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**Leukocyte coping capacity to measure infection/vaccination related stress in weaning
piglets**

Diploma Thesis

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1 Abstract

The assessment of stress and pain in animals presents a persistent challenge in veterinary medicine, often lacking a universally accepted golden standard. The Leukocyte Coping Capacity (LCC) is an innovative functional assay measuring the ability of polymorphonuclear neutrophils (PMNLs) to produce reactive oxygen species (ROS) upon stimulation. Low LCC values have been associated with increased stress, while high values suggest a reduced stress burden. This study investigated the utility of LCC as an indicator of immune response to infection and vaccination related stress in weaned piglets.

Fifty-two commercial weaned piglets were randomly assigned to four experimental groups (G1, G2, G3 Control G4). G1 and G2 were infected intranasally with two distinct Porcine Reproductive and Respiratory Syndrome Virus 1 (PRRSV1) isolates, G3 was vaccinated intramuscularly with a modified live virus vaccine, and G4 served as the control group. Blood samples were collected eight times over 28 days to measure LCC Area Under the Curve (AUC) and the Myeloperoxidase Index (MPXI). A linear mixed-effects model was used for statistical analysis.

The LCC assay successfully produced a clear, measurable effect. While infected groups (G1, G2) and the control group (CG4) showed relatively stable LCC AUC values throughout the trial, the vaccinated group (G3) exhibited a rapid and substantial increase in LCC AUC, peaking around day 14 at mean values exceeding 7,000–8,000, followed by a sharp decline. This elevation suggests a transient hyperactivation of neutrophil function in response to vaccine. Conversely, no significant differences were observed in MPXI values between groups. Total leukocyte and neutrophil counts across all groups showed a progressive, age-related decline over the 28-day period.

This study demonstrates that the LCC assay is a valuable functional tool for assessing neutrophil oxidative activity in weaned piglets, particularly highlighting a potent, measurable immune response following vaccination. This approach can provide standardized and objective insights into the immune status of swine and supports LCC as a promising technique for evaluating the physiological impact of interventions, such as vaccination and infection, in a field setting.

2 Zusammenfassung

Die Evaluierung von Stress und Schmerz bei Tieren bleibt eine zentrale Herausforderung der Veterinärmedizin, da ein universeller Goldstandard bislang fehlt. Die Leukocyte Copping Capacity (LCC) stellt hierbei ein innovatives Verfahren dar: Dieser funktionelle Assay misst die Produktion reaktiver Sauerstoffspezies (ROS) durch polymorphkernige neutrophile Granulozyten (PMNL) nach gezielter Stimulation. Während niedrige LCC-Werte mit hohem Stress korrelieren, deuten hohe Werte auf eine reduzierte Stressbelastung hin. Ziel dieser Studie war es, die LCC als Indikator für die Immunantwort auf Infektionen sowie auf impfbedingten Stress bei Absetzferkeln zu untersuchen.

Im Rahmen der Untersuchung wurden 52 Absetzferkel aus konventioneller Haltung zufällig auf vier Gruppen verteilt (G1, G2, G3 und die Kontrollgruppe G4). Die Gruppen G1 und G2 wurden intranasal mit zwei verschiedenen Isolaten des Porzinen Reproductiven und Respiratorischen Syndrom Virus 1 (PRRSV1) infiziert, während G3 eine intramuskuläre Impfung mit einem modifizierten Lebendimpfstoff erhielt. Über 28 Tage wurden insgesamt acht Blutproben pro Tier entnommen, um die LCC-Fläche unter der Kurve (AUC) und den Myeloperoxidase-Index (MPXI) zu bestimmen. Die Analyse der Daten erfolgte über ein lineares gemischtes Modell.

Die Ergebnisse belegen, dass der LCC-Assay deutliche und messbare Effekte liefert. Während die Infektionsgruppen und die Kontrolle über den Versuchszeitraum stabile LCC-AUC-Werte aufwiesen, zeigte die vakzinierte Gruppe (G3) einen rasanten Anstieg der LCC-AUC mit Spitzenwerten von durchschnittlich 7.000–8.000 um den 14. Tag. Diese Dynamik spricht für eine transiente Hyperaktivierung der Neutrophilenfunktion infolge der Vakzinierung. Im Gegensatz dazu wies der MPXI keine signifikanten Gruppenunterschiede auf, während die Gesamtleukozyten- und Neutrophilenzahlen in allen Gruppen altersbedingt sanken.

Zusammenfassend belegt diese Studie, dass die LCC ein wertvolles funktionelles Werkzeug zur Messung der oxidativen Neutrophilenaktivität bei Ferkeln ist. Insbesondere die starke Immunantwort nach einer Impfung lässt sich damit objektiv darstellen. Die Methode bietet somit das Potenzial, den Immunstatus von Schweinen standardisiert zu erfassen und die physiologischen Auswirkungen von Infektionen oder Interventionen unter Praxisbedingungen zu bewerten.

3 Introduction

A variety of techniques is used to evaluate stress and pain in vertebrate biology, including fields such as human medicine, animal husbandry, and wildlife ecology (1). Determining the levels of pain and stress experienced by animals has long represented a major challenge in scientific research (2). Various approaches have been developed to quantify these parameters, and numerous studies have investigated a broad range of methodologies, including the measurement of cortisol levels as well as physiological, hematological, and behavioral indicators (1, 2). Despite significant research efforts, a universally accepted golden standard for the evaluation of pain and stress in animals has not yet been established so far (2).

In general, LCC is described as a measure of leukocyte functional activity, specifically their ability to produce oxygen free radicals upon stimulation by bacterial peptides binding to their cell membranes, by activation of protein kinase C through phorbol 12-myristate 13-acetate (PMA) and stress-related stimuli (1, 2). This measurement, which focuses on the examination of polymorphonuclear neutrophils (PMNLs) from stressed animals, indicates that the results vary depending on the level of stress: low LCC values are associated with increased stress, whereas high LCC levels reflect a reduced stress burden (2, 3). Additionally, studies indicate that stress can influence the production of reactive oxygen species (ROS) by leukocytes, which further supports the use of LCC as an indicator of physical stress (1).

PMNLs, commonly referred to as neutrophils, represent a key cellular component of the innate immune system (4). They are continuously generated in the bone marrow and typically constitute approximately 20–45% of all circulating leukocytes in peripheral porcine blood (4). They play a pivotal role as first-line defenders against invading pathogens owing to their high motility and ability to be rapidly recruited to sites of bacterial, fungal and viral infection (4–6). With over 150 different receptors, PMNLs can sense a wide variety of stress-associated signals in the organism. Such signals involve endocrine substances in circulation, changes in blood composition and red blood cell dynamics, shifts in cytokine concentrations, and mediators produced through activation of both the hypothalamic–pituitary–adrenal-axis (HPA) and the sympathetic nervous system (SNS) (7).

In recent years, LCC has gained attention as an indicator of the immune response in animals (1). The ability to measure and understand the LCC could lead to better management of animal

health, especially under stress or infection (1). Previous research has primarily concentrated on species such as cattle (2), wildlife (7) and humans (6), resulting in a limited understanding of LCC in pigs.

The Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is one of the most significant pathogens affecting pigs (8). Infection is associated with respiratory issues and reduced growth rates in weaned piglets and fattening pigs. In breeding animals, the virus primarily causes reproductive disorders, such as stillbirths, abortions, and weak-born piglets (9). PRRSV is a single-stranded, enveloped RNA virus from the family *Arteriviridae* and exhibits high genetic variability (8). There are two main genotypes: the former European genotype 1 (PRRSV-1) and the North American genotype 2 (PRRSV-2). It is known for its high mutation rate and constant genetic changes due to the lack of proofreading activity in its RNA-dependent RNA polymerase (10). Furthermore, new recombinant strains continuously emerge, further increasing the virus's complexity and adaptability (9).

Pigs are the only established natural host of PRRSV. The virus exhibits a marked tropism for cells of the mononuclear phagocyte system, with porcine alveolar macrophages being the primary site of viral replication. There is also evidence suggesting that dendritic cells may contribute to supporting PRRSV replication (11). Upon binding of PRRSV glycoproteins to sialic acid-containing receptors on porcine macrophages, the virus enters the host cell via clathrin-mediated endocytosis. This is followed by viral transcription, assembly, and release (11, 12). Furthermore, PRRSV infection has been observed to significantly suppress the cytotoxic activity of natural killer (NK) cells (11).

The development of PRRSV infection is generally described as occurring in three separate and well-characterized phases: acute infection, persistence, and extinction. PRRSV has been shown to replicate for up to 250 days post infection, indicating its potential to establish a possibly lifelong infection in pigs (11).

PMNLs are activated either through binding to bacterial peptides or via stress-induced activation of glucocorticoid, α -, and β -adrenoceptors (3). Among their numerous effector functions, neutrophils generate reactive oxygen species (ROS) through a process known as the oxidative burst, which is mediated by the nicotinamide adenine dinucleotide phosphate

(NADPH) oxidase complex, from both, the membrane complex and intracellular pools. These reactive molecules not only activate granular proteases but also play a crucial role in the formation of neutrophil extracellular traps (NETs). The production of ROS triggers a range of downstream cellular response, including the release of cytoplasmic granules and the stimulation of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF α) and macrophage inflammatory protein-2 (MIP-2) (6).

ROS contribute to antimicrobial defense by penetrating pathogen membranes, especially those of bacteria, and inducing damage to critical cellular structures including nucleic acids, proteins, and membrane components. Consequently, ROS play a fundamental role in the host's innate immune response against a broad spectrum of pathogens (6). Excessive ROS production can lead to tissue damage, highlighting the need for precise regulation of the oxidative burst; in this context, studies have shown that ROS can act in both directions, either helping to contain infection or, conversely, exacerbating disease progression, depending on the intensity and nature of the immune response (5, 6).

The regulation and impact of this oxidative burst, particularly in the context of neutrophil activity, can be assessed through various markers. The myeloperoxidase index (MPXI) is a parameter that reflects the balance between myeloperoxidase (MPO) synthesis during the promyelocyte stage and the consumption of peripheral blood neutrophils during infection and respiratory burst activity. In humans, the myeloperoxidase index is considered a potential marker of inflammation. Most studies indicate that MPXI values are decreased in case of sepsis accompanied by severe inflammatory response. Comparative studies in domestic animals are limited; however, one study in horses demonstrated that lower MPXI values were associated with increased mortality (13).

This study investigated the LCC in pigs in the context of an experimental infection using an economically important porcine virus to explore how various factors influence the immune system's response. It is hypothesized that infection with a virus, such as PRRSV in the present study, leads to a measurable impairment in the functional competence of neutrophilic granulocytes (14). This study further investigated the immune responses and changes in leukocyte coping capacity (LCC) among pigs assigned to four distinct experimental groups including one group with vaccination with a modified live virus vaccine and control group.

4 Material and Methods

The experimental infection study took place from November to December 2024. A total of 52 commercial weaned piglets (*Sus scrofa domesticus*), aged 28 days with an approximate weight of 6–8 kg, were used. The piglets originated from a PRRSV-free farm in Lower Austria, specifically the Teaching and Research Farm VetFarm Medau, Berndorf, Austria. The animals were housed in the biosafety level 2 isolation unit of the University of Veterinary Medicine Vienna and were randomly allocated to the four compartments of the stable: infection group 1 (G1), infection group 2 (G2), vaccination group 3 (G3) and control group 4 (CG4). Before the trial started, the animals had a one-week acclimatization period, were fed *ad libitum* a commercial diet, and had permanent access to fresh water.

4.1 Infection and Treatment

Infection and treatment (vaccination or administration of cell culture medium) were carried out as following: G1 and G2 were infected with a dose of 5×10^5 TCID₅₀ of PRRSV1 isolate AUT18_2488 and PRRSV1 isolate Ger22_1510 intranasally. G3 was vaccinated with 2 ml vaccine intramuscularly as recommended by the manufacturer (Porcilis® PRRS, Intervet GesmbH, Vienna, Austria). CG4 only received 2 ml cell culture medium intranasally, which was used for virus propagation in G1 and G2.

4.2 Sample collection

For four consecutive weeks, rectal body temperature was measured, and general behaviour was assessed daily. Only animals that were euthanized 28 days post treatment were included in this LCC study. Blood samples (serum, lithium-heparinized blood) and nasal swabs were collected from all piglets on study days (SD) zero (day of infection), two, four, seven, ten, 14, 21, and 28 and analyzed using RT-qPCR, which was used for another part of the study that is not included in this diploma thesis. Li-heparinized blood was additionally used for determination of a total blood cell count, using the analyzer at the Central Laboratory of the University of Veterinary Medicine Vienna, which also included the MPXI. Total blood cell count was necessary for correction of LCC for neutrophils.

The preparation of the chemicals for the LCC involved several key steps. First, a PMA stock solution was prepared by dissolving 5 mg of PMA in 8.106 ml of DMSO, creating a dilution of 10^{-3} M. From this stock solution, 0.1 ml aliquots were transferred into Eppendorf tubes and then stored at -20°C . To create the PMA working solution, one of the frozen 0.1 ml aliquot of the stock solution was defrosted and 9.9 ml of PBS was added, achieving a final dilution of 10^{-5} M. For the Luminol stock solution, 0.0177 g of Luminol was weighed and dissolved in 1 ml of DMSO. As Luminol is highly light-sensitive, it was essential to keep the solution protected from light. After dissolution, 9 ml of PBS (pH 7.4) was added, resulting in a final concentration of 10^{-2} M. The solution was then divided into 0.5 ml aliquots, transferred into Eppendorf tubes, wrapped in aluminium foil for light protection, and stored at -20°C . To prepare Luminol working solution, a 0.5 ml aliquot of the frozen stock solution was defrosted, and 49.5 ml of PBS (pH 7.4) was added, obtaining a final dilution of 10^{-4} M. As with the stock solution, the working solution was kept wrapped in aluminium foil to prevent light exposure and ensure chemical stability.

The preparations for each sampling day were as follows: The frozen chemicals were removed from the freezer at -20°C and left to thaw. The samples were then opened, ensuring that the pellet was at the bottom of the tube and not on the lid. After arranging both solutions, they were gently swirled. As previously described, blood was collected on specific days from the pigs via puncture of the *Vena cava cranialis* or the *Vena jugularis externa* using syringe and needle. Blood samples were collected using specialized lithium heparine-coated microvettes (Microvette® 200 Lithium Heparin LH, 200 μl , SARSTEDT AG & Co. KG, Nürnbrecht, Germany) and were processed for LCC measurement immediately after collection.

For each pig, two labelled tubes were used to distinguish between the challenge and control samples. In each tube, 10 μl of heparinized blood was added, followed by 90 μl of Luminol. Before adding the blood, it was important to swivel the lithium-heparinized tubes to prevent clotting. Next, 10 μl of the PMA working solution was added to the challenge tubes, and the tubes were gently swirled to ensure proper mixing. The samples were then ready for LCC measurement. Both the challenge tube and the control tube were measured in parallel with two chemiluminometers simultaneously for about 20 seconds every 5 minutes over a total duration of 50 minutes. During the measurement intervals, the samples were kept in a block heater at

39.5°C (upper level of mean rectal body temperature of weaners) and protected from light using a lightproof pasteboard.

4.3 Data duration

Data collection took place from 18th November to December 17th, 2024. Prior to the trial, the animals underwent a 10-day acclimatization period in the new environment. Blood samples were collected on specific days, as previously described in the methods section, and were analysed within a maximum of one to two hours after collection. All data used in this thesis, including LCC measurements and clinical observations, are based on this defined sampling period.

4.4 Statistical analyses

The analyses and partial effect plots were conducted in R, following an already published approach (15): A linear mixed-effects model was applied to the variable of interest, LCC, including group, day of sampling, and the quadratic term of day of sampling as fixed effects, and ID as a random effect (16). For this purpose, the R package, Linear and Nonlinear Mixed Effects Models (nlme) were used. Using the Multi-Model Interference package (MuMIn), model comparison based on the corrected Akaike Information Criterion (AICc) (17, 18) was performed to evaluate whether the linear term for day of sampling was sufficient, or if including a quadratic term would improve model fit (19). The statistical values presented in the results section are based on a Type II ANOVA conducted on the respective best-fitting model, as determined by AICc comparisons (20). Marginal (excluding random effects) and conditional (including random effects) R^2 values were calculated using the MuMIn package (19). If a variable (e.g., MPXI) showed a significant effect, a post-hoc analysis was conducted to determine between which groups the differences occurred (21). These comparisons are visualized in the figures by horizontal bars indicating significant group differences.

All models were validated using histograms and Q-Q plots, confirming that the residual followed a normal distribution.

4.5 Language Editing and Artificial Intelligence-Based Writing Support

Artificial intelligence-based language tools were utilized to enhance the linguistic quality and coherence of the written content in this thesis.

5 Results

5.1 Overview

The results presented in this section summarize the clinical, haematological, and immunological findings obtained from the experimental investigation. Data are organized according to the parameters assessed, including clinical observations, LCC, the MPXI

and immune cell population dynamics. Statistical analyses were conducted to identify significant effects of treatment, time and their interactions, as outlined in the Material and Methods.

5.2 Clinical Observations

Throughout the observation period, no severe clinical symptoms were observed in most pigs. There was one exception where one pig developed polyserositis, polyarthritis and pleuritis. Additionally, a slight increase in internal body temperature was observed in several pigs. However, this was most likely attributable to stress by handling the animals rather than the infection itself.

5.3 Leukocyte Coping Capacity (LCC)

Figure 1: G1 (PRRSV1 isolate AUT18_2488), G2 (PRRSV1 isolate Ger22_1510), and CG4 (cell culture medium, control group) demonstrated relatively stable LCC AUC values, with minor fluctuations and a slight downward trend observed in some cases. Throughout the observation period, values in these groups generally remained below 5,000, indicating limited variation in neutrophil functional response.

In marked contrast, G3 (vaccinated with Porcilis® PRRS) exhibited a distinct trajectory. While initial LCC AUC values were comparable to those of the other groups, a rapid and substantial increase was observed beginning in the early phase of the trial. This response culminated in a pronounced peak around day 14, with mean LCC AUC values exceeding 7,000–8,000. Subsequently, a sharp decline followed, with values returning to near-baseline levels by the finalisation of the study. This transient yet significant elevation suggests a short-term hyperactivation of neutrophil function in this group.

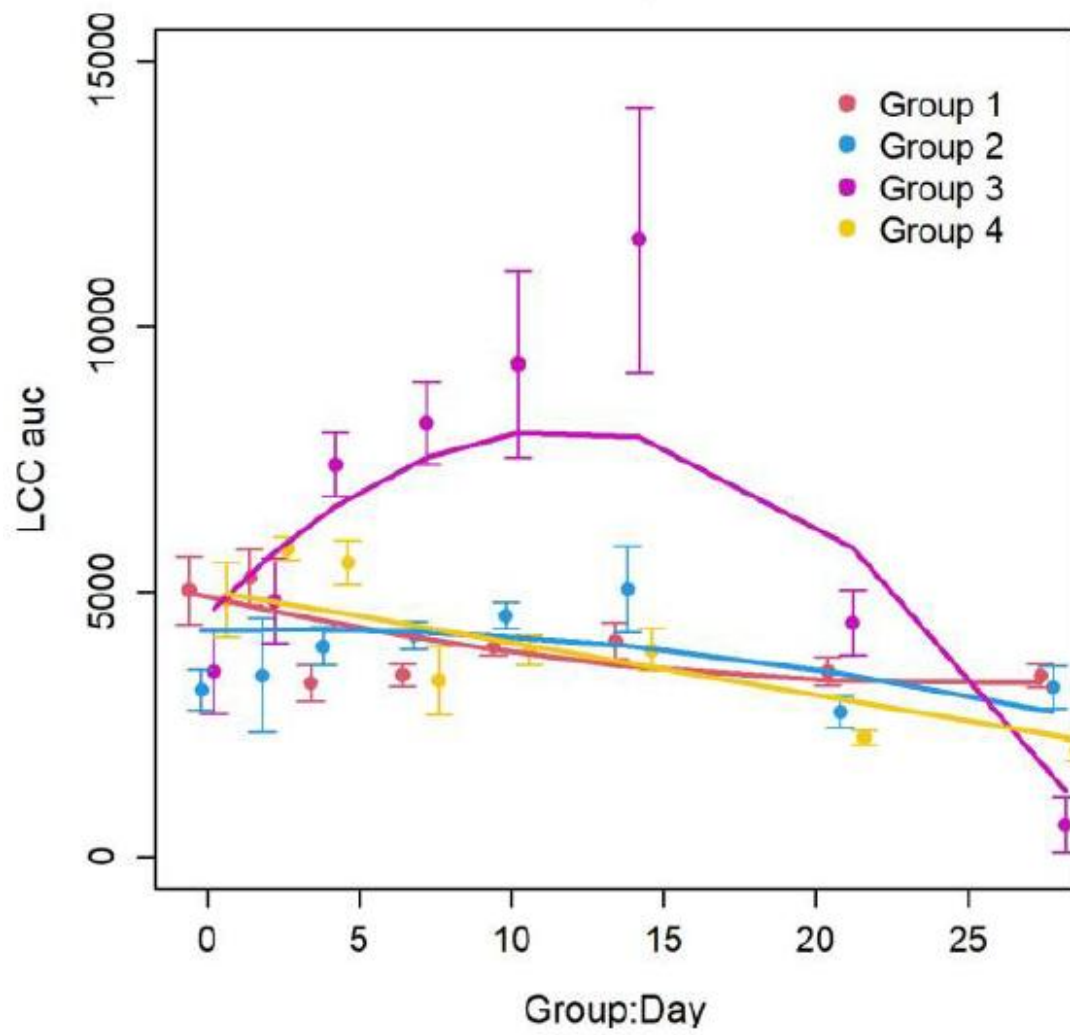


Figure 1 Temporal progression of the leukocyte coping capacity (LCC) area under the curve (AUC) in the four experimental groups over the duration of the study

Figure 2: The data demonstrate a strong, positive linear correlation between the two variables peak LCC and LCC AUC, with both values increasing proportionally. The close clustering of data points along the fitted regression line indicates a high degree of association, suggesting that higher peak LCC responses strongly linked to a greater cumulative leukocyte activity over time.

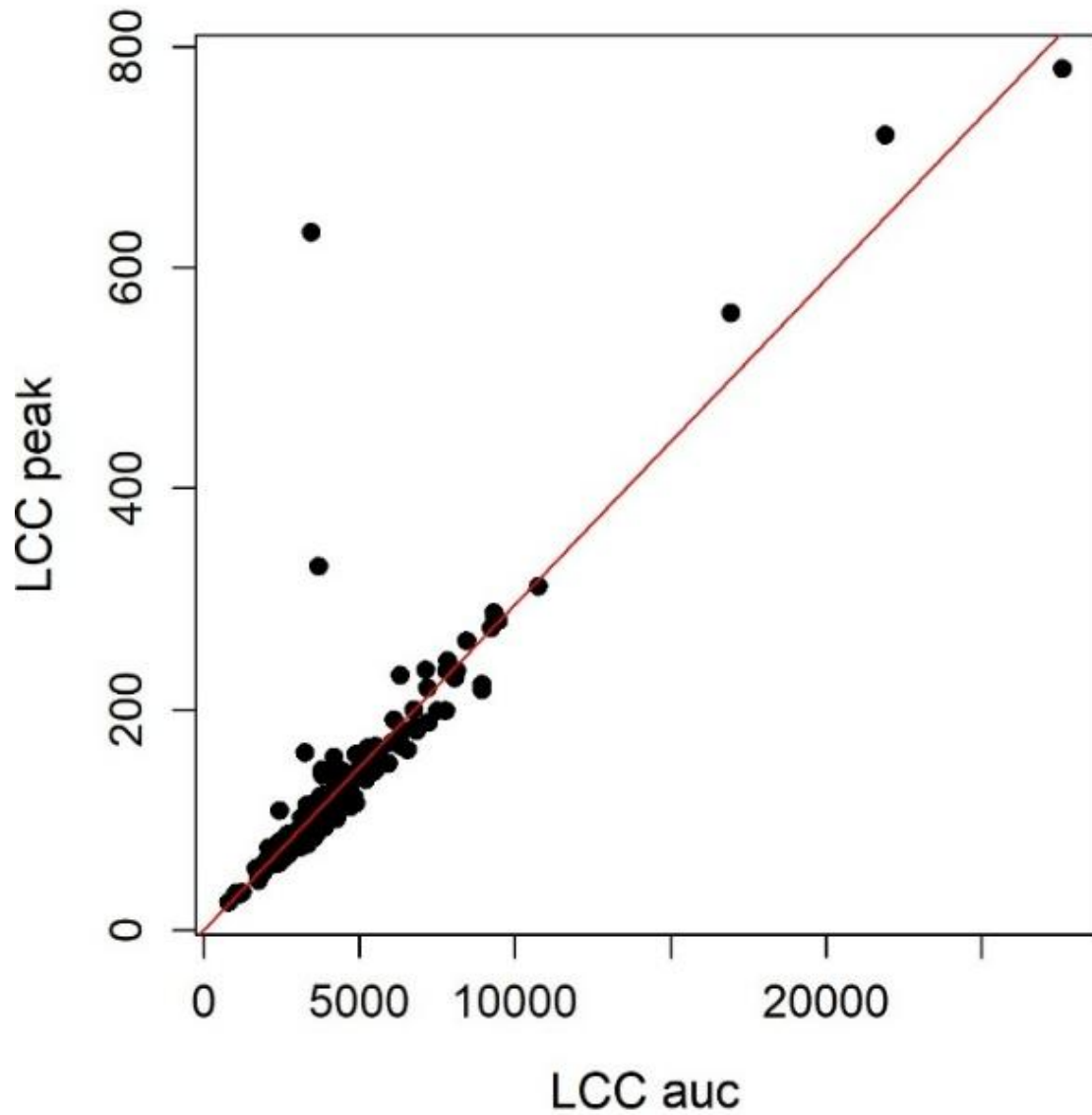


Figure 2 Relationship between the LCC peak values (y-axis, ranging from zero to 800) and the corresponding LCC area under the curve (AUC) values (x-axis, ranging from zero to 30000).

Figure 3: The longitudinal analysis of LCC reveals that all four experimental groups started at a similar baseline on SD0, with values primarily ranging between 0.25 and 0.45. While the PRRSV-infected groups (G1: AUT18-248 and G2: Ger22-1510) and the G4 (Control) remained relatively stable with only minor fluctuations throughout the 28-day period, G3 (Porcilis) exhibited a distinct upward trend starting after SD4. This vaccinated group reached a peak LCC value of approximately 0.65 between SD10 and SD14, a phase characterized by significantly higher inter-individual variability as indicated by the expanded error bars. Following this peak, the LCC levels for G3 declined, and by SD21, all four cohorts converged back to a uniform baseline that persisted until the final observation on SD28.

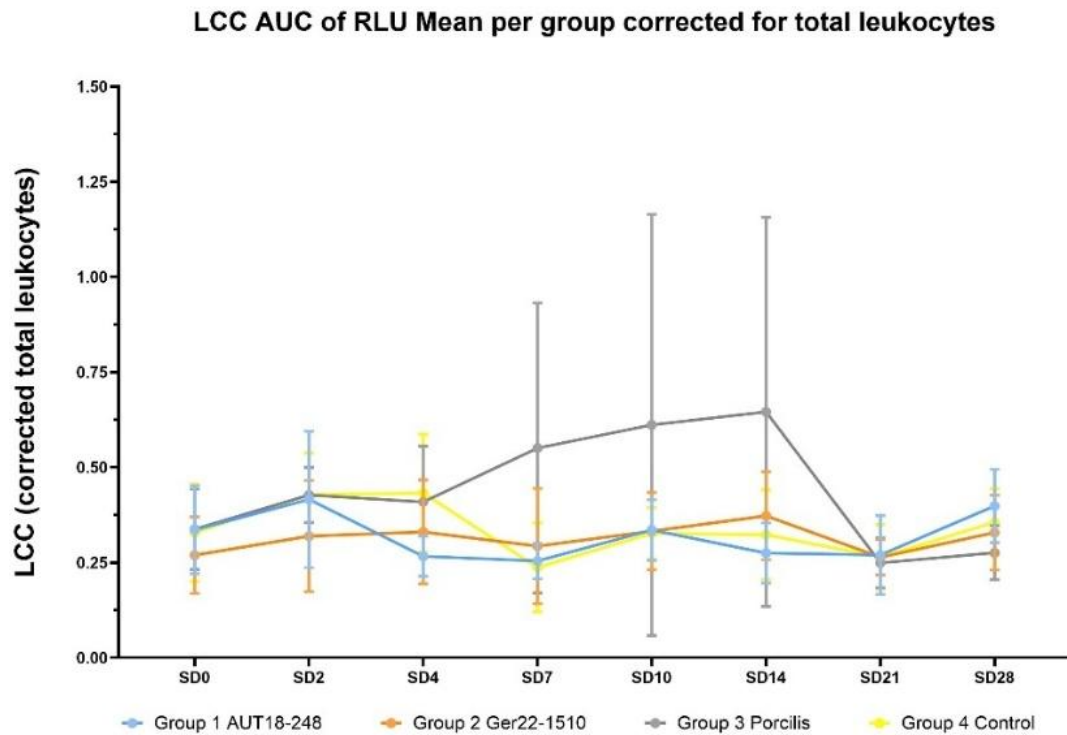


Figure 3 Data are presented as the Area Under the Curve (AUC) of the Relative Light Unit (RLU) mean, corrected for total leukocyte counts, over a 28-day study period (SD0 to SD28). The groups represent two PRRSV-infected cohorts (Group 1: AUT18-248; Group 2: Ger22_1510

5.4 Neutrophil Functional Assays

Figure 4: A progressive decline in neutrophil numbers was observed across the entire observation period, with initial values averaging approximately 8000 cells/ μ L and decreasing around 4500 cells/ μ L by the final sampling days. Notably, the rate of decline was steeper during the first half of the study, indicating a pronounced initial immune, followed by a more gradual reduction in the latter half.

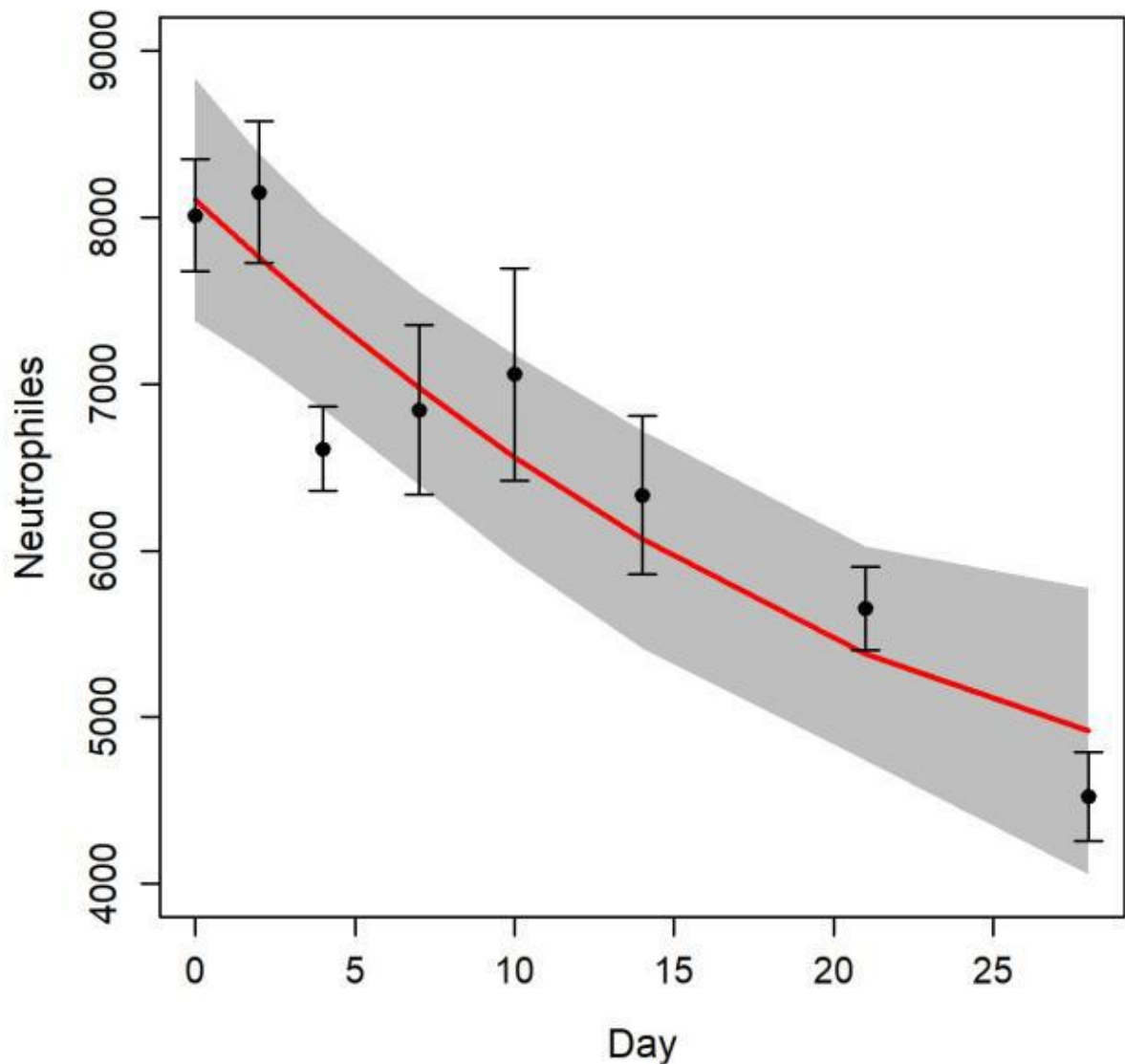


Figure 4 The corresponding graph depicts the temporal development of peripheral blood neutrophil counts from the animals throughout the study period. Days are plotted on the x-axis, while absolute neutrophil counts (cells/ μ L) are represented on the y-axis.

Figure 5: At the beginning of the study, the leukocyte count was at its highest level, approximately 23,000 cells/ μL . Over the course of the four-week period, the count declined steadily, reaching its lowest value of about 13,000 cells/ μL on the final sampling day. The red trend line illustrates this consistent downward trajectory, indicating a relatively constant rate of decrease. The individual black dots represent the mean leukocyte count measured on each sampling day. While these data points closely follow the trend line, they also reflect natural biological variation. The vertical error bars accompanying each dot indicate the variability of the measurements for that day.

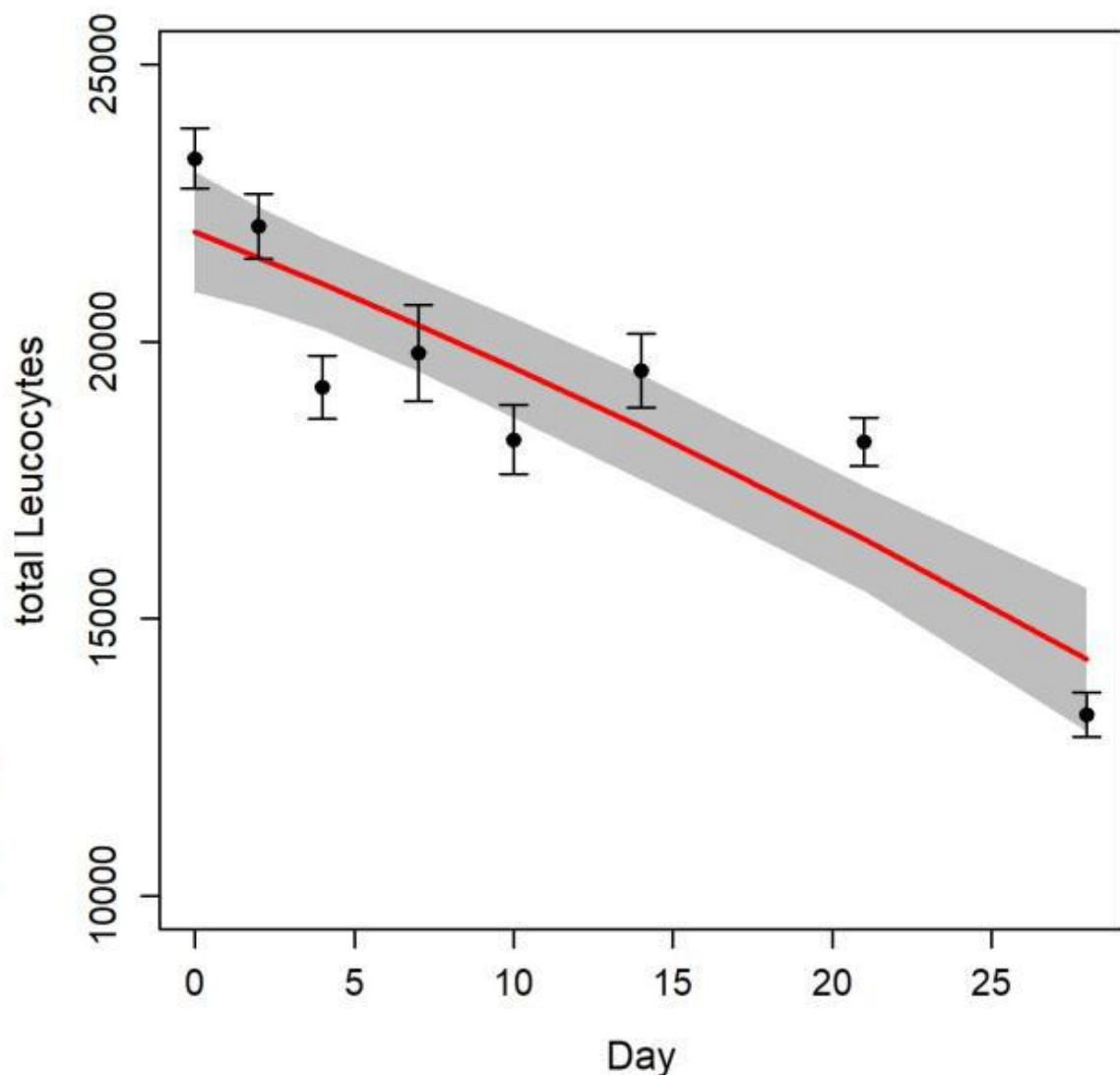


Figure 5 This graph illustrates the total leukocyte count on the y-axis measured at several time points over 28 days on the x-axis

5.5 Myeloperoxidase Index (MPXI)

Figure 6: The distribution of values reveals a weak inverse association, whereby increases in LCC AUC are accompanied by a slight decrease in MPXI. The broad dispersion of individual data points relative to the fitted trend line suggests a low correlation strength between these two parameters.

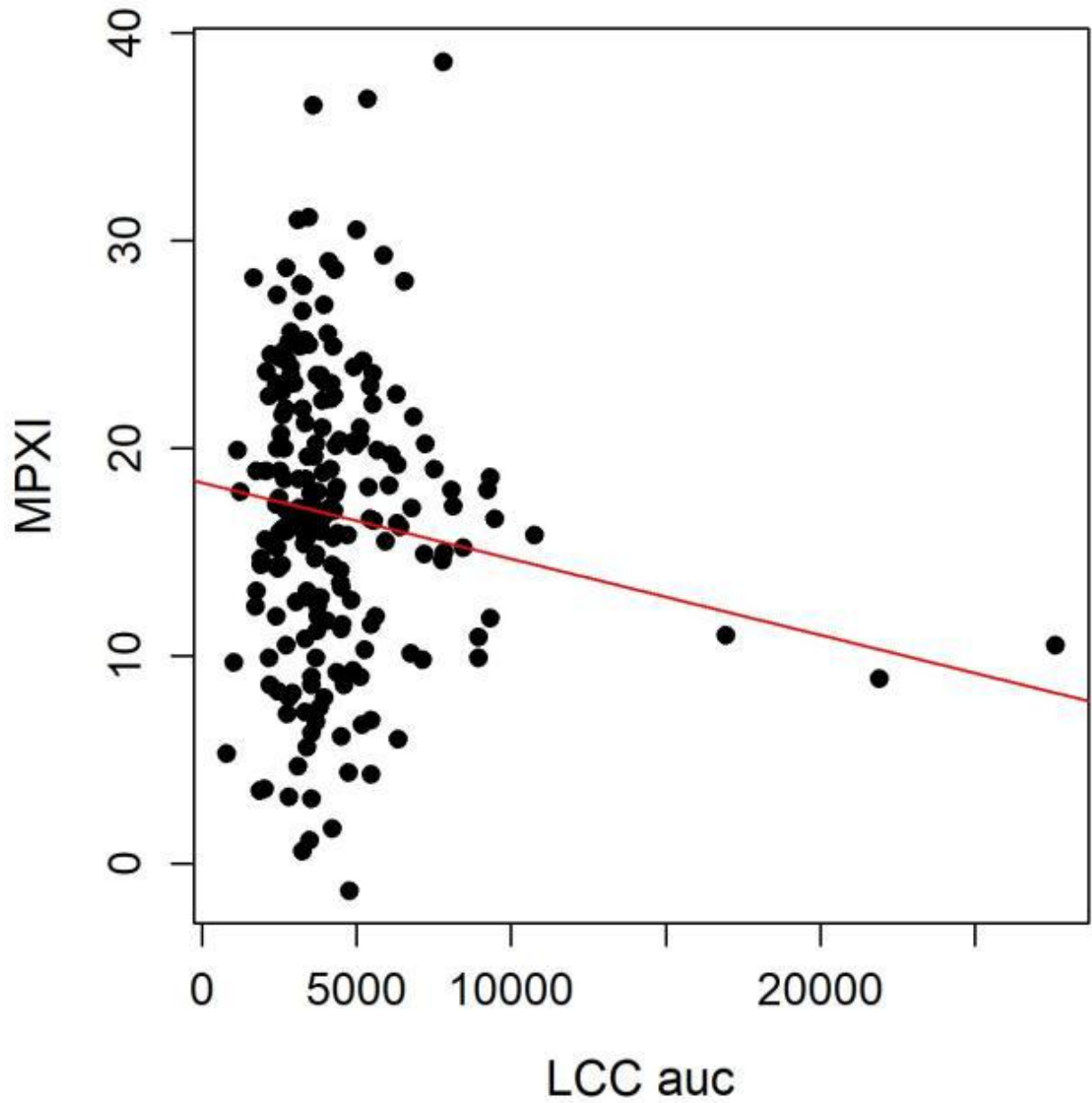


Figure 6 presents an analysis of the relationship between MPXI and LCC auc, with LCC auc values plotted along the x-axis and MPXI values on the y-axis.

Figure 7: At baseline (SD 0), MPXI values started at approximately 15–16. An upward trend was observed across all groups, reaching a peak of around 20 by day 15. Following this peak, MPXI values declined steadily through the remainder of the study period.

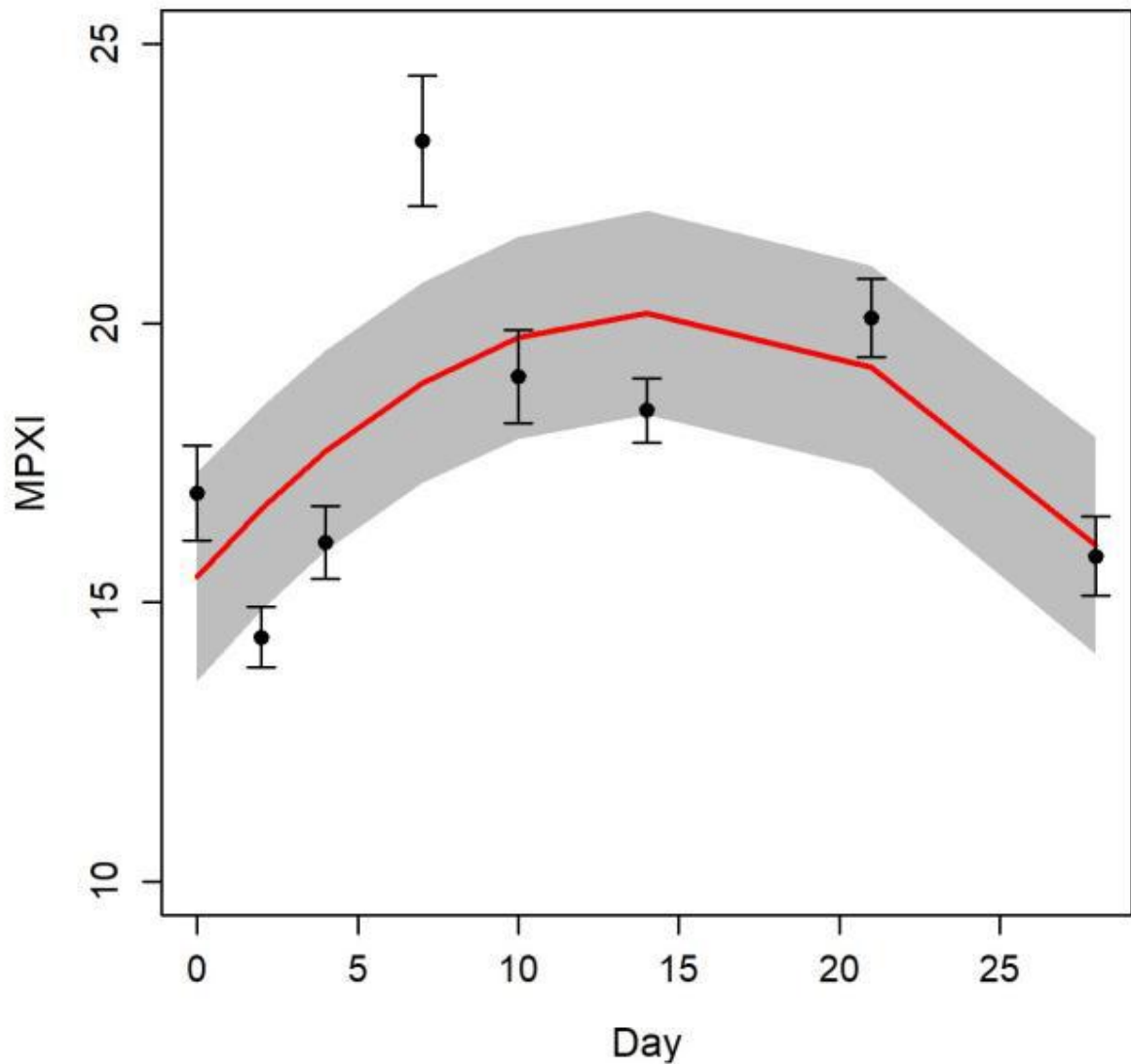


Figure 7 The graph illustrates the progression of the myeloperoxidase index (MPXI) over the course of the trial, with time (days) represented on the x-axis and MPXI values on the y-axis.

5.6 Results in Summary

The LCC assay produced a clear measurable effect, with the vaccinated group exhibiting the greatest increase in LCC AUC. No significant differences were observed in monocytes or MPXI values. Furthermore, no severe pathological or clinical symptoms were detected in the infected groups, apart from a single pig in each group that showed reduced general behaviour.

6 Discussion

Immune responses are modulated by various stressors through the actions of cortisol and other neuroendocrine mechanisms. Elevated cortisol concentrations can suppress lymphocyte proliferation, diminish natural killer cell activity, and interfere with the chemotactic function of neutrophils. In pigs, exposure to social stressors, for example, mixing with unfamiliar individuals, is associated with increased cortisol levels and a corresponding negative impact on immunity (22). This highlights how systemic factors, such as stress induced hormonal changes, can influence innate immune cells including neutrophils, which plays a central role in the initial defense against infection (22, 23).

Neutrophils represent a predominant population of circulating leukocytes, accounting for approximately 50 to 70% under steady state conditions and serve as key effectors of the innate immune system. During infection or tissue injury, a range of local cell types, including endothelial cells, fibroblasts, stromal cells and resident immune cells release diverse cytokines and chemokines that collectively orchestrate the production and mobilization of neutrophils. These mediators stimulate emergency granulopoiesis in the bone marrow, rapidly increasing neutrophil numbers and their release into the circulation to address the elevated immune demand (23). Simultaneously, chemokine gradients and upregulation of endothelial adhesion molecules facilitate neutrophil extravasation and directed migration toward the site of infection. (24). Once neutrophils reach the affected tissue, they deploy effector mechanisms including phagocytosis, degranulation, production of ROS, and in some cases the formation of NETs to eliminate pathogens. Furthermore, neutrophils can themselves secrete cytokines and chemokines, reinforcing the recruitment of additional immune cells and modulating the local inflammatory environment (23).

Building on these functions, markers such as myeloperoxidase (MPO) provide a useful measure of early neutrophil activation. Taken together, while MPO reflects early innate immune activation and neutrophil antimicrobial functions, the broader adaptive immune system appears to be less acutely and directly affected by stress-induced cortisol elevation and the associated inflammatory milieu. In line with this, although PRRSV affects the thymus and bone marrow, infection does not necessarily impair leukocyte differentiation or maturation, nor does it cause functional loss, suggesting that the host's adaptive immune response remains largely intact (12).

The LCC method was successfully applied in this study as a functional assay to evaluate neutrophil oxidative activity, providing explicit insights into the innate immune status of the animals across different experimental groups. LCC proved effective on piglets at a PMA concentration of 10^{-5} and may serve as a useful tool for assessing infections in terms of the natural immune response. In this trial, the field isolates PRRSV1 AUT18_2488 and PRRSV1 Ger22_1510 induced only mild or no clinical and pathological signs, which were analyzed for another part of the study that is not included in this diploma thesis, suggesting that either the isolates lacked pathogenicity, were attenuated, or the infection did not establish efficiently due to undetermined factors. Nevertheless, ELISA results measuring antibodies directed against non-structural proteins of PRRSV confirmed that an immune response occurred, indicating that at least partial infection and immunological activation took place.

Another hypothesis to explain the progressive decline in LCC in Group 1,2 and 4 is that the progression of LCC values across the groups might be explained by an age-related effect. As pigs age, they may undergo immunological alterations that influence LCC levels, thereby contributing to the observed results. Longitudinal blood sampling from pigs aged three to 21 weeks revealed an age dependent decline in neutrophils, type III monocytes and double-negative T-cells, whereas eosinophils, type II monocytes and double-positive T cells exhibited a progressive increase during this developmental period (25, 26). Numerous factors are known to influence hematological parameters in pigs. Metabolic adjustments after birth play an important role, and additional variation arises from nutrition, growth rate, genotype, breed and age. Health status, stress and exposure to pathogens further modify leukocyte profiles and related blood values. Notably, the weaning process can induce substantial and measurable changes in the hematology of piglets, reflecting both physiological stress and adaptations of the developing immune system. Analysis of piglets at one and 21 days, revealed that total leukocyte values remain unchanged, despite a decrease in cortisol levels by day 21. (26).

Another plausible explanation for the difference of LCC in Group 3, compared to the other groups is that these findings may be related to the vaccine formulation itself, which includes adjuvant components such as vitamin E. Vitamin E has well-established immunomodulatory properties and can influence immune cell activation partly through the regulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Under both physiological and

inflammatory conditions, it exerts context-dependent effects on immune function. Experimental and clinical data indicate that vitamin E supplementation can enhance lymphocyte proliferation, increase immunoglobulin concentrations, augment antibody responses, stimulate natural killer cell activity, and promote interleukin-2 production. Together, these effects are associated with improved host resistance to a variety of pathogens (27) and possibly activation of neutrophil burst activity.

In this context, the specific isoform DL- α -tocopherol, as used in the vaccine in our study, is an immunologically active bioavailable form of vitamin E that serves as key component of the AS03 adjuvant system may play a particularly relevant role. DL- α -tocopherol bridges innate and adaptive immune mechanisms. At the innate level, its administration has been associated with increased transcription of genes encoding pro-inflammatory cytokines and chemokines involved in immune cell recruitment. In murine models, AS03 induces marked transcriptional changes as early as four hours after injection, both at the injection site and within draining lymph nodes (dLNs). These early responses, largely attributable to the α -tocopherol fraction, involve upregulation of chemokines implicated in the recruitment of dendritic cells, monocytes, neutrophils, and eosinophils. Monocytes and macrophages appear to be the principal cellular targets of α -tocopherol and are major contributors to chemokine production. Notably, gene expression changes within dLNs are transient, diminishing by 24 hours post-administration, consistent with a rapid, localized innate activation phase.

Beyond these early innate events, DL- α -tocopherol also shapes downstream adaptive immune responses. Although one study reported an association between modulation of innate signaling and a reduced overall antibody response, α -tocopherol has been shown to enhance antigen-specific CD4⁺ T-cell responses. This T-cell support may facilitate the maintenance of neutralizing antibody titers and promote the generation of memory B cells, thereby contributing to sustained humoral immunity. Collectively, these findings suggest that DL- α -tocopherol drives an early, transient pro-inflammatory transcriptional program - primarily through monocyte and macrophage activation that subsequently influences the quality and durability of antigen-specific adaptive responses (28, 29).

A further assumption for the represented data could be, that the different routes of administration contributed to the observed data. This warrants consideration of whether the

outcomes might have differed if the mode of application, whether intramuscular or intranasal, had been standardized across all groups. Potential effects of stress induced by sample collection can not be fully excluded, although standardized handling protocols were employed for all animals to minimize variability between groups.

LCC represents a valuable tool for assessing pain and stress across a wide range of species, including livestock animals, and even wildlife (2, 7). Evaluating pain in nonverbal animals remains a persistent challenge in veterinary practice. Currently, the assessment of pain in these patients relies primarily on indirect methods, such as behavioral observation using species-specific pain scales or scoring systems (2). In addition, white blood cell counts and their distribution are also affected by stress, providing a complementary physiological measure to support welfare and pain assessment (30).

A notable advantage of the used method to determine the oxidative burst activity in our study is that leukocytes remain within their native milieu, allowing them to maintain physiological interactions with other blood constituents. Furthermore, the method obviates the need for centrifugation, a procedure that may alter cellular responsiveness and consequently affect functional measurements. The assay enables real-time monitoring through quantitative chemiluminescence measurements, providing immediate and dynamic information on leukocyte activity (7). Given the established relationship between stress and immune function, LCC represents a valuable tool not only in wildlife research but also in pigs and other livestock species, providing meaningful indicators of an individual's physiological status (2, 7).

Additionally, the technique is highly practical and suitable for field application, as it requires only minimal blood volume and yields results within minutes. The assay relies on relatively simple equipment, thereby limiting potential sources of error, and its evaluation does not depend on baseline values obtained from unstressed individuals (1, 7). Moreover, the method allows measurements to be conducted during, immediately before, or directly after stressful events, offering a flexible and context specific assessment of immune responsiveness (1).

The described approach has already demonstrated considerable practical value and is expected to gain broader application in the future, not only for assessing stress and pain in animals but also in human medicine. Its relevance to infection medicine is further supported by studies

involving patients with COVID-19 and sepsis. These investigations indicate that viral infections can differentially regulate reactive oxygen species production depending on whether the response is initiated at the plasma membrane or within intracellular granules, and the excessive ROS generation, in combination with neutrophilia, may lead to tissue damage in affected individuals. Additionally, bacterial infections have been shown to influence the regulation of ROS production, highlighting the central role of leukocyte oxidative response in host pathogen reactions (6).

LCC is also of significant interest for future applications, as it may aid in predicting disease outcomes and informing treatment strategies during acute infections. This method provides a robust means of assessing the functional capabilities of neutrophils. In addition, the approach has potential for guiding the implementation of immunomodulatory therapies, with the aim of enhancing innate immune responses and improving clinical prognosis (6).

The LCC is a functional assay that can be viably implemented in field settings, provided specific operational parameters are maintained. It constitutes a novel methodological framework for infectivity studies, enabling the standardized and objective assessment of a virus isolate's phenotype.

A limitation of the LCC assay is its sensitivity to various stressors, such as infection or handling, which may confound the results. This sensitivity can also be advantageous, as it reflects real-time influences on immune function. A reduction in LCC values can therefore serve as an indicator of increased stress exposure and may signal a concomitant decrease in immune competence of the individual (7).

Although LCC has demonstrated increasingly promising results, it is recommended to integrate it with complementary assessment methodologies to optimize the reliability and power of interpretation of the findings. Moreover, this approach provides a more precise quantification of the physiological impact of human interventions, thereby informing more effective on planning and execution of such procedures (30).

7 Literature

1. McLaren GW, Macdonald DW, Georgiou C, Mathews F, Newman C, Mian R. Leukocyte coping capacity: a novel technique for measuring the stress response in vertebrates. *Exp Physiol* 2003; 88(4):541–6. doi: 10.1113/eph8802571.
2. Gaudio E, Bordin S, Lora I, Lora M, Massignani M, Benedictis GM de. Leukocyte coping capacity chemiluminescence as an innovative tool for stress and pain assessment in calves undergoing ring castration. *J Anim Sci* 2018; 96(11):4579–89. doi: 10.1093/jas/sky342.
3. Huber N, Fusani L, Ferretti A, Mahr K, Canoine V. Measuring short-term stress in birds: Comparing different endpoints of the endocrine-immune interface. *Physiol Behav* 2017; 182:46–53. doi: 10.1016/j.physbeh.2017.09.017.
4. Mosca F, Trachtman AR, Hattab J, Marruchella G, Tiscar PG. Effect of hydrogen peroxide on the oxidative burst of neutrophils in pigs and ruminants. *Vet World* 2020; 13(9):1934–9. doi: 10.14202/vetworld.2020.1934-1939.
5. Gabriel C, Her Z, Ng LFP. Neutrophils: neglected players in viral diseases. *DNA Cell Biol* 2013; 32(12):665–75. doi: 10.1089/dna.2013.2211.
6. Veenith T, Martin H, Le Breuilly M, Whitehouse T, Gao-Smith F, Duggal N et al. High generation of reactive oxygen species from neutrophils in patients with severe COVID-19. *Sci Rep* 2022; 12(1):10484. doi: 10.1038/s41598-022-13825-7.
7. Huber N, Marasco V, Painer J, Vetter SG, Göritz F, Kaczensky P et al. Leukocyte Coping Capacity: An Integrative Parameter for Wildlife Welfare Within Conservation Interventions. *Front Vet Sci* 2019; 6:105. doi: 10.3389/fvets.2019.00105.
8. Cho JG, Dee SA. Porcine reproductive and respiratory syndrome virus. *Theriogenology* 2006; 66(3):655–62. doi: 10.1016/j.theriogenology.2006.04.024.
9. Zimmerman JJ, Dee SA, Holtkamp DJ, Murtaugh MP, Stadejek T, Stevenson GW et al. Porcine Reproductive and Respiratory Syndrome Viruses (Porcine Arteriviruses). In: Zimmerman JJ, Hrsg. *Diseases of swine*. 11th edition. Hoboken, NJ: Wiley-Blackwell; 2019. S. 685–708.
10. Lauber C, Goeman JJ, Del Parquet MC, Nga PT, Snijder EJ, Morita K et al. The footprint of genome architecture in the largest genome expansion in RNA viruses. *PLoS Pathog* 2013; 9(7):e1003500. doi: 10.1371/journal.ppat.1003500.
11. Lunney JK, Fang Y, Ladinig A, Chen N, Li Y, Rowland B et al. Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): Pathogenesis and Interaction with the Immune System. *Annu Rev Anim Biosci* 2016; 4:129–54. doi: 10.1146/annurev-animal-022114-111025.

12. Cai H, Zhang H, Cheng H, Liu M, Wen S, Ren J. Progress in PRRSV Infection and Adaptive Immune Response Mechanisms. *Viruses* 2023; 15(7). doi: 10.3390/v15071442.
13. Celliers A, Rautenbach Y, Hooijberg E, Christopher M, Goddard A. Neutrophil Myeloperoxidase Index in Dogs With Babesiosis Caused by *Babesia rossi*. *Front Vet Sci* 2020; 7:72. doi: 10.3389/fvets.2020.00072.
14. Yang JW-J, Wang F-I. Impairment of oxidative burst in porcine neutrophils induced by pseudorabies virus. *Vet Immunol Immunopathol* 2004; 101(1-2):123–30. doi: 10.1016/j.vetimm.2004.04.018.
15. R: A language and Environment for Statistical Computing: R Foundation for Statistical Computing. Vienna, Austria: R Core Team; 2024. Verfügbar unter: <https://www.R-project.org/>.
16. nlme: Linear and Nonlinear Mixed Effects Models: R Core Team; 2024. Verfügbar unter: <https://CRAN.R-project.org/package=nlme>.
17. Akaike H. Information Theory and an Extension of the Maximum Likelihood Principle. In: Parzen E, Tanabe K, Kitagawa G, Hrsg. *Selected Papers of Hirotugu Akaike*. New York, NY: Springer New York; 1998. S. 199–213.
18. HURVICH CM, TSAI C-L. Regression and time series model selection in small samples. *Biometrika* 1989; 76(2):297–307. doi: 10.1093/biomet/76.2.297.
19. MuMIn: Multi-Model Inference: R package; 2024. Verfügbar unter: <https://CRAN.R-project.org/package=MuMIn>.
20. An {R} Companion to Applied Regression. Thousand Oaks {CA}; 2019. Verfügbar unter: <https://www.john-fox.ca/Companion/>.
21. Package multcomp: Simultaneous Inference in General Parametric Models; 2024. Verfügbar unter: <http://multcomp.R-forge.R-project.org>.
22. Groot J de, Ruis MA, Scholten JW, Koolhaas JM, Boersma WJ. Long-term effects of social stress on antiviral immunity in pigs. *Physiol Behav* 2001; 73(1-2):145–58. doi: 10.1016/s0031-9384(01)00472-3.
23. Tsioumpekou M, Krijgsman D, Leusen JHW, Olofsen PA. The Role of Cytokines in Neutrophil Development, Tissue Homing, Function and Plasticity in Health and Disease. *Cells* 2023; 12(15). doi: 10.3390/cells12151981.
24. Kolaczowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol* 2013; 13(3):159–75. doi: 10.1038/nri3399.
25. Blanc F, Prévost-Blondel A, Piton G, Bouguyon E, Leplat J-J, Andréoletti F et al. The Composition of Circulating Leukocytes Varies With Age and Melanoma Onset in the

- MeLiM Pig Biomedical Model. *Front Immunol* 2020; 11:291. doi: 10.3389/fimmu.2020.00291.
26. Cincović M, Mirkov M, Radović I, Belić B, Lakić I. Reference Values of Hematological, Biochemical and Endocrinological Parameters in the Blood of Piglets Aged 1 and 21 Days. *Contemporary Agriculture* 2020; 69(3-4):34–40. doi: 10.2478/contagri-2020-0006.
 27. Lee GY, Han SN. The Role of Vitamin E in Immunity. *Nutrients* 2018; 10(11). doi: 10.3390/nu10111614.
 28. Harandi AM. Systems analysis of human vaccine adjuvants. *Semin Immunol* 2018; 39:30–4. doi: 10.1016/j.smim.2018.08.001.
 29. Shi S, Zhu H, Xia X, Liang Z, Ma X, Sun B. Vaccine adjuvants: Understanding the structure and mechanism of adjuvanticity. *Vaccine* 2019; 37(24):3167–78. Verfügbar unter: <https://www.sciencedirect.com/science/article/pii/S0264410X19305298>.
 30. Esteruelas NF, Huber N, Evans AL, Zedrosser A, Cattet M, Palomares F et al. leukocyte coping capacity as a tool to asses capture- and handling-induced stress in scandinavian brown bears (*ursus arctos*). *J Wildl Dis* 2016; 52(2 Suppl):S40-53. doi: 10.7589/52.2S.S40.

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