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## Innate immune response in neonate Holstein heifer calves fed fresh or frozen colostrum



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### ABSTRACT

The aim of this research was to evaluate the influence of maternal cells from colostrum on the development and function of the innate immune response in Holstein calves. Calves were divided into 2 groups: COL+ ( $n = 10$ ) received fresh colostrum; and COL− ( $n = 10$ ) which received frozen colostrum containing no viable cells. The calves were assessed before colostrum intake (D0), 48 h of age (D2), and weekly from D7 up to D28. Blood samples were collected for analysis of the distribution of leukocytes, cellular phenotype and in vitro granulocyte function. COL+ calves tended to have a high number of neutrophils on D7 ( $p = 0.073$ ). COL− calves took up significantly more *Escherichia coli* (measured as MFI) on D7 ( $p = 0.034$ ). Endogenous production of radicals (as percentage of cells) tended to be higher in COL− calves on D14 ( $p = 0.061$ ). The intensity of endogenous reactive oxygen species (ROS) produced by granulocytes tended to be higher in COL+ calves on D21 ( $p = 0.094$ ). Overall, ROS production (percent of cells, and MFI) induced by *Staphylococcus aureus* and *Escherichia coli* were higher in COL+ calves than COL− calves. It was our observation that COL+ calves developed an innate immune response more quickly and efficiently after natural exposure to pathogens after birth. In contrast, COL− calves mounted an innate response more slowly that yielded a persistent inflammatory response after natural exposure to these bacteria agents. This research provides evidence of an advantage to the calf of receiving fresh colostrum on the development and function of the innate immune system.

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### 1. Introduction

Newborn calves are born with a reduced capacity to mount innate and adaptive immune response relative to older cattle, and are agammaglobulinemic due to the impermeability of bovine placenta. The innate and adaptive immune response capacity develops, progressively, from birth to puberty. The development is mediated by the exposure to environmental microorganisms, making the survival of the neonate dependent on the rapid population and functional development of the innate immune system in concert with the transfer of maternal immune cells, antibody and other factors in colostrum (Kelly and Coutts, 2000; Chase et al., 2008).

Failure of passive transfer, caused by inadequate volume or poor colostrum management, are associated with high prevalence (19%) and

incidence (21%) of neonatal diarrheal disease caused by *Escherichia coli*, Rotavirus, Coronavirus and *Cryptosporidium parvum* (Windeyer et al., 2013; Meganck et al., 2014).

Colostrum contains high concentrations of nutrients, hormones, cytokines, antibodies, and many viable and compromised maternal leukocytes that appear to support the growth and maturation of the calf by enhancement of in the physiological environment. Colostrum is also important to neonatal immune protection. The role of colostrum antibodies in newborn protection has been well established. In contrast, the role of maternal leukocytes transferred with colostrum, and the cytokines contained in colostrum have not been clearly established.

Bovine colostrum contains between  $1 \times 10^6$  and  $2.5 \times 10^6$  somatic cells/mL. About 32% of these cells are viable (Liebler-Tenorio et al., 2002; Gomes et al., 2011). The population of leukocytes in first milking colostrum consists of about 13% of neutrophils, 16% of lymphocytes, 70% large cells (monocytes/macrophages, and epithelial cells), and 0.3% of eosinophils (Gomes et al., 2011). Meganck et al. (2014) evaluated the distribution of colostrum leukocytes and obtained 25% of T lymphocytes, 3% B lymphocytes, and 33% of macrophages.

Many of the cells from colostrum pass through the intestinal epithelium of calves and migrate to Peyer's patches and mesenteric lymph

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nodes (Liebler-Tenorio et al., 2002). Maternal cells were also found in the bloodstream of newborns, detecting peak 24 h after colostrum intake. (Reber et al., 2006) Then, after 36 h, maternal cells disappeared from the bloodstream, due to their migration to other tissues and secondary lymphoid organs to help provide protection to the newborn (Reber et al., 2006).

There is evidence that maternal cells, in combination with maternal antibody and cytokines, have direct antimicrobial effect, or can stimulate the endogenous innate immune response to infection caused by enteropathogenic *Escherichia coli* (Riedel-Caspari, 1993). Further, maternal cells appear to enhance the development of innate and adaptive immune function in neonatal calves (Donovan et al., 2007; Reber et al., 2008a; Reber et al., 2008b; Langel et al., 2015).

With respect to the innate immune response, only three papers have been published that suggest an enhancing effect of fresh colostrum containing maternal cells on the innate immune response in calves (Riedel-Caspari and Schmidt, 1991; Reber et al., 2008a; Stieler et al., 2012).

Colostrum is frequently preserved for future use by refrigeration for a limited period, freezing, or even storage at ambient temperatures after fermentation or chemical treatment to produce curd. Cold storage facilities are common on dairy farms and the use of frozen colostrum is on the increase. Freezing results in virtually no loss of basic nutrients (protein, energy and antibody) during storage, but requires extra handling and careful thawing for proper activity (Stieler et al., 2012). The immunoglobulin content of colostrum has been shown to be unaffected by the freezing process. However, the maternal leukocytes are lysed during freezing and thaw, and lost (Novo et al., 2014; Langel et al., 2015).

The hypothesis driving this research is that maternal cells transferred with colostrum enhance the development of the innate immune response in dairy calves relative to a natural exposure to enteric bacterial pathogens after birth. The aim of this research was to evaluate if supplying fresh colostrum, with viable cells, would demonstrate an enhanced number and function of blood granulocytes over the first month of life in calves relative to calves that received frozen colostrum.

## 2. Material and methods

### 2.1. Farm and animals

This research was approved by University of São Paulo Animal Care and Use Committee number 2934/2013. The experiment was conducted on a commercial farm located in São Paulo- Brazil between July to October of 2014.

Holstein cows were moved from the dry-cow pasture to the maternity barn 30 days before the expected delivery date of each cow. Births were monitored to prevent natural suckling. Healthy calves from eutocic deliveries were select following a screening clinical examination.

Holstein heifer calves were distributed in two groups: COL+ ( $n = 10$ ) which received whole colostrum containing immune cells from their own dams, and COL- ( $n = 10$ ) which received pooled frozen colostrum containing no viable cells from 10 donor cows.

Dams and donors were milked immediately after delivery using a portable milking machine at the maternity unit. Colostrum was collected after cleaning teats with soap and water, dipping in a 1% iodine solution, and drying with fresh paper towels. Initially, colostrum was screened using a colostrometer and a Brix refractometer. Only colostrum with  $\geq 50$  g/L and  $\geq 21\%$ , respectively were included in the study.

COL+ calves were fed up to 6 h after birth by bottle. Each calf received two litters of fresh colostrum from her own dam. A second 2 L bottle of whole colostrum was held at 4 °C for a second feeding about 6 h later, and warmed to 37 °C immediately before feeding.

Four liters of donor colostrum was stored for each COL- calf. The colostrum was split into two plastic 2 L bottles before freezing at  $-20$  °C.

Frozen colostrum was stored between 24 h and 3 months prior to thawing and use. The frozen colostrum was slowly warmed to 37 °C in a water bath. One bottle was fed to each COL- calves within 3 h of birth. A second bottle was fed about 6 h later.

Aliquots of fresh and frozen colostrum (50 mL) were taken from each bottle to allow for evaluation of the composition of what was fed. The somatic cell count (SCC) was estimated for fresh colostrum only, as freezing and thaw induced universal leukocyte lysis. The viability of cells in fresh colostrum was measured in a sample diluted 1:1 in Phosphate Buffer Saline (PBS), centrifuged at  $800 \times g$  at 4 °C for 15 min. The centrifugation allowed for separation of colostrum into three phases: fat, whey and cells. The fat and whey were removed and disposed of. The cells were washed in 20 mL of PBS, then the viability was assessed using Trypan blue stain on a hemocytometer (Novo et al., 2014).

Twenty milliliters of colostrum was used to prepare whey according to the procedure described by Klaus et al. (1969). The total protein content and quantity of albumin were assessed using an automatic biochemical analyzer (RX Daytona, Randox®). Commercial kits were used as described by the manufacturer (TP 4001, AB 3800, Randox®).

Finally, total plate counts (TPC) and total coliform counts (TCC) were performed using five 1:10 serial dilutions of each sample in sterile PBS that were each spread on Petri plates containing MacConkey's agar for TCC or Tryptic Soy Agar for TPC, as described by Godden et al. (2012). The plates were incubated for 48 h at 37 °C and the number of colonies were counted. The number of colonies was calculated as cfu/mL of the original sample using the sample dilution multiplied by the mean colony count for each sample.

COL- and COL+ calves were moved to individual pens. These calves were maintained in this housing for 28 days. Routine farm management was applied. After colostrum intake, calves received two feedings of 3 L of raw milk from the research dairy herd per day with the addition of started feed (ad libitum - Rumileite 20®, Guabi) and water ad libitum.

Despite good hygiene practices being applied, the occurrence of diarrhea observed was high in both the COL+ calves (78%) and the COL- calves (70%) during the 30 days of neonatal period. The diarrhea peaked on week + 1 and week + 2. COL- calves appeared to develop more severe disease than COL+ calves. The COL- calves had increased respiratory rate and higher temperatures during the 2nd week of life. Hematological examination showed anemia and hypoferrremia in the COL- calves during third and fourth week of life. Only one calf from COL- group was diagnosed with bronchopneumonia on week 4. Navel inflammation was observed in three COL- calves, but none in the COL+ calves (Complete description the GI disease and clinical profile can be found in Novo et al., 2017, Effect of maternal cells transferred with colostrum on the health in neonatal calves. Accepted for publication in Research in Veterinary Science).

### 2.2. Blood samples

Calves were assessed before colostrum intake (D0); at 24–48 h after birth (D2); on day 7 (D7); 14 (D14); 21 (D21) and 28 after birth (D28). Four milliliters of whole blood was collected in vacutainer tubes containing 7.4 mg of EDTA and sodium heparin by external jugular puncture.

### 2.3. Blood cell profile

Absolute total leukocyte count (WBC) was obtained using an automatic cell counter (ABC Vet, ABX®). Leukocyte differential (lymphocyte, monocytes, basophils, eosinophils and granulocyte-neutrophils per 100 WBC) was performed using microscopic cells morphology of stained cells on standard slides at  $1000\times$  magnification.

## 2.4. Phenotyping

The phenotype of leukocytes was evaluated using bovine-specific monoclonal antibodies for cell surface receptors associated with differentiation and innated cell function. Antibodies concentrations were optimized using a step-wise dilution series. The highest dilution yielding saturation (peak) signal intensity was used for each antibody. The mouse monoclonal antibodies utilized were CH138 (an IgM isotype for neutrophil identification – catalog number CH138A) and CD62L (an IgG1 isotype for identification of L-selectin-catalog number CC32) from (Washington State University).

One hundred microliters of whole blood was distributed into individual flow cytometer tubes (12 × 75 mm polystyrene, Falcon, BD Biosciences, San Jose, CA, USA), for each staining test. The red blood cells were lysed using 900 µL of FACSlyse solution according to the recommendation of manufacturer (BD Biosciences, San Jose, CA, USA). The samples were centrifuged at 4 °C, 290 ×g for 8 min. The supernatant was discarded. The cells were suspended in 1 mL of PBS and washed twice by centrifugation as above.

Cells were co-incubated with 100 µL of each primary antibody for 30 min at 4 °C. Cells were washed three times with PBS as described above, then incubated with 100 µL of isotype-specific secondary antibody conjugate for 30 min at 4 °C. The IgM specific detection antibody was conjugated with APC (Allophycocyanin) (INVITROGEN, U.S.A.) and IgG1 specific detection antibody was conjugated with rPE (Phycoerythrin) (BENCTON DICKINSON, U.S.A.). Finally, cells were washed twice as described above, and suspended in 300 µL of cold sterile PBS.

The compensation settings used to minimize color cross-talk between the detection antibody pairs was established by the use of the CH138 and CD62L single color saturation stained samples. Compensation was set in FL4 against FL2 for CH138 and in FL2 against FL4 for CD62L.

Samples were analyzed using a FACS Calibur cytometer (BD Biosciences, San Jose, CA, USA). Gate windows in forward angle and 90° light scatter were established to allow for inclusion of the granulocyte population and exclusion of mononuclear cells based on size and granularity.

Flowjo analysis software (Treestar Inc., San Carlos, USA) was used to parse and analyze the populations within the samples. At least 10,000 cells were analyzed per sample. The analysis was based on assignment of the polymorphonuclear region defined in the forward scatter and side scatter two parameter profile. This population was analyzed for the expression of specific fluorescence for CH138 and CD62L cells.

## 2.5. Phagocytosis and assessment of radical oxygen species

Assays for the assessment of the intracellular production of ROS and for phagocytosis by blood leukocytes were performed using flow cytometry. The blood cells were incubated with *Staphylococcus aureus* or *Escherichia coli* stained with propidium iodide (PI; catalog no. P4170, Sigma-Aldrich, St. Louis, MO). The labeling of bacteria was performed as described by Hasui et al. (1989), with subsequent modifications (Batista et al., 2015). The assays were performed in tubes made of polypropylene that are suitable for flow cytometry. To perform the assay, 100 µL of heparinized blood containing approximately  $2 \times 10^5$  cells was added to each flow cytometry tube. Then, 200 µL of 0.3 mM 2,7 dichlorofluorescein diacetate (DCFH-DA an ROS indicator, Sigma Aldrich, St. Louis, EUA, n° cat. D6883) was added to the assay medium. To perform the phagocytosis test, 50 µL of PI labeled *S. aureus* and *E. coli* were added to the appropriate tubes. The mixture was incubated at 37 °C for 30 min, kept in suspension using a tube rotator. The assay was stopped by addition of 2 mL of cold 3 mM EDTA per tube. Red blood cells were lysed by adding 1 mL of distilled water to the pellet. The tubes were vortexed for 15 s and 1 mL of PBS 2× concentrate was immediately added to each tube. The samples were diluted with 3 mL of PBS. The tubes were centrifuged for 5 min at 4 °C and 783 ×g. The

supernatant was discarded. The pellet was suspended in 1 mL PBS. The tubes were centrifuged again under the same conditions. Finally, the pellet was suspended in 300 µL of PBS for flow assessment. The assessment was performed using a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Diego, CA). For each sample, 20,000 events were acquired in the granulocyte forward angle and 90 light scatter gate using the CellQuest® software (Becton Dickinson Immunocytometry Systems, San Diego CA).

The recorded data were analyzed using Flow Jo software, version 7.6.1 for Windows (Tree Star Inc., Ashland, OR). The granulocyte gated was established in samples containing cells only. The gate was copied to the other assays from the same animal for consistency. The production of endogenous ROS was determined as the signal in FL1 for unstimulated cells in the presence of the ROS substrate. Bacterial phagocytosis was assessed in FL2 for samples containing only cells and bacteria (*Staphylococcus aureus* or *Escherichia coli*). Compensation was set for DCFH-DA for FL1 against FL2 and bacteria for FL2 against FL1. Finally, after the thresholds based on the untreated controls were established for ROS and phagocytic activity. These were used for the analysis of samples containing DCFH-DA and bacteria. Concomitant measure of phagocytosis and ROS production by the same cells were analyzed.

## 2.6. Statistical analysis

Statistical analyses were performed using the software Statistical Package for the Social Sciences (SPSS) version 19.0 (IBM Corporation, Armonk, NY).

The normality of the data was tested using the Shapiro-Wilk test, and if the assumptions of normality were not satisfied, these variables were transformed logarithmically ( $\log_{10}$ ). Data were recorded as mean and standard deviation for each variable set. Student t-test for independent samples was performed for comparison between groups (COL+ and COL−) at individual assessment points, and Two-way analysis of variance (ANOVA) coupled with Bonferroni post-hoc test when comparison over time was assessed within the each experimental group.

It was impossible to transform total solids, SCC and TCC from colostrum to serve as parametric data. Therefore, these parameters were analyzed between COL+ and COL− using Mann-Whitney U test.

All analyzes were considered statistically significant at  $p < 0.05$  and to show a statistical tendency when  $p > 0.05$  and  $p < 0.1$ .

## 3. Results

The composition of fresh (COL+) and frozen (COL−) colostrum were similar with respect to IgG, total solids, total protein, albumin, total plate count (TPC), and total coliform count (TCC) (Table 1). The median SCC was  $1.25 \times 10^6$  ( $0.25\text{--}5.90 \times 10^6$  cells/mL). A significant difference between groups was observed only in cellular viability (COL+ =  $24 \pm 8\%$ ; COL− = 0%,  $p = 0.003$ ) (Table 1).

The results for absolute total WBC count and the percentage of granulocytes and neutrophils, and the phenotype assessments are shown in Fig. 1A–C. Calves of both groups showed maximum peak of WBC on D7 (Fig. 1A). The absolute number of total WBC declined at subsequent observations. There was no significant difference between treatment groups during the study. However, a significant reduction was observed when comparing D0 and D28 in COL, P calves (Fig. 1A,  $p = 0.031$ ).

The percent of neutrophil measured using classical differential morphology and staining (Ne) tended to be different between groups on D7, with a larger fraction of Ne observed for COL+ calves (Fig. 1B,  $p = 0.073$ ).

Overall, the % of Ne in both treatment groups declined over the course of the study, with each having a peak of about 70% around the time of birth and a decline to a more adult-like %NE of about 40% by 28 days of age. A two-way ANOVA analysis demonstrated a significant

**Table 1**  
Composition of fresh and frozen colostrum fed to COL+ and COL– calves.

| Parameters                            | Variables | Fresh colostrum (COL+) | Frozen colostrum (COL–) | Significance |
|---------------------------------------|-----------|------------------------|-------------------------|--------------|
| Colostrometer (g/mL)                  | Mean      | $9.4 \times 10^3$      | $10.3 \times 10^3$      | 0.225        |
|                                       | SD        | $\pm 1.8 \times 10^3$  | $\pm 1.7 \times 10^3$   |              |
| Total solids (%)                      | Median    | 29                     | 29                      | 0.588        |
|                                       | Minimum   | 23                     | 23                      |              |
|                                       | Maximum   | 32                     | 32                      |              |
|                                       | Mean      | 10                     | 10                      |              |
| Total protein (mg/mL)                 | SD        | $\pm 2$                | $\pm 2$                 | 0.749        |
|                                       | Mean      | 1.3                    | 1.2                     |              |
| Albumin (mg/mL)                       | SD        | $\pm 0.4$              | $\pm 0.2$               | 0.720        |
|                                       | Mean      | $9.5 \times 10^5$      | $39.4 \times 10^5$      |              |
| TPC (cfu/mL)                          | SD        | $\pm 9.3 \times 10^5$  | $\pm 51.4 \times 10^5$  | 0.124        |
|                                       | Mean      | $6.3 \times 10^4$      | $9.9 \times 10^4$       |              |
| TCC (cfu/mL)                          | Median    | $0.6 \times 10^4$      | $0.2 \times 10^4$       | 0.427        |
|                                       | Minimum   | $18.1 \times 10^4$     | $221 \times 10^4$       |              |
|                                       | Maximum   | –                      | –                       |              |
| SCC ( $\times 10^6$ /mL) <sup>a</sup> | Median    | 1.25                   | –                       | –            |
|                                       | Minimum   | 0.25                   | –                       |              |
|                                       | Maximum   | 5.90                   | –                       |              |
| Viability (%)                         | Mean      | 24                     | 0                       | 0.003        |
|                                       | SD        | $\pm 8$                | 0                       |              |

SD: standard deviation; TPC: total plate count; TCC: total coliform count; SCC: somatic cell count.

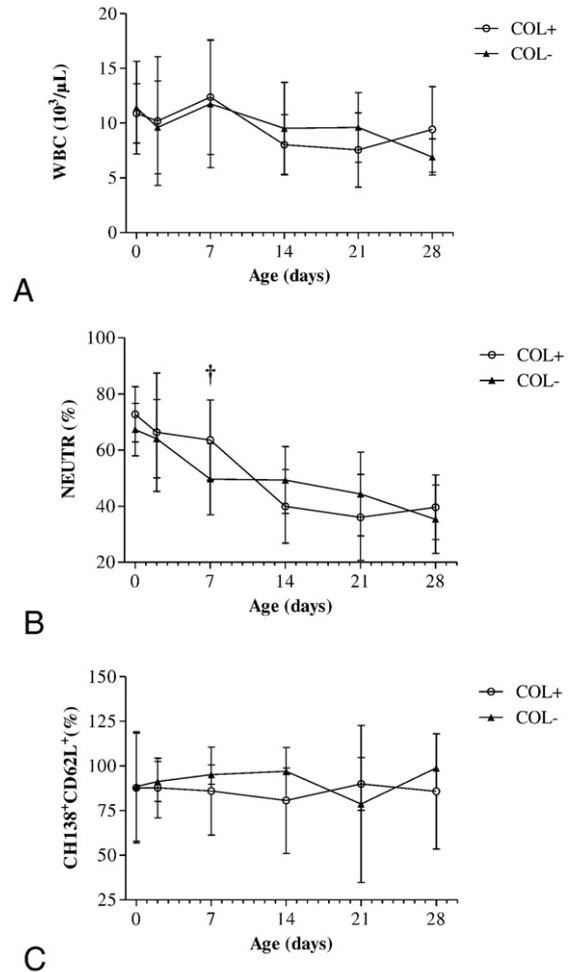
<sup>a</sup> SCC was performed only in the fresh colostrum due to the lysis of somatic cells during freezing process.

difference in the relative number of Ne (%) when D0 was compared to D14, D21 or D28 (Fig. 1B,  $p \leq 0.008$ ), D2 to D21 (Fig. 1B,  $p = 0.013$ ), D7 to D1 or D28 (Fig. 1B,  $p \leq 0.036$ ) for COL+ calves, and when D0 was compared to D21 or D28 (Fig. 1B,  $p \leq 0.032$ ) for COL– calves using the Bonferroni post test to confirm the differential time points.

The fraction (%) of CH138<sup>+</sup>CD62L<sup>+</sup> appeared to increase slightly in COL– calves from over the course of the study, with the exception of the D21 measurement. The drop observed at D21 was not statistically significant relative to any of the other measurement of COL– calves. COL+ calves showed no change in CH138CD62L+ expression during the study (Fig. 1C).

The results of the phagocytosis assays are shown in Fig. 2. The percent phagocytosis (%) of *Staphylococcus aureus* and *Escherichia coli* were not different between COL+ and COL– calves at any time during the study. (Fig. 2A–B). There appeared to be an overall increase in the % of phagocytic granulocytes during the course of the study period in both treatment groups. A two-way ANOVA analysis demonstrated a significant difference in the percent of cells taking up *Staphylococcus aureus* in the COL+ calves when D0 was compared with D2 or D28 (Fig. 2A,  $p \leq 0.012$ ) and in COL– calves when D0 was compared with D2, D7 or D21 (Fig. 2A,  $p \leq 0.020$ ) by application of the Bonferroni post test. A time dependent tendency was also observed in COL– calves when D2 was compared with D21 (Fig. 2A,  $p = 0.095$ ) in COL– group. A tendency toward significance was detected for the percent of cells taking up *Escherichia coli* in the COL+ group when D0 was compared with D2 (Fig. 2B,  $p = 0.054$ ), and in COL– when D0 was compared with D2 and D7 (Fig. 2B,  $p \leq 0.055$ ). A significant difference also was observed in COL– when D2 as compared with D21 (Fig. 2B,  $p = 0.045$ ).

The average phagocytic avidity (expressed as mean fluorescence intensity, MFI) for *Staphylococcus aureus* and *Escherichia coli* phagocytosis were not significantly different between treatment groups. The only statistical significant differences observed were for COL– calves on D7 for *Escherichia coli* phagocytosis relative to COL+ calves (Fig. 2D,  $p = 0.034$ ). Overall, MFI of phagocytosis increased for both treatment groups over the course of the study. A two-way ANOVA analysis demonstrated significant differences in *Staphylococcus aureus* phagocytosis MFI in the COL+ calves when D0 was compared with D2, D7 or D28 (Fig. 2C,  $p \leq 0.010$ ) and when D2 was compared with D21 (Fig. 2C,  $p = 0.041$ ) using the



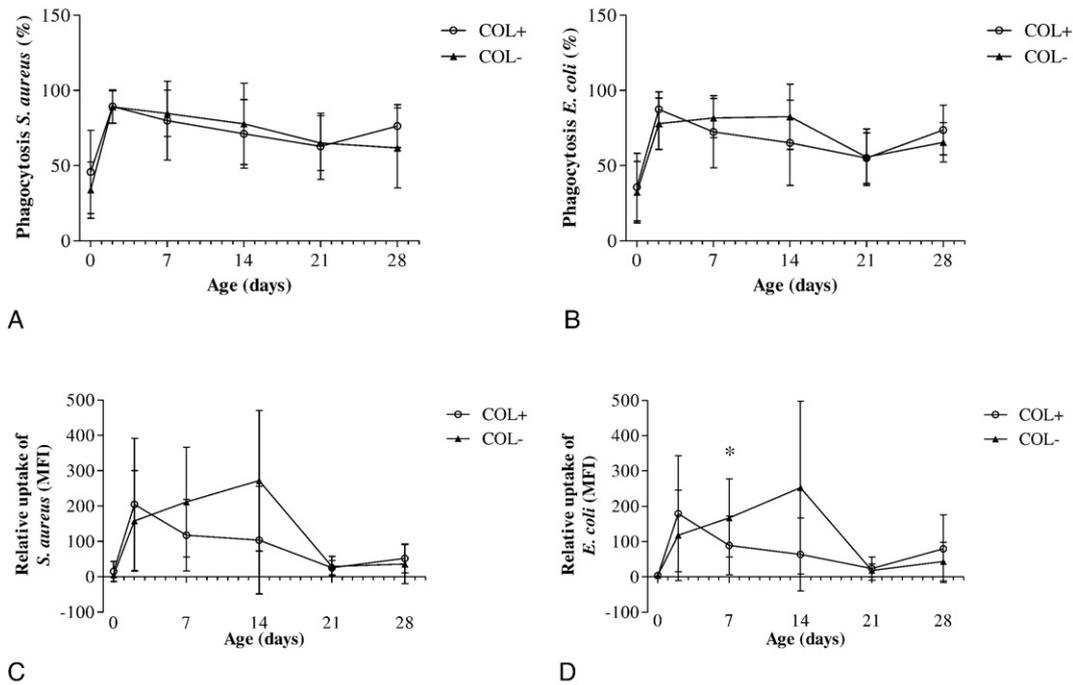
**Fig. 1.** Total white blood cells (WBC), neutrophils (NEUTR), and the percent of CH138 (granulocytes) expressing L selectin (CD62L) in Holstein heifers fed whole fresh (COL+) or frozen colostrum (COL–) during the first month of life. Legend: † indicates a tendency toward significance between the two groups ( $p < 0.1$ ) at the indicated sampling time.

Bonferroni post-test assessments. Similarly, COL– calves showed a significant difference in phagocytic MFI by two-way ANOVA assessment when D0 was compared with D2, D7, D14, D21 or D28 (Fig. 2C,  $p \leq 0.05$ ). A time dependent tendency was also observed in both groups for *Escherichia coli* phagocytosis MFI, observing higher values on D0 than D2, D7, D21 and D28 (Fig. 2D,  $p \leq 0.024$ ). The phagocytic MFI value observed on D21 also was higher than D28 in COL– group ( $p = 0.004$ ).

The percent of cells with endogenous ROS activity is shown in Fig. 3A. The profile of ROS production by cells from COL+ and COL– calves showed the same pattern and almost universal detection of endogenous ROS production by all cells. A tendency for a difference between treatments on D14 was observed, but appears to be a weak finding (Fig. 3A,  $p = 0.061$ ).

COL+ and COL– calves showed a common level of endogenous cellular ROS production (MFI) over the course of the study. On D21, a tendency toward an enhanced level of cellular endogenous ROS production was observed in COL+ calves, but this was a single observation (Fig. 3B,  $p = 0.094$ ).

COL+ and COL– calves demonstrated a similar pattern in the percent of cells producing ROS after *Staphylococcus aureus* stimulation over the whole course of the study. An isolated significant difference indicated that the COL+ calves had higher percentage of ROS producing cells than the COL– calves on D7 (Fig. 3C,  $p = 0.008$ ). The cellular



**Fig. 2.** The percentage of granulocytes engulfing bacteria (%) and the mean intensity of fluorescence (MFI) of granulocytes by COL+ COL- calves. Legend: \* indicates difference between groups and † indicates trend between groups in that moment ( $p > 0,05$  and  $< 0,1$ ).

intensity of ROS production (MFI) after *Staphylococcus aureus* stimulation progressively increased in calves under both treatments from birth (at about 500 MFI units) to D28 (about 950 MFI units). There were no significant differences observed between groups.

The percent of cells producing ROS after *Escherichia coli* stimulation was relatively consistent during the study. It was observed that the COL+ calves had a higher percent of ROS producing cells after *Escherichia coli* stimulation on D28 than COL- calves (Fig. 3E,  $p = 0.040$ ). The cellular intensity of ROS production (MFI) after *Escherichia coli* stimulation did not change significantly in either treatment group over the course of the study. While a slightly higher mean MFI for ROS production was recorded on D21 in the COL+ calves than COL- calves, it was not significant (and is probably not relevant).

#### 4. Discussion

This research examined the role of whole maternal colostrum containing live maternal cells on the development and function of the innate immune response in neonatal calves. The colostrum treatments were essentially similar, except for the presence of live maternal cells in the fresh colostrum. The fresh and frozen colostrum had similar IgG concentrations, total solids, total protein, albumin, total bacteria (per TPC) and total coliforms (per TCC). On examination, we did not find any viable cells after the freezing-thaw process of the pool colostrum from the same farm used in this study. Previous papers using a similar design did not describe the composition of colostrum comprehensively. Past characterization seems to have been limited to the presence or absence of viable cells (Reber et al., 2006, 2008a, 2008b, Donovan et al., 2007, Langel et al., 2015). Reber et al. (2006), Donovan et al. (2007) and Langel et al. (2015) also found absence of viable cells after freezing process.

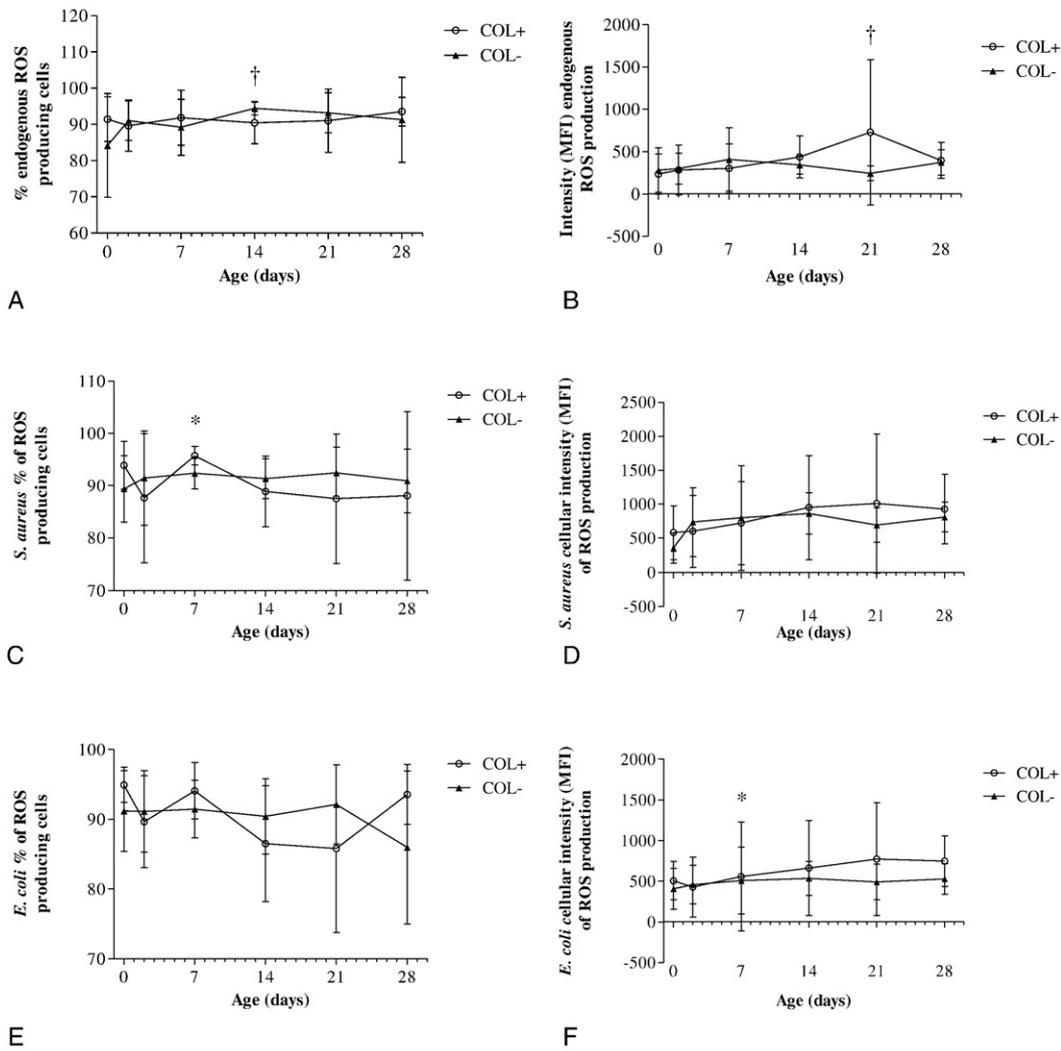
At birth, an immunologically immature and naïve calf is naturally confronted with a massive microbial exposure. Neonatal calves are dependent on the function of their innate immune activities directed by maternal antibody, cells and protein immune modulating factors transferred with colostrum. These interactions modulate the microbial

colonization of the intestine and the lungs of calves, and provide targeting and priming activation of innate responses in the neonate (Griebel, 2009; Liang et al., 2015).

Total absolute WBC counts of calves under both treatments showed slight variation, and appeared to decline, but not significantly, over the course of this trial. We did observe that the fraction (%) of Ne tended to be higher in COL+ calves than COL- calves on D7. The percentage of Ne dropped in COL+ calves over the rest of the study, but COL- calves appeared to hold a percentage of neutrophils more similar to that at birth. Neither observation proved to be significant. The difference we observed, with respect to the differential % of NE, might be related to greater efficiency of neutrophils from COL+ calves to migrating from blood to gut after exposure to diarrheal pathogens on D7 as previously described (Zwahlen and Roth, 1990).

The fraction (%) of CH138<sup>+</sup>CD62L<sup>+</sup> increased slightly from D0 up to D28 in COL- calves, with the exception of the D21 measurement. The proportion of these cells in COL+ group was constant over the course of the study. There is some previous evidence that calves deprived of maternal cells from colostrum have an inflammatory phenotype when colonized with bacteria with higher expression of CD11a, CD11c, CD43 and CD62L in mononuclear cells (Reber et al., 2006, 2008a, 2008b). The CD11a and CD43 are important receptors related to cell migration from the gut across the epithelium to the bloodstream, and CD62L is related to the signaling of leukocytes so they reduce the flow velocity and to adhere to the blood vessel endothelial cells to facilitate the adhesion and migration (Reber et al., 2006).

The percent of cells taking up *Staphylococcus aureus* and *Escherichia coli* by phagocytosis were similar between the two groups. However, the relative number of bacteria taken up by each granulocyte (expressed as MFI) was higher in COL- calves on D7 than the COL+ calves (Fig. 2A and B). This fact points out that granulocytes from COL- calves took up a larger number of bacteria into the cytoplasm. Stieler et al. (2012) found a trend toward a higher fraction of phagocytic cells taking up *Escherichia coli* in calves fed with frozen than fresh colostrum compared to frozen colostrum on D2 after birth. However, the number of bacteria per cell was



**Fig. 3.** The responding fraction (%) and the mean fluorescence intensity (MFI) of ROS production by granulocytes from COL + and COL – calves. Legend: \* indicates a significant difference ( $p > 0,05$ ) between groups and † indicates a tendency toward significance between the two groups ( $p < 0,1$ ) between groups at the indicated sampling time.

similar between the treatment groups. Our experiment agrees with Stielier et al. (2012) that the percent of phagocytic cells was higher in calves fed frozen colostrum. Stielier et al. (2012) believed that cytokines and/or transfer factor (and products of cells destroyed by freezing and thawing colostrum) were likely to be responsible for the difference observed between groups. In our view, the difference between COL + and COL – calves is mediated by the reduced capacity of COL – calves to clear bacteria after birth, in part due to their reduced capacity to reach the target tissue, resulting in a prolonged inflammatory response.

The profile presented for the endogenous production of ROS by granulocytic leukocytes from both treatment groups was similar. The fraction of cells producing endogenous ROS tended to be higher in the COL – calves on D14 (Fig. 3A). Further, COL + calves tended to produce more ROS per cell on D21 than COL – calves (Fig. 3B). The fraction of cells producing ROS after *Staphylococcus aureus* or *Escherichia coli* stimulation was essentially unchanged in both groups over the course of the study when considering the variation in measurements overall (Fig. 3C and E). The production of ROS per cells after stimulating with *Staphylococcus aureus* and *E. coli* was also very similar for calves in both groups. The cellular production of ROS was lower at birth for calves in both treatment groups on D0 after *Staphylococcus aureus* stimulation than during the rest of the study in each group (Fig. 3D). Cellular ROS production after

*Escherichia coli* stimulation did not really change during the study (Fig. 3F).

Riedel-Caspari and Schmidt (1991) reported that bacterial stress after birth of colostrum cell deprived calves induced an increased number of circulating neutrophils on D2 that showed an association with a higher index of *Streptococcus agalactiae* phagocytosis index for colostrum cell deprived calves on D5 relative to those receiving whole colostrum. She found that production of ROS (measured by NBT reduction) was similar between the two treatment groups.

The percent of phagocytic granulocytes was essentially similar for both groups over the course of the study. We observed great variation in the ROS production for both COL – and COL + calves over the course of the study (Fig. 3E and F). It also appeared to us that COL + calves could produce more ROS per cells than COL – calves (Fig. 3E and F).

Data generated by this study may help to explain the differential clinical picture observed for COL + and COL – calves relative to GI bacterial infection and disease. The frequency of diarrhea observed was high for calves in both treatment groups in this study. However COL – calves presented a more severe total set of clinical signs and stronger impact of disease during the study. The COL – calves had more reported fever during the 2nd week of life followed by a number of cases of anemia and hypoferrremia during the third and fourth week of the study. Moreover, only COL – calves were diagnosed with bronchopneumonia

or navel inflammation during the study (Complete description the GI disease and clinical profile can be found in [Novo et al., 2017](#), Effect of maternal cells transferred with colostrum on the health in neonatal calves. Accepted for publication in Research in Veterinary Science).

Overall, the innate immune system of COL+ neonate calves appeared to have some enhanced capacity to control diarrhea pathogens after natural exposure to microbes and to have slightly enhance innate immune development.

## 5. Conclusions

Calves fed whole fresh colostrum (COL+) appeared to have somewhat enhanced neonatal innate immune response against natural exposure to diarrhea causing microorganisms based on the differential disease symptoms observed as part of the parent study generating the immune data presented here. In contrast, calves fed frozen, viable cell free colostrum had more severe and protracted clinical symptoms after natural bacterial exposure. The data with respect to the development and function of neonatal innate immunity generated by this study offers some indications that feeding viable maternal cells in colostrum may slightly enhance innate immune development. These findings may help to explain the generally poorer clinical course of COL– calves after GI pathogen exposure. This research indicates a possible advantage of feeding fresh colostrum with live maternal cells on the development and function of the innate immune system and in offering a benefit to the health of neonate calves.

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