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Immunity to *Lawsonia intracellularis* vaccination in pigs

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*Submitted to the University of Sydney in fulfilment of the requirements
for the degree of Doctor of Philosophy*



Faculty of Veterinary Science

The University of Sydney

2013

*“All our dreams can come true,
if we have the courage to pursue them”*

Walt Disney

STATEMENT OF ORIGINALITY

“The material in this thesis has not been previously submitted for a degree in any university, and is original to the best of my knowledge. It does not contain material previous published or written by any other person except where due acknowledgement is made in the thesis itself.”

Mariana Gomes Nogueira
24/02/2014

ABSTRACT

Lawsonia intracellularis is the causative agent of proliferative enteropathy (PE). PE is an important disease of weaner and grower pigs causing degrees of diarrhoea and negative effects on feed intake and weight gain. In-feed antibiotics are routinely used to control PE disease outbreaks. However, increasing restrictions to antibiotic use is being implemented in various pig producing countries. Therefore, alternatives to improve resistance while promoting growth performance are ideal. To limit the establishment of infection and increase profitability of pig production, disease prevention needs both nutritional and immunological strategies and well as effective sanitary measures. Since 2006, in the Australian market, a live attenuated *Lawsonia intracellularis* vaccine has been used to reduce clinical signs and PE lesions and reduce *L. intracellularis* shedding in faeces. However, the systemic and local immunological responses to a standard vaccine dose are poorly characterised. In the absence of proof that vaccinated pigs are protected from PE, veterinarians are unwilling to remove antibiotic medication for fear of acute haemorrhagic PE in adult pigs. Additionally, feeding strategies as alternatives to antibiotics, such as beta-glucan have been proposed as a possible option, but no study has investigated the effect of phytase on immune responses in growing pigs. The research in this thesis addresses studies to immune-based investigation of factors affecting the induction of immune responses following vaccination with *L. intracellularis*. Results revealed that the use of an oral standard and ten times dose of *L. intracellularis* vaccine protects pigs against PE disease by reducing lesions, shedding in faeces and clinical signs. However, intramuscular delivery also protected pigs against PE. Immunological responses to *L. intracellularis* vaccination, particularly IgG and cytokines response were observed after oral, intraperitoneal and intramuscular *L. intracellularis* vaccination. However these were highly variable, highlighting the difficulties in finding suitable biomarkers. The effect of adding *S. cerevisiae* yeast beta-glucan and microbial phytase to weaner diet affected on mucosal and systemic *L. intracellularis* vaccination local immune response.

ACKNOWLEDGEMENTS

I wish to thank the many people who have helped, encouraged, provided financial support and advised me during my PhD.

I would like to express my sincere gratitude to my supervisor Prof. David Emery and advisors Dr. Alison Collins and Dr. Stuart Wilkinson. Their guidance, support and vast knowledge in the field of immunology, swine health and nutrition have made this thesis far more than it would have been without their input. They have been an invaluable source of knowledge, skills training, guidance and encouragement throughout the entire period of my project. They have gone out of their way on innumerable occasions to provide explanations, assistance, and support above and beyond their call of duty. They also have given an enormous amount of their time to reading and editing my draft material.

I would like to thank the Australian Research Council, New South Wales Department of Primary Industries, The Sydney University and Boehringer Ingelheim for their financial support for the vaccination trial project. A special thanks to Dr. Roel Lising, Dr. Bernd Grosse, Dr. Oliver Duran, Dr. Jeremy Kroll and Dr. Mike Roof. Thanks Boehringer Ingelheim for the invitation and funding tickets and accommodation to the Boehringer Ingelheim Research & Development Meeting in United States 2012.

I would like to acknowledge the assistance of veterinarian Dr. Hugo Dunlop for his expertise in pig health and input for the on farm trial. Thank you to the farm owner, manager and staff for the invaluable help during the trial. I would also like to thank Patrick Daniel and Dr. Tony Fahy from the Department of Primary Industries Pig Health Research Unit, Bendigo, Victoria for assistance with necropsy.

I would like to thank AB Vista for their generous financial support and opportunity to run a trial with their feed additives, special thanks to Carrie Walk. Also special thanks to the Sydney University Associate Professors Dr. Aaron Cowieson and Dr. Navneet Dhand on their input on nutrition and statistical analysis to this research.

Thank you to all of the wonderfully helpful Sydney University staff, with special thanks to Greg McNamara with help with pigs. As well as I gratefully appreciate the help from the Elizabeth Macarthur Institute staff, special thanks to Shayne Fell, Jocelyn Gonsalves, Dr. Graeme Eamens, Lauren Wolley, Dr. Ian Marsh, Francesca Galea, Dr. David Read and Dr. Rodney Reece.

I'd like to thank my Brazilian colleagues Juliana Calveyra and Dr. Patricia Schwartz from Boehringer Ingelheim (Brazil) for supplying internal manuals and papers. Also like to thank Prof. Roberto Guedes for permitting me to use his pictures in this thesis.

I would like to acknowledge the Australian Pork Limited (APL) and Pork CRC, especially Pat Mitchell for their generous invitation to present at Australasian Pig Science Association (APSA) conference 2011 and funding for accommodation.

I would also like to express my immense gratitude to my best friend and partner Matt for all the love, tolerance during periods of stress and the understanding given during my absence. Daily phone calls and occasional weekend flights have been part of our life in the last three years, and I'm excited about the future, wherever it may lead, and I am grateful that you are going to be there with me. As well as, thank you to my beloved Australian family David, Diane, Ben and Louise for extensive emotional and financial support. Thanks Uncle Kim, it is definitely an advantage to have a family member inside the University.

At last but not least, I'd like to thank my parents, Roberto and Aurea, and also my sisters Aline and Caroline for the constant source of support – emotional, moral and of course financial. I am honoured to be part of this family and this thesis would certainly not have existed without them. Thank you! Obrigada!

Peer-reviewed publications

Nogueira, MG; Emery, D; Collins AM (2013). Impact of route of administration on the mucosal immune response to *Lawsonia intracellularis* vaccine in pigs. *Australian Veterinary Journal*. Submitted.

Nogueira, MG; Emery, D; Collins, AM; Walk, CL; Wilkinson, SJ; Cowieson, AJ (2013). Dietary effects of *Saccharomyces cerevisiae* β -glucan yeast on vaccine immune response and performance of pigs. In: *Manipulation in pig production IXV*, p.113. ed. Pluske, J & Pluske, J. Australasian Pig Science Association, Werribee, Australia.

Nogueira, MG; Emery, D; Collins, AM; Walk, CL; Wilkinson, SJ; Cowieson, AJ (2013). The impact of reduced dietary phytate on immune responses and performance of pigs In: *Manipulation in pig production IXV*, p.114. ed. Pluske, J & Pluske, J. Australasian Pig Science Association, Werribee, Australia.

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Nogueira, MG; Collins, AM; Donahoo, M; Emery D. (2013) Immunological responses to vaccination following experimental *Lawsonia intracellularis* virulent challenge. *Veterinary Microbiology*. 164:131-138.

Nogueira, MG; Collins, AM; Emery, D. (2011). The immune response to a ten-times oral or intramuscular immunisation with *Lawsonia* vaccine in weaner pigs. In: *Manipulation in pig production IV*, p.47. eds van Barneveld, RJ. Australasian Pig Science Association, Werribee, Australia.

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Nogueira, MG; Collins, AM; Emery D. (2012) Immune characterization to *Lawsonia* vaccination in experimentally challenged pigs. 22nd International Pig Veterinary Society Congress (IPVS) *proceedings*. p.595, Jeju, South Korea.

Nogueira, MG; Emery D.; Wilkinson, S; Collins, AM; (2012) Dietary effects of phytate concentrations and β -glucan yeast immune stimulation to vaccination in pigs. Sydney Emerging Infections and Biosecurity Institute Colloquium. 51p, Sydney, Australia.

Oral presentations

Nogueira MG. (2012) Immunological responses to Enterisol® Ileitis vaccination. Boehringer Ingelheim Research & Development (BIRD) Meeting, Kansas City, U.S.

Nogueira MG, Collins AM, Emery D. (2011). The immune response to a ten-times oral or intramuscular immunisation with *Lawsonia* vaccine in weaner pigs. 13th biennial Conference of the Australasian Pig Science Association, Adelaide, Australia.

Nogueira MG. (2011). Infection and immunity to *Lawsonia intracellularis* in pigs. APL/Pork CRC Student Workshop, Adelaide, Australia.

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LIST OF ABBREVIATIONS

ADG	average daily weight gain
AID	Apparent ileal digestibility coefficients
AU\$	Australian dollar
BW	bodyweight
CD	Cell cluster of differentiation marker
cm	centimetre
DNA	deoxyribonucleic acid
dpi	days post-inoculation/challenge
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
FCR	Feed conversion ratio = feed intake divided by weight gain
FI	Feed intake
FTU	Phytase activity units
h	hour
H&E	Haematoxylin and eosin stain
HRP	Horseradish peroxidase
IEC	Intestinal epithelial cells
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IM	intramuscular
IP	Intraperitoneal
IPMA	Immunoperoxidase monolayer assay
kg	kilogram
km	kilometre
L	litre
LP	Lamina propria
LPS	Lipopolysaccharide
m	metre
mg	milligram
min	minute

mL millilitre
M cells Microfold cell
mg Micrograms
MHC Major Histocompatibility complex
MLN Mesenteric lymph node
NK cells Natural killer cells
OD Optical density
PAMPS Pathogen Associated Molecular Patterns
PBS Phosphate buffered saline
PCR polymerase chain reaction
PLN Pre-scapular lymph node
PE Proliferative enteropathy
PHE Proliferative haemorrhagic enteropathy
PI Percent inhibition
PIA Porcine Intestinal Adenomatosis
PMBC peripheral mononuclear blood cells
PMN Polymorphonuclear cells
PP Peyer's patches
ppm part per million
REML Restricted maximum likelihood
rRNA ribosomal ribonucleic acid
sec second
SEM Standard error of the mean
sIgA Secretory immunoglobulin A
Taq *Thermus aquaticus* enzyme
Tc Cytotoxic T cell
TCID₅₀ Tissue culture infective dose at 50%
TGF Tumor growth factor
Th T helper cell
TMB 3,3',5,5-tetramethylbenzidine
TNF Tumor necrosis factor
μL microlitre
μM micromolar

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Chapter 1 *Introduction*

Diarrhoea and enteric diseases that principally affect young weaner and grower pigs are significant problems facing the pork industry worldwide. They directly affect the profitability of pig production, not only due to the death of some animals, but also due to the negative impact on growth, feed conversion efficiency and increasing production and medication costs incurred. To limit the establishment of infection and safeguard the profitability of pig production as in-feed antibiotics are progressively withdrawn, disease prevention needs both nutritional and immunological strategies as well as effective sanitary measures. The research in this thesis investigates factors affecting the induction of mucosal immune responses following vaccination with *Lawsonia intracellularis*.

Proliferative enteropathy (PE) or ileitis, is caused by the obligate intracellular bacterium *Lawsonia intracellularis* (Lawson *et al.*, 1993). It is one of the most important causes of diarrhoea in weaner and grower pigs (Jacobson, 2003). The disease commonly causes a range of clinical signs from acute haemorrhage to chronic diarrhoea (Lawson and Gebhart, 2000). Clinical and subclinical cases (with no apparent clinical signs) are commonly accompanied by a decrease in feed intake, slower rate of weight gain and poorer feed conversion rate (Collins *et al.*, 2010b; Paradis *et al.*, 2012). The economic losses to pig producers are not only from poorer performance but are also due to the costs of controlling the disease. Previous economic models have estimated that PE costs pig producers around US\$ 20 million dollars annually or around AU\$ 7.00 per pig (Holyoake *et al.*, 1996; Bronsvort *et al.*, 2001). However, these reports did not take into account the economic impact of subclinical PE and the cost of controlling the disease. More recently, subclinical *L. intracellularis* infection was estimated to reduce profitability by AU\$ 8.33 per pig if 80% of the herd were affected, while clinical infection reduced profitability by AU\$ 13.0 per pig when as few as 16% of pigs are clinically affected (Holyoake *et al.*, 2010a). In contrast, the cost of controlling PE by vaccination (Enterisol® Ileitis, Boehringer Ingelheim) was estimated to be AU\$ 2.70 per pig.

Post-mortem diagnosis of PE relies on the combination of gross lesions and histopathological lesions (e.g. stained with haematoxylin and eosin, HE or with a monoclonal antibody against *L. intracellularis*). However, recovery from PE within three to four weeks means that pathology is only a useful means of diagnosis in pre-slaughter pigs or very sick animals. Ante-mortem diagnostic tests, such as serology and PCR, are now routinely used for diagnosis, but are also useful for estimating the prevalence of *L. intracellularis* infection in pig herds. The prevalence of *L. intracellularis* in faeces (by PCR) has been reported to range from 20 to 75% in positive herds of large pig producing countries such as the United States, Brazil and Denmark (Chiriboga *et al.*, 1999; Stege *et al.*, 2004; Armbruster *et al.*, 2007). However, 4.5%, 30% and 37.6% of the herds in Spain, Taiwan and Norway were also tested as PE positive, respectively (Chang *et al.*, 1997; Pozo *et al.*, 1998; Flo *et al.*, 2000). Positive faecal PCR results are usually indicative of *L. intracellularis* presence, either currently or in recovery stages while high seroprevalence indicates that pigs were previously exposed to *L. intracellularis*. Serological analysis by indirect immunofluorescence (IFAT) or ELISA tests revealed that approximately 60 to 100% of the farms in the United States, the European Union and Australia were positive for *L. intracellularis* antibodies (Chouet *et al.*, 2003; Class and Bilkei, 2004; Holyoake *et al.*, 2010b).

In Australia, PE is traditionally controlled in pig herds with in-feed antibiotic medication (Holyoake *et al.*, 2010b). Although antibiotics are effective in controlling PE outbreaks, care needs to be exercised in the use of antibiotics to avoid the emergence of bacterial resistance to antimicrobials. Over the last 2 decades the European Union (EU) has increasingly restricted the use of antimicrobial growth promoters in animal production. Currently, the United States is also expanding its program of restricting the use of antibiotics while in Australia specific recommendations for the appropriate use of antibiotic in food producing animals have been established (JETACAR, 1999; USDA, 2007; NARMS, 2010). Therefore, in order to prevent PE infection an alternative to antibiotics is required.

Such an alternative to *L. intracellularis* infection may be through vaccination of pig herds with a commercially available oral live vaccine (Enterisol® Ileitis, Boehringer Ingelheim). However, while this vaccine has been demonstrated to reduce clinical signs and lesions of PE (Kroll *et al.*, 2004; McOrist and Smits, 2007), the

systemic and local immune responses to a standard vaccine dose are poorly characterised (Donahoo, 2009). In the absence of proof that vaccinated pigs are protected from PE, some producers are unwilling to remove antibiotic medication for fear of acute haemorrhagic PE in adult pigs. Therefore, in the first part of this doctorate (**Chapters 4 and 5**) trials were designed with the objective being to measure the immune response of vaccinated and challenged pigs in an effort to identify immune markers for vaccination and protection.

Additionally, nutritional strategies have been suggested as alternatives to antibiotic growth promoters (Gallois *et al.*, 2009; Heo *et al.*, 2013; Pluske, 2013). The addition of β -glucan in pigs diets has been shown to improve growth performance (Dritz *et al.*, 1995; Decuypere *et al.*, 1998), increase functional activity of macrophages and neutrophils (Hiss and Sauerwein, 2003; Sonck *et al.*, 2010) and increase release of pro-inflammatory cytokines (Young *et al.*, 2001; Xiao *et al.*, 2004). Conversely, some components of pig diets such as the phytate-P can reduce growth performance. The addition of the enzyme phytase to pig diets can improve phytate-P digestibility, nutrient absorption and increase pig growth rates (Selle and Ravindran, 2008). However, the information on its effects on the immune response to vaccination is limited, but increases in serum lymphocyte numbers have been observed when phytase was added to nutritionally marginal broiler diets (Liu *et al.*, 2008). It is possible that the increased bioavailability of nutrients in the gut (with phytase) will allow additional nutrients to be redirected for immune cell growth and replication. Thus, this trial was designed to observe the effect of phytase and beta-glucan in weaner pigs diets on the immune response to *L. intracellularis* vaccination and growth performance (**Chapter 6**).

AIMS

The work presented in this thesis is from a series of animal studies intended to investigate the dynamics of immunity for *L. intracellularis* using a commercial vaccine and its effects of nutritional additives. The first objective was to determine the immunological responses of weaner pigs to a *L. intracellularis* vaccine at the local intestine mucosa and systemically in serum. The second objective was to investigate whether different routes and dose concentrations of a *L. intracellularis* vaccine provide equivalent protection to pigs against a virulent *L. intracellularis* challenge. Characterisation of an associated immune response that might predict successful induction of protection was also a part of this objective. In a following study the aim was to determine whether any immune response detected after different routes of vaccination are correlated with successful induction of protection (prior to challenge). A third study, investigated the effects of dietary yeast and phytase on the local and systemic immune responses of pigs after vaccination with *L. intracellularis*.

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Part 1- Proliferative enteropathy in pigs

2.1 *Background and Aetiology to proliferative enteropathy disease*

Twenty years after the association of porcine proliferative enteropathies with the presence of an intracellular bacterium within the ileal epithelial cells by electron microscopy (Rowland and Lawson, 1973), the causative agent was successfully cultured *in vitro* (Lawson *et al.*, 1993). This intracellular bacterium, previously known as *Campylobacter*-like organism, *Ileal symbiont intracellularis*, and *Ileobacter intracellularis* was then established in a new genus *Lawsonia* and species *intracellularis* (McOrist *et al.*, 1995a). The final classification was achieved using molecular taxonomic methods on the 16S rRNA gene, positioning the causal organism (*Lawsonia intracellularis*) with 81% similarities to *Desulfovibrionacea* family from the delta subdivision of Phylum Proteobacteria (Gebhart *et al.*, 1993). It has unique characteristics as a Gram-negative bacterium (around 1.5 µm in length and 0.34 µm in width), with curved or sigmoid rod shape (Lawson *et al.*, 1993), single and unipolar flagellum, but no fimbriae or spores (McOrist *et al.*, 1995a).

2.2 *Clinical aspects of the proliferative enteropathy disease*

Proliferative enteropathy has been reported in a number of animals, but clinical signs differ between them. Most species are affected with diarrhoea and poor growth. However, clinical signs such as fever, peripheral oedema and colic are present in affected horses (Pusterla and Gebhart, 2009), but not in pigs or other affected animals. While the clinical signs may slightly differ between horses and pigs, post-mortem examinations show similar thickening of sections of the small intestinal wall in both species (Vannucci *et al.*, 2012a).

In pigs, proliferative enteropathy (PE) can affect animals from post weaning until adult life. Clinical signs can range from acute to chronic and subclinical, presenting as various degrees of diarrhoea and reduced growth rates (Ward and Winkelman, 1990; Paradis *et al.*, 2012). The acute presentation of proliferative haemorrhagic enteropathy (PHE) affects mainly adult pigs between 4 and 12 months of age (Ward and Winkelman, 1990). Pregnant sows may abort following infection

and mortality can reach 50% in severe outbreaks (Love *et al.*, 1977; Mauch and Bilkei, 2005). This haemorrhagic syndrome can start with pigs becoming pale and having severe diarrhoea and malaena and if it is not treated may result in death (Love and Love, 1979). However, sudden death may also occur without symptomatic signs (Love and Love, 1979). Other pathologies during the finisher to adult phase can present similar signs of severe diarrhoea such as swine dysentery (*Brachyspira hyodysenteriae*) and Salmonellosis (*Salmonella Typhimurium*) (Straw *et al.*, 2006). In addition, non-pathogenic occurrences can lead to haemorrhagic diarrhoea such as in chronic intestinal bleedings (McOrist and Gebhart, 2006).

Porcine intestinal adenomatosis (PIA) commonly affects weaners and growers observed as persistent chronic diarrhoea (Ward and Winkelman, 1990), but can cause mortality (Rowland, 1975). PIA presents as reduced weight gains and non-uniformity in weight among 6 to 12 weeks old pigs (Lawson and Gebhart, 2000). Gogolewski *et al.*, (1991) described a marked ill-thrift (weight gain less than half of the weekly mean weight gain from non-infected pigs) and diarrhoea in grower pigs affected with PIA. PIA needs to be differentiated from other diseases that cause diarrhoea during the weaning and growing phase such as post-weaning diarrhoea (*Escherichia coli*), Spirochetal colitis (*Brachyspira pilosicoli*) and Salmonellosis (Jacobson, 2003).

Clinical signs of proliferative enteropathy have been reported to improve gradually after a week (Yates *et al.*, 1979), however, pigs can also progress to necrotic enteritis and regional ileitis which can lead to death (Ward and Winkelman, 1990). In necrotic enteritis the mucosa is destroyed resulting in extensive coagulative necrosis of the epithelium (Rowland, 1975), with yellowish-grey lesions on the mucosal surface (Rowland and Hutchings, 1978). The animals that survive this episode of necrotic enteritis may progress to regional ileitis (Rowland, 1975; Lawson and Gebhart, 2000). Regional ileitis is a progressive granulation tissue proliferation in the lamina propria and submucosa (Rowland, 1975; 1978).

The subclinical form of PE is considered to be the most common in pigs, but is difficult to recognize due to the absence of clinical signs. However, production parameters are negatively affected, such as reduced feed intake, lower rate of daily

live weight gain and poor feed conversion efficiency (FCE)(Collins *et al.*, 2010b; Paradis *et al.*, 2012). Poor growth in affected pigs results in an increased number of days to slaughter (Brandt *et al.*, 2010). The dose of *L. intracellularis* pigs are exposed to will impact on the severity of clinical signs, including weight gain and FCE. In a study by Paradis *et al.*, (2012), six different groups of pigs were challenged with 10 fold dilutions of mucosal homogenate *L. intracellularis* between 10^4 to 10^8 and their performance compared to uninfected controls. Excretion of *L. intracellularis* was detected in all pigs, starting from 14 days post-challenge. Consistently poor performance was observed in all challenged groups, even those given the lowest dose, 10^4 *L. intracellularis*, with a 37% reduction in average daily weight gain and a 27% increase in FCE during the 21 day trial period relative to non-challenged pigs (Paradis *et al.*, 2012). Similarly, Collins *et al.*, (2010b) reported a reduction in feed intake and large variation in final body weight of pigs that were subclinically infected (without diarrhoea and positive for *Lawsonia*-PCR and immunofluorescence antibody test (IFAT)) after an experimental challenge with 5.9×10^9 *L. intracellularis* compared with an uninfected cohort.

2.3 Epidemiology

2.3.1 Pig to pig transmission and risk factors

The principal mode of transmission of *Lawsonia intracellularis* is direct contact between affected and susceptible pigs (Jordan *et al.*, 2004) and through the faecal-oral route (Collins *et al.*, 2000). Transmission also occurs through contact with *L. intracellularis* contaminated environments (Collins *et al.*, 2013), but may also be transmitted by rodents (Collins *et al.*, 2011). Pigs clinically affected with PE can shed at least 3×10^8 *L. intracellularis* per gram of faeces (Collins *et al.*, 2011), so less than one gram of infected faeces is required to infect naive pigs (Collins *et al.*, 2001). The infection of a single pig within a group or pen is likely to result in the infection of susceptible pigs that are in contact, as sentinel pigs became infected 8 days after they were housed in contact with pigs inoculated with 10^5 of pure culture *L. intracellularis* (Jordan *et al.*, 2004). Similarly, in a natural PHE outbreak, *L. intracellularis* infection was transmitted between breeding stock to young adult pigs, where the movement of breeding stock between units was performed (Love *et al.*, 1977). *L. intracellularis*

infection was observed in naive pigs after they were introduced to dirty pens (Collins *et al.*, 2013) or dosed orally with 10g of faeces containing 10^6 to 10^7 *L. intracellularis* from naturally infected pigs (Collins *et al.*, 2000). In both of these studies it was observed that *L. intracellularis* survived in faeces in contaminated pig pens for at least 2 weeks at temperatures between 9°C and 18°C. Therefore, the combination of pigs shedding large numbers of bacteria in faeces, the prolonged survival of the bacteria in the environment, together with the small doses required to initiate an infection strongly favours the transmission of infection within the herd.

Farm management factors such as animal grouping, feed management, buying replacement stock, stocking density, age, and hygiene have been shown to influence the risk of *L. intracellularis* infection (Smith *et al.*, 1998; Bronsvort *et al.*, 2001; Stege *et al.*, 2001; Collins and Love, 2003). For instance, Collins and Love (2003) observed the risk of *L. intracellularis* infection was six times greater in a continuous flow management pig production system when compared to an all-in-all-out management program. Additionally, an all-in-all-out management system has often been shown to provide protective factors against other post-weaning intestinal pathogens infections (Madec *et al.*, 1998). Proper hygiene such as cleaning and disinfecting pens between pig groups has been shown to eliminate *L. intracellularis* and prevent transmission of infection to a second group of naive pigs introduced to cleaned pens (Collins *et al.*, 2013).

Smith *et al.* (1998) studied risk factors for PE using a postal survey of 319 British herds. Breeding herd size greater than 500 sows, concurrent enzootic pneumonia, replacement boars from selected nucleus herds and use of slatted floors above deep sunken pits were important factors associated with owner-reported PE on the farm during the three year survey. However, the authors' point out that possibly the farm owners relied only on slatted floors to clean pens. Another study, which included a questionnaire survey, production records and faecal PCR analysis, demonstrated that the use of new buildings and recent mixing of pigs were associated with PE outbreaks by grouping affected and susceptible pigs (Bane *et al.*, 2001). A cross sectional study in Danish pigs herds showed that the use of commercial feed products increased the risk of *L. intracellularis* infection when compared to batch production systems using home-mixed feed (Stege *et al.*, 2001).

Bronsvoort *et al.*, (2001) surveyed 184 herds in the United States by collecting serum from breeding sows and grower to finisher pigs and testing them for *L. intracellularis* antibodies (by IFAT) and linked the results with questionnaire data from respective farms. Risk factors associated with PE outbreaks in breeding herds included seropositivity to *L. intracellularis* during the grower-finisher phase (48.9% positive herds), a continuous flow system for the farrowing unit, and younger sow parity. On the other hand, risk factors for a PE outbreak in grower-finisher herds included seropositive status of the breeding unit (66.9% positive herds), a high number of pigs entering the facilities, the use of concrete slats as flooring, and intensive indoor management.

2.3.2 Mechanical and vector transmission

Others sources of infection, such as mechanical and biological vectors may also be of significant consequence for the re-introduction of *L. intracellularis* to new herds (Jensen *et al.*, 2005). The potential for *L. intracellularis* transmission via fomites like boots, overalls, brooms and shovels has not been reported. However, transmission of *L. intracellularis* infection has been reported despite preventive measures, such as separate clothing and disinfecting footbaths being used (Winkelman *et al.*, 1998; Jordan *et al.*, 2004).

Transmission of *L. intracellularis* infection is also likely via biological vectors where wild and domestic animals shedding *L. intracellularis* in their faeces could transmit the infection to naive pigs, if they come into contact with infected faeces. Lesions of PE have been reported in the intestines of hamsters (Jonas *et al.*, 1965), rabbits (Hotchkiss *et al.*, 1996), ferrets (Fox and Lawson, 1988), lambs (Cross *et al.*, 1973), horses (Duhamel and Wheeldon, 1982), guinea pigs (Muto *et al.*, 1983), deer (Cooper *et al.*, 1997), macaques (Klein *et al.*, 1999), dogs (Leblanc *et al.*, 1993), cows, giraffes and porcupines (Herbst *et al.*, 2003). However, it is more likely that animals, such as wild pigs, rodents, insects and birds, are possible vectors due to their close contact with pig farms. Highlighting the potential for external vectors, Tomanova *et al.*, (2002) described the detection of *L. intracellularis* in 29.6% of ileal tissues from wild pigs using nested PCR and 51.6% of these were seropositive for *L. intracellularis* in the Czech Republic. In Australia, *L. intracellularis* antibodies have been detected in feral pigs (91.5% of pigs tested were positive) within 10 km of two large scale

commercial piggeries in southern Queensland state (Pearson, 2012). On the other hand, Pusterla *et al.*, (2008) was not able to detect *L. intracellularis* infection in Brewer's blackbird (*Euphagus cyanocephalus*) found around livestock farms. Experimental infections with *L. intracellularis* in chickens (*Gallus gallus*) and sparrows (*Passer domesticus*) did not cause detectable histological lesions of PE (Collins *et al.*, 1999; Viott *et al.*, 2013). However, proliferative lesions have been detected in other avian species such as ostrich (*Struthio camelus*) and emu (*Dromaius novaehollandiae*) (Cooper *et al.*, 1997; Lemarchand *et al.*, 1997).

The presence of *L. intracellularis* in the intestines of rodent captured on PE positive farms implicates rodents as potential biological vectors (Friedman *et al.*, 2008). Collins *et al.*, (2011) also reported *L. intracellularis* excretion from 70.6% of rodents captured on PE positive pig farms, with up to 10^{10} organisms excreted per gram of rat faeces. However, although these studies could not prove if rats were the infective source for pigs or vice versa, it is clearly an important avenue for contamination, particularly where rodents are in close contact with pigs. Similarly, the potential mechanism of disease spread within herds through positive flies has been raised since the detection of *Lawsonia*-DNA from 22% to 75% of all adult flies collected from 14 seropositive pig farms in England (McOrist *et al.*, 2011). The *Musca domestica* (house fly) and *Eristalis* sp. (hoverfly or flower fly) were the most common positive species captured among farms (and in closest proximity to pigs). However, although the study did not determine the amount of viable DNA or the flight distance of these flies, it indicates the potential for spread of *L. intracellularis* contamination within herds.

2.3.3 Prevalence of PE

PE is an endemic disease that is widespread across every continent involved in pork production and occurs in many different production systems. Prevalence is determined either by the use of *L. intracellularis*-specific serology assays such as indirect immunofluorescent antibody test (IFAT), immunoperoxidase monolayer assay (IPMA) or by molecular assays such as the polymerase chain reaction (PCR) (Guedes *et al.*, 2002b; Jensen *et al.*, 2005).

Infection prevalence differs depending on the sample and assay used. For instance, the amplification of *L. intracellularis* DNA from faeces by PCR was demonstrated between 14 and 40 days after 10^7 *L. intracellularis* challenge (Collins and Love, 2007). While after the same challenge the detection of serum IgG antibodies against *L. intracellularis* were observed two weeks after (28 days) and persisted until 70 days post-challenge (Collins and Love, 2007). These assays may be used as a tool to estimate the timing of infection (Guedes, 2008). Molecular testing (PCR) has been reliably used to detect *L. intracellularis* DNA in actively infected pigs, while the *L. intracellularis* specific antibodies indicate previous exposure to infection (Jacobson *et al.*, 2004).

In Australia, a cross-sectional study of finisher pigs from 63 herds across all states determined that 100% of the herds tested were positive for *L. intracellularis* antibodies (Holyoake *et al.*, 2010b). Similarly, a longitudinal study of natural *L. intracellularis* infection in five large Danish grower pig herds also revealed that seroconversion had occurred in all herds, and that 75% of pigs examined by faecal PCR were actively infected (Stege *et al.*, 2004). Specific *L. intracellularis* serum antibodies were also found in 100% of the grower-finished herds tested in Korea (Lee *et al.*, 2001) and 90.9% of 174 pig farms in United States (Armbruster *et al.*, 2007). In a US study, faecal shedding of *L. intracellularis* occurred most commonly in grower and finisher pigs, with the reported prevalence of *L. intracellularis* infection ranging from 8 to 67% of pigs positive (Armbruster *et al.*, 2007).

The prevalence of lesions during slaughter from seropositive farms with subclinical infection has been reported as 1.5% (Brandt *et al.*, 2010). The low gross lesion prevalence at slaughter age (at 26 weeks of age) was likely due to lesion resolution, as severe histopathological changes were observed in five euthanized 8 weeks old pigs (Brandt *et al.*, 2010). In more severe outbreaks of PHE, thickening of the mucosa was observed in 72.8% of slaughter age pigs (van der Heijden *et al.*, 2004). Jensen *et al.*, (1999) recommend monitoring of slaughter pigs (110 kg) by visual and palpatory demonstration to identify pigs with increased thickening of the ileum. These techniques correlate with the presence of *L. intracellularis* (by IHC), but are not a reliable guide to the prevalence of PE in herds.

2.4 *Lawsonia intracellularis* entry and recovery

The presence of intracellular *L. intracellularis* bacteria within the apical cytoplasm of enterocytes is highly correlated with proliferation of cells as observed by electronic microscopy (Rowland, 1975; McOrist *et al.*, 1995b). The preferential localization of *L. intracellularis* in the ileum has been demonstrated by IHC (Boutrup *et al.*, 2010), but *L. intracellularis* can spread and colonise other sections of the intestinal tract such as the jejunum, colon and caecum (Smith and Lawson, 2001; Jensen *et al.*, 2006). Possible factors for the preferential colonisation of the ileum sections might include the presence of specific receptors, a favourable physiological environment for *L. intracellularis* or simple mechanical reasons such as longer exposure to the intestinal epithelium (Boutrup *et al.*, 2010). The ability of some Gram-negative intracellular bacteria, like *Yersinia enterocolitica* (Autenrieth and Firsching, 1996) and *Salmonella* spp. (Clark *et al.*, 1994) to exploit M cells (Microfold cells) to penetrate the host epithelium, and the presence of early lesions of PE in the mucosa overlying the Payer's patches (PP) (Lomax *et al.*, 1982) suggests that *L. intracellularis* could also utilise a similar strategy. Alternatively, *L. intracellularis* could enter crypt enterocytes directly by forming single membrane bound vacuoles (McOrist *et al.*, 2006a), as well as through loose membrane junctions (McOrist *et al.*, 1995b).

The identification of specific receptors or adhesins for *Lawsonia intracellularis* remains speculative, with proposed entry mechanisms inferred by extrapolation from other pathogens. McCluskey *et al.*, (2002), identified an outer membrane protein (OMP) *Lawsonia* surface antigen (LsaA) from cultured *L. intracellularis* and from bacteria present in ileal tissues of infected animals. They demonstrated that the *LsaA* gene was expressed in early infection and the protein was synthesized by *L. intracellularis* during infection (McCluskey *et al.*, 2002). In addition, the analysis of the genetic sequence of *L. intracellularis* identified some homologous regions to membrane factors of *Yersinia* sp. (Yop and LvrV) and these may indicate that the type III secretion system (T3SS) is present in *L. intracellularis* and is expressed during infection (Hueck, 1998; Alberdi *et al.*, 2009). This secretion system is common in enteropathogenic Gram negative bacteria, presenting as a needle-like protein structure that has important pathogenic role by forming pores in the host cell

membrane, as well as evading host's innate immunity by down-regulating inflammation (Autenrieth and Firsching, 1996).

Under normal conditions, enterocytes lining the intestine divide by mitosis and mature as they migrate from the crypt to the tip of the villus, where they are involved in absorption of nutrients as they mature (Friendship, 1989). It is thought that *L. intracellularis* preferentially adheres to the immature epithelial cells. However, the mechanism by which *L. intracellularis* inhibits normal crypt cell differentiation is not yet understood. During recovery from infection, the restoration of normal epithelium occurs by elimination of the infected enterocytes and multiplication of uninfected adjacent cells (McOrist *et al.*, 1996; MacIntyre *et al.*, 2003). Resolution of lesions starts approximately three to four weeks after infection (Smith and McOrist, 1997; Winkelman *et al.*, 2002) and is dependent on the initial infection dose. Higher average PE lesion length (171cm) 20 days after pigs were inoculated with 10^{10} *L. intracellularis* was observed in comparison with lower length (5cm) lesions from pigs inoculated with 10^8 *L. intracellularis* (5cm) (Guedes *et al.*, 2003). The presence of *L. intracellularis* antigen has been reported in the intestinal lumen following resolution of *L. intracellularis* induced lesions where infected cells have been lysed and shed (van der Heijden *et al.*, 2004).

2.5 Pathology of the porcine proliferative enteropathies

The thickening of the intestinal mucosa is the most noticeable macroscopic alteration of all forms of PE. Intestines affected with PHE may also have a solid clot of blood in the intestinal lumen (Figure 1a), as well as thickening of mucosa and oedema. In PIA cases, the macroscopic lesions can present as severe thickening of the mucosa ("cerebroid aspect") and relatively free from inflammation as shown in Figure 1b. By comparison, enteric bacteria such as *Escherichia coli* can affect small intestines and present severe areas of inflammation (Fairbrother and Gyles, 2012). In more severe cases of coagulative necrotic enteritis, clearly defined fibrin deposits will be present in the intestinal lumen (Love *et al.*, 1977). In mild cases, the changes may be subtly characterized by oedema associated with focal proliferative lesions.

1a)



1b)

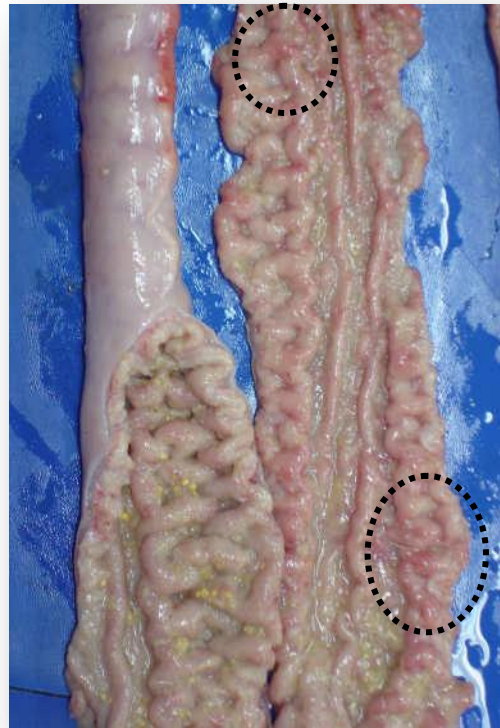


Figure 1: Porcine small intestine. (1a): Proliferative haemorrhagic enteropathy (PHE). Observe mucosa thickened and presence of blood in the lumen (arrow). (1b): Porcine intestinal adenomatosis (PIA), with marked thickening of intestinal mucosal with small reddening area (circle). (Photographs courtesy of Prof. Dr. Roberto M. C. Guedes, Department of Veterinary Clinic and Surgery, Minas Gerais Federal University, Belo Horizonte, Brazil).

Lesions of PE at the ultra-structural level, show the presence of vibrio-shaped intracellular bacteria in the apical cytoplasm of enterocytes, which can be visualised with Warthin-Starry silver staining, monoclonal antibodies to *L. intracellularis* or electron microscopy (Rowland, 1975; McOrist *et al.*, 2006a). The villus-crypt structures are elongated with proliferation of enterocytes (Figure 2), marked reduction or loss of goblet cells and/or presence of exudate in the crypt lumen (Lawson *et al.*, 1993; Lawson and Gebhart, 2000). Compared with normal crypts, which are a single layer of cells, affected crypts are often 5, 10 or more cells deep (McOrist *et al.*, 2006a). Electron microscopic studies of experimentally and naturally infected pigs (Rowland, 1975; Love *et al.*, 1977) have shown that highly infected enterocytes usually have short, irregular microvilli compared with healthy animals.

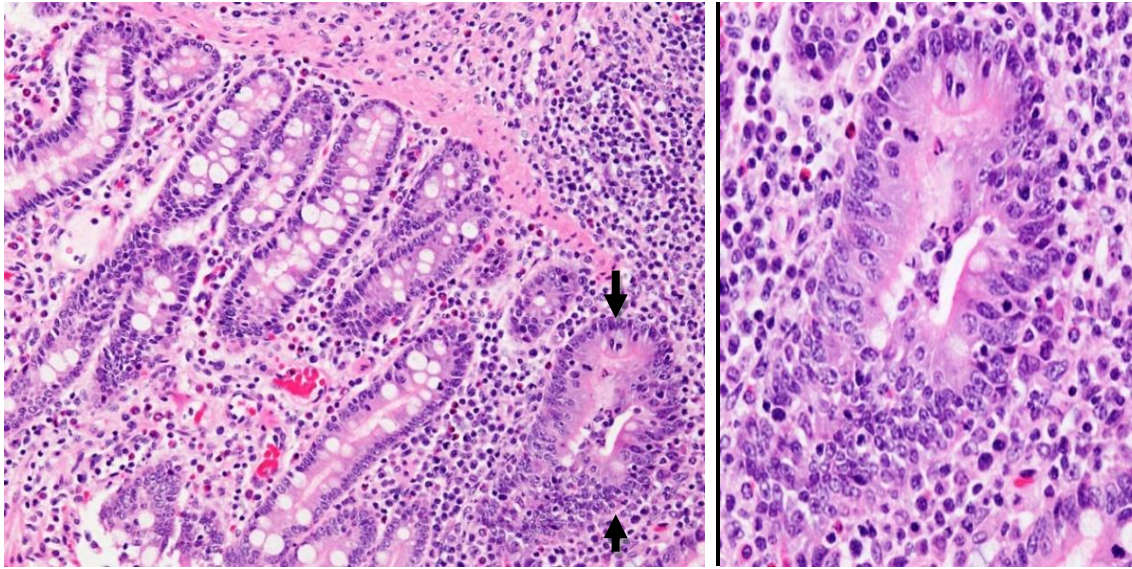


Figure 2: Ileal section of a pig experimentally infected with *L. intracellularis*: H&E staining, left figure (10 x magnifications) showing normal tissue with areas of characteristic PE lesions of crypt enterocyte proliferation (arrows). Right figure the proliferation of enterocytes with loss of goblet cells and exudate within crypt lumen (100 x mags.).

2.6 Diagnostic test for *Lawsonia intracellularis* infection

Diagnosis of PE by routine culture from faeces is not feasible because *L. intracellularis* is an obligate intracellular bacterium that is fastidious in its culture requirements (Lawson *et al.*, 1993). The non-specific nature of clinical signs of PE makes a differential diagnosis difficult. Other enteric infections such as *Brachyspira hyodysenteriae*, *Brachyspira pilosicoli*, haemolytic *E. coli* and *Salmonella* spp. may also cause diarrhoea and reduced weight gains in growing pigs (Jacobson, 2003).

Diagnosis of PE is based on a combination of gross lesions and histopathology lesions associated with more specific immunohistochemistry. Although severe lesions in the terminal jejunum and ileum are easily seen, the more common moderate to mild lesions may be harder to detect (Guedes *et al.*, 2002c). The presence of proliferation of enterocytes on routine haematoxylin and eosin (H&E) staining is a good indication of PE infection. However, in recovering pigs PE lesions may not be present. Immunohistochemistry of intestinal tissues from the ileum using an antibody specific for *L. intracellularis* antigen allows the visualization of the bacteria within intestinal crypts early in the infection and in the lamina propria late in the course of disease (Guedes and Gebhart, 2003a). However, the monitoring of PE

prevalence in herds through post-mortem tests of slaughter age pigs have the tendency to be underestimated because most lesions in the infected pigs have recovered by slaughter time (van der Heijden *et al.*, 2004; Brandt *et al.*, 2010). Therefore, an ante-mortem diagnostic test, such as serology and PCR, specific for *L. intracellularis* is beneficial for diagnosing *L. intracellularis* infection in pig herds.

An indirect fluorescent antibody test (IFAT) using *L. intracellularis* antigen from PE affected mucosa or cultured bacteria has been used to detect IgM, IgA and IgG responses in serum and mucosa of naturally and experimentally infected pigs (Lawson *et al.*, 1988; Guedes and Gebhart, 2003b; Donahoo, 2009). A modified IFAT using cultured *L. intracellularis* and peroxidase labelled antibody (immunoperoxidase monolayer assay, IPMA), was used to detect *L. intracellularis* specific serum IgG in field and experimentally challenged pigs (Guedes *et al.*, 2002b; Marsteller *et al.*, 2003). However, for larger numbers of diagnostic samples a high throughput diagnostic technique is practical. Epidemiology studies have employed a commercial ELISA (Bioscreen® Enterisol Ileitis ELISA, GmbH, Münster, Germany) to determine the prevalence of *L. intracellularis* antibodies (Holyoake *et al.*, 2010b; Jacobson *et al.*, 2011b; Collins *et al.*, 2012). This blocking ELISA detects serum IgG antibodies to *L. intracellularis* (Keller *et al.*, 2006). To ensure high specificity, the blocking ELISA is a direct sandwich ELISA with *L. intracellularis* specific monoclonal antibodies used to bind *L. intracellularis* to wells. Specific antibodies in pig serum are captured with a peroxidase-conjugated *L. intracellularis* monoclonal antibody (Keller *et al.*, 2006). This commercial ELISA is reported to have a sensitivity of 90.5% and specificity of 83% relative to an indirect fluorescent antibody test (IFAT) in pigs experimentally challenged with *L. intracellularis* (Collins *et al.*, 2012).

The polymerase chain reaction (PCR) assay is a diagnostic test to detect the presence or absence of *L. intracellularis* DNA in faeces and tissues. Several studies using PCR assays have been used to monitor the dynamics of disease in both experimental and naturally infected pigs (Jones *et al.*, 1993; Knittel *et al.*, 1998). The lowest quantity of *L. intracellularis* in faeces that conventional PCR has been reported to detect is 10^3 *L. intracellularis* per gram of faeces (Jones *et al.*, 1993). The diagnostic sensitivity has been reported to be highly variable (between 36 and 100%) (Pedersen *et al.*, 2010). This may be due to sample quality and the presence of inhibitory factors

in faeces (Nathues and Beilage, 2008). However, this test is unable to quantify the numbers of *L. intracellularis* in faecal samples. More recently real-time PCR (qPCR) methods have been developed that allow quantification of *L. intracellularis* relative to faecal standards seeded with known numbers of *L. intracellularis* (Lindecrona *et al.*, 2002; Nathues *et al.*, 2009; Collins *et al.*, 2011). In a previous study, Collins *et al.* (2011) used qPCR to detected between 10^4 and 10^8 *L. intracellularis* per gram of pig faeces on farms. The correlation between quantification of bacterial load and indication of clinical disease and histological findings have been studied by Pedersen *et al.*, (2012). They demonstrated a positive correlation between histopathology and *L. intracellularis* numbers in faeces of pigs with diarrhoea.

2.7 *Lawsonia intracellularis* dynamics of infection

2.7.1 Infective doses

The dynamics and severity of *L. intracellularis* infection are closely related to the initial challenge dose (Guedes *et al.*, 2003; Collins and Love, 2007). The minimal infective dose was evident in a study by Collins *et al.*, (2001), where groups of weaner pigs were challenged with either 10^3 , 10^5 , 10^7 , 10^{10} *L. intracellularis* and compared with a negative control group. Doses as low as 10^3 *L. intracellularis* induced faecal shedding of the bacteria from day 26 until day 54 post-challenge (by PCR), even though the serum *L. intracellularis* IgG response (IFAT) was delayed up to 56 days after challenge. Similarly, pigs inoculated with 10^5 *L. intracellularis* by experimental challenge (Collins and Love, 2007) or orally vaccinated (Guedes and Gebhart, 2003b) also showed delayed bacterial faecal shedding (from 19 to 63 days) and delayed serological responses (from 35 until 91 days) when compared with pigs inoculated with higher doses of *L. intracellularis* (10^{10} and 10^9 , respectively).

The earliest serological response to *L. intracellularis* was 14 days post challenge and faecal shedding after one week in pigs inoculated with higher doses of 10^9 and 10^{10} *L. intracellularis* (Collins *et al.*, 2001; Riber *et al.*, 2011b). Higher doses (10^9) of *L. intracellularis* from intestinal homogenates (Guedes *et al.*, 2003; Collins *et al.*, 2007) and pure culture (Vannucci *et al.*, 2012b) also induce clinical signs of PE including diarrhoea and reduced weight gains from 14 to 21 days post-challenge. In moderate

challenges with 10^7 *L. intracellularis*, clinical signs of diarrhoea were still observed, but with less severe diarrhoea over a shorter duration (day 19-21) (Collins and Love, 2007). Faecal shedding of *L. intracellularis* was observed between day 14 and day 40, and antibodies between day 28 and day 70 after challenge (Collins *et al.*, 2001; Collins and Love, 2007). Four pigs challenged with a pure culture of *L. intracellularis* containing 10^6 organisms remained clinically healthy throughout the trial but two pigs had gross PE lesions and all four pigs had IHC evidence of *L. intracellularis* antigen 22 days after challenge (McOrist *et al.*, 1993).

2.7.2 Immunity to re-infection

A number of studies have demonstrated that once pigs recover from infection, they are immune to re-infection with *L. intracellularis* (Collins and Love, 2007; Riber *et al.*, 2011b; Cordes *et al.*, 2012). In one trial, by Collins and Love (2007), groups of pigs challenged with 10^5 to 10^{10} *L. intracellularis* were monitored for 70 days to determine when faecal shedding ceased. At 70 days post primary challenge, all groups were given a second challenge with 10^{10} *L. intracellularis*. All previously challenged pigs were protected from re-colonization regardless of the initial dose of *L. intracellularis*. On the other hand, naive controls exhibited persistent faecal shedding of *L. intracellularis* (from 7 to 23 days), diarrhoea and a serum IgG response (Collins and Love, 2007). The replication of B and T cells, the production of antibodies against *L. intracellularis* after first exposure, and the generation of memory cells have been speculated as the mechanism to inactivate the antigen prior to entry and avoid colonization after a second challenge (Collins and Love, 2007; Cordes *et al.*, 2012). Accumulation of secretory IgA has been demonstrated (by IHC) in the apical cytoplasm of proliferating enterocytes, macrophage-granulocytes and in cell debris in the crypt lumen of PE-affected pigs (McOrist *et al.*, 1992). An increasing *L. intracellularis* IgG serum immune response were also detected in re-challenged pigs (Collins and Love, 2007). In a later re-challenge study, Riber *et al.*, (2011b) initially challenged pigs with 10^9 *L. intracellularis* from PE-affected mucosae followed by antibiotic (tiamulin) treatment. Seven weeks post-challenge, pigs were re-challenged with 10^{10} *Lawsonia intracellularis*. Pigs were protected from re-infection as demonstrated by the absence of faecal shedding of *L. intracellularis* and low concentrations of antigen in intestinal mucosa of re-inoculated pigs compared with

infection infected controls. In addition, no increases in acute phase protein (C-reactive protein and haptoglobin) concentrations were observed. In a similar study, whole blood IFN- γ concentrations increased ten-fold in re-challenged pigs, indicating a memory recall immune response to *L. intracellularis* (Cordes *et al.*, 2012). Therefore, the protection against PE disease re-exposure provides the basis for vaccine strategies formulation utilized to control PE disease.

Part 2- Porcine immunological responses to infection

2.8 Innate and adaptive immunity to enteric pathogens in pigs

Within the intestinal tract, the mucosal layer consists of secretory and absorptive epithelium and is the primary physical barrier against external antigens. Innate immunity is constitutive of an array of lectins, C-reactive proteins, β -defensins, macroglobulins and complement system (Sauerwein *et al.*, 2007). It is the first differentiation between self from non- self products by recognition of conserved microbial structures (pathogen associated molecular patterns, PAMPs)(Kumar *et al.*, 2009). The main leucocytes involved in this process are granulocytes (e.g. neutrophils), phagocytes (monocytes and macrophages) and natural killer cells (NK). These cells and also enterocytes possess membrane receptors called Toll-like receptors (TLRs) to recognize PAMPs on infectious agents, such as the outer membrane lipopolysaccharide (LPS) molecules in Gram-negative bacteria and β -glucan from fungi and yeast (Abreu and Targan, 1996). Specifically, TLR subsets have been reported to recognise extracellular bacterial components (TLR 1, 2, 4, 5, 6 and 10), bacterial flagellin (TLR 5) and intracellular components (TLR 3, 7, 8) in pigs (Uenishi and Shinkai, 2009; Emery and Collins, 2011). The role of TLRs in immunity has been elucidated by identifying functional single-nucleotide polymorphisms (SNPs), TLRs and immunity against specific pathogens. For instance, Toka *et al.*, (2009) demonstrated *in vitro* that TLR7 was associated with increased IFN- γ production and activation of porcine NK cells against foot and mouth disease virus.

The consequence of binding TLRs to epithelial cells and phagocytosis of antigens results in the secretion of a range of inflammatory cytokines (IFN- γ , IL-6, TNF- α , IL-12) by T and B lymphocytes (Bailey, 2009; Abreu, 2010). For instance, increasing movements of macrophages (by MIF), neutrophils (by IL-8) and dendritic cells (CCL20) were expressed in ileal sections 3 h after pigs were orally challenge with pathogenic *Salmonella* sp (Skjolaas *et al.*, 2006). These pro-inflammatory cytokines also stimulate the liver to produce acute-phase proteins, increase the release of amino acids from muscle tissue, and may induce fever and loss of appetite (Murtaugh *et al.*, 2009). Eventually, migration of macrophages and dendritic cells (antigen presenting cells, APC's) into the lymphoid tissues (e.g. Payers patches and lymph nodes) occurs with consequent antigen presentation to T and B lymphocytes and activation of adaptive immunity.

Innate immunity is antigenic nonspecific and produces a short lived response. On the other hand, while the adaptive immunity takes longer to be induced (5 to 7 days) it provides a more effective and antigenic specific response (Rothkötter *et al.*, 1999). The classical anamnestic response to adaptive immunity is by developing antigenic memory and protection (and boost immunity) against re-exposure to the same pathogen (Figure 3). The adaptive immune response enables the production of antibodies against specific antigens by B lymphocytes (humoral immune response) and the generation of cytotoxic and helper T lymphocytes in infected cells (cell-mediated immune response) (Ogra *et al.*, 2005; Abbas *et al.*, 2010).

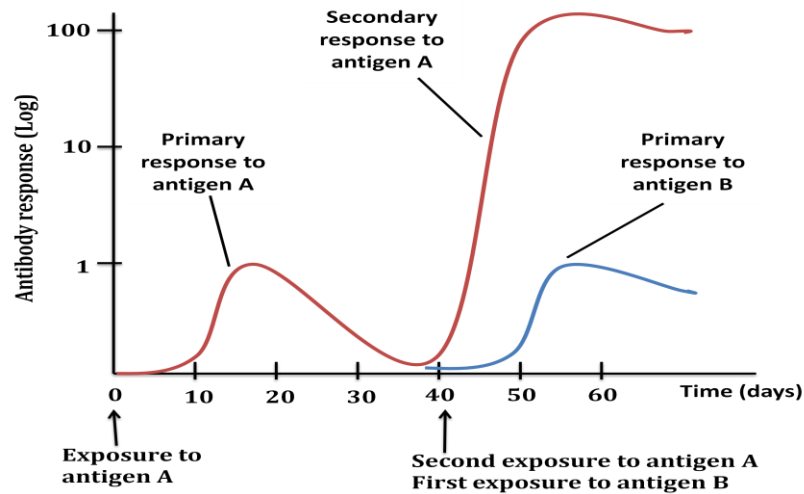


Figure 3: Primary and secondary antibody responses induced by a first and second vaccine exposure. Note that the secondary response is faster and greater than the primary response and it is specific for antigen A.

The presence of large clusters of immune cells (B and T lymphocytes), as well as dendritic cells and macrophages, along the intestinal epithelium and lamina propria (LP) have been described in pigs (Stokes *et al.*, 1994; Burkey *et al.*, 2009). In naive animals, the specific immune response will initiate once antigen presenting cells (APC's) bind and present antigens to naive or sensitised T cells by expressing the major histocompatibility complex (MHC) in organised lymphoid tissues (Stokes *et al.*, 1994; Ogra *et al.*, 2005). APC's and T cells in the mucosa can migrate to the mesenteric lymph nodes (MLN's) via the afferent lymphatic circulation, where clonal expansion will occur and memory antigen-specific cells can be generated. In other words, upon encountering with specific antigen, B cells are stimulated (by T cells) to divide into plasma and memory cells (Moser and Leo, 2010). Sensitised cells then re-circulate in the body via blood and fluids reaching the infection site. The pathogen is then attacked by phagocytosis or cytotoxicity and apoptosis through local production of cytokines (T cells) or antibodies (B cells) (Bailey, 2009; Moser and Leo, 2010).

2.8.1 Antibody and cellular mediated immunity (CMI)

Antibodies (immunoglobulin) are basically Y shaped proteins, produced by B cells, to recognize and eliminate a given antigen. The antibody class (e.g. IgM, IgG, IgD) is important to determining the capacity of a given antibody to reach the site of infection and recruit the adequate effector mechanism (Janeway *et al.*, 2001). The most direct way in which antibodies can protect from pathogens or toxic products is

by binding to them and thereby blocking their attachment to the epithelium and avoiding antigen colonization (Stokes *et al.*, 1994). The distribution of different antibodies classes within ileal section has been observed in an earlier study where IgA (42%) and IgM (7.5%) and occasional IgG (2.5%) staining (IHC) was detected in ileal crypt and lamina propria of adult pigs (Butler *et al.*, 1981). After initial exposure the antigen-antibodies, such as IgM and IgG are observed locally and systemically. For instance, *Taenia solium* specific IgM response on intestinal mucus was observed by Western blotting one week after pigs were infected with 10.000 *Taenia* eggs (Tsang *et al.*, 1991). Following IgM disappearance, IgG increases were observed after 3 weeks (Tsang *et al.*, 1991). Similarly, anti- *E. coli* IgM antibody response in serum increased and declined rapidly in the first 2 weeks after weaner pigs were challenged, following by gradual increase in serum concentrations of IgG antibodies (Porter and Hill, 1970). IgM possess high avidity and is effective in activating the complement system (C3b)(Wellek *et al.*, 1976). IgG antibody has a more amplified function such as binding to antigen via agglutination and also opsonising pathogens, as well as activating the complement system (Wellek *et al.*, 1976). In addition, IgA presence within fluids and membrane mucosa in adult pigs have been described (Lamm *et al.*, 1996) and are attributed to disease protection (Jemmott and McClelland, 1989). For instance, the use of a dendrimeric peptide suspension protected pigs against challenge with foot-and-mouth disease virus and induced high titres of specific virus neutralizing antibodies and IgA response locally and systemically compared with non-infected controls pigs (Cubillos *et al.*, 2008).

The generation of cytotoxic and helper T lymphocytes in infected cells, includes a series of mutual complex interaction to control infection as summarized in Figure 4. CD4⁺ T cells subpopulation stimulate CD8⁺ cytotoxic T cells or macrophage to kill cells through intracellular pathogens (Th1 pathway). The Th2 pathway is essential to control immune activation, prevent auto-immune destruction and activate allergic responses to parasites (Charerntantanakul and Roth, 2006; Moser and Leo, 2010). Th1 and Th2 pathways are mostly mediated by the secretion of cytokines. CD4⁺ Th1 cells produce mainly IL-2, IFN- γ and TNF- α promoting cell-mediated immune responses against intracellular pathogens, while the CD4⁺ Th2 subset produces IL-4, IL-5, IL-6 and IL-10 cytokines activate the antibody specific production (IgE) by B

cells to aid in the clearance of extracellular bacteria and parasites (Ogra *et al.*, 2005; Emery and Collins, 2011). The IL-2, IFN- γ , and TNF- α and also IL-1 β mediate the initial inflammatory response to the acute phase proteins, increases blood flow and vascular permeability (Burke-Gaffey and Keenan, 1993). The expression of mRNA encoding for IFN- γ and TNF- α in lung tissues have been demonstrated one day after pigs were experimentally infected with porcine respiratory reproductive syndrome (PRRS) virus (Shi *et al.*, 2010). On the other hand, up-regulation of Th2-type genes (CCR3, ARG1, MUC5AC, IL-4, IL-5, IL-13) in colonic sections was observed in pigs 5 weeks after *Trichuris suis* nematode infection (Kringel *et al.*, 2006). However, interactions are more complex than this, as for instance, in porcine B cells cultures, the secretion of IgG isotypes (IgG1 and IgG2) in presence of Th1 and Th2 type cytokines, confirmed that IL-10 promotes IgG1 and IFN- γ promotes IgG2 (Crawley *et al.*, 2003).

Transforming growth factor- β (TGF- β) controls the initiation and resolution of inflammatory responses through the regulation of lymphocyte activation, promoting T cell survival and inducing IgA class switching in B cells (Schluesener *et al.*, 1990). For instance, mice injected with anti-TGF- β 1 in spleen and liver became susceptible to listeriosis, whereas the administration of human TGF- β 1 enhanced their resistance, even though level of IFN- γ , TNF- α and IL-6 were reduced in these animals (Nakane *et al.*, 1996). Therefore, together with immunoregulatory T cells (T-reg) and Th-17 cells, TGF- β is involved in the tolerance and regulation in the gut (Moser and Leo, 2010). T-reg are CD4⁺T cells that secrete predominantly TGF- β and IL-10 (Fontenot and Rudensky, 2005) and contribute to dampening macrophage function and maintaining homeostasis (Bailey, 2009). T-reg also express the transcription factor FoxP3 and the surface receptor CD25 (the IL-2 receptor α -chain), through involvement with NK, T and B cells, control the immune response against self or exogenous antigen (Fontenot and Rudensky, 2005). Meanwhile, Th17 cells are related to $\gamma\delta$ -T cells and produce IL-17 and IL-22 which influence neutrophilia, tissue remodelling and repair (Emery and Collins, 2011). CD4⁺ effector T cells and Th17 cells have been found in mucosa surfaces and act against bacterial and fungal infections by secreting IL-17 and expressing CCR6 and IL-23R signalling receptors (Weaver *et al.*, 2007).

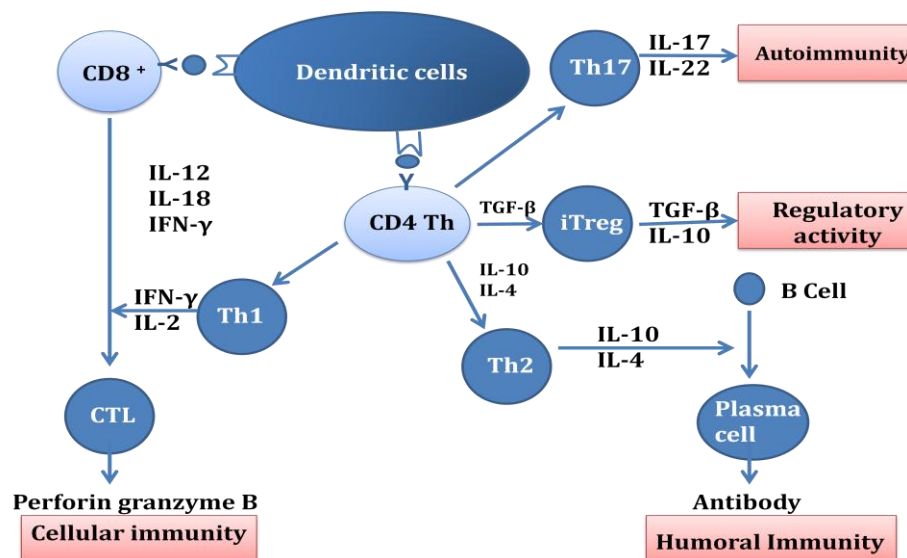


Figure 4: The role of T helper (Th) cells in immunity. CD4⁺ Th cells play a multiple role as: Th1 cells by secreting IL-2 and IFN- γ cytokine, Th2 by secreting IL-4 and IL-10 cytokines. As well as stimulating a regulatory activity by releasing of TGF- β , IL-17 cytokines.

2.9 Immune responses to *Lawsonia intracellularis* vaccination and virulent challenge

The *L. intracellularis* immune response is most probably initiated after the pathogen is captured by dendritic cells and macrophages and presented to T-lymphocytes in lymphoid tissues. Consequently, dendritic cells and monocytes recruitment leads to activation of CD4⁺ Th1 cells and later production of secretory IgA (Abreu, 2010). Humoral and cellular immune responses in pigs naturally or experimentally infected with *L. intracellularis* have been measured by immunoassays and gene expression studies (Collins *et al.*, 2001; Guedes and Gebhart, 2003b; Collins and Love, 2007; Jacobson *et al.*, 2011a; Riber *et al.*, 2011a; Cordes *et al.*, 2012). In an initial study using serological immunofluorescence tests with a crude filtered *Campylobacter*-like antigen suspension, it was demonstrated that growing pigs with naturally severe PHE lesions had predominantly IgM responses, which persisted for 8 weeks, with less marked IgG and IgA responses (Lawson *et al.*, 1988). Similarly, Holyoake *et al.*, (1994), struggled to detect significant differences in IgG titres between uninfected and challenged pigs (mucosal homogenate from PHE-affected pigs) using an IgG ELISA test with whole *L. intracellularis* as the antigen. This work was additionally hampered by the difficulty in identifying naive pigs for negative

control sera. A subsequent study was able to improve the sensitivity and specificity of IgG detection, employing an immunofluorescent (IFAT) test based on *L. intracellularis* co-cultured in IEC-18 mammalian cells (Knittel *et al.*, 1998). The IFAT was able to detect serum specific IgG antibodies in 90% of pigs 3 weeks after virulent challenge with cultured *L. intracellularis* (Knittel *et al.*, 1998). More recently, the reliability of a commercial ELISA to detect *Lawsonia intracellularis* IgG antibodies in pigs serum with subclinical PE after experimental challenge (10^9) has been also described during the 35 days trial (Collins *et al.*, 2012). Similarly, *L. intracellularis* specific IgG antibodies have been detected (1:25 titre) in ileal mucosa 21 days after piglets were vaccinated with attenuated *L. intracellularis* suspension (Donahoo, 2009).

Secretory IgA is promoted as an important defence mechanism against enteropathogenic bacteria by blocking antigen adhesion to the mucosal epithelium (Lawson *et al.*, 1988; Jemmott and McClelland, 1989; Husband *et al.*, 1996). Accumulations of IgA have been demonstrated (by IHC) in the apical cytoplasm of proliferating enterocytes in the crypt lumen and Peyer's patches from pigs with clinical cases of PIA and PHE (Lawson *et al.*, 1979; McOrist *et al.*, 1992). However, minimal concentrations (1:4 titre) of *L. intracellularis* specific IgA (by IPMA) were detected in intestinal lavage 22 days after 10^8 cultured *L. intracellularis* were given orally to weaner pigs (Guedes and Gebhart, 2010). Similarly, total faecal IgA was also minimal (<200ng/mg of faeces) in 10 week old pigs after primary (10^9) and re-challenge (10^{10} *L. intracellularis*) inoculation (Cordes *et al.*, 2012). Additionally, the *L. intracellularis* specific IgA in serum increased slowly with peak 4 weeks (100 OD%) after primary challenge, being undetectable after 7 weeks (<5 OD%) (Cordes *et al.*, 2012). However, significant increase in *L. intracellularis* specific IgA in serum of pigs following 10^{10} *L. intracellularis* re-inoculation was not detectable (Cordes *et al.*, 2012).

Early lesions of PE contain few infiltrating inflammatory cells, indicating the initial epithelial cell nature of the infection and the lack of an inflammatory stimulus (McOrist *et al.*, 1992). The lack of inflammation associated with non-haemorrhagic PE in pigs is possibly due to the limited numbers of CD8⁺CD25⁺ T lymphocytes observed within intraepithelial lymphocytes cells (IELs) and the lamina propria (McOrist *et al.*, 1992; MacIntyre *et al.*, 2003). Descriptive immunocytological studies of intestinal

tissues sections of pigs clinically affected with PE reveal a mild infiltration of cytotoxic CD8⁺ T cells, macrophages and B lymphocyte carrying MHC class II structure 14 days after 10⁸ *L. intracellularis* challenge (MacIntyre *et al.*, 2003). However, moderate infiltrations of CD8⁺CD25⁺T lymphocytes have been observed in pigs suffering from severe PHE (McOrist *et al.*, 1987), suggesting that more severe cases can induce infiltration of cytotoxic cells. However, the expansion of CD8⁺ and CD4⁺ cell populations (reduced ratio of non specific CD4:CD8 T cells) have been observed in ileal samples 3 to 4 weeks after experimental *L. intracellularis* challenge (10⁹) (Cordes *et al.*, 2012). In addition, high concentrations of IFN- γ were associated with increased numbers of *L. intracellularis* specific IFN- γ producing CD8⁺ cells in the serum of PE infected pigs (Cordes *et al.*, 2012).

A significant role for IFN- γ in immunity against PE disease has been implicated in mouse and pig challenge models (Smith *et al.*, 2000; Guedes and Gebhart, 2003b). Mice without IFN- γ receptor (IFN- γ R⁻) were substantially more susceptible to PE disease outbreaks and more extensive lesions as observed by immunohistochemistry compared with wild type mice (IFN- γ R⁺) after oral infection with 10⁷ cultured *L. intracellularis* (Smith *et al.*, 2000). The generation of antigen-sensitised (CD4⁺ and CD8⁺) lymphocytes and the production of *L. intracellularis* specific IFN- γ following incubation of *L. intracellularis* antigen with peripheral blood mononuclear cells (PBMC) has been reported in pigs within 14 days of challenge with 10⁹ virulent *L. intracellularis* in 12 weeks old pigs (Guedes and Gebhart, 2010; Cordes *et al.*, 2012). Guedes *et al.*, (2003b) also detected *L. intracellularis* specific IFN- γ producing cells by ELISPOT, in porcine PBMC until 13 weeks post-vaccination with 10⁵ *L. intracellularis*. In horses, IFN- γ gene expression in all vaccinated foals was significantly higher after 60 days following oral vaccine administration compared to IFN- γ gene expression in control unvaccinated foals (Pusterla *et al.*, 2012a). *L. intracellularis* as intracellular organism stimulate IFN- γ , leading to the clearance of infection or imputing disease severity (Cruz *et al.*, 2006). However, cytokine gene expression was limited and did not correlate with clinical signs, lesions or antibody response in naturally infected PHE and PIA pigs (Jacobson *et al.*, 2011a). But the gene encoding for insulin-like growth factor binding protein 3 (IGFBP-3) was up-regulated in two pigs with

prominent mucosal proliferation (PIA and PHE) and also in *L. intracellularis* infected McCoy cells (Oh *et al.*, 2010).

Delayed-type hypersensitivity (DTH) responses can be induced by a number of intracellular bacteria, such as *Brucella abortus*, *Listeria monocytogenes* and *Mycobacterium bovis* (Aboureyeh *et al.*, 1992; Bercovich, 2000). The mechanism by which DTH responses are observed is due to secondary exposure to the pathogen and activation of antigen-specific Th1 T cells to secrete especially IL-2 and IFN- γ that will mediate the hypersensitivity reaction (Aboureyeh *et al.*, 1992). In a *L. intracellularis* trial by Guedes *et al.* (2010), DTH were detected 20 days after pigs experimentally infected with 10^8 cultured *L. intracellularis*. The DTH reactions were observed in a dose dependent manner 24 hours after intradermal injections with 10^7 to 10^9 *L. intracellularis* treated by formalin fixation, sonication or extracts of outer membrane proteins (Guedes and Gebhart, 2010). In other diseases, such as *Brucella abortus*, responses are typically within 48 to 72 hours in sensitized hosts no matter the dose used (Bercovich, 2000).

Part 3- Principles of mucosal vaccination

2.10 Introduction to porcine mucosal vaccination

The activation, replication and differentiation of T and B cells lymphocytes leading to the generation of memory cells which respond and provide protection against specific pathogens are desirable for successful immunization (Meeusen *et al.*, 2004). Mucosal immunity refers literally to the immunity induced at the mucus-covered epithelial surfaces typically present in the gastrointestinal, upper respiratory and lower reproductive tracts (McGhee and Kiyono, 1994; Meeusen *et al.*, 2004; Sedgmen *et al.*, 2004). Additionally, identifying an immune marker that correlates to the protective mechanism to be induced by mucosal vaccines is ideal to facilitate vaccine design (Emery and Collins, 2011). Therefore, for the specific formulation of a vaccine, it is fundamental the information about the pathogen, such as how it infects the cells, and how the immune system responds to it, as well as practical considerations (e.g. costs of production and delivery). On the other hand, the maternal antibodies may also need to be considered, since their interference has

been related to vaccination failure (Hodgins *et al.*, 2004). Additionally, factors to be considered in vaccine formulation include the administration route (parenteral vs. oral), antigen dosage and formulation (killed vs. live), the presence of adjuvants (saline vs. oil) (McGhee and Kiyono, 1994; Sedgmen *et al.*, 2004). To induce protective responses against (extracellular) tetanus toxin, high titres of systemic antibody are required (Tregoning *et al.*, 2005), while for mycobacterial infections such as tuberculosis, macrophage activated cell-mediated immunity and for influenza viruses, cytotoxic T cell responses and IFN- γ are important (Brodin *et al.*, 2004).

The success of live vaccines has been attributed to a mimic of natural infection without causing “severe” disease. Live vaccines are normally produced by attenuating an agent from field disease outbreaks by continuous sub-cultivation or chemical/radiation attenuation (Meeusen *et al.*, 2004; Sedgmen *et al.*, 2004). This produces an ability of the antigen to replicate and produce subclinical disease (depending dose) generating immune responses similar to the natural infection. Examples of available live vaccines for pigs include the porcine reproductive and respiratory syndrome (PRRS) vaccine (Porcilis® PRRS, MSD Animal Health) and *Salmonella Choleraesuis* vaccine (Enterisol® SC-54, Boehringer Ingelheim). These vaccines have been demonstrated to elicit a strong and rapid antibody and cellular immune responses (dominated by CD8⁺ T cell) resulting in lifelong immunity (at least 3 months) with only one dose (Husa *et al.*, 2009; Martelli *et al.*, 2009). However, if an attenuated live vaccine contains insufficient live organisms or replication is compromised (e.g. temperature of refrigeration), it may not provide sufficient stimulus to the immune system to induce a measurable response or immunity (Meeusen *et al.*, 2007).

Inactivated vaccines can contain antigen specific epitopes, fractions of protein, toxoids, subunits, DNA or whole viruses or bacteria (Meeusen *et al.*, 2004). The immune response to an inactivated vaccines is mostly humoral (Watson, 1987), except when they are added with specific adjuvants (e.g. Complete Freund's adjuvant/CFA) (Holmgren *et al.*, 2003). One of the benefits of using inactivated vaccines is the ability to amplify the responses against specific pathogens strains by combining different pathogens or different virulent factors in one unique suspension. For instance, the *Actinobacillus pleuropneumoniae* (App) vaccine (Porcilis® APP, MSD

Animal Health) is based on the outer membrane protein (OMP) with an additional three toxoids ApxI, ApxII and ApxIII, that are produced by different strains found around the world (Tumamao *et al.*, 2004). Another commercially available *Clostridium perfringens* Type C and *Escherichia coli* (K88, K99, 987P) bacterin (Scourmune-C®, Merck Animal Health) for use in pregnant gilts and sows have been shown to reduce neonatal diarrhoea in piglets when compared with unvaccinated sows (Cunningham *et al.*, 2005). However, during inactivated vaccine development the addition of adjuvants or multiple immunisations are often required to achieve sufficient stimulus to generate suitable and sustained protection (Pavot *et al.*, 2012). The use of recombinant DNA (or vector) vaccines is beneficial due to addition of specific pathogen genes. For example, an inactivated vaccine containing a killed baculovirus vector carrying a protective ORF2 antigen against Porcine Circovirus 2 (PCV2) is commercially available (Porcilis® PCV, Merck Animal Health). Live vector and DNA vaccines have been shown to be effective against intracellular pathogens (bacterial and viral) at mucosal surfaces (Fort *et al.*, 2008), possibly because they elicit intracellular antigen production and induce cell-mediated immunity which can inactivate or destroy infected target cells or prevent pathogen replication.

2.11 The maternal immunity interference on vaccination

Non-responsiveness to vaccination in young piglets to active immunisation has been previously associated with maternal immunity interference (Mengeling *et al.*, 1992; Lawhorn *et al.*, 1994; Hodgins *et al.*, 1999). The maternal antibody half-life for *L. intracellularis* in serum has been estimated to be between 3 to 6 weeks in serum of piglets (Holyoake *et al.*, 1994; Guedes *et al.*, 2002a). Investigation on the efficacy of the *L. intracellularis* vaccine (Enterisol®Ileitis) when administered to 2 to 6 days old suckling piglets (from sows that were naturally exposed to *L. Intracellularis*) and challenged (10^8 organism) at 7 weeks of age, demonstrated the reduction on the duration of *L. intracellularis* faecal shedding but did not reduce intestinal lesions relative to non-vaccinated pigs (Donahoo, 2009). It is hypothesised that when live vaccines are administered orally to suckling pigs the antigen are not readily available due to neutralisation by maternal antibodies present in the intestinal tract of suckling animals (Siegrist, 2003). Therefore, piglets active immunisation strategies are normally delayed until passive immunity is declining but to not create a window of

opportunity for infecting diseases (around 4 to 6 weeks) (Emery and Collins, 2011). Alternatively, strategies to avoid this interference include using inactivated vaccines or alternative routes of vaccination such as passive vaccination (Emery and Collins, 2011; Chase and Lunney, 2012).

2.12 Route of vaccination on mucosal immune response

The ultimate goal of vaccination is to induce a specific immune response at the local site of pathogen adherence and proliferation. Therefore, it is only logical that to avoid mucosal pathogens colonization, a mucosal vaccine delivery is the most likely to be successful in protecting against disease. The oral dosing is often related as the main mucosal vaccination route (Curtiss *et al.*, 1996; Mirchamsy *et al.*, 1996; Husa *et al.*, 2009). This is based on the premise that the induction of IgA/IgG plasma cells will occur following antigen uptake at inductive sites (e.g. Peyer's patches) in the gut mucosa (Mestecky *et al.*, 2005; Roth, 2010). Pigs orally vaccinated with *Salmonella* Typhimurium live attenuated vaccine showed reduced intestinal lesions and increasing antigen specific IgG in serum after pigs were inoculated with 10^9 virulent *Salmonella* sp. compared with the unvaccinated group (Husa *et al.*, 2009). However, alternative mucosal routes such as intranasal, intraperitoneal and intrarectal vaccinations have been also successful in protecting livestock against mucosal diseases (Muir *et al.*, 1998; Pusterla *et al.*, 2010; Riddle *et al.*, 2011). Similar to oral vaccination, intrarectal and intranasal routes induce IgA responses at the local site of infection following antigen uptake within lymphoid follicles present at these sites (Sedgmen *et al.*, 2004). In humans, intranasal delivery with a *Shigella flexneri* 2a lipopolysaccharide 50 subunit vaccine (LPS, IpaB, IpaC and IpaD) induced a fivefold increase in the concentrations of antigen-specific intestinal faecal IgA and IgG (Riddle *et al.*, 2011). Similarly, intra-rectal vaccination using a live attenuated *L. intracellularis* vaccine has been found to induce increased specific *L. intracellularis* IgG responses in serum of horses (Pusterla *et al.*, 2009).

In addition, the intraperitoneal route has also been demonstrated to induce mucosal immune responses against specific pathogens. For instance, intraperitoneal vaccination of a crude tetanus toxoids with either Freund's adjuvants or oil-in-water adjuvants demonstrated a markedly improved anti-tetanus IgA antibody responses in

jejunum of chickens compared with an orally vaccinated group (Muir *et al.*, 1995). In a subsequent study in broilers, the intraperitoneal vaccination against *Salmonella* sp. produced protection and increased IgG and IgA serum responses that were comparable to those from orally vaccinated birds (Muir *et al.*, 1998). Antigens delivered by intraperitoneal route act by reaching the serosal surface through diffusing across the peritoneal membrane and are absorbed via the lymphatic system and consequently drain to the mesenteric lymph nodes (Lukas *et al.*, 1971). Pigs vaccinated intraperitoneally at 30 days and 60 days old with formalin killed *Mycoplasma hyopneumoniae* plus adjuvant had significantly fewer lung lesions and increasing serum *M. hyopneumoniae* specific IgG response than the non-vaccinated controls (Sheldrake *et al.*, 1993).

By contrast, non-mucosal routes of vaccination have also been related to vaccine protection against mucosal pathogens. For instance, *Salmonella* Typhimurium autogenous bacterin reduced the rate of pigs positive to *Salmonella* sp. shedding from 24% prior to intramuscular vaccination (d0) to 8.5% in marketing-age pigs (d49) (Farzan and Friendship, 2010). Presumably the mechanism by which intramuscular vaccination protects against mucosal pathogen is through the homing of activated immune cells from somatic sites and lymph nodes to mucosal tissues (Daynes *et al.*, 1996), or more likely, by the diffusion of circulating antibody into mucosal tissues. In *L. intracellularis* trials, Dale *et al.*, (1997) observed a 98.5% reduction in faecal *L. intracellularis* counts in four pigs that were vaccinated twice intramuscularly, 3 weeks apart, with killed *L. intracellularis* in incomplete Freund's adjuvant. The additional pigs were then inoculated with recombinant GroEL-Like protein and exhibited reduced faecal bacteria compared to infected controls.

2.13 *Lawsonia intracellularis* vaccines

Recently, a patent (WO/2009/127684) have has been registered presenting an inactivated *Lawsonia intracellularis*, *Mycoplasma hyopneumoniae* and porcine circovirus (PCV2) vaccine (Jacobs *et al.*, (2011); Intervet/Schering-Plough Animal Health). This invention vaccine is constituted with inactivated *Lawsonia intracellularis* whole cells (1.7×10^8 cells/mL), PCV2 antigen with ORF2 expressed in baculovirus and inactivated whole *M. hyopneumoniae* antigen in an oil-in-water

adjuvant. The whole cell *Lawsonia intracellularis* vaccine was derived from a live isolate from a pig with PE and was inactivated by high temperature (100°C) and 0.01% beta-propiolactone (Jacobs *et al.*, 2011). In the first experimental trial using this vaccine, 2mL of the *L. intracellularis* whole antigen plus 19/21kD, 37kD and 50kD outer membrane proteins (OMP) suspension was given twice intramuscularly (4 weeks apart) to 6 weeks old pigs. At 7 weeks post vaccination pigs were then orally challenged with a 10⁸ *L. intracellularis* mucosa homogenate. Results showed higher *L. intracellularis* antibody concentrations (by IFAT) 4 weeks after vaccination comparing with the unvaccinated pigs. After the *L. intracellularis* challenge, pigs vaccinated showed significant reductions in *L. intracellularis* shedding and lesions relative to control animals. However, this vaccine is not yet commercially available.

In the Australian market, a lyophilised *Lawsonia intracellularis* vaccine for pigs (Enterisol® Ileitis, Boehringer Ingelheim Vetmedica) has been available since 2006. The Enterisol® Ileitis is a live attenuated vaccine containing a *L. intracellularis* isolate derived from the ileum of a Danish sow with acute PHE (Kroll *et al.*, 2004). The *L. intracellularis* isolate (B3903) was co-cultured in McCoy mouse fibroblast cells with repeated subculturing for attenuation (Knittel and Roof, 1999). The culture is harvested and the number of viable *L. intracellularis* is estimated using the dilution assay to quantify the number of bacteria required to infect 50% of cell hosts and induce pathological changes (TCID₅₀). According to the Enterisol® Ileitis vaccine label information, one dose of reconstituted vaccine contains a minimum concentration of 1x 10^{4.9} TCID₅₀ and a maximum of 1x 10^{6.1} TCID₅₀ *L. intracellularis* (Kroll and Roof, 2007).

The Enterisol® Ileitis vaccine is recommended for use in weaned pigs 3-4 weeks of age, and given via drinking water or drench gun (McOrist *et al.*, 2006b). Since it is a live vaccine it is recommended that antibiotics should be removed from feed 2 days prior and 3 days post vaccination (Walter *et al.*, 2005; McOrist *et al.*, 2006b). The manufacture's also recommends, when giving the vaccine in drinking water, adding skimmed milk or sodium thiosulphate solution as a stabilizer prior to adding the vaccine (McOrist *et al.*, 2006b). There is no withdrawal period prior to slaughter. The *L. intracellularis* vaccine has been shown to protect 3 week old pigs and older pigs against clinical signs and intestinal lesions (28 days) (Kroll *et al.*, 2004;

McOrist and Smits, 2007). Increased herd uniformity and improved average daily weight gains of individual pigs between 20 to 34 g per pig per day have also been reported (Almond and Bilkei, 2006; Bak and Rathkjen, 2008). The onset of protection occurs as early as 3-4 weeks post-vaccination and lasts for at least 17 weeks (Guedes *et al.*, 2003); however, a direct correlation between immune responses and the level of protection have not been clearly established and is the focus of this thesis.

Part 4- Nutrition and mucosal immunology

2.14 Nutrition of the young pig

The basic physiology of the digestive system comprises the physical and chemical break down of complex nutrients in feed into simple molecules and absorption of these molecules across the intestinal epithelium (Yen, 2001). The absorption of nutrients from the lumen of the gut begins with their transport, active or passive, across the membrane of enterocytes lining the mucosal surface, followed by passage across the cells or metabolism within cells and entry into the blood or lymphatic system (Yen, 2001; Böhme, 2002). Concomitantly the presence of immune cells, mucus and microbiota along intestinal epithelium act against attachment and colonization of pathogens (Bailey *et al.*, 2005). Therefore, an efficient physiology of the gastrointestinal tract, such as motility, chemical digestion and absorption of essential nutrients, is critical to ensure optimum health and maintain growth and development of immunity. However, during post-partum and at weaning, piglets have to cope with many environmental and nutritional changes (Pluske *et al.*, 1997).

Initially the colostrum and milk intake in neonate piglets plays a crucial role in the piglets survival and development by providing energy (Le Dividich *et al.*, 2007) and enabling maternal antibody immune transfer (Rooke and Bland, 2002). Colostrum and milk have high fat (21.9% and 62%, respectively) and protein (68.04% and 56%) contents (Lin *et al.*, 2009). The intake of colostrum is directly correlated with piglet survival rate and growth performance (Le Dividich *et al.*, 2007). Lin *et al.*, (2009) observed that 98.3% of proteins in the colostrum were utilised by neonatal piglets in the form of the amino acids; lysine, glutamine, leucine,

and threonine. Amino acids are known to be utilized in a range of processes, such as the formation of secretory mucins, biosynthesis of amino acid, glutathione peptide formation and nucleic acid composition (Halas *et al.*, 2006; Stoll, 2006).

Weaning piglets around 21 to 35 days of age is part of the routine farm management of intensive pig production systems. Weaning is a critical period in the young pig's life as they are abruptly forced to adapt to new nutritional, immunological and psychological changes (Pluske *et al.*, 1997). The innate and adaptive immune system of weaner pigs develop while passive immunity from sows is declining at weaning. Additionally, piglets have to adapt from sow's milk that is highly digestible to a dry and less digestible starch based diet containing complex protein and carbohydrates that also include anti-nutritional factors (Williams, 2003; Lalles *et al.*, 2009). Dietary changes induce villus atrophy and crypt hyperplasia leading to reduced nutrient absorption and changes in the digestive enzymes profiles (Pluske *et al.*, 1997). Therefore it is no surprise that increased pig morbidity and mortality occurs post-weaning with increased pathogen attachment and proliferation along the intestinal tract. Common endemic pathogens at this stage include *E. coli*, *Salmonella* spp. and *L. intracellularis* and rotavirus (Jacobson, 2003). All of these pathogens cause diarrhoea and reduced growth performance of piglets. Therefore, strategies to overcome these post-weaning challenges are important. Feed antibiotic growth promoters and/or mineral compounds such as zinc (ZnO) are known to be beneficial in reducing disease outbreaks and mortality (Verstegen and Williams, 2002). However, due to food safety concerns and issues surrounding antibiotic resistance in humans, alternative strategies and feed additives are being investigated (de Lange *et al.*, 2010; Kim *et al.*, 2012; Heo *et al.*, 2013; Pluske, 2013). These include using diet composition to offset some of the nutritional challenges presented at weaning (e.g. protein/amino acid content of diets)(de Lange *et al.*, 2010) as well as the supply of immunomodulators (e.g. β -glucans, spray-dried plasma)(Halas *et al.*, 2006); and addition of feed additives (e.g. ZnO, organic acids, prebiotics and probiotics)(Pluske, 2013). For instance, the use of β -glucans to increase and modulate immune cells, protect against disease and increase growth performance has been discussed as a viable alternative to antibiotic use in mammals (Bohn and BeMiller, 1995; Brown and Gordon, 2003; Gallois *et al.*, 2009).

2.14.1 Impact of *Lawsonia intracellularis* infection on digestion and absorption of nutrients

The small intestine is around 4m long in neonatal pigs, 12m in weaner pigs and 20m long in adult pigs (Yen, 2001). Morphologically, the presence of villi projecting into the intestinal lumen are lined with epithelial cells covered with a continuous single-layer of absorptive cells termed enterocytes (Böhme, 2002). Under normal conditions, enterocyte cells actively divide by mitosis, migrate and mature along the crypt-villus to the tip of the villi where they are involved in absorption of nutrients across the intestinal wall and into the bloodstream (Friendship, 1989). Eventually, mature cells are sloughed off into the lumen and replaced with new cells.

Histopathology of *L. intracellularis* infected pigs shows proliferation of enterocytes and a marked reduction or loss of goblet cells (Lawson *et al.*, 1993; Lawson and Gebhart, 2000). Therefore, it is expected that disturbance can also lead to reduction in nutrient absorption. Hamsters experimentally infected with 10^9 *L. intracellularis* were associated with a reduction in the absorption of protein, amino acids and Cl^- and K^+ at 26 days post-challenge (Vannucci *et al.*, 2010). The authors' speculate that the effect of PE may have reduced expression or production of sodium-dependent glucose transporter 1 (SGLT1) (Vannucci *et al.*, 2010), therefore lowering intestinal absorption of nutrients and redirecting water into the intestinal lumen. Wong *et al.*, (2009) also demonstrated impaired intestinal absorption of glucose in four PE affected foals using an oral glucose absorption test. Although this test is not specific, it clearly does suggest that *L. intracellularis* infection impairs the intestinal absorption of nutrients. Supporting this notion, when compared to healthy pigs, PE lesions in infected pigs resulted in an approximate 7% reduction in the amino acid digestibility in the terminal ileum of pigs (Rowan and Lawrence, 1982). Similarly, PE lesions have also been related to changes in enzyme activity of the brush border. For instance, pigs infected with PE showed reduced concentrations of Mg-ATPase and acid phosphatase activity in crypt cells (Eriksen and Landsverk, 1988). Similarly, PE severity has been directly correlated to reduced activity of alkaline phosphatase and amino peptidase enzymes in the ileum of pigs severely affected with PE compared with control pigs (Collins *et al.*, 2009). Alkaline phosphatase and amino-peptidase enzymes have been demonstrated as essential in fat absorption and digestion of amino acids, respectively (Danielsen *et al.*, 1995; Hansen *et al.*, 2007). Therefore, *L.*

intracellularis infection possibly leads to alteration in the intestinal morphology and function, with possible impairment of nutrient digestion and absorption.

2.15 Effect of diet on immune stimulation

The immune system is dependent upon the redirection of dietary nutrients to meet the demand of immune cells for growth and replication, and this impacts directly on pig health and performance (Klasing, 2007). The deficiency of macronutrients or micronutrients, such as zinc, selenium, iron, and antioxidant vitamins can lead to clinical disease, and alter immunocompetence and increase the risk of infection (Scrimshaw, 2007). Specifically, nutritional deficiencies can impair phagocyte function of innate immunity and cytokine production, as well as adversely affecting certain aspects of humoral and cell-mediated immunity (Klasing and Korver, 1997; Korver and Klasing, 1997; Kidd, 2004). Impairment of these responses can compromise the integrity of the immune system, thereby increasing the animal's susceptibility to infection. For instance, Zn deficiency affects many lymphocyte functions, antibody and cytokine synthesis (Peterson *et al.*, 2008), recruitment of naive T cells and reduces the resistance to infection (McMurray *et al.*, 1990). Therefore, nutritionists aim to feed pigs to optimize the supply of nutrients to guarantee growth and performance, health as well as improve resistance to disease. Antigen or cytokine receptor functions (such as CD79, CD21, CD23) on lymphocytes and phagocytic cells can be influenced by nutrients that affect membrane structure or transmembrane signalling functions (Scrimshaw and SanGiovanni, 1997; Scrimshaw, 2007). Kegley *et al.*, (2001) fed 3 weeks old piglets with one of four corn-soybean meal based treatment diets containing 0.16, 0.24, 0.32 or 0.40% available P to investigate the effect on immune function. Pigs were challenged with lipopolysaccharide (LPS) and the researchers demonstrated that increasing supplemental dietary available P increased the production of lymphocytes 21 days after challenge. In addition, dietary depletion of vitamin A in C57BL/6J mice decreased lymphoid dendritic cells, memory CD8⁺ cells and CD4⁺ T lymphocytes in spleen using a multicolour flow cytometry test (Duriancik and Hoag, 2010). Therefore, this result suggests that diet could not only be a cofactor to infection

resistance but may also be able to potentially influence immune responses to vaccination.

2.15.1 β -glucan as immunomodulators for pigs

Beta-glucans (β -glucan) are the most abundant polysaccharides found inside cell walls of yeast, bacteria, cereals, seaweed and fungi (Bohn and BeMiller, 1995). They contain D-glucose as structural components and, depending on the source, are linked by either 1,3 or/and 1,6 β -glycosidic bonds (Sonck *et al.*, 2011). β -glucans extracted from the cell wall of *Saccharomyces cerevisiae* yeast is the most common source of commercial preparations for use in pigs' diet (e.g. Macrogard[®], Glucagen[®], EnergyPlus[®] and AntafermMG[®]). β -glucan derived from the *Saccharomyces cerevisiae* yeast is composed mainly of β -1-3-glucan linkages (around 85%) and about, 3% β -1-6-glucan linkages (Manners *et al.*, 1974). In contrast, the structure of cereal derived glucans consist of β -1-3 and β -1-4 linkage, while bacteria presents only β -1-3 linkages (Bohn and BeMiller, 1995). Besides the differences in the type of linkage, β -glucans can vary in solubility, molecular mass and polymer charge (Volman *et al.*, 2008). The high molecular weight of β -glucans from fungi directly activate leukocytes, while low molecular weight β -glucans only modulate the response of cells when they are stimulated with cytokines (Brown and Gordon, 2005). The type of isolation of β -glucan, even if is derived from the same source, can influence the lymphocyte responses (Sonck *et al.*, 2010). Therefore, variation between β -glucans will influence their physiological function and immune modulating effects. Sonck *et al.*, (2010) incubated pig peripheral mononuclear blood cells (PMBCs) with each of seven β -glucans (100 μ g/mL) from different sources. Interestingly, the two products originating from *S. cerevisiae* stimulated proliferation of lymphocytes, neutrophils and increased significantly concentrations of TNF- α and IL-1 β (Sonck *et al.*, 2010). Conversely, β -glucan from the bacterium *Agrobacterium biovar* did not activate monocytes or neutrophils, but stimulated lymphocyte proliferation and IL-10 cytokine production (Sonck *et al.*, 2010). More recently the same authors, in a similar experimental design demonstrated an enhancement of porcine dendritic cell (DCs) maturation by *S. cerevisiae* beta-glucan (Sonck *et al.*, 2011). It is possible that the extra-branching of the *S. cerevisiae* (1, 3 and 1, 6) may increase the recognition by dendritic cells and macrophage receptors and be an important determining factor on

their stimulatory capacity (Volman *et al.*, 2008). Variation in β -glucan supplementation responses can be also influenced by inclusion rate to diet. For example, while pigs fed diets with 0.25% *S. cerevisiae* β -glucan (Macrogard®) increased average daily weight gain after 28 days, with no affect in cohort pigs supplemented with 1% β -glucan (Dritz *et al.*, 1995).

The activation of the mononuclear phagocyte system and protective inflammatory cytokines by β -glucans in humans and pigs has been directly correlated to innate immune responses (Decuypere *et al.*, 1998; Brown and Gordon, 2001). Human whole blood incubated with soluble yeast β -glucan showed an increase in the production of pro-inflammatory cytokines, TNF- α , IL-6, IL-8 and monocyte tissue factor (Adachi *et al.*, 1994; Young *et al.*, 2001). At least four receptors have been identified for initial recognition of beta-glucans. With there being complement receptor 3 (CR3), lactosylceramide, scavenger receptors and dectin-1 (Brown and Gordon, 2001; Brown *et al.*, 2003; Brown and Gordon, 2005). Between them, dectin-1, a C type lectin was described as the most important receptor in pigs (Sonck *et al.*, 2009). Dectin-1 belongs to the large family of pattern recognition receptors (PRRs) which identify conserved PAMPs (Brown and Gordon, 2001). These are expressed by various antigen-presenting cells, such as dendritic cells, neutrophils, macrophages and some T and B cells (Bohn and BeMiller, 1995), and modulate innate immunity by increasing cytokine production (Adachi *et al.*, 1994). And possibly could lead to infection resistance. Glucan therapy by inoculating 1,3- β -D-glucan (150 mg/kg) intraperitoneally in mice prior to a 10^8 *E. coli* challenge, showed increased blood leukocyte activity and enhanced phagocytosis of *E. coli* (Williams *et al.*, 1988).

In pigs, an increase in PRRS specific IFN- γ concentrations (by ELISPOT) was detected in PBMCs of infected pigs after incubation with a β -1,3-1,6-glucan preparation (50 μ g/mL) (Xiao *et al.*, 2004). These authors suggest that β -glucan possibly enhanced Th1 specific immune responses, by increasing the maturational or activation state of sensitised T cells, as the effect *in vitro* was delayed until 50 days after infection (Xiao *et al.*, 2004). In agreement with this notion, Shen *et al* (2009) also observed an increase in CD4⁺ T cells and IFN- γ secretion in the gut mucosa 21 days after piglets were fed diets containing yeast culture (5g/ kg). However, concentrations of IgM, IgA or CD4⁺ and CD8⁺ T-cells in ileal tissues (by IHC) of

finishing pigs were not modified by β -glucan supplementation at 0.03% or 0.3% (Sauerwein *et al.*, 2007). However, this may be related because a 2.5% β -glucan daily dose, given to newborn piglets for two weeks caused a significantly increased expression of TNF- α and IL-1 β mRNA in ileal sections (Eicher *et al.*, 2006). Additionally, seven day old piglets supplemented with a β -glucan derived from seaweed *Laminaria digitata* and *Laminaria hyperborean*, and yeast *Saccharomyces cerevisiae* (650g β -glucan per kg of diet) did not express any pro- or anti-inflammatory cytokines markers in the ileal epithelial cells (Sweeney *et al.*, 2012). On the other hand, after an *ex vivo* LPS challenge, incubation of PBMC with *Laminaria digitata* beta-glucan showed enhanced expression of the IL-8 cytokine marker. This was also site-related, as the effect was reduced in the colon (Sweeney *et al.*, 2012). All of these findings imply that the effects of β -glucan on porcine immunity depends on the source and processing (related to structure), dose and age of recipient, as well as site of analysis / infection in the gut and the duration of feeding. Therefore it is essential to decipher the literature carefully, but also essential to fully trial the effects of supplementation in vaccination and disease models to have a relevant and functional read-out.

2.15.2 Microbial phytase potential effect on immunity

Phytate salts (myo-inositol hexaphosphate; IP-6) occur naturally in plants and serves as the storage form of phosphorus P (Sharpe *et al.*, 1950). The primary storage compartment of phytate in plants is seeds, which are used extensively in pig diets. However, phytate-P has a low bioavailability in monogastric animals (Simons *et al.*, 1990). Different feed ingredients vary in their concentration of phytate-P as well as the proportion of P that is present in phytate. For instance, the proportion of phosphorus present as phytate-P ranges from 67 to 86% in barley, maize, oats, canola, soybean, sorghum and wheat in Australian sourced pig and poultry feed ingredients (Selle *et al.*, 2003). Therefore the composition of the diet influences the amount of available-P in the diet (Table 1).

Table 1: The mean of total P and proportion of phytate-P in total P and bioavailability of total P for pigs in common feed ingredients ^a.

<i>Feed ingredient</i>	<i>Samples (n)</i>	<i>Total P (g/Kg)</i>	<i>Phytate-P. proportion of total P (%)</i>	<i>P bioavailability for pigs (%)</i>
Barley	41	3.21	61.0	30.0
Maize	45	2.62	71.6	13.0
Sorghum	64	3.42	77.8	20.0
Wheat	97	3.07	71.6	49.0
Canola meal	28	9.72	66.4	21.0
Cottonseed meal	21	10.02	77.1	10.0
Soybean meal	89	6.49	59.9	27.0
Rice bran	37	17.82	79.5	25.0
Wheat bran	25	10.96	76.3	41.0

^a Table adapted from Selle *et al.*, 2011.

The main anti-nutritional effect of phytate occurs by interference with digestibility and chelation to nutrients directly affecting the absorption and utilization of nutrients (Simons *et al.*, 1990; Selle *et al.*, 2011). The formation of an insoluble Ca₅-K₂-phytate complex occurs at pH 5 (Simons *et al.*, 1990) and Cu²⁺, Pb²⁺, Zn²⁺ phytate complexes have been observed in small intestines of pigs (Cigala *et al.*, 2010). In addition, phytate has been shown to negatively affect the activity of α -amylase and trypsinogen (Deshpande and Cheryan, 1984; Caldwell, 1992).

The use of a phytase enzyme to break down phytate chelation releases nutrients bound to phytate and potentially increases their digestion and utilization in the gut. Phytase enzyme can be derived from intrinsic plant phytase (e.g. wheat), endogenous mucosal phytase (e.g. phytase generated by small intestines of some animals), gut microbiota or exogenous microbial phytase (e.g. feed enzyme) (Selle and Ravindran, 2008). The production of phytases, their characteristics, and manipulation of thermo-tolerance and gastric resistance are beyond the scope of this thesis but have been described in detail elsewhere (Ha *et al.*, 2000; Pandey *et al.*, 2001). Briefly, microbial phytases have been demonstrated to degrade phytate-P in the acidic conditions of the stomach and small intestine of pigs (Yi and Komegay, 1996), by a series of dephosphorylation reactions and generation of a series of lower myo-inositol phosphate esters, resulting in inositol and inorganic P (Schlemmer *et al.*, 2001). The addition of phytase to diets of monogastric animals has been shown to increase availability of P (Cromwell *et al.*, 1995) and as well as other minerals (Lei *et al.*, 1993;

Adeola *et al.*, 1995; Biehl and Baker, 1996). Cromwell *et al.* (1995) observed an improvement in P absorption and an decrease of P excreted in faeces of growing pigs fed with soybean meal supplemented with *Aspergillus niger* derived phytase (5,000 phytase units (FTU)/g). Similarly, Igbasan *et al.*, (2000) demonstrated that two phytases derived from *Aspergillus niger* and *E. coli* both improved the total digestibility of P (33-34%) and Ca (18-20%) to similar extents in the intestines of weaner pigs offered maize-soy diets. Weaner pigs fed with a corn soybean meal diet supplemented with microbial phytase at 1350 FTU/g feed had improved bioavailability of Zn comparing with control groups without supplemented phytase (Lei *et al.*, 1993). However, although the correlation with growth performance improvement by phytase has been confirmed (Brana *et al.*, 2006), the increased bioavailability of protein and amino acids by adding phytase to diets is not as consistent. Brana *et al.* (2006), compared the efficacy of phytases derived from *A. niger* or *E. coli* on P in growing pigs fed inadequate maize-soy diets for 130 days. At 500 phytase units (FTU)/kg, both phytases improved weight gain (9.9%), feed efficiency (8%) and fibular ash (6.5%). Bohlke *et al.*, (2005) reported greater amino acid digestibility in growing pigs fed low-phytate corn meal with phytase than responses from pigs fed normal corn meal and the enzyme. On the other hand, Johnston *et al.*, (2004) and Woyengo *et al.* (2009) indicated that phytase had a limited affect on the bioavailability of protein and amino acids at the terminal ileum of weaner pigs.

Studies on the effects of phytase on immunological parameters and related to nutrition are very limited. Theoretically, the increased bioavailability of chelated nutrients should also increase the availability of nutrients for immune cell growth and replication. Lower molecular weight inositol phosphatase added to human cell lines (insulin secreting cells) increased Ca^{2+} channel activity by initiating the uptake and phosphorylation of glucose and regulation of adenosine triphosphate (ATP) activity as well as increasing the bioavailability of calcium (Larsson *et al.*, 1997). Additionally, the free inositol IP6 molecule has been speculated to regulate cell division and differentiation (Menniti *et al.*, 1993) and to stimulate respiratory burst and bacterial killing by releasing IL-8, TNF- α and IL-6 cytokines (Eggleton, 1999). Broilers fed a high phytate diet (0.44% phytate P) supplemented with *E. coli*-derived

phytase (1,000 phytase units (FTU)/kg feed) had a 3-fold increase in numbers of erythrocyte rosette forming cells at day 21 compared to the control group (high phytate diet 0.44% P). The percentage of CD4⁺ and CD8⁺ T lymphocytes subsets were also increased by phytase on day 28 after supplementation (Liu *et al.*, 2008). These effects could also lead to a protective response. As observed previously, broilers fed a marginal diet supplemented with 500FTU/kg of phytase (0.35% P) and challenged with *Eimeria acervulina* (1,000 oocysts) showed less lesions when compared with infected birds fed control diets (Shaw *et al.*, 2011). In addition, increased IL-17 mRNA gene expression in the duodenum in phytase supplemented broilers correlated with decreased pathogenicity on day 18 after challenge (Shaw *et al.*, 2011). Ghahri *et al.*, (2012) fed 14 to 42 day old broilers either an adequate or low calcium and nonphytate P (Ca-nPP) diet supplemented with a microbial phytase (600-1000 FTU/g feed, Natuphos®10000). They observed neutralising antibody titres against Newcastle disease vaccine in the marginal but not the adequate C-nPP diets, 14 and 42 days after vaccination (but not at other weekly intervals) (Ghahri *et al.*, 2012). A search of the literature failed to identify and research investigating the effect of phytase on immune responses in growing pigs.

Chapter 3 *General Methods and Materials*

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3.1 *Housing conditions and husbandry*

Two experimental trials were conducted at Elizabeth Macarthur Agricultural Institute research unit (EMAI, NSW-DPI, Menangle, Figure 5). The experimental building has four rooms, with separated external and internal access and controlled waste management. Room temperatures were monitored during trials to maintain ideal ambient temperature by an automatic air conditioning system. The facility has a concrete slatted-floor and pens were constructed using interlocking, portable metal partitions and gates. Each pen was fitted with at least one nipple drinker and hopper feeder.



Figure 5: Pig research unit at Department of Primary Industries (DPI-NSW), Australia. Right figure shows internal room structure ready for the start of an experimental trial.

Biosecurity measures were instigated and followed prior to and during trials. Each room and equipment were cleaned prior with a pressure hose and disinfected with Virkon® (Antec International, Sudbury, UK) to eliminate any possible bacterial and viral contamination. The protocols also minimised the risk of external spreading of *Lawsonia intracellularis* into the rooms, between rooms and vice versa. Before entry to the facility, personnel were required to change into a pair of protective boots, for internal use only and to use clean overalls daily. Other protective equipment (such as gloves, masks, glasses and earmuffs) was also used when required. In addition, to reduce cross-contamination between pens with different treatments, the sampling and treatment routines occurred in the order from the less to the most contaminated pens.

3.2 Vaccine preparation

The lyophilized Enterisol® Ileitis vaccines (Boehringer Ingelheim Pty Ltd.) were reconstituted according to the manufacturer's instructions to either a standard (1x) dose ($10^{4.9}TCID_{50}$) or ten times this ($10^{5.9}TCID_{50}$). Briefly, 100 mL of sterile diluent was mixed thoroughly with lyophilized *L. intracellularis* antigen containing approximately $10^{4.9} TCID_{50}$ *L. intracellularis* organism. For the ten times dose the antigen was diluted using 10 mL of sterile diluent and mixed ($10^{5.9} TCID_{50}$). Vaccination occurred within 30 minutes of reconstitution and left over of vaccine was discarded. The same batch vaccine was used for each experimental trial.

3.3 Preparation of *L. intracellularis* challenge inoculum

The inoculum of *L. intracellularis* was prepared using *L. intracellularis* infected intestinal mucosa and quantified by Dr. Alison Collins at the EMAI-Microbiology laboratory (Collins *et al.*, 1996; Collins and Love, 2007). Briefly, the intestinal mucosa derived from infected pigs with *L. intracellularis* (clinical PHE) was scraped and homogenized gently in sterile PBS. The extracellular material was separated by repeated washing in PBS and 0.5M EDTA and centrifugation until the supernatant was clear. The supernatant was retrieved and stored on ice in a sterile container until challenge. Prior to inoculation, the presence of the bacteria was confirmed by modified Ziehl Neelsen (MZN) staining. The number of *L. intracellularis* was calculated per dose by counting bacteria under the microscope after immunofluorescence antibody (IFA) staining. The inoculum suspensions were serially diluted with PBS from 1:2 to 1:64 and each dilution was fixed on slides. A *L. intracellularis* monoclonal antibody (IG4) was diluted 1:200 in PBS and incubated with the inoculum at 37°C for 30 minutes. Non-specific binding was removed by washing slides in PBS, and the slides were then incubated with 1:50 dilution of sheep anti-mouse IgG conjugated with FITC (Sigma, US). After incubation, slides were washed in water and examined by fluorescent microscopy at 1000X magnification. The number of fluorescing comma-shaped bacteria was counted for ten field diameters at two different dilutions and in duplicate wells. The number of bacteria per mL of inoculum was calculated using specific formula (Equation 1).

Equation 1- Quantification of inocula

$$\text{Bacterial per mL} = \frac{\text{Mean number of bacteria per field diameter} * \text{dilution of inoculum}}{5.94 * 10^{-6}}$$

3.4 Clinical scores

During experimental trials, animals were assessed daily recording their clinical score: faecal consistency, body condition and behaviour (Appendix I). Faecal consistency was scored as, 1=normal, 2= soft (semi-solid), 3= watery, 4= presence of blood. Behaviour was scored as 1=normal, 2= moderately depressed, standing, 3= severely depressed, lying down. Body condition score as 1=normal, 2= mildly gaunt (thin, hips and backbone noticeable), severely gaunt (hips and backbone very prominent).

3.5 Sampling procedures

3.5.1 Blood and faeces

Individual pigs were restrained in a cradle (pigs less than 15kgs) or by using a nasal snare (and standing) depending on their size. Blood was captured by venipuncture of the jugular vein using serum clot separator vacuum tubes (Vacuette®, Greiner BioOne, Austria). After collecting around 5 mL of blood, samples were left in 4°C cold room overnight and then centrifuged at 2000g for 20 minutes to separate blood clot. Serum samples were then poured off to sterile individual containers and stored frozen at -20°C until analysis. Around two grams of faecal matter were individually collected directly from the rectum using clean gloves for each animal. Samples were then stored in sterile containers and frozen at -20°C until further processing.

3.5.2 Tissue section

Intestinal tissues were collected at necropsy from the ileum 10cm proximal from the ileal caecal valve (ICV), the jejunum 40cm proximal to the ICV and the caecum, 5cm distal to the ICV. Tissues were divided with one part kept in 10% neutral-buffered formalin fixative and another in RNA stabilization suspension (RNA later, Ambion®, Life Technologies, US) at room temperature until analysis. A third tissue segment was snapped frozen using the fresh tissue freezing technique with optimum cutting temperature (OCT) compound (Tissue-Tek®, Sakura, US). Tissues were placed in a plastic mold containing OCT and quickly placed in a steel bowl on the top of dry ice pellets. To the bowl, isopentane (2-methyl butane) was slowly added and once frozen; the tissue moulds were stored in a -80°C freezer.

3.5.3 Mucosa secretions

Samples were collected 10cm from the ileal-caecal valve (ICV). The ileum was slit longitudinally and the intestinal mucosa was gently scraped with a sterile scalpel blade to collect the surface secretions. These were placed into 2 mL phosphate-buffered saline (PBS, pH 7.2) containing 0.2M EDTA. Each scraping was stored at -20°C. Prior to assay, mucosal scrapings were thawed and adjusted to a protein concentration of 25mg/mL. In this method, each sample was thawed in ice and transferred into sterile 2mL microfuge tubes containing approximately 200µL of

0.1 mm silicon beads (Biospec Products, Bartlesville, USA) for tissue disruption. Then, samples were vortex for 1 min five times, to solubilise the antibody in the tissue. Following extraction, samples were centrifuged at 11000g for 15 minutes at 6°C to separate the cellular debris and tissue from the soluble protein. After the supernatant was collected, the total protein concentration was estimated by reading the absorbance of each sample diluted 1:100 in sterile PBS using spectrophotometer set at 280nm (BioRad®, xMark™ Microplate Absorbance, US). All samples were standardised to 25mg/mL of total protein using sterile PBS and re-frozen until the day of analysis.

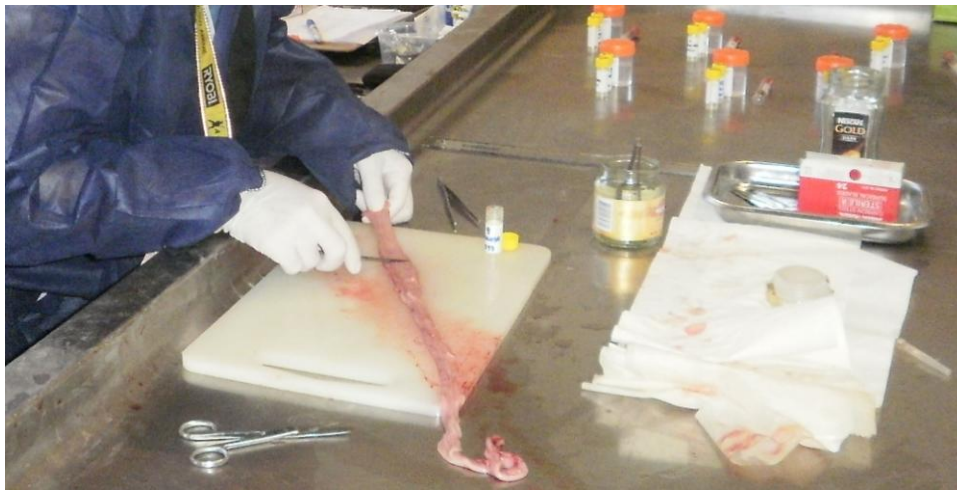


Figure 6: Collection of ileal mucosa secretion by scraping gently with sterile scalpel blade and adding to sterile containers with PBS+ EDTA.

3.6 Laboratory procedures

3.6.1 *Lawsonia intracellularis* competitive ELISA test

Sera and mucosal scrapings were assayed for *Lawsonia intracellularis* specific IgG antibody using a commercial competitive (blocking) ELISA kit (Bioscreen® Ileitis, GmbH, Münster, Germany). The principle of the test consists of serum and mucosa samples that contain *L. intracellularis* antibodies (mainly IgG) will bind to the *L. intracellularis* antigen coated to the plates (Kroll *et al.*, 2005). And after a washing step in PBS, an anti- *L. intracellularis* monoclonal antibody (Mab) peroxidase conjugate is added. The conjugate will bind only to the free antigen epitopes (where *Lawsonia*-antibodies from the sample did not bind). After excess conjugate is eliminated in a second wash step, the addition of a substrate may react

with peroxidase linked to *Mab* forming a colour reaction. In this case, the absence of antibody will have an intense coloured reaction while the presence of anti-*Lawsonia* antibodies in the samples diminishes the coloured reaction in proportion to the antibody titre. The optical density (OD) of the wells was read bichromatically at 450 and 630nm using a microplate spectrophotometer (BioRad®, xMark™ Microplate Absorbance, US). OD values were then used to validate run (negative controls ≥ 0.500 , and the percentage inhibition of positive controls are $\geq 40\%$) and to calculate the percentage inhibition value (PI value) of the samples, determined by using the following formula:

Equation 2- The percentage inhibition estimation equation for Bioscreen®.

$$\text{Percentage inhibition (PI)} = \frac{\text{OD of negative control} - \text{OD of sample well}}{\text{OD of negative control}} * 100$$

According to manufacturer's instructions, PI value greater than 30% were interpreted as positive to *L. intracellularis* antibodies and value less than 20% were negative. The sensitivity and specificity has been previously tested as 90.5% and 83%, respectively Collins *et al.*(2012).

3.6.2 "In house" modified direct ELISA test

Lawsonia intracellularis specific IgM, IgG and IgA titres were assayed using an experimental modified direct ELISA developed specific for this project (Donahoo, 2009) and re-validated for each trial in this thesis. Samples calculated by plotting OD values versus the dilution of standard controls the unknown samples were then estimated using a regression equation from the standard curve in each plate. The re-validation of the ELISA efficiency, the R² (coefficient of determination) parameter was calculated (R² ≥ 0.98). Briefly, *L. intracellularis* coated plates (Bioscreen®Ileitis, GmnH, Münster, Germany) were blocked with 1.5% w/v skim milk powder for 2h at room temperature before washing three times in phosphate-buffered saline (PBS, pH 7.2) plus 0.05% v/v Tween®20 (Sigma-Aldrich, US) washing buffer in plate washer (BioTek® ELX405™, US). Serum or mucosal scrapings were serially diluted in blocking solution before adding (100µl) to wells in the plate. Samples were gently rocked for 1h at 37°C before washing five times in washing solution. Subsequently,

labelled (HRP, Horseradish peroxidase) goat anti-porcine IgM, IgA and IgG (AAI39P/AAI40P/AAI41F, AbD Serotec, UK) polyclonal conjugate were added separately to relevant plates for 1.5 h at 37°C before washing. The colour reaction was developed by adding substrate (TMB, Sigma-Aldrich, US) for 10 min before the reaction was stopped with 10µl of 2M sulphuric acid (Sigma-Aldrich, US). The optical density of the wells was read at 450nm using a microplate spectrophotometer (BioTek Instruments, Inc., US), with an established cut-off point of 0.250 OD, which was 2-fold higher than the negative sample (<0.120 OD).

3.6.3 Porcine cytokines sandwich ELISA

The estimation of each cytokine, in mucosal scrapings or in serum, was analysed using commercial sandwich ELISA kits (porcine Quantikine® ELISA, R&D systems, Minneapolis, USA) according to manufacturer's directions. Basically, this assay is a quantitative sandwich immunoassay with a monoclonal antibody specific for the respective cytokine, pre-coated to the plate. After the addition of samples, control and standards, a second monoclonal antibody is added. The intensity of colour measured is in proportion to the amount of cytokine bound in the initial step. Using GraphPad®Prism software (v.4.02, 2004), the cytokine concentrations in pictograms (pg)/mL were calculated by plotting the OD readings bichromatically at 450nm and 540nm against a titration curve produced from dilution of the standard control provided with the kit. The significance assessed by manufactures is summarized in the table below (Table 2).

Table 2- The minimum detection dose range (MDD) recovery %, of porcine cytokines in plasma according to the manufactures of the Quantikine® Elisa test kits (R&D Systems, US).

Cytokines	MDD (pg/mL)	Recovery (%)	Code
IFN-γ	2.7-11.2	98.2	PIF00
IL-6	0.68-4.3	96.5	P6000B
TNF-α	1.8-5.5	99.1	PTA00
TGF-β1	2.8-5.0	99.7	PMB100B
IL-10	1.7-15.4	94.8	P1000



Figure 7: Porcine Quantikine® Elisa kit used for cytokine evaluation.

3.6.4 Real-time PCR detection of *L. intracellularis* in faeces

Extraction and purification of bacterial DNA from faecal samples (0.1g) was conducted using the MagMax DNA extraction kit (AM1939, A&B Applied Biosystems, California, US). A test sample of around 0.05-0.1g of faeces was diluted in 700 μ L of PBS, vortexed and centrifuged (100g for 3 secs). The main characteristic of this kit is the use of microspherical paramagnetic beads, which bind to nucleic acid. The beads with nucleic acids are captured by magnets, and other contaminants are washed away. The procedure delivers very consistent high quality RNA and DNA, as much as 50 μ L (AM1939 protocol, A&B Applied Biosystems).

Extraction products were analysed by quantitative PCR (qPCR) using primers (Li-ubi-F 5'GCT CAT ACC GAT TGT GTA ATG CA 3'; Li-ubi-R 5'GAA AAA CAG GCC GTA TCC TTG A 3') and Taq Man probe (Li-ubi-probe 5'FAM-TAG CCA CAT CAA GTG TTC CAG CTG CAA G- TAMRA 3') as described by Nathues *et al.*, (2009). Briefly, PCR amplifications were carried out for each sample in 25 μ L volume. Each reaction contained 12.5 pmol of each primer and 5 pmol of the probe using a commercial real-time PCR reagent (TaqMan® Universal PCR Master Mix). The PCR conditions involved a holding step at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds in a 7500 Real-Time PCR thermocycler (Applied Biosystems, US). The sensitivity and specificity has been previously reported in this laboratory as 99% and 97%, respectively (Collins *et al.*, 2011).

Amplification was plotted for each sample to demonstrate the intersection between the fluorescence reaction (ΔRn) and the PCR cycle number (Figure 8). Then the CT value line was drawn at initial point of the linear phase, for better reliability and to accurately estimate bacterial numbers (Nadkarni *et al.*, 2002). The amount of amplified product approximately doubles in each cycle. As the reaction proceeds, enough amplified product accumulates to yield a detectable fluorescent signal (threshold cycle, CT). And to quantify numbers of the unknown sample a range of standards with known quantity were added to each run. Quantification standards were used by seeding five negative faeces with 10^6 , 10^5 , 10^4 , 10^3 , 10^2 *L. intracellularis* / g respectively. And by plotting CT value versus the number of *L. intracellularis* seeded, the unknown samples were then estimated using a regression equation from the standard curve. To evaluate the PCR efficiency, the R^2 parameter was calculated to provide a good confidence value. When $R^2 \leq 0.98$, results were not considered and the test repeated.

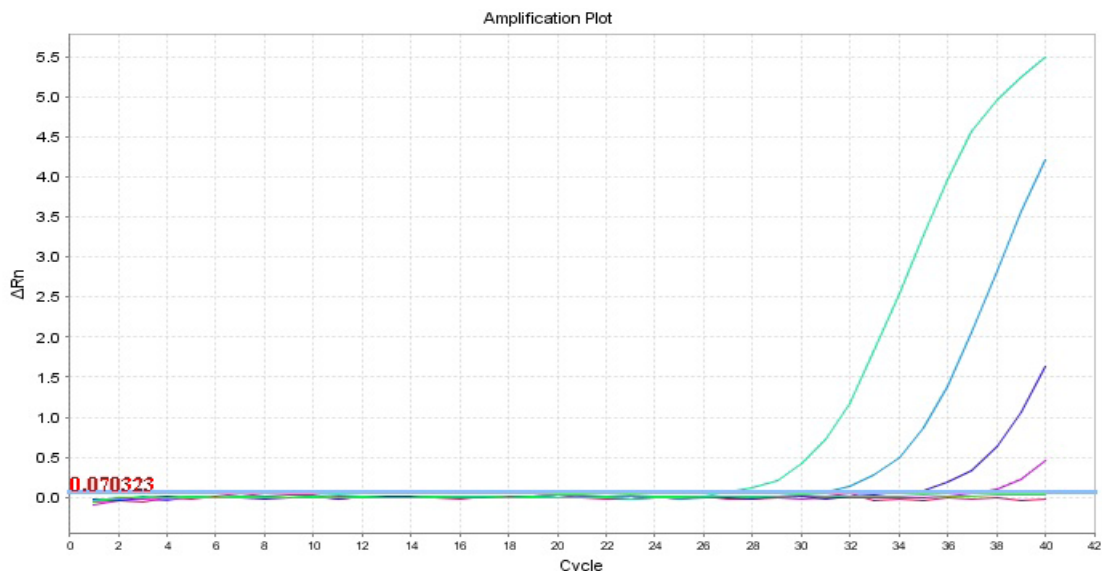


Figure 8: Graphic representation of real-time PCR standards. ΔRn is the fluorescence signal strength plotted against PCR cycle number. The blue line indicates the threshold cycle value (CT=0.07032) at the initial point of the linear phase.

3.6.5 Haematoxylin and eosin staining (HE)

The tissue sections were processed at the veterinary pathology diagnostic services laboratory at University of Sydney. Briefly, the formalin fixed tissue was embedded in paraffin and sectioned at 4 μ m in duplicate. Slides were then dewaxed in three steps with 100%, 95% and 75% ethanol and rehydrated with running tap

water. Tissue were then stained in Whitlock's Haematoxylin for 3 minutes, then in Scott's Blueing solution (alkaline solution of Mg_2SO_4 and $NaCO_3$) for 3 minutes, rinsed under running tap water for 2 minutes and rinsed with 70% ethanol. Subsequently, the sections were counterstained in alcoholic Eosin Y for 30 seconds, dehydrated rapidly through 70%, 95% and 100% ethanol, cleared in xylol and mounted in DPX mounting media and cover-slipped (Figure 9).

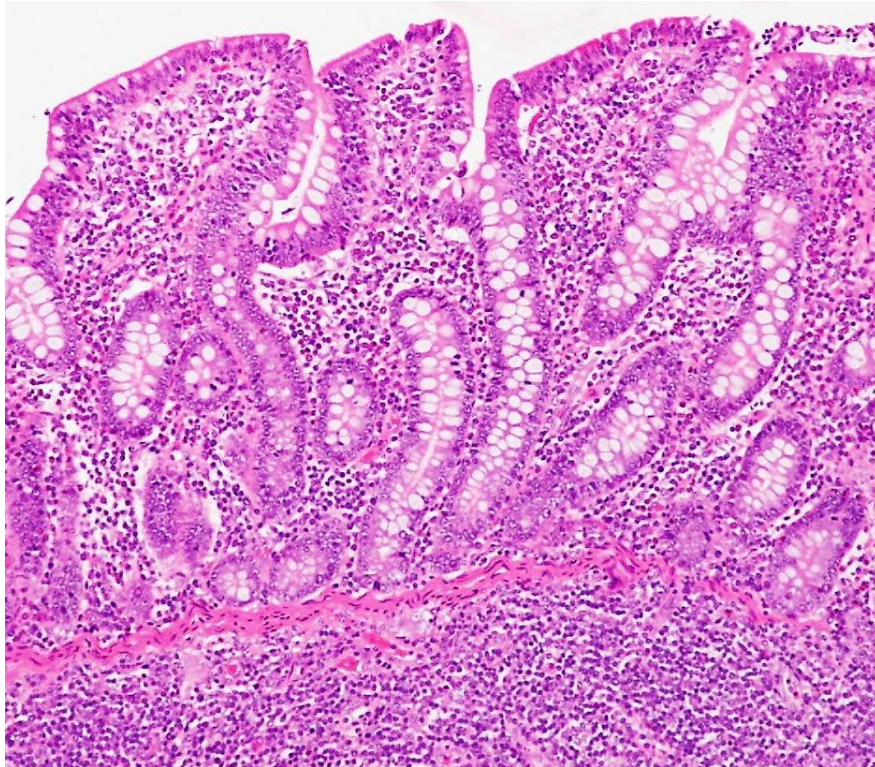


Figure 9: Small intestine HE staining, at 10x magnification with clear overall look at ileum villus crypt and goblet cells.

3.6.6 Immunohistochemistry (IHC)

Formalin-fixed tissues were stained by immunohistochemistry (IHC) with VPM53 *Lawsonia intracellularis* monoclonal antibody (Boehringer Ingelheim Vetmedica, Ames, USA), and using the method described by Jensen *et al.*, (1997). In each batch of IHC, a positive and negative control section was included. Initially, sections were dewaxed twice in xylene and once in absolute ethanol, following by blocking solution containing methanol peroxidase (30% v/v hydrogen peroxidase) for 10 minutes to inhibit endogenous peroxidase activity in the cells before being washed in reverse osmosis (RO) water. Sections were incubated in pre-warmed

trypsin digest (0.2% w/v Trypsin 250, 0.1% w/v calcium chloride dehydrate made up to 200mL with milliQ water) for 30 minutes at 37°C. Sections were washed twice in RO water, once in PBS, drained and placed in humidity chambers. To inhibit non-specific binding, 150µL of blocking solution (0.5% w/v bovine serum albumin-BSA, 1% v/v goat serum made up to volume with PBS) was added to each section and incubated at room temperature for 20 minutes. A plastic coverslip was placed over the top to ensure contact between the tissue section and the solution. Following rinsing of slides in PBS to remove the coverslip and draining, the VPM53 monoclonal antibody was diluted in PBS at 1:200. 15µL of antibody was applied to each slide with a coverslip and incubated for one hour at 37°C. The coverslip were removed in PBS, the slides flooded with PBS, drained and a HRP-labelled secondary antibody (Dako Cytomation Envision, Dako, Carpinteria, US) was applied at 150µL per slide with a coverslip and re-incubated for one hour at 37°C.

Chromagen working solutions were pre-prepared and stored. The 3-amino-9-ethylcarbazole (AEC) was made into solution by adding 0.04g of AEC powder to 4mL of N, N-dimethylformamide (DMF) and was stored in a light-proof container at room temperature. A 0.05M acetate buffer (5pH) was prepared by adding 0.82 grams of anhydrous sodium acetate to 200mL of milliQ water. At the end of the incubation period, the chromagen substrate was prepared by adding 150µL of AEC in DMF to 7.5 mL of acetate buffer. Coverslips were removed in PBS and drained, following by 150 µL chromagen applied with coverslip. Colour development and stoped after exactly 12 minutes by washing sections twice in RO water and removing the coverslip. Section were stained in Mayer's Haematoxylin for 3 minutes, washed twice in RO water and set to drain. They were mounted with aqueous mounting solution (Dako, Carpinteria, US), set to dry and placed in the dark until read (Figure 10).

The blinded slides were scored by one operator under 10x magnification light microscope to estimate the proportion of intestinal crypt hyperplasia (H&E) and presence of *L. intracellularis* -specific antigen (IHC) within crypts. A total of fifteen fields was analysed for each section.

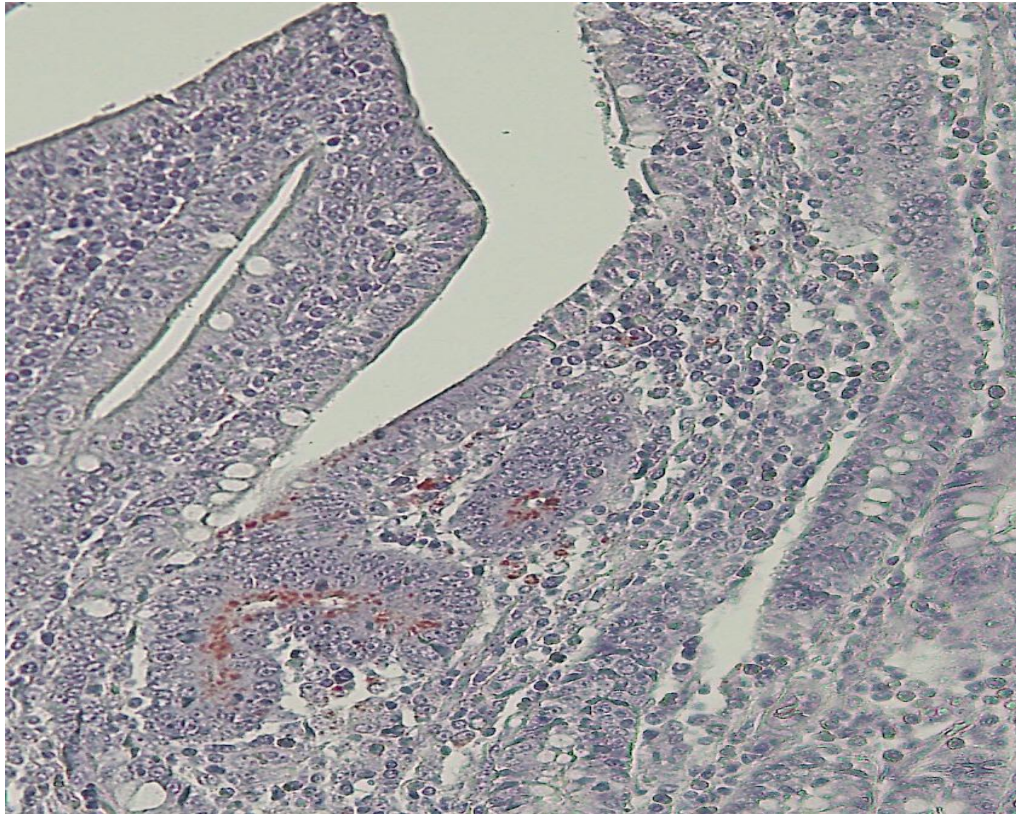


Figure 10: Immunohistochemistry staining (40x magnification) with positive areas of *L. intracellularis* antigen presence (brown) in ileal crypts.

3.7 Statistical analysis

Statistical analysis was performed in GenStat 13th ed. (2010). Raw data were initially screened with graphical observations and data summaries to locate overall tendencies, outliers and sample distribution using Microsoft Office Excel 2007-2010. Treatment differences were analysed by restricted maximum likelihood test (REML) and testing residuals for normality distribution. The REML test (linear mixed model) was chosen for its ability to analyse a wide range of data that involve more than one source of error variation and unbalance designs (Steel, 1997). Variables that did not fitted to the residual normality were log transformed to fulfil linearity assumptions. Pens, pigs ID and room were included as random effects when required. Unpaired variables were tested using Mann-Whitney U (Wilcoxon rank-sum) test correlations within groups at a 95% confident level ($p < 0.05$). Qualitative data were categorised into binary data and then analysed using Generalized Mix Models (GLMM) for repetitive measures and two-sample binomial test for single measure (GenStat).

Chapter 4 Systemic and mucosal immune responses following vaccination and challenge with *Lawsonia intracellularis*

(Modified from: Nogueira MG, Collins AM, Donahoo M, Emery, D; (2013). Immunological responses to vaccination following experimental *Lawsonia intracellularis* virulent challenge. *Veterinary Microbiology*, 164:131-138.)

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4.1 Introduction

The Gram-negative intracellular bacterium *Lawsonia intracellularis* causes proliferative enteropathy (PE), characterized by diarrhoea and poor performance in growing pigs and severe haemorrhagic diarrhoea in finisher and breeding animals (Lawson and Gebhart, 2000). The most consistent macroscopic pathology of PE is thickening of the intestinal mucosa which is associated histologically with proliferation of immature enterocytes (Lawson and Gebhart, 2000). The clinical signs and lesions can be controlled using a commercial oral live attenuated vaccine (Enterisol®Ileitis, Boehringer Ingelheim) (Kroll *et al.*, 2004; McOrist and Smits, 2007). This vaccine contains an attenuated *L. intracellularis* isolate (B3903; 10^{4.9}

TCID₅₀/dose) originally isolated from the ileum of a Danish pig with acute proliferative haemorrhagic enteropathy (PHE) (Kroll *et al.*, 2004). Protective immunity against re-infection is also apparent in recovered pigs (Collins and Love, 2007; Riber *et al.*, 2011b; Cordes *et al.*, 2012).

A conventional immunological approach to induce mucosal immunity against gut pathogens has involved oral vaccination or intraperitoneal inoculation (Muir *et al.*, 1998). However, where antibody provides mucosal protection, high concentrations of serum IgG engendered by systemic vaccination have been effective against some intracellular bacteria. For example, *Salmonella enterica* serovar Typhimurium bacterin administered intramuscularly (IM) reduced lesions and shedding in naturally-infected pigs (Farzan and Friendship, 2010). Similarly, an intramuscular *L. intracellularis* killed bacterin induced significant protection to PE after virulent challenge (Dale *et al.*, 1997), and in a patent description, protection was induced after immunisation IM with killed *L. intracellularis* (Jacobs *et al.*, 2011).

To identify immune responses which might indicate the successful induction of protective immunity following vaccination with Enterisol® Ileitis, this study measured local mucosal and systemic immune responses in the first 3 weeks after immunisation. Since local mucosal responses were marginal following a conventional single dose vaccination in a preliminary trial 1 (published in the paper Nogueira *et al.*, 2013, but not in this thesis), pigs in trial 2 were vaccinated orally with a ten times dose (10x) of Enterisol® Ileitis. Local mucosal and systemic antibody and selected cytokine responses were measured and compared with those from piglets given the same dose of vaccine IM. To determine whether these responses could foreshadow protection, the remaining vaccinated cohorts were challenged and immunity was assessed by serological assays, reduction of clinical signs and intestinal lesions and the duration and magnitude of bacterial shedding in faeces.

4.2 Materials and Methods

4.2.1 Animal Ethics

All animal experiments were performed according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and approved by the University of Sydney and Elizabeth Macarthur Agricultural Institute Animal Ethics Committees (M09/10).

4.2.2 Experimental design

For trials 1 and 2, a total of 40 and 62 Landrace x Large White pigs, respectively, aged 3-4 weeks, were purchased from a commercial herd clinically and serologically negative for *L. intracellularis*. Piglets were transferred to a controlled environment research facility, with access to water and fed on weaner/grower diet (DE: 15.3MJ/kg; CP: 20%; Fibre: 2.5%; Vella Stock Feeds, Australia) with no medication. Pigs were weighed (6.0 ± 0.5 kg) and randomly allocated into respective treatment groups (Table 3; trial 2) housed in separate pens under strict quarantine conditions. Pigs were monitored daily for body condition, clinical signs and abnormal behaviour.

4.2.3 Lawsonia vaccination

Prior to vaccination (d0) at 5 weeks of age, pigs were bled by jugular venipuncture and faeces were collected from individual pigs to confirm the absence of *L. intracellularis* or the presence of maternal antibodies that might interfere with vaccination. The lyophilised vaccine (Enterisol® Ileitis, Boehringer Ingelheim Pty Ltd., SA-122A-323) was reconstituted according to the manufacturer's instructions to either standard (1x) dose ($10^{4.9}$ TCID₅₀) or ten times ($10^{5.9}$ TCID₅₀) concentrations. Pigs received the appropriate vaccine dose orally in 2.0mL diluent by drenching gun or by intramuscular inoculation (IM) in the right deltoid muscle (cervical area). Negative and positive control pigs were not vaccinated.

Table 3- Experiment design and groups in trial 2*.

Experiment	Group	Li dose	Route	Li challenge	N° pigs
2a	10xOral	10 ^{5.9} TCID ₅₀	Oral	No	6
	10xIM	10 ^{5.9} TCID ₅₀	IM	No	6
	Control	-	-	No	4
2b	PC	-	-	Yes	10
	1xOR	10 ^{4.9} TCID ₅₀	Oral	Yes	10
	1x IM	10 ^{4.9} TCID ₅₀	IM	Yes	10
	10xOR	10 ^{5.9} TCID ₅₀	Oral	Yes	10
	NC	-	-	No	6

*Li: *Lawsonia intracellularis*; IM: intramuscular; PC: Positive control; NC: Negative control.

4.2.4 Ten times vaccination trial with challenge infection

4.2.4.1 Experiment 2a: Analysis of immune responses after vaccination

Fifteen pigs aged 4 weeks were randomly assigned into three treatment groups: six pigs in each of groups 1 and 2 were orally vaccinated and IM vaccinated, respectively, with 10x dose of Enterisol®Ileitis vaccine and three pigs in group 3 were not vaccinated. Sera were obtained from each pig by venipuncture of the jugular vein before vaccination and necropsy. Seven pigs were randomly selected (3 from groups 1 and 2, and one from group 3) and euthanized on d9 post vaccination, and the remainder on d17. At necropsy, the left and right pre-scapular lymph nodes were weighed and blood taken from the jugular vein. Ileal and jejunal tissue was sampled at 10cm and 1m from the proximal ileo-caecal valve (ICV), respectively, together with adjacent mesenteric lymph nodes (MLN). Each tissue and MLN sample (ca 2x2mm) was stored in 10% neutral-buffered formalin fixative until analysis. In the adjacent segment, the intestinal mucosal secretions were collected by gently scraping the ileal mucosa with a sterile scalpel and placed into 2ml phosphate-buffered saline (PBS, pH 7.2) containing 0.2M EDTA on ice. The scrapings were each stored at -20°C. Prior to assay, mucosal scraping samples were thawed and adjusted to a protein level of 25mg of protein ml⁻¹.

4.2.4.2 Experiment 2b: Immune responses after challenge infection

Forty-six pigs were randomly allocated into 5 treatment groups. Groups 1-3 (10 pigs each) were vaccinated with 1x oral (1xOR- group1), 1x IM (1xIM- group 2), 10x oral (10xOR- group3), respectively, while 10 positive control pigs (PC- group 4) remained unvaccinated. The vaccination procedures were the same as described for

experiment 2a. Six pigs were kept unvaccinated and unchallenged as negative controls (NC- group 5). Four weeks after vaccination, each pig in groups 1-4 was dosed orally with *L. intracellularis* infected intestinal mucosa inoculum prepared and quantified as described elsewhere (Collins *et al.*, 1996; Collins and Love, 2007). Briefly, pigs were fasted overnight, sedated with 0.2mg/kg of azaperone (Stresnil®, Fivet, Australia) and intubated with an oro-gastric tube to enable administration of 25mL of the bacterial suspension containing around 10^9 *L. intracellularis*. Pigs were euthanized at a local abattoir 21 days post infection (21pi) where the gut was collected for analysis.

Pig live weights were recorded before vaccination, at challenge and at necropsy to determine the average daily live weight gain. Sera were collected from each pig on days 0pi, 7pi, 14pi and 21pi while faecal samples from individual animals were collected on days 0pi, 7pi, 10pi, 14pi, 17pi and 21pi for a quantitative *L. intracellularis* PCR (qPCR). At necropsy, ileal samples were collected as for experiment 2a and preserved in 10% buffered formalin prior to staining with H&E and immunohistochemistry (IHC). Following histopathological analysis, 24 pigs were selected from all groups according to the percentage affected area and *Lawsonia intracellularis* shedding numbers. There were four negative controls, ten severely affected and ten infected but without lesions, and also their sera from days 0pi and 21pi were tested for cytokines using porcine Quantikine® assay kits (Chapter 3).

4.2.5 Antibodies and cytokines analysis

Sera and mucosal scrapings were assayed in duplicate for the detection of *L. intracellularis* -specific IgM, IgG, IgA and concentrations of the cytokines, interferon-gamma (IFN- γ), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor-alpha (TNF- α) and transforming growth factor-beta1 (TGF- β 1). *L. intracellularis*-specific antibody was determined using a commercial competitive (blocking) ELISA kit (Bioscreen® Ileitis, GmbH, Münster, Germany) at a positive- negative cut-off of 30% inhibition as recommended by the manufacturers. In addition, *L. intracellularis*-specific IgM, IgG and IgA titres were assayed using an experimental modified direct ELISA developed for this project (Donahoo, 2009). The quantity of each of the cytokines IFN- γ , IL-6, IL-10, TNF- α and TGF- β 1 in mucosal scrapings or serum was analysed using commercial sandwich ELISA kits (porcine Quantikine® ELISA, R&D

systems, Minneapolis, USA) according to the manufacturer's directions (Chapter 3). The minimum limits of detection were 2.7 pg/mL for IFN- γ , 10 pg/mL for IL-6, 1.8 pg/mL for IL-10, 2.8 pg/mL for TNF- α and 19.5pg/mL for TGF- β 1. Concentrations were estimated by generating a logistic parameter curve using GraphPad®Prism software (v.4.02, 2004).

4.2.6 Correlation of serum cytokines with PE disease severity

The 20 infected pigs were selected from challenged groups according to the percentage affected area on IHC staining and *L. intracellularis* shedding in faeces (from subclinical to severe infection, Table 4). Cytokine concentrations were correlated with average weight gain, ileal lesions and quantities of *Lawsonia intracellularis* shed on days 14pi, 17pi and 21pi.

Table 4- Groups selected for serum cytokine analysis and results correlated to respective percentage affected area of PE lesions(IHC and HE) and numbers of *L. intracellularis* shedding (*Li g/faeces*) on day 21 pi.

Groups	IHC and HE	<i>Li</i> shedding (g/ faeces)	No. samples
Severe infection	>10%	10 ⁴ -10 ⁶	10
Subclinical infection	0-5%	10 ² -10 ⁴	10
Negative controls	0%	0	4

*IHC: Immunohistochemistry expressed as percentage affected area; HE: Haematoxylin and eosin;

4.2.7 *L. intracellularis* real-time PCR

Extraction and purification of bacterial DNA from individual faecal samples (0.1g) were made using the MagMax DNA extraction kit (A&B Applied Biosystems, California, USA) (Chapter 3). Extraction products were analysed by real time PCR (7500 Real-Time PCR System; Applied Biosystems) using primers and Taq Man probe as described by Nathues *et al.*, (2009). The sensitivity and specificity of the test in our laboratory have been reported by Collins *et al.* (2011) as 99% and 97%, respectively .

4.2.8 Histological lesion scores in intestinal tissue

Formalin-fixed tissues were stained with haematoxylin and eosin (H&E) (Luna, 1968) and by immunohistochemistry (IHC) with VPM53 *L. intracellularis* monoclonal antibody (Boehringer Ingelheim Vetmedica, Ames, USA) using the method described by Jensen *et al.*, (1997). The blinded slides were scored by one operator under 10x magnification light microscope to estimate the proportion of intestinal crypt

hyperplasia (H&E) and presence of *L. intracellularis* -specific antigen (IHC) within crypts. A total of fifteen fields was analysed for each section.

4.2.9 Skin testing for delayed type-hypersensitivity

Delayed type hypersensitivity reactions were examined in individual pigs 20 days after vaccination by intradermal (ID) injections of 0.2mL of sterile phosphate buffer saline (PBS, pH 7.2) (control) or around 10^5 *L. intracellularis* in 0.2ml of 10x Enterisol® Ileitis in two areas between nipples. The sites were examined at 24h and 48h and reactions were recorded as the diameter of erythema at the injection sites (Figure 11).





Figure 11: Delayed type hypersensitivity reaction test with intradermal injections of sterile phosphate buffer saline (control) or *L. intracellularis* vaccine.

4.2.10 Statistical analysis

Statistical differences were analysed among treatment groups and days post-vaccination and challenge using Restricted Maximum Likelihood (REML, GenStat Release 13th Ed., Oxford, UK) at a 95% confidence level ($P < 0.05$). Individual pigs were included as random effects in every analysis. Correlations between two unpaired variables were analysed using the Mann Whitney U test.

4.3 Results

4.3.1 Experiment 2a: Immune response to a ten times oral or intramuscular immunisation

Prior to vaccination, all pigs were PCR negative and seronegative for *L. intracellularis* and did not exhibit diarrhoea or clinical signs of PE after vaccination. Pigs gradually increased weights between days 0 and 17 post-vaccination, however, without differences between treatments groups (Figure 12). There was no evidence of gross or histopathological lesions of PE in ileal sections at necropsy (Figure 17). No significant differences between weights from left and right pre-scapular lymph nodes ($P > 0.05$) and delayed type hypersensitivity reaction were minimal between animals at 20 days after vaccination (Table 5).

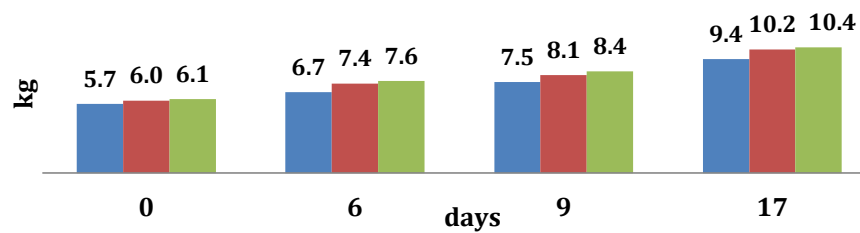


Figure 12: Predicted means for weight at each time point post-vaccination (days). (■) Negative Control (NC, blue), (■) ten-times oral (10xOR, red) and (■) ten-times intramuscular (10xIM, green).

Table 5- Mean weights from the left and right pre-scapular lymph nodes (PLN, grams) and delayed type hypersensitivity (DTH) reactions measurements after *L. intracellularis* (Li) vaccination.

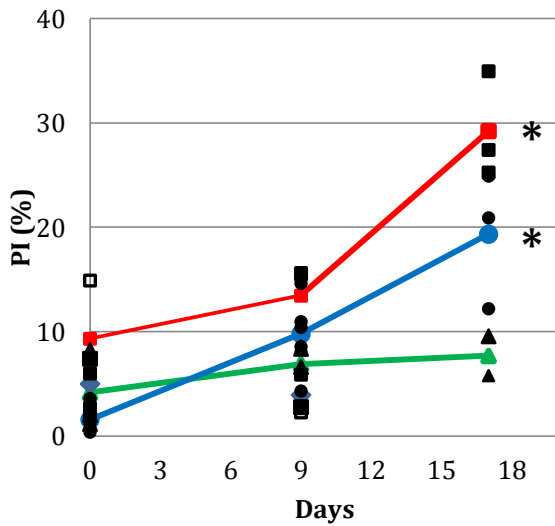
Treatment	Left PLN (grams)	Right PLN (grams)	DTH control PBS (mm)	DTH Li vaccine (mm)
10xOR	1.39	1.33	-	<0.5
10xIM	1.70	1.91	-	<0.5
NC	1.51	1.12	-	-

Increased concentrations of serum *L. intracellularis* IgG (direct) were detected in the orally and IM vaccinated pigs compared with the control group 3 between d9 and d17 ($P < 0.001$, Figure 13b). The serum IgG titres reached 85 (direct) and 24% inhibition (Bioscreen®) at day 17, and while similar values were detected in both vaccinated groups, they were significantly higher than control animals (33 and 7% inhibition, respectively in the two assays) (Figure 13). The orally vaccinated group produced serum IgM titres of 148 that was significantly higher ($P = 0.03$) than those expressed by pigs in the IM and control groups (62 and 55, respectively, Figure 14). *L. intracellularis* -specific IgA titres in sera and in mucosal secretions from all pigs were not different and all data titres less than 4 using the modified ELISA test.

In mucosal scrapings, higher titres of anti - *L. intracellularis* IgM on day 9 (270) were found in ileal scrapings from pigs vaccinated orally ($P < 0.001$) and IgG on day 17 (14%) for both oral and IM vaccinated animals (58 and 70, respectively) compared to control group (Figure 16a). Serum cytokines exhibited a large variation within animals from the same treatment group and no significant differences ($P > 0.05$) between treatments were apparent (Figure 15). In contrast, the concentrations of all five cytokines (IFN- γ , IL-6, IL-10, TNF- α , TGF- β 1) in mucosal secretions from oral vaccinates significantly increased between days 9 and 17 over values in control and

IM vaccinates (Figure 16). The quantity of TNF- α and TGF- β 1 in mucosal scrapings from oral vaccinates was significantly higher ($P < 0.05$) than the concentrations found in IM and control pigs on day 17.

a) Bioscreen®



b) direct

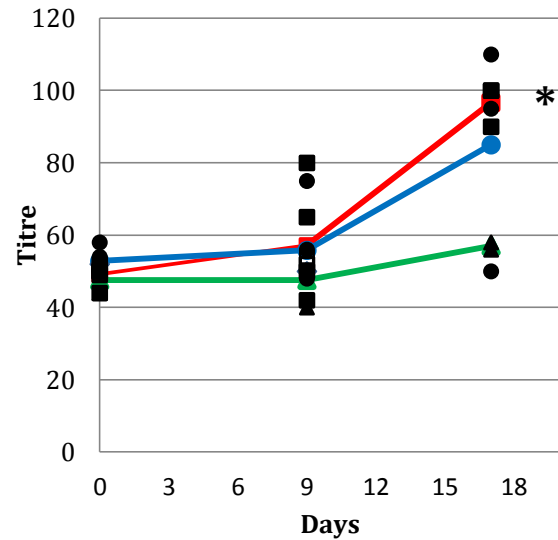
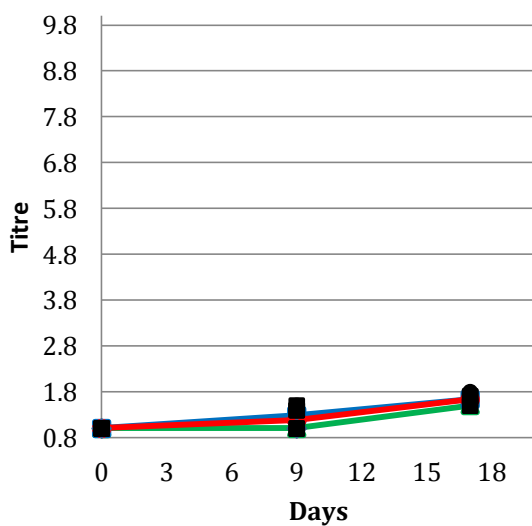


Figure 13: Humoral immune response to *L. intracellularis* Bioscreen ELISA (a), Direct ELISA for IgG (b). Lines correspond to the mean percentage of inhibition (PI) for (a) and mean titre for (b) for each treatment: (\blacktriangle) Negative control (green, $n=4$); (\bullet) 10xOral (red, $n=5$); (\blacksquare) 10xIM (Blue, $n=6$). * Superscript next to lines identifies significant difference between treatment and control groups ($P < 0.05$).

a) IgA



b) IgM

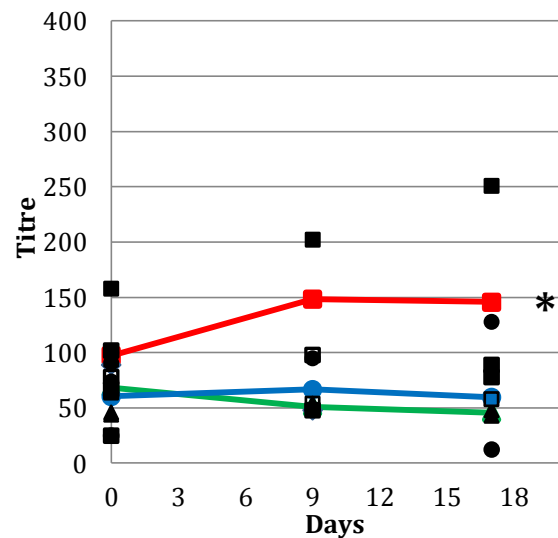


Figure 14: Humoral immune response to *L. intracellularis* IgA (a) and IgM (b). (\blacktriangle) Negative control (green, $n=4$); (\bullet) 10xOral (red, $n=5$); (\blacksquare) 10xIM (Blue, $n=6$). * Superscript next to lines identifies significant difference between treatment and control groups ($P < 0.05$).

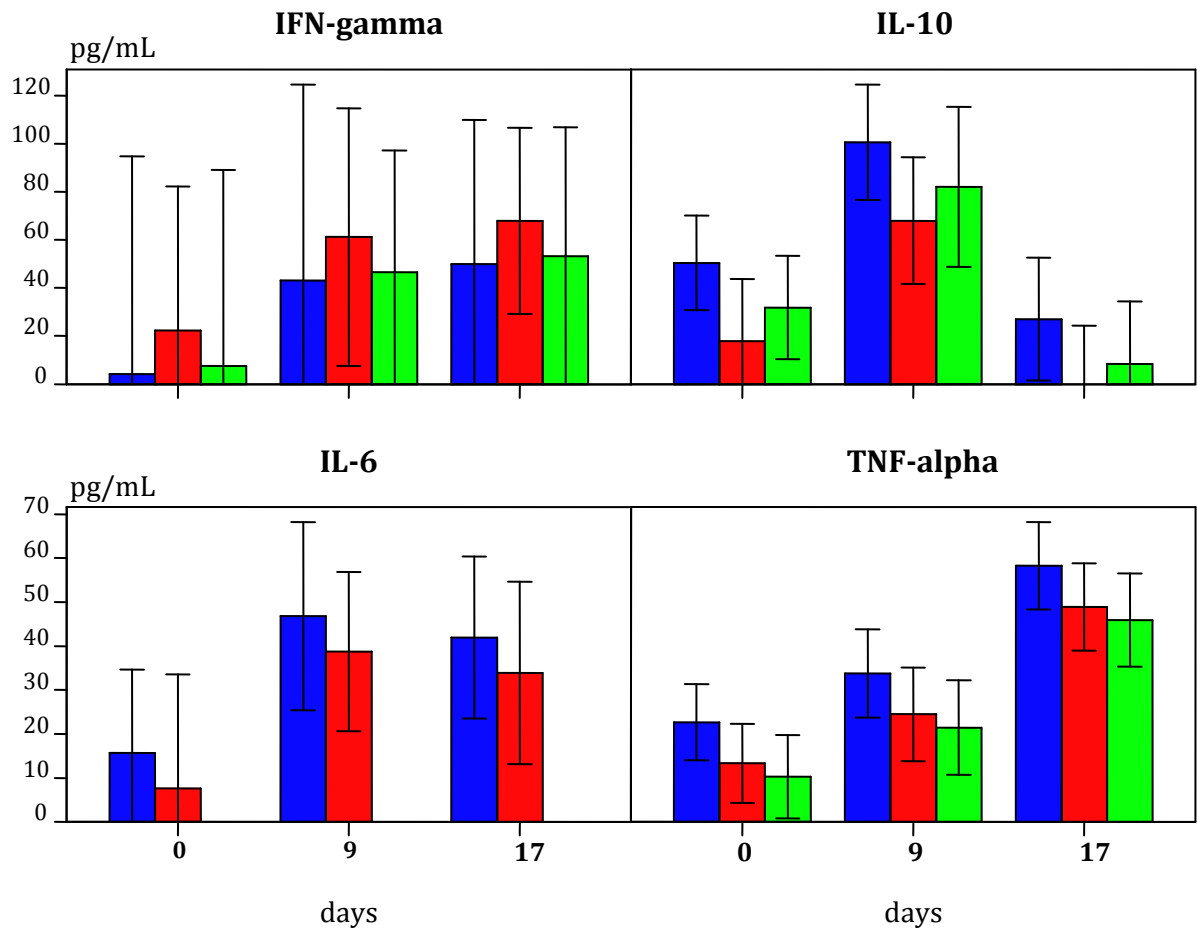


Figure 15: The concentration of circulating (serum) cytokines (pg/mL) on days 0, 9 and 17 after *L. intracellularis* vaccination within treatments: Negative control (green, n=4); 10xOral (red, n=5); 10xIM (blue, n=6).

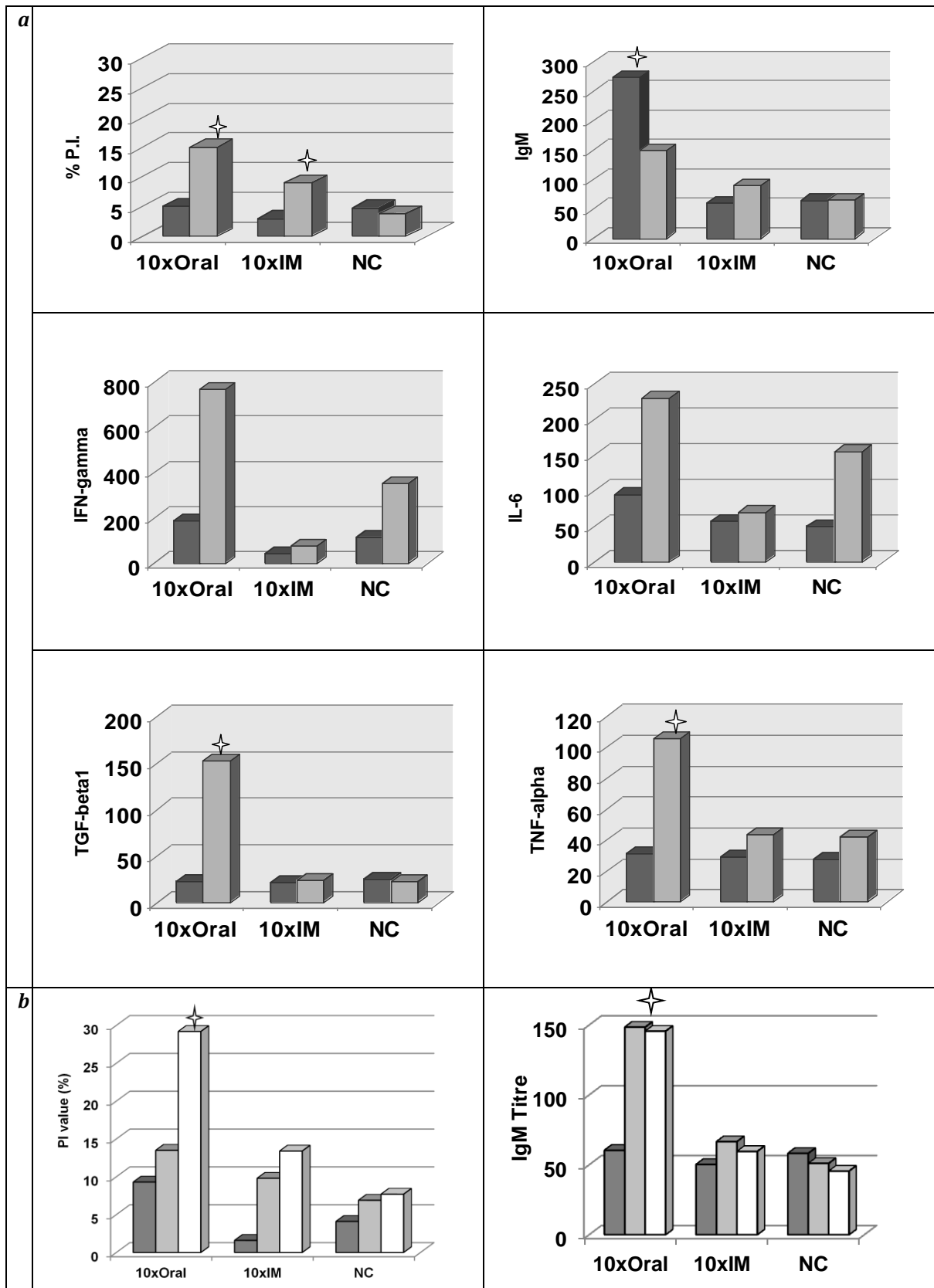


Figure 16: Mean concentrations for cytokines (pg/ml) and IgG (PI value) and IgM (Titre), *L. intracellularis* specific antibodies in (a) mucosal scrapings on days 9 (dark) and 17 (clear) and (b) in serum on days 0 (dark), 9 (shade) and 17 (clear) after ten-times oral (10xOral), intramuscular (10xIM) vaccination and unvaccinated (NC). Columns with a star on the top indicate statistical difference ($P < 0.05$).

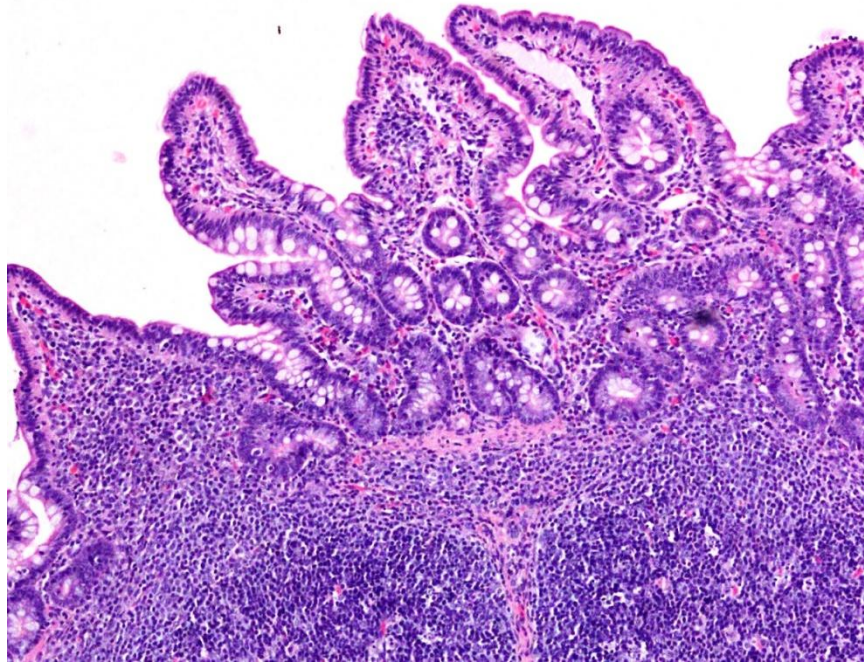


Figure 17: Ileum section of a pig (#B53), in HE staining, after 9 days orally vaccinated (10xOR) with *L. intracellularis*.

4.3.2 Experiment 2b: Protective immunity to Proliferative Enteropathy

All 40 pigs appeared clinically normal after challenge and there were no significant differences in average weight gain between groups over the 3 weeks following vaccination or challenge and over the entire 6 weeks of the trial (Table 6). Similar kinetics for the significant increases ($P < 0.001$) in serum *L. intracellularis* IgG and IgA titres were observed in challenged animals (groups 1-4) compared to negative control animals in group 5 (Figure 18). There were no significant differences ($P > 0.05$) in antibody titres between challenged groups. At day 21pi, all infected pigs were serologically positive for *L. intracellularis* -antibodies, with a mean P.I. of 48%.

Table 6- Average weight gains (kg) and approximate (\pm) standard errors (SE) of pigs between periods pre-challenge, post-challenge and total period of trial (d0: vaccination; d21: *L. intracellularis* challenge; d42: necropsy).

Treatment	Pre-challenge (d0-d21)	Post-challenge (d21- d42)	Total period (d0-d42)
Positive Control	6.67 \pm 0.52	9.44 \pm 0.72	16.12 \pm 0.91
1xOR	6.09 \pm 0.50	9.90 \pm 0.68	16.00 \pm 0.86
1xIM	6.28 \pm 0.50	8.82 \pm 0.68	15.10 \pm 0.86
10xOR	6.76 \pm 0.50	10.85 \pm 0.68	17.61 \pm 0.86
Negative Control	6.40 \pm 0.50	8.64 \pm 0.70	15.04 \pm 0.86
P (<0.05)	0.877	0.206	0.272

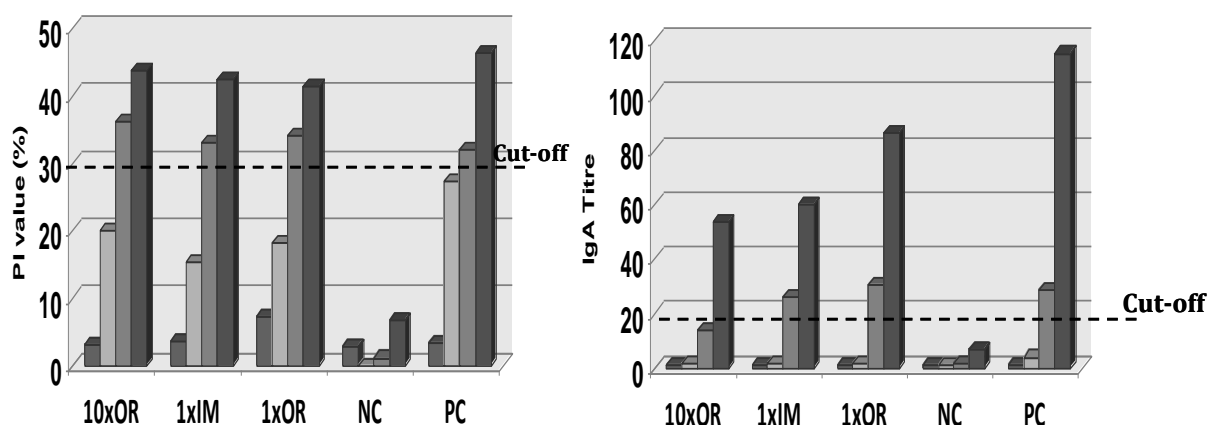


Figure 18: Predicted means for serum *L. intracellularis*-IgG (percentage inhibition, PI; left) and IgA titres (right) on days 0, 7, 14 and 21 in each group after *L. intracellularis* virulent challenge between groups ten-times oral (10xOR), one-time oral (1xOR) and intramuscular (1xIM), negative control (NC) and positive control (PC).

There was a range of pathology noted at necropsy (Figure 20) and determined by subsequent histological (Figures 21 and 22) and immunohistological analyses (Figures 23 and 24). Assessments of protection based on estimations of the numbers of *L. intracellularis* shed in faeces and the % affected area in ileal sections revealed that the vaccination significantly ($P < 0.001$) reduced *L. intracellularis* shedding when compared to positive control group. Pigs given the 10x oral vaccine were significantly ($P < 0.05$) more protected than the other 2 vaccinated groups (Figure 19 and 25).

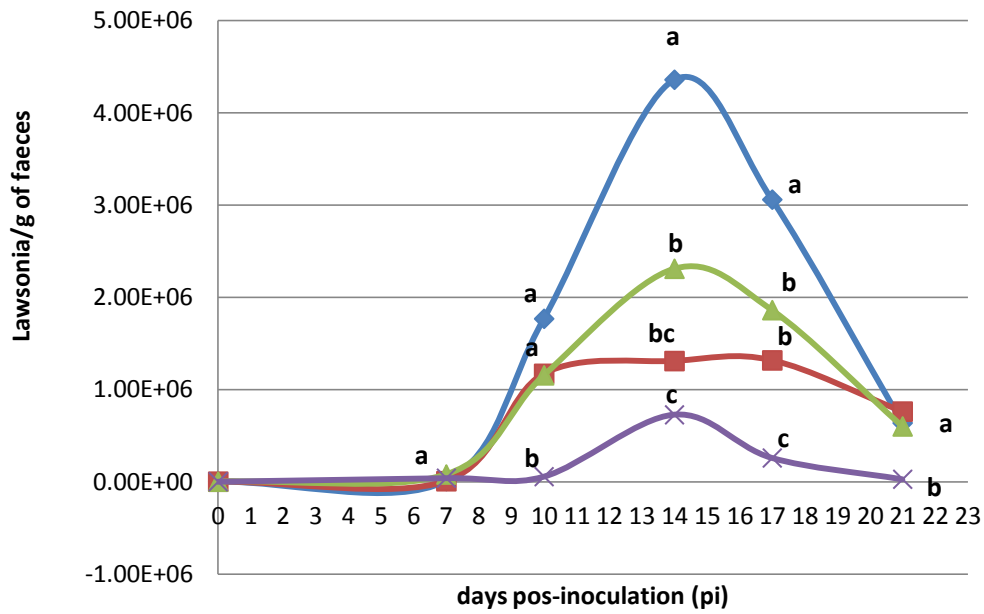


Figure 19: Estimation of *Lawsonia intracellularis* per gram of faeces at each time point post-infection for each group (♦, blue) Positive Control (PC); (■, red) 1x Oral; (▲, green) 1xIM, and (X, purple) 10xOral. On the same sampling values without common superscripts (a-c) are significantly different ($P < 0.05$).



Figure 20: Small intestine section from a positive control pig (#Y67), unvaccinated, with characteristic proliferation 21 days after *Lawsonia intracellularis* challenge (arrow).

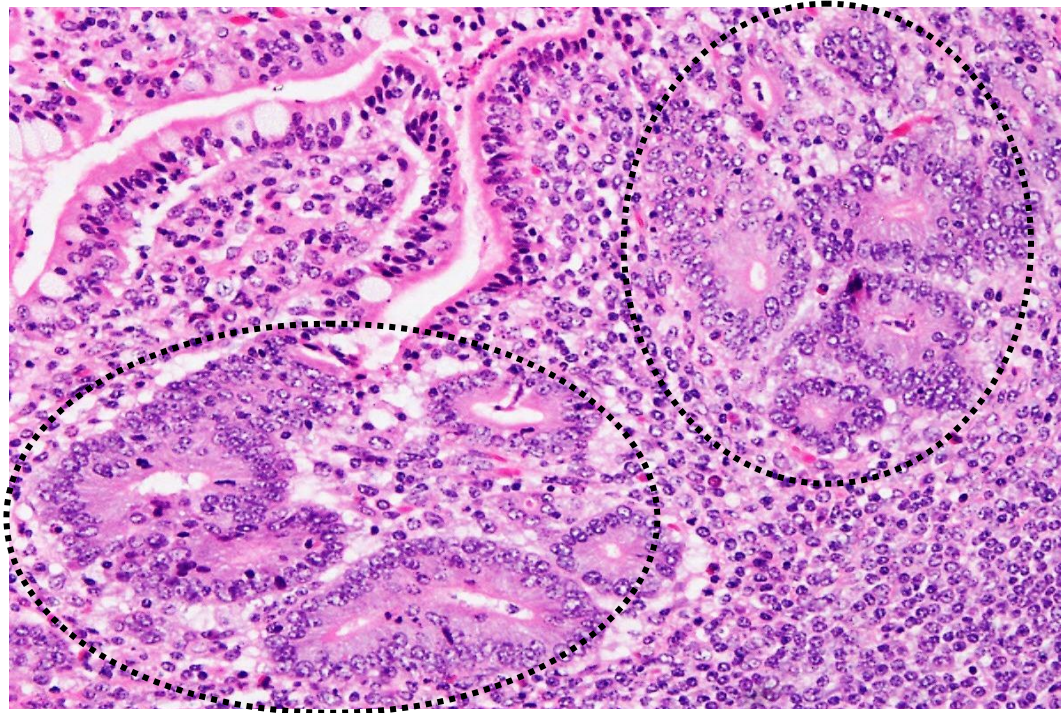


Figure 21: Ileal section from a pig (#G99) from group 1xIM presenting extensive crypt enterocytes proliferation and loss of goblet cells, 21 days after *Lawsonia intracellularis* challenge (circle).

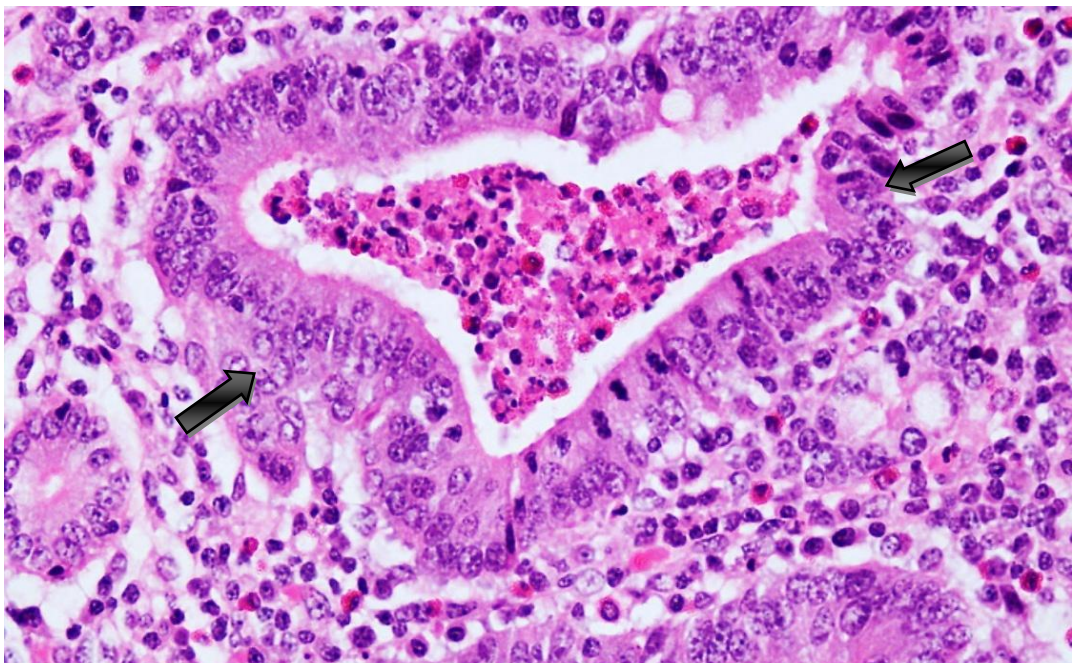


Figure 22: Proliferation of enterocytes in ileal crypt with loss of goblet cells and presence of exudate in the lumen (40 x mags) from pig (#075) orally vaccinated (1xOR) and challenged (arrow).

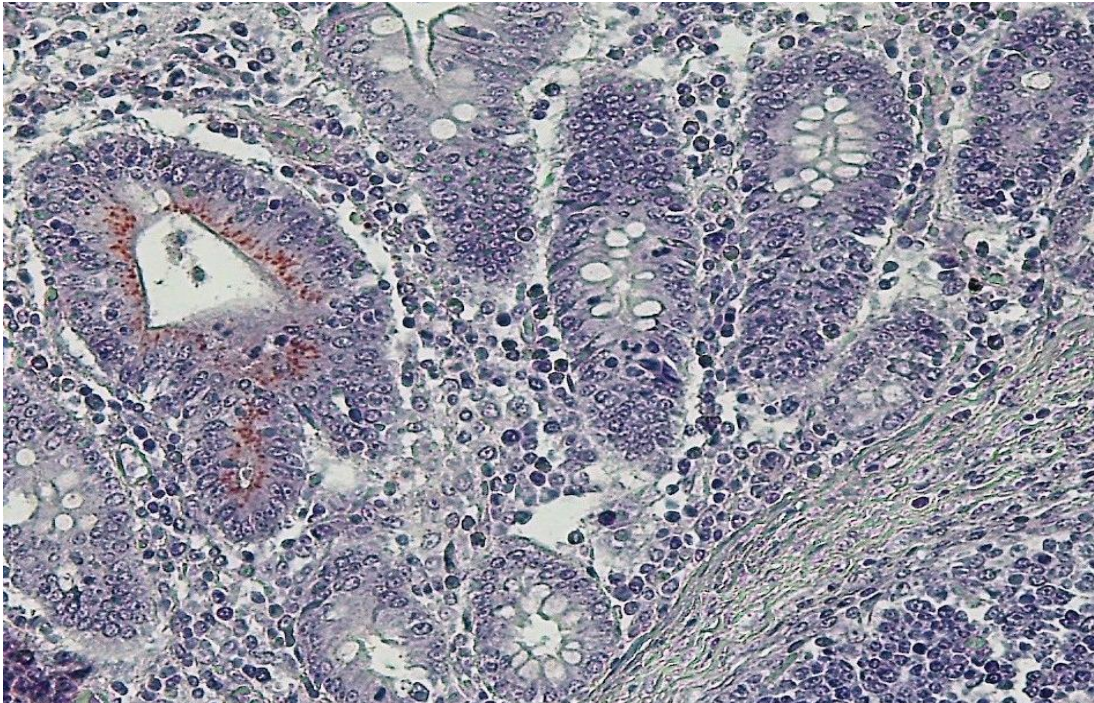


Figure 23: *Lawsonia intracellularis* antigen staining (brown, 20x mag.) in ileal section by immunohistochemistry from pig (#Y77), unvaccinated, from positive control group 21 days after challenge.

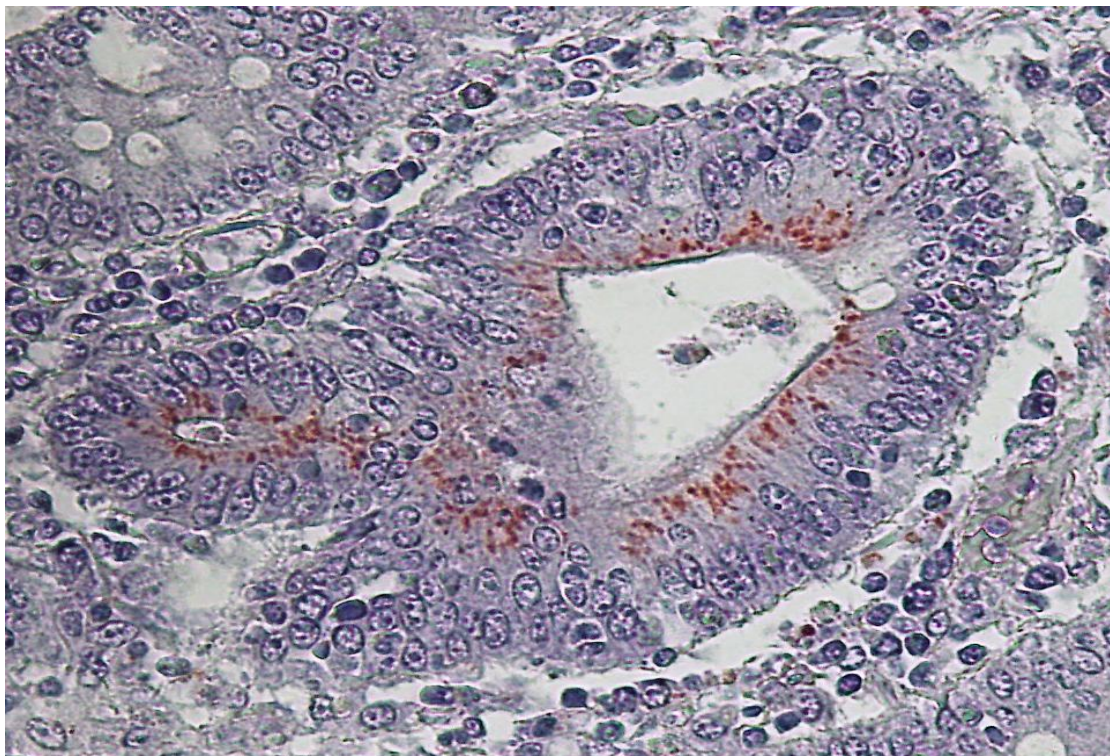


Figure 24: Ileal section in 40 times magnification from pig (#Y77), unvaccinated and challenged (positive control) with *Lawsonia intracellularis* antigen staining in the apical area of enterocytes (brown).

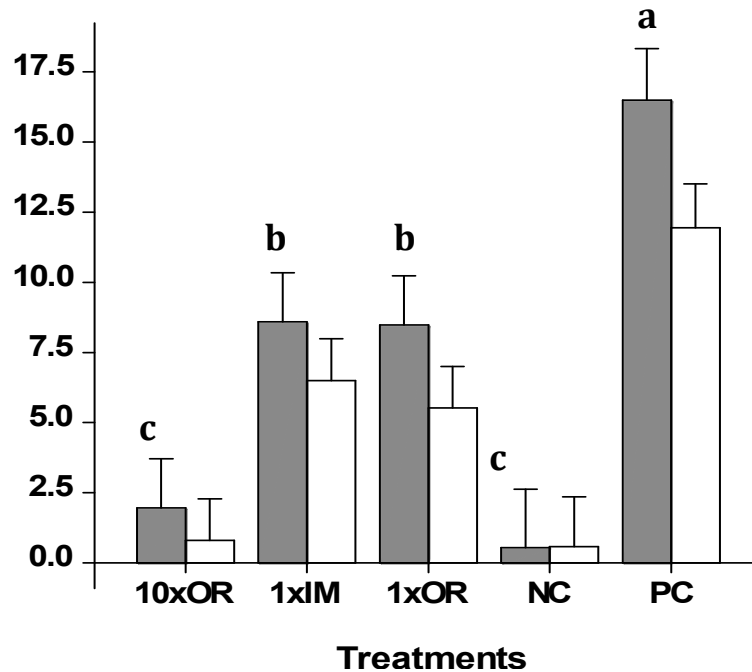


Figure 25: Mean percentage affected area (\pm SD) in the ileum for each group as determined by haematoxylin and eosin (shaded) and immunohistochemistry (clear) staining. ^{a-c} different letters differ significantly ($P < 0.05$).

4.3.3 Correlations between disease severity and serum cytokines

The serum cytokine concentrations for IFN- γ , IL-6, IL-10 and TNF- α were variable and while there was a significant ($P < 0.05$) increase in the level of each cytokine between days 0 and 21 post challenge in each of the 24 pigs tested, there was no correlation between cytokine concentrations and disease severity ($P > 0.05$; Table 7).

Table 7- Serum cytokines concentrations (pg/mL) in individual pigs from groups (G) PE severe infection (S), sub-clinical infection (SB) and negative controls (NC) and the correlations between group*lesions*cytokines, group*Li-shedding-in-faeces*cytokines, group*ADG*cytokine.

G	ID	Lesions		ADG kg	Li g/f	IFN- γ pg/mL		IL-6 pg/mL		IL-10 pg/mL		TNF- α pg/mL	
		HE %	IHC %			0pi	21pi	0pi	21pi	0pi	21pi	0pi	21pi
S	G99	35	10	22.2	10 ⁵⁻⁶	49.9	12.5	9.4	7.2	<	44.5	44.6	87.9
S	O81	31	15	16.0	10 ⁵⁻⁶	9.1	70.9	16.6	50.9	<	50.4	<	149.1
S	G64	27	10	14.8	10 ⁵	18.1	27.5	11.5	58.4	9.5	10.2	68.8	112.4
S	Y63	27	9	15.6	10 ⁵	<	29.6	<	98.4	57.5	119.5	47.3	326.9
S	Y68	24	2	17.7	10 ⁴⁻⁵	33.3	51.8	8.3	8.8	<	30.0	51.9	92.3
S	Y77	23	9	17.3	10 ⁴⁻⁶	14.3	60.6	36.2	77.0	22.9	77.9	89.3	196.5
S	Y70	20	2	18.4	10 ⁵	27.5	23.7	12.6	21.4	11.7	15.3	83.6	92.8
S	O54	19	7	18.4	10 ⁴	126	133.9	12.1	60.1	<	16.9	18.0	60.8
S	B81	14	0	22.8	10 ⁵	31.2	46.3	9.4	19.1	8.7	21.1	21.9	55.2
S	O100	11	1	14.5	10 ⁶	29.3	3.2	3.6	58.4	<	79.0	28.4	109.9
S	G100	10	5	17.2	10 ⁶	87.6	1.4	9.4	14.8	23.1	89.2	52.9	111.4
Mean		22	6	18.0	10 ⁵	43	42	13	43	22	50	51	127
SEM		5.8	5	0.8		22.5	38.9	16.7	15.9	8.6	89.6	26.8	42.6
SB	Y83	5	0	17.4	10 ⁴	<	<	43.4	20.8	47.6	54.48	28.4	58.9
SB	Y65	2	0	9.7	10 ⁵	5.1	29.6	7.2	75.4	<	17.56	48.7	363.2
SB	Y71	3	0	17.1	10 ⁵	23.7	1.4	11.0	9.9	<	<	18.0	62.6
SB	O77	0	0	14.2	10 ⁵	31.2	46.3	9.9	9.4	23.8	38.5	21.0	206.2
SB	G98	0	0	9.5	10 ⁴	<	<	8.8	9.4	<	79.2	33.3	90.8
SB	Y73	2	0	17.5	10 ⁴	27.5	12.5	90.8	4.1	45.3	78.4	38.7	96.7
SB	G53	0	0	15.7	10 ³	3.2	31.2	10.4	11.5	<	<	30.7	157.7
SB	O43	0	0	15.5	10 ⁴	1.4	<	14.8	37.1	<	56.4	40.0	69.7
SB	B98	0	0	17.9	10 ²	12.5	20.1	16.4	74.2	<	44.4	84.6	71.6
SB	O73	0	0	17.5	10 ³	35.1	20.1	13.1	21.9	85	97.1	33.3	118.5
Mean		1	0	15.0	10 ⁴	17	23	23	27	50	58	38	130
SEM		0.2	0	0.5		11.9	19.7	26.7	20.0	30.5	32.8	29.6	52.9
NC	B60	0	0	14.0	0	<	128.4	12.1	136.4	35.2	36.9	141	139.2
NC	B71	0	0	16.1	0	18.1	89.5	14.2	16.4	<	<	46.9	115.0
NC	O88	0	0	11.3	0	23.7	133.4	24.7	45.1	<	47.2	37.3	84.6
NC	W81	0	0	19.6	0	<	27.5	13.1	5.7	<	52.1	60.8	101.1
Mean		0	0	15.0	0	21	95	16	51	35	45	72	110
SEM		0	0	0.6	0	12.6	92.6	9.8	57.8	28.1	42.5	94.5	16.9
P (<0.05)		<0.05		NS	<0.05	NS	NS	NS	NS	NS	NS	NS	NS
G* lesions						NS	NS	NS	NS	NS	NS	NS	NS
G*Li						NS	NS	NS	NS	NS	NS	NS	NS
G*ADG						NS	NS	NS	NS	NS	NS	NS	NS

NS: Not significant; Li: L. intracellularis; ADG: average daily weight gain; IHC: immunohistochemistry; HE: Haematoxylin and eosin;

4.4 Discussion

This study monitored and detected dose-dependent increases in mucosal and serum IgM, IgG, IgA and cytokines following vaccination with Enterisol® Ileitis and subsequent challenge. Dose-dependent induction of protection against virulent *L. intracellularis* challenge was demonstrated by significant reduction in PE lesion scores and the magnitude and duration of bacterial shedding in faeces in all vaccinated pigs. As anticipated, oral vaccination induced local mucosal immunity with increased IgG and cytokine responses and tended to generate more comprehensive protection than IM vaccination.

The analysis of protective immune responses to *L. intracellularis* is an area of considerable interest for the control of PE. Initial studies on the development of humoral and cell-mediated immunity (involving analysis of IFN- γ responses) have described similar kinetics to those found in the current study. Dose-dependency of humoral responses is well documented. Following vaccination with $10^{4.9}$ TCID₅₀ *L. intracellularis*, serum antibody responses were either undetectable (Kroll *et al.*, 2004) or restricted to modest concentrations of *L. intracellularis*-specific IgG in ileal scrapings. Higher doses ($10^{5.9}$ TCID₅₀ in this study) of vaccine or primary infection with 10^5 - 10^{10} *L. intracellularis* induced detectable concentrations of *L. intracellularis*-specific IgG within 14 days after inoculation (Collins and Love, 2007; Riber *et al.*, 2011b). Consistent with previous analyses (Guedes and Gebhart, 2010; Jacobs *et al.*, 2011), mucosal IgA responses were of lesser magnitude and while the highest serum titres were generated by challenge control pigs (group 4), IgA titres were not boosted substantially by challenge of immunised animals. This could be dose related, as IgA accumulations have been demonstrated previously, by IHC, in apical cytoplasm of proliferating enterocytes from pigs with clinical cases of PIA and PHE (Lawson *et al.*, 1979; McOrist *et al.*, 1992). The timing and duration of humoral responses is also well studied and relatively consistent. In the current study, a 10x dose oral vaccination elicited increased concentrations of IgM, IgG in ileal mucosal scrapings and elevated concentrations of IgM and IgG in serum. The systemic and local IgM immune response after 9 days post vaccination precedes IgG (Lawson *et al.*, 1988) and has similar kinetics to increases in serum haptoglobin and C-reactive protein following primary infection with *L. intracellularis* (Riber *et al.*, 2011b). The induction of IgA was not detected in this study after vaccination with $10^{5.9}$ TCID₅₀ *L. intracellularis*, but

was apparent in serum from all pigs by 14 days after challenge with 10^9 *L. intracellularis*. The results agree with the detection of ileal IgA (McOrist *et al.*, 1992) and *L. intracellularis* -specific IgA in intestinal lavage by 21 days after *L. intracellularis* infection (Guedes and Gebhart, 2010).

In assessing the development and role of cell-mediated immunity in *L. intracellularis* immunity, immediate and delayed skin reactions to intradermal *L. intracellularis* antigen were not detected in immunised pigs in this and previous reports (Guedes and Gebhart, 2003b, 2010). However, in contrast to a 1x dose or following IM vaccination, the local intestinal response induced by the 10x oral vaccine activated the production of each of the five cytokines assayed in ileal scrapings (IFN- γ , IL-6, IL-10, TNF- α and TGF- β 1) with significant concentrations of TNF- α and TGF- β 1 detectable by day 17 after vaccination. In mice, IFN- γ receptor knock-out animals (IFN- γ R⁻) had failed to resolve *L. intracellularis* infection after 35 days whereas normal 129 mice were clear after 21 days (Smith *et al.*, 2000). This indicated a role for IFN- γ in immunity but while elevated, individual variations in the concentrations of IFN- γ found in immunised pigs in this trial precluded significant differences compared to control animals. Given the intracellular pathogenesis of *L. intracellularis* infection, subsequent studies have concentrated on the role of IFN- γ as evidence of cellular immunity and protection against re-infection (Guedes and Gebhart, 2010; Cordes *et al.*, 2012). In pigs, the generation of antigen-sensitised (CD4⁺ and CD8⁺) lymphocytes was evident from the production of *L. Intracellularis* specific IFN- γ in peripheral blood mononuclear cells (PBMC) in 12-13 week-old pigs, within 14 days after infection. But was delayed in appearance in 5-6 week-old animals (Guedes and Gebhart, 2010; Cordes *et al.*, 2012). It was noted that the pigs in the current trial were 5 weeks old when vaccinated and produced elevated cytokine responses within 17 days. The differences in age-related responses could be explained by a number of factors including breed and nutrition, but may also reflect the time after vaccination (14 vs. 17 days). *L. intracellularis* specific IFN- γ producing cells were also detected by ELISPOT, in porcine PBMC, 4 to 13 weeks post vaccination (Guedes and Gebhart, 2003b) and after 15 days in horses vaccinated rectally with 10^6 attenuated *L. intracellularis* (Pusterla *et al.*, 2009). Three weeks after infection with 5×10^9 *L. intracellularis*, Riber *et al.*, (2011a) detected increased amounts of IFN- γ in

the supernatant fluid after incubation of whole porcine blood with *Lawsonia* antigen and the inflammatory cytokine, IL-18. In contrast, limited microarray studies of pigs with varying concentrations of intestinal pathology did not reveal substantial activation of cytokine genes (Jacobson *et al.*, 2011a). While the synthesis of the three “inflammatory” cytokines (IFN- γ from NK cells, IL-6, and TNF- α) would result from local infection and serve to activate the acquired immune system, including IFN- γ -producing T-cells and IgG production. The quantities of IL-10 and TGF- β 1 protein were also increased in the ileal scrapings. This may indicate induction of local immunoregulation through T-reg cells (Chen *et al.*, 2007; Bailey, 2009), and local restoration, given the role of TGF- β 1 in wound healing in pigs (Quaglino *et al.*, 1991). Increased concentrations of IL-10 and TGF- β 1 are also consistent with detection of insulin-like growth factor binding protein 3 (IGFBP-3) during the immune priming following oral infection or vaccination with *L. intracellularis* (Jacobson *et al.*, 2011a).

To induce protective immune responses at mucosal surfaces, oral vaccination with live attenuated or killed/ subunit vaccines is the preferred protocol to block pathogen attachment or to neutralize local virulence factors by specific IgA or IgG antibody (Mestecky and McGhee, 1987; Husband *et al.*, 1996). Attenuated live vaccines are used to generate protective immunity against intracellular pathogens. For *L. intracellularis*, oral administration has been effective, but rectal administration of $10^{6.3}$ TCID₅₀ *L. intracellularis* generated *L. intracellularis* specific IgG detectable responses in horses (Pusterla *et al.*, 2009) and intraperitoneal (IP) vaccination against *Salmonella* Typhimurium has effectively immunised livestock (Muir *et al.*, 1998). When the quantitative and qualitative aspect of *L. intracellularis* challenge was determined by real-time PCR in faeces, the ten times oral vaccination ($10^{5.9}$ TCID₅₀) elicited the greatest protection but the 1x intramuscular and 1x oral vaccines were also efficacious. Since *L. Intracellularis* enters and multiplies in immature (crypt) enterocytes (Lawson and Gebhart, 2000), the 1x IM protocol suggests that serum antibody can significantly reduce the number of *L. Intracellularis* available in the gastrointestinal tract prior to cellular entry. Results with inactivated or subcellular *L. intracellularis* vaccines would support this mechanism. Dale *et al* (1997) reported that faecal *L. intracellularis* counts were reduced by 98.5% in four pigs vaccinated twice intramuscularly (IM), 3 weeks apart, with killed *L. intracellularis* in incomplete

Freunds adjuvant. Additional pigs inoculated with recombinant GroEL-like protein exhibited reduced faecal bacteria counts compared to infected controls (Dale *et al.*, 1997). In their patent, Jacobs *et al.*, (2011) also reported the induction of significant protection against *L. intracellularis* challenge after two IM inocula were given 4 weeks apart, of 2.8×10^8 killed *L. intracellularis* in oil adjuvant. These researchers reported significant reductions in *L. intracellularis* shedding after initial IM vaccination of pigs with 50µg each of recombinant 19/21 and 37kDa outer membrane proteins (OMPs) or putative LPS in adjuvant. Dose dependent protection was achieved with vaccines containing killed organisms where 5×10^7 *L. intracellularis* elicited protection and 1.25×10^7 *L. intracellularis* was ineffective (Jacobs *et al.*, 2011). These results and the fact that IM administration of Enterisol® would be expected to kill the live *L. intracellularis* rapidly after inoculation, indicate that with sufficient antigenic stimulus, antibodies against *L. intracellularis* cells and components can protect against infection. Given the elevated serological titres at challenge, this presumably occurs by preventing intracellular entry. That significant mucosal protection can be induced by relatively small doses of live oral *L. intracellularis* without substantial serological titres (Kroll *et al.*, 2004) would indicate both a dose-dependent and site-specific response.

In the search for readily accessible biomarkers for immunity to *L. Intracellularis*, antibody responses have not been informative (Kroll *et al.*, 2004; Cordes *et al.*, 2012), with IgA responses low and ephemeral (Cordes *et al.*, 2012). Skin tests are also unreliable (Guedes and Gebhart, 2010), as was found in this trial. In addition, the measurement of cytokine concentrations in body fluids such as serum and mucosal secretions is costly and is complicated by the dynamic responses to *L. intracellularis* in individual pigs where the variations between individuals are often greater than the responses within the treatment groups (Jacobson *et al.*, 2011a; Cordes *et al.*, 2012). Unlike cases where clinical signs have been correlated with serum IFN-γ, IL-6 and TNF- α in pigs experimentally infected with *B. hyodysenteriae* (Kruse *et al.*, 2008), pigs with clinical signs of diarrhoea after natural infection with *L. intracellularis* showed high variability of TNF-α and TGF-β1 concentrations in sera and poor cytokine expression in intestinal tissues (Jacobson *et al.*, 2011a). In contrast, this study documented increased TNF-α and TGF-β1 in mucosal secretions in pigs from

the 10x orally vaccinated group while individual variations in IFN- γ , IL-6 and IL-10 precluded significant results. However, in this trial, an attempt was made to define an immune correlate between vaccination and protection. A sequential serological analysis of antibody and TNF- α in 24 pigs with varying concentrations of immunity as assessed by bacterial excretion and ileal histopathology did not reveal any significant associations. The results concurred with previous reports (Jacobson *et al.*, 2011a; Cordes *et al.*, 2012). These contrasting findings highlight the difficulties associated with finding suitable systemic biomarkers which illuminate the immune status of mucosal tissues.

In conclusion, this study was able to demonstrate the dose-dependent correlation to increased local and systemic IgG and IgM responses after vaccination and protection by reducing lesions and *L. intracellularis* shedding. Although protective immunity was also elicited when Enterisol®Ileitis was administered IM, suggesting antibody-mediated neutralisation, the precise mechanisms and immune correlates underscoring the more comprehensive protection following oral vaccination are still not completely resolved.

Chapter 5 The effect of route of vaccination with *Lawsonia intracellularis* on immune responses in weaner pigs

(Modified from: Nogueira, MG; Emery, D; Collins AM (2013). Impact of route of administration on the mucosal immune response to *Lawsonia intracellularis* vaccine in pigs. *Australian Veterinary Journal*. Submitted.)

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5.1 Introduction

Lawsonia intracellularis is an obligate intracellular Gram negative bacterium that causes PE in pigs (McOrist *et al.*, 1993). The major effects of PE infection in pigs includes poor growth, diarrhoea or sudden death (Lawson and Gebhart, 2000). It is a financially significant production problem estimated to reduce profitability from AU\$ 8 to 13.0 per pig (Holyoake *et al.*, 2010a), requires continuous inclusion of antibiotics in the feed of grower and finisher pigs on farms to prevent the occurrence of clinical disease. The trial in the previous chapter has documented the humoral and cell mediated immune responses after experimental exposure with pathogenic *L. intracellularis*, and has been reported in other experimental and field trials (Guedes *et al.*, 2002a; Collins and Love, 2007). Oral administration of live *L. intracellularis* vaccine reduces clinical signs and lesions of PE (Kroll *et al.*, 2004), without generating consistently detectable systemic immune responses. Without evidence of vaccine induced protection, many veterinarians are unwilling to recommend the removal of antibiotic medication for fear of outbreaks of the haemorrhagic form of PE. Oral delivery of mucosal vaccines reliably induces immunity against enteric pathogens,

but protection has also been demonstrated after intraperitoneal (IP) or intramuscular (IM) administration (Djordjevic *et al.*, 1997; Muir *et al.*, 1998).

As part of routine farm management, piglets are often vaccinated while they're still suckling to overcome the need to remove antibiotics for 5 days around vaccination. In some herds, this leads to reduced vaccine efficacy because maternal antibodies transferred to the piglet in the sow's milk can inactivate the vaccine (Hodgins *et al.*, 1999). Efficacy data on intramuscular or intraperitoneal administration of the vaccine may allow producers to vaccinate and protect weaner pigs from ileitis without needing to remove antibiotics to treat other infections. In the previous experimental challenge trial, protection was successfully induced by IM vaccination against challenge with *L. intracellularis* (Nogueira *et al.*, 2013). Potential correlates for protection were identified, but their specificity in the field needed to be tested. This is because weaner pigs face multiple challenges that would stimulate the immune system at the same time as *L. intracellularis* vaccination, including the introduction of solid feed and other infections such as haemolytic *E. coli*. Therefore, efficient strategies to induce mucosal protection to a range of enteric diseases in young pigs is necessary, especially to complement nutritional additives and to replace the need for antibiotics in feed. To ensure that vaccines have effectively immunised animals prior to exposure to pathogens, knowledge of the protective immune response is also highly beneficial. In the search for an immune correlate with protection, and to further define the interaction between the routes of inoculation, this investigation compared local and systemic immune responses after oral, IM and IP inoculation of *L. intracellularis* vaccine in a trial on farm.

5.2 Methods

5.2.1 Code of Ethics

This research was approved by the Animal Ethics Committees of the Elizabeth Macarthur Agricultural Institute (Department of Primary Industries-DPI) and the Sydney University (M12/03), Australia.

5.2.2 Farm health status and pre-screening

The trial was conducted on a commercial piggery located in the state of Victoria, Australia between months of March and April of 2012. According to reports from the farm veterinarian (Dr. Hugo Dunlop, Chris Richards & Associates), the commercial farm consisted of 650 farrowing to finish batch unit, with an additional 2 grow-out sites. Pre and post weaning mortality were below 10% and 3.5%, respectively. Gilts and sows were routinely vaccinated against pathogenic neonatal *E. coli*, Glässer's Disease and *Streptococcus suis* at introduction to the herd and 3 weeks prior to farrowing. The farm was free of *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* (sv. 1, 5, 7, 15). Historically, *Brachyspira hyodysenteriae* and *Ascaris suum* have been present on this farm, but based on regular farm visits and slaughter inspections of pigs there has been no clinical evidence of their presence for the last 5 years. Clinical outbreaks of Glässer's disease and *Streptococcus suis* occasionally occur from 11 weeks of age; they are treated using water medication. Post-weaning diarrhoea and PE infections has not been a significant problem of this farm, however, preventive antibiotics are used at strategic times post-weaning. In the routine management of the herds, progeny are normally vaccinated against proliferative enteropathy and porcine circovirus type 2 three days prior to weaning. Piglets are normally weaned at 22-24 days of age into an all-in-all-out room with controlled ventilation until they are 8 weeks of age and then are moved into a naturally ventilated grower facility until 16 weeks of age, where they stay until sale.

Prior to the trial, a pre-screening selection was performed to observe the dynamics of *Lawsonia intracellularis* infection of this farm. Blood was collected from 12 to 16 weeks old pigs for serological analysis (Bioscreen® Elisa) and pen pooled faeces from 24, 37, 53, 70 and 98 day old pigs were collected and screened to estimate numbers of *L. intracellularis* in faeces (qPCR). Results indicated that at 12 weeks of age, 67% of the pigs tested were seropositive for *L. intracellularis* but this prevalence had declined to 13.3% at 16 weeks of age. The presence of *L. intracellularis* was observed in pooled faeces from 37, 53 and 70 days old pigs (from 10^3 to 10^4 *L. intracellularis* per gram of faeces).

5.2.3 Experimental design

Forty Landrace/ Large White crossbred piglets were selected at 21 days of age (5 days pre-weaning). Grouping of piglets was performed by randomly selecting four offspring from one sow and allocating one pig to each of the four treatment groups: oral (OR), intramuscular (IM), intraperitoneal (IP) and unvaccinated control groups (Figure 26). This was repeated for ten sows until each group had ten piglets. At 26 days of age (just after weaning), 10 pigs were orally vaccinated with 2.0 mL of a ten times dose concentrate ($10^{5.9}$ TCID₅₀) of *L. intracellularis* vaccine via drench gun (Enterisol® Ileitis, Boehringer Ingelheim, US). A second group was given the same 2.0 mL dose IM via syringe in the right shoulder (21G needle) and the third group was vaccinated IP through the inguinal region in the lower half of the abdomen using a 23G needle (d0) (Figure 27). The remaining 10 pigs were kept unvaccinated as a negative control group. Routine herd management was continued with sows and piglets given water and feed *ad libitum*. Antibiotics were removed from feed and water 3 days prior and 2 days after vaccination.



Piglets	Vaccination	Vaccination route	N° of <i>Li</i> per dose	N° pigs
1	Yes	Oral	$10^{5.9}$ TCID ₅₀	10
2	Yes	Intramuscular	$10^{5.9}$ TCID ₅₀	10
3	Yes	Intraperitoneal	$10^{5.9}$ TCID ₅₀	10
4	No	-	-	10

Figure 26: On farm route of *Lawsonia intracellularis* (*Li*) vaccination.

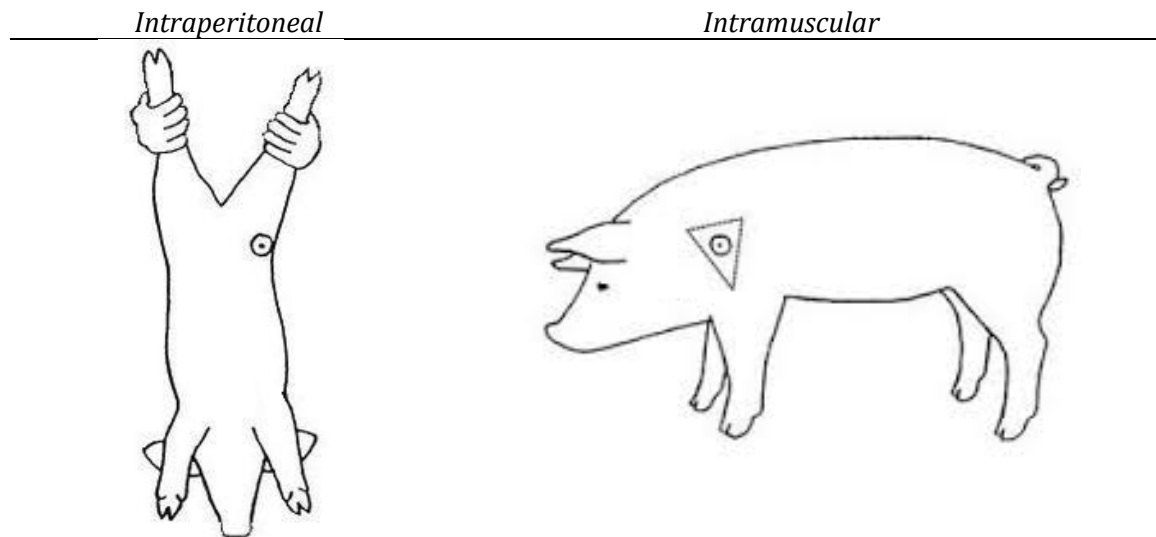


Figure 27: Local of intraperitoneal and intramuscular routes of vaccine administration (Anonymous, 2010).

5.2.4 Sample collection and analyses

Blood was collected from individual pigs on day 0, 8 and 17 post-vaccination (pi) for further serological analysis. Necropsy of the pigs was performed 17 days after vaccination. All pigs were transported to Department of Primary Industries regional laboratory necropsy room (Bendigo, Victoria), where pigs were sedated (Xylazil IM 1.5ml/pig) and euthanized (Lethobarb IV 1 mL/pig) by a DPI veterinarian. All post-mortem sampling was conducted essentially as described in Chapters 3. Ileal tissue sections were placed in 10% formalin for histopathology and mucosal scrapings were collected and processed as previous described. Blood and mucosal secretions were assessed for *L. intracellularis* specific IgG antibodies (as percentage inhibition, PI%) using the Bioscreen® Ileitis ELISA (GmnH, Münster, Germany) and IgA was assessed with an *in-house* modified direct ELISA. PI values for greater than 30% were interpreted as positive for *L. intracellularis* antibodies and values less than 20% were deemed negative. The quantities of IFN- γ , TNF- α , IL-6, IL-10 and TGF- β 1 cytokines in ileal secretions were analysed (Quantikine®ELISA kit; R&D Systems, US), with minimum limits of detection for this trial in pg/mL were; 3.8 (IFN- γ); 2.7 (TNF- α); 15.3 (IL-6); 3.2 (IL-10); 2.8 (TGF- β 1).

5.1 Statistical analysis

The IgA titres and cytokines concentrations (pg/mL) were estimated by generating a logistic parameter curve using GraphPad®Prism software (2004). Statistical analysis was performed in GenStat 13th ed. (2010) using restricted maximum likelihood test (REML), testing each group vs. the controls; and also testing differences between vaccinated groups. Sow parity and individual pigs were included as random effects.

5.2 Results

5.2.1 Antibody responses

Prior to vaccination, all piglets were seronegative for *L. intracellularis* antibodies. Increased *Lawsonia intracellularis* specific IgG in serum was detected in each vaccinated group (Figure 28; $P < 0.05$) between day 0 and 17 post-vaccination (pv). By day 17 pv, pigs in the oral (28.9%), IP (11.7%) and IM (15.8%) vaccinated groups had produced a significant increase in *L. intracellularis* IgG antibody compared to the unvaccinated pigs (3.7%). However, only orally vaccinated pigs (9/10) were above the seropositive cut-off (30% PI).

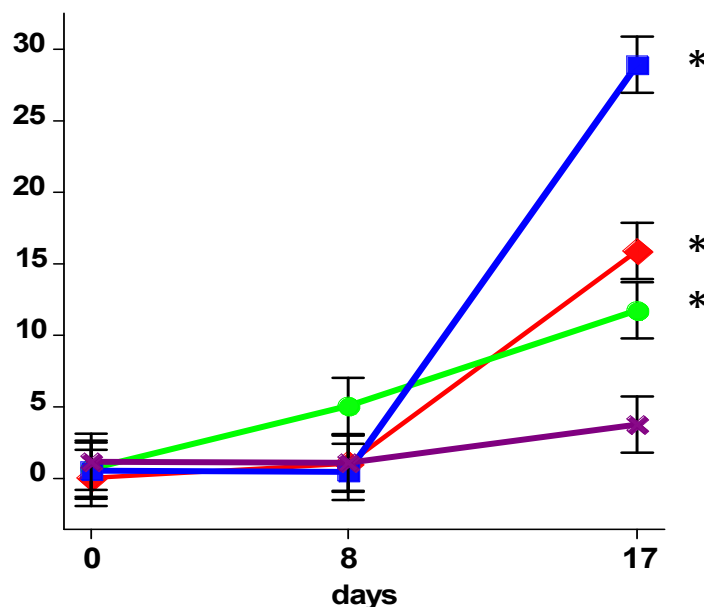


Figure 28: Predicted mean and standard errors for serum *Lawsonia intracellularis* IgG percentage inhibition (P.I.) value on days 0, 8 and 17 post-vaccination with Enterisol® via intramuscular (IM, ◆, red), intraperitoneal (IP, ●, green), oral (■, blue) and unvaccinated (×, purple) groups. On day 17 means with different superscripts differ significantly (*, $P < 0.05$).

In the ileal mucosa, the majority of pigs in all vaccinated groups were above the cut-off value (PI: 30%) at day 17 pv, with positive values in oral (8/10), IP (8/10), IM (7/10), but also unvaccinated pigs (6/10) for *L. intracellularis* IgG antibody (Figure 29). However, vaccinated pigs generated significant increases in *L. intracellularis*-IgG concentrations in the ileal mucosa when compared with unvaccinated pigs at day 17 post-vaccination (Figure 30). In addition, oral and IP vaccination also generated significant ($P<0.05$) mucosal increases in *L. intracellularis* IgA, but the humoral immune response to IM vaccination was limited to increased IgG (Figure 30).

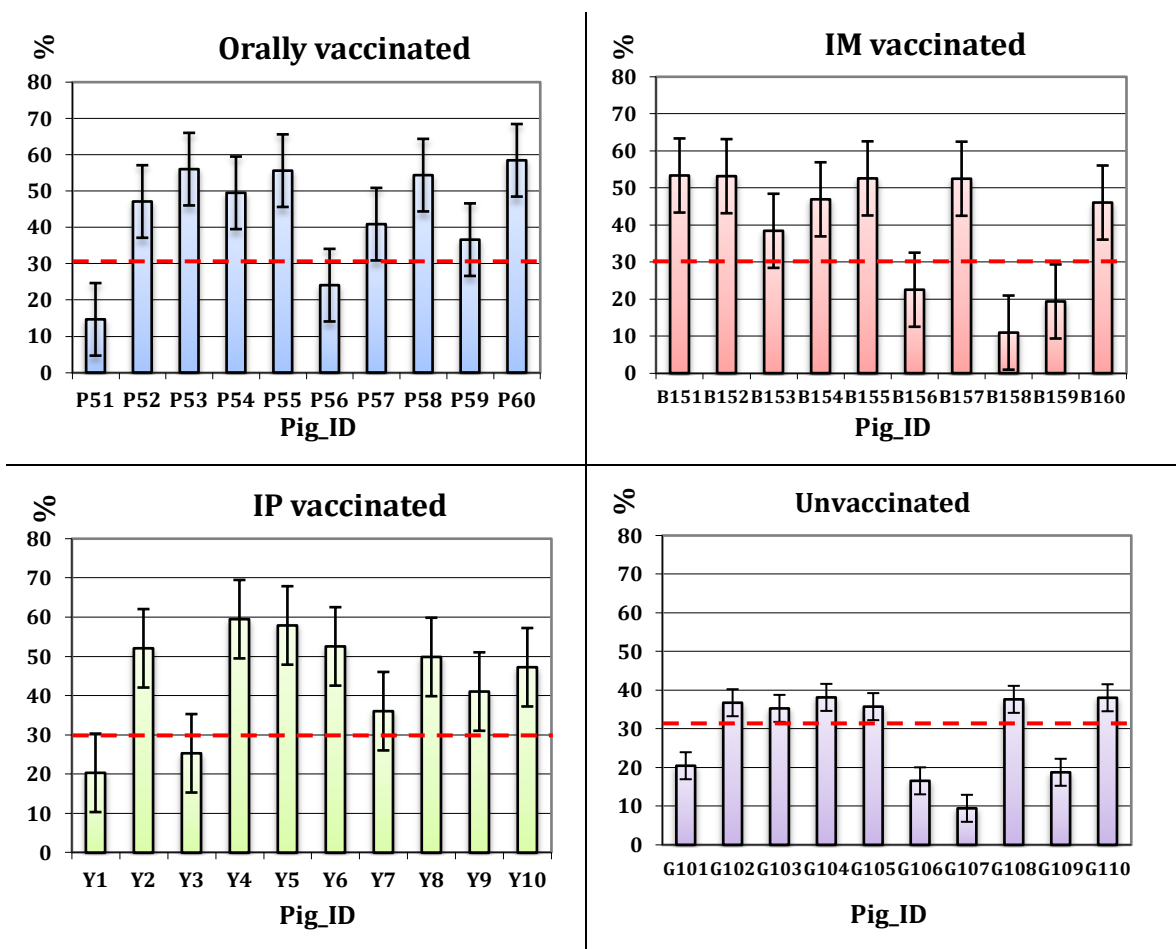


Figure 29: Lawsonia intracellularis specific IgG antibody, as percentage inhibition values (% PI value) in ileal mucosa of individual pigs, 17 days after vaccination with *L. intracellularis*. The cut-off value of 30% is highlighted (red line).

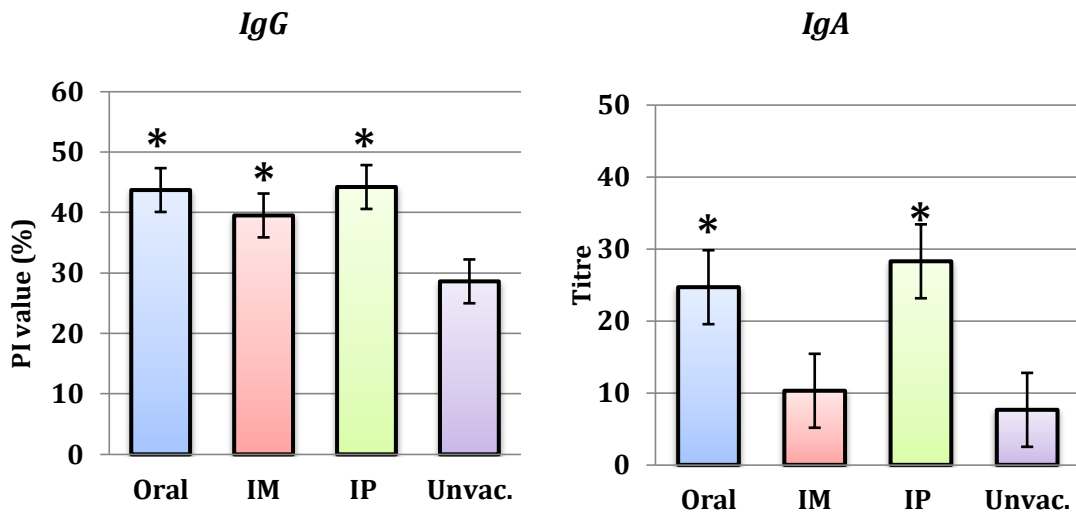
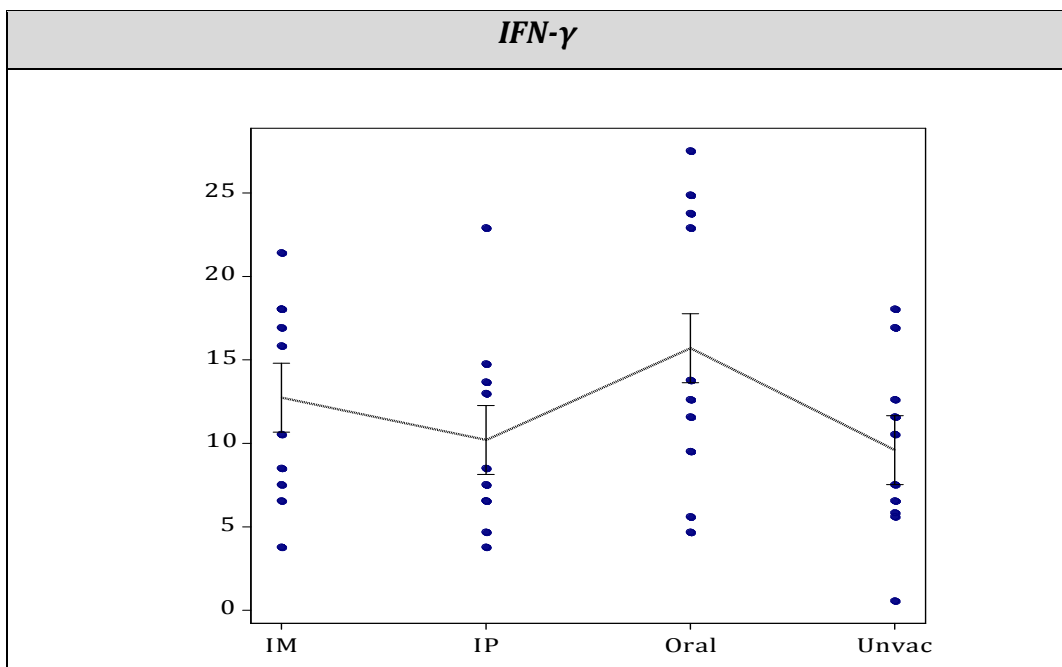


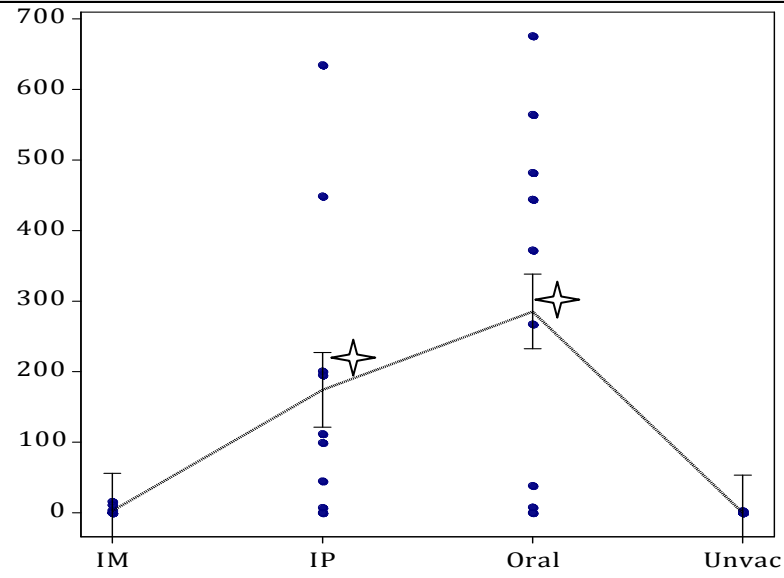
Figure 30: Predicted means and standard errors for concentrations of *Lawsonia intracellularis* IgG (percentage inhibition, PI, %) and IgA (Titre) in ileal mucosa secretions at day 17 post-vaccination. * Significant differences between treatment groups vs. controls.

5.2.1 Mucosal cytokine responses

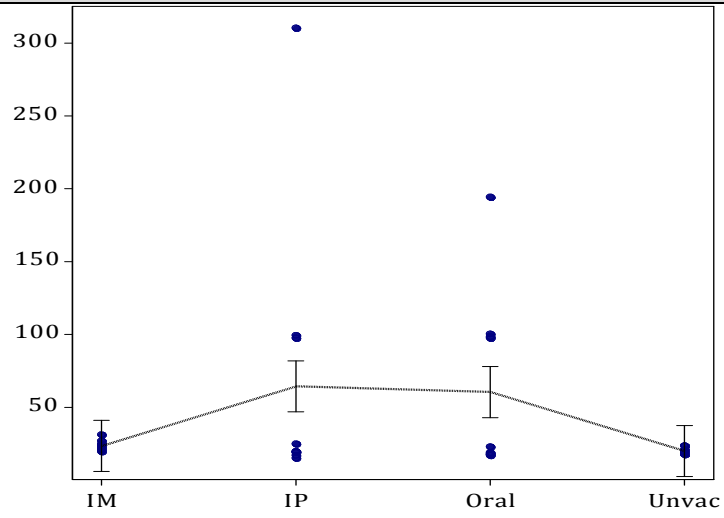
The mucosal cytokines responses for individual pigs within groups are summarized in the Figure 31. In this on farm study, piglets generated significant ($P < 0.05$) increases in mucosal concentrations of TNF- α after oral and IP immunisation and TGF- $\beta 1$ after oral vaccination ($P < 0.05$).



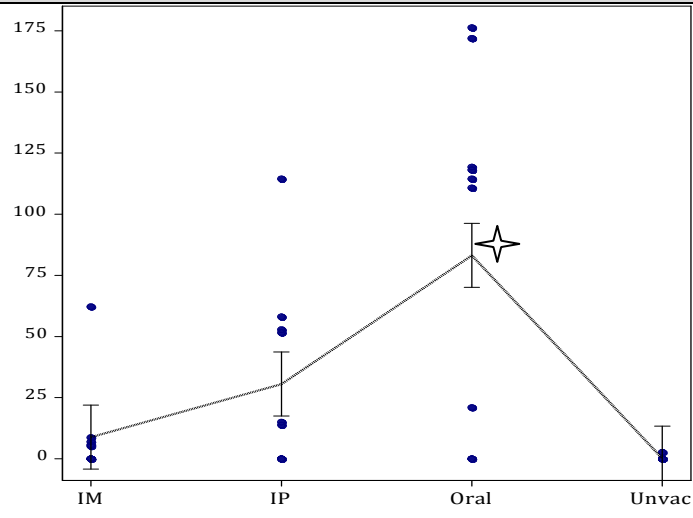
TNF- α



IL-6



TGF- β 1



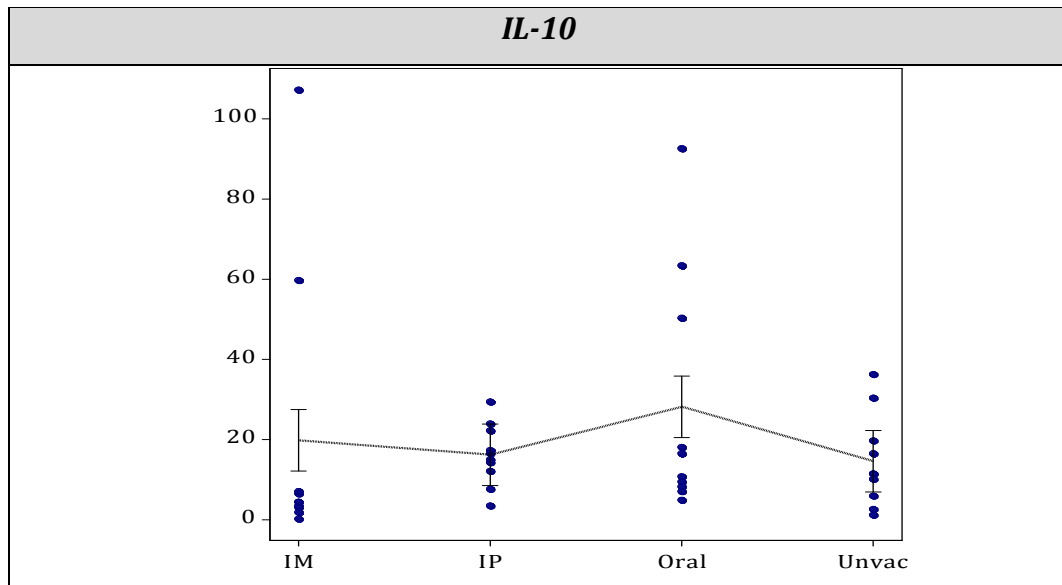


Figure 31- Mean and individual concentrations of cytokines Interferon-gamma (IFN- γ), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Tumour Necrosis factor-alpha (TNF- α) and Transforming Growth factor-beta1 in ileal mucosal secretions. Significant differences ($P < 0.05$) between means for the unvaccinated and treated pigs is shown by the star superscript.

5.3 Discussion

This study demonstrated that systemic and local mucosal immune responses after IP inoculation of the *L. intracellularis* vaccine paralleled those induced by oral vaccination. *L. intracellularis* specific IgG titres in serum significantly increased ($P < 0.05$) from days 0 to 17 post vaccination in each vaccinated group. Oral dosing with live or attenuated vaccines has been shown to induce protective immune responses at mucosal surfaces which block pathogen attachment or neutralise local virulence factors by specific IgA or IgG antibody, but IP immunisation has been equally efficacious (Mestecky and McGhee, 1987; Husband *et al.*, 1996). In this study, oral vaccination consistently elicited the highest mucosal responses. The oral route is often prescribed as the principal route to induce effective mucosal vaccination (Curtiss *et al.*, 1996; Mirchamsy *et al.*, 1996; Husa *et al.*, 2009) on the premise that the induction of IgA/IgG plasma cells will occur following antigen uptake at inductive sites (e.g. Peyer's patches) in the gut mucosa (Mestecky *et al.*, 2005; Roth, 2010). For instance, pigs orally vaccinated with *Salmonella* Typhimurium live attenuated vaccine showed reduced intestinal lesions and increasing antigen specific IgG in serum after inoculation with 10^9 virulent *Salmonella* sp. compared with unvaccinated group (Husa *et al.*, 2009). However, it was also anticipated from previous reports (Hall *et al.*, 1989; Husband *et al.*, 1996; Muir *et al.*, 1998) that the serosal surface of the intestine

and draining mesenteric lymph nodes may be accessed by IP delivery. This happens especially if the antigen is accompanied by an inflammatory adjuvant or bacterial components, and induces mucosal immunity with secretion of bioactive products into the intestinal lumen. Previously, IP vaccination with either Freund's adjuvant or oil-in-water emulsion adjuvants had been shown to protect pigs from lung lesions due to *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* (Hall *et al.*, 1989; Djordjevic *et al.*, 1997). Administration of antigen IP have been reported as being effective in stimulating IgA producing lymphocytes at Payer's patches of pigs (Sheldrake *et al.*, 1993). In other species, IP vaccination of crude tetanus toxoids preparation with either Freund's adjuvants or oil-in-water adjuvants demonstrated a markedly improve anti-tetanus IgA antibody responses in the jejunum of chickens compared with orally vaccinated groups (Muir *et al.*, 1995). Similar the results of our study, when Muir *et al.*, (1998) vaccinated chickens against *Salmonella* sp. IP, the IgG and IgA serum responses were increased and comparable to oral vaccination and induced protection against disease. In addition, other accessible mucosa, such as intranasal and intrarectal approaches have been explored as alternative sites for delivering mucosal vaccines. For instance, foals were seropositive for *Lawsonia*-specific IgG (by IPMA) 21 days after intra-rectal administration of an attenuated live vaccine of *L. intracellularis* (Enterisol®Ileitis) (Pusterla *et al.*, 2010). In humans, intranasal delivery with *Shigella flexneri* 2a lipopolysaccharide 50 subunit vaccine (LPS, IpaB, IpaC and IpaD) increased by five-fold, the concentrations of antigen-specific intestinal faecal IgA and IgG (Riddle *et al.*, 2011). However, the requirement for mucosal immunization to generate protective immunity against mucosal pathogens it is not essential, as this and the previous trials have demonstrated that *L. intracellularis* specific IgG immune responses were detectable at mucosal sites and in serum following systemic delivery (IM). This is consistent with other studies where pigs vaccinated intradermally with live attenuated porcine reproductive and respiratory syndrome (PRRS) virus and then naturally exposed to a heterologous European field strain showed reduced clinical signs and the development of cell-mediated immunity (reactive NK cells, and antigen-specific γ/δ T and cytotoxic T lymphocytes) in the blood (Martelli *et al.*, 2009). Protection from mucosal challenges with *L.intracellularis* has been also reported. For instance, Dale *et al.*, (1997), reduced faecal *L.intracellularis* counts by 98.5% in four pigs vaccinated twice IM, 3

weeks apart, with killed *L. intracellularis* in incomplete Freund's adjuvant (IFA). In that study, additional pigs inoculated with recombinant GroEL-like protein also exhibited reduced faecal bacteria counts compared to infected controls. Presumably the mechanism by which IM vaccination protects against mucosal pathogens is through the homing of activated immune cells from somatic sites and lymph nodes to mucosal tissues (Daynes *et al.*, 1996), or more likely, by the diffusion of circulating antibody into mucosal tissues. As observed in the earlier study (Chapter 4), pigs vaccinated IM elicited increased *L. intracellularis* IgG in the ileal mucosa providing by protection against a challenge with 10^8 virulent *L. intracellularis*. In this study, the majority of the pigs from all groups (including some in the unvaccinated group) were seropositive for *L. intracellularis* IgG antibodies, however, higher titres were derived in the vaccinated, compared to unvaccinated pigs.

In this on-farm study, ileal antibody and cytokine responses generated following IP immunisation resembled those induced by oral, rather than IM routes of vaccination. The quantities of the inflammatory cytokines IFN- γ and IL-6 (16 and 60 pg/mL) in the ileal secretions following oral vaccination were reduced by comparison with the previous pen trial in Chapter 4 (750 and 225 pg/mL), whereas concentrations of TNF- α and TGF- β 1 were comparable. In this trial, significantly ($P < 0.05$) increased mucosal concentrations of TNF- α and TGF- β 1 were exhibited after oral and IP immunisation. Interestingly, cytokine concentrations were 2- to 200-fold lower in unvaccinated pigs relative to their vaccinated cohorts. Differences in immune responses between pen and farm trials are expected, as on farm, the environment is less controlled and concomitant infections are expected. Caged hens exhibited a significantly higher level of total antibody production (5.30 ± 0.23) for the 3 weeks after inoculation with killed Newcastle disease viral vaccine compared to free-range hens (2.82 ± 0.26) (Hoffman *et al.*, 2009). The prevalence of a soil fungus infection *Paracoccidioides braziliensis* was also increased in free-range chickens, as measure by ELISA titres to the gp43 antigen (Oliveira *et al.*, 2011). Although protein concentrations of mucosal secretions were standardised between studies and pigs did not show signs of diarrhoea and were healthy, the range of mucosal responses of individual pigs does vary substantially in on-farm trials (Hall *et al.*, 1989). However, in this situation, the reduced responses for IFN- γ and IL-6 may reflect the use of

antibiotics, the different gastrointestinal microflora in these piglets or the timing of sampling, but the aim was to highlight the response to the *L. intracellularis* vaccine. Cytokines such as IFN- γ have been involved in protective immunity against intracellular infections and this has been implicated for *L. intracellularis*. Specific IFN- γ producing cells were detected by ELISPOT, in porcine peripheral blood mononuclear cells (PBMC), 4 to 13 weeks after vaccination with *L. intracellularis* (Guedes and Gebhart, 2003b). This method is more sensitive than analysis of mucosal secretions as it measures the response of primed T-lymphocytes after a second exposure to *L. intracellularis* antigen *in vitro*. Similarly, knockout mice for IFN- γ receptor (IFN- γ R⁻) were substantially more susceptible to disease outbreaks and more extensive lesions as observed by immunohistochemistry compared with wild type mice (IFN- γ R⁺) after oral infection with 10⁷ cultured *L. intracellularis* (Smith *et al.*, 2000). This implies a specific and direct protective effect of IFN- γ . In horses, IFN- γ gene expression in all Enterisol[®] vaccinated foals was significantly higher, 60 days following oral vaccine administration compared to IFN- γ gene expression in control foals (Pusterla *et al.*, 2012a). The specificity of the cytokine response to vaccination in this trial was also supported by higher concentrations of TGF- β 1, possibly related to local mucosal repair and immune regulation resulting from the limited infection associated with vaccine “take”. The TGF- β controls the initiation and resolution of inflammatory responses through the regulation of lymphocytes activation, promoting T cell survival and inducing IgA class switching in B cells (Schluesener *et al.*, 1990). In pigs, a transient decrease in TGF- β 1 concentrations at the intestinal villi epithelium was observed and associated with obvious intestinal villi atrophy and marked reduction of mucosal digestive enzyme activities (Mei and Xu, 2005), suggesting that changes would be expected with its intestinal infection like that caused by *L. intracellularis*. Consistent with its’ anti-inflammatory and regulatory role, porcine TGF- β 1 suppressed human T-helper (Th)-cell activation through the inhibition of IL-12 secretion and the induction of Th-cell apoptosis (Palmer *et al.*, 2002).

The comparable spectrum of immune responses generated after vaccination and the subsequent protection after challenge in the previous pen trial (Chapter 4), suggests that significant protection against *L. intracellularis* would be anticipated in each of the vaccinated groups in this study. The results from both trials would indicate that if specific antibody responses can be detected following vaccination, a protective level of immunity may have been induced against a moderate level of challenge. However, this would be expected to wane with time as the interval between vaccination and challenge increased.

Chapter 6 *Effect of phytase and beta-glucan in feed on immune responses of growing pigs to vaccination with *Lawsonia intracellularis**

(Modified from a confidential report submitted to AB Vista)

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6.1 Introduction

In pig herds, the presence of proliferative enteropathy (PE) disease is associated with a range of clinical signs from acute haemorrhage to chronic diarrhoea and death (Lawson and Gebhart, 2000). The aetiologic agent is *Lawsonia intracellularis* an obligate intracellular bacterium. Histologically, the lesions in

infected animals consist of proliferating immature enterocytes containing intracellular *L. intracellularis* bacterial antigen (Lawson and Gebhart, 2000). Clinical outbreaks and subclinical cases (with no apparent clinical signs) are also accompanied by decreased feed intake, reduced weight gain and poorer feed conversion efficiency (Paradis *et al.*, 2012). PE is also associated with reduced activity of digestive enzymes in the small intestine of pigs which may lead to decreased digestion and absorption of proteins from the lumen of the intestine (Rowan and Lawrence, 1982; Collins *et al.*, 2009). Therefore, controlling PE is important to improve productivity, reduce animal losses and increase herd profitability. PE is traditionally controlled in pig herds with in-feed antibiotic medication which have been effective in controlling PE outbreaks (Tzika *et al.*, 2009). However, with a complete ban on in-feed antibiotics in place inside the EU since 2006, pig producers are seeking alternatives to in feed antibiotics to control pathogens in pigs. An alternative to in-feed antibiotics for the control of PE is the commercially available attenuated live *L. intracellularis* vaccine (Enterisol® Ileitis, Boehringer Ingelheim). This vaccine has been shown to reduce the clinical signs and lesions of PE as well as the amount of *L. intracellularis* that is shed in faeces (Kroll *et al.*, 2004). The Enterisol® Ileitis vaccine has been shown to increase herd uniformity and provide additional weight gains of between 20 to 34 g per pig/day (Almond and Bilkei, 2006). In the previous chapters, systemic and local immune responses were monitored and detected after vaccination by various routes with a ten times vaccine dose (Chapters 4 and 5). The result showed an increase in *L. intracellularis* specific IgG antibody response in serum and mucosa, which could not be detected following administration of standard doses (Donahoo, 2009). The variability in local and systemic immune responses negated the detection of an immune-marker which correlated with herd or individual protection in pigs after vaccination. However, it was apparent that detection of either specific antibody or significantly increased concentrations of IFN- γ following oral or IM vaccination would indicate protection from a field challenge. Pig producers are hesitant to remove preventive in-feed antibiotics without the guarantee of protection against PE. Therefore it was reasoned that feed additives which are proposed to boost immune responses could enhance the development of mucosal immune responses to detectable and consistent concentrations.

Phytase is frequently added to pig diets to improve phytate-P (inositol hexaphosphate, IP6) digestibility. However, other benefits to the use of phytase include increased nutrient absorption, growth rates and possible enhanced immunity (Selle and Ravindran, 2008). These results are hypothesised to be associated with the extra phosphoric effects of phytase where the benefit to animals fed phytase extends beyond the role of just liberating phytate-P (Liu *et al.*, 2008; Shaw *et al.*, 2011). While the precise mechanism of action is unclear, phytase could possibly increase immunity by releasing the nutrient-bound-phytate, increasing its bioavailability and absorption and promote growth or proliferation of immune cells (Scrimshaw, 2007). The inositol ring has also been shown to reduce pro-cancerous intestinal cell proliferation by increasing pro-inflammatory cytokine production (Wawszczyk *et al.*, 2013) and inducing cellular apoptosis (Ferry *et al.*, 2002) which may influence the pathogenesis of PE. Increases in blood lymphocyte numbers have been observed after 18 days when 500 FTU/kg of a microbial phytase was added to a high phytate (0.44% P) broiler diets (Liu *et al.*, 2008). From the available literature, the effects of phytase on the mucosal immune responses in pigs have not been examined and will be investigated in this chapter.

Another feed additive that has gained recent attention for its possible immunological benefits for animals is beta glucan (β -glucans). They have been identified as potential dietary supplements to offset the removal of antibiotic growth promoters. Results in nursery pigs have shown improved growth performance and increased production of lymphocytes when diets were supplemented with 5g/kg of *Saccharomyces cerevisiae* yeast culture (Shen *et al.*, 2009). Glucans have been reported to increase the phagocytic activity of macrophage and neutrophils in human and porcine peripheral blood mononuclear cells (PBMCs) (Williams *et al.*, 1986; Sherwood *et al.*, 1987; Sonck *et al.*, 2010). However, depending on their source and structure, the type of β -glucan used influences the immune responses of animals (Sonck *et al.*, 2011). For instance, reduced clinical signs and increased PRRS specific IFN- γ in PBMCs incubated with high molecular β -1,3-1,6-glucan preparation were observed 50 days after a challenge infection with PRRS virus (Xiao *et al.*, 2004). Pigs fed diets supplemented with 600 ppm β -glucan prior to an ex-vivo *E. coli* lipopolysaccharide challenge showed increased cytokine production when compared

to intestinal tissues from non-supplemented pigs (Sweeney *et al.*, 2012). The effect of β -glucan on specific mucosal responses to vaccines such as *L. intracellularis* has not been determined. Therefore, this trial assessed the individual and potential synergistic effects of adding phytase and β -glucan to diets on immune responses of pigs when given an attenuated *L. intracellularis* oral vaccine.

6.2 Materials and Methods

6.2.1 Animal Ethics

All procedures involving the use of animals were approved by the Elizabeth Macarthur Agricultural Institute Animal Ethics Committee (M12-02), Australia.

6.2.2 Experimental design and diets

The experiment was conducted as a 2 x 2 x 2 factorial arrangement with phytase, β -glucan and vaccination as the main factors. Ninety-nine entire, male, Landrace X Large White crossbred weaner pigs were randomly allocated by weight (6.9 ± 1.7 kg; BW \pm SE) to one of eight treatment groups (Table 9).

Table 8 - Experimental design and treatment groups.

Groups	Phytase (2000 FTU/kg)	β -Glucan (100ppm)	Vaccination ($10^{5.9}$ TCID ₅₀)	N° of pigs
G1	Yes	Yes	Yes	12
G2	No	Yes	Yes	12
G3	Yes	No	Yes	12
G4	No	No	Yes	12
G5	Yes	Yes	No	12
G6	No	Yes	No	13
G7	Yes	No	No	13
G8	No	No	No	13
Total				99

Diets (Table 10) were offered in mash form without antibiotic growth promoters and were formulated to meet or exceed the requirements for all nutrients (NRC, 2012). Groups **G1**, **G3**, **G5** and **G7** had a third generation *E. coli*-phytase (Quantum® Blue phytase, AB Vista, UK) added (mean analysed activity of 2,007 phytase units (FTU)/kg). The diets containing β -glucan (groups **G1**, **G2** and **G5**, **G6**) were supplemented at the rate of 1g/kg of feed (AB Vista Feed Ingredients, Marlborough, UK) according to the manufacturer's instructions. The β -glucan preparation (AB Vista, UK) was extracted from baker's yeast, *Saccharomyces*

cerevisae and isolated by methods described by Haldar *et al.*, (2011). To calculate the apparent ileal digestibility (AID) coefficients for crude nitrogen (N) and minerals, an indigestible marker Celite 281 (Filchem Australia Pty Ltd, Australia) was added to diets at a concentration of 20 g/kg.

Table 9- Ingredient and nutrient specification (g/kg) of the basal diet fed to grower pigs (as-fed basis).

Ingredient	g/kg
Wheat (fine)	557.3
Soybean meal	153.3
Canola meal	80.0
Whey	80.0
Millrun (Manildra)	66.6
Spent oil	26.7
Dicalcium phosphate	13.3
Limestone (fine)	10.0
Lysine HCl	5.23
Vitamin-mineral premix ¹	2.52
Betafin (betaine)	2.00
L-threonine	1.22
Salt	1.1
Celite	20.0
Calculated composition ²	
Moisture, %	9.89
DM, %	89.6
CP	199.5
Fibre	36.6
Fat	49.5
Ca	9.35
Total P	7.23
Phytate-P	3.01
Ca:P	1.29
Cal/D:P	2.05
Total Sulphur	0.12
Threonine	8.40
Lysine	0.137
Methionine,	0.041
DE, MJ/kg	14.25

¹ Vitamin premix provided per kilogram of diet: Mg, 20mg; Na, 18.2mg; Cl, 23.2mg; Fe, 257mg; Mn, 114mg; Cu, 198mg; Se, 714µg; Mo, 1.0mg; Co, 932µg; I, 125mg; Vitamin B6, 103mg; Vitamin B12, 25µg; niacin, 78mg; folic, 322 µg; biotin, 252mg; choline, 398µg; potassium, 6 mg; tyrosine, 6 mg; tryptophane, 23mg; arginine, 115mg; isoleucine, 8.2mg; phenylalanine, 8.7mg. ²Composition was calculated on the requirements for weaner pigs (NRC);

6.2.3 Pig housing and treatments

Pigs aged between 3 to 4 weeks were purchased from a commercial herd (Young, NSW) clinically and serologically free of PE and housed in an environmentally controlled research facility. Pigs were placed in groups of 3 to 4 animals in 4.0 x 4.5 m pens equipped with slatted-floors, a feeder and nipple drinker to provide *ad libitum* access to feed and water, respectively. The temperature in the room was controlled between 24 and 28°C as recommended by the Australian animal welfare standard and guidelines (Animal Welfare Working Group, 2007). After one week on the respective diets, pigs in G1, G2, G3 and G4 groups were orally vaccinated (d 0) via drench gun with 2.0 mL of *Lawsonia intracellularis* vaccine containing 10^{5.9} TCID₅₀ organisms (Enterisol®Ileitis, Boehringer Ingelheim, US). The remaining groups (G5-G8) were not vaccinated.

6.2.4 Sampling schedule

On days 0, 7 and 25 post-vaccination (pv), individual pigs were bled by jugular venipuncture (5.0 mL) using serum clot separator vacuum tubes (Vacurette®, Geiner Bio-one, Australia) for serological analysis. On these days, faeces (approximately 2 g) were collected from individual pigs using clean gloves and stored in sterile containers (Techno-Plas®, Australia) and frozen at -20 °C until further analysis (Table 11).

Table 10- Experimental days and sampling schedule

	-7	-6	0	1	7	8	15	22	25	28	29
		Feed starts	Vacc.								Necropsy
Faeces			√		√				√		
Blood			√		√				√		
Feed intake		√ _{w0}		√ _{w1}		√ _{w2}	√ _{w3}	√ _{w4}		√ _{w5}	√ _{w5}
Weight		√		√		√	√	√		√	√
Necropsy											
• Ileal Mucosa											
• Ileal Digesta										√	√
• Ileal section											
• Carcass weight											

After 35 days of feed consumption (d 28 pv), pigs from all groups were slaughtered at a local abattoir (Wollondilly, NSW) over two consecutive days. Entire gastrointestinal tracts were collected, placed in individually-labelled bags and transported on ice to Elizabeth Macarthur Agricultural Institute necropsy room for tissue collection. Briefly, ileal digesta was obtained by massaging caudally from 2 m to 10 cm anterior to the ileo-caecal valve (ICV) and placed in sterile containers and

kept at -20 °C until freeze-dried. Subsequently, a section of ileal tissue was cut and around 1 cm segment stored in 10 % neutral-buffered formalin fixative for histopathology. The adjacent segment was cut longitudinally and the intestinal mucosal secretions were collected by gently scraping the ileal mucosa with a sterile scalpel as previously described (Chapter 3).

6.2.5 Production parameters

Pigs were monitored daily for body condition, clinical signs of PE and abnormal behaviour. Pigs were individually weighed in electronic scales (Adam Equipment, South Africa) at the time of arrival and weekly during the trial to determine average daily live weight gain (ADG). Weekly feed consumption was calculated as the difference in the weight of feed supplied minus the feed remaining and was used to estimate the average daily feed intake (ADFI) and feed conversion ratio (FCR). At necropsy, the percentage dress weight for each pig was calculated as the hot standard carcass weight (HSCW) divided by the final live weight prior to slaughter multiplied by 100.

6.2.6 Sample analyses

Sera and mucosal scrapings were assayed in duplicate for the detection of *L. intracellularis* specific antibodies (IgG) using a commercial blocking ELISA (Bioscreen® Ileitis, GmbH, Münster, Germany). *L. intracellularis*-IgA was assayed using an experimentally modified direct ELISA (as described in Chapter 3). The quantities of cytokines IFN- γ , IL-6, IL-10, TNF- α and TGF- β 1 in ileal mucosal secretions were analysed using a commercial sandwich ELISA Kit (porcine Quantikine® ELISA, R&D systems, Minneapolis, USA). The minimum limits of detection for this study were 2.0 pg/mL for IFN- γ , 1.7 pg/mL for IL-6, 17.2 pg/mL for IL-10, 1.7 pg/mL for TNF- α and 8.1 pg/mL for TGF- β 1. Concentrations were estimated by generating a logistic parameter curve using GraphPad®Prism software. To quantify bacterial excretion, purification of bacterial DNA from faecal samples (0.1g) was performed with the MagMax DNA extraction kit (A&B Applied Biosystems, California, USA). Extraction products were analysed by real-time PCR (7500 Real-Time PCR System, Applied Biosystems) using primers and Taq Man probe as described by Nathues *et al.*, (2009). The sensitivity and specificity of the test has been reported by Collins *et al.*, (2011) as 99% and 97%, respectively.

6.2.7 Histological procedures

Formalin-fixed tissues were stained with haematoxylin and eosin (H&E) (Luna, 1968). The blinded slides were scored by one operator under 40x magnification light microscope to count the number of goblet cell crypts using a manual cell counter (Bantex, USA). A total of ten fields were analysed for each slide, with the first chosen randomly and then every second field to the right. The presence of proliferative affected area (%) was also noted and when positive an immunohistochemistry (IHC) stain, using a VPM53 *L. intracellularis* monoclonal antibody (Boehringer Ingelheim, US) was performed to confirm the presence of *L. intracellularis* -specific antigen within crypts (Jensen *et al.*, 1997).

6.2.8 Chemical analysis of diet and ileal digesta

The ileal digesta samples were freeze-dried and all samples were ground (Retsch ZM 100, Retsch GmbH, Germany) to pass through a 0.5 mm screen prior to chemical analyses. The nitrogen (N) contents of the minerals P, Ca, Cu, K, Mg, Mn, Na, Sr, Fe and Zn were determined in feed and dried digesta samples. Nitrogen concentration was determined by the Dumas method using a FP-428 nitrogen analyser (method 968.06, LECO® Corporation, St. Joseph, MI, USA) as described by Sweeney (1989). Samples were wet acid digested using nitric acid and hydrogen peroxide prior to the determination of mineral concentration by Inductively Coupled Plasma-Optical Emission Spectroscopy using a Perkin Elmer OPTIMA 7300 (Perkin Elmer Inc, Waltham, MA, USA) at the Mark Wainwright analytical centre (The University of New South Wales, Kensington, 2052). The acid insoluble ash component of dried diets and ileal digesta samples were determined according to the method of Siriwan *et al.*, (1993). The apparent ileal digestibility (AID) coefficient of crude protein (CP) and minerals were calculated as described by Ravindran *et al.*, (2001). Phytase activity was tested in the diet using an ELISA test kit (Envirologix, USA) as designated by the ESC Standard Analytical Method (#SAM099, ESC laboratories, UK). Phytase recovered in the diets was determined (Enzyme Services and Consultancy, Ystrad Mynach, UK) according to the method of Engelen *et al.* (2001), modified to determine activity specifically from the exogenous phytase. One phytase unit is defined as the amount of enzyme required to liberate 1 mol of inorganic P/min from phytic acid at pH 4.5 and 60 °C.

6.2.9 Statistical analysis

The experiment was a 2 x 2 x 2 factorial arrangement, with dietary phytase, β -glucan and vaccination being the main factors. The data were subjected to randomized complete block design using a linear mixed model procedure of Restricted Maximum Likelihood (REML, GenStat Release 13th Ed, UK) with a 95% confidence level ($P < 0.05$) (Steel, 1997). Pigs were the experimental unit for all immune responses and pathology lesions. Pen was the experimental unit for growth performance and nutrient digestibility metrics. Clinical observation data were categorized into binary data and then analysed using a two sample binomial test comparison (Steel, 1997).

6.3 Results

6.3.1 Room temperature and clinical health

Overall, pigs were healthy during the entire trial. Occasional scouring was observed and treated orally with 200mL electrolytes (Vytrate, Jurox, Rutherford, Australia) for affected pigs. However, scouring was not attributed to any specific diet.

6.3.2 Production performances

Performance data is shown in Table 12. At the start of the trial (d-7) pigs weighed an average of 8 kg \pm 0.65 (kg \pm SE) with no differences between the total bodyweights in each randomised group. Overall, vaccinated pigs demonstrated lower average weight gain during weeks 4 and 5 (694.6 and 746 g/day, respectively) when compared with unvaccinated pigs (724.1 and 803.7 g/day). This might be due to the fact that unvaccinated pigs had higher daily feed intake (1383 g/ day) than the vaccinated groups (1184 g/ day).

Prior to slaughter, live bodyweight averaged 27.8 kg across all groups with pigs from the group G2 (glucan and vaccinated) showing the lowest average weights (26.3 kg). This group (G2) also had the lowest hot standard carcass weights (HSCW) of 19.2 kg, but there were no significant differences across groups (20.5 kg). Similarly, no differences were found for carcass dressing percentage. The respective carcass dressing percentage for the 8 groups were 73.2% (G1), 72.9% (G2), 72.9% (G3), 74.1% (G4), 74.5% (G5), 72.8% (G6), 73.5% (G7), 72.5% (G8).

Table 11- Individual average daily weight gain (ADG, g/day), average daily feed intake (FI, g/day) and feed weigh: gain ratio (FCR, g feed/g weight) over the 5 week trial (week 1=vaccination) within groups.

	Vaccinated groups				Non vaccinated groups				SEM	P (<0.05)
	G1 (+P+B)	G2 (-P+B)	G3 (+P-B)	G4 (-P-B)	G5 (+P+B)	G6 (-P+B)	G7 (+P-B)	G8 (-P-B)		
Week 1										
ADG	319.6	318.2	344.5	385.8	369.4	323.2	343.5	383.5	31.9	NS
FI	463.9	433.1	518.7	491.4	487.7	454.6	503.0	464.0	32.2	NS
FCR	1.46	1.37	1.5	1.29	1.32	1.41	1.47	1.23	0.08	NS
Week 2										
ADG	465.2 ^b	417.9 ^a	471.2 ^b	526.2 ^c	473.7 ^b	497.4 ^{bc}	493.0 ^{bc}	478.0 ^b	38.6	0.034
FI	660.8 ^a	625.3 ^a	760.1 ^c	715.1 ^{bc}	677.6 ^{ab}	663.7 ^a	705.3 ^b	688.3 ^b	44.2	<0.001
FCR	1.42	1.51	1.61	1.37	1.43	1.34	1.44	1.45	0.08	NS
Week 3										
ADG	611.9 ^b	535.0 ^a	579.2 ^a	559.5 ^a	609.5 ^b	548.2 ^a	603.3 ^b	560.4 ^a	43.2	0.015
FI	947.0 ^c	829.7 ^a	967.2 ^c	916.7 ^b	956.3 ^c	922.2 ^b	961.3 ^c	925.7 ^b	51.2	<0.001
FCR	1.56	1.55	1.67	1.64	1.57	1.7	1.61	1.65	0.07	NS
Week 4										
ADG	717.9 ^{ab}	664.3 ^a	666.7 ^a	729.8 ^b	694.0 ^a	745.8 ^c	739.9 ^{bc}	725.3 ^b	37.2	0.027
FI	1114.4 ^a	1078.5 ^a	1124.8 ^a	1143.5 ^b	1151.5 ^b	1186.4 ^b	1251.4 ^b	1146.4 ^b	54.8	<0.001
FCR	1.58	1.63	1.69	1.57	1.66	1.59	1.7	1.58	0.06	NS
Week 5										
ADG	840.9 ^c	759.1 ^b	752.4 ^b	699.0 ^a	773.2 ^b	814.5 ^c	847.1 ^c	838.4 ^c	23.1	<0.001
FI	1428.2	1342.3	1382.6	1377.6	1418.3	1486.3	1479.8	1386.1	162.2	NS
FCR	1.75 ^a	1.77 ^{ab}	1.83 ^b	1.99 ^b	1.84 ^b	1.85 ^b	1.76 ^{ab}	1.67 ^a	0.08	0.047
Overall main effects										
			ADG	FI	FCR					
Vaccinated										
			Vacc +	620.0	1137	1.84				
			Vacc -	647.8	1175	1.81				
			P (<0.05)	NS	NS	NS				
Phytase										
			Phytase +	641.5	1178	1.83				
			Phytase -	626.3	1134	1.81				
			P (<0.05)	NS	NS	NS				
Glucan										
			Glucan +	630.7	1137	1.81				
			Glucan -	637.0	1175	1.85				
			P(<0.05)	NS	NS	NS				
Interactions (P<0.05)										
			P*V	NS	NS	NS				
			V*B	NS	NS	NS				
			B*P	NS	NS	NS				
			P*V*B	NS	NS	NS				

¹ Means in the same row without common superscripts are significantly different (P<0.05); ² NS: Not significant (P>0.05); P: Phytase; B: β-glucan; V: vaccination

6.3.3 Nutrient digestibility

The effects of dietary treatment on the apparent ileal digestibility (AID) of nitrogen (N) and the minerals Ca, P, Mg, Na, K, Fe, Cu, Mn, Zn and Sr are presented in Table 13. No main effects of phytase, β -glucan or vaccination were found. However, three way interactions were found for the AID of Mg, K, Fe, and Cu with the highest AID of Mg observed for the phytase supplemented and vaccinated groups G1 and G3. Similarly, the highest AID of K was detected in phytase supplemented groups (G1, G3, G5 and G7) and for AID of Fe the group G5 had the highest coefficient.

Table 12- The apparent ileal digestibility coefficients of nitrogen (N) and minerals in pigs vaccinated (V) and unvaccinated, with or without β -glucan yeast (+/- B) and phytase (+/- P)^{1,2}.

Groups	N	Ca	P	Mg	Na	K	Fe	Cu	Mn	Zn	Sr
G1 (+P+B)	0.48	0.78	0.61	0.48 ^b	-0.07	0.63 ^{ab}	0.33 ^a	0.28 ^b	0.47	0.52	0.69
G2 (-P+B)	0.46	0.76	0.57	0.42 ^a	-0.07	0.55 ^a	0.44 ^b	0.22 ^{ab}	0.41	0.49	0.60
G3 (+P-B)	0.54	0.79	0.63	0.50 ^b	-0.08	0.67 ^b	0.25 ^a	0.30 ^b	0.38	0.43	0.54
G4 (-P-B)	0.52	0.76	0.59	0.43 ^a	-0.06	0.59 ^a	0.36 ^{ab}	0.24 ^b	0.32	0.39	0.45
G5 (+P+B)	0.43	0.76	0.55	0.43 ^a	-0.03	0.68 ^b	0.47 ^b	0.19 ^a	0.37	0.40	0.47
G6 (-P+B)	0.42	0.73	0.51	0.37 ^a	-0.09	0.57 ^a	0.35 ^a	0.13 ^a	0.31	0.37	0.39
G7 (+P-B)	0.49	0.76	0.56	0.42 ^a	-0.10	0.72 ^b	0.38 ^{ab}	0.21 ^{ab}	0.28	0.31	0.61
G8 (-P-B)	0.48	0.73	0.52	0.35 ^a	-0.06	0.61 ^a	0.27 ^a	0.15 ^a	0.22	0.27	0.52
SEM	0.08	0.05	0.05	0.08	0.11	0.08	0.09	0.07	0.06	0.07	0.07
P(<0.05)	NS	NS	NS	0.046	NS	0.045	0.032	0.038	NS	NS	0.068
Vaccinated											
Vacc +	0.49	0.78	0.65	0.48	0.10	0.71	0.30	0.29	0.31	0.31	0.57
Vacc -	0.44	0.77	0.62	0.47	0.14	0.69	0.37	0.28	0.27	0.29	0.58
P (<0.05)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Phytase											
Phytase +	0.47	0.79	0.66	0.48	0.03	0.72	0.38	0.31	0.30	0.37	0.60
Phytase -	0.46	0.76	0.61	0.47	0.02	0.68	0.25	0.25	0.28	0.24	0.55
P (<0.05)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Glucan											
Glucan +	0.44	0.79	0.64	0.50	0.03	0.70	0.36	0.28	0.30	0.33	0.60
Glucan -	0.50	0.77	0.63	0.45	0.02	0.70	0.35	0.29	0.28	0.27	0.55
P (<0.05)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Interactions (P<0.05)											
P*V	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
V*B	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
B*P	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
P*V*B	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

¹ Values in the same column without common superscripts are significant different (P<0.05); NS: Not significant (P>0.05); P: Phytase; B: β -glucan; V: vaccination

6.3.4 Systemic immune responses

Pigs were seronegative at day 0 and 7 post-vaccination (Table 14). After 25 days pv, vaccinated pigs had higher *L. intracellularis*-IgG responses than unvaccinated pigs (PI: 35% and 8.8%, respectively). The highest percentage inhibitions (PI) for *Lawsonia intracellularis* specific IgG were observed on day 25 in groups G1, G3 (41 and 35.5%) when compared to other two vaccinated groups (G2 and G4, 10.8% and 16.2%). Vaccinating pigs increased specific IgG concentrations when compared to non-vaccinated pigs. In addition, a tendency for higher IgG responses were also observed in phytase and β -glucan supplemented pigs, leading to a phytase- β -glucan-vaccination interaction (Table 14).

Table 13 – Mean concentrations for the main effects and interactions of *Lawsonia intracellularis* IgG in serum, as percentage inhibition (PI, %), within groups vaccinated and unvaccinated, with or without β -glucan yeast (+/- B) and phytase (+/- P).

	Treatment	IgG (PI, %) ^{1,2}		
		day 0	day 7	day 25
<i>Vaccinated</i>	G1 (+P+B)	-1.0	1.3	41.0 ^c
	G2 (-P+B)	-0.9	-0.1	10.8 ^a
	G3 (+P-B)	-6.5	-4.2	35.6 ^c
	G4 (-P-B)	-6.3	-5.6	16.2 ^{ab}
<i>Unvaccinated</i>	G5 (+P+B)	-9.5	-7.4	18.2 ^b
	G6 (-P+B)	-11.2	-2.0	22.8 ^b
	G7 (+P-B)	-4.3	6.6	7.4 ^a
	G8 (-P-B)	-6.0	5.0	23.4 ^b
	SEM	2.0	5.3	8.4
	P (<0.05)	NS	NS	<0.001
Main effects				
Vaccination				
	Vacc +	-11.1	1.8	35.0 ^a
	Vacc -	-8.4	0.6	8.3 ^b
	P (<0.05)	NS	NS	<0.001
Phytase				
	Phytase +	-10.8	-1.0	22.2
	Phytase -	-8.7	3.2	20.4
	P (<0.05)	NS	NS	NS
Glucan				
	Glucan +	-8.7	-1.4	26.6
	Glucan -	-10.7	0.4	16.1
	P (<0.05)	NS	NS	NS
Interactions				
	P*V	NS	NS	NS
	V*B	NS	NS	NS
	B*P	NS	NS	NS
	P*V*B	NS	NS	0.024

¹ Negative percentage means indicates that the unknown sample has less antibody concentration than the negative control available on the kit. ² Values in the same column without common superscripts are significant different ($P < 0.05$); ³ NS: Not significant ($P > 0.05$);

6.3.5 Local mucosal immune responses

Local immune responses are summarized in Table 15. In unvaccinated groups, β -glucan supplementation substantially increased concentrations of IFN- γ , TNF- α and TGF- β 1 (group 6) compared to un-supplemented group 8 while phytase had no significant effect (group 7), unless animals were vaccinated (group 3, Table 15). Vaccinated pigs had higher mucosal IgG immune concentrations than unvaccinated pigs at day 28 pv (PI: 21% and 13.4%, respectively). Pigs supplemented with phytase generated higher *L. intracellularis* specific IgG concentrations in the ileal mucosal scrapings compared to non-supplemented pigs (PI: 24.7% and 10.3%, respectively). In addition, pigs fed β -glucan had higher mucosal IgG antibody responses than non-supplemented pigs (20.4 and 14.6 pg/ml, respectively). Similarly, mucosal IgA titres were increased in pigs supplemented with β -glucan (22.3 pg/mL) when compared with the non-supplemented cohort (16.7 pg/mL). Interactions between phytase and vaccination were observed, as vaccinating pigs had increased *L. intracellularis* IgG responses when compared to non-vaccination and titres were further increased by adding phytase to the pig diets.

Cytokine immune responses revealed that vaccinated pigs produced significantly higher mucosal concentrations of TNF- α , IL-6 and IL-10 (TNF- α , 20.4; IL-6, 18.9 and IL-10, 85.5) than unvaccinated animals (TNF- α , 9.3; IL-6, 14.3 and IL-10, 67.0) (Table 15). Vaccinated pigs also tended to have higher concentrations of mucosal IFN- γ and TGF- β 1 in mucosal secretions than control animals (IFN- γ ; 113.2 vs. 87.4 pg/mL, respectively; TGF- β 1; 143.3 vs. 103.1 pg/mL, respectively). Pigs supplemented with β -glucan also had higher concentrations of IFN- γ (147.1pg/ml) and TNF- α (20.6) cytokines in ileal mucosal scrapings when compared with unsupplemented groups (IFN- γ =53.2 and TNF- α TNF=8.9 pg/ml). *L. intracellularis* vaccination increased TNF- α response in ileal mucosa when compared with unvaccinated pigs and a further increased response was observed when β -glucan was added, leading to a vaccination- β -glucan interaction. In addition, pigs consuming diets with added phytase generated higher IFN- γ concentrations (118. 8 pg/mL) in mucosal secretions compared to pigs without phytase in the diet (81.4pg/mL). These results and also the increasing IFN- γ responses associated with supplementation of phytase in pigs feed resulted in a phytase- β -glucan interaction.

Table 14- The mean concentrations for the main effects and interactions of *Lawsonia intracellularis* IgG (PI, %) and IgA (Titre) and cytokines (pg/mL) in ileal mucosa within groups vaccinated and unvaccinated, with or without β -glucan yeast (+/- B) and phytase (+/- P).

	Groups	Cytokines ^{1,2}					Li-Antibody		
		IFN- γ	TNF- α	TGF- β 1	IL-10	IL-6	IgG	IgA	
<i>Vaccinated</i>	G1 (+P+B)	199.3 ^c	26.,3	292.8 ^c	80.4	21.2	36.78 ^a	22.52	
	G2 (-P+B)	121.7 ^c	26.7	78.1 ^b	84.2	19.7	24.18 ^b	17.23	
	G3 (+P-B)	65.7 ^a	14.2	145.1 ^c	60.3	18.2	16.39 ^c	17.75	
	G4 (-P -B)	66.1 ^a	14.6	55.4 ^a	63.8	16.7	9.75 ^d	16.88	
<i>Unvaccinated</i>	G5 (+P+B)	174.6 ^c	15.0	54.7 ^a	87.1	16.8	20.48 ^b	21.22	
	G6 (-P+B)	174.5 ^c	15.5	79.2 ^b	90.6	15.3	18.26 ^{bc}	27.92	
	G7 (+P-B)	41.0 ^a	3.0	60.5 ^a	66.9	13.7	8.98 ^d	15.28	
	G8 (-P-B)	97.0 ^b	3.4	65.1 ^a	70.5	12.3	6.69 ^d	17.23	
	SEM	20.5	8.9	17.6	14.1	6.3	4.8	7.2	
	P (<0.05)	<0.001	NS	<0.001	NS	NS	<0.001	NS	
Main factor									
Vaccination									
	Vacc +	113.2	20.4 ^a	144.3	85.5 ^a	18.9 ^a	21.78 ^a	20.4	
	Vacc -	87.4	9.3 ^b	107.1	67.0 ^b	14.3 ^b	13.47 ^b	18.59	
	P (<0.05)	NS	0.02	NS	0.04	0.04	<0.001	NS	
Phytase									
	Phytase +	118.8	14.6	139.9	74.1	17.3	24.79 ^a	19.93	
	Phytase -	81.4	14.8	120.4	77.9	15.8	10.35 ^b	19.11	
	P (<0.05)	NS	NS	NS	NS	NS	0.002	NS	
Glucan									
	Glucan +	147.1	20.6	116.6	72.5	18.2	20.42 ^a	22.34 ^a	
	Glucan -	53.2	8.9	143.0	79.4	15.0	14.63 ^b	16.76 ^b	
	P (<0.05)	<0.001	0.006	NS	NS	NS	<0.001	0.03	
Interactions									
	P*V	NS	NS	NS	NS	NS	0.045	NS	
	V*B	NS	0.004	NS	NS	NS	NS	NS	
	B*P	0.008	NS	NS	NS	NS	NS	NS	
	P*V*B	NS	NS	NS	NS	NS	NS	NS	

¹ Values in the same column without common superscripts are significant different ($P < 0.05$); ² NS: Not significant ($P > 0.05$);

6.3.6 *L. intracellularis* shedding

At day 0, pigs were not shedding *Lawsonia intracellularis*. However, by day 25 two pigs from room 1 (pen G2 and G4) and two pigs from room 4 (pen G6 and G7) were shedding between 5.9×10^3 and 3.1×10^3 *L. intracellularis* per gram of faeces. However, ileal sections were negative for *L. intracellularis* antigen staining (IHC) from these animals at necropsy after 28 days pv. Although strict quarantine procedures were followed it may be possible that several pigs may have been recently infected on-farm prior to transport and this resulted in low shedding, no lesions and a relative low *L. intracellularis* IgG response in the serum.

6.3.7 Pathology results

Pathology results from goblet cells counts (Figure 32) showed small differences between groups (Figure 33). Stained ileal sections from groups G1 (112.1), G2 (116.3) and G5 (111.9) showed the highest per field counts of goblet cells per field, and the lowest counts were found in groups G3 (91.5), G4 (100.4) and G5 (105.5).

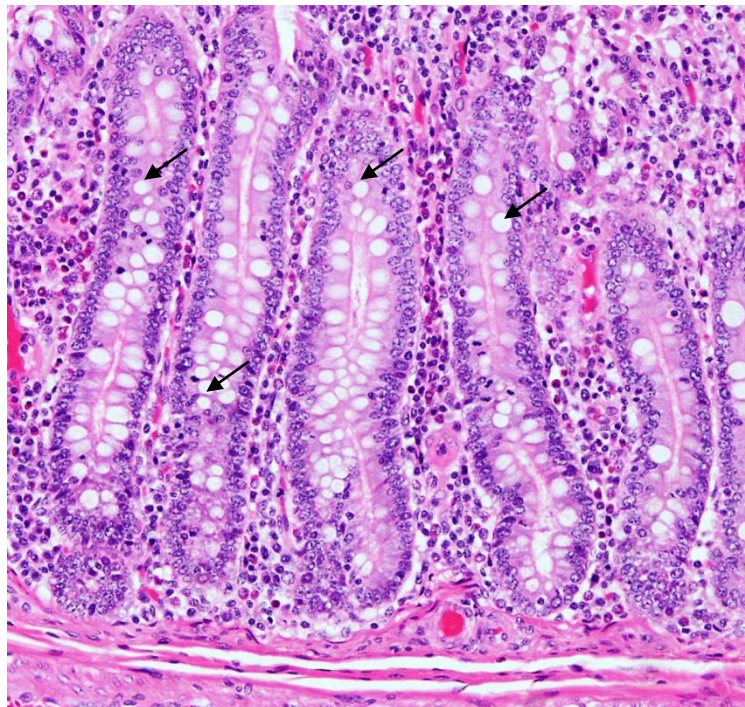


Figure 32: Ileal section (40x mag.), showing visualization of goblets cells (arrows) within enterocytes of pig (#B171) from G2 group, supplemented with β -glucan and *L. intracellularis* (vaccinated) after 28 days post-vaccination.

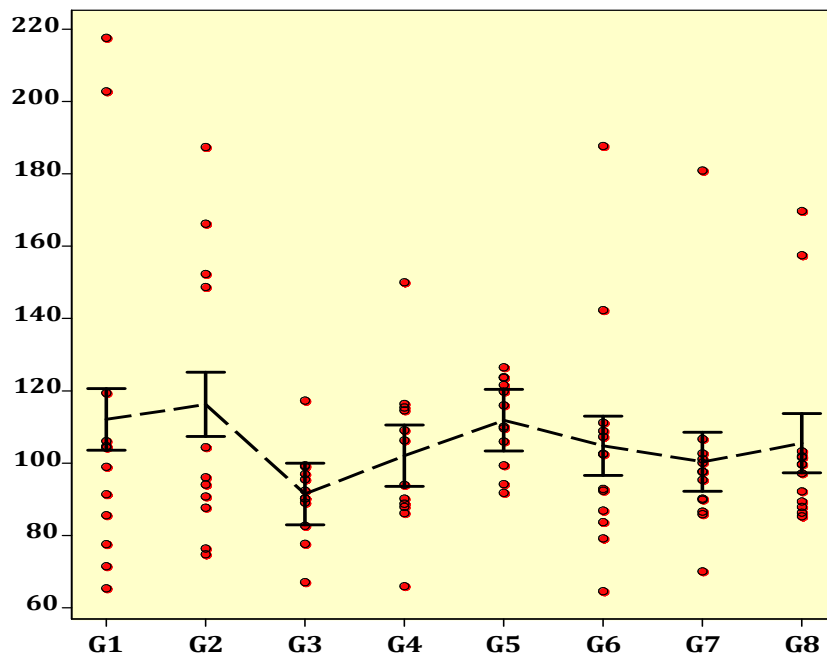


Figure 33: Mean goblet cells counts in ileal sections and mean from each group (G1-G8).

6.4 Discussion

In this study, the supplementation of 100 ppm *Saccharomyces cerevisiae* yeast beta-glucan and 2,000 FTU/kg microbial phytase to weaner diets increased the mucosal immune response to an attenuated *Lawsonia intracellularis* vaccine, as evidenced by the significant increases in *L. intracellularis* specific IgG and the cytokines, IFN- γ and TNF- α in ileal mucosa. From the available literature, this is the first study to evaluate the response to *L. intracellularis* vaccination in pig's fed diets supplemented with both these additives. Although, the effect of β -glucan on humoral and cellular immune cells is well studied, the effect of microbial phytase on immune response in pigs is relatively limited. As expected, vaccination enhanced the concentrations of *L. intracellularis* specific IgG in the serum and mucosa and these serological results are consistent with previous findings reported in Chapters 4 and 5.

6.4.1 Potential effect of phytase on vaccination

In this study, pigs consuming diets with phytase generated higher *L. intracellularis* specific IgG concentrations in mucosal scrapings compared with pigs fed the diets without phytase. This increase may possibly be associated with the activation of the innate immunity due to increased nutritional availability. Phytate can interfere with digestibility and chelate to nutrients, directly affecting its

absorption and utilization of nutrients (Simons *et al.*, 1990). Therefore, the use of an enzyme phytase to break down phytate is used to release nutrients bound to phytate and potentially increase their digestion and utilization in the gut. It is possible that the increased bioavailability of nutrients in the gut (with phytase) will allow additional nutrients to be redirected for immune cell growth and replication. For instance, Zn is known to affect the recruitment of naïve T cells and cytokine synthesis (Peterson *et al.*, 2008). As well as, the free inositol molecule has been speculated to somehow up-regulate cell division and differentiation (Menniti *et al.*, 1993) and to stimulate respiratory burst, bacterial killing and recovery by releasing IL-8, TNF- α and IL-6 and TGF- β 1 cytokines (Eggleton, 1999). In the current study we observed higher pro-inflammatory cytokine IFN- γ concentrations in ileal mucosa (118.8 pg/mL) in pigs fed diets that were supplemented with phytase than pigs fed the un-supplemented diet (81.4pg/mL). Pro-inflammatory cytokines have been shown to orchestrate the mechanisms responsible for supply of nutrients for proliferation of lymphocyte and macrophage population during periods of immune challenge (Klasing and Korver, 1997; Spurlock, 1997). Liu *et al.*, (2008) evaluated the effect of phytase (500 FTU/kg) on the immune responses of broilers that were vaccinated with Newcastle disease virus (NDV) and fed a high phytate diet (0.44% phytate P). Their results showed that phytase supplementation increase intestinal secretory IgA, as well as increased CD3⁺CD4⁺ and CD3⁺CD8⁺ T lymphocyte numbers in serum on day 28 after vaccination (Liu *et al.*, 2008).

The CD4⁺ Th1 cells produce pro-inflammatory IL-2, IFN- γ and TNF- α cytokines promoting cell mediated immune responses against intracellular pathogens, whereas CD8⁺ T mediate cytotoxic killing of infected cells (Emery and Collins, 2011). However, mucosa scrapings from the duodenum, jejunum and ileum of birds fed diets supplemented with 2,000 FTU/ kg did not express cytokines (IL1 β and IL-6) nor innate immunity receptors TLR 2 and TLR 4 genes (Olukosi *et al.*, 2013). In their study, birds were not challenged or vaccinated. Therefore it is plausible that if any changes in cytokine or innate immunity receptors did occur these may have been too subtle to detect. Shaw *et al.*, (2011) reported that birds fed 500FTU/kg phytase added to broilers diets following an *Eimeria* sp. challenge had no effect on oocyst shedding. However, the average lesion scores of infected birds fed phytase were reduced in the caecum by 18 days post challenge. In addition, cytokine IL-17 sRNA expression in the

duodenum was increased in phytase supplemented broilers. IL-17 and T-reg cells are normally involved in the inflammatory regulation in the gut which accompanies neutrophil recruitment and activity against pathogens (Basso *et al.*, 2009), as well as promoting secretion of cytokines TGF- β and IL-10 by CD4⁺ Th2 cells (Emery and Collins, 2011). Since IL-17 is produced from T-reg T-cells, and activates production of TGF- β , these results may concur with our findings that phytase supplemented pigs exhibited a trend to higher TGF- β concentrations (139pg/mL) than non-supplemented animals (120 pg/mL). Therefore, the results in this trial could be related to local gut restoration and wound healing as a consequence of the vaccine “stimulation” in the gut lumen.

6.4.2 Diet effect on growth performance and digestibility

This study did not observe any increases in growth performance (ADFI, ADG and FCR) of pigs supplemented with phytase or β -glucan. Similarly, previous studies observed minimal effects on pig performance (FI, ADG and FCR) when pigs were fed β -glucan (0.25%) from either *Laminaria digitata* or *Saccharomyces cerevisiae* β -glucan (Boling *et al.*, 2000; Woyengo *et al.*, 2008). The observations of growth performance effects of β -glucan could also be related to the source, the length and dose of supplementation. The available commercial *E. coli* phytases increased growth performances (ADG) by 33% when given to nursery pigs until 3 weeks (Jones *et al.*, 2010). Similarly, supplementing *E. coli*-phytase (2,500 FTU/kg) to P deficient diets (0.15%P) had increased ADG and FCR in weaner pigs during the 28 days trial (Veum *et al.*, 2006). However, modest changes on ADG and FCR were observed in weaner pigs supplemented with 2,000 FTU/kg of *E. coli* derived phytase added to P deficient corn based diets (0.53% P) (Beaulieu *et al.*, 2007). Possibly subtle differences in these phytase responses on growth performance could be due to differences in experimental designs and the effects are more pronounced when dietary deficiencies are corrected.

In this study, no main effects of phytase, β -glucan or vaccination were found in nutrient digestibility in pigs. However, three way interactions were found for the AID of Mg, K, Fe, and Cu with the highest AID of Mg observed for the phytase supplemented and vaccinated groups G1 and G3. Madrid *et al.*, (2013) investigated the effect of microbial phytase (500 FTU/kg) addition to low total phosphorus diet on

apparent faecal mineral digestibility. Results demonstrated that pigs supplemented with phytase had improved growth performance, however, increases in mineral digestibility retention was limited to P and Cu (39% and 33%, respectively). Adeola *et al.* (1995) also observed that supplemental phytase did not affect the retention, as percentage of intake, of either Zn or Mg.

6.4.3 Diet effect on general health

During this study, pigs were overall healthy and with occasional scouring, but without lesions and overall without *L. intracellularis* shedding after vaccination. The anti-nutritive compound phytate has been related to increase mucin excretion and it is suggested that the enzyme phytase could reverse this effect (Onyango *et al.*, 2009; Selle *et al.*, 2012). The quantity and quality of mucin produced by goblet cells, within the intestinal lumen has been directly correlated to protection against mechanical and chemical damage and pathogen invasions (Belley *et al.*, 1999). Therefore, while excessive mucin excretion may compromise intestinal mucosa integrity, the opposite is also true, the absence of mucin could lead to increased pathogen attachment (Kim and Ho, 2010). Walk *et al.*, (2011) observed no effect of in feed *E. coli* 6-phytase (1,000 FTU/kg) and *Eimeria* sp. vaccination on the number of goblet cells in the duodenum of birds 7 days post-vaccination. Similarly, in this study, no effect of numbers of goblet cells within the ileal mucosa was observed by histopathology. A tendency for increased number of goblet cells in β -glucan supplemented pigs was observed in ileal mucosa (mean 111) compared with the un-supplemented cohort (100). Smith *et al.*, (2011) demonstrated that dietary supplementation of 300ppm of seaweed derived *Laminarin* sp. in pigs led to significant increases in MUC2 and MUC4 gene expression in ileum. The mechanisms by which glucans influence mucin production are not yet understood, but it has been speculated that alterations in the intestinal microbiota could influence mucin synthesis and secretion (Enss *et al.*, 1994; Rice *et al.*, 2005).

6.4.4 Effect of β -glucan on *L. intracellularis* vaccination

The activation of the mononuclear phagocyte system and protective inflammatory cytokines by β -glucans in humans and pigs has been directly correlated with innate immune responses (Decuyper *et al.*, 1998; Brown and Gordon, 2001). Human whole blood incubated with soluble yeast β -glucan demonstrated an increase

in the production of pro-inflammatory cytokines TNF- α , IL-6, IL-8 and monocyte tissue factor (Adachi *et al.*, 1994; Young *et al.*, 2001). Similarly, Sonck *et al.*, (2010) observed proliferation of lymphocytes, neutrophils and monocytes, with TNF- α and IL-1 β production after porcine PMBCs were incubated with *S. cerevisiae* β -glucans (200 μ g/mL). In the current study, pigs fed β -glucan had raised mucosal *L. intracellularis* IgG antibody responses when compared with non-supplemented pigs (20.4 and 14.6 pg/ml, respectively). Mucosal IgA (22.3 and 16.7 pg/mL) concentrations were similar. The presence of immunoglobulin A and G antibodies in the intestinal lumen is often associated with increased protection against enteric pathogens (Jemmott and McClelland, 1989; Husband *et al.*, 1996). Purified β -glucan from the same source (AB Vista), produced higher haemagglutination inhibition (HI) titres against Newcastle disease 14 days post-challenge in yeast supplemented broilers, compared with un-supplemented animals (Haldar *et al.*, 2011). However, the effects of β -glucans on immunity are not reliably repeatable and so their ability to act against specific diseases cannot be predicted with any certainty. The administration of 90 mg/kg of purified yeast (1,3/1,6)-D-glucan to neonatal piglets that were vaccinated against human influenza, had no effect on intestinal or immune development of T cell phenotypes or cytokine gene expression and did not improve the antibody response to vaccination during the 21 day trial (Hester *et al.*, 2012). Conversely, Stuyven *et al.*, (2009), observed that in-feed β -glucan (Macrogard[®], 500g/ton) protected piglets against ETEC-F4 (*E. coli*) infection, and induced increased concentrations of F4 specific serum antibody. However, further research is required to specifically associate the effect *S. cerevisiae* yeast β -glucan to proliferative enteropathy protection in pigs.

Dendritic cell- associated C type lectin-1 (dectin-1) has been implicated as the pattern recognition receptor mostly expressed in porcine monocytes and other APCs that bind β -glucans (Sonck *et al.*, 2009). The subsequent activation of NF- κ B in monocytes and linkage of TLR2, results in enhanced phagocytosis, oxidative burst and cytokine production (Brown and Gordon, 2001; Sonck *et al.*, 2009). These are in agreement to our findings of higher concentrations of IFN- γ (147.1pg/ml) and TNF- α (20.6) cytokines in ileal mucosa from pigs supplemented with β -glucan compared with un-supplemented groups (IFN=53.2 and TNF=8.9 pg/ml). Possibly yeast

activation of the innate immune function could lead to greater induction of acquired immunity and protection against specific diseases. For instance, reduced clinical signs and increased PRRS specific IFN- γ in PBMCs incubated with a high molecular β -1,3-1,6-glucan preparation *in vitro* was observed 50 days after a challenge infection with PRRS virus (Xiao *et al.*, 2004). However, the β -glucan structure and chemical composition can modulate their effects on growth performance, health and immunity (Sonck *et al.*, 2011). Additionally, variations in the immune responses studied could be also be related to the dose of supplementation. Concentrations of IgM, IgA or CD4⁺ and CD8⁺ T-cells in ileal tissues (by IHC) of finishing pigs were not modified by the addition of *S. cerevisiae* β -glucan (at 0.03% and 0.3%, AntafermMG®) (Sauerwein *et al.*, 2007). However when 2.5% *S. cerevisiae* β -glucan (EnergyPlus®) was given to 7 days old piglets, an increased in TNF- α and IL- β 1 mRNA expression in ileal tissues was detected (Eicher *et al.*, 2006).

In conclusion, the supplementation of *Saccharomyces cerevisiae* yeast beta-glucan and microbial phytase added to weaner diet affected the mucosal and systemic *Lawsonia intracellularis* local immune response after vaccination. This study showed that *L. intracellularis* vaccination with an attenuated infection to growing pigs may be influenced by diet. It is possible that dietary components influence the efficacy of concomitantly innate and acquired immune responses against specific pathogen and protection. However, further research is required to confirm these finding and relate to specific disease protection.

Chapter 7 General discussion

As the current limitations on the use of in-feed antibiotics become more widely accepted and implemented, sanitary methods and vaccination assume a greater importance to maintain the productivity and profitability of pig herds. Currently, control of proliferative enteropathy relies on antibiotic medication and vaccination. As pressures to reduce antimicrobial growth promoters continue, the use of vaccination is considered the best alternative to protect herds from disease. The results in this thesis provide novel information on the effects of vaccine dose response, route of immunisation and in-feed additives on the systemic and local mucosal responses to vaccination with an attenuated bacterium. The experimental design also addressed whether an immune response could be confidently correlated with successful vaccination (i.e. the magnitude of the responses is predictive of protective immunity). The investigations in this thesis have expanded the limited information available in the immunological responses to the attenuated *Lawsonia intracellularis* vaccine Enterisol® ileitis in pigs.

If they are to remove in-feed antibiotics and rely on vaccines to prevent disease, producers need to be assured that pigs are protected after vaccination. To define the protective immune responses and the adequate level of protection induced in experimental and on-farm vaccination with ten-times dose of *L. intracellularis* ($10^{5.9}$ TCID₅₀) were performed. The dose reliably induces detectable local and systemic antibody and cytokine responses (Chapters 4 and 5). From the data in chapter 4, the induction of these responses indicates that protective immunity is generated. Since sampling of mucosal responses is not practical, the detection of *Lawsonia*-specific serum antibodies is a feasible indicative of protective immunity after infection (Collins and Love, 2007). Additionally, using a standard dose of *L. intracellularis* ($10^{4.9}$ TCID₅₀) induced low concentrations of *L. intracellularis* antibody in the ileal mucosa and did not stimulate detectable concentrations of mucosal cytokines (Nogueira *et al.*, 2013). So, from the results in this thesis and from other approaches such as gene expression (Jacobson *et al.*, 2011a) and flow cytometry (Cordes *et al.*, 2012), a reliable immune correlate or biomarker for on-farm use could not be recommended. The dose-dependent induction of protection against virulent *L. intracellularis* challenge was demonstrated by a significant reduction of PE lesion scores and the

magnitude and duration of bacterial shedding in faeces in all vaccinated pigs. Previous studies have related the lower initial oral challenge dose (10^3 *L. intracellularis*) with minimal clinical expression and delayed bacterial shedding in faeces and reduced histological changes when compared with higher doses (10^{10} *L. intracellularis*) (Collins *et al.*, 2001).

Additionally, there were significant variations in the concentrations of responses depending on the individual pig, experiment or herd (pen or on-farm) and the timing of the sample collection. Variation in antibody titres generated after natural infection is well-described as being affected by sampling time and original infective dose (related to severity of the primary infection) (in chapters 4 and 5; Guedes and Gebhart, 2003b; Collins and Love, 2007). Timing of sampling is critical, especially when attempts to account for individual pig variation require a quantum of animals to be sacrificed to determine mucosal responses. The studies included in this thesis was limited to sampling prior to 21 days after vaccination (days 7, 9-10 or 17) since previous reports documented detectable serum responses by this time (Kroll *et al.*, 2004; Walter *et al.*, 2005). It was gratifying to detect significant differences in mucosal cytokine responses at 9 and 17 days after vaccination, even though the spectrum of responses appeared to reflect the dynamics of immune activity. Here, some pigs appeared by day 17 to be resolving pathology (higher IL-10) while others remained activated with increased TNF- α and IFN- γ . The *Lawsonia intracellularis* specific IgG concentrations in ileal mucosal secretions also ranged from 14% and 45% in experimental and on farm trials after 17 days post-vaccination; and around 21% after 28 days post- vaccination.

L. intracellularis pathogenesis in the intestinal mucosa is not understood, but individual differences in the gut microflora of these piglets could explain some variability in infection rates and immune reactivity (Molbak *et al.*, 2008). The description of the proportion of microbial and *L. intracellularis* populations in the pigs in this study was thwarted by technical difficulties in optimizing a 16S microbial population real-time PCR. However, given the effects of *L. intracellularis* on carcass characteristics (Collins *et al.*, 2009; Collins *et al.*, 2010a), it would be a useful adjunct to this work in attempting to define the effects of infection on gastrointestinal microflora and vice-versa.

As part of routine farm management, piglets are often vaccinated while they're still suckling to overcome the need to remove antibiotics for a week around vaccination. In some herds, this leads to reduced vaccine efficacy because maternal antibodies transferred to the piglet in the sow's milk can inactivate the vaccine. Efficacy data on alternative sites for delivering mucosal vaccines, such as intranasal, intraperitoneal and intrarectal vaccination may allow producers to vaccinate and protect weaner pigs from ileitis without needing to remove antibiotics used to treat other infections. This thesis demonstrated the success of intramuscular (IM) vaccination and noted the systemic and local immunological responses after intraperitoneal (IP) inoculation of the *L. intracellularis* vaccine paralleled those induced by oral vaccination. These IM and IP routes would presumably act by neutralising antibody and blocking *L. intracellularis* attachment and colonisation. In horses, intra-rectal vaccination with Enterisol® Ileitis induced high titres (120) of *L. intracellularis* IgG responses by IPMA (Pusterla *et al.*, 2012b). The intraperitoneal route of vaccination has been previously used to protect against disease in pig herds (Sheldrake *et al.*, 1993; Djordjevic *et al.*, 1997). An intraperitoneal vaccination against *Salmonella* sp. observed protection and increased IgG and IgA serum responses that were comparable to those in orally vaccinated birds (Muir *et al.*, 1998). As noted in chapter 5, the comparable spectrum of immune responses generated after vaccination and the subsequent protection after challenge seen in Chapter 4 data strongly suggested that significant protection against *L. intracellularis* would occur after intraperitoneal vaccination. In fact, anecdotal industry personal communication would indicate that this may occur.

It was highly likely that the inoculation of *L. intracellularis* intramuscular or intraperitoneal would probably kill the organism quickly. With inactivated or subunit vaccines (or killed attenuated vaccines), the addition of an adjuvant to the *L. intracellularis* vaccine would possibly induce greater mucosal immune responses and further protection. As observed previously, killed *L. intracellularis* in incomplete Freund's adjuvant vaccine given intramuscularly to pigs reduced faecal *L. intracellularis* counts by 98.5% (Dale *et al.*, 1997). Additionally, whereas *L. intracellularis* oral live vaccine is very efficient in protecting pigs against proliferative enteropathy disease and increasing growth performances, an inactivated killed

vaccine could also induce protection. One of the benefits of using inactivated vaccines is the ability to amplify the responses against specific pathogens strains by combining different pathogens in one unique suspension. However, type of antigen to be used, cost of production and practicability of administration needs to be also considered. Recently, a patent has been registered presenting an inactivated *Lawsonia intracellularis*, *Mycoplasma hyopneumoniae* and PCV2 combo vaccine given intramuscularly. These initial studies demonstrated to be positive in protecting pigs against PE disease (reducing lesions and clinical signs), however, still information regarding field efficiency is needed.

Another approach to the replacement of in-feed antibiotics is to uniformly and non-specifically, heighten the immune “competence” to combat pathogens within pig herds by in-feed additives. Chapter 6 was the first approach to evaluate the effect of *Saccharomyces cerevisiae* derived β -glucan and microbial phytase on the responses to vaccination with *Lawsonia intracellularis*. Any synergic effect of immunomodulators in feed to amplify responses after oral vaccination is particularly appealing given the significance of gastrointestinal pathogens on pig production. This type of approach and its effect on productivity has been an area of increasing research in international pig production (Gallois *et al.*, 2009; Heo *et al.*, 2013). In this study, pigs consuming a weaner diet with added β -glucan raised mucosal *L. intracellularis* specific IgG antibody responses when compared with non-supplemented pigs, again indicating immunity against *L. intracellularis* challenge (in line with chapter 4). However, results from one trial are not sufficient and further investigation would be required to make predictive assertions about the interaction of diet and their application towards improving mucosal immunity in pigs. The effects of β -glucan are mediated through innate immunity by receptors-mediated interactions with macrophages, neutrophils and/or monocytes (Brown and Gordon, 2005), and were supported from the study in Chapter 6 which also found higher concentrations of IFN- γ and TNF- α in ileal secretions.

Phytases have been used in poultry and pigs herds to degrade plant phytate-P to release P, which would otherwise be expelled in the faeces. And since phytate is

able to chelate many cations, its hydrolysis has been associated with improved mineral utilization. Pigs consuming a weaner diet plus phytase generated higher *L. intracellularis* specific IgG concentrations in ileal mucosa secretions compared with pigs eating the standard diet. Again, comparing titres with those reported in chapter 4, it would anticipate protection against *L. intracellularis* disease in all groups vaccinated. The detection of specific IgG as an indicator of vaccine stimulation and the generation of protective immunity, possibly the supplementation with β -glucan could increase the antibody response generated by a standard dose of Enterisol® Ileitis to a detectable level and provide the relevant immune biomarker. The continual activation of the innate immune system in a state of readiness by feed additives (such as β -glucan in chapter 6) could possibly require greater energy input and the enhanced inflammatory sensitivity may detract from weight gains. A better method might be to increase the efficiency of digestion with phytase which did not promote inflammatory cytokine production (group 7 pigs in chapter 6) and enhance the availability of nutrients for immunity without compromising growth.

The implementation of strategies to induce the development of immunity to *L. intracellularis* by vaccination, as part of a farm health plan, leads to improved animal health, welfare and productivity while reducing reliance on antibiotics for preventing disease outbreaks. The results in this thesis have provided confirmation that alternative routes of vaccination as well as different approaches to nutritional strategies could provide alternative means to augment the induction of mucosal immune responses after vaccination.

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Appendix I Clinical observation record

Clinical Observation Record

Study Number:

Room #

Group	Pig ID	Diarrhoea Score	Behaviour	Body Condition	Score
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	
		1 2 3 4	1 2 3 4	1 2 3	

Diarrhoea Score

- 1 = none
- 2 = semi-solid, no blood
- 3 = watery, no blood/dark faeces
- 4 = blood tinged faeces, loose or formed

Behaviour

- 1 = normal
- 2 = slight to moderately depressed, will stand
- 3 = severely depressed or recumbent
- 4 = excitable

Body Condition

- 1 = normal
- 2 = mild to moderately gaunt (thin)
- 3 = severely gaunt

Note: If an animal is dead, record "found dead" in comments column and assign total score of 20

Observed by _____ Date _____

Recorded by _____ *Date* _____

Appendix II Published papers
