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Adjuvant-driven antibody response to use cows as biofactories of anti-SARS-CoV-2 neutralizing antibodies in colostrum

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ABSTRACT

Cows produce a substantial amount of immunoglobulin in the colostrum, and nutraceutical products derived from these antibodies are gaining attention for their potential role in human viral disease prevention. The objective of our study was to develop an immunization schedule for pregnant cows to produce hyperimmune colostrum with antibodies presenting high avidity and neutralizing activity against SARS-CoV-2. The recombinant spike receptor-binding domain (RBD) from SARS-CoV-2, expressed using the Expi293F system and purified via Ni-affinity chromatography, was solubilized in (1) saponin (QuilA) or (2) a suspension of potassium and aluminum hydroxide (Alum). Vaccination of pregnant cows and serum sample collection were performed 45, 30, and 15 d before the expected calving date. Serum and colostrum were also collected on the day of parturition. Anti-RBD IgG, IgG1, and IgG2 production, viral neutralization, and antibody avidity were evaluated by ELISA. Cows immunized with recombinant RBD with the QuilA adjuvant produced higher amounts of all antibody subclasses than cows in the Alum group. The viral neutralization index from serum samples was also higher in the QuilA group. Significant differences were not observed in the avidity of antibodies, except for that of IgG2, which was higher in the serum of cows receiving the Alum formulation. As the IgG1 antibody subclass and its avidity are crucial for SARS-CoV-2 neutralization, QuilA might be the optimal adjuvant for producing hyperimmune colostrum in cows. These findings support the use of cows as biofactories of neutralizing antibodies against SARS-CoV-2 or any future emerging and re-emerging viral diseases, with the possibility of simply substituting the subunit antigen in the vaccine formulation. Further tests must be done to evaluate the efficacy of using hyperimmune colostrum as a nutraceutical or purified bovine antibodies as a pharmacological approach for COVID-19 prevention.

Key words: bovine colostrum, COVID-19, adjuvant, neutralizing antibody

INTRODUCTION

The emergence of novel infectious diseases, particularly COVID-19, caused by SARS-CoV-2, has underscored the urgent need for sustainable and effective prevention strategies. In this context, neutralizing antibodies have gained significant attention due to their potential to provide immediate protection against viral infections. Given the high homology of bovine and human immunoglobulins, cow colostrum has gained attention as a natural source of neutralizing antibodies for many viral and bacterial diseases (Pirro et al., 1995; Kramski et al., 2012; Burke et al., 2020; Kangro et al., 2022). As bovine colostrum can present ranges from 50 to 150 g/L of immunoglobulins (Stelwagen et al., 2009; Hurley and Theil, 2011), it is reasonable to propose the use of cows as biofactories generating high neutralizing antibody titers. Indeed, after the COVID-19 pandemic, the production of hyperimmune colostrum has emerged

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as a promising approach for preventing SARS-CoV2 infection (Duman and Karav, 2023).

Neutralizing antibodies are characterized by their ability to specifically bind to viral epitopes, preventing the virus from infecting host cells. Within the IgG class, different subclasses, notably IgG1 and IgG2, exhibit distinct functions and mechanisms of action in response to viral infections, especially those related to SARS-CoV-2 (Morales- Núñez et al., 2021; Moura et al., 2021; Chen et al., 2022). IgG1 is particularly effective in neutralizing viruses and mediating antibody-dependent cellular cytotoxicity, whereas IgG2 plays a critical role in complement activation and opsonization, enhancing the ability of the immune system to target and eliminate pathogens (Morales-Núñez et al., 2021). The balance between these subclasses can significantly influence the overall antiviral efficacy, making their optimal production essential for developing effective immunotherapeutic approaches. When combined with the divergence in protocols used to immunize cows to obtain hyperimmune colostrum against COVID-19, this highlights the importance of optimizing immunization protocols and characterizing the subclasses, neutralization capacity, and avidity of anti-SARS-CoV-2 antibodies.

Adjuvants are widely studied for enhancing immune responses in vaccination protocols, particularly in the production of neutralizing antibodies. Saponin-based adjuvants, such as QuilA, are known for stimulating Th1 and Th2 immune responses, which are essential for producing of long-lasting and high-affinity antibodies (Cibulski et al., 2018). In contrast, the aluminum-based adjuvant Alum, which is most commonly used in human vaccines, predominantly induces a strong humoral response boosting IgG1 antibody production (He et al., 2015; Gatt et al., 2023). Many studies have shown that vaccines incorporating QuilA (Dalsgaard and Jensen, 1977), Alum (Guasconi et al., 2012), or both enhance the production of neutralizing antibodies and protection against many diseases in cattle (Shu et al., 2000; Boerhout et al., 2018; Chelliah et al., 2020).

In this study, we aimed to evaluate production of total IgG, IgG1, and IgG2 antibody production after vaccination using the recombinant spike receptor-binding domain (**RBD**) from SARS-CoV-2 (**S-RBD**) solubilized in 2 distinct commercial adjuvants: a saponin-based adjuvant (QuilA) and a suspension of potassium and aluminum hydroxide (Alum).

MATERIALS AND METHODS

Cow vaccination and sample collection took place at the Experimental Field José Henrique Bruschi, Embrapa Dairy Cattle Multi-User Laboratory for Livestock Bioefficiency and Sustainability (LMBS) in Coronel Pacheco, MG, Brazil. In compliance with Brazilian federal law, the Animal Ethics Committee of Embrapa Dairy Cattle approved all experimental procedures and activities (protocol code 1915290721). The expression, purification, and characterization of recombinant S-RBD were done at Adolfo Lutz Institute, São Paulo, SP, Brazil. ELISA for detection of antibody subclasses, their avidity, and viral neutralization was performed at Embrapa Dairy Cattle, Juiz de Fora, MG, Brazil.

Expression and Purification of Recombinant S-RBD

Expression and purification of recombinant S-RBD were performed according to da Costa et al. (2023). Briefly, plasmid containing the RBD gene sequence was transfected in HEK 293F cells (Thermo Fisher Scientific, Waltham, MA) using an ExpiFectamine 293 Transfection Kit (Thermo Fisher Scientific, Waltham, MA) to overexpress recombinant proteins. Supernatants (~100 mL) from cultures of transfected cells were harvested 3 to 5 d post-transfection by centrifugation at 4,000 × *g* for 20 min at 4°C and concentrated using an Amicon Ultra Centrifugal Filter (Merck Millipore, Burlington, MA) to a final volume of 5 mL. Then, 20 mL of buffer A (20 m*M* sodium phosphate, 500 m*M* NaCl, pH 7.4) was added to the supernatant, and the sample was clarified in a 0.22-µm syringe filter. First, the recombinant proteins were purified by immobilized metal ion affinity chromatography using HisTrap Excel columns (GE Healthcare Life Sciences, Chicago, IL) on a fast protein liquid chromatography system (ÄKTA Pure, Cytiva Life Sciences, Marlborough, MA). The supernatant was applied to the column, previously equilibrated with buffer A, at a flow rate of 1 mL/min. Unbound proteins were washed away with 10 column volumes of 20 m*M* imidazole. Bound fractions were eluted using a gradient with 20 column volumes of buffer B (20 m*M* sodium phosphate, 500 m*M* NaCl, 500 m*M* imidazole, pH 7.4). The peak corresponding to the protein of interest was collected, concentrated in Amicon centrifugal units (Merck Millipore, Burlington, MA), and resuspended in 2 mL of PBS for size-exclusion chromatography using a Superdex 75 column (Cytiva Life Sciences, Marlborough, MA). The fractions were collected and analyzed by 10% SDS-PAGE to check protein integrity and western blot to identify the recombinant RBD expression. For western blot, after 10% SDS-PAGE, proteins were transferred overnight to a 0.22-µm nitrocellulose filter membrane (Bio-Rad, Hercules, CA). Each membrane was stained with Ponceau red, divided into 4 strips, blocked with 5% skim milk (wt/vol) diluted in PBS, and further incubated with a rabbit anti-His-tag antibody conjugated to horseradish peroxidase (**HRP**; 1:1,000, Sigma-Aldrich, St. Louis, MO). Subsequently, the strips were washed 3 times with 0.1% PBS containing

0.05% Tween-20 (PBS-T), and the protein bands were visualized using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA), following the manufacturer's instructions. Images were recorded using an iBright CL1500 Imaging System (Thermo Fisher Scientific, Waltham, MA). The single-batch production of recombinant S-RBD was quantified by Bradford assays (Thermo Fisher Scientific), and 750 µg per vial of this protein was aliquoted and kept frozen at −80°C until use.

Formulations

The QuilA saponin adjuvant formulation was derived from *Quillaja saponaria* bark (Sigma-Aldrich S4521, St. Louis, MO; batch 031M7018V, equivalent to 27% QuilA). This was diluted in PBS (pH 7.4) under sterile conditions to 1 mg/mL of QuilA. The solution was sealed with parafilm and incubated in a water bath at 35°C during sonication at 50 W for 5 min to ensure complete solubilization. This solution was filtered through sterile 0.22-µm filters and kept at 4°C until use. The recombinant RBD antigen (750 µg per formulation) was added to a final volume of 5 mL of either QuilA solution or a ready-to-use aluminum hydroxide-based suspension (AlumInject; Thermo Fisher Scientific, Waltham, MA) followed by 5 min of vortexing to ensure antigen solubilization homogeneity and complete adsorption of the protein into the adjuvant solutions.

Animals, Immunization Protocol, and Sample Collection

The controlled, randomized study design used Holstein cows that were impregnated by fixed-time artificial insemination, which allows for prediction of the date of calving. They were divided into homogeneous groups according to parity, milk production, and the estimated date of calving. The cows were kept in a compost barn structure with daily management to verify clinical alterations that might interfere with the experiment. All cows were immunized intramuscularly in the neck region with 150 µg of recombinant S-RBD solubilized in QuilA (QuilA group; n = 5) or InjectAlumen (Alum group; n = 5) vaccine formulations. Cows inoculated with sterile saline solution were used as the negative control group (**NC**; n = 5). The number of animals per group was determined through Monte Carlo simulations using SAS Macros software (SAS version 9.4M6; https://www.sas.com). The calculations were based on values representing a 50% difference in the average antibody levels between animals immunized with recombinant spike RBD in different adjuvant formulations and those inoculated with saline buffer (NC group). The vaccine inoculation was administered 45, 30, and 15 d before the estimated calving dates.

Peripheral blood samples were collected immediately before each immunization and on the calving date by jugular venipuncture with a vacutainer system coated with a clotting agent (Becton Dickinson, São Paulo, Brazil), followed by incubation at room temperature for 30 min and refrigeration at 4°C for 1 h. Serum was obtained by centrifugation of the blood vacutainer tubes at 3,000 × *g* for 10 min at room temperature, aliquoted, and kept frozen at −20°C until use. Serum and colostrum samples were also collected on the date of calf birth within a maximum of 6 h after parturition. For colostrum collection, cows were individually milked using a mechanical milking machine that recorded the colostrum yield in liters. The colostrum was homogenized, immediately aliquoted, and frozen at −20°C until use.

ELISA for Quantification of Total IgG, IgG1, and IgG2 Antibodies in Bovine Serum and Colostrum

The production of total IgG and the subclass IgG1 and IgG2 antibodies in bovine serum and colostrum was evaluated by indirect ELISA using anti-bovine secondary antibodies (Bethyl Laboratories Inc., Waltham, MA). Indirect ELISA protocols were previous standardized using a widely known checkerboard titration assay (Koliaraki et al., 2009; Perera and Murray, 2009; Berrizbeitia et al., 2012). Colostrum samples were thawed and centrifuged at 4,500 × *g* for 20 min at 4°C to remove fat, which concentrated at the top, and cellular debris, which concentrated at the bottom after centrifugation. The serum and colostrum were then homogenized and diluted in PBS-T. All samples were tested in duplicate. Briefly, for indirect ELISA, high-binding 96-well ELISA plates (Corning) were coated with recombinant S-RBD (2 µg/mL) diluted in coating buffer (0.05 *M* carbonate-bicarbonate buffer, pH 9.6) at 4°C overnight. Plates were washed 4 times with PBS-T and then blocked with 5% nonfat dry milk (Accumedia, Prolab, São Paulo, Brazil) in PBS-T for 2 h at 37°C to prevent nonspecific binding. Serum and colostrum samples were diluted at 1:200 in PBS-T, except for IgG2 testing, for which they were diluted at 1:400. Then, 100 µL of each sample was added to the plates in duplicate and incubated for 1 h at 37°C. After washing the plates 3 times, 100 µL of HRP-conjugated anti-bovine IgG (1:20,000), IgG1 (1:10,000) or IgG2 (1:10,000) secondary antibody (Bethyl Laboratories Inc., Waltham, MA), diluted in PBS, was added to each well and incubated for 1 h at 37°C. Following 4 additional washes with PBS-T, 100 µL of o-phenylenediamine dihydrochloride (Sigmafast, Sigma-Aldrich) substrate solution was added to each well. The reaction was stopped after 15 min by adding 50 µL of 3 *N* sulfuric

acid, and the absorbance was measured at 492 nm using a microplate reader (BioTek). All data were normalized by background subtraction to account for nonspecific binding. A pool of positive serum and colostrum, collected at the calving date, from cows immunized with recombinant S-RBD + QuilA was used to estimate the positivity percentage to normalize errors and calculate intra- and inter-assay CV. This pool underwent 6 rounds of serial dilution using a base-2 scheme, starting at 1:50 and progressing to 1:1600. Absorbance measurements generated an exponential curve, with the 1:200 dilution set as 100%. The positivity percentage (%) was then calculated based on the curve equation. The mean and SD of the positivity percentage were calculated for each immunized group and transformed by $\log_2$ positivity (%).

Antibody Avidity Rate Assessment

To evaluate the avidity rate of the produced antibodies, a modified ELISA protocol was employed as described by Bjorkman et al. (1999). This assay incorporates urea (6 *M*; Sigma-Aldrich), a chaotropic agent that facilitates the dissociation of low-avidity antigen-antibody complexes, causing a measurable change in optical density (**OD**) at 492 nm. Briefly, the ELISA conditions described above were used, and after incubation with serum or colostrum samples, an additional step was introduced using 6 *M* urea for 5 min at room temperature. The urea-modified and nonmodified assays were performed simultaneously on the same plate to minimize intrasample and interplate variation. After washing with PBS-T, the subsequent steps were performed as already described. The mean avidity rate was calculated for each group, and the avidity rate (%)was calculated using the following formula:

$$\text{Avidity rate } (\%) = \frac{\text{Treated urea OD}_{492\text{ nm}}}{\text{Nontreated urea OD}_{492\text{ nm}}} \times 100.$$

Viral Neutralization Assay

A viral neutralization assay was performed using a competitive ELISA method with the cPass SARS-CoV-2 Neutralization Antibody Detection Kit (NL Diagnostica, São Paulo, Brazil), following the manufacturer's protocol. Briefly, serum and colostrum samples, along with the positive and negative controls provided in the kit, were preincubated with the recombinant RBD of the SARS-CoV-2 virus conjugated to HRP. The mixture was then transferred to ACE2 receptor-coated plates and incubated at 37°C. After incubation, the plates were washed, and enzymatic development was carried out using 3,3′,5,5′-tetramethylbenzidine substrate. The reaction was stopped with 3 *N* sulfuric acid, and OD was measured at 450 nm using a microplate spectrophotometer (BioTek). The percentage of viral neutralization was calculated relative to the negative control using the following formula:

$$\text{Viral neutralization rate}(\%) = \left(1 - \frac{\text{Sample OD}_{450\text{ nm}}}{\text{NC OD}_{450\text{ nm}}}\right) \times 100.$$

According to the manufacturer's guidelines, a neutralization rate equal to or greater than 20% indicates the presence of neutralizing antibodies against SARS-CoV-2 in the tested samples.

Statistical Analysis

The mean and SD of normalized OD 492 nm, avidity, and viral inhibition rates were calculated for each experimental group individually. To determine statistical significance, the nonparametric Mann-Whitney test was employed for comparisons between 2 groups, and the Kruskal-Wallis test followed by Dunn's post hoc test were performed for multiple comparisons among groups. The Tukey test was used followed by the Sidak correction to adjust for multiple comparisons. $P < 0.05$ was considered statistically significant. Data were analyzed using GraphPad Prism software (version 7.0, GraphPad Software, San Diego, CA).

RESULTS

Expression and Purification of Recombinant S-RBD Protein

A satisfactory degree of purity and high yield concentration of recombinant RBD was achieved after purification (Figure 1), as evidenced by the clear 30-kDa protein bands observed after SDS-PAGE (Figures 1A and 1B) and western blotting (Figure 1C). The amount of purified recombinant RBD was sufficient to produce all formulations on the day of cow inoculation, thus avoiding variations, especially in the integrity of the recombinant protein and probably precipitation or adjuvant-activity decay, and ensuring greater uniformity in the tests.

Serum and Colostrum Levels of IgG, IgG1, and IgG2 Antibodies against Recombinant S-RBD Induced by Vaccine Formulations with QuilA or Alum as an Adjuvant

The results of indirect ELISA to verify IgG, IgG1, and IgG2 antibody production in bovine serum after each inoculation of recombinant RBD protein from SARS-CoV-2 formulated with QuilA or Alum as an adjuvant

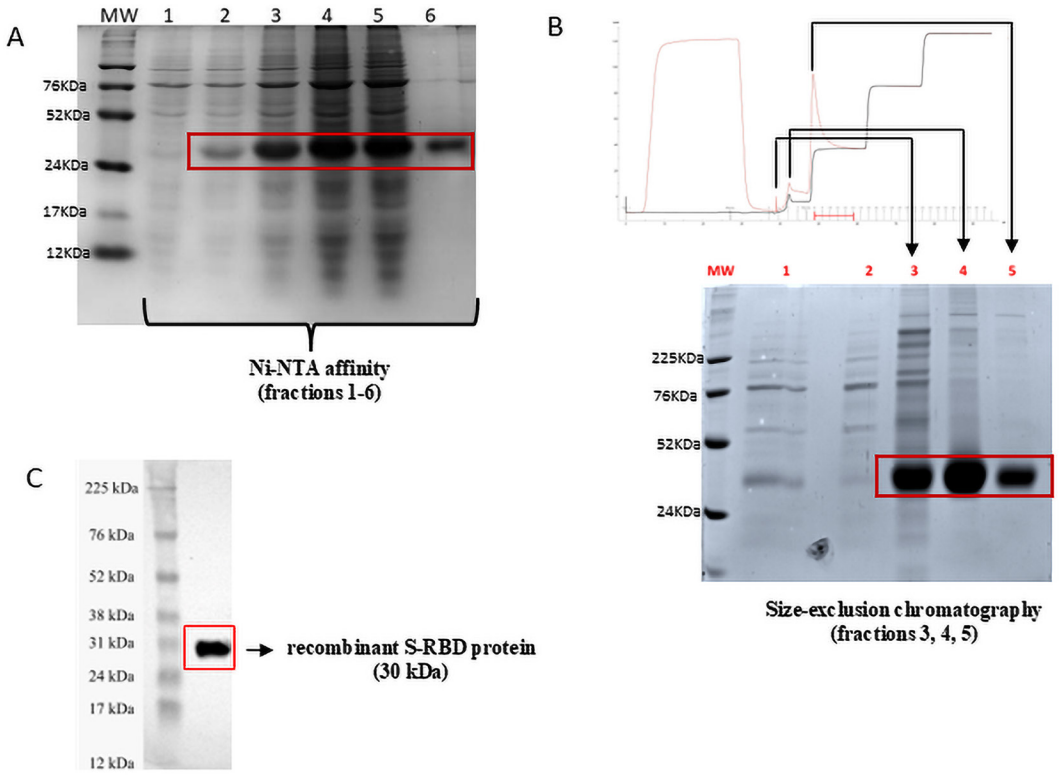

Figure 1. Expression and purification of SARS-CoV-2 recombinant spike RBD protein. Representative scheme of supernatant fractions of the Expi293F expression system purified through (A) 15% SDS-PAGE and Ni-NTA affinity (fractions 1–6) and (B) size-exclusion chromatography and respective 15% SDS-PAGE of eluted peaks (3–5). (C) Western blot of purified recombinant S-RBD protein using anti-His-tag-HRP antibody. Red boxes = recombinant S-RBD protein (predictive molecular weight = 30 kDa). MW = molecular weight marker.

are shown in Figure 2. The intra-assay CV was lower than 2% for serum and 3% for colostrum, considering all subclasses analyzed. The interassay CV was lower than 5.96% for serum and 4.75% for colostrum, confirming the reproducibility of the ELISA results. As expected, at the beginning of the experiment (45 d before calving), all groups presented the same background levels of all antibody subclasses analyzed in serum (Figure 2A–C). A significant increase in the levels of total IgG and its subclasses, IgG1 and IgG2, compared with the NC and Alum groups, was observed in the QuilA group 15 d before calving ($P < 0.05$; Dunn's multiple comparisons test; Figure 2A–C). By the calving date, IgG (Figure 2A) and IgG1 (Figure 2B) percent IgG positivity in the QuilA group had decreased but remained significantly ($P < 0.05$) higher (108.37 ± 39.13) than those in the NC group (16.25 ± 2.01) and the Alum group (22.67 ± 5.572). Although cows vaccinated with formulations containing Alum adjuvant did not show notable results in serum (Figure 2A–C), significantly higher percent positivity ($P < 0.05$; Dunn's multiple comparisons test) of IgG (13.73 ± 39.13; Figure 2D) and IgG1 (18.06 ± 19.16; Figure 2E) antibodies against recombinant RBD were detected in colostrum from cows in this group than cows in the NC group (0.79 ± 1.16 for IgG and 4.75 ± 0.96 for IgG1; $P < 0.05$). Once again, the QuilA group demonstrated the highest percent IgG positivity (177.38 ± 26.8; Figure 2D), IgG1 (66.37 ± 19.54; Figure 2E; $P < 0.05$), and IgG2 (556.12 ± 172.93; Figure 2F) compared with the Alum and NC groups. The colostrum yield, in liters, did not significantly vary among groups (NC = 2.28 ± 0.708 L; QuilA = 2.16 ± 1.352 L; Alum = 2.22 ± 0.776 L; $P > 0.05$). It is noteworthy that one cow in the QuilA group presented lower yields of colostrum and antibodies in it, which might be associated with the occurrence of a dystocic calf birth.

Avidity Rates in Serum and Colostrum from Cows Vaccinated with Recombinant S-RBD Formulated with QuilA or Alum as an Adjuvant

The avidity rate indicates the strength and stability of antibody-antigen binding, and it was recorded at calving after the 3 vaccine doses. The results in Figure 3 show that cows produced IgG1 anti-recombinant RBD antibodies with significantly higher avidity in serum than in colostrum ($P < 0.01$; Mann-Whitney test; Figure 3B). Surprisingly, cows vaccinated with the Alum-based formulation

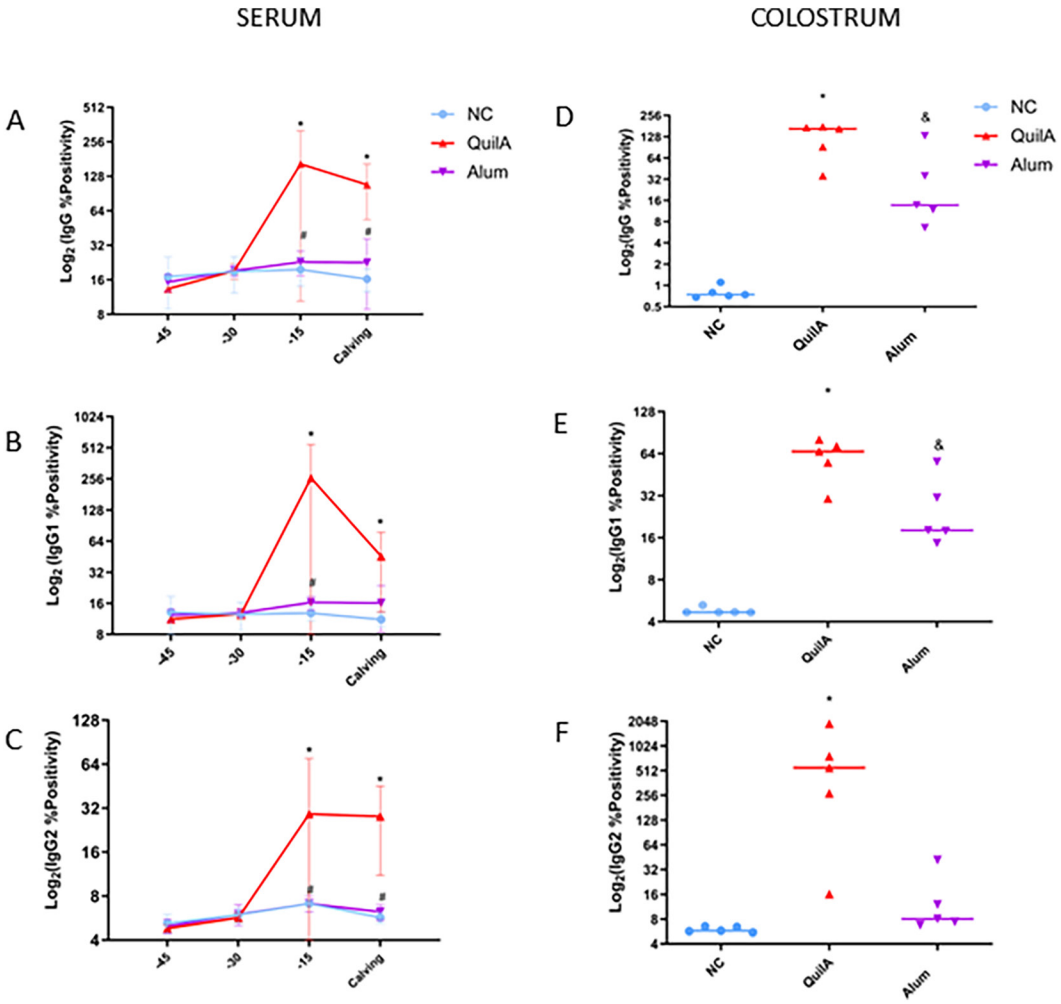

Figure 2. Levels of IgG, IgG1, and IgG2 antibodies against recombinant S-RBD induced by vaccine formulations with QuilA or Alum as an adjuvant. Indirect ELISA to detect IgG, IgG1 and IgG2 in serum (A, B, and C, respectively) and colostrum (D, E, and F, respectively). Samples were collected from cows immediately before inoculation with saline solution (control group; NC; n = 5) and vaccines containing recombinant SARS-CoV2 RBD formulated with QuilA (n = 5) and InjectAlumen (Alum; n = 5). Measurements were taken on d 45, 30, and 15 before giving birth and on the day of calving. Data are expressed as $\log_2$(mean ± SD of the positivity [%]). Differences were considered significant at $P < 0.05$ (Mann-Whitney test). * Indicates comparison between Alum and NC groups; # indicates comparison between QuilA and NC groups; & indicates comparison between QuilA and Alum groups.

presented higher anti-recombinant RBD IgG2 avidity in serum ($P < 0.05$; Mann-Whitney test; Figure 4C).

Viral Neutralization Rates in Serum and Colostrum from Cows Vaccinated with Recombinant S-RBD Formulated with QuilA or Alum as an Adjuvant

The viral neutralization results are shown in Figure 4. In serum (Figure 4A), the QuilA group exhibited significantly higher percent of neutralization rates (83.18 ± 26.44%) than both the NC group (0.77 ± 0.03%; $P < 0.05$) and the Alum group (43.63 ± 20.26%; $P < 0.05$). In colostrum (Figure 4B), both the QuilA (96.66 ± 1.67%) and Alum (91.86 ± 7.57%) groups showed significantly higher percent of neutralization activity than the NC group (−8.91 ± 1.2%; $P < 0.05$, Mann-Whitney test).

DISCUSSION

The ongoing global health challenges exposed by emerging viral pathogens, such as SARS-CoV-2, highlight the urgent need for advancements in vaccine development and passive immunity strategies. Bovine colostrum has gained importance as a potential source of neutralizing antibodies to prevent viral infections in humans (Kangro et al., 2022; Nili et al., 2022; Duman and Karav, 2023) due to the immunoglobulin evolutionary homology (Kacskovics, 2004). Nevertheless, cow vac-

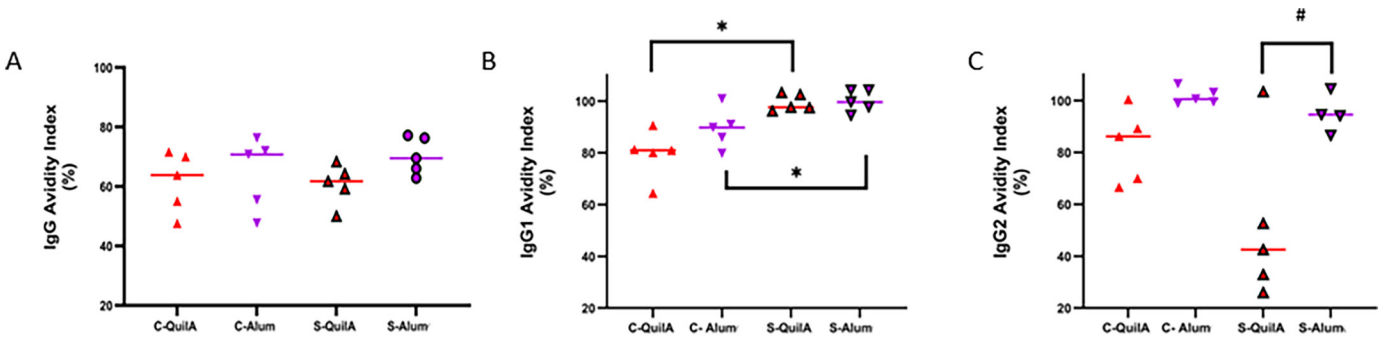

Figure 3. Avidity index of antibodies produced in serum and colostrum from cows immunized with the recombinant S-RBD. A modified ELISA protocol was used to evaluate the avidity index (%) of (A) IgG, (B) IgG1, and (C) IgG2. Serum (S) and colostrum (C) were collected on the day of calving after 3 doses of the vaccine formulations based on QuilA or Alum as an adjuvant were administered. Bars represent the mean avidity index calculated for each group. Differences were considered significant at $P < 0.05$ (Tukey test followed by Sidak correction). * Indicates comparison between serum and colostrum; # indicates comparison between QuilA and Alum groups.

cination protocols need to be improved to modulate the production of specific antibody subclasses, particularly IgG1 and IgG2, which play a central role in combating viral infections and activating the complement system (Morales-Núñez et al., 2021). Indeed, this study aimed to address these gaps by evaluating the efficacy of recombinant RBD protein-based vaccines formulated with different adjuvants (QuilA or Alum) in inducing immune responses in cows, with a focus on antibody levels, avidity, and viral neutralization.

To ensure the minimum divergence caused by different batches of antigen or by cow genetics and management on hyperimmune colostrum production, we successfully expressed and purified the recombinant RBD protein using the Expi293F expression system. The high yields and purity obtained through nickel-nitrilotriacetic acid (**Ni-NTA**) affinity and size-exclusion chromatography were confirmed via SDS-PAGE and western blotting, where the expected 30-kDa bands were observed with minimal nonspecific signals. This level of protein purity is essential for generating reliable data in subsequent immunological assays and demonstrates the scalability and efficacy of this production platform. Considering the known influences of cow breed, number of pregnancies, and milk production on colostrum quality and quantity (Shivley et al., 2018; Moretti et al., 2020; Soufleri et al., 2021), these factors were taken into account to set up experimental groups. Recent literature has also shown that cows vaccinated during the late-gestation period produce greater and more consistent levels of specific antibodies in colostrum (Reppert et al., 2019), justifying our choice to vaccinate during this period with a short interval between immunizations. The dose of recombinant S-RBD used for immunization was also based on the literature, with levels varying between 50 and 200 µg per dose (Kangro et al., 2022; Nili et al., 2022; Jacobson et al., 2023).

The immunogenicity results showed that QuilA, a saponin-based adjuvant, significantly enhanced the production of total IgG and both subclass IgG1 and IgG2 antibodies in serum and colostrum compared with Alum and the control. This finding is consistent with the known efficacy of QuilA in stimulating humoral immunity through the activation of both T-cell-dependent and T-cell-independent pathways (Cibulski et al., 2018; Fleck et al., 2019). The marked increase in antibody levels following the second inoculation, particularly in the QuilA group, highlights its potential for inducing sustained and robust immune responses. The superior performance of QuilA, even though antibody levels decreased slightly by the time of calving, underscores its role as a potent adjuvant that can significantly improve vaccine efficacy.

The avidity assays revealed important insights into the maturity of the antibodies produced. Avidity, which reflects the strength of antigen-antibody binding, is a critical factor in determining the efficacy of neutralizing antibodies and can be influenced by adjuvants and the number of booster doses in vaccination protocols (Budroni et al., 2021; Tang et al., 2022). Moreover, a relation between IgG avidity and SARS-Cov-2 viral neutralization has already been demonstrated (Gaspar and De Gaspari, 2021). Although the avidity of anti-RBD IgG1 was higher in serum than in colostrum, the mean avidity in colostrum was considered high (above 50%) for all subclasses (Chackerian et al., 2001). This indicates that the systemic immune response triggered by the vaccine formulations utilizing colostrum as a source of antibodies was both robust and effective for passive immune transfer (Chackerian et al., 2001). Interestingly, cows vaccinated with the Alum-based formulation showed similar levels of antibody avidity compared with those in the QuilA group and significantly higher IgG2 avidity in serum despite lower antibody titers. This indicates that Alum-based adjuvants may enhance

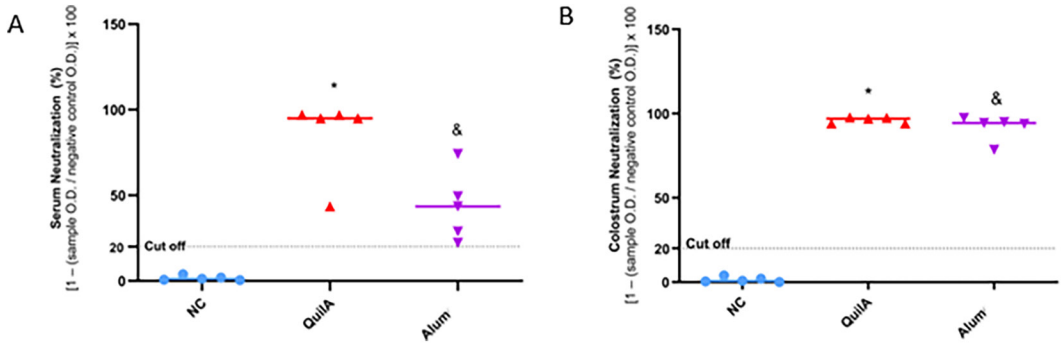

Figure 4. Viral neutralization rates in serum and colostrum from cows vaccinated with recombinant S-RBD formulated with QuilA or Alum as an adjuvant. Viral neutralization (%) determined using the cPass SARS-CoV-2 Neutralization Antibody Detection Competitive ELISA Kit (NL Diagnostica) with (A) serum and (B) colostrum from cows inoculated with saline solution (control group; NC; n = 5) or vaccines containing recombinant SARS-CoV2 RBD formulated with QuilA (n = 5) or InjectAlumen (Alum; n = 5). Measurements were taken from samples collected on the day of calving. Viral rate neutralization data were calculated according to the manufacturer's instructions. Bars represent the mean viral neutralization rate calculated for each group. Differences were considered significant at $P < 0.05$ (Mann-Whitney test). * Indicates comparison between QuilA and NC groups; & indicates comparison between QuilA and Alum groups. O.D. = optical density at 492 nm.

the efficiency of IgG2-neutralizing antibody subclasses, even when the overall quantity of these molecules is lower than that produced by QuilA-based formulations. Because the ability of Alum to elicit high-quality immune responses is widely known (He et al., 2015), it might be a valuable adjuvant for improving specific long-term immunity in livestock.

Finally, the viral neutralization assay results emphasize the potency of QuilA as an adjuvant for producing hyperimmune bovine colostrum, as the SARS-CoV-2 neutralization rate was significantly higher in serum, whereas the colostrum from cows immunized with Alum presented a similar neutralization index with lower overall antibody titers. Although our study did not analyze IgA antibodies, which are present in high amounts in bovine colostrum and are known to play a critical role in viral neutralization, especially against SARS-CoV2 (Stelwagen et al., 2009; Cakebread et al., 2015; Sterlin et al., 2021) it is possible that the Alum-based formulation induced high levels of IgA, contributing to the neutralization activity observed. Future studies will be conducted to evaluate this issue. Although bovine IgG shows evolutionary homology to human IgG, its interaction with human Fc receptors is limited (Kacskovics, 2004). The large-scale bovine IgG production from hyperimmune colostrum further supports its use for passive immunity control. Bovine IgG is abundant and safe and can provided localized immunity in the gastrointestinal and oropharyngeal mucosa to reduce viral loads and transmission risk. Although its systemic action may be limited due to weak interaction with human Fc receptors, its localized effects make it effective for prophylaxis against mucosal infections such as those caused by SARS-CoV-2 (Kangro et al., 2022; Nili et al., 2022). It is noteworthy that bovine colostrum contains many immunomodulatory and antimicrobial molecules that are useful for humans and might play essential roles in health and diseases (Guberti et al., 2021; Duan et al., 2024). The use of adjuvant combinations with the same antigen in a prime-boost protocol using QuilA followed by boosts using Alum might enhance antibody avidity and neutralization efficacy and be valuable for producing hyperimmune colostrum to treat or prevent COVID-19.

CONCLUSIONS

The immune response elicited by vaccine formulations against SARS-CoV-2 containing QuilA or Alum as an adjuvant in pregnant cows modulated the production, avidity, and neutralization index of antibodies in both serum and colostrum. This research contributes to the broader field of livestock immunization by emphasizing the importance of selecting adjuvants that enhance antibody production and improve the quality of hyperimmune colostrum production to prevent COVID-19 or future emerging and re-emerging viral diseases.

NOTES

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Nonstandard abbreviations used: HRP = horseradish peroxidase; MW = molecular weight; NC = negative control; Ni-NTA = nickel-nitrilotriacetic acid; OD = optical density; RBD = receptor-binding domain (of the SARS-CoV-2 spike protein); S-RBD = recombinant spike receptor-binding domain (RBD) from SARS-CoV-2.

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