ABSTRACT

Dairy cattle are subjected to oxidative stress, inflammation, and altered immune function during the transition to lactation. The objective of this study was to evaluate the effects of a dietary *Saccharomyces cerevisiae* fermentation product (SCFP; NutriTek, Diamond V) on oxidative status, inflammation, and innate and adaptive immune responses during the transition period. Holstein cows were blocked by parity, expected calving date, and previous milk yield and then randomly assigned to treatment within block. Treatment was a control total mixed ration (n = 30) or SCFP total mixed ration (n = 34) fed from −29 ± 5 to 42 d relative to calving (RTC). Blood was sampled during wk −4, −2, 1, 2, and 5 and liver tissue at wk −3 and 2 RTC. Oxidative status was evaluated in plasma by retinol, α-tocopherol, and malondialdehyde concentrations, glutathione peroxidase activity, and Trolox equivalent antioxidant capacity, and in liver by mRNA abundance of nuclear factor E2-related factor 2 (*NFE2L2*), metallothionein 1E (*MT1E*), and glutathione peroxidase 3 (*GPX3*). Inflammation was evaluated in plasma by haptoglobin (HP) and serum amyloid A (SAA) concentrations and in liver by mRNA abundance of *HP*, serum amyloid A3 (*SAA3*), and nuclear factor kappa-light-chain-enhancer of activated B cells (*NFκB1*). Innate immune response was measured by stimulated oxidative burst of polymorphonuclear cells (neutrophils) isolated from blood. Ovalbumin (OVA) was administered with adjuvant on d 7 and 21 RTC, and adaptive immune response was evaluated by serum anti-OVA IgG content on d 28 and 35. Mixed models were used to assess effects of treatment, time, parity, and all interactions. We previously reported that SCFP had limited effects on productivity in this cohort, although milk fat yield was transiently increased and subclinical ketosis incidence was increased. Supplementation with SCFP did not affect overall oxidative, inflammatory, or immune parameters. The only treatment × week interaction detected was for plasma α-tocopherol concentration, which tended to be greater in control cows during wk 2 RTC. A tendency for a treatment × parity interaction was detected for serum anti-OVA IgG titer, which tended to be greater for SCFP than for controls among primiparous cows. Plasma inflammatory biomarkers were not affected by SCFP but, unexpectedly, plasma HP was elevated at both prepartum time points and plasma SAA was elevated during wk −2 RTC compared with the expected increases in both biomarkers postpartum. In this cohort of transition cows with low disease incidence, SCFP generally did not affect oxidative, inflammatory, or immune parameters.

Key words: *Saccharomyces cerevisiae* fermentation product, transition period, oxidative stress, immunology

INTRODUCTION

Dairy cattle experience drastic changes in metabolic and physiological demands during the transition from late pregnancy to early lactation (Goff and Horst, 1997; Drackley, 1999). This transition period is also marked by altered immune function (Mallard et al., 1998), inflammation (Bradford et al., 2015), and oxidative stress, which occurs when the production of pro-oxidants, such as reactive oxygen species (ROS), surpasses the neutralizing capacity of antioxidants (Sordillo and Aitken, 2009). Oxidative stress damages lipids, DNA, proteins, and other macromolecules, ultimately disrupting cellular membranes, interfering with several cellular processes, and impairing immune defenses, thereby increasing susceptibility to health disorders (Sordillo and Aitken, 2009). These challenges may contribute to the characteristic increase in susceptibility to metabolic and infectious diseases observed during this period (Goff and Horst, 1997; Drackley, 1999). Thus, much emphasis has been placed on improving the management of peripar-
turient cattle, particularly by implementing nutritional and immunomodulatory strategies.

Various yeast products, including *Saccharomyces cerevisiae* fermentation products (SCFP), have been shown to improve ruminal fermentation and milk production (Acharya et al., 2017), alter feeding behavior (DeVries and Chevaux, 2014), and increase DMI (Popp et al., 2012). Recently, yeast products have also been shown to modulate oxidative status and immune response in dairy calves and lactating cows (Alugongo et al., 2017; Al-Qaisi et al., 2020; Mahmoud et al., 2020), but few studies have evaluated such effects in transition dairy cattle (Zaworski et al., 2014; Yuan et al., 2015; Knoblock et al., 2019). Zaworski et al. (2014) found that supplementing SCFP during the transition period induced inflammatory biomarkers, potentially priming the immune system, but did not affect antibody production; however, the authors attributed the latter to a lack of specific vaccine challenge. When transition dairy cows were vaccinated against an innocuous protein, increasing doses of enzymatically hydrolyzed yeast increased antigen-specific antibody production (Yuan et al., 2015). Although Knoblock et al. (2019) did not observe any differences with adaptive immune response to a vaccine challenge, SCFP supplementation altered antioxidant capacity and inflammation postpartum.

Although studies such as those described above have provided proof-of-principle that dietary *Saccharomyces cerevisiae* products can influence immune responses of animals, the mechanism of action remains somewhat unclear. In simplified in vitro systems, however, it has been clearly demonstrated that yeast cell components such as β-glucan can potently stimulate macrophage activation at concentrations that are physiologically plausible (Sivinski et al., 2020). Another possible effect of yeast cells in the gut is their ability to agglutinate pathobionts such as enterotoxigenic *Escherichia coli*, which could prevent such bacteria from causing infection and necessitating an immune response (Zanello et al., 2011). Whether acting directly on gastrointestinal microbiota or on the host immune system, the possibility of using yeast products to improve animal resilience to disease is an appealing one.

The objective of the current study was to evaluate the effects of a new dietary SCFP containing antioxidants and polyphenols on oxidative status, inflammation, and innate and adaptive immunity in transition dairy cattle. We hypothesized that dietary SCFP would enhance antioxidant status, innate immune response, and adaptive immune response and alleviate excessive inflammation during the transition period compared with control cows.

### MATERIALS AND METHODS

Experimental procedures were approved by the Kansas State University Institutional Animal Care and Use Committee (protocol #3759.2).

### Design and Treatments

Sixty-four Holstein cows (50 multiparous, 14 primiparous) were enrolled in a transition study from –29 ± 5 to 42 d relative to calving (RTC; enrolled June–April). An initial publication from this study reported design details as well as production and feeding behavior outcomes (Olagaray et al., 2019). Cows were blocked by parity, expected calving date, and previous 305-d mature-equivalent milk yield, and then randomly assigned to treatment within block. Treatments were either control (n = 30) or 16.0 ± 0.7 g/d prepartum and 18.9 ± 0.5 g/d postpartum SCFP (NutriTek, Diamond V; n = 34), which was incorporated into a TMR. These dosages were fed based on the manufacturer’s recommended dose of 19 g of SCFP/d and formulated with expected average DMI of 11.1 kg/d prepartum and 20 kg/d postpartum. Treatments were identified by color-coding feed bins and revealing only the color assignment of each cow to handlers. Samples collected from cows were labeled only with number assigned at enrollment and time of collection relative to calving. Enrollment and allocation of treatments was performed by the principal investigators. The number of cows per treatment was determined by a power calculation using variance data from a previous study in our laboratory (Yuan et al., 2015). We determined adaptive immune response via IgG response to an innocuous protein (ovalbumin; OVA) similarly to Yuan et al. (2015). Based on those data, we determined that 30 cows per treatment would provide 90% power (α = 0.05) to detect a 0.06 difference in optical density (OD) for anti-OVA IgG.

Cows were fed 3×/d prepartum and 2×/d postpartum and were milked 2×/d postpartum. Diets were formulated to meet NRC (2001) requirements. Prepartum diets consisted of 43% NDF and 15% starch, and postpartum diets consisted of 31% NDF and 23% starch; full diet details are provided in Olagaray et al. (2019). Cows were housed in a bedded-pack barn prepartum and in a tiestall barn postpartum and monitored for health daily. A total of 59 cows completed the trial, whereas 5 cows were removed from the study shortly after parturition and only prepartum data were used for analysis. Reasons for removal of these 5 cows and all other health incidence data have been reported previously (Olagaray et al., 2019).
Sample Analyses

Oxidative Status Parameters in Plasma. Retinol and α-tocopherol concentrations were measured using HPLC with some modifications to the methods of Greaves et al. (2010). Tocopherol acetate (0.03 g/L in 95% ethanol) was used as the internal standard. Samples of plasma (0.5 mL) were mixed with 0.5 mL of internal standard and were handled in opaque tubes throughout the process to prevent photooxidation. Hexane (1.0 mL) was added to extract the lipid-soluble vitamins. After centrifugation at 1,000 × g, the hexane layer was recovered and dried at 20°C with nitrogen gas. Samples were reconstituted in the mobile phase (0.4% acetic acid in acetonitrile) and separated isocratically by HPLC at 0.5 mL/min through a Kinetex 2.6-μm XB-C18, 100 mm × 4.6 mm column with a guard column (cat. no. 001D-4496-E0; Phenomenex). Retinol, α-tocopherol, and tocopherol acetate were detected by absorption at 290 nm with retention times of 3.5, 12.8, and 15 min, respectively. α-Tocopherol was also evaluated based on the ratio of plasma α-tocopherol to plasma cholesterol to account for changes in lipid mobilization, and thus changes in α-tocopherol transport (Herdt and Smith, 1996).

Glutathione peroxidase (GPx) enzyme activity was measured using a colorimetric assay kit (#K762-100; Biovision) based on the methods of Paglia and Valentine (1967). Trolox equivalent antioxidant capacity (TEAC) was measured by a colorimetric assay kit (no. 709001; Cayman Chemical). Malondialdehyde (MDA) concentration was measured using colorimetric detection with a commercial kit (#ab118970; Abcam) via adduction of MDA and thiobarbituric acid.

Inflammatory Biomarkers in Plasma. Serum amyloid A (SAA) concentration was measured in plasma using a sandwich ELISA kit (cat. no. TP-802; Tridelta Development Ltd.). Controls (multi-species control, cat no. TP-802-CON; Tridelta Development Ltd.) were run on each ELISA plate. Haptoglobin (HP) concentration was measured in plasma based on differences in hydrogen peroxidase activity through measuring absorbance of complexes formed by HP and hemoglobin (Cooke and Arthington, 2012). Briefly, 10 μL of plasma was added to an o-dianisidine solution at pH 4.1 in a 16 × 100 mm borosilicate tube. Immediately afterward, 25 μL of hemoglobin solution was added to the tube. Following a 45-min incubation at 37°C, 100 μL of a freshly prepared hydrogen peroxide solution was added. Tubes were then incubated at room temperature for 1 h. A 200-μL aliquot from each tube was transferred to a well in a 96-well plate, and absorbance was measured at 450 nm. A blank was run using water in place of plasma, which was subtracted from all OD values of plasma samples. Plasma samples from a previous study were analyzed using a bovine-specific HP ELISA kit (cat. no. HAPT-11; Life Diagnostics Inc.), and the sample with the greatest HP concentration
was used as a stock standard. This stock standard was serially diluted in plasma from the least concentrated sample and used as the standard curve on each plate for the biochemical assay.

### Quantitative Real-Time PCR

Liver biopsy samples were evaluated for mRNA abundance of oxidative stress response mediators [nuclear factor E2-related factor 2 (NFE2L2; commonly known as NRF2)], metallothionein 1E (MT1E), glutathione peroxidase 3 (GPX3), inflammatory mediators [haptoglobin (HP), serum amyloid A3 (SAA3), and nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB1)], and 2 internal control genes [β-actin (ACTB) and 40S ribosomal protein S15 (RPS15)]. Messenger RNA abundance was calculated as (2 × PCR efficiency)−ΔCt, where Ct = cycle threshold (Pfaffl, 2001), and data were log-transformed for statistical analysis. Results were back-transformed and scaled such that the wk −3 control mean equaled 1 for each transcript.

### Innate Immunity

Polymorphonuclear cells were isolated from jugular blood samples as reported previously by Oh et al. (2008) with some modifications. Briefly, whole blood was first layered 1:1 with Ficoll-Paque (cat. no. 17-1440-03; ThermoFisher Scientific) in another tube and centrifuged at 1,700 × g for 45 min at 4°C. The top layers containing plasma, peripheral blood mononuclear cells, and Ficoll-Paque were discarded and the remaining PMN were mixed with cold Hanks’ Balanced Salt Solution without calcium or magnesium (HBSS; cat. no. 14175079; ThermoFisher Scientific). Tubes were centrifuged again at 2,500 × g for 5 min at 4°C and the supernatant was discarded. Cell pellets were then mixed with ammonium-chloride-potassium lysing buffer (cat. no. A1049201; ThermoFisher Scientific) to lyse red blood cells, followed by another centrifugation at 2,500 × g for 5 min at 4°C. Cell pellets were resuspended in more lysing buffer and HBSS and centrifuged again under the same conditions. Cells were resuspended in HBSS, centrifuged under similar conditions, and then resuspended in HBSS with calcium and magnesium (HBSS; cat. no. 14025126; ThermoFisher Scientific) and 2% BSA (cat. no. BP9704100; Fisher Scientific).

Isolated PMN were then counted using a Countess Automated Cell Counter (cat. no. C10281; Invitrogen). Isolated PMN were used to determine oxidative burst

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**Table 1. Gene primers for quantitative reverse transcription PCR of liver samples**

| Transcript | Primer | Primer sequence (5′ to 3′) | Accession number | Efficiency (%) |
|------------|--------|----------------------------|------------------|---------------|
| NFE2L2     | Forward| CTCAGCAATGGGACTTGAGAG     | NM_00101678.2    | 81            |
|            | Reverse| CTCATGCCTCTCTGTGGTG        |                  |               |
| MT1E       | Forward| TCGATCGGGTGTCTTCTCT        | NM_001078134.2   | 116           |
|            | Reverse| GAGAGATGGGAGCGTTTAATTG     |                  |               |
| GPX3       | Forward| GGAGCACTCTGCTGAACCCCT      | NM_174077.4      | 142           |
|            | Reverse| TACGCGGTCTTCTGTTG          |                  |               |
| HP         | Forward| TGCCGGTGCTGTTGTA           | NM_000104070.2   | 98            |
|            | Reverse| TACGCGGTCTTCTGTTG          |                  |               |
| SAA3       | Forward| GTCTGCGCCTCGATAAGTA        | NM_00124573.1    | 81            |
|            | Reverse| GACCCGCTCTCGAGCGCATCAG     |                  |               |
| NFKB1      | Forward| ATCTGTCCTGCAAACTCAG        | NM_001076409.1   | 89            |
|            | Reverse| GACGGGCTGCTGCAGCGCATCAG    |                  |               |
| ACTB       | Forward| GACGACATGGGAAGATCGTG       | NM_173979.3      | 109           |
|            | Reverse| GACGACATGGGAAGATCGTG       |                  |               |
| RPS15      | Forward| GTGCGGATGGAGAACAGAG        | NM_001024541.2   | 96            |
|            | Reverse| GTGCGGATGGAGAACAGAG        |                  |               |

1NFE2L2 = nuclear factor E2-related factor 2; MT1E = metallothionein 1E; GPX3 = glutathione peroxidase 3; HP = haptoglobin; SAA3 = serum amyloid A3; NFKB1 = nuclear factor kappa-light-chain-enhancer of activated B cells; ACTB = β-actin; RPS15 = 40S ribosomal protein S15.

2From the National Center for Biotechnology Information Nucleotide Database (https://www.ncbi.nlm.nih.gov/nucleotide/).
under the stimulation of phorbol 12-myristate 13-acetate (PMA), essentially as described by Llamas Moya et al. (2008), modified as a plate-based assay using a kit (kit #ABEL-16MM; ABEL). Briefly, 50 μL of PMN cell suspension at 1 × 10^6 cells/mL was added to each of 6 wells in a black-walled, 96-well plate. All 6 wells also received 60 μL of the assay buffer, 20 μL of an adjuvant to increase chemiluminescent signal, and 50 μL of pholasin, a photoprotein that emits light in the presence of ROS. To activate PMN to produce ROS through the NADPH oxidase system, 20 μL of PMA solution was added to half of the wells, referred to as “PMA-stimulated PMN oxidative burst.” The other half of the wells was left untreated, referred to as “un-stimulated PMN oxidative burst.” The plate was incubated at 37°C for 10 min and chemiluminescence was measured (Synergy HTX; BioTek Instruments Inc.). Additionally, concentrations of tumor necrosis factor-α in basal and PMA-stimulated PMN media samples were analyzed using a commercial kit (kit no. EBTNF; Thermo-Scientific), but concentrations were below the limit of detection.

**Adaptive Immunity**

Serum samples collected on d 7, 21, 28, and 35 postpartum were analyzed for anti-OVA IgG content by ELISA using a protocol previously described (Yuan et al., 2015). Briefly, 96-well plates were coated with 100 μL of a coating solution that consisted of 15.4 mg of OVA (cat. no. A5503; Sigma-Aldrich) diluted in 11 mL of 0.05 M carbonate-bicarbonate buffer (pH 9.4). This buffer was prepared by mixing 4 mL of a 0.2 M anhydrous sodium carbonate solution with 46 mL of a 0.2 M sodium bicarbonate solution and 150 mL of water. Plates were incubated with the coating solution overnight. The next day, plates were washed 5 times with a wash buffer, which consisted of PBS and 0.05% Tween solution (pH 7.4). Next, 200 μL of blocking buffer, which consisted of 4% BSA and 5% sucrose in PBS filtered at 0.2 μm, was added to each well and incubated at 24°C for 2 h. Plates were then washed again with the wash buffer.

Afterward, serum samples diluted 1:200 in PBS, blank controls (wash buffer), negative controls (pooled serum samples from d –7 diluted 1:200), and positive controls (pooled serum samples from d 28 diluted 1:200) were added to duplicate wells. Plates were incubated at 20°C for 1 h and then washed 5 times again. Anti-bovine IgG peroxidase (cat. no. A5295; Sigma-Aldrich) diluted in 10 mM PBS at 1:30,000 was then added in 100-μL aliquots to each well. After a 1-h incubation, plates were washed 5 times with wash buffer, and 100 μL of 3,3′,5,5′-tetramethylbenzidine substrate solution (TMB solution; ThermoFisher Scientific) was added to each well. After 5 min of incubation at 20°C, 100 μL of stop solution (0.18 M sulfuric acid) was added to each well. Absorbance was measured by OD adjusted for the blank control at 450 nm. Final OD was corrected between plates to reach a positive control OD of 0.5 by multiplying the original OD by 0.5 and then dividing by the positive control OD for that plate.

**Statistical Analyses.**

Results were analyzed using the Mixed Procedure of SAS (v. 9.4, SAS Institute Inc.). Models included fixed effects of treatment, time, parity, and 2- and 3-way interactions with treatment, and the random effects of block and cow. Interactions with parity were tested and removed from the model when P > 0.20. Anti-ovalbumin IgG content was analyzed using d-7 values as co-variants. Repeated measures within cow were modeled with autoregressive or heterogeneous autoregressive covariance structures when data points were equally spaced, with covariance structure selected based on the least Bayesian information criterion value. Unequally spaced data points (e.g., plasma data) were modeled with spatial power covariance structures. Outliers were excluded when the conditional Studentized residuals exceeded an absolute value of 4. If residual distributions were not normal, data were natural log-transformed for analysis and then back-transformed for presentation of results. Significance was declared when P < 0.05, and tendencies at 0.05 ≤ P < 0.10.

Regression techniques were used to explore relationships among oxidative parameters (JMP v. 16.2, SAS Institute Inc.), and outliers were removed from analysis based on Cook’s distance >0.14.

**RESULTS**

Metabolic and production responses from this study were reported previously (Olagaray et al., 2019). Briefly, we reported that SCFP supplementation increased meal frequency before and after parturition but did not affect DMI. Supplementation with SCFP did not affect milk protein or lactose yields but increased milk fat yield in wk 4 postpartum, and had some effects on lipid metabolism in the liver, as evidenced by tendencies for decreased liver cholesterol and increased plasma cholesterol, as well as an increased incidence of subclinical ketosis. The results reported herein build on these findings by exploring treatment effects on oxidative and immune status of periparturient cows supplemented with SCFP.
Table 2. Oxidative status parameters and inflammatory biomarkers in plasma of control cows and cows supplemented with *Saccharomyces cerevisiae* fermentation product (SCFP) from 29 ± 5 d prepartum to 42 DIM

| Variable | Control | SCFP | SEM | Trt | Week |  Trt × Week | Parity | Trt × Parity | Trt × Wk × Parity |
|----------|---------|------|-----|-----|------|------------|--------|--------------|------------------|
| Retinol, μg/mL | 0.33 | 0.34 | 0.01 | 0.66 | <0.001 | 0.21 | 0.11 | <0.001 | <0.001 |
| α-Tocopherol, μg/mL | 4.75 | 4.61 | 0.21 | 0.56 | <0.001 | 0.74 | 0.14 | NS | NS |
| TEAC, mmol/L | 0.69 | 0.68 | 0.01 | 0.54 | <0.001 | 0.72 | 0.62 | NS | NS |
| GPx, mU/mL | 0.93 | 0.99 | 0.07 | 0.32 | <0.001 | 0.44 | 0.03 | NS | NS |
| SAA, ng/mL | 10.0 | 12.0 | 1.72 | 0.33 | <0.001 | 0.72 | 0.62 | NS | NS |
| HP, mg/mL | 514 | 575 | 44.0 | 0.18 | 0.07 | 0.51 | 0.75 | NS | NS |

1MDA = malondialdehyde; TEAC = Trolox equivalent antioxidant capacity; GPx = glutathione peroxidase; SAA = serum amyloid A; HP = haptoglobin.

2Pooled standard error of the mean.

3Trt = treatment. For NS results, interaction not included in final model because *P* > 0.20.

Oxidative Status in Plasma and Liver

The effects of SCFP on plasma measurements of oxidative status are summarized in Table 2. A tendency for treatment × week interaction pointed to marginally decreased α-tocopherol concentrations 2 wk before expected parturition in SCFP cows (*P* = 0.06; Figure 1A). Treatment effects on plasma retinol concentrations were dependent on parity (*P* < 0.001) as plasma retinol was greater in primiparous cows supplemented with SCFP (0.37 vs. 0.33 ± 0.02 μg/mL; *P* = 0.02) but lesser in multiparous cows supplemented with SCFP (0.30 vs. 0.33 ± 0.01 μg/mL; *P* < 0.01) compared with parity-matched controls. Additionally, the 3-way interaction of treatment, parity, and week (*P* < 0.001) indicated greater retinol concentrations in primiparous SCFP cows during wk −2 (Figure 1C) but lesser in multiparous SCFP cows during wk 2 (Figure 1D). Similarly, we detected a treatment × parity × week interaction for TEAC (*P* < 0.001). Although TEAC was greater in primiparous control cows than in primiparous SCFP cows during wk 1 (Figure 1E), there were no treatment differences across time for multiparous cows (Figure 1F). There was no evidence of treatment effects on plasma activity of GPx (Figure 1B) or plasma concentration of MDA (*P* ≥ 0.32). Multiparous cows had greater plasma α-tocopherol concentration and plasma GPx activity than primiparous cows (5.02 vs. 4.34 ± 0.23 μg/mL and 1.10 vs. 0.83 ± 0.08 mU/mL, respectively; *P* ≤ 0.05). The ratio of α-tocopherol to cholesterol tended to be greater for control versus SCFP cows (0.0038 vs. 0.0034 ± 0.0002; *P* < 0.10), but control cows also had lesser plasma cholesterol concentrations than SCFP cows (*P* = 0.02; Olagaray et al., 2019).

Supplementation with SCFP did not affect hepatic mRNA abundance of *NFE2L2*, *MT1E*, or *GPX3* (*P* ≥ 0.57; Table 3). Although mRNA abundance of *NFE2L2* did not differ by week, relative abundance of 2 of its target genes, *MT1E* and *GPX3*, increased postpartum (*P* ≤ 0.02). Hepatic mRNA abundance of *GPX3* and *MT1E* also tended to be greater in multiparous cows than primiparous cows (2.62 vs. 1.12 ± 0.48, *P* = 0.06 and 1.83 vs. 1.03 ± 0.31, *P* < 0.10, respectively).

Acute Phase Response

Supplementation with SCFP during the transition period did not affect plasma concentrations of SAA or HP (*P* ≥ 0.18; Figures 2A and 2B, respectively). The effect of week for plasma SAA (*P* < 0.001) was caused by a spike at −2 wk RTC. Plasma HP tended to differ by week (*P* = 0.07) but was largely elevated throughout the transition period, rather than only postpartum, as is typically reported. Similar to plasma results, SCFP supplementation did not affect hepatic mRNA abundance of *SAA3*, *HP*, or *NFKB1* (*P* ≥ 0.55; Table 3). Relative abundance of both *HP* and *NFKB1* was greater postpartum than prepartum (*P* < 0.05), whereas that of hepatic *SAA3* did not differ with time (*P* = 0.10).

Immune Response

Measures of innate and adaptive immune responses are reported in Table 4. Primiparous cows tended to have greater PMN oxidative burst than multiparous cows (7.441 vs. 5.572 ± 784 mV relative light units; *P* < 0.10), but SCFP supplementation did not affect basal or PMA-stimulated oxidative burst of neutrophils (*P* = 0.47; Figure 3A).

As expected, anti-OVA IgG titers increased over time (*P* < 0.001), but there was no effect of SCFP or treatment × time interaction (*P* ≥ 0.25; Figure 3B). However, we did observe a tendency for a treatment ×
Figure 1. Measures of oxidative status in periparturient cows supplemented with *Saccharomyces cerevisiae* fermentation product (SCFP) or not (control) from d −29 ± 5 relative to calving through 42 DIM. (A) Plasma α-tocopherol concentration was not affected by treatment, but a tendency for a treatment × week interaction was detected, with a marginal effect during wk −2 (*P* = 0.06). (B) Plasma glutathione peroxidase (GPx) activity was not affected by treatment. (C) Plasma retinol was greater in primiparous SCFP cows compared with parity-matched controls during wk −2 (*P* < 0.001). (D) Plasma retinol was lesser in multiparous SCFP cows compared with parity-matched controls during wk 2 (*P* < 0.001). (E) Plasma Trolox equivalent antioxidant capacity (TEAC) concentrations were greater in primiparous control cows than primiparous SCFP cows during wk 1 (*P* < 0.001). (F) Plasma TEAC was not affected by treatment across time points for multiparous cows. Treatment differences are indicated by †(*P* < 0.05) and ‡(*0.05 ≤ P < 0.10). Error bars show SEM. Full statistical details are provided in Table 2.
parity interaction \((P = 0.07)\) as anti-OVA IgG tended to be greater in primiparous SCFP cows compared with primiparous control cows \((0.36 \text{ vs. } 0.28 \pm 0.08 \text{ OD}; \ P = 0.08)\). Anti-OVA IgG was not affected by treatment among multiparous cows \((P = 0.61)\).

**DISCUSSION**

**Oxidative Status in Plasma and Liver**

Oxidative stress occurs when ROS, produced through normal cellular functions and increased with greater metabolic activity, overcome the neutralizing capacity of antioxidants. This stress can lead to tissue damage, immunosuppression, increased disease prevalence, and decreased performance (Miller et al., 1993a; Sordillo and Aitken, 2009; Abuelo et al., 2015). Animals consume antioxidants in the diet and produce their own proteins with antioxidant functions, particularly in the liver (Abuelo et al., 2015). Exogenous antioxidant, such as α-tocopherol and retinol, and endogenous antioxidants, such as GPx, collectively work to detoxify ROS, and measures of total antioxidant capacity attempt to quantify the net neutralizing capacity of these molecules (Miller et al., 1993a,b).

Trolox equivalent antioxidant capacity represents the total antioxidant capacity of both lipid- and water-soluble antioxidants using Trolox, an α-tocopherol analog, as a standard (Miller et al., 1993b). This measurement more accurately reflects antioxidant capacity within an animal compared with individual antioxidant measurements, as deficiency of one antioxidant may not decrease overall neutralizing capacity (Abuelo et al., 2015). In our study, TEAC was greater than that previously observed in periparturient cows (Gessner et al., 2015; Winkler et al., 2015). Assuming this accurately reflects a high antioxidant status in this cohort of cows, this may have limited the potential for SCFP supplementation to further enhance antioxidant capacity.

In wk 1 postpartum, however, SCFP supplementation decreased TEAC in primiparous cows compared with parity-matched controls. Although SCFP could have suppressed antioxidants not measured in this study, neither the individual antioxidants measured nor MDA were affected by treatment or its interactions at this time point. Similarly, a recent study showed that SCFP tended to decrease total antioxidant capacity at d 1 and 7 postpartum but did not alter plasma concentrations of reactive oxygen metabolites or MDA (Knoblock et al., 2019). Results from both studies suggest that the decrease in TEAC within the first week postpartum signified effective neutralization of prooxidants in SCFP-supplemented cows (Abuelo et al., 2015; Knoblock et al., 2019), leading to questions about how treatment may have influenced demand for antioxidants. The TEAC results from our study suggest that primiparous cows may be more sensitive to SCFP supplementation, but more research is needed in this area.

Hepatic responses to oxidative stress are largely stimulated by the transcription factor NFE2L2, which induces several antioxidant and cytoprotective genes (Ma, 2013). Although mRNA abundance of NFE2L2 did not differ by week, abundance of 2 of its target

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### Table 3. Hepatic mRNA abundance of oxidative stress mediator genes and inflammatory mediator genes was measured in control cows and cows supplemented with *Saccharomyces cerevisiae* fermentation product (SCFP) from 29 ± 5 d prepartum to 42 DIM

| Gene1 | Treatment (Trt) | Wk −3 | Wk 2 | SEM2 | Trt | Week | Trt × Wk | Parity | Trt × Parity | Trt × Wk × Parity |
|-------|----------------|-------|------|------|-----|------|----------|--------|-------------|------------------|
| Oxidative stress mediator genes | | | | | | | | | | |
| NFE2L2 | Control | 1 | 1.68 | 0.31 | 0.79 | 0.11 | 0.22 | 0.53 | NS3 | NS |
| | SCFP | 1.47 | 1.58 | 0.36 | 0.74 | 0.02 | 0.73 | 0.10 | 0.42 | 0.10 |
| MT1E | Control | 1 | 2.10 | 0.48 | | | | | | |
| | SCFP | 0.98 | 1.71 | 0.43 | | | | | | |
| GPX3 | Control | 1 | 3.42 | 0.68 | 0.57 | <0.001 | 0.45 | 0.06 | NS | NS |
| | SCFP | 0.71 | 3.56 | 0.66 | | | | | | |
| Inflammatory mediator genes | | | | | | | | | | |
| HP | Control | 1 | 2.69 | 1.15 | 0.92 | 0.01 | 0.41 | 0.22 | NS | NS |
| | SCFP | 0.62 | 3.97 | 1.44 | | | | | | |
| SAA3 | Control | 1 | 1.96 | 0.50 | 0.55 | 0.10 | 0.58 | 0.63 | NS | NS |
| | SCFP | 0.97 | 1.35 | 0.40 | | | | | | |
| NFKB1 | Control | 1 | 1.66 | 0.38 | 0.96 | <0.05 | 0.91 | 0.17 | NS | NS |
| | SCFP | 1.04 | 1.64 | 0.39 | | | | | | |

1 NFE2L2 = nuclear factor E2-related factor 2; MT1E = metallothionein 1E; GPX3 = glutathione peroxidase 3; HP = haptoglobin; SAA3 = serum amyloid A3; NFKB1 = nuclear factor kappa-light-chain-enhancer of activated B cells.

2 Pooled standard error of the mean.

3 For NS results, interaction not included in final model because \(P > 0.20\).
genes, MT1E and GPX3, increased postpartum, suggesting that cows were experiencing some level of oxidative stress at 2 wk postpartum; however, SCFP supplementation did not affect hepatic mRNA abundance of NFE2L2, MT1E, or GPX3, or plasma GPx activity. Similar to the lack of significant treatment results in our study, feeding a different SCFP source did not affect plasma GPx activity in dairy calves during weaning (Alugongo et al., 2017).

Both α-tocopherol and retinol are diet-derived lipid-soluble vitamins with antioxidant functions that can be stored in adipose tissue (Debier et al., 2005). α-Tocopherol is often associated with lipoproteins (as is cholesterol) for transport through circulation (Bjørneboe et al., 1990); thus, α-tocopherol-to-cholesterol ratios are often used as proxy for true changes in α-tocopherol concentrations versus increases in lipid mobilization (Herdt and Smith, 1996). However, the tendency for SCFP to decrease α-tocopherol concentration at wk −2 was not attributed to a decrease in plasma cholesterol concentration (Olagaray et al., 2019). Instead, this tendency could be explained by increased utilization of α-tocopherol for neutralization of oxidants or an increase in α-tocopherol storage with SCFP supplementation, both of which would appear as a decrease in circulating concentrations (Debier et

![Figure 2](image-url)
al., 2005; Abuelo et al., 2015). Regardless, TEAC and MDA, a marker of lipoperoxidation, did not differ at wk –2, indicating that overall antioxidant status was not affected by the lesser α-tocopherol concentrations. Retinol is also mobilized into circulation from adipose stores but is instead associated with retinol binding protein for this process (Frey and Vogel, 2011). Although retinol and α-tocopherol both originate from the diet, differences in carrier proteins (Frey and Vogel, 2011) and specific antioxidant functions (Miller et al., 1993a) could explain different observations for these vitamins.

Multiparous cows generally experience more severe negative energy balance and mobilize more tissue energy stores at the onset of lactation compared with primiparous cows. Greater lipid mobilization in multiparous cows could also subject them to greater lipid peroxidation and ROS production compared with primiparous cows (Friggens et al., 2007). As antioxidants neutralize prooxidants, less antioxidants are measurable in circulation (Miller et al., 1993a). Thus, differences in retinol responses to SCFP supplementation between parities may have been due to lesser oxidative stress experienced in first-lactation cows, allowing for greater circulating concentrations of the antioxidant (Debier et al., 2005). Postpartum, when oxidative stress is known to be elevated (Abuelo et al., 2015), retinol could have been more effectively used to neutralize the greater levels of oxidation in multiparous cows, represented by lesser concentrations in circulation (Miller et al., 1993a). In fact, as previously reported (Olagaray et al., 2019), multiparous cows fed SCFP had increased plasma β-hydroxybutyrate concentrations relative to parity-matched controls in wk 2 postpartum, the same time point at which SCFP reduced plasma retinol in multiparous cows. This may point to greater lipid oxidation and need for lipid peroxide neutralization in multiparous cows fed SCFP.

Alternatively, SCFP supplementation could have promoted retinol storage for multiparous cows, thus decreasing available concentrations in serum (Miller et al., 1993a; Debier et al., 2005), which is particularly relevant as multiparous cows tend to have greater adipose tissue available for lipid-soluble antioxidant storage (Bjørneboe et al., 1990; Frey and Vogel, 2011). In our study, hepatic mRNA abundance of GPX3 and MT1E also tended to be greater in multiparous cows than in primiparous cows (2.62 vs. 1.2 ± 0.48, P = 0.06, and 1.83 vs. 1.03 ± 0.31, P < 0.10, respectively). Because transcription of these antioxidant genes is induced by NFE2L2 signals from oxidative stress (Gessner et al., 2013; Ma, 2013; Han et al., 2018), these differences between parities further suggest that multiparous cows underwent more oxidative stress than primiparous cows. Greater oxidative stress in multiparous cows could also
have signaled an increase in endogenous antioxidant enzyme production (Tüzün et al., 2002; Bierl et al., 2004), leading to greater GPx production in multiparous cows compared with primiparous cows as observed in our study. In contrast to the implication that multiparous cows experience greater oxidative stress than primiparous cows, TEAC did not differ between parities ($P = 0.21$). However, endogenous antioxidant signals and antioxidant stores most likely allowed multiparous cows to overcome a decrease in overall antioxidant capacity, even with increased lipid peroxidation and ROS production.

We also explored the contributions of the individual antioxidants measured in our study to the overall antioxidant measure. Retinol, $\alpha$-tocopherol, and GPx did not strongly predict TEAC during wk −2, 1, or 5 (all $R^2 < 0.20$); however, GPx was a stronger predictor during wk 2 ($R^2 = 0.21; P < 0.001$: Figure 4). During wk 2 postpartum, GPx activity below 0.5 mU/mL appeared to reflect deficient TEAC, but TEAC did not change with GPx activity above 1.0 mU/mL (Figure 4). This quadratic relationship suggests that GPx activity ≥0.8 mU/mL (breakpoint in the curve) may be necessary for maximal TEAC.
If antioxidants are unable to neutralize pro-oxidants, lipid peroxidation can occur, producing MDA. Determination of MDA has been criticized for being nonspecific, but it is still considered a sensitive biomarker of oxidative stress (Abuelo et al., 2015). Consistent with a lack of changes in overall antioxidants, plasma MDA was not affected by SCFP supplementation ($P = 0.52$). Similarly, supplementing dairy calves with SCFP during weaning did not affect different plasma MDA concentrations (Alugongo et al., 2017). In our study, concentrations of MDA increased at wk $-2$, indicating increased lipid peroxidation at this prepartum time point. This result contradicts previous reports that observed lesser MDA during late pregnancy compared with early lactation (Bernabucci et al., 2005; Liu et al., 2013). This prepartum time point was also characterized by an unexpected spike in plasma SAA, an inflammatory biomarker. Although both plasma MDA and SAA shared similar unexpected increases during wk $-2$, the 2 were not significantly correlated at this time point ($R = -0.20; P = 0.13$). Unexpectedly (Bernabucci et al., 2005; Liu et al., 2013), MDA levels decreased 1 wk after calving, suggesting that cows either experienced less lipid peroxidation even with elevated lipid mobilization, or that antioxidants were able to detoxify lipid peroxides.

**Inflammatory Biomarkers in Plasma and Liver**

Nuclear factor κB, SAA, and HP are all considered biomarkers of inflammation. Hepatic mRNA abundance of both $HP$ and $NFKB1$ was greater postpartum than prepartum ($P = 0.01$ and $P < 0.05$, respectively), as expected from previous reports in transition dairy cattle (Gessner et al., 2013; Han et al., 2018), but hepatic $SAA3$ was not significantly different prepartum versus postpartum ($P = 0.10$). Although we expected lesser $SAA3$ abundance at 3 wk prepartum (Gessner et al., 2013), we observed an unexpected spike in plasma SAA at wk $-2$. Although these 2 biomarkers did not correlate at the prepartum time point ($R = -0.22; P = 0.12$), it is possible that another isoform of SAA was more relevant to the circulating concentrations.

Supplementation with SCFP increased plasma concentrations of SAA within the first week postpartum in one transition dairy herd (Zaworski et al., 2014) but had no effect on SAA and decreased HP in another (Knoblock et al., 2019). In another challenge model, SCFP supplementation tended to decrease peak SAA in heat-stressed lactating dairy cows (Al-Qaisi et al., 2020). In our study, SCFP treatment did not affect plasma concentrations of SAA ($P = 0.33$; Figure 2A) or HP ($P = 0.18$; Figure 2B), or hepatic mRNA abundance of $SAA3$, $HP$, or $NFKB1$ ($P = 0.55$, 0.92, and 0.96 respectively). Patterns of both plasma SAA and HP were unlike other reports in transition dairy cattle (Zaworski et al., 2014; Yuan et al., 2015). Plasma SAA differed by week ($P < 0.001$), spiking 2 wk before calving, instead of within the first week postpartum as was expected (Zaworski et al., 2014). Plasma HP was elevated throughout the transition period, rather than only postpartum as typically reported (Zaworski et al., 2014; Yuan et al., 2015). These unusual acute phase protein patterns in our study are puzzling and may reflect the stress of adapting to the automated feeding system used to deliver treatments prepartum. On the other hand, we found no evidence of abnormally low DMI in cows during the first week on the study (Olagaray et al., 2019), suggesting that cows were not excessively stressed.

**Innate Immune Response: PMN Oxidative Burst**

Polymorphonuclear leukocytes, primarily consisting of neutrophils, are abundant innate immune cells that respond quickly to infection or tissue damage. These cells phagocytose pathogens and produce ROS as a killing mechanism. This oxidative burst is used as an indicator of PMN function (Llamas Moya et al., 2008). Although SCFP decreased oxidative burst of stimulated cells in vitro (Jensen et al., 2008), SCFP treatment did not affect basal or PMA-stimulated oxidative burst of neutrophils in our study ($P = 0.47$; Figure 3A). Previous reports have described impaired neutrophil and macrophage innate immune cell function in periparturient cows, particularly around calving (Kehrli et al.,

![Figure 4. Plasma glutathione peroxidase (GPx) enzyme activity significantly predicted plasma Trolox equivalent antioxidant capacity (TEAC), a measure of total antioxidant capacity in blood, during wk 2 postpartum. At this time point, GPx activity below ~0.8 mU/mL was associated with decreased TEAC, suggesting that GPx activity ≥0.8 mU/mL may be necessary for optimal antioxidant capacity. n = 57; 2 outliers were removed based on Cook’s distance >0.14.](image)
Adaptive Immune Response: Anti-Ovalbumin IgG Response

The adaptive immune system consists of cytotoxic and humoral responses to remember and defend against specific antigens. The humoral pathway depends on B lymphocytes, which produce immunoglobins that bind specific antigens of pathogens or other foreign bodies for effective recognition and killing (Butler, 1998). Anti-OVA IgG titers increased after injections of the foreign protein ($P < 0.001$; Figure 3B), as expected (Butler, 1998; Yuan et al., 2015).

Supplementing SCFP or other yeast products has been shown to modulate adaptive immune response previously in several animal models. Transition dairy cattle fed mannan oligosaccharide, a component of yeast cell walls, during the last 3 wk of the dry period had greater antibody response to rotavirus vaccinations at parturition; however, the product did not affect serum IgA or total serum immunoglobulin titers (Franklin et al., 2005). Dairy calves challenged with a respiratory virus had reduced lung pathology, incidence of secondary bacterial infection, viral shed, and viral load in lungs when supplemented with SCFP compared with their unsupplemented counterparts (Mahmoud et al., 2020). In broilers, yeast increased antibody response to Newcastle disease virus after vaccination (Muthusamy et al., 2011). This finding was further investigated by Chou et al. (2017), who found that SCFP increased mature B-cell markers in the spleen and mature T-cell subset counts in the thymus. Additionally, SCFP increased secretory tonsil IgA counts in broilers challenged with pathogenic Eimeria tenella (Gao et al., 2009). In vitro trials with human immune cells showed that incubation with SCFP enhanced B-lymphocyte activation and natural killer cell cytotoxic response (Jensen et al., 2008).

Similar to a recent study supplementing SCFP to transition dairy cows (Knoblock et al., 2019), SCFP supplementation did not affect overall anti-OVA IgG titers in serum in our study ($P = 0.25$). However, we did observe a tendency for a treatment $\times$ parity interaction ($P = 0.07$) as anti-OVA IgG tended to be greater in primiparous SCFP cows compared with primiparous control cows (0.36 vs. 0.28 $\pm$ 0.08 OD; $P = 0.08$). Anti-OVA IgG was not affected by treatment among multiparous cows ($P = 0.61$). Interestingly, transition dairy cows in their second parity supplemented with mannan oligosaccharide presented greater serum rotavirus neutralization titers after vaccination than control cows of the same parity, whereas transition cows beyond their second parity were unaffected by supplementation for this measure (Franklin et al., 2005). Though our cohort of multiparous cows included cows in their second parity and beyond, our findings, along with those of Franklin et al. (2005), suggest a greater adaptive immune response to yeast product supplementation with lesser parity. One potential explanation for this is that primiparous cows had greater energy balance than
multiparous cows ($P < 0.001$; Olagaray et al., 2019), although there were no differences in energy balance between treatments in our study. Natural antibody production was positively correlated with energy balance in early lactation dairy cows (van Knevel et al., 2007). B Lymphocytes from primiparous cows could have had more energy to respond to SCFP stimulation when the product was supplemented, resulting in greater production of anti-OVA IgG.

Treatment with SCFP may be able to affect the adaptive immune response in the gut as well. In preweaning dairy calves, SCFP reduced the intensity of Cryptosporidium infection on a commercial farm (Vélez et al., 2019). Pisoni and Relling (2020) found that plasma IL-1β tended to be greater for calves supplemented with SCFP, although no difference in gut permeability was found between treatments. Although supplementation with SCFP did not affect serum anti-OVA IgG titers, Magalhães et al. (2008) observed decreased incidence of mortality due to diarrhea in supplemented dairy calves compared with control-fed calves. In transition dairy cattle, hydrolyzed yeast fed at low and medium doses increased fecal IgA, the main immunoglobin from mucosal tissues of the digestive tract (Yuan et al., 2015). Additionally, IFN-γ concentrations in the gut, but not plasma, were greater in nursery pigs fed SCFP than in control pigs and pigs treated with antibiotics (Shen et al., 2009). In addition, many studies have demonstrated the ability of yeast cells and components to bind to and interact with pathogens, thus potentially preventing them from invading intestinal tissues and causing infection (Badia et al., 2012; Tiago et al., 2012). Supplementation of SCFP may have altered other aspects of the adaptive immune system not measured in this study, as demonstrated previously with T cells (Chou et al., 2017) and natural killer cells (Jensen et al., 2008).

**CONCLUSIONS**

In this study, we added measures of oxidative and immune status to broaden our previous report showing that SCFP increased meal frequency and transiently increased milk fat yield in periparturient cows, although it also increased the incidence of subclinical ketosis. In general, SCFP supplementation did not affect measures of oxidative status, inflammation, innate immunity, or adaptive immunity in this relatively healthy cohort of transition dairy cattle, with some exceptions. Uncharacteristic patterns of inflammatory biomarkers in plasma were observed before parturition but were not affected by SCFP. Supplementation of SCFP may enhance adaptive immune response in primiparous cows, as antibody production tended to be greater for SCFP-supplemented primiparous cows than for control primiparous cows. Future research is warranted to determine effects and mechanisms of SCFP supplementation on oxidative status, inflammation, and immune responses during the transition period, particularly in more disease-challenged dairy cattle.

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