Implications of Intranasal Polyvalent Vaccination for Pulmonary Alveolar Macrophage and Neutrophil Functions in Calves

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Abstract

Background: Vaccines undoubtedly represent a promising and much needed approach to combatting infectious diseases, but there is also increasing evidence that they exert nonspecific effects, and these effects may be beneficial but also sometimes detrimental. Thus, here we sought to explore the effect of intranasal vaccination on the functions of pulmonary alveolar macrophages and neutrophils in calves of different ages.

Results: Respiratory clinical signs and impaired microbicidal and phagocytic activities were observed for the alveolar neutrophils and vacuolized macrophages in the vaccinated calves compared with those in the unvaccinated calves, particularly at 15 days of age.

Conclusions: Our results indicated that the use of some polyvalent attenuated and modified live virus vaccines in early stage of calves' life, especially those under 15 days of age, should be restricted to farms in which the cattle are at a higher risk of contracting these viral infections, particularly considering the importance of alveolar macrophages and neutrophils against bacterial pathogens in a period of intense pulmonary challenge.

Background

Vaccines against a variety of infectious diseases represent one of the greatest triumphs of medicine, mainly due to disease-specific effects [1]. On the other hand, there is increasing evidence that vaccination also can exert nonspecific effects, and these effects may be beneficial but sometimes detrimental. The nonspecific effects of vaccines have long been dismissed or minimized, but these biological phenomena should not be ignored, particularly considering that the innate immune system reveals some adaptive characteristics. Thus, it is now urgent that the effects of vaccination should be viewed in a much more systematic and open-minded manner [2, 3]. Worryingly, a transitory state of immunosuppression subsequently to vaccination has been described in several studies [4–9].

With this in mind, few studies have focused on immunity in calves receiving polyvalent vaccinations [10, 11], although there are some concerns if immunosuppression coincide with the time of vaccination, and if the vaccine may hinder a satisfactory immune response [12], predisposing the animal to coinfections or increasing the severity of a latent disease. Nevertheless, calves are usually vaccinated with polyvalent vaccines.

When using combined vaccines, sundry interactions between the attenuated viruses must also be considered. Nevertheless, there are still many gaps into the mechanisms underlying the apparent immunosuppression of healthy calves after polyvalent vaccination [13]. Moreover, vaccine-virus interactions and their inherent immunosuppressive properties in calves are not well understood. Thus, vaccinations should be considered from different perspectives.

Sentinel cells of the innate immune system, such as macrophages and neutrophils, represent the first line of defense against a drastic exposure to infectious pathogens in neonates. The innate immune system in turn stimulates the adaptive immune response, and as a result the stimulation of immune memory responses [3, 14, 15]. Thus, another factor that should be investigated is the optimal age for vaccination [11, 16–19] because age has an impact on an animal's immune response [20, 21]. While colonization of the neonate with distinct microorganisms is influenced by the immediate environment, the use of vaccines to assess the immune pathways provides an opportunity to study specific types of antigens exposure and examine their downstream effects [3].

By all indications, the most effective method for inducing mucosal immunity in the upper respiratory tract is intranasal immunization, particularly with vaccines containing live attenuated or modified virus antigens; however, most of the available vaccines used to fight costly respiratory diseases in cattle are parenteral neglecting the mucosal immunity [22]. The nasopharynx-associated lymphoid tissue has been regarded an attractive site for vaccination through nasal immunization and the induction of antigen-specific immunity in the oral cavity and respiratory tract [23, 24].

Altogether, the scenarios described above highlight the larger issue that only a limited number of articles [11, 25, 26] have focused on: the interactions of multiple agents within the calf respiratory system and the fact that the conditions that influence the interactions, benefits, risks and losses associated with the use of numerous live viral vaccines and bacterins are either unknown or poorly understood [27, 28]. Thus, to focus on the nonspecific effects of vaccination, our study was performed to evaluate the behavior of resident pulmonary alveolar macrophages and neutrophils after polyvalent vaccination in calves at different ages.

Results

In the clinical examination of calves in the group A, auscultation detected the presence of pulmonary crackles and an indirect endoscopic inspection detected the presence of large or small pulmonary secretions in three calves (50%) beginning on day 36. In these calves, direct inspection and palpation revealed the presence of a positive cough reflex in two calves (33%) beginning at 36 d. In the group B animals, direct inspection, palpation or auscultation detected the presence of spontaneous cough in one calf (20%), polipneic respiration in one calf (20%), lung crepitation in one calf (20%), wheezing in two calves (40%) and a positive cough reflex in three calves (60%) after day 66. In four calves (80%) from group B, an indirect endoscopic inspection detected the presence of large or small pulmonary secretions beginning on day 66. In the control group C, it was found that just one (20%) animal presented clinical respiratory symptoms with the presence of polypneic respiration on inspection, and wheezing and crackles on lung auscultation after 36 d. In the endoscopic examination, three calves of control group C (60%) had large or small pulmonary secretions beginning on day 66.

An increase in the geometric mean fluorescence intensity (GMFI) of intracellular reactive oxygen species (ROS) production by the pulmonary alveolar vacuolized macrophages from the unvaccinated control

calves (group C) was observed over time (day 36 to day 57; $P = 0.01$) (Fig. 2) and was not observed in the calves vaccinated on days 15 and 36 (group A; $P = 0.71$) (Fig. 3). The calves that were vaccinated on day 45 (group B) showed a decrease in the GMFI of intracellular ROS production over time (day 45 to day 87 d; $P = 0.01$) (Fig. 5) that was not observed in the unvaccinated calves ($P = 0.15$) (Fig. 4). Furthermore, in group B, we observed a time-dependent decrease in the percentage of pulmonary alveolar vacuolized macrophages that phagocytosed *S. aureus* (day 45 to day 66; $P = 0.05$) (Fig. 7) that was not encountered in the unvaccinated control calves ($P = 0.62$) (Fig. 6).

The functions of monocyte-like macrophages showed a significant difference only in terms of the percentage of intracellular ROS production when a decrease over time was observed in calves that were vaccinated on day 15 (group A) (day 15 to day 57; $P = 0.03$), differing from the unvaccinated control group on day 57 ($P = 0.02$). There was no significant difference in functions of monocyte-like macrophages in calves of group B and C.

The percentage of neutrophils from the unvaccinated control calves that produced ROS was higher on day 57 than on day 15 ($P = 0.006$) (Fig. 8), and this was not observed in the vaccinated calves (group A; $P = 0.64$) (Fig. 9). The percentage of phagocytosing neutrophils and the GMFI of the propidium iodide-labeled heat-killed *S. aureus* phagocytized by neutrophils were reduced in the vaccinated calves of group A on day 57 ($P = 0.02$, Fig. 11; $P = 0.03$, Fig. 13), and these values were not altered in the unvaccinated control calves ($P = 0.29$, Fig. 10; $P = 0.40$, Fig. 12). The calves that were vaccinated on day 45 did not show alterations in the phagocytosis of *S. aureus* or intracellular ROS production by the alveolar neutrophils compared with their respective controls.

Discussion

Here, it could be observed that intranasal polyvalent vaccination of calves at early stages of life could exert undesirable non-specific effects, especially in innate immunity, which was critically showed in animals under 15 days of age, which could predispose animals to opportunistic infections. In this regard, it has been proposed that the immunologic response of an animal to vaccination is similar to its response to the natural disease and involves both nonspecific and specific aspects of the immune system [5]; this effect may partially explain the reduced functions of the pulmonary alveolar vacuolized macrophages and neutrophils observed in this study, particularly apparent in calves from group A that received the first vaccination dose on day 15, a result that is likely related to the maturation of the calf's own immune system and/or the presence of maternal antibodies.

Until 35 days of life, colostral immunity, is extremely important in preventing disease, only after the 42 days of life, colostral immunity declined and phagocytosis increased. Thus, healthy calves showed a reduced capacity of phagocytosis of alveolar macrophages between 21 and 35 days of life [20].

Studies have shown that the functions of macrophages are impaired during a natural infection by a viral disease, such as virus that compound the attenuated and modified live polyvalent vaccine used here. For instance, some studies have described a significant reduction in the phagocytosis and microbicidal

activities of the bovine pulmonary alveolar macrophages in calves experimentally inoculated with bovine viral diarrhea virus (BVDV) [29], parainfluenza type-3 virus (PI3) [25, 30], bovine respiratory syncytial virus (BRSV) [31] and infectious bovine rhinotracheitis virus (IBR) [32]. In addition, an impairment in the bovine neutrophil function has been described in PI3, IBR [25] and BVD [33] virus-infections. Although the mechanisms by which the animals respond to vaccines are similar to those deployed in the presence of a pathogen, the use of a polyvalent vaccine can trigger a different response to a single viral infection. Nevertheless, a study of the effects of BRSV and non-cytopathic BVDV [34] showed that both single and combined viral infections significantly impaired the microbicidal and phagocytic ability of pulmonary alveolar macrophages.

In face of, our findings of the impaired functions of pulmonary alveolar vacuolized macrophages and neutrophils should be highlighted especially regarding the crucial role of these cells against bacterial infections, the slow maturation of the immune system in neonates that befall up to 30 days of age [20, 35], and the anatomical and physiological factors of the bovine respiratory tract allow pathogens to remain in the airways and contribute to the susceptibility of cattle to pneumonia [20, 36]. Experimentally BRSV induced infections showed that bovine neonates assembled a less robust inflammatory response, and viral elimination was inefficient compared with animals above one month of age [17, 37]. Our findings regarding the functions of the alveolar neutrophils and vacuolized macrophages in vaccinated calves are corroborated with clinical findings found here.

Also where observed that a modified live vaccine strain of BVDV is capable of suppressing lymphocyte and neutrophil functions (the activity of the myeloperoxidase-H_2O_2-halide antibacterial system and antibody-dependent, cell-mediated cytotoxicity) in a manner similar to the virulent BVDV, which is consistent with our results in both vaccinated groups, especially in group A. In this context, was conducted a field study [27] and observed a negative association between the use of vaccines (as used in the field) and mortality (i.e., by fibrinous pneumonia and bronchopneumonia), with the highest mortality rate occurring in the groups that received the IBR/PI3/BVDV combined vaccine. At the ages of vaccination in our study, the immune response is mainly a humoral or T_H2 response; however, much of the immune protection in calves appears to be conferred by the cellular or T_H1 response [37], and for this response to be effective, the cells must exert their normal functions or be activated.

Another factor that should not be ruled out is the possibility of the modified live vaccine virus strains replicating in the respiratory system, which may have led to the local immune dysfunctions observed in both vaccinated calves groups in this study, particularly because the modified live vaccine virus (i.e., PI-3, IBR and BRSV) was administered intranasally. For instance, bovine herpesvirus type-1 has reportedly been isolated from feedlot calves with bovine respiratory disease shortly after they received an intranasal application with a modified live virus vaccine [38]. In addition, was recently described [39] four bovine herpesvirus type-1 clinical isolates that were identical to a vaccine strain, with three from post-vaccination abortion episodes showing typical herpetic lesions from dams that had received the modified live virus vaccine during pregnancy. Thus, further studies must be performed to determine whether the viral vaccines used here replicate in the respiratory system.

Another explanation for our results may be due to macrophage polarization. For instance, a characteristic that appears to be shared by most of these regulatory cells is that anti-inflammatory activity is induced by two stimuli. The first signal (i.e., immune complexes) commonly has little or no stimulatory function on its own. Conversely, when combined with a second stimulus, such as toll-like receptor ligands [40], including those present in adjuvants [41], the two signals reprogram the macrophages to produce interleukin-10, which in turn result in the inhibition of pro-inflammatory cytokines and consequently in poor microbicidal activity, which is a typical characteristic of regulatory macrophages [40]. This finding indicates that the presence of regulatory macrophages could be negatively correlated with the vaccine response, which requires the induction of pro-inflammatory cytokine production. With this in mind, further studies examining the fine-tuned characterization of macrophage polarization in response to vaccination in in this age group could be helpful.

Conclusions

Thus, regarding our results, although vaccination is a promising and much-needed approach to preventing infectious diseases, particularly for immunosuppressive viral diseases that can also enhance the susceptibility to other opportunistic infections, their use should be restricted to farms in which the cattle are at a higher risk of contracting these viral infections. In addition, our study demonstrated that the vaccine used intrasally in calves especially under the age of 45 days can favor immunosuppression determined by the decrease in the microbicidal and phagocytic activity of defense alveolar cells, which was even worse in animals vaccinated at 15 days of age. More importantly, the results highlighted the occasional dangers associated with the use of attenuated, modified live virus vaccines in the presence of another infectious agent because many infectious agents may interact with the modified live or attenuated vaccine. However, it is necessary to clarify that these results were observed for the intranasal route and specific pathogens and at a particular age.

Methods

Animals and experimental design

The animals were born on a dairy farm and were transported to the University of São Paulo on the first day of life after receiving the main neonatal care and ingesting colostrum corresponding to at least 10% of their live weight along with a consent document from the owner for use of the animals in the study.

After arriving at the university, to ensure the successful transfer of passive immunity, the total serum protein should be greater than 5.0 g/dL [42] and glutaraldehyde agglutination test faster than five minutes [43]. Furthermore, a complete blood count was performed, and they should be within normal ranges for their respective age and specie [44]. In the meantime, all animals were also subjected to physical examination [45], which include the following parameters: animal's general condition, respiratory and heart rates, body temperature, intestinal motility rate, degree of body hydration, apparent color of

mucous and palpation of lymph nodes. If any alteration was found, the animal was excluded from the experiment.

Sixteen male Holstein dairy calves with median weight of 31,5 kg (range, 23 to 40 kg) were all housed in individual suspended iron cages that included a shelter and outdoor access. They were feeded milk and calf feed twice a day until the day 30 and after this day tifton hay and *ad libitum* access to water.

Calves were randomized assigned to three groups on the day of birth (day 0). Calves in group A (n = 6) were inatranasally inoculated as previously proposed [11, 18, 46] with 1 mL of a polyvalent vaccine on days 15 and 36, calves in group B (n = 5) were inatranasally inoculated on days 45 and 66, and calves in group C (n = 5) were not inoculated and served as a control group. Bronchoalveolar lavage (BAL) samples were obtained by endoscopy [20] from calves in groups A and C on days 15, 36 and 57 and from calves in groups B and C on days 45, 66 and 87.

Mucosal immunization was implemented because it was shown to be effective in calves and had less interference from maternal antibodies [18, 47, 48]. The commercial vaccine (CattleMaster Gold FP 5/L5, Pfizer Animal Health, Montreal, Canada) used contains a lyophilized preparation of modified live strains of IBR, PI3, and BRSV, inactivated BVDV types 1 and 2 and inactivated cultures of five *Leptospira* sp. serovars in a liquid preparation together with an adjuvant Quil-A.

After the study, the animals were donated to a dairy farmer, who kept the animals in the production system.

Clinical examination

Firstly, the respiratory and heart rates, body temperature, rumen motility rate, degree of body hydration, apparent color of mucous and palpation of lymph nodes were performed. Then, the calves were subjected to specific examination of the respiratory system by the direct inspection, palpation, percussion, and auscultation. The presence of secretion in the respiratory system was assessed by endoscopy using a flexible video gastroscope.

Bronchoalveolar lavage sampling

The animals were sedated with 0.02 mg/kg of xylazine (Rompun 2%, Bayer Animal Health) and after a few minutes were placed in a lateral recumbent position, and then approximately 50 mL of BAL was recovered by endoscopy. To maintain cell viability, all of the samples were kept on ice until processing, which did not exceed a maximum of 4 h after collection. The samples were subjected to two washes with phosphate buffered saline, centrifuged and resuspended to a concentration of 2×10^6 viable cells mL^{-1}, as previously described [20].

Identification of neutrophils, monocyte-like macrophages and vacuolized macrophages

The neutrophils, monocyte-like macrophages and vacuolized macrophages were identified by flow cytometry (FACSCalibur™, Becton Dickinson Immunocytometry System™, San Diego, EUA) using the procedure proposed by Sladek and Rysanek [49] with some modifications. Briefly, the region distribution of BAL cells in dot plots was differentiated by their forward scatter and side scatter parameters into neutrophils, monocyte-like macrophages and vacuolized macrophages (Fig. 1A). After identifying each leukocyte population, the neutrophils ($CD14^-$ cells; Fig. 1B), vacuolized macrophages ($CD14^+$ cells; Fig. 1C) and monocyte-like macrophages ($CD14^+$ cells; Fig. 1D) were analyzed based on their cytoplasmic granularity and mean fluorescence intensity following a two-step fluorescent immunolabeling protocol using primary anti-bovine monoclonal antibody specific for CD14 cells (cat. no. MM61A, VMRD Pullman Inc. Corp, Pullman, USA) and secondary goat anti-mouse IgG1 conjugated to phycoerythrin-Cy5 (cat. no. M32018, Life Technologies, San Diego, USA) [20]. An unstained control, secondary antibody control and single stained samples were also prepared as compensation controls. FlowJo software® (TreeStar Inc., Ashland, OR, USA) was used to analyze the data.

Intracellular reactive oxygen species production

Reactive oxygen species production by alveolar neutrophils, monocyte-like macrophages and vacuolized macrophages was evaluated by flow cytometry using 2',7'-dichlorofluorescein diacetate (cat. n. D6883, Sigma Aldrich, St. Louis, MO, USA) as a probe [50–54]. For this assay, 10,000 to 15,000 BAL cells were examined per sample. An unstained control and single stained samples were also prepared as compensation controls. FlowJo software® (TreeStar Inc., Ashland, OR, USA) was used to analyze the data.

Phagocytosis assay

The phagocytosis assay of alveolar neutrophils, monocyte-like macrophages and vacuolized macrophages was performed by flow cytometry using propidium iodide-labeled *Staphylococcus aureus* as previously described [20, 50–53] at multiplicity of infection of 120. For this assay, 10,000 to 15,000 BAL cells were examined per sample. An unstained control and single stained samples were also prepared as compensation controls. FlowJo software® (TreeStar Inc., Ashland, OR, USA) was used to analyze the data.

Statistical analysis

Firstly, the results of the flow cytometry were corrected for autofluorescence by substraction, which was defined as the fluorescence that was associated with unlabeled freshly cells from the same calf. The

statistical analysis was performed using an especific software (GraphPad Software, Inc., San Diego, CA, USA). First, the Gaussian distribution was verified by the Kolmogorov-Smirnov test. To compare the differences between groups (vaccinated vs unvaccinated), Student's t-test for unpaired data was used for the data with Gaussian distributions, and the Mann-Whitney test was used for the non-parametric data. To evaluate differences among the days in each group, a repeated measures ANOVA and the post hoc Newman-Keuls test were used for the data with a normal distribution. The Kruskal-Wallis test and Dunn's post hoc test were applied to the non-parametric data. Significance was set at $P < 0.05$ unless indicated otherwise.

Abbreviations

GMFI: geometric mean fluorescence intensity; ROS: reactive oxygen species; BAL: bronchoalveolar lavage; IBR: infectious bovine rhinotracheitis virus; PI3: parainfluenza type-3 virus; BRSV: bovine respiratory syncytial virus; BVDV: bovine viral diarrhea virus.

Declarations

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Authors' contribution

RCG designed the experiments, performed all analysis, draft and edited the manuscript. CFB designed the experiments and performed all analysis. FNS provided technical help, supervised studies, draft and edited the manuscript. KRS and HGB participated in all analysis. EMP, CPR and AMMPDL designed the experiments and supervised studies. All authors have read and approved the manuscript.

Availability of data and materials

All data generated or analyzed will be available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The study was approved by the University of São Paulo Ethic Committee in Use of Animals (2799/2012) and was conducted at the Research Center of the University of São Paulo.

Consent for publication

Not applicable.

Competing interest

The authors have no conflicts of interest to declare.

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Figures

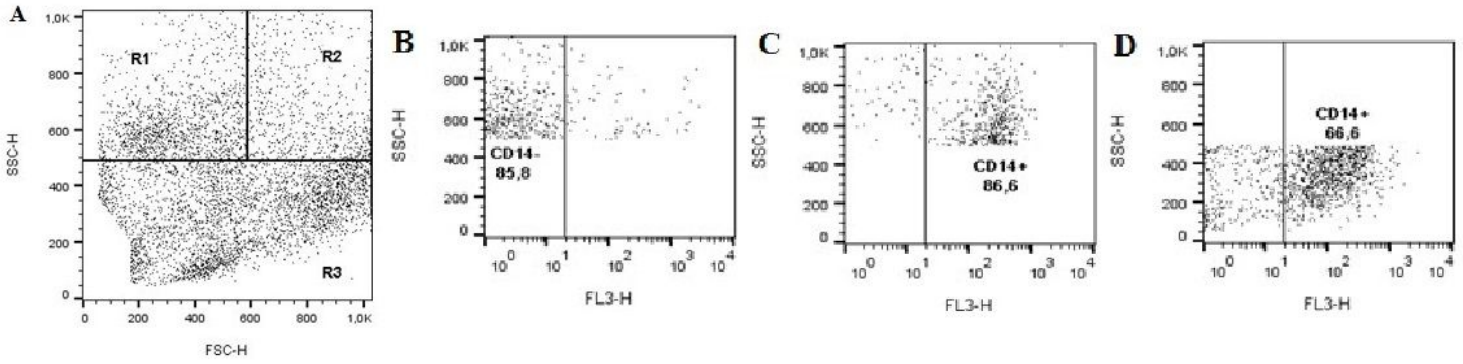

Figure 1

Flow cytometry analysis of the bronchoalveolar cells. The representative dot plot (A) shows the distribution of cells that were differentiated by their forward scatter and side scatter parameters into neutrophils (R1), vacuolized macrophages (R2) and monocyte-like macrophages (R3). The cell populations were identified by the expression of the cluster of differentiation 14 (CD14+) into neutrophils (CD14-; B), monocyte-like macrophages (CD14+; C) and vacuolized macrophages (CD14+; D).

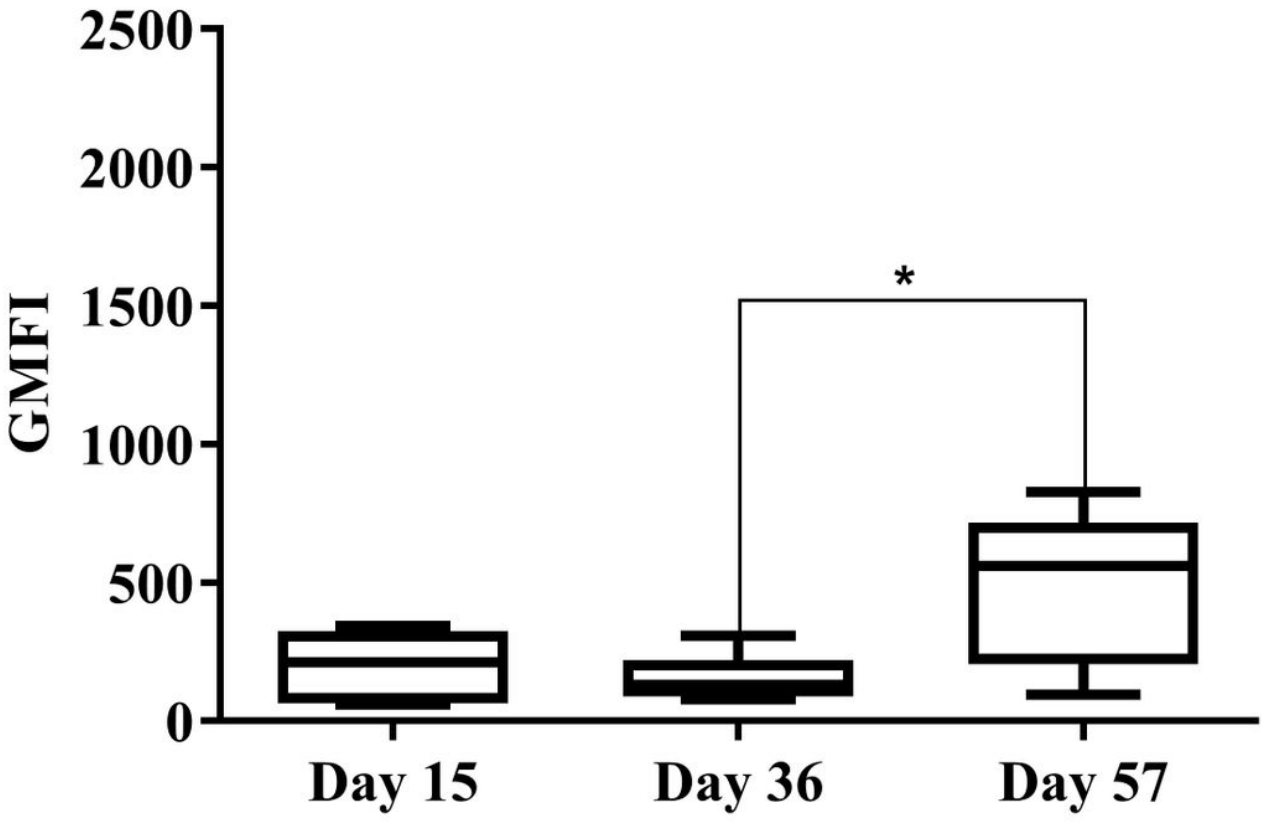

Figure 2

Geometric mean fluorescence intensity of the production of intracellular reactive oxygen species by pulmonary vacuolized macrophages from the bronchoalveolar lavages of the unvaccinated control calves on days 15, 36 and 57. * $P \leq 0.05$.

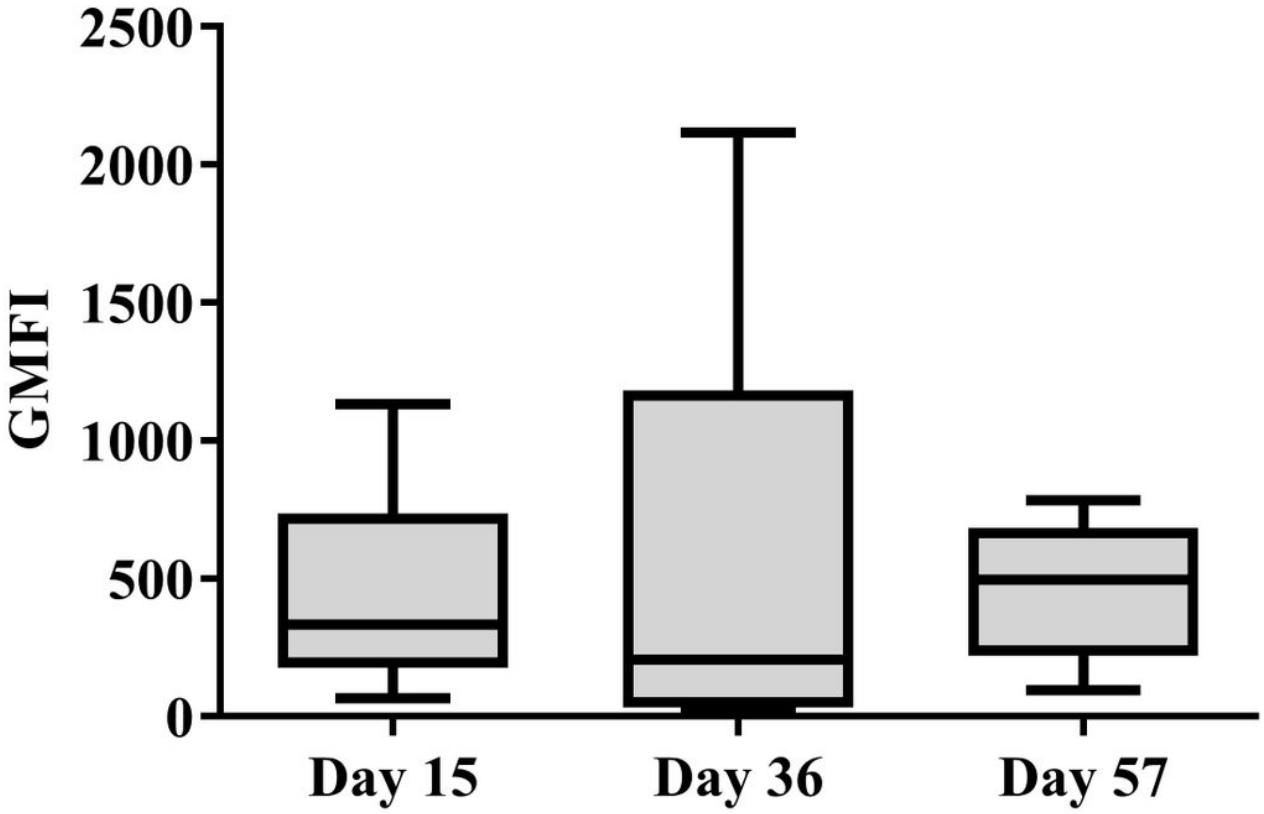

Figure 3

Geometric mean fluorescence intensity of the production of intracellular reactive oxygen species by pulmonary vacuolized macrophages from the bronchoalveolar lavages of the vaccinated calves on days 15, 36 and 57.

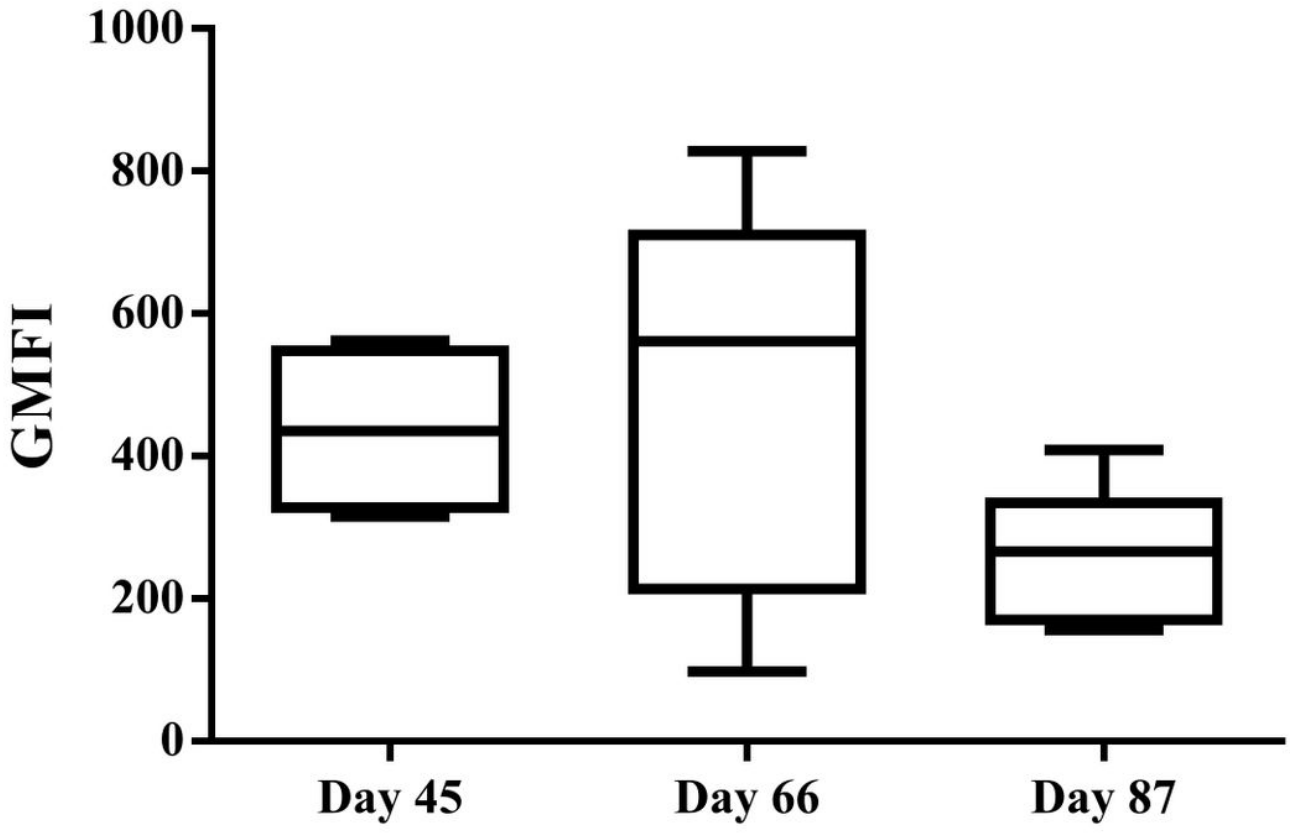

Figure 4

Geometric mean fluorescence intensity of the production of intracellular reactive oxygen species by pulmonary vacuolized macrophages from the bronchoalveolar lavages of the unvaccinated control calves on days 45, 66 and 87.

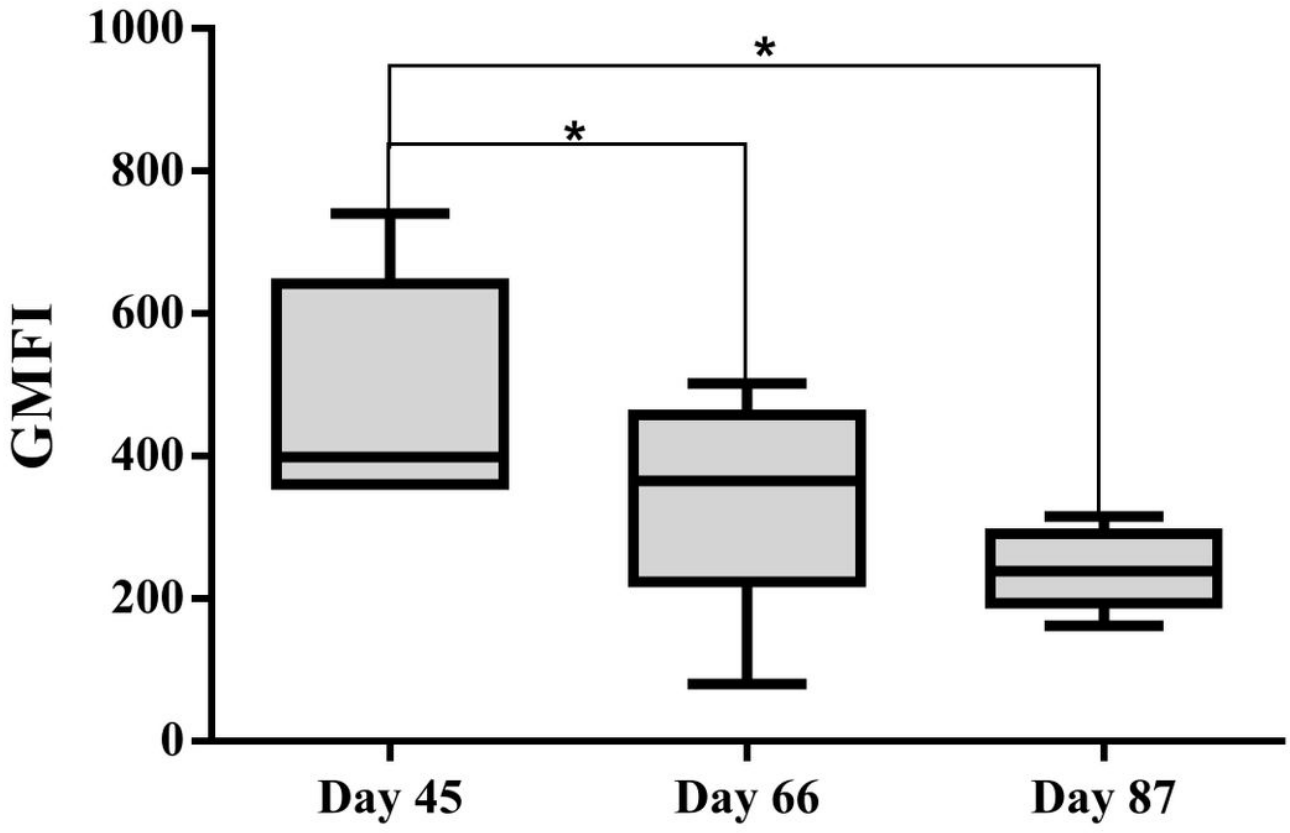

Figure 5

Geometric mean fluorescence intensity of the production of intracellular ROS by pulmonary vacuolized macrophages from the bronchoalveolar lavages of the vaccinated calves on days 45, 66 and 87. * $P \leq 0.05$.

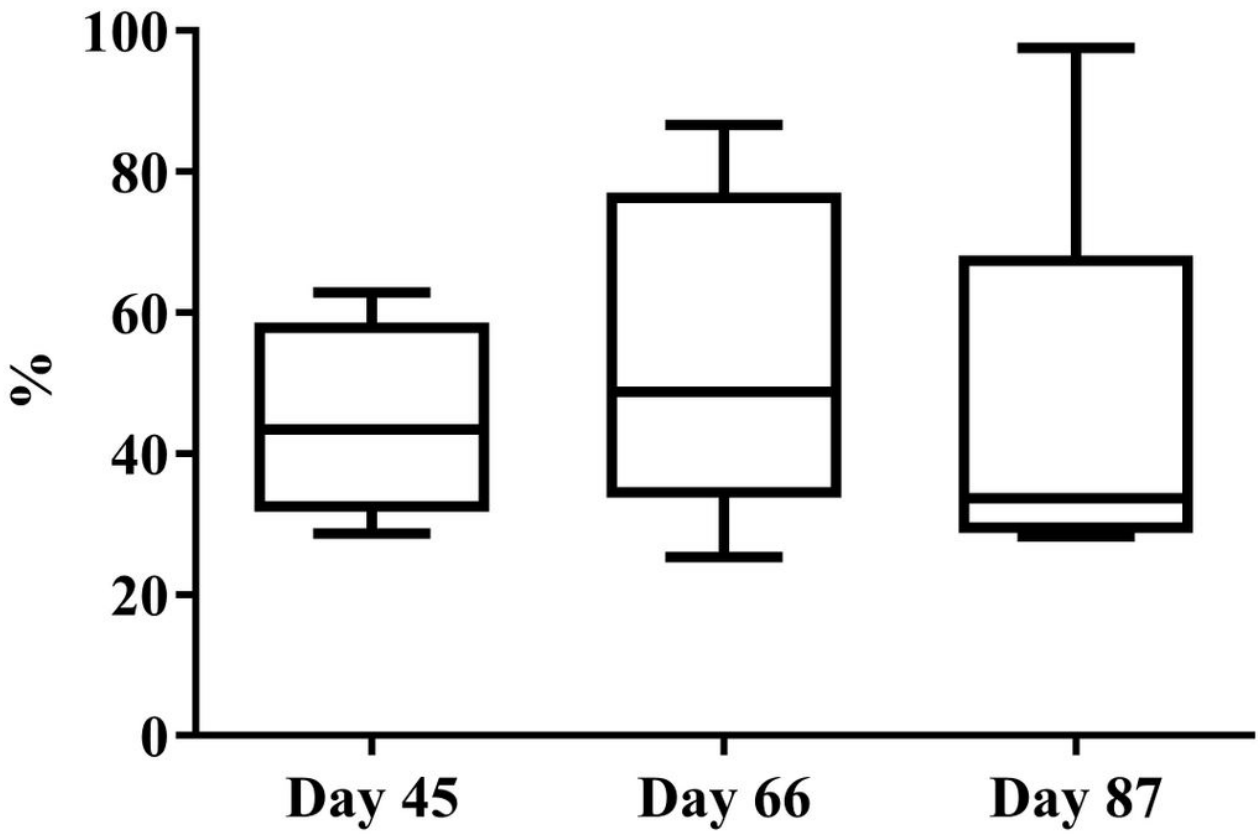

Figure 6

Percentage of Staphylococcus aureus phagocytosis by pulmonary vacuolized macrophages from the bronchoalveolar lavages of the unvaccinated control calves on days 45, 66 and 87.

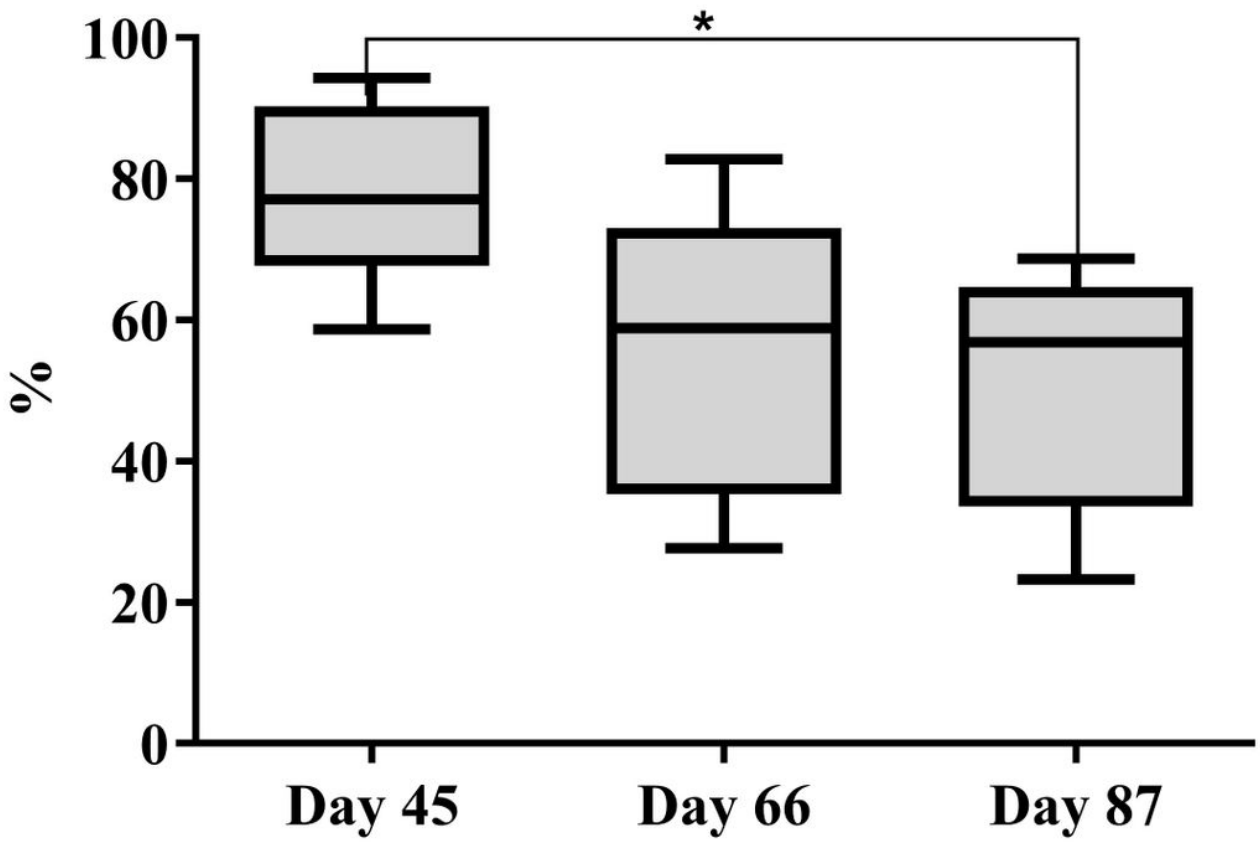

Figure 7

Percentage of Staphylococcus aureus phagocytosis by pulmonary vacuolized macrophages from the bronchoalveolar lavages of the vaccinated calves on days 45, 66 and 87. * $P \leq 0.05$.

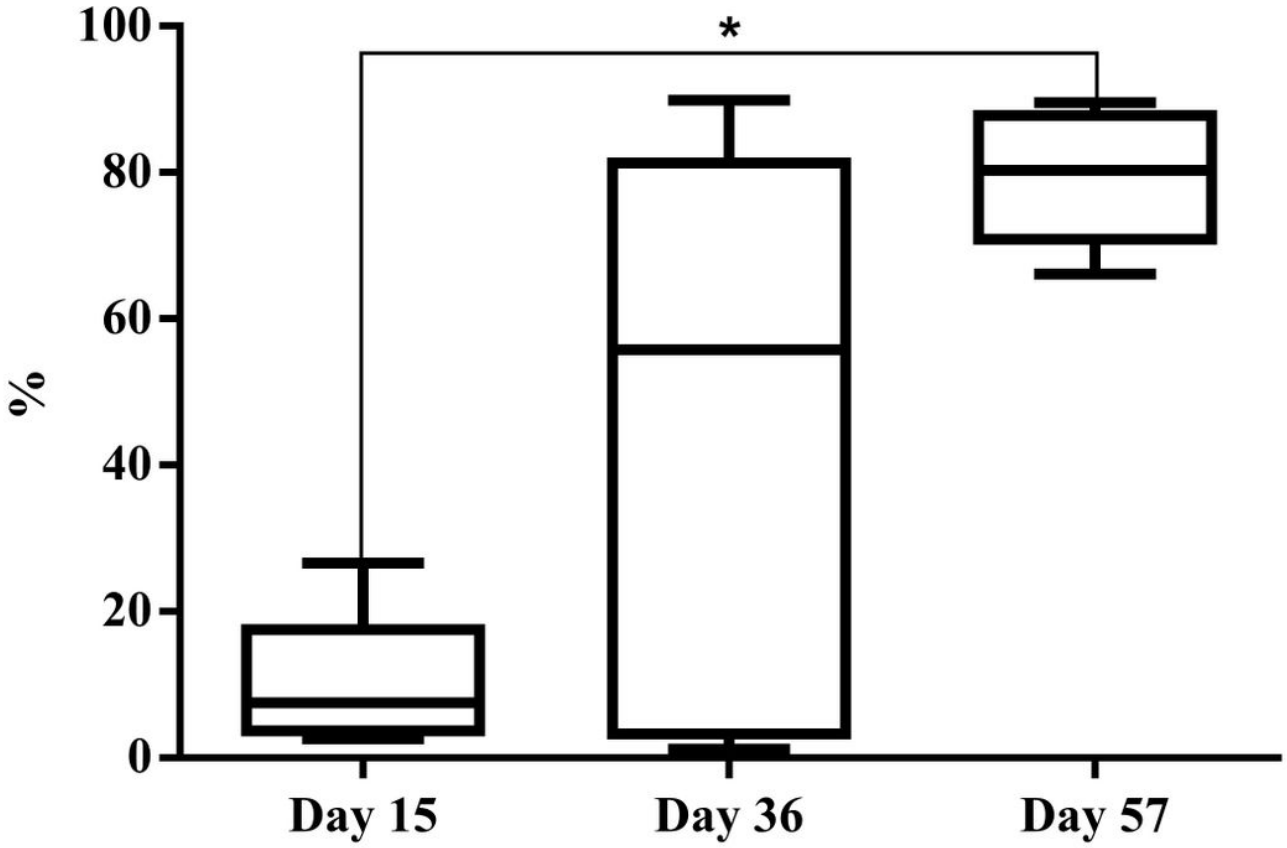

Figure 8

Percentage of intracellular reactive oxygen species produced by pulmonary neutrophils from the bronchoalveolar lavages of the unvaccinated control calves on days 15, 36 and 57. * $P \leq 0.05$.

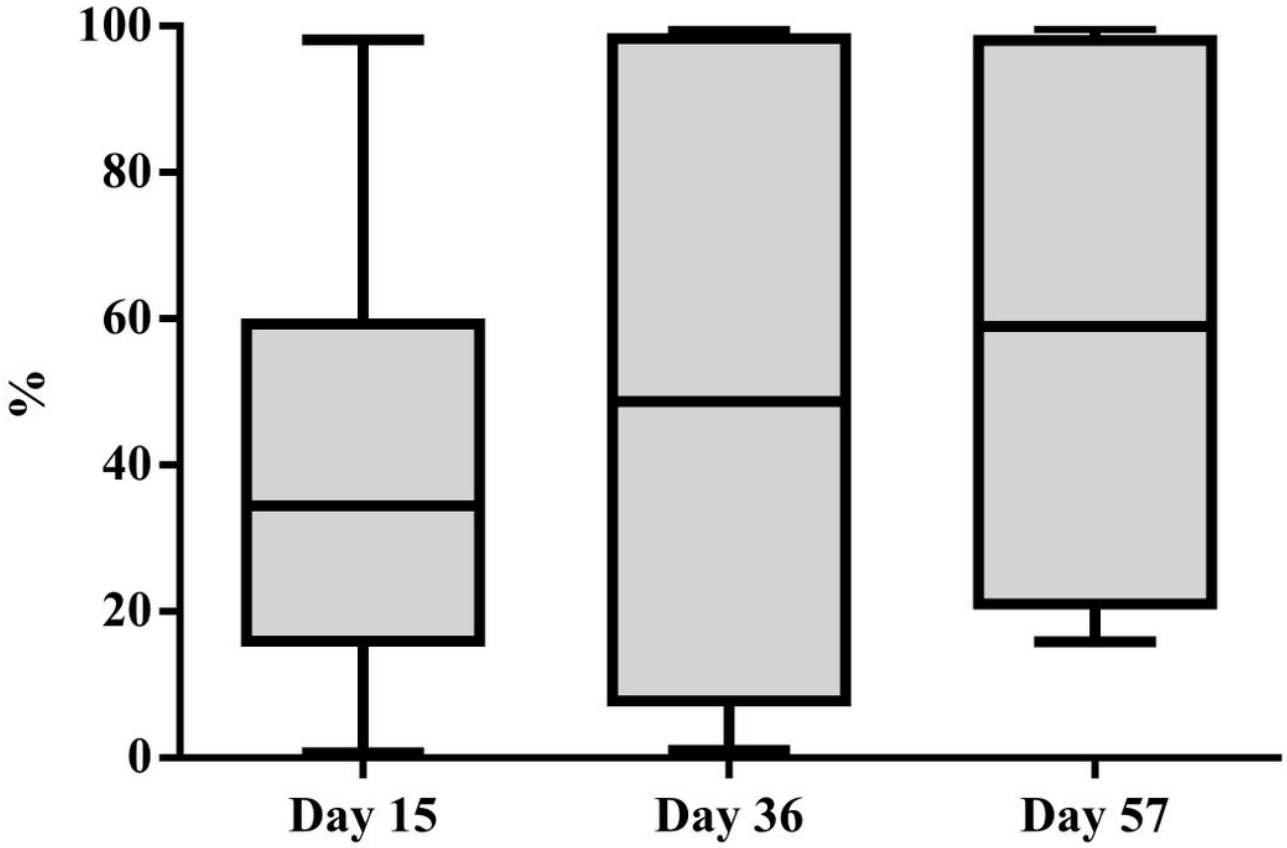

Figure 9

Percentage of intracellular reactive oxygen species produced by pulmonary neutrophils from the bronchoalveolar lavages of the vaccinated calves on days 15, 36 and 57.

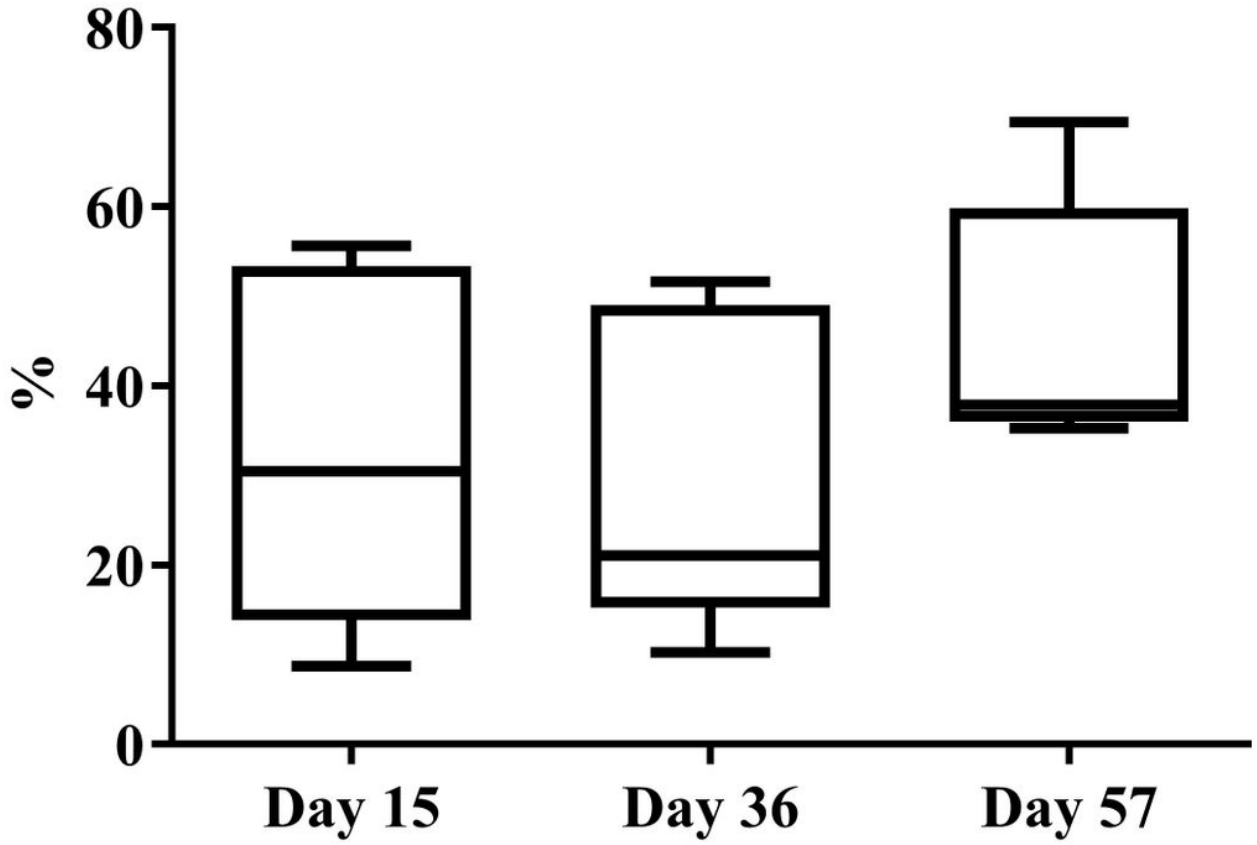

Figure 10

Percentage of Staphylococcus aureus phagocytosis by pulmonary alveolar neutrophils from the bronchoalveolar lavages of the unvaccinated controls on days 15, 36 and 57.

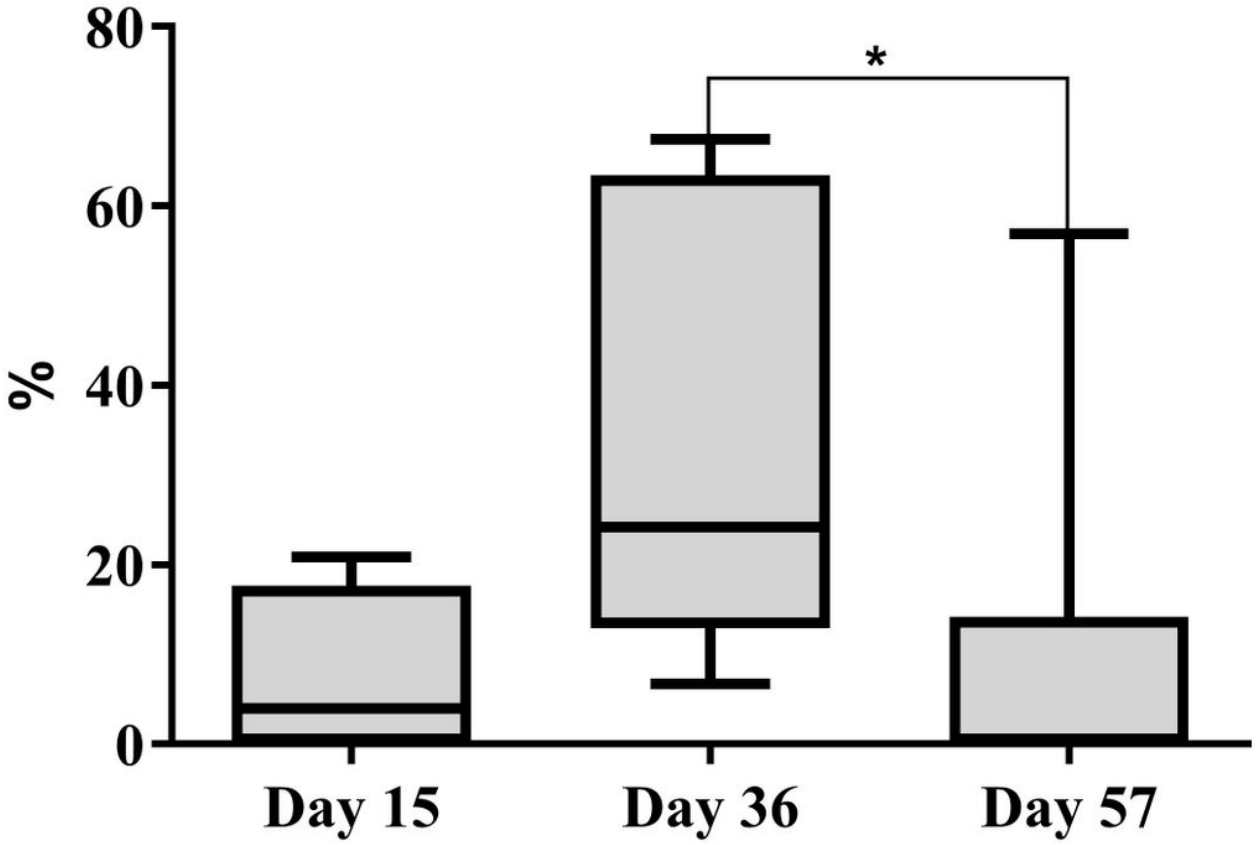

Figure 11

Percentage of Staphylococcus aureus phagocytosis by pulmonary alveolar neutrophils from the bronchoalveolar lavages of the vaccinated calves on days 15, 36 and 57. * $P \leq 0.05$.

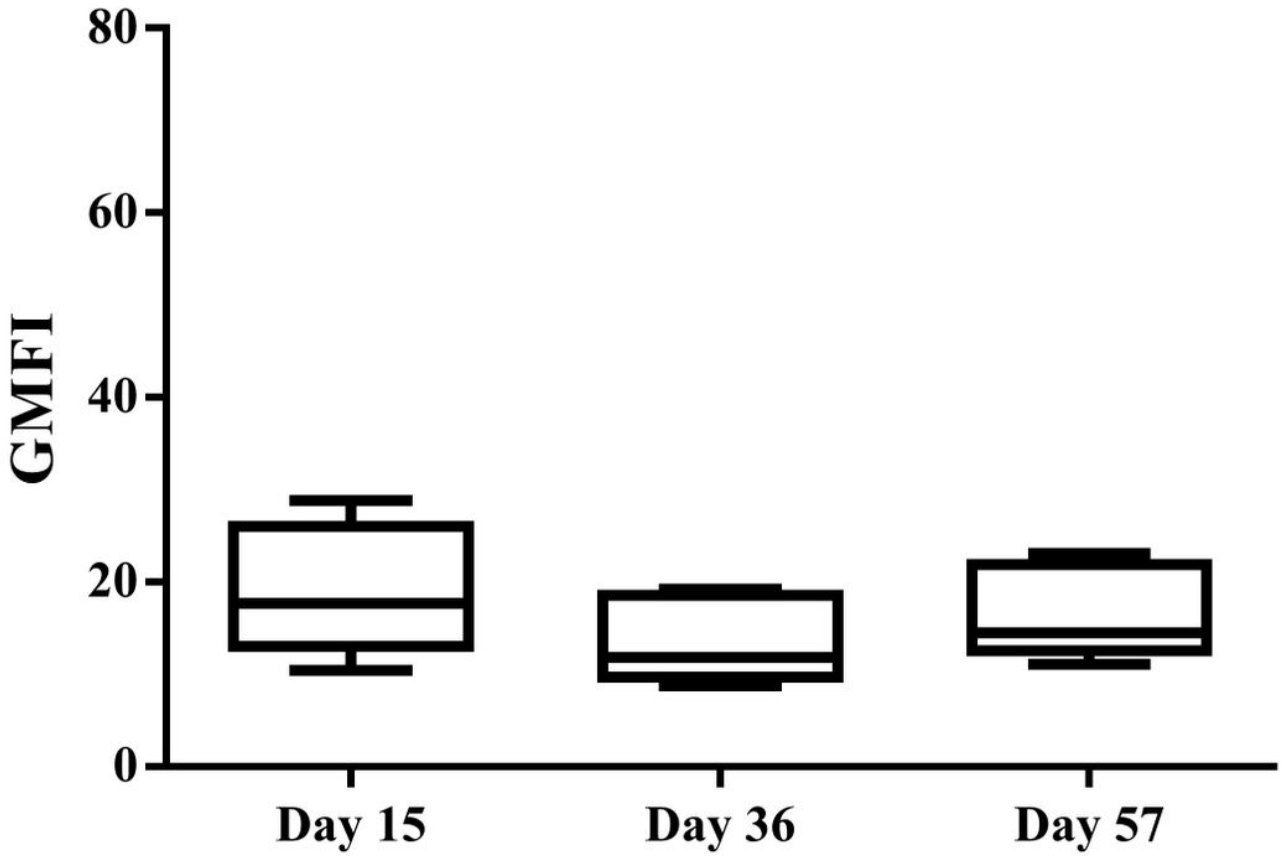

Figure 12

Geometric mean fluorescence intensity of Staphylococcus aureus phagocytosis by the pulmonary alveolar neutrophils from the bronchoalveolar lavages of the unvaccinated control calves on days 15, 36 and 57.

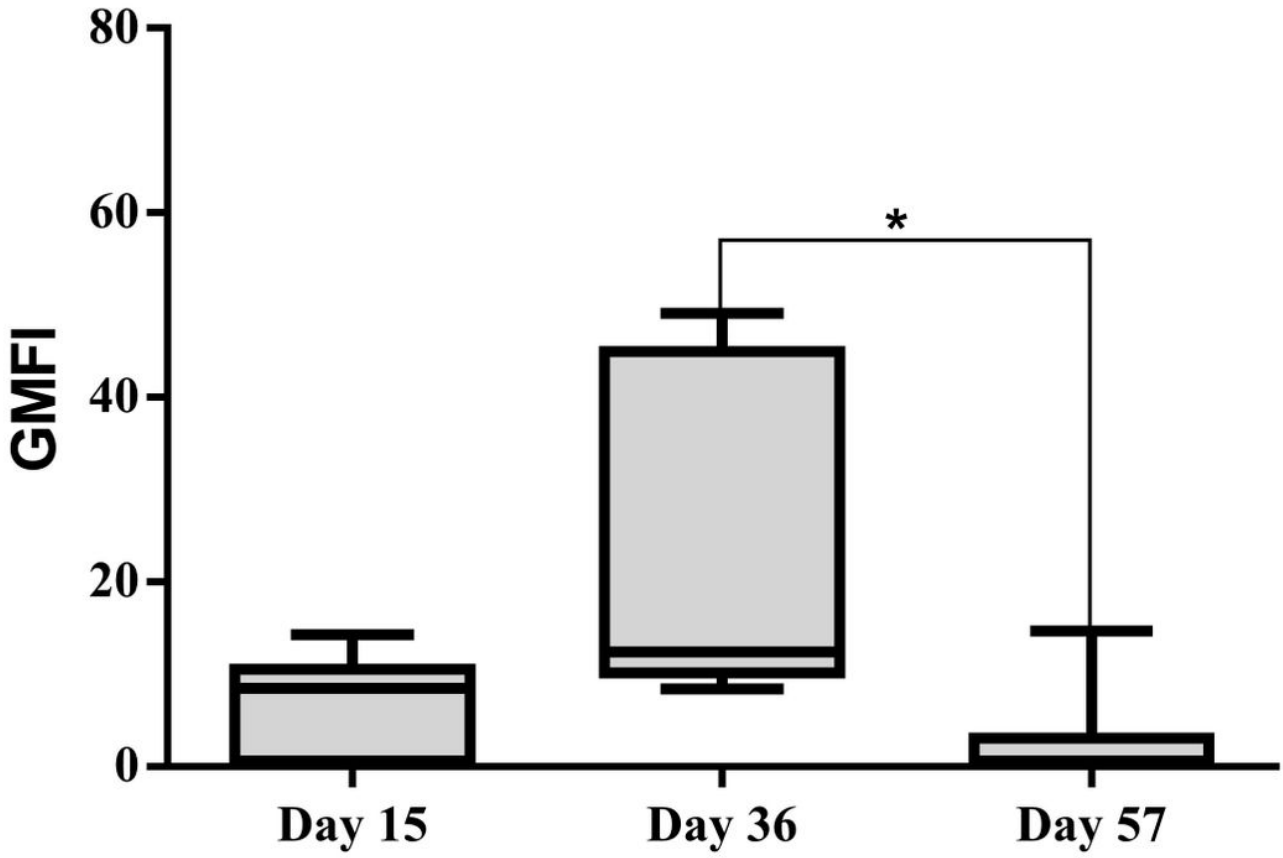

Figure 13

Geometric mean fluorescence intensity of Staphylococcus aureus phagocytosis by the pulmonary alveolar neutrophils from the bronchoalveolar lavages of the vaccinated calves on days 15, 36 and 57. * $P \leq 0.05$.

Supplementary Files

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- NC3RsARRIVEGuidelinesChecklist2020.pdf