

# VACCINATION AGAINST *LAWSONIA INTRACELLULARIS* DECREASES SHEDDING OF *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM IN CO-INFECTED PIGS AND CHANGES THE HOST GUT MICROBIOME

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## **Introduction**

*Salmonella enterica* is a leading cause of foodborne illness in the world. Attribution studies have suggested pork as a major source for human salmonellosis in different countries (Mughini-Gras et al., 2013, Pires et al., 2014). In the United States, efforts to reduce the incidence of salmonellosis have mainly remained ineffective, the incidence of salmonellosis has not been reduced appreciably since 1996 (Boore et al., 2015). There is a consensus in the field that there is a lack of on farm cost-effective strategies to reduce the prevalence of salmonellosis (Dickson & Hurd, 2013). The source of *S. enterica* that contaminates pork products are the animals themselves (Dickson & Hurd, 2013) and novel intervention strategies are much needed.

Like *S. enterica*, *Lawsonia intracellularis* is a common porcine intestinal pathogen and is prevalent in pig production sites worldwide with prevalence ranging from 48 to 100% in different swine producing countries (Dors et al., 2015). In the United States, it has been estimated that *L. intracellularis* is present in more than 90% of swine farms (Armbruster et al., 2007). The last study conducted by the USDA found that *S. enterica* was present in 52.6% of swine productions sites (USDA, 2009), thus it is reasonable to assume that co-infection with both pathogens occurs frequently. *L. intracellularis* causes porcine proliferative enteropathy (PPE). This disease more commonly occurs in post-weaned pigs and leads to decreased weight gain, diarrhea and is often subclinical. Transmission of this organism also occurs by the fecal oral route and lesions are marked by a thickening of the mucosa of the ileum and colon (Lawson & Gebhart, 2000).

The association between *L. intracellularis* infection and increased shedding of *Salmonella* was first demonstrated by Beloeil et al., 2004, who performed an epidemiological study and found a significant association between seroconversion to *L. intracellularis* and increased prevalence of pigs shedding *S. enterica*. In this study, we hypothesized that vaccination against *L. intracellularis* would decrease shedding of *S. enterica* in co-infected animals.

## **Materials and methods**

### **Animals and experimental design**

In this study a total of five treatment groups were used. The treatment groups were: 1) challenged with *S. Typhimurium* alone, 2) challenged with both *S. Typhimurium* and *L. intracellularis*, 3) challenged with *S. Typhimurium* and vaccinated against *L. intracellularis*, 4) challenged with both *S. Typhimurium* and *L. intracellularis* and vaccinated against *L. intracellularis*, and 5) a non-infected control. Each treatment group was comprised of 9 pigs housed in three separate isolation rooms with three

animals per room. The non-challenge control group was comprised of 6 animals divided among two rooms with three animals per room. These rooms were distributed between two isolation buildings. The groups that were vaccinated against *L. intracellularis* received the single dose oral live attenuated vaccine Enterisol Ileitis (Boehringer Ingelheim) at three weeks of age. Twenty-one days post vaccination, animals were challenged with a pure culture of *L. intracellularis* ( $2 \times 10^9$  organisms per pig) (strain PHE/MN1-00). One week post *L. intracellularis* challenge, pigs were challenged orally with *S. Typhimurium* (strain 798) ( $1 \times 10^8$  organisms per pig). Fecal samples from pigs were obtained on the day of challenge and two days post *S. Typhimurium* challenge and weekly thereafter until 49 days post infection.

### **Salmonella quantification**

To quantify the amount of *Salmonella* shed in feces of pigs, a most probable number (MPN) enrichment method was used as in Borewicz et al., 2015. Briefly, one gram of feces was suspended in 9 ml tetrathionate broth (TTB) and incubated at 41°C for 48 hours. One hundred  $\mu$ l was then transferred to 900  $\mu$ l Rappaport-Vassiliadis R10 broth and incubated for 24 hours at 41°C. Cultures were then inoculated on to XLT4 agar plates containing 100 $\mu$ g/ml of nalidixic acid (NA) to quantify the challenge strain which is NA resistant. A duplicate inoculation was performed on XLT4 agar without antibiotic to quantify any other potential *Salmonella* strains the animals could harbor. Colonies with typical *Salmonella* morphology were confirmed by PCR using primers specific for the gene *invA* (Singer et al., 2006).

### **DNA extraction and 16S sequencing**

For microbiome analysis, DNA was extracted from fecal samples using the MoBio PowerSoil DNA extraction kit. DNA concentration was measured by Nanodrop. The V1-V3 region of the 16SrRNA gene was amplified following a dual indexing approach as described in Gohl et al., 2016. Quality filtered sequences were analyzed with QIIME (Caporaso et. al, 2010).

### **Statistical analysis**

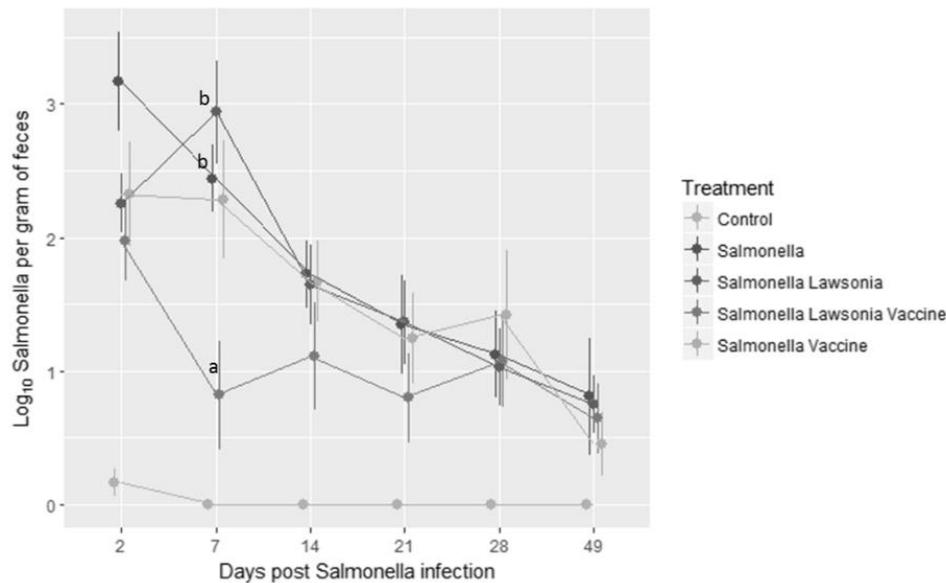
To test for differences in MPN between treatments over time, a linear mixed model was used with  $\log_{10}$ (MPN) as the response, treatment, day, and the treatment/day interaction as fixed effects, barn as a fixed block effect, and pen, pig, and day within pen as random effects. Reported are least square means for treatment by day, and pairwise comparisons between treatments for each day, with p-values corrected for multiple comparisons.

## **Results and discussion**

### ***L. intracellularis* vaccination reduces *S. Typhimurium* shedding in co-infected animals**

No animals had detectable levels of the challenge strain prior to challenge and detection levels of *Salmonella* were similar in XLT4 with *S. Typhimurium*. The greatest difference in shedding level between groups was found at 7 days post infection. At this time point, the co-challenged non-vaccinated group shed 2.94  $\log_{10}$  *S. Typhimurium*

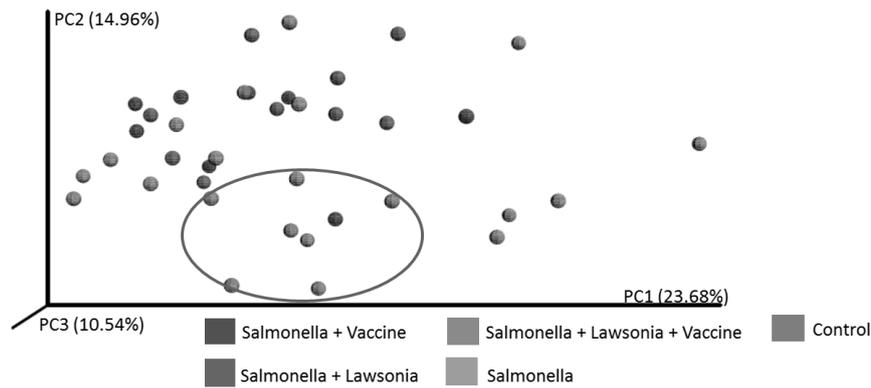
organisms per gram of feces while the vaccinated co-challenged group shed 0.82 log<sub>10</sub> *S. Typhimurium* organisms per gram of feces ( $p=0.003$ ) (Figure 1). The co-challenged vaccinated group also shed significantly less *S. Typhimurium* than the singly infected *S. Typhimurium* group which shed 2.44 Log<sub>10</sub> *S. Typhimurium* organisms per gram ( $p=0.03$ ). *L. intracellularis* vaccination did not have a significant impact on *S. Typhimurium* shedding when animals were singly infected with *S. Typhimurium*.



**Figure 1.** Fecal shedding of *Salmonella enterica* serovar Typhimurium measured by the MPN method. Line graph of *S. Typhimurium* shedding by group in different time points. Significant differences between treatment groups are designated by different letters ( $P < 0.05$ ).

### ***L. intracellularis* vaccination alters the microbiome in co-infected animals**

To investigate microbiome differences between treatment groups, beta diversity analysis was performed using the weighted UniFrac distance which measures dissimilarity in microbiomes based on their phylogenetic composition (Lozupone et al., 2007). Investigating the 7 day post infection timepoint, different treatment groups had significant differences in their microbiome community structure (ANOSIM  $p < 0.05$ ). The co-infected vaccinated group clustered apart from all other treatment groups. Again, this effect was dependent on an animal receiving both *S. Typhimurium* and *L. intracellularis* as well as prior *L. intracellularis* vaccination (Figure 2).



**Figure 2.** Beta diversity analysis, Principal coordinate analysis plot of weighted UniFrac distance among different treatment groups at 7 days post *S. Typhimurium* infection (ANOSIM  $p < 0.05$ ).

## Conclusion

Vaccination against *L. intracellularis* significantly reduced *S. Typhimurium* shedding ( $p < 0.05$ ) in co-infected animals in comparison to the co-infected group without vaccination and the group challenged with *S. Typhimurium* alone. Significant differences in beta diversity were found (ANOSIM  $p < 0.05$ ) and the co-challenged vaccinated group had a distinct community structure from other groups demonstrating that co-challenge and vaccination lead to different changes in the microbiome compared to single or dual infection and vaccination without challenge. These results indicate that vaccination against *L. intracellularis* impacts the microbiome and reduces shedding of *S. Typhimurium* in co-infected animals. This evidence suggests that *L. intracellularis* vaccination may be used as novel tool to aid in the control of *Salmonella* on swine farms as well as an alternative to reduce the need for antibiotic treatment of pigs and improve food safety.

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