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Changes in Analytes Related to Immunity in the Saliva of Pigs After Vaccination Against *Lawsonia intracellularis*

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Abstract: *Lawsonia intracellularis* is a Gram-negative, intracellular bacterium that can infect several animal species. In pigs, the bacteria cause porcine proliferative enteropathy, or ileitis. The wide spread of the pathogen produces a large impact on pig production worldwide. Saliva is a source of biomarkers that can help to monitor changes in the immune system after vaccination. The purpose of this study was to study the changes in haptoglobin (Hp), immunoglobulin G (IgG), and adenosine deaminase (ADA) in saliva after vaccination against *Lawsonia intracellularis*. In addition, productivity parameters were analysed to evaluate if vaccination and changes in salivary analytes could be associated with changes in these parameters. The pigs vaccinated against Lawsonia showed an improvement in the productive parameters and a reduction in food conversion and frequency of diseases. In addition, they showed lower values of Hp ($p = 0.011$), IgG ($p < 0.01$), and ADA ($p < 0.003$) in saliva during the first two months of the fattening period compared to non-vaccinated pigs. It could be concluded that in our experimental conditions, the vaccination against *Lawsonia intracellularis* produced a significant decrease in biomarkers of the immune response in saliva compared with the non-vaccinated pigs. This would indicate a reduction in the activation of the immune system, which could be postulated to be due to the increased defence ability of the organism against pathogens. This reduced activation of the immune system can lead to better food conversion and an increase in the productive parameters of these pigs. Overall, this report opens a new window for the possible use of saliva for non-invasive evaluation of the immune system after vaccination in pigs.

Keywords: Lawsonia; saliva; vaccine; ADA; haptoglobin; IgG

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1. Introduction

Lawsonia (L.) intracellularis is a Gram-negative, intracellular bacterium that in pigs affects the gastrointestinal tract with a specific tropism for the terminal ileum, producing porcine proliferative enteropathy (PPE), or ileitis. The clinical appearance consists mainly of diarrhoea, poor growth, and increased mortality. This pathogen affects pig production worldwide since *L. intracellularis* is reported to affect 57–100% of herds globally [1].

For the treatment of *L. intracellularis* infections, antibiotics from the macrolide and pleuromutilin groups can be used. For prophylaxis, inactivated and live-attenuated vaccines are being used in the pig industry against the disease. An example is an inactivated injectable one-dose vaccine, Porcilis® Lawsonia, that has an inactivated freeze-dried bacterial antigen and a duration of immunity of at least 21 weeks [2]. This vaccine reduced the intestinal colonisation and the severity and duration of faecal shedding of this bacteria, leading to a decrease in the incidence and severity of clinical signs of ileitis due to *L. intracellularis* and improving gut function [3]. In addition, vaccination reduces the feed conversion ratio and antimicrobial consumption and increases average daily weight gain [4].

Saliva analysis is gaining importance in pigs, especially due to its easy collection, which is much less stressful and painful than blood sampling. In saliva, there are some analytes, such as haptoglobin (Hp), immunoglobulin G (IgG) and adenosine deaminase (ADA), that can be used to gain information about the immune system [5]. Hp is a moderate acute-phase protein (APP). APPs show changes in their concentrations in response to any inflammatory stimulus, are highly sensitive to detecting inflammation, and are early biomarkers for this process [6]. IgG concentrations in saliva are mainly derived from blood and are related to its concentration in serum, being considered a biomarker of humoral acquired immunity [7]. Increases in salivary IgG have been described in infectious diseases such as porcine reproductive and respiratory syndrome [5]. ADA is an enzyme related to cell-mediated immunity and differentiation of T lymphocytes in humans [8]. In pigs, ADA activity is over 100-fold higher in saliva than in serum [9], and increases in ADA activity in saliva have been found in infectious diseases such as *Streptococcus Suis* [10] or *Escherichia coli* infections [11], possibly reflecting immune activation.

The hypothesis of this report is that vaccination against *Lawsonia intracellularis* could produce changes in analytes related to the immune system in saliva. Therefore, the purpose of this study was to study the changes in Hp, IgG, and ADA in saliva after vaccination against *Lawsonia intracellularis* in finishing pigs. In addition, productivity parameters and antimicrobial consumption were analysed to evaluate if vaccination and changes in salivary analytes could be associated with changes in these parameters.

2. Materials and Methods

2.1. Animals

This study was conducted on a commercial farm located in the municipality of Lorca (Region of Murcia, Spain) from November 2023 to March 2024. Regarding the health status, the farm was positive for the porcine reproductive and respiratory syndrome (PRRS) but stable, negative for toxigenic *Pasteurella multocida*, and positive for *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* (serotype 17). Two groups of pigs were made, a vaccinated group and a control group, the animals of each group being randomly selected. The group of vaccinated pigs received the vaccine against porcine proliferative enteropathy (*Lawsonia intracellularis*) before weaning. The two groups were monitored from the beginning of the fattening phase (10 weeks old) until their transfer to the slaughterhouse (approximately 4 months after). In each group, productive data were collected in 630 pigs, whereas blood Lawsonia titters and analytes in saliva were evaluated in 15 pigs per group.

In the farm of the study, the production system was a multi-site production, in which each of the production phases (reproduction, weaning, and fattening) was located at three independent sites. Both the control group and the study group barns were filled in a single day, following the all-in, all-out management program established by the farms' protocol. The capacity of each barn is 630 animals, all of which came from the same source. In both groups, the piglets were vaccinated at 2 days of age against Edema Disease and 5 days before weaning (which took place at 4 weeks of age) against *Mycoplasma hyopneumoniae* and

Porcine Circovirus (PCV2). At this time, additionally, the vaccinated group received a dose of vaccine against porcine proliferative enteropathy (*Lawsonia intracellularis*), administered along with the *M. hyopneumoniae* and PCV2 vaccine by separate injections intramuscularly.

2.2. Data Collection

Once the vaccine was administered, the piglets were observed for three weeks at 2–3 day intervals to identify any local reactions at the injection site or any systemic change. After this, pigs were monitored daily in both study groups. In these monitorizations, routine clinical observation and external evaluation were performed in animals, recording pigs with lower bodyweight gain (individual bodyweight was not measured) and those that had a decrease in feed intake since they did not perform the normal feeding behaviours. In addition, clinical signs of disease were monitored, and in case of the appearance of respiratory symptoms, the presence of animals with cough and dyspnoea was recorded. In cases of clinical signs of diarrhoea, severe diarrhoea was considered when it appeared with other systemic clinical signs such as prostration, weakness, and wasting, whereas mild diarrhoea was considered when it appeared without other evident clinical signs.

The average daily weight gain (ADWG, gr), feed conversion ratio (FCR, kg), antimicrobial consumption (AM), and mortality rate (number of dead relative to the total number of pigs) were calculated for each of the groups, taking into account all animals present in the barn (630 pigs), and were extracted from the routine herd database software package (SIP Consultors). The data included were from the day of the entrance to the fattening unit to the transportation to the slaughterhouse (4 months later). In the case of ADWG, it was calculated by weighing the pigs on the day of transferring to the finishing unit and the day of transferring to the slaughterhouse and dividing by the number of days at the finishing unit.

For data on antimicrobial consumption during the period of this study, only antimicrobials administered orally were recorded. The use of antimicrobials was performed exclusively in the case of the occurrence of clinical disease, and the choice of treatment was at the discretion of the farm veterinarians. The cost of medicines was calculated per animal.

Blood and saliva samples were collected from 15 randomly selected pigs of each group from the beginning of the fattening phase at 1-month intervals for 2 months, with the first sample taken at the beginning of the fattening and the other two samples taken one and two months later. Therefore, bleeding and saliva sampling were performed at three different time points: T1 at 10 weeks of age at the entrance to the fattening unit, T2 at 14 weeks of age, and T3 at 18 weeks of age.

2.3. Vaccine

The vaccine used in this study contained inactivated *L. intracellularis* bacteria in Emunade® adjuvant (Porcilis® Lawsonia from MSD Animal Health). The vaccine is an oil-in-water emulsion. Just prior to use, Porcilis® Lawsonia lyophilizate was dissolved in Porcilis® PCV M Hyo (associated mixed use) and was administered to three-week-old pigs under typical field conditions.

2.4. Lawsonia Serological Analysis

Serum samples were analysed for Lawsonia titers with a Lawsonia antibody ELISA Svanovir® L. intracellularis/Ileitis-Ab, Sweden. This is a blocking Enzyme-Linked Immunosorbent Assay (ELISA) for the detection of *Lawsonia intracellularis (L. intracellularis*) specific antibodies in porcine serum and plasma samples. The kit procedure is based on a solid phase-blocking ELISA.

The optical density (OD) was measured at 450 nm. ELISA results were expressed as percentage of *Lawsonia intracelularis* detected by pig IgG (PI).

2.5. Saliva Analysis

Haptoglobin concentration was measured by an assay based on AlphaLisa technology previously used in pig saliva [12].

IgG was measured by a commercially available assay that has been previously validated in pigs [13].

ADA was analysed with a commercially available automated spectrophotometric assay (Adenosine Deaminase assay kit, Diazyme Laboratories) previously validated for porcine saliva [9].

2.6. Statistical Analysis

Statistical analysis was performed by using SPSS v 26 software (IBM, Chicago, IL, USA). The Shapiro–Wilk test was used for assessing the normal distribution of considered variables. As all these considered variables showed significant deviations from a normal distribution, they were submitted to log10 transformation prior to any comparison. To examine potential variations in concentrations among independent groups (control and vaccinated) over time, a two-way repeated measures analysis of variance (ANOVA) was conducted. For all analyses, statistical significance was set at a threshold of $p < 0.05$. The F-statistic is used to assess whether the variability between groups is significantly greater than the variability within groups, and it has been included in the statistical results. When detecting significant differences across multiple comparisons, we employed pairwise comparisons with Bonferroni's correction. To evaluate the association between clinical signs and mortality with the vaccination status, a Chi-square test was performed. First, a global analysis was conducted to determine whether there was a significant association across all clinical signs combined. Subsequently, individual Chi-square tests were performed for each clinical sign to identify specific associations between vaccination status and the presence of the sign. $p < 0.05$ was considered statistically significant for all analyses.

This study's statistical power for the saliva analysis was calculated using the following parameters: a significance level (α) of 0.05, an effect size of 0.8 (representing a biologically meaningful difference of 10 units on a logarithmic scale), the means and standard deviations of each group, and the sample size per group. The analysis was performed using online software (https://www.statskingdom.com accessed on 24 September 2024) and plots using the GraphPad Prism (GraphPad software version 10.3.0, Boston, MA, USA). Based on these calculations, a minimum number of 15 animals for the saliva analysis was recommended for each group.

3. Results

3.1. Clinical and Productivity Data

When piglets were examined for three weeks at intervals of 2–3 days after vaccination to identify any local reactions at the injection site, no adverse local reactions, such as redness, inflammation, or abscess formation, were observed. Significant systemic reactions were also not noted. Some of the piglets, after the administration of the vaccine (approximately at 26 days of age), appeared somewhat more apathetic but did not stop nursing.

A significant global association was observed between vaccination status and the prevalence of clinical signs during the fattening period, with non-vaccinated animals showing a higher prevalence of clinical signs compared to vaccinated animals ($p < 0.001$). In the vaccinated group, no severe diarrhoea was observed in any of the animals, whereas in the control group, 12% of the animals presented, throughout the study period, severe diarrhoea that was sometimes accompanied by digested blood (dark appearance). The presence of severe diarrhoea was significantly higher in non-vaccinated pigs compared to vaccinated pigs ($p < 0.001$). The affected animals reduced their feed intake, which

resulted in a loss of body condition. The pigs with severe diarrhoea were apathetic, and the body condition in the pens was very heterogeneous, which complicated and prolonged the loading process for slaughter. In addition, in the control group, 22% of the animals also had mild diarrhoea, whereas 2% had mild diarrhoea in the vaccinated group. The presence of mild diarrhoea was significantly higher in non-vaccinated pigs compared with vaccinated pigs ($p < 0.001$). In some of the animals with diarrhoea in the non-vaccinated group, respiratory symptoms appeared, with cough (15%) and dyspnoea (4%), whereas in the vaccinated group, respiratory signs were milder, and only 5% of animals had coughs ($p < 0.001$). Cases of tail biting were higher in the control group (2%) versus vaccinated (0.5%) ($p = 0.007$).

Regarding the losses suffered (Table 1), a total of 24 losses occurred in the vaccinated group, whereas the control group had a total of 37 losses. In the vaccinated group, 12 losses were due to respiratory problems, 6 due to diarrhoea, and 6 due to other causes (3 due to problems in the locomotor system, 2 due to *C. perfrigens* infection, and 1 due to meningitis due to *S. suis* infection). In the control group, 19 losses were due to diarrhoea, 9 due to respiratory problems, and 9 due to other causes (3 due to meningitis due to *S. suis* infection, 3 due to *C. perfrigens* infection, 2 due to problems in the locomotor system, and 1 due to gastric ulcers). There was a statistical tendency to increase the number of losses in non-vaccinated pigs compared with vaccinated pigs ($p = 0.088$).

Table 1. Productive data. ADWG: average weight gain. FCR: food conversion rate.

	Number of Entered Animals	Removals	Days in Fattening	ADWG (gr/Day)	FCR (kg)
Vaccinated Study Batch	630	24	122	755	2.35
Unvaccinated Control Batch	630	37	133	683	2.49

The vaccinated pigs showed a higher average weight gain of 755 g per day compared to 683 g in the unvaccinated group (Table 1). The better conversion in the vaccinated group (2.35 versus 2.49, that in the average price of kg of feed during the study period, representing an impact of 2.97 euros) and low antibiotic consumption (1.7 versus 3.1 euros per animal) had an economic impact of 4.37 euros per animal for the vaccinated group compared to the control group. The percentages of antimicrobial treatment per cause and per group were 39% and 61% in the control group in respiratory disease and digestive disease, respectively, whereas in the vaccinated group, there were 80% and 20% in respiratory disease and digestive disease, respectively.

3.2. Serological Data

Results of the percentage of *Lawsonia intracelularis* detected by pig IgG in the commercial kit used in our report, which would be indicative of the concentration of *L. intracellularis* IgG antibodies in serum, appear in Table 2.

Table 2. Percentage of *Lawsonia intracelularis* detected by pig IgG in the serum of pigs at different sampling times.

Time	Group	Median	Deviation
T1	Control	2.47	3.63
	Vaccinated	20.3	11.3
T2	Control	8.51	16.5
	Vaccinated	19.2	9.23
T3	Control	20.3	12.6
	Vaccinated	23.7	16.2

When the two-way mixed ANOVA was applied, a significant interaction was found between time and group (F = 3.916, $p = 0.026$).

When the simple effect of the group was evaluated, highly significant differences appeared between the two groups at T1 ($p < 0.001$) and T2 ($p = 0.036$), with higher concentrations of antibodies in the vaccinated group. At T3, no significant differences were detected between the groups ($p = 0.517$). When only the effect of time was evaluated, significant differences did exist in the control group ($p = 0.001$) between T1 and T3, with a significantly higher mean concentration of antibodies in T3.

The vaccinated group had no significant differences between the sampling times ($p = 0.311$).

3.3. Effect of Vaccination on the Salivary Haptoglobin, Immunoglobulin G, and Adenosine Deaminase Concentrations

Hp concentrations showed significant changes at the different times depending on the group considered (F = 4.844, $p = 0.011$) (Figure 1). A significant main effect of time, depending on the group, was observed in time 2 (F = 31.754, $p < 0.001$) and time 3 (F = 31.754, $p < 0.001$). In the case of time 2, the results showed that vaccinated pigs had lower haptoglobin concentrations (1.17 ± 1.08 mg/L) than non-vaccinated pigs (5.21 ± 10.05 mg/L). Also, at time 3, haptoglobin concentrations were significantly lower in vaccinated pigs (1.25 ± 0.96 mg/L) compared with controls (8.50 ± 10.22 mg/L).

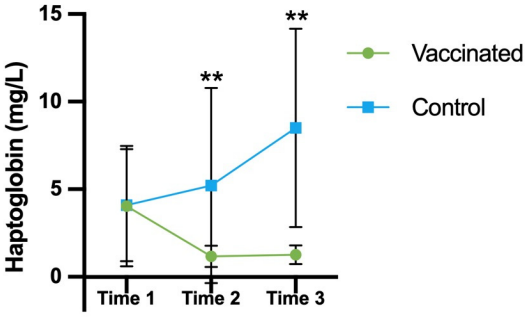

Figure 1. Mean and standard deviation of haptoglobin concentrations at different sampling times in vaccinated (green line) and non-vaccinated (control) pigs (blue line). ** Significant differences between groups ($p < 0.01$).

The sampling time significantly affected the vaccinated group (F = 3.689, $p = 0.038$), but no significant differences were detected between specific times after Bonferroni's correction in multiple comparisons. The control group had no differences between the sampling times (F = 2.785; $p = 0.079$).

IgG concentrations showed significant changes in their dynamics at different times, being different depending on the group considered (F = 18.805, $p < 0.01$) (Figure 2). At time 2, the results showed that vaccinated pigs exhibited lower IgG salivary concentrations (1.0 ± 0.1 mg/L) than control pigs (7.1 ± 9.2 mg/L) (F = 9.083, $p = 0.006$). In the case of time 3, the pigs from the vaccinated farm also showed lower IgG concentrations (1.4 ± 1.3 mg/L) than control pigs (93.9 ± 144.5 mg/dL) (F = 135.754, $p < 0.001$).

When comparing each group individually, the effect of time was significant in the vaccinated pigs (F = 14.616, $p < 0.001$), indicating a significant decrease at time 2 (1 ± 0.1 mg/L) ($p = 0.004$) and time 3 (1.4 ± 1.3 mg/L) ($p = 0.007$) compared with time 1 (16.1 ± 24.8 mg/L). In the control group, a significant effect of time was found (F = 25.455, $p < 0.001$); in this case, mean IgG concentrations were significantly reduced at time 2 (7.1 ± 9.2 mg/L) ($p = 0.011$) but were significantly increased at time 3 (93.9 ± 144.5 mg/L) ($p = 0.011$) compared with

time 1 (14.8 ± 9.7 mg/L). Compared with time 2, IgG concentrations significantly increased at time 3 ($p \leq 0.001$).

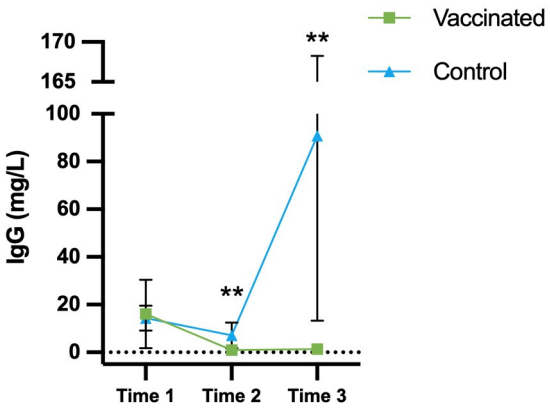

Figure 2. Mean and standard deviation of immunoglobulin G concentrations at different sampling times in vaccinated (green line) and non-vaccinated (control) pigs (blue line). ** Significant differences between groups ($p < 0.01$).

For ADA, the time behaved in the same way regardless of the group considered (F = 0.316, p = 0.73). Whatever the time considered, there were significant differences between groups (F = 10.298, p = 0.003), ADA concentrations being higher in the control group (Figure 3).

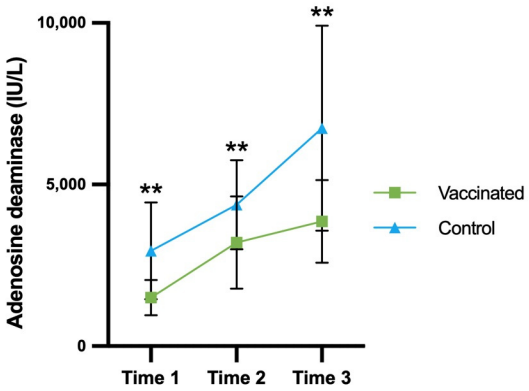

Figure 3. Mean and standard deviation of adenosine deaminase concentrations at different sampling times in vaccinated (green line) and non-vaccinated (control) pigs (blue line). ** Significant differences between groups ($p < 0.01$).

When comparing each group individually, the effect of time was significant in both groups (F = 16.361, $p < 0.001$), indicating a significant increase at time 2 (p = 0.003) and time 3 ($p < 0.001$) compared with time 1.

4. Discussion

In this report, the changes in three analytes, Hp, IgG, and ADA, related to the immune system and that can be measured non-invasively in saliva were studied after a vaccination against *Lawsonia intracellularis*.

The pigs vaccinated against *Lawsonia intracellularis* showed an improvement in the productive parameters and a decrease in food conversion. This is in agreement with a previous report, and it would indicate that in our experimental conditions, the vaccination was effective [4]. In addition, they showed a lower incidence of diseases, which is in line with a previous study made with this vaccine [3]. This could be possibly due to

the reduction in bacterial load and increase in ileum integrity of the vaccinated animals compared to the control non-vaccinated [3].

The results of the serology indicated that in the vaccinated animals, there was an antibody response due to the vaccine that was observed in the first sample taken 6 weeks after vaccination. It can be postulated that in the vaccinated group, the antibody response could be related to vaccination since, at this first sample collection, there were already 46.7% of positive animals in this group versus 0% in the control group that was not vaccinated. In the control group, the positive animals do not appear until T2, which could indicate the circulation of the agent in the fattening units, in which the pigs had entered 4 weeks before. This is in line with the fact that Lawsonia-specific IgG and IgM responses peak in serum at 3–4 weeks and can be detectable for up to 13 weeks post-infection [14].

This study provides additional evidence about changes produced in saliva analytes related to the immune system after *Lawsonia intracellularis* vaccination. Hp is an acute-phase protein that can measured in the saliva of pigs, being correlated with serum values, and it is considered a very sensitive biomarker to detect inflammation [5]. Therefore, the lower values found in pigs after vaccination compared to those non-vaccinated could indicate a lower incidence of inflammation. Our results agreed with previous reports that lower Hp values were associated with improved productive results in pigs treated with immunomodulators [15,16]. In addition, this will align with previous data that indicate that lower Hp concentrations in serum are associated with increased productivity performance in the pig [17]. On the other hand, the high values of Hp found in the control group can be related to the increased incidence of inflammation and/or infection, which leads to a reduction in the productivity of the pigs. The way in which high Hp concentrations could be associated with a decreased performance is because the synthesis of Hp and other acute-phase proteins that are produced in inflammation produced a shift in the amino acids that are diverted from the synthesis of muscle protein to the generation of these acute-phase proteins [18].

IgG in saliva is related to the concentration of serum, which is mainly derived from blood [5]. Although specific IgGs are used more for the detection of antibody production against an infectious agent [19], the measurement of their total values has clinical value, and an increase in their concentration can indicate the presence of an infectious disease [5]. Therefore, the presence of an increase in IgG in the non-vaccinated pigs in our study would indicate the existence of a higher incidence of infectious diseases in this group.

ADA is a ubiquitously expressed enzyme that is abundant in pig saliva [5]. It is related to the differentiation of T lymphocytes in humans. Its measurement in serum has been used as a biomarker of cell-mediated immunity [8]. In pigs, it has been described to be increased in gastrointestinal [11] and other diseases in pigs, such as *S. suis* infection [10]. In addition, it has been correlated with acute-phase proteins, and therefore, it could be affected by inflammation [9]. It could be postulated that the decrease in ADA in the group that was vaccinated compared to the control group could be related to lower activation of cellular immunity and inflammation due to the lower incidence of pathogens affecting these pigs. In the case of *Lawsonia intracellularis*, it could be influenced by their reduced ileal colonization and the severity and duration of faecal shedding of this bacteria [3] that vaccination produced.

It is interesting to point out that the three biomarkers analysed in our study can increase in a non-specific way in regard to the nature of the inciting agent [5,17]. So, overall, in addition to the lower incidence of *Lawsonia intracellularis*, the lower incidence of other diseases that were found in the vaccinated group could lead to the postulation that the vaccination reduced the incidence of other diseases and, therefore, the immune system activation against them.

This study has various limitations. One is that it was made in a herd with a history of outbreaks of diarrhoea caused by *Lawsonia intracellularis* at levels which affect productivity results. Therefore, vaccination was made with a previous expectation of positive results. Further studies should be conducted on the changes in the analytes of this study in farms with a lower incidence of this disease. In addition, ideally, an additional sample should have been performed at month 4, but this was not possible due to logistic reasons. However, this approach raised an interesting topic, which is the fact that lower previous values on immune markers could predict increases in productive performance, this being in agreement with previous reports, in which the concentration of serum Hp obtained five weeks before could predict pigs that would have a high weight gain as opposed those which would have a low weight gain [17]. Also, ideally, the individual animals should have been weighted and their productive parameters monitored in order to be able to make a statistical analysis of the results, although in studies in which a large number of pigs were evaluated, the same approach as our report was taken [20]. Finally, it would be of interest in the future to evaluate how other analytes related to the immune system, such as S100 proteins, other acute-phase proteins, or IgA that can be measured in saliva, behave in vaccination.

5. Conclusions

It could be concluded that in our experimental conditions, the vaccination against *Lawsonia intracellularis* produced a decrease in Hp, IgG, and ADA in saliva compared with the non-vaccinated pigs, which would indicate a reduction in the activation of the immune system, probably due to the increased defence ability of the organism against pathogens. This reduced activation of the immune system can lead to better food conversion and an increase in the productive parameters of these pigs.

Author Contributions: Conceptualization, M.T., J.J.C., and R.M; methodology, A.M.-M., M.T., J.J.C., E.G., and A.M.-P.; software, R.M., M.T.T., and A.M-P.; validation, A.M.-M., J.J.C., and A.M.-P.; formal analysis, A.M.-M. and A.M.-P.; investigation, M.T., A.F., J.J.C., E.G., and A.M.-P.; resources, M.T., J.J.C., E.G., and A.M.-P.; data curation, A.M.-M., R.M., M.T.T., and A.M-P.; writing—original draft preparation, A.M.-M., R.M., and J.J.C.; writing—review and editing, A.M.-M., M.T., E.R., S.G., A.F., J.J.C., R.M., M.T.T., E.G., and A.M.-P.; visualization, S.G., A.F., J.J.C., and A.M.-P.; supervision, M.T., J.J.C., E.G., and A.M.-P.; project administration, J.J.C. and A.M.-P.; funding acquisition, J.J.C. and R.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All procedures were approved by the Ethical Committee on Animal Experimentation (CEEA) of the University of Murcia (protocol code CEEA 563/2019).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of this study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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