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Exploring the distinct immunological reactions of bovine neutrophils towards major and minor pathogens responsible for mastitis

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ABSTRACT

Bovine mastitis is primarily caused by a group of bacteria known as *Staphylococcus* and *Streptococcus*. However, additional types of bacteria, such as bovine non-aureus staphylococci and mammaliicocci (NASM) as well as lactic acid bacteria (LAB), are considered minor pathogens and have less impact on cows. Modulating bovine neutrophil activities and gene expressions in response to bacterial stimuli prompted the cells to execute effector functions to combat udder infections. Although neutrophils can manage major mastitis-causing bacteria, this strategy has not been tested against minor pathogens, i.e. NASM, *Weissella* spp. Our main objective was to investigate how neutrophils interacted with major and minor pathogens during *in vitro* bacterial stimulation. The results reveal that neutrophils performed offensive duties regardless of the type of bacteria encountered. Neutrophils generated high levels of reactive oxygen species, efficiently phagocytosed both types of bacteria, and facilitated extracellular killing by releasing NET structures against all bacteria. In addition, neutrophils migrated preferentially towards the majors rather than the minors, although myeloperoxidase (MPO) degranulation did not differ substantially across bacteria. Furthermore, the killing capacity of neutrophils was not dependent on any particular bacterium. The correlation of effector functions is intimately linked to the up-regulation of genes associated with the above functions, except for *IL6*, which was down-regulated. Furthermore, neutrophil apoptosis can be modulated by altering apoptosis-associated genes in response to harmful stimuli. These findings provide valuable information on how neutrophils react to major and minor mastitis-causing bacteria. However, future research should explore the interplay between minor pathogens and the host's responses.

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1. Introduction

Bovine mastitis is a highly prevalent and costly disease affecting the global dairy industry. Intra-mammary infections (IMIs) caused by microorganisms in the udder/skin are the main drivers of mastitis. Three types of bacteria can infect bovine mammary glands: contagious, opportunistic, and environmental pathogens [1]. Contagious pathogens, including *S. aureus*, *S. agalactiae*, and *Mycoplasma bovis*, reside on the udder and are spread during milking. Environmental pathogens, such as *S. dysgalactiae*, *S. uberis*, and *E. coli*, live in the cow's environment and enter the teat canal, causing IMIs [1].

Bovine non-aureus staphylococci and mammaliicocci (NASM) are a diverse group of the skin microbiota of cows [2–4]. They have been isolated from various cows' anatomical sites and the dairy environment [2,4,5]. Although previously considered insignificant pathogens, NASM, for example, *S. chromogenes, S. epidermidis*, *S. simulans*, *S. xylosus*, *S. haemolyticus*, and *Mammaliicoccus fleurettii*, may pose a severe threat to the health of bovine udders [2,3,6,7]. NASM can potentially cause persistent IMIs [8–10], resulting in increased somatic cell count (SCC) and reduced milk production [6,7,10]; however, the impact of NASM on udder health is still a topic of discussion. The presence of NASM has played a role in the suppression of other Staphylococci, including the biofilm dispersion and regulation of quorum sensing [5,11]. Being discovered and well-known for their probiotic properties, various genus of Gram-positive lactic acid bacteria (LAB), including *Enterococcus*, *Lactobacillus*, and *Weissella*, were detected in raw

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milk, forages, and cow manure [12–14]. Furthermore, certain strains of *W. paramesenteroides* and *W. cibaria* were isolated from milk samples obtained from cows with clinical mastitis [15].

Intramammary infections are characterized by elevated SCC in milk, comprising neutrophils, lymphocytes, macrophages, and less epithelial cell content. The cow's immune response is pivotal in determining its susceptibility to mastitis. Pathogenic bacteria engage with the host's innate immunity at the teat canal, triggering a rapid response from innate immune cells that migrate to the site of infection [16]. Bovine neutrophils play a vital role in phagocytosis, generating reactive oxygen species (ROS) and releasing granule enzymes and antimicrobial peptides, all leading to eliminating pathogens [8,17–23]. Additionally, neutrophils release extracellular traps (NETs) consisting of chromatin and granule proteins, which ensnare and eradicate mastitis-causing bacteria nearby [24–26]. However, despite extensive research, comprehensive information regarding the diverse functional capacities of bovine neutrophils against both major and minor mastitis-causing bacteria in cows remains limited.

This study sought to provide new insight into the role of bovine neutrophils in the intracellular and extracellular defence mechanisms against major and minor mastitis-causing bacteria, including LAB strains. In this work, we analysed neutrophil movement, degranulation, phagocytosis, ROS production, intracellular bacterial clearance, and NETs release *ex vivo* from purified blood neutrophils. In addition to the functional experiments, we also performed real-time PCR analyses of gene expression.

2. Materials and methods

2.1. Animals, blood collection, and bovine polymorphonuclear neutrophil (PMN) isolation

Blood was collected from adult Holstein Friesian cows (*Bos taurus*) that were not pregnant and were in good health. For this study, a total of fifteen cows were used throughout three independent experiments, each using five cows. All cows were housed at local farms in Mae Wang district, Chiang Mai, Thailand. Using jugular venipuncture, about 40 mL of whole blood was obtained into a sterile syringe containing 10 mL of 1× acid citrate dextrose solution for bovine polymorphonuclear neutrophil (PMN) isolation within 2 h after blood collection. Bovine neutrophil isolation was performed as previously described [18]. Briefly, blood tubers were inverted many times to mix and then centrifuged at 1,000 ×*g* at 4°C for 20 min (Allegra X-15 R Centrifuge, Beckman Coulter, Brea, CA, USA) in order to obtain the buffy coat layer and separate the packed RBCs beneath. To restore isotonicity, the remaining RBCs were lysed using a hypotonic water solution before being treated with hypertonic phosphate-buffered saline solution, then centrifuged at 600 ×*g* for 10 min. Pellets were rinsed twice with cold Hanks' Balanced Salt Solution (HBSS) and resuspended in cold Roswell Park Memorial Institute 1640 (RPMI 1640) medium containing 1% heat-inactivated foetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The Trypan blue exclusion method and cytospin were used to assess the viability and to determine the neutrophil purity, respectively [19]. The results show a viability rate of over 95%, while the purity of the cells was more than 85%. Subsequently, the cell density was adjusted to approximately 3×10^6 cells per mL. The conducted animal experiments were approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine, Chiang Mai University (FVM-ACUC) under the reference number S26/2562.

2.2. Bacterial growth condition

As major causes of bovine mastitis, *Staphylococcus aureus* (SAU), *Streptococcus agalactiae* (SAG) [19], and *Streptococcus uberis* (SUB) [27] were selected for the current study. Bovine non-aureus staphylococci and mammaliicocci (NASM); *Staphylococcus hominis* (SHO) and *Staphylococcus chromogenes* (SCH); lactic acid bacteria (LAB); and two isolates of *Weissella paramesenteroides* (WPA1 and WPA2) with antimicrobial activity against *S. aureus* or *Escherichia coli* were used as minor pathogen-causing bovine mastitis in the entire experiment. Bacteria were originally from field isolates and stored at −20°C in glycerol/Tryptic soy broth (TSB, HIMEDIA, Mumbai, India). Before use in the actual experiments, an aliquot of bacteria from the frozen collection was thawed. It was inoculated onto Tryptic soy agar (TSA, HIMEDIA) plates with 5% bovine blood and grown for 24 h at 37°C. *Weissella paramesenteroides* (WPA) was stored at −20°C in glycerol/MRS broth. The frozen collection was thawed and inoculated onto Lactobacillus MRS broth (MRS, HIMEDIA) plates with Lactobacillus MRS agar and grown anaerobically at 37°C for 24 h before use. The bacteria number was adjusted to approximately 10^8 CFU/mL in the experiment before use.

2.3. Fluorescent labelling and opsonization of bacteria

Live bacteria from Section 2.2 were grown to the log phase, suspended in a Hanks' balanced salt solution (HBSS) solution (Sigma-Aldrich, St. Louis, MO, USA), and heat-killed at 70°C for 60 min [19,28]. Heat-killed bacteria were resuspended at a 10^8 CFU/mL density in 1 µg/mL goat anti-mouse IgG (H+L) antibody, Alexa Fluor® 488 (Invitrogen, Thermo Fisher Scientific) for

SAU, SAG, and SCH. Propidium iodide (PI) was used for SUB and WPA at 10 µg/mL. Bacteria were fluorescently labelled for 30 min at 4°C. Then, they were washed extensively with HBSS to remove the free dye, adjusted to 10^6 CFU/mL with HBSS, and stored at 4°C until use. Fluorescently labelled bacteria were opsonized with 10% heat-inactivated autologous bovine serum for 20 min at 37°C before using in the phagocytosis assay.

2.4. Measurement of intracellular reactive oxygen species (ROS)

Bovine neutrophils (3×10^5 cells) were seeded onto 96-well, flat bottom cell culture plates in duplicated wells and then activated to produce ROS with each live bacteria at the multiplicity of infection (MOI) of 10 in HBSS. The cells were then incubated for 30 min at 37°C with 5% CO_2. Then, the cells were washed with HBSS, centrifuged at 1,200 rpm for 3 min, and the supernatant was discarded. Then, 10 µM H_2DCF-DA (Invitrogen, Thermo Fisher Scientific) was loaded into each well to stain the intracellular H_2O_2 [18,19]. Cells were incubated in the dark for 15 min, then washed with cold HBSS. A DxFLEX Flow Cytometer (Beckman Coulter, Brea, CA, USA) was used to collect data (10,000 events) from ROS-containing cells (10,000 cell events) and later analysed using FlowJo 10 (Treestar, Ashland, OR, USA) [29].

2.5. Phagocytosis

The phagocytosis of each bacterium was assessed via flow cytometry. In brief, bovine neutrophils (3×10^5 cells) were mixed with either heat-killed, opsonized FITC labelled SAU, SAG, and SCH or heat-killed, PI-labelled SUB and WPA at the MOI of 10 in duplicate wells of a 96-well, flat bottom cell culture plate. To promote the uptake of bacteria, the cell mixture was centrifuged at 1,200 rpm for 3 min, and the bovine neutrophils were allowed to process the bacteria for 45 min at 37°C, 5% CO_2 [19,28]. After incubation, cells were quenched with 0.4% trypan blue and washed extensively with ice-cold HBSS. The sample acquisitions (10,000 cell events) were acquired on DxFLEX Flow Cytometer and analysed using FlowJo software.

2.6. Myeloperoxidase (MPO) degranulation assay

Isolated bovine neutrophils (3×10^5 cells) were plated onto a 96-well flat bottom cell culture plate and stimulated with each live bacteria at the multiplicity of infection of 10 (MOI of 10) or 3×10^6 CFU at 37°C with 5% CO_2 for 30 min. Plates were centrifuged at 1,200 rpm for 3 minutes to remove and collect the supernatant. Three microlitres of cell pellet mixture were spotted onto 0.45 µm pore – size nitrocellulose (NC) membrane (Bio-Rad, Hercules, CA, USA) and dried at RT for 30 min. The dried NC membranes were washed once with Tris-buffered saline with Tween® 20 (TBST) and rocked for 5 min. After washing, the membrane surfaces were covered with an ample amount of the BioFX® TMB One Component HRP Membrane Substrate (Surmodics, Eden Prairie, MN, USA) and rotated for 10 min at RT or until an insoluble permanent dark blue reaction product was obtained. Then, the reaction was stopped by rinsing the membrane with distilled water. After complete drying, the membrane was photographed, followed by spot intensity measurement using Image Studio™ Lite (v.5.2) Quantification Software (LI-COR Biosciences, Lincoln, Nebraska, USA). One hundred microlitres of supernatant was transferred onto a 96-well, flat bottom cell culture plate, followed by adding 50 µL of TMB Chromogen Solution (Thermo Fisher Scientific), and thoroughly mixed. The plate was kept in the dark and incubated for 5 min at RT. Colorimetric detection was performed at a wavelength of 650 nm (Anthos Labtec 2010 Microplate Reader, Anthos Labtec, Heerhugowaard, The Netherlands) [30,31].

2.7. Bacterial killing (MTT) assay

The bactericidal activity of bovine neutrophils was assessed using a semi-quantitative MTT test to determine the percentage of bacterial viability [18,19]. Bacteria were propagated in accordance with the procedure described in Section 2.2. Isolated bovine neutrophils (3×10^5 cells) were plated onto a 96-well flat bottom cell culture plate and stimulated with opsonized live bacteria at a 1:10 ratio. The plate was centrifuged (1,200 rpm, 3 min) and placed in an incubator at 37°C with 5% CO_2 for 45 min. After incubation, the plate was again centrifuged to remove non-ingested bacteria. Hypotonic solution (diH_2O) was used for releasing internalized bacteria from cells (5 min at RT). After lysing, Tryptic soy broth (TSB) or Lactobacillus MRS broth (MRS) with 2 µg/mL of Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma-Aldrich) was supplemented to all wells. Plates were incubated for a total of 90 min at 37°C. The MTT-insoluble formazan was solubilized to coloured crystals by adding dimethyl sulphoxide (DMSO). Colorimetric detection was performed at a wavelength of 570 nm (Anthos Labtec 2010 Microplate Reader). In each experiment, optical density (OD) from MTT solution only (Blank) was included to indicate that no live bacteria were present. The percentage of bacterial killing was calculated by substituting measured OD values into the following formula [18]:

$$\%\ \text{of killing} = 100 - \left[\left(OD_{\text{sample}} - OD_{\text{Blank}}\right) \times 100\right]$$

2.8. Under-agarose cell migration assay

We adapted the previously reported methods to examine the direct migration of bovine neutrophils towards living bacteria [32,33]. To begin, 0.5 g agarose powder (Bio Basic Inc., Ontario, Canada) was dissolved in 10 mL sterile, distilled water via boiling to thoroughly dissolve the agarose powder. After cooling to 48°C in a water bath, the agarose was combined with a prewarmed cell culture medium (20 mL RPMI 1640 supplemented with 5% FBS +10 mL of 1× HBSS) to obtain the desired final volume of 40 mL (Agarose medium). Seven millilitres of agarose medium was added to each 90 × 15 mm SPL Petri dish (SPL Life Sciences, Gyeonggi-do, Korea) and left to solidify at RT for 15 min. Using a hole punch made of stainless steel, three series of seven wells, each measuring 3.5 mm in diameter, were punched into each plate so that the wells could be configured as desired (Figure 2a). A pointed-tip tweezer was used to clear the agarose plugs from the wells. Gel plates were equilibrated at 37°C, 5% CO_2 for 30 min. Twenty microlitres of bovine neutrophils (60,000 cells), live bacteria (2×10^5 bacteria), or RPMI 1640 (non-chemotactic control medium) was loaded onto the corresponding wells (Figure 2a). The dishes were incubated at 37°C, 5% CO_2 for 2 h. After incubation, the cells were fixed with agarose *in situ* for 5 min by adding 6 mL of methanol (Sigma-Aldrich). After fixation, the dishes were stained with DipQuick (RVL Supply, Bangkok, Thailand), washed twice with H_2O, and dried completely at RT. The migration patterns (areas between the neutrophils-containing wells and bacteria-containing wells or RPMI 1640) were captured at a 4× and 10× magnification using Olympus B×53Upright Microscope (Olympus Corporation, Tokyo, Japan). Migrated cells were quantified and analysed with ImageJ v 1.52a software with a cell counter plug-in (National Institutes of Health (NIH), Bethesda, MD, USA).

2.9. Quantification and visualization of neutrophil extracellular traps (NETs) release

Isolated bovine neutrophils, at a concentration of 3×10^5 cells per well, were plated in duplicate in a 96-well flat bottom plate. After that, 3×10^6 viable bacteria were placed in each of the wells. Cells stimulated with HBSS served as control. Additionally, all wells were supplemented with HBSS with Ca_2^+ and Mg_2^+ before incubation at 37°C, with 5% CO_2 for 150 min. After activation, plates were centrifuged at 1,200 rpm for 3 min, and the supernatant was discarded. Each well was filled with ice-cold RPMI 1640 medium and carefully mixed with a pipette, and then centrifuged. The supernatant containing extracellular DNAs was transferred onto new plates. NET-DNA was quantified using a fluorescent dye (Hoechst 33,342) at 5 mg/mL [19,34]. The fluorescence measurement of stained NETs was measured with a Synergy™ HT Multi-Detection Microplate Reader using an excitation wavelength at 360 nm and emission at 470 nm. The relative fluorescence units (RFUs) were recorded [19,29]. As a prior procedure, NET structures were validated via light microscopy by staining the NET structure with DipQuick [19]. In brief, 8-well chamber slides (SPL Life Sciences) were filled with bovine neutrophils (3×10^4 cells), and live bacteria (3×10^5 bacteria) or HBSS served as control. Cells were left to be stimulated for 150 min at 37°C, 5% CO_2. The visualization and image capture were performed at 10× objectives.

2.10. Apoptosis assay using flow cytometry

Bovine neutrophils (1×10^5 cells) were seeded onto 96-well, flat bottom cell culture plates in duplicated wells and then stimulated with each live bacterium at the MOI of 10 in HBSS. The cells were then incubated for 45 min at 37°C with 5% CO_2. For viability analysis, cells were stained with FITC Annexin V apoptosis detection kit with Propidium Iodide (PI) (BioLegend, San Diego, CA, USA) in Annexin V Binding Buffer (BioLegend), according to the manufacturer's instructions. Cells were recorded on DxFLEX Flow Cytometer (Beckman Coulter), and data were analysed using FlowJo. The percentage of apoptotic cells was quantified via Annexin V^+/PI^- (early apoptosis) and Annexin V^+/PI^+ cells (late apoptosis), as shown in Figure 4d.

2.11. Gene expression using real-time PCR (qPCR)

The effects of major and minor pathogens in the pathogenesis of bovine mastitis on bovine neutrophils were investigated utilizing gene expression patterns after the cells were exposed to bacteria. Bovine neutrophils (1×10^6 cells) were placed into 1.5 mL microcentrifuge tubes, and then live bacteria (1×10^7 bacteria) were induced to express genes for 2 h at 37°C with 5% CO_2. Control cells were stimulated with HBSS. Following the incubation, bacteria and other debris were removed by pelleting the cells (8,000 rpm, 5 min) and washing them with HBSS. To preserve RNA integrity, cell pellets were treated with RNA*later* (Invitrogen). RNAs were extracted using RNAzol®RT following the manufacturer's instructions [19]. The cDNAs were synthesized using two µg of total RNA using the Tetro cDNA Synthesis Kit (Bioline, Taunton, MA, USA), and 100 ng samples of cDNA from neutrophils were quantitatively evaluated for the mRNA transcripts of pathogen recognition and cellular functions: *IL1B*, *IL6*, *TNF*, *CXCL8*, *NOX1*, *SOD1*, *LAMP1*, *CORO1A*, *TLR1*, *TLR2*, and *TLR6*.

For apoptotic genes, *BAX*, *BCL2*, *BCL2L1* (also called Bcl-xL), *CFLAR* (CASP8 and FADD Like Apoptosis Regulator), *FAS*, *CASP3*, and *CASP9* were chosen to identify genes implicated in cellular apoptosis. The real-time RT-PCR (qPCR) reactions were run using a HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) on ABI Prism 7300 real-time PCR (Applied Biosystems, Thermo Fisher Scientific). Gene expression levels normalized to *ACTB* as endogenous control were calculated using the $2^{-\Delta Ct}$ method [35] and expressed as mean ± SEM relative to non-stimulated condition (control). The primer information used in the current study is listed in detail in Supplementary Table S1.

2.12. Data analysis

The normality of the data through Gaussian distribution from three independent experiments was verified with the Shapiro – Wilk normality test. Data with normal distribution and equal variances were analysed with one-way ANOVA with Tukey's multiple comparisons or the honestly significant difference (HSD) test. Otherwise, a nonparametric test, Kruskal – Wallis with Dunn's multiple comparisons tests, was used. Statistical analysis was performed with GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA) or R version 4.2.1 (R Core Team, 2013). Bubble plots, principal component analysis (PCA) plots, and correlation matrix were generated via R using the packages *gplot2*, *ggfortify*, and *corrplot* [36–38]. Statistical significance was accepted where $p < 0.05$. Data presentations are displayed as mean with standard error (mean ± SE).

3. Results

3.1. Major and minor pathogen-causing bovine mastitis robustly induced bovine neutrophils to produce intracellular ROS

To assess the effects of major (SAU, SAG, SUB) and minor pathogen-causing bovine mastitis (SHO, SCH, WPA1, WPA2) on the generation of intracellular reactive oxygen species (ROS), we measured primarily intracellular hydrogen peroxide (H_2O_2) with fluorescent dye (H_2DCF-DA) and analysed using flow cytometry (Figure 1a, b). Flow cytometry data (ROS Index or fold-change) showed that the cells stimulated with both major and minor bacteria could significantly induce a tremendous amount of ROS production compared with cells that received HBSS as control cells ($p = 0.011$, Figure 1b). The findings of this study revealed the levels of ROS for each group as follows: SAU exhibited a fold change of 3.13 ± 0.51, SAG

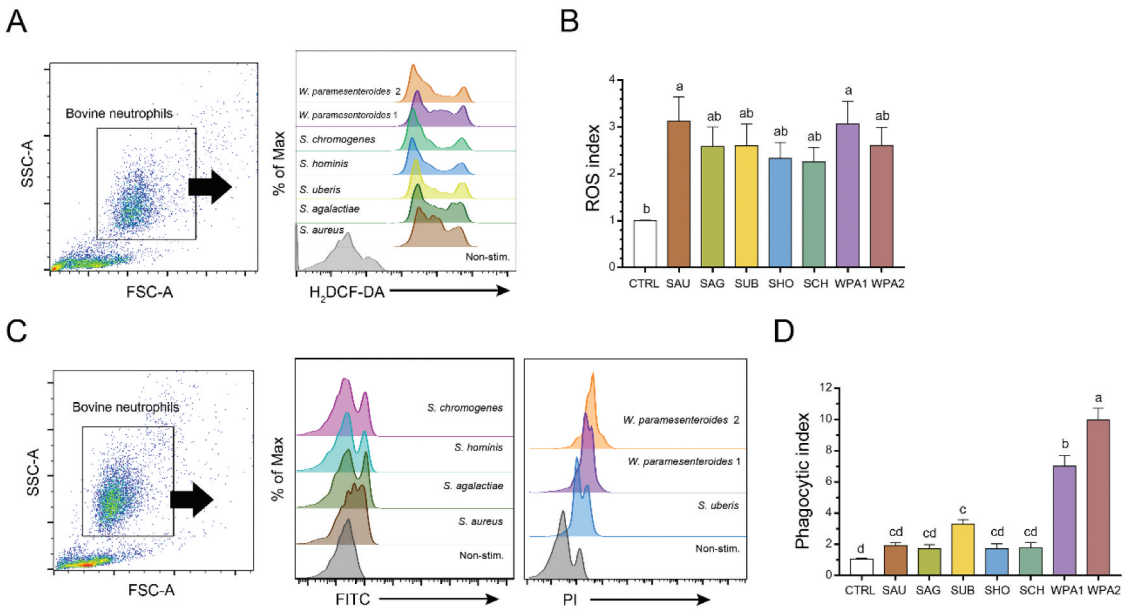

Figure 1. Major and minor bacteria-causing mastitis induce an increase in ROS production and phagocytosis in bovine neutrophils. (a) Flow cytometric analysis showing intracellular ROS generation (H_2DCF-DA) of non-stimulated cells and bacteria-stimulated bovine neutrophils by half offset histograms. (b) Quantification of intracellular ROS generation in bovine neutrophils stimulated with bacteria as ROS index (mean ± SEM). Data are representative of twelve cows ($n = 12$) of three independent experiments. Statistical analysis was performed using one-way ANOVA and Tukey's multiple comparison test. (c) Histograms depict flow cytometric analyses of bovine neutrophil phagocytosis of bacteria-causing mastitis. (d) Phagocytic index of bovine neutrophil phagocytosis by flow cytometric data (mean ± SEM). Data are representative of ten cows ($n = 10$) of two independent experiments. Statistical analysis was performed using one-way ANOVA and Tukey's multiple comparison test. Different letters indicate statistically significant differences at $p < 0.05$. Bars with no common letters are significantly different ($p < 0.05$). Note: CTRL = non-stimulated cells, SAU = *Staphylococcus aureus*, SAG = *Streptococcus agalactiae*, SUB = *Streptococcus uberis*, SHO = *Staphylococcus hominis*, SCH = *Staphylococcus chromogenes*, WPA = *Weissella paramesenteroides*

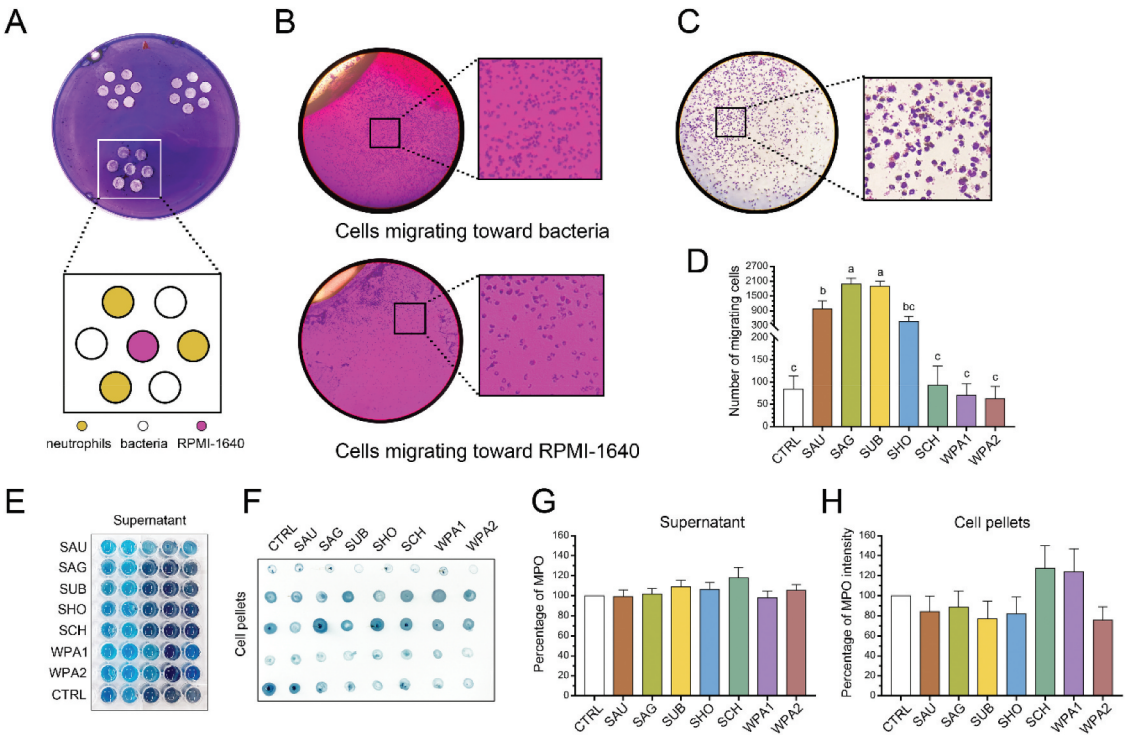

Figure 2. The number of migrating bovine neutrophils towards major pathogens of mastitis increased significantly while the degree of MPO degranulation remained unaltered across all bacteria. (a) Gel cut template set up for under-agarose cell migration assay and schematic illustration of assay wells. Cells/bacteria/RPMI-1640 were seeded into the wells as depicted. (b) Representative cell migration images of migrating neutrophils towards bacteria (upper panel) or towards RPMI-1640 (lower panel). The areas of migrating cells are magnified for clarity (black squares). (c) Representative image of cells arrived at the site of bacteria-containing assay well. (d) Number of migrating cells in the respective bacteria-containing wells or RPMI-1640 or CTRL (mean ± SEM). Data are representative of ten cows ($n = 10$) of two independent experiments. Statistical analysis was performed using one-way ANOVA and Tukey's multiple comparison test. (e-f) Representative results of MPO degranulation from cell supernatant in microtiter plate (e) or dot-blot assay from cell pellet mixture (f). (g-h) MPO degranulation assays show no significant degranulation both from supernatant (g) and cell pellet mixture (h). Data are present as mean ± SEM from ten cows ($n = 10$) of two independent experiments, analysed using one-way ANOVA. Bars with no common letters are significantly different ($p < 0.05$). Note: CTRL = non-stimulated cells, SAU = *Staphylococcus aureus*, SAG = *Streptococcus agalactiae*, SUB = *Streptococcus uberis*, SHO = *Staphylococcus hominis*, SCH = *Staphylococcus chromogenes*, WPA = *Weissella paramesenteroides*

showed a fold change of 2.59 ± 0.41, SUB had a fold change of 2.60 ± 0.47, SHO displayed a fold change of 2.33 ± 0.34, SCH presented a fold change of 2.26 ± 0.31, WPA1 showed a fold change of 3.07 ± 0.48, and WPA2 exhibited a fold change of 2.60 ± 0.38. Based on the multiple comparisons, it was evident that the major pathogen, SAU, and the minor bacteria, WPA1, exhibited distinct and significant variations in the production of ROS when compared to the non-stimulated control (Figure 1b). Conversely, no significant differences in ROS production between the major and minor bacteria were found.

3.2. The process of internalization and phagocytosis of bacteria by bovine neutrophils was markedly elevated

The phagocytosis of FITC-labelled SAU, SAG, SHO, SCH, and PI-labelled SUB, WPA1, and WPA2 by bovine neutrophils was studied *in vitro*. The bovine neutrophils appeared to ingest SUB, WPA1, and WPA2 robustly compared with control cells receiving HBSS (Figures 1c, d). The encounter of bacteria resulted in significantly enhancing phagocytosis of SUB (3.30 ± 0.28-fold), WPA1 (7.00 ± 0.69-fold), and WPA2 (9.95 ± 0.77-fold) compared with control cells, respectively ($p < 0.0001$, Figure 1d). As with the bacteria above phagocytosis, SAU, SAG, SHO, and SCH again appeared robust in every experiment. The fold-increase in phagocytosis is as follows: SAU (1.93 ± 0.17-fold), SAG (1.72 ± 0.25-fold), SHO (1.73 ± 0.30-fold), and SCH (1.79 ± 0.34-fold), respectively.

3.3. Bovine neutrophils migrated extensively towards major pathogens but not minor pathogens

We next assessed the dynamic cell motility towards live major (SAU, SAG, SUB) and minor pathogens (NASM and WPA) for bovine mastitis using under-agarose migration assays (Figures 2a, b). There was a significant migration of bovine neutrophils towards the major pathogens but not the minor ones. The average numbers of migrated cells with each stimulant

were 84.3 ± 28.9 cells (RPMI), 990.4 ± 319.2 cells (SAU), 1,989 ± 236.9 cells (SAG), 1,893 ± 205.1 cells (SUB), 479.4 ± 199.8 cells (SHO), 93.3 ± 43.06 cells (SCH), 70.8 ± 25.18 cells (WPA1), and 63 ± 27.4 cells (WPA2), respectively (Figure 2d). Overall, the migration of cells towards major pathogens presented in this report has significantly differed from the non-chemotactic control medium (RPMI) ($p < 0.0001$, Figure 2d). For minor pathogens, the number of migrated cells seemed to be equal to that of the control medium.

The patterns in Figure 2b were obtained by incubating bovine neutrophils in the centre well for 2 h with bacteria in the outer wells. The directions in the migration pattern are characteristic of responses to bacteria and have been a consistent observation. However, no differences were seen in the numbers of cells when RPMI-1640, SCH, WPA1, and WPA2 were tested. Figure 2b shows the cells *en route* from wells receiving major bacteria at higher magnification (×10). After incubation, neutrophils of some samples dwelled in their original location without migrating. On the contrary, some bovine neutrophils are eager to find and encounter bacteria in the bacteria wells (Figure 2c).

3.4. Reduced MPO degranulation by bovine neutrophils upon contact with bacteria

After bacterial challenges by major and minor bacteria, MPO degranulation by bovine neutrophils was measured semi-quantitatively using changes in an enzymatic substrate (TMB). MPO releases were active in both the supernatant and the cell pellet portions. Detectable amounts of MPO were released in the supernatants of all bacteria-stimulated cells; however, most cells released MPO with equal amounts of non-stimulated control ($p = 0.946$, Figure 2e, g). The percentage of MPO activity released during SCH stimulation was much higher than that released from non-stimulated cells (118% ± 10.39% versus non-stimulated control equivalent to 100%).

Using a spot assay, the MPO granule proteins were quantified in cellular components following the retention of MPO within cell pellets (Figures 2f, h). The percentage of MPO content in cells stimulated with different bacteria did not differ from the non-stimulated control ($p = 1.441$, Figure 2H). In SAU, SAG, SUB, SHO, and WPA2, the remaining MPO contents in the cells were diminished, whereas in SCH and WPA1, the MPO contents were preserved to a much greater extent than in the control group (127.5% ± 22.6% and 124% ± 2.93%, respectively) (Figure 2h).

3.5. The ability of bovine neutrophils to kill bacteria was not affected, regardless of the different types of bacteria

After incubating bovine neutrophils with either major or minor pathogens in bovine mastitis, the MTT assay was used to evaluate bacterial cell proliferation. In the MTT bactericidal assay for major pathogens, 68.38 ± 2.80% of *Staphylococcus aureus* (SAU) were killed, whereas 60.63 ± 3.64% of *Streptococcus agalactiae* (SAG) and 71.06 ± 1.74% of *Streptococcus uberis* (SUB) were killed when incubated with bovine neutrophils (Figure 3a). The percentages of SHO, SCH, WPA1, and WPA2 killed by bovine neutrophils against minor pathogens were 67.78 ± 3.52%, 70.16 ± 4.33%, 65.31 ± 3.76%, and 71.10 ± 2.22%, respectively (Figure 3a). The overall percentage of killing activities among the bacteria did not show statistical significance when analysed using a one-way ANOVA ($p = 0.249$, Figure 3a); however, different bacteria displayed different levels of vulnerability, as well as interaction with bovine neutrophils. In other words, bovine neutrophils had varying levels of effectiveness against different bacterial strains, with SAG and WPA1 being the most susceptible and SAU, SUB, SHO, SCH, and WPA2 being more resistant to neutrophil-mediated killing.

3.6. Major and minor pathogens in bovine mastitis were able to trigger neutrophil extracellular trap (NET) formation

When extracellular pathogens are present, bovine neutrophils may release structures containing genetic materials, granule enzymes, and noxious chemicals to devastate microbes in their vicinity. As with other bovine neutrophils, we used HBSS as a control or bovine mastitis-causing bacteria to induce neutrophil extracellular trap (NET) formation under culture conditions. The NET releases were then measured using a fluorescent plate reader. The release of NETs into the supernatant was determined to be 127.4% ± 8.86% (SAU), 117.7% ± 5.32% (SAG), 121.3% ± 6.30% (SUB), 121.2% ± 5.04% (SHO), 126.7% ± 6.10% (SCH), 128.9% ± 5.45% (WPA1), and 140.6% ± 15.65% (WPA2), respectively (Figure 3b). The NET formed spontaneously by non-stimulated cells (100% ± 1.86%) was statistically significant when compared to cells stimulated with bacteria ($p = 0.0011$, Figure 3b). As expected, our DipQuick stained slides showed that some bovine neutrophils activated with bacteria disseminated NET-like fibrous structures (Figure 3c).

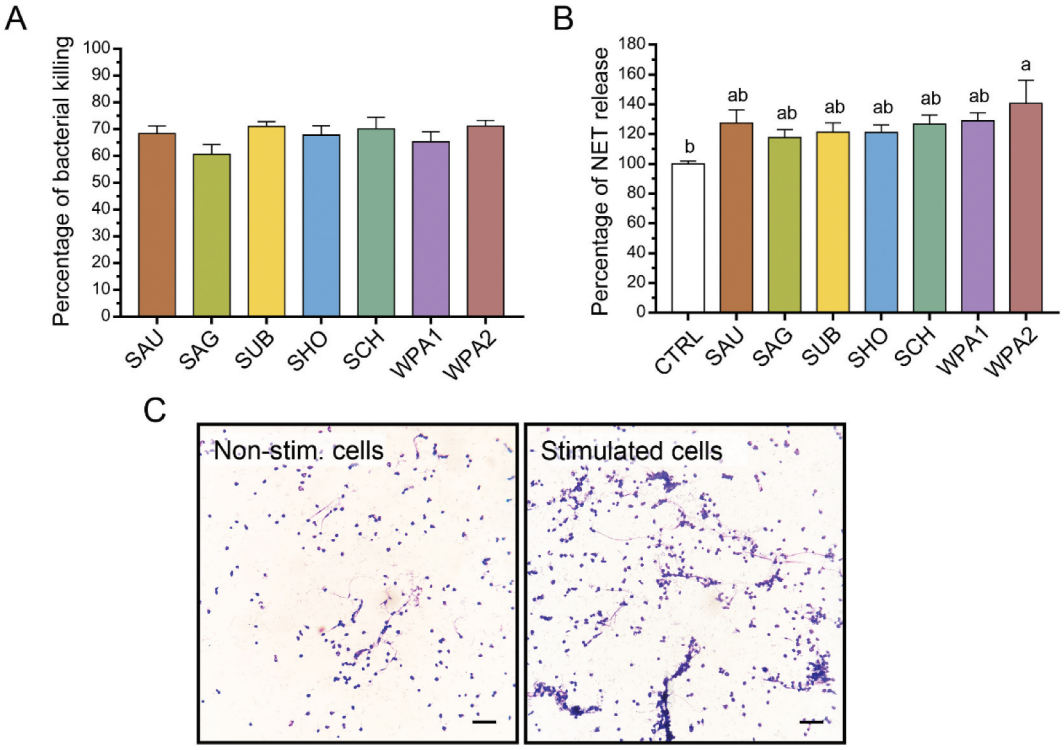

Figure 3. The killing ability of bovine neutrophils did not differ substantially among bacteria-causing mastitis but was significantly higher in extracellular NET release. (a) The percentages of bacterial killing as measured by the MTT assay are above 60% for all bacteria examined. Data are present as mean ± SEM from ten cows ($n = 15$) of three independent experiments, analysed using one-way ANOVA. (b) Quantification of bovine NET in supernatant following various bacterial stimulation. The percentage of NET release significantly increased in all bacteria examined compared with cells received HBSS. Data are present as mean ± SEM from ten cows ($n = 12$) of three independent experiments, analysed with Kruskal-Wallis test followed by Dunn's multiple comparison. (c) Representative images of bovine NETs induced *in vitro* by HBSS (Left) or live *Streptococcus uberis*. NETs are visualized by staining with DipQuick (magnification × 10, scale bar = 100 µm). Different letters indicate statistically significant differences at $p < 0.05$. Bars with no common letters are significantly different ($p < 0.05$). Note: CTRL = non-stimulated cells, SAU = *Staphylococcus aureus*, SAG = *Streptococcus agalactiae*, SUB = *Streptococcus uberis*, SHO = *Staphylococcus hominis*, SCH = *Staphylococcus chromogenes*, WPA = *Weissella paramesenteroides*

3.7. Major and minor pathogens mediated transcriptional responses of bovine neutrophils in cellular functions and apoptosis in response to harmful stimuli

We described here altered patterns of gene expression in bovine neutrophils following the induction of major and minor pathogens in bovine mastitis based on measurements of mRNA levels. The genes involved in pro-inflammation (*IL1B*, *IL6*, *TNF, CXCL8*), ROS (*NOX1*, *SOD1*), phagocytosis (*LAMP1, CORO1A*), chemotactic factor (*CXCL8*), pathogen recognition/activation of innate immunity (*TLR1*, *TLR2*, *TLR6*), and programmed cell death or apoptosis (*FAS*, *BAX*, *BCL2*, *BCL2L1*, *CASP3*, *CASP9*, *CFLAR*) were analysed for the levels of expression among bacterial challenges. Due to the fact that bacterial challenge causes the expression of pro-inflammatory genes, we found a significant difference in the overexpression of pro-inflammatory genes (*IL1B, TNF, CXCL8*), which are required to maintain the characteristics of innate immune responses at the udder immunity *in vivo* ($p < 0.05$, Table 1 and Figure 4a). Notably, IL6, known for stimulating acute phase protein synthesis and pro-inflammation, was down-regulated in most bacteria challenges, but the significance was observed compared with non-stimulated cells ($p < 0.05$, Table 1 and Figure 4a). Pathogen recognition receptor (TLR) activations for a wide spectrum of TLR stimuli, in this case, genes for Gram-positive bacteria, namely *TLR1*, *TLR2*, and *TLR6*, were determined. As a prerequisite for innate immune cells to recognize the microbe-associated molecular pattern, the *TLR1*, *TLR2*, and *TLR6* genes were significantly up-regulated in bovine neutrophils challenged with either major or minor pathogens compared to non-stimulated cells ($p < 0.05$, Table 1 and Figure 4a).

To further investigate genes involved in ROS production, we selected the NADPH oxidase (*NOX1*) and superoxide dismutase (*SOD1*) genes in this study. The results confirmed that the transcript levels of *NOX1* and *SOD1* significantly increased in the expression levels following encounters with different types of bacterial species committed to bovine mastitis ($p < 0.05$, Table 1 and Figure 4a). Phagocytosis is an

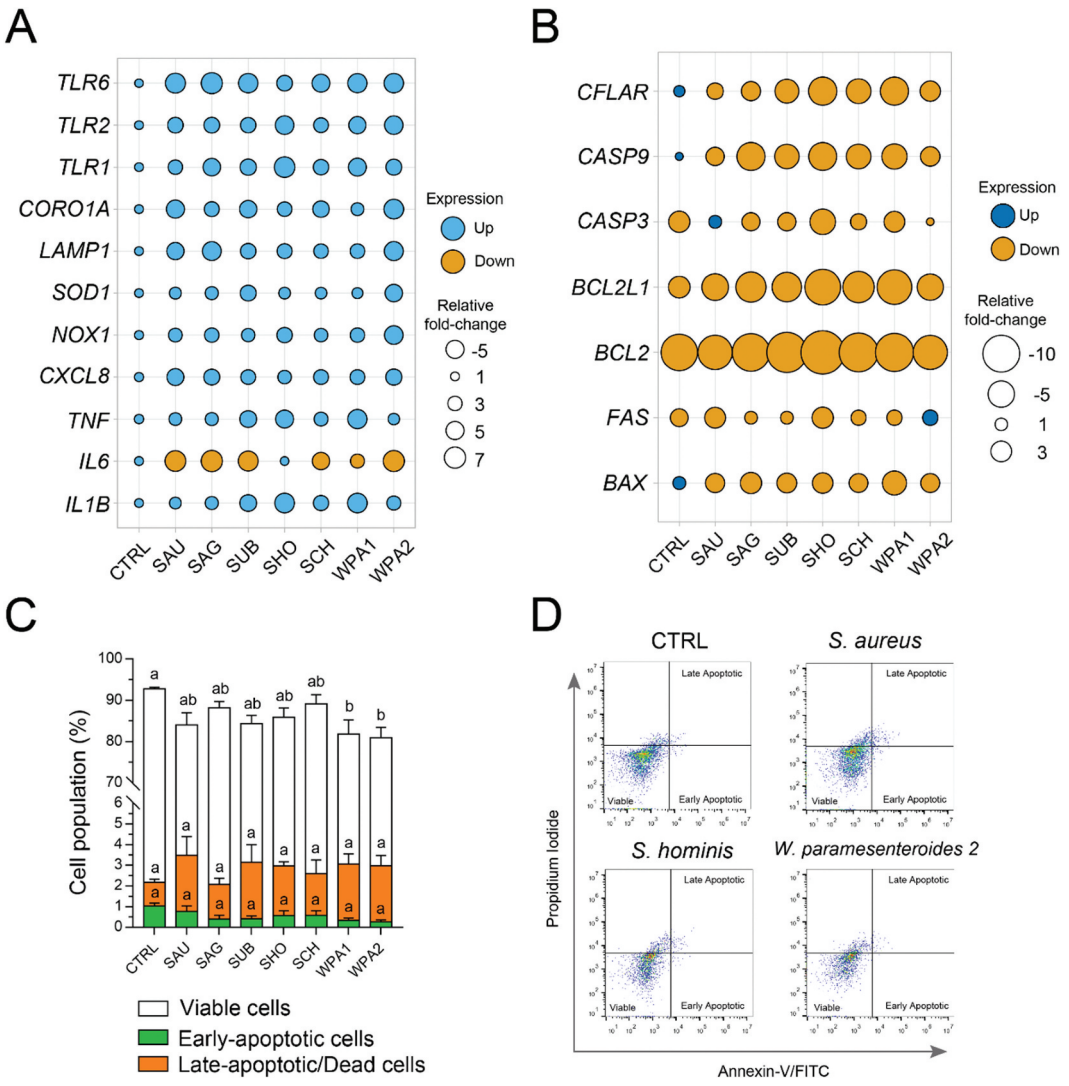

Figure 4. Bovine neutrophils up-regulated genes in functional categories in response to bacterial stimulation; however, they had no effect on genes implicated in cellular apoptosis. (a) Bubble plot showing up-regulated and down-regulated expression of genes related to functions in bovine neutrophils stimulated with various bacteria or non-stimulated (CTRL). Pro-inflammatory genes: *IL1B*, *IL6*, *TNF*, *CXCL8*. ROS-related genes: *NOX1*, *SOD1*. Phagocytosis-related genes: *LAMP1*, *CORO1A*. Migration-related gene: *CXCL8*. Pathogen recognition genes: *TLR1*, *TLR2*, *TLR6*. Scaled relative fold-change is shown by dot size. Dot colours represent expression type. (b) Bubble plot showing the relative fold-change (depicted by dot size) and expression type (depicted by dot colour) of apoptosis-related genes in bovine neutrophils stimulated with various bacteria or control cells. Pro-apoptotic gene: *BAX*. Anti-apoptotic genes: *BCL2*, *BCL2L1*. Cell surface death receptor gene: *FAS*. Execution-phase of cell apoptotic genes: *CASP3*, *CASP9*, *CFLAR*. (c) Proportions of viable (Annexin V^-PI^-), early-apoptotic (Annexin V^+PI^-), and late-apoptotic/dead cells (Annexin V^+PI^+) of bovine neutrophils as determined by flow cytometry after incubation with various bacteria or left untreated (CTRL). Data are present as mean ± SEM from ten cows ($n = 10$) of two independent experiments. Statistical analysis was performed using one-way ANOVA and Tukey's multiple comparison test. (d) Representative flow cytometry staining for Annexin V and Propidium Iodide (PI) on bovine neutrophils exposed to bacteria or left unstimulated for 45 min, the same data as of (c). Bars with no common letters are significantly different ($p < 0.05$). Note: CTRL = non-stimulated cells, SAU = *Staphylococcus aureus*, SAG = *Streptococcus agalactiae*, SUB = *Streptococcus uberis*, SHO = *Staphylococcus hominis*, SCH = *Staphylococcus chromogenes*, WPA = *Weissella paramesenteroides*

essential component of the innate immune response. Next, we monitored the differences in the expression of genes involved in phagocytosis, *LAMP1*, and *CORO1A* to assess their function in phagolysosome biogenesis. Upon encountering those bacteria, bovine neutrophils significantly up-regulated the expression of specific genes involved in phagocytosis ($p < 0.05$, Table 1 and Figure 4a).

Phagocytosis, followed by the intracellular destruction of engulfed bacteria, is a crucial defence mechanism of bovine neutrophils during mastitis pathogenesis. This aforementioned event may lead to the apoptotic cell death of neutrophils. Therefore, we speculate that the altered expression of genes involved in cellular apoptosis may contribute to the longevity of bovine neutrophils during mastitis. The expression profiles of genes involved in cellular apoptosis were examined in pro- or anti-apoptotic regulators (*BAX*, *BCL2*, *BCL2L1*), cell surface death receptor (*FAS*), and execution-phase of cell apoptosis (*CASP3*, *CASP9*, *CFLAR*) of non-stimulated and stimulated bovine neutrophils. Cell death may result

Table 1. Results of the one-way ANOVA and Tukey's multiple comparison tests of mRNA expression of bovine neutrophil function-associated genes by real-time PCR performed on bovine neutrophils stimulated with various mastitis-causing pathogens.

Gene	CTRL	SAU	SAG	SUB	SHO	SCH	WPA1	WPA2	*P*-value
IL1B	0.99 ± 0.56^{b}	1.94 ± 0.99^{b}	2.46 ± 1.09^{b}	4.16 ± 0.37^{ab}	5.99 ± 0.30^{a}	3.39 ± 0.44^{ab}	5.94 ± 0.61a	2.86 ± 1.11ab	0.0001
IL6	1.02 ± 1.00^{ab}	-6.67 ± 1.34^{c}	-7.07 ± 1.57^{c}	-6.17 ± 1.05^{c}	0.98 ± 1.44^{ab}	-4.60 ± 1.89^{bc}	2.83 ± 0.94a	−6.93 ± 1.58c	<0.0001
TNF	1.14 ± 0.31^{c}	2.31 ± 0.73^{bc}	2.43 ± 1.10^{bc}	4.20 ± 0.64^{abc}	4.91 ± 0.29^{ab}	2.79 ± 0.34^{abc}	5.71 ± 0.38a	1.82 ± 1.24bc	0.0005
CXCL8	1.09 ± 0.16^{c}	4.36 ± 0.20^{a}	3.75 ± 0.48^{ab}	3.17 ± 0.34^{ab}	2.64 ± 0.26^{b}	3.22 ± 0.62^{ab}	3.22 ± 0.22ab	3.90 ± 0.23ab	<0.0001
NOX1	1.06 ± 0.15^{c}	2.74 ± 0.43^{bc}	3.03 ± 0.76^{abc}	2.56 ± 0.63^{bc}	3.48 ± 0.61^{ab}	2.63 ± 0.65^{bc}	3.04 ± 0.48abc	5.33 ± 0.21a	0.0006
SOD1	0.98 ± 0.33^{c}	2.00 ± 0.24^{bc}	2.46 ± 0.40^{bc}	3.73 ± 0.55^{ab}	1.93 ± 0.59^{bc}	1.89 ± 0.21^{bc}	1.64 ± 0.35c	4.66 ± 0.56a	0.0001
LAMP1	1.00 ± 0.23^{c}	4.53 ± 0.31^{ab}	5.48 ± 0.40^{a}	3.52 ± 0.57^{ab}	3.25 ± 0.79^{b}	3.07 ± 0.52^{bc}	3.12 ± 0.54bc	5.59 ± 0.32a	<0.0001
CORO1A	1.01 ± 0.36^{d}	5.02 ± 0.23^{ab}	3.26 ± 0.82^{bcd}	4.57 ± 0.30^{abc}	3.09 ± 0.84^{bcd}	4.02 ± 0.23^{abc}	2.35 ± 0.71 cd	6.01 ± 0.27a	<0.0001
TLR1	1.01 ± 0.22^{c}	3.04 ± 0.68^{bc}	4.68 ± 0.67^{ab}	3.91 ± 0.54^{b}	6.62 ± 0.23^{a}	3.50 ± 0.54^{bc}	4.92 ± 0.43ab	3.75 ± 0.95b	<0.0001
TLR2	1.01 ± 0.26^{b}	3.61 ± 0.49^{ab}	3.63 ± 0.99^{ab}	3.46 ± 0.69^{ab}	5.31 ± 0.55^{a}	3.19 ± 0.62^{ab}	4.50 ± 0.42a	5.25 ± 0.55a	0.0007
TLR6	0.92 ± 0.35^{c}	6.20 ± 0.50^{a}	7.02 ± 0.79^{a}	5.86 ± 0.47^{a}	3.60 ± 0.45^{b}	4.90 ± 0.49^{ab}	5.70 ± 0.52ab	5.85 ± 0.19a	<0.0001

CTRL = non-stimulated cells, SAU = *Staphylococcus aureus*, SAG = *Streptococcus agalactiae*, SUB = *Streptococcus uberis*. SHO = *Staphylococcus hominis*, SCH = *Staphylococcus chromogenes*, WPA = *Weissella paramesenteroides*.

[a]Any means within a row with shared superscript letters are not significantly different from each other at $p < 0.05$ with Tukey's multiple comparison test.

from an increase in death receptors, pro-apoptotic genes, and the execution phase of cell apoptosis. To detect the changes in the aforementioned gene expressions, we measured the mRNA levels of *FAS*, *BAX*, CASP3, *CASP9*, and *CFLAR* with real-time PCR (qPCR). As shown in Figure 4b and Table 2, all samples with *BAX*, *CASP9*, and *CFLAR* mRNA levels was significantly decreased after bacterial induction in bovine neutrophils ($p < 0.05$). On the other hand, most samples in *FAS* and *CASP3* were significantly down-regulated, except for the samples from SAU in *CASP3* and SAU and WAP2 in *FAS* that were up-regulated in respective genes (Table 2 and Figure 4b). In terms of gene expression for the suppression of apoptosis, *BCL2* and *BCL2L1* were significantly down-regulated in all sample testes compared with non-stimulated cells ($p < 0.05$, Table 2 and Figure 4b). To recapitulate the expression patterns described above, bubble plots generated using the qPCR data in Table 2 of relative mRNA abundance after exposure of cells to each bacterium were illustrated (Figure 4b).

In this work, flow cytometry analysis revealed that the bacterial stimulation of bovine neutrophils had no discernible effects on early ($p = 0.087$, Figure 4c) or late apoptosis ($p = 0.388$, Figure 4c), as compared to the non-stimulated control. The mean percentages of bacteria-stimulated cells undergoing early and late apoptosis were less than 1% and 3%, respectively. These findings potentially point to a good correlation between the proportion of apoptotic cells as quantified using flow cytometry (Figure 4d) and a set of genes implicated in pro- and anti-apoptosis, as well as the execution phase of apoptosis, as noted earlier (Figure 4c).

3.8. Correlation and principal component analysis (PCA) based on functional responses and gene expression data indicated that parameters were highly correlated, resulting in the identification of bacterial groupings that clustered together

Figure 5 5a depicts the overlap between neutrophil cellular activities and gene expressions studied *in vitro* challenges with major and minor mastitis-causing bacteria. Although the correlation coefficient has a broad range of numbers, the majority of the cellular function groups – including ROS, phagocytosis, MTT bacterial killing, and NET – have substantial correlations ($r = 0.25$ to 0.86, $p < 0.05$) with the associated functional gene expressions (*CXCL8*, *NOX1*, *SOD1*, *LAMP1*, *CORO1A*). Pathogen recognition (*TLR1*, *TLR2*, *TLR6*, $r = 0.1$ to 0.88, $p < 0.05$) and pro-inflammatory genes (*IL1B*, *TNF*, *CXCL8*, $r = 0.13$ to 0.55, $p < 0.05$) exhibited similar patterns, with the exception of IL6, which exhibited a negative association ($r = -0.56$ to -0.38, $p < 0.05$). Not surprisingly, the apoptotic gene expressions (*BAX*, *FAS*, *BCL2*, *BCL2L1*, *CFLAR*, *CASP3*, *CASP9*) were among the groups with the strongest negative correlation to

Table 2. Results of the one-way ANOVA and Tukey's multiple comparison tests of mRNA expression of apoptosis-associated genes by real-time PCR performed on bovine neutrophils stimulated with various mastitis-causing pathogens.

Gene	CTRL	SAU	SAG	SUB	SHO	SCH	WPA1	WPA2	*P*-value
BAX	1.06 ± 0.35^{a}	-2.57 ± 0.52^{b}	-3.19 ± 0.33^{bc}	-2.74 ± 0.29^{bc}	-2.95 ± 0.29^{bc}	-2.46 ± 0.20^{b}	−4.14 ± 0.24c	−2.49 ± 0.42b	<.0001
BCL2	-9.79 ± 0.20^{ab}	-8.66 ± 0.49^{a}	$-10.46 + 0.30^{b}$	-11.96 ± 0.31^{c}	-14.01 ± 0.30^{d}	-11.18 ± 0.30^{bc}	−1.77 ± 0.27bc	−8.55 ± 0.42a	<.0001
BCL2L1	-3.32 ± 0.84^{a}	-5.14 ± 0.15^{ab}	-6.63 ± 0.30^{bc}	-6.32 ± 0.33^{bc}	-9.39 ± 0.29^{e}	-7.17 ± 0.42^{cd}	−8.82 ± 0.28de	−4.97 ± 0.37ab	<.0001
FAS	2.19 ± 0.69^{a}	3.04 ± 0.37^{a}	-1.05 ± 0.30^{b}	-1.05 ± 0.32^{b}	-3.18 ± 0.28^{c}	-1.53 ± 0.22^{bc}	−1.55 ± 0.10bc	1.58 ± 0.46a	<.0001
CASP3	3.17 ± 0.44^{a}	1.07 ± 0.46^{b}	-2.26 ± 0.32^{d}	-2.31 ± 0.41^{d}	-4.69 ± 0.35^{e}	-1.75 ± 0.21^{cd}	−3.03 ± 0.13d	−.32 ± 0.28bc	<.0001
CASP9	0.35 ± 0.47^{a}	-2.32 ± 0.36^{b}	-5.72 ± 0.35^{d}	-4.28 ± 0.43^{c}	-5.67 ± 0.15^{cd}	-4.38 ± 0.24^{cd}	−4.59 ± 0.26 cd	−2.64 ± 0.12b	<.0001
CFLAR	0.77 ± 0.67^{a}	-1.82 ± 0.73^{ab}	-2.56 ± 0.28^{b}	-4.06 ± 0.52^{bc}	-5.81 ± 0.42^{c}	-4.39 ± 0.98^{bc}	−5.77 ± 0.98c	−2.88 ± 0.86bc	<.0001

CTRL = non-stimulated cells, SAU = *Staphylococcus aureus*, SAG = *Streptococcus agalactiae*, SUB = *Streptococcus uberis*. SHO = *Staphylococcus hominis*, SCH = *Staphylococcus chromogenes*, WPA = *Weissella paramesenteroides*.

[a]Any means within a row with shared superscript letters are not significantly different from each other at $p < 0.05$ with Tukey's multiple comparison test.

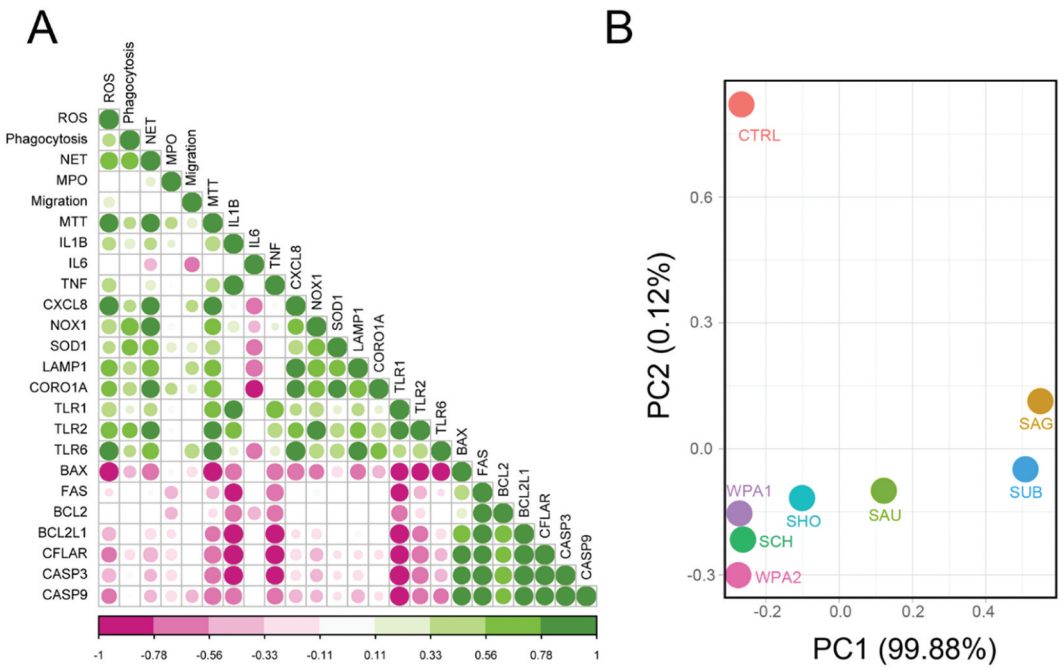

Figure 5. Gene expression patterns connect with neutrophil activities, and principal component analysis (PCA) identifies unique clusters of mastitis-causing bacteria. (a) Correlation matrix between variables (average value of bovine neutrophil functional assays: ROS, phagocytosis, NET, MPO, migration, MTT and mean expression value of each individual gene: *IL1B*, *IL6*, *TNF*, *CXCL8*, *NOX1*, *SOD1*, *LAMP1*, *CORO1A*, *TLR1*, *TLR2*, *TLR6*, *BAX*, *FAS*, *BCL2*, *BCL2L1*, *CFLAR*, *CASP3*, *CASP9*) of cells stimulated with various bacteria and non-stimulated cells. Green circles represent positive correlations and pink circles represent negative correlations. Size of circles indicate the strength of the correlation, while in the matrices without circle indicate no statistical significance of correlations ($p > 0.05$). The colour bar at the bottom provides a scale of the correlation coefficients analysed with Person's correlation test. To provide a clean presentation, the matrix views show only the lower portion of the matrix for clarity. (b) Principal Component Analysis (PCA) of variable data derived from bovine neutrophils stimulated with various bacteria or left non-stimulated, the same data as of (A). Position of neutrophil-stimulated with each of bacteria or CTRL in the space defined by the first two principal components. The proportion of variance captured is given as a percentage for both the 1st (PC1) and 2nd component (PC2). The first two PCs accounted for more than 99% of variance. PCA reveals that all minor pathogens (SHO, SCH, WPA1, WPA2) of mastitis cluster closely, major pathogens (SAG, SUB) cluster together, one major pathogen (SAU) cluster between those two groups, and CTRL is distinct from the others. Note: CTRL = non-stimulated cells, SAU = *Staphylococcus aureus*, SAG = *Streptococcus agalactiae*, SUB = *Streptococcus uberis*, SHO = *Staphylococcus hominis*, SCH = *Staphylococcus chromogenes*, WPA = *Weissella paramesenteroides*

cellular functioning ($r = -0.67$ to 0, $p < 0.05$). The correlation coefficient for the entire correlation matrix is provided in Supplementary Figure S1. Principal component analysis, produced from the data for correlation analysis, indicated that practically all of the minor pathogens (SHO, SCH, WPA1, and WPA2) in bovine mastitis grouped independently from the major pathogens, but SAU was more closely associated with those from the minor pathogens (Figure 5b).

4. Discussion

Controlling bacterial strains responsible for initiating IMI is crucial for balancing pathogen invasions that lead to mastitis. The solid innate immune responses of the host are another essential factor in reducing the harmful effects of mastitis-causing pathogens. Previous studies have shown that bovine neutrophil activities can influence major mastitis-causing bacteria [18,19,22,23,28]. These findings highlight the significant impact of bovine neutrophils on the growth and pathogenesis of primary mastitis agents, but their complete neutrophil responses to minor pathogens remain unclear. Our findings indicate that two strains of *W. paramesenteroides* being investigated in the same experimental setting have a reduced array of defence mechanisms against neutrophil phagocytosis compared to other bacteria. Certain bacteria possess defence mechanisms that allow them to resist intracellular killing by phagocytes. These mechanisms include biofilm production (e.g. *S. aureus*, *S. chromogenes*, *S. hominis*, *S. simulans*, and *W. paramesenteroides*) and the synthesis of polysaccharide/protein capsules (e.g. *S. aureus* and *S. uberis*) to prevent complement activation and killing [11,15,39–42].

Staphylococcus aureus and *S. agalactiae* possess virulence factors associated with the invasion of host and immune cells, unlike the LAB strains, which lack mastitis-related virulence factor genes [40,42,43]. In our study, *S. uberis* with a capsular phenotype was used, and despite its potential capsule production, it exhibited a higher phagocytosis percentage than other major mastitis pathogens. However, this variation may

not remain true in an *in vivo* investigation. On the other hand, both NASM strains used in this study (*S. chromogenes* and *S. hominis*) showed a lower percentage of phagocytosis, suggesting their potential for biofilm formation, although the mechanisms require further investigation. Certain NASM strains (e.g. *S. chromogenes*) have demonstrated the ability to suppress major mastitis-causing bacteria through the secretion of antimicrobial peptides, specifically bacteriocin [7,44]. Bacterial activation prompts neutrophils to release antimicrobial peptides for mastitis-causing bacteria elimination. Neutrophils also secrete many inflammatory cytokines to recruit other immune cells to the udder [16,45].

During our study, we observed the release of myeloperoxidase (MPO) towards all pathogens. Interestingly, we discovered that MPO was notably secreted against *S. hominis*, despite being a minor pathogen. Additionally, when stimulated by *S. chromogenes* and *W. paramesenteroides*, we observed sustained or increased intracellular MPO levels (Figure 2f). The observed cell migration in our studies may be linked to the findings of MPO degranulation. Increased intracellular levels of MPO may negatively regulate neutrophil migration and degranulation [46]. Consistent with previous neutrophil functional assays, we observed activated bovine neutrophils displaying similar migratory patterns towards the major bacteria responsible for mastitis and one minor bacterium (*S. hominis*). Based on our findings, minor bovine mastitis pathogens exhibited limited ability to attract neutrophils. While certain virulence factors are absent in LABs [43], we still require a deeper understanding of alternative mechanisms and signalling pathways that facilitate neutrophil recruitment through interactions with pathogens. The regulation of immunological responses by NASM and LAB strains under field conditions may contribute to this phenomenon, but further investigation is needed to establish the underlying facts.

Since its discovery nearly two decades ago, NETs have emerged as a crucial extracellular microbiological killing mechanism in bovine mastitis pathogenesis. The formation and function of bovine NETs against the primary pathogens causing mastitis have been documented [18,19,47]. However, it remains unclear whether activated bovine neutrophils would release NETs when encountering minor mastitis pathogens in similar conditions. We demonstrated, for the first time, that minor mastitis pathogens (*S. hominis*, *S. chromogenes*, and *W. paramesenteroides*) induce the formation of NETs, similar to major mastitis-causing pathogens. NET release requires ROS production via the NADPH oxidase pathway activation and up-regulation of anti-apoptotic proteins [48]. Moreover, H_2O_2-producing LAB can impact NET formation in conjunction with neutrophil death [43]. As previously mentioned, all mastitis-causing bacteria in our study generate significant amounts of ROS, suggesting that ROS may play a crucial role in mediating NET formation in both major and minor pathogens.

Pro-inflammatory cytokines mediate pain and inflammation in the mammary gland, both locally and systemically [49]. Our findings indicate that bovine neutrophils differentially regulate pro-inflammatory cytokines (IL1B, IL6, TNF, and CXCL8) in response to the examined microorganisms. Previous studies have also demonstrated that key mastitis-causing bacteria are associated with the expression of pro-inflammatory genes and the modulation of apoptotic cell death through Fas, Casp3, CFLAR, Bcl-2, Bcl2l1, and ROS [18,19,50]. Our *IL-6* gene expression results reveal a distinct down-regulation pattern in neutrophils stimulated by major and select minor bacteria. This observation can be attributed to the different role of IL-6 in the inflammatory process compared to IL-1β. IL-6 facilitates the transition from neutrophil influx to monocytes and mitigates the adverse effects of neutrophils while other pro-inflammatory processes are still active [49]. These changes in gene expression are vital for optimizing cellular function and managing damage. We have identified a correlation between persistent gene expression patterns and the regulation of cellular activity (Figure 5a). In our current study, all bacterial stimuli created favourable conditions for the majority of the gene expressions (*NOX1*, *SOD1*, *CXCL8*, *LAMP1*, *CORO1A*, *TLR1*, *TLR2*, and *TLR6*), which contributed to positive effects on cellular functioning. Various downstream cellular processes, regulated by these related genes, depend on activating NF-κB and MAPK signalling pathways for signal transduction [42,51,52]. However, the complete molecular mechanisms governing ROS, phagocytosis, migration, pathogen recognition, and other cellular functions in response to major and minor bacterial stimuli in bovine neutrophils remain incompletely understood.

Apoptosis of neutrophils is crucial in resolving inflammation in humans and cows [45]. Within the context of bovine mastitis, our knowledge of bovine neutrophil cell death after being stimulated by pathogens continues to be constrained. The stimulation of TLR4 via LPS is connected to lowered cell death in bovine neutrophils, as reported by Bassel and Caswell (2018) [17]. In contrast, the immune response is slow, and cell death is postponed when encountering Gram-positive bacteria, since they either fail to activate or activate to a lesser extent in TLR signalling [1,42]. Our findings regarding apoptotic-related gene expressions align with the results mentioned above. We demonstrate that both major and minor pathogens associated with bovine mastitis suppress neutrophil apoptosis. Specifically, they affect receptor and pro-apoptotic

(*FAS*, *BAX*), anti-apoptotic (*BCL2*, *BCL2L1*), and execution-phase genes involved in cell apoptosis (*CASP3*, *CASP9*, *CFLAR*). Delaying neutrophil apoptosis can potentially prolong their lifespan, allowing them more time to exert antimicrobial activities during the early stages of infection [17]. However, this delay may also increase the risk of neutrophil-mediated tissue damage. Accelerating neutrophil apoptosis may offer advantages once the infectious agent has been cleared.

Our results show that blood neutrophils are a valuable tool for evaluating their functions against different major and minor mastitis-causing bacteria; nevertheless, it is important to be aware that milk neutrophils may present more accurate and reliable results. Therefore, it is necessary to acknowledge and address the limitations in our study.

5. Conclusions

Major mastitis-causing pathogens, such as *S. aureus*, in conjunction with minor pathogens, such as *S. hominis* and *Weissella paramesenteroides*, can contribute to bovine mastitis, depending on the circumstances. Bovine neutrophils must be able to effectively recognize bacteria and respond appropriately to changes in the microbiome within the mammary gland. Our data showed that bovine neutrophils migrated preferentially towards major pathogens, i.e. *Staphylococcus* and *Streptococcus*, but had more robust intracellular defence mechanisms against *Weissella*, including phagocytosis, ROS generation, degranulation, and NET discharge. In addition, our results showed that minor pathogens (*S. hominis* and *W. paramesenteroides*) induced specific changes in the gene expressions involved in pathogen recognition and inflammation (*IL1B*, *IL6*, *TNF*, *TLR1*, *TLR2*). These changes may be linked to the ability of bovine neutrophils to carry out the effector functions mentioned above. Our results also indicated that genes involved in apoptosis (*BAX*, *BCL2*, *BCL2L1*, *FAS*, *CASP3*, *CASP9*, *CFLAR*) were greatly reduced in bovine neutrophils incubated with minor pathogens. This suggests that bovine neutrophils prioritize their antimicrobial functions over initiating apoptosis. The regulation of cellular longevity appears to be influenced during the response to bacteria; however, further investigation is necessary to fully understand this phenomenon. In conclusion, our supporting evidence and perspective have clearly revealed that neutrophil responses to minor pathogens (*Weissella* and *S. hominis*) were essentially comparable to their response towards major pathogens. Our findings imply that studying minor bacteria is essential and should not be neglected in the context of bovine mastitis research.

Disclosure statement

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