute or chronic) of the disease. The absence of this genomic island in the equine isolate at low passage reveals an important genomic variation related to species-specificity of *L. intracellularis* isolates. The availability of only one hamster isolate that has been cultivated over 100 times *in vitro* restricts a more confident conclusion regarding the prevalence of the prophage in this species. Nevertheless, this is the first study describing a significant genomic variation among *L. intracellularis* isolates, since to date *L. intracellularis* has been considered a monotypic organism (Kroll et al., 2005).

In addition to the porcine isolates, the presence of the prophage DLP12-associated genomic island was confirmed in fecal samples from PE-affected pigs and its absence confirmed in horses and rabbits infected with *L. intracellularis*. Despite the fact that the loss of this genetic element was coincident with the loss of virulent phenotype in one porcine isolate (PHE/MN1-00) (Vannucci et al., 2013), the consistent lack of this island in infected horses shows that this prophage-associated island is not essential for virulence

in *L. intracellularis* infections. However, our results show that this genomic region is indeed associated with host-adapted *L. intracellularis* variants.

Supporting the genotypic variation among the species origin of the bacterial isolate described here, a previous study phenotypically demonstrated the host adaptation of *L. intracellularis* in pigs and horses (Vannucci et al., 2012d). In a cross-species experimental model using pure culture of *L. intracellularis* at low passages, this study showed that pigs and foals infected with their species-specific isolates shed the bacterium in the feces for longer periods of time and exhibited stronger serologic immune responses. The previously demonstrated evidence of host adaptation in pigs and horses seems to extend to experimental infection models using hamsters and rabbits. Sampieri et al (2013) showed that rabbits are more susceptible to equine-derived isolates than to porcine variants and hamsters more susceptible to porcine-derived isolates than to equine variants. These results suggest that *L. intracellularis* variants evolved to be adapted to more than one host species. The evolutionary ecology responsible for driving host-adapted variants remains to be determined. Although our study failed to specifically associate the prophage DLP12 genomic region with virulent phenotype, we hypothesize that this genetic element contributes to the adaptation of *L. intracellularis* in the porcine host. Based on evidence showing the role of this defective prophage in helping *E. coli* cope with adverse environment and form biofilms, this genetic element may drive an ecological specialization in pig-adapted *L. intracellularis* variants. In a future perspective, the whole genome sequencing of horse isolates could allow a more

comprehensive conclusion regarding the genetic traits related to the host-adapted *L. intracellularis* variants.

Recently, the analysis of whole genome sequences of *Staphylococcus aureus* ST398 strains derived from livestock and humans showed the presence of the prophage φ3 in human but not animal-derived isolates (Uhlemann et al., 2012). Interestingly, this prophage contains the human-specific immune evasion cluster genes *chp* and *scn* that modulate the innate immune response and are readily transmitted within households, independent of animal contact. The authors suggest that these differences at the genome level are able to drive host-specific adaptation in the ST398 strains. Additionally, the prophage φ3 seems to be lost after jumping from humans to livestock.

In summary, the present study showed a prophage-associated genomic island present specifically in pathogenic porcine *L. intracellularis* isolates. The identification of this genetic element may be applied as a molecular marker to determine the species origin of the bacterial isolates and to trace the evolutionary history related to the host species in *L. intracellularis* infections. Finally, this first study showing significant genomic variation in *L. intracellularis* isolates associated with the previously reported host-adapted phenotype may support the characterization of novel *L. intracellularis* subspecies or species-specific genotypes.

CHAPTER 4

Comparative transcriptional analysis of homologous pathogenic and non-pathogenic *Lawsonia intracellularis* isolates in infected porcine cells

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Summary

Lawsonia intracellularis is the causative agent of proliferative enteropathy. This disease affects various animal species, including nonhuman primates, has been endemic in pigs, and is an emerging concern in horses. Non-pathogenic variants obtained through multiple passages *in vitro* do not induce disease, but bacterial isolates at low passage induce clinical and pathological changes. The present study used high-throughput sequencing technology to characterize the transcriptional profiling of a pathogenic and a non-pathogenic homologous *L. intracellularis* variant during *in vitro* infection. A total of 401 genes were exclusively expressed by the pathogenic variant. Plasmid-encoded genes and those involved in membrane transporter, adaptation and stress response were the categories mostly responsible for this wider transcriptional landscape. The entire gene repertoire of plasmid A was repressed in the non-pathogenic variant suggesting its relevant role in the virulence phenotype of the pathogenic variant. Of the 319 genes that were commonly expressed in both pathogenic and non-pathogenic variants, no significant difference was observed by comparing their normalized transcription levels (fold change$\pm$2; $p<0.05$). Unexpectedly, these genes demonstrated a positive correlation ($r^2=0.81$; $p<0.05$), indicating the involvement of gene silencing (switching off) mechanisms to attenuate virulence properties of the pathogenic variant during multiple cell passages. The complexity of the virulence phenotype was demonstrated by the diversity of genes exclusively expressed in the pathogenic isolate. The results provide the basis for prospective mechanistic studies regarding specific roles of target genes involved in the pathogenesis, diagnosis and control of proliferative enteropathy.

4.1. Introduction

Lawsonia intracellularis is a fastidious intracellular bacterium and the etiologic agent of proliferative enteropathy (PE), an intestinal hyperplasic disease characterized by thickening of the mucosa of the intestine due to enterocyte proliferation (Gebhart and Guedes, 2010). Cell proliferation is directly associated with bacterial infection and replication in the intestinal epithelium (McOrist et al., 1996a). As a result, mild to severe diarrhea is the major clinical sign described in infected animals (Lawson and Gebhart, 2000). Since the 1990s, PE has been endemic in swine herds and has been occasionally reported in various other species, including nonhuman primates, wild mammalians and ratite birds (Cooper et al., 1997b; Lafortune et al., 2004). Outbreaks among foals began to be reported on breeding farms worldwide within the last decade (McGurrin et al., 2007; Pusterla et al., 2009b). Therefore, PE is now considered an emerging disease in horses (Pusterla and Gebhart, 2009).

Although PE was first reported in 1931 (Biester and Schwarte, 1931), the causative bacterium was primarily isolated only in 1993 using rat small intestinal cells (IEC-18) in strict microaerophilic environmental conditions (Lawson et al., 1993). Since then, various cell lines have supported *L. intracellularis* growth *in vitro*, including insect and avian cell lines (Evans et al., 2011; Guedes and Gebhart, 2003c; Knittel and Roof, 1999; McOrist et al., 1997a). To date, growth of the bacteria in axenic (cell-free) media has not been reported. Regardless of the cell type, the dynamics of the infection *in vitro* requires actively dividing cells in a microaerophilic atmosphere with the peak of infection at six to seven days post-inoculation (Evans et al., 2011; Lawson et al., 1993; McOrist et

al., 1995b). McOrist et al (1995) chronologically described the dynamics of the infection and bacterial replication in intestinal porcine epithelial cells (IPEC-J2). Most events closely resembled those observed at the cellular level in infected animals, including multiplication of the bacteria freely in the cell cytoplasm. While the dynamics of the infection have been well-characterized, little is known so far about the genetic basis for the virulence, pathogenesis or physiology of *L. intracellularis* (Jacobson et al., 2010).

Spontaneously attenuated isolates obtained through multiple passages in cell culture have not been successful at inducing typical PE lesions or reversing its virulence in experimentally-infected pigs (Knittel and Roof, 1999). Conversely, bacterial isolates at low passage induce clinical and pathological changes typical of PE (Guedes and Gebhart, 2003b). Various standard DNA-based typing techniques, such as pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and variable number tandem repeat (VNTR) have shown identical genotypes in both pathogenic (low passage) and non-pathogenic (high passage) variants (Beckler et al., 2004; Kelley et al., 2010; Oliveira and Gebhart, 2008). As a result, we believe their phenotypic properties occur at the transcriptional level. Bacterial genes differentially expressed between pathogenic (low passage) and non-pathogenic (high passage) homologous isolates have not been reported and this information may help to elucidate genes encoding the major bacterial virulence factors involved in the pathogenesis of PE.

We hypothesize that genes differentially expressed between pathogenic (passage 10) and non-pathogenic (passage 60) homologous *L. intracellularis* isolates encode potential bacterial virulence factors. High-throughput technology (RNA-seq) was used to

characterize and compare the transcriptional profile of a pathogenic and a non-pathogenic variant. Plasmid-encoded genes, regulatory factors and ATP-binding cassette (ABC) transporters associated genes were important for contributing to the wider transcriptional landscape observed in the pathogenic isolate. Additionally, the present study provided novel information for studying specific mechanisms of target genes and their potential usefulness for the diagnosis and control of PE.

4.2 Materials and Methods

Cell culture and infection in vitro

The intestinal piglet epithelial cell line IPEC-J2 is a non-transformed columnar cell type derived from neonatal piglet mid-jejunum (Berschneider, 1989). The cells were maintained in T_{75} cell culture flasks with Dulbecco's MEM/F12 nutrient mix (1:1) supplemented with 5% Fetal Bovine Serum, 5ng/ml Epidermal Growth Factor (Sigma-Aldrich) and 5ng/ml Insulin-Transferrin-Selenium mixture (BD Biosciences) without antibiotics at 37ºC in a humidified atmosphere of 5% CO_2, as previously described (McOrist et al., 1995b).

L. intracellularis isolate PHE/MN1-00 (ATCC PTA-3457) previously isolated from a pig with the hemorrhagic form of PE was used for passage 6 (pathogenic variant) and 56 (non-pathogenic variant) in cell culture. The pathogenic and non-pathogenic properties of these two variants were confirmed by experimental inoculation in pigs, performed in a previous study (Vannucci et al., 2013). A pure culture of the bacteria was thawed and grown in IPEC-J2 for three continuous passages in order to allow the bacteria

to recover from frozen storage. T_{75} cell culture flasks containing 30% confluent IPEC-J2 cell monolayer were infected with 10^3 pathogenic (passage 10) and non-pathogenic (passage 60) *L. intracellularis* organisms derived from the isolate (PHE/MN1-00). Inoculated cultures were placed in an anaerobic chamber, which was evacuated to 500 mmHg and refilled with hydrogen gas. Infected cultures were then incubated for seven days in a Tri-gas incubator with 83.2% nitrogen gas, 8.8% carbon dioxide, 8% oxygen gas and a temperature of 37^{o}C (Lawson et al., 1993).

The infection was monitored daily by counting the number of heavily infected cells (HIC) using immunoperoxidase staining with polyclonal antibody specific for *L. intracellularis*, as previously described (Guedes et al., 2002). A parallel infection was also monitored using 16-well tissue culture plates, as described previously (Vannucci et al., 2012e). The inoculated doses in this parallel monitoring system were proportional to those used in the T_{75} flasks according to the number of IPEC-J2 cells previously passed on the day before the infection. The monitoring was achieved by counting the number of HIC daily in eight wells (replicates) infected with the pathogenic and eight with the non-pathogenic variant. Cells containing 30 or more intracellular bacteria were considered to be HIC (McOrist et al., 1995c; Wattanaphansak et al., 2009). Additionally, quantitative PCR (qPCR) based on the copy number of the aspartate ammonia lyase gene of *L. intracellularis* was performed daily as a second parameter of monitoring, as described elsewhere (Vannucci et al., 2012e). Direct counting of infected cells and qPCR were used to confirm the exponential phase of the bacterial growth and to standardize the amount of *L. intracellularis* used as starting material for RNA isolation. All procedures used in the

present study were approved by the Institutional Sponsored Projects Administration of the University of Minnesota.

RNA isolation and enrichment

On the fourth continuous passage, the infection was passed using two replicates from each variant (pathogenic and non-pathogenic) and a total of four infected monolayers were harvested. A negative control using non-infected IPEC-J2 cells was conducted by treating the monolayers with sterile complete media. On day five post-inoculation the supernatants were removed and the infected monolayers were washed with RNAprotect® Bacterial Reagent (Qiagen). The infected cells were scraped and passed through a 20-gauge needle five times. Total RNA were extracted using RNeasy® Mini Kit (Qiagen) with an additional step for removing residual DNA using DNase I (Qiagen). Total RNA was assessed by a NanoDrop ND-8000 Spectrophotometer and Agilent Bioanalyzer for quality and integrity. Only samples with RNA Integrity Numbers (RIN) $\geq$ 8.0 were used in subsequent mRNA purification steps.

The bacterial mRNA was enriched from the total extracted RNA (mixture of cells and bacterial RNA) by subtractive hybridization using MicrobEnrich™ and MicrobExpress™ kits (Ambion Inc). Briefly, oligonucleotides specific to the mammalian RNAs (18S rRNA, 28S rRNA and polyadenylated mRNAs) and to the bacterial ribosomal RNA (16S and 23S) were hybridized and captured by magnetic beads. Equal amount of input RNA was used following the manufacturer's instructions for both pathogenic and non-pathogenic *L. intracellularis* variants. Internal controls provided in

both kits were performed in all enrichments. The effectiveness of rRNA depletions was evaluated using an Agilent Bioanalyzer 2100 (mRNA Nano Series Assay).

Library preparation and sequencing

The library preparation and sequencing were conducted in the core facility of the Biomedical Genomics Center at the University of Minnesota. Briefly, 100ng of the enriched mRNAs from both pathogenic and non-pathogenic *L. intracellularis* variants were fragmented and the first and second strand cDNAs were synthetized and ends repaired. The cDNA template was enriched by PCR and validated using a High Sensitivity Chip on the Agilent2100 Bioanalyzer. Following quantification of the cDNA generated for the library using PicoGreen Assay, the samples were clustered and loaded on the Illumina® Genome Analyzer GA IIx platform which generated on average 6,190,522 single reads with 76 bp. Base calling and quality filtering were performed following the manufacturer's instructions (Illumina® GA Pipeline).

Analysis and mapping the sequence reads

Data analysis including quality control, trimming and mapping were performed in the Galaxy platform (Giardine et al., 2005). Initially, FastQC tool was applied on the raw sequence data followed by FastQ Trimmer (Blankenberg et al., 2010). Using these tools, ten base pairs were trimmed from the 5'end and six from the 3'end. As a result, trimmed sequences containing 60 bp were used in the gene expression analysis. The sequence reads showed more than 25 phred quality score (Ewing et al., 1998) and were then mapped onto the *L. intracellularis* isolate PHE/MN1-00 reference genome obtained from

the NCBI database using Bowtie short read aligner (Langmead et al., 2009), with no more than two mismatches.

The number of reads that were mapped within each annotated coding sequence (CDS) was calculated in order to estimate the level of transcription for each gene. The Cufflinks tool was used to estimate the relative abundances of the transcript reads in each gene (Trapnell et al., 2010). For comparison of the expression levels between the pathogenic and non-pathogenic *L. intracellularis* variants, the read counts were normalized based on the number of reads per kilobase of coding sequence per million mapped reads (RPKM).

Differential expression analysis

Following the quantitative analysis of expressed genes, the differential expression between pathogenic and non-pathogenic variants was assessed using the CuffDiff tool (Trapnell et al., 2010). The total read count was determined for each gene by combining data from the two replicate sequencing runs. RPKM values were expressed in $\log_2$ (RPKM) to allow for the statistical comparison of transcription levels. As a result, $\log_2$-fold change in abundance of each transcript was obtained by $\log_2$ ($RPKM_{[pathogenic]}$ / $RPKM_{[non\text{-}pathogenic]}$). *P*-values were calculated and adjusted for multiple comparisons using false discovery rate (FDR) (Benjamini and Hochberg, 1995). Significant differential expression was determined in genes with FDR-adjusted *p*-values < 0.05 and fold change $\pm$ 2 in the comparison of transcription levels between pathogenic and non-pathogenic variants.

Quantitative reverse transcriptase PCR

The validation of the expression data identified by RNA-seq was performed by qRT-PCR from a specific set of genes: LI0005 (superoxide dismutase); LI0035 (fur regulator); LI0447 (hypothetical protein); LI0614 (thioredoxin); LI0902 (outer membrane protein); LIA017 (Fe-S oxidoreductase); LIC056 (hypothetical protein); LIB024 (chromosome partitioning ATPase); LI0825 (lipid A core-O-antigen ligase) and LI0959 (30S ribosomal protein S10). Specific primers were designed using Roche Universal Probe Library (UPL) generating a UPL probe number (Supplementary Table 4.1). RNA samples were synthesized to first-strand cDNA using SuperScript® II RT (Invitrogen, Carlsbad, CA). Duplicate qRT-PCR reactions from each primer probe set were validated by five serial dilutions of cDNA on the ABI7900HT instrument (Applied Biosystems, Foster City, CA). After validation, quantitative PCR was performed in duplicate using 15ng of cDNA per sample with the following conditions: 60°C for 2 min, 95°C for 5 min and 45 cycles (95°C/10 sec and 60° for 1 min). Averages of relative transcriptional levels were calculated and $\log_2$ transformed in order to be compared to the RNA-seq expression levels. Linear regression model was used to evaluate the correlation between average RPKM and qRT-PCR data.

4.3 Results

Mapping and differential expression

The sequence reads representing the RNA transcripts derived from the pathogenic and non-pathogenic homologous *L. intracellularis* PHE/MN1-00 isolates were mapped

onto the complete genome sequence of the same bacterial strain available at the National Center for Biotechnology Information (NCBI) (accession: NC_008011). The circular *L. intracellularis* genome has 1,719,014 base pairs (bp) comprised of one chromosome (1,457,619 bp) and three plasmids (plasmid A: 27,048 bp, plasmid B: 39,794 bp and plasmid C: 194,553 bp). From a total of 1,391 computationally predicted genes in the annotated PHE/MN1-00 genome, 1,340 are protein coding. Combining the pathogenic and non-pathogenic transcript reads, 731 protein coding genes were mapped onto the reference DNA sequence of the *L. intracellularis* PHE/MN1-00 isolate. Sequence reads mapped against the bacterial genome were used to quantify the gene expression levels based on the number of reads per kilobase of coding sequence per million mapped reads (RPKM). The expression data were sufficiently reproducible by showing a positive correlation between the two biological replicates of the pathogenic ($r^2 = 0.86$) and non-pathogenic ($r^2 = 0.81$) variants which was obtained using a linear regression model ($p< 0.05$). Genes with low expression levels showed lesser agreement between replicates (Supplementary Figure 4.1). This observation was reported using Illumina® RNA-seq data from human brain RNA (Bullard et al., 2010) and using ABI SOLiD™ platform in a transcriptome study of *Neisseria gonorrhoeae* (Isabella and Clark, 2011). Following the default parameters of the CuffDiff software (see Material and Methods) our study used ten sequenced reads as the minimum number of alignments in a locus needed to conduct agreement and comparison testing within and between the biological replicates.

A total of 720 and 330 genes were expressed by the pathogenic and non-pathogenic variants, respectively (Supplementary Figure 4.2). The wider transcriptional

landscape observed in the pathogenic variant was characterized by 401 genes uniquely expressed by this variant. Genes with the highest transcription levels according to their biological function categories are shown in Table 4.1. Only 11 genes were expressed exclusively by the non-pathogenic variant (Table 4.2). Differential mapping and distribution of the expressed genes into the *L. intracellularis* chromosome and its three plasmids are summarized in Figure 4.1. Plasmid-encoded genes significantly contributed to this broader profile of gene expression exhibited by the pathogenic isolate.

Narrowing the analysis to the putative biological gene functions, the number of genes expressed by the pathogenic variant was consistently higher throughout the functional categories (Figure 4.2). The transcriptional landscape was most significantly reduced in those genes involved in the membrane transport (72%), general predicted function (64%), cell membrane and motility (61%) and adaptation and stress response (61%) categories. In comparing the transcription levels of all 319 genes commonly expressed in both the pathogenic and non-pathogenic variants, there was no significant difference (fold change $\pm$ 2; adjusted p-value < 0.05 – Supplementary Table 4.2). When the expression levels from both variants were plotted against each other (Figure 4.3), $\log_2$ (RPKM) values unexpectedly showed a positive correlation (0.809) in a linear regression model. These results revealed the importance of gene silencing (switching off) mechanisms to attenuate virulence properties of *L. intracellularis* throughout passages *in vitro*.

Gene expression data generated by RNA-seq were validated using quantitative reverse transcriptase PCR (qRT-PCR). The reliability of RNA-seq results was confirmed

based on the relative quantification of 10 unlinked genes: four expressed in both variants, four expressed by the pathogenic and two expressed by the non-pathogenic (see Materials and Methods). The average log-transformed from two qRT-PCR replicates was plotted against the $\log_2$ (RPKM) (Figure 4.4). The transcript levels were positively correlated on a linear regression model (p-value < 0.05; $r^2 = 0.827$). Therefore, the RNA-seq data were consistent in quantitatively estimating the transcription levels of *L. intracellularis* during infection *in vitro*. The validation of RNA-seq data using qRT-PCR has also been previously reported in yeast (Nagalakshmi et al., 2008) and other bacterial transcriptomes (Isabella and Clark, 2011; Oliver et al., 2009; Yoder-Himes et al., 2009).

Cell division and macromolecule biosynthesis

From a total of 43 ribosomal protein-encoding genes present in the *L. intracellularis* reference genome, we identified 31 genes expressed by both the pathogenic and non-pathogenic variants and eight additional genes exclusively expressed by the pathogenic one. Furthermore, the operon responsible for orchestrating bacterial cell division that contained the *FtsA* and *FtsZ* genes (Adams and Errington, 2009) was also identified in both variants (Supplementary Table 4.2). The expression of RNA polymerase α and β subunits has also been associated with growth rate in that their synthesis increases in response to a rich nutrient medium (nutritional shift-up) (Shepherd et al., 1980). All *L. intracellularis* genes encoding RNA polymerase subunits previously predicted from the annotated genome were expressed in both variants.

Energy production and conversion

Pathogenic bacteria are frequently able to couple virulence pathways with general metabolic functions such as energy production, cellular signaling and molecular biosynthesis (Dalebroux et al., 2010). The operon comprising F_0-F_1 ATP synthase subunits, which was expressed in both pathogenic and non-pathogenic *L. intracellularis* variants (Supplementary Table 4.2), is essential for life in many bacterial species since it maintains pH homeostasis of the cell (Ryan et al., 2008). Concomitantly, it plays a critical role in the acid stress response, specifically in enteric bacteria which encounter low pH conditions transiting through stomach and in the phagosome of host cells (Gahan and Hill, 1999). Although we intended to harvest *L. intracellularis* freely multiplying in the cell cytoplasm (five days post-infection) that would fully represent its exponential growth phase, the identification of the F_0-F_1 operon in both variants suggests that a fraction of harvested intracellular bacteria was in the transient phagosome phase.

Along with these observations, the expression data also indicated the presence of free intracellular bacteria in the cell cytoplasm by detecting high transcriptional levels (Supplementary Table 4.2) of rickettsia-like ATP/ADP translocase gene in both variants.

Cellular processes and small molecule biosynthesis

Although *L. intracellularis* and other obligate intracellular bacteria depend on their hosts for certain nutrients (e.g. cytoplasmic ATP, see above), a wide spectrum of biosynthetic pathway-encoding genes are critical for fulfilling their essential functions. The 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway is primarily involved in the biosynthesis of structural and functional isoprenoid molecules but the genes encoding the

final two enzymes, *ispG* and *lytB*, have also been related to intracellular survival and induction of cellular immune response (Heuston et al., 2012). Six genes of the *Lawsonia* MEP pathway previously predicted in the Kyoto encyclopedia of gene and genomes (KEGG) database were exclusively expressed by the pathogenic variant. However, the final remaining two enzymes (*ispG* and *lytB*) were transcribed by both the pathogenic and non-pathogenic variants. We suggest that *ispG* and *lytB* may be essential genes in *L. intracellularis*, as previously reported in several other bacterial pathogens (Heuston et al., 2012). Based on this vital characteristic of the MEP pathway, it has recently been used as a drug target (Obiol-Pardo et al., 2011). Fosmidomicyn showed the ability to inhibit the second enzyme of the MEP pathway (*Dxr*) and has been used in clinical trials (Oyakhirome et al., 2007). Interestingly, our study identified expression levels of the *Dxr*-encoding gene ($\log_2$ [RPKM] = 10.1) exclusively in the pathogenic variant, suggesting its potential use for drug targeting.

Membrane transport

From a total of 14 ABC transporter operons, 10 cassettes were specifically expressed by the pathogenic variant, including those involved in resistance to organic solvents, membrane transport of polyamines (spermidine), phosphates, nitrates, amino acids (glutamine and branched-chain), metallic cations (cobalt and nickel), lipopolysaccharides and lipoproteins. We specifically observed the highest expression levels in ABC uptake systems for amino acids (Table 4.1).

The ABC transporter involving polyamine uptake (*PotABCD* operon) and uniquely expressed in the pathogenic variant at moderate levels ($\log_2$ [RPKM] = 11.1)

has also been important for virulence in other bacterial organisms (Ware et al., 2006). Corresponding with the glutamine ABC transporters discussed above, mutations in the *PotABCD* operon of *S. pneumoniae* showed no effect on the growth rates *in vitro*, but the mutant strain showed significant attenuation of virulence within murine models regardless of the inoculation route (Ware et al., 2006).

Protein turnover and Chaperones

Chaperone-encoded genes (*DnaK, DnaJ, GroEL and GroES*) were highly expressed by the pathogenic and non-pathogenic variants. This observation corroborates the up-regulation of this gene category identified in a variety of bacterial organisms growing inside eukaryotic cells (La et al., 2008). Furthermore, bacterial chaperones are known to be essential for overcoming bacterial stress by ensuring the proper folding of proteins. Three additional genes (*CbpA*, *CrpE* and *ClpA*) involving the synthesis of chaperone molecules were exclusively expressed by the pathogenic variant and their specific biological functions remain to be determined.

Cell membrane and motility

A broader diversity of genes encoding cell wall molecules were observed in the pathogenic *L. intracellularis* variant, suggesting more extensive options for remodeling of the bacterial envelope compared with the non-pathogenic variant. This characteristic is important for altering the bacterial cell surface during different steps of the infectious process (La et al., 2008). The wider variety of this gene category in the pathogenic variant was predominantly comprised of genes encoding glycosyltransferase enzymes that have been implicated in posttranslational processing of proteins responsible for

modifying the lipopolysaccharide composition. Modifications of the glycosylation composition of the cell wall were depicted in *Campylobacter jejuni* during its intestinal life cycle. These events were then considered part of the strategy used by the bacterium to resist and evade the host immune responses (Stintzi et al., 2005).

In the mapping of genes involved in the flagellar assembly pathway, there were no consistent differences between the pathogenic and non-pathogenic variants. From a total of 30 predicted genes in the KEGG database, ten were expressed in both variants (Supplementary Table 4.2), eight were exclusively identified in the pathogenic and one was unique from the non-pathogenic variant (Table 4.2). Our study detected similar expression levels of the *FliC* gene in both variants (Supplementary Table 4.2). The flagellin encoded by this gene has been well studied regarding its ability to induce an immune response (Bobat et al., 2011). *S. enterica* Typhimurium expressed this protein in the membrane-bound compartment and it was then translocated into the host cytoplasm to be detected by cytosolic Nod-like receptors which are able to mount an innate immune response (Sun et al., 2007). Studies characterizing the immune response of cells infected with pathogenic and non-pathogenic *L. intracellularis* would be important to detect specific host factors associated with these two variants.

Adaptation and stress response

The number of genes encoding proteins involved in adaptation and stress response was one of the gene categories with significant reduction (61%) in the non-pathogenic variant (Figure 4.2). Ten of the 28 genes uniquely expressed by the pathogenic variant in this category were transcriptional regulatory factors. A gene encoding the major

transcription factor involved in the iron homeostasis (*fur* regulator) was uniquely expressed by the pathogenic variant (Table 4.1) and its transcription levels were confirmed by qRT-PCR (Figure 4.4; Supplementary Table 4.1).

Predicted/unknown function

Our study identified four genes encoding hypothetical proteins in the ten most highly expressed genes commonly identified in the pathogenic and non-pathogenic variants. Among all commonly expressed genes, the LI0447 gene demonstrated the highest transcription levels (Supplementary Table 4.2) which was confirmed by qRT-PCR (Figure 4.4; Supplementary Table 4.1). The protein encoded by the LI0447 gene was computationally predicted to be a transmembrane protein with 50.7% identity at the amino acid level to other hypothetical proteins identified in the *Mannheimia haemolytica* genome (MHA_1476 – accession: ZP_04978003.1). Regarding the genes uniquely expressed by the pathogenic variant, the hypothetical protein encoded by the LI0259 gene was the third most highly expressed chromosomal gene (Table 4.1). This protein (accession: YP_594636) has a conserved putative domain of 41 amino acids found in a wide range of bacteria and is noted as a regulatory factor included in the FmdB family.

From a total of 35 plasmid-encoded genes uniquely expressed by the pathogenic variant (Figure 4.1), 19 genes were predicted to encode hypothetical proteins. Genes showing the highest transcription levels in the plasmids are summarized in Table 4.3. The LIC056 gene was the most highly expressed gene identified uniquely in the pathogenic variant and its transcription levels were confirmed by qRT-PCR (Figure 4.4; Supplementary Table 4.1). This gene encodes a predicted transmembrane protein

containing a conserved autotransporter beta-domain. This class of proteins has been implied to mediate secretion by translocation of bacterial proteins across the outer membrane. Another intriguing observation was the expression of the LIC091 gene exclusively in the pathogenic variant (Table 4.3). This gene has been predicted to encode one of the largest proteins among all bacterial organisms containing 8746 amino acids. Eight different protein families have been proposed for this molecule including a transmembrane protein with 99.5% of its amino acid sequence belonging to an extracellular domain.

Table 4.1. Chromosomal genes showing highest transcript levels exclusively expressed by the pathogenic variant of the *L. intracellularis* isolate PHE/MN1-00 according to the putative biological function.

Locus *Biological function*	Gene	Description	Log_2 (RPKM)
Cell division and macromolecule biosynthesis			
LI0372		putative cell division protein *FtsB*	12.8
LI0844		tRNA and rRNA cytosine-C5-methylases	14.1
LI0962	*rplW*	50S ribosomal protein L23	13.5
Energy production and conversion			
LI0442	*hyaD*	processing of HyaA and HyaB proteins	10.9
LI1176		nitroreductase	11.1
Cellular processes and small molecule biosynthesis			
LI0154	*ribH*	riboflavin synthase beta-chain	12.6
LI0361		quinolinate synthetase	12.7
LI0735	*ychB*	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	14.5
Membrane transport			
LI0338	*livH*	branched chain amino acid ABC transporter (permease)	12.5
LI0754	*glnH*	amino acid ABC transporter substrate-binding protein	13.9
LI0995	*oprM*	outer membrane efflux protein	12.1
Protein turnover and chaperones			
LI0375	*pcm*	protein-L-isoaspartate carboxylmethyltransferase	12.2
LI0225	*smpB*	SsrA-binding protein	11.4
LI0618		ATPases with chaperone activity, ATP-binding subunit ClpA	11.4
Cell membrane and motility			
LI0825		Lipid A core-O-antigen ligase and related enzymes	13.2
LI0947		cell wall-associated hydrolases	12.6
LI1141	*cheW*	chemotaxis signal transduction protein	11.0
Adaptation and stress response			
LI0035	*fur*	Fe2+/Zn2+ uptake regulation proteins	11.8
LI0301	*recO*	DNA repair protein RecO (recombination protein O)	13.0
LI0457	*rpoN*	Sigma54-like protein	11.7
General predicted function			
LI0140		glycosyltransferase	11.5
LI0246	*hypA*	zinc finger protein	11.6
LI0880	*sfsA*	DNA-binding protein, stimulates sugar fermentation	11.4
Hypothetical / Unknown function			
LI0259		hypothetical protein	13.9
LI0917		hypothetical protein	12.9
LI1056		hypothetical protein	12.3

Table 4.2. Genes exclusively expressed by the non-pathogenic variant of the *L. intracellularis* isolate PHE/MN1-00.

Locus *Biological function*	Gene	Description	Log_2 (RPKM)
Cell division and macromolecule biosynthesis			
LI0702	*maf*	nucleotide-binding protein involved in septum formation	11.7
LIB024	*parA*	ATPases involved in chromosome partitioning	11.1
Energy production and conversion			
LI0614		Thiol-disulfide isomerase and thioredoxins	12.1
LI1001	*napC*	cytochrome c nitrite reductase small subunit	12.2
Cellular processes and small molecule biosynthesis			
LI0709	*ribF*	FAD synthase involved in riboflavin metabolism	12.2
Membrane transport			
LI0540	*yscN*	type III secretion system ATPase	11.3
LI1163	*YscJ*	type III secretion protein	10.8
Cell membrane and motility			
LI0747	*flgK*	flagellar hook-associated protein	11.2
Predicted and unknown function			
LI0427		hypothetical protein	9.8
LI0554		hypothetical protein	10.8
LI0583		hypothetical protein	10.1

Table 4.3. Plasmid-encoded genes with highest transcript levels exclusively expressed by the pathogenic variant of the *L. intracellularis* isolate PHE/MN1-00.

Locus *Biological function*	Gene	Description	Log_2 (RPKM)
Plasmid A			
LIA022		cell wall biosynthesis glycosyltransferase	12.1
LIA025		plasmid stabilization system protein	11.7
LIA017	*bchE*	Fe-S oxidoreductase	11.2
Plasmid B			
LIB002		PbsX family transcriptional regulator	13.5
LIB017		hypothetical protein	12.1
LIB019		hypothetical protein	13.3
Plasmid C			
LIC047		hypothetical protein	12.8
LIC056		hypothetical protein	16
LIC079		hypothetical protein	11.4
LIC091		hypothetical protein	11.4

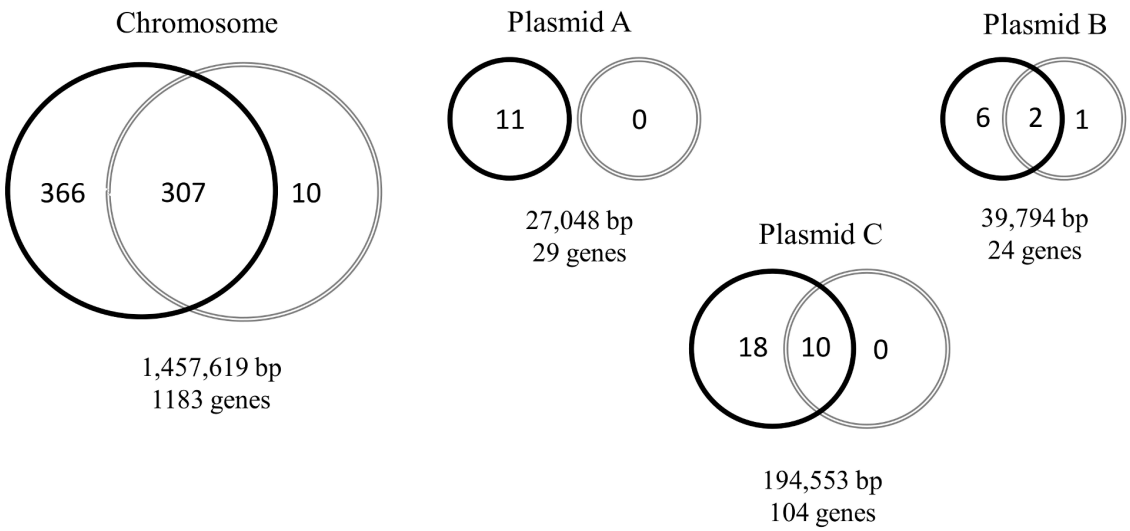

Figure 4.1. Schematic representation of the *L. intracellularis* genome. Distribution of genes expressed by the pathogenic (black circles) and the non-pathogenic (gray circles) variants. Overlapping zones represent genes expressed in both variants.

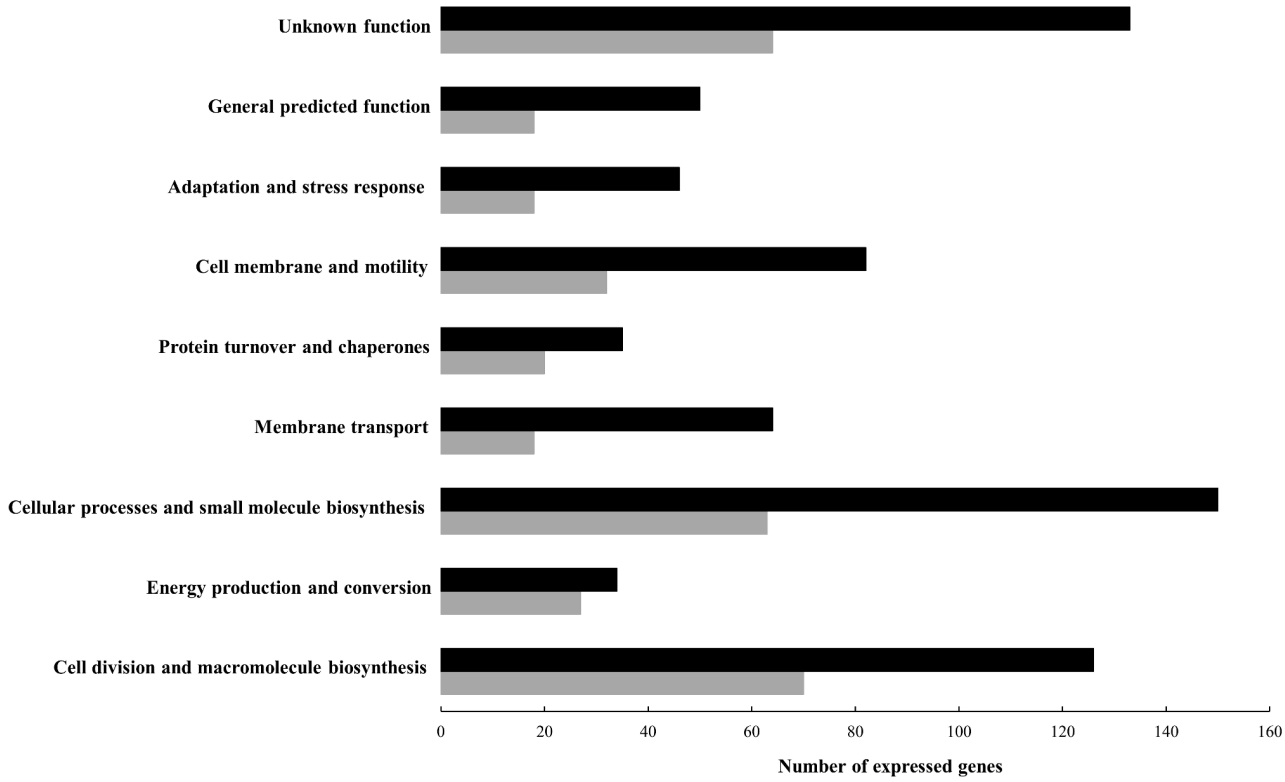

Figure 4.2. Functional categories of genes expressed by the pathogenic and non-pathogenic homologous *L. intracellularis* isolate PHE/MN1-00. Black and gray bars represent the number of genes expressed by the pathogenic and non-pathogenic variants, respectively.

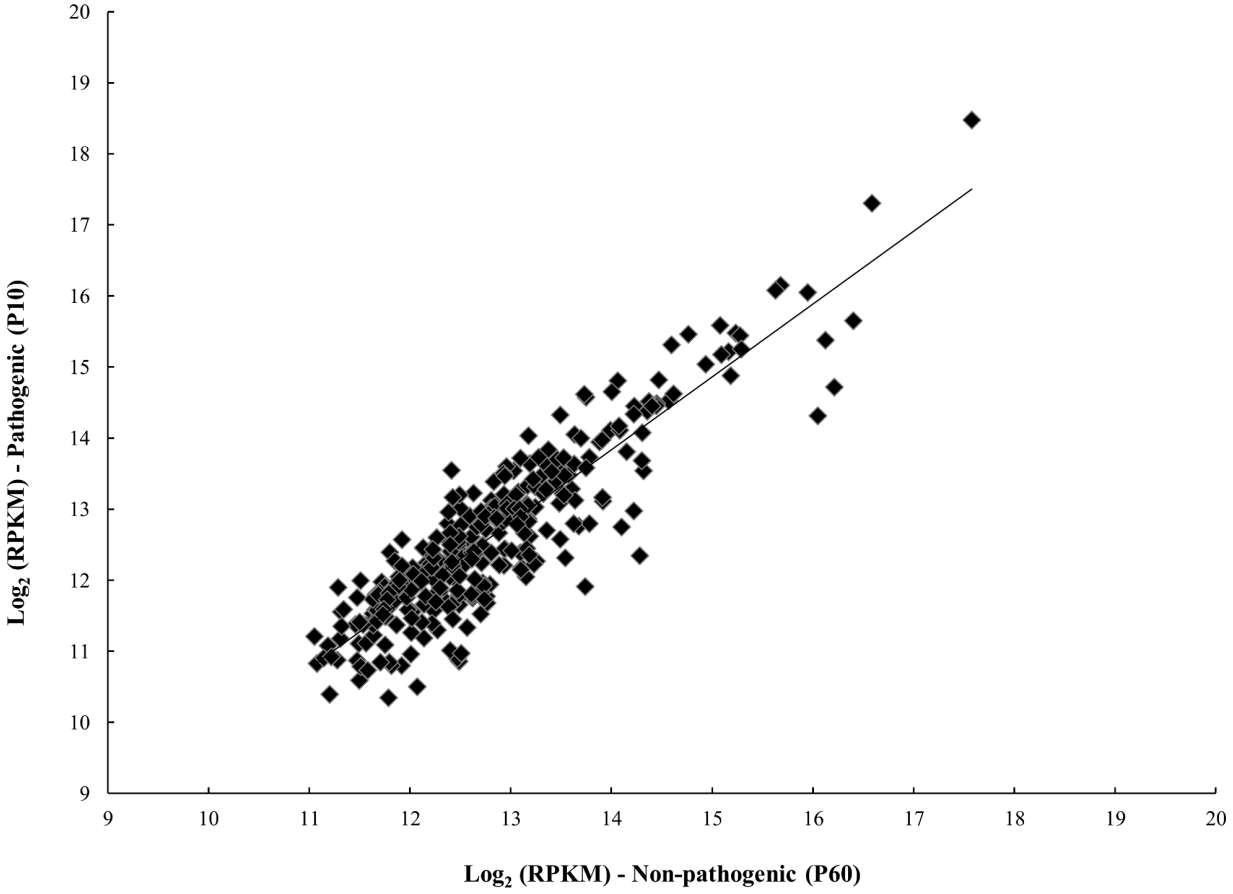

Figure 4.3. Average log-transformed RPKM ($\log_2$ [RPKM]) of the 319 genes commonly expressed by the pathogenic (y-axis) and non-pathogenic (x-axis) variant. The trend line represents a linear regression model (p-value < 0.05; $r^2 = 0.809$).

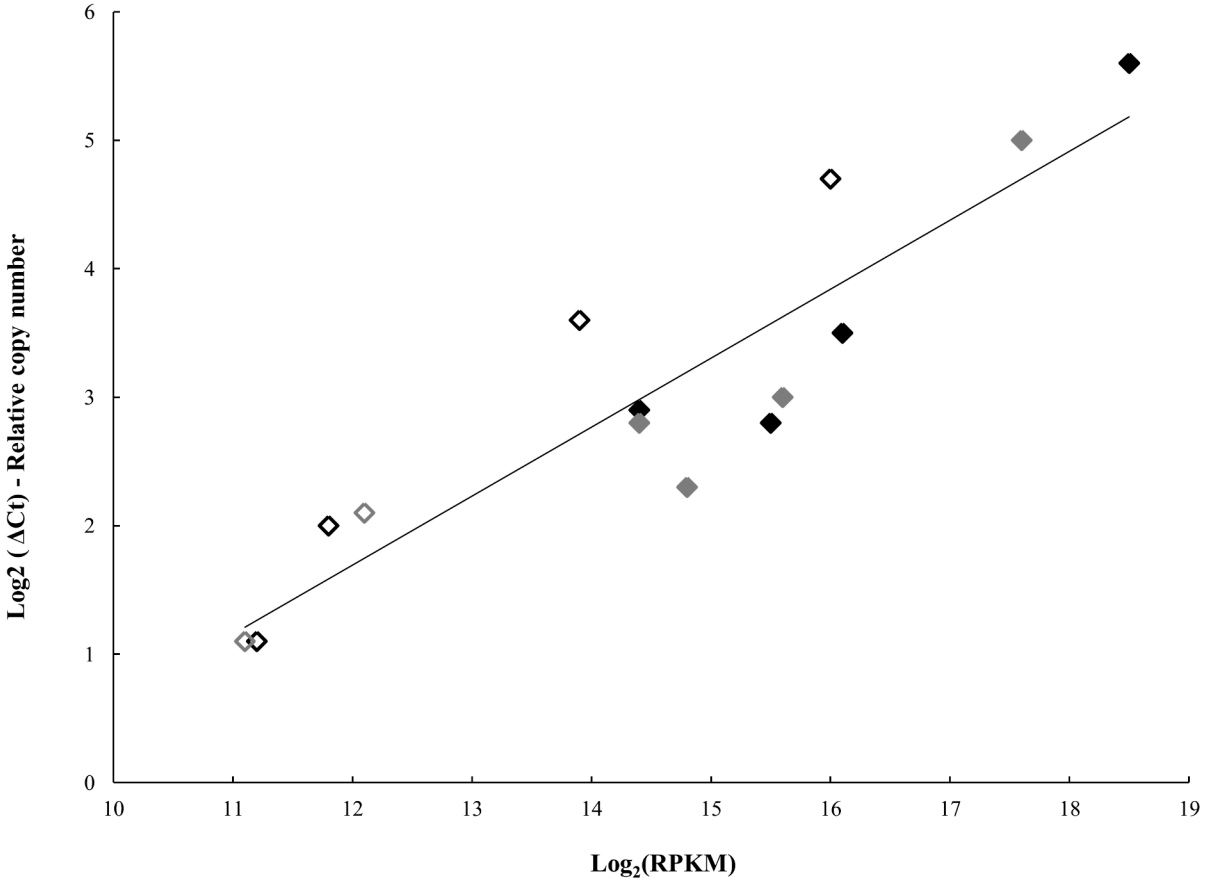

Figure 4.4. Correlation between RNA-seq and qRT-PCR. Plot demonstrating the relative quantification of 10 unlinked genes by qRT-PCR (y-axis) and the transcript levels generated by RNA-seq (x-axis). Genes commonly expressed by the pathogenic (♦) and non-pathogenic (♦) variants. Genes exclusively expressed by the pathogenic (◊) and non-pathogenic (◊) variants.

4.4 Discussion

Whole-transcriptome profiling of bacterial organisms has been widely studied to understand global changes in gene expression *in vitro* and *in vivo* (Virtaneva et al., 2005; Wehrly et al., 2009). Hybridization-based approaches have been successfully applied for years (Behr et al., 1999), but it has limited use in obligate intracellular bacteria since it needs a reference purified sample from cultivated bacteria. High-throughput technologies have overcome these limitations by providing high resolution data to describe the bacterial transcripts in different experimental conditions (Isabella and Clark, 2011; Yoder-Himes et al., 2009). The present study used RNA-seq to qualitatively and quantitatively characterize the transcriptional profile of pathogenic and non-pathogenic homologous *L. intracellularis* isolates during *in vitro* infection. Since this is the first comprehensive gene expression analysis regarding this organism, the findings are discussed in a comparative pathogenomic approach based on the information available from other related bacterial organisms.

The entire transcriptional repertoire of the plasmid A was suppressed in the non-pathogenic isolate (Figure 4.1) suggesting its potential contribution in the *L. intracellularis* virulence. Transcription factors located in bacterial chromosomes have been shown to positively regulate virulence factors in the plasmid of *Shigella flexneri* and *Pseudomonas syringae* (Alarcón-Chaidez et al., 2003; Zhu et al., 2010). Our study also identified many regulatory factors exclusively expressed by the pathogenic *L. intracellularis* variant which potentially regulate plasmid-encoded genes.

A correlation between infectivity and loss of DNA contents from linear and/or circular plasmids during serial passages *in vitro* has been well established in *Borrelia burgdorferi* infections (Biškup et al., 2011; Purser and Norris, 2000). Since there are a large number of different plasmids found in *Borreliae* genomes, their stability and loss *in vitro* vary between and within species (Glöckner et al., 2006). Although our study did not identify any transcriptional activities in the plasmid A of the non-pathogenic variant, the DNA contents of this plasmid were identical in both pathogenic and non-pathogenic variants (data not shown). Additionally, transcription levels were detected in three and ten genes present in the plasmid B and C of the non-pathogenic variant, respectively (Figure 4.1). These findings demonstrated the occurrence of transcriptional activities and, consequently, the presence of these two plasmids in this variant. These observations support the hypothesis that global changes in gene expression are able to drive the loss of the virulence phenotype in cultivated *L. intracellularis* without altering the genomic DNA.

Regulation of gene expression during *in vitro* cultivation has been widely reported in various bacterial organisms (Fux et al., 2005). *Bordetella pertussis* switches off the expression of type III secretion system (TTSS) proteins in laboratory-adapted strains (Fennelly et al., 2008). One of the TTSS components, protein Bsp22, ceases to be expressed between passages three and four (Gaillard et al., 2011). Interestingly, these authors also showed the reversible re-expression of this protein after contact with the host *in vivo*. The expression of TTSS proteins has been demonstrated during *L. intracellularis* infection (Alberdi et al., 2009). However, our study failed to demonstrate consistent

differences in the expression of TTSS proteins between pathogenic and non-pathogenic variants. Additionally, reversibility of virulence has not been reported in animals infected with laboratory-adapted *L. intracellularis*.

Transcriptional regulation has also been associated with mutations characterized by non-synonymous substitutions at the DNA level in *Escherichia coli* cultivated in glucose-limited medium for 20,000 generations (Cooper et al., 2003). Regulatory changes in gene expression following by mutations in 12 lines of *E. coli* were driven by the specific environment *in vitro* evolving an ecological specialization in laboratory-adapted strains (Cooper and Lenski, 2000). Narrower transcriptional profiling of the non-pathogenic *L. intracellularis* passed 60 times *in vitro* was observed in our study; this could represent earlier stages of adaptation to a specialized *in vitro* environment. As in the case of *E. coli*, unnecessary functions that are costly to fitness in the *in vitro* condition might then be eliminated after hundreds or thousands of generations. According to ecological specialization models, organisms genetically adapted to one environment may lose fitness in other environments (Cooper and Lenski, 2000; Futuyma and Moreno, 1988). Corroborating with this principle, the lack of reversible virulence in animals infected with laboratory-adapted *L. intracellularis* suggests that improving fitness in a specialized environment *in vitro* adversely affects performance in other complex substrates *in vivo*.

Synthesis of proteins involved in cell division and ribosome biogenesis has been closely related to bacterial growth rate (Nomura et al., 1984; Weart and Levin, 2003). The genes associated with these functions were observed in both pathogenic and non-

pathogenic variant. These observations demonstrated that both variants grew at the same rate and were harvested during exponential phase at time of RNA harvesting (five days post-infection). Likewise, the results corroborate previous studies that described an increase in the number of *Lawsonia*-infected cells from two to seven days after infection (McOrist et al., 1995b; Vannucci et al., 2012e). Adverse effects in bacterial growth have been related to the reduced expression of RNA polymerase subunits and ribosomal proteins in *Neisseria gonorrhoeae* cultivated in an oxygen-limited environment (Isabella and Clark, 2011). The microaerophilic environment provided in our study was first described by Lawson et al (1993) and it has been shown to be an optimal atmosphere for cultivation of *L. intracellularis* (Lawson et al., 1993; Vannucci et al., 2012e).

Despite the similarities in the expression of ribosomal proteins and RNA polymerase subunits between pathogenic and non-pathogenic variants described previously, our study also identified the expression of *relA* gene exclusively in the pathogenic variant. This gene encodes an enzyme responsible for synthesizing guanosine tetraphosphate (ppGpp). This small signal molecule, also called stringent factor, is involved in various cellular processes and affects the control of growth rates by binding to RNA polymerase and reduces synthesis of ribosomal proteins and tRNA molecules during nutritional stress (Jain et al., 2006; Potrykus et al., 2011). Although the transcript reads of *relA* gene were exclusively identified in the pathogenic variant, its expression levels were relatively low ($\log_2$ [RPKM] = 9.7) suggesting that it was not sufficient to affect the expression of genes involved in macromolecule biosynthesis.

Based on the empirical observation that high-passage variants of *L. intracellularis* have higher growth rates than low passages *in vitro*, we believe that *relA*-dependent accumulation of ppGpp may control the bacterial growth rate at some point during the course of the infection *in vitro*. However, chronological transcriptional analysis is needed in order to elucidate this question. Supporting this speculation, Cooper et al (2003) demonstrated an increased fitness of laboratory-adapted *E. coli* (after 20,000 generations *in vitro*) associated with reduction in the concentration of ppGpp. These authors did not observe any mutation in the DNA sequence of *relA* gene. Similarly, the suppression of *Lawsonia relA* gene in the non-pathogenic variant was not associated with any mutations in the *relA* DNA sequence (data not shown).

The stringent response mediated by ppGpp also plays an important role in bacterial virulence, especially to adapt to conditions encountered in the intracellular host environment (Dalebroux et al., 2010). A *relA* mutant strain of *Listeria monocytogenes* showed no virulence *in vivo* using murine infection models (Bennett et al., 2007). However, the same study shows no difference between the wild type and Δ*relA* mutant strain in their ability to escape from the phagosome and polymerize host cell actin *in vitro* using Caco-2 cells. Comparable phenotypic differences between *in vitro* and *in vivo* infections have also been observed in *L. intracellularis*. Low passage and high passage variants have similar abilities to infect cells *in vitro* but they demonstrated differences in pathogenic and non-pathogenic phenotypes *in vivo* (Vannucci et al., 2013).

Our study identified the expression of the F_0-F_1 operon in both variants suggests that a fraction of harvested intracellular bacteria was in the transient phagosome phase.

But we also found the expression of the rickettsia-like ATP/ADP translocase that is typically expressed in the cytoplasm of intracellular bacteria. This transmembrane protein was first identified in a few obligate intracellular bacteria, including *Chlamydiales*, *Rickettsiales* and amoebal symbionts (Schmitz-Esser et al., 2004), and more recently in *L. intracellularis* (Schmitz-Esser et al., 2008). A remarkable adaptation of these organisms to an intracellular microenvironment allows them to import cytoplasmic ATP generated in their hosts into the prokaryotic cell across the bacterial cell membrane (Winkler and Neuhaus, 1999). In an exchange mode, the bacterial ADP is exported back into the host cytosol. This exploitation of the host's energy pool is also referred to as energy parasitism. In agreement with the exponential growth phase of *L. intracellularis*, we also identified the expression of cytochrome *bd* operon in the pathogenic and non-pathogenic variant (Supplementary Table 4.1). This oxygen reductase is used in the bioenergetic pathway in a variety of bacterial organisms under O_2-limited conditions (Borisov et al., 2011).

The majority of genes encoding ABC transporters identified in the *L. intracellularis* genome were shown to be expressed in the pathogenic variant. Elevated transcription levels of the glutamine transport system (*glnHPQ* operon) were shown to be essential for virulence in *Salmonella enterica* Serovar Typhimurium (Klose and Mekalanos, 1997) and *Streptococcus pneumoniae* (Härtel et al., 2011). Both studies demonstrated significant attenuation of the virulence *in vivo* using mouse models infected with mutant strains (Δ*glnHPQ*). Despite the attenuated phenotype, the mutant strain of *S. enterica* Typhimurium provided a protective immune response against challenge with the

wild-type. Additionally, the authors showed that *glnHPQ* operon is positively regulated by the sigma factor σ^{54} (RpoN). This regulatory factor was also exclusively expressed by the pathogenic *L. intracellularis* variant in our study (Table 4.1). The expression of σ^{54} and other transcription factors are discussed later in the subsection related to bacterial adaptation and stress response.

The *in vitro* growth kinetics of *S. enterica* Typhimurium and *S. pneumoniae* *glnHPQ* mutants were not affected in either broth or agar supplemented with glutamine or peptides (Härtel et al., 2011; Klose and Mekalanos, 1997). On the other hand, both studies showed reduced intracellular survival of these mutants in macrophage cell lines. There is no information regarding the intracellular survival of pathogenic or non-pathogenic *L. intracellularis* in infected macrophages and no impairment of intracellular survival has been reported in *L. intracellularis* continually cultivated using epithelial or fibroblastic-like cells (McOrist et al., 1995b; Vannucci et al., 2012e). Although the IPEC-J2 cell culture media used in our study was not supplemented with glutamine, the glutamine ABC transporters were consistently expressed only by the pathogenic variant in the two *glnHPQ* operons present in the *L. intracellularis* genome.

The differential mapping of other bacterial transport systems including TTSS and general secretory (Sec) pathways was not consistently different between the pathogenic and non-pathogenic variants. The role of TTSS proteins has been well established at earlier stages of infection and has been required for the invasion of various bacterial organisms (Knodler and Steele-Mortimer, 2003). Our study detected the intracellular expression of five of a total of 15 *L. intracellularis* genes involved in the synthesis of

TTSS previously identified in the KEGG database. This unexpected intracellular expression of TTSS proteins was reported in *S. enterica* Typhimurium and plays a role in the survival of this organism within the *Salmonella*-containing vacuole (Hautefort et al., 2008; Hensel et al., 1998).

The expression of genes encoding structural components of TTSS (*YscN*, *YscO* and *YscQ*) was previously identified by RT-PCR in three *L. intracellularis* isolates infecting rat small intestinal cells (IEC-18) (Alberdi et al., 2009). However, the study did not provide information about the number of cell passages the isolates had undergone or the time points in which the bacterial RNA was harvested from the infected cell monolayers. Our study identified two (*YscQ* and *YscN*) of the three major TTSS components cited above. The *YscQ* gene was expressed in both the pathogenic and non-pathogenic variant and *YscN* were uniquely identified in the non-pathogenic variant (Table 4.2). These variable results regarding the expression of TTSS proteins suggest that further studies are required in order to elucidate the role of TTSS in *L. intracellularis* infection *in vitro*.

Various transcriptional factors related to adaptation and stress response were uniquely expressed by the pathogenic variant. These molecules regulate bacterial gene expression by acting globally or at specific DNA regions to active or repress transcription or modulate DNA topology. The σ^{70} is the "housekeeping" sigma factor and has been required for cell growth in the majority of bacterial organisms (Kazmierczak et al., 2005). In agreement with this, we observed similar expression levels of σ^{70} in the pathogenic and non-pathogenic variants (Supplementary Table 4.2). Conversely, the global regulator

σ^{54} was uniquely expressed in the pathogenic variant (Table 4.1) and has been reported to control the transcription of virulence genes in a variety of bacteria species (Kazmierczak et al., 2005). Specifically in *S. enterica* Typhimurium, the role of σ^{54} is to coordinate the transcription of the glutamine ABC transporter, *glnHPQ* operon, during the intracellular life cycle. The coincidental and exclusive co-expression of σ^{54} and *glnHPQ* by the pathogenic variant supports the hypothesis that this sigma factor may also coordinate the glutamine uptake operon in *L. intracellularis* to ensure an adequate supply of this crucial amino acid during its intracellular life. Additionally, the consistently higher number of ABC transporter-encoding genes expressed by the pathogenic, but not the non-pathogenic, variant begs the question whether σ^{54} is also able to positively regulate other ABC transporters (e.g. polyamines and branched-chain amino acid transporters). The obligate intracellular nature of *L. intracellularis* has imposed considerable limitations in elucidating this and other specific regulatory mechanisms. To date, the construction of recombinant plasmids appears to be an alternative model for overcoming these barriers.

The *fur* regulator can act as either a repressor or an activator in a variety of bacterial organisms (Carpenter et al., 2009). The wider gene expression profile identified in the pathogenic variant of *L. intracellularis* suggests the role of *fur* as an activator in our experimental conditions. Supporting this hypothesis, *fur*-activated genes have been reported in other enteric pathogens (e.g. *S. enterica* Typhimurium) and bacteria that replicate freely in the host cytoplasm (e.g. *L. monocytogenes*) (Carpenter et al., 2009; McLaughlin et al., 2011). Although this transcription factor is typically related to the iron metabolism in response to iron availability, its role in the stress response and virulence *in*

vivo has also been well established (Carpenter et al., 2009). For instance, *fur* mutants of *L. monocytogenes* and *C. jejuni* showed reduced virulence in experimental models (Palyada et al., 2004; Rea et al., 2004). Furthermore, the *in vitro* growth rates in a *fur* mutant strain of *Desulfovibrio vulgaris*, one of the closest bacterial species genetically related to *L. intracellularis* (Bender et al., 2007; McOrist et al., 1995a), were not affected.

In addition to its role in virulence, *fur* acts in the regulation of genes involved in oxidative stress, such as superoxide dismutase (*sod*). However, the unique *sod* gene previously annotated in the *L. intracellularis* genome (*sodC* gene) was expressed at high levels by both pathogenic and non-pathogenic variants (Supplementary Table 4.2). Its transcription levels were validated by qRT-PCR (Figure 4.4; Supplementary Table 4.1). This finding indicates that a *fur*-independent mechanism potentially regulates *sodC* expression *in vitro*. In agreement with this speculation, the literature describes *fur* regulation of *sodA* (Mn^{2+}-containing *sod*) and *sodB* (Fe^{2+}-containing *sod*) but not *sodC* (Cu-Zn^{2+}-containing *sod*) (Carpenter et al., 2009). In addition, *fur* mutation had no effect on the regulation of *sodC* in *E. coli* (Lynch and Kuramitsu, 2000). Regardless of specific regulatory mechanisms, the expression of the *sodC* gene is critical for intracellular survival of pathogenic bacteria by catalyzing reactive host-derived superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2) (McCord and Fridovich, 1969). In our study, this mechanism also appears to be essential for both pathogenic and non-pathogenic *L. intracellularis* during *in vitro* infection. The gene (*rubA*) encoding rubredoxin 2 (Rb-2) was computationally predicted in the *L. intracellularis* genome and has been described to complement the catalytic activity of *sodC* against oxidative stress

in *Desulfovibrio* species. Our study identified *rubA* as the second most commonly expressed gene by both pathogenic and non-pathogenic variants (Supplementary Table 4.2). According to a model proposed by Lumppio et al (2001), the superoxide dismutase acts in the periplasm fraction and the Rb-2 neutralizes superoxide radicals in the bacterial cytoplasm (Lumppio et al., 2001).

Genes encoding hypothetical proteins comprise approximately 27.2% of the 1340 protein encoding genes previously predicted in the reference DNA sequence of the *L. intracellularis* PHE/MN1-00 isolate. This significant number becomes more evident within the plasmid A (55.2%), plasmid B (58.3%) and plasmid C (63.5%). An autotransporter protein (LatA) was recently characterized as a prominent antigen during infection of IEC-18 cells with the *L. intracellularis* isolate LR189/5/83 (Watson et al., 2011). However, the authors did not specify the number of *in vitro* passages that bacterial isolate had. Our study identified the gene encoding this protein, referred to as LI0649, in both the pathogenic and non-pathogenic variants (Supplementary Table 4.2). Our study provides evidence regarding the potential role of genes encoding hypothetical proteins during *L. intracellularis* infection *in vitro*, but their specific biological functions remain to be elucidated. Additionally, the substantial number of these genes previously identified in the *L. intracellularis* genome and now associated with its transcriptional profiling suggests that this organism may adopt unique mechanisms of survival and pathogenesis among bacterial pathogens.

The present study is the first report characterizing the transcriptional profile of *L. intracellularis* and comparing the gene expression levels of a pathogenic and a non-

pathogenic homologous isolate. The wider transcriptional landscape identified in the pathogenic variant was consistent throughout the gene categories and had significant contributions of plasmid-encoded genes. High expression levels of genes encoding ABC transporters and specific transcriptional regulators were uniquely identified in the pathogenic variant and suggest specific metabolic adaptation of *L. intracellularis*, including substrate acquisition that allows its efficient proliferation in the infected host. The dynamics of the genetic changes in laboratory-adapted bacterial organisms have developed a new ecological specialization which results in different bacterial phenotypes (Cooper and Lenski, 2000). In our study, the lack of selective pressure during multiple cell passages *in vitro* might be the reason for the narrower transcriptional profile observed in the non-pathogenic variant and loss of pathogenicity *in vivo* by gene silencing (switching off) mechanisms.

CHAPTER 5

Laser microdissection coupled with RNA-seq analysis of porcine enterocytes infected with an obligate intracellular pathogen (*Lawsonia intracellularis*)

Vannucci F.A.; Foster D.; Gebhart C.J. (2013) Laser microdissection coupled with RNA-seq analysis of porcine enterocytes infected with an obligate intracellular pathogen (*Lawsonia intracellularis*). *BMC Genomics* (In review)

Summary

Lawsonia intracellularis is an obligate intracellular bacterium and the etiologic agent of proliferative enteropathy. Cell proliferation is associated with bacterial replication in the enterocyte cytoplasm, but the molecular basis of the host-pathogen interaction is unknown. We used laser capture microdissection coupled with RNA-seq technology to characterize the transcriptional responses of infected enterocytes and the bacterial interactome. Three thousand each of *Lawsonia*-infected and non-infected enterocytes were microdissected. Amplified cDNA prepared by random priming was sequenced using an Illumina® platform. Sequence reads were mapped against porcine and *L. intracellularis* reference genomes to characterize the host transcriptome and the bacterial interactome. Proliferative enterocytes were associated with activation of transcription, protein biosynthesis and genes acting in the G_1 phase of the host cell cycle (Rho family). The lack of differentiation in infected enterocytes was demonstrated by the repression of membrane transporters related to nutrient acquisition. The activation of the copper uptake transporter by infected enterocytes was associated with high expression of the Zn/Cu superoxide dismutase by *L. intracellularis*. This suggests that the intracellular bacteria incorporate intracytoplasmic copper and express a sophisticated mechanism to cope with oxidative stress. The feasibility of coupling microdissection and RNA-seq was demonstrated by characterizing the host-bacterial interactome from a specific cell type in a heterogeneous tissue. High expression of *L. intracellularis* genes encoding hypothetical proteins and activation of host Rho genes infers the role of unrecognized bacterial cyclomodulins in the pathogenesis of proliferative enteropathy.

5.1. Introduction

Cell proliferation concomitant with bacterial infections has been associated with carcinogenesis in chronic diseases caused by *Helicobacter pylori, Salmonella typhi* and *Citrobacter rodentium* (Lax and Thomas, 2002). Proliferative changes resulting in a hyperplastic but non-carcinogenic process is induced in *Lawsonia intracellularis* and *Bartonella* spp. infections (Lax and Thomas, 2002). The inflammatory mediators generated during the chronic gastritis in *H. pylori* infections have been related with oxidative DNA damage and cell transformation (Baik et al., 1996; Ikeno et al., 1999). However, other gram negative pathogenic bacteria (e.g. *L. intracellularis* and *C. rodentium*) are able to promote enterocyte proliferation with minimal inflammatory responses (Luperchio and Schauer, 2001; Smith and Lawson, 2001). *C. rodentium* induces proliferation of mouse colonic enterocytes when it attaches to the apical membrane (Sellin et al., 2009). *L. intracellularis* escapes from the endosome after internalization and multiplies freely in the cytoplasm of undifferentiated intestinal crypts promoting their proliferation and progressive replacement of the differentiated intestinal epithelium by immature infected enterocytes. These unusual pathological changes characterized by the presence of a large number of intracellular bacteria and proliferation of enterocytes suggests that *L. intracellularis* has adopted mechanisms of survival and pathogenesis that are unique among bacterial pathogens (Smith and Lawson, 2001). To date, hypotheses and speculations have been discussed regarding the pathogenesis of this infection but, the underlying mechanisms by which *L. intracellularis* induces proliferative changes have not been addressed.

Lawsonia intracellularis is an obligate intracellular bacterium and the etiologic agent of proliferative enteropathy (PE) (Gebhart and Guedes, 2010). Mild to severe diarrhea is the major clinical sign described in infected animals and is directly associated with cell proliferation and replication of the bacteria in the intestinal epithelium (Lawson and Gebhart, 2000). PE is endemic in swine herds, an emerging disease in horses and has been reported in various other species, including non-human primates, wild mammals and ratite birds (Cooper et al., 1997a; Klein et al., 1999; Lavoie and Drolet, 2009; Pusterla et al., 2008b).

The disease was first reported in 1931 but, due to its fastidious properties, porcine *L. intracellularis* was first isolated in 1993 using rat small intestinal cells cultured in a strictly defined microaerophilic environment (Biester and Schwarte, 1931; Lawson et al., 1993). Since then, the dynamics of infection *in vitro* and *in vivo* have been well characterized (Guedes and Gebhart, 2003a; McOrist et al., 1995b). While proliferative changes *in vivo* follow an increase in the number of intracellular bacteria, the bacterium is not able to induce proliferation in infected cells *in vitro* (Vannucci et al., 2012e). This intriguing observation associated with the fastidious properties of this bacterium has limited the studies on the pathogenesis of *L. intracellularis*. Additionally, the adaptation of this microaerophilic, but obligate intracellular organism to grow freely in the cytoplasm of metabolically active enterocytes suggests that this organism has properties that are novel and unique among bacterial pathogens.

In the present study, we hypothesized that genes expressed by *L. intracellularis* in the host cytoplasm are capable of inducing proliferation and preventing differentiation of

immature enterocytes by altering cell cycle-associated pathways. We established a method integrating laser capture microdissection (LCM) and RNA-seq technology to characterize the host transcriptome and constructed the bacterial interactome *in vivo*. Activation of transcription, protein synthesis and Rho family genes in infected enterocytes characterized the transcriptional mechanisms involved in the cell proliferation. The ability of *L. intracellularis* in preventing enterocyte differentiation and maturation was proved by the consistent down-regulation of apical membrane transporters related to nutrient acquisition by infected enterocytes. The bacterial interactome showed a high level of expression of a sophisticated oxidative protection mechanism which involves redox enzymes and a rubrerythrin-rubredoxin operon (*rubY-rubA*). Rho genes expressed by the host and associated with the high expression of bacterial genes encoding hypothetical proteins implies a potential role of unrecognized bacterial effector proteins that modulate eukaryotic cell cycle (cyclomodulins) in the pathogenesis of PE.

5.2 Materials and Methods

Preparation and quantification of L. intracellularis inoculum

The porcine pathogenic *L. intracellularis* strain PHE/MN1-00 was previously isolated and continuously grown in cell culture using murine fibroblast-like McCoy cells (ATCC CRL 1696) for 10 passages (Guedes and Gebhart, 2003b). The bacteria were then pelleted, suspended in sucrose-potassium glutamate (pH 7.0; 0.218 M sucrose, 0.0038 M KH_2PO_4, 0.0072 M K_2HPO_4 and 0.0049 M potassium glutamate) solution with 10% fetal

bovine serum and stored at -80^{o}C until the day of infection. The number of *L. intracellularis* organisms was assessed by direct counting after immunoperoxidase staining (Guedes et al., 2002) and by quantitative PCR (qPCR), as described elsewhere (Vannucci et al., 2012d).

Experimental infection

Twelve Duroc-Landrace cross pigs at 3-weeks-of-age were divided into two groups: infected and control (n = 6/group). The animals were obtained from a herd with no history of PE and each treatment group was housed in a different pen. Prior to the study, blood and fecal samples from all animals were collected and tested for *L. intracellularis*-specific antibodies by immunoperoxidase monolayer assay (IPMA) and for the presence of *Lawsonia* DNA in the feces in order to confirm their negative status (Guedes et al., 2002; Pusterla et al., 2008b). The animals were allocated in the isolation barns at the College of Veterinary Medicine of the University of Minnesota and fed with non-medicated nursery feed and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Minnesota. The infected group was orally inoculated with 30 ml of *L. intracellularis* culture at 10^{8} organisms per ml. The non-infected group was orally treated with sodium-phosphate-glutamate (SPG) solution. Fecal samples were collected every other day and analyzed by qPCR for the presence *L. intracellularis* DNA (Pusterla et al., 2008b).

Tissue collection and preservation

All animals were euthanized 21 days post-infection (PI) approaching the peak of the clinical disease, as described previously (Vannucci et al., 2013). Immediately after

euthanasia, the ileal mucosa was washed three times with PBS solution containing 2.0 U/µl RNAse inhibitor (Roche Applied Science) (Brown and Smith, 2009). A total of six tissue samples from each animal were collected, placed into plastic cryomold cassette (Tissue-Tek® Sakura Finetek) and embedded in optimal cutting temperature (OCT) compound (Tissue-Tek® Sakura Finetek). The samples were then placed onto blocks of dry ice until the tissues and OCT were frozen to a solid white. All samples were stored at -80ºC until use.

Laser capture microdissection

Five serial 8-µm frozen sections were cut at -20ºC using RNAse-free blades and mounted on StarFrost® RNAse-free slides (Fisher Scientific). The first and fifth cryosections (reference slides) were evaluated by immunohistochemistry (IHC) using the streptavidin method with polyclonal antibodies specific for *L. intracellularis* (Guedes and Gebhart, 2003c). In these two reference slides the levels of infection were assessed based on the amount of positively labeled antigen present in the intestinal sections: Grade 0 (-) = no positive antigen labeled; Grade 1 (+) = one isolated focal area of antigen labeled; Grade 2 (++) = multi-focal areas of antigen labeled; Grade 3 (+++) = majority of the mucosa has positive antigen labeled; and Grade 4 (++++) = all of the mucosa has positive antigen labeled (Guedes and Gebhart, 2003a). The series of slides containing the two flanking pairs of reference slides most severely infected (grade 3 or 4) was selected for LCM from each animal. Similar anatomic portions of the ileum were collected from the control group and stained by IHC to confirm the negative status of the samples. One non-

infected series of cryosections was selected for LCM from the similar anatomic portion of those selected in the infected group.

The LCM procedures were performed in the second, third and fourth serially-cut slides using Histogene™ staining (Life Technologies). Briefly, the specimens were thawed at room temperature for 30s, fixed in nuclease-free 75% ethanol, rehydrated in nuclease-free water for 30s, stained with HistoGene® solution for 20s and rinsed in water for 30s, then dehydrated by sequential immersion into 75%, 95% and 100% ethanol for 30s each. All microdissection procedures were performed at the College of Biological Sciences Imaging Center at the University of Minnesota using PixCell® II System (Life Technologies). Single-cells were captured from the slides using CapSure LCM caps (Life Technologies) with laser spot diameter 7.5 μm, power 60 mW and duration 650 μs. One thousand cells were captured from the bottom to the upper part of the intestinal crypts (Figure 5.1) in each of the three stained slides in approximately ten minutes, incubated at 42ºC for 30 min in 50 μl extraction buffer and stored at -80ºC. The microdissected cells from each of the three serially-cut slides were pooled, totaling 3000 cells, and used for the RNA extraction.

RNA isolation and amplification

Total RNA was isolated from microdissected tissues using a PicoPure™ Kit (Life Technologies) according to the manufacturer's instructions. Genomic DNA was digested using DNase I (Qiagen). RNA samples were then assessed with a NanoDrop ND-8000 Spectrophotometer and Agilent Bioanalyzer 2100. Amplified cDNA was prepared from 100ng of total RNA using the Ovation® RNA-Seq System V2 (Nugen®), following the

manufacturer's instructions. The amplification was initiated at the 3' end as well as randomly throughout the sample which allows the amplification of eukaryotic and prokaryotic RNA transcripts. The amplified cDNA generated was then purified using MinElute Reaction Cleanup Kit (Qiagen) for library preparation.

Library preparation and sequencing

The library preparation and sequencing were conducted in the core facility of the Biomedical Genomics Center at the University of Minnesota. Briefly, 100ng of the amplified cDNA was fragmented, ends repaired, enriched by PCR and validated using High Sensitivity Chip on the Agilent2100 Bioanalyzer. Following quantification of the cDNA generated for the library using PicoGreen Assay, the samples were clustered and loaded on the Illumina® Genome Analyzer GA IIx platform which generated on average 22,136,064 paired reads of 100 bp. Base calling and quality filtering were performed following the manufacturer's instructions (Illumina® GA Pipeline).

Filtering and mapping of sequence reads

Quality control, trimming and mapping were performed in the Galaxy platform (Giardine et al., 2005). Initially, FastQC tool was applied to the raw sequence data followed by FastQ Trimmer (Blankenberg et al., 2010). As a result, 15 base pairs were trimmed from the 5'end and ten from the 3'end of each raw sequence read. Sequences containing 75 bp and a phred quality score of more than 20 were used in the gene expression analysis (Ewing et al., 1998). Using Bowtie short read aligner with no more than two mismatches, the filtered sequences were mapped onto the pig genome *S. scrofa* 10.2 and the *L. intracellularis* isolate PHE/MN1-00 reference genome, both obtained

from the National Center for Biotechnology Information (NCBI) database (Langmead et al., 2009). The number of reads mapped within each annotated transcript was calculated in order to estimate the level of transcription for each gene. Cufflinks tool was used to estimate the relative abundances of the transcript reads for each gene (Trapnell et al., 2012). For comparing the levels of gene expression between infected and non-infected enterocytes and characterizing the abundance of *L. intracellularis* transcripts expressed in the host cytoplasm, the read counts were normalized based on the number of reads per kilobase of coding sequence per million mapped reads (RPKM).

In order to determine any potential co-infection associated with the intracytoplasmic *L. intracellularis*, the sequence reads were screened against 4,279 viral sequences available at NCBI database. Additionally, MetaPhlAn (Metagenomic Phylogenetic Analysis) was performed to identify other bacterial genome sequences in the experimental samples (Segata et al., 2012).

Differential gene expression

The differential gene expression between infected and non-infected enterocytes was assessed using the CuffDiff tool. This tool defines the total read count for each gene by combining the expression data from all the replicates in each experimental group and testing for differential regulation in expressed transcripts present in at least three replicates. RPKM values were expressed in $\log_2$ (RPKM) to allow for statistical comparison. As a result, $\log_2$-fold change in abundance of each transcript was obtained by $\log_2$ (RPKM [infected]/RPKM [non-infected]). *P*-values were calculated and adjusted for multiple comparisons using false discovery rate (FDR) (Benjamini and Hochberg,

1995). Significant differential expression was determined in genes with FDR-adjusted p-values < 0.05 and fold change $\pm$ 2.

Functional gene ontology and pathway analysis

Biological functions and interactions of the genes differentially expressed were determined using the database for annotation visualization and integrated discovery (DAVID v6.7) and the Ingenuity pathway analysis (IPA) (Ingenuity® Systems). While the DAVID knowledge database was used for functional clustering, the mammalian knowledge database of the IPA was used for pathway and network analyses of the differentially expressed genes.

Predictive analysis regarding the ontology and motifs of gene-encoding hypothetical proteins highly expressed by *L. intracellularis* were performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Additionally, Phyre^2 (Protein Homology/analogY Recognition Engine) (Kelley and Sternberg, 2009) and ConFunc (Protein Function Prediction Server) (Wass and Sternberg, 2008) software were used to predict their biological functions and three-dimensional structure.

Reverse transcription PCR

In addition to the assessment of RNA quality based on the bacterial and eukaryotic ribosomal RNAs using Agilent Bioanalyzer 2100, one-step RT-PCR was applied to the RNA extracted from microdissection tissues in order to evaluate the quality of bacterial mRNA for RNA-seq analysis. The one-step RT-PCR (Qiagen®) was used with specific primers (Supplementary Table 5.1) targeting three housekeeping genes of *L. intracellularis*.

Quantitative RT-PCR (qRT-PCR) was performed for validating the expression data identified by RNA-seq using a specific set of genes from the host and bacterium (Supplementary Table 5.1). RNA samples were synthesized to first-strand cDNA using SuperScript® II RT (Invitrogen). Duplicate qRT-PCR reactions from each primer probe set were validated by five serial dilutions of cDNA on the ABI7900HT instrument (Applied Biosystems). After validation, quantitative PCR was performed in duplicate using 15ng of cDNA per sample with the following conditions: 60°C for 2 min, 95°C for 5 min and 45 cycles (95°C/10 sec and 60° for 1 min). The differential expression for the host transcriptome were described as relative fold-change between infected and non-infected enterocytes based on Ct values ($2^{-\Delta\Delta Ct}$), as previously described (Bookout et al., 2006). The suitability of four porcine housekeeping genes (Beta actin, Cyclophilin A, Hypoxanthine phosphoribosyltransferase, Ribosomal protein L4) for qRT-PCR analysis was evaluated.

Based on consistent Cyclophilin A gene expression throughout the biological replicates, this gene was used to normalize the expression data. For the bacterial transcriptome, averages of relative transcriptional levels normalized by the 30S ribosomal gene were calculated and compared to the RNA-seq expression levels. A linear regression model was used to evaluate the correlation between average RPKM and qRT-PCR data, as described previously (Vannucci et al., 2012c).

5.3 Results

Technical Characterization of the LCM Coupled with RNA-seq Analysis

Based on previous studies reporting the chronological course of the *L. intracellularis* infection *in vivo* (Guedes and Gebhart, 2003b; Vannucci et al., 2013), experimentally-infected pigs in our study were monitored every other day regarding clinical signs (diarrhea and general attitude) and quantitative fecal shedding of *L. intracellularis* DNA until the end point of the study (21 PI). The negative control group was also monitored and its negative status was consistent throughout the study period. One animal from the infected group exhibiting only mild to moderate lesions typical of PE associated with grade 1 and 2 in the IHC grading scheme was discarded from the experiment. In order to have the same number of replicates in both infected and control groups, one animal was randomly discarded from the negative control group. As a result, five biological replicates (animals) from each group were included in the LCM procedures and RNA-seq analysis.

The quality of RNA samples extracted from microdissected tissues was evaluated by assessing bacterial and eukaryotic ribosomal RNAs using Agilent Bioanalyzer 2100 (Figure 5.1C). The successful recovery of high quality eukaryotic RNA from microdissected cells using the LCM procedures described in our study has been well-established (Mikulowska-Mennis et al., 2002). Since studies using prokaryotic RNA from microdissected cells have not been well-explored, the feasibility of using *L. intracellularis* mRNA for sequencing was confirmed by one-step RT-PCR using specific primers targeting three housekeeping genes (Figure 5.1D).

The sequence reads representing the RNA transcripts derived from the host cells and the intracellular bacteria were mapped onto both pig (*S. scrofa* 10.2) and the *L. intracellularis* (PHE/MN1-00) reference genomes available at NCBI. From a total average of 22,136,064 reads generated, 82% (18,280,138) and 4% (1,000,440) were mapped against the porcine and the bacterium genome, respectively (Supplementary Table 5.2). A total of 11,778 genes were expressed in the porcine transcriptome and met the criteria for differential expression analysis (see Materials and Methods). Up-regulation of 119 and down-regulation of 46 protein coding genes were identified in infected enterocytes (fold-change ≥2.0; $p<0.05$) (Supplementary Table 5.3).

In the bacterial transcriptome analysis, 754 protein coding genes had at least one mapped read against the reference DNA sequence of the *L. intracellularis* PHE/MN1-00 isolate, which was the same isolate used in the experimental infection. The top 20 genes highest expressed by *L. intracellularis* and their respective normalized transcription levels based on the RPKM values are described in Table 5.1.

In order to screen for the presence of any other microbial organisms in the experimental samples, the sequence reads were also mapped against viral, bacterial and archaeal genome databases. While no detectable hits were found against the viral and archaeal genomes, 0.052% and 0.084% of the sequence reads from the infected and control group, respectively, were mapped against the bacterial database. Three unculturable bacterial species (*Candidatus Zinderia*, *Candidatus Carsonella ruddii*, *Candidatus* Sulcia muelleri) and *Propionibacterium acnes* were commonly found in both

groups. An uncharacterized species of the genus *Peptoniphilus* previously reported in the oral cavity of a human was exclusively found in the control group.

The RNA-seq expression data were validated by qRT-PCR based on the relative quantification of 16 differentially expressed genes from the host and 10 bacterial genes (Supplementary Table 5.1). The averages of fold-change in gene expression from infected enterocytes and the relative transcriptional levels of *L. intracellularis* genes were plotted against the $\log_2$ (RPKM) (Figure 5.2). The $\log_2$ transformed fold-change from the host gene and transcript levels from the bacterial genes were positively correlated on a linear regression model (p-value < 0.05). Based on the qRT-PCR validation, the RNA-seq data properly estimated the fold-change expression in infected enterocytes and the transcription levels of *L. intracellularis* genes.

Protein Biosynthesis and Transcription

The 165 porcine genes differentially expressed in *Lawsonia*-infected enterocytes were analyzed regarding their biological functions and molecular networks based on the mammalian gene expression information available in the Ingenuity® System. The system knowledge database recognized and analyzed 144 differentially expressed genes. The IPA system associates the set of differentially expressed genes with cellular networks (focus genes) and creates a score based on the number of network eligible genes they contain (Table 5.2). The protein biosynthesis network was most correlated with the set of genes differentially expressed and up-regulated in *Lawsonia*-infected enterocytes. Figure 5.3A shows the molecular interaction of this network that mainly includes ribosomal proteins and mRNA translation factors. The eukaryotic initiation factor (IEF2) signaling

was the canonical cell pathway (CP) more significantly associated (*p*-value 2.76E-22) with the genes differentially expressed (Figure 5.3B). A remarkable influence of IEF2 signaling on the protein biosynthesis was identified by merging both network and pathway analysis (Figure 5.3A).

Functional clustering analysis using DAVID knowledge database confirmed the involvement of the 27 differentially expressed genes in the protein biosynthesis (data not shown) and also showed the association of four genes acting in the positive regulation of transcription (Table 5.3). The activation of these two cellular processes (transcription and protein biosynthesis) revealed a global increasing in the cell metabolism in response to the *L. intracellularis* infection and it has also been described to occur during the gap phase 1 (G_1) of the host cell cycle (Schafer, 1998).

Cell Cycle and Apoptosis

In addition to the positive regulation of transcription, DAVID clustering analysis identified genes associated with cell cycle and apoptotic events differentially expressed in infected enterocytes (Table 5.3). Cell cycle-associated genes mainly represented by Ras homolog proteins were significantly induced in association with Cyclin-dependent kinase 2 (CDK2). The aberrant activation of Rho-genes including those described in our study has been well-described in oncogenesis by causing deregulation of cell cycle progression and promoting cell proliferation (Pruitt and Der, 2001). Additionally, the co-expression of Rho proteins and Cyclin-dependent kinases (CDKs) specifically act by stimulating the entry and progression of the G_1 phase of the host cell cycle (Coleman et al., 2004).

The functional clustering analysis revealed 14 differentially expressed genes associated with pro- and anti-apoptotic events (Table 5.3). All seven genes related to anti-apoptotic events were up-regulated in infected cells. Among the pro-apoptotic genes, four were down-regulated and three up-regulated. Interestingly, two of these pro-apoptotic genes that are also involved in the cellular immune response against intracellular pathogens (Signaling lymphocytic activation molecule 7 and Tumor necrosis factor ligand 10) were down-regulated. On the other hand, significant up-regulation of genes encoding the major histocompatibility complex class I (MHC-I) was identified in *Lawsonia*-infected enterocytes (Figure 5.4A).

Nutrient Acquisition and Electrolyte Secretion

Consistent down-regulation of numerous genes expressed in the apical membrane of enterocytes that are involved in nutrient acquisition was observed in *Lawsonia*-infected cells (Figure 5.4A). These membrane transporters are involved in the absorption of carbohydrates (sodium/glucose co-transporter and sucrase-isomaltase), amino acids (cationic amino acid transporter), bile acid (sodium/bile acid co-transporter), lipids (lipid phosphate phosphohydrolase) and Vitamin B_{12} (cubilin receptor). Additionally, the intracellular infection also affects the electrolyte secretion by decreasing expression of the chloride channel gene (CLCA1). The reduction of both nutrient acquisition and electrolyte secretion indicates that *L. intracellularis* may be able to prevent cell differentiation in immature enterocytes.

In contrast to the down-regulation of genes expressed on the apical membrane, the gene encoding the glucose transporter 1 (also known as solute carrier family 2, glucose

transporter member 1) was highly up-regulated in infected enterocytes. This transporter was the third highest expressed gene (Supplementary Table 5.3) and its expression also has been reported on the basolateral membrane of human enterocytes *in vitro* (Harris et al., 1992) and rat jejunum *in vivo* (Boyer et al., 1996). Additionally, significant up-regulation of the high-affinity copper uptake protein (CTR1) involved in copper absorption was also found in infected enterocytes (Figure 5.4A).

Bacterial Interactome

The transcriptional landscape of the intracellular bacteria was determined by classifying all genes with at least one mapped read into one of three levels of expression: low, moderate and high. As expected, genes encoding ribosomal-related proteins were the functional category most associated with the mapped reads and the majority of these genes exhibited moderate to high expression. The *L. intracellularis* interactome was characterized and discussed based on the most highly expressed bacterial genes (Table 5.1).

The functional categories of bacterial genes that were highly expressed included those involved in protein folding (e.g. *groES-groEL* operon and chaperone *dnaK*) and biosynthesis (e.g. elongation factor Tu), oxidative stress (e.g. Cu-Zn superoxide dismutase, *rubY-rubA* operon and dioxygenases), secretion system effector-related proteins (*PcrH-SseC*-LI1159 operon) and various hypothetical proteins. Since we were not able to distinguish free bacteria in the cell cytoplasm from those organisms within cell endosomes, the interactome was built and discussed considering both scenarios. Figure 5.4B illustrates the biological activities of the bacterial proteins encoded by highly

expressed genes considering the intracellular microenvironment of *Lawsonia*-infected enterocytes.

While redox enzymes catalyze reduction of O_2 derived from the cell cytoplasm, Cu-Zn superoxide dismutase C (*sodC*) and rubrerythrin-rubredoxin operon (*rubY-rubA*) neutralize reactive oxygen species (O_2^- and H_2O_2) generated in the endosome or from the reduced O_2 molecule (Figure 5.4B) (Lumppio et al., 2001). An operon genetically related to the *Salmonella* pathogenicity island 2 (SPI2) composed of three genes, a chaperone (*PcrH*), an effector protein (*SseC*) and a hypothetical protein LI1159 (referred as *Sse?*) was highly expressed (Figure 5.4B). Interestingly, genes encoding the type III secretion system (TIIISS) apparatus whereby these effector proteins would be delivered to the cell cytoplasm showed only moderate expression (data not shown) and were located downstream of the SPI2-related operon on the *L. intracellularis* chromosome.

Among the ten genes most highly expressed by the intracellular bacterium, five represented hypothetical proteins. A summary of the predictive analyses evaluating their structures and biological functions are described in the supplemental material (Supplementary Table 5.4). Two different families of proteins were predicted for the gene locus LI0447 (aminomethyltransferase beta-barrel and growth factor receptor domain). Additionally, transmembrane proteins (porin and autotransporter), proline isomerase, extracellular protease and secretory factor were also identified based on predictive motifs.

Table 5.1. The top 20 highly expressed genes by *L. intracellularis* in the enterocyte cytoplasm.

Locus	Gene product	Predicted function	Log_2 (RPKM)
LI0461	Hypothetical protein	Uknown	15.0
LI0625	Chaperonin GroEL	Protein folding	14.9
LI1159	Hypothetical protein	Uknown	13.7
LI0447	Hypothetical protein	Uknown	13.6
LI0935	Elongation factor Tu (*tufA*)	Protein biosynthesis	13.5
LI0624	Co-chaperonin GroES	Protein folding	13.5
LI0005	Cu-Zn superoxide dismutase precursor (*sodC*)	Oxidative stress	13.3
LIC060	Hypothetical protein	Uknown	12.9
LI0267	Hypothetical protein	Uknown	12.8
LI1075	Dioxygenases related to 2-nitropropane dioxygenase	Oxidative stress	12.7
LI0809	Hypothetical protein	Uknown	12.5
LI0912	Molecular chaperone DnaK	Protein folding	12.4
LI0439	Hydrogenase-1 small subunit	Cell metabolism	12.4
LI0902	Outer membrane protein related to OmpA-OmpF porin	Membrane transport	12.4
LI1158	Hypothetical protein related to secretion system effector C	Intracellular survival	12.3
LI1005	Pseudouridine synthase (*truB*)	Protein biosynthesis	12.2
LI0559	50S ribosomal protein L13	Protein biosynthesis	12.2
LI0697	Rubrerythrin (*rubY*)	Oxidative stress	12.2
LI0043	Hypothetical protein	Uknown	12.0
LIC103	Methyl-accepting chemotaxis protein (*pilJ*)	Chemotaxis	11.9

Table 5.2. The four cell networks most associated with the genes differentially expressed in *Lawsonia*-infected enterocytes.

Associated network functions	Score*	Focus genes
Protein Synthesis, Cellular Assembly and Organization, Molecular Transport	59	27
RNA Post-Transcriptional Modification, Cancer, Hematological Disease	42	21
Cellular Movement, Cancer, Reproductive System Disease	31	17
Cell Death and Survival, Carbohydrate Metabolism, Molecular Transport	31	18

* Based on the number of network eligible genes within the referred network.

Table 5.3. Functional clustering associated with genes differentially expressed in *Lawsonia*-infected enterocytes.

Gene Symbol	Gene product	ln(fold change)
Clustering		
Positive regulation of transcription		
TEF1	Transcriptional enhancer factor 1	2.2
SMARCC2	SWI/SNF complex subunit SMARCC2-like	2.3
SOX-9	Transcription factor SOX-9	2.6
FHL2	Four and a half LIM domains 2	2.1
Mitotic cell cycle-associated genes		
CDK2	Cyclin-dependent kinase 2	2.6
RHOA	Ras homolog family member A	2.2
RHOB	Ras homolog family member B	2.2
RRAGA	Ras-related GTP-binding protein A	2.3
RPL24	60S ribosomal protein L24-like	2.7
SKA2	Spindle and kinetochore-associated protein 2-like	2.1
PSMA1	Proteasome subunit alpha type-1-like	2.4
Pro-apoptosis-related genes		
SLAM7	Signaling lymphocytic activation molecule 7	-4.6
C6	Complement component C6	2.3
C9	Complement component C9	3.5
PRDX1	Peroxiredoxin 1	2.2
SST	Somatostatin	-3.4
TNFSF10	Tumor necrosis factor ligand 10	-2.5
VAV2	Vav 2 guanine nucleotide exchange factor	-2.1
Anti-apoptosis-related genes		
DAD1	Defender against cell death 1	2.3
NME2	Non-metastatic cells protein	2.9
PRNP	Prion protein	2.6
PPP2CB	Protein phosphatase 2, catalytic subunit, beta isozyme	2.1
RHOA	Ras homolog family member A	2.2
TPT1	Translationally-controlled tumor protein-like	2.7
ERBB3	Receptor erythroblastic leukemia viral oncogene	2.2

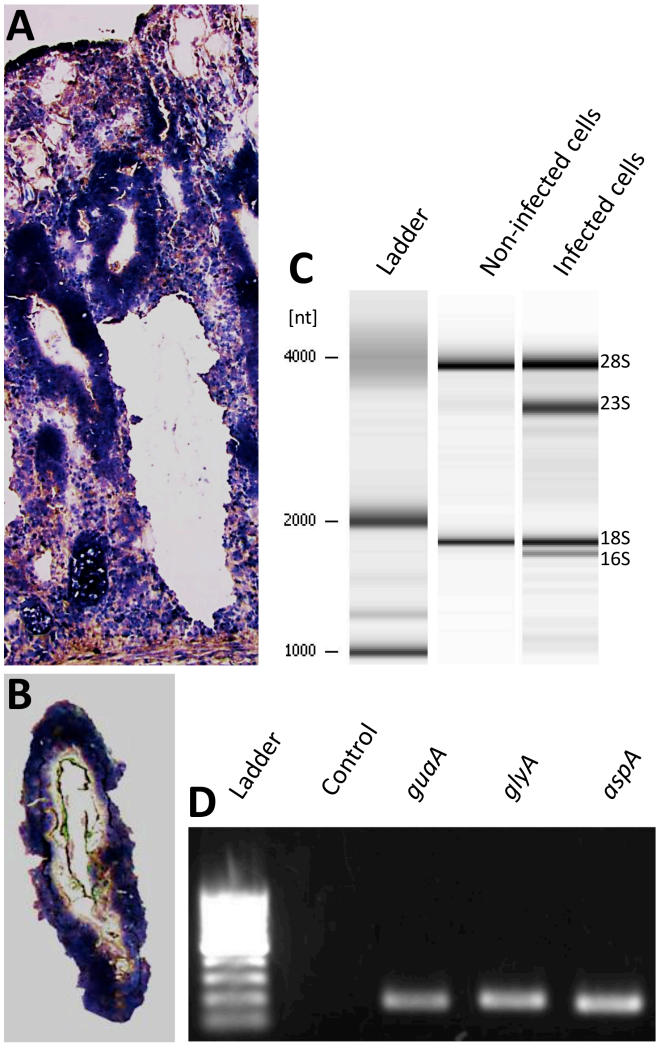

Figure 5.1. Laser capture microdissection of an intestinal crypt infected with *L. intracellularis* (A-B) and evaluation of RNA quality from microdissected cells (C-D). (A) Hematoxylin stained cryosection of infected ileal mucosa. (B) Microdissected intestinal crypt captured in the thermoplastic film of the LCM cap prior to RNA isolation. (C) Agilent Bioanalyzer data showing bacterial and eukaryotic ribosomal RNA in infected and non-infected cells. (D) One-step RT-PCR products of three protein-encoding genes of *L. intracellularis*.

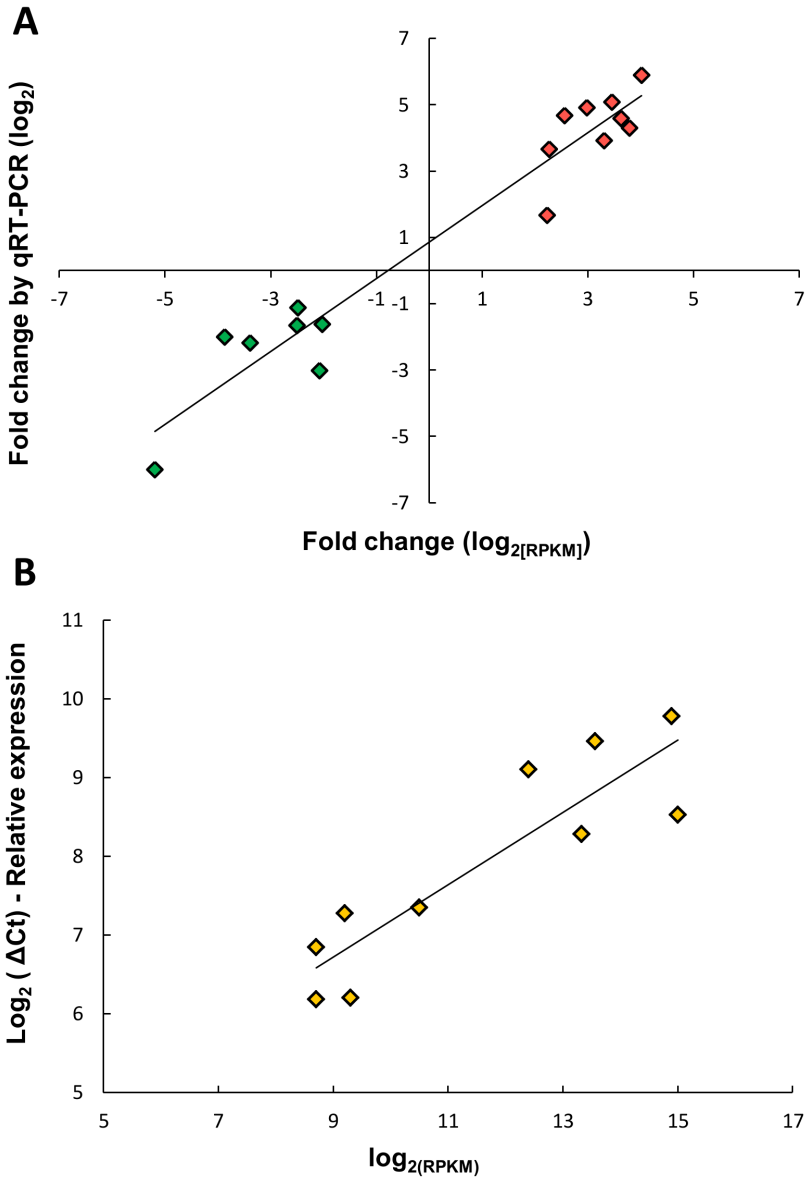

Figure 5.2. Correlation between RNA-seq and qRT-PCR expression data. (A) Plot of the relative fold-change in gene expression of 16 porcine genes from infected enterocytes by RNA-seq (x-axis) and qRT-PCR (y-axis). R^2 = 0.92 (p<0.05). (B) Plot of the relative quantification of ten *L. intracellularis* genes by RNA-seq (x-axis) and the transcript levels generated by qRT-PCR (y-axis). R^2 = 0.81 (p<0.05).

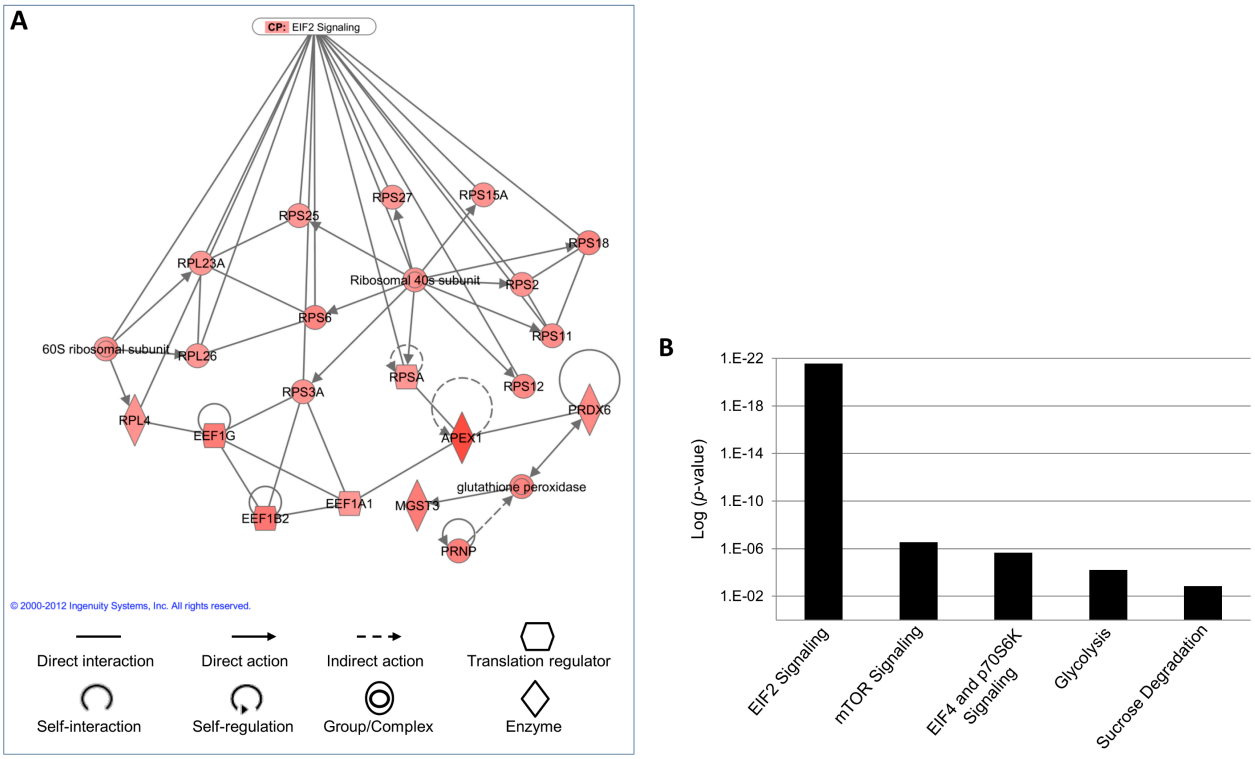

Figure 5.3. Cellular network and canonical pathway analysis of genes differentially expressed by enterocytes infected with *L. intracellularis*. (A) Molecular interaction representing part of the protein biosynthesis network which was most correlated with the set of genes significantly up-regulated in infected cells. Strong interaction between the main canonical pathway (CP: EIF2 signaling) identified in the differentially expressed genes and the protein biosynthesis network. (B) Canonical pathways (x-axis) most associated with genes differentially expressed based on the -log of *p*-value calculated by Fisher's exact test (y-axis).

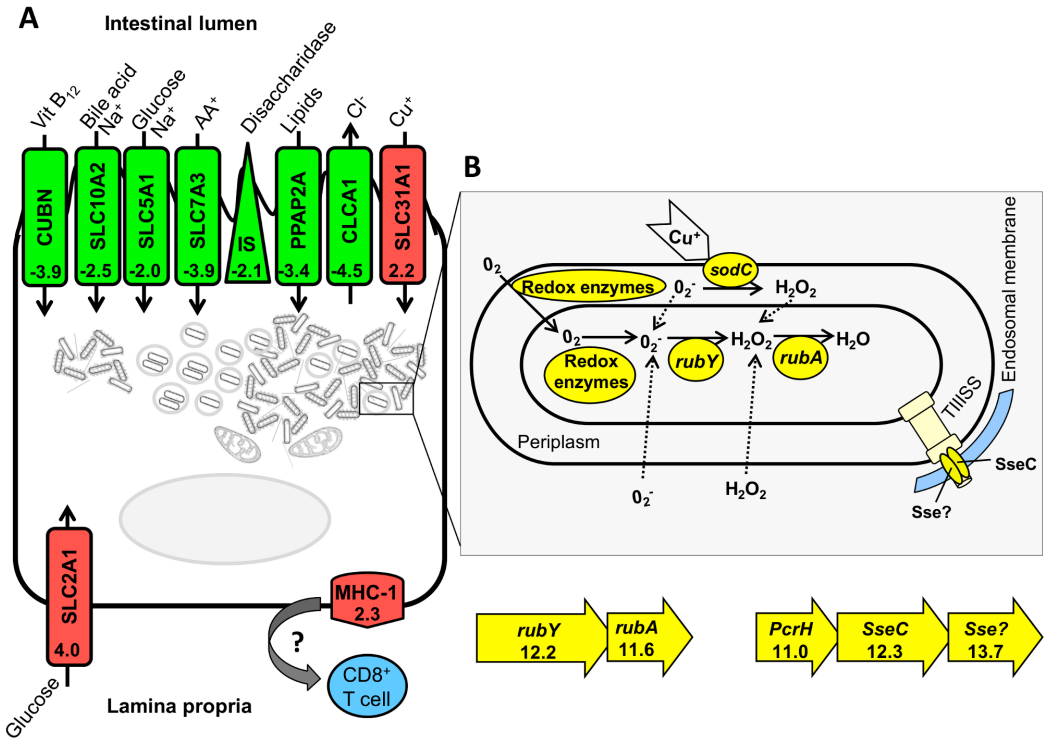

Figure 5.4. Proposed model for host-pathogen interaction in porcine enterocytes infected with *L. intracellularis*. (A) Infected enterocyte. Apical membrane exhibiting down-regulation of genes involved in nutrient acquisition and electrolyte secretion (green) and up-regulation of copper uptake protein (red). Basolateral membrane exhibiting up-regulation of glucose transporter (SLC2A1) and MHC class I genes. (B) Intracellular bacterium. High expression (yellow) of genes included in the oxidative stress protection system: redox enzymes, Cu-Zn superoxide dismutase (*sodC*) and rubrerythrin-rubredoxin (*rubY-rubA*) operon (Adapted from Lumppio et al. 2001). Moderate expression genes encoding the basal body of the type III secretion system (TIIISS) (light yellow) and high expression of TIIISS effector proteins (*PcrH-SseC-Sse?* operon). Genetic organization and gene expression ($\log_{2\,[RPKM]}$) of the *rubY-rubA* and *PcrH-SseC-Sse* operons.

5.4 Discussion

The present study used LCM technology to microscopically dissect enterocytes from pigs experimentally infected with *L. intracellularis* and to characterize the cell-specific transcriptional landscape associated with the bacterial interactome using high throughput sequencing (RNA-seq). The results demonstrated the usefulness of coupling LCM and RNA-seq techniques to study the host-pathogen interaction in a specific cell population present in a heterogeneous tissue. The simultaneous evaluation of the gene expression changes in both the host and the pathogen has been recently designated dual RNA-seq (Westermann et al., 2012). The challenge in studying both RNA transcriptomes from a common sample arises mainly because of the extensive variety of the bacterial organisms regarding their genomic compositions (e.g. %CG contents) and the particular characteristics of each infectious process at the cellular level. Since the abundance of eukaryotic RNA is significantly higher compared to the prokaryotic RNA in an infected cell, a sufficient number of bacteria per host cell followed by an unbiased RNA amplification step are crucial for studying the cell-bacteria interactome (Westermann et al., 2012). In light of this, we established the endpoint of our study (21 days PI), based on the chronological course of the *L. intracellularis* infection previously described (Guedes and Gebhart, 2003a; McOrist et al., 1996a). Confirming these previous observations, our study showed at 21 days PI, numerous bacteria in proliferative enterocytes indicating the active stage of the infectious process. We hypothesized that the disease at this point was approaching its peak when the intracellular bacteria were exhibiting their virulence factors and the host cells were responding to the pathogen at appropriate levels to allow

us to evaluate the changes in gene expression. We believe that earlier endpoints would not provide a sufficient amount of RNA to be recovered and later stages of the disease would not represent the logarithmic phase of the bacterial growth. Additionally, the increasing presence of *L. intracellularis* within the lamina propria along the disease progression would also reduce the number of bacteria in enterocytes.

A previous chronological analysis using microarray technology evaluated the host response of fibroblastic cells *in vitro* at three time points (24, 48 and 72 hours PI) (Oh et al., 2010). Altered transcription of genes related to cell cycle and cell differentiation were described (Oh et al., 2010). However, cellular proliferation which is the main phenotypic characteristic of the *L. intracellularis* infections *in vivo* has not been reproduced in *in vitro* models (Lawson et al., 1993; Vannucci et al., 2012e). Furthermore, the transcriptional response of mesenchymal cells which are not the natural target cells for *L. intracellularis* needs to be interpreted with caution.

A single time point analysis of the host transcriptome using intestinal tissues from pigs naturally affected with PE was recently described (Jacobson et al. 2011). Although this study provided an interesting snapshot of the transcriptional host response, it used field cases of diarrhea where the samples were co-infected with porcine circovirus type 2 which may be a confounding factor in the evaluation of the expression of genes especially related to the immune response (Jacobson et al. 2011). Additionally, the microarray was performed using entire intestinal tissues. As a result, the specific characterization of the transcriptional host response was impaired by either the heterogeneity of the cell population included in the intestinal tissues or the virus

infection. By applying the LCM technique to isolate ileal enterocytes in our study, we confirmed the identification of several genes specifically expressed in the intestinal epithelium of the ileum (e.g. ileal sodium/bile acid cotransporter) corroborating with a recent comprehensive study describing the gene expression atlas of the domestic pig (Freeman et al., 2012).

Gene network and pathway analyses revealed that protein biosynthesis and activation of transcription were the host cellular events most associated with the genes differentially expressed in *Lawsonia*-infected enterocytes (Figure 5.3A). In agreement with these molecular findings, ultrastructural studies *in vivo* using electron microscopy identified the numerous bacteria occupying the apical cytoplasm of infected enterocytes which was otherwise composed almost entirely of free ribosomes and scattered mitochondria (Jasni et al., 1994a; Johnson and Jacoby, 1978). These morphological findings associated with our molecular results suggest a global increase in the cell metabolism in response to *L. intracellularis* infection. Taking the host cell cycle events into consideration, an increase in protein synthesis and transcription is required during the G_1 phase in order to prepare the cell for subsequent division (Schafer, 1998). Interestingly, this protein synthesis network was also associated with cell proliferation at an early stage of infection with human immunodeficiency virus (Navare et al., 2012).

The differential expression of cell cycle-associated genes identified by the functional clustering analysis (Table 5.3) showed specific activation of Rho family genes (RhoA, RhoB and Rho GTPase). These molecules play a key role in carcinogenesis through their aberrant activation that results in cell proliferation (Pruitt and Der, 2001).

Rho family proteins specifically act on the G_1-checkpoint of the cell cycle when the transition to commit the cell to the proliferative stage occurs. If the signals responsible for promoting this transition are not present then the cells enter into the non-proliferative phase (G_0) (Oswald et al., 2005). Additionally, a gene encoding Rho GTPase was also highly up-regulated during *L. intracellularis* infection *in vitro* (Oh et al., 2010), suggesting that exacerbated activation of the G_1 phase is an important mechanism involved in the proliferative changes induced by *L. intracellularis* in infected enterocytes.

In addition to their roles in cancer development, Rho proteins are also pathologically activated by bacterial toxins also known as cyclomodulins (Lax and Thomas, 2002; Nougayrède et al., 2005; Oswald et al., 2005). Cytotoxic necrotizing factor (CNF) found in uropathogenic *Escherichia coli*, *Pasteurella multocida* toxin (PMT) and dermonecrotic toxin of *Bordetella* spp. act directly on Rho family proteins to bring about their irreversible activation (Lemonnier et al., 2007). Furthermore, CNF-expressing *E. coli* establish a persistent intracellular infection in the urogenital tract and suppress apoptosis by affecting the transcription levels of Bcl-2 family genes (Lemonnier et al., 2007). It is thought that apoptosis inhibition in target cells may favor bacterial persistence at the epithelium surface, thereby favoring bacterial replication and spread inside the host cell (Mulvey et al., 1998). Although our study showed a predominant activation of anti-apoptotic-related genes compared with pro-apoptotic events, the Bcl-2 gene was not significantly activated in infected cells ($\log_2$-fold change = 1.06). Therefore, other mechanisms may be involved in the predominant activation of anti-apoptotic genes identified in the present study.

The contribution of apoptotic mechanisms for the pathogenesis of *L. intracellularis* infections has been speculated over the years and still needs to be elucidated. Initially, a temporary reduction in apoptosis was hypothesized to be an important mechanism involved in the cell proliferation (McOrist et al., 1996a). Later studies suggested an increase in apoptosis based on the Caspase-3 immunohistochemical staining (Boutrup, 2008; Gebhart and Guedes, 2010). One of these studies described the dynamic of the Caspase-3 staining through the chronological evaluation of experimentally-infected animals and showed variations on the pattern of Caspase-3 staining within different parts of the intestinal mucosa on day 19 PI (Boutrup, 2008). Our study showed activation of the gene-encoding Caspase-3 (log_2-fold change = 1.12) in infected enterocytes, but the level did not reach the parameters for statistical significance. Considering the complexity and dynamism of the apoptosis process, the stage of the infection at the cellular level may directly influence the apoptotic gene network. Therefore, an *in vitro* model displaying the proliferative phenotype would be an ideal starting point to address this question.

In association with pro-apoptotic events, we identified down-regulation of two genes involved in the cellular immune response against intracellular pathogens (SLAM7 and TNFSF10) (Table 5.3). The apparent poor immune response in *L. intracellularis* infections has been indicated over the years (McOrist et al., 1992; Rowland and Lawson, 1974; Smith and Lawson, 2001). A microarray study also identified poor activation of immune response-related genes in general in field cases of PE (Jacobson et al.). Despite the reduced expression of these two immune response-associated genes identified in our

study, we observed significant up-regulation of MHC-I genes in infected cells, indicating that *Lawsonia*-derived antigen is presented to the lamina propria through the basal membrane of infected enterocytes (Figure 5.4A).

Although the physiopathology of diarrhea in *L. intracellularis* infections remains to be elucidated (Moeser and Blikslager, 2007), the significant down-regulation of numerous genes related to nutrient acquisition observed in the present study indicates that malabsorptive diarrhea represents the major mechanism involved in the poor performance and growth of affected animals. Supporting our observations, a lower absorption of glucose and electrolytes was reported using a hamster experimental model of PE (Vannucci et al., 2010). The reduced expression of nutrient acquisition-related genes also indicates the lack of cell differentiation in infected enterocytes.

The deficiency in nutrient acquisition described above seems to contrast with the increase in the cell metabolism characterized by the activation of transcription and protein synthesis-related genes also described here. As proposed in Figure 5.4A, the high expression of the glucose transporter SLC2A1 on the basolateral membrane appears to compensate for this lack of nutrient acquisition from the intestinal lumen, since it has been described as being expressed in the basolateral membrane of human enterocytes *in vitro* (Harris et al., 1992) and rat jejunum *in vivo* (Boyer et al., 1996).

The only gene significantly up-regulated in infected cells which is involved in nutrient acquisition and is physiologically expressed on the apical membrane is the high-affinity copper uptake gene (Sharp, 2003). Copper is an essential metal used by eukaryotic cells as a biochemical cofactor especially during the process of oxygen

reduction by cytochrome *c* oxidase which leads to the production of ATP (Festa and Thiele, 2012). The production of ATP by the eukaryotic host is crucial for metabolism of *Lawsonia* and two other groups of obligate intracellular bacteria (*Chlamydiales* and *Rickettsiales*). These organisms express ATP/ADP translocase that catalyzes the exchange of bacterial ADP for host ATP allowing bacteria to exploit their hosts' energy pool, a process referred to as energy parasitism (Schmitz-Esser et al., 2008; Schmitz-Esser et al., 2004). In addition to the ubiquitous essentiality for host cells, an increase in copper uptake has also been reported as a defense mechanism against intracellular pathogens because of its toxic properties. Free intracellular copper can lead to oxidative stress whereby cycling of copper oxidation and reduction produce reactive oxygen species through the Fenton reaction (Festa and Thiele, 2012). Interestingly, our study identified *L. intracellularis* genes related to oxidative stress highly expressed within the host cytoplasm (Figure 5.4B).

Along with the comprehensive host transcriptome, three to five percent of the sequence reads generated in our study also mapped against the *L. intracellularis* genome. We believe the reduced bias introduced during the RNA amplification step using random primers associated with the generation of amplified cDNA through SPIA (single primer isothermal amplification) technology was crucial in the enrichment of bacterial transcripts. However, the detection of prokaryote transcripts may vary among bacterial species especially due to the wide variation in genomic composition and the amount of organisms in infected cells (Westermann et al., 2012). Less than 0.1% of the sequence reads mapped against four bacterial species commonly found in both infected and non-

infected groups, when screened for the presence of virus and other bacteria. While three of these species represent unculturable organisms (*Candidatus*) related to insect symbionts, one (*Propionibacterium acnes*) is part of the normal flora of the skin, oral cavity, large intestine, the conjunctiva and the external ear canal of humans (Perry and Lambert, 2011). The identification of the genus *Peptoniphilus*, found exclusively in the control group, may be due to human contamination during the experimental procedures, since it was reported in the oral cavity through the human microbiome project (NCBI accession: PRJNA52051).

L. intracellularis genes belonging to the oxidative stress protection were the functional cluster most associated with genes highly expressed in the host cytoplasm. Although this fastidious organism requires a strict microaerophilic environment for cultivation in cell culture, *Lawsonia* is often located close to the cell mitochondria, where the transport of oxygen is continuous due to the oxidative phosphorylation. Additionally, this intracellular location is important to exploit the host energy pool by exchanging bacterial ADP for host generated ATP, as discussed earlier. Based on this scenario, our study demonstrated that *L. intracellularis* displays a sophisticated mechanism to survive in this microenvironment by coping with the oxidative stress and potentially incorporating intracytoplasmic copper taken by the host to combat the intracellular infection (Figure 5.4A).

Our study identified high expression of an operon in which the first two genes (*PcrH* and *SseC*) were genetically related to the SPI2 and the last gene (hypothetical protein LI1159) was the third highest expressed gene by *L. intracellularis*. The proteins

included within this operon act as a translocon attached to the phagosomal membrane to allow translocation of effector proteins into the cell cytoplasm (Nikolaus et al., 2001). These effector proteins interfere with the intracellular trafficking favoring bacterial survival (Uchiya et al., 1999). Since we could not distinguish intracellular bacteria free in the cytoplasm from those organisms present within endosomes, we hypothesize that this operon was highly expressed by *L. intracellularis* organisms in the cell endosome (Figure 5.4B).

We found five genes encoding hypothetical proteins among the ten highest expressed by *L. intracellularis* (Table 5.1). From the host transcriptome, we identified significant activation of Rho family genes which are crucial for the progression of the G_1 phase of the host cell cycle and are targeted by bacterial toxins (Lax and Thomas, 2002). Taken together, this evidence suggests the presence of an unrecognized cyclomodulin encoded by those highly expressed genes of *L. intracellularis*. Our results form the basis for future studies focusing on the specific properties and functions of these genes.

SECTION III

PROLIFERATIVE ENTEROPATHY – HOST ADAPTATION

CHAPTER 6

An Alternative Method for Cultivation of *Lawsonia intracellularis*

Vannucci F.A.; Wattanaphansak S.; Gebhart C.J. (2012) An Alternative Method for Cultivation of *Lawsonia intracellularis*. *J Clin Microbiol* 50(3): 1070–1072.

Summary

The aim of this study was to describe an alternative protocol for cultivation of *L. intracellularis* in cell monolayers providing necessary growth conditions without Tri-gas incubators. The isolate PHE/MN1-00 was previously grown in murine fibroblast-like McCoy cells and stored at -72°C until use. The infected cells were incubated in two different conditions. For the conventional protocol, the cells were placed in the Tri-gas incubator with 83.2% nitrogen, 8.8% carbon dioxide and 8% oxygen and a temperature of 37^{o}C. For the alternative protocol, the flasks were placed in a plastic bag (Original Space Bag®) which was then hermetically closed, inflated with a mixture of gas containing 10% hydrogen, 10% carbon dioxide, and 80% nitrogen, incubated at 37^{o}C for eight days. The CyQuant® cell proliferation assay was used to monitor the growth rate of infected and non-infected cells in both incubation protocols. *L. intracelluaris* growth and infection monitoring were performed by immunocytochemistry (ICC) and quantitative PCR. In the alternative protocol, the daily CO_2 and O_2 levels were between 7.0-8.0% and 5.5-6.5%, respectively. Non-infected and infected cells had similar growth rates in both incubation protocols. There was no significant difference in the numbers of heavily infected cells, counted by ICC, between the two protocols. Furthermore, quantitative PCR showed similar growth curves reaching a peak concentration (10^6 *L. intracellularis*/ml) seven days after infection. The flexibility of this protocol allows testing of various environmental conditions for *L. intracellularis* cultivation and development of diagnostic techniques. This affordable technology gives an opportunity to engage more research institutes in this area.

6.1. Introduction

Lawsonia intracellularis is a fastidious and obligate intracellular bacterium and the causative agent of proliferative enteropathy or ileitis. The disease has been reported in a variety of animal species including nonhuman primates, but it has been best described in hamsters, pigs and horses (Gebhart and Guedes, 2010). Dividing eukaryotic cells in culture and strict environmental conditions are required for isolation and cultivation of *L. intracellularis in vitro* (Lawson et al., 1993). The conventional method for isolation and cultivation of this bacterium in monolayer is well established using various methods of supplying hydrogen for infection followed by incubation in a Tri-gas incubators with 83.2% nitrogen, 8.8% carbon dioxide and 8% oxygen at 37^{o}C (Guedes and Gebhart, 2003c; McOrist et al., 1995a). The cost of these requirements has limited the maintenance of this microorganism *in vitro* to only a few research institutes. Furthermore, since this disease was first reported (Biester and Schwarte, 1931), there have been only a dozen or so cultured *L. intracellularis* isolates worldwide so far. This study describes an alternative method for cultivation of *L. intracellularis* in cell monolayers providing necessary atmospheric conditions for growth without Tri-gas incubators. This alternative protocol presents new opportunities for testing different environmental conditions for isolation and cultivation of this organism. Additionally, it also allows the development of diagnostic approaches including immunoperoxidase monolayer assay, indirect immunofluorescence and minimum inhibitory concentration.

6.2 Materials and Methods

Bacteria culture

The *L. intracellularis* isolate PHE/MN1-00 **(**ATCC PTA-3457) previously isolated from a pig with the hemorrhagic form of proliferative enteropathy was used for evaluating its growth and *in vitro* infection under two different environmental conditions (conventional and alternative). This isolate was grown in murine fibroblast-like McCoy cells (ATCC CRL 1696), maintained in a cell culture system and stored at -72°C until use, as described previously (Guedes and Gebhart, 2003c). Frozen bacteria were thawed and grown in cell culture for three continuous passages in order to allow the bacteria to recover from the frozen stage. In these three passages, the bacteria were grown using both the conventional and alternative method (Figure 6.1), as described below. The infection was monitored during every passage using immunoperoxidase staining with polyclonal antibody specific for *L. intracellularis* (Guedes and Gebhart, 2003c). After three passages, 16-well, glass-bottom tissue culture plates containing one-day-old McCoy (30% confluence) cells were infected (Day 0) with bacterial suspensions containing approximately 10^4 *L. intracellularis* organisms/well. Murine fibroblast-like McCoy cells were grown in Dulbecco's Modified Eagles Medium (DMEM; Gibco Invitrogen Corporation) with 1% L-glutamine (Gibco Invitrogen Corporation), 7% fetal bovine serum (FBS; Sigma Chemical) and 0.5% amphotericin B (Cellgro; Mediatech), without antibiotics or medium replacements throughout the study (Guedes and Gebhart, 2003c).

Incubation

The infected cells were incubated using two different methods. For the conventional method, the cells were placed in the Tri-gas incubator with 83.2% nitrogen gas, 8.8% carbon dioxide and 8% oxygen gas at a temperature of 37^{o}C (Lawson et al., 1993). Tissue culture plates were removed and flushed with hydrogen gas daily. For the alternative method, the plates were placed in an Original Space Bag® (Storage Packs, San Diego, CA, USA, [http://www.spacebag.com]) measuring 54 cm x 85 cm which was hermetically closed. The air inside the bag was then removed by vacuum pump to a pressure of 100 mm Hg. Afterward, the bag was inflated through a cuff containing a 0.22 µm filter connected to a gas cylinder containing 10% hydrogen, 10% carbon dioxide, and 80% nitrogen gas. Finally, the bag was incubated at 37^{o}C for eight days (Figure 6.1). The atmosphere inside the bag was replaced as described above every 24 hours. Carbon dioxide and oxygen gas percentages were monitored in both protocols using CO_2 and O_2 indicators (FYRITE® Gas Analyzer) at the initiation of incubation and every 24 hours during the eight days. The CO_2 and O_2 levels were demonstrated to be stable in the bag when they were measured daily after inflating and before replacing the gas mixture.

Monitoring of infection and cell growth

The CyQuant® cell proliferation assay, a fluorescence-based approach for determining numbers of cultured cells (Jones et al., 2001), was used to monitor the cell growth of infected and non-infected cells for both incubation methods. In addition, the population doubling time (days) of the McCoy cells was calculated using the algorithm provided by http://www.doubling-time.com (Widera et al., 2009), based on the intensity

of the fluorescence demonstrated in the CyQuant® assay. Infection and growth monitoring of *L. intracelluaris* were performed by direct counting of heavily infected cells (HIC), identified by immunocytochemistry staining with polyclonal antibody specific for *L. intracelluaris* (Guedes and Gebhart, 2003c), and by quantitative PCR (qPCR), as previously described (Wattanaphansak et al., 2010). The level of infection was also monitored by calculating the estimated population doublings of the HIC, which were previously counted. The number of IHC and the number of *L. intracellularis* organisms were quantified using four replicates (wells) in 16-well tissue culture plates. The average from four replicates was used in the statistical analysis. Wilcoxon signed-rank test was performed using SAS software (9.1) to assess differences between both incubation methods. A value of $p<0.05$ was considered significant.

6.3 Results and Discussion

During the eight days of incubation, the carbon dioxide and oxygen gas were constant (8.8% CO_2; 8.0% O_2) in the Tri-gas incubator (conventional method). In the alternative method, CO_2 and O_2 levels ranged between 7.0-8.0% and 5.5-6.5%, respectively (Table 6.1). The CyQuant® cell proliferation assay was used to measure cellular DNA via fluorescent dye binding in non-infected and infected cells every 24 hours for eight days of incubation. Non-infected cells had similar growth rates and no significant difference on average of estimated population doubling (calculated by day) between the conventional protocol at 5% CO_2/37ºC (1.83 ± 0.18), the Tri-gas incubator (1.79 ± 0.06) and the plastic bag (1.76 ± 0.08). These results showed that non-infected

McCoy cells are able to be grown in these bags and able to support the cultivation of *L. intracellularis*. The bag described previously in this study was able to support up to 12 T_{25} (Figure 6.1) or six T_{175} tissue culture flasks (Corning®). However, we have observed that larger bags from the same manufacturer support higher number of flasks.

Enterocyte proliferation is the primary lesion associated with *L. intracellularis* infection *in vivo* (Gebhart and Guedes, 2010). Although previous experiments have not reported cellular proliferation *in vitro* to date, there is no information regarding the cell growth during *in vitro* infection (McOrist et al., 2006; Oh et al., 2010). Using the CyQuant® cell proliferation assay, the growth curves of infected and non-infected cells were compared for the conventional and alternative methods (Figure 6.2). The results did not show statistical differences ($p<0.05$) between infected and non-infected in either incubation method. Oh et al (2010) described up-regulation of cell cycle genes in infected McCoy cells; however, cell growth was not measured in this experiment. Furthermore, epithelial growth factors and their interactions with the lamina propria during *in vivo* infections can play a critical role in the pathogenesis of the disease, which is still poorly understood.

The number of heavily infected cells increased progressively and reached the peak on day seven post-infection in both the conventional and alternative incubation methods. There was no significant difference ($p<0.05$) in the number HIC throughout the days of incubation (Figure 6.3). No significant difference was found in the estimated population doubling of HIC using the conventional (2.8 ± 0.2) and the alternative (3.1 ± 0.3) protocols. Similar to the immunocytochemistry results, the greatest number of

L. intracellularis organisms per well was observed on day seven post-infection by quantitative PCR (Figure 6.3). In addition, the quantitative PCR showed no significant difference between the conventional and alternative methods of incubation. There was no positive reaction for the non-infected cell cultures (negative control) in the qPCR. Previous studies have described *L. intracellularis* cultivation in five to seven days using a Tri-gas incubator, including experiments to validate diagnostic approaches and to investigate the pathogenesis of proliferative enteropathy (Guedes et al., 2002; Oh et al., 2010). However, these studies failed to quantify the numbers of *L. intracellularis* organisms or the number of HIC in the infected cultures.

Similar to the conventional method, incubation in the bag provided environmental conditions that enable *L. intracellularis* to infect and multiply in the cells. Based on these results, we believe this approach can be used for the static cultivation and, potentially, isolation of this bacterium without requiring a Tri-gas incubator. In addition, our experience has shown no difference in the cell growth or level of the infection when the gas inside the bag is replaced at least two times (on second and fifth days post-infection) throughout the period of incubation. This alternative method has been also successfully reproduced in an independent trial (J. S. V. Oliveira and R. M. C. Guedes, unpublished data). This fact has confirmed the usefulness and feasibility of the present protocol.

The flexibility of this methodology allows for the testing of various environmental conditions for *L. intracelluaris* cultivation and production of antigens for the development of diagnostic techniques. Additionally, this affordable technology gives to

research institutes an opportunity to explore this bacterial proliferative disease, which has intriguing and unique properties among bacterial pathogens.

Table 6.1. Carbon dioxide and oxygen gas levels during eight days of incubation in a conventional Tri-gas incubator and the Original Space Bag®.

	Days of incubation							
Incubation Gas levels	1	2	3	4	5	6	7	8
Tri-gas incubator								
$\%O_2$	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
$\%CO_2$	8.8	8.8	8.8	8.8	8.8	8.8	8.8	8.8
Original Space Bag®								
$\%O_2$	6.0	5.5	6.0	6.0	6.5	6.5	6.0	6.0
$\%CO_2$	7.0	7.0	7.5	7.5	8.0	8.0	7.5	7.5

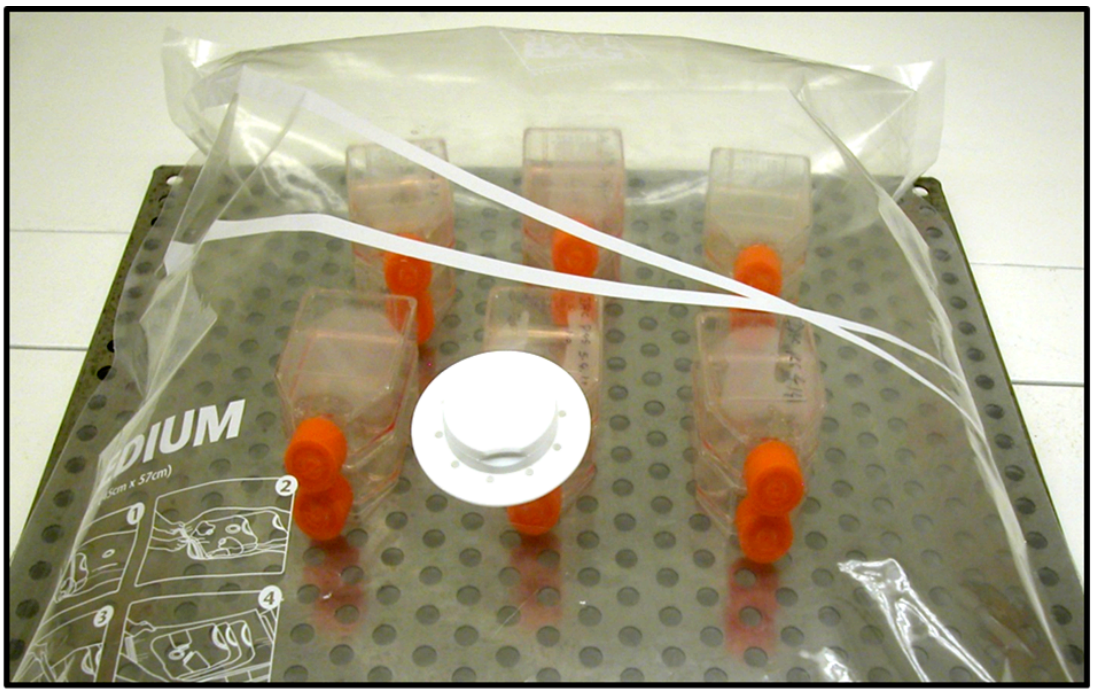

Figure 6.1. Alternative incubation with eight T_{25} tissue culture flasks in the Original Space Bag®.

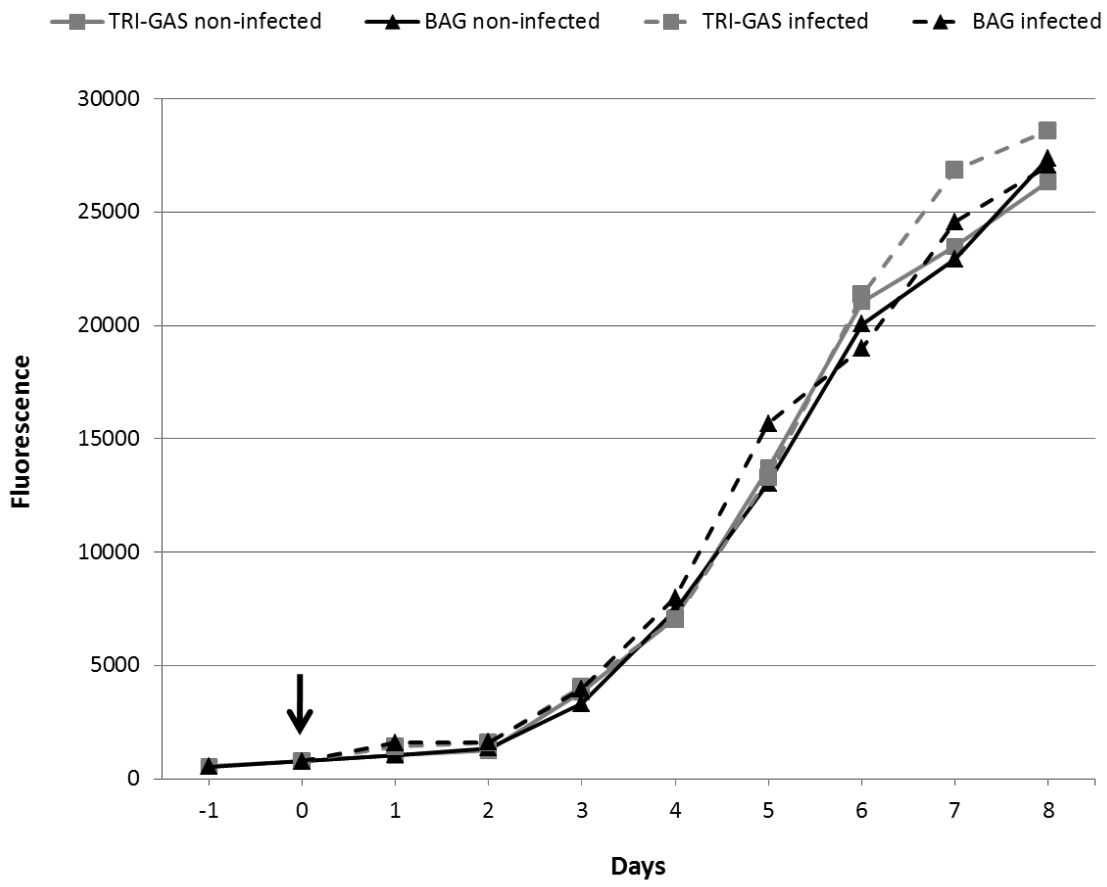

Figure 6.2. Quantification of infected and non-infected cells in the tri-gas and bag incubation systems using a CyQuant® cell proliferation assay. Fluorescence intensities are based on cellular DNA content during days of incubation. Infected cells were measured from day 0 (arrow).

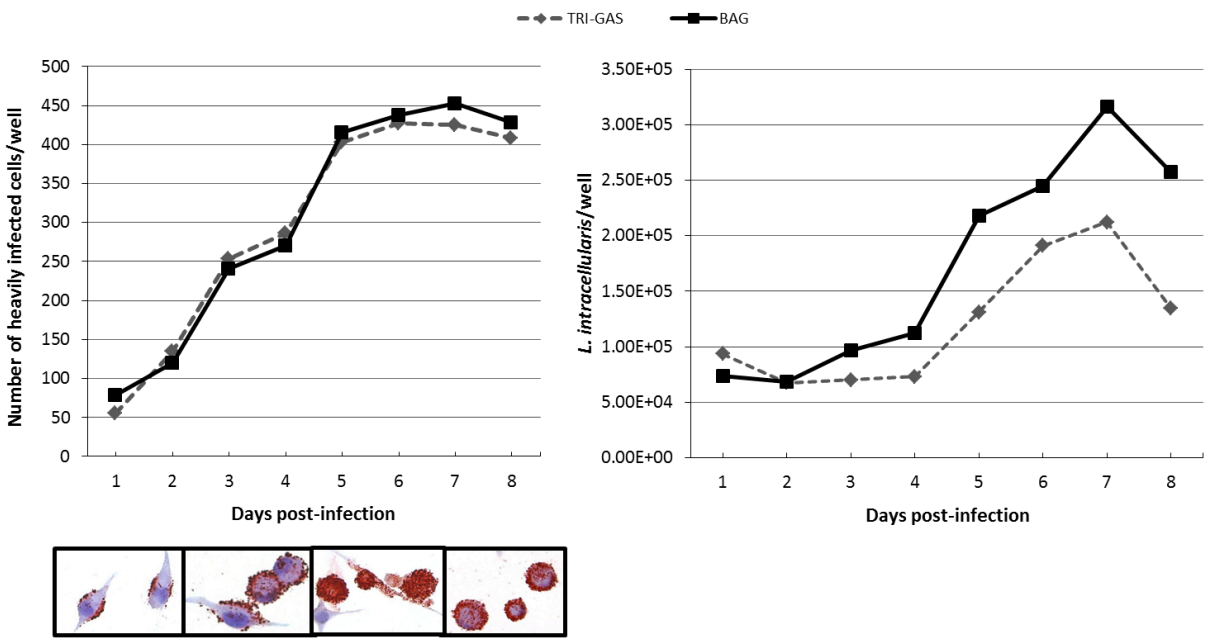

Figure 6.3. Infection and growth monitoring of *L. intracellularis* in cell culture plates during 8 days of incubation. (Left) Direct counting of heavily infected cells by immunocytochemistry, with infected cells in the alternative bag incubation system over time shown below the graph. (Right) Copies of the aspartate ammonia lyase gene of L. intracellularis measured by quantitative PCR.

CHAPTER 7

Evidence of host adaptation in *Lawsonia intracellularis* infections

Vannucci F.A.; Pusterla N.; Mapes S.M.; Gebhart C.J. (2012) Evidence of host adaptation in *Lawsonia intracellularis* infections. *Vet Res* (2012) 43:53

Summary

Lawsonia intracellularis is the causative agent of proliferative enteropathy, an endemic disease in pigs and an emerging concern in horses. Enterocyte hyperplasia is a common lesion in every case but there are differences regarding clinical and pathological presentations among affected species. The objective of this study was to evaluate the susceptibilities of pigs and horses to *L. intracellularis* infection using either a porcine or an equine isolate. Twelve foals and eighteen pigs were equally divided into three groups and infected with either a porcine or an equine isolate, and a saline solution (negative control group). The animals were monitored regarding clinical signs, average of daily weight gain, fecal shedding of the bacteria by PCR and humoral serological response. Foals infected with the equine isolate developed moderate to severe clinical signs and maintained a lower average of weight gain compared to control foals. Fecal quantitative PCR in equine isolate-infected foals revealed higher amounts of bacterial DNA associated with longer duration of shedding compared with porcine isolate-infected foals. All four foals infected with the equine isolate demonstrated higher IgG titers in the serum compared with porcine isolate-infected foals. In the pig trial, diarrhea and seroconversion were only observed in animals infected with the porcine isolate. Pathological changes typical of proliferative enteropathy were observed in one foal infected with the equine isolate and in two pigs infected with the porcine isolate. Evident clinical signs, longer periods of bacterial shedding and stronger serologic immune responses were observed in animals infected with species-specific isolates. These results show that host susceptibility is driven by the origin of the isolated *L. intracellularis* strain.

7.1. Introduction

Lawsonia intracellularis is an obligate intracellular bacterium and the etiologic agent of proliferative enteropathy (PE), an intestinal hyperplasic disease characterized by thickening of the mucosa of the intestine due to enterocyte proliferation (Gebhart and Guedes, 2010). The disease has been reported in a variety of animal species, including nonhuman primates, wild mammals and ratite birds (Cooper et al., 1997b; Lafortune et al., 2004). Since the 1990s, it has been endemic in pigs and one of the most economically important diseases in the swine industry (Lawson and Gebhart, 2000). In the last decade, the disease also has been frequently reported in weanling foals worldwide, and now is described as an emerging disease in the horse population (Drolet, 2009; Frazer, 2008; Guimarães-Ladeira et al., 2009; Stämpfli and Oliver, 2006).

Although hyperplasic lesions are present in every case of PE, there are some differences regarding clinical and pathological presentations among affected species. In pigs, there are two major clinical forms: a sporadic, acute, hemorrhagic diarrhea and a chronic, mild diarrhea (Gebhart and Guedes, 2010). A hemorrhagic form has also been reported in macaques but not in any other susceptible species (Klein et al., 1999). Infected horses develop acute but non-hemorrhagic diarrhea. Furthermore, hypoproteinemia is an important clinical sign of PE in horses but it has not been reported in pigs. These observations demonstrate important host-specific characteristics of this infection.

Isolation and cultivation of *L. intracellularis* has only been achieved by using dividing cells in culture under strict microaerophilic conditions. These fastidious

properties restrict opportunities to study the dynamics of inter-species transmission, potential reservoirs for the bacterium and host susceptibilities to different bacterial isolates. The disease has been experimentally reproduced in hamsters, pigs and horses using species-specific isolates or intestinal homogenate derived from infected animals (Guedes and Gebhart, 2003a; Jacoby, 1978; Pusterla et al., 2010). Results from cross-species experimental infections in hamsters and mice models using intestinal homogenates or porcine *L. intracellularis* isolates have consistently reproduced subclinical disease and mild lesions in infected animals (Jasni et al., 1994b; Smith et al., 2000; Vannucci et al., 2010). Therefore, the bacterium seems to adapt and persist differently depending on the species origin of the isolate. The susceptibility of pigs to equine isolates or vice-versa has not been reported and may provide relevant information about host adaptation or specificity of *L. intracellularis* infections.

We hypothesize that host adaptation to *L. intracellularis* infection is capable of driving the susceptibilities of pigs and horses depending on the species origin of the isolate. The objective of this study was to evaluate the susceptibilities of horses and pigs to *L. intracellularis* infection using porcine and equine isolates. The present study reports clinical signs, longer periods of fecal shedding of bacteria and stronger serologic immune responses in pigs and foals infected with their species-specific isolates.

7.2 Materials and Methods

Challenge isolates and preparation

The present study used a porcine (PHE/MN1-00) and an equine (E40504) *L. intracellularis* strain isolated from a gilt and a foal, respectively, both affected with the acute form of PE. The pathogenicity of each of these isolates was previously established in a porcine and an equine experimental model (Guedes and Gebhart, 2003a; Pusterla et al., 2010). Both strains were isolated and grown in murine fibroblast-like McCoy cells (ATCC CRL 1696), as described elsewhere (Guedes and Gebhart, 2003b; Wattanaphansak et al., 2005). Briefly, one-day-old McCoy cells growing in T_{175} cell culture flasks containing Dulbecco's Modified Eagles Medium with 1% L-glutamine, 0.5% amphotericin B and 7% fetal bovine serum (FBS) were infected with 2 ml of *L. intracellularis* (with approximately 10^6 organisms). Infected flasks were placed in an anaerobic jar, from which the atmospheric air was evacuated by a vacuum pump to 500 mm Hg and replaced with hydrogen gas. The infected flasks were then placed in the Tri-gas incubator with 83.2% nitrogen gas, 8.8% carbon dioxide and 8% oxygen gas and incubated at a temperature of 37^{o}C for seven days (Lawson et al., 1993). After a total of ten serial cell passages *in vitro*, the bacteria were pelleted, suspended in sucrose-potassium glutamate (pH 7.0; 0.218 M sucrose, 0.0038 M KH_2PO_4, 0.0072 M K_2HPO_4 and 0.0049 M potassium glutamate) solution with 10% FBS and stored at -80^{o}C until the day of infection (Guedes and Gebhart, 2003b). The inocula for both horse and pig experiments were identically prepared at the College of Veterinary Medicine of the University of Minnesota, using the same protocols for isolation and cultivation of *L.*

intracellularis. For the horse trial, porcine and equine isolates were separately preserved in dry ice and shipped to the University of California-Davis Center for Equine Health. For both equine and porcine isolates the molecular identities were determined by multi-locus variable number tandem repeat (VNTR) analysis (Beckler et al., 2004).

The number of *L. intracellularis* organisms was assessed by direct counting after immunoperoxidase staining of serial 10-fold dilutions prepared in sterile phosphate-buffered saline (PBS) using polyclonal *L. intracellularis*-specific antibody (Guedes and Gebhart, 2003b). Additionally, quantitative PCR (qPCR) was performed using an aliquot from each inoculum in order to validate the direct counting and standardize the challenge doses used in the foal and pig experimental infection models (Pusterla et al., 2008b).

Animals

Twelve Quarter Horse foals between 4 and 5 months of age were randomly divided into three groups: Horse/Porcine isolate (n = 4); Horse/Equine isolate (n = 4) and Horse/Negative control (n = 4). Similarly, 18 Duroc-Landrace cross pigs at 3-weeks-of-age were allocated into three groups: Pig/Porcine isolate (n = 6); Pig/Equine isolate (n = 6) and Pig/Negative control (n = 6). The animals were obtained from a herd with no history of PE and each treatment group was housed in a different pen. Prior to study commencement, all animals were evaluated for any signs of illness by a full physical examination. In addition, blood and fecal samples from all animals were collected and tested for *L. intracellularis*-specific antibodies by immunoperoxidase monolayer assay (IPMA) and qPCR in order to document the negative status for each animal. Throughout the study, the foals were housed at the University of California-Davis Center for Equine

Health and fed with free choice of grass and alfalfa hay and water and were supplemented daily with a commercial foal supplement. The pigs were housed in the isolation barns at the College of Veterinary Medicine of the University of Minnesota and fed with non-medicated nursery feed and water *ad libitum*. Horse and pig pens were cleaned once daily. All procedures were approved by the Institutional Animal Care and Use Committees of the University of California (horse study) and University of Minnesota (pig study). The horse and pig studies were conducted in the summer of 2010 and 2011, respectively.

Experimental infection

On the challenge day (day 0), the frozen inoculum was thawed at 37°C and administered within 1 hour of thawing. The foals were sedated with detomidine hydrochloride (0.01 mg/kg BWT) and inoculated with 50 ml of the inoculum via nasogastric intubation (Pusterla et al., 2010). The pigs were restrained and inoculated with 30 ml of the inoculum using a stomach tube (Guedes and Gebhart, 2003b). Control foals and pigs received sucrose-potassium glutamate solution by the same route of inoculation.

Monitoring and sample collection

Monitoring and sample collection were conducted similarly in the horse and pig studies. All animals were observed daily for general attitude and appetite for 56 days post-inoculation (PI). Once weekly, the weight of each animal was recorded in order to determine average daily weight gains throughout the study period. Feces were collected directly from the rectum every other day and submitted for qPCR (Pusterla et al., 2008b).

Blood samples were collected on days 0, 7, 14, 21, 28, 42 and 56 PI to determine serum concentration of total solids and for serologic analysis by IPMA (Guedes et al., 2002). Due to the non-terminal design of the horse study, foals developing either moderate to severe acute (fever, depression, anorexia, colic, diarrhea) or chronic (weight loss, peripheral edema) signs and/or hypoproteinemia (< 5.0 g/dL) were treated with doxycycline hyclate (10 mg/kg, PO, q 12 h) for 10 days. Additional supportive treatment including intravenous flunixine meglumine (1.1 mg/kg, IV, q 12 h) and replacement fluids (4-6 ml, IV, q 1h) were given based on the patient's need.

Quantitative PCR and serological IgG response

All fecal samples were analyzed by qPCR for *L. intracellularis* DNA at the School of Veterinary Medicine of the University of California-Davis, as described elsewhere (Pusterla et al., 2008b). Briefly, DNA purification was performed using an automated nucleic acid extraction system[e], according to the manufacturer's recommendations. Absolute quantification was calculated using a standard curve for *L. intracellularis* and expressed as copy numbers of the *aspA* gene of *L. intracellularis* per gram of feces. The standard curve was determined by using 10-fold dilutions of *L. intracellularis* derived from cell culture in McCoy cells added to *L. intracellularis*-free equine feces. Furthermore, a qPCR assay targeting a universal sequence of the bacterial 16S rRNA gene was used as quality control (i.e. efficiency of DNA purification and amplification) and as an indicator of fecal inhibition (Mapes et al., 2007).

Blood samples for the collection of serum were drawn from all animals in order to determine the concentration of total solids using a refractometer and to measure anti-*L.*

intracellularis specific IgG by IPMA at the College of Veterinary Medicine of the University of Minnesota, as previously reported (Guedes et al., 2002). Positive serum samples (titer ≥ 60) were tested to endpoint dilution and titers were reported as the reciprocal of the dilution.

Post-mortem examination, histology and immunohistochemistry

Two pigs from each group were euthanized on day 21 PI and evaluated for typical PE lesions. Intestinal samples from jejunum, ileum, cecum and colon were collected, fixed in 10% buffered formalin, processed routinely for histology, embedded in paraffin, and sectioned 5 μm thick. Two sections were prepared: one section was stained by haematoxylin and eosin (Luna, 1968) and the other by immunohistochemistry (IHC) using the streptavidin method with polyclonal antibodies to *L. intracellularis* (Guedes and Gebhart, 2003c). The level of infection was assessed by IHC based on the amount of positive labeled antigen present in the intestinal sections: Grade 0 = no positive antigen labeled; Grade 1 = one isolated focal area of antigen labeled; Grade 2 = multi-focal areas of antigen labeled; Grade 3 = majority of the mucosa has positive antigen labeled; and Grade 4 = all of the mucosa has positive antigen labeled (Guedes and Gebhart, 2003a). Histology and IHC procedures were conducted in the Veterinary Diagnostic Laboratory of the University of Minnesota.

Statistical analysis

Descriptive analyses were used to describe clinical findings among the different groups due to the limited number of animals. Statistical analysis was performed by use of Wilcoxon-Mann-Whitney tests to assess differences in daily weight gain among the

different groups. The area under curve (AUC) for the amount and duration of fecal shedding, as well as for the magnitude and duration of measureable IgG titers against *L. intracellularis*, were calculated using trapezoid rule in the SAS software (version 9.2). The Wilcoxon-Mann-Whitney test was used to compare the experimental groups based on AUC. Statistical significance was defined at values of $p < 0.05$.

7.3 Results

Experimental infection and pathological findings

The challenge doses were standardized for the horse and pig trials in order to avoid any influence of dose effect on the clinical, pathological or immune response. Based on the quantification of *L. intracellularis* organisms, which was confirmed by qPCR, each animal received 10^9 bacteria intragastrically. In addition, the molecular identities of the porcine and equine isolates were confirmed after the experimental infection by VNTR typing using PCR positive samples.

Diarrhea and significant lower daily weight gain ($p<0.05$) were observed in pigs infected with the porcine isolate and in foals infected with the equine isolate, as shown in the Table 7.1. Three equine isolate-infected foals developed moderate to severe clinical signs typical of equine PE, including depression, anorexia, colic and peripheral edema. Hypoproteinemia (<5.0 g/dL) was also observed in these foals (ranging from 4.1 to 4.7 g/dL) between 21 and 28 days PI. Hypoproteinemia was not observed in any infected pigs. Because of the severe clinical signs, one equine isolate-infected foal was treated according to the protocol previously described, but it did not respond to antimicrobial and

supportive treatment and it was then humanely euthanized 24 days PI. A full necropsy revealed severe and diffuse (from duodenum to cecum) thickening of the intestinal mucosa associated with the presence of large numbers of intracellular *Lawsonia*-specific antigen identified by IHC (Figure 7.1A and B). Based on the typical clinical signs of PE, the peak of the experimental infection occurred between the third and fourth week PI in both pigs and foals. Foals infected with the porcine isolate, pigs infected with the equine isolate and negative control groups failed to show clinical signs, hypoproteinemia (foals), and lower average of weight gain or pathological changes.

The experimental infection in pigs revealed macroscopic and histologic lesions typical of porcine PE in the two animals infected with porcine isolate and euthanized 21 days PI. These lesions were associated with the presence of *Lawsonia*-specific antigen in the intestinal epithelium (Figure 7.1C and D). Neither the two pigs infected with the equine isolate nor the two negative controls which were euthanized 21 days PI showed any clinical or pathological changes (Figure 7.1E and F).

Quantification of fecal L. intracellularis *DNA*

Results of fecal shedding of *L. intracellularis* throughout the study period are summarized in Figure 7.2. Positive PCR signals for the universal bacterial 16S rRNA gene were detected in all fecal samples demonstrating the efficiency of the DNA extraction protocol. *L. intracellularis* DNA was observed in the feces of foals infected with the equine isolate from 12 to 38 days PI. Three foals infected with the porcine isolate shed bacteria. However, *L. intracellularis* DNA was detected in these animals at only four time points and no more than 10^4 bacterial organisms per gram of feces was

detected (Figure 7.2A). The mean areas under the "time – *L. intracellularis* organisms/g" curve (Figure 7.2A) were significantly different ($p<0.05$) between foals infected with equine and porcine isolates. As a result, equine isolate-infected foals revealed higher amounts of bacterial DNA in the feces associated with longer duration of shedding compared with porcine isolate-infected foals.

In the pig trial, animals infected with the porcine isolate showed higher and longer shedding of bacteria in the feces throughout the study period ($p<0.05$) (Figure 7.2B). In these animals, PCR detection of *L. intracellularis* lasted from day 2 to 38 PI. Two pigs infected with the equine isolate shed the bacteria at low levels (10^2 bacteria/g of feces) on day 2 PI. All foals and pigs in the negative control group remained negative for the entire study period.

Serological IgG response

All four equine isolate-infected foals demonstrated higher IgG titers ($\geq$ 3840) against *L. intracellularis* in the serum compared with the porcine isolate-infected foals ($\leq$ 1920) (Figure 7.3A). One foal infected with the porcine isolate showed no measurable serologic response during the entire study period. The mean areas under the time – titer curve of the IPMA titers were not significantly different (p <0.05) between foals infected with equine and porcine isolates. However, because there was no serum from day 28 PI in the foal euthanized on day 21, the area under the curve for this animal was underestimated in the analysis. Simulating IgG titers of at least 960 on days 28 and 42 for this foal, significant difference (p <0.03) would be observed in the time – titer curve of the IPMA between foals infected with porcine and equine isolates.

The serologic responses in the pig experiment are summarized in Figure 7.3. There was no detectable serologic response in pigs infected with the equine isolate at any time point. On the other hand, the majority of porcine isolate-infected pigs demonstrated IgG titers (> 120) against *L. intracellularis* from day 21 PI. The serological responses were persistent throughout the study in foals and pigs infected with species-specific isolates. However, foals infected with the equine isolate had much stronger immune responses (p= 0.057) than those pigs infected with the porcine isolate.

Table 7.1 - Average of daily weight gain (mean ± standard error) throughout the entire study period.

Infected species	Control	*L. intracellularis* isolate	
		Porcine	Equine
Pig (n=6)	0.59 ± 0.09 [a]	0.32 ± 0.07 [b]	0.54 ± 0.11 [a]
Horse (n=4)	0.90 ± 0.04 [a]	0.75 ± 0.04 [a]	-0.07 ± 0.28 [b]

Pairwise comparisons are within columns: means that do not share letters are significantly different ($p < 0.05$).

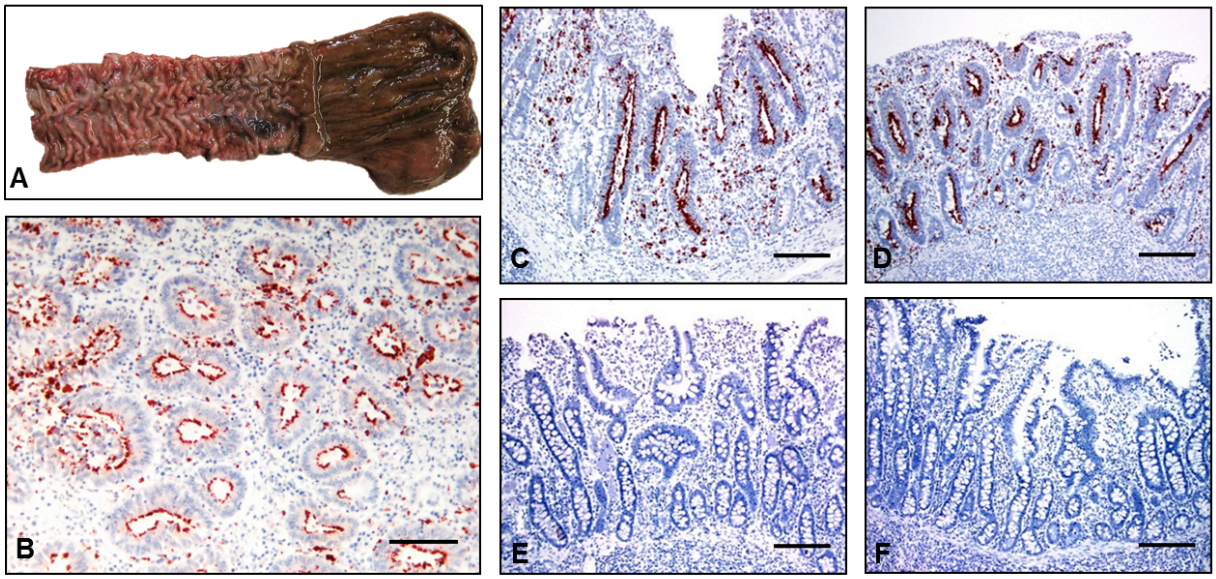

Figure 7.1 - Pathological findings. A) Terminal ileum. Equine isolate-infected foal euthanized on day 24 PI. Hyperaemia associated with diffuse and severe thickening of the intestinal mucosa compatible with PE. B) Histologic section of ileum. Equine isolate-infected foal euthanized 24 days PI. L. intracellularis antigen-specific staining with AEC substrate-chromogen and counterstained with Mayer's hematoxylin. Diffuse hyperplasia of immature enterocytes and severe reduction in the number of goblet cells associated with the diffuse presence of intracellular bacteria in the apical cytoplasm. Scale bar, 100 µm. C – D) IHC staining. Ileal mucosa of porcine isolate-infected pigs euthanized 21 days PI. Diffuse presence of the bacteria in the apical cytoplasm of the hyperplastic enterocytes and in the lamina propria. Scale bar, 200 µm. E – F) IHC staining. Ileal mucosa of equine isolate-infected pigs euthanized 21 days PI. Absence of hyperplastic enterocytes or L. intracellularis antigen associated with diffuse presence of goblet cells in the intestinal epithelium. Scale bar, 200 µm.

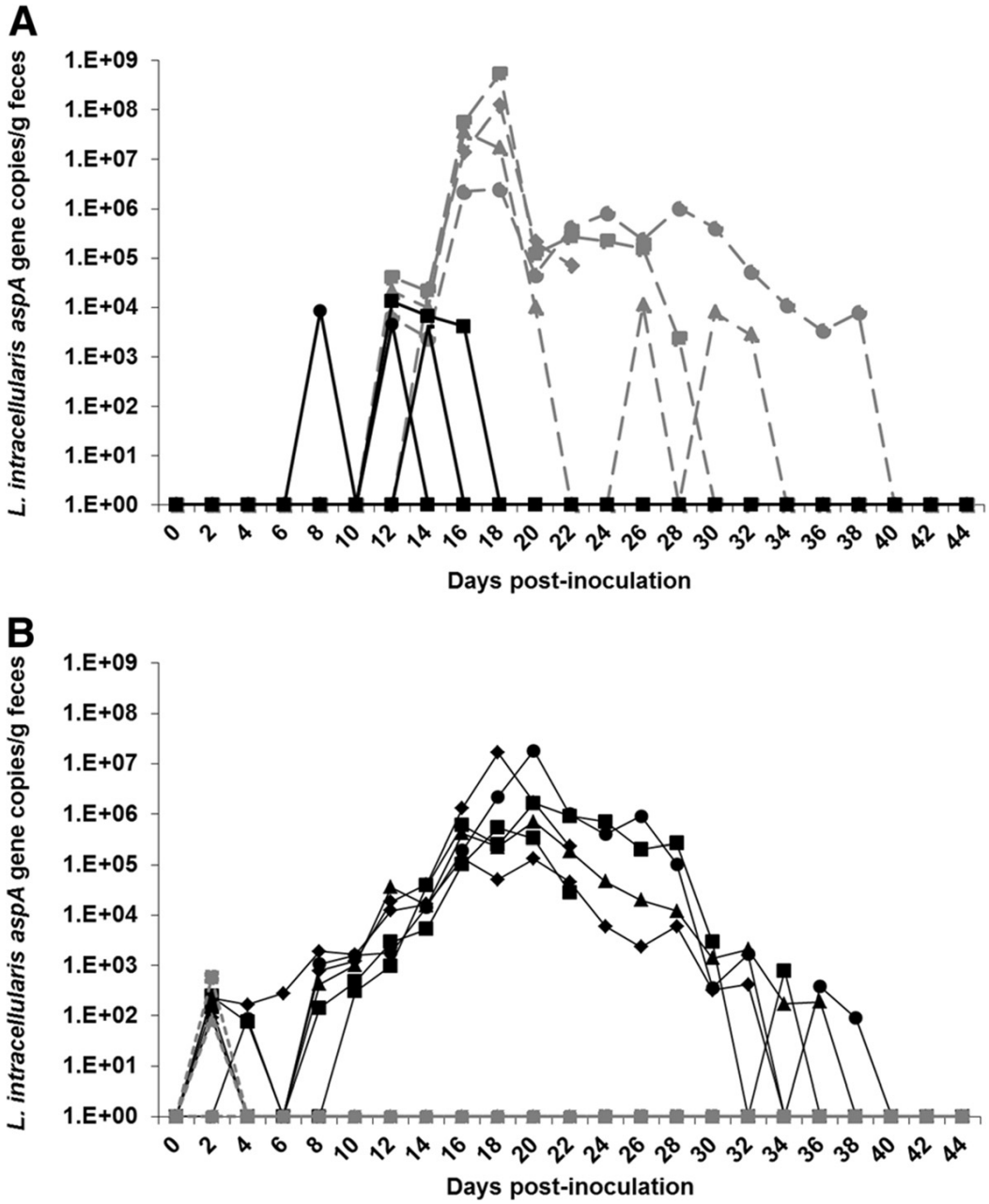

Figure 7.2 – Quantification of the *L. intracellularis* DNA in the feces performed by qPCR. The results are expressed as copy numbers of *aspA* gene copies of *L. intracellularis* per gram of feces throughout the study period. A) Foal experiment. B) Pig experiment. Black-solid lines represent animals infected with the porcine isolate and grey-dashed lines represent animals infected with the equine isolate.

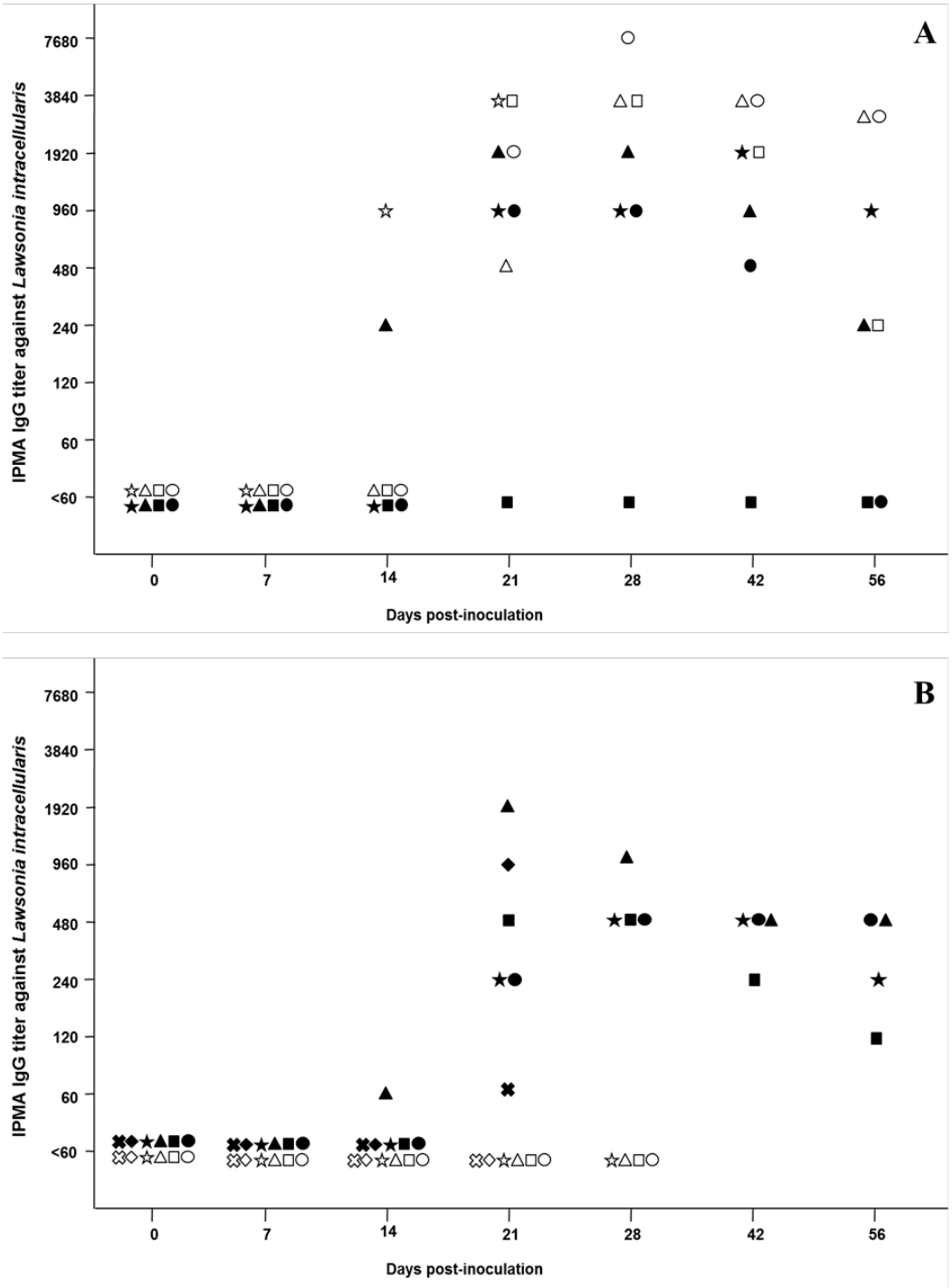

Figure 7.3 – Serologic response against *L. intracellularis* throughout the study period. A) Foal experiment. B) Pig experiment. White points represent animals infected with the equine isolate and black points represent animals infected with the porcine isolate.

7.4 Discussion

Marked clinical signs and pathological changes typical of PE associated with longer periods of bacterial shedding and stronger serologic immune responses were observed in pigs and foals infected with species-specific isolates. These observations were made despite the identical doses of *L. intracellularis* used in the horse and pig experimental infections and support the hypothesis that host susceptibilities are driven by the origin of the isolated strain. Pigs and foals demonstrated a similar pattern of fecal shedding, which lasted until 39 days PI, but the bacterial DNA was identified earlier in pigs (day 2 PI) compared to foals (day 8 PI). In those animals infected with species-specific isolates, foals exhibited a more robust serological IgG response compared with pigs which may reflect a characteristic related to the host response, regardless of the *L. intracellularis* isolate. Additionally, hypoproteinemia was an important sign of PE in the affected horses. Despite the phenotypic differences regarding the susceptibilities of these two species, gross and histological lesions characterized by thickening of intestinal mucosa due to proliferation of enterocytes were identical in those animals infected with species-specific isolates.

PE has been previously reproduced using pure cultures or intestinal mucosa homogenates derived from infected animals (Guedes and Gebhart, 2003a; Jacoby et al., 1975; Pusterla et al., 2010). Since *L. intracellularis* is a fastidious organism and extremely difficult to isolate and propagate, mucosal homogenate challenge models have been reported in pigs using intestinal mucosa from PE-affected pigs (Guedes et al., 2009; Guedes et al., 2003; McOrist et al., 1996b). Hamsters and mice infected with intestinal

homogenates from PE-affected pigs have consistently developed subclinical PE associated with mild lesions, mainly found in the cecum and colon (Murakata et al., 2008; Vannucci et al., 2010). However, dehydration and profuse diarrhea were observed in hamsters inoculated with intestinal homogenate or filtrate derived from PE affected hamsters (Jacoby, 1978; Jacoby et al., 1975). Studies using mice experimentally infected with pure cultures of porcine *L. intracellularis* isolates have revealed inconsistent results. Smith et al. (Smith et al., 2000) described pathological changes in wild-type 129-Sv-Ev and IFN-γ receptor knockout (IFN-γ R^-) mice infected with a porcine isolate. Go et al. (2005) reported gross and histological lesions in IFN-γ R^- mice but not in three wild-type mice (ICR, BALB/c and C57BL/6) . A more recent study showed mild histological lesions in four mice strains, including BALB/c and C57BL/6 (Viott et al., 2010). As described above, the lack of availability of *L. intracellularis* isolates from different host species has limited the experimental infection studies to the use of porcine isolates. Recently, our research group isolated a new equine strain and its virulence was confirmed in a foal experimental model (Pusterla et al., 2010). This allowed us to perform the present study which is the first cross-species experimental model using pure cultures of *L. intracellularis* and to evaluate different host susceptibilities and adaptation to species-specific bacterial isolates.

The challenge models previously described have contributed to advances in the prevention and control of PE by determining the efficacy of different antimicrobial drugs and vaccination protocols (Guedes and Gebhart, 2003b; McOrist et al., 1997b; McOrist et al., 1996b). Nevertheless, little is known about the epidemiology and ecology of *L.*

intracellularis infection, especially regarding inter-species transmissions, source or reservoir hosts of *L. intracellularis* and potential biological or mechanical vectors. Based on the pattern of bacterial shedding (Figure 7.2), foals infected with the porcine isolate and pigs infected with the equine isolate showed low levels *L. intracellularis* DNA in the feces during a short period of time at the earlier stages of the experimental infection. Based on these findings and the uncommon opportunity of pigs and foals to share the same environment in the modern pig production system or horse breeding farms, any direct cross-species transmission in the field between these two species is unlikely.

Free-living animals have been speculated to be a reservoir of PE by allowing the maintenance of *L. intracellularis* in the wild population with successive introductions into domestic pig or horse farms. Pusterla et al. (Pusterla et al., 2008b) detected bacterial DNA by fecal PCR in a variety of domestic and wild species, including dogs, cats, mice, rabbits, opossums, skunks and coyote, on horse breeding farms with documented occurrence of EPE. The involvement of wild animals as biological vectors for *L. intracellularis* in the pig production system has also been investigated. Using quantitative PCR, Collins et al (Collins et al., 2011) recently identified rats trapped in endemic pig farms shedding less than 10^5 *L. intracellularis*/g of feces. However, these authors also reported that a small proportion of rats shed more than 10^8 bacteria per gram of feces. Associating these observations with the results from our study leads us to speculate on the presence of different sources or reservoir species for *L. intracellularis* infections in horses and pigs. In addition, the lack of evidence supporting inter-species transmission

between horses and pigs helps confirm the host adaptation to species-specific isolates demonstrated in the present study.

In order to support these hypotheses, the phenotypic observations described in the present study may be linked with genotypic differences between *L. intracellularis* isolates. Comparison of the 16S ribosomal DNA sequences showed a high degree of similarity among *L. intracellularis* from pigs, hamsters, deer and ostriches (Cooper et al., 1997a). Four sets of primers targeting hypervariable regions of the *L. intracellularis* genome were used in the development of the VNTR technique (Beckler et al., 2004). This technique has shown unique and distinct VNTR profiles from epidemiologically unrelated outbreaks in pigs and horses and it was performed to confirm the molecular identity of the porcine and equine isolates used in the present study. A comprehensive analysis involving whole-genome sequencing of equine, as well as porcine, *L. intracellularis* isolates is crucial to identify any genomic variations associated with different host adaptations and susceptibilities.

In conclusion, the present study demonstrated marked clinical signs, longer periods of bacterial shedding and stronger serologic immune responses in foals and pigs experimentally infected with their species-specific isolates. This evidence of host adaptation in these species suggests that host susceptibilities to PE are driven by the origin of the *L. intracellularis* isolate. Comparative genomic analysis is a promising route to associate phenotypic characteristics with potential genomic variations between porcine and equine isolates and may help to characterize species-specific *L. intracellularis* strains and potentially novel bacterial subspecies or genotypes.

SECTION IV

GENERAL CONCLUSIONS

The present thesis studied the pathogenesis of proliferative enteropathy (PE) evaluating phenotypic traits, genomic features and gene expression profiles during *L. intracellularis* infection *in vitro* and *in vivo* (Section II). Studies on host adaptation (Section III) were motivated by the differences in the disease presentation among PE-affected species and the genomic variations between porcine and equine *L. intracellularis* isolates described in Chapter 3.

Following a literature review focusing on the pathogenesis of PE (Chapter 1), the second chapter phenotypically described the loss of virulence in a porcine *L. intracellularis* isolate continuously grown in cell monolayer. While clinical PE was reproduced in pigs infected with the homologous isolate grown for 10 and 20 passages *in vitro*, no clinical signs, pathological changes or serological IgG responses were observed in pigs infected with the same isolate grown for 40 passages. This result indicates that significant attenuation of virulence occurs between 20 and 40 passages *in vitro*.

In Chapter 3, a comparative genome analysis between a pathogenic (low passage) and a non-pathogenic (high passage) variant from the same isolate revealed the loss of a prophage-associated genomic island in the non-pathogenic variant. This chromosomal island located downstream from the DLP12 prophage integrase gene was conserved among pathogenic porcine isolates and PE-affected pigs, but it was not identified in

equine isolates or PE-affected horses. This genetic element represents the major genomic variation described among the *L. intracellularis* isolates and suggests that it is involved in a potential ecological specialization of pig-adapted variants.

The gene expression profiling of a pathogenic porcine *L. intracellularis* isolate *in vitro* and *in vivo* did not show expression of this genomic island, suggesting that this element is not essential for the virulent phenotype. Comparing the bacterial transcriptome between pathogenic and non-pathogenic variants, Chapter 4 showed a wider transcriptional landscape in the pathogenic isolate indicating that the virulent phenotype is more complex than previously thought. Additionally, the transcriptional profiling of the pathogenic *L. intracellularis* isolate *in vitro* and *in vivo* demonstrated high expression of genes encoding hypothetical proteins and activation of a sophisticated mechanism to cope with oxidative stress.

Chapter 5 describes the transcriptional host response in *Lawsonia*-infected enterocytes *in vivo*. The proliferative changes in infected cells were associated with activation of transcription, protein biosynthesis and genes acting on the G_1 phase of the host cell cycle (Rho family). High expression of *L. intracellularis* genes encoding hypothetical proteins and activation of host Rho genes infers the role of unrecognized bacterial cyclomodulins in the pathogenesis of proliferative enteropathy. The lack of differentiation in infected enterocytes was demonstrated by the repression of membrane transporters related to nutrient acquisition, indicating that malabsorptive diarrhea represents the major mechanism involved in the poor performance and growth of affected animals.

Section III describes an alternative protocol for cultivation of *L. intracellularis* (Chapter 6) that was developed for amplifying the bacterial inoculum to be used in the cross-species experimental infection study reported in Chapter 7. The susceptibility of pigs and horses to *L. intracellularis* infection was evaluated using a porcine or an equine isolate. The lack of clinical and pathological findings in animals infected with non-species-specific isolates showed phenotypic evidence of host adaptation in porcine and equine-derived isolates.

Throughout the studies included in this thesis, the phenotypic, genomic and transcriptomic evaluations were mostly based on one porcine *L. intracellularis* isolate, PHE/MN1-00. This fact represents the major limitation in the studies presented here. *L. intracellularis* has been considered a monotypic organism with highly conserved genotypic especially because of its obligate intracellular characteristic. However, we showed in Chapter 3 a considerable genomic variation within the same isolate continuously grown *in vitro*. This observation leads to the question whether the results presented in this thesis can be extrapolated to all *L. intracellularis* isolates. On the other hand, the similarity of the proliferative lesions in *L. intracellularis* infections, regardless the bacterial isolate, suggests common bacterial properties among different isolates, especially regarding the gene expression pattern exhibited by intracellular pathogenic *Lawsonia*.

Another important limitation of the studies described here is the assumption that highly expressed genes represent proteins exhibiting their respective biological functions. With this in mind, the characterization of the proteins encoded by the genes highly

expressed in our studies would be necessary in order to evaluate their roles in the pathogenesis of PE and their immunogenic properties for diagnosis and vaccine purposes.

Although Chapter 3 showed a consistent loss of the prophage-associated genomic island in the porcine *L. intracellularis* isolates at high passage *in vitro*, a better characterization of this event needs to be further evaluated in order to determine the specific number of passages required for its excision from the bacterial DNA. It also would be interesting to attempt isolation of a phage particle in the supernatant of cultured *L. intracellularis* and the potential reintegration of this genetic element into DNA of bacteria at high passage or equine isolates.

Finally, a comparative analysis involving the whole genome sequence of a porcine and an equine isolate may complement the phenotypic study showing the host adaptation of *L. intracellularis* in these two species. Besides the absence of the prophage-associated genomic island in the equine isolates, the inclusion of other genomic variations in horse-derived isolates may reveal aspects related to bacterium fitness and allow characterization of novel *L. intracellularis* subspecies or genotypes.

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APPENDIX

APPENDIX 1 – SUPPLEMENTAL MATERIAL

Chapter 4

Supplementary Table 4.1. Primers used for validation of RNA-seq expression data by quantitative reverse transcriptase PCR assay.

Locus	Gene product	Entrez Gene ID	Primers
LI0005	superoxide dismutase	4059803	TCATATCCATGAAGGAGGATCA GGAGTCCAGCTGTTAGATGTCC
LI0035	fur regulator	4060312	CAAGCAACTGTTTACCGTACTATGA TCACTAAAACGTACTTCTCGTGCTA
LI0447	hypothetical protein	4060194	CCCAGCGTTTTACCCTATGA TCGCTGCATATCCTTTTATGG
LI0614	thioredoxin	4059703	TCATAACAGTTTTTGCAACATGG GCAATAATCCCAGGCATTTC
LI0902	outer membrane protein	4059372	TGCATGAAACAGTCATTAATCAAA CCCTAAATTATTATGACCATCAGTGAG
LIA017	Fe-S oxidoreductase	4073414	AAGATCAAAAATTATTACCAGTTTGCT GCCATAATGCAACCATGTACC
LIC056	hypothetical protein	4073409	GGGAAATCCTTGCCCTTATC AAAACACCTGCGACAGTACCTAA
LIB024	chromosome partitioning ATPase	4073430	AAAGCACCACAGCATTAGCA CATCTAGGTCAATGAAAAGTACGC
LI0825	lipid A core-O-antigen ligase	4059468	CATCTGAGTGGTTAGCTGCAA CACAAAAGCTAAAACAATCCCTCT
LI0959	30S ribosomal protein S10	4059852	CAGCAAGAAATACAGGTGCAAG TTGCTCACGGGACTTTTTATC