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Research Article

Enhancing Herd Immunity: The Indirect Immune Effects of the *M. bovis*-BoHV-1 Combined Vaccine

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Bovine respiratory disease (BRD) is a complex illness driven by the interplay of various bacteria and viruses, often resulting in co-infection. It stands as one of the most significant and costly challenges in the cattle industry. The development of vaccines targeting BRD pathogens has garnered substantial attention, particularly for their ability to induce indirect immune protection in unvaccinated animals through the immune effects of vaccinated individuals. In this study, we comprehensively evaluated the indirect immune effect of our developed attenuated and marker *Mycoplasma bovis*-BoHV-1 combined vaccine. Our results demonstrated that the combined vaccine effectively activates the innate immune response in animals cohabitating with immunized individuals. This was evidenced by a significant increase in serum lysozyme levels, blood lymphocyte counts, and elevated cytokine levels. Furthermore, these cohabitating animals exhibited effective activation of the humoral immune response, as indicated by the elevated levels of specific antibodies against various pathogens. Notably, *M. bovis* serum ELISA antibodies and BoHV-1 neutralizing antibodies in all calves from the co-housing group turned positive by the second week, exceeding the threshold values of 41% and 1:8, respectively. In addition, serum levels of total IgA and IgG antibodies were significantly elevated compared to the blank control group. In conclusion, the attenuated and marker *M. bovis*-BoHV-1 combined vaccine we developed shows a notable indirect immune effect, which is essential for controlling the spread of infection and enhancing calf survival. This study greatly facilitated the sustainable growth of the cattle industry.

Keywords: bovine respiratory disease; combined vaccine; indirect immune effect

1. Introduction

Bovine respiratory disease (BRD) presents a multifaceted challenge in the cattle industry. It is primarily caused by the individual or co-infection of various pathogens in the upper respiratory tract [1–3]. With BRD being the primary cause of illness and mortality in feedlot cattle in North America, this has major negative effects on health and causes substantial financial losses [4]. In the United States, BRD is the most common disease reported by over 90% of large feedlots, with an estimated $1–3 billion in annual economic effect [5].

In China, the primary pathogens associated with BRD include *Mycoplasma bovis* (*M. bovis*) and bovine herpesvirus type 1 (BoHV-1). Since its isolation in 2008, *M. bovis* has led to alarming high morbidity rates of 50%–100% and mortality rates ranging from 10% to 50% in affected herds [6, 7]. Molecular epidemiological studies suggest that BoHV-1 is responsible for over 40% of the viral pathogens associated with BRD [8], with a national prevalence of ~40% [9]. This underscores the urgent need for effective vaccination strategies.

Vaccination plays a crucial role in preventing BRD, particularly in densely populated feedlot environments where the

Table 1: Animal immunization information.

Group	Vaccination content	Age	No
1 (Vaccinated group)	*M. bovis*-BoHV-1 combined vaccine	2–4 months	3
2 (Co-housing group)	PBS	2–4 months	3
3 (Blank control group)	PBS	2–4 months	3

indirect immune response is essential for controlling disease transmission and improving calf health and survival. However, existing inactivated vaccines often fail to induce strong indirect immune responses, necessitating multiple doses to achieve sufficient protection [10]. Although combined vaccines hold potential, there is currently a lack of comprehensive data regarding the efficacy of their indirect immune effects.

In our previous study, we successfully developed an attenuated and marker *M. bovis*-BoHV-1 combined vaccine, which exhibited notable immunogenicity and protective efficacy against wild-type strains [11]. The present study aims to provide evidence of the vaccine's ability to enhance the indirect immune response, a critical factor in improving calf survival and overall health outcomes. By addressing this important aspect, our research aims to advance vaccination strategies and contribute to the long-term sustainability of the livestock industry.

2. Materials and Methods

2.1. Cells and Viruses. The wild-type BoHV-1 HB06 strain (GenBank accession number: AJ004801.1), BoHV-1 gG-/tk- strain, as well as *M. bovis* HB0801 and *M. bovis* HB150 strains were preserved at the National Key Laboratory of Agricultural Microbiology. Briefly, the *M. bovis* HB0801 strain was a wild-type strain isolated from the clinical sample, and the *M. bovis* HB150 strain was a vaccine strain weakened by continuous in vitro passage based on the *M. bovis* HB0801 strain. China Institute of Veterinary Drug Control provided the Madin–Darby bovine kidney cells (MDBK).

2.2. Culture of M. bovis and BoHV-1. M. bovis and BoHV-1 were cultivated similarly to those previously reported studies [12, 13]. In short, the *M. bovis* HB150 strain was cultivated for 40–48 h at 37°C in PPLO complete medium in an incubator with 5% CO_2. The BoHV-1 strains, HB06 and BoHV-1 gG-/tk-, were cultivated using MDBK cells at 37°C in an incubator with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

2.3. Animal Experiments. A total of nine Holstein dairy cows, ranging in age from 2 to 4 months, were selected for the study. The calves were randomly assigned to three different groups. Prior to the study, all calves were confirmed to be seronegative for both *M. bovis* and BoHV-1. The detailed study design is presented in Table 1.

The preparation of the combined vaccine is as follows: In short, the same volume of the two antigens *M. bovis* HB150 and BoHV-1 gG-/tk-, of which the contents were 2.0×10^8 CFU and $1.0 \times 10^{6.3}$ $TCID_{50}$, respectively, was mixed with lyophilized protective substances and lyophilized. Then stored at −20°C.

Calves in group 1 received a single dose of a combined *M. bovis*-BoHV-1 vaccine. The vaccine was dissolved in 2 mL of saline solution, with 1 mL administered into each nasal cavity of the calve using a 2 mL syringe. Calves in groups 2 and 3 were inoculated with 2 mL of phosphate-buffered saline (PBS) using the same procedure. In all three experimental groups, group 2 was co-housed with group 1 in the same cattle pen, which was ~8 m wide × 15 m long in size, and group 3 was kept in isolation, with their pen which was ~5 m wide × 10 m long in size, located at a straight-line distance of 30 m from the pen in which groups 1 and 2 were located, to prevent cross-infection.

2.4. Clinical Evaluation and Sample Collection. Clinical signs were continuously monitored throughout the duration of the experiment, including rectal temperature, mental status, and respiratory status. Rectal temperature was measured by inserting a thermometer ~15 cm into the rectum and recording the reading once it stabilized.

For 14 days following vaccination, nasal swabs were collected daily. Samples were fully vortexed in tubes containing 1 mL sterile PBS, passed through a 0.45-μm filter, and preserved at −20°C for subsequent polymerase chain reaction (PCR) analysis.

Nasal lavage fluid (NALF) was collected based on previous experience [14]. One person held the cattle head upright while another injected 5 mL of PBS containing 0.1% BSA into each nostril. The nasal cavity was gently rinsed for ~15 s, after which the cow's head lowered the effluent to drain and be collected. This procedure was repeated three times to obtain sufficient NALF.

Blood samples, including procoagulant blood, anticoagulation blood containing sodium heparin, and anti-coagulated blood containing ethylenediaminetetraacetic acid (EDTA), were collected on a weekly basis until the experiment ended for the detection of lysozyme, lymphocyte proliferation, and antibodies. Additional blood samples were taken on days 1 and 3 after immunization for cytokine detection.

Serum samples were prepared as follows: whole blood samples containing anticoagulant were centrifuged at 4°C and 4000 rpm for 10 min. The resulting supernatant was carefully collected and designated as serum samples.

2.5. Virus and Bacteria Detection. To determine the presence of *M. bovis* HB150 and BoHV-1 gG-/tk- excretion, genomic DNA from nasal swabs was extracted and subsequently amplified by PCR. For *M. bovis* detection, the uvrC and 0732 genes were targeted, while BoHV-1 detection focused on the glycoprotein G (gG) and thymidine kinase (tk) genes. The PCRs were performed under conditions described in previous studies [13, 15].

The PCR conditions for the *M. bovis* uvrC gene were as follows: 95°C for 3 min; followed by 35 cycles of 95°C for 15 s,

Table 2: Primers used in this study.

Target gene	Primer sequence (5′→3′)	Target gene length
uvrC	Forward: TAATTTAGAAGCTTTAAATGAGCGC Reverse: CATATCTAGGTCAATTAAGGCTTTG	238 bp
0732	Forward: AGCGACCAAAATACTAGAC Reverse: TCGTTGCCACTGTATTCA	HB150 strain:\Wildtype strain: 140 bp
gG	Forward: CCGACCGCCTCCTACACCAGATGCT Reverse: GGGTGTAGGCAAGCTCACCGCAACG	Deleted strain: 524 bp Wildtype strain: 1859 bp
tk	Forward: ACGGGCTGGGAAAGACAACAACGG Reverse: GCGGACACGTCCAGCACGAACA	Deleted strain: 235 bp Wildtype strain:868 bp

Abbreviations: gG, glycoprotein G; tk, thymidine kinase.

55°C for 15 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. For the *M. bovis* 0732 gene, the conditions were: 95°C for 3 min; followed by 35 cycles of 95°C for 15 s, 52.5°C for 15 s, and 72°C for 30 s; with a final extension at 72°C for 5 min.

For the BoHV-1 gG gene, the PCR conditions were: 95°C for 3 min; followed by 35 cycles of 95°C for 15 s, 60.5°C for 15 s, and 72°C for 40 s; with a final extension at 72°C for 5 min. Lastly, the BoHV-1 tk gene was amplified under the following conditions: 95°C for 3 min; followed by 35 cycles of 95°C for 15 s, 60.5°C for 15 s, and 72°C for 30 s; and a final extension at 72°C for 5 min.

The primers used in this study are listed in Table 2.

2.6. Routine Blood Examination. The EDTA anti-coagulated blood samples collected post-vaccination were subjected to routine blood examinations using an automatic five-class blood cell analyzer (Mindray Biomedical Electronics Co., Ltd., Shenzhen, China).

2.7. Lymphocyte Proliferation Assay. Peripheral blood mononuclear cells (PBMCs) were isolated using the following procedure: Anticoagulated blood samples were collected and centrifuged at room temperature for 10 min to separate the blood components. The middle cell layer was carefully collected, and then 4–6 times the volume of erythrocyte lysate was added at 4°C for 2 min. This solution was centrifuged for 5 min, and the process was repeated until all erythrocytes were lysed. The supernatant was discarded, and PBMCs were washed twice with 10 mL of 1640 complete medium.

Isolated PBMCs (1.0×10^6) were cultured in each well of a 96-well cell culture plate. Subsequently, 10 μL of the medium containing 25μg/mL of *M. bovis* protein, inactivated BoHV-1, and concanavalin A was added to each well, respectively. The plates were then incubated at 37°C for 48 h. Following incubation, 10 μL of CCK8 reagent was dispensed into each well, and the plates underwent a further incubation period at 37°C for an additional 4 h. The absorbance was then read at a wavelength of 450 nm. The stimulation index (SI) was calculated using the formula:

$$\text{SI} = \text{Mean OD in test wells} / \text{Mean OD in unstimulated wells.}$$

2.8. Assessment of Cytokines and ELISA Antibodies and Serum Biochemical Parameters. Changes in serum levels of cytokine and antibodies, including IL-12, TNF-α, IFN-γ, sIgA, IgG, and BoHV-1 gB antibodies, as well as lysozyme levels, were detected using commercial ELISA kits from Meimian Industrial Co., Ltd. (Jiangsu, China) and Jiancheng Bioengineering Institute (Nanjing, China).

2.9. Detection of M. bovis Serum Antibodies. A competitive ELISA assay was used to detect serum antibodies specific to *M. bovis*. The procedure was as follows. The test serum, subjected to a fourfold dilution, along with positive and negative serum controls, was mixed with HRP-labeled monoclonal antibodies. This composite was then applied to a microplate precoated with *M. bovis* p579 protein and maintained at 37°C for a duration of 60 min. After incubation, the plate was washed. 100 μL of substrate chromogenic solution was added and incubated at room temperature, protected from light for 10 min; the $OD_{450\,nm}$ value was read immediately after stopping the reaction.

The blocking rate (PI value) was calculated using the formula:

$$\text{Blocking rate} = (1 - S/N) \times 100\%, \text{ where } S = \text{sample } OD_{450\,nm}; N = \text{mean } OD_{450\,nm} \text{ of negative control serum.}$$

$$\text{Test Conditions}: 0.65 < OD_{450\,nm} \text{ negative control} < 2.0, PI_{\text{positive control}} > 60\%.$$

$$\text{Interpretation}: PI_{\text{sample}} \geq 41\% \text{ indicates a positive result; } PI_{\text{sample}} < 41\% \text{ indicates a negative result.}$$

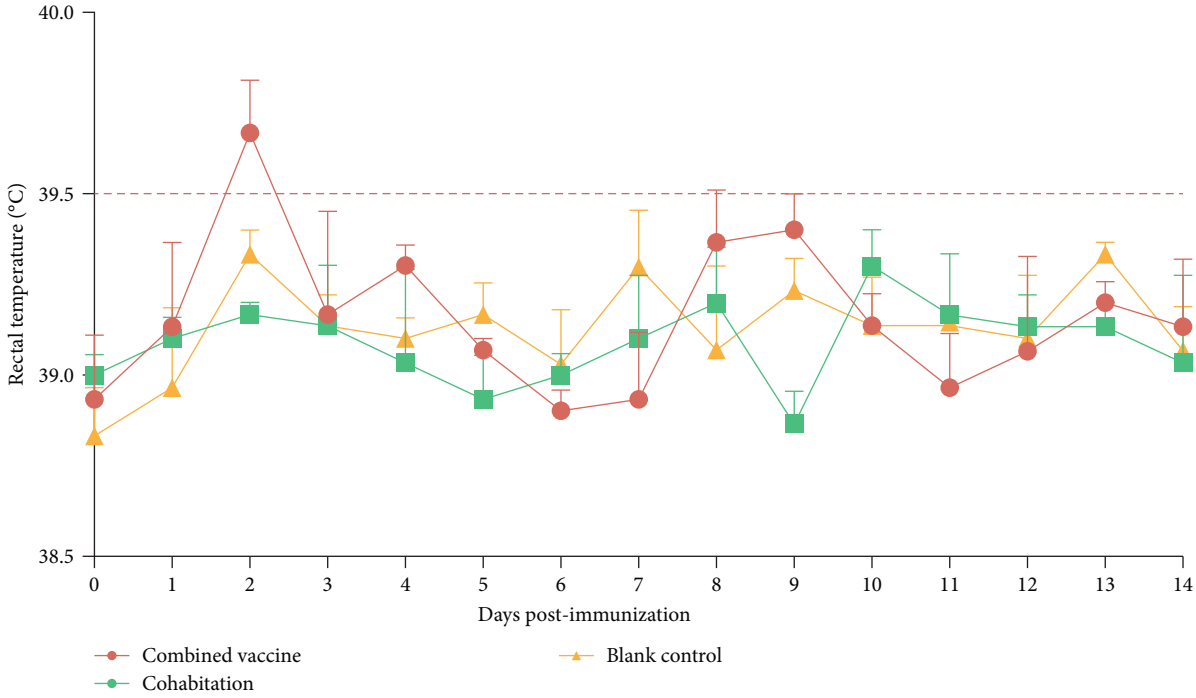


FIGURE 1: Rectal temperature changes. The red line represents the upper threshold of normal rectal temperature.

2.10. Neutralization Assay. The serum was serially diluted in a 96-well cell culture plate after inactivation by heating at 56°C for 30 min. Four replications were done for each sample at each dilution. The diluted serum was subsequently incubated with 100 $TCID_{50}$ of BoHV-1 HB06 virus at 37°C in a 5% CO_2 incubator for 1 h. Following the incubation, the serum–virus mixture was transferred to a separate 96-well cell culture plate containing MDBK cells and maintained in the same condition mentioned above for 3 days. The Reed–Muench method was used to determine neutralizing antibody titers, characterized as the highest serum dilutions that prevent BoHV-1 infection.

2.11. Ethics Statement. The animal experiment received approval from the Animal Experiment Ethics Committee of Huazhong Agricultural University and was carried out in strict compliance with the Guidelines for the Care and Use of Laboratory Animals of Wuhan, Hubei, China (Huazhong Agricultural University Ethics Approval Number: HZAUCA-2024-0046).

2.12. Statistical Analysis. Student's *t*-test and one-way ANOVA were utilized using GraphPad Prism 9.5.1 to identify significant differences among groups. Statistical significance was defined as follows: $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), or $p<0.0001$ (****). Error bars represent the standard error of the mean.

3. Results

3.1. Indirect Immune Effect of the Combined Vaccine Is Safe for Co-Housing Calves. During the observation period, calves in the inoculated group showed a mild increase in rectal temperature on the second day, which subsequently returned to normal. This temperature rise is a typical and generally acceptable side effect of vaccination. In contrast, calves in the co-housing group did not show any abnormalities in their rectal temperature (Figure 1). In addition, all calves in both experimental groups remained mentally alert and showed no signs of respiratory distress. These observations suggest that the combined vaccines are safe for co-housed calves and do not adversely affect their health.

3.2. Detection of M. bovis and BoHV-1 Excretion in Immunized and Co-Housed Calves. We assessed the excretion patterns of *M. bovis* HB150 and BoHV-1 gG-/tk- in calves following a single dose of the combined vaccine. Remarkably, excretion of both *M. bovis* HB150 and BoHV-1 gG-/tk- was observed in more than 50% of the vaccinated calves within the first 9 days post-vaccination. However, excretion ceased by days 11 and 14 for *M. bovis* HB150 and BoHV-1 gG-/tk-, respectively.

In contrast, the co-housing group did not exhibit detectable excretion of *M. bovis* HB150 until day 4 and BoHV-1 gG-/tk- until day 3. Notably, over half of the animals in this group consistently exhibited excretion from days 4 to 9 post-vaccination. The duration of excretion for *M. bovis* HB150 and BoHV-1 gG-/tk- in the co-housing group was considerably shorter, lasting 6 and 8 days, respectively. Importantly, in the blank control group, neither *M. bovis* HB150 nor BoHV-1 gG-/tk- excretion was detected throughout the observation period (Table 3).

These findings indicate that the combined vaccine not only triggers the initial excretion of vaccine strains in immunized calves but also promotes indirect immune effects in non-immunized calves. Although the duration of excretion is slightly shorter in the co-housing group, the effect still plays

Table 3: Detection of *M. bovis* HB150 and BoHV-1 gG-/tk- following vaccination in 2- to 4-month-old calves.

Group		Days post-immunization (%)													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*M. bovis*	3/3 (100)	3/3 (100)	3/3 (100)	2/3 (66.7)	3/3 (100)	2/3 (66.7)	3/3 (100)	3/3 (100)	2/3 (66.7)	1/3 (33.3)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)
	BoHV-1	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)	2/3 (66.7)	2/3 (66.7)	3/3 (100)	1/3 (33.3)	2/3 (66.7)	1/3 (33.3)	1/3 (33.3)	0/3 (0)
2	*M. bovis*	0/3 (0)	0/3 (0)	0/3 (0)	2/3 (66.7)	2/3 (66.7)	3/3 (100)	3/3 (100)	2/3 (66.7)	2/3 (66.7)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)
	BoHV-1	0/3 (0)	0/3 (0)	1/3 (33.3)	2/3 (66.7)	2/3 (66.7)	3/3 (100)	3/3 (100)	2/3 (66.7)	3/3 (100)	1/3 (33.3)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)
3	*M. bovis*	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)
	BoHV-1	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)

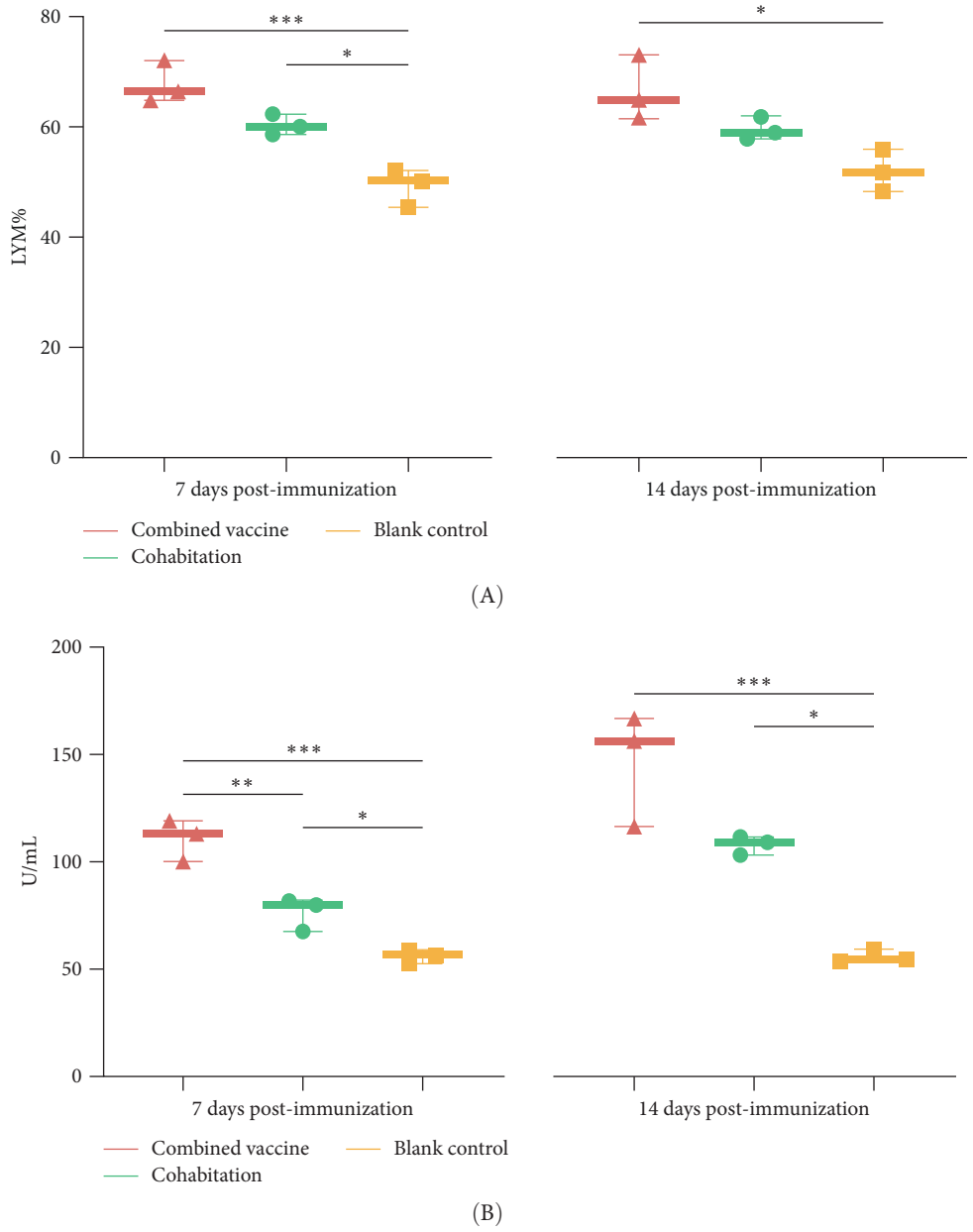


FIGURE 2: (A) Blood samples were analyzed for lymphocyte percentage using a five-classification blood routine. (B) The concentration of lysozyme in serum.

a significant role in boosting herd immunity and decreasing overall pathogen circulation within the population.

3.3. Detection of Lymphocyte Percentages Post-Immunization. Lymphocytes are crucial for developing an effective immune response against pathogens. In this study, we investigated the alterations in lymphocyte percentages after immunization with the *M. bovis*-BoHV-1 combined vaccine. Blood samples from the both combined vaccine and co-housing groups were collected at 7 and 14 days post-vaccination and analyzed using a five-class blood cell analyzer.

The results indicated that, at both time points, the percentage of lymphocytes in the vaccinated and co-housing groups was significantly higher compared to the blank control group ($p < 0.05$) (Figure 2A). Notably, there were no statistically significant differences observed between the vaccinated group and the co-housing group across the entire observation period, implying that co-housing does not interfere with the immune response induced by vaccination.

These findings highlight the success of the immunization strategy in enhancing a strong adaptive immune response, which is essential for boosting disease resistance and overall herd health.

3.4. Lysozyme Detection after Immunization. Lysozyme levels are a key indicator of a heightened innate immune response to

bacterial infections. 7 days after administration, lysozyme concentrations were measured in both the immunized and co-housing groups. The result showed that the serum lysozyme levels in these two groups were significantly higher compared to the blank control group ($p < 0.05$). In addition, the lysozyme levels in the combined vaccine group were significantly greater than those in the co-housing group ($p < 0.01$).

By 14 days post-immunization, there was a significant increase in lysozyme secretion in the experimental groups. Serum lysozyme concentrations were notably higher in the two experimental groups compared to the blank control group ($p < 0.05$) (Figure 2B), highlighting the effectiveness of immunization in promoting lysozyme production.

The consistently high levels of lysozyme in the experimental groups suggest a strong and prolonged immune activation following vaccination. This indicates the potential effectiveness of the immunization strategy, showing that it not only enhances innate immune responses but also supports the overall health of the calves.

3.5. Lymphocyte Proliferative Response. Assessing lymphocyte proliferation following immunization is crucial for understanding the immune response induced by the combined vaccine. Upon stimulation with concanavalin A (ConA), increased lymphocyte proliferation was observed at both 7 and 14 days post-immunization across all groups. However, no significant differences were found between the experimental groups and the blank control group when exposed to nonspecific stimuli (Figure 3A).

When stimulated with *M. bovis* protein, the immunized group showed a significantly greater proliferation than the co-housing group, which also exhibited a notable increase compared to the blank control ($p < 0.001$) (Figure 3B). This result supports the idea that the immunization approach effectively boosts lymphocyte activation in response to specific antigens.

The trends observed after stimulation with inactivated BoHV-1 closely resembled those seen with *M. bovis* protein, especially at the 7-day mark, where the immunized group exhibited significantly higher proliferation. By day 14, the immunized group still maintained a significant advantage over the co-housing group ($p < 0.05$), which, in turn, continued to show significantly higher proliferation compared to the blank control group ($p < 0.001$) (Figure 3C).

This sustained increase in lymphocyte proliferation in the immunized group highlights the vaccine's capacity to induce a robust and long-lasting immune response, which is essential for effective disease resistance.

In summary, the findings underline the strategy of the immunization protocol in boosting lymphocyte proliferation across various antigens, confirming the hypothesis that vaccination fosters strong and lasting immune activation. The observed differences between the immunized and co-housing groups suggest that the vaccine effectively primes the immune system, providing enhanced benefits in specific antigen recognition and memory response, which are critical for long-term protection against infections.

3.6. Cytokine Expression Levels. The levels of expression for IFN-γ, TNF-α, and IL-12 were assessed following immunization. At days 1, 3, 7, and 14 post-immunization, the concentration of IFN-γ in the immunized group was markedly elevated compared to that in the blank control group ($p < 0.0001$). By days 7 and 14, the co-housing group also showed significantly higher levels compared to the blank control group ($p < 0.05$) (Figure 4A).

Likewise, the concentrations of TNF-α in the immunized group were considerably enhanced compared to those in the blank control group ($p < 0.001$ at day 1 and 3, $p < 0.0001$ at day 7 and 14). The co-housing group also exhibited significant increases compared to the blank control group on days 7 and 14 ($p < 0.01$ and $p < 0.001$) (Figure 4B).

For IL-12, the expression in the immunized group was significantly higher than that in the blank control group, with p values of <0.001 on days 1 and 3, and <0.0001 on days 7 and 14. The co-housing group also demonstrated significant increases compared to the blank control group on days 7 and 14, with p values of <0.01 and <0.001, respectively (Figure 4C).

Specifically, the level of IFN-γ and TNF-α showed a consistent increase throughout the immunization period, peaking at 200 and 81 pg/mL on days 3 and 7 post-inoculation, respectively. In contrast, IL-12 levels displayed a variable pattern, with the highest concentration of 41.3 pg/mL observed on day 3 after vaccination.

Overall, these results highlight a robust immune response induced by the vaccination, marked by elevated concentrations of critical pro-inflammatory cytokines.

3.7. Assessment of Antibody Responses against M. bovis, Neutralizing Antibodies to BoHV-1, and BoHV-1 gB Antibody Responses. The antibody response of *M. bovis*, BoHV-1 neutralizing antibody, and BoHV-1 gB antibody responses were evaluated in vaccinated calves and those co-housing with them using competitive ELISA. Within the first week following immunization, all calves in the combined vaccine group tested positive for *M. bovis* antibodies. Interestingly, one calf from the co-housing group also tested positive during this period. By the second week post-vaccination, all calves in the co-housing group had seroconverted.

Subsequently, serum *M. bovis* antibody levels continued to rise significantly in both the vaccinated and co-housing groups, reaching peak levels of 92.5% and 85.4% observed at 5 and 6 weeks post-vaccination, respectively. Throughout the study, the vaccinated group consistently exhibited significantly higher *M. bovis* antibody levels compared to the blank control group ($p < 0.05$). In addition, the co-housing group showed significantly elevated antibody levels compared to the blank control group from weeks 2 to 6 ($p < 0.01$) (Figure 5A).

Neutralizing antibody levels were measured in the serum, showing that all animals in the combined vaccine group reached high titers by the first week, with an average of1:15.3. In contrast, the neutralizing antibody titer in the co-housing group surpassed 1:8 by the second-week post-immunization. While the neutralizing antibody titer in the combined vaccine group fluctuated within a specific range over time, the co-housing group peaked at 1:21.3 in the third week after cohabitation, after which it began to decrease. Throughout the observation period, neutralizing antibody levels in both groups

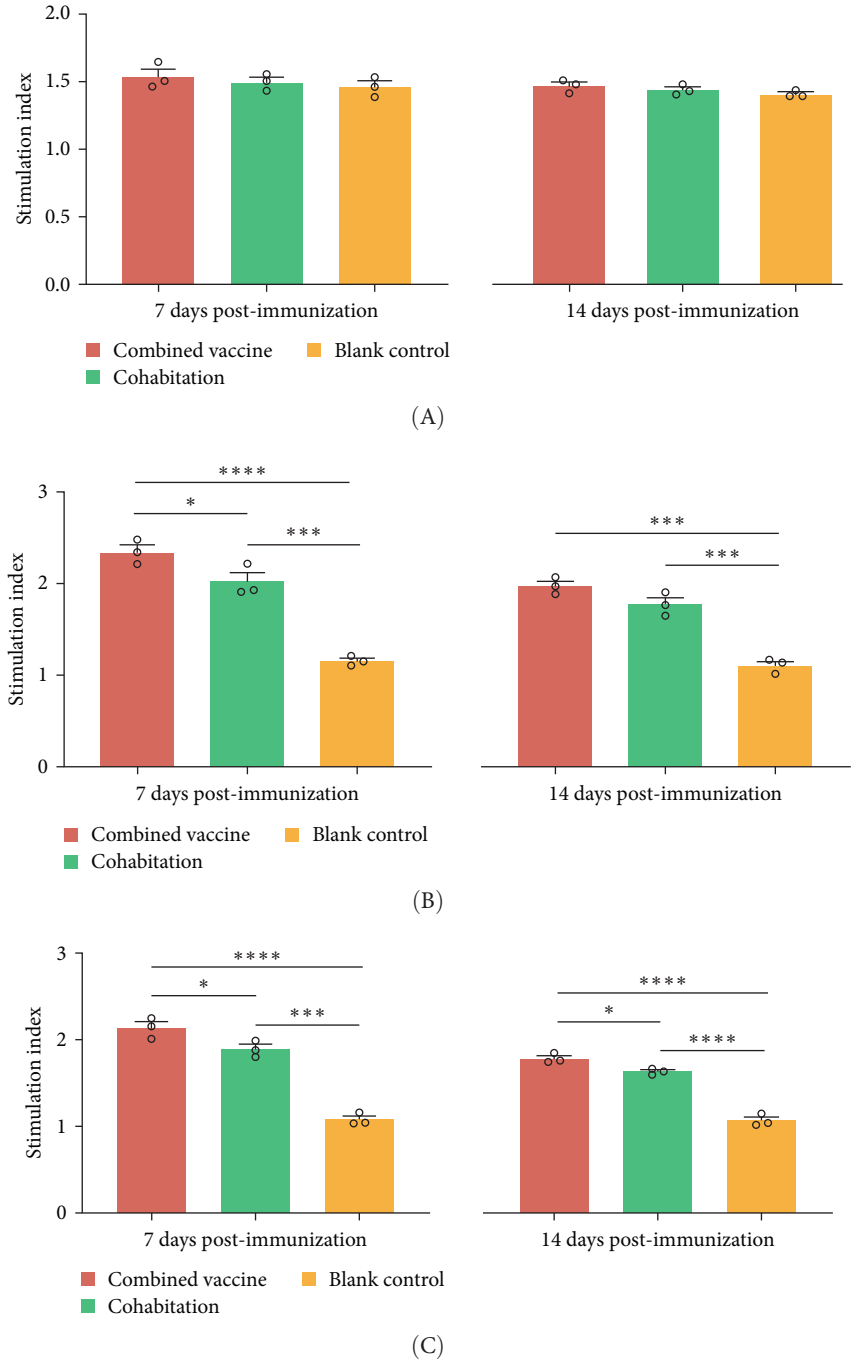


Figure 3: Lymphocyte proliferation assay using PBMCs. (A) Con A was used as positive control to measure lymphocyte proliferation after vaccination. (B) *M. bovis* protein and (C) inactivated BoHV-1 were used as stimuli.

remained significantly higher than those in the blank control group ($p < 0.05$ and $p < 0.05$) (Figure 5B).

In the first 4 weeks following immunization, gB antibody levels in the combined vaccine group rose sharply before beginning to decline. By the 4th week, the peak gB antibody level in this group reached 0.351. All calves in the combined vaccine group maintained positive gB antibody levels until the sixth week post-inoculation. In contrast, the co-housing group showed a gradual increase in gB antibodies over the course of the experiment, with all individuals testing positive for gB antibodies by the fifth week of cohabitation. Statistically significant differences in gB antibody levels were observed between the two experimental groups and the blank control group throughout the observation period ($p < 0.01$ and $p < 0.05$) (Figure 5C).

3.8. Detection of IgA and IgG Antibody Responses. To assess the immune systemic response, total IgA and IgG antibody levels in serum and NALF were measured in both the combined vaccine and co-housing groups. Inoculation resulted in a

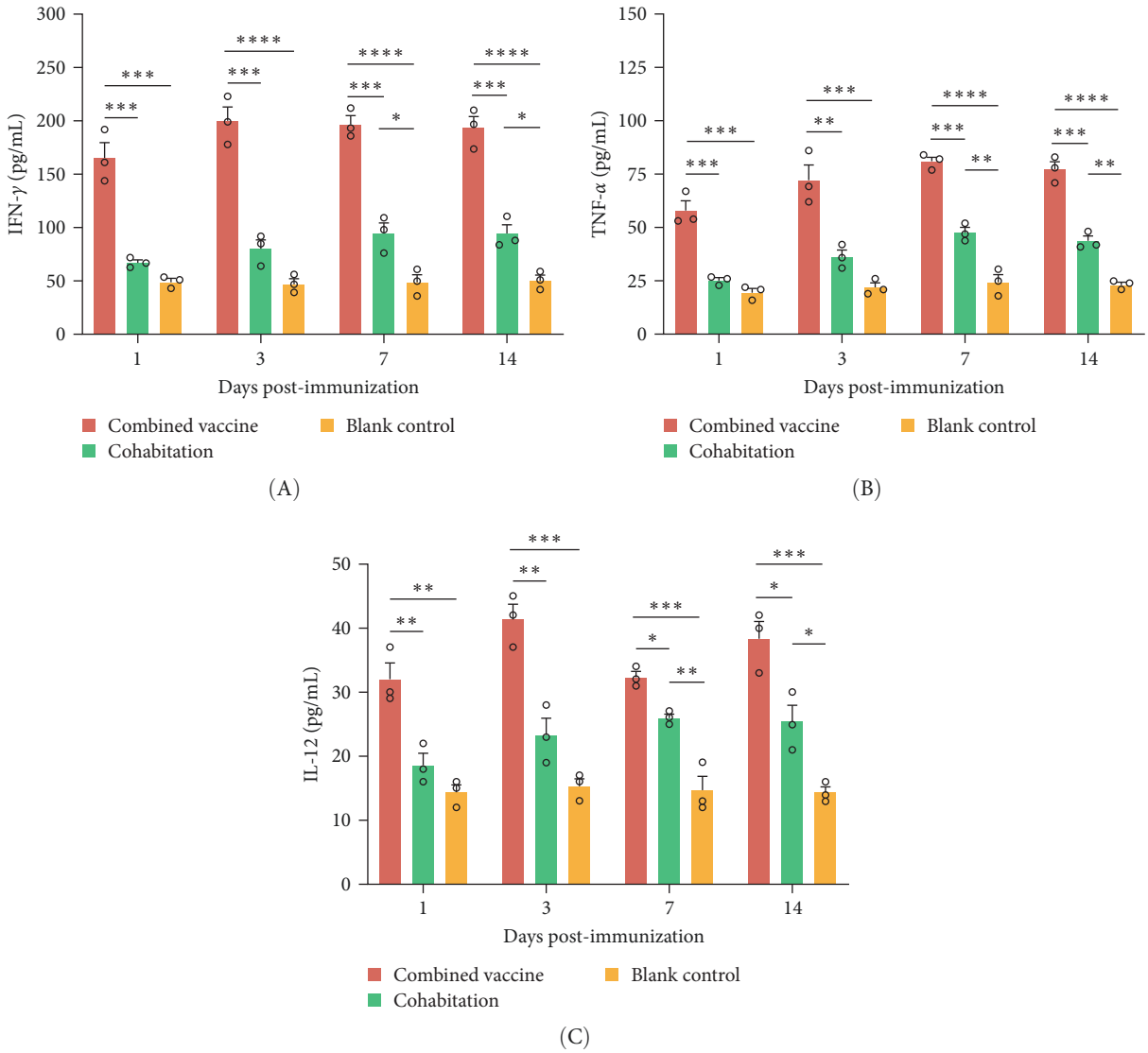


FIGURE 4: Cellular immunity in calves immunized with *M. bovis*-BoHV-1 combined vaccine and those in co-housing group. (A), (B) and (C) represent the level of IFN-γ, TNF-α, and IL-12, respectively.

significant increase in serum IgA antibodies, with the combined vaccine group reaching a peak of 55.3 pg/mL during the first week, which was significantly higher than the blank control group ($p<0.001$). However, serum IgA levels in the combined vaccine group declined sharply afterward, resulting in no significant difference when compared to the blank control group.

In contrast, the co-housing group exhibited a gradual increase in serum IgA level during the first 2 weeks, reaching significantly higher levels than the blank control group by week 2 ($p<0.05$). However, the levels subsequently decreased and remained relatively lower afterward (Figure 6A).

IgA levels in NALF remained low across both experimental groups. However, the levels of IgA in these groups were notably elevated compared to those in the blank control group during the initial week following administration ($p<0.001$ and $p<0.05$). A significant difference in IgA levels between the combined vaccine group and the blank control group continued in the second-week post-vaccination ($p<0.05$) (Figure 6A).

IgG, which serves as a vital component in the secondary immune response, significantly increased in calves vaccinated with the combined vaccine, reaching a peak of 211.7 pg/mL by the second-week post-vaccination. Although IgG levels gradually declined between weeks 3 and 6, levels in this group remained significantly elevated throughout the study compared with those in the blank control group ($p<0.001$). In addition, calves cohabiting with the combined vaccine group also exhibited higher IgG titer in the first-week post-cohabitation, with sustained elevated levels from week 2 to 6, showing significant differences compared to the blank control group ($p<0.05$) (Figure 6B).

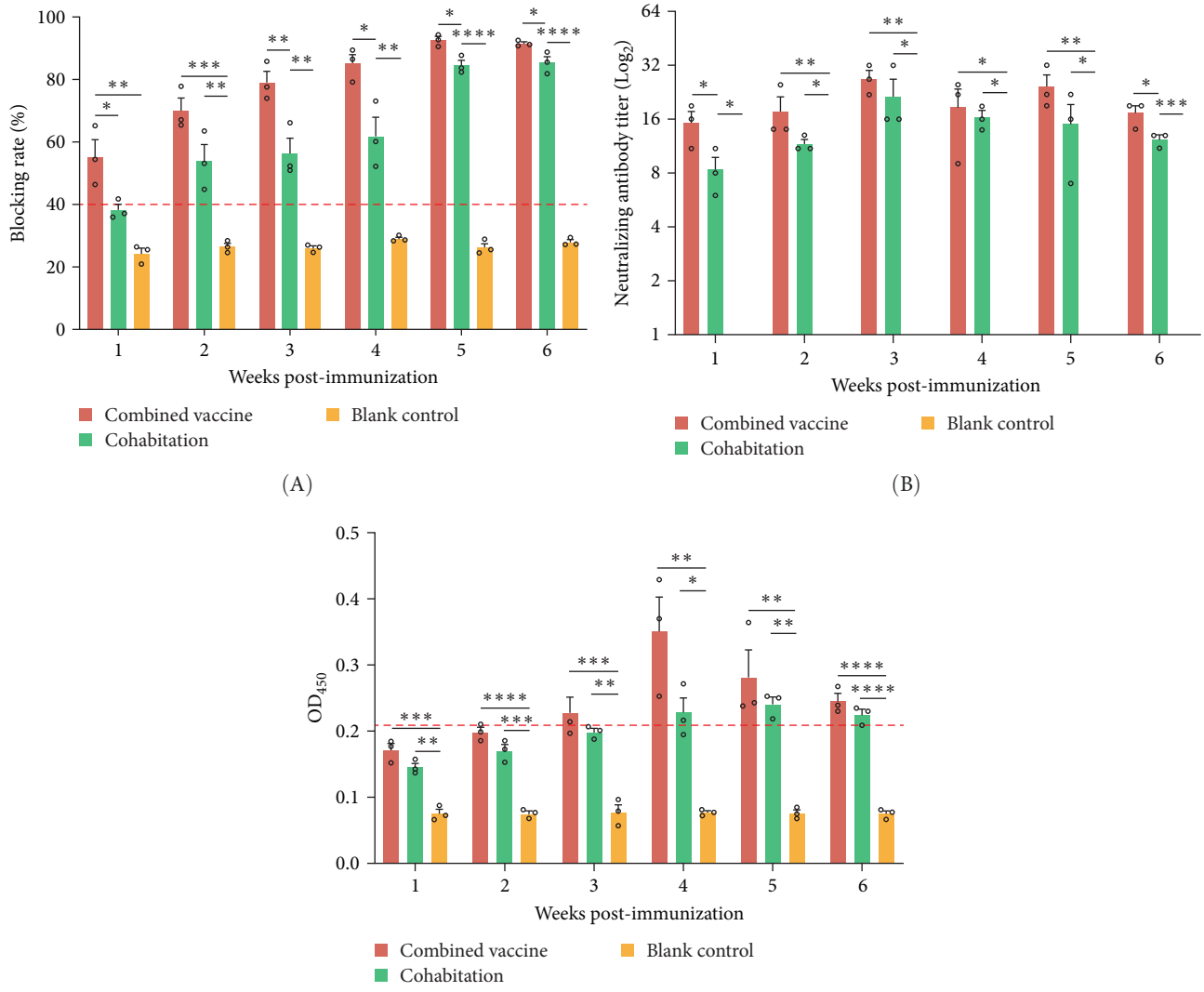


FIGURE 5: Specific humoral immune responses in *M. bovis*-BoHV-1 combined vaccine immunized group and co-housing group. (A) *M. bovis* serum specific ELISA antibody, (B) BoHV-1 neutralizing antibody titers, and (C) BoHV-1 gB antibody were monitored. The red line indicates the negative-positive threshold for *M. bovis* ELISA antibody and gB antibody.

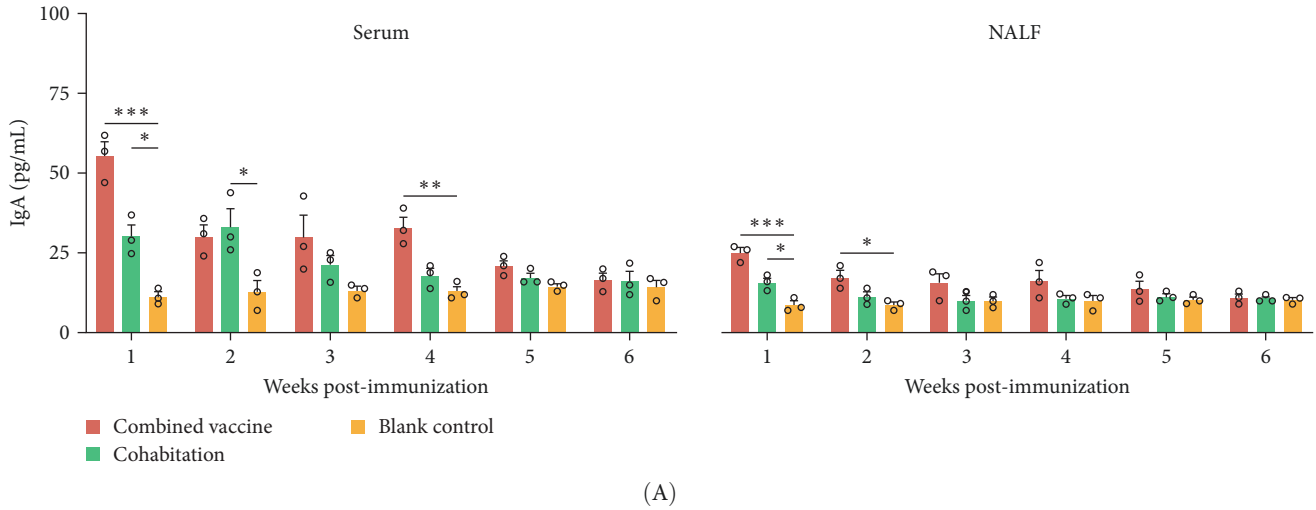
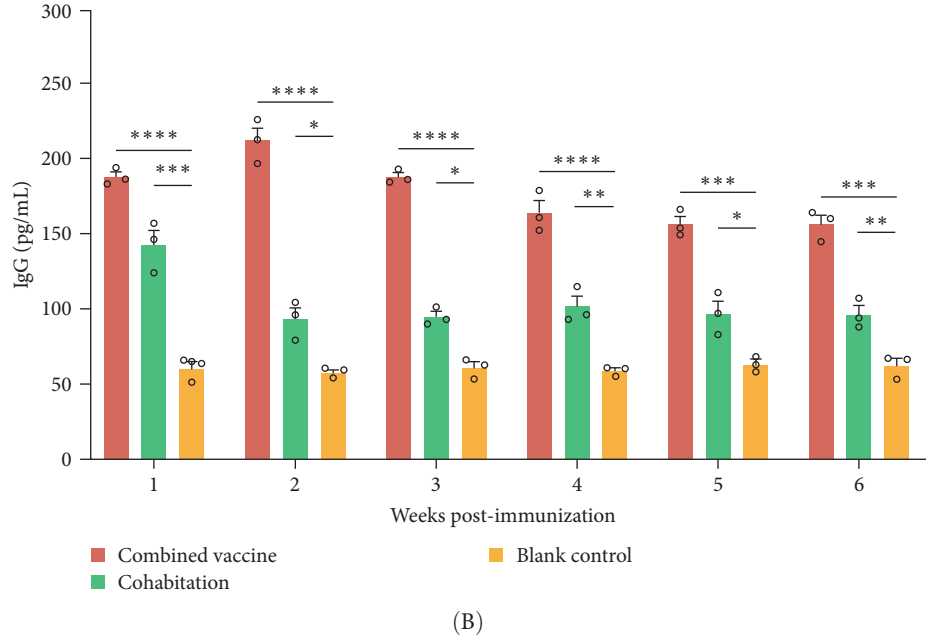


FIGURE 6: Continued.



(B)

FIGURE 6: Total IgA and IgG concentrations were assayed in vaccinated group and co-housing group. (A) Serum IgA levels, IgA levels of nasal lavage fluid (NALF), and (B) serum IgG antibody levels were monitored.

4. Discussion

BRD presents a serious threat to cattle health and results in substantial economic losses for the agricultural industry. The pathogenesis of BRD is often driven by intricate interactions between multiple pathogens, including both bacterial and viral agents. Our study emphasizes the promising effectiveness of a novel combined vaccine targeting *M. bovis* and BoHV-1, demonstrating its potential to improve indirect immune responses.

The findings show that this vaccine not only triggers strong immune responses but also promotes an indirect immune effect in non-immunized animals. This feature is especially important in densely populated agricultural environments, where the vaccine's ability to enhance herd immunity can greatly reduce the spread of pathogens, leading to a decrease in BRD incidence [16, 17].

Cattle, being ruminants, often transfer rumen contents into their oral cavities, which can impact the respiratory microbiota. Particularly, Holstein cows and calves engage in frequent licking and oral interactions, making their environment a significant source of infection [18, 19]. This behavior highlights the vulnerability of dairy herds to the lateral transmission of pathogens, underlining the crucial role of the indirect immune effects of live vaccines on non-immunized individuals.

Evidence from human studies supports the concept that live rotavirus vaccines, once administrated, can be isolated from the feces of vaccinated individuals [20] and subsequently transmitted to unvaccinated siblings [21]. Similarly, the mumps vaccine strain has shown potential for lateral spread within populations [22]. In poultry, the use of live attenuated *Mycoplasma gallisepticum* vaccine has been demonstrated to significantly reduce bacterial shedding among contact birds [23]. However, despite these valuable insights, research into the indirect immune effects of live vaccines in ruminants remains limited. Addressing this gap is crucial for refining vaccination strategies in cattle and improving overall herd health outcomes.

Our findings demonstrate that non-immune calves can effectively trigger their innate immune responses after cohabiting with vaccinated cattle. This is particularly demonstrated by the increase in serum lysozyme levels and enhanced lymphocyte proliferation. Lysozyme, a natural antibacterial agent, plays a crucial role in the body's first line of defense against pathogens and has antiviral properties against various viruses, including those affecting domesticated and wild ungulates, as well as human influenza and respiratory viruses [24–26]. Previous research has highlighted lysozyme's essential role in natural immunity and its immunomodulatory functions in response to infections [27, 28]. The marked rise in lymphocyte percentages and proliferation in cohabiting calves, when compared to the control group, support the activation of both cellular and humoral immune responses.

Furthermore, the observed activation of the innate immune response highlights the broader, indirect immune effects of the *M. bovis*-BoHV-1 combined vaccine, which extend beyond basic innate immunity. Our findings demonstrate that calves living in proximity to vaccinated individuals exhibited significantly higher levels of key cytokines, including IFN-γ, TNF-α, and IL-12, shortly after the initiation of the experiment. The levels of cellular immune response in calves in the co-housing group remained significantly different from those in the control group until day 14 after cohabitation. These cytokines are crucial in coordinating protective immune responses. Specifically, IFN-γ, a central marker of cell-mediated immunity (CMI), plays critical roles in antiviral defense, tumor suppression, and immunomodulation, serving as a primary regulator of

immune reactions [29, 30]. Similarly, TNF-α and IL-12 are vital for effective infection control and have potential implications in cancer therapy [31–33]. Collectively, these results underscore the importance of the indirect immune effects within a herd, demonstrating how vaccination can enhance overall herd immunity and highlighting the critical role of live vaccines in controlling BRD.

Moreover, the ability of cohabiting calves to generate specific humoral immune responses highlights the potent indirect immune effects of the *M. bovis*-BoHV-1 combined vaccine. Although the intensity and duration of these responses in cohabiting individuals were less pronounced compared to those in directly vaccinated cattle, they still exhibited significantly higher titers of neutralizing antibodies against both *M. bovis* and BoHV-1, as well as gB antibodies, when compared to the control group. Notably, antibody levels in cohabiting calves became detectable by the second week of cohabitation, with average titers of BoHV-1 neutralizing antibodies surpassing the protective threshold of 1:8 [34]. Also, in the sixth week after the trial, calves in the co-housing group were still stimulated to produce significantly higher titers of these three specific antibodies than those in the blank control group due to the indirect immune effect of the combined vaccine. This robust antibody response further supports the beneficial indirect immune effect of the *M. bovis*-BoHV-1 combined vaccine.

In addition, we observed a significant increase in serum IgA antibody production in cohabiting calves, with notably higher mucosal IgA levels detected in NALF compared to the control group within the first week of cohabitation. IgA antibodies are essential for mucosal immunity, offering protection against pathogens and playing a key role in preventing respiratory infections [35, 36]. Furthermore, the vaccinated individuals seemed to enhance IgG antibody production in cohabiting calves. IgG is critical for systemic humoral immunity and secondary immune responses, and the sustained IgG response in cohabiting calves is vital for combating respiratory infection-related diseases [37]. These findings provide strong evidence for the effective indirect immune effect of the *M. bovis*-BoHV-1 combined vaccine.

These findings confirmed the favorable indirect immune effect of the combined vaccine, and we were able to effectively differentiate between vaccinated and passively immunized individuals by the time of nasal antigen excretion and the titer and duration of serum-specific antibodies. However, there are still some limitations that warrant further research. Further challenge experiments are required to explore the true protective efficacy of the indirect immune effect produced by the combined vaccine in passively immunized individuals. In addition, although combined vaccines have a promising indirect immune effect on a small scale, large-scale trials are needed to assess the ability of vaccination to produce herd immunity. Besides, it leaves questions about the durability of humoral and cellular immunity in cohabitating individuals induced by indirect immune effect, so longer post-immunization monitoring should also be considered in the next experiment.

In summary, our study demonstrates that the combined *M. bovis*-BoHV-1 vaccine not only elicits strong immune responses in vaccinated calves but also promotes notable indirect immune effects in non-vaccinated animals. This finding holds significant implications for vaccination strategies designed to improve herd immunity and control BRD in cattle populations. Further research is needed to investigate the mechanisms behind these indirect effects and to refine vaccine formulations for enhanced efficacy across diverse agricultural environments.

5. Conclusion

In conclusion, our study highlights the significant potential of the *M. bovis*-BoHV-1 combined vaccine in inducing indirect immune responses. Vaccinated individuals successfully stimulate both cellular and humoral immune responses in cohabiting calves. These results underscore the vaccine's immunogenicity in unimmunized calves, providing immediate protection against infections. This research offers strong evidence to support the ongoing development and use of the *M. bovis*-BoHV-1 combined vaccine, paving the way for improved disease management and better overall herd health in cattle.

Data Availability Statement

The data supporting the findings of this study are available within the article. Further inquiries can be directed to the corresponding authors.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Sen Zhang, Guoxing Liu, Jianguo Chen, Aizhen Guo, and Yingyu Chen: conceptualization. **Sen Zhang and Guoxing Liu**: formal analysis. **Jianguo Chen, Aizhen Guo, and Yingyu Chen**: funding acquisition. **Sen Zhang**: investigation. **Sen Zhang and Guoxing Liu**: methodology. **Sen Zhang, Guoxing Liu, Jianguo Chen, Aizhen Guo, and Yingyu Chen**: project administration. **Sen Zhang and Guoxing Liu**: resources. **Sen Zhang**: software. **Aizhen Guo and Yingyu Chen**: supervision. **Guoxing Liu**: validation. **Sen Zhang**: visualization. **Sen Zhang**: writing – original draft. **Jianguo Chen, Aizhen Guo, and Yingyu Chen**: writing – review and editing. All authors have read and approved the final work. Sen Zhang and Guoxing Liu contributed equally to this work and should be listed as co-first authors.

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