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Maternal Supplementation of *Saccharomyces cerevisiae var. boulardii* to Sows from Late Gestation through Lactation Impacts the Neutrophil Function of the Sow and the Innate Immune Status of Progeny Short-Term

Lily P. Hernandez, Janeen L. Salak-Johnson*

Department of Animal and Food Sciences, Oklahoma State University, Stillwater, USA
Email: *janeen.johnson@okstate.edu

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Abstract

The primary objective of this study was to evaluate the potential immunomodulatory effect of maternal supplementation of *Saccharomyces cerevisiae var. boulardii* (Scb) from late gestation until the end of lactation on the immune phenotype of her progeny. Eighteen sows were fed 2 boluses per day of *Saccharomyces cerevisiae var. boulardii* CMCN-1079 (probiotic; PRO, n = 9) or placebo (CON, n = 9) starting at gestational day (GD) 84 and continuing until 21 days post-farrowing (end of lactation). Sow blood samples were collected every 7 days post-supplementation during gestation and 24-h post-farrowing and end of lactation. Blood samples were taken from 84 female pigs (n = 42 per sow treatment group) at 1, 7, 14, 21, 28, and 35 days old to assess innate and adaptive immune measures. Minimal effects of Scb supplementation were found on sow immune status during gestation and lactation, except for PRO-treated sows that had enhanced neutrophil function ($P < 0.05$) overall and mitogen-induced lymphocyte proliferation after 51 days of treatment ($P < 0.0001$). Overall, pigs from PRO-treated sows had higher C5a- and IL-8-induced neutrophil chemotaxis, NK cytotoxicity, and mitogen-induced B-lymphocyte proliferation than those from CON sows ($P < 0.05$; TRT). Supplementation had minimal effect on the sows but postnatal maternal exposure to *Saccharomyces cerevisiae var. boulardii* supplementation modulated the immune status of the progeny beyond the lactation period resulting in those from PRO-treated sows having more enhanced neutrophil function and B-cell proliferative response in the short term. Therefore, these data imply that including yeast probiotics in maternal diets may have carry-over effects in priming offspring's immune func-

tion, especially neutrophil function and B-cell proliferation in the short term.

Keywords

Immune, Maternal Supplementation, Piglet, Sow, Yeast Probiotic

1. Introduction

Antibiotics have been found to improve feed efficiency, growth rate, and reproductive performance and reduce mortality and morbidity when fed at low levels [1] [2] [3]. Livestock producers adopted feeding low levels (subtherapeutic) of antibiotics as part of their feeding programs, thus leading to non-judicious use. As a result, worldwide concern over antimicrobial resistance has increased [4] [5], as have consumer concerns about drug residue in animal products. Regarding these concerns, the United States Food and Drug Administration fully implemented the Veterinary Feed Directive (VFD) in 2017, following the European Union ban on including antibiotics in swine diets as routine growth promoter. The VFD aims to govern the use of medically necessary antimicrobials and reduce the therapeutic use in food-producing animals to minimize the potential co-selection of genes involved in broad-spectrum drug resistance [6]. Unfortunately, minimizing the co-selection of genes that lead to resistance is tenacious when considering the worldwide impact that antimicrobial-resistant organisms have on mortality. It has been estimated that 700,000 people die yearly from drug-resistant strains of common bacterial infections [7]. Therefore, recent research has focused on developing alternative antibiotics to maintain swine health and performance while potentially minimizing the impact of antimicrobial resistance.

In the past decade, probiotics have emerged as one of the most notable alternatives to the subtherapeutic use of antibiotics due to reported health benefits for humans and animals [8]. Probiotics are yeast or bacteria, and variations within the two classes are attributed to the strains used. By increasing the percentage of active phagocytic cells and increasing CD57+ expression, bacterial-derived probiotics are known to modulate the cellular immune system of the host [9] [10]. Bacterial strains are commonly found in the gut, preventing disruption of the normal gut microbiome while increasing competition for pathogenic bacteria from colonizing. The susceptibility of bacterial species to antibiotics is still a concern [11], as there are potential antibiotic-resistant mechanisms of the most common strains found in bacterial probiotics [12]. However, yeast strains are generally not found in the host's gut, indicating that they cannot integrate fully with the natural flora. As a result, yeast has a more remarkable ability to travel through the gastrointestinal tract freely, allowing it to affect the intestines, such as their means of altering cytokine production through modulation of cellular signal transduction pathways [13].

Many yeast strains have been used solely or with others to determine their im-

pact on immune function. Yeast-derived probiotics have modulated the microflora in poultry [14] [15] and immune responsiveness to enteric pathogens in swine [16]. One of the most common yeast-derived strains, *Saccharomyces cerevisiae,* has been shown to impact health and performance in livestock [17]. Various studies have utilized portions or the complete strain of *S. cerevisiae* in diets to determine its health and performance benefits in many livestock species. Kiros *et al.* [18] found that administering a highly concentrated solution of *S. cerevisiae* every other day to suckling piglets from 1 to 28 days of age improved average daily gain, while those given a low concentration had reduced microbial richness of the cecum. Lipopolysaccharide (LPS) challenged feedlot heifers fed an *S. cerevisiae*-supplemented diet had reduced cortisol and IL-6 concentrations compared to control heifers [19]. Even the inclusion of live *S. cerevisiae* in diets of *E. coli*-challenged broilers denoted its ability to obstruct the increased expression of IL-1β, TLR4, and NF-κB [20]. These proteins could induce damage to the intestines due to an extreme influx of inflammation. A reduced inflammatory response following the challenge may indicate that animals fed the yeast strain may have been better equipped to fight against infection without many activations of the acute phase response. Although data illustrated the health and performance benefits, the effectiveness of *S. cerevisiae* as an immunomodulator varies considerably, highlighting that superior yeast strains need to be identified for use in animal agriculture.

The use of *Saccharomyces cerevisiae var. boulardii* (Scb) in human health has demonstrated it as a considerable strain for use in livestock. When fed to broilers and pigs, Scb reduced the incidence of diarrhea and enhanced the growth rate [21] [22] [23] [24]. Adding Scb in nursery diets before an LPS challenge with newly weaned barrows induced a hastened peak production of tumor necrosis factor-α. In contrast, peak production of IL-1β and IL-6 was less than in control animals [25]. Despite limited research, these findings imply that yeast-based probiotics could improve performance and modulate the immune system. Most studies focus on yeast-based products' impact on the newly weaned pig, even when sows are supplemented [26] [27] [28]. However, utilizing probiotics during gestation and lactation to program offspring's immune function may prove more economically friendly than supplementing piglets individually. Xia *et al.* [29] illustrated that including *S. cerevisiae* in maternal diets at gestation and lactation altered serum immunoglobulin concentration of the progeny post-wean; however, the effects of Scb and other immune measures are unknown. Thus, this study aimed to determine if maternal supplementation of *Saccharomyces cerevisiae var. boulardii* in late gestation through lactation could modulate not only the immune status of the sow but also her progeny from birth to 35 days old.

2. Materials and Methods

2.1. Animals and Experimental Design

Eighteen parity-2 sows (Genetiporc maternal line) were individually housed in standard gestation stalls (61 cm × 216 cm) in a mechanically-ventilated gestation

barn at at the University of Illinois Swine Research Center in Urbana-Champaign (U.S.A.). Sows were hand-fed two boluses of either probiotic (PRO; n = 9 sows) or placebo (CON; n = 9 sows) every morning (0600 h) starting at gestational day (GD) 84 through the end of lactation (21-day period). The probiotic bolus (2×10^9 CFU/g) was composed of monogastric specific yeast known as Levucell SB® (*Saccharomyces cerevisiae* var. *boulardii* (**Scb**) CNCMI-1079; Lallemand Animal Nutrition, Montreal, Quebec, Canada). The CON bolus was sugar-based and anatomically the same size and shape as the probiotic bolus. At gestational day, 112 sows were moved to mechanically ventilated farrowing-rooms and kept in individual farrowing crates until the end of the 21-day lactation period. Sows were nose-snared and bled at days 7, 14, 21, 28 (before moving to farrowing), 31 (24 h post-farrowing), and 51 (weaning) of post-initial supplementation (Table 1). Samples were obtained via jugular venipuncture using sterile heparinized syringes after nose snaring the sows. The procedure lasted < 2 minutes. Blood samples were placed in a cooler on ice and transported to the laboratory, where they were immediately processed.

The entire litter was collected, dried, and weighed at farrowing. Piglets were kept with their dams from birth until 21 days of age (weaning). They were weaned and moved as littermates to nursery pens and fed *ad libitum,* a standard nursery diet formulated to meet or exceed NRC requirements [30]. Each pen was equipped with one nipple waterer. Lights came on at 0700 h and went off at 1700 h.

Table 1. Timeline for blood collection and assay analysis for sows and piglets.

Study Time Points	Sow Blood Collection and Assay Analysis	Piglet Blood Collection and Assay Analysis
Gestational day 84	First day of treatment	
Gestational day 91	X	
Gestational day 98	X	
Gestational day 105	X	
Gestational day 112	X	
Lactation day 1 (1 day old)	X	
Lactation day 7 (7 days old)		X
Lactation day 14 (14 days old)		X
Lactation day 21 (21 days old)	X Last day of Treatment	X
7 Days post-wean (28 days old)		X
14 Days post-wean (35 days old)		X

Eighty-four female pigs (n = 42 pigs/treatment) were chosen for assessing dam treatment effects. Blood samples were taken at 7, 14, and 21 days old to assess potential developmental effects during the suckling phase (Table 1). Again, at 28 and 35 days old, assess the potential long-term effects of maternal treatment (Table 1). All blood samples were obtained via jugular venipuncture using vacutainers containing sodium heparin or EDTA while pigs were held in a supine position. The procedure lasted < 2 mins. Blood samples were placed in a cooler on ice and transported to the laboratory, where they were immediately processed.

2.2. Total White Blood Cell Counts (WBC) and Leukocyte Differentials

Whole blood was used to determine total white blood cell (WBC) and leukocyte differential counts. Total WBC counts were made electronically using a Coulter Z1 particle counter (Beckman Coulter, Miami, FL, U.S.A.). Ten µl of whole blood was added to 10 ml of Isoflow® (Beckman Coulter, Miami, FL, U.S.A.), then three drops of ZAP-OGLOBIN® (Beckman Coulter, Miami, FL, U.S.A.) were added to lyse the red blood cells, and tubes were mixed. The sample cup was placed in the counting chamber to determine the total WBC count. Whole blood smears were made, fixed in methanol, and then stained with the Hema-3 staining system (Fisher Scientific, Houston, TX, U.S.A.). Slides were viewed under a light microscope, and 100 cells per slide were visually counted to determine leukocyte differential percentages.

2.3. Cell Isolation and Plasma Analysis

Whole blood samples were centrifuged at 700 × g for 30 min at 4˚C. Plasma was aspirated, transferred to tubes, and stored at −80˚C until further analysis. Buffy coat was diluted with Roswell Park Memorial Institute (RPMI 1640; Gibco, Carlsbad, CA, U.S.A.) complete medium which contains 20 mM Hepes and L-glutamine with sodium bicarbonate and then layered over Histopaque-1077 (density = 1.077 g/mL; Sigma, St. Louis, MO, U.S.A.) and −1119 (density = 1.119 g/mL; Sigma, St. Louis, MO, U.S.A.), and centrifuged at 700 × g for 30 min at 25˚C. Lymphocytes were removed from the 1077 layer, washed twice in RPMI, resuspended, and counted. Neutrophils were removed from the 1119 layer and washed once, and red blood cells were then lysed from the neutrophil fraction, washed in RPMI, and counted. Cell concentrations were adjusted with RPMI based on the immune assay requirements.

Plasma cortisol and interleukin-12 (IL-12) were analyzed following the manufacturer's protocols. Commercial radioimmunoassay validated for porcine cortisol was measured. Plasma samples from heparin-treated whole blood were assayed for cortisol using a Coat-A-Count cortisol kit, following the manufacturer's protocol (Diagnostic Products Corp., Los Angeles, CA, U.S.A.). Briefly, 25 µL of sample or standard were added to antibody-coated tubes in duplicate. Radiolabeled (I125) cortisol was added to tubes and incubated for 45 min at 37˚C

in a water bath. The liquid phase was decanted, and radioactivity was counted with a gamma counter. A standard curve was 0, 10, 50, 100, 200, and 500 μg/mL. Intra- and inter-assay coefficients of variations were 7.0% and 16.5%, respectively. This assay's minimal detectable cortisol concentration was approximately 2 ng/mL. A Porcine IL-12/IL-23 Quantikine kit was used to measure IL-12 in plasma samples (R & D Systems, Minneapolis, MN, U.S.A.). On average, the minimal detectable concentration of IL-12/IL-23 using this kit was 9.0 pg/mL.

2.4. Immune Assays

Neutrophil chemotaxis and phagocytosis assays were performed to assess the functional aspects of neutrophils. Chemotaxis was measured using an assay previously described by Salak *et al.* [31]. Assay medium was used to assess random migration (chemokinesis) and recombinant human complement-5a (C5a; 10-7 M; Sigma Aldrich) and interleukin-8 (IL-8; 100 ug/mL; Sigma Aldrich) to assess directed migration (chemotaxis) of neutrophils [31]. Briefly, neutrophils were adjusted to a cell concentration of 3×10^6 cells/mL, and 50 μl was added in duplicate to the top wells of the chemotaxis chamber and incubated for 1-h at 37˚C in a humidified chamber (5% CO_2). The polycarbonate (pore size 5 μm) filter was fixed and stained using LeukoStat® I and II (Fischer Scientific, Houston, TX). A technician counted cells that migrated to the underside of the filter without knowledge of treatments. Four fields/well were counted at 100x with a light microscope, and valid duplicates were averaged. Neutrophil phagocytosis was measured using a flow cytometry-based assay previously described by Jolie *et al.* [32], with minor modifications described by Niekamp *et al.* [33]. Briefly, neutrophil concentrations were adjusted to a cell concentration of 2×10^6 cells/mL in RPMI. Fluorescent beads were pre-incubated for 30-min in non-heat-inactivated porcine serum and then added to the samples at a 10:1 (beads-to-neutrophils) ratio. Samples were incubated at room temperature for 45-min in the dark and then centrifuged at 1000 × g for 5-min. Following centrifugation, samples were washed once, decanted, and finally resuspended in 1 mL of RPMI and fixed in 4% paraformaldehyde. The percentage of engulfment of beads by neutrophils was evaluated using a flow cytometer.

Natural killer cell (NK) cytotoxicity and mitogen-induced lymphocyte proliferation assays assessed the functional aspects of innate and adaptive immune function. A commercially available non-radioactive cytotoxicity detection kit (Roche Diagnostics, Indianapolis, IN, U.S.A.) was used for NK cytotoxicity, as described previously by Sutherland *et al.* [34]. Briefly, porcine lymphocytes were used as effector cells, and K-562 chronic human myelogenous leukemia cells (American Tissue Type Culture Collection, Manassas, VA, U.S.A.) were used as target cells. Lymphocytes were adjusted to 1×10^7 cells/ml, and K-562 cells were adjusted to 10,000 cells per well. Samples were run in triplicate at effector (lymphocytes)-to-target-cell (K-562) ratios of 12.5:1, 25:1, 50:1, and 100:1, respectively. Results were measured using a microplate reader (Bio-Tek, Winooski, VT, U.S.A.) at

490 nm and a reference wavelength of 690 nm. The assay was valid if the maximum release divided by spontaneous release was ≤20%. A non-radioactive cell proliferation assay kit (CellTiter96®, Promega, Madison, WI, U.S.A.) was used as described previously by Sutherland *et al.* [34] with minor modifications. Briefly, lymphocytes were used at a 5 × 106 cells/ml concentration and placed in a sterile 96-well flat-bottom plate. Concanavalin A (ConA) and lipopolysaccharide (L.P.S.) were used as mitogens (Sigma, St. Louis, MO, U.S.A.) to stimulate T and B cells, respectively, at a concentration of 0, 0.2, 2.0, and 20 μg/mL. Plates were incubated for 68 h at 37˚C in a 5% CO_2 humidified incubator, and then 15 μL of dye was added to each well. Plates were incubated for 4 h, and the reaction was stopped by adding 100 μL of stop solution to each well. Plates were read using a microplate reader (BioTek Instruments, Winooski, VT, U.S.A.) at 550 nm with a reference wavelength of 690 nm. Results are expressed as a proliferation index (P.I.):

$$\text{P.I.} = \frac{\text{Optical Density}(550/690\text{ nm})\text{stimulated cells}}{\text{Optical Density}(550/690\text{ nm})\text{non-stimulated cells}}$$

2.5. Statistical Analysis

Data were analyzed using the MIXED and General-Linear-Model (GLM) procedures of SAS 9.4 with repeated measures (SAS Inst. Inc., Cary, NC, U.S.A.). All traits were tested for departure from a normal distribution. Both main and interactive effects of Treatment × Day (TRT × Day) for sows or maternal Treatment × Age (TRT × Age) for piglets were analyzed. The model included fixed effects of maternal treatment (placebo or probiotics), periods (gestation or lactation), and age of piglets (birth, 1, 7, 14, 21, 28, and 35). Significance was set at $P < 0.05$, but trends were discussed at $P > 0.05$ to ≤ 0.10.

3. Results

3.1. Immune and Cortisol Traits of Dam during Treatment

3.1.1. Gestational Treatment Period

After 7 days of treatment, sows fed PRO boluses tended to have higher neutrophil-to-lymphocyte ratios (N:L) than CON sows ($P = 0.068$; TRT × Day; **Table 2**), and after 14 days, plasma cortisol was higher among sows fed PRO boluses than CON fed sows ($P < 0.05$; TRT × Day; **Table 2**). However, by 28 days of treatment, natural killer (NK) cell cytotoxicity ($P = 0.08$) and neutrophil phagocytosis ($P < 0.05$) were both higher among those fed the PRO boluses than the placebo ones (**Table 2**).

Moreover, at the end of the 28-day treatment period in gestation, overall, the PRO-treated sows tended to have higher total WBC counts ($P < 0.10$) and percentages of peripheral neutrophils ($P < 0.05$) but lower peripheral lymphocytes ($P < 0.05$) resulting in a higher N:L ratio ($P < 0.05$; **Table 3**). Interestingly, these sows also had greater neutrophil chemotaxis (IL8 and C5a) than CON sows ($P < 0.05$; **Table 3**). All other immune parameters were similar between treatment groups at the end of the gestational treatment period.

Table 2. Interactive effects of maternal supplementation on plasma cortisol and descriptive and functional immune measures during the gestational period for sows.[1,2,3]

Measures	Treatment		TRT × Day, P-value
	Control	Probiotic	
Plasma Cortisol, ng/mL			0.042
Day 7	32.8 ± 6.68	38.5 ± 6.30	
Day 14	31.2 ± 6.26[a]	51.0 ± 6.26[b]	
Day 21	45.0 ± 5.99	36.9 ± 5.99	
Day 28	45.6 ± 5.99	47.7 ± 6.30	
White Blood Cell (WBC), 10^7/10 μL			0.643
Day 7	1.56 ± 0.30	1.75 ± 0.30	
Day 14	1.36 ± 0.31	1.93 ± 0.31	
Day 21	1.38 ± 0.30	1.79 ± 0.30	
Day 28	1.66 ± 0.30	1.74 ± 0.31	
Neutrophils, %			0.179
Day 7	37.7 ± 4.87	53.3 ± 4.87	
Day 14	41.5 ± 5.03	47.3 ± 5.03	
Day 21	43.1 ± 4.87	48.1 ± 4.87	
Day 28	51.2 ± 4.87	50.6 ± 5.04	
Lymphocytes, %			0.747
Day 7	50.5 ± 4.11	43.3 ± 4.11	
Day 14	56.3 ± 4.31	47.9 ± 4.31	
Day 21	52.3 ± 4.11	45.7 ± 4.11	
Day 28	45.8 ± 4.11	45.2 ± 4.31	
Monocytes, %			0.328
Day 7	9.18 ± 2.71	2.18 ± 2.71	
Day 14	0.77 ± 2.84	1.72 ± 2.84	
Day 21	3.18 ± 2.71	3.62 ± 2.71	
Day 28	3.18 ± 2.71	3.36 ± 2.84	
Eosinophils, %			0.918
Day 7	5.22 ± 1.59	5.56 ± 1.59	
Day 14	5.24 ± 1.65	7.17 ± 1.65	
Day 21	5.44 ± 1.59	6.56 ± 1.59	
Day 28	3.89 ± 1.59	5.15 ± 1.66	
Neutrophil-to-Lymphocyte ratio			0.068
Day 7	0.82 ± 0.20[x]	1.58 ± 0.20[y]	
Day 14	0.93 ± 0.21	1.08 ± 0.21	
Day 21	0.97 ± 0.20	1.14 ± 0.20	
Day 28	1.24 ± 0.20	1.21 ± 0.21	

Continued

Chemotaxis-C5a, no./5 fields			0.960
Day 7	99.1 ± 21.9	136.0 ± 22.2	
Day 14	73.5 ± 22.8	124.2 ± 22.7	
Day 21	116.9 ± 23.8	155.2 ± 25.2	
Day 28	121.2 ± 21.4	153.4 ± 23.0	
Chemotaxis-IL8, no./5 fields			0.452
Day 7	85.1 ± 27.5	179.8 ± 27.1	
Day 14	63.1 ± 29.1	95.2 ± 30.0	
Day 21	85.9 ± 30.8	133.9 ± 26.4	
Day 28	89.2 ± 26.3	133.7 ± 26.8	
Neutrophil phagocytosis, %			0.042
Day 7	31.9 ± 5.78	38.66 ± 6.06	
Day 14	55.8 ± 5.93	51.85 ± 6.11	
Day 21	53.9 ± 5.78	49.32 ± 5.78	
Day 28	44.6 ± 5.85[a]	59.80 ± 6.28[b]	
Natural killer cell cytotoxicity, %			0.080
Day 7	27.6 ± 11.3	18.0 ± 11.3	
Day 14	36.5 ± 11.0	21.9 ± 11.1	
Day 21	22.9 ± 9.22	34.9 ± 9.88	
Day 28	23.5 ± 10.8[x]	54.5 ± 10.9[y]	
Concanavalin-A proliferation, index			0.937
Day 7	1.13 ± 0.35	1.17 ± 0.35	
Day 14	0.71 ± 0.36	0.62 ± 0.36	
Day 21	1.14 ± 0.35	0.93 ± 0.35	
Day 28	0.88 ± 0.37	1.04 ± 0.43	
Lipopolysaccharide proliferation, index			0.467
Day 7	1.51 ± 0.41	2.51 ± 0.41	
Day 14	0.96 ± 0.44	1.59 ± 0.44	
Day 21	2.10 ± 0.41	1.81 ± 0.41	
Day 28	1.31 ± 0.51	1.64 ± 0.72	
Interleukin-12, pg/mL			0.265
Day 7	160.2 ± 26.5	183.6 ± 26.5	
Day 14	139.8 ± 27.6	201.8 ± 27.7	
Day 21	199.0 ± 26.5	180.4 ± 26.6	
Day 28	180.4 ± 26.5	163.8 ± 27.8	

[1]Sow treatments were controls = sugar-based placebo and probiotics = *Saccharomyces cerevisiae var. boulardii*. [2]Data are shown as least-square means ± standard error means. [3]Collections occurred every 7d post-treatment, and days correspond to gestational days 91, 98, 105, and 112. [a,b]Within a row, means without a common superscript letter differ at $P \leq 0.05$. [x,y]Within a row, means without a common superscript letter differs at $P \leq 0.10$.

Table 3. Mean plasma cortisol and descriptive and functional immune values for sows at the end of the 28-day supplementation period during gestation.[1,2,3]

Measures	Control	Probiotics	P-value
Plasma Cortisol, ng/mL	38.7 ± 4.10	43.5 ± 4.10	0.403
White Blood Cell (WBC), 10^7/10 µL	1.49 ± 0.25^{x}	1.80 ± 0.25^{y}	0.098
Neutrophils, %	43.4 ± 3.84^{a}	49.8 ± 3.85^{b}	0.038
Lymphocyte, %	51.2 ± 2.70^{a}	45.5 ± 2.72^{b}	0.048
Monocytes, %	4.08 ± 1.72	2.72 ± 1.74	0.476
Eosinophils, %	4.95 ± 1.15	6.11 ± 1.16	0.480
Neutrophil-to-Lymphocyte ratio	0.99 ± 0.15^{a}	1.25 ± 0.15^{b}	0.035
Chemotaxis-IL8, no./5 fields	80.3 ± 22.0^{a}	135.6 ± 21.8^{b}	0.003
Chemotaxis-C5a, no./5 fields	102.7 ± 16.7^{a}	142.2 ± 17.6^{b}	0.003
Neutrophil phagocytosis, %	46.6 ± 4.77	49.9 ± 4.91	0.410
Natural killer cell cytotoxicity, %	27.7 ± 6.03	32.3 ± 6.07	0.463
Concanavalin-A proliferation	0.96 ± 0.25	0.94 ± 0.26	0.912
Lipopolysaccharide proliferation	1.47 ± 0.21	1.89 ± 0.25	0.212
Interleukin-12, pg/mL	169.9 ± 18.3	182.4 ± 18.4	0.630

[1]Data are shown as least-square means ± standard error means. [a,b]Within a row, means without a common superscript letter differ at $P \leq 0.05$. [x,y]Within a row, means without a common superscript letter differs at $P < 0.10$.

3.1.2. Lactational Treatment Period

Interestingly, it was not until the end of 51 days of treatment that adaptive immune measures were affected (Table 4). The sows fed PRO boluses had greater concanavalin-A-induced lymphocyte proliferation index than CON sows ($P < 0.05$; TRT × Day; Table 4). Although no other measures were different at the end of the lactation period ($P > 0.10$; TRT × Day; Table 4), it is important to note that the lipopolysaccharide-induced lymphocyte proliferation index was 33% higher among sows fed the PRO boluses than the CON sows at 51 days on treatment.

3.1.3. Entire Treatment Period

Across the entire treatment period, only neutrophil chemotaxis in response to C5a and IL-8 were affected. PRO-treated sows had greater chemotaxis than the CON sows ($P < 0.05$; TRT; Figure 1). All other immune parameters were similar between the sows fed PRO boluses, and those fed the placebo ($P > 0.10$).

3.2. Immune Measures for Progeny from 7 to 35 Days Old

We assessed leukocyte populations and innate and adaptive immunity measures by assessing the potential carryover effect of maternal treatment during late gestation (prenatal, in utero exposure) and the 21-day lactation period (postnatal, suckling) on the progeny immune response. At 7 and 35 days old, pigs from

Table 4. Interactive effects of maternal supplementation on plasma cortisol and descriptive and functional immune measures during the lactational period in sows.[1,2]

Measures	Treatment		TRT × Day, P-value
	Control	Probiotics	
Plasma Cortisol, ng/mL			0.815
Day 31	88.5 ± 13.5	57.8 ± 13.5	
Day 51	58.6 ± 13.5	32.8 ± 13.5	
White Blood Cell (WBC), 10^7/10 μL			0.354
Day 31	1.46 ± 0.28	2.39 ± 0.28	
Day 51	1.92 ± 0.28	2.31 ± 0.28	
Neutrophils, %			0.739
Day 31	61.5 ± 7.25	56.2 ± 7.25	
Day 51	58.7 ± 7.46	49.9 ± 7.25	
Lymphocytes, %			0.758
Day 31	39.9 ± 5.84	43.2 ± 5.84	
Day 51	42.5 ± 6.08	49.2 ± 5.84	
Monocytes, %			0.153
Day 31	1.72 ± 1.22	1.39 ± 1.22	
Day 51	1.28 ± 1.25	1.05 ± 1.22	
Eosinophils, %			0.680
Day 31	−0.74 ± 1.38	−0.63 ± 1.38	
Day 51	0.56 ± 1.41	1.26 ± 1.38	
Neutrophil-to-Lymphocyte Ratio			0.349
Day 31	2.01 ± 0.34	1.31 ± 0.34	
Day 51	1.36 ± 0.35	1.13 ± 0.34	
Chemotaxis-IL8, no./5 fields			0.389
Day 31	82.4 ± 18.9	175.5 ± 21.8	
Day 51	80.0 ± 21.5	146.5 ± 17.5	
Neutrophil phagocytosis, %			0.966
Day 31	58.8 ± 5.82	59.1 ± 5.64	
Day 51	54.1 ± 6.00	54.1 ± 5.49	
Concanavalin-A proliferation			0.0001
Day 31	1.87 ± 0.20	1.97 ± 0.18	
Day 51	0.57 ± 0.23[a]	1.84 ± 0.22[b]	
Lipopolysaccharide proliferation			0.312
Day 31	1.45 ± 0.21	1.71 ± 0.19	
Day 51	1.44 ± 0.25	2.01 ± 0.26	
Interleukin-12, pg/mL			0.532
Day 31	69.0 ± 32.1	81.4 ± 31.3	
Day 51	124.8 ± 31.3	162.5 ± 31.3	

[1]Data are shown as least-square means ± standard error means. [2]Days of collection correspond to lactational days 4 and 21. [a,b]Within a row, means without a common superscript letter differ at $P \leq 0.05$. [x,y]Within a row, means without a common superscript letter differs at $P < 0.10$.

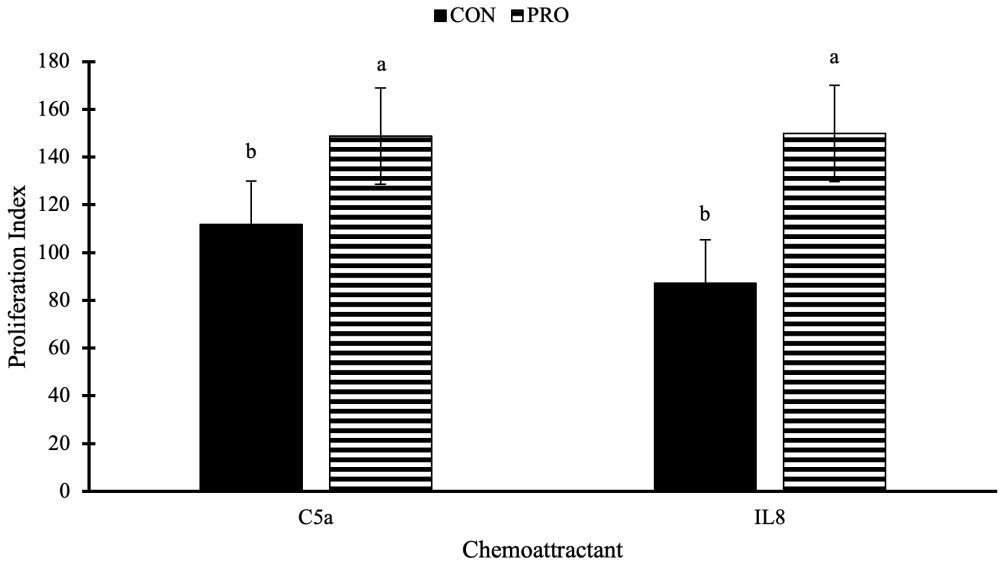

Figure 1. Effect of maternal treatment on neutrophil chemotaxis for the entire supplementation period of the study. Means without a common superscript letter a,b differ at P ≤ 0.05.

PRO-treated sows tended to have higher percentages of lymphocytes (P < 0.10) than those from CON sows (P < 0.10; TRT × Age; Table 5). In addition, those from CON sows had higher lymphocyte numbers at 21 days old (9.0 ± 1.5 vs. 4.3 ± 1.5, 10^7/mL) but higher neutrophil numbers at 35 days old (7.3 ± 0.47 vs. 5.2 ± 0.46, 10^6/mL) than those from PRO-treated sows. At 7 and 35 days old, pigs born to PRO-treated sows had a lower N:L ratio than those born to CON ones (P = 0.001, TRT × Age; Table 5). However, at 14 days old, the N:L ratio was reduced by 57% in pigs born to CON sows but increased by 43% at 35 days old (TRT × age; P < 0.001). It should be noted that the N:L ratio fluctuated over time in both groups, with the highest ratio recorded at 7 days old for those from PRO-treated sows and 35 days old for those from CON ones.

In addition, at 14 days old, NK cytotoxicity was greater (P < 0.05) in those from PRO-treated than CON ones and still higher at 35 days old (Table 5). Concanavalin-A-induced lymphocyte proliferation index was highest ($P < 0.05$) at 21 days old among the pigs from CON sows than those from PRO-treated sows. It was not until 28 days old that those from PRO-treated sows had the highest proliferation index (P < 0.05; Table 5). No interactive effects of maternal treatment and piglet age were observed for other recorded measures.

Moreover, within treatment groups, NK cytotoxicity was highest (P < 0.05) at 14 days old in pigs born to PRO-treated, while in pigs in the CON group, NK cytotoxicity was highest at 21 days old. It should also be noted that at 21 days old, the CON-A proliferation index was higher than at 7 and 14 days old among pigs from CON sows (P < 0.05), while for pigs from PRO-treated sows, the index was greatest at 28 days old than at 7 and 21 (P < 0.05). At the same time, the LPS-induced proliferation index was higher among those from PRO-treated sows at all ages except for 28 days old than those born to CON sows (P < 0.05).

Table 5. Interactive effects of maternal treatment on plasma cortisol and descriptive and functional immune measures for the progeny from 7 to 35 days of age.[1,2,3]

Measures	Maternal Treatment		Trt × Age P-value
	Control	Probiotics	
Plasma Cortisol, ng/mL			0.512
7 days old	44.2 ± 4.4	42.6 ± 4.4	
14 days old	37.9 ± 4.5	45.8 ± 4.4	
21 days old	43.9 ± 4.4	50.0 ± 4.4	
28 days old	24.4 ± 4.4	30.3 ± 4.4	
35 days old	25.6 ± 4.4	24.0 ± 4.4	
White Blood Cell (WBC), 10^7/10 mL			0.702
7 days old	6.28 ± 0.54	6.02 ± 0.54	
14 days old	3.40 ± 0.54	3.25 ± 0.54	
21 days old	1.97 ± 0.54	2.28 ± 0.54	
28 days old	2.36 ± 0.54	2.14 ± 0.54	
35 days old	3.43 ± 0.54	3.59 ± 0.54	
Neutrophils, %			0.146
7 days old	53.6 ± 2.2	44.1 ± 2.3	
14 days old	37.6 ± 2.3	32.5 ± 2.2	
21 days old	32.5 ± 2.2	32.3 ± 2.2	
28 days old	36.6 ± 2.2	34.1 ± 2.2	
35 days old	49.2 ± 2.2	41.4 ± 2.2	
Lymphocytes, %			0.085
7 days old	44.0 ± 2.2^x	53.3 ± 2.3^y	
14 days old	60.2 ± 2.3	64.6 ± 2.3	
21 days old	66.2 ± 2.2	65.3 ± 2.3	
28 days old	59.5 ± 2.2	70.0 ± 2.3	
35 days old	48.2 ± 2.2^x	55.9 ± 2.3^y	
Monocytes, %			0.146
7 days old	2.46 ± 0.51	2.23 ± 0.52	
14 days old	1.92 ± 0.51	1.16 ± 0.51	
21 days old	1.66 ± 0.51	2.06 ± 0.51	
28 days old	3.01 ± 0.51	3.85 ± 0.51	
35 days old	1.68 ± 0.51	1.83 ± 0.51	
Eosinophils, %			0.262
7 days old	0.27 ± 0.15	0.39 ± 0.16	
14 days old	0.47 ± 0.16	0.52 ± 0.15	
21 days old	0.41 ± 0.15	0.36 ± 0.15	
28 days old	0.53 ± 0.15	1.05 ± 0.15	
35 days old	0.96 ± 0.15	0.91 ± 0.15	

Continued

Neutrophil-to-Lymphocyte Ratio			0.001
7 days old	1.62 ± 0.11[a]	0.96 ± 0.11[b]	
14 days old	0.70 ± 0.11	0.60 ± 0.11	
21 days old	0.54 ± 0.11	0.60 ± 0.11	
28 days old	0.69 ± 0.11	0.63 ± 0.11	
35 days old	1.20 ± 0.11[a]	0.82 ± 0.11[b]	
Chemotaxis-C5a, no./5 fields			0.227
7 days old	26.9 ± 10.1	65.0 ± 12.4	
14 days old	39.0 ± 9.8	99.9 ± 9.6	
21 days old	44.2 ± 12.0	80.9 ± 11.1	
28 days old	42.1 ± 11.6	67.0 ± 10.6	
35 days old	24.1 ± 15.5	89.2 ± 16.9	
Chemotaxis-IL8, no./5 fields			0.198
7 days old	27.2 ± 12.7	48.6 ± 14.6	
14 days old	55.0 ± 11.6	109.2 ± 11.2	
21 days old	53.5 ± 12.9	62.8 ± 13.4	
28 days old	35.2 ± 15.7	72.3 ± 12.9	
35 days old	44.6 ± 19.0	97.3 ± 22.0	
Neutrophil phagocytosis, %			0.063
7 days old	67.4 ± 2.2	67.6 ± 2.2	
14 days old	63.4 ± 2.2	60.5 ± 2.2	
21 days old	56.1 ± 2.2[x]	62.6 ± 2.2[y]	
28 days old	60.5 ± 2.2	62.5 ± 2.2	
35 days old	66.2 ± 2.2	68.6 ± 2.2	
Natural killer cell cytotoxicity, %			<0.0001
7 days old	71.2 ± 4.1	69.9 ± 3.9	
14 days old	63.7 ± 6.3[a]	112.9 ± 5.4[b]	
21 days old	77.5 ± 4.6	69.1 ± 4.7	
28 days old	60.7 ± 4.4	52.9 ± 4.4	
35 days old	57.1 ± 3.7	64.5 ± 3.7	
Concanavalin-A proliferation			0.026
7 days old	2.25 ± 0.26	1.85 ± 0.29	
14 days old	1.95 ± 0.26	2.10 ± 0.29	
21 days old	2.84 ± 0.24[a]	2.07 ± 0.26[b]	
28 days old	2.12 ± 0.25[a]	2.78 ± 0.25[b]	
35 days old	2.17 ± 0.34	2.01 ± 0.33	

Continued

Lipopolysaccharide proliferation			0.056
7 days old	1.01 ± 0.17[a]	1.53 ± 0.18[b]	
14 days old	1.21 ± 0.19[a]	1.51 ± 0.20[b]	
21 days old	1.30 ± 0.16[a]	1.51 ± 0.16[b]	
28 days old	1.13 ± 0.16	1.10 ± 0.16	
35 days old	1.48 ± 0.11[a]	1.89 ± 0.10[b]	
Interleukin-12, pg/mL			0.514
7 days old	162.5 ± 25.5	154.5 ± 25.7	
14 days old	257.3 ± 25.6	277.0 ± 25.7	
21 days old	293.1 ± 25.5	311.0 ± 25.7	
28 days old	422.1 ± 25.6	411.4 ± 25.7	
35 days old	315.8 ± 25.5	343.5 ± 25.7	

[1]Measurements collected at 7, 14, and 21 days old occurred prior to weaning, and 28 and 35 days old occurred post-weaning. [2]Data are shown as least-square means ± standard error means. [a,b]Within a row, means without a common superscript letter differ at $P \leq 0.05$. [x,y]Within a row, means without a common superscript letter differs at $P < 0.10$.

3.3. Maternal Treatment Effects on Overall Immune Status of Progeny

The main effect of maternal supplementation fed to sows starting in late gestation and through the 21-day lactation period resulted in pigs born to PRO-treated sows having higher percentages of lymphocytes, while those from CON sows had higher percentages of neutrophils resulting in pigs born to CON sows having a greater N:L ratio ($P < 0.05$; Table 6). Interestingly, pigs from PRO-treated sows had a more stimulated immune status, as indicated by the greater neutrophil chemotactic response to C5a- and IL-8 ($P \leq 0.008$), NK cytotoxicity ($P < 0.05$), and lipopolysaccharide-induced lymphocyte proliferation index ($P < 0.05$) than those from CON sow (Table 6). Overall, there were no maternal treatment differences in plasma cortisol, leukocyte differentials, neutrophil phagocytosis, or IL-12 concentration ($P > 0.10$; Table 6).

4. Discussion

Sub-therapeutic levels of antibiotics have been used as growth promoters in pig production resulting in higher productive efficiency and animal growth [35]. The excessive use of antibiotics has resulted in a loss of effectiveness and the development of multi-drug-resistant microorganisms in farm animals. Thus, searching for other strategies to improve animal health and well-being is necessary. One potential strategy is probiotic supplementation, which has been shown to promote the general health of pigs [36]. However, most studies have focused on the effects of feeding probiotics on post-weaning outcomes [37] [38] [39]. Here, we focused on the effects of feeding *Saccharomyces cerevisiae var. boulardii*

Table 6. Mean maternal treatment affects plasma cortisol and descriptive and functional immune measures in the progeny from birth through 35 days old.[1]

Measures	Maternal Treatment		P-value
	Control	Probiotics	
Plasma Cortisol, ng/mL	35.2 ± 3.1	38.5 ± 3.12	0.447
White Blood Cell (WBC), 10^7/10 mL	3.4 ± 0.48	3.5 ± 0.48	0.985
Neutrophils, %	42.0 ± 1.3	36.8 ± 1.3	0.011
Lymphocytes, %	55.5 ± 1.3	60.0 ± 1.3	0.021
Monocytes, %	2.15 ± 0.41	2.22 ± 0.42	0.894
Eosinophils, %	0.53 ± 0.09	0.65 ± 0.09	0.325
Neutrophil-to-Lymphocyte Ratio	0.95 ± 0.07	0.72 ± 0.07	0.018
Chemotaxis-C5a, no./5 fields	35.2 ± 7.2	80.4 ± 7.5	<0.0001
Chemotaxis-IL8, no./5 fields	43.1 ± 8.8	78.0 ± 9.4	0.008
Neutrophil phagocytosis, %	62.8 ± 1.6	64.4 ± 1.6	0.490
NK Cytotoxicity, %	64.0 ± 2.8	74.0 ± 2.7	0.047
Concanavalin-A proliferation	2.32 ± 0.19	2.21 ± 0.19	0.584
Lipopolysaccharide proliferation	1.20 ± 0.08	1.78 ± 0.08	0.017
Interleukin-12, pg/mL	190.1 ± 21.5	299.5 ± 21.7	0.760

[1]Average measurements do not include birth and 24-h post-farrow datapoints but include d7, 14, and 21 of lactation and d28 and 35 of the post-wean period.

to pregnant sows during late gestation and through the 21-day lactation period on her immune status and the potential carry-over effects on her progeny. Overall, we found that this yeast-based probiotic had limited effects on the sow's immune status; neutrophil chemotaxis was enhanced in sows by the end of the treatment period with minimal effect on T- or B-cell proliferation. At the same time, carry-over effects were found on the progeny. More specifically, progeny from the PRO-treated sows had a more stimulated innate and adaptive immune status, indicating that maternal supplementation can have immunomodulatory effects on selected immune parameters in the progeny up to 35 days old.

Saccharomyces cerevisiae var. boulardii supplementation in the sows had limited effects on immune measures, but at the end of the 28-day treatment period, neutrophil function was enhanced. It was still enhanced at the end of the treatment period. It is plausible that this yeast-derived probiotic may directly enhance neutrophil function, especially since this is the only immune measure that was still enhanced at the end of the entire treatment period in the sows but also affected progeny's response. These findings agree with others that reported including *S. cerevisiae* fermented product in sow diets for the entire gestation and lactation resulted in reduced WBC and neutrophil numbers [40]. Although including a yeast probiotic may reduce leukocyte presence, the decreased number does not indicate diminished function. Araki *et al.* [41] found that the continuous feeding of Active Egg White Product, a probiotic produced from the *S.*

cerevisiae fermentation of egg whites to weaned pigs for a week, enhanced neutrophilic function. Thus, the continued dosage of Scb to sows may have been effective in enhanced function.

Conversely, we did not find the effects of Scb supplementation on measures of adaptative immunity until day 51 of supplementation (end of lactation), in which the mitogen-induced T-cell proliferation index was enhanced. At this time, it is uncertain what may have resulted in this impact of sow adaptive immunity. The effect of Scb probiotic supplementation may take longer to impact adaptive immune measures, or a different probiotic may be more appropriate. Moreover, it is plausible that the supplementation period may not have been long enough to affect the overall immune status of the sow.

Despite limited effects on the sow's immune status, there were in utero effects [42] and carry-over effects on the progeny during lactation and up to 35 days old. We previously reported that at birth, pigs born to sows fed probiotics had lower cortisol but higher percentages of neutrophils and lower lymphocytes than those born to control sows. However, there were no differences in either cortisol or leukocyte populations 24-h later [42]. Here, we found limited differences during the 21-day lactation period on innate and adaptive immune measures, except NK cytotoxicity being enhanced at 14 days old among those born to PRO-treated sows, but at 21 days old, mitogen-induced T cell proliferation index was less than those born to CON sows. At the same time, the LPS-mitogen-induced lymphocyte proliferation index was more significant at all ages except at 28 days old in pigs from PRO-treated sows. Regardless, these data show that we found that postnatal maternal exposure to *Saccharomyces cerevisiae var. boulardii* supplementation modulated the immune status of the progeny beyond the lactation period resulting in those born to PRO-treated dams having more enhanced neutrophil function and B-cell proliferative response. Offspring exposure to the yeast strain via the milk may have partly contributed to the more stimulated B-cell response. Chevaux *et al.* [43] found that Scb supplemented in sow diets during peripartum and farrowing periods improved colostrum quality and milk, directly affecting the well-being of pigs during nursing. Jurgens *et al.* [44] observed that the inclusion of Saccharomyces cerevisiae CNCM I-4407, a strain of *S. cerevisiae* closely related to the probiotic fed here, increased antibody concentration in both colostrum and milk, resulting in enhanced piglet immunity in the postnatal period. Donovan *et al.* [45] even observed that including a different strain than used in this study, Brewer's yeast, in sow diets from late gestation throughout lactation increased the composition of IgG in colostrum. Direct exposure of the offspring via these potential pathways allowed specific descriptive and functional immune measures to differ later in the suckling phase.

When observed by day of age, the altered immune measures in progeny provide insight into the capability of the yeast probiotics when exposed indirectly. Due to the variation at which the immune system's functionality differed from control animals, exposure to the probiotic may have primed progeny's immune

response to be more reactive than the immune system development impacted. Neutrophils or polymorphonuclear leukocytes are one of the most prominent components of the innate immune system and their ability to eliminate invading pathogens [46]. Neutrophils occupy various functionalities, all aiming to eliminate invading pathogens and foreign objects in the body. Probiotic progeny had more reactive or enhanced innate functions, such as phagocytosis and chemotaxis, for the entirety of the study period. The non-specificity of neutrophil action [46] may be linked to the observed enhancement of the innate system. The yeast components of Scb are generally not located within the host's gut, making them foreign, resulting in a more pronounced response within the offspring. By continuous exposure, the potential of priming or preparing the offspring's innate response may have occurred. It could result in more pronounced responses to future exposures to pathogens than if they were not exposed indirectly to the probiotic. Unlike the sow, the adaptive immune system aspect was found to be enhanced in progeny. Of the two lymphocyte subsets measures in this study, B cells were observed to have been more pronounced within offspring of the probiotic-fed sows than their control counterparts. As part of the humoral immune response, B lymphocytes produce antibodies, particularly immunoglobins [47]. As the current goal of including probiotics in livestock diets is to act as replacements for antibiotics, enhancing lymphocyte subsets that drive antibody production may be advantageous. Xia *et al.* [29], when feeding a live yeast probiotic to the dam during gestation and lactation, discovered that offspring from those treated sows had elevated concentrations of IgG and IgA at birth and well into the post-wean period. Higher levels of these two immunoglobulins may indicate an improved functionality of the adaptive immune response to fight off infection, as more antibodies are readily available to bind and begin to fight off the infection. An enhanced aspect of adaptive immune function illustrates the notion that probiotics have the potential to be better alternatives to antibiotics as antibody production is elevated in offspring even later in an age when no longer nursing. Overall, exposure to the components of probiotics from the dam may prime or prepare portions of the progeny's innate and adaptive functions.

5. Conclusion

Although including a probiotic in maternal diets resulted in minimal alterations to the dam immune status, progeny's innate and adaptive immune response was modified. Piglet exposure to the probiotic did not delay or accelerate overall development; instead, data illustrates its potential of priming offspring's immune response. Boosted innate function observed in the progeny of Scb dams illustrates the potential for probiotics to influence neutrophils, a vital cellular component of the innate immune system. The enhancement of antibody-producing cells illustrates the potential success of probiotics as an alternative to antibiotics without directly feeding the probiotic to the animal. Overall, results from this study demonstrate that maternal supplementation presents the opportunity to

modulate the immune functionality of both arms of the immune system of her progeny.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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