Dietary Bovine Lactoferrin Reduces *Staphylococcus aureus* in the Tissues and Modulates the Immune Response in Piglets Systemically Infected with *Staphylococcus aureus*

Elizabeth A. Reznikov[1], Sarah S. Comstock[3], Jennifer L. Hoeflinger[2], Mei Wang[2], Michael J. Miller[1, 2], Sharon M. Donovan[1,2*]

[1]Division of Nutritional Sciences and [2]Department of Food Science and Human Nutrition, University of Illinois, Urbana, Illinois, [3]Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Michigan.

*** Address Correspondence:** 339 Bevier Hall, 905 S. Goodwin Avenue, University of Illinois, Urbana, IL 61801, Phone: 217-333-2289; E-mail: sdonovan@illinois.edu

Supported by: This research was supported by USDA Hatch Project ILLU-698-311.

Disclosure Statement: EAR, SSC, JLH, MW, MJM, SMD no conflict of interest.

Running Title: Lactoferrin protects against *S. aureus* infection

Category of Study: Basic Science

Supplementary Material: Supplemental Tables 1-3 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://cdn.nutrition.org.

Abbreviations used: BI, group receiving *B. infantis; Bifidobacterium longum biovar infantis*, *B. infantis*, bLF, bovine lactoferrin; CFU, colony forming units; CON, control formula group; COMB, piglets fed bLF and receiving *B. infantis;* LF, bLF formula group; *Staphylococcus aureus*, *S. aureus;* TLR2, toll-like receptor 2

ABSTRACT:

Background: Bovine lactoferrin (bLf) reduces *Staphylococcus aureus* infection in premature infants and promotes the growth of *Bifidobacterium infantis*, a predominant infant gut species. We hypothesized bLf in combination with *B. infantis* would reduce the severity of systemic *S. aureus* infection.

Objective: To determine the effects of oral administration of bLf and *B. infantis* on the course of systemic *S. aureus* infection.

Methods: Colostrum-deprived piglets consumed formulas containing 4g/l whey (CON) or bLf (LF). Half were gavaged with *B. infantis* (10^9 CFU/d) resulting in two additional groups (BI or COMB). On d7, piglets were intravenously injected with *S. aureus*. Blood samples were collected pre-infection and every 12h post-infection for immune analyses. Tissue samples were collected on d12 for analysis of bacterial abundance and gene expression.

Results: Pre-infection, LF piglets had lower serum IL-10, higher percent lymphocytes and lower percent neutrophils than BI or COMB. After infection, dietary bLf increased piglet weight gain, reduced staphylococcal counts in kidney and tended to lower staphylococcal counts in lung and heart. Dietary bLf also decreased kidney IL-10 and increased lung IFNγ mRNA. *B. infantis* increased splenic IFNγ expression. Renal toll-like receptor 2 was upregulated in BI piglets, but not in COMB piglets. Post-infection, BI piglets had increased serum IL-10 and decreased memory T cell populations. LF and COMB piglets had fewer circulating monocytes and B-cells than CON or BI piglets.

Conclusions: Dietary bLf and *B. infantis* produced independent and tissue-specific effects. Piglets fed bLf alone or in combination with *B. infantis* mounted a more effective immune response and exhibited lower bacterial abundance, whereas *B. infantis* administration was

associated with greater tissue bacterial abundance. This study provides biological underpinnings to the clinical benefits of bLf observed in preterm infants, but does not support *B. infantis* administration during *S. aureus* infection.

Key Words: lactoferrin, infection, probiotic, immune system, sepsis, neonate

INTRODUCTION

Staphylococcus aureus infection is the most common fatal bacterial infection in neonates worldwide (1). The infection can result in endocarditis, pneumonia, osteomyelitis, and septic shock (2). In recent years, antibiotic-resistant strains of *S. aureus* have emerged, limiting treatment options for life-threatening *S. aureus* infections in infants (2). A clinical trial (3) and subsequent meta-analysis (4) showed that orally-administered bovine lactoferrin (bLf) reduced the incidence of late-onset sepsis in very low birth weight infants colonized with gram-positive bacteria, including *Staphylococcus* species (3).

The role of lactoferrin in the immune response and infection has recently been reviewed (5, 6). These effects likely occur in the intestinal lumen as well as through direct effects on immune cells as bLF is resistant to proteolytic digestion (7, 8) and because both piglet and human intestinal epithelial cells express lactoferrin receptors (9-11). Although the mechanism of action whereby dietary bLf reduces *S. aureus* infection is unknown, several modes of action are plausible. First, lactoferrin is an iron-binding glycoprotein, which may exert antimicrobial effects by preventing microbes from accessing adequate iron. Lactoferrin is also released by neutrophils at the sites of injury or infection. The release of lactoferrin inhibits infiltration of inflammatory neutrophils while attracting monocytes, suggesting that lactoferrin plays an important role in regulating inflammation thereby preventing sepsis without inhibiting the original immune response (12). In addition to having effects on the early immune response, reports in the literature suggest that lactoferrin is important in generating a Th1 adaptive immune response in mice (13, 14) and piglets (15). A Th1 adaptive response would favor a cellular-immune response to intracellular *S. aureus* that would increase bacterial clearance from tissues. Lactoferrin also promotes the growth of beneficial commensal bacteria of the *Bifidobacteria* spp., especially *Bifidobacterium infantis* (16). Foxp3 expressing intestinal and splenic T regulatory cells were

53 increased in *B. infantis*-fed mice (17) and humans (18). This evidence suggests that lactoferrin
54 and *B. infantis* may act synergistically to influence regulatory immune function in addition to
55 having independent effects on the innate and adaptive immune responses resulting in an
56 improved immune response to a pathogenic challenge.

57 This study was designed to analyze the immune response to a systemic *S. aureus*
58 infection in piglets fed bLf alone, *B. infantis* alone, or bLf and *B. infantis* in combination.
59 Because bLf and *B. infantis* promote key innate and adaptive immune responses, we
60 hypothesized that bLf in combination with *B. infantis* would be more efficacious at improving
61 the anti-bacterial immune response and bacterial clearance during a blood-borne infection than
62 either bLF or *B. infantis* alone.

63

64 **MATERIALS and METHODS**

65 *Animal Protocol and Diets*

66 All animal care and experimental procedures were approved by the Institutional Animal
67 Care and Use Committee at the University of Illinois. Pregnant sows (n=15) at the University of
68 Illinois Swine Research Center were monitored for farrowing beginning on d110 of gestation and
69 female piglets (n=49) were removed prior to ingestion of colostrum. Due to the close proximity
70 of the umbilicus and urethral opening in male piglets, females were selected to avoid soaking of
71 bandages and urethral catheters with urine. Piglets were randomized to treatment group based on
72 birth weight and litter. All pigs weighed ~1.5kg at birth. To provide passive immunity, sow
73 serum was administered to the piglets via oral gavage at a volume of 5 ml/kg BW at birth, 12, 24
74 and 36h postpartum. Piglets were individually housed in cages in environmentally-controlled
75 rooms (25°C). Plastic heating pads in each enclosure were used to maintain an ambient

temperature of 30°C. Piglets were fed a non-medicated, sow-milk replacer formula (Liqui-Wean Advance, Milk Specialties Global Animal Nutrition, Carpentersville, IL) with either 4g/l of supplemental protein as whey protein (CON; Provon 192; Glanbia) or bLf (LF; Bioferrin 200; Glanbia, Kilkenny, Ireland). This dose of bLf was chosen based on our previous research (15, 19) and whey was added to the CON diet to keep the diets isonitrogenous. Piglets were fed 20-times daily totaling 360 ml/kg BW/d. Half of the piglets in each group were further randomized to receive *Bifidobacterium longum biovar infantis* (*B. infantis;* 3 x 10^9 CFU/day): Whey + *B. infantis* (BI) and bLf + *B. infantis* (COMB). Resulting in four treatment groups: LF, CON, BI, COMB.

B. infantis Preparation, Storage, and Administration

B. infantis ATCC 15697 (ATCC, Manassas, VA) was grown from a frozen stock in deMan, Rogosa, Sharpe broth (Difco, Livonia, MI) supplemented with cysteine (0.05%) and incubated anaerobically as previously described (20). Mid-exponential phase *B. infantis* was harvested by centrifugation, resuspended in sterile PBS and cryogenically preserved in a 1:1 cell to glycerol (25%) suspension. Each batch of *B. infantis* inoculum was validated for viability on Reinforced Clostridial Agar. On average, each stock contained 5 x 10^8 colony forming units (CFU) per ml. Prior to administration, *B. infantis* was washed in PBS, and the bacterial pellet was resuspended in PBS for administration. Piglets in the BI and COMB groups were orally gavaged with 2 ml of the washed *B. infantis* thrice daily for a total dosage of ~3 x 10^9 CFU/day. This dose was selected based on typical probiotic dosing regimens (21-23).

99

100 *Umbilical Catheterization*

101 Piglets underwent a surgical procedure within 12 h of birth to place two umbilical
102 catheters using established methods (24). One catheter was used for administering the *S. aureus*
103 and the other for blood sampling. Briefly, piglets were lightly sedated with 2% isoflurane
104 (IsoFlo, Abbott Laboratories, North Chicago, IL). The abdomen was washed and an iodine
105 disinfectant applied to the umbilicus and surrounding region. To numb the umbilicus, lidocaine
106 (Henry Schein, Melville, NY) was injected subcutaneously into multiple sites surrounding the
107 umbilical stump. One catheter (3.5 french polyvinyl chloride catheter; Tyco Healthcare Group,
108 Mansfield MA) was inserted 22 cm into the dorsal aorta to a position near the heart. A second
109 catheter for blood sampling was inserted and advanced 20 cm. Catheters were sutured to the
110 umbilical stump and secured to the body with suture and elastic tape. Piglets were fitted into a
111 jacket to protect the catheterization site while allowing free movement within the cages. Catheter
112 patency was maintained by flushing twice daily with heparinized saline (10 IU heparin/ml in
113 0.9% NaCl). Piglets received one systemic dose of Enrofloxacin (2.5 mg/kg; Bayer, Shawnee
114 Mission, KS) immediately after surgery.

115

116 *S. aureus Infection*

117 *S. aureus* strain S54F9 was a gift from Dr. Bent Aalbaek, (University of Copenhagen,
118 Denmark) (25). *S. aureus* was cultured in Brain Heart Infusion broth aerobically at 37°C. Prior to
119 administration, stationary phase *S. aureus* cells were harvested by centrifugation, resuspended in
120 0.9% sterile isotonic saline and diluted to 10^5 CFU/ml. On d7, all animals were administered *S.*
121 *aureus* at 1ml per kg body weight via the umbilical catheter. This dose was chosen based on a

pilot study in which the temporal clinical signs and immune responses to two *S. aureus* doses (10^3 and 10^5 CFU) were evaluated compared to non-infected piglets (26).

Sample Collection

Body weight and formula intake were assessed daily. Rectal temperature and activity were assessed every 12h post-infection. Blood samples were collected into non-heparinized vacuum tubes via the sampling umbilical catheter prior to infection and every 12h post-infection for cytokine analyses. Heparinized blood was collected at 72h and 120h post-infection for PBMC isolation. Prior to infection and at 120h post-infection, fresh blood smears were collected for complete blood counts (CBC)(Clinical Pathology Laboratory, Hematology Department, University of Illinois College of Veterinary Medicine, Urbana, IL). At 120h post-infection, piglets were euthanized by an intravenous injection of sodium pentobarbital (72 mg/kg BW, Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI).

Measuring B. infantis in Ascending Colon Contents and Feces

DNA Extraction: Total genomic DNA was isolated from ascending colon (AC) contents and feces using a combination of bead beating and the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germantown, MD). Approximately 200 mg of AC or feces was combined with 1 ml of InhibitEx buffer in a 2 ml Lysing Matrix B tube. Tubes were shaken at 6 m/s for 30s using the Fastprep 24 System (MP Biomedicals, Solon, OH). Samples were incubated at 95°C for 5 min and centrifuged at 20,800 x *g* for 1 min. DNA was purified from 200 µl of supernatant using the QIAmp Fast DNA Stool Mini Kit according to the manufacturer's instructions. Isolated DNA

was quantified with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

RT qPCR: The absolute abundance of *B. infantis* in AC contents and feces was quantified by qPCR using primers BiINF-1 and BiINF-2 (27). PCR was performed with an Applied Biosystems 7900HT Fast Real-Time PCR System using SYBR Green assays. PCR was run in triplicate with a reaction volume of 10 µl: 5 µl of 2X Power SYBR Green PCR Master mix (Applied Biosystems), 1 µl bovine serum albumin (1mg/ml, New England Biolabs, Ipswich, MA), 0.5 µM of each primer and 10 ng of template DNA. The cycling conditions were 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles of 95˚C for 15s, 55 ˚C for 20s and 72˚C for 60s. Following amplification, a dissociation step was included to analyze the melting profile of the amplified products. Standard curve (10^2–10^7 16S rRNA gene copies per reaction) was generated using purified pCR 4 TOPO-TA plasmids (Thermo Fisher Scientific) containing the 16S rRNA genes of *B. infantis*. Data were processed with SDS v2.3 software (Thermo Fisher Scientific).

Flow Cytometry to Identify PBMC subpopulations

PBMC were obtained by Ficoll-Hypaque centrifugation of heparinized blood. Cells were resuspended in flow staining buffer (PBS, 1% BSA, 0.1% sodium azide). Cell populations were assessed by flow cytometry using fluorescently-labeled antibodies as described (28). Cell staining antibody cocktails are presented in **Supplemental Table 1** (online).

T lymphocyte populations were expressed as a percent of CD3+ events for $CD3^+CD4^+CD8^-$ (T helper cells) and $CD3^+CD4^-CD8^+$ (Cytotoxic T cells) and $CD3^+CD4^+CD8^+$ (Memory T cells). NK cells were identified as $CD3^-CD4^-CD8^+$ events and expressed as a percent of $CD3^-$ events. Monocytes were identified as $CD14^+CD163^+CD172a^+$ events and expressed as a percent of

CD172a^{+} events. B cells were identified as CD21^{+}MHCII^{+} events and expressed as a percent of total lymphocytes.

Serum Cytokines

Serum was obtained by centrifugation of blood samples and was analyzed using porcine-specific ELISA kits for interferon-gamma (IFNγ) (limit of detection [LOD]: 62.5 pg/ml), IL-6 (LOD: 125 pg/ml) and IL-10 (LOD: 23.4 pg/ml) (R&D Systems, Minneapolis, MN). IL-10 was measured in blood samples taken pre-infection and every 12 h post-infection. IFNγ was measured in blood samples taken pre-infection and 24, 36 and 72h post-infection. IL-6 was measured in blood samples taken pre-infection and 72h post-infection. For samples with concentrations below the LOD of the assay (the lowest point on the standard curve), the value was set to one-half the LOD (29).

Detecting S. aureus in Blood and Tissue Samples

Immediately after euthanasia, blood was collected by cardiac puncture into heparin-laced vials. Five grams of whole organ cross sections for the kidney (each section included: left, cortex and medulla), lung (each section included: left, caudal lobe), heart (each section included: apex, left and right ventricles) and spleen were collected. Tissues were homogenized at a 1:5 dilution in sterile PBS using a stomacher (Stomacher 80 Biomaster, Seward Laboratory Systems Inc., Port St. Lucie, FL). For each tissue sample, three cultures were started on mannitol salt agar (MSA) (BD, Franklin Lakes, NJ), a selective and differential media for *S. aureus:* 200 µl was hand-plated at 1:5, 50 µl was spiral plated (Neu-Tec Group Inc., Farmingdale, NY) at 1:5, and 50

µl was spiral plated at 1:10. Blood samples were plated undiluted on MSA. All tissue samples were plated in triplicates at each dilution. Plates were incubated for 48h aerobically at 37 °C before being counted. Colony counts were averaged for each set of triplicates and corrected for the dilution factor. Final results for *S. aureus* load are expressed as CFU/g of tissue.

Tissue Cytokine Analysis

RNA Extraction: Frozen kidney, lung and spleen samples (100 mg) were homogenized with 1 mL of TRIzol reagent per the manufacturer's instructions (Thermo Fisher Scientific). RNA was dissolved in 20 µl of nuclease-free water (Thermo Fisher Scientific) and quantified using a NanoDrop 1000 instrument (Thermo Fisher Scientific). Samples were diluted to an RNA concentration of ≤500 ng/µl, and RNA quality was determined using a 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA).

RT qPCR: Samples with an RNA integrity number (RIN) > 6 were transformed into cDNA utilizing a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific). PCR was performed using TaqMan gene expression assays for *IFNG* (Ss03391053_g1), *IL10* (Ss03382372_u1) and *TLR2* (Ss03381278_u1). The expression of the 60S ribosomal protein L19 (*RPL19*; Ss03375624_g1) gene was used as an endogenous control. The relative standard curve method was used for quantitation. Standard curves consisted of dilutions of cDNA created from spleen mRNA pooled from animals in all of the treatment groups. Normalized values for each target were calculated by dividing the target quantity mean by the RPL-19 quantity mean. A fold-difference was calculated for each measurement by dividing the normalized target values by the normalized calibrator sample. Animals fed whey (CON) were used as the calibrator in each instance.

Statistical Analysis

Statistical analyses were performed using SAS Version 9.2 (Cary, NC). Body weight, rectal temperatures, serum cytokines and immune cells were compared among groups by repeated-measures ANOVA using PROC MIXED with treatment (bLf or no bLf), probiotic (received or did not receive *B. infantis*) and time as fixed effects. The model also included all interactions treatment by time, treatment by probiotic, and probiotic by time. Because some pre-infection measurements were missing, CBC/differential data were analyzed by 1-way ANOVA (treatment) using PROC GLM. RT qPCR data was analyzed by 1-way ANOVA (treatment) using PROC GLM. The statistical model for this data included fixed effects for treatment (bLf or no bLf), probiotic (received or did not receive *B. infantis*), and the interaction between treatment and probiotic. Staphylococcal load was analyzed using a nonparametric Wilcoxon Rank Sum on CFU/g, where the one-sided Z-statistic was used due to a sample size greater than 10. In the event of a significant main effect, a post hoc Least Significant Difference test was used. The abundances of *B. infantis* are presented as means (Log_{10} copies/g of content) ± SD. The number of 16S rRNA gene copies was log_{10} transformed prior to analysis. To compare *B. infantis* abundance, statistical analysis was performed using the PROC MIXED procedure of SAS version 9.2 (SAS Institute) with Tukey post-hoc tests. Fixed effects included diet, probiotic and the interaction of diet and probiotic. Replicate was included as random effect. When the abundance of *B. infantis* was below the LOD (2×10^5 copies of 16S rRNA genes/g of content), ½ value of the LOD was used. The occurrence of *B. infantis* among treatments was analyzed by Fisher's exact test. Statistical significance was defined as $P<0.05$ and trends reported as $P<0.10$. Unless otherwise stated, data are presented as mean ± SEM.

RESULTS

Formula Intake, BW and Survival

All piglets were fed an additional 4g/l protein. CON and BI pigs received whey protein. LF and COMB pigs received bLf. Formula intake was unaffected by treatment or infection and averaged 793 ± 30 ml/d on d1-d7 and 1092 ± 27 ml/d following infection between d7-12 postpartum. Thus, mean supplemental protein intake was 4 g/day at d7 and 4.8 g/day at d12. Prior to infection all piglets had similar body weights. The addition of the probiotic *B. infantis* to either diet had no effect on body weight, therefore, values were pooled by bLf supplement: bLf-supplemented (LF and COMB) and non-bLf supplemented (CON and BI) (**Figure 1**). Following infection, bLF-supplemented piglets weighed more than non-bLf supplemented piglets (treatment x time, $P<0.0001$). At the end of the study, bLf-supplemented piglets weighed 3.7 ± 0.1 kg and non-bLf-supplemented piglets weighed 3.4 ± 0.1 kg (Figure 1). Four piglets died during the experiment (1 LF, 2 BI and 1 COMB) and were not included in the analyses. The LF piglet had failure to thrive throughout the experiment. The other three piglets died 1, 2 or 3 days post-infection. While these post-infection deaths could be attributed to the *S. aureus* infection, their growth and rectal temperatures post-infection did not differ from that of piglets that survived the study.

Rectal Temperature

Rectal temperatures peaked 36h post-infection and remained elevated until euthanasia at 120 h post-infection. *B. infantis* had no effect on rectal temperatures, so temperatures were pooled by bLf supplement: bLf-supplemented (LF and COMB) and non-bLf-supplemented (CON and BI). Post-infection, there was a significant overall effect where bLf-supplemented animals had greater rectal temperatures than non-bLf-supplemented piglets (P=0.01) with no interaction between protein supplement and hour. On average, post-infection rectal temperatures in bLf-supplemented pigs were 0.21°C higher than those of non-bLf-supplemented animals. Rectal temperatures differed by time (P<0.0001). All animals had elevated rectal temperatures at 36 h post-infection compared with baseline, 12 or 24h post-infection. All animals continued to have elevated rectal temperatures until euthanasia at 120h post-infection. At baseline, just prior to infection, animals had rectal temperatures of 39.2 ± 0.2, 39.1 ± 0.2, 39.0 ± 0.1, and 39.1 ± 0.1 °C for CON, BI, LF and COMB, respectively. At 36h post-infection, animals had rectal temperatures of 39.3 ± 0.1, 39.5 ± 0.2, 40.0 ± 0.2 and 39.7 ± 0.2 °C for CON, BI, LF and COMB, respectively.

Detection of B. infantis in AC and Feces

B. infantis was detected more frequently in the AC and feces from piglets inoculated with *B. infantis* than in non-inoculated pigs (94.4% vs 4.2% in AC; 100% vs. 5% in feces; $P < 0.0001$) (Supplemental **Table 2**). The occurrences of *B. infantis* were similar in BI and COMB (the treatment groups that included probiotic) pigs. The occurrences *B. infantis* were significantly higher in BI and COMB pigs compared to CON and LF (the treatment groups without probiotic) pigs in both sampling sites. Bovine Lf had no effect on the abundance of *B. infantis. B. infantis* abundance was greater in both AC and feces of BI (7.0 ± 1.1 and 7.2 ± 1.0 $\log_{10}$ copies/g,

respectively) and COMB (7.5 ± 1.4 and 7.4 ± 1.0 $\log_{10}$ copies/g, respectively) compared to CON and LF (*B. infantis* was detected in only one LF AC sample and one CON fecal sample). In both AC and fecal samples, the abundance of *B. infantis* did not differ between BI and COMB groups.

Peripheral Blood Cell Populations

Blood cell populations were assessed pre- and 120h post-infection using cell counts from whole blood smears. *B. infantis* administration had no effect on whole blood cell populations. However, bLf influenced circulating cell populations (**Supplemental Table 3**). Prior to infection, the peripheral blood from bLf-supplemented animals (LF and COMB) had more lymphocytes and tended to have fewer neutrophils than the blood from non-bLf-supplemented animals (CON and BI). Post-infection, only one pig (in the BI group) met the immature-to-total neutrophil ratio cut-off (>0.25) indicative of sepsis (30). On average, bLf-supplemented piglets had 43 ± 3.7% and non-bLf-supplemented piglets had 31 ± 3.0% blood lymphocytes. bLf-supplemented pigs had 53 ± 3.8% and non-bLf-supplemented pigs had 62 ± 3.4% blood neutrophils. Following infection, nucleated red blood cell (NRBC) populations were lower (P=0.047) in bLf-supplemented piglets compared to non-bLF-supplemented piglets. On average, NRBC were 3.5 ± 0.9% and 7.3 ± 1.7% of total blood cells in bLf-supplemented and non-bLF supplemented piglets, respectively.

Using peripheral blood mononuclear cells (PBMC) isolated by density gradient centrifugation, B cell, T cell, NK cell, and monocyte/macrophage cell populations were assessed at 72h and 120h post-infection.

303 B cell populations were similar for all piglets within each time point (**Table 1**). The
304 percentage increase in PBMC B cells from 72h to 120h post-infection differed by protein
305 supplement group, with bLf-supplemented piglets experiencing a smaller increase in B cell
306 population size than CON piglets (P=0.04).
307 Monocyte populations were similar for piglets in all four treatment groups at both 72 and
308 120h post-infection (Table 1). The percent difference in PBMC monocytes from 72h to 120h
309 post-infection in bLf-supplemented piglets tended (P=0.07) to be smaller than that in non-bLf-
310 supplemented animals (Table 1).
311 T cell subpopulations: Neither T helper (70.2 ± 8.4%) nor cytotoxic T cell (5.3 ± 0.6%)
312 populations differed among treatment groups at either time point. However, in an analysis
313 measuring only the effect of treatment at 120h post-infection, memory T cell populations were
314 smaller (P=0.03) in piglets receiving *B. infantis* than in piglets that did not receive the probiotic
315 (Table 1). The percent difference in PBMC memory T cell populations from 72h to 120h post-
316 infection in probiotic supplemented piglets was lower (P=0.03) than the percent difference in
317 monocytes from 72 to 120h post-infection in non-probiotic-supplemented animals.
318 NK cell populations were similar for all piglets at each timepoint, but NK cell populations
319 were larger 120h post-infection (5.8 ± 1.0%) than 72h post-infection (2.6 ± 0.4%).
320
321 *Serum Cytokines*
322 Of the three serum cytokines analyzed, only IL-10 was detected in the serum of piglets at
323 multiple time points. Neither IFN-γ nor IL-6 was detectable in piglets prior to infection nor were
324 these two cytokines detectable in the majority of piglets at 72h post-infection. Prior to infection
325 on postpartum d7, there was no effect of probiotic, therefore data were pooled by bLF (LF and

326 COMB) vs non-bLf (CON and BI)-supplemented piglets. Piglets fed formula with bLf had 3-fold
327 lower (P=0.01) serum IL-10 than non-bLf-supplemented piglets (**Figure 2a**). Following
328 infection, serum IL-10 concentrations changed significantly over time (P= 0.03). The highest
329 concentrations of IL-10 were observed at 96h (58.2 ± 24.0 pg/ml) and 108h (45.4 ± 16.7 pg/ml)
330 post-infection (**Figure 2b**). There was no effect of bLf on serum IL-10 post-infection. Therefore,
331 data were pooled by probiotic treatment. Probiotic-treated animals (BI and COMB) had higher
332 serum IL-10 (P=0.03) concentrations than piglets that did not receive *B. infantis* (Figure 2b).
333
334 *S. aureus in blood and tissues*
335 Bacterial number: *S. aureus* was detected in the kidneys, lungs, hearts and spleens, but
336 not in the blood. There was no effect of BI on *S. aureus* content in blood or tissues, therefore
337 data was pooled by bLf supplement (**Figure 3**). bLf-supplemented pigs had lower numbers
338 (CFU) of *S. aureus* in the kidney (P=0.02) and tended to have lower numbers in lung (P=0.07)
339 and heart (P=0.06) compared to piglets who consumed diets without bLf. There was no effect of
340 bLf on *S. aureus* numbers in the spleen. Overall, dietary bLf decreased kidney, lung and heart *S.*
341 *aureus* load by 7.3-, 4- and 1.8-fold, respectively.
342
343 *Immune Gene Expression in the Tissues*
344 Kidney: Piglets exposed to *B. infantis* in the context of a whey protein diet (BI) had the
345 highest (P=0.01) kidney TLR2 mRNA expression. However, piglets exposed to *B. infantis* in the
346 context of a bLf diet (COMB) had renal TLR2 mRNA expression similar to that of CON or LF
347 piglets (**Table 2**). Pigs consuming diets with bLf (LF and COMB) had decreased renal IL-10
348 expression (P=0.03). Piglets receiving *B. infantis* in the absence of dietary bLf (BI) tended

(P=0.09) to have the highest renal IL-10 expression. Renal IFNγ expression was similar in all piglets.

Lung: IFNγ expression was significantly higher in LF and COMB piglets compared to CON and BI piglets (Table 2). No significant differences in TLR2 or IL-10 mRNA expression in the lung were observed.

Spleen: Piglets exposed to *B. infantis* had higher (P=0.02) splenic IFNγ mRNA expression (Table 2) than piglets that were not given the probiotic. Splenic TLR2 and IL-10 mRNA expression was similar in all piglets.

DISCUSSION

The goal of this study was to investigate the individual and combined effects of bLf and *B. infantis* on the clinical course of a systemic *S. aureus* infection in the neonatal piglet. This research was predicated on previous clinical trials demonstrating the orally-administered bLf reduced the incidence of late-onset sepsis in very low birth weight infants colonized with gram-positive bacteria, including *Staphylococcus* species (3). Furthermore, *B. infantis* has been shown to be immunomodulatory (17, 31) and bLf may promote the growth of *B. infantis* (16), suggesting the potential for synergistic effects. Based on our previous research (15, 19), by the 7th day of life, bLf improved gastrointestinal development and immune system development compared to piglets fed formula alone. Thus, these early effects on the gastrointestinal and immune systems likely give the bLf-fed piglets an advantage over their formula fed peers upon systemic challenge with *S. aureus*.

Despite promising clinical outcomes, the immune response is difficult to assess in human infants, thus the systemic and tissue immune responses over the course of *S. aureus* infection

were assessed using the newborn piglet. The piglet is an excellent preclinical model for this research for the following reasons: *S. aureus* is a dominant cause of widespread septicemia in pigs and humans (32); swine physiology, immune response and anatomy are highly similar to those of humans (33); and, most importantly, pigs are capable of reproducing the gradual pathophysiologic changes and clinical characteristics of neonatal sepsis (34), including body weight and temperature, which are easily monitored in piglets. The use of bLf in this appropriate pre-clinical model for human infants was deliberate as this is the same compound that can used in human infant formulas. In the current study, dietary bLf and *B. infantis* each influenced the response to *S. aureus* infection in the neonatal piglet, but the combination of bLf and *B. infantis* did not exert synergistic effects. Overall, the severity of *S. aureus* infection was reduced in piglets fed dietary bLf, with limited beneficial effects of *B. infantis.*

Dietary bLf improved weight gain following *S. aureus* infection. The beneficial effect of bLf on growth cannot be attributed to the presence of additional protein, as the CON and BI diet were supplemented with 4 g/l whey protein to maintain similar protein content to the LF and COMB diets (supplemented with 4g/l bLf. Previous studies have shown that bLf improves weight gain in piglets (35) and human infants (36). The increase in body weight has been partially attributed to its proposed role in regulating the immune system and providing protection against microbial infection. Wherein, animals that more effectively respond to infection have a reduced period of cachexia and can apportion more energy toward growth versus directing that energy toward a sustained immune response.

A key finding of this study was that dietary bLf significantly reduced bacterial abundance in the kidneys and tended to lower the *S. aureus* counts in the lungs and hearts of infected animals. The improved bacterial clearance in the presence of bLf was likely due in part to an enhanced

Th1 immune response in bLf-supplemented pigs. In our study, bLf-supplementation increased IFNγ mRNA expression in lung following infection, indicating a Th1 response. Furthermore, bLf-supplemented pigs had less serum IL-10 on d7 post-partum just prior to *S. aureus* infection. A high level of IL-10 is known to inhibit Th1 immune responses (37). Others have shown that Lf-transgenic mice demonstrated an enhanced Th1 response to *S. aureus* infection. These mice showed greater IFN-γ response and increased ability to clear the bacterial infection (13).

Accordingly, *S. aureus* persistence was affected by dietary treatment. As in previous studies (25, 38), *S. aureus* was rapidly cleared from the blood of infected pigs but persisted in other tissues. Compared to supplementation with whey protein (CON, BI), supplementation with bLf (COMB, LF) significantly reduced *S. aureus* load. This was expected based on previous reports in rodents where bLf supplementation reduced *S. aureus* load in the kidneys of *S. aureus* challenged mice (14) and where Lf-transgenic mice demonstrated an increased ability to clear *S. aureus* following a challenge (13). Importantly, bLf-supplementation decreased *S. aureus* counts in the kidney and tended to decrease counts in the lung and heart.

In contrast, the probiotic *B. infantis* had no effect on bacterial clearance when administered alone. This may be related to increased TLR2 message and IL-10 message as well as IL-10 protein in these animals. *In vitro* work has shown that *S. aureus* down-regulates the inflammatory T cell response by triggering IL-10 production by monocytes via TLR2 activation (39, 40). The increased IL-10 dampens the immune response enabling *S. aureus* to colonize the host (40). Consistent with the IL-10 serum cytokine data, probiotic-treated animals also had fewer circulating memory T cells with significantly fewer memory T cells compared to non-probiotic-treated pigs at 120h post-infection. In the kidney, the enhanced TLR2 and IL-10 mRNA expression in response to probiotic treatment was decreased when bLf was present. In

418 fact, the presence of bLf also decreased renal IL-10 message compared to the CON diet.
419 However, as the effects were not consistent across tissues (kidney, lung, spleen, blood), other
420 mechanisms may be at work. TLR2 signaling regulates additional inflammatory cytokine
421 responses (41, 42), and *S. aureus* stimulates additional immune receptors so effects of bLf and *B.*
422 *infantis* on other cytokines are also important. For instance, bLf-supplementation increased IFNγ
423 expression in the lung. It is important to note that bLf may not only have decreased bacterial
424 load, but also may have impaired *S. aureus* viability leading to these lower tissue counts.

425 We hypothesized that bLf would stimulate a robust Th1 immune response. Because *B.*
426 *infantis* is known to down-regulate inflammatory responses (17), we hypothesized *B. infantis*
427 would provide protection in the later phase of infection. In addition, bLf has been shown to
428 promote growth of *B. infantis in vitro* (16), suggesting a potential mechanism by which these
429 dietary components could synergize. In this experiment, bLf did not support the growth of *B.*
430 *infantis* over that seen in pigs exposed to *B. infantis* in the absence of bLf. However, we
431 observed that bLf-supplementation was most important for *S. aureus* bacterial clearance. Post-
432 infection, *B. infantis* had an overall effect of increased serum IL-10, which is consistent with
433 what has been observed in mice and humans supplemented with *B. infantis* (17, 18). High
434 concentrations of IL-10 have consistently been shown to be a strong indicator of septic shock
435 and predictor of mortality during infection due to its broadly immunosuppressive function (43).
436 Consistent with this, the only pig to have reached the diagnostic immature-to-mature neutrophil
437 ratio for sepsis (44) was in the BI group. Furthermore, probiotic-treated pigs had higher post-
438 infection NRBC counts. NRBCs are another cell population that has been associated with
439 septicemia (45). It has been shown that in addition to promoting a Th1 response through
440 increased IFNγ production, bLf can also increase the IL-12-to-IL-10 ratio in lipopolysaccharide

(LPS) stimulated splenocytes to promote the Th1 response (46). The presence of IL-12, a cytokine that promotes the Th1 response, in addition to IFNγ, leads to significantly decreased production of IL-10 (46). However, in our studies, we did not measure IL-12 and no IFNγ was detected in the serum. One possible explanation for this could be that IFNγ is an intracellular cytokine and its presence may be too low in the serum to detect. Previously, we found that spleen cells isolated from pigs fed bLf produced more IFNγ and TNFα than cells isolated from pigs fed a control diet (15). Although NK cells are the most likely potential sources of IFNγ, it may be that peripheral memory T cells contain intracellular IFNγ, both of these cells could release the IFNγ when needed during an immune response. Future experiments should use intracellular cytokine staining in combination with flow cytometry or ELISpot assays to determine the IFNγ production potential of cells isolated from areas local to the infection.

Despite the novelty of the model, the study described herein has several limitations. Neither iron status nor levels of bLf in stool and serum were measured. Therefore, the current study could not assess the fate of ingested bLf nor determine if the effects of bLf on *S. aureus* infection were due to bLf sequestration of iron from *S. aureus*. This study was designed to test the regulatory and adaptive immune response to *S. aureus* infection, based on the hypothesis that those would be the stages of the immune response during which the effects of combining probiotic and bLf treatment would be most efficacious at reducing the severity of *S. aureus* infection. Therefore, most details about the effects of the dietary treatments on the early immune response *S. aureus* infection are unknown. However, blood was collected pre-infection and every 12h post-infection for some analyses. IFN-γ was measured pre-infection and at 24, 36, and 72h post-infection. IL-6 was measured pre-infection and at 72h post-infection. These time points were chosen based on a pilot study. Because a limited volume of blood could be sampled (due to

464 piglet size and the repeated sampling design), blood was used for cytokine analyses rather than *S.*
465 *aureus* quantification at the early time points post-infection. Thus, conclusions about the effects
466 of the dietary treatment on *S. aureus* levels in the blood at early time points post infection cannot
467 be drawn. Finally, although the presence of *B. infantis* was determined in feces and ascending
468 colon content, no attempt was made to test for *B. infantis* in the blood or other tissues. Therefore,
469 any effects that the potential translocation of the probiotic from the gut to the bloodstream could
470 have on the immune response to infection are unknown. Future studies should measure bLf in
471 fecal matter, serum and urine, and should carefully assess the iron status (ferritin, transferrin, free
472 iron) of subjects. Additional studies, should also examine the effects of probiotic and bLf on
473 early inflammatory and innate immune responses to infection.This preliminary animal study
474 provides mechanistic insight supporting clinical studies showing the effectiveness of orally
475 administered bLf in the prevention of systemic *S. aureus* infections and suggests that future
476 studies investigating lactoferrin's role in treating *S. aureus* are warranted. IL-10 is a critically
477 important predictor of mortality during infection. Therefore, the observation that bLf lowers
478 serum IL-10 pre-infection and BI increases serum IL-10 post-infection provides evidence that
479 modulation of IL-10 is one potential mechanism by which bLf reduces tissue bacterial
480 abundance in this model of systemic *S. aureus* infection. Furthermore, another indicator of
481 sepsis, NRBC counts, were lower in pigs fed bLf. Although NRBC counts were not affected by
482 the presence of *B. infantis*, exposure to the probiotic increased serum IL-10 levels post-infection
483 potentially indicating that this probiotic should be avoided by neonates at risk of systemic *S.*
484 *aureus* infection. However, due to its ability to induce IL-10, *B. infantis* may be a useful therapy
485 in already septic neonates. In conclusion, this study suggests that colostrum and mother's milk,

two liquids with abundant Lf concentrations (47, 48), may protect neonates from *S. aureus* infections and from the complications caused by such infections.

Acknowledgements

We thank Gianna Vella for assistance with conducting the RT-qPCR analyses. We thank Dr. Barbara Pilas in the Roy J. Carver Biotechnology Center Flow Cytometry Core at the University of Illinois for her guidance. The authors' responsibilities were as follows—EAR: designed the research, conducted the research, analyzed data, performed statistical analyses, wrote the paper; SSC: designed the research, analyzed data and performed statistical analyses, and wrote the paper; JLH: provided technical assistance; MW: provided technical assistance; MJM provided essential reagents and materials as well as technical assistance; SMD: directed the research and has primary responsibility for the final content. All authors have read and approved the final manuscript.

REFERENCES

1. Park DA, Lee SM, Peck KR, Joo EJ, Oh EG. Impact of methicillin-resistance on mortality in children and neonates with *Staphylococcus aureus* bacteremia: A meta-analysis. Infect Chemother 2013;45:202-10.
2. Maraqa NF, Aigbivbalu L, Masnita-Iusan C, Wludyka P, Shareef Z, Bailey C, Rathore MH. Prevalence of and risk factors for methicillin-resistant Staphylococcus aureus colonization and infection among infants at a level III neonatal intensive care unit. Am J Infect Control 2011 Feb;39:35-41.
3. Manzoni P, Rinaldi M, Cattani S, Pugni L, Romeo MG, Messner H, Stolfi I, Decembrino L, Laforgia N, Vagnarelli F, et al. Bovine lactoferrin supplementation for prevention of late-onset sepsis in very low-birth-weight neonates: a randomized trial. JAMA 2009;302:1421-8.
4. Pammi M, Suresh G. Enteral lactoferrin supplementation for prevention of sepsis and necrotizing enterocolitis in preterm infants. Cochrane Database Syst Rev 2017;6:CD007137.
5. Manzoni P. Clinical benefits of lactoferrin for infants and children. J Pediat 2016;173 Suppl:S43-52.
6. Drago-Serrano ME, Campos-Rodriguez R, Carrero JC, de la Garza M. Lactoferrin: Balancing ups and downs of inflammation due to microbial infections. Intl J Molec Sci 2017;18. doi: 10.3390/ijms18030501.
7. Davidson LA, Lönnerdal B. Persistence of human milk proteins in the breast-fed infant. Acta Paediatr Scand 1987;76:733-40.
8. Hutchens TW, Henry JF, Yip TT. Structurally intact (78-kDa) forms of maternal lactoferrin purified from urine of preterm infants fed human milk: identification of a trypsin-like proteolytic cleavage event in vivo that does not result in fragment dissociation. Proc Natl Acad Sci USA 1991;88:2994-8.
9. Harada E, Itoh Y, Sitizyo K, Takeuchi T, Araki Y, Kitagawa H. Characteristic transport of lactoferrin from the intestinal lumen into the bile via the blood in piglets. Comp Biochem Physiol A Mol Integr Physiol 1999;124:321-7.
10. Kawakami H, Lonnerdal B. Isolation and function of a receptor for human lactoferrin in human fetal intestinal brush-border membranes. Am Journal Physiol 1991;261:G841-6.
11. Liao Y, Lopez V, Shafizadeh TB, Halsted CH, Lonnerdal B. Cloning of a pig homologue of the human lactoferrin receptor: expression and localization during intestinal maturation in piglets. Comp Biochem Physiol A Mol Integr Physiol 2007;148:584-90.
12. de la Rosa G, Yang D, Tewary P, Varadhachary A, Oppenheim JJ. Lactoferrin acts as an alarmin to promote the recruitment and activation of APCs and antigen-specific immune responses. J Immunol 2008180:6868-76.
13. Guillen C, McInnes IB, Vaughan DM, Kommajosyula S, Van Berkel PH, Leung BP, Aguila A, Brock JH. Enhanced Th1 response to *Staphylococcus aureus* infection in human lactoferrin-transgenic mice. J Immunol 2002 Apr 15;168:3950-7.
14. Bhimani RS, Vendrov Y, Furmanski P. Influence of lactoferrin feeding and injection against systemic staphylococcal infections in mice. J Appl Microbiol 1999;86:135-44.

15. Comstock SS, Reznikov EA, Contractor N, Donovan SM. Dietary bovine lactoferrin alters mucosal and systemic immune cell responses in neonatal piglets. J Nutr 2014;144:525-32.
16. Rahman MM, Kim WS, Ito T, Kumura H, Shimazaki K. Examination of bovine lactoferrin binding to bifidobacteria. Prikl Biokhim Mikrobiol 2008;44:529-32.
17. O'Mahony C, Scully P, O'Mahony D, Murphy S, O'Brien F, Lyons A, Sherlock G, MacSharry J, Kiely B, Shanahan F, et al. Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-kappaB activation. PLoS Pathogens 2008;4:e1000112. doi: 10.1371/journal.ppat.1000112.
18. Konieczna P, Groeger D, Ziegler M, Frei R, Ferstl R, Shanahan F, Quigley EM, Kiely B, Akdis CA, O'Mahony L. *Bifidobacterium infantis* 35624 administration induces Foxp3 T regulatory cells in human peripheral blood: potential role for myeloid and plasmacytoid dendritic cells. Gut 2012;61:354-66.
19. Reznikov EA, Comstock SS, Yi C, Contractor N, Donovan SM. Dietary bovine lactoferrin increases intestinal cell proliferation in neonatal piglets. J Nutr 2014;144:1401-8.
20. Francl AL, Hoeflinger JL, Miller MJ. Identification of lactose phosphotransferase systems in *Lactobacillus gasseri* ATCC 33323 required for lactose utilization. Microbiology 2012;158:944-52.
21. Athalye-Jape G, Deshpande G, Rao S, Patole S. Benefits of probiotics on enteral nutrition in preterm neonates: a systematic review. Am J Clin Nutr 2014;100:1508-19.
22. Lyseng-Williamson K. *Bifidobacterium infantis* 35624 as a probiotic dietary supplement: a profile of its use. Drugs Ther Perspect 2017 2017;33:368-74.
23. Ouwehand AC. A review of dose-responses of probiotics in human studies. Benef Microbes 2017;8:143-51.
24. Donovan SM, Chao JC, Zijlstra RT, Odle J. Orally administered iodinated recombinant human insulin-like growth factor-I (125I-rhIGF-I) is poorly absorbed by the newborn piglet. J Pediatr Gastroenterol Nutr 1997;24:174-82.
25. Leifsson PS, Iburg T, Jensen HE, Agerholm JS, Kjelgaard-Hansen M, Wiinberg B, Heegaard PM, Astrup LB, Olsson AE, Skov MG, et al. Intravenous inoculation of *Staphylococcus aureus* in pigs induces severe sepsis as indicated by increased hypercoagulability and hepatic dysfunction. FEMS Microbiol Lett 2010;309:208-16.
26. Reznikov EA, Hoeflinger JL, Monaco MH, Miller MJ, Donovan SM. Development of a piglet model of neonatal systemic *Staphylococcus aureus* infection. FASEB J. 2013;27 (Suppl 1):1083.2.
27. Matsuki T, Watanabe K, Tanaka R, Fukuda M, Oyaizu H. Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. Appl Environ Microbiol 1999;65:4506-12.
28. Comstock SS, Wang M, Hester SN, Li M, Donovan SM. Select human milk oligosaccharides directly modulate peripheral blood mononuclear cells isolated from 10-d-old pigs. Br J Nutr 2014;111:819-28.
29. Chung MK, Riby J, Li H, Iavarone AT, Williams ER, Zheng Y, Rappaport SM. A sandwich enzyme-linked immunosorbent assay for adducts of polycyclic aromatic hydrocarbons with human serum albumin. Anal Biochem 2010;400:123-9.

30. Caserta MT. Infections in Neonates. Neonatal Sepsis. The Merck Manual Online Medical Library Database Merck Manual Professional Pediatrics, 2015 [cited July 20, 2017]; Available from: http://www.merckmanuals.com/professional/pediatrics/infections-in-neonates/neonatal-sepsis
31. Underwood MA, Arriola J, Gerber CW, Kaveti A, Kalanetra KM, Kananurak A, Bevins CL, Mills DA, Dvorak B. Bifidobacterium longum subsp. infantis in experimental necrotizing enterocolitis: alterations in inflammation, innate immune response, and the microbiota. Pediatr Res 2014;76:326-33.
32. Jensen HE, Nielsen OL, Agerholm JS, Iburg T, Johansen LK, Johannesson E, Moller M, Jahn L, Munk L, Aalbaek B, et al. A non-traumatic *Staphylococcus aureus* osteomyelitis model in pigs. *In vivo* 2010;24:257-64.
33. Meurens F, Summerfield A, Nauwynck H, Saif L, Gerdts V. The pig: a model for human infectious diseases. Trends Microbiol 2012;20:50-7.
34. Kato T, Hussein MH, Sugiura T, Suzuki S, Fukuda S, Tanaka T, Kato I, Togari H. Development and characterization of a novel porcine model of neonatal sepsis. Shock 2004;21:329-35.
35. Wang Y, Shan T, Xu Z, Liu J, Feng J. Effect of lactoferrin on the growth performance, intestinal morphology, and expression of PR-39 and protegrin-1 genes in weaned piglets. J Anim Sci 2006;84:2636-41.
36. Hernell O, Lönnerdal B. Iron status of infants fed low-iron formula: no effect of added bovine lactoferrin or nucleotides. Am J Clin Nutr 2002;76:858-64.
37. Fiorentino DF, Zlotnik A, Vieira P, Mosmann TR, Howard M, Moore KW, O'Garra A. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J Immunol 1991;146:3444-51.
38. Nielsen OL, Iburg T, Aalbaek B, Leifsson PS, Agerholm JS, Heegaard P, Boye M, Simon S, Jensen KB, Christensen S, et al. A pig model of acute *Staphylococcus aureus* induced pyemia. Acta Vet Scand 2009;51:14.
39. Takeuchi O, Hoshino K, Akira S. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. J Immunol 2000;165:5392-6.
40. Chau TA, McCully ML, Brintnell W, An G, Kasper KJ, Vines ED, Kubes P, Haeryfar SM, McCormick JK, Cairns E, et al. Toll-like receptor 2 ligands on the staphylococcal cell wall downregulate superantigen-induced T cell activation and prevent toxic shock syndrome. Nature Med 2009;15:641-8.
41. Yimin, Kohanawa M, Zhao S, Ozaki M, Haga S, Nan G, Kuge Y, Tamaki N. Contribution of toll-like receptor 2 to the innate response against Staphylococcus aureus infection in mice. PloS One 2013;8:e74287. doi: 10.1371/journal.pone.0074287
42. Nandi A, Dey S, Biswas J, Jaiswal P, Naaz S, Yasmin T, Bishayi B. Differential induction of inflammatory cytokines and reactive oxygen species in murine peritoneal macrophages and resident fresh bone marrow cells by acute *Staphylococcus aureus* infection: Contribution of toll-like receptor 2 (TLR2). Inflammation 2015;38:224-44.
43. Heper Y, Akalin EH, Mistik R, Akgoz S, Tore O, Goral G, Oral B, Budak F, Helvaci S. Evaluation of serum C-reactive protein, procalcitonin, tumor necrosis factor alpha, and interleukin-10 levels as diagnostic and prognostic parameters in patients with community-

acquired sepsis, severe sepsis, and septic shock. Eur J Clin Microbiol Infect Dis 2006;25:481-91.

44. Engle WD, Rosenfeld CR, Mouzinho A, Risser RC, Zeray F, Sanchez PJ. Circulating neutrophils in septic preterm neonates: comparison of two reference ranges. Pediatrics 1997;99:E10.
45. Cremer M, Roll S, Graf C, Weimann A, Buhrer C, Dame C. Nucleated red blood cells as marker for an increased risk of unfavorable outcome and mortality in very low birth weight infants. Early Hum Dev 2015;91:559-63.
46. Hwang SA, Wilk KM, Bangale YA, Kruzel ML, Actor JK. Lactoferrin modulation of IL-12 and IL-10 response from activated murine leukocytes. Med Microbiol Immunol 2007;196:171-80.
47. Masson PL, Heremans JF. Lactoferrin in milk from different species. Comp Biochem and Physiol B 1971;39:119-29.
48. Hirai Y, Kawakata N, Satoh K, Ikeda Y, Hisayasu S, Orimo H, Yoshino Y. Concentrations of lactoferrin and iron in human milk at different stages of lactation. J Nutr Sci Vitaminol 1990;36:531-44.

Tables

Table 1. Immune cells in isolated PBMC at 72 and 120h after *S. aureus* infection in piglets fed control formula (CON), control formula with *B. infantis* administration (BI, $3x10^9$ CFU/d) or formula with 4 g/L bLf alone (LF) or with *B. infantis* administration (COMB).

	B-Cells ($CD21^+MHCII^+$) [a]			**Monocytes($CD14^+CD163^+CD172a^+$)[b]**			**Memory T-Cells ($CD4^+CD8^+CD3^+$) [c]**		
	72h	**120h**	**% Difference[d]**	**72h**	**120h**	**% Difference**	**72h**	**120h**	**% Difference**
CON	4.2±0.8	10.0±2.9	240 ± 59	18.5±5.2	24.9±7.5	192±74	9.6±1.2	10.7±1.2	119±15
BI	6.1±1.3	11.2±2.6	177±20	20.3±4.2	23.3±5.2	201±71	9.5±0.9	8.3±0.6*	92±9*
LF	6.0±1.1	8.4±1.8	151±25*	23.5±4.3	18.6±4.6	102±25†	9.2±1.1	10.8±1.2	124±17
COMB	5.3±1.0	6.6±2.0	112±18*	22.3±4.6	18.7±5.1	104±31†	10.2±1.4	9.0±0.8*	91±9*

Abbreviations: CON, control; BI, *Bifidobacterium infantis*; LF, bovine lactoferrin; COMB, both *Bifidobacterium infantis* and bovine lactoferrin; *S. aureus*, *Staphylococcus aureus* strain S54F9

Data are expressed as Mean ± SEM

[a] Expressed as a % of total PBMC

[b] Expressed as a % of $CD172^+$ cells

[c] Expressed as a % of $CD3^+$ cells

[d] Immune cell percentage at 120h divided by the Immune cell percentage at 72h x 100

*Means in a column without a common superscript differ by treatment, $P<0.05$; †Means in a column without a common superscript showed a trend to differ by treatment, $p=0.07$

Table 2. Immune-related gene expression in kidney, lung and spleen at 120h after *S. aureus* infection in piglets fed control formula (CON), control formula with *B. infantis* administration (BI, $3x10^9$ CFU/d) or formula with 4 g/L bLf alone (LF) or with *B. infantis* administration (COMB).

	Kidney			**Lung**			**Spleen**		
	IFNγ	**TLR2**	**IL-10**	**IFNγ**	**TLR2**	**IL-10**	**IFNγ**	**TLR2**	**IL-10**
CON	1.0±1.0	1.0±0.16	1.0±0.3	1.0±0.3	1.0±0.2	1.0±0.2	1.0±0.2	1.0±0.1	1.0±0.1
BI	1.2±1.1	2.5±0.6*	2.3±1.0	0.9±0.3	0.9±0.2	1.0±0.3	2.5±2.1*	1.2±0.1	1.0±0.1
LF	0.6±0.5	1.1±0.1	0.4±0.1*	2.0±0.5*	0.9±0.1	1.6±0.5	1.1±0.3	1.4±0.2	1.1±0.2
COMB	1.2±1.3	1.3±0.2	0.7±0.2*	1.5±0.4*	1.2±0.3	1.4±0.6	2.7±0.8*	1.2±0.3	1.0±0.2

Abbreviations: CON, control; BI, *Bifidobacterium infantis*; IFNγ, interferon-gamma; IL, Interleukin; LF, bovine lactoferrin; COMB, both *Bifidobacterium infantis* and bovine lactoferrin; *S. aureus*, *Staphylococcus aureus* strain S54F9; TLR, toll-like receptor

A fold-difference was calculated for each measurement by dividing the normalized target values (calculated by dividing the target quantity mean by the RPL-19 quantity mean) by the normalized calibrator values. For samples from the same tissue, values for CON animals were used as the calibrator. *Means in a column without a common superscript differ by treatment, $P<0.05$

Figure Legends

Figure 1. Body weight of piglets fed formula from birth and infected with *S. aureus* on d 7 postpartum. Prior to infection all piglets had similar body weights. The addition of the probiotic *B. infantis* had no effect on body weight, therefore, groups were pooled by bLf supplement: bLf-supplemented (bLf; LF and COMB) and non-bLf supplemented (No bLf; CON and BI) piglets. Following infection, on d10, 11 and 12, bLf-supplemented piglets weighed more than non-bLf supplemented (treatment x time, P<0.0001) piglets. Values are means ± SEM. *P<0.05 between no bLF and bLF. Abbreviations: bLF, bovine lactoferrin.

Figure 2. Serum IL-10 concentrations measured prior to *S. aureus* infection and every 12h post-infection. Pre-infection, bLf, but not *B. infantis* significantly affected serum IL-10, therefore groups were pooled by bLf (LF and COMB; n=26) vs. non-bLf (CON and BI; n=23). Prior to infection bLf-supplemented pigs had lower (P=0.01) serum IL-10 concentrations than non-bLf-supplemented pigs (**panel a**). Following *S. aureus* infection, there was a main effect of time (p=0.03), where animals produced the highest IL-10 concentration at 96 and 108h post-infection (**panel b**). There was also a main effect of probiotic (P=0.03), but notbLf, therefore values were pooled for probiotic (BI and COMB; n=26) and no probiotic (CON and LF; n=23). Probiotic treated piglets had greater serum IL-10 than non-probiotic treated piglets. Values are means ± SEM, n=11-13 per group. Abbreviations: IL, interleukin; LF, lactoferrin alone; BI, *B. infantis* alone; CON, control; COMB, combined; bLF, bovine lactoferrin.

Figure 3. *S. aureus* counts (CFU/g) in heart, kidney, lung, and spleen 120h after *S. aureus* infection in 12-day-old piglets fed control formula (CON), control formula with *B. infantis* administration (BI, 10^9 CFU/d) or formula with 4 g/L bLf alone (LF) or with *B. infantis* administration (COMB). There was no effect of *B. infantis*, therefore values were pooled by bLF supplement: bLf-supplemented (LF and COMB; bLf; n=26) and non-bLf-supplemented (CON and BI; No bLf; n=23). No differences were detected at the spleen. Piglets fed diets containing bLf had decreased *S. aureus* counts in the kidney and tended to have decreased CFU/g in the lung and heart. Values are means ± SEM. * $P<0.05$ and † $0.05>P<0.1$ between no bLf and bLf. Abbreviations: bLf, bovine lactoferrin; LF, lactoferrin alone; BI, *B. infantis* alone; CON, control; COMB, combined; CFU, colony forming units.

Figure 1

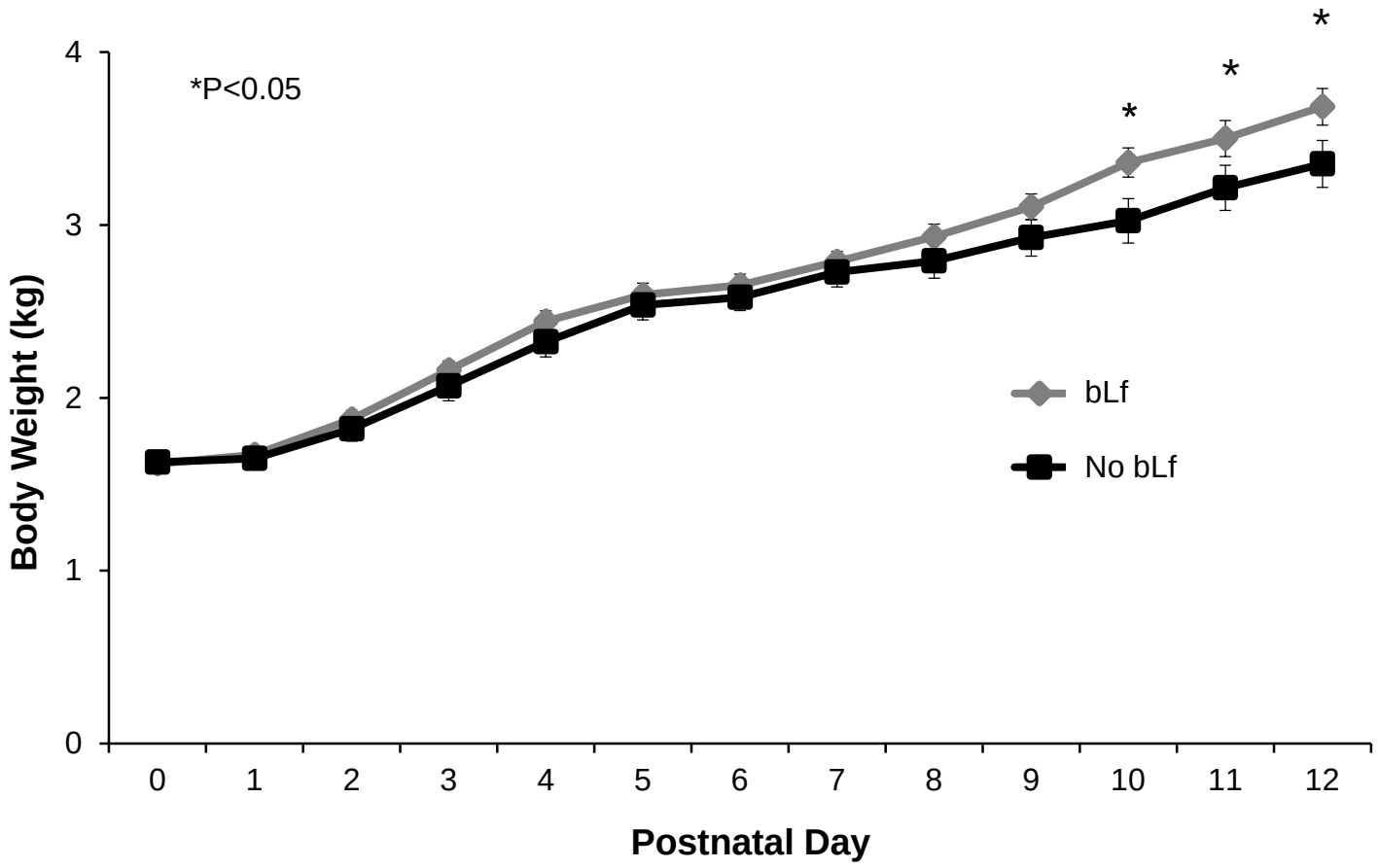

*P<0.05
*
*
*
Body Weight (kg)
0
1
2
3
4
0
1
2
3
4
5
6
7
8
9
10
11
12
Postnatal Day
bLf
No bLf

Figure 2

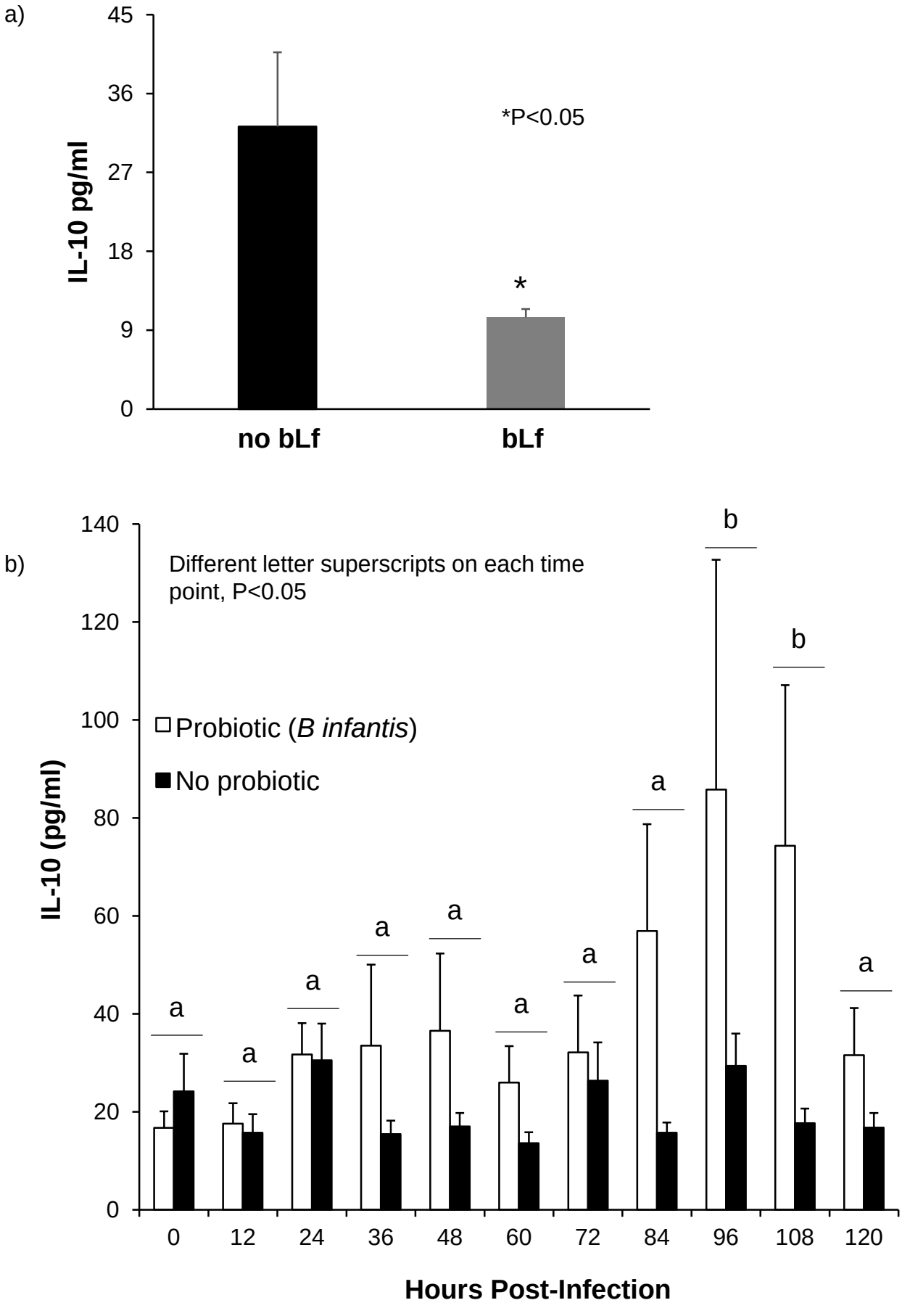
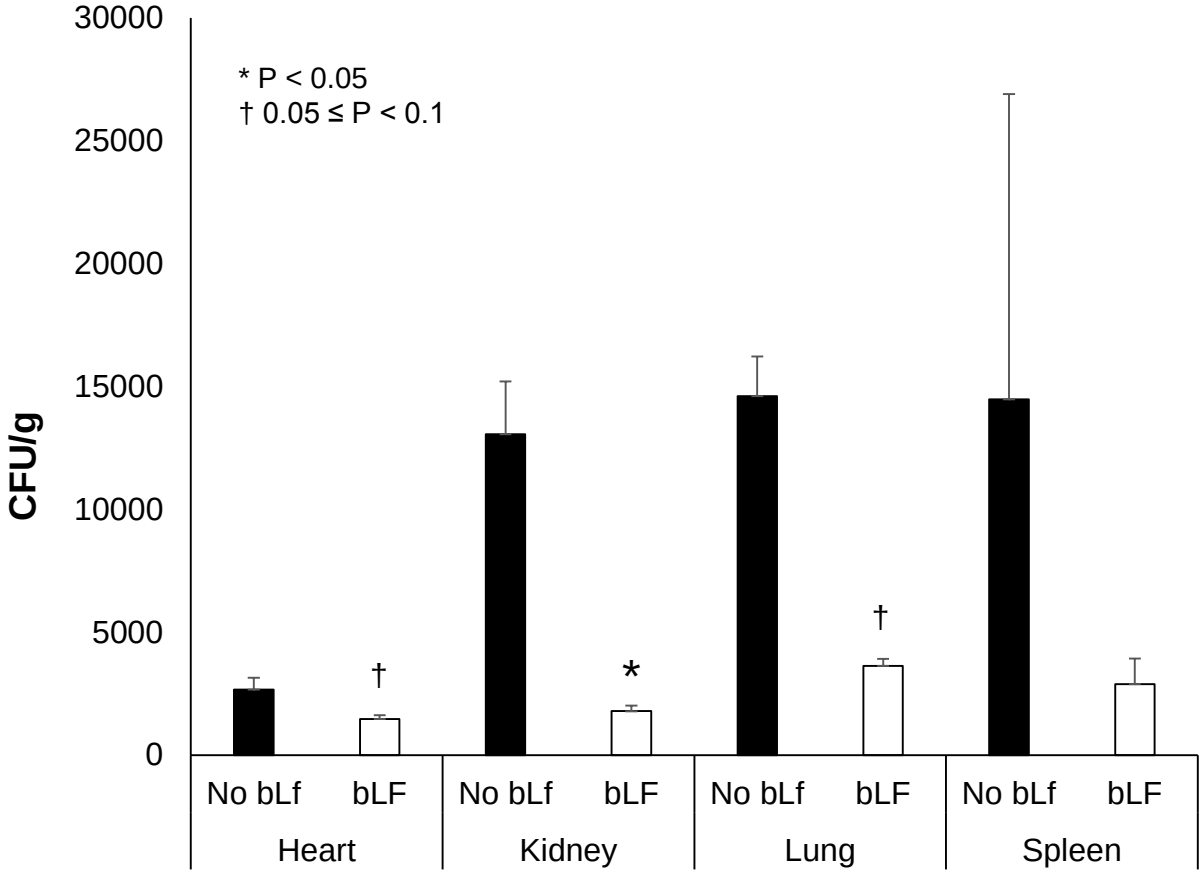


Figure 3


30000
25000
20000
15000
10000
5000
0
CFU/g
* P < 0.05
† 0.05 ≤ P < 0.1
†
*
†
No bLf
bLF
Heart
Kidney
Lung
Spleen

SUPPLEMENTAL DATA FOR PUBLICATION

Supplemental Table 1. Cocktails of Antibodies used to Detect Immune Cells by Flow Cytometry

Target Cell Populations	Antibodies in Cocktail (Clone)
T cells/Natural Killer Cells	Anti-CD3 (PPT3)[a]
	Anti-CD4 (74-12-4)[a]
	Anti-CD8 (76-2-11)[a]
Monocytes/Macrophages	Anti-CD172a (74-22-15)[a]
	Anti-CD163 (2A10/11)[b]
	Anti-CD14 (MIL2)[b]
B cells	Anti-CD21 (BB6-11C9.6)[a]
	Anti-SLA class II DR (2E9/13)[b]

[a]Southern Biotech (Birmingham, AL, USA)

[b]AbD Serotec, a Bio-Rad Company (Raleigh, NC, USA).

Supplemental Table 2. *B. infantis* in ascending colon contents and feces at 120h after *S. aureus* infection in piglets fed control formula (CON), control formula with *B. infantis* administration (BI, $3x10^9$ CFU/d) or formula with 4 g/L bLf alone (LF) or with *B. infantis* administration (COMB).

	Occurrence		**Abundance**	
	# positive/total # of animals, (%)		**Log_{10} copies/g**	
	AC	**Feces**	**AC**	**Feces**
CON	0/12, (0)	1/9, (11)	BLD	BLD
LF	1/12, (8)	0/11, (0)	BLD	BLD
BI	9/10, (90)*	8/8, (100) *	7.0 ± 1.1*	7.2 ± 1.1*
COMB	12/13, (92) *	9/9, (100) *	7.5 ± 1.4*	7.4 ± 1.0*

Abbreviations: AC, ascending colon; *B. infantis*, *Bifidobacterium longum* subsp. *infantis* ATCC 15697; BLD, below level of detection; CON, control; BI, *B. infantis*; LF, bovine lactoferrin; COMB, both *B. infantis* and bovine lactoferrin; *S. aureus*, *Staphylococcus aureus* strain S54F9

Data are expressed as Mean ± SD

*Means in a column without a common superscript differ by treatment, P<0.0001

Supplemental Table 3. Whole blood cell populations and percentages determined by CBC/differential analysis in 7-day-old piglets (pre-infection) or 12-day-old piglets (5 days post-infection with *S. aureus*) fed formula with 4 g/l whey (CON), control formula with *B. infantis* administration (BI, 10^9 CFU/d) or formula with 4 g/l bLf alone (LF) or with *B. infantis* administration (COMB).

	CON	LF	BI	COMB
Hemoglobin, g/dL				
Pre-Infection	7.5 ± 0.7	8.2 ± 0.4	7.5 ± 0.5	7.7 ± 0.4
Post-Infection	6.6 ± 0.3	6.3 ± 0.2	6.2 ± 0.3	6.5 ± 0.2
NRBC, per 200 WBC				
Pre-Infection	2.0 ± 0.9	2.5 ± 1.1	3.2 ± 0.9	3.0 ± 1.1
Post-Infection	5.4 ± 2.1	3.9 ± 1.5*	9.2 ± 2.6	3.2 ± 1.1*
WBC Count, per µL				
Pre-Infection	8.9 ± 2.1	10.9 ± 1.3	11.1 ± 1.0	9.7 ± 1.9
Post-Infection	10.0 ± 2.9	10.8 ± 2.0	11.4 ± 2.2	10.8 ± 1.6
Platelets, x 10^3 per µL				
Pre-Infection	486 ± 65.6	545 ± 55.9	487 ± 29.4	456 ± 66.8
Post-Infection	748 ± 71.5	723 ± 46.9	681 ± 90.3	745 ± 46.0
Seg, %				
Pre-Infection	66.9 ± 4.4	54.2 ± 5.1†	59.4 ± 5.0	51.9 ± 5.9†
Post-Infection	57.1 ± 5.8	57.3 ± 3.2	60.7 ± 4.7	60.5 ± 4.1
Band, %, (n detected / total n)				
Pre-Infection	0.9 ± 0.9 (1/7)	0.6 ± 0.5 (2/10)	7.0 ± 5.2 (5/10)	1.1 ± 0.7 (3/10)
Post-Infection	1.4 ± 0.5 (6/11)	0.9 ± 0.3 (7/13)	3.0 ± 1.9 (7/11)	1.0 ± 0.5 (5/13)
Lymph, %				
Pre-Infection	30.1 ± 4.4	42.6 ± 5.4*	31.5 ± 4.1	43.2 ± 5.5*
Post-Infection	37.2 ± 6.1	36.4 ± 3.1	31.6 ± 3.1	34.5 ± 3.6

Data are expressed as Mean ± SEM

*Means in a row without a common superscript differ by treatment, $P<0.05$; † Means in a row without a common superscript showed a trend to differ by treatment, $0.05 \geq P<0.10$.

Abbreviations: Lymph, lymphocytes; Band, immature neutrophils; Seg, neutrophils; WBC, white blood cells; NRBC, nucleated red blood cells.