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Selective IgG binding to the LPS glycolipid core found in bovine colostrum, or milk, during *Escherichia coli* mastitis influences endotoxin function

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Abstract

The dynamic interplay between intramammary IgG, formation of antigen-IgG complexes and effector immune cell function is essential for immune homeostasis within the bovine mammary gland. We explore how changes in the recognition and binding of anti-LPS IgG to the glycolipid "functional" core in milk from healthy or clinically diagnosed *Escherichia coli (E. coli)* mastitis cows' controls endotoxin function. In colostrum, we found a varied anti-LPS IgG repertoire and novel soluble LPS/IgG complexes with direct IgG binding to the LPS glycolipid core. These soluble complexes, absent in milk from healthy lactating cows, were evident in cows diagnosed with *E. coli* mastitis and correlated with endotoxin-driven inflammation. *E. coli* mastitis milk displayed a proportional reduction in anti-LPS glycolipid core IgG compared to colostrum. Milk IgG extracts showed that only colostrum IgG attenuated LPS induced endotoxin activity. Furthermore, LPS-stimulated reactive oxygen species (ROS) in milk granulocytes was only suppressed by colostrum IgG, while IgG extracts of neither colostrum nor *E. coli* mastitis milk influenced N-formylmethionine-leucyl-phenylalanine (fMLP)-stimulated ROS in LPS primed granulocytes. Our findings support bovine intramammary IgG diversity in health and in response to *E. coli* infection generate milk anti-LPS IgG repertoires that coordinate appropriate LPS innate-adaptive immune responses essential for animal health.

Keywords

Anti-LPS IgG, bovine colostrum, *E. coli* mastitis, endotoxin function, LPS/IgG complexes

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Introduction

During infectious disease, the adaptive humoral immune response generates specific immunoglobulin (Ig) G (IgG) to coordinate defence processes.[1] The formation of immune complexes through antigen binding to the IgG-Fab region, and recognition of IgG-Fc gamma receptors (FcγRs) presented on immune cells directs the well characterised effector immune cell functions, such as complement activation, opsonisation, toxin/viral neutralisation, antibody-dependent cell-mediated cytotoxicity, and the regulation of the inflammatory response.[1–3] The dynamics within the IgG repertoire during a microbial infection will therefore play a vital role not only in determining the function of immune complexes but also during disease progression to ensure the appropriate outcome and return to immune homeostasis.[4–6]

Epithelial cells, during late-stage pregnancy, lining the bovine mammary gland undergo transformation to deliver a unique secretion product, colostrum, to the neonatal calf.[7,8] Colostrum contains a range of essential nutritional and defence compounds. This includes prophylactic IgG, IgA, and IgM antibodies, of which IgG (primarily the IgG1 subclass) being the principal antibody (up to 95% of total Ig).[5] The ingestion of colostrum by the calf is essential to its survival as it supports intestinal and systemic immune functions until maturation of its own immunity in approximately 3–4 weeks.[7,8] The prophylactic nature of colostrum IgG possesses the ability to primarily target molecules within the functional domains of invading microbes to

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form complexes that mask or reduce pathogenicity. Indeed, this has been shown in the neutralisation of endotoxins, although attempts to enhance this passive protective property via immunisation programmes targeting the functional domains of LPS have had mixed outcomes.[9–13] In addition, post-translational changes in the IgG-Fc region during the transformation of colostrum to mature milk appear to actively support the maturation of the calf's immunity and gut microbiota.[14,15] These findings suggest that IgG in milk is dynamic and tailored to mitigate endotoxin inflammation and facilitate maturation of calf immunity.

In adult cows, coordination between innate and adaptive immunity within the mammary gland maintains an immune environment that can quickly respond to any invading pathogens.[16–18] Repeat exposures to microbes, for example *Escherichia coli (E. coli)*, cause a robust adaptive immune response that is accompanied by an influx of IgG antibodies into the mammary gland, paralleling the inflammatory response and the severity of mastitis. *E. coli* infections causing acute mastitis, are primarily found during early lactation. *E. coli* pathogenicity is due to a diverse range of toxins, hemolysins, adhesins, and LPS compounds in the outer bacteria membrane that ultimately underlie endotoxic shock. In addition, the shedding of LPS from gram-negative bacteria during an infection not only has the ability to augment inflammation via activation of the Toll-like receptor 4 (TLR4)/myeloid differentiation factor 2(MD2) (TLR4/MD2) complex present in the epithelial cells lining the mammary gland, but also prime resident intramammary granulocytes to inflammatory signals.[19] Therefore, proportional changes in the *E. coli* mastitis milk antibody repertoire that recognise the LPS glycolipid "functional" core will elicit immune responses to LPS that affect the ability to control coliform infection within the mammary gland.

In this fundamental study, we explore how intramammary IgG diversity in healthy and clinical *E. coli* mastitis infections creates milk antibody repertoires with distinct proportional changes in IgG for the recognition of the LPS "functional" glycolipid core. Furthermore, we explore the subsequent impact on endotoxin activity and the regulation of the oxidative status in milk granulocytes.

Results

Bovine colostrum contains a wide repertoire of IgG that specifically recognises and binds to a variety of gram-negative bacterial shed LPS

We utilised the ionic and hydrophobic attractive forces within the conserved LPS glycolipid core for the antibiotic polymyxin B to characterise milk IgG binding to LPS compounds and its impacts on endotoxin function. Our initial experiments focused on the binding ability of colostrum IgG (from different commercial sources) to LPS using *E. coli* mutant K12-LPS (Figure 1Ai). We observed the recognition and binding of IgG within 3 pooled colostrum extracts (0.1 mg/ml[w/v] total IgG) to *E. coli* mutant K12-LPS in a dose-dependent manner (0.09–10 μg/ml) with a detection limit of ~ 0.31 μg/ml. The 2.5 μg/ml dose of *E. coli* mutant K12-LPS showed mean absorbances (± SD) of 0.87 ± 0.1, 0.92 ± 0.1, and 0.53 ± 0.12 for colostrum #1, #2, and #3, respectively. In contrast, an equivalent dilution (0.1 mg/ml [w/v]) of a pasteurised skim milk preparation at the same dose showed a mean absorbance of 0.01 ± 0.01. The detection specificity and sensitivity of shed LPS from gram-negative bacteria (Figure 1Aii) was explored using bacterial strains commonly identified in bovine mastitis.[20] Utilising heat-induced shed LPS from selected gram-negative bacteria, a dose-dependent (0.001–1 μg/ml, endotoxin activity equivalents) increase in shed LPS was detected, using an IgG enriched colostrum preparation (0.1 mg/ml [w/v] total IgG). The limit of detection was ~ 0.005 μg/ml and a 0.5 μg/ml dose showed mean absorbances (± SD) for LPS shed from *E. coli* of 1.47 ± 0.14, *Serratia marcescens (S. marcescens); 1*.3 ± 0.06, *Klebsiella oxytoca (K. oxytoca);* 1.14 ± 0.23, and *Pseudomonas aeruginosa* (*P. aeruginosa);* 0.75 ± 0.12. In contrast, we failed to detect antigens present in whole bacterial lysates prepared from gram-positive bacteria primarily causing clinical mastitis using this ELISA (*Streptococcus uberis* [*S. uberis*], *Staphylococcus aureus* [*S. aureus*] and *coagulase-negative staphylococci* [CNS]). A 0.5 μg/ml (equivalent [w/v]) dose of these lysates showed mean absorbances ± SD of 0.00 ± 0.01, 0.00 ± 0.01, 0.01 ± 0.01, respectively (Figure 1Aii). Although not the focus of this study, we have detected the presence of IgG antibodies within this colostrum preparation that recognised and bound to antigens within these gram-positive bacterial lysates in a direct ELISA format.

Assay specificity of LPS detection in the ELISA was examined by interfering with the capture of *E. coli* mutant K12-LPS by the immobilised polymyxin B (Figure 1B). Preincubation of polymyxin B (0.75–50 μg/ml) with *E. coli* mutant K12-LPS (2.5 μg/ml) prior to performing the ELISA caused a dose-dependent reduction in *E. coli* mutant K12-LPS detection, with a dose of 12.5 μg/ml showing a ~75% decrease in absorbance ± SD (0.22 ± 0.11) from the *E. coli* mutant K12-LPS control (0.86 ± 0.32), Figure 1Bi. Similarly, a 30 min preincubation of *E. coli* mutant K12-LPS (5 μg/ml) with whole milk (1:10000–1:10 dilutions) caused a dose-dependent decrease in *E. coli* mutant K12-LPS detection (Figure 1Bii). A 1:10 dilution of whole milk reduced *E. coli* mutant K12-LPS detection by 80% absorbance ± SD (0.32 ± 0.21) compared to *E. coli* mutant K12-LPS diluted in buffer alone (1.48 ± 0.37). The binding of colostrum IgG to LPS were further explored by comparing the detection of LPS (i) after being captured by the immobilised polymyxin B ELISA

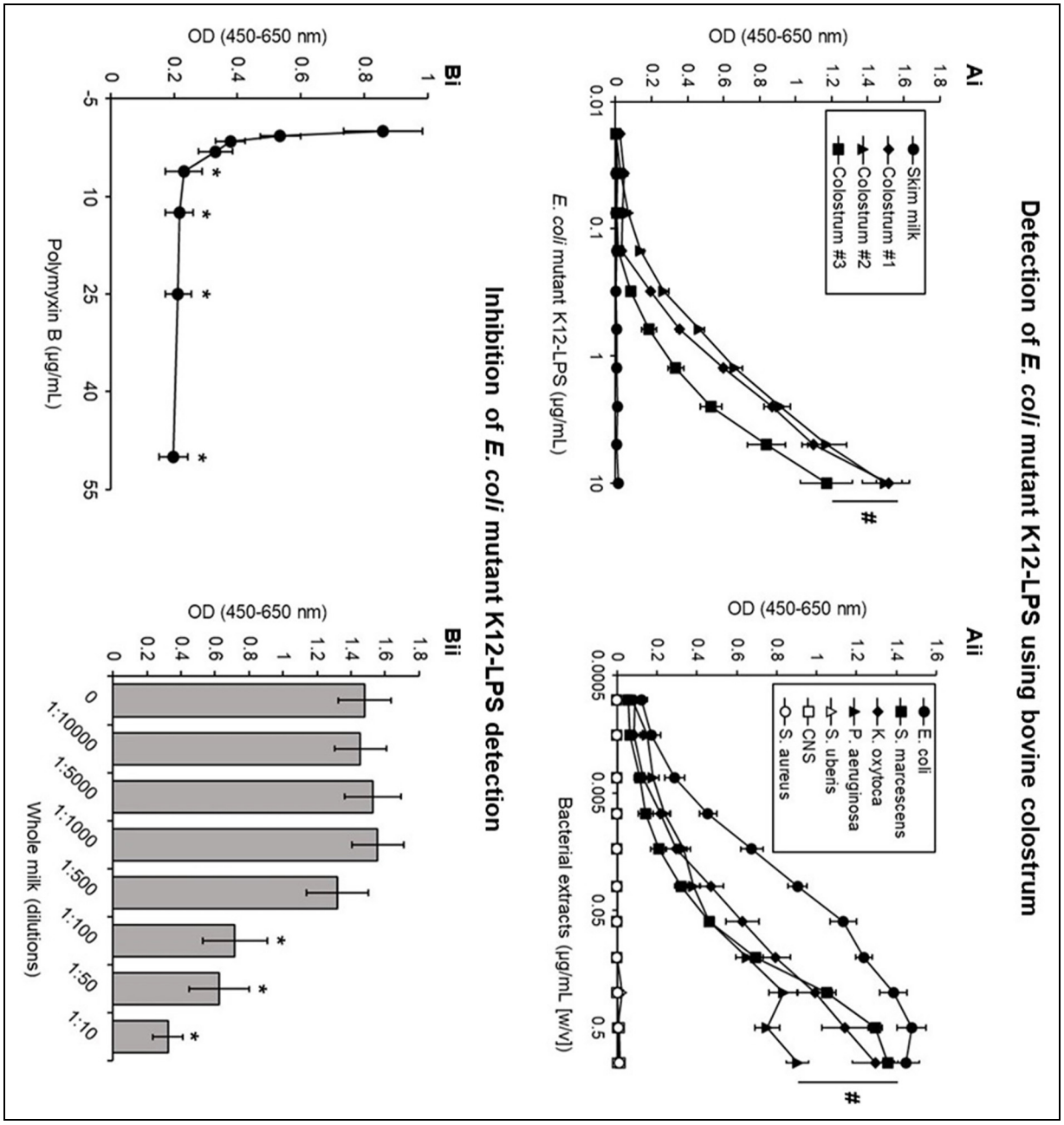

Figure 1. Bovine colostrum IgG binds to LPS from a variety of gram-negative bacteria. [Ai] Detection of *E. coli* mutant K12-LPS (0.009–10 µg/ml), by 3 different commercial bovine colostrum pooled powders (0.1 mg/ml [w/v] total IgG); #1(♦), #2 (▲) or #3 (■) or mg/ml [w/v equ.] antibody-free skimmed milk powder (●), or [Aii] specific detection of gram-negative bacteria shed LPS (0.001–1 µg/ml endotoxin equivalents); *E. coli (*●), *S. marcescens* (■), *K. oxytoca* (♦), *P. aeruginosa* (▲), but not gram-positive bacteria lysate antigens (0.001–1 µg/ml [w/v]); *S. uberis* (Δ), CNS (□), *S. aureus* (○), measured by ELISA. Data is expressed as mean absorbance ± SEM from 6 separate experiments, where # represents statistical ($P < 0.01$) difference from skimmed milk or gram-positive bacteria lysate antigen values, respectively. Assay specificity of LPS detection [Bi] after a 30 min preincubation of polymyxin B (0.75–50 µg/ml) with *E. coli* mutant K12-LPS (2.5 µg/ml) or [Bii] 30 min preincubation with whole milk (dilutions 1:10–1: 10,000) with *E. coli* mutant K12-LPS (5 µg/ml) measured by ELISA. Data is expressed as mean absorbance ± SEM, respectively, from 4 separate experiments; * represents statistical significance ($P < 0.01$) from respective *E.coli* mutant K12-LPS controls.

matrix and (ii) after a 10 min preincubation with colostrum prior to performing the ELISA (Figure 2). We found that *E. coli* shed LPS (0.0009–1 µg/ml) captured by immobilised polymyxin B prior to its detection by a colostrum preparation (0.2 mg/ml [w/v] total IgG) showed a dose-dependent increase in absorbance (± SD) (Figure 2Ai), which became

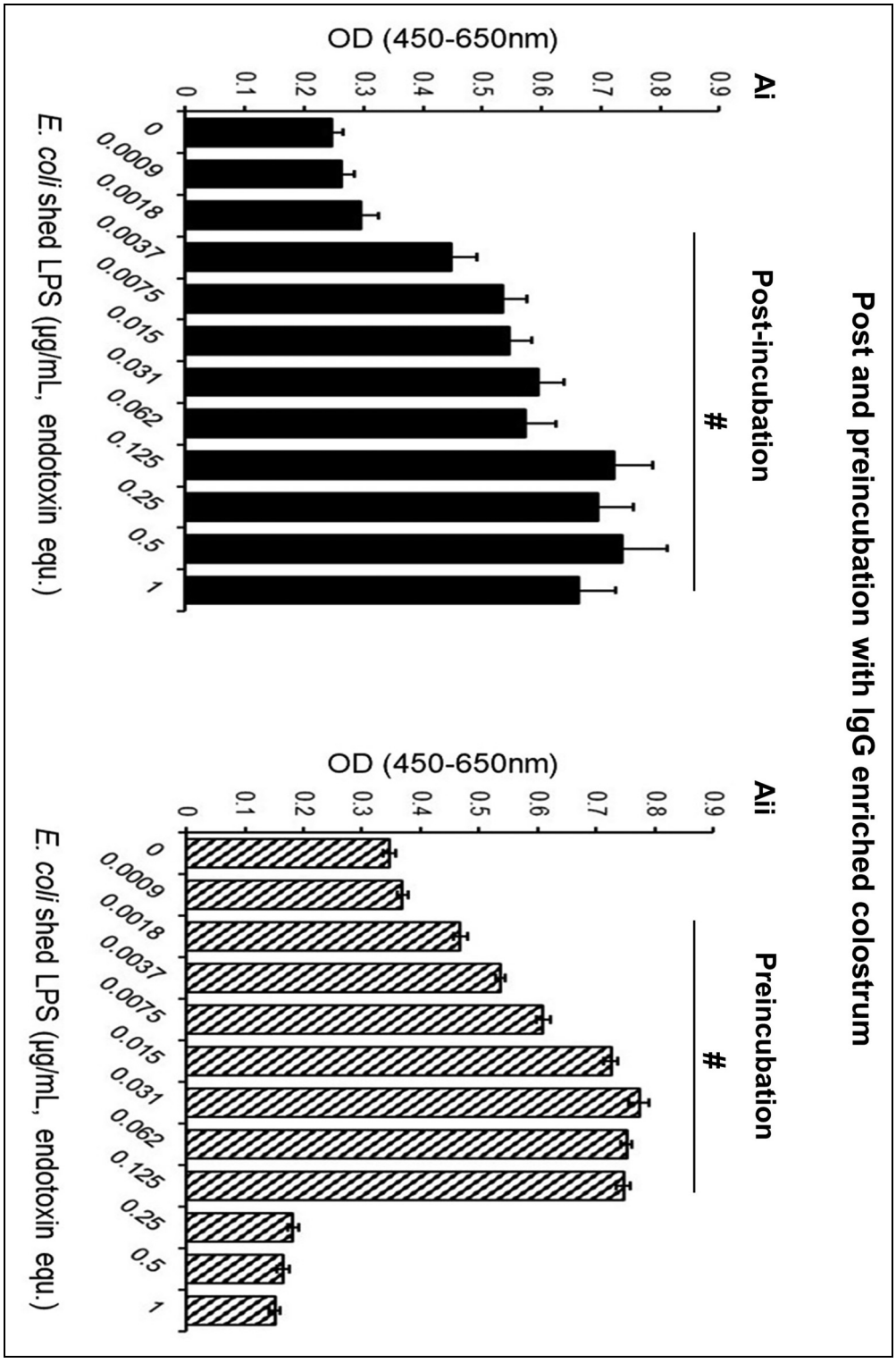

Figure 2. Colostrum IgG forms soluble complexes with *E. coli* shed LPS that are detectable using a polymyxin B capture ELISA. Detection of *E. coli* shed LPS (0.0009–1 µg/ml), using ELISA, [Ai] by colostrum IgG followed by protein G-HRP after initial capture by polymyxin B matrix, or [Aii] using only protein G-HRP after preincubation with colostrum IgG prior to polymyxin B capture. Data is expressed as mean absorbance ± SEM from 6 separate experiments, where # represents statistical ($P < 0.05$) difference from control (no *E. coli* shed LPS).

maximal using 0.125 μg/ml shed LPS (0.75 ± 0.16). In contrast, a preincubation of the shed LPS with the same colostrum preparation prior to its capture by the polymyxin B matrix, and subsequent detection using protein G-HRP only, revealed a bell-shaped detection pattern (Figure 2Aii). Whilst the baseline absorbance (± SD) was elevated (0.34 ± 0.03) compared to capturing the LPS first (0.24 ± 0.04), a dose-dependent (0.0018 to 0.125 μg/ml. $P < 0.05$) increase in absorbance was found when the shed LPS was preincubated with the colostrum. Maximal absorbance (± SD) was observed using 0.031 μg/ml (0.77 ± 0.04). No change in absorbance was identified using shed LPS doses of 0.25 μg/ml and greater.

Soluble LPS/IgG complexes are present in bovine colostrum, but not in the milk of healthy lactating cows

We next examined the presence of soluble LPS/IgG complexes in bovine colostrum and milk collected from healthy lactating cows (Figure 3). Soluble LPS/IgG complexes were detected in 4 out of the 5 individual commercial bovine pooled colostrum preparations (2.5 mg/ml [w/v] total IgG) tested (Figure 3Ai). These 4 pooled colostrum preparations showed a mean absorbance (± SD) of 0.46 ± 0.05 (4 replicates/sample) and ranged between 0.29 ± 0.04 and 0.78 ± 0.05, which were significantly ($P < 0.01$) above the baseline (buffer only) absorbance (0.02 ± 0.02). Assessment of LPS endotoxin activity in the same 5 colostrum preparations, utilising the LAL bioassay (Figure 3Aii), showed a large variation in LAL activity (in un-boiled colostrum preparations, ranging from zero to 3969.74 EU/ml (mean ± SD 1171.76 ± 1686.21 EU/ml). Heat denaturation of the colostrum proteins by boiling (>98 °C) increased the LAL bioactivity within all of colostrum samples, showing an overall 5-fold increase above un-boiled milk levels (5820.23 ± 6650.28 EU/ml, $P < 0.05$). We next evaluated for the presence of soluble LPS/IgG complex levels in aseptically collected milk from healthy lactating cows (Figure 3Bi). Soluble LPS/IgG complex levels in colostrum and milk collected from healthy cows during a 43-week lactation period (used by majority of commercial dairy herds in New Zealand) were only observed in colostrum samples (0.35 ± 0.23). No soluble LPS/IgG complexes were identified in milk collected at early (0.023 ± 0.01), mid (0.021 ± 0.01), or late (just before drying off, 0.025 ± 0.02) lactation time points. Measurement of LAL activity in milk samples from healthy lactating cows (Figure 3Bii) demonstrated a variable endotoxin driven LAL activity (EU/ml ± SD) with 6.9 ± 9.58, 2.5 ± 0.28, 11.48 ± 13.31 EU/ml (± SD), early, mid, or late lactation, respectively. Boiling the milk caused an elevation in milk LAL activity; 45.67 ± 94.10 ($P = 0.11$), 6.31 ± 6.27 ($P = 0.041$), 86.14 ± 151.32 ($P < 0.05$), early, mid, and late lactation, respectively. LAL activity was also evident in the raw colostrum samples tested where un-boiled colostrum levels (13.55 ± 7.27 EU/ml) increased to 189.55 ± 120.15 EU/ml ($P = 0.016$) after boiling. Detection of soluble colostrum LPS/IgG complex by ELISA was further examined using commercial pooled colostrum preparation #4 (Figure 3C). This colostrum preparation showed a dose-dependent (0.075–1.25 mg/ml [w/v] total IgG) presence of soluble LPS/IgG complexes (Figure 3Ci) with a dose of 1.25 mg/ml showing an absorbance (± SD) of 0.88 ± 0.15. Heat denaturation and removal of colostrum proteins reduced the detection of soluble LPS/IgG complex by ~ 96% (0.039 ± 0.02). Furthermore, preincubation of colostrum (0.62 mg/ml [w/v] total IgG) with polymyxin B (0.62–500 μg/ml) for 30 min prior to performing the ELISA (Figure 3Cii) caused a dose-dependent decrease in soluble LPS/IgG detection. A preincubation of colostrum with 500 μg/ml polymyxin B showed a ~ 67% decrease in absorbance (± SD) from 0.64 ± 0.068 to 0.21 ± 0.02, respectively.

Milk collected from cows diagnosed with clinical E. coli mastitis contains soluble LPS/IgG complexes that mirror endotoxin and LDH activity

Since IgG recruited during the onset of intramammary *E. coli* infections correlate with the severity of mastitis,[21] we selected to use clinical *E. coli* mastitis as a model to identify the presence of soluble milk LPS/IgG complexes and their relationship to endotoxin activity. Soluble LPS/IgG complexes in milk (Figure 4A) collected from cows (2–6 week postpartum) diagnosed "on-farm" with clinical *E. coli* mastitis on day 1 were detected by ELISA and showed absorbance (± SD) of 0.47 ± 0.63 (n = 17). Soluble milk LPS/IgG complex levels remained detectable in the milk for the following 2 days (day 2 [0.48 ± 0.6], day 3 [0.42 ± 0.5]) but were negligible ($P < 0.01$) in milk collected from cows that had eventually recovered from clinical *E. coli* mastitis (0.03 ± 0.01). LPS endotoxin activity (using the LAL activity [EU/ml ± SD]) in these milk samples was elevated in milk collected on days 1–3 after mastitis diagnosis (Figure 4B). Although a sequential decrease in LAL activity was observed in milk collected on days 2 (90.24 ± 213.39) and 3 (73.67 ± 124.89), they were not statistically different from day 1 (166.88 ± 372.33). The detection of LAL activity in milk, however, collected from cows that completely recovered from *E. coli* mastitis (26.61 ± 18.0) was statistically ($P < 0.05$) reduced from day 1. Heat denaturation of proteins (boiling [>98 °C] for 10 min) in these milk samples caused a significant ($P < 0.01$) elevation in LAL activity (685.74 ± 939.13, 646.42 ± 1591.9 and 291.61 ± 747.12, days 1, 2 and 3, respectively), which was also found in milk collected from cows that ultimately recovered from clinical mastitis (75.89 ± 92.14). Despite the large variation

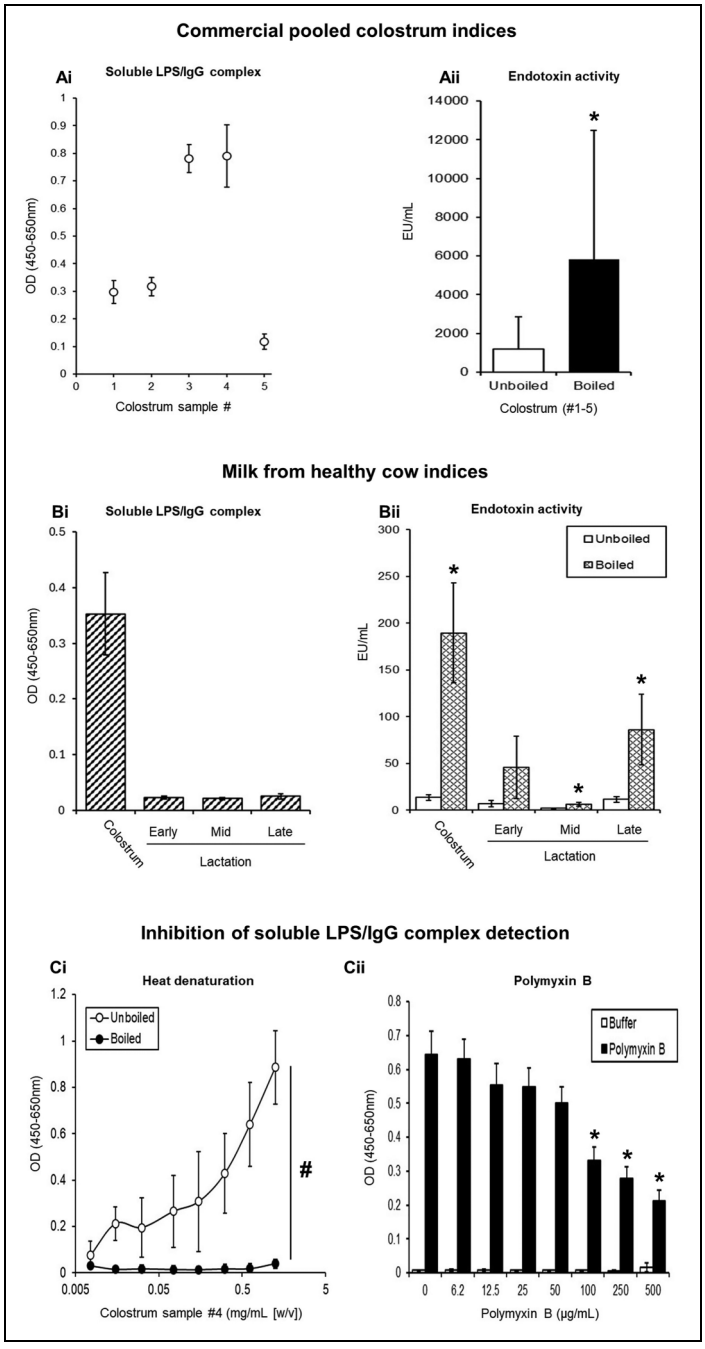

Figure 3. Bovine colostrum contains soluble LPS/IgG complexes. [Ai] Variation in soluble LPS/IgG complex levels amongst 5 (#1–5) separate commercial colostrum preparations (2.5 mg/ml [w/v] total IgG) measured by ELISA. Data is expressed as mean absorbance ± SD from 4 replicates of each colostrum preparation. [Aii] Colostrum endotoxin activity, using LAL bioassay, measured before (unfilled bars) and after boiling (filled bars) for 10 min (>98 °C). Data is expressed as mean EU/ml ± SD of 5 commercial bovine colostrum preparations (#1–5); * represents a statistical ($P < 0.05$) elevation from un-boiled colostrum values. Raw colostrum (n = 5 individual cows) and milk collected at specific times during the lactation period; early (2–6 weeks, n = 8 individual cows), mid (16–20 weeks, n = 10 individuals) and late (~32 weeks, n = 16 individuals cows), measured [Bi] soluble LPS/IgG complex levels, and [Bii] endotoxin activity before (unfilled bars) and after boiling (>98 °C) for 10 min (crossed bars). Data is expressed as mean absorbance or EU/ml ± SEM, respectively, where * represents statistical ($P < 0.05$) increase from un-boiled milk endotoxin activity. [C] Soluble LPS/IgG complex levels (0.075–1.25 mg/ml [w/v] total IgG) present in bovine colostrum (preparation #4) measured [Ci] before (○) and after boiling (>98 °C) for 10 min (●), or [Cii] after a 30 min preincubation of polymyxin B (6.2–500 µg/ml) with colostrum (0.62 mg/ml [w/v] total IgG, preparation #4, filled bars) or buffer (mg/ml w/v equivalent, unfilled bars) measured by ELISA. Data is expressed as mean absorbance ± SD from 6 replicates, where # denotes statistical ($P < 0.01$) increase from boiled colostrum values and * signifies statistical ($P < 0.01$) decrease from control (no polymyxin B).

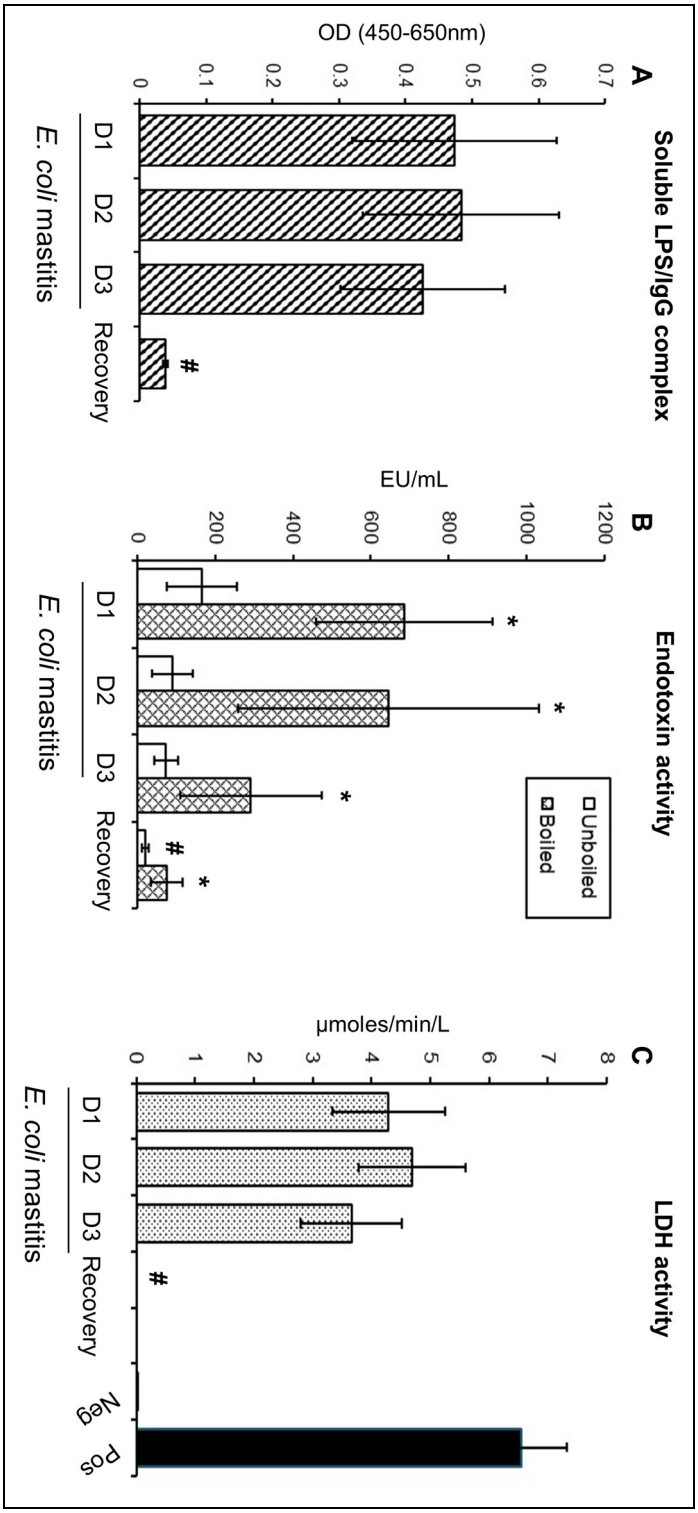

Figure 4. The presence of soluble milk LPS/IgG complexes in postpartum *E. coli*-induced clinical mastitis mirror increases in endotoxin activity and LDH activity. Milk collected days 1–3 upon the diagnosis of postpartum *E. coli* clinical mastitis (n = 17 individuals cows) and after a period of recovery (~ 20 weeks, n = 5) measured [A] soluble LPS/IgG complex levels by ELISA, [B] endotoxin activity before (unfilled bars) and after boiling (>98 °C) for 10 min (crossed bars) using LAL bioassay, and [C] LDH activity using an enzyme kinetic assay: Pooled raw milk samples from healthy or clinical *E. coli* mastitis cows represent negative (unfilled bar) and positive (filled bar) LDH activity controls, respectively. Data is expressed as mean absorbance, EU/ml, or µmoles/min/l ± SEM, respectively, where * representing statistical ($P < 0.05$) increase from un-boiled milk endotoxin activity, and # denotes a statistical ($P < 0.05$) reduction in milk soluble LPS/IgG complex levels, endotoxin activity (un-boiled) or LDH activity after a period of recovery.

in the indices tested, a positive correlation ($R^2 = 0.66$, $P = 0.03$) between milk soluble LPS/IgG complex levels and LAL bioactivity in un-boiled milk measured on day 1 after the initial diagnosis of clinical *E. coli* mastitis was identified. No relationship was found between soluble LPS/IgG complex levels and LAL activity in boiled milk samples ($R^2 = 0.062$). LDH activity (μmoles/min/l ± SD) in milk samples collected on days 1–3 from cows upon the diagnosis of clinical *E. coli* mastitis were also elevated (Figure 4C). LDH activity was 4.29 ± 3.95, 4.69 ± 3.72 and 3.66 ± 3.54, days 1, 2 and 3, respectively. Milk collected from cows that completely recovered showed no detectable LDH activity. A positive relationship ($R^2 = 0.76$, $P = 0.04$) was also identified between soluble LPS/IgG complex levels and LDH activity measured in milk collected on day 1 of the clinical *E. coli* mastitis diagnosis.

Intramammary milk anti-LPS IgG repertoire during E. coli mastitis displays reduced binding to the LPS glycolipid core

Next, we evaluated the binding ability of IgG within *E. coli* mastitis milk to (i) LPS shed from a wild-type *E. coli* strain commonly found in clinical gram-negative mastitis (shed-LPS) and (ii) LPS generated from an *E. coli* mutant bacterium that contains the glycolipid core but lacks species specific O-antigens (*E. coli* mutant K12-LPS), Figure 5. A representative SDS-PAGE image (Figure 5A) highlights the structural differences in the LPS used in these experiments, with shed LPS displaying a range of ladder-like pattern of molecular weight bands, representing various O-antigen repeats together with a low molecule weight band indicating the glycolipid moiety. In contrast, *E. coli* mutant K12-LPS showed only the low molecular weight bands of the glycolipid molecules. Initial ELISA experiments showed a dose-dependent increase in the detection of both shed LPS (0.009–1 μg/ml), and *E. coli* mutant K12-LPS (0.009–5 μg/ml) using a bovine commercial colostrum preparation (0.1 mg/ml[w/v] total IgG) (Figure 5Bi). Using this information, we selected doses of shed LPS (0.1 μg/ml) and *E. coli* mutant K12-LPS (2.5 μg/ml) that showed similar absorbance values (± SD), 0.71 ± 0.12 and 0.73 ± 0.09 ($P = 0.37$), respectively, in the ELISA (Figure 5Bii), to identify any changes in the binding ability of anti-LPS IgG present in the raw colostrum and *E. coli* mastitis milk samples to the LPS glycolipid core (*E. coli* mutant K12-LPS).

Analysis of milk collected from 40 individual cows diagnosed with *E. coli* clinical mastitis showed a ~55% reduction (absorbance ± SD) in the ability of IgG to bind to *E. coli* mutant K12-LPS (2.5 μg/ml) compared to shed-LPS (0.1 μg/ml); 0.58 ± 0.52 vs.1.3 ± 0.49 ($P < 0.01$), respectively, (Figure 5Ci). A frequency analysis plot of absorbance values for the 40 milk samples (Figure 5Cii) revealed a high percentage (~79%) of IgG within these milk samples bound to *E. coli* mutant K12-LPS molecules with an absorbance value ≤0.8, although a small number (~20%) bound to *E. coli* mutant K12-LPS with an absorbance value ≥0.8. In contrast, the percentage of milk IgG bound to shed LPS with an absorbance value ≤0.8 was ~28%, with ~73% binding to shed LPS with an absorbance value ≥0.8. Similarly (Figure 5Ciii), the binding ability of anti-LPS IgG present within aseptically collected milk from cows diagnosed with *E. coli* clinical mastitis (days 1–3) showed a reduced ($P < 0.01$) ability to bind *E. coli* mutant K12-LPS (absorbance ± SD, 0.73 ± 0.35, 0.62 ± 0.32, 0.61 ± 0.34, D1-D3, respectively, compared to shed LPS 0.92 ± 0.44, 0.83 ± 0.42, 0.82 ± 0.41, D1-D3, respectively). However, milk collected from cows who recovered from clinical mastitis showed similar (although at lower absorbance levels) IgG binding to *E. coli* mutant K12-LPS and shed LPS (0.3 ± 0.31 vs. 0.33 ± 0.25 respectively, $P = 0.45$). Furthermore, IgG present within aseptically collected raw colostrum samples displayed similar binding profiles to both *E. coli* mutant K12-LPS and shed LPS; 1.11 ± 0.13 and 1.19 ± 0.11 ($P = 0.07$), respectively.

The distinct binding properties of IgG present in colostrum and E. coli mastitis milk to the LPS glycolipid core has a contrasting impact on endotoxin activity

To elucidate how differences in the binding of milk IgG to the LPS glycolipid core influences endotoxin activity, IgG extracts from selected colostrum or clinical *E. coli* milk samples, that reflected these differences, were prepared. Preliminary assessment of an IgG extract from a clinical *E. coli* mastitis milk sample showed a significant ($P < 0.05$) reduction in (i) soluble LPS/IgG complex levels (0.39 ± 0.08 vs. 0.07 ± 0.01, absorbance ± SD [Figure 6Ai]), (ii) endotoxin activity (920 ± 146 vs. 52.9 ± 47.2, EU/ml ± SD [Figure 6Aii]) and (iii) LDH activity (6.89 ± 1.55 vs. undetectable levels, μmoles/min/l ± SD [Figure 6Aiii]) compared to original milk sample (IgG extract vs. original milk, respectively). The selected colostrum sample (a commercial pooled preparation) contained negligible levels of soluble LPS/IgG complex levels in both the original colostrum sample and its IgG extract; 0.11 ± 0.01 and 0.09 ± 0.01, absorbance ± SD, respectively. Similarly, endotoxin activity (original colostrum, 0.01 ± 0.05 and IgG extract, undetectable, EU/ml) and LDH activity (original colostrum, 25.1 ± 13.8 and IgG extract, undetectable, μmoles/min/l) in the samples were also minimal (Figures 6Ai-iii).

With regards to milk anti-LPS IgG binding profiles, the anti-LPS IgG present in the original colostrum sample (0.1 mg/ml [w/v] total IgG) showed a similar ($P = 0.3$) binding pattern to both the shed LPS (0.1 μg/ml) and *E. coli* mutant K12-LPS (2.5 μg/ml), absorbance ± SD, 1.23

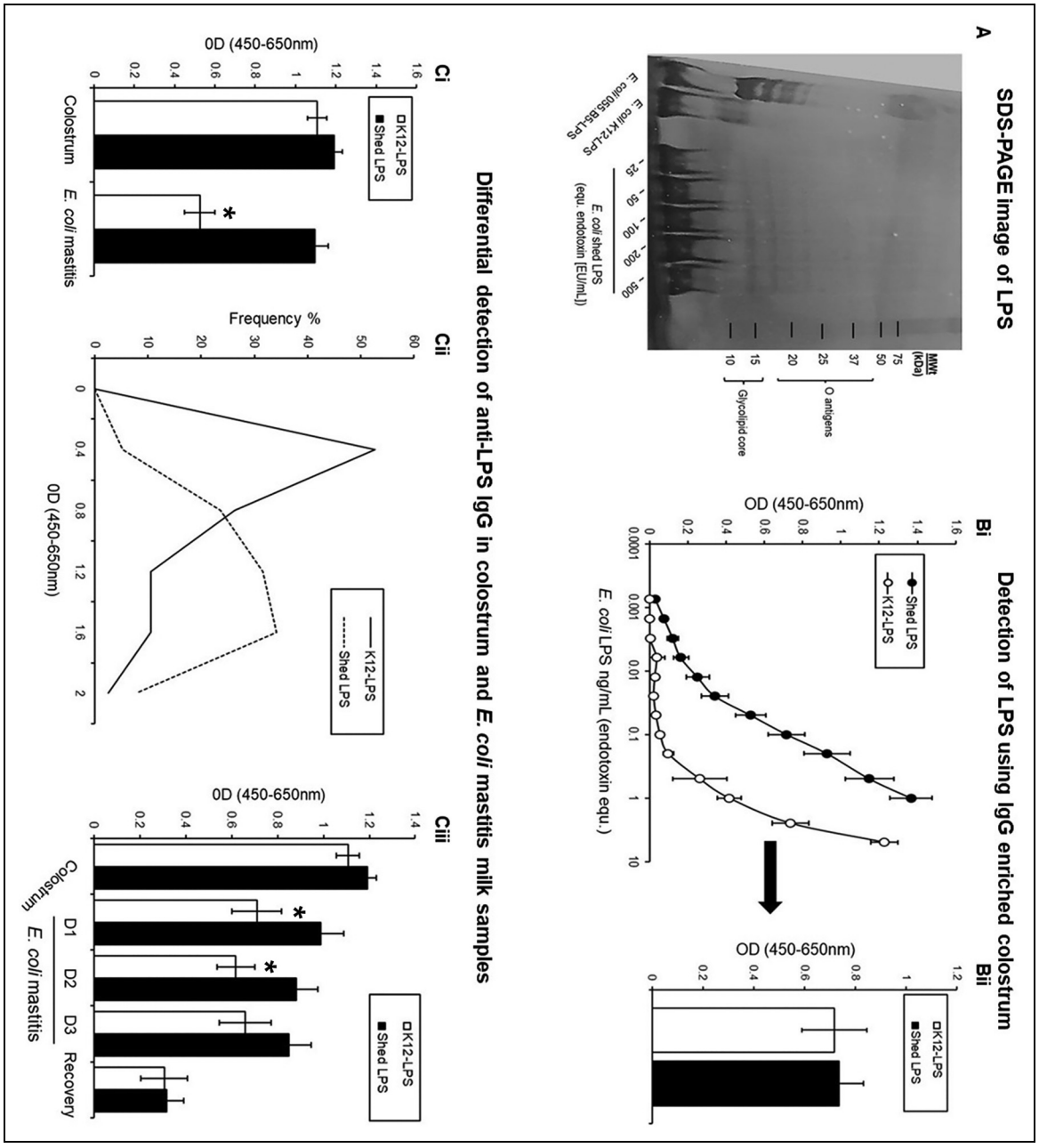

Figure 5. The IgG repertoire in *E. coli* mastitis milk shows reduced binding to the LPS glycolipid core. [A] represents a gel image of purified *E. coli* O55.B5 LPS (5 µg, lane 1), *E. coli* mutant K12-LPS (5 µg, lane 2), *E. coli* shed LPS (~ 25–500 ng endotoxin equ., lanes 4–8) and pre-stained protein markers (with defined molecular mass [kDa], lane 10) separated by SDS-PAGE and visualised using zinc sulphate/imidazole reverse staining. LPS glycolipid core molecules are the lower bands, whilst the upper ladder-like patterns represent various number of O-antigen repeating units within the LPS. Commercial bovine colostrum enriched in IgG (0.1 mg/ml [w/v] total IgG) ability to detect [Bi] 001–1 µg/ml *E. coli* shed LPS (●) or 0.01–5 µg/ml *E. coli* mutant K12-LPS (○), and [Bii] selected doses of *E. coli* shed LPS (0.1 µg/ml, filled bars) and *E. coli* mutant K12-LPS (2.5 µg/ml, unfilled bars), in an ELISA. Data is expressed as mean absorbance ± SD of at least 4 separate experiments. Dashed line represents doses of shed LPS and K12-LPS that show similar absorbance values in the ELISA. [C] Detection of *E. coli* mutant K12-LPS (2.5 µg/ml, unfilled bars) or *E. coli* shed LPS (0.1 µg/ml, filled bars) by IgG present in milk from clinical *E.coli* mastitis infected quarters (n = 40) and pooled raw (first milk) colostrum (n = 5 separate cows). Data is expressed as either [Ci] mean absorbance ± SEM or [Cii] a frequency distribution plot of ELISA absorbance values for the recognition of either *E. coli* mutant K12-LPS (solid line) or *E. coli* shed LPS (dashed line); * signifies a statistical ($P < 0.01$) reduction in milk IgG that bind to *E. coli* mutant K12-LPS. [Ciii] Detection of *E. coli* mutant K12-LPS (2.5 µg/ml, unfilled bars) or *E. coli* shed LPS (0.1 µg/ml, filled bars) by IgG present in raw colostrum (n = 5), milk collected 1–3 days (D1-D3) upon the detection of clinical *E. coli* mastitis (n = 17), or milk collected after recovery (n = 5). Data is expressed as mean absorbance ± SEM, where * represents statistical ($P < 0.05$) reduction in milk IgG that binds to *E. coli* mutant K12-LPS.

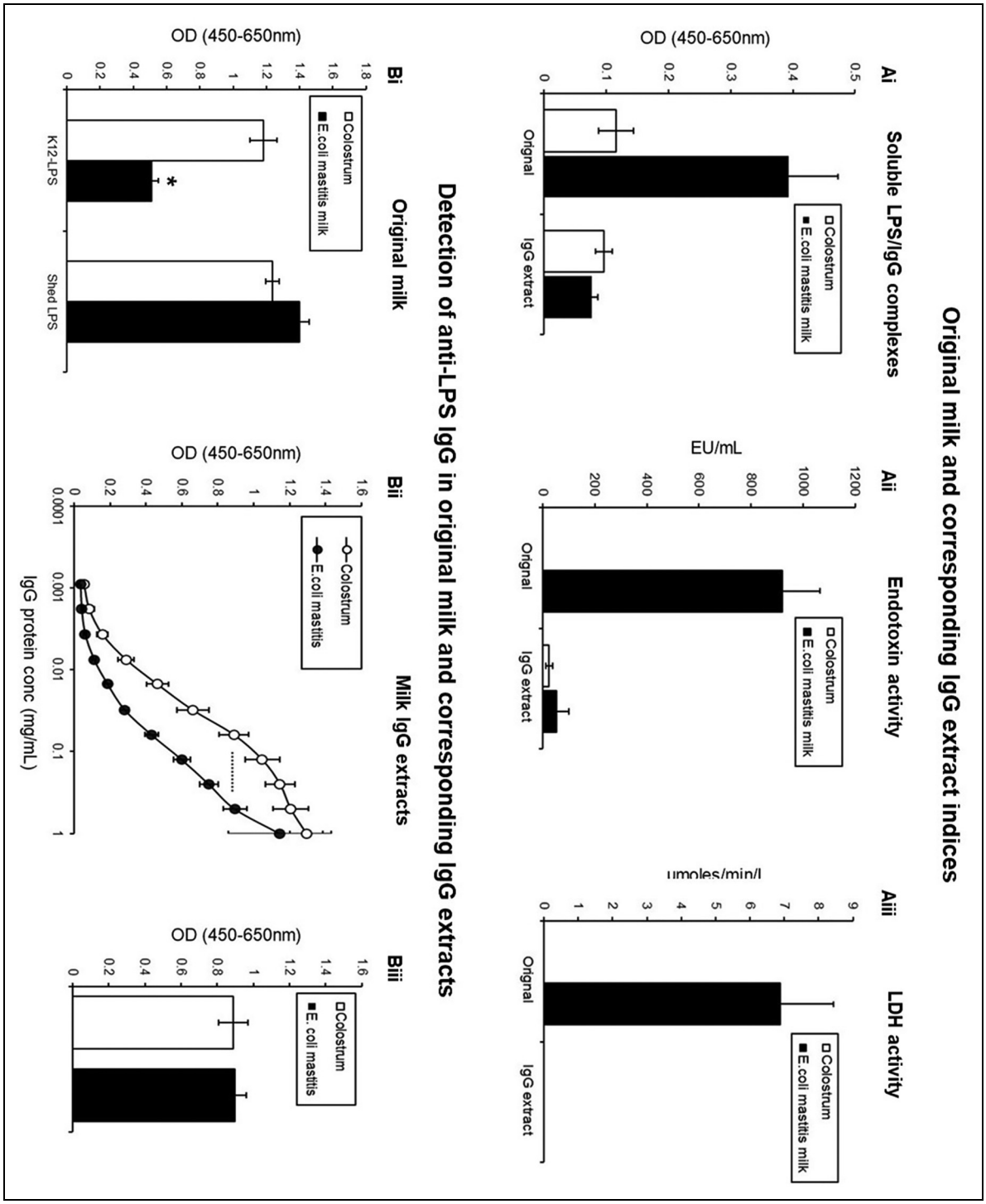

Figure 6. IgG extracted from selected colostrum and clinical *E. coli* mastitis milk samples show differential binding to the LPS glycolipid core. [Ai] Soluble LPS/IgG immune complex levels, [Aii] endotoxin bioactivity and [Aiii] LDH activity and in selected colostrum (unfilled bars) and *E. coli* mastitis milk (filled bars) samples (original) and their respective IgG extracts (IgG extract). Data is expressed as mean absorbance, EU/ml, µmoles/min/l ± SD, respectively, of at least 4 replicates. [Bi] IgG present in original colostrum (unfilled bars) or *E. coli* mastitis milk (filled bars) samples ability to bind to *E. coli* mutant K12-LPS (2.5 µg/ml) or *E. coli* shed LPS (0.1 µg/ml) using ELISA. Data is expressed as absorbance ± SD of 4 separate experiments where * represents statistical ($P < 0.01$) reduction in milk IgG that binds to *E. coli* mutant K12-LPS. IgG extracts from colostrum or *E. coli* mastitis milk ability to bind to *E. coli* shed LPS (0.1 µg/ml) measured by ELISA; [Bii] IgG titre (0.0009–1 mg/ml) or [Biii] selected milk IgG concentrations (0.062 mg/ml and 0.5 mg/ml, respectively). Data is expressed as absorbance ± SD of 4 separate experiments. Dashed line represents doses of milk IgG extract that show similar absorbance values in the ELISA.

± 0.08 vs. 1.18 ± 0.2, respectively (Figure 6Bi). In contrast, anti-LPS IgG present in the original clinical *E. coli* mastitis milk sample showed a reduced ($P < 0.01$) ability to bind (absorbance $\pm$ SD) to *E. coli* mutant K12-LPS (2.5 μg/ml) compared to shed LPS (0.1 μg/ml); 0.51 ± 0.08 vs. 1.39 ± 0.13, respectively. After IgG extraction, both colostrum and *E. coli* mastitis milk preparations (0.009–1 mg/ml, total protein) retained their ability to bind to 0.1 μg/ml shed LPS in an ELISA (Figure 6Bii). By selecting doses of colostrum (0.062 mg/ml) and *E. coli* mastitis milk (0.5 mg/ml) IgG extracts, we were able to achieve similar absorbance values by ELISA when binding to 0.1 μg/ml shed-LPS; 0.88 ± 0.08 and 0.89 ± 0.07, respectively, $P = 0.43$ (Figure 6Biii).

Subsequent application of these milk IgG extract concentrations to assess binding to either shed LPS (0.9–500 ng/ml) or *E. coli* mutant K12-LPS (2.2–2500 ng/ml) revealed a similar dose-dependent binding pattern to shed LPS for both colostrum and *E. coli* mastitis milk IgG (Figure 7Ai). Both colostrum and *E. coli* mastitis milk IgG extracts showed absorbance ($\pm$ SD) values of 1.09 ± 0.18 and 1.03 ± 0.05 ($P > 0.05$), respectively, when binding to shed LPS at a dose of 500 ng/ml. Similarly, colostrum IgG extract (0.062 mg/ml) showed a dose-dependent (2.2–2500 ng/ml) increased binding to *E. coli* mutant K12-LPS (Figure 7Aii). However, this was not observed for the *E. coli* mastitis milk IgG extract (0.5 mg/ml), which showed a significant ($P < 0.01$) reduction in the ability to bind to *E. coli* mutant K12-LPS doses between 125 ng/ml and 2500 ng/ml compared to colostrum IgG. Absorbance ($\pm$ SD) values for 620 ng/ml *E. coli* mutant K12-LPS were 0.61 ± 0.11 and 0.03 ± 0.07 ($P < 0.01$) when using colostrum and *E. coli* mastitis milk IgG extracts, respectively.

These experimental conditions were then applied to assess endotoxin activity (Figure 7Bi). A 1 h incubation of shed LPS (3.1–200 ng/ml) with either colostrum (0.062 mg/ml) or *E. coli* mastitis milk (0.5 mg/ml) IgG extracts resulted in different LAL bioactivities. Shed LPS alone showed a dose-dependent increase in LAL activity. Whilst a 1 h incubation of shed LPS with IgG extracted from *E. coli* mastitis milk mirrored the LAL activity pattern of shed LPS alone, incubation with colostrum IgG extract ameliorated endotoxin activity, causing a significant ($P < 0.05$) reduction in the endotoxin activity of shed LPS (25 to 100 ng/ml). Preincubation of 50 ng/ml shed LPS with colostrum IgG extracts decreased shed LPS-induced LAL activity by $\sim$ 70% (30.15 ± 23.4 vs. 103.54 ± 45.3, EU/ml $\pm$ SD), whereas IgG extracted from *E. coli* mastitis milk IgG had no effect (112.22 ± 45.03 vs. 103.54 ± 45.3 EU/ml $\pm$ SD). Furthermore, soluble LPS/IgG immune complexes were also detected after a 1 h incubation of shed LPS (3.1–200 ng/ml) with both milk IgG extracts (Figure 7Bii). A small, yet significant ($P < 0.05$), dose-dependent increase in soluble LPS/IgG complex levels was detected when 2.5–100 ng/ml shed LPS was incubated with either colostrum or *E. coli* mastitis milk IgG extracts. No significant ($P = 0.094$) difference between the levels of soluble LPS/IgG complexes generated by the 2 milk IgG extracts was observed. Plus, no change in baseline absorbance was found when shed LPS (3.1–200 ng/ml) was assayed alone in the ELISA.

Exposure of milk granulocytes to shed LPS pretreated with colostrum IgG reduces baseline reactive oxygen species (ROS) generation but not the augmentation of fMLP-mediated ROS

To explore how differences in milk anti-LPS IgG binding to the LPS glycolipid core influences endotoxin function, we utilised the ability of shed LPS to alter the oxidative status of resident milk granulocytes in healthy cows. *E. coli* shed LPS (3.1–200 ng/ml) caused a dose-dependent increase in baseline ROS generation from milk granulocytes loaded with H_2DCFH (Figure 8A). Shed LPS doses > 50 ng/ml caused a significant ($P < 0.05$) elevation in fluorescence indicating ROS generation (201.03 ± 17.4, 226.98 ± 22.9 and 247.46 ± 25.2 ΔFI, 50, 100 and 200 ng/ml LPS) above baseline (131.35 ± 13.9 ΔFI). In a separate experiment, N-acetyl cysteine (NAC), a known ROS scavenger, was used to verify that the observed increases in fluorescence using H_2DCFH were due to an elevation of cellular ROS. We found a 30 min preincubation of milk granulocytes with NAC (0.5–10 mg/ml) caused a dose dependent decrease in shed LPS-stimulated ROS generation, with a NAC dose of 2 mg/ml causing an $\sim$ 47% decrease in ROS from the LPS control; mean ΔFI $\pm$ SD of 120.21 ± 12.18 vs. 225.34 ± 15.28, NAC vs. shed LPS control, respectively.

We subsequently used this cell model to examine the direct effect of milk IgG on ROS generation in milk granulocytes (Figure 8B). Although a gradual increase of ROS within granulocytes was observed over 20 min, no overall significant ($P > 0.05$) increase (fluorescence $\pm$ SD) above baseline (129.3 ± 46.7) was recorded in the presence of IgG extracted from either colostrum (113.1 ± 12.5) or *E. coli* mastitis milk (161.2 ± 47.4). The ability of shed LPS to generate ROS by granulocytes was examined after a 1 h incubation with buffer or IgG extract doses of colostrum (0.062 mg/ml) or *E. coli* mastitis milk (0.5 mg/ml) previously used in the shed LPS binding and LAL bioactivity experiments. Preincubation of shed LPS (3.1–200 ng/ml) with buffer caused a dose-dependent increase in ROS in H_2DCFH loaded milk granulocytes (Figure 8C). A similar dose-dependent increase in cellular ROS was recorded after a 1 h preincubation of shed LPS with IgG extracted from *E. coli* mastitis milk. In contrast, a 1 h preincubation of shed LPS (3.1–200 ng/ml) with IgG extracted from colostrum reduced ROS generation in granulocytes

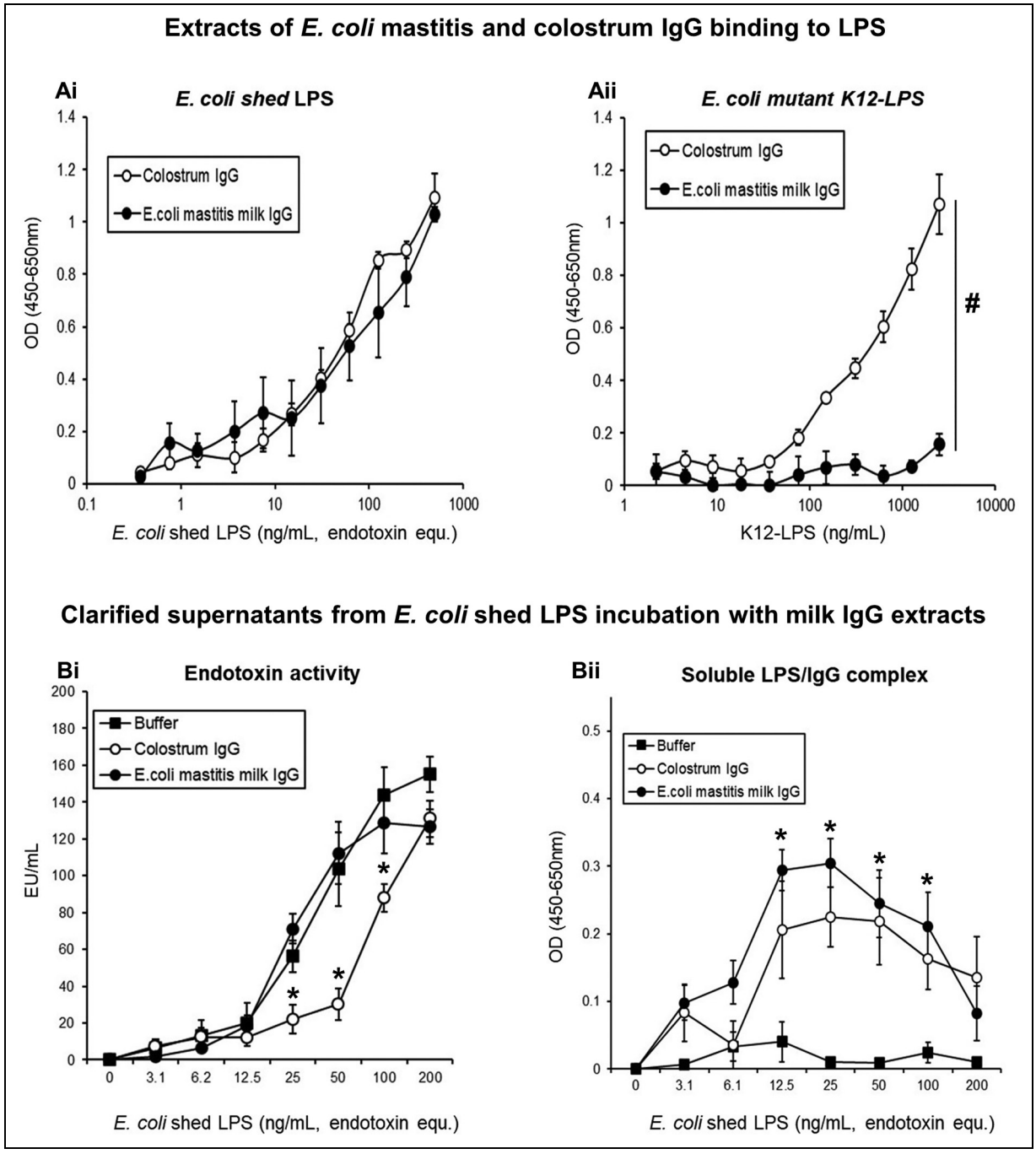

Figure 7. Colostrum IgG extract binds to the LPS glycolipid core to reduce endotoxin activity. [A] IgG extracts of the selected colostrum (0.062 mg/ml, ○) or *E. coli* mastitis milk (0.5 mg/ml, ●) samples ability to detect [Ai] *E. coli* shed LPS (0.9–100 ng/ml) or [Aii] *E. coli* mutant K12-LPS (2.2–2500 ng/ml) by ELISA. Data is expressed as mean absorbance ± SEM of 6 separate experiments; # denotes a statistical ($P < 0.01$) reduction in the ability of IgG present in the *E. coli* mastitis milk extract to detect K12-LPS. [B] Clarified supernatant from a 1 h incubation of *E. coli* shed LPS (3.1–200 ng/ml) with buffer (■) or IgG extracts of colostrum (○) or *E. coli* mastitis milk (●) assessed for [Bi] endotoxin activity using the LAL bioassay or [Bii] soluble LPS/IgG complex levels measured by ELISA. Data is expressed as mean EU/ml or absorbance ± SEM, respectively, of 4 separate experiments; * denotes a statistical ($P < 0.05$) increase from buffer (*E. coli* shed LPS alone) values.

stimulated with doses of shed LPS between 25–100 ng/ml. Preincubation of 50 or 100 ng/ml shed LPS with colostrum IgG caused a significant ($P < 0.01$) ~ 90 and 64% decrease in fluorescence (± SD) (4.78 ± 15.0 and 35.66 ± 18.7, 50 and 100 ng/ml shed LPS, respectively) in H_2DCFH-loaded granulocytes compared to shed LPS

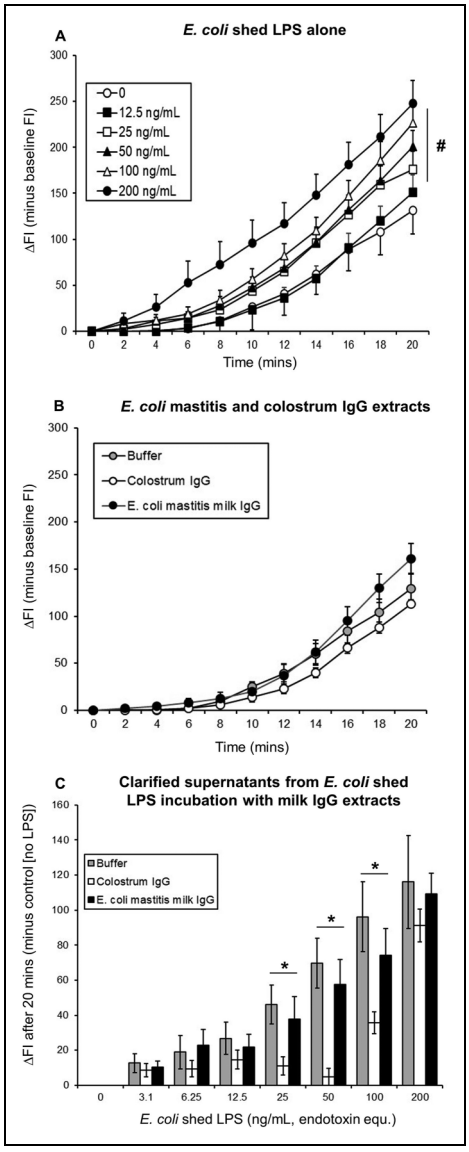

Figure 8. Bovine colostrum IgG attenuates *E. coli* shed LPS stimulated ROS in milk granulocytes. [A] Milk granulocytes loaded with H_2DCFH assessed [Ai] *E. coli* shed LPS (12.5–200 ng/ml)-induced ROS generation by measuring the change in fluorescence (excitation 492 nm, emission 527 nm) over 20 min. Data is expressed as mean change in fluorescent intensity (ΔFI) minus baseline FI over 20 min ± SEM, respectively, of at least 4 separate experiments; # represents a statistical ($P < 0.05$) increase from buffer only. [B] ROS generation in H_2DCFH loaded milk granulocytes over 20 min in response to buffer (●), IgG extracts of selected colostrum (0.062 mg/ml, ○) or *E. coli* mastitis milk (0.5 mg/ml, ●) samples. Data is expressed as (ΔFI) minus baseline FI over 20 min ± SEM of 5 separate experiments. [C] ROS generation in H_2DCFH loaded milk granulocytes after a 20 min incubation with the clarified supernatant generated from a 1 h incubation of *E. coli* shed LPS (3.1–200 ng/ml) with buffer (grey bars) or IgG extracts of colostrum (unfilled bars) or *E. coli* mastitis milk (filled bars). Data is expressed as ΔFI after 20 min minus respective baseline FI of 5 separate milk granulocytes experiments; * represents a statistical ($P < 0.05$) decrease from *E. coli* shed LPS alone (Buffer) values.

alone (69.7 ± 42.1 and 96.21 ± 60.2, 50 and 100 ng/ml shed LPS, respectively).

Since LPS is capable of priming neutrophils to enhance their responsiveness to early inflammatory mediators such as fMLP,[22] we examined the impact of milk IgG on the priming ability of shed LPS to facilitate fMLP-stimulated ROS in milk granulocytes. Initial experiments using H_2DCFH loaded milk granulocytes showed that a 10 min exposure with fMLP alone caused a dose-dependent increase in ROS generation (Figure 9A). Doses of fMLP > 0.1 μM caused a significant ($P < 0.05$) increase in ROS (fluorescence ± SD) above baseline (740.54 ± 399.37 and 1066.32 ± 485.93, 0.1 and 0.5 μM, respectively). The ability of fMLP (0.1 μM) to stimulate ROS in milk granulocytes was initially examined after exposure to the milk IgG extracts alone (Figure 9B). A 20 min preincubation of IgG extracts; colostrum or *E. coli* mastitis milk IgG with H_2DCFH loaded milk granulocytes had no significant ($P = 0.19$) impact on the ability of 0.1 μM fMLP to induce ROS generation: ΔFI (± SD) values were 649.99 ± 241.82, 671.17 ± 153.97, and 856.6 ± 142.39, control, colostrum, and *E. coli* IgG extracts respectively. All of these were significantly ($P < 0.05$) greater than baseline ROS levels (459.53 ± 210.99). We also found that a 1 h preincubation of shed LPS with IgG extracted from colostrum or *E. coli* mastitis milk had no influence on the ability of shed LPS to enhance the responsiveness of H_2DCFH loaded granulocytes to fMLP (Figure 9C). Shed LPS caused a dose-dependent (50–200 ng/ml) enhancement of fMLP-evoked ROS ($P < 0.05$). Whilst baseline ROS (fluorescence ± SD) levels were slightly ($P = 0.193$) higher in *E. coli* mastitis milk IgG after 10 min fMLP stimulation (856.6 ± 142.39) compared to colostrum IgG (671.17 ± 153.97) or buffer alone (649.99 ± 241.82), the responses following exposure to fMLP were similar and not significantly augmented. For example, a 100 ng/ml dose of shed LPS caused a 1.44-fold increase above baseline after fMLP stimulation (939.81 ± 255.59) which was similar to the 1 h preincubation with IgG extracted from either colostrum (1.35-fold; 907.9 ± 207.32) or *E. coli* mastitis milk (1.42-fold; 1221.63 ± 180.85).

Discussion

Here we utilise *in situ* milk IgG repertoires found in distinct physiological scenarios (first milk colostrum and milk from cows with clinical *E. coli* mastitis), to support the notion that proportional differences within milk IgG that target "functional" antigen regions modulate the function of antigen-IgG complexes. These subsequentially will serve to coordinate appropriate innate-adaptive immune responses essential for animal health.

The mammary glands of cows are constantly exposed to environmental pathogens, especially coliforms, and since small amounts of free endotoxins activate oxidative stress/

inflammation, it is important that secretions into this gland (that constitute colostrum and mature milk) contain compounds to minimise initiation of these events.[23–25] We found the endotoxin activity (measured using the LAL bioassay) of raw milk collected from healthy lactating cows, although varied throughout the lactation cycle (early, mid and late), were similar to the endotoxin activity levels found in raw milk from other studies.[26,27] Heat denaturation of milk proteins, however, caused a ~ 2–3 fold increase in LAL bioactivity in these healthy milk samples, demonstrating that endotoxins present within the udder are most likely sequestered by milk proteins, most notably casein micelles.[23] Interestingly, we found ~ 10-fold increase in LAL bioactivity in boiled raw colostrum compared to un-boiled, which displayed similar LAL activity to that of the healthy milk samples, indicating a sequestering role for colostrum proteins. Bovine colostrum, as opposed to adult milk from healthy cows, also contains a broad repertoire of IgG (50–60% of colostrum total protein), which provides a vital prophylactic role in the neonatal calf to minimise the toxic effects of invading pathogens. Since the primary role of colostrum is to protect the calf,[28,29] it is unclear whether this transient elevation in intramammary IgG offers any protection to the cow itself. Periparturient cows are immunocompromised and highly susceptible to acute coliform mastitis, so whilst it seems plausible this elevation in intramammary IgG (independent of pathogen infection) may form LPS/IgG complexes to sequester endotoxin activity, deficits in both immune and nutritional processes within the cow during this period may negate any potential health benefits.

We found that the IgG repertoire within bovine colostrum recognises and binds to shed LPS from a range of gram-negative bacteria and forms soluble LPS/IgG complexes that were captured by a polymyxin B ELISA matrix. Whilst the presence of milk compounds interfered with the capture of free LPS by the polymyxin B matrix, this was not evident when capturing soluble LPS/IgG complexes. We were able to detect soluble LPS/IgG complexes in bovine colostrum and *E. coli* mastitis milk samples in the presence of other milk components.[25–27] This suggests that the formation of these soluble LPS-IgG complexes can occur in the presence of other sequestering milk components. Our identification of soluble LPS/IgG complexes in bovine postpartum colostrum together with our findings that colostrum anti-LPS IgG sequester endotoxin activity supports a possible prophylactic role to sequester intramammary LPS. In a recent study by Gonzáalez-Cabrera and colleagues,[30] an intramammary administration of LPS (50 μg) at parturition in goats showed minimal endotoxin mastitis. Since IgG (predominantly IgG1) is a major milk component (colostrum) during this lactation period,[28,29] a rapid formation of LPS-IgG complexes, in addition to LPS/IgM and LPS/IgA complexes, in this scenario may have contributed to reducing endotoxin-driven inflammation observed. Although not the focus of this present study, we have also detected soluble LPS/IgA and LPS/IgM complexes in colostrum using our ELISA (unpublished observation), highlighting specific properties of these antibody subclasses for LPS in bovine colostrum. In addition to the opsonisation/agglutination action of colostrum IgG, *in vitro* studies have reported colostrum IgG binding to, and neutralisation of *E. coli* toxins to prevent cytotoxic damage and facilitate appropriate *E. coli* bacteria colonisation in the calf's gut mucosa supporting a regulatory role for bovine colostrum IgG on LPS function.[31] Whilst the consumption of an IgG enriched colostrum to alleviate gastrointestinal infections has shown some successes,[32] numerous attempts using immunisation to generate IgG that targets the LPS "functional" core to enhance protection for the calf and/or reduce the severity of postpartum clinical gram-negative bacteria mastitis have produced mixed results.[11–13] The difficulties encountered in altering intramammary IgG dynamics to produce colostrum that favours recognition and binding to the LPS core suggests that the secretion of IgG that targets these functional regions are tightly regulated, largely to ensure delivery of the appropriate immune benefits to the calf.

Mammary gland infections with *E. coli* are frequently caused by strains residing in the intestine as commensal flora with an appropriate immune memory already developed and stimulate a local adaptive antibody response.[18] This will quickly generate specific IgG that predominantly target LPS O-antigens to facilitate an immune response that aims to remove the invading pathogens. We found that milk collected from cows diagnosed with clinical *E. coli* mastitis showed parallel elevation in the inflammatory biomarkers LDH activity and LAL bioactivity with *E. coli* mastitis severity. IgG antibody influx into the mammary gland also simultaneously occurs with the onset of mastitis, and although it has been shown to correlate with LDH activity,[33,34] we found no correlation between milk IgG that bound to shed LPS and LDH activity. In addition, we found an increase in soluble LPS/IgG complex levels in milk samples collected upon the diagnoses of clinical *E. coli* mastitis and over the following 3 days, whereas soluble LPS/IgG levels in milk collected from healthy cows during this early lactation period were undetectable. We initially formed the assumption that the formation of LPS/IgG complexes serves to sequester endotoxin activity and agglutinate with other milk proteins to passively remove gram-negative bacteria and endotoxins. However, a positive correlation between soluble milk LPS/IgG complex levels with LDH activity and LAL bioactivity suggests changes to intramammary IgG recruitment and secretion and subsequent LPS/IgG complex formation which may not favour this outcome. Furthermore, the shift in intramammary IgG dynamics during *E. coli* mastitis to producing a milk IgG repertoire to recognise specific O-antigens may proportionally outweigh IgG that targets

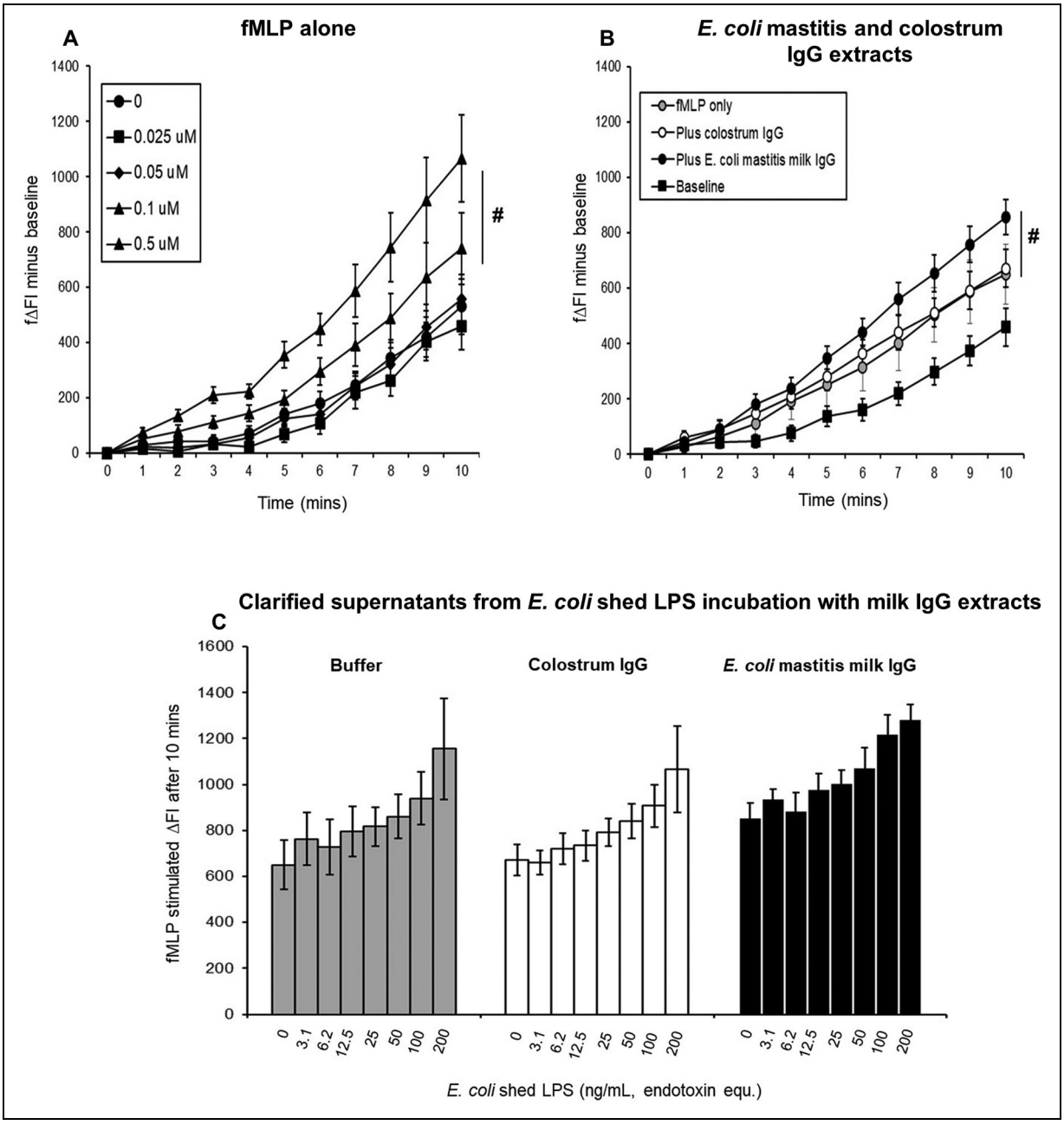

Figure 9. IgG extracts from colostrum or *E. coli* mastitis milk had no effect on *E. coli* shed LPS augmentation of fMLP-stimulated ROS in milk granulocytes. Milk granulocytes loaded with H_2DCFH assessed [A] fMLP (0.025–0.5 μM)-induced ROS by measuring the change in fluorescence (excitation 492 nm, emission 527 nm) over 10 min. Data is expressed as a mean change in fluorescence intensity (ΔFI) minus baseline FI over 10 min ± SEM, respectively, of at least 5 separate experiments; # represents a statistical ($P < 0.05$) increase from buffer only. [B] Baseline (Buffer, ■) or fMLP (0.1 μM)-stimulated ROS generation in H_2DCFH loaded milk granulocytes measured over 10 min after a 20 min preincubation with either buffer (fMLP only, ●) or IgG extracts of colostrum (0.062 mg/ml, ○) or *E. coli* mastitis milk (0.5 mg/ml, ●). Data is expressed as mean ΔFI minus baseline FI over 10 min ± SEM of 4 separate experiments, where # signifies statistical ($P < 0.05$) difference from baseline ΔFI (baseline). [C] fMLP (0.1 μM) stimulated ROS generation within H_2DCFH loaded milk granulocytes for 10 min after a 20 min preincubation with clarified supernatants that had been generated from a 1 h incubation of *E. coli* shed LPS (3.1–200 ng/ml) with buffer (grey bars) or IgG extracts of colostrum (unfilled bars) or *E. coli* mastitis milk (filled bars). Data is expressed as mean fMLP-stimulated ΔFI after 10 min ± SEM of 5 separate granulocyte experiments.

the LPS "functional" glycolipid core to sequester endotoxin-driven inflammation. We found an overall decrease (~ 50%) in IgG that recognised and bound to the glycolipid core in milk from cows diagnosed with clinical *E. coli* mastitis compared to raw colostrum from healthy cows. In addition, by forming shed LPS/IgG complexes utilising IgG extracted from colostrum and *E. coli* mastitis milk we were able to detect changes in shed LPS endotoxin activity and oxidative status in milk granulocytes. The notion of elevating intramammary IgG to target the LPS glycolipid core has been explored to generate gram-negative bacteria vaccines. However, while the severity of clinical mastitis was initially alleviated, the efficacy of the IgG antibodies generated only lasted for a few months.[12]

The differential effect of IgG from colostrum and *E. coli* mastitis milk on endotoxin activity supports the regulation of LPS function through proportional changes in the milk IgG repertoire that targets the LPS glycolipid core. Although the mechanism underlying this process is unknown, the integral role of IgG steric hinderance to sequester LPS function does suggest a likely mechanism.[35] However, LPS can elicit a range of biological actions, including the activation of neutrophil TLR4/MD2 signalling pathways, and so it is also plausible that the interaction between colostrum IgG and the LPS glycolipid antigen may only affect selective LPS functions.[36–39] Utilising bovine milk granulocytes, we found that whilst colostrum IgG attenuated *E. coli* shed LPS's ability to generate ROS, this had no significant impact on the enhanced responsiveness of granulocytes to the potent pro-inflammatory peptide fMLP after LPS priming. These findings therefore indicate that colostrum IgG interaction with LPS does not just involve a passive covering of the LPS glycolipid core. In contrast, although we found that *E. coli* mastitis milk IgG extracts could form LPS/IgG complexes with *E. coli* shed LPS, they had no impact on LPS activation of factor C in the LAL bioassay or interfered with the ability of LPS to generate a pro-oxidant environment in milk granulocytes. Since we observed a positive correlation between soluble milk LPS/IgG complex levels and endotoxin-driven inflammation, it is feasible that a primary role of IgG recruited into the mammary gland during clinical *E. coli* mastitis is to opsonise LPS. However, since LPS activation of cellular TLR4/MD2 is an essential innate process to ensure the sequential activation of pro- and anti-inflammatory events during the onset of *E. coli* mastitis,[40] it is possible that suppression of LPS function by IgG (targeting the glycolipid "functional" region) would hinder these early inflammatory events ultimately prolonging *E. coli* mastitis. Furthermore, while we focused here on differences in total milk IgG *in situ*, differences within IgG subclasses present in colostrum (predominantly IgG1) and *E. coli* mastitis milk (equal levels of IgG1, IgG2, IgG3), may also exhibit distinct effects on endotoxin function in leukocytes.[41] We would also like to speculate that, with the recent identification of post-translational IgG modifications that occur systemically during the progression of an infection[42] (as well as during the maturation of colostrum into milk),[14] that environmentally driven intra-mammary post-translational modifications of IgG (subclass dependent or independent) may contribute to the delivery of a milk IgG repertoire that underlies appropriate antigen-IgG functional responses during the progression of *E. coli* mastitis and a return to immune homeostasis.

In conclusion, our novel findings support the importance of bovine intramammary IgG diversity, during health and disease, to generate an anti-LPS milk IgG repertoire that ensures the appropriate functionality of LPS-IgG complexes.

Materials and methods

Bovine milk sampling

(i) *Source and collection of raw milk*. Milk samples from healthy Holstein-Friesian cows were collected over a 43-week lactation (early [2–6 weeks, n = 8], mid [16–20 weeks, n = 10] or late [30–32 weeks, n = 16]) from a single dairy herd (Galaxy Dairies, Linton, Manawatu, NZ). All cows produced milk with a < 100,000 somatic cell count (SCC) /ml as determined by routine herd testing, displayed no symptoms of mastitis and standard microbiology testing of the milk showed no bacterial growth. From the same lactation cycle, milk from cows diagnosed with clinical *E. coli* mastitis during early lactation (2–6 weeks, n = 17) period were collected and then again later during the same lactation cycle if recovered (~ 20 weeks, n = 5). Clinical acute mastitis was diagnosed "on-farm" using clinical and mammary gland assessments. Foremilk testing revealed physical changes to milk. Milk was collected within 24 h (day 1) from the diagnosis of acute mastitis and for the following 2 days (days 2 & 3), and then again later in the lactation period. In addition, one-off milk samples from cows (Holstein-Friesians) diagnosed with clinical *E. coli* mastitis were collected from local veterinary services (n = 40). Samples for which the presence of *E. coli* were verified using standard microbiology testing followed by Matrix-Assisted Laser Desorption Ionisation Time Of Flight Mass Spectrometry (MALDI-TOF MS) analysis, were selected. Bovine colostrum was collected from healthy cows (Holstein-Friesian) at the first milking after removal of teat plug from 5 separate

commercial dairy herds within the Manawatu area, NZ.

(ii) *Preparation of milk*. Raw milk and colostrum was collected aseptically from cows and either kept at room temperature (RT) for microbiological testing or immediately chilled on ice for preparation of obtaining fat-free milk. Milk and colostrum samples, in this instance were centrifuged (3500 *g*, 10 min, 10 °C) and filtered through several layers of gauze. Fat-free samples were then frozen at −20 °C for future biochemical analysis.

(iii) *Ethical approval*. Collection and use of milk and colostrum from healthy cows only required approval from dairy farmers (as samples were collected during their routine milking). No additional ethical consideration was required. Ethical approval for the collection and analysis of acute mastitis milk samples was obtained from Animals Ethics Committee, Massey University / Te Kunenga ki Pūrehuroa, Palmerston North, NZ. Study entitled "Evaluating an ELISA for identifying whether acute mastitis is caused by *E. coli*": Ref #AEC 22/41, approved 19/08/2022.

Biochemical analysis

(i) *Soluble LPS/IgG complex levels*. Milk, colostrum and LPS in the absence or presence of milk IgG extracts were assessed for the presence of soluble LPS/IgG complexes by ELISA. The development, characterisation and application of this ELISA is described below.

(ii) *Endotoxin activity*. The ToxinSensor™ chromogenic LAL endotoxin assay kit (GeneScript USA Inc., Piscataway, NJ, USA) that measured LPS/endotoxin activation of factor C as part of the coagulation cascade,[43] was used to assess the endotoxin activity of milk, colostrum and LPS in the absence or presence of milk IgG extracts. Milk / colostrum endotoxin activity was assessed in the supernatant after a 10 min incubation at either 4 °C or 98 °C (heat protein denaturation) followed by centrifugation (6000 *g* for 5 min, 10 °C). The endotoxin activity of Gram-negative bacteria shed LPS or LPS ± IgG extracts were assessed un-boiled. Prior to measurement of LAL activity, all samples were adjusted (if necessary) to pH 6–8 and appropriately diluted in endotoxin-free water to fit on an LPS standard curve between 0.1–5 EU/ml (ng/ml). The LPS standard (part of the kit) was diluted in endotoxin-free water and assayed at the same time as the test samples. Briefly, reconstituted LAL reagent was added to the LPS standards and diluted test samples, incubated (37 °C) for specific time (determined by kit batch #), followed by a 6 min incubation (37 °C) with the chromogenic substrate. The reaction was stopped by the sequential addition of a stop solution and 2 colour stabilisers, and then the absorbance of the solution was measured at wavelength (λ) 545 nm. The endotoxin activity of test samples was calculated using the LPS standard curve and presented as either EU/ml or ng/ml endotoxin equivalents.

(iii) *Lactate dehydrogenase (LDH) activity*. Milk LDH activity was measured using a standard kinetic enzyme assay.[44,45] Milk, colostrum and IgG extracts were diluted in 30 mM Tris/HCl buffer pH 8.5 containing 1 mM EDTA plus or minus 1 mM oxalic acid to achieve a final assay dilution of 2.5% (v/v). To start the enzyme reaction, sodium pyruvate (final 4 mM) and reduced NADH (final 0.15 mM) was added and the change in absorbance (λ 340 nm) was monitored over 10 min at 21 °C. Milk LDH activity was calculated using the extinction coefficient for NADH ($\epsilon 340 = 6220\ M^{-1}cm^{-1}$) and absorbance after subtraction of corresponding oxalic acid values over 10 min and expressed as μmoles/min/l.

Development of LPS ELISA

(i) *Basic ELISA format*. A polymyxin B matrix (endotoxin free) ELISA, designed to captured LPS and LPS-associated compounds (i.e., immunoglobulins), was developed and validated utilising methodologies reported by others.[46–48] Microplates were coated with polymyxin B sulphate; 50 μg/ml (Fort Richards Laboratories, Auckland, NZ) dissolved in 0.01 M PBS, pH 7.4, containing 0.1% (v/v) PEG-20 (Sigma-Aldrich, Merck Pty. Ltd, Auckland, NZ) for ~16 h at room temperature (RT). After washing plates with PBS, LPS samples were added and incubated for 90 min at 37 °C. Plates were then washed (x3) with PBS containing 0.1% (v/v) Tween-20 (PBST), followed by blocking non-specific binding sites with 2% (w/v) BSA/PBS (fatty acid-free, pH Scientific Ltd, Auckland, NZ) for 30 min at RT. Colostrum or milk samples diluted in PBST were added to wells and incubated for 60 min at 37 °C, followed by washing with PBST (4x). Detection of captured LPS/IgG antibody complexes was achieved using HRP-conjugates of protein G or sheep anti-bovine IgG (Invitrogen, Thermo-Fisher, Auckland, NZ),

which was added to wells and incubated for 45 min at 37 °C. Plates were washed with PBST (4x), and peroxidase activity measured using TMB solution (Invitrogen, Thermo-Fisher, Auckland, NZ). The reaction was then stopped with 10% (v/v) sulphuric acid and the absorbance read at 450 nm and 650 nm in a SpectroMax plate reader (Molecular Devices, Bio-Strategy, Auckland, NZ). Results were calculated and expressed as absorbance values read at 450–650 nm minus background absorbance from HRP-conjugate solutions only.

(ii) *Detection of free LPS by IgG present in pooled colostrum preparations*. LPS samples were diluted in 0.1 M sodium citrate buffer, pH 5.4 containing 0.025% (v/v) Triton x100 (TTx100) and incubated at RT for 30 min prior to performing the ELISA. Detection of LPS capture by the polymyxin B matrix was initially examined using *E. coli* mutant K12-LPS (InvivoGen, San Diego, CA, USA) together with three commercial bovine pooled colostrum concentrates containing known amounts of IgG antibodies: (i) Colexan premium grade colostrum; 60% total IgG (Colostrum Biotec GmbH, Königsbrunn, Germany), (ii) Peptide Ignition colostrum; 20% total IgG (TBR Labs, Spanish Fork, UT, USA) and (iii) Westland colostrum; 20% total IgG (Westland Milk Products, Hokitika, NZ). A commercial pasteurised fat-free pooled skimmed milk powder (Anchor, Fonterra, NZ) was used as a source of immunoglobulin-free milk.

(iii) *Preparation of bacteria shed LPS and whole lysates*. Milk collected from cows with clinical mastitis were cultured on blood agar/MacConkey plates (Fort Richards laboratories, Auckland, NZ) using standard microbiological techniques, followed by identification using MALDI-TOF MS analysis. Shed LPS from selected gram-negative bacteria, *E. coli*, *S. marcescens, K. oxytoca*, and *P. aeruginosa* was generated by heating bacterial cultures at 80 °C for 30 min, followed by sonication for 1 min and removal of bacterial debris by centrifugation (4000 *g*, 10 min, RT).[49] Endotoxin activity was determined using LAL bioassay. Whole bacterial lysates of selected mastitis causing gram-positive bacteria, *S. uberis, CNS and S. aureus* were generated by microfluidisation.

(iv) *Specificity of free LPS detection using colostrum IgG*. *(a) Competition with polymyxin B.* Prior to the ELISA, *E. coli* mutant K12-LPS (2.5 μg/ml) diluted in the sodium citrate/TTx100, pH 5.4 buffer was incubated with soluble polymyxin B (0.75–50 μg/ml) for 30 min at RT. Samples were then added to the polymyxin B ELISA matrix and incubated for 90 min at 37 °C. *(b) Milk interference*. *E. coli* mutant K12-LPS (2.5 μg/ml) was diluted in sodium citrate/TTx100, pH 5.4 buffer containing 1: 10,000–1:10 dilution of whole raw milk (aseptically collected from a local dairy farm), followed by a 30 min incubation at RT. In these assays, samples were analysed using the ELISA described above. *(c) LPS-colostrum IgG binding interactions. E. coli* shed LPS (0.0009–1 μg/ml) was diluted in sodium citrate/TTx100, pH 5.4 buffer, incubated for 30 min at RT and then either analysed in the ELISA, or prior to the ELISA mixed with an equal volume of colostrum preparation #1 (0.2 mg/ml [w/v] total IgG) for 10 min at RT. In this scenario, the HRP conjugate was directly used to detect LPS/IgG complex binding to the polymyxin B matrix.

Soluble LPS/IgG complex detection by ELISA

(i) *Detection of soluble milk LPS/IgG complexes*. Soluble LPS/IgG complexes were detected by ELISA with the modification that the HRP-conjugated protein G was added after blocking the wells with 2% BSA/PBS. The presence of soluble LPS/IgG complexes was assessed in five commercial bovine pooled colostrum extracts containing known amounts of IgG antibodies and diluted in ELISA sample to achieve the required dilution: (i) Launchpad 18 colostrum; 18% total IgG (AgriVantage, Chemiplas (NZ) Limited, NZ, (ii) Westland colostrum, (iii) Colexan premium grade colostrum, (iv) NZ pure colostrum; 20% total IgG (NZ Pure Health, Auckland, NZ), and (v) Peptide Ignition colostrum. Raw milk and colostrum samples collected for this study were diluted 1:1 (v/v) in ELISA sample buffer prior to the ELISA, similarly, experiments where LPS/IgG complexes were assessed were also diluted 1:1(v/v) in sample buffer.

(ii) *Characterisation of soluble LPS/IgG complex detection*. This was examined using commercial Colexan premium grade colostrum: *(a) Heat denaturation of colostrum proteins.* A 10 mg/ml dose ([v/w] total IgG using manufacturers' specification values) of this colostrum preparation, un-boiled or boiled (~98 °C) for 10 min was assessed by the ELISA. *(b) Competition with polymyxin B.* Colostrum (0.62 mg/ml [w/v] total IgG) was diluted in citrate buffer containing 0.62–500 μg/ml polymyxin B sulphate and incubated for 30 min at RT prior to performing the ELISA.

Evaluation of IgG present in colostrum and milk that targets the LPS glycolipid core

(i) *Detection of free LPS* Initial experiments utilised a pooled colostrum enriched in IgG (but was shown to contain minimal soluble LPS/IgG complexes) to evaluate IgG binding to (i) *E. coli* mutant K12-LPS or (ii) shed LPS (from a wild-type *E. coli* strain commonly found in clinical gram-negative mastitis milk). *E. coli* mutant K12-LPS (0.009–5 μg/ml) and shed LPS (0.009–1 μg/ml) were analysed by ELISA using an IgG enriched colostrum (0.1 mg/ml [w/v] total IgG). Next selected doses of *E. coli* mutant K12-LPS (2.5 μg/ml) or shed LPS (0.1 μg/ml) were analysed by ELISA using either raw colostrum or milk collected from cows diagnosed with clinical *E. coli* mastitis. Data were calculated as differences in absorbance values between 450–650 nm and shown as bar charts or a histogram depicting the frequency number (#) of the absorbance values for colostrum or milk IgG binding to either *E. coli* mutant K12-LPS or *E. coli* shed LPS.

(ii) *Characterisation of LPS by SDS-PAGE*. LPS samples; *E. coli* mutant K12-LPS (5 μg), *E. coli* 055.B5 LPS (5 μg, Sigma-Aldrich, Merck Pty. Ltd, Auckland, NZ) and *E. coli* shed LPS (25–500 ng, based upon LAL endotoxin activity) were reduced and separated by SDS-PAGE (16% separating gel) using standard protocols. The gel was then reversed stained using zinc sulphate/imidazole protocol described by Castellanos-Serra & Hardy.[50] The gel image was recorded by digital photography, with the gel placed upon a glass plate and held just above a black surface with lighting from the sides. The image was recorded using Microsoft power-point with brightness/contrasting adjusted to highlight the LPS O-antigen ladder.

Generation of milk IgG extracts to examine LPS binding properties and endotoxin activity

(i) *Enrichment of milk IgG antibodies*. Initial experiments were performed to select colostrum and *E. coli* mastitis milk samples that exhibited *in situ* a differential anti-LPS IgG binding to the glycolipid core (i.e., *E. coli* mutant K12-LPS) in the ELISA. In addition, these milk samples were assessed for soluble LPS/IgG complex levels by ELISA, LAL endotoxin activity and LDH activity. IgG content was enriched using a salting-out technique described by Skalka, and colleagues,[51] so as maintain the same IgG proportions reflected in the selected colostrum and *E. coli* mastitis milk *in situ* LPS-IgG binding profiles. Briefly, after the removal of milk fat and contaminating LPS compounds (and associated compounds, using a purafix ET-R syringe filter [Filtrox-AG, St Gallen, Switzerland]), IgG antibodies were precipitated with ammonium sulphate (final saturation ~ 45%), followed by dialysis of the IgG preparation against PBS and concentration using protein concentrators MWCO 30 kDa (Thermo-Fisher, Auckland, NZ). Protein content was measured using a BCA protein assay (Pierce, Thermo-Fisher, Auckland, NZ). Preparative qualitative analysis of milk IgG extracts by SDS-PAGE verified the presence of IgG in the extracts against a bovine IgG standard (1 ug, purified bovine sera IgG, MPI biomedicals, pH Scientific Ltd, Auckland, NZ). Levels of anti-LPS IgA and IgM in colostrum and *E. coli mastitis* milk extracts were also assessed. In these ELISA shed LPS (100 ng/ml) was used to verify the undetectability of these antibodies using specific HRP conjugated anti-IgA (HRP conjugated sheep anti-bovine IgA, Invitrogen, Thermo-Fisher, Auckland, NZ) or anti-IgM (HRP conjugated rabbit anti-Bovine IgM, Sapphire Bioscience Pty, Ltd, Redfern, Australia) antibodies. In addition, the absence of LPS-IgA and LPS-IgM complexes in the milk IgG extracts was verified using modified LPS/IgG complex ELISA using the HRP conjugated antibodies listed above.

(ii) *Standardisation of milk IgG extracts for LPS binding and functional experiments*. Detection of shed LPS (0.1 μg/ml) by ELISA was used to standardise the amount (mg/ml) of IgG extract. Data were calculated as differences in absorbance values between 450–650 nm, and the results were used to select concentrations of milk IgG extracts for LPS binding and functional experiments. Next the IgG binding profiles of the milk IgG extracts to shed LPS (0.9–500 ng/ml) and *E. coli* mutant K12-LPS (2.2–2500 ng/ml) were examined by ELISA . In these ELISAs, LPS binding was detected using fixed concentrations of colostrum (0.062 mg/ml) or *E. coli* mastitis milk (0.5 mg/ml) IgG extracts.

(iii) *Preincubation of shed LPS with milk IgG extracts*. Shed LPS (3.1–200 ng/ml) was diluted in PBS, pH 7.4, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and incubated with buffer or fixed concentrations of colostrum (0.062 mg/ml) or *E. coli* mastitis milk (0.5 mg/ml) IgG extracts for 1 h at 37 °C. The mixture was then clarified by centrifugation at 6000 *g* for 5 min (RT) and supernatants collected, diluted in appropriate assay buffer, and assessed

for LAL activity or the presence of soluble LPS/IgG complexes by ELISA.

Milk IgG modulation of shed LPS stimulated ROS generation by milk granulocytes

(i) *Preparation of milk granulocyte.* Milk granulocytes were isolated from pooled raw milk, collected within 2 h of milking and quickly chilled to 4 °C, using a modified method described by Mehrzad and co-workers[52] that preserved cell viability. Milk was diluted (1:1) in ice-cold 10 mM PBS, pH 7.2, and centrifuged at 600 *g* for 5 min at 10 °C. The supernatant and fat were removed, and the pellet washed in PBS at 300 *g* for 10 min, followed by a final wash at 200 *g* for 5 min. The final pellet was re-suspended in PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.5 mg/ml gelatin adjusted to 2×10^6 cells/ml, kept on ice, and used within 2 h of isolation. Quality/viability of milk granulocytes were assessed using microscopy and trypan blue staining. Only milk granulocyte preparations showing minimal cell debris and viability > 90% were used to assess oxidative status in milk granulocytes. Changes in milk granulocyte oxidative status (changes in ROS generation) were monitored using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) as described by others.[52,53] Changes in cellular fluorescence intensity (ΔFI); excitation 492 nm, emission 527 nm, at 37 °C over a specified time (mins) were measured in a SpectroMax M5 plate reader (Molecular Devices, San Jose, CA, USA). Data were calculated as ΔFI minus background (buffer only). To verify that a change in fluorescence within H_2DCFH loaded milk granulocytes represented changes in ROS, a ROS scavenger N-acetyl cysteine NAC (0.5–10 mg/ml) was incubated with H_2DCFH loaded cells for 30 min (RT), prior to being stimulated with LPS.

(ii) *LPS stimulated ROS generation.* Shed LPS (12.5–200 ng/ml) was added to H_2DCFDA loaded granulocytes (0.5×10^6 cells/ml), placed in a preheated 37 °C plate reader where the fluorescence was monitored over 20 min. The effect of colostrum or *E. coli* mastitis milk IgG extracts (final concentration 0.062 or 0.5 mg/ml, respectively) were assessed by adding them directly to H_2DCFDA-loaded milk granulocytes and monitoring the ΔFI over 20 min. In experiments examining the impact of these milk IgG extracts on shed LPS-induced ROS, shed LPS (3.1–200 ng/ml) was initially preincubated with either buffer or fixed concentration colostrum or *E. coli* mastitis milk IgG extracts (final concentration 0.062 or 0.5 mg/ml, respectively) for 1 h to generate clarified supernatants. These supernatants were then directly added to H_2DCFDA-loaded milk granulocytes (0.5×10^6 cells/ml) and the ΔFI was then monitored over 20 min.

(iii) *fMLP stimulated ROS generation.* fMLP (0.25–0.5 μM) was added to H_2DCFDA loaded granulocytes (0.5×10^6 cells/ml) and ΔFI was monitored over 10 min. The impact of colostrum or *E. coli* mastitis milk IgG extracts (final concentration 0.062 or 0.5 mg/ml, respectively) on fMLP stimulated ROS was assessed by preincubating the milk IgG extracts with H_2DCFDA-loaded milk granulocytes for 20 min (37 °C) prior to fMLP (0.1 μM) stimulation for a further 10 min. In these experiments, baseline ΔFI was also measured in H_2DCFDA-loaded milk granulocytes to verify that the granulocytes after the 20 min incubation period were still responsive to fMLP. Next, we examined the impact of the milk IgG extracts on the ability of LPS to augment fMLP-stimulated ROS. Shed LPS (3.1–200 ng/ml) was initially incubated with either buffer or fixed concentrations of colostrum or *E. coli* mastitis milk IgG extractions (final concentration 0.062 or 0.5 mg/ml, respectively) for 1 h to generate clarified supernatants. These supernatants were then added to H_2DCFDA-loaded milk granulocytes (0.5×10^6 cells/ml), incubated for 20 min at 37 °C and then stimulated with fMLP (0.1 μM) for a further 10 min. Inclusion of H_2DCFDA-loaded milk granulocytes incubated with buffer alone was used to verify granulocytes were responsive to fMLP stimulation.

Statistical analysis

Data were analysed using Microsoft Excel Analysis ToolPak statistical software or power-point image software (2021). Results using milk indices, specific milk IgG antibodies alone or combination with LPS are expressed as mean ± standard deviation (SD) and/or standard error of the mean (SEM). Statistical significance for the comparison between these data variables were assessed using a two-way analysis of variance. A significant result ($P < 0.01$) in this test was followed by a Tukey post-*hoc* analysis set at $P < 0.01$ (95% confidence level). A paired Student's *t*-test, set at $P < 0.05$ (95% confidence level) was used to identify statistical differences between 2 indices within the sample group, e.g., LAL activity in un-boiled vs. boiled milk samples. Linear regression analysis between soluble LPS/

IgG immune complex levels and the two independent variables; endotoxicity or LDH activity, was set at $P < 0.01$ with a confidence level of 95%.

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Author contribution

SH, DF and AP conceived the study and provided experimental design. SH, DF and RK conducted experiments, validated assays, and analysed results. SH and AP wrote the manuscript.

Declaration of conflicting interests

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: The authors, SH, DF and RK are or were (DF) employees of Koru Diagnostics Ltd, confirm there is no individual personal financial relationship and declare that research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest. AP is a shareholder of Koru Diagnostics Ltd and as CSO was involved in conceptualisation, supervision, investigation and writing of the original draft. No payment or services from a third party was received for any aspect of the submitted work. In addition, selective concepts and data described in this manuscript are part of a patent submission by Koru Diagnostics Ltd, entitled "Method for Detecting Microorganisms and Uses Thereof" in November 2022. Patent Ref: PCT/NC2022/050136, #KDL1007PC. The raw data supporting the conclusions of this study are not readily available as they are proprietary and part of a pending patent submission. However, requests to access specific data should be directed to Koru Diagnostics Ltd info@korudiagnostics.com.

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