Research paper

Milk supplemented with immune colostrum: Protection against rotavirus diarrhea and modulatory effect on the systemic and mucosal antibody responses in calves experimentally challenged with bovine rotavirus

V. Parreño a,*, G. Marcoppido a, C. Vega a, L. Garaicoechea a, D. Rodriguez a, L. Saif b, F. Fernández a

a Instituto de Virología, CICV y A - INTA, CC 25, 1712 Castelar, Bs. As., Argentina
b Food Animal Health Research Program (FAHRP), The Ohio Agricultural Research and Development Center, The Ohio State University, USA

1. Introduction

Group A bovine rotavirus (BRV) is the major cause of neonatal calf diarrhea worldwide. As a preventative strategy, we evaluated the protection and immunomodulation in two groups of BRV-inoculated calves. All calves received control colostrum (CC; VN = 65,536; IgG1 = 16,384) prior to gut closure followed by the milk supplemented with immune colostrum (VN = 1,048,576; IgG1 = 262,144), twice a day, for 14 days. Calves received milk supplemented with 0.8% immune colostrum [(Gp 1) VN = 16,384; IgG1 = 4096] or milk supplemented with 0.4% immune colostrum [(Gp 2) VN = 1024; IgG1 = 1024]. Calves receiving CC or colostrum deprived calves (CD) fed antibody (Ab) free milk served as controls (Gp 3 and 4). Calves were inoculated with virulent BRV IND at 2 days of age. Group 1 calves (milk IgG1 4096) showed 80% protection against BRV diarrhea and significantly reduced virus shedding. At 21 post-inoculation days (PID), the antibody secreting cell (ASC) responses of Gp 1 calves were limited mainly to duodenal and jejunal lamina propria (LP) with limited or no responses in systemic sites (spleen and PBL) and mesenteric lymph nodes. The profile of serum and fecal Ab responses as well as the ASC responses was also modulated by the presence of passive IgG1 Abs and probably other colostrum components, toward higher titers of IgA Ab in serum and feces and a greater number of IgA ASC in the proximal intestine, reflecting positive modulation by colostrum toward this isotype associated with optimal protection of the intestinal mucosa. After challenge, at PID 21, all calves in Gp 1 and 2 were fully protected against diarrhea and only 1 of 5 calves in Gp 1 shed virus asymptomatically, indicating that the passive Ab treatment for 14 days was effective in protecting most of the animals after a first and a second virus exposure. The final outcome was a positive modulation of the mucosal immune responses and a high protection rate against diarrhea and virus shedding during the period of peak susceptibility to BRV infection.

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* Corresponding author. Tel.: +54 11 4481 3006; fax: +54 11 4481 3006.
E-mail addresses: vparreno@cnia.inta.gov.ar, vivipar@fibertel.com.ar (V. Parreño).

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the presence of passively transferred antibodies (Abs) (IgG1 and IgA) in the gut lumen derived from unabsorbed colostrum and milk plays an important role in protection against BRV infection and disease (Fernandez et al., 1998; Saif et al., 1983, 1987). High titers of passive circulating Abs, derived from colostrum intake, play a complementary role in protection against BRV diarrhea, since IgG1 is also transferred from serum to the intestine of neonatal calves (Besser et al., 1988a,b; Saif et al., 1983, 1987). Serum Abs are essential in dairy calves which are separated from their dams after colostrum intake and subsequently fed with milk replacers lacking Abs. However, we previously demonstrated that BRV-specific circulating Abs derived from colostrum intake suppress the development of the normal ASC responses of neonatal calves, in a dose-dependent manner, at both, systemic and mucosal levels (Parreño et al., 2004). The protocol for animal management and euthanasia met the requirements of The Institutional Animal Care Committee (IACC) of the Veterinary College (University of Buenos Aires) and OLAW, NIH. All calves received 1 l of the pooled control colostrum (CC) within the first 6 h of life. Calves were randomly assigned to one of the following feeding groups: Gp 1: CC + 0.8% immune colostrum supplemented milk with a final BRV IgG1 Ab titer of 4096 (n = 10); Gp 2: CC + 0.4% immune colostrum supplemented milk with a final BRV IgG1 Ab titer of 1024 (n = 8) (Table 1). Milk used in the experiment was commercial sterilized bovine milk for human consumption (Mastellone Hnos., Bs. As., Argentina). In addition, two groups of calves from trials previously conducted in our laboratory were considered as control groups and received one dose of CC (Gp 3; n = 8) or were CD calves (Gp4; n = 5) and thereafter were fed with milk without BRV Abs (Parreño et al., 2004). After the initial intake of control colostrum, calves from Gp 1 and 2 were fed twice a day with 2 l of each supplemented milk, for 14 days. From day 15 onwards, all calves were fed with milk without supplemental (immune colostrum) Abs. All animals were orally inoculated with 103.35 FFU of virulent IND (P[5]G6) BRV between the third and fourth feeding (approximately 36 h after colostrum intake; 0 post-inoculation day – PID). This viral dose was previously confirmed to cause diarrhea and virus shedding in 100% of inoculated CD calves. Half of the calves in Gp 1 and 2 and all the calves from Gp 3 and 4 were euthanized at 21 PID to study the primary Ab responses to BRV infection, while the calves from Gp 1 and 2 that remained alive were further challenged with the same dose of virus at 21 PID and euthanized at 35 PID to evaluate protection and the Ab responses to a second exposure to the virus.

2.1.2. Clinical observations and sample collection
Calves were examined daily for the development of diarrhea and virus shedding after BRV inoculation and challenge. To estimate the severity of the diarrhea, fecal consistency was scored as follows: 0, normal; 1, pasty; 2, semi-liquid; 3, liquid. A score equal to or greater than 2 was considered diarrhea. The presence of elevated rectal temperatures was recorded. Prior and after BRV inoculation and challenge, fecal samples were collected daily to assess virus shedding. Serum samples were collected before the colostrum feeding (within 2 h after birth), at inoculation, and then weekly (7, 14, 21, 28, 35 PID). Serum

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume of IC added to 2 l of Ab-free milk</th>
<th>Isotype ELISA Ab titer against BRV IND P[5]G6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>VN&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control colostrum (CC)</td>
<td>–</td>
<td>65,536</td>
</tr>
<tr>
<td>Immune colostrum (IC)</td>
<td>–</td>
<td>1,048,576</td>
</tr>
<tr>
<td>Gp 1 Colostrum supplemented milk</td>
<td>32 ml&lt;sup&gt;c&lt;/sup&gt; (0.8%)</td>
<td>16,384</td>
</tr>
<tr>
<td>Gp 2 Colostrum supplemented milk</td>
<td>8 ml&lt;sup&gt;c&lt;/sup&gt; (0.4%)</td>
<td>1024</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by fluorescence focus forming unit reduction assay.
<sup>b</sup> Isotype antibody titers were determined by ELISA.
<sup>c</sup> Volume (percentage) of immune colostrum (IC) added to 2 l of commercial milk to obtain the indicated final IgG<sub>1</sub> Ab titer against RV.

Table 1
Virus neutralization (VN) and ELISA isotype antibody titers to BRV IND in the colostrum supplemented milks and the colostrum pools from control (non-immunized cows) and immunized cows used to feed the experimental calves.

2. Materials and methods

2.1. Experimental design

2.1.1. Colostrum feeding and calf inoculation
Eighteen newborn Holstein male calves procured from the same dairy farm were removed from their mothers at birth prior to suckling (colostrum deprived, CD) and transported to isolation facilities within the first 4 h of life. The animals were housed in individual isolation rooms under a strict management protocol as previously described (Parreño et al., 2004). The protocol for animal management and euthanasia met the requirements of The Institutional Animal Care Committee (IACC) of the Veterinary College (University of Buenos Aires) and OLAW, NIH. All calves received 1 l of the pooled control colostrum (CC) within the first 6 h of life. Calves were randomly assigned to one of the following feeding groups: Gp 1: CC + 0.8% immune colostrum supplemented milk with a final BRV IgG1 Ab titer of 4096 (n = 10); Gp 2: CC + 0.4% immune colostrum supplemented milk with a final BRV IgG1 Ab titer of 1024 (n = 8) (Table 1). Milk used in the experiment was commercial sterilized bovine milk for human consumption (Mastellone Hnos., Bs. As., Argentina). In addition, two groups of calves from trials previously conducted in our laboratory were considered as control groups and received one dose of CC (Gp 3; n = 8) or were CD calves (Gp4; n = 5) and thereafter were fed with milk without BRV Abs (Parreño et al., 2004). After the initial intake of control colostrum, calves from Gp 1 and 2 were fed twice a day with 2 l of each supplemented milk, for 14 days. From day 15 onwards, all calves were fed with milk without supplemental (immune colostrum) Abs. All animals were orally inoculated with 10³.35 FFU of virulent IND (P[5]G6) BRV between the third and fourth feeding (approximately 36 h after colostrum intake; 0 post-inoculation day – PID). This viral dose was previously confirmed to cause diarrhea and virus shedding in 100% of inoculated CD calves. Half of the calves in Gp 1 and 2 and all the calves from Gp 3 and 4 were euthanized at 21 PID to study the primary Ab responses to BRV infection, while the calves from Gp 1 and 2 that remained alive were further challenged with the same dose of virus at 21 PID and euthanized at 35 PID to evaluate protection and the Ab responses to a second exposure to the virus.

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Abs against BRV were detected by ELISA and virus neutralization (VN). Additionally, the presence of coproantibodies was evaluated by ELISA. Peripheral blood lymphocytes (PBL) were sampled at 0 PID and then weekly, in order to assess ASC responses by ELISPOT assay. At 21 or 35 PID, the animals were euthanized and the number of BRV ASC was also quantified by ELISPOT in the following mucosal-associated lymphoid tissues (MALT): Duodenum, Jejunum and ileum lamina propria (LP), Jejunum and ileum Peyer’s patches (PP), Jejunum and cecal mesenteric lymph nodes (MLN), and in systemic lymphoid tissues (spleen and PBL). Large (LIC) and small (SIC) intestinal contents from all the calves were collected at necropsy for Ab detection by ELISA (Parreño et al., 2004).

2.1.3. Virus

Virulent IND BRV (P[5]G6) inoculum used for calf experimental infection consisted of the intestinal contents of a CD calf that had been orally inoculated with a fecal suspension containing virulent BRV, previously obtained from a gnotobiotic calf (kindly supplied by Dr L. Saif, The Ohio State University, USA). The pool had an infectious titer of 10^{6.3} FFU/ml. The cell–culture-adapted IND BRV was propagated in monkey kidney cells (MA-104) for its use in ELISPOT, ELISA and VN assays (Parreño et al., 2004).

2.1.4. Immune and control colostrum pools

The immune and control colostrum pools used in this study were derived from vaccinated and non-vaccinated dairy cows, respectively, as previously described and the collected colostrum pools were prepared in our laboratory, and stored at –20°C until use (Parreño et al., 2004).

2.1.5. Pilot dose–response study and preparation of supplemented milk

To establish the BRV Ab titer needed in the milk diet of neonatal calves to protect them against diarrhea and virus shedding after BRV experimental infection, supplemented milks were prepared by the addition of increasing volumes of liquid immune colostrum (from previously frozen colostrum pools). Four sets of milks containing final BRV IgG1 titers of 1024, 2048, 4096 and 16,384 were initially tested in a pilot study. Most calves fed with milk with a final IgG1 Ab titer of 4096 or higher were protected against diarrhea and virus shedding (data not shown). Hence, for the present study, and specially to address potential interference effects of local passive Ab treatments, we tested the supplemented milk that induced no protection (BRV IgG1 final Ab titer of 1024) and the milk supplemented with the lowest amount of colostrum able to induce high protection rates (final IgG1, Ab titer of: 4096). The BRV Ab titers of each supplemented milk, colostrum and the amount of immune colostrum used for its preparation are detailed in Table 1.

2.1.6. Rotavirus antigen detection

Rotavirus shedding was detected in fecal samples using an antigen capture ELISA as previously described (Cornaglia et al., 1989).

2.1.7. Cell culture immunofluorescence assay (CCIF)

Virus infectious titer was assessed by a cell culture immunofluorescence (CCIF) assay (Hodgins et al., 1999). Briefly, calf fecal samples were diluted 1:4 in MEM and clarified by centrifugation for 20 min at 1500 × g. Four-fold serial dilutions of each sample were assayed in duplicate on 96-well plates containing MA-104 cell monolayers. After 48 h incubation at 37°C, the cells were fixed with 70% acetone. Fluorescent foci were visualized using a FITC-labeled hyperimmune bovine antisera to IND BRV, and fluorescent foci-forming cells were counted using a fluorescence microscope. Titers were expressed as the number of fluorescent focus forming units per millilitres (FFU/ml).

2.1.8. Isotype-specific antibody ELISA

The IgM, IgA, IgG1 and IgG2 Ab titers to IND BRV were quantitated in colostrum pools, calf sera, feces, LIC and SIC. Specific IgG1 and IgG2 were detected by an indirect ELISA using the reagents and protocol previously described (Fernandez et al., 1998; Parreño et al., 2004). Specific IgM and IgA were measured by capture ELISAs standardized in our laboratory, using an anti-bovine IgM MAb (kindly supplied by Dr L.J. Saif, The Ohio State University, USA) or an affinity purified sheep anti-bovine IgA polyclonal antibody (Bethyl Laboratories Inc., Montgomery, Texas, USA). Negative samples at a dilution of 1:4 were assigned a titer of 1:2 for the calculation of geometric mean titers (GMTs) (Parreño et al., 2004).

2.1.9. Fluorescent focus reduction virus neutralization (FFN) test

Virus neutralizing (VN) Ab titers to IND BRV in colostrum pools and supplemented milks were determined by a fluorescent focus neutralization (FFN) test as previously described (To et al., 1998). The VN titer was expressed as the reciprocal of the highest sample dilution that resulted in >80% reduction in the number of fluorescent foci.

2.1.10. Isolation of mononuclear cells (MNC)

Approximately 15 cm of tissue samples of Duodenum, Jejunum, and ileum were collected. Discrete Peyer’s patches (n = 3) were identified at different points along the mucosal surface of the Jejunum. The surrounding lamina propria was carefully separated and the PPs were collected. A segment of intestine corresponding to a portion of the distal ileum continuous Peyer’s patch (10 cm cranial to the ileo-caecal junction) was also obtained. To investigate the number of BRV ASC settled in the mesenteric lymph nodes (MLN) draining the small and large intestines, MLN corresponding to the Jejunum and ileal-caecal regions were collected and processed separately. To evaluate ASC responses in systemic lymphoid tissues, PBL and spleen were also collected and processed. Thereafter, MNC from all samples were extracted as previously described by Parreño et al. (2004).

2.1.11. ELISPOT assay for BRV-specific ASC

An ELISPOT assay for quantification of anti-BRV IgM, IgA, IgG1, and IgG2 ASC was conducted to evaluate effector
B cell responses from all calves. The assay was adapted from that conducted in pigs (Hodgins et al., 1999; Yuan et al., 1996). Briefly, IND BRV infected MA-104 cells in 96-well plates were fixed with 70% acetone, air-dried, and stored at −20°C until use. Only plates showing ≥80% BRV infection by CCIF were used. Single-cell suspensions of MNC from each tissue were added to duplicate wells at the following concentrations: 5 × 10^4, 5 × 10^6, and 5 × 10^3 cells/well. After centrifugation at 500 × g for 5 min, plates were incubated for 12–14 h at 37°C and 5% CO₂. The plates were washed with PBS−0.05% Tween-20 to remove adherent cells. The plates were incubated with a set of commercial peroxidase-labeled goat polyclonal Abs to bovine IgM (1:4000); IgG1 (1:10,000); IgG2 (1:2000) and IgA (1:1000) (Bethyl Laboratories Inc., Montgomery, Texas, USA) for 2 h at room temperature and the spots were developed with a tetramethylbenzidine peroxidase substrate system (KPL, Gaithersburg, MD, USA).

2.2. Statistical analysis

Fisher’s exact test was used to compare proportions of calves with diarrhea and virus shedding among groups. The Kruskal–Wallis rank sum test (non-parametric) was used to compare days to onset and duration of diarrhea and virus shedding, cumulative diarrhea scores, cumulative titers of virus in shed and days with high rectal temperature, among groups. Neutralizing and isotype-specific Ab titers were log_{10}-transformed prior to statistical analysis. Differences in Ab titers among groups were evaluated by general analysis of variance (ANOVA) followed by DGC multiple comparison test on repeated measures throughout time. At 21 PID, the ASC numbers followed by DGC multiple comparison test on repeated measures analysis. At 21 PID, the ASC numbers followed by DGC multiple comparison test on repeated measures analysis.

3. Results

3.1. Virus neutralization and isotype-specific ELISA antibody titers against IND BRV in colostrum pools and supplemented milks used for calf feeding

Titers of VN and isotype-specific Abs to IND BRV in bovine colostrum pools and the colostrum supplemented milks used for calf feeding are summarized in Table 1. As IgG1 was the main isotype present in colostrum pools, it was also the primary Ab isotype present in the supplemented milks. The milk used to feed calves assigned to Gp 1 and 2, was prepared by adding 32 or 8 ml of immune colostrum to 2 l of commercial sterilized milk, respectively, in order to render a final IgG1 Ab titer to BRV of 4096 and 1024, respectively. The volume of colostrum added to the milks represented 0.8 and 0.4% of the total feeding volume, respectively. The addition of Colostral IgG1 Ab also conferred virus neutralizing activity to the milks (16,384 and 1024, respectively). As indicated in Section 2, results from a pilot study indicated that supplemented milk from Gp 2 was not protective, while supplemented milk from Gp 1 provided high protection against BRV diarrhea.

3.2. Protection against diarrhea and virus shedding after primary inoculation and challenge

Results of the various parameters associated with BRV infection and disease are summarized in Table 2. Fig. 1 depicts the patterns of virus shedding by each calf assigned to the different treatment groups (Gp 1–4) after inoculation and challenge (only Gp 1 and 2).

After inoculation with BRV, 80% (8/10) and 37.5% (3/8) of the calves fed with milk supplemented with immune colostrum for 14 days were protected against disease (Gp 1 and 2, respectively), whereas 100% of calves receiving control colostrum (Gp 3) or colostrum deprived calves (Gp 4), fed with Ab-free milk, developed severe diarrhea. The onset of the clinical signs was delayed in both treated groups (Gp 1 and 2) in comparison with the control calves.

### Table 2
Diarrhea and fecal virus shedding in calves after oral inoculation with BRV IND (P5[G6]).

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>n</th>
<th>Inoculum infectious titer (FFU/ml)</th>
<th>Diarrhea</th>
<th>Virus shedding</th>
<th>Days with fever (&gt;39°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>% affected calves</td>
<td>Mean onset (PID)</td>
<td>Mean duration (days)</td>
</tr>
<tr>
<td>Gp 1: CC + IgG1</td>
<td>10</td>
<td>10^{18}</td>
<td>20%</td>
<td>4.0A</td>
<td>1.3</td>
</tr>
<tr>
<td>4096 milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gp 2: CC + IgG1</td>
<td>8</td>
<td>10^{15}</td>
<td>62.5%AB</td>
<td>5.2B</td>
<td>3.88BC</td>
</tr>
<tr>
<td>1024 milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gp 3: CC +</td>
<td>8</td>
<td>10^{4.4}</td>
<td>100%A</td>
<td>2.4A</td>
<td>7AB</td>
</tr>
<tr>
<td>Ab-free milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gp 4: CD +</td>
<td>5</td>
<td>10^{3.9}</td>
<td>100%A</td>
<td>1.2A</td>
<td>10.8</td>
</tr>
<tr>
<td>Ab-free milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CC = control colostrum/CD = colostrum deprived. Calves were fed with 2 l of milk, twice a day, for 14 days, with or without the addition of the corresponding volume of BRV-immune colostrum according to the group.

- Diarrhea duration was defined as the number of days with fecal score ≥2. Stool consistency was scored daily (0 = normal; 1 = pasty; 2 = semi-liquid; 3 = liquid).
- Means in the same column with different superscript upper case letters differ significantly (Kruskal–Wallis rank sum test; p < 0.05).
- Determined by ELISA and CCIF.
- Proportions in the same column with different superscript upper case letters differs significantly (Fisher’s exact test, p < 0.05).
- Mean cumulative scores from 0 to 21 PID calculated as a measure of severity of diarrhea (sum daily fecal score)/n.
Fig. 1. Curve of virus shedding for each calf in the four treatment groups: (a) Gp 1: calves fed with control colostrum (CC) followed by BR-immune colostrum supplemented milk with a final BRV IgG 1 Ab titer of 4096 were subdivided in: (a1) calves without virus shedding and diarrhea after inoculation and (a2) calves shedding virus after inoculation, 33% of these calves also developed mild diarrhea. (b) Gp 2: calves receiving CC followed by BRV-immune colostrum supplemented milk with a final BRV IgG 1 Ab titer of 1024. (c) Gp 3: calves receiving CC. (d) Gp 4: colostrum deprived calves, both fed with Ab-free milk. Each line represent the infectious virus titers measured daily in each calf, expressed as the log_{10} of the FFU/ml determined by CCIF.
(Gp 3 and 4). Moreover, compared with control groups (Gp 3 and 4), in the calves receiving the highest supplementation in milk (Gp 1), the duration and severity of diarrhea were significantly diminished in the two calves with diarrhea. In addition, these calves showed a significant reduction in the number of days with hyperthermia associated with diarrhea (0.8 days) in comparison to the control groups (Gp 3: 6.9 days; Gp 4: 5.6 days).

Regarding BRV infection, both groups of calves receiving supplemented milk had a significantly delayed onset of virus shedding. Forty percent (4/10) of the animals from Gp 1 (IgG 1 Ab 4096) did not shed detectable virus either by ELISA or by CCIF, generating a significant reduction in the duration, peak and mean titer of virus shed in the group. Calves in Gp 1 were further divided into two subgroups for the analysis: the animals that were fully protected against diarrhea and virus shedding (4/10) (Fig. 1a1) and the ones that shed detectable virus (6/10) after the first inoculation (Fig. 1a2). From the four animals in the subgroup protected at inoculation, two were challenged at 21 PID, and only one shed virus asymptotically from 23 PID/2 PCD for 7 days. The behavior of the six animals shedding virus after the first inoculation was variable. Most of them showed intermittent shedding and only two animals had mild diarrhea. Of those animals with diarrhea, one shed virus for 6 days after diarrhea onset. The other one had diarrhea at 5 PID with only 1 day of BRV shedding (7 PID) and then shed again for 4 days after the cessation of milk supplementation. The 3 calves from this group that were challenged were fully protected against a second exposure to the virus.

As expected from the results obtained in the pilot study, the BRV IgG 1 Ab 1024 treatment (Gp 2) failed to prevent virus shedding. All calves were infected after the first inoculation shedding virus continuously for long periods of time. The half of the animals in the group, challenged at 21 PID, were protected after a second exposure to the virus (Fig. 1b).

Calves fed control colostrum followed by Ab-free milk (Gp 3) had a high intra-group variability in the amount and duration of virus shedding, with some calves shedding virus for a long period of time (Fig. 1c).

The pattern of virus shedding in CD calves (Gp 4) was homogeneous between calves and characterized by a fast onset, high amount of virus shed and a fast resolution at 5.4 PID (Table 2 and Fig. 1d).

In all animals shedding the virus, the sample corresponding to the peak of shedding was analyzed by Heminested Multiplex RT-PCR and was typed as P[5]G6, in concordance with the type of the inoculated strain BRV IND (data not shown).

3.3. Passive antibodies in neonatal calves after colostrum intake

After colostrum administration prior to gut closure, all calves in Gp 1–3, which received 1 l of control colostrum within the first 6 h of life, had optimal level of total serum proteins (5.2–9.4 g/100 ml) and statistically similar geometric mean VN (0 PID, Gp 1: 338; Gp 2: 197; Gp 3: 362) and IgG 1 (0 PID, Gp 1: 1776; Gp 2: 724; Gp 3: 1024) Ab titers to BRV in the sera, indicative of proper colostrum intake (Fig. 2, supplementary data, Table 1).

In contrast to the similarity observed in serum, the IgG 1 Ab titers detected in feces of each calf after the initial dose of colostrum were highly variable. No IgG 1 Abs were detected in feces from 54% of the animals, while 46% had IgG 1 Abs ranging from 64 to 65,536. Geometric mean IgG 1 Ab titers for each treatment group were: Gp 1: 45.3; Gp 2: 6.7; Gp 3: 117 (Fig. 3; supplementary data Table 2).

3.4. Isotype-specific and VN Ab responses to IND BRV in serum after BRV inoculation

Isotype-specific and VN Ab responses to IND BRV in serum and the number of anti-BRV ASC in PBL (detected weekly) are depicted in Fig. 2 (statistical analysis detailed in supplementary data, Table 1).

Colostrum deprived calves (Gp 4) developed a specific serum Ab response against BRV with a peak IgM response in serum at 7 PID, and then switching to IgG 1 and, in lower magnitude, to IgA, toward 21 PID. Virus neutralizing Ab responses were also detected from 14 PID.

All calves receiving the first feeding of control colostrum (Gp 1–3), maintained the initial serum titers of passively acquired IgG 1 and neutralizing Abs. Calves in Gp 3 developed significantly lower IgM and IgA Ab responses against BRV than CD calves, while Gp 2 calves fed CC followed by IgG 1 1024 supplemented milk developed strong IgM and IgA Abs responses but the peak of both isotypes was detected at 14 PID, related to the delay in the onset of virus shedding and diarrhea in that group. Calves fed CC and IgG 1 4096 supplemented milk showed a clear tendency to develop higher IgA than IgM Ab titers, with significantly higher IgA Ab titers at 14 and 21 PII compared to Gp 3.

The profiles of virus shedding (Fig. 1) and the profile of the Ab responses in calves fed with colostrum supplemented milks (Gp 1 and 2) (Fig. 2a1, a2, b, c) were more heterogeneous than that observed in CD calves, where all the animals responded more uniformly (Fig. 2d).

Half of the calves from Gp 1 and 2 were challenged at 21 PID. After challenge, only slight increments in the IgG 1 and IgA Ab titers were detected at 28/7 PID or 35 PID/14 PID in both groups.

No IgG 2 Ab responses were detected in any animal during the experimental period.

3.5. The active ASC responses in PBL

No BRV ASC were detected in PBL from any of the calves at the onset of the experiment (0 PID). A BRV-specific IgM ASC peak response was detected in PBL of calves fed milk without Abs (Gp 3 and 4), at 7 PID. IgA ASC were detected from 7 PID on, followed by circulation of a few IgG 1 ASC at 14 and 21 PID. Lower numbers of ASC (7 PID) were associated with the presence of passive circulating maternal Abs in Gp 3 calves, although they were not significantly lower at any PID. In contrast, in the groups fed IgG 1 supplemented milk (Gp 1 and 2), a significant reduction in the ASC circulating in PBL, compared to Gp 3 or 4 calves was evident (Fig. 2). After challenge, both
Fig. 2. Left axis: geometric mean isotype and neutralizing Ab titer (GMT) against IND BRV in serum collected weekly (lines); right axis: number of RV-specific ASC/5 x 10^5 MNC in PBL (bars; error bars: SD) from: (a) Gp 1: calves fed with control colostrum (CC) followed by BRV-immune colostrum supplemented milk with a final BRV IgG1 Ab titer of 4096; Group 1 was subdivided into two groups: (a1) calves without virus shedding and diarrhea after inoculation and (a2) calves shedding virus after inoculation, 33% of this calves also developed diarrhea. (b) Gp 2: calves receiving CC followed by BRV-immune colostrum supplemented milk with a final BRV IgG1 Ab titer of 1024. (c) Gp 3: calves receiving CC. (d) Gp 4: colostrum deprived calves, both fed with Ab-free milk. All calves were orally inoculated with IND BRV at 0 PID, half of the animals in Gp 1 and 2 were further challenged at 21 PID. Bars corresponding to ASC of the same isotype with different upper case superscript letters indicate significant differences among groups (Kruskal–Wallis non-parametric sum rank test, p < 0.05). Statistical analysis of the Ab titers in serum are detailed in supplementary data, Table 1 (ANOVA followed by DGC multiple comparison test through time, p < 0.05).
Fig. 3. Geometric mean isotype Ab titers (GMT) to IND BRV detected daily in feces of: (a) Gp 1: calves fed control colostrum (CC) followed by BR-immune colostrum supplemented milk with a final BRV IgG, Ab titer of 4096; Group 1 was subdivided into two groups: (a1) calves without virus shedding and diarrhea after inoculation and (a2) calves shedding virus after inoculation, 33% of this calves also developed diarrhea. (b) Gp 2: calves receiving CC followed by BR-immune colostrum supplemented milk with a final BRV IgG, Ab titer of 1024. (c) Gp 3: calves receiving CC. (d) Gp 4: colostrum deprived calves, both fed Ab-free milk. All animals were orally inoculated at 2 days of age/36 h after colostrum intake [0 days post-inoculation (0 PID)], and euthanized at 21 PID; Gp 1–4. Half of the calves in Gp 1 and 2 were further challenged at 21 PID and euthanized at 35 PID. SIC, small intestinal contents; and LIC, large intestinal contents, were collected at 21 and 35 PID. GMT Ab titers for the same isotype in SIC and LIC with different lower case letter, differ significantly (one way ANOVA, Tukey, p < 0.05). Statistical analysis of the Ab titers in feces analyzed every 7 days is detailed in supplementary data, Table 2 (ANOVA followed by DGC multiple comparison test trough time, p < 0.05).
a) Gp 1: CC+milk BRV-IgG1 Ab 4096 (n=10) subdivided in calves without virus shedding (1) and calves with virus shedding after inoculation (2)

**a 1. Calves without virus shedding and diarrhea after inoculation (n=4)**

- IgM
- IgA
- IgG1

**Time (post inoculation days)**

**a 2. Calves shedding virus after inoculation (n=6)**

- IgM
- IgA
- IgG1

**Time (post inoculation days)**

- BRV inoculation at 0 PID
- BRV challenge at 21 PID
- Milk supplementation
groups (Gp 1 and 2) showed BRV-specific ASC in PBL at 35 PID/14 PCD (Fig. 2a1 and a2).

3.6. Isotype-specific Abs to IND BRV detected in feces and intestinal contents after inoculation

Isotype-specific Ab responses to IND BRV in feces (evaluated daily), and in LIC and SIC (determined on 21 PID/35 PID) are shown in Fig. 3 (statistical analysis detailed in supplementary data, Table 2).

Colostrum deprived calves (Gp 4) developed strong IgM, IgA and IgG1 coproantibody responses. Gp 3 calves also developed strong IgM and IgA Ab responses; however the IgG1 Ab response in feces, at 21 PID was significantly lower than in CD calves.

Calves in Gp 2 (IgG1 1024 milk) developed strong IgM and IgA responses similar to those of the control groups, but the peaks were delayed, in association with the delay in the onset of virus shedding, compared with CD calves. Half of the calves in Gp 2 had IgG1 Abs in feces only along the diet supplementation period, but the titers were variable. Once the treatments finished, no BRV IgG1 Abs were detected in feces and intestinal contents collected at 21 PID.

In calves from Gp 1, receiving the highest supplementation (IgG1 4096 milk), IgM and IgA Ab responses in feces were greatly reduced. As observed in serum, IgM was the dominant isotype. The IgG1 Abs were detected in higher titers than those observed for Gp 2, in concordance with the higher dose of colostrum supplementation in the diet. The Ab responses in feces of calves from this group were very heterogeneous, since 40% of the animals were fully protected against diarrhea and virus shedding, while 60% became asymptomatically infected. The pattern of fecal Ab response for each subgroup was further studied separately (Fig. 3a1 and a2).

Two of the calves that initiated the experiment with high passive Ab titers in feces, did not shed detectable virus by ELISA and did not develop fecal Ab responses. The two calves developed low IgM and IgA Ab responses reflected in the peak detected at 9 PID in this subgroup (Fig. 3a1). The Ab responses in the subset of calves that shed virus varied from the absence of response, 4 out of 6 animals, to strong responses in the animal that developed severe infection including RV antigemia after experimental inoculation (Blutt et al., 2003).

At 21 PID, CD calves (Gp 4) showed a strong response against BRV, characterized by IgG1 ASC, followed by IgA ASC, in all tissues analyzed. In this group, the highest ASC counts were registered in Duodenum, which were significantly higher than the numbers of ASC detected in the rest of the tissue. The higher numbers of IgG1 ASC detected in systemic sites and the GALT were in concordance with the higher IgG1 Ab titers detected in serum, feces and intestinal contents for this group at this time point, and with the fact that CD calves developed a severe infection including RV antigemia after experimental inoculation (Blutt et al., 2003).

In calves from Gp 3 (Ab-free milk) the IgM and IgA ASC responses were the principal isotypes followed by IgG1, and in a lower magnitude, IgG2. The IgG1 ASC were reduced in all the sites studied, compared with numbers in CD calves, except for Duodenum and jejunum lamina propria.

Calves fed with supplemented milk developed high ASC responses in the GALT, but a very low (Gp 2) to almost absent (Gp 1) response in systemic sites and MLN draining the small and large intestine. So, the highest ASC responses were detected in the BRV affected tissues, decreasing progressively along the intestinal tract, and were characterized by the predominance of IgA ASC, followed by IgG1. Both groups also developed an IgG2 ASC response, preferentially in Duodenum and Jejunum.

Collectively, the results obtained indicate that milk supplementation with BRV-specific IgG1 Abs derived from immune colostrum fed for the first 14 days of life seemed to diversify and enhance the local immune responses against BRV, with IgA as the dominant ASC isotype instead of IgG1.

3.7. Active ASC responses in systemic and intestinal lymphoid tissues at 21 PID

The magnitude and profile of BRV ASC responses, evaluated by ELISPOT in PBL, spleen and MLN are depicted in Fig. 4. The ASC responses in the intestinal mucosal are depicted in Fig. 5. Rotavirus ASC responses were detected in all the lymphoid tissues tested. The majority of ASC were detected in the intestinal lamina propria (Duodenum, Jejunum and Ileum), Jejunum and Ileum Peyer’s patches, and MLN draining both the small and large intestine, with fewer ASC in systemic organs, such as spleen and PBL. Most ASC responses occurred in the proximal intestinal lamina propria (Duodenum, Jejunum) and, in lower magnitude, in Peyer’s patches, independently of the treatment. This finding is in agreement with the site of BRV infection and replication in the small intestine and represents the main GALT effector and inductive sites, respectively.

At 21 PID, CD calves (Gp 4) showed a strong response against BRV, characterized by IgG1 ASC, followed by IgA ASC, in all tissues analyzed. In this group, the highest ASC counts were registered in Duodenum, which were significantly higher than the numbers of ASC detected in the rest of the tissue. The higher numbers of IgG1 ASC detected in systemic sites and the GALT were in concordance with the higher IgG1 Ab titers detected in serum, feces and intestinal contents for this group at this time point, and with the fact that CD calves developed a severe infection including RV antigemia after experimental inoculation (Blutt et al., 2003).

In calves from Gp 3 (Ab-free milk) the IgM and IgA ASC responses were the principal isotypes followed by IgG1, and in a lower magnitude, IgG2. The IgG1 ASC were reduced in all the sites studied, compared with numbers in CD calves, except for Duodenum and jejunum lamina propria.

Calves fed with supplemented milk developed high ASC responses in the GALT, but a very low (Gp 2) to almost absent (Gp 1) response in systemic sites and MLN draining the small and large intestine. So, the highest ASC responses were detected in the BRV affected tissues, decreasing progressively along the intestinal tract, and were characterized by the predominance of IgA ASC, followed by IgG1. Both groups also developed an IgG2 ASC response, preferentially in Duodenum and Jejunum.

Collectively, the results obtained indicate that milk supplementation with BRV-specific IgG1 Abs derived from immune colostrum fed for the first 14 days of life seemed to diversify and enhance the local immune responses against BRV, with IgA as the dominant ASC isotype instead of IgG1.

3.8. Active ASC responses in systemic and intestinal lymphoid tissues post-challenge at 35 PID/14 PCD

At 35 PID/14 PCD, in both groups of calves fed supplemented milk, the distribution of ASC was similar to that observed after primary inoculation but of higher...
Fig. 4. Mean numbers of BRV ASC per $5 \times 10^5$/MNC obtained from systemic lymphoid tissues (PBL and spleen) and MLN draining the small and large intestine, at 21 PID. (a) Gp 1: calves fed with control colostrum (CC) followed by BRV-immune colostrum supplemented milk with a final BRV IgG1 Ab titer of 4096. (b) Gp 2: calves receiving CC followed by BRV-immune colostrum supplemented milk with a final BRV IgG1 Ab titer of 1024. (c) Gp 3: calves receiving CC. (d) Gp 4: colostrum deprived calves, both fed with Ab-free milk. For each tissue, when comparing mean ASC numbers of the same isotype, among treatment groups: bars with different letter indicate a significant difference (Kruskal–Wallis rank sum test, \( p < 0.05 \)). \( n \) = number of calves in each group. Error bars indicate SD.
Fig. 5. Mean numbers of BRV ASC per $5 \times 10^5$/MNC obtained from the intestinal mucosa associated lymphoid tissues (GALT): Duodenum, Jejunum LP and PP, Ileum LP and PP, at 21 PID. (a) Gp 1: calves fed with control colostrum (CC) followed by BRV-immune colostrum supplemented milk with a final IgG1 Ab titer of 4096. (b) Gp 2: calves receiving CC followed by BRV-immune colostrum supplemented milk with a final IgG1 Ab titer of 1024. (c) Gp 3: calves receiving CC. (d) Gp 4: colostrum deprived calves, both fed with Ab-free milk. For each tissue, when comparing mean ASC numbers of the same isotype, among treatment groups: bars with different letters indicate a significant difference (Kruskal–Wallis rank sum test, $p < 0.05$). $n =$ number of calves in each group. Error bars indicate SD.
magnitude for some small intestinal regions, as expected for a secondary response. High levels of IgG ASC were observed, especially in Gp 1 calves. The ASC were mainly localized to the intestinal mucosa, with the highest numbers registered in Duodenum and Jejunum lamina propria (Fig. 6). ASC responses in the systemic tissues (spleen and PBL) were low. However the numbers of ASC in MLN of Gp 1 calves where higher at 35 PID/14 PCD than at 21 PID (Fig. 2). At 35 PID/14 PCD, corresponding to 21 days after cessation of milk supplementation in both groups of calves (Gp 1 and 2) the numbers of IgG ASC were comparable or higher than IgA, similar to the profiles observed in CD calves (Gp 4), and also with more participation of the IgG2 isotype (Fig. 6).

4. Discussion

In the present study, we evaluated modulation of the development of active serum and fecal Ab responses to BRV infection and the BRV-specific ASC responses in systemic and mucosal-associated lymphoid tissues in conventional calves fed milk supplemented with BRV-immune colostrum, corresponding to 0.8 and 0.4% of the feeding volume (Gp 1 and Gp 2). As IgG1 was the main isotype for some small intestinal regions, as expected for a secondary response. High levels of IgG2 ASC were observed, especially in Gp 1 calves. The ASC were mainly localized to the intestinal mucosa, with the highest numbers registered in Duodenum and Jejunum lamina propria (Fig. 6). ASC responses in the systemic tissues (spleen and PBL) were low. However the numbers of ASC in MLN of Gp 1 calves were higher at 35 PID/14 PCD than at 21 PID (supplementary data, Fig. 2). At 35 PID/14 PCD, corresponding to 21 days after cessation of milk supplementation in both groups of calves (Gp 1 and 2) the numbers of IgG ASC were comparable or higher than IgA, similar to the profiles observed in CD calves (Gp 4), and also with more participation of the IgG2 isotype (Fig. 6).
isotype present in colostrum, the milks were enriched in this immunoglobulin.

The doses of colostrum selected for the experiments were obtained from a pilot study whereby calves fed milk with IgG1 Ab titer of 1024 were partially protected against diarrhea and milk IgG, with Ab titer of 4096 provided high protection against diarrhea and drastically reduced virus shedding. Our goal was to provide passive Abs in the gut to prevent the disease, reduce virus replication and neutralize the shed virus, thereby still evoking a subclinical infection that would prime the neonatal mucosal immune response to BRV.

Passive Abs present in the gut, although associated with protection against RV diarrhea, also interfered with the development of active immune responses in neonatal germfree pigs (Hodgins et al., 1999; Parreño et al., 1999). Hence, in this study, we evaluated protection and immune modulation after a first BRV inoculation at 2 days of age (0 PID) followed by a second challenge (21 PID) after the cessation of feeding supplemented milk in conventional calves. The administration of the supplemented milk twice a day, for 14 days resembles management practices in Argentinian dairy farms, where farmers collect the colostrum of dairy cows and use it to supplement the milk replacer used to feed the calves under artificial rearing.

The supplementation of milk diets with immune colostrum induced protection against BRV diarrhea after inoculation in a dose-dependent manner. This protection was associated with the presence of passive IgG1, in the feces of these calves.

The group of calves fed with colostrum supplemented milk at a final IgG1 Ab titer to BRV of 4096 showed 80% protection against BRV diarrhea and 40% protection against virus shedding, after inoculation. The duration of diarrhea and virus shedding was also significantly lower compared with control groups. These finding are similar to those reported by Fernandez et al. (1998), in calves fed milk supplemented with immune colostrum from cows vaccinated with SA11 2/6/4/7 and 2/6 VLP vaccines.

The supplementation of milk with a lower amount of colostrum (Gp 2, IgG1, Ab 1024 milk), failed to protect calves against infection, but protected 37.5% of the calves against diarrhea. The onset, duration and severity of the illness was significantly reduced compared to CD calves (Gp 4).

Moreover, all calves receiving supplemented milk (Gp 1 and 2) were fully protected against diarrhea and only one shed virus asymptomatically, after a second exposure to the virus, 7 days after the cessation of the supplemented milk feeding.

An important point to remark was that not only the specific Ab titers, but also the duration of the supplemented milk feeding were key factors to obtain high protection during the period of peak susceptibility to BRV diarrhea in this study. Previous investigators have studied colostrum supplementations in calves for only 5–7 days and they detected virus shedding and mild to severe diarrhea immediately at the end of the supplement feeding (Fernandez et al., 1998; Saif et al., 1983; Tsunemitsu et al., 1988).

Collectively, the obtained results indicate that the practice of feeding supplemental colostrum from BRV-vaccinated cows should be highly effective to control BRV diarrhea in endemic dairy farms where neonatal calf diarrhea represents an important economic problem (Foster and Smith, 2009). Long term impact of this practice might be reduced virus loads in the environment and reduced virus dissemination and transmission.

Immune modulation by homologous passive Abs has been studied comprehensively in germfree pigs (Hodgins et al., 1999; Nguyen et al., 2007, 2006; Parreño et al., 1999). In calves, initial studies focused on the kinetics of the VN and isotype-specific Ab responses in CD calves fed milk supplemented with 1% BRV-immune colostrum (Fernandez et al., 1998; Saif et al., 1987). Subsequently, the magnitude and distribution of ASC in the systemic and mucosal-associated lymphoid tissues was studied in CD and calves fed one initial feeding of colostrum from non-vaccinated and vaccinated cows followed by Ab-free milk (Parreño et al., 2004). In the present work we studied the additional immune modulation caused by the supplementation of the milk with immune colostrum.

After inoculation, the presence of passive IgG1 Abs with neutralizing activity in the serum of the calves receiving one initial colostrum feeding significantly suppressed active systemic Ab responses in terms of Ab titers in serum and ASC circulating in PBL. Milk supplementation with 0.8% of colostrum (Gp 1) enhanced this effect, inducing a delay in the Ab responses according to the delay in the onset of the infection and also modified the isotype profile of the active Abs responses toward higher IgA than IgM Ab titers in serum. The inverse relationship observed between the magnitude of the Ab response in serum, the numbers of ASC detected in PBL and the presence of passive maternal antibodies in serum and milk agrees with the results reported by Parreño et al. (1999) and Hodgins et al. (1999) who, working in gnotobiotic pigs fed with milk with similar Ab titers, described a suppression in the serum Ab response and did not detect ASC in PBL at 21 PID. In concordance with the results obtained in serum, again fecal Ab responses to BRV infection in Gp 2–4 calves were similar in profile and magnitude; while fecal the Ab responses of calves fed milk with the highest supplementation (Gp 1) were reduced in magnitude. Similar suppression was observed in calves fed milk supplemented with 1% immune colostrum of high Ab titer (IgG1; 1,048,576) from cows vaccinated with VLP (Fernandez et al., 1998).

The study of the distribution of BRV-specific ASC in different systemic and mucosal-associated lymphoid tissues at 21 PID showed that BRV ASC were present in all analyzed tissues, as reported previously for piglets (Saif et al., 1996). Independently of the treatment, the intestinal lamina propria (especially Duodenum) was the main site of IgA and IgG1 ASC localization, in agreement with the main site of BRV replication in the small intestine (Bridger, 1994).

In agreement with the results obtained in serum, the supplementation of the milk with 0.8% of BRV-immune colostrum also induced a suppressive effect in the ASC responses in systemic tissues, since almost no ASC were

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detected in spleen and MLN of Gp 1 calves. Similar results were reported in gnotobiotic pigs vaccinated with human RV, in the presence of passive circulating Abs (Nguyen et al., 2006), and infected in the presence of circulating and local passive maternal Abs (Hodgins et al., 1999). This effect might be related to the more limited infection in the animals of this group, to the neutralization of viremia by the passive circulating Abs.

In contrast to the suppressive effect observed in systemic sites (PBL and spleen) and MLN, the supplementation of milk with BRV-immune colostrum enhanced and diversified the profile of the ASC responses in the intestinal mucosa, toward higher numbers of IgA ASC in the proximal intestinal mucosa and increasing numbers of IgG2 ASC.

High numbers of BRV-specific ASC, mainly IgA, in the intestinal mucosa at 21 PIDs were associated with protection against challenge. This hypothesis is supported by the fact that the only calf shedding virus after challenge in Gp 1 was not infected and did not develop a detectable Ab response in serum and feces after first inoculation.

Our results agree with those obtained in mice and pigs, which described a correlation between the fecal IgA Ab titers, the number of IgA ASC in the intestinal mucosa and protection against challenge (Feng et al., 1994; Saif et al., 1996; Yuan et al., 1998). It has been speculated that the differential effect caused by passive Abs in different compartments of the immune system with suppression at the systemic level and positive modulation with the production of IgA in the GALT might be related with a T-independent pathway of IgA production in the gastrointestinal mucosa (Litinskiy et al., 2002; Macpherson et al., 2000).

To study the ASC response to a second exposure to the virus in the absence of passive Abs, calves were challenged at 21 PIDs, 7 days after the end of milk supplementation, and euthanized at 35 PIDs/14 PCD. As expected, the secondary ASC response was of higher magnitude than the primary response, involving mainly the proximal small intestine and MLN. The profile was characterized by high and similar numbers of IgA and IgG2 ASC, recovering the profile detected in CD calves, and also higher numbers of IgG2, according to the older age of the animals (Corbeil et al., 1997; Pastoret, 1998).

Colostrum is composed of high Ab titers of maternal Abs, especially IgG1, IgA and also other molecules with anti-microbial activity, grow factors and cytokines that are transferred from the serum or locally produced in the mammary gland and secreted into colostrum. These molecules have been reported to have immunomodulatory properties influencing neonatal immunity, stimulating cytokine production, B cell proliferation and promoting the maturation of neonatal mucosal immune response and isotype switching to IgA (Bottcher et al., 2000; Boudry et al., 2007a,b; Donnet-Hughes et al., 2000; Kehrl and Harp, 2004; Kelly, 2003). Specifically for RV, the impact of different concentrations of the two main colostral cytokines (TGF-β1 and IL-4) on the B cell responses in vitro, showed that high levels of TGF-β1 (10 ng/ml), similar to those levels detected in serum of piglets fed colostrum, suppressed B cell responses of all isotypes, while low concentrations (0.1 ng/ml), similar to those acting locally in the gut, increased the IgM and IgA ASC responses to RV (Nguyen et al., 2007). Our results in calves are in agreement with this finding. The first effect could explain the negative modulation of the ASC response in all calves receiving colostrum (Gp 1–3) and the second effect may explain the predominance of IgA ASC responses in the gut in Gp 1 and 2 calves. It also had been reported that these immunomodulatory molecules present in colostrum are resistant to freezing temperatures (Aldridge et al., 1998), then future experiments will be focused on evaluation of cytokine profiles in colostrum, milk and calf samples obtained in this study to evaluate their potential impact in the profile of the ASC responses to BRV infection in neonatal calves.

5. Conclusions

The administration of milk supplemented with BRV-immune colostrum for the first 14 days of life induced high protection rates against rotavirus diarrhea in calves during the period of peak susceptibility to BRV infection and a positive modulation of the neonatal immune responses toward higher numbers of IgA ASC and greater isotype diversity in the intestinal mucosa.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetimm.2010.01.003.

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