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Bovine-associated staphylococci and mammaliicocci trigger T-lymphocyte proliferative response and cytokine production differently

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ABSTRACT

We examined whether distinct staphylococcal and mammaliicoccal species and strains trigger B- and T-lymphocyte proliferation and interleukin (IL)-17A and interferon (IFN)-γ production by peripheral blood mononuclear cells in nulliparous, primiparous, and multiparous dairy cows. Flow cytometry was used to measure lymphocyte proliferation with the Ki67 antibody, and specific monoclonal antibodies were used to identify CD3, CD4, and CD8 T lymphocyte and CD21 B lymphocyte populations. The supernatant of the peripheral blood mononuclear cell culture was used to measure IL-17A and IFN-γ production. Two distinct, inactivated strains of bovine-associated *Staphylococcus aureus* [one causing a persistent intramammary infection (IMI) and the other from the nose], 2 inactivated *Staphylococcus chromogenes* strains [one causing an IMI and the other from a teat apex), as well as an inactivated *Mammaliicoccus fleurettii* strain originating from sawdust from a dairy farm, and the mitogens concanavalin A and phytohemagglutinin M-form (both specifically to measure lymphocyte proliferation) were studied. In contrast to the "commensal" *Staph. aureus* strain originating from the nose, the *Staph. aureus* strain causing a persistent IMI triggered proliferation of $CD4^+$ and $CD8^+$ subpopulations of T lymphocytes. The *M. fleurettii* strain and the 2 *Staph. chromogenes* strains had no effect on T- or B-cell proliferation. Furthermore, both *Staph. aureus* and *Staph. chromogenes* strains causing persistent IMI significantly increased IL-17A and IFN-γ production by peripheral blood mononuclear cells. Overall, multiparous cows tended to have a higher B-lymphocyte and a lower T-lymphocyte proliferative response than primiparous and nulliparous cows. Peripheral blood mononuclear cells of multiparous cows also produced significantly more IL-17A and IFN-γ. In contrast to concanavalin A, phytohemagglutinin M-form selectively stimulated T-cell proliferation.
Key words: mastitis, *Staphylococcus aureus*, non-*aureus* staphylococci, *Mammaliicoccus*, lymphocyte proliferation

INTRODUCTION

Staphylococcus and *Mammaliicoccus* (formerly *Staphylococcus*; Madhaiyan et al., 2020) species are part of the skin microbiota of mammals and have been isolated from various body sites of dairy cows and from the dairy environment. They are also the most common cause of bovine IMI (De Visscher et al., 2016; Leuenberger et al., 2019; Wuytack et al., 2019). Among the genus *Staphylococcus*, *Staphylococcus aureus* is considered a major mastitis pathogen that represents a real threat to bovine udder health (Rainard et al., 2018). Nonetheless, the impact of *Staph. aureus* markedly depends on the genotype, as adapted to a particular host and even to a particular body site (Leuenberger et al., 2019).

Bovine non-*aureus* staphylococci and mammaliicocci (**NASM**) are a heterogeneous group of bacteria that cover a broad range of ecological habitats, varying from primarily the environment of dairy cows (e.g., *Mammaliicoccus fleurettii*) to the cow's udder (e.g., *Staphylococcus chromogenes*; De Visscher et al., 2014; Vanderhaeghen et al., 2014; De Buck et al., 2021). The significance of NASM for bovine udder health has come

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under attention because of the vast range of species and strain-specific differences in virulence and host interactions (Souza et al., 2016, 2022b; Piccart et al., 2016). Previous studies regarded NASM as true, yet minor mastitis pathogens that can cause both subclinical and mild clinical mastitis (Supré et al., 2011; Piessens et al., 2012; De Buck et al., 2021) but they usually have limited effects on milk SCC and milk quality and often no effect on milk yield (Tomazi et al., 2015; Valckenier et al., 2020). Others have classified NASM as part of a commensal microbiota of the bovine mammary gland or teat apices with potential protective effects against mastitis caused by major mastitis bacteria (Piepers et al., 2010; Isaac et al., 2017; Toledo-Silva et al., 2021a).

There is a gap in our understanding of how distinct staphylococcal and mammaliicoccal species and strains orchestrate the bovine immune response and the underlying, potentially parity-related mechanisms, in the clearance of mastitis pathogens. T and B lymphocytes play a central role in cellular and humoral immunity of the mammary gland, as they are capable of eliminating pathogens through direct cytotoxicity, producing antibodies and cytokines, activating macrophages, or recruiting neutrophils. For instance, lymphocytes produce critical cytokines such as IL-17A and IFN-γ that promote the defense of the bovine mammary gland (Ezzat Alnakip et al., 2014; Rainard et al., 2020).

Thus, we investigated (1) the in vitro blood-derived B- and T-lymphocyte proliferative responses, and (2) the production of IL-17A and IFN-γ by peripheral blood mononuclear cells (**PBMC**) stimulated by distinct staphylococcal and mammaliicoccal strains in nulliparous, primiparous, and multiparous dairy cows.

MATERIALS AND METHODS

This study was approved by the Animal Research Ethics Committee of the Faculty of Veterinary Medicine and Animal Science, University of São Paulo, Brazil (protocol number: 8609270815).

Bacterial Strains and Growth Conditions

In the study, we used 2 distinct strains of *Staph. aureus* (Supplemental Table S1, https://doi.org/10.5281/zenodo.7215435; Souza et al., 2022a), the first isolated from a persistent subclinical IMI (*spa* type t605; Cunha et al., 2020; Santos et al., 2020) and the other isolated from a nose (*spa* type t089; Santos et al., 2020). The same NASM strains (Supplemental Table S1) as we used previously (Breyne et al., 2015; Piccart et al., 2016; Souza et al., 2016; Toledo-Silva et al., 2021a,b, 2022) were included in the present experiments: 2 dissimilar *Staph. chromogenes* strains ("IM" and "TA") and 1 strain of *Mammaliicoccus fleurettii* (**MF**). The *Staph. chromogenes* "TA" isolate was cultured from a heifer's teat apex and has in vitro inhibitory effects against *Staph. aureus*, *Streptococcus uberis*, and *Streptococcus dysgalactiae* ("C2" in De Vliegher et al., 2004), whereas the other *Staph. chromogenes* strain ("IM"; multilocus sequence type 1, "Chromo-MAS" in Huebner et al., 2021) originated from a persistent IMI (Supré et al., 2011) capable of colonizing quarters of dry quarters (Beuckelaere et al., 2021). The so-called environmental MF strain was isolated from sawdust on a dairy farm (Piessens et al., 2011).

The bacterial isolates were kept at −80°C and then thawed at 37°C and cultivated on 5% sheep blood agar at the start of the experiments. Subsequently, fresh colonies from each isolate were grown overnight in 37°C brain-heart infusion (**BHI**) broth. Afterward, all isolated were diluted 1:1,000 in new BHI broth and cultivated until late exponential growth. Then, the bacterial broth was centrifuged and washed twice with sterile saline solution (0.9% NaCl). The isolates were then resuspended in RPMI-1640 (R7638, Sigma-Aldrich). Using 10-fold serial dilutions, 10 μL of each diluted isolate sample was poured onto BHI agar until live bacteria were counted reliably to determine the number of colony-forming units. After 60 min at 60°C, the bacteria were heat-killed, and inactivation was confirmed by pouring 100 μL of each bacterial inoculum on 5% sheep blood agar (Souza et al., 2016), which was validated by the absence of bacterial growth.

Dairy Cows and Sampling. Peripheral blood samples (approximately 40 mL per cow) were taken aseptically in heparin tubes from 18 clinically healthy Holstein cows and heifers at the dairy farm of the University of São Paulo (Pirassununga, Brazil). These animals were divided as follows: 6 nulliparous (age: 17.65 + 0.36 mo; BW: 434.2 + 20.71 kg), 6 mid-lactating primiparous (age: 33.16 + 2.85 mo; BW: 599.7 + 13.04 kg; milk yield: 24.58 + 0.70 kg; DIM: 156.8 + 15.48 d; milk SCC: 79,820 + 16,460 cells mL^{-1}), and 6 mid-lactating multiparous (age: 58.64 + 4.63 mo; BW: 685.7 + 15.01 kg; milk yield: 31.58 + 1.88 kg; DIM: 141.2 + 10.31 d; milk SCC: 91,000 + 24,490 cells mL^{-1}; parity: 2.83 + 0.54) dairy cows. Subsequently, the blood samples were coded and randomized, and further analyses were carried out in which the researcher was blinded to the animal parity of the animals from which the samples was collected.

Isolation of PBMC. For lymphocyte proliferation, the PBMC were isolated using Ficoll-Paque Plus (density gradient 1.077 g mL^{-1}; cat. no. 17-1440-03, GE Healthcare) following the manufacturer's instruc-

tions. The PBMC were then placed in 1 mL of proliferation medium composed of RPMI-1640 nutrition medium (R7638, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal calf serum (**FCS**; F9665, Sigma-Aldrich), 5×10^{-2} m*M* 2-mercaptoethanol (cat. no. 21985-023, Invitrogen), GlutaMAX supplement (cat. no. 35050-061, GIBCO/Life Technologies), and antibiotic-antimycotic solution (cat. no. 15240-062, Life Technologies). Cell viability was determined by trypan blue exclusion (cat. no. T8154, Sigma-Aldrich), which was always >98%, and PBMC were counted in a Neubauer chamber and their concentration was adjusted to 2.2×10^6 viable cells mL^{-1}.

PBMC Culture. The PBMC (2×10^5 cells per well) were cultured in triplicate wells of 96-well culture plates for 72 h at 37°C in a 5% CO_2 incubator in the presence of different potential mitogens: (1) concanavalin A type IV-S (**Con-A**, C0412, Sigma-Aldrich; 10 μg mL^{-1}), (2) phytohemagglutinin M-form (**PHA-M**, cat. no. 10576015, GIBCO/Life Technologies) with (3) the 5 heat-killed staphylococcal and mammaliicoccal strains (multiplicity of infection = 10; Alekseeva et al., 2013; Santos et al., 2021), or (4) without stimuli (baseline, unstimulated control cells), respectively. After 72 h, the cells were harvest and centrifuged at 250 × *g* for 8 min at room temperature, and the supernatant were collected and stored at −80°C for further processing for measurement of cytokines (i.e., IFN-γ and IL-I7-A).

Proliferation Measurement and Identification of Lymphocyte Subpopulations. After PBMC culture, the cells were centrifuged at 250 × *g* for 8 min. Then, the cells were resuspended in 100 μL of PBS supplemented with 1% (vol/vol) heat-inactivated FCS and incubated for 30 min with a mix of the following primary mAb: CD3, CD4, CD8, and CD21. The following combinations of primary mAb against bovine lymphocytes were used: (1) mouse IgG1 anti-bovine CD3 (BOV2009; T lymphocytes, Washington State University), (2) mouse IgG2a anti-bovine CD4 (BOV2010; Washington State University), and (3) mouse anti-bovine CD8 conjugated with phycoerythrin (cat. no. MCA837, Bio-Rad); and (4) mouse anti-bovine CD21 conjugated with phycoerythrin (B lymphocytes; cat. no. MCA1424, Bio-Rad). After incubation, 1 mL of PBS was added to the cell suspensions and centrifuged at 250 × *g* for 8 min. Then, cells were incubated for 30 min with the labeled secondary antibodies (Abs) resuspended in PBS with 1% (vol/vol) FCS. The following secondary Abs were used: (1) goat anti-mouse IgG1 conjugated with allophycocyanin (cat. no. A10541, Invitrogen) and (2) goat anti-mouse IgG2a conjugated with Brilliant violet 421 (BD Horizon). After incubation, 1 mL of PBS was added to the cell suspension and centrifuged at 250 × *g* for 8 min. Afterward, the cells were fixed with paraformaldehyde and permeabilized using a permeabilization solution (0.1% saponin in PBS with 1% FCS and 0.09% sodium azide). Then, the samples were incubated for 1 h at 4°C with 10 μL of anti-Ki67 antibody (diluted 1/100 in PBS with 1% FCS and 0.09% sodium azide; cat. no. ab15580, Abcam) to quantify lymphocyte proliferation (Soares et al., 2010; Lašťovička et al., 2016; Santos et al., 2021). After incubation, 1 mL of permeabilization solution was added to the cell suspension and centrifuged at 250 × *g* for 8 min. Next, the cells were incubated for 30 min with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (1/100 in PBS with 1% FCS and 0.09% sodium azide; cat. no. ab6717, Abcam). Finally, 1 mL of permeabilization solution was added to the cell suspension and centrifuged at 250 × *g* for 8 min resuspended in PBS with 1% (vol/vol) FCS, and resuspended in 300 μL of PBS and analyzed by flow cytometry (BD FACS Aria II, Becton Dickinson Immunocytometry System). Here, 20,000 cells, excluding most of the cellular debris, were analyzed. An unstained control and single-stained samples were also prepared as compensation controls. Negative control samples were stained with conjugated isotype control Abs. In addition, cells were stained with fluorescence minus one (FMO) controls. Doublets were excluded using forward scatter (**FSC**) area versus FSC height. Our gating strategy analysis for B- and T-lymphocyte subsets is illustrated in Figure 1; the obtained data (percentage of $ki67^+$ cells) were analyzed with FlowJo software (TreeStar Inc.).

IL-17A and IFN-γ Quantification. Quantification of IL-17A (DIY0673B-003, Kingfisher Biotech Inc.) and IFN-γ (KBC1231, ThermoFisher Scientific Inc.) from the supernatant of the cell cultures was performed using commercial bovine ELISA kits, using the protocol supplied by the manufacturers.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9.4 (GraphPad Software Inc.). First, the outcome variables were tested for normality of the distribution using the Shapiro-Wilk test. Normally distributed data were subjected to 2-way ANOVA followed by Tukey's test (concentrations of IFN-γ). Outcome variables that were not normally distributed ($CD3^+$, $CD4^+$, $CD8^+$ T-lymphocyte and $CD21^+$ B-lymphocyte proliferation; IL-17A) were analyzed using the Kruskal-Wallis test followed by the Student-Newman-Keuls test. The interaction terms between parity and the stimuli (in-

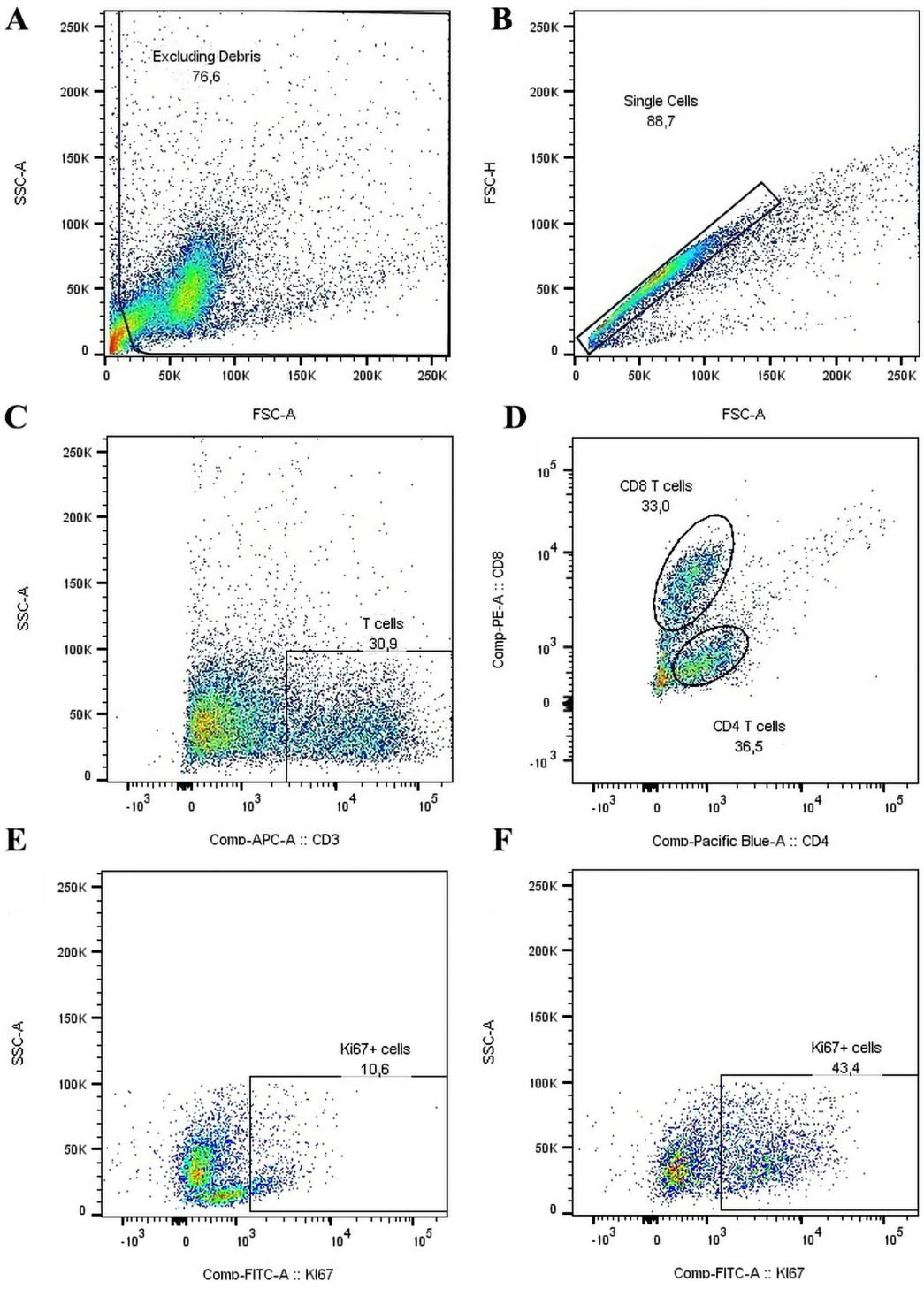

Figure 1. Successive gating scheme for identifying the $CD3^+$ T-lymphocyte subset. First, most debris was excluded (A). Subsequently, doublets were excluded using forward scatter area (FSC-A) versus FSC height (FSC-H; B). Then, $CD3^+$ lymphocytes were analyzed based on their cytoplasmic granularity and mean fluorescence intensity following a 2-step fluorescent immunolabeling protocol using primary anti-bovine mAb specific for T-cell identification (CD3) and secondary Abs coupled to long-wavelength fluorescent probe (APC). The recordings of the side angle light scatter (SSC-H) and fluorescent properties (APC) for $CD3^+$-gated cells (T lymphocytes) were determined (C). Then, the T-lymphocyte subsets were divided according to their expression of CD8 (phycoerythrin, PE) and CD4 (Brilliant violet 421, BV421) molecules (D). Finally, the expression of Ki-67 was quantified in unstimulated (E) and stimulated (F; concanavalin A type IV) samples. An unstained control and single-stained samples were also prepared as compensation controls. Negative control samples were also stained with conjugated isotype control antibodies. In addition, cells were stained with fluorescence minus one (FMO) controls. SSC-A = side scatter area.

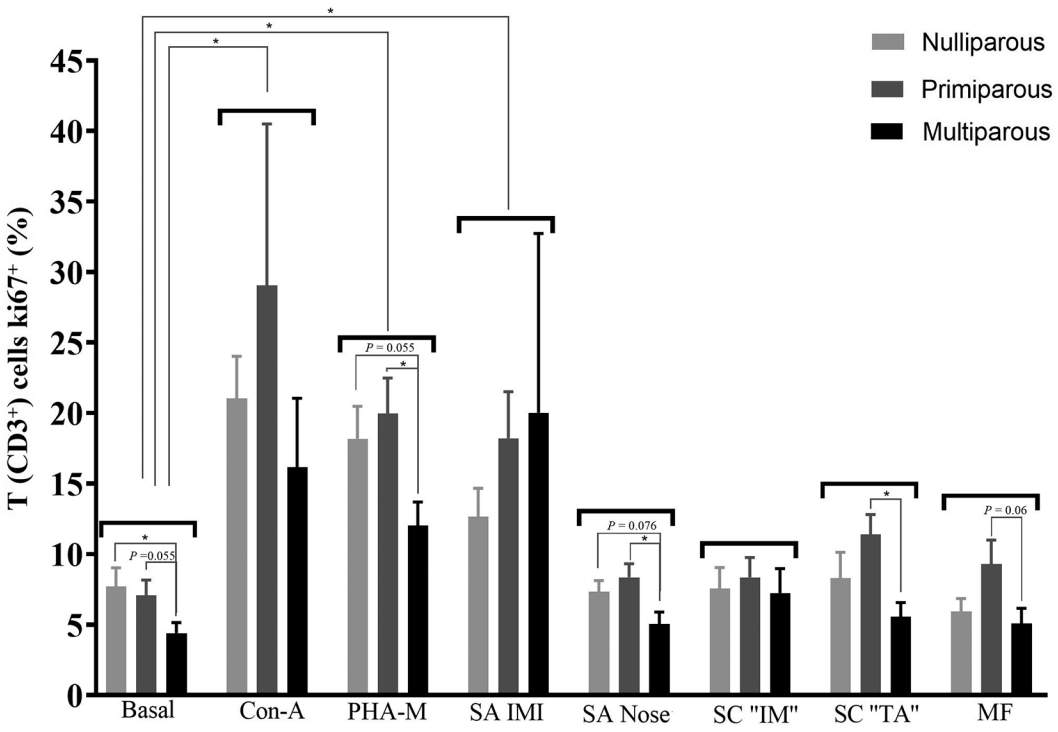

Figure 2. Blood T-lymphocyte ($CD3^+$) proliferation (median with interquartile range) after incubation of peripheral blood mononuclear cells with medium only (unstimulated basal), the mitogens concanavalin A (Con-A) and phytohemagglutinin M-form (PHA-M), and distinct staphylococcal and mammaliicoccal strains in nulliparous (n = 6), primiparous (n = 6), and multiparous (n = 6) dairy cows. SA IMI = *Staphylococcus aureus* isolated from a persistent IMI; SA Nose = *Staph. aureus* isolated from a nose; SC "IM" = *Staphylococcus chromogenes* isolated from a persistent IMI; SC "TA" = *Staph. chromogenes* isolated from the teat apex; and MF = *Mammaliicoccus fleurettii* (MF) isolated from sawdust. $ki67^+$ indicates positive staining for Ki67 antibody. $^*P < 0.05$.

activated isolates and Con-A and PHA-M) were never significant. $P \leq 0.05$ was considered significant.

RESULTS

T-Lymphocyte Proliferation

In the present study, PHA-M behaved as a specific mitogen for T lymphocytes ($CD3^+$), whereas Con-A was mitogenic for both T ($CD3^+$) and B ($CD21^+$) lymphocytes. The $CD3^+$, $CD4^+$, and $CD8^+$ T-lymphocyte proliferation stimulated with Con-A, PHA-M, and *Staph. aureus* isolated from a persistent IMI was higher than under the baseline condition (no stimulus, control; Figures 2, 3, and 4). No significant difference was observed in $CD3^+$, $CD4^+$, or $CD8^+$ T-lymphocyte proliferation between the other staphylococcal and mammaliicoccal species and strains and the basal control (Figures 2, 3, and 4).

The overall proliferative response of $CD3^+$ $CD4^+$, and $CD8^+$ T-lymphocyte subsets were higher in primiparous and nulliparous cows than in multiparous cows (Figures 2, 3, and 4).

B-Lymphocyte Proliferation

In the present study, Con-A was the only stimulus that led to greater B-cell proliferation (Figure 5). Multiparous cows had a higher B-lymphoproliferative response than primiparous ($P < 0.0001$) and nulliparous ($P = 0.0007$) dairy cows. Furthermore, nulliparous dairy cows had higher B-lymphocyte proliferation than primiparous cows ($P < 0.0001$).

IL-17A and IFN-γ Production

Production of IL-17A by lymphocytes was higher under *Staph. aureus* originating from a persistent IMI and *Staph. chromogenes* IM (Figure 6) compared with the unstimulated values. Higher levels of IL-17A were found in multiparous cows than in primiparous ($P = 0.016$) and nulliparous ($P = 0.0007$) cows, for all stimuli (Figure 6).

Production of IFN-γ by lymphocytes was higher than that of unstimulated control when stimulated with *Staph. aureus* originating from an IMI and *Staph. chromogenes* IM (Figure 7). Higher levels of IFN-γ were

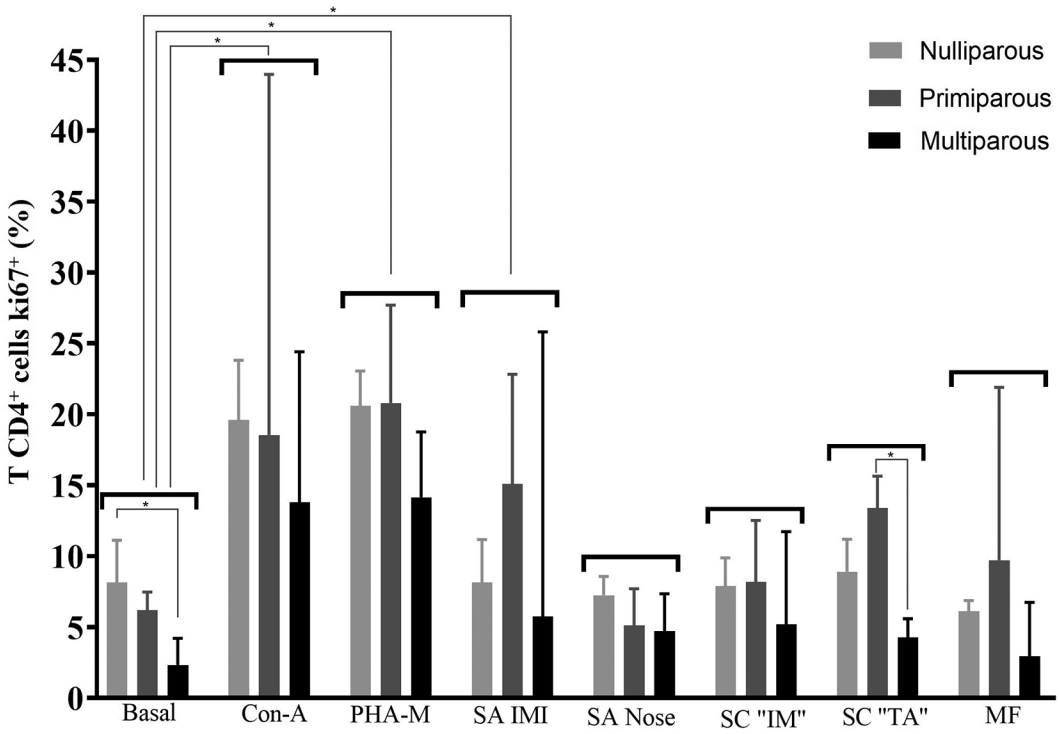

Figure 3. Blood T-lymphocyte ($CD4^+$) proliferation (median with interquartile range) after incubation of peripheral blood mononuclear cells with medium only (unstimulated basal), the mitogens concanavalin A (Con-A) and phytohemagglutinin M-form (PHA-M) and distinct staphylococci species and strains in nulliparous (n = 6), primiparous (n = 6), and multiparous (n = 6) dairy cows. SA IMI = *Staphylococcus aureus* isolated from a persistent IMI; SA Nose = *Staph. aureus* isolated from a nose; SC "IM" = *Staphylococcus chromogenes* isolated from a persistent IMI; SC "TA" = *Staph. chromogenes* isolated from the teat apex; and MF = *Mammaliicoccus fleurettii* (MF) isolated from sawdust. $ki67^+$ indicates positive staining for Ki67 antibody. $^*P < 0.05$.

found in multiparous cows than in nulliparous ($P = 0.002$) and primiparous ($P = 0.075$) cows, for all stimuli.

DISCUSSION

In vitro lymphocyte proliferation in response to antigen-specific or nonspecific stimulation is a widely used approach to test for immunological memory and general immune function. During a proper immune response, lymphocyte proliferation is the first step toward the development of effector and memory lymphocytes, both of which are necessary to eliminate a current antigen and ensure that future responses to an antigen are faster and stronger than the first encounter with the antigen (Desforges et al., 2016). In the present study, we evaluated blood lymphocyte proliferation using Ki67 expression because it showed comparable results to lymphocyte proliferation evaluated using carboxyfluorescein succinimidyl ester but was more robust and sensitive (Lašťovička et al., 2016). Apart from one study that investigated the mitogen-induced blood T-lymphocyte proliferative capacity in primiparous and multiparous dairy cows (Mehrzad and Zhao, 2008), no study has investigated B- and T-lymphoproliferative responses using distinct staphylococcal and mammaliicoccal strains.

Interactions between distinct staphylococcal and mammaliicoccal species and strains and dairy cows' mammary gland immunity remain widely uncharacterized, although these interactions could dictate the course of the disease. Here, we found a marked variation in T-lymphocyte proliferation between inactivated *Staph. aureus* originating from a persistent IMI, a true major mastitis pathogen, and a "commensal" *Staph. aureus* strain originating from the nose. In agreement with our results, previous studies have shown that the immune response to *Staph. aureus* is genotype-dependent (Pereyra et al., 2017; Murphy et al., 2019; Niedziela et al., 2021). In fact, the IMI-associated *Staph. aureus* was the only isolate that induced proliferation of T lymphocytes, including the $CD4^+$ and $CD8^+$ subpopulations.

It is conceivable that the ancient co-evolution of hundreds of microorganisms and the host immune system will lead to the development of sophisticated immune controls that can distinguish between pathogenic bacteria and beneficial commensal microorganisms (Sriniva-

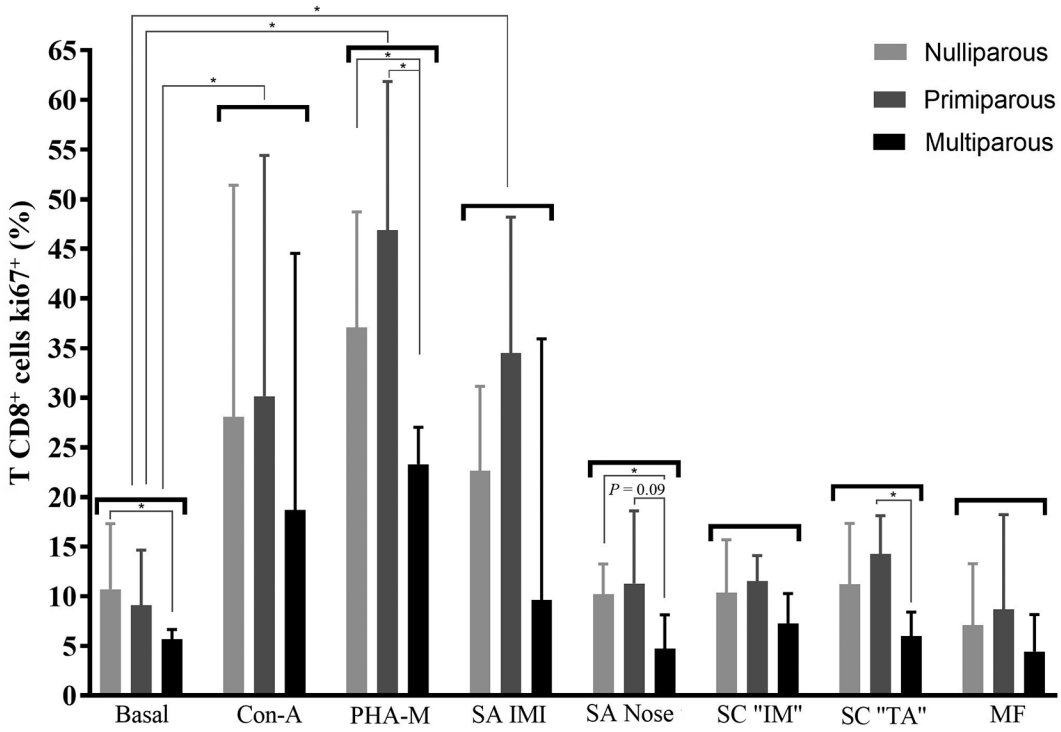

Figure 4. Blood T-lymphocyte ($CD8^+$) proliferation (median with interquartile range) after incubation of peripheral blood mononuclear cells with medium only (unstimulated basal), the mitogens concanavalin A (Con-A) and phytohemagglutinin M-form (PHA-M) and distinct staphylococci species and strains in nulliparous (n = 6), primiparous (n = 6), and multiparous (n = 6) dairy cows. SA IMI = *Staphylococcus aureus* isolated from a persistent IMI; SA Nose = *Staph. aureus* isolated from a nose; SC "IM" = *Staphylococcus chromogenes* isolated from a persistent IMI; SC "TA" = *Staph. chromogenes* isolated from the teat apex; and MF = *Mammaliicoccus fleurettii* (MF) isolated from sawdust. $ki67^+$ indicates positive staining for Ki67 antibody. $^*P < 0.05$.

san, 2010; Littman and Pamer, 2011). In this scenario, it is widely accepted that commensal bacteria build up mechanisms to maintain immunological tolerance, such as by sustaining regulatory T lymphocytes (Littman and Pamer, 2011). For instance, levels of T regulatory cells coincide with *Staphylococcus epidermidis* skin colonization in humans (Parlet et al., 2019; Lunjani et al., 2021). In this context, there is growing evidence that a core bovine mammary gland microbiome exists, in which staphylococci emerge as a protagonist (Porcellato et al., 2020). Thus, we speculate that the commensal *Staph. aureus* strain and the NASM, also potentially assigned as commensals, could endow immunological tolerance. In contrast to the IMI-associated *Staph. aureus*, they promote commensal-specific regulatory T lymphocytes that, in response, suppress T-lymphocyte proliferation (Dowling et al., 2018). Indeed, the 3 NASM strains included in this work induced only minimal or nondetectable levels of IL-1β, IL-8, and tumor necrosis factor-α in milk after an experimental challenge in dairy heifers (Piccart et al., 2016).

The supernatant of the *Staph. aureus* strain originating from a persistent IMI had higher levels of IL-17A and IFN-γ. In line with our findings, Zielinski et al. (2012) found that T lymphocytes stimulated with inactivated *Staph. aureus* in humans predominantly foster T helper ($\mathbf{T_H}$)1 and T_H17 lymphocyte subsets, and, to a lesser extent, T_H2 lymphocytes. Furthermore, Tebartz et al. (2015) showed that T lymphocytes proliferated vigorously when exposed to *Staph. aureus* ex vivo during acute infection. However, this response was abolished in the chronic phase, mainly due to myeloid-derived suppressor cells with a minor contribution from regulatory T lymphocytes. This response exerts a solid immunosuppressive effect in the later T-lymphocyte response, which endows the chronic infection status.

Apart from the 4 NASM strains, the *Staph. chromogenes* IM strain induced substantial production of IL-17A and IFN-γ, comparable to the *Staph. aureus* strain originating from the persistent IMI. In agreement with our findings, Beuckelaere et al. (2021) showed that quarters inoculated with and colonized by this particular IM strain resulted in higher concentrations of IFN-γ during the dry period and early lactation in dairy cows. We hypothesize that the signature cytokines triggered by this strain could reinforce bovine mammary

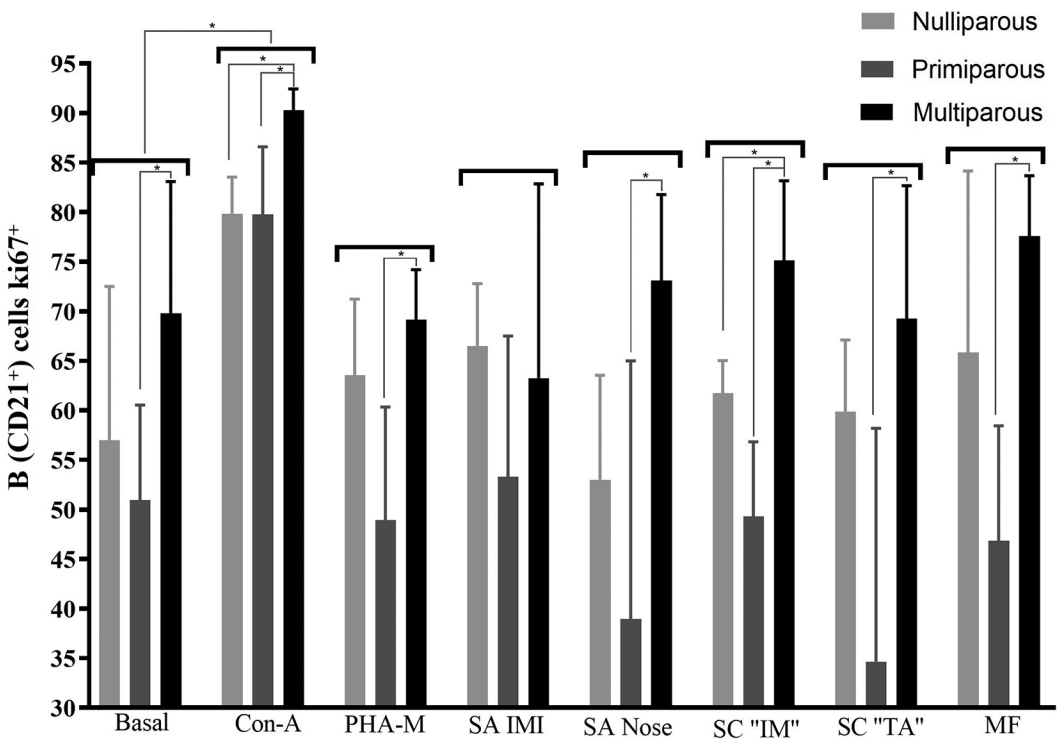

Figure 5. Blood B-lymphocyte ($CD21^+$) proliferation (median with interquartile range) after incubation of peripheral blood mononuclear cells with medium only (unstimulated basal), the mitogens concanavalin A (Con-A) and phytohemagglutinin M-form (PHA-M) and distinct staphylococci species and strains in nulliparous (n = 6), primiparous (n = 6), and multiparous (n = 6) dairy cows. SA IMI = *Staphylococcus aureus* isolated from a persistent IMI; SA Nose = *Staph. aureus* isolated from a nose; SC "IM" = *Staphylococcus chromogenes* isolated from a persistent IMI; SC "TA" = *Staph. chromogenes* isolated from the teat apex; and MF = *Mammaliicoccus fleurettii* (MF) isolated from sawdust. $ki67^+$ indicates positive staining for Ki67 antibody. $^*P < 0.05$.

gland defenses through immune training. The type 3 immunity is actually associated with IL-17A, which is marked by the recruitment of neutrophils, antigen-specific neutrophilic inflammation, and antimicrobial defenses triggered on the epithelial layer (Rainard et al., 2020). On the other hand, type 1 immunity is associated with IFN-γ, which induces cell-mediated immunity and activates phagocytes, improving their phagocytic and bactericidal activities (Ezzat Alnakip et al., 2014). Nonetheless, further longitudinal studies are needed to investigate whether (1) quarters colonized with the *Staph. chromogenes* IM strain sustain a mammary gland protective immunity by in vivo production of IFN-γ and especially IL-17A, or (2) myeloid-derived suppressor cells or regulatory T lymphocytes will override such a positive effect with negative implications of udder health (as is true for *Staph. aureus*).

In the current study, a lower T-lymphocyte proliferative capacity and a higher B-cell response was seen in multiparous dairy cows than in younger counterparts. In line with our findings, Mehrzad and Zhao (2008) reported that the proliferation of overall blood lymphocytes stimulated with Con-A, which they regarded as specific to T lymphocytes, was lower in multiparous than in primiparous dairy cows. Thus, we can hypothesize that immunosenescence could explain the differences between multiparous dairy cows and their younger counterparts and may reflect the capacity of the T lymphocytes to respond to stimuli, pathogens, and commensals. Furthermore, no difference in T-lymphocyte proliferation was identified between nulliparous (but nonlactating) and primiparous (lactating) dairy cows, indicating that lactation did not affect the T-proliferative responses of young dairy cows. Similar to our findings, Mehrzad and Zhao (2008) reported that primiparous dairy cows responded less to LPS, assumed to be a B-cell-specific mitogen. This could be attributed to the presence of primed and activated B lymphocytes in multiparous cows, which have been significantly more exposed to antigens over the course of their lifetime (Mehrzad and Zhao, 2008).

In addition, multiparous cows are prone to have a T_H17 and T_H1 response, which could explain, at least in part, why these animals have a lower incidence of clinical mastitis during early lactation than primiparous cows (De Vliegher et al., 2012), as bovine mammary

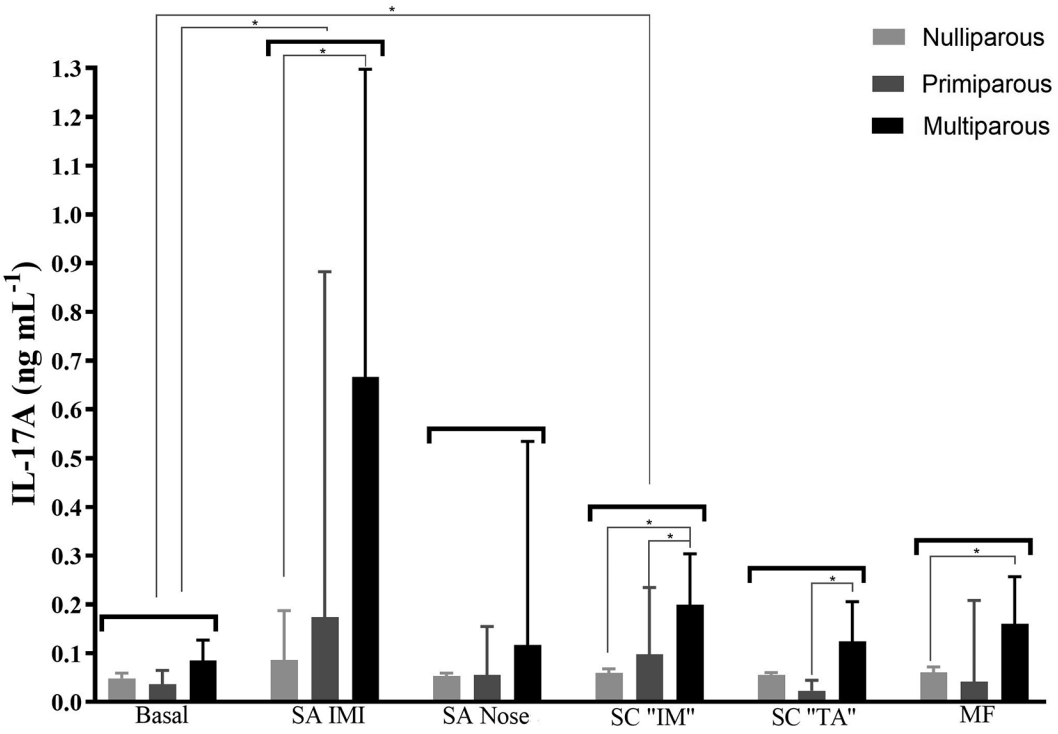

Figure 6. Concentration of IL-17A (ng mL^{-1}; median with interquartile range) of the supernatant of peripheral blood mononuclear cells after incubation with medium only (unstimulated basal) and the distinct staphylococci species and strains in nulliparous (n = 6), primiparous (n = 6), and multiparous (n = 6) dairy cows. SA IMI = *Staphylococcus aureus* isolated from a persistent IMI; SA Nose = *Staph. aureus* isolated from a nose; SC "IM" = *Staphylococcus chromogenes* isolated from a persistent IMI; SC "TA" = *Staph. chromogenes* isolated from the teat apex; and MF = *Mammaliicoccus fleurettii* (MF) isolated from sawdust. $^*P < 0.05$.

gland defense relies mainly on neutrophils and epithelial cells (Rainard et al., 2020). On the other hand, this neutrophilic and proinflammatory response may predispose multiparous cows to systemic rather than local clinical mastitis cases compared with primiparous dairy cows, as previously described (Van Werven et al., 1997; Mehrzad et al., 2009). Similar to our results, Ohtsuka et al. (2010) showed that the ratio of IFN-γ to IL-4 was significantly lower in colostrum mononuclear cells from primiparous cows than in third-calving dairy cows. Nevertheless, the IFN-γ and IL-17A concentrations in our samples were relatively low, so caution is warranted when interpreting our findings.

Although Con-A has been extensively used as a selective T-cell mitogen in human (Palacios, 1982; Weiss et al., 1987) and murine (Kanellopoulos et al., 1985) lymphocyte proliferative studies, we demonstrated that Con-A did not behave as a selective T-cell mitogen in dairy cows, as initially presumed (Mehrzad and Zhao, 2008; Erskine et al., 2011; Nieto Farias et al., 2018). Nonetheless, our studies confirmed that PHA-M is a mitogen that only affects T lymphocytes (Kanellopoulos et al., 1985).

CONCLUSIONS

In this study, we demonstrated a notable difference in blood T-lymphocyte proliferation among a pathogenic *Staph. aureus* strain, a "commensal" *Staph. aureus* strain, and well-studied NASM strains. This provides us with new insights into the complex host-staphylococcal and mammaliicoccal species and strain relationships. The data generated on the *Staph. chromogenes* IM strain support the hypothesis of a protective bovine mammary gland immune response. Beyond that, parity has a significant effect on T- and B-lymphocyte proliferation, in addition to the production of critical cytokines. Finally, we demonstrated that PHA-M is more appropriate as a selective mitogen for bovine T lymphocytes than the commonly used Con-A.

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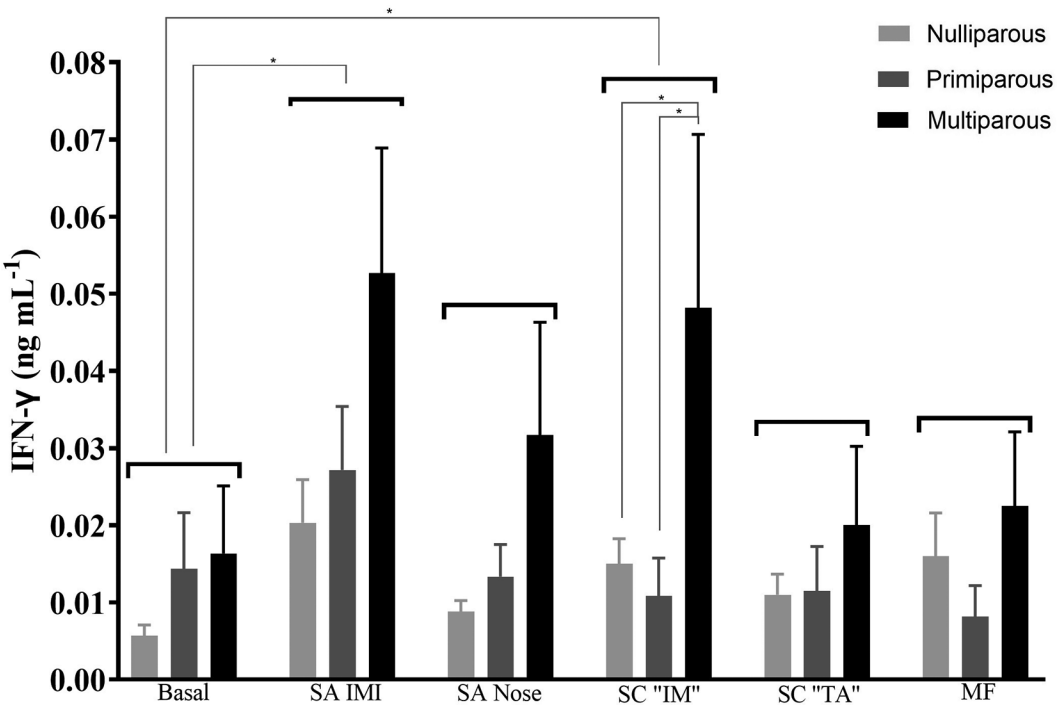

Figure 7. Concentration of IFN-γ (ng mL^{-1}; mean + SEM) of the supernatant of peripheral blood mononuclear cells after incubation with medium only (unstimulated basal) and the distinct staphylococci species and strains in nulliparous (n = 6), primiparous (n = 6), and multiparous (n = 6) dairy cows. SA IMI = *Staphylococcus aureus* isolated from a persistent IMI; SA Nose = *Staph. aureus* isolated from a nose; SC "IM" = *Staphylococcus chromogenes* isolated from a persistent IMI; SC "TA" = *Staph. chromogenes* isolated from the teat apex; and MF = *Mammaliicoccus fleurettii* (MF) isolated from sawdust. $^*P < 0.05$.

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REFERENCES

Alekseeva, L., L. Rault, S. Almeida, P. Legembre, V. Edmond, V. Azevedo, A. Miyoshi, S. Even, F. Taieb, Y. Arlot-Bonnemains, Y. Le Loir, and N. Berkova. 2013. *Staphylococcus aureus*-induced G2/M phase transition delay in host epithelial cells increases bacterial infective efficiency. PLoS One 8:e63279. https://doi.org/10.1371/journal.pone.0063279.

Beuckelaere, L., A. De Visscher, F. N. Souza, E. Meyer, F. Haesebrouck, S. Piepers, and S. De Vliegher. 2021. Colonization and local host response following intramammary *Staphylococcus chromogenes* challenge in dry cows. Vet. Res. 52:137. https://doi.org/10.1186/s13567-021-01007-8.

Breyne, K., S. De Vliegher, A. De Visscher, S. Piepers, and E. Meyer. 2015. Technical note: A pilot study using a mouse mastitis model to study differences between bovine associated coagulase-negative staphylococci. J. Dairy Sci. 98:1090–1100. https://doi.org/10.3168/jds.2014-8699.

Cunha, A. F., H. M. Andrade, F. N. Souza, L. C. Fialho Júnior, D. L. S. O. Rosa, E. M. R. Ramos Sanchez, M. Gidlund, H. Goto, M. A. V. P. Brito, A. S. Guimarães, A. P. Lage, L. C. Reis, A. M. M. P. Della Libera, M. B. Heinemann, and M. M. O. P. Cerqueira. 2020. Comparison of antibody repertories against *Staphylococcus aureus* in healthy and infected dairy cows with distinct mastitis history and vaccinated with a polyvalent mastitis vaccine. J. Dairy Sci. 103:4588–4605. https://doi.org/10.3168/jds.2019-17084.

De Buck, J., V. Ha, S. Naushad, D. B. Nobrega, C. Luby, J. R. Middleton, S. De Vliegher, and H. W. Barkema. 2021. Non-*aureus* staphylococci and bovine udder health: Current understanding and knowledge gaps. Front. Vet. Sci. 8:658031. https://doi.org/10.3389/fvets.2021.658031.

De Visscher, A., S. Piepers, F. Haesebrouck, and S. De Vliegher. 2016. Teat apex colonization with coagulase-negative *Staphylococcus* species before parturition: Distribution and species-specific risk factors. J. Dairy Sci. 99:1427–1439. https://doi.org/10.3168/jds.2015-10326.

De Visscher, A., K. Supré, F. Haesebrouck, R. N. Zadoks, V. Piessens, E. Van Coillie, S. Piepers, and S. De Vliegher. 2014. Further evidence for the existence of environmental and host-associated species of coagulase-negative staphylococci in dairy cattle. Vet. Microbiol. 172:466–474. https://doi.org/10.1016/j.vetmic.2014.06.011.

De Vliegher, S., L. K. Fox, S. Piepers, S. McDougall, and H. W. Barkema. 2012. Invited review: Mastitis in dairy heifers: Nature of the disease, potential impact, prevention, and control. J. Dairy Sci. 95:1025–1040. https://doi.org/10.3168/jds.2010-4074.

De Vliegher, S., G. Opsomer, A. Vanrolleghem, L. A. Devriese, O. C. Sampimon, J. Sol, H. W. Barkema, F. Haesebrouck, and A. de Kruif. 2004. *In vitro* growth inhibition of major mastitis pathogens by *Staphylococcus chromogenes* originating from teat apices of dairy heifers. Vet. Microbiol. 101:215–221. https://doi.org/10.1016/j.vetmic.2004.03.020.

Desforges, J. P., C. Sonne, M. Levin, U. Siebert, S. De Guise, and R. Dietz. 2016. Immunotoxic effects of environmental pollutants in marine mammals. Environ. Int. 86:126–139. https://doi.org/10.1016/j.envint.2015.10.007.

Dowling, M. R., A. Kan, S. Heinzel, J. M. Marchingo, P. D. Hodgkin, and E. D. Hawkins. 2018. Regulatory T cells suppress effector T cell proliferation by limiting division destiny. Front. Immunol. 9:2461. https://doi.org/10.3389/fimmu.2018.02461.

Erskine, R. J., C. M. Corl, J. C. Gandy, and L. M. Sordillo. 2011. Effect of infection with bovine leukosis virus on lymphocyte proliferation and apoptosis in dairy cattle. Am. J. Vet. Res. 72:1059–1064. https://doi.org/10.2460/ajvr.72.8.1059.

Ezzat Alnakip, M., M. Quintela-Baluja, K. Böhme, I. Fernández-No, S. Caamaño-Antelo, P. Calo-Mata, and J. Barros-Velázquez. 2014. The immunology of mammary gland of dairy ruminants between healthy and inflammatory conditions. J. Vet. Med. 2014:659801. https://doi.org/10.1155/2014/659801.

Huebner, R., R. Mugabi, G. Hetesy, L. Fox, S. De Vliegher, A. De Visscher, J. W. Barlow, and G. Sensabaugh. 2021. Characterization of genetic diversity and population structure within *Staphylococcus chromogenes* by multilocus sequence typing. PLoS One 16:e0243688. https://doi.org/10.1371/journal.pone.0243688.

Isaac, P., L. P. Bohl, M. L. Breser, M. S. Orellano, A. Conesa, M. A. Ferrero, and C. Porporatto. 2017. Commensal coagulase-negative *Staphylococcus* from the udder of healthy cows inhibit biofilm formation of mastitis-related pathogens. Vet. Microbiol. 207:259–266. https://doi.org/10.1016/j.vetmic.2017.05.025.

Kanellopoulos, J. M., S. De Petris, G. Leca, and M. J. Crumpton. 1985. The mitogenic lectin from *Phaseolus vulgaris* does not recognize the T3 antigen of human T lymphocytes. Eur. J. Immunol. 15:479–486. https://doi.org/10.1002/eji.1830150512.

Lašťovička, J., M. Rataj, and J. Bartůňková. 2016. Assessment of lymphocyte proliferation for diagnostic purpose: Comparison of CFSE staining, Ki-67 expression and ^{3}H-thymidine incorporation. Hum. Immunol. 77:1215–1222. https://doi.org/10.1016/j.humimm.2016.08.012.

Leuenberger, A., C. Sartori, R. Boss, G. Resch, F. Oechslin, A. Steiner, P. Moreillon, and H. U. Graber. 2019. Genotypes of *Staphylococcus aureus*: On-farm epidemiology and the consequences for prevention of intramammary infections. J. Dairy Sci. 102:3295–3309. https://doi.org/10.3168/jds.2018-15181.

Littman, D. R., and E. G. Pamer. 2011. Role of the commensal microbiota in normal and pathogenic host immune responses. Cell Host Microbe 10:311–323. https://doi.org/10.1016/j.chom.2011.10.004.

Lunjani, N., S. Ahearn-Ford, F. S. Dube, C. Hlela, and L. O'Mahony. 2021. Mechanisms of microbe-immune system dialogue within the skin. Genes Immun. 22:276–288. https://doi.org/10.1038/s41435-021-00133-9.

Madhaiyan, M., J. S. Wirth, and V. S. Saravanan. 2020. Phylogenomic analyses of the *Staphylococcaceae* family suggest the reclassification of five species within the genus *Staphylococcus* as heterotypic synonyms, the promotion of five subspecies to novel species, the taxonomic reassignment of five *Staphylococcus* species to *Mammaliicoccus* gen. nov., and the formal assignment of *Nosocomiicoccus* to the family *Staphylococcaceae*. Int. J. Syst. Evol. Microbiol. 70:5926–5936. https://doi.org/10.1099/ijsem.0.004498.

Mehrzad, J., L. Duchateau, and C. Burvenich. 2009. Phagocytic and bactericidal activity of blood and milk-resident neutrophils against *Staphylococcus aureus* in primiparous and multiparous cows during early lactation. Vet. Microbiol. 134:106–112. https://doi.org/10.1016/j.vetmic.2008.09.001.

Mehrzad, J., and X. Zhao. 2008. T lymphocyte proliferative capacity and $CD4^{+}/CD8^{+}$ ratio in primiparous and pluriparous lactating cows. J. Dairy Res. 75:457–465. https://doi.org/10.1017/S0022029908003439.

Murphy, M. P., D. A. Niedziela, F. C. Leonard, and O. M. Keane. 2019. The *in vitro* host cell immune response to bovine-adapted *Staphylococcus aureus* varies according to bacterial lineage. Sci. Rep. 9:6134. https://doi.org/10.1038/s41598-019-42424-2.

Niedziela, D. A., P. Cormican, G. Foucras, F. C. Leonard, and O. M. Keane. 2021. Bovine milk somatic cell transcriptomic response to *Staphylococcus aureus* is dependent on strain genotype. BMC Genomics 22:796. https://doi.org/10.1186/s12864-021-08135-7.

Nieto Farias, M. V., F. N. Souza, P. A. Lendez, L. Martínez-Cuesta, K. R. Santos, A. M. M. P. Della Libera, M. C. Ceriani, and G. L. Dolcini. 2018. Lymphocyte proliferation and apoptosis of lymphocyte subpopulations in bovine leukemia virus-infected dairy cows with high and low proviral load. Vet. Immunol. Immunopathol. 206:41–48. https://doi.org/10.1016/j.vetimm.2018.10.012.

Ohtsuka, H., S. Terasawa, C. Watanabe, M. Kohiruimaki, M. Mukai, T. Ando, K. R. Petrovski, and S. Morris. 2010. Effect of parity on lymphocytes in peripheral blood and colostrum of healthy Holstein dairy cows. Can. J. Vet. Res. 74:130–135.

Palacios, R. 1982. Concanavalin A triggers T lymphocytes by directly interacting with their receptors for activation. J. Immunol. 128:337–342.

Parlet, C. P., M. M. Brown, and A. R. Horswill. 2019. Commensal staphylococci influence *Staphylococcus aureus* skin colonization and disease. Trends Microbiol. 27:497–507. https://doi.org/10.1016/j.tim.2019.01.008.

Pereyra, E. A. L., S. C. Sacco, A. Duré, C. Baravalle, M. S. Renna, C. S. Andreotti, S. Monecke, L. F. Calvinho, and B. E. Dallard. 2017. Immune response of *Staphylococcus aureus* strains in a mouse mastitis model is linked to adaptive capacity and genotypic profiles. Vet. Microbiol. 204:64–76. https://doi.org/10.1016/j.vetmic.2017.04.009.

Piccart, K., J. Verbeke, A. De Visscher, S. Piepers, F. Haesebrouck, and S. De Vliegher. 2016. Local host response following an intramammary challenge with *Staphylococcus fleurettii* and different strains of *Staphylococcus chromogenes* in dairy heifers. Vet. Res. 47:56. https://doi.org/10.1186/s13567-016-0338-9.

Piepers, S., G. Opsomer, H. W. Barkema, A. de Kruif, and S. De Vliegher. 2010. Heifers infected with coagulase-negative staphylococci in early lactation have fewer cases of clinical mastitis and higher milk production in their first lactation than noninfected heifers. J. Dairy Sci. 93:2014–2024. https://doi.org/10.3168/jds.2009-2897.

Piessens, V., S. De Vliegher, B. Verbist, G. Braem, A. Van Nuffel, L. De Vuyst, M. Heyndrickx, and E. Van Coillie. 2012. Intra-species diversity and epidemiology varies among coagulase-negative *Staphylococcus* species causing bovine intramammary infections. Vet. Microbiol. 155:62–71. https://doi.org/10.1016/j.vetmic.2011.08.005.

Piessens, V., E. Van Coillie, B. Verbist, K. Supré, G. Braem, A. Van Nuffel, L. De Vuyst, M. Heyndrickx, and S. De Vliegher. 2011. Distribution of coagulase-negative *Staphylococcus species* from milk and environment of dairy cows differs between herds. J. Dairy Sci. 94:2933–2944. https://doi.org/10.3168/jds.2010-3956.

Porcellato, D., R. Meisal, A. Bombelli, and J. A. Narvhus. 2020. A core microbiota dominates a rich microbial diversity in the bovine udder and may indicate presence of dysbiosis. Sci. Rep. 10:21608. https://doi.org/10.1038/s41598-020-77054-6.

Rainard, P., P. Cunha, R. P. Martins, F. B. Gilbert, P. Germon, and G. Foucras. 2020. Type 3 immunity: A perspective for the defense of the mammary gland against infections. Vet. Res. 51:129. https://doi.org/10.1186/s13567-020-00852-3.

Rainard, P., G. Foucras, J. R. Fitzgerald, J. L. Watts, G. Koop, and J. R. Middleton. 2018. Knowledge gaps and research priorities in *Staphylococcus aureus* mastitis control. Transbound. Emerg. Dis. 65(Suppl 1):149–165. https://doi.org/10.1111/tbed.12698.

Santos, K. R., F. N. Souza, E. M. Ramos-Sanchez, C. F. Batista, L. C. Reis, W. F. Fotoran, M. B. Heinemann, H. Goto, M. Gidlund, A. F. Cunha, A. R. Faria, H. M. Andrade, A. P. Lage, M. M. O. P. Cerqueira, and A. M. M. P. Della Libera. 2021. *Staphylococcus aureus* protection-related type 3 cell-mediated immune response elicited by recombinant proteins and GM-CSF DNA vaccine. Vaccines (Basel) 9:899. https://doi.org/10.3390/vaccines9080899.

Santos, R. P., F. N. Souza, A. C. D. Oliveira, A. F. de Souza Filho, J. Aizawa, L. Z. Moreno, A. F. da Cunha, A. Cortez, A. M. M. P. Della Libera, M. B. Heinemann, and M. M. O. P. Cerqueira. 2020. Molecular typing and antimicrobial susceptibility profile of *Staphylococcus aureus* isolates recovered from bovine mastitis and nasal samples. Animals (Basel) 10:2143. https://doi.org/10.3390/ani10112143.

Soares, A., L. Govender, J. Hughes, W. Mavakla, M. de Kock, C. Barnard, B. Pienaar, E. Janse van Rensburg, G. Jacobs, G. Khomba,

L. Stone, B. Abel, T. J. Scriba, and W. A. Hanekom. 2010. Novel application of Ki67 to quantify antigen-specific in vitro lymphoproliferation. J. Immunol. Methods 362:43–50. https://doi.org/10.1016/j.jim.2010.08.007.

Souza, F. N., S. Piepers, A. M. M. P. Della Libera, M. B. Heinemann, M. M. O. P. Cerqueira, and S. De Vliegher. 2016. Interaction between bovine-associated coagulase-negative staphylococci species and strains and bovine mammary epithelial cells reflects differences in ecology and epidemiological behaviour. J. Dairy Sci. 99:2867–2874. https://doi.org/10.3168/jds.2015-10230.

Souza, F. N., K. R. Santos, J. A. Ferronatto, E. M. Ramos Sanchez, B. Toledo-Silva, M. B. Heinemann, S. De Vliegher, and A. M. M. P. Della Libera. 2022a. *Staphylococcus aureus* and non-aureus staphylococci and mammaliicocci strains (NASM) used in our study. [Data set]. Zenodo. https://doi.org/10.5281/zenodo.7215435.

Souza, R. M., F. N. Souza, C. F. Batista, S. Piepers, A. De Visscher, K. R. Santos, P. C. Molinari, J. A. Ferronatto, A. Franca da Cunha, M. G. Blagitz, G. G. da Silva, F. P. Rennó, M. M. O. P. Cerqueira, M. B. Heinemann, S. De Vliegher, and A. M. M. P. Della Libera. 2022b. Distinct behavior of bovine-associated staphylococci species in their ability to resist phagocytosis and trigger respiratory burst activity by blood and milk polymorphonuclear leukocytes in dairy cows. J. Dairy Sci. 105:1625–1637. https://doi.org/10.3168/jds.2021-20953.

Srinivasan, N. 2010. Telling apart friend from foe: Discriminating between commensals and pathogens at mucosal sites. Innate Immun. 16:391–404. https://doi.org/10.1177/1753425909357577.

Supré, K., F. Haesebrouck, R. N. Zadoks, M. Vaneechoutte, S. Piepers, and S. De Vliegher. 2011. Some coagulase-negative *Staphylococcus* species affect udder health more than others. J. Dairy Sci. 94:2329–2340. https://doi.org/10.3168/jds.2010-3741.

Tebartz, C., S. A. Horst, T. Sparwasser, J. Huehn, A. Beineke, G. Peters, and E. Medina. 2015. A major role for myeloid-derived suppressor cells and a minor role for regulatory T cells in immunosuppression during *Staphylococcus aureus* infection. J. Immunol. 194:1100–1111. https://doi.org/10.4049/jimmunol.1400196.

Toledo-Silva, B., L. Beuckelaere, A. De Visscher, C. Geeroms, E. Meyer, S. Piepers, D. Thiry, F. Haesebrouck, and S. De Vliegher. 2022. Novel quantitative assay to describe in vitro bovine mastitis bacterial pathogen inhibition by non-*aureus* staphylococci. Pathogens 11:264. https://doi.org/10.3390/pathogens11020264.

Toledo-Silva, B., F. N. de Souza, K. Mertens, S. Piepers, F. Haesebrouck, and S. De Vliegher. 2021b. Bovine-associated non-*aureus* staphylococci suppress *Staphylococcus aureus* biofilm dispersal *in vitro* yet not through *agr* regulation. Vet. Res. 52:114. https://doi.org/10.1186/s13567-021-00985-z.

Toledo-Silva, B., F. N. de Souza, S. Piepers, K. Mertens, F. Haesebrouck, and S. De Vliegher. 2021a. Metabolites of bovine-associated non-*aureus* staphylococci influence expression of *Staphylococcus aureus agr*-related genes *in vitro*. Vet. Res. 52:62. https://doi.org/10.1186/s13567-021-00933-x.

Tomazi, T., J. L. Gonçalves, J. R. Barreiro, M. A. Arcari, and M. V. dos Santos. 2015. Bovine subclinical intramammary infection caused by coagulase-negative staphylococci increases somatic cell count but has no effect on milk yield or composition. J. Dairy Sci. 98:3071–3078. https://doi.org/10.3168/jds.2014-8466.

Valckenier, D., S. Piepers, A. De Visscher, and S. De Vliegher. 2020. The effect of intramammary infection in early lactation with non-aureus staphylococci in general and *Staphylococcus chromogenes* specifically on quarter milk somatic cell count and quarter milk yield. J. Dairy Sci. 103:768–782. https://doi.org/10.3168/jds.2019-16818.

Van Werven, T., E. N. Noordhuizen-Stassen, A. J. Daemen, Y. H. Schukken, A. Brand, and C. Burvenich. 1997. Preinfection *in vitro* chemotaxis, phagocytosis, oxidative burst, and expression of CD11/CD18 receptors and their predictive capacity on the outcome of mastitis induced in dairy cows with *Escherichia coli*. J. Dairy Sci. 80:67–74. https://doi.org/10.3168/jds.S0022-0302(97)75913-7.

Vanderhaeghen, W., S. Piepers, F. Leroy, E. Van Coillie, F. Haesebrouck, and S. De Vliegher. 2014. Invited review: Effect, persistence, and virulence of coagulase- negative *Staphylococcus* species associated with ruminant udder health. J. Dairy Sci. 97:5275–5293. https://doi.org/10.3168/jds.2013-7775.

Vangroenweghe, F., L. Duchateau, and C. Burvenich. 2004. Moderate inflammatory reaction during experimental *Escherichia coli* mastitis in primiparous cows. J. Dairy Sci. 87:886–895. https://doi.org/10.3168/jds.S0022-0302(04)73233-6.

Weiss, A., R. Shields, M. Newton, B. Manger, and J. Imboden. 1987. Ligand-receptor interactions required for commitment to the activation of the interleukin 2 gene. J. Immunol. 138:2169–2176.

Wuytack, A., A. De Visscher, S. Piepers, F. Boyen, F. Haesebrouck, and S. De Vliegher. 2019. Non-*aureus* staphylococci in fecal samples of dairy cows: First report and phenotypic and genotypic characterization. J. Dairy Sci. 102:9345–9359. https://doi.org/10.3168/jds.2019-16662.

Zielinski, C. E., F. Mele, D. Aschenbrenner, D. Jarrossay, F. Ronchi, M. Gattorno, S. Monticelli, A. Lanzavecchia, and F. Sallusto. 2012. Pathogen-induced human TH17 cells produce IFN-γ or IL-10 and are regulated by IL-1β. Nature 484:514–518. https://doi.org/10.1038/nature10957.