Different durations of whole raw soybean supplementation during the prepartum period: Measures of cellular immune function in transition cows

R. Gardinal,* G. D. Calomeni,* F. Zanferari,* T. H. A. Vendramini,* C. S. Takiya,* H. G. Bertagnon,†
C. F. Batista,† A. M. M. P. Della Libera,† and F. P. Renno*‡¹

*Department of Animal Nutrition and Production, University of Sao Paulo, Pirassununga, Brazil, 13635-900

†Department of Internal Medicine, University of Sao Paulo, Sao Paulo, Brazil, 05508-270

‡Bursar 1-C of the National Council of Scientific and Technologic Development, Brasilia, Brazil, 71605-001

ABSTRACT

The objective of this study was to evaluate different durations of whole raw soybean (WS) supplementation (diet rich in n-6 fatty acid) during the prepartum period on cellular immune function of dairy cows in the transition period and early lactation. Thirty-one Holstein cows were used in a completely randomized design and assigned to 4 experimental groups (G) [G90, G60, G30, and G0 (control)] supplemented with a diet containing 12% of WS from 90, 60, 30 and 0 d relative to the calving date, respectively. Cows were dried off 60 d before the expected calving date. After parturition, all cows were fed a diet containing 12% of WS until 84 DIM. Blood samples were collected before the morning feeding (d -56 ± 2 , -28 ± 2 , -14 ± 2 , -7 ± 2 , at the day of partum, 7 ± 2 , 14 ± 2 , 28 ± 2 , and 56 ± 2 relative to parturition). Cell phenotyping and phagocytosis assays were carried out using monoclonal antibodies and flow cytometry technique. Duration of WS supplementation linearly increased the percentage of blood CD3⁺ cells, as well as increased the percentage of blood CD8⁺ cells in the postpartum period, notably in G30, whereas the lowest values were observed in G0. Further, the duration of WS supplementation linearly increased the reactive oxygen species median fluorescence intensity of CH138⁺ cells after phagocytizing *Staphylococcus aureus* in the postpartum period. Longer periods of WS supplementation linearly increased phagocytosis median fluorescence intensity of CH138⁺ cells in the prepartum period of cows. Duration of WS supplementation linearly increased the percentage of blood CD14⁺ cells producing reactive oxygen species when stimulated either by *Staph. aureus* or *Escherichia*

coli in the postpartum period. In conclusion, longer periods of WS supplementation during late lactation and the dry period (beginning on d 90 of the expected calving date) alter the leukocyte population and improve neutrophil immune response in the postpartum period with no detrimental effects on cow performance.

Key words: adaptive immunity, innate immunity, oxidative burst, linoleic acid, phagocytosis

INTRODUCTION

The immune system is composed of the innate and the adaptive systems that operate in a synchronized, sequential, and complementary manner to prevent diseases (Carroll and Forsberg, 2007). The innate immune system is responsible for combating the first infection stages and delays pathogen propagation into the organism. If the innate immune system fails to eliminate the pathogen, the adaptive system is triggered and it develops a specific response including the production of antigen-specific lymphocytes and memory cells able to identify antigenic determinants of a pathogen (Tizard, 2014; Sordillo, 2016).

Hormonal and physiological changes occurring in the transition period and early lactation, such as increased blood cortisol and nonesterified fatty acid (NEFA) concentration, and decreased energy balance often jeopardize the immune response of cows. These changes reduce immune cellular activity (Cai et al., 1994; Zerbe et al., 2000), impair cellular migration to peripheral tissue (Kimura et al., 1999), and decrease cell bactericidal and phagocytic activities (Kehrli et al., 1989) and reactive oxygen species (ROS) production, especially in neutrophils and monocytes (Mehrzhad et al., 2001; Singh et al., 2008).

Research evaluating the supplementation of PUFA to dairy cows has found PUFA may either stimulate (pro-inflammatory; Grummer et al., 2004) or inhibit (anti-inflammatory; Calder, 2012) the immune system. Poly-

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¹Corresponding author: francisco.renno@usp.br

unsaturated fatty acids may alter the fatty acid (FA) profile of cell membranes, modifying the production of pro- or anti-inflammatory molecules including cytokines, IL, and leukotrienes. In addition, PUFA influence the expression of adhesion molecules, and proliferation and activation of immune cells (Contreras and Sordillo, 2011). The different outcomes of PUFA supplementation on the immune system are mostly attributed to the type of FA provided. Polyunsaturated FA of n-3 and n-6 series may modulate immune response (Gandra et al., 2016a) depending on the processing method of FA (rumen protected or as a free oil) and their proportion in the diet (Onetti and Grummer, 2004; Greco et al., 2015). The n-3 FA have anti-inflammatory properties, whereas the n-6 FA possess pro-inflammatory properties (Silvestre et al., 2011; Calder, 2012).

Linoleic acid is a FA from the n-6 series, commonly supplemented in animal and human diets. In the immune cells, linoleic acid acts as a precursor for arachidonic acid synthesis, a major component of pro-inflammatory molecules and activator of immune cells (Sordillo et al., 2009). In humans and rodents, linoleic acid supplementation directly influences innate immunity by increas-

ing the adhesion molecule expression in neutrophils, enhancing phagocytic activity, promoting bacterial killing by increasing ROS production in polymorphonuclear cells, and altering the synthesis of intracellular signaling molecules in immune cells (Contreras and Sordillo, 2011; Poorani et al., 2016). Studies that evaluated the supplementation of rumen-protected sources of linoleic acid to dairy cattle have shown similar responses to linoleic acid provision to humans regarding the immune system (Silvestre et al., 2011; Gandra et al., 2016a).

The major nutrient in whole raw soybean (WS) is protein; however, it is also rich in linoleic acid. Further, oilseeds have a protein surrounding their lipid content that partially protects FA against ruminal biohydrogenation (Lock and Bauman, 2004; Barletta et al., 2016). The partial biohydrogenation of linoleic acid produces *trans*-11 C18:1 FA and *cis*-9,*trans*-11 C18:2 CLA (Jenkins et al., 2008). However, recent in vivo or ex vivo studies have demonstrated that CLA has a minor effect on immune response of cows (Petzold et al., 2015; Eger et al., 2017).

Although the effects of n-6 FA supplementation have been extensively investigated, research evaluating their

Table 1. Percentage of blood granulocytes and mononuclear cells expressing specific adhesion molecules in dairy cows fed whole raw soybeans during different periods (mean $\pm$ SD)

Item ¹	Group ²				P-value ³				
	G90	G60	G30	G0	Group	Time	Group $\times$ time	Linear	Quadratic
Granulocytes									
Prepartum	32.3 $\pm$ 2.70	30.2 $\pm$ 2.47	36.4 $\pm$ 3.54	37.6 $\pm$ 3.65	0.287	0.343	0.760	0.129	0.600
Postpartum	35.9 $\pm$ 2.84	40.5 $\pm$ 2.77	37.2 $\pm$ 3.28	28.6 $\pm$ 3.04	0.040	0.162	0.962	0.059	0.030
CH138 ⁺									
Prepartum	85.6 $\pm$ 3.46	82.5 $\pm$ 3.34	80.3 $\pm$ 4.56	83.8 $\pm$ 4.55	0.818	0.073	0.814	0.687	0.431
Postpartum	82.4 $\pm$ 3.07	88.4 $\pm$ 2.62	80.4 $\pm$ 3.14	84.5 $\pm$ 2.99	0.241	0.009	0.001	0.898	0.753
Mononuclear cells									
Prepartum	60.2 $\pm$ 3.16	59.6 $\pm$ 2.91	56.9 $\pm$ 4.09	55.4 $\pm$ 4.18	0.776	0.090	0.970	0.341	0.898
Postpartum	60.9 $\pm$ 2.98	54.6 $\pm$ 2.82	58.1 $\pm$ 3.34	60.4 $\pm$ 3.09	0.124	0.283	0.984	0.143	0.235
CD14 ⁺									
Prepartum	17.4 $\pm$ 4.46	21.9 $\pm$ 4.11	24.0 $\pm$ 5.89	23.6 $\pm$ 5.98	0.767	0.093	0.789	0.407	0.644
Postpartum	16.9 $\pm$ 2.16	19.6 $\pm$ 2.03	17.5 $\pm$ 2.49	15.7 $\pm$ 2.16	0.611	0.110	0.009	0.551	0.311
CD3 ⁺									
Prepartum	35.5 $\pm$ 2.59	31.2 $\pm$ 2.58	32.9 $\pm$ 3.03	31.5 $\pm$ 3.02	0.392	<0.001	0.132	0.515	0.392
Postpartum	31.4 $\pm$ 2.92	30.7 $\pm$ 2.85	29.9 $\pm$ 3.37	21.6 $\pm$ 3.12	0.091	0.234	0.011	0.028	0.220
CD4 ⁺									
Prepartum	2.2 $\pm$ 0.89	3.0 $\pm$ 0.91	3.0 $\pm$ 1.17	2.7 $\pm$ 1.19	0.928	0.551	0.549	0.735	0.625
Postpartum	1.7 $\pm$ 0.6	1.8 $\pm$ 0.6	3.6 $\pm$ 0.7	1.3 $\pm$ 0.7	0.114	0.175	0.521	0.867	0.071
CD8 ⁺									
Prepartum	13.8 $\pm$ 1.26	10.9 $\pm$ 1.35	14.4 $\pm$ 1.86	13.4 $\pm$ 1.85	0.356	<0.001	0.062	0.736	0.567
Postpartum	7.4 $\pm$ 1.08	7.3 $\pm$ 1.04	9.8 $\pm$ 1.20	5.0 $\pm$ 1.11	0.046	0.004	0.223	0.359	0.038
CD62L ⁺									
Prepartum	52.3 $\pm$ 8.55	38.5 $\pm$ 8.03	49.6 $\pm$ 10.8	39.9 $\pm$ 10.9	0.622	0.162	0.692	0.554	0.834
Postpartum	79.4 $\pm$ 5.9	77.2 $\pm$ 5.65	75.6 $\pm$ 6.05	69.8 $\pm$ 5.92	0.692	0.096	0.106	0.258	0.757

¹Granulocytes (% of total leukocytes); CH138⁺ (% of total granulocytes); mononuclear cells (% of total leukocytes); CD14⁺ (% of total mononuclear cells); CD3⁺ (% of total mononuclear cells); CD4⁺ (% of total mononuclear cells); CD8⁺ (% of total mononuclear cells); CD62L⁺ (% of total leukocytes).

²G0 = group 0, cows did not receive whole raw soybeans (WS) throughout the prepartum period; G30, G60, and G90 = group that received diets containing WS from 30, 60, and 90 d from the expected calving date, respectively.

³Probabilities for group, time (week), group by time, linear, and quadratic effects.

period of supplementation on the immune response of cows in transition and early lactation is lacking. Thus, this study was designed to evaluate different durations of WS supplementation in the prepartum period on leukocytes population and phagocytic and ROS production of neutrophils and monocytes in dairy cows. Our hypothesis was that the longer the period of WS supplementation, the greater would be the improvements on leukocyte phagocytosis ability and ROS production in early lactating cows.

MATERIALS AND METHODS

This paper discusses the influence of different durations of WS supplementation in the prepartum period on measures of cellular immune function of transition and early lactating dairy cows. Other data from this experiment are described in a companion paper (Gardinal et al., 2018), including the nutrient intake and digestibility, energy balance, milk yield and FA profile, blood metabolites, and oocyte and embryo quality. This study was approved by the Ethics Committee of the School of Veterinary Medicine and Animal Science, University

of Sao Paulo, and it was conducted at the Dairy Cattle Research Laboratory, Pirassununga, Brazil.

Cows, Experimental Design, and Diets

Detailed descriptions of cows, management, and diet compositions are shown in the study by Gardinal et al. (2018). Forty-four Holstein cows, in late lactation (20.3 ± 1.00 kg/d milk yield, 332 ± 26 DIM) and with 90 d from the expected day of parturition, were used in a completely randomized design study, balanced for milk production and BCS of cows. Cows were dried off 60 d before the expected calving date. At the beginning of the study, 11 cows were assigned to each treatment; however, because of the occurrence of metabolic or infectious disorders not related to experimental treatments (3 abortions, 3 displaced abomasum, 3 foot disorders, and 4 dystocia), 13 cows were removed from the study, and only data of healthy cows were evaluated. Cows were distributed to one of the following groups (G): **G90** ($n = 8$), **G60** ($n = 10$), **G30** ($n = 6$) and **G0** (control, $n = 7$). The groups were supplemented with a diet rich in n-6 FA (12% WS on a DM basis) from 90,

Figure 1. Blood granulocytes (% of total leukocytes) of cows supplemented whole raw soybeans (WS) for different durations in the prepartum period. G0 ($n = 7$) = cows fed a diet containing 12% WS after calving; G30 ($n = 6$), G60 ($n = 10$), and G90 ($n = 8$) = cows fed a diet containing 12% WS beginning at 30, 60, and 90 d before the expected calving date, respectively. Error bars represent SE.

60, 30 and 0 d relative to the expected calving date, respectively. After parturition, cows were fed a diet containing 12% of WS on a DM basis until 90 DIM.

Immune Cell Phenotyping in Blood

Whole blood (10 mL/cow) was collected by puncture of coccygeal vessels into sterile vacutainers (BD Vacutainer systems, Becton, Dickinson and Company, Franklin Lakes, NJ) containing anticoagulant solution (heparin). Blood samples (10 mL/cow) were collected before the morning feeding (d -56 ± 2 , -28 ± 2 , -14 ± 2 , -7 ± 2 , partum, 7 ± 2 , 14 ± 2 , 28 ± 2 , and 56 ± 2 relative to parturition). Blood samples were transported on ice to the Department of Internal Medicine of the University of Sao Paulo.

Identification of leukocytes population was performed according to their cytoplasmic granularities (side scatter cells) and median fluorescence intensity (MFI) in a 2-step fluorescence immunolabeling protocol (Brown and Witter, 2000) using primary (targeting cell of interest) and secondary monoclonal antibodies with fluorescent probes (except for $CD62L^+$ cells). Cells

were washed with PBS and incubated with primary monoclonal antibody for 30 min on ice. Then, 1 mL of PBS was added to the cell reconstitution and centrifuged at $250 \times g$ for 8 min at room temperature. Afterwards, secondary monoclonal antibody was added to the cell suspension and incubated on ice for 30 min. Finally, cells were washed in PBS and analyzed by flow cytometry (20,000 leukocytes). Cell populations were determined using forward scatter and side scatter (Hart et al., 2011). For granulocytes and mononuclear cells, the calculation of the percentage of cells was based on the cell dispersion cytogram according to size (forward scatter) and cytoplasmic granularities (side scatter cells). For the neutrophil and mononuclear cells, the calculation of the percentage of cells bearing each marker was performed by subtracting the percentage of positive cells for detection antibody from the entire percentage of the cells classified as granulocytes and mononuclear cells, respectively.

For the $CD62L^+$ cells, the calculation of the percentage of cells bearing each marker was performed by subtracting the percentage of positive cells for detection antibody from the entire percentage of the cells

Figure 2. Percentage of granulocytes in blood expressing $CHI38^+$ molecules of cows supplemented whole raw soybeans (WS) for different durations in the prepartum period. G0 ($n = 7$) = cows fed a diet containing 12% WS after calving; G30 ($n = 6$), G60 ($n = 10$), and G90 ($n = 8$) = cows fed a diet containing 12% WS beginning at 30, 60, and 90 d before the expected calving date, respectively. Error bars represent SE; an asterisk indicates means in the same time point differ significantly.

classified as granulocytes. For the T-lymphocytes, the calculation of the percentage of cells bearing each marker was performed by subtracting the percentage of positive cells for detection antibody from the cells classified as mononuclear cells. For T-helper cells and T-cytotoxic cells, the calculation of the percentage of cells bearing each marker was performed by subtracting the percentage of positive cells for detection antibody from the cells classified as T-lymphocytes cells. Pairs of antibodies used, amounts added, and their specificity are described by Blagitz et al. (2015).

Function and ROS Production of CH138⁺ Granulocytes and CD14⁺ Mononuclear Cells

Assays for population quantification, for the assessment of the intracellular production of ROS, and for phagocytosis of blood monocytes and neutrophils were performed using flow cytometry (Hasui et al., 1989; Kampen et al., 2004a,b). The assays were performed in polypropylene tubes suitable for flow cytometry. To assess the basal intracellular ROS production, 100 μ L of total blood from each animal was added to each tube

and incubated at 37°C for 30 min with 200 μ L of 0.3 mM 2,7-dichlorofluorescein diacetate. To conduct the phagocytosis tests, 10 μ L of *Staphylococcus aureus* or *Escherichia coli* (both containing 3×10^5 /mL), labeled with Alexa 594 (Sigma-Aldrich, St. Louis, MO, cat. no. D6883), was added to those tubes.

After centrifuging ($250 \times g$ for 8 min at 4°C) and discarding the supernatant, 1 μ L of monoclonal primary antibody was added to the tubes according to the population to be identified, and the tubes were left standing at room temperature for 30 min. After the incubation period, 1,000 μ L of ice-cold PBS was added, and then another centrifugation ($250 \times g$ for 8 min at 4°C) was performed. The supernatant was discarded, and 1 μ L of monoclonal secondary antibody was added to the tubes to label according to the population to be identified. The tubes were kept at room temperature in the dark for 30 min. An additional washing was performed, and the samples were resuspended in 400 μ L of ice-cold PBS + BSA (0.1%) and kept in the dark until they were read in a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Diego, CA). The cytometer was connected to a computer with

Figure 3. Percentage of mononuclear cells in blood expressing CD14⁺ molecules of cows supplemented whole raw soybeans (WS) for different durations in the prepartum period. G0 (n = 7) = cows fed a diet containing 12% WS after calving; G30 (n = 6), G60 (n = 10), and G90 (n = 8) = cows fed a diet containing 12% WS beginning at 30, 60, and 90 d before the expected calving date, respectively. Error bars represent SE; an asterisk indicates means in the same time point differ significantly.

the software CellQuest (Becton Dickinson Immunocytometry Systems).

Statistical Analysis

Data were subjected to SAS (Version 9.1.3, 2004, SAS Institute Inc., Cary, NC), verifying the normality of residuals and homogeneity of variances by PROC UNIVARIATE. Outliers were removed to achieve normality of residuals when necessary. Data were analyzed by PROC MIXED as repeated measures (Littell et al., 2006) according to the model below:

$$Y_{ijk} = \mu + G_i + T_j + G_i \times T_j + a_k + e_{ijk},$$

where Y_{ijk} = dependent variable; μ = overall mean; G_i = fixed effect of group; T_j = fixed effect of time; $G_i \times T_j$ = fixed effect of group by time interaction; a_k = random effect of animal; and e_{ijk} = residual. The degrees of freedom were calculated according to the Satterthwaite's method (ddfm = satterth). The covariance structure for each parameter was determined based on the smallest Akaike's information criterion values. Data from the first sampling date of blood parameters were

added as a covariate adjustment to prepartum period statistical analysis. Data were independently analyzed for each period (pre- and postpartum) to establish the effect of duration of WS supplementation in the prepartum period on the early lactation. Differences among treatments were tested by polynomial simple regression. Significance was set at $P < 0.05$.

RESULTS

Immune Cell Phenotypes

Duration of WS supplementation had a quadratic positive effect on the percentage of granulocytes in blood of postpartum cows ($P = 0.030$) but had no effect on the percentage of blood CH138⁺ cells (Table 1). Although the total percentage of mononuclear cells was not affected by the duration of WS supplementation, the percentage of CD3⁺ cells linearly increased ($P = 0.028$) and the percentage of CD8⁺ cells exhibited a quadratic positive effect ($P = 0.038$) according to the period of WS supplementation. Neither time nor group by time effects ($P \geq 0.162$) were observed for the total percentage of granulocytes in blood of cows (Figure 1).

Figure 4. Percentage of mononuclear cells in blood expressing CD3⁺ molecules of cows supplemented whole raw soybeans (WS) for different durations in the prepartum period. G0 (n = 7) = cows fed a diet containing 12% WS after calving; G30 (n = 6), G60 (n = 10), and G90 (n = 8) = cows fed a diet containing 12% WS beginning at 30, 60, and 90 d before the expected calving date, respectively. Error bars represent SE; an asterisk indicates means in the same time point differ significantly.

A group by time interaction effect was noticed ($P = 0.001$) for the percentage of CH138⁺ cells at 28 DIM in cows (Figure 2). A group by time interaction effect was also observed ($P = 0.009$) for the percentage of CD14⁺ cells at 28 and 56 DIM (Figure 3). A group by time interaction effect ($P = 0.011$) was observed on the percentage of CD3⁺ cells in blood of cows at 28 and 56 DIM (Figure 4). Finally, a time effect was observed ($P \leq 0.004$) for the percentage of CD8⁺ cells in the pre- and postpartum periods of cows (Figure 5).

Phagocytosis and ROS Production by CH138⁺ Granulocytes

Duration of WS supplementation quadratically affected ($P = 0.029$) the percentage of CH138⁺ producing ROS when stimulated by *E. coli* in the postpartum period, wherein the highest value was found when WS supplementation began at 30 d from the expected calving date (Table 2). In addition, the length of WS supplementation linearly increased ($P = 0.001$) the ROS MFI of CH138⁺ after phagocytizing *Staph. aureus* in the postpartum period. No differences were found among

experimental groups on the percentage of CH138⁺ cells able to phagocytize *Staph. aureus* or *E. coli*, but WS supplementation length linearly increased ($P = 0.039$) the phagocytosis MFI of CH138⁺ cells when stimulated by *E. coli* in the prepartum period. A time effect was found ($P \leq 0.009$) in the pre- and postpartum periods for ROS MFI of CH138⁺ cells when stimulated by *Staph. aureus* (Figure 6). A time effect was noticed ($P = 0.029$) on phagocytosis MFI of CH138⁺ cells when stimulated by *E. coli* during the prepartum period (Figure 7).

Phagocytosis and ROS Production by CD14⁺ Mononuclear Cells

The length of WS supplementation linearly increased ($P \leq 0.048$) the percentage of CD14⁺ cells producing ROS when stimulated by *Staph. aureus* and *E. coli* in the postpartum period (Table 3). Finally, no time and group by time interaction effects were observed for the percentage of CD14⁺ producing ROS when stimulated by *Staph. aureus* or *E. coli* (Figure 8 and Figure 9, respectively).

Figure 5. Percentage of mononuclear cells in blood expressing CD8⁺ molecules of cows supplemented whole raw soybeans (WS) for different durations in the prepartum period. G0 (n = 7) = cows fed a diet containing 12% WS after calving; G30 (n = 6), G60 (n = 10), and G90 (n = 8) = cows fed a diet containing 12% WS beginning at 30, 60, and 90 d before the expected calving date, respectively. Error bars represent SE.

DISCUSSION

Whole raw soybean supplementation during the dry period and transition period increases abomasal flow of linoleic acid (Bettero et al., 2017) and linoleic acid content in milk of cows (Gandra et al., 2016b), suggesting that WS supplementation augments linoleic acid absorption in the gut of cows. Zachut et al. (2010) supplemented extruded flaxseed (rich in n-3 FA), an oilseed such as WS, to transition cows and found an increase in blood n-3 FA content. In addition, the current study showed that WS supplementation had a positive quadratic effect on *cis*-9,*trans*-11 C18:2 FA and total PUFA content in milk of cows, in which the highest values were found in G30, and the lowest values were found in G90 (Gardinal et al., 2018). Moreover, Barletta et al. (2016) concluded that supplying WS is a better strategy to protect PUFA against ruminal biohydrogenation in comparison with supplying calcium salts of FA to lactating cows. Thus, it is reasonable to consider the provision of WS, or other oilseeds, as a way to increase blood PUFA concentration in dairy cows.

Positive effects of WS supplementation to transition cows on measures of immune system were reported in literature by Gandra et al. (2016a), but these authors provided WS from 35 d of the expected calving date to 84 DIM. In this experiment, we evaluated different durations of WS supplementation including -90, -60, -30, and 0 d relative to the calving date until 90 DIM. Rumen microbiota of cows fed PUFA for longer periods has shown enhancements of biohydrogenation efficiency (Hobson and Stewart, 2012), which affects linoleic acid and its products of biohydrogenation availability in the intestine.

Changes in leukocyte population profile were observed when supplementing WS in the prepartum period to dairy cows. Blood granulocyte percentage increased when supplementing WS beginning at 30 or 60 d before the expected calving date, but no differences were detected in neutrophils percentage. Granulocytes in blood are composed of eosinophils and basophils, besides neutrophils. The role of basophils is still not clear, but they are thought to play a role in host defense against parasites (Stone et al., 2010). The activation of basophils leads to IL-4 and IL-13 release, which stimu-

Table 2. CH138⁺ granulocyte production of reactive oxygen species (ROS) and phagocytic responses of dairy cows to different periods of whole raw soybean supplementation (mean $\pm$ SD)

Item	Group ¹				P-value ²				
	G90	G60	G30	G0	Group	Time	Group $\times$ time	Linear	Quadratic
Cells producing ROS (%)									
<i>Staphylococcus aureus</i>									
Prepartum	77.6 $\pm$ 4.15	86.3 $\pm$ 3.75	86.2 $\pm$ 5.73	84.9 $\pm$ 5.82	0.486	0.201	0.352	0.381	0.363
Postpartum	87.8 $\pm$ 2.16	80.3 $\pm$ 2.13	85.4 $\pm$ 2.47	84.7 $\pm$ 2.37	0.106	0.336	0.254	0.698	0.140
<i>Escherichia coli</i>									
Prepartum	88.2 $\pm$ 3.10	87.1 $\pm$ 2.84	88.5 $\pm$ 3.98	88.6 $\pm$ 4.07	0.895	0.786	0.326	0.881	0.873
Postpartum	91.1 $\pm$ 2.65	92.3 $\pm$ 2.60	93.9 $\pm$ 2.72	84.4 $\pm$ 2.80	0.112	0.517	0.528	0.315	0.029
ROS median fluorescence intensity									
<i>Staphylococcus aureus</i>									
Prepartum	436 $\pm$ 70.5	416 $\pm$ 70.9	447 $\pm$ 87.6	424 $\pm$ 87.4	0.723	0.009	0.085	0.852	0.795
Postpartum	715 $\pm$ 81.4	569 $\pm$ 84.4	584 $\pm$ 96.3	274 $\pm$ 92.3	0.006	0.004	0.874	0.001	0.363
<i>Escherichia coli</i>									
Prepartum	325 $\pm$ 97.6	525 $\pm$ 89.2	424 $\pm$ 125.2	461 $\pm$ 128.2	0.505	0.822	0.391	0.545	0.465
Postpartum	655 $\pm$ 85.5	527 $\pm$ 83.4	441 $\pm$ 95.8	408 $\pm$ 89.7	0.219	0.854	0.743	0.065	0.603
Phagocytosis (%)									
<i>Staphylococcus aureus</i>									
Prepartum	48.3 $\pm$ 3.60	39.7 $\pm$ 3.70	43.3 $\pm$ 5.51	45.8 $\pm$ 5.40	0.380	0.111	0.080	0.818	0.186
Postpartum	45.5 $\pm$ 3.88	46.5 $\pm$ 3.72	45.7 $\pm$ 4.40	49.2 $\pm$ 4.16	0.916	0.238	0.572	0.759	0.745
<i>Escherichia coli</i>									
Prepartum	34.4 $\pm$ 5.29	30.4 $\pm$ 5.22	36.4 $\pm$ 6.64	42.3 $\pm$ 6.73	0.497	0.828	0.825	0.226	0.356
Postpartum	38.2 $\pm$ 3.07	36.9 $\pm$ 2.90	33.7 $\pm$ 3.42	38.7 $\pm$ 3.17	0.722	0.584	0.058	0.907	0.331
Phagocytosis median fluorescence intensity									
<i>Staphylococcus aureus</i>									
Prepartum	384 $\pm$ 65.7	289 $\pm$ 56.7	238 $\pm$ 91.9	225 $\pm$ 91.0	0.493	0.091	0.100	0.203	0.624
Postpartum	214 $\pm$ 33.3	215 $\pm$ 33.0	227 $\pm$ 37.7	262 $\pm$ 35.5	0.746	0.993	0.062	0.322	0.633
<i>Escherichia coli</i>									
Prepartum	562 $\pm$ 54.4	528 $\pm$ 47.8	259 $\pm$ 65.1	104 $\pm$ 64.7	0.344	0.029	0.204	0.039	0.412
Postpartum	154 $\pm$ 25.7	202 $\pm$ 25.0	194 $\pm$ 29.2	188 $\pm$ 29.8	0.237	0.561	0.370	0.087	0.691

¹G0 = group 0, cows did not receive whole raw soybeans (WS) throughout the prepartum period; G30, G60, and G90 = group that received diets containing WS from 30, 60, and 90 d from the expected calving date, respectively.

²Probabilities for group, time (week), group by time, linear, and quadratic effects.

lates the differentiation of naïve T-cells and triggers allergic inflammation, respectively. Eosinophils, when activated, release several pro-inflammatory molecules, such as eicosanoids, cytokines, and granule-stored cationic proteins; eosinophils are usually elevated in peripheral blood during allergic reactions and helminthic infections (Hogan et al., 2008). The duration of WS supplementation did not affect granulocyte population, because neutrophils accounted for over 80% of total granulocytes in peripheral blood of cows in this study.

The longer was the period of WS supplementation, the higher was the percentage of blood $CD3^+$ mononuclear cells (T-lymphocytes) and $CD8^+$ mononuclear cells (T-cytotoxic) in the postpartum period. T-lymphocytes may respond to a stimulus and further differentiate in subsets including T-helper or T-cytotoxic cells. T-cells play a role in the adaptive immune system by mediating certain attributes such as specific, diversity, memory, and recognition of self and non-self molecules (Sordillo, 2016). Activation of T-helper cells results in cytokines production with a range of immune responses (Sordillo and Streicher, 2002), and T-cytotoxic ($CD8^+$)

cells destroy altered self cells. We found an increase in percentage of blood $CD3^+$ cells, but the literature frequently reports that PUFA supplementation causes alterations in lymphocyte membranes associated with decreased T-cell proliferation in humans (Curi et al., 1993; Larbi et al., 2005). Data are lacking in the literature regarding PUFA supplementation on lipid membrane composition in lymphocytes of bovine, and thus, outcomes may differ from those expected for humans. The different responses may be related to the dietary concentration of linoleic acid supplied, because linoleic acid is toxic to T-cells when provided in high concentration (Cury-Boaventura et al., 2006), but linoleic acid can induce proliferation when supplied in nontoxic concentrations (Ioan-Facsina et al., 2013).

In addition, the lack of WS effects in the prepartum period and their positive responses in the postpartum period are partially related to blood NEFA concentration of the cow. Whereas cows supplemented with WS before the partum date (G90, G60, and G30) exhibited an average blood NEFA concentration of 0.42 mmol/L in the postpartum period, cows in G0 had a blood

Figure 6. Median fluorescence intensity (MFI) of the reactive oxygen species (ROS) production in blood $CH138^+$ granulocytes when stimulated by *Staphylococcus aureus* in cows supplemented whole raw soybeans (WS) for different durations in the prepartum period. G0 ($n = 7$) = cows fed a diet containing 12% WS after calving; G30 ($n = 6$), G60 ($n = 10$), and G90 ($n = 8$) = cows fed a diet containing 12% WS beginning at 30, 60, and 90 d before the expected calving date, respectively. Error bars represent SE.

NEFA concentration of 0.51 mmol/L (Gardinal et al., 2018). In vitro studies have reported that an equivalent NEFA concentration of periparturient cows in vivo decreases peripheral blood mononuclear cell proliferation (Ster et al., 2012).

The innate immune response is primarily mediated by neutrophil and macrophage phagocytosis capacity and ROS production. Neutrophils (CH138⁺ granulocytes) are the first line of cellular defense against antigens. Neutrophils contain numerous cytoplasmic ROS granules to destroy bacteria, store large amounts of glycogen as an energy source, and have a convoluted surface that facilitates phagocytosis and intracellular vacuole formation (Paape et al., 2003). Researchers have demonstrated that WS supplementation to transition cows can increase the capacity of neutrophils to engulf *Staph. aureus* in in vitro challenges (Gandra et al., 2016a). In the current study, we demonstrated that the duration of WS supplementation increased the ROS MFI of blood neutrophils phagocytizing *Staph. aureus* in early lactating cows. In other words, WS supplementation likely increases the capacity of neutrophils to de-

stroy *Staph. aureus*. Further, we also demonstrated that WS supplementation increased the phagocytosis MFI of neutrophils after phagocytizing *E. coli* in prepartum cows. This means that WS supplementation increases the capacity of neutrophils to engulf *E. coli* in the prepartum period. Therefore, the results of phagocytosis and oxidative burst suggest that longer periods of WS supplementation (notably from 90 d of the expected calving date) positively influence the response and activation of neutrophils in both the pre- and postpartum periods.

The exact mechanism by which WS supplementation may enhance blood neutrophils function is still unknown. One potential mechanism may be related to linoleic acid conversion to arachidonic acid, with consequent production of pro-inflammatory mediators such as IL-1, IL-2, IL-6, tumor necrosis factor α , and IFN- γ (Yaqoob and Calder, 1995; Beutler et al., 2003; Greco et al., 2015). Silvestre et al. (2011) reported that the n-6 FA content in the membrane of neutrophils tended to increase after supplementing safflower oil (linoleic acid source, 63.6 g/100 g of FA) to periparturient cows.

Figure 7. Median fluorescence intensity (MFI) of the phagocytosis in blood CH138⁺ granulocytes when stimulated by *Escherichia coli* in cows supplemented whole raw soybeans (WS) for different durations in the prepartum period. G0 (n = 7) = cows fed a diet containing 12% WS after calving; G30 (n = 6), G60 (n = 10), and G90 (n = 8) = cows fed a diet containing 12% WS beginning at 30, 60, and 90 d before the expected calving date, respectively. Error bars represent SE.

Increased proportions of n-6 and n-3 FA in neutrophil and monocyte membranes were positively correlated with phagocytosis capacity and ROS production in humans (Kew et al., 2003). Changes in FA profile of the leukocyte membrane may also alter the gene expression of lipid mediators related to inflammation (Calder, 2006). Among the lipid mediators, eicosanoids (prostaglandins, prostacyclin, leukotrienes, lipoxin, and thromboxane) are known regulators of acute and chronic inflammation responses, activating endothelial cells and leukocyte populations (Contreras and Sordillo, 2011). Further, CLA isomers such as *cis*-9,*trans*-11 C18:2 FA have improved the cellular immunity in pigs by increasing the cytotoxic potential of peripheral blood lymphocytes (Bassaganya-Riera et al., 2001).

The duration of WS supplementation increased the percentage of monocytes producing ROS against *Staph. aureus* and *E. coli* during the postpartum period of cows, without enhancing ROS production inside the cells and without affecting the ability of monocytes to engulf bacteria. Our findings suggest that WS supplementation during the prepartum period does not in-

crease the capacity of macrophages to destroy bacteria. These results support previous findings where cows supplemented with WS in the pre- and postpartum periods did not show differences in monocyte phagocytosis capacity against *Staph. aureus*, whereas WS supplementation increased the percentage of phagocytic monocytes (Gandra et al., 2016a).

We need to stress that positive effects on the immune system may also be related to the greater blood concentration of *trans* FA and CLA (not measured in this study), because they are products of partial biohydrogenation of lipids in WS. Although it is difficult to attribute the positive effects of WS supplementation on the immune system to specific FA, this study clearly demonstrated that WS supplementation modulates the immune system of transition cows. Finally, note that although WS supplementation in the pre- and postpartum periods modulated the immune system of cows, this study did not demonstrate effects of WS supplementation on productive performance, energy balance, and blood NEFA concentration in early lactating cows (Gardinal et al., 2018); thus, the influences of WS sup-

Table 3. CD14⁺ mononuclear cell production of reactive oxygen species (ROS) and phagocytic responses of dairy cows to different periods of whole raw soybean supplementation (mean $\pm$ SD)

Item	Group ¹				P-value ²				
	G90	G60	G30	G0	Group	Time	Group $\times$ time	Linear	Quadratic
Cells producing ROS (%)									
<i>Staphylococcus aureus</i>									
Prepartum	52.1 $\pm$ 5.30	47.6 $\pm$ 5.02	46.7 $\pm$ 6.69	43.5 $\pm$ 6.89	0.788	0.372	0.265	0.338	0.924
Postpartum	64.8 $\pm$ 4.18	56.6 $\pm$ 4.12	63.2 $\pm$ 4.74	45.5 $\pm$ 6.59	0.012	0.056	0.761	0.010	0.284
<i>Escherichia coli</i>									
Prepartum	48.2 $\pm$ 6.20	43.5 $\pm$ 5.77	46.2 $\pm$ 7.72	38.4 $\pm$ 7.78	0.789	0.784	0.135	0.400	0.820
Postpartum	62.1 $\pm$ 4.27	59.7 $\pm$ 4.10	66.4 $\pm$ 4.85	46.8 $\pm$ 4.49	0.020	0.237	0.369	0.048	0.056
ROS median fluorescence intensity									
<i>Staphylococcus aureus</i>									
Prepartum	86.3 $\pm$ 21.0	91.0 $\pm$ 20.1	80.3 $\pm$ 24.1	68.6 $\pm$ 26.7	0.918	0.711	0.076	0.549	0.725
Postpartum	138 $\pm$ 24.2	120 $\pm$ 24.2	164 $\pm$ 27.6	72.0 $\pm$ 26.0	0.104	0.026	0.584	0.179	0.152
<i>Escherichia coli</i>									
Prepartum	66.7 $\pm$ 18.5	83.3 $\pm$ 16.9	89.7 $\pm$ 23.0	81.6 $\pm$ 1.39	0.864	0.507	0.130	0.587	0.553
Postpartum	105 $\pm$ 16.7	76.6 $\pm$ 17.0	119 $\pm$ 18.9	78.1 $\pm$ 17.3	0.266	0.005	0.266	0.614	0.725
Phagocytic cells (%)									
<i>Staphylococcus aureus</i>									
Prepartum	49.6 $\pm$ 7.09	47.8 $\pm$ 6.91	49.8 $\pm$ 8.61	54.5 $\pm$ 8.77	0.945	0.124	0.396	0.643	0.681
Postpartum	43.0 $\pm$ 4.6	45.3 $\pm$ 4.49	42.8 $\pm$ 5.30	44.6 $\pm$ 5.09	0.978	0.743	0.068	0.913	0.956
<i>Escherichia coli</i>									
Prepartum	38.1 $\pm$ 5.75	44.3 $\pm$ 5.59	43.3 $\pm$ 7.05	53.9 $\pm$ 6.99	0.396	0.435	0.701	0.115	0.741
Postpartum	48.6 $\pm$ 3.31	48.6 $\pm$ 3.31	44.0 $\pm$ 3.85	47.3 $\pm$ 3.57	0.717	0.049	0.321	0.444	0.543
Phagocytosis median fluorescence intensity									
<i>Staphylococcus aureus</i>									
Prepartum	50.8 $\pm$ 8.42	43.6 $\pm$ 7.12	41.5 $\pm$ 9.51	30.1 $\pm$ 9.61	0.094	0.463	0.632	0.086	0.672
Postpartum	52.2 $\pm$ 5.50	41.7 $\pm$ 5.77	45.3 $\pm$ 5.93	43.8 $\pm$ 6.12	0.582	0.057	0.188	0.412	0.444
<i>Escherichia coli</i>									
Prepartum	52.7 $\pm$ 11.3	57.5 $\pm$ 9.55	34.7 $\pm$ 15.7	35.7 $\pm$ 15.6	0.550	0.340	0.341	0.296	0.894
Postpartum	66.4 $\pm$ 10.6	61.8 $\pm$ 11.06	62.2 $\pm$ 11.7	70.7 $\pm$ 12.5	0.947	0.081	0.080	0.798	0.573

¹G0 = group 0, cows did not receive whole raw soybeans (WS) throughout the prepartum period; G30, G60, and G90 = group that received diets containing WS from 30, 60, and 90 d from the expected calving date, respectively.

²Probabilities for group, time (week), group by time, linear, and quadratic effects.

Figure 8. Percentage of CD14⁺ mononuclear cells producing reactive oxygen species (ROS) when stimulated by *Staphylococcus aureus* in cows supplemented whole raw soybeans (WS) for different durations in the prepartum period. G0 (n = 7) = cows fed a diet containing 12% WS after calving; G30 (n = 6), G60 (n = 10), and G90 (n = 8) = cows fed a diet containing 12% WS beginning at 30, 60, and 90 d before the expected calving date, respectively. Error bars represent SE.

Figure 9. Percentage of CD14⁺ mononuclear cells producing reactive oxygen species (ROS) when stimulated by *Escherichia coli* in cows supplemented whole raw soybeans (WS) for different durations in the prepartum period. G0 (n = 7) = cows fed a diet containing 12% WS after calving; G30 (n = 6), G60 (n = 10), and G90 (n = 8) = cows fed a diet containing 12% WS beginning at 30, 60, and 90 d before the expected calving date, respectively. Error bars represent SE.

plementation on immune measures are related to the period of supplementation and FA profile metabolized by cows.

CONCLUSIONS

Different periods of WS supplementation, notably 60 and 30 d from the expected calving date, influenced the leukocyte population of cows by increasing the percentage of blood granulocytes, T-lymphocytes, and T-cytotoxic cells in the postpartum period. Furthermore, longer periods of WS supplementation enhanced neutrophil activity supported by an increase in ROS production against *Staph. aureus* and an augment in the capacity to phagocytize *E. coli* in the postpartum period. In general, WS supplementation starting at 90 d from the expected calving date altered the leukocyte population and improved neutrophil immune response in the postpartum period of dairy cows. Whole raw soybean supplementation during the prepartum period may be a strategy to improve the immune status of early lactating cows and potentially decrease infectious diseases in this period.

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