



AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Colostrum from cows immunized with a veterinary vaccine against bovine rotavirus displays enhanced in vitro anti-human rotavirus activity

This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1700967 since 2019-05-06T16:11:09Z

Published version:

DOI:10.3168/jds.2018-16016

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)





This is the author's final version of the contribution published as:

Andrea Civra, Alessandra Altomare, Rachele Francese, Manuela Donalisio, Giancarlo Aldini, and David Lembo. Colostrum from cows immunized with a veterinary vaccine against bovine rotavirus displays enhanced in vitro anti-human rotavirus activity.J Dairy Sci. 2019 Apr 10. pii: S0022-0302(19)30344-3. doi: 10.3168/jds.2018-16016.

The publisher's version is available at:

https://www.journalofdairyscience.org/article/S0022-0302(19)30344-3/fulltext

When citing, please refer to the published version.

Link to this full text: http://hdl.handle.net/

This full text was downloaded from iris-Aperto: https://iris.unito.it/

1 Interpretive summary

Human rotaviruses (HRoV) are a major cause of severe diarrheal disease in infants and young 2 children. Whilst the vaccination of pregnant cows with HRoV boosts the release of HRoV-specific 3 IgGs in bovine colostrum (BC), it raises regulatory and safety issues. In this study, we demonstrate 4 that the conventional BRoV vaccine is sufficient to enhance the anti-HRoV protective efficacy of BC, 5 thus providing a conservative approach to produce hyperimmune BC, making it exploitable as a 6 7 functional food. 8 9 HYPERIMMUNE BOVINE COLOSTRUM: A NOVEL APPROACH 10 11 Colostrum from cows immunized with a veterinary vaccine against bovine 12 rotavirus displays enhanced in vitro anti-human rotavirus activity 13

14 15

Andrea Civra^{*‡}, Alessandra Altomare^{†‡}, Rachele Francese^{*}, Manuela Donalisio^{*}, Giancarlo Aldini^{†1},
David Lembo^{*1}

- 18
- 19

23

30

38

²⁰ [‡]AC and AA contributed equally to this work

¹Corresponding Authors

e-mail: david.lembo@unito.it

- ^{*}Department of Clinical and Biological Sciences, University of Turin, Orbassano, Italy
- [†]Department of Pharmaceutical Sciences, Università degli Studi di Milano, Milan, Italy.
- Prof. David Lembo 31 Prof. Giancarlo Aldini 24 Dept. of Clinical and Biological Sciences 32 Dept. of Pharmaceutical Sciences 25 University of Turin 33 Università degli Studi di Milano 26 Regione Gonzole, 10 Via Mangiagalli, 25 34 27 20133 Milano (Milan), ITALY 10043 Orbassano (Turin), ITALY 35 28 Phone: (+39) 0116705484 36 Phone: (+39) 0250319296 29

37 e-mail: giancarlo.aldini@unimi.it

39 ABSTRACT

Human rotaviruses represent a major cause of severe diarrheal disease in infants and young children. 40 The limited impact of oral vaccines on global estimates of rotavirus mortality, and the suboptimal use 41 of oral rehydration, justify the need for alternative prophylactic and therapeutic strategies, especially 42 43 for immunocompromised hosts. The protective effects of colostrum - i.e. the first milk produced during the initial 24-48 hours post-parturition - is well documented in literature. In particular, the 44 ingestion of hyperimmune bovine colostrum has been proposed as an alternative preventive approach 45 against human rotavirus gastroenteritis. Whilst the immunization of pregnant cows with human 46 47 rotavirus boosts the release of specific immunoglobulins G (IgGs) in bovine colostrum, it raises regulatory and safety issues. In this study, we demonstrate that the conventional bovine rotavirus 48 49 vaccine is sufficient to enhance the anti-human rotavirus protective efficacy of bovine colostrum, thus 50 providing a conservative approach to produce hyperimmune bovine colostrum, making it exploitable 51 as a functional food.

- 52
- 53

54 **Keywords:** rotavirus, colostrum, cows, hyperimmune, immunoglobulins

56 INTRODUCTION

Viral gastroenteritis represents a relevant economic and public health burden, causing high morbidity 57 and mortality rates, mainly in the poorest countries (Das, Salam, & Bhutta, 2014). Human rotaviruses 58 (HRoVs) are a major cause of severe diarrheal disease in infants and young children, and the second 59 cause of death in children less than 5 years old (Marcotte & Hammarström, 2016). Since no specific 60 antiviral drug is available, the conventional treatment for HRoV acute gastroenteritis is largely 61 symptomatic and involves fluid and electrolyte replacement and maintenance of nutrition. Despite 62 the introduction of oral HRoV vaccines that significantly reduced the incidence of the disease in 63 developed countries (Payne et al., 2013), the impact of this active prophylaxis on global estimates of 64 65 HRoV mortality has been limited (Tate et al., 2016). The reason is mainly ascribable to the inadequate immunization coverage in lower income countries, where the burden of diarrheal disease is higher, 66 67 and vaccines are mostly needed. As a matter of fact, oral vaccines are less immunogenic when given to infants in low-income compared with high-income countries, due to transplacental maternally-68 69 acquired antibodies, breastfeeding, histo blood group antigens, malnutrition, microbiota dysbiosis 70 and environmental enteropathy. Moreover, the scarce availability of vaccines in these areas, along 71 with their contraindications in immunodeficient patients (Babji & Kang, 2012; Glass et al., 2014; 72 Binder et al., 2014; Gaspar et al., 2014) leave between a third to a half of children unprotected from 73 severe HRoV disease (Babji & Kang, 2012). These hindrances, together with the suboptimal use of 74 therapeutic oral rehydration solutions, justify the need for development of effective alternative prophylactic and therapeutic approaches to prevent and control HRoV gastroenteritis disease, 75 76 especially for immunocompromised hosts.

Colostrum is the first milk produced by mammary glands during the initial 24–48 hours postparturition (Tokuyama et al., 1990; Stelwagen et al., 2009), and represents a unique source of highly
concentrated nutritional components (Macy et al., 1949), and growth factors (Pakkanen et al., 1997)
for the gastrointestinal development of mammalian newborns.

81 More importantly, colostrum provides neonates with the essential passive immunity against infectious diseases (Ogra and Ogra, 1978; Cohen, 2006; Morris et al., 1980; Ebina et al., 1992; Majumdar and 82 83 Ghose, 1982; Stephan et al., 1990; Tokuyama et al., 1990; Stelwagen et al., 2009). In particular, 84 bovine colostrum (BC) has evolved into a highly effective host defense mechanism (Rainard & Riollet, 2006). In ruminants no transplacental exchange of immune factors occurs in utero, therefore 85 colostrum and, to a lesser extent, mature milk provide protection through a high immunoglobulin (Ig) 86 87 content, without which the newborn would not survive (Larson et al., 1980). The Igs present in BC 88 are IgG1, IgG2, IgA, and IgM (Ogra and Ogra, 1978). The abundance of different Ig classes in colostrum and milk varies among species, with IgA being the predominant Ig in human mammary
secretions. By contrast, in cow's colostrum IgG1 is the most represented (Barrington et al., 1997),
while IgA and IgM are present at much reduced concentrations. The BC Igs, in conjunction with the
ability of the ruminant neonatal gut to allow unrestricted passage of the large Ig molecules, provide

the young animal with passive immunization (Bush & Staley, 1980; Moore et al., 2005).

Although the effect of colostrum is species-specific, a growing body of literature documented the
protective effect of BC against several viral infections in humans (Benson et al., 2012; Ng et al., 2010;
Inagaki et al., 2014; El-Fakharany et al., 2017; Bojsen et al., 2007).

97 In particular, vaccination of cows against specific human pathogens results in polyclonal pathogenspecific antibodies in BC. The antibodies purified from this hyperimmune BC (HBC) have 98 99 successfully been exploited for the treatment of a variety of gastrointestinal infections caused by pathogenic bacteria (Hammarström and Weiner, 2008; Kelly, 2003; Playford et al., 2000; Struff and 100 101 Sprotte, 2008) or virus (Korhonen et al., 2000; Mehra, 2006; Ng et al., 2010; Kramski et al., 2012a,b; Byakwaga et al., 2011; Inagaki et al., 2010; Ingaki et al., 2013) indicating HBC as an alternative 102 103 source for low-cost virus-specific antibodies. These evidences, together with the high titer content of antimicrobial peptides and proteins, such as lactoferrin, lactoperoxidase and lysozyme, that can 104 105 stimulate innate antiviral pathways and adaptive immune responses (Smolenski et al., 2007; 106 Stelwagen et al., 2009; Tharpa, 2005) indicate BC as a functional food to provide protection against viral infections. 107

Consistently, ingestion of HBC has been proposed as an alternative prophylactic approach against 108 109 HRoV gastroenteritis (Ebina et al., 1992; Sarker et al., 1998). HBC containing HRoV-specific, neutralizing IgGs has been produced so far by immunizing pregnant cows with HRoV and harvesting 110 colostrum after delivery. However, the additional costs and regulatory and safety issues derived from 111 the use of a HRoV vaccine, make impossible to generate large-scale amounts of HBC. The alternative 112 use of BC from non-immunized cows (NHBC) may bypass these limits but, as expected, literature 113 has clearly shown that NHBC is endowed with a significant lower ant-HRoV efficacy (Inagaki et al., 114 2010). 115

In this study, we present proof of concept data disclosing the protective effect against different HRoV genotypes of HBC from cows vaccinated with a conventional bovine rotavirus strain (BRoV). These results show that HBC generated by immunizing cows with the routinely-used bovine vaccine has a significantly higher anti-HRoV activity if compared to the one of NHBC, and contains crossreactive IgGs able to neutralize the infectivity of different HRoV strains, thus representing a functional food providing an alternative feasible and cost-effective strategy to manage HRoV infections.

124 MATERIALS AND METHODS

125 Chemicals

Laemmli buffer, molecular mass standards and electrophoresis apparatus for one-dimensional electrophoresis were supplied by Bio-Rad Laboratories, Inc., Hercules CA. β-mercaptoethanol, dithiothreitol (DTT), acetonitrile (ACN), sodium dodecyl sulphate (SDS), iodoacetamide (IAA), formic acid (FA), and all other chemicals used throughout the experimental work were current pure analytical grade products and purchased from Sigma-Aldrich Corporation, St Louis, MO. Water and acetonitrile (OPTIMA® LC/MS grade) for LC/MS analyses were purchased from Fisher Scientific, (Loughborough, UK).

133

134 Bovine colostrum collection

135 Fresh BC and HBC samples were supplied by the company Advances in Medicine (AIM, Bologna, IT). According to the supplier, colostrum was collected from both non-vaccinated and vaccinated 136 137 pregnant dairy Holstein cows. Three cows were immunized by subcutaneous inoculation of the inactivated trivalent vaccine Trivacton 6 (MERIAL, Italia SpA) to maintain a maximum rate of 138 139 antibodies in colostral secretions against Escherichia coli, Rotavirus and Coronavirus, which are implied in the establishment of neonatal diarrhea. Vaccination was performed with a two-injections 140 schedule, administered two months and four weeks before parturition. Colostrum from the three 141 vaccinated cows was collected until the fifth hour after birth, pooled and immediately frozen at -20°C. 142 Concentrations of IgGs in whole colostra was 50 mg/ml (protein content: 12%), as assessed by 143 previously described methods (Sacerdote et al., 2013). 144

After a suitable dilution with demineralized water, (1 volume), the suspension was introduced into a 145 sterile beaker (controlled continuous stirring) and heated at $\sim 38^{\circ}$ C for about 1 hour. The suspension 146 was then subjected to the skimming step, then caseins were removed by adjusting the pH to their 147 isoelectric point (pH 4.6 with HCl 1M). After 1 hour, the product was centrifuged at 4000 rpm to 148 149 definitively remove caseins. Low molecular weight components including salts and lactose were then removed by using hollow fiber cross flow filtration cartridges with 4000 NMWC (Nominal Molecular 150 151 Weight Cutoff) coupled to a tangential flow filtration system equipped with a peristaltic pump essential to keep the flow recirculation continuous (Kross Flo®- Tangential flow Filtration System 152 153 Research III). The pH of the retentate was then adjusted with NaOH 1M to pH 7.0 \pm 0.2, and the neutralized sample centrifuged at 8500 rpm and the supernatant retained. The next steps consisted of
 clarification through 0:45 and 0:22 µm filters followed by lyophilisation.

156

157 Bovine IgG purification

Affinity Chromatography – IgG were purified by affinity chromatography, using protein G from 158 Streptococci as stationary phase, immobilized in a preparative chromatographic column. In more 159 detail, the affinity column was prepared by packing 400 mL of Protein G Sepharose 4 Fast Flow resin 160 (GE Healthcare) in a column support HiScale [™] 50 (GE Healthcare) which was connected to an 161 FPLC system (ÄKTAprime plus, GE Healthcare line-up). The chromatographic purification started 162 by eluting the column with 5 volumes (5 x chromatographic bed volume) of buffer A (Binding Buffer: 163 20 mM sodium phosphate, pH 7), and then the sample was loaded at a flow rate of 20 ml min⁻¹. The 164 eluate was monitored by a UV detector at 280nm, a conductivity meter (0.001-999.9 mS/cm), and a 165 pH-meter; all the fractions characterized by a significant UV absorption were automatically collected 166 (IgG depleted fractions). The subsequent step consisted of recovering the IgG fractions (IgG enriched 167 fractions) by eluting the column with 100% of Elution Buffer (1 M glycine hydrochloride pH 2.5). 168 The column regeneration was carried out by eluting 5 volumes (5 x chromatographic bed volume) of 169 170 20% ethanol.

171 Tangential Flow Filtration -IgG enriched fractions as well as IgG depleted fractions obtained as above reported were mixed and subjected to concentration and desalting using hollow fiber cross flow 172 filtration cartridges with 3000 NMWC (Nominal Molecular Weight Cutoff) and a surface area of 650 173 cm^2 (GE Healthcare) coupled to a tangential flow filtration system equipped with a peristaltic pump 174 essential to keep the flow recirculation continuous (Kross Flo®- Tangential flow Filtration System 175 Research III). The IgG and IgG-depleted fractions were concentrated 20/30 times, dialyzed with 5 176 volumes of water, filtered through 0.22 micron membranes in sterile conditions, and finally 177 lyophilized. 178

179

180 Separative methods of proteins on polyacrylamide gel

One-dimensional analysis (SDS-PAGE) - Protein separation was performed under reducing conditions; aliquots of 10μ L of samples containing 20-25 µg of proteins were mixed with 10 µL of Laemmli sample buffer containing 50 mM DTT and heated at 95°C for 5 minutes. Samples and the standard proteins mixture (Precision Plus Protein Standards) were loaded on precast gels (Any KD Mini Protean® TGX TM) and then placed in the electrophoresis cell (Mini-PROTEAN Tetra) and run at 200V (constant) for a variable time of about 30-40 min. Staining was carried out using
Coomassie blue stain (Biosafe G250 Stain, Bio-Rad) and the images acquired by using the Bio-Rad
GS800 densitometer and analyzed by using the software quantity One 1-D.

189

190 IgG and IgM analysis by SEC-UV

The IgG content of the IgG enriched and depleted fractions were then determined by a size exclusion 191 chromatography (SEC) according to the method reported by Altomare and colleagues (Altomare et 192 al., 2016a); SEC was performed on a Thermo Finnigan Surveyor HPLC system (ThermoFinnigan 193 194 Italia, Milan, Italy) equipped with a variable wavelength detector and an auto-sampler, controlled by Xcalibur software (version 2.0.7 Thermo Fisher Scientific, Rodano, MI, Italy). The SEC separation 195 was performed on a 4.6 × 300 mm Phenomenex Yarra[™] 3u SEC-3000, with a 4 × 3 mm GFC4000 196 pre-column, by running an isocratic flow of mobile phase containing 0.1M sodium phosphate bibasic, 197 0.025% sodium azide pH 6.8, at a constant flow rate of 0.5 mL/min. The autosampler temperature 198 was set at 8°C and UV detection was conducted at a wavelength of 280 nm, typical wavelength for 199 protein detection. Due to its low molecular weight, the dipeptide Tyrosine-Histidine (TH) was chosen 200 as internal standard (IS), since it marks the racing front, being the smallest analyte. All the samples 201 202 to be analyzed were added by a fixed amount of the internal standard, the TH (0.3mM).

203

204 Cell lines and viruses

205 African green monkey kidney epithelial cells (MA104) and human epithelial adenocarcinoma HeLa cells (ATCC® CCL-2TM) were propagated in Dulbecco's modified Eagle's medium (DMEM) (Gibco-206 BRL, Gaithersburg, MD) supplemented with heat-inactivated 10% fetal bovine serum (FBS) (Gibco-207 BRL) and 1% antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin, 208 Germany), at 37°C in an atmosphere of 5% of CO₂. African green monkey kidney cells (Vero) (ATCC 209 CCL-81) were grown as monolayers in Eagle's minimal essential medium (MEM) (Gibco/BRL, 210 Gaithersburg, MD) supplemented with 10% FBS and 1% antibiotic-antimycotic solution. HRoV 211 strains Wa (ATCC® VR-2018), HRV 408 (ATCC® VR-2273), HRV 248 (ATCC® VR-2274), and 212 BRoV strain NCDV (ATCC[®] VR-1290) were activated with 5µg/ml of porcine pancreatic trypsin 213 214 type IX (Sigma) for 30 min at 37°C and propagated in MA104 cells using DMEM containing 0.5µg of trypsin per ml as described previously (Civra et al., 2015). Viral titers are expressed as focus-215 forming unit (FFU) per ml. A serologic characterization of RoVs exploited in this study is provided 216 in Table 1. Human rhinovirus (HRhV) 1A (ATCC® VR-1559), was propagated in HeLa cells, at 217

34°C, in a humidified 5% CO₂ incubator. Clinical isolates of human herpes virus type 1 (HSV1) and 218 type 2 (HSV2) were kindly provided by Prof. M. Pistello, University of Pisa, Italy. Human 219 cytomegalovirus (HCMV) strain Towne was kindly provided by Prof. W. Brune, Heinrich Pette 220 Institut, Hamburg, Germany. Vesicular stomatitis virus (VSV; ATCC® VR-1238), HCMV and HSV1 221 and HSV2 were propagated in Vero cells, at 37°C in a humidified 5% CO₂ incubator. When the full 222 cytopathic effect (CPE) developed, cells and supernatants were harvested, pooled, frozen and thawed 223 three times, clarified and aliquoted. Viruses were stored at -70°C. Viral titers were determined by 224 the standard plaque method as described previously (Civra et al., 2014; Cagno et al., 2017), and 225 226 expressed as plaque-forming units (PFU) per ml.

227

228 Focus reduction assays

Antiviral activity of NHBC, HBC, or IgGs against RoV Wa, HRV408, HRV248, and NCDV was 229 230 determined by focus reduction assay or plaque reduction assays. Assays of inhibition of rotavirus infectivity were carried out with confluent MA104 cell monolayers plated in 96-well trays, as 231 described elsewhere (Civra et al., 2014). Cells were treated for 2 hours at 37°C with serial dilutions 232 of colostrum, at protein concentrations ranging from 0.02 to 3340µg protein/ml in serum-free medium 233 prior to virus addition. HRoV infection was performed at a multiplicity of infection (MOI) of 0.02 234 FFU/ml for 1 hour at 37°C, in presence of the colostra. Infected cells were washed with serum-free 235 236 medium, fresh methanol extract was added, and cells were incubated in this medium at 37°C in a humidified incubator in 5% (vol/vol) CO₂-95% (vol/vol) air. After 16 hours of incubation, infected 237 cells were fixed with cold acetone-methanol (50:50), and viral titers determined by indirect 238 immunostaining by using a mouse monoclonal antibody directed to human rotavirus VP6 (0036; 239 240 Villeurbanne, France), and the secondary antibody peroxidase-conjugated AffiniPure F(ab')₂ Fragment Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories Inc., 872 W. 241 Baltimore Pike, West Grove, PA 19390). 242

243

244 Plaque reduction assay

HeLa or Vero cells were first seeded (at 8×10^4 cells/well) in 24 well plates. 24 hours later the HBC, NHBC, or IgGs were serially diluted in medium (from 0.02 to 3340µg protein/ml) and added to cell monolayers. After 2 hours of incubation (37°C, 5% CO₂), medium was removed and infection was performed with 200 µL/well with HRhV 1A, VSV, HSV-1, HSV-2, or HCMV at a MOI of 0.0002 PFU/ml in presence of colostra. The infected cells were incubated at 34°C for HRhV infections or 250 37°C for the other viruses for 1 hour, then washed with medium, and overlaid with a 1:1 combination 251 of 1.6% SeaPlaque Agarose and 2X DMEM containing the colostra. Control wells (100% of 252 infection) were prepared by treating cells with equal volumes of culture medium. The plates were 253 incubated at 34°C or 37°C for 3 days. After incubation, the plates were fixed with 7.5% formaldehyde 254 (Fluka) and stained with crystal violet (Sigma, St. Louis, Mo.). The number of plaques formed was 255 counted.

256

257 Rotavirus (RoV) neutralization assay

IgG precipitated with ammonium sulphate at respective 90% effective concentration (EC₉₀) or equal volume of culture medium or ammonium sulphate supernatant were added to $2x10^5$ FFU/ml of trypsin-activated RoV suspension and mixed in a total volume of 200 µl. The virus-compound mixtures were incubated for 1 hour at 37 °C then serially diluted to the non-inhibitory concentration of test IgG, and the residual viral infectivity was determined as previously described (Civra et al., 2014).

264

265 **RoV-cell binding assay**

RoV-cell binding assays were performed as described previously (Civra et al., 2015). Trypsin-266 activated RoVs Wa, HRV248, HRV408, and NCDV were treated as described for neutralization 267 assays. After 1 hour, cells were washed with fresh medium and cooled on ice. RoVs were then cooled 268 to 4°C, and allowed to attach to cells for 1 h (MOI=3 FFU/cell) at 4°C. After a wash with cold DMEM, 269 cells were subjected to two rounds of freeze-thawing and then incubated at 37°C for 30 minutes with 270 10 µg/ml porcine trypsin to release bound virus. The lysates were then clarified by low speed 271 272 centrifugation for 10 min, and cell-bound virus titers were determined by indirect immunostaining as above. 273

274

275 Cell viability assay

Cells were seeded at a density of 5 x 10^3 /well in 96-well plates and treated the next day with HBC, NHBC, IgGs or ammonium sulfate supernatant at concentrations ranging from 0.02 to 7140µg/ml to generate dose-response curves. Control samples (100% of viability) were prepared by treating cells with culture medium. After 24 or 72 hours of incubation, cell viability was determined using a CellTiter 96 Proliferation Assay Kit (Promega, Madison, WI, USA) and following the manufacturer's instructions. Absorbances were measured using a Microplate Reader (Model 680, Bio-Rad
Laboratories, Hercules, CA, USA) at 490nm. Viability of treated cells is expressed as a percentage
relative to cells incubated with culture medium.

284

285 Statistical assessment

Blockades of viral infectivity are expressed as mean $\% \pm$ standard deviation (SD). Where possible, 286 anti-viral effective concentration (EC₅₀) values were calculated by regression analysis using the dose-287 response curves generated from the experimental data, using PRISM 4 (GraphPad Software, San 288 Diego, CA, U.S.A.). The 50% cytotoxic concentration (CC₅₀) was determined using logarithmic 289 viability curves. Where possible, a selectivity index (SI) was calculated dividing the CC_{50} by the EC_{50} 290 value. One-way ANOVA, followed by Bonferroni test, was used to assess the statistical significance 291 of the differences between treated and untreated samples. EC₅₀ values were compared using the sum-292 of-squares F test. Significance was set at the 95% level. 293

- 294
- 295

296 **RESULTS**

297 1D gel electrophoresis

Figure 1 displays the SDS-PAGE profile of colostrum proteins (reducing conditions) relative to the 298 following samples: colostrum from non-immunized (lane 2) and immunized (lane 3) cows and the 299 corresponding defatted / casein-depleted / dialyzed / filtered samples (lanes 4 and 5). As expected, all 300 the protein profiles show two intense bands at ca. 50 and 25 kDa, representing the heavy and light 301 302 IgG chains, respectively. The other bands can be ascribable to the classical set of high-abundance species normally found in just about any type of milk of animal origin (although in colostrum IgGs 303 304 alone represent ca. 80% of the total protein mass), including: α-lactalbumin (14.1 kDa), βlactoglobulin (19.9 kDa), serum transferrin (77.7 kDa) and a2-macroglobulin (167.5 kDa). As 305 306 expected, the protein patterns relative to colostrum samples differ in respect to the corresponding defatted / casein-depleted / dialyzed / filtered ones, by a significant reduction of the bands attributable 307 308 to the caseins (lane 2/3, figure 1).

309

310 Bovine IgG purification by affinity chromatography

Purification of IgG from colostrum collected from non-immunized and immunized cows was achieved by using a preparative affinity chromatography system based on protein G from Streptococci which is highly selective against the bovine IgG.

The affinity chromatogram trace, recorded at 280nm, displayed two peaks: the first, not retained, corresponding to the non-immunoglobulin protein fraction, and the second to the IgG fraction (Figure 2 - panel a) whose peak accounts for the 80%. The electrophoretic patterns obtained for the two collected fractions (Figure 2 - panel b), indicate a good depletion of immunoglobulins, whose characteristic bands at 160-50-25 kDa, are very weak in the aliquot eluted within the first peak, and clearly much more intense in the fraction of the second peak corresponding to the immunoglobulins.

320

321 SEC-UV for IgG analysis

A SEC-UV method was then optimized to achieve a reproducible separation of the most abundant analytes in bovine colostrum; the method was then applied to verify the purity of the fractions obtained: the non-immunoglobulin protein fraction and the IgG eluted fraction.

Figure 3 represents typical chromatograms recorded by setting the UV detector at 280nm and relative to the IgG-depleted (panel a) and IgG-enriched (panel b) fractions, each spiked with the internal standard TH (0.3mM). The typical retention times for IgG and the dipeptide TH are 9.15min and 11.57min, respectively.

The peak relative to IgG is well evident only in the IgG enriched fraction and absent in the depleted IgG fraction which is characterized by other peaks such as those relative to IgM and betalactoglobulin

332

333 Antiviral activity assessment

After their biochemical characterization, colostra and IgG samples were tested for antiviral activity. 334 The results summarized in table 2 clearly show that nor NHBC neither HBC are endowed with 335 antiviral activity against human pathogens such as HSV-1, HSV-2, HCMV, and HRhV. Not 336 surprisingly, NHBC is effective against bovine pathogens such as BRoV NCDV ($EC_{50}=61.5\mu g$) 337 protein/ml) and VSV (EC₅₀=64µg protein/ml) (table 2). Of note, NHBC shows non-strain restricted 338 antiviral efficacy against the human RoV strains Wa ($EC_{50}=2.3\mu g$ protein/ml), HRV248 ($EC_{50}=4.2\mu g$ 339 protein/ml), and HRV408 (EC₅₀=12.6µg protein/ml) with percentages of inhibition to maxima of 340 100% (figure 4). To test the hypothesis that colostrum from cows immunized with a veterinary anti 341

BRoV vaccine may exert a higher anti-HRoV activity than the one from non immunized cows, a 342 second set of antiviral assays was performed. As expected, HBC exerts a significantly (p_{Ftest}<0.0001) 343 higher antiviral activity against BRoV NCDV ($EC_{50}=5.5\mu g$ protein/ml), if compared with the one of 344 non-immunized cows (table 2). This result indicates that vaccination elicited an anti-BRoV immune 345 response. Notably, HBC is significantly (0.0001pFtest<0.0005</pre>) more effective than NHBC also 346 against Wa (EC₅₀=0.3µg protein/ml), HRV248 (EC₅₀=1.6µg protein/ml), and HRV408 (EC₅₀=2.1µg \pm 347 protein/ml) human strains (table 2), suggesting a high titer of cross-reactive IgG in HBC. Therefore, 348 a third set of experiments was performed to test the presence of anti-HRoV IgG in HBC-derived IgGs. 349 350 The results shown in table 3 and figure 5 demonstrate that these IgGs inhibit the infectivity of BRoV strain NCDV ($EC_{50}=6.2\mu g$ protein/ml), but more interestingly are endowed with a strong antiviral 351 352 activity also against HRoV strains Wa (EC₅₀=1.9µg protein/ml), HRV248 (EC₅₀=0.7µg protein/ml), and HRV408 (EC₅₀=1.8µg protein/ml) with percentages of inhibition to maxima of 100%. As a 353 354 negative control to IgG antiviral assays, we treated cells with equal volumes of IgG-depleted ammonium sulfate supernatant; results shown in table 3 and figure 5 clearly show that the IgG-355 356 depleted ammonium sulfate supernatant is not endowed with a significant antiviral activity. Notably, treatment of the different cell lines with NHBC, HBC and IgG do not affect cell viability even at high 357 358 concentrations, showing that the antiviral activity we observed is not ascribable to non-specific cytotoxic effects. 359

Mechanism of action experiments show that these IgGs neutralize virus infectivity by significantly ($0.0103 < p_{ANOVA} < 0.0155$) inhibit RoV-cell binding (figure 6, panels A, B, and C). More importantly, the significant ($0.0005 < p_{ANOVA} < 0.0183$) neutralization of viral infectivity observed in virus inactivation assays (figure 6, panel D, E, and F) is consistent with a neutralizing activity of virusspecific antibodies targeting the RoV surface antigens, rather than cellular receptors.

365 366

367 **DISCUSSION**

The supportive properties of BC when consumed by other mammalian species, including pigs and humans, are well documented in the medical literature (Boudry et al., 2007; Bridger & Brown, 1981; Gopal & Gill, 2000; He et al., 2001; Pakkanen & Aalto, 1997; Solomons, 2002; Struff and Sprotte, 2007; Uruakpa et al., 2002). Emerging evidences indicate BC as a promising nutraceutical which can prevent or mitigate various diseases in newborns and adults (Bagwe et al., 2015), in particular gastrointestinal infections. Consistently with these findings, in this study we confirmed the protective activity of NHBC against HRoV, which is well documented in literature (Inagaki et al., 2010; Inagaki et al., 2013). Of note, NHBC shows antiviral activity also against several HRoV strains and, as
expected, against BRoV strain NCDV, at concentrations comparable to the ones previously showed
(Inagaki et al., 2013).

In our experimental setting NHBC showed no significant antiviral activity against three different viral pathogens, namely HRhV, HCMV, HSV-1, and HSV-2. These results show that BC is not a "broad spectrum antiviral", but it rather exerts specific antiviral activities. It is likely that this antiviral specificity and potency most probably reflects the immunological status of the animal.

Boosting the natural concentrations of immune components in colostrum and milk through 382 383 vaccination of cows offers great potential for their use as prophylactic or therapeutic products in humans. HBC from cows vaccinated with HRoV showed to be an effective therapeutic in reducing 384 the duration and severity of RoV-caused diarrhea in a double-blind controlled clinical study with 385 infants of 6 to 24 months of age (Mitra et al., 1995). In a second study, Davidson and colleagues 386 produced a HBC by introducing a vaccine containing four HRoV into pregnant Freisian cows. They 387 demonstrated that this HBC administered orally mediated protection by preventing infection of HRoV 388 infection (Davidson et al., 1989). Efficacy of passive immunization with HBC-derived IgG is 389 documented in literature. Hilpert and colleagues (Hilpert et al., 1987) treated infants hospitalized with 390 acute diarrhea with anti-rotavirus immunoglobulin concentrate without a significant decrease in 391 392 duration of diarrhea or excretion of virus, while Turner and colleagues demonstrated a reduction in incidence and duration of diarrhea in treated infants (Turner and Kelsey, 1987). In 1998, Sarker and 393 394 colleagues produced a HBC by immunizing pregnant cows with HRoV strains, i.e. Wa, RV3, RV5 and ST3, representing serotypes 1 to 4, respectively. HBC-purified IgGs were then administered in a 395 396 double blind placebo-controlled trial to children with diagnosed HRoV diarrhea (Sarker et al., 1998). Children who received HBC-IgGs had significantly less daily and total stool output and stool 397 398 frequency and required a smaller amount of oral rehydration solution than did children who received 399 placebo. However, it is difficult to generate large-scale amounts of HBC by immunizing cows with a 400 non-scheduled and thus non-conventional HRoV vaccine; indeed, the yield is not enough to 401 successfully cover the global requirement since over 500,000 deaths occur across the world every 402 year due to rotavirus-induced diarrhoea (Bagwe et al., 2015). To overcome these limitations we put forward and tested the hypothesis that HBC from cows immunized with a conventional veterinary 403 404 vaccine against BRoV would exert a higher anti HRoV activity compared to NHBC due to a high titer of cross-reactive anti HRoV IgG. Indeed this study confirms this hypothesis and propose an 405 easier and cheaper approach for the production anti-HRoV HBC or IgG in cows. Our results show an 406 in vitro HRoV inhibition efficacy (i.e. EC50) of HBC comparable with the one that Inagaki and 407

colleagues obtained by treating MA104 cells with skimmed and concentrated bovine late colostrum
(SCBLC) from HRoV-immunized cows (Inagaki et al., 2010; Inagaki et al., 2013). Notably, we
demonstrated that HBC and its IgGs can inhibit the infectivity of four RoVs having four different GP
genotypes (Table 1), thus suggesting that vaccination with BRoV stimulates the production of crossreactive neutralizing antibodies.

With a view to exploit HBC as source of anti-HRoV IgG, quality assessment procedures would be necessary in order to monitor the IgG content; nevertheless, these techniques are already available and well documented in literature (Altomare et al., 2016b). Moreover, cows produce BC in large excess respective to the amount needed to feed their calves (cows produce about 33 liters of colostrum each day in the first days after parturition, while just 4-6 liters per day are administered to the calf

418 during the first two days) (Devery-Pocius and Larson, 1983).

Overall, this study demonstrate that the conventional BRoV vaccine is sufficient to boost the antiHRoV protective efficacy of BC. This is by itself a conservative, feasible, and not yield-limiting
approach to produce HBC exploitable as a functional food to prevent and treat HRoV infections.

423 ACKOWLEDGEMENTS

- 424 This work was supported by a grant from "Ricerca finanziata dall'Università degli Studi di Torino"
- 425 (Grant number: RILO15).

426

428 **References**

- ^aAltomare, A., Regazzoni, L., Parra, X. M. P., Selmin, F., Rumio, C., Carini, M., and G. Aldini. 2016.
 Set-up and application of an analytical approach for the quality control of purified colostrum as
 food supplement. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 1028:130-144.
- 432 ^bAltomare A., Fasoli E., Colzani M., Paredes Parra X. M., Ferrari M., Cilurzo F., Rumio C.,
- 433 Cannizzaro L., Carini M., Righetti P. G., and G. Aldini. 2016. An in depth proteomic analysis
- based on ProteoMiner, affinity chromatography and nano-HPLC-MS/MS to explain the potential
- health benefits of bovine colostrum. J. Pharm. Biomed. Anal. 121:297-306.
- Babji, S., and G. Kang. 2012. Rotavirus vaccination in developing countries. Curr. Opin. Virol.
 2:443–448.
- Bagwe, S., Tharappel, L. J., Kaur, G., and H. S. Buttar. 2015. Bovine colostrum: an emerging
 nutraceutical. J. Complement. Integr. Med. 12:175-185.
- Barrington, G. M., Besser, T. E., Davis, W. C., Gay, C. C., Reeves, J. J., and T. B. McFadden. 1997.
 Expression of immunoglobulin G1 receptors by bovine mammary epithelial cells and mammary
 leukocytes. J. Dairy Sci. 80:86–93.
- Benson, K. F., Carter, S. G., Patterson, K. M., Patel, D., and G. S. Jensen. 2012. A novel extract from
 bovine colostrum whey supports anti-bacterial and anti-viral innate immune functions in vitro and
 in vivo: I. Enhanced immune activity in vitro translates to improved microbial clearance in animal
 infection models. Prev. Med. 54:S116-123.
- Binder, H. J., Brown, I., Ramakrishna, B. S., and G. P. Young. 2014. Oral rehydration therapy in the
 second decade of the twenty-first century. Curr. Gastroenterol. Rep. 16:376–384.
- Bojsen A., Buesa J., Montava R., Kvistgaard A. S., Kongsbak M. B., Petersen T. E., Heegaard C. W.,
 and J. T. 2007. Rasmussen. Inhibitory activities of bovine macromolecular whey proteins on
 rotavirus infections in vitro and in vivo. J. Dairy Sci. 90:66-74.
- Boudry, C., Buldgen, A., Portetelle, D., Collard, A., Thewis, A., and J. P. Dehoux. 2007. Effects of
 oral supplementation with bovine colostrum on the immune system of weaned piglets. Res. Vet.
 Sci. 83:91–101.
- Bridger, J. C., and J. F. Brown. 1981. Development of immunity to porcine rotavirus in piglets
 protected from disease by bovine colostrum. Infect. Immun. 31:906–910.

Bush, L. J., and T. E. Staley. 1980. Absorption of colostral immunoglobulins in newborn calves. J.
Dairy Sci. 63:672–680.

Byakwaga, H., Kelly, M., Purcell, D. F., French, M.A., Amin, J., Lewin, S. R, Haskelberg, H.,
Kelleher, A.D., Garsia, R., Boyd, M.A., Cooper, D.A., and S. Emery. 2011. CORAL Study Group.
Intensification of antiretroviral therapy with raltegravir or addition of hyperimmune bovine
colostrum in HIV-infected patients with suboptimal CD4+ T-cell response: a randomized
controlled trial. J. Infect. Dis. 204:1532-1540.

Cagno, V., Civra, A., Rossin, D., Calfapietra, S., Caccia, C., Leoni, V., Dorma, N., Biasi, F., Poli, G.,
and D. Lembo. 2017. Inhibition of herpes simplex-1 virus replication by 25-hydroxycholesterol
and 27 hydroxycholesterol. Redox Biol. 12:522-527.

467 Civra, A., Cagno, V., Donalisio, M., Biasi, F., Leonarduzzi, G., Poli, G., and D. Lembo. 2014.
468 Inhibition of pathogenic non-enveloped viruses by 25-hydroxycholesterol and 27469 hydroxycholesterol. Sci. Rep. 4:7487.

- Civra, A., Giuffrida, M. G., Donalisio, M., Napolitano, L., Takada, Y., Coulson, B. S., Conti, A., and
 D. Lembo. 2015. Identification of Equine Lactadherin-derived Peptides That Inhibit Rotavirus
 Infection via Integrin Receptor Competition. J. Biol. Chem. 290:12403-12414.
- 473 Cohen, S. M. 2006. Jaundice in the full-term newborn. Pediatr. Nurs. 32:202–208.
- 474 Das, J. K., Salam, R. A., and Z. A. Bhutta. 2014. Global burden of childhood diarrhea and
 475 interventions. Curr. Opin. Infect. Dis. 27:451–458.
- Davidson, G. P., Whyte, P. B., Daniels, E., Franklin, K., Nunan, H., McCloud, P. I., Moore, A. G.,
 and D. J. Moore. 1989. Passive immunisation of children with bovine colostrum containing
 antibodies to human rotavirus. Lancet. 2:709-712.
- 479 Devery-Pocius, J. E., and B. L. Larson. 1983 Age and previous lactations as factors in the amount of
 480 bovine colostral immunoglobulins. J. Dairy Sci. 66:221-226.
- Ebina, T., Ohta, M., Kanamaru, Y., Yamamoto-Osumi, Y., and K. Baba. Passive immunizations of
 suckling mice and infants with bovine colostrum containing antibodies to human rotavirus. 1992.
 J. Med. Virol. 38:117–123.

- El-Fakharany, E. M., Uversky, V. N., and E. M. Redwan. 2017. Comparative Analysis of the Antiviral
 Activity of Camel, Bovine, and Human Lactoperoxidases Against Herpes Simplex Virus Type 1.
 Appl. Biochem. Biotechnol. 182:294-310.
- Gaspar, H. B., Hammarström, L., Mahlaoui, N., Borte, M., and S. Borte. 2014. The case for
 mandatory newborn screening for severe combined immunodeficiency (SCID). J. Clin. Immunol.
 34:393–397.
- Glass, R. I., Parashar, U., Patel, M., Gentsch, J., and B. Jiang. 2014. Rotavirus vaccines: Successes
 and challenges. J. Infect. 68:9–18.
- Gopal P. K., and H. S. Gill. 2000. Oligosaccharides and glycoconjugates in bovine milk andcolostrum. Br. J. Nutr. 84:S69-74.
- Hammarström, L., and C. K. Weiner. 2008. Targeted antibodies in dairy-based products. Adv. Exp.
 Med. Biol. 606:321–343.
- He, F., Tuomola, E., Arvilommi, H., and S. Salminen. 2001. Modulation of human humoral immune
 response through orally administered bovine colostrum. FEMS Immunol. Med. Microbiol. 31:93–
 96.
- Hilpert, H., Brüssow, H., Mietens, C., Sidoti, J., Lerner, L., and H. Werchau. 1987. Use of bovine
 milk concentrate containing antibody to rotavirus to treat rotavirus gastroenteritis in infants. J.
 Infect. Dis. 156:158-166.
- Inagaki, M., Yamamoto, M., Xijier, Cairangzhuoma, Uchida, K., Yamaguchi, H., Kawasaki, M.,
 Yamashita, K., Yabe, T., and Y. Kanamaru. 2010. In vitro and in vivo evaluation of the efficacy
 of bovine colostrum against human rotavirus infection. Biosci. Biotechnol. Biochem. 74:680-682.
- Inagaki, M., Yamamoto, M., Cairangzhuoma, Xijier, Yabe, T., Uchida, K., Kawasaki, M., Nakagomi,
 T., Nakagomi, O., Minamoto, N., and Y. Kanamaru. 2013. Multiple-dose therapy with bovine
 colostrum confers significant protection against diarrhea in a mouse model of human rotavirus induced gastrointestinal disease. J. Dairy Sci. 96:806-814
- Inagaki, M., Muranishi, H., Yamada, K., Kakehi, K, Uchida, K., Suzuki, T., Yabe, T., Nakagomi, T.,
 Nakagomi, O., and Y. Kanamaru. 2014. Bovine κ-casein inhibits human rotavirus (HRV) infection
 via direct binding of glycans to HRV. J. Dairy Sci. 97:2653-2661.

- 512 Kelly, G. S. 2003. Bovine colostra: a review of clinical uses. Alt. Med. Rev. 8:378 394.
- Korhonen, H., Marnila, P., and H. S. Gill. 2000. Bovine milk antibodies for health. Br. J. Nutr.
 84:S135–S146.
- ^aKramski, M., Center, R. J., Wheatley, A. K., Jacobson, J. C., Alexander, M.R., Rawlin, G., and D.
 F. Purcell. 2012. Hyperimmune bovine colostrum as a low-cost, large-scale source of antibodies
 with broad neutralizing activity for HIV-1 envelope with potential use in microbicides.
 Antimicrob. Agents Chemother. 56:4310-4319.
- ^bKramski, M., Lichtfuss, G. F., Navis, M., Isitman, G., Wren, L., Rawlin, G., Center, R. J.,
 Jaworowski, A., Kent, S. J., and D. F. Purcell. 2012. Anti-HIV-1 antibody-dependent cellular
 cytotoxicity mediated by hyperimmune bovine colostrum IgG. Eur. J. Immunol. 42:2771-2781.
- Larson, B. L., Heary, H. L., and J. E. Devery. 1980. Immunoglobulin production and transport by the
 mammary gland. J. Dairy Sci., 63:665–671.
- 524 Macy, I.G. 1949. Composition of human colostrum and milk. Am. J. Dis. Child. 78:589–603.
- Majumdar, A. S., and A. C. Ghose. 1982. Protective properties of anti-cholera antibodies in human
 colostrum. Infect. Immun. 36:962–965.
- Marcotte, H., and L. Hammarström. 2016. Immunodeficiencies: Significance for gastrointestinal
 disease. Pages 47-72 in Viral gastroenteritis. Molecular epidemiology and pathogenesis. L.
- 529 Svensson, U. Desselberger, H. B. Greenberg, and M. K. Estes, ed Academic Press, London, UK.
- 530 Mehra, R. 2006. Milk Ig for health promotion. Int. Dairy J. 16:1262–1271.
- Mitra, A. K., Mahalanabis, D., Ashraf, H., Unicomb, L., Eeckles, R., and S. Tzipori. 1995.
 Hyperimmune cow colostrum reduces diarrhoea due to rotavirus: A double-blind, controlled
 clinical trial. Acta Paediatr. 84:996.
- Moore, M., Tyler, J. W., Chigerwe, M., Dawes, M. E, and J. R. Middleton, J. R. 2005. Effect of
 delayed colostrum collection on colostral IgG concentration in dairy cows. J. Am. Vet. Med.
 Assoc. 226:1375–1377.
- Morris, J. A., Wray, C., and W. J. Sojka. 1980. Passive protection of lambs against enteropathogenic
 Escherichia coli: role of antibodies in serum and colostra. J. Med. Microbio. 13:265–271.

- Ng, W. C., Wong, V., Muller, B., Rawlin, G., and L. E. Brown. 2010. Prevention and treatment of
 influenza with hyperimmune bovine colostrum antibody. PLoS One. 5:e13622.
- Ogra, S. S., and P. L. Ogra. 1978. Immunological aspects of human colostrum and milk. J. Pediatr.
 92:550–555.
- Pakkanen, R., J. Aalto. 1997. Growth factors and antimicrobial factors in bovine colostrum. Int. Dairy
 J. 7:285–297.
- Parrón, J. A., Ripollés, D., Sánchez, A. C., Pérez, M. D., Calvo, M., López, S., Arias, C. F., and L.
 Sánchezm. 2018. Antirotaviral activity of bovine milk components: Extending the list of inhibitory
 proteins and seeking a better understanding of their neutralization mechanism. J. Funct. Foods,
 44:103-111.
- Payne, D. C., Boom, J. A., Staat, M. A., Edwards, K. M., Szilagyi, P. G., Klein, E. J., and U. D.
 Parashar. 2013. Effectiveness of pentavalent and monovalent rotavirus vaccines in concurrent use
 among US children<5 years of age, 2009–2011. Clin Infect Dis. 57:13–20.
- Playford, R. J., Macdonald, C. E., and W. S. Johnson. 2000. Colostrum and milkderived peptide
 growth factors for the treatment of gastrointestinal disorders. Am. J. Clin. Nutr. 72:5–14.

Rahman, S., Higo-Moriguchi, K., Htun, K. W., Taniguchi, K., Icatlo, F. C. Jr., Tsuji, T., Kodama, Y.,
Nguyen, S. V., Umeda, K., Oo, H. N., Myint, Y. Y., Htut, T., Myint, S. S., Thura, K., Thu, H. M.,
Dwi Fatmawati, N.N., and K. Oguma. 2012. Randomized placebo-controlled clinical trial of
immunoglobulin Y as adjunct to standard supportive therapy for rotavirus-associated diarrhea
among pediatric patients. Vaccine. 30:4661–4669.

- Rainard P., and C. Riollet. 2006. Innate immunity of the bovine mammary gland. Vet. Res. 37:369400.
- Sacerdote P., Mussano F., Franchi S., Panerai A. E., Bussolati G., Carossa S., Bartorelli A., and B.
 Bussolati. Biological components in a standardized derivative of bovine colostrum. J Dairy Sci.
 96:1745-1754.
- Sarker, S. A., Casswall, T. H., Mahalanabis, D., Alam, N. H., Albert, M. J., Brussow, H., Fuchs, G.
 J., and L. Hammerstrom. 1998. Successful treatment of rotavirus diarrhea in children with
 immunoglobulin from immunized bovine colostrum. Pediatr. Infect. Dis. J. 17:1149–1154.

- Smolenski, G., Haines, S., Kwan, F. Y., Bond, J., Farr, V., Davis, S. R., Stelwagen, K., and T. T.
 Wheeler. 2007. Characterisation of host defence proteins in milk using a proteomic approach. J.
 Proteome Res. 6:207–215.
- Solomons, N. W. 2002. Modulation of the immune system and the response against pathogens with
 bovine colostrum concentrates. Eur. J. Clin. Nutr. 56:S24–S28.
- Stelwagen, K., Carpenter, E., Haigh, B., Hodgkinson, A., and T. T. Wheeler. 2009. Immune
 components of bovine colostrum and milk. J. Anim. Sci. 87:3–9.
- Stephan, W., Dichtelmuller, H., and R. Lissner. 1990. Antibodies from colostrum in oral
 immunotherapy. J. Clin. Chem. Clin. Biochem. 28:19–23.
- 576 Struff, W. G., and G. Sprotte. 2007. Bovine colostrum as a biologic in clinical medicine: a review.
- Part I: biotechnological standards, pharmacodynamic and pharmacokinetic characteristics and
 principles of treatment. Int. J. Clin. Pharmacol. Ther. 45:193–202.
- 579 Struff, W. G., and G. Sprotte. 2008. Bovine colostrum as a biologic in clinical medicine: a review—
 580 part II: clinical studies. Int. J. Clin. Pharmacol. Ther. 46:211–225.
- Tate, J. E., Burton, A. H., Boschi-Pinto, C., and U. D. Parashar. 2016. Global, regional, and national
 estimates of rotavirus mortality in children<5 years of age, 2000–2013. Clin. Infect. Dis. 62:96–
 105.
- 584 Tharpa, B. 2005. Health factors in colostrum. Ind. J. Pediatr. 72:579.
- Tokuyama, H., Tokuyama, Y., and S. Migita. 1990. Isolation of two new proteins from bovine
 colostrum which stimulate epidermal growth factor-dependent colony formation of NRK-49f cells.
 Growth Factors. 3:105–114.
- Turner, R. B., and D. K. Kelsey. Passive immunization for prevention of rotavirus illness in healthy
 infants. 1993. Pediatr. Infect. Dis. J. 12:718-722.
- 590 Uruakpa, F. O., Ismond, M. A. H., and E. N. T. Akobundu. 2002. Colostrum and its benefits: a review.
 591 Nutr. Res. 22:755–767.
- 592

Strain	Origin	Serotype
Wa	Human	G1(P8)
HRV 408	Human	Natural reassortant G3(P?)
HRV 248	Human	Natural reassortant G4(P4)
NCDV	Bovine	G6(P1)

Table 1. Rotavirus strains used in this study.

597 (P?) means P genotype is unknown

598 The characteristics of the rotavirus strains are from Parrón et al., 2017 and Rahman et al., 2012.

	Virus	EC50 (µg/ml) (95% С.І.)	EC90 (µg/ml) (95% C.I.)	CC50 (µg/ml)	SI
Bovine not immune					
colostrum	HSV-1	n.a.	n.a.	> 6680	-
	HSV-2	n.a	n.a	> 6680	-
	HCMV	n.a.	n.a.	> 6680	-
	HRhV	n.a.	n.a.	> 6680	-
	HRoV (#Wa)	2.3 (1.6-3.5)	16.3 (6.8-39.1)	> 6680	>2855
	HRoV (#HRV 408)	12.6 (8.6-18.7)	90 (38-215)	> 6680	>529
	HRoV (#HRV 248)	4.2 (2.4-7.3)	134 (39-454)	> 6680	>1591
	BRoV (#NCDV)	61 (44-86)	143 (97-211)	> 6680	>109
	VSV	64 (55-75)	343 (252-467)	> 6680	> 104
Bovine hypermmune					
colostrum	HSV-1	n.a	n.a.	>7140	-
	HSV-2	6018 (2560-14110)	n.a.	>7140	> 1.18
	HCMV	n.a.	n.a.	>7140	-
	HRhV	n.a.	n.a.	> 7140	-
	HRoV (#Wa)	0.3 (0.3-0.5)	4.5 (2.4-8.5)	> 7140	>21000
	HRoV (#HRV 408)	2.1 (1.7-2.6)	7.6 (4.7-12.5)	> 7140	>3449
	HRoV (#HRV 248)	1.6 (1.2-2.8)	8.1 (5.6-11.6)	> 7140	>4519
	BRoV (#NCDV)	5.5 (3.7-8.2)	51 (21-122)	>7140	>1301
	VSV	89 (60-132)	489 (200-1198)	>7140	>80

6@able 2. Antiviral activities of BCs.

603

604 erpes simplex type 1 (HSV-1); herpes simplex type 2 (HSV-2); human cytomegalovirus (HCMV); human 60 finovirus (HRhV); human rotavirus (HRoV); bovine rotavirus (BRoV); vesicular stomatitis virus (VSV). EC₅₀ half-60 faximal effective concentration; CI confidence interval; EC₉₀ 90% effective concentration; CC₅₀ half maximal 60 y totoxic concentration; SI selectivity index; n.a. not assessable 608

	Virus	EC50 (µg/ml) (95% C.I.)	EC90 (µg/ml) (95% C.I.)	CC50 (µg/ml)	SI
IgG	HRoV (#Wa) HRoV (#HRV 408) HRoV (#HRV 248)	1.9 (1.4-2.6) 1.8 (0.8-3.9) 0.7 (0.6-0.9)	19.7 (10.4-37.1) 12.6 (2.5-63.2) 8.2 (4.9-13.9)	> 5910 > 5910 > 5910	> 3110 > 3283 > 8443
	BRoV (#NCDV)	6.2 (5.2-7.4)	30.2 (19.7-49.0)	> 5910	> 953
AS supernatant	HRoV (#Wa) HRoV (#HRV 408) HRoV (#HRV 248) BRoV (#NCDV)	n.a. n.a. n.a. n.a.	n.a. n.a. n.a. n.a.	> 5910 > 5910 > 5910 > 5910 > 5910	n.a. n.a. n.a. n.a.

609 Table 3. Antiviral activities of IgGs from HBC.

610

611 Herpes simplex type 1 (HSV-1); herpes simplex type 2 (HSV-2); human cytomegalovirus (HCMV);

human rhinovirus (HRhV); human rotavirus (HRoV); bovine rotavirus (BRoV); vesicular stomatitis

virus (VSV). EC₅₀ half-maximal effective concentration; CI confidence interval; EC₉₀ 90% effective

614 concentration; CC₅₀ half maximal cytotoxic concentration; SI selectivity index; n.a. not assessable

615



618 Figure 1.





Figure 2.





Figure 4.









Figure 6.

641 Legends

Figure 1. SDS-PAGE profiling of colostrum proteins. Lane 1. Precision Plus Protein Standards
(BIORAD); lane 2. Untreated - Bovine Colostrum; lane 3. Untreated Hyperimmune Bovine
Colostrum; lane 4. Bovine Colostrum Defatted/Casein-depleted/Dialyzed/Filtered; lane 5.
Hyperimmune Bovine Colostrum Defatted/Casein-depleted/Dialyzed/Filtered.

Figure 2. IgG purification by affinity chromatography. Panel a) FPLC-UV chromatogram of the untreated-Bovine Colostrum (casein-depleted and defatted bovine colostrum) obtained by using protein G as affinity stationary phase; peaks 1 and 2 are attributed to the IgG-depleted and IgG fractions as demonstrated by the SDS-gel electrophoresis patterns. Panels b) reports the gel electrophoretic pattern of peak 1 (lane 3) and 2 (lane 4) compared to the untreated bovine colostrum pattern, run in reducing conditions. The characteristic bands at 25 and 50 Da are well evident in peak 2 and only in negligible amounts in peak 1.

Figure 3. IgG analysis by SEC-UV; panels a) shows the SEC-UV chromatogram of IgG - Depleted
Bovine Colostrum sample, using TH as internal standard; panel b) shows the SEC-UV chromatogram
of IgG purified fraction from bovine colostrum.

Figure 4. Antiviral activity of colostrum from immunized cows (HBC, solid circles) and colostrum from non-immunized cows (NHBC, open squares) against HRoV strains Wa (A), HRV408 (B), HRV248 (C), and NCDV (D) on MA104 cells. Cells were treated for 2h with increasing concentrations of colostra and then infected in presence of colostrum. Viral infections were detected as described in the Material and Methods section. The percentage infection was calculated by comparing treated and untreated wells. The results are means and SEM for duplicates.

Figure 5. Antiviral activity of hyperimmune colostrum (HBC)-derived immunoglobulin G (IgG, solid diamonds) or ammonium sulfate (AS, open diamonds) supernatant against HRoV strains Wa (A), HRV408 (B), HRV248 (C), and NCDV (D) on MA104 cells. Viral infections were detected as described in the Material and Methods section. The percentage infection was calculated by comparing treated and untreated wells. The results are means and SEM for duplicates.

Figure 6. Mechanism of action of HBC-derived IgGs. Panels A, B, and C show the effect of HBCderived IgGs respectively on Wa, HRV248, and NCDV binding to the MA104 cell surface. Panels
D, E, and F show the results of virus inactivation assays on infectious particles respectively of Wa,
HRV248, and NCDV. On the y axis, the Wa infectious titers are expressed as focus-forming units per

- 671 ml (FFU/ml). On the y axis, the infectious titer of Wa bound to cells is expressed as a % of the titer
- bound to control untreated MA104 cells. Error bars represent the SEM of independent experiments.
- 673 *panova<0.05 **panova<0.001 ***panova<0.0001