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

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1 **Interpretive summary**

2 Human rotaviruses (HRoV) are a major cause of severe diarrheal disease in infants and young
3 children. Whilst the vaccination of pregnant cows with HRoV boosts the release of HRoV-specific
4 IgGs in bovine colostrum (BC), it raises regulatory and safety issues. In this study, we demonstrate
5 that the conventional BRoV vaccine is sufficient to enhance the anti-HRoV protective efficacy of BC,
6 thus providing a conservative approach to produce hyperimmune BC, making it exploitable as a
7 functional food.

8

9 **HYPERIMMUNE BOVINE COLOSTRUM: A NOVEL APPROACH**

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

14

15

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38

39 **ABSTRACT**

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

55

56 INTRODUCTION

57 Viral gastroenteritis represents a relevant economic and public health burden, causing high morbidity
58 and mortality rates, mainly in the poorest countries (Das, Salam, & Bhutta, 2014). Human rotaviruses
59 (HRoVs) are a major cause of severe diarrheal disease in infants and young children, and the second
60 cause of death in children less than 5 years old (Marcotte & Hammarström, 2016). Since no specific
61 antiviral drug is available, the conventional treatment for HRoV acute gastroenteritis is largely
62 symptomatic and involves fluid and electrolyte replacement and maintenance of nutrition. Despite
63 the introduction of oral HRoV vaccines that significantly reduced the incidence of the disease in
64 developed countries (Payne et al., 2013), the impact of this active prophylaxis on global estimates of
65 HRoV mortality has been limited (Tate et al., 2016). The reason is mainly ascribable to the inadequate
66 immunization coverage in lower income countries, where the burden of diarrheal disease is higher,
67 and vaccines are mostly needed. As a matter of fact, oral vaccines are less immunogenic when given
68 to infants in low-income compared with high-income countries, due to transplacental maternally-
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
75 prophylactic and therapeutic approaches to prevent and control HRoV gastroenteritis disease,
76 especially for immunocompromised hosts.

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

81 More importantly, colostrum provides neonates with the essential passive immunity against infectious
82 diseases (Ogra and Ogra, 1978; Cohen, 2006; Morris et al., 1980; Ebina et al., 1992; Majumdar and
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 &
85 Riollet, 2006). In ruminants no transplacental exchange of immune factors occurs in utero, therefore
86 colostrum and, to a lesser extent, mature milk provide protection through a high immunoglobulin (Ig)
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

89 colostrum and milk varies among species, with IgA being the predominant Ig in human mammary
90 secretions. By contrast, in cow's colostrum IgG1 is the most represented (Barrington et al., 1997),
91 while IgA and IgM are present at much reduced concentrations. The BC Igs, in conjunction with the
92 ability of the ruminant neonatal gut to allow unrestricted passage of the large Ig molecules, provide
93 the young animal with passive immunization (Bush & Staley, 1980; Moore et al., 2005).

94 Although the effect of colostrum is species-specific, a growing body of literature documented the
95 protective effect of BC against several viral infections in humans (Benson et al., 2012; Ng et al., 2010;
96 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 pathogen-
98 specific antibodies in BC. The antibodies purified from this hyperimmune BC (HBC) have
99 successfully been exploited for the treatment of a variety of gastrointestinal infections caused by
100 pathogenic bacteria (Hammarström and Weiner, 2008; Kelly, 2003; Playford et al., 2000; Struff and
101 Sprotte, 2008) or virus (Korhonen et al., 2000; Mehra, 2006; Ng et al., 2010; Kramski et al., 2012a,b;
102 Byakwaga et al., 2011; Inagaki et al., 2010; Inagaki et al., 2013) indicating HBC as an alternative
103 source for low-cost virus-specific antibodies. These evidences, together with the high titer content of
104 antimicrobial peptides and proteins, such as lactoferrin, lactoperoxidase and lysozyme, that can
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
107 viral infections.

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

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

122

123

124 **MATERIALS AND METHODS**

125 ***Chemicals***

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

133

134 ***Bovine colostrum collection***

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

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

154 neutralized sample centrifuged at 8500 rpm and the supernatant retained. The next steps consisted of
155 clarification through 0:45 and 0:22 µm filters followed by lyophilisation.

156

157 ***Bovine IgG purification***

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

171 Tangential Flow Filtration –IgG enriched fractions as well as IgG depleted fractions obtained as
172 above reported were mixed and subjected to concentration and desalting using hollow fiber cross flow
173 filtration cartridges with 3000 NMWC (Nominal Molecular Weight Cutoff) and a surface area of 650
174 cm² (GE Healthcare) coupled to a tangential flow filtration system equipped with a peristaltic pump
175 essential to keep the flow recirculation continuous (Kross Flo®- Tangential flow Filtration System
176 Research III). The IgG and IgG-depleted fractions were concentrated 20/30 times, dialyzed with 5
177 volumes of water, filtered through 0.22 micron membranes in sterile conditions, and finally
178 lyophilized.

179

180 ***Separative methods of proteins on polyacrylamide gel***

181 One-dimensional analysis (SDS-PAGE) - Protein separation was performed under reducing
182 conditions; aliquots of 10µL of samples containing 20-25 µg of proteins were mixed with 10 µL of
183 Laemmli sample buffer containing 50 mM DTT and heated at 95°C for 5 minutes. Samples and the
184 standard proteins mixture (Precision Plus Protein Standards) were loaded on precast gels (Any KD
185™ Mini Protean® TGX™) and then placed in the electrophoresis cell (Mini-PROTEAN Tetra) and

186 run at 200V (constant) for a variable time of about 30-40 min. Staining was carried out using
187 Coomassie blue stain (Biosafe G250 Stain, Bio-Rad) and the images acquired by using the Bio-Rad
188 GS800 densitometer and analyzed by using the software quantity One 1-D.

189

190 ***IgG and IgM analysis by SEC-UV***

191 The IgG content of the IgG enriched and depleted fractions were then determined by a size exclusion
192 chromatography (SEC) according to the method reported by Altomare and colleagues (Altomare et
193 al., 2016a); SEC was performed on a Thermo Finnigan Surveyor HPLC system (ThermoFinnigan
194 Italia, Milan, Italy) equipped with a variable wavelength detector and an auto-sampler, controlled by
195 Xcalibur software (version 2.0.7 Thermo Fisher Scientific, Rodano, MI, Italy). The SEC separation
196 was performed on a 4.6 × 300 mm Phenomenex Yarra™ 3u SEC-3000, with a 4 × 3 mm GFC4000
197 pre-column, by running an isocratic flow of mobile phase containing 0.1M sodium phosphate bibasic,
198 0.025% sodium azide pH 6.8, at a constant flow rate of 0.5 mL/min. The autosampler temperature
199 was set at 8°C and UV detection was conducted at a wavelength of 280 nm, typical wavelength for
200 protein detection. Due to its low molecular weight, the dipeptide Tyrosine-Histidine (TH) was chosen
201 as internal standard (IS), since it marks the racing front, being the smallest analyte. All the samples
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
206 cells (ATCC® CCL-2™) were propagated in Dulbecco's modified Eagle's medium (DMEM) (Gibco-
207 BRL, Gaithersburg, MD) supplemented with heat-inactivated 10% fetal bovine serum (FBS) (Gibco-
208 BRL) and 1% antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin,
209 Germany), at 37°C in an atmosphere of 5% of CO₂. African green monkey kidney cells (Vero) (ATCC
210 CCL-81) were grown as monolayers in Eagle's minimal essential medium (MEM) (Gibco/BRL,
211 Gaithersburg, MD) supplemented with 10% FBS and 1% antibiotic-antimycotic solution. HRoV
212 strains Wa (ATCC® VR-2018), HRV 408 (ATCC® VR-2273), HRV 248 (ATCC® VR-2274), and
213 BRoV strain NCDV (ATCC® VR-1290) were activated with 5µg/ml of porcine pancreatic trypsin
214 type IX (Sigma) for 30 min at 37°C and propagated in MA104 cells using DMEM containing 0.5µg
215 of trypsin per ml as described previously (Civra et al., 2015). Viral titers are expressed as focus-
216 forming unit (FFU) per ml. A serologic characterization of RoVs exploited in this study is provided
217 in Table 1. Human rhinovirus (HRhV) 1A (ATCC® VR-1559), was propagated in HeLa cells, at

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

227

228 *Focus reduction assays*

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

243

244 *Plaque reduction assay*

245 HeLa or Vero cells were first seeded (at 8×10^4 cells/well) in 24 well plates. 24 hours later the HBC,
246 NHBC, or IgGs were serially diluted in medium (from 0.02 to 3340µg protein/ml) and added to cell
247 monolayers. After 2 hours of incubation (37°C, 5% CO₂), medium was removed and infection was
248 performed with 200 µL/well with HRhV 1A, VSV, HSV-1, HSV-2, or HCMV at a MOI of 0.0002
249 PFU/ml in presence of colostrum. 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 colostrum. 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***

258 IgG precipitated with ammonium sulphate at respective 90% effective concentration (EC₉₀) or equal
259 volume of culture medium or ammonium sulphate supernatant were added to 2x10⁵ FFU/ml of
260 trypsin-activated RoV suspension and mixed in a total volume of 200 µl. The virus-compound
261 mixtures were incubated for 1 hour at 37 °C then serially diluted to the non-inhibitory concentration
262 of test IgG, and the residual viral infectivity was determined as previously described (Civra et al.,
263 2014).

264

265 ***RoV-cell binding assay***

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

274

275 ***Cell viability assay***

276 Cells were seeded at a density of 5 x 10³/well in 96-well plates and treated the next day with HBC,
277 NHBC, IgGs or ammonium sulfate supernatant at concentrations ranging from 0.02 to 7140µg/ml to
278 generate dose-response curves. Control samples (100% of viability) were prepared by treating cells
279 with culture medium. After 24 or 72 hours of incubation, cell viability was determined using a
280 CellTiter 96 Proliferation Assay Kit (Promega, Madison, WI, USA) and following the manufacturer's

281 instructions. Absorbances were measured using a Microplate Reader (Model 680, Bio-Rad
282 Laboratories, Hercules, CA, USA) at 490nm. Viability of treated cells is expressed as a percentage
283 relative to cells incubated with culture medium.

284

285 *Statistical assessment*

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

294

295

296 **RESULTS**

297 *ID gel electrophoresis*

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

309

310 *Bovine IgG purification by affinity chromatography*

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

314 The affinity chromatogram trace, recorded at 280nm, displayed two peaks: the first, not retained,
315 corresponding to the non-immunoglobulin protein fraction, and the second to the IgG fraction (Figure
316 2 – panel a) whose peak accounts for the 80%. The electrophoretic patterns obtained for the two
317 collected fractions (Figure 2 – panel b), indicate a good depletion of immunoglobulins, whose
318 characteristic bands at 160-50-25 kDa, are very weak in the aliquot eluted within the first peak, and
319 clearly much more intense in the fraction of the second peak corresponding to the immunoglobulins.

320

321 *SEC-UV for IgG analysis*

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

325 Figure 3 represents typical chromatograms recorded by setting the UV detector at 280nm and relative
326 to the IgG-depleted (panel a) and IgG-enriched (panel b) fractions, each spiked with the internal
327 standard TH (0.3mM). The typical retention times for IgG and the dipeptide TH are 9.15min and
328 11.57min, respectively.

329 The peak relative to IgG is well evident only in the IgG enriched fraction and absent in the depleted
330 IgG fraction which is characterized by other peaks such as those relative to IgM and beta-
331 lactoglobulin

332

333 *Antiviral activity assessment*

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

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

360 Mechanism of action experiments show that these IgGs neutralize virus infectivity by significantly
361 ($0.0103 < p_{\text{ANOVA}} < 0.0155$) inhibit RoV-cell binding (figure 6, panels A, B, and C). More importantly,
362 the significant ($0.0005 < p_{\text{ANOVA}} < 0.0183$) neutralization of viral infectivity observed in virus
363 inactivation assays (figure 6, panel D, E, and F) is consistent with a neutralizing activity of virus-
364 specific antibodies targeting the RoV surface antigens, rather than cellular receptors.

365

366

367 **DISCUSSION**

368 The supportive properties of BC when consumed by other mammalian species, including pigs and
369 humans, are well documented in the medical literature (Boudry et al., 2007; Bridger & Brown, 1981;
370 Gopal & Gill, 2000; He et al., 2001; Pakkanen & Aalto, 1997; Solomons, 2002; Struff and Sprotte,
371 2007; Uruakpa et al., 2002). Emerging evidences indicate BC as a promising nutraceutical which can
372 prevent or mitigate various diseases in newborns and adults (Bagwe et al., 2015), in particular
373 gastrointestinal infections. Consistently with these findings, in this study we confirmed the protective
374 activity of NHBC against HRoV, which is well documented in literature (Inagaki et al., 2010; Inagaki

375 et al., 2013). Of note, NHBC shows antiviral activity also against several HRoV strains and, as
376 expected, against BRoV strain NCDV, at concentrations comparable to the ones previously showed
377 (Inagaki et al., 2013).

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

382 Boosting the natural concentrations of immune components in colostrum and milk through
383 vaccination of cows offers great potential for their use as prophylactic or therapeutic products in
384 humans. HBC from cows vaccinated with HRoV showed to be an effective therapeutic in reducing
385 the duration and severity of RoV-caused diarrhea in a double-blind controlled clinical study with
386 infants of 6 to 24 months of age (Mitra et al., 1995). In a second study, Davidson and colleagues
387 produced a HBC by introducing a vaccine containing four HRoV into pregnant Freisian cows. They
388 demonstrated that this HBC administered orally mediated protection by preventing infection of HRoV
389 infection (Davidson et al., 1989). Efficacy of passive immunization with HBC-derived IgG is
390 documented in literature. Hilpert and colleagues (Hilpert et al., 1987) treated infants hospitalized with
391 acute diarrhea with anti-rotavirus immunoglobulin concentrate without a significant decrease in
392 duration of diarrhea or excretion of virus, while Turner and colleagues demonstrated a reduction in
393 incidence and duration of diarrhea in treated infants (Turner and Kelsey, 1987). In 1998, Sarker and
394 colleagues produced a HBC by immunizing pregnant cows with HRoV strains, i.e. Wa, RV3, RV5
395 and ST3, representing serotypes 1 to 4, respectively. HBC-purified IgGs were then administered in a
396 double blind placebo-controlled trial to children with diagnosed HRoV diarrhea (Sarker et al., 1998).
397 Children who received HBC-IgGs had significantly less daily and total stool output and stool
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
403 forward and tested the hypothesis that HBC from cows immunized with a conventional veterinary
404 vaccine against BRoV would exert a higher anti HRoV activity compared to NHBC due to a high
405 titer of cross-reactive anti HRoV IgG. Indeed this study confirms this hypothesis and propose an
406 easier and cheaper approach for the production anti-HRoV HBC or IgG in cows. Our results show an
407 in vitro HRoV inhibition efficacy (i.e. EC50) of HBC comparable with the one that Inagaki and

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

413 With a view to exploit HBC as source of anti-HRoV IgG, quality assessment procedures would be
414 necessary in order to monitor the IgG content; nevertheless, these techniques are already available
415 and well documented in literature (Altomare et al., 2016b). Moreover, cows produce BC in large
416 excess respective to the amount needed to feed their calves (cows produce about 33 liters of colostrum
417 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).

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

422

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426

427

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594

595 **Table 1. Rotavirus strains used in this study.**

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)

596

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.

599

600

Table 2. Antiviral activities of BCs.

	Virus	EC₅₀ (µg/ml) (95% C.I.)	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 hyperimmune 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

604 Herpes simplex type 1 (HSV-1); herpes simplex type 2 (HSV-2); human cytomegalovirus (HCMV); human
605 adenovirus (HRhV); human rotavirus (HRoV); bovine rotavirus (BRoV); vesicular stomatitis virus (VSV). EC₅₀ half-
606 maximal effective concentration; CI confidence interval; EC₉₀ 90% effective concentration; CC₅₀ half maximal
607 cytotoxic concentration; SI selectivity index; n.a. not assessable

609 **Table 3. Antiviral activities of IgGs from HBC.**

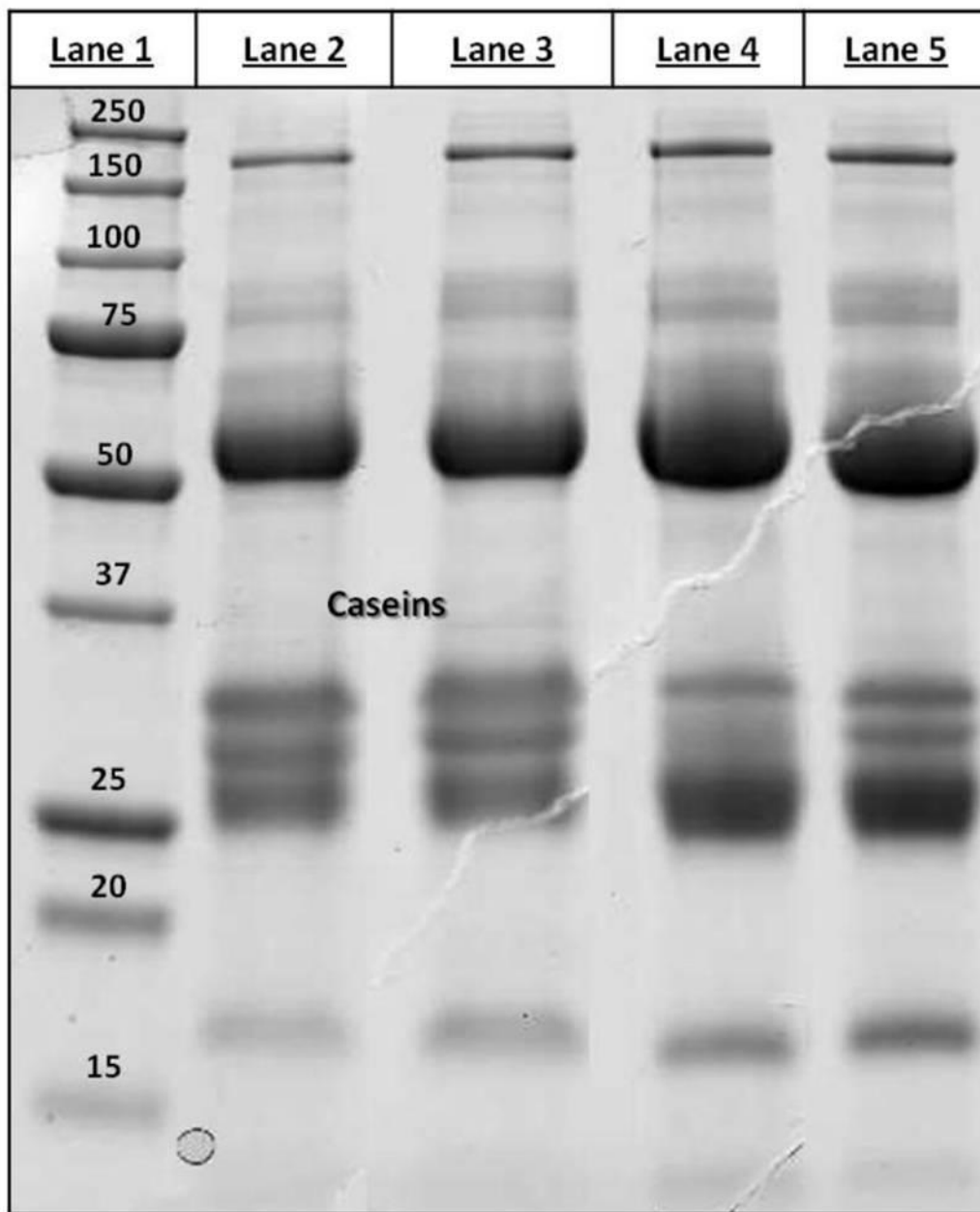
	Virus	EC₅₀ (µg/ml) (95% C.I.)	EC₉₀ (µg/ml) (95% C.I.)	CC₅₀ (µg/ml)	SI
IgG	HRoV (#Wa)	1.9 (1.4-2.6)	19.7 (10.4-37.1)	> 5910	> 3110
	HRoV (#HRV 408)	1.8 (0.8-3.9)	12.6 (2.5-63.2)	> 5910	> 3283
	HRoV (#HRV 248)	0.7 (0.6-0.9)	8.2 (4.9-13.9)	> 5910	> 8443
	BROV (#NCDV)	6.2 (5.2-7.4)	30.2 (19.7-49.0)	> 5910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

610

611 Herpes simplex type 1 (HSV-1); herpes simplex type 2 (HSV-2); human cytomegalovirus (HCMV);
 612 human rhinovirus (HRhV); human rotavirus (HRoV); bovine rotavirus (BROV); vesicular stomatitis
 613 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

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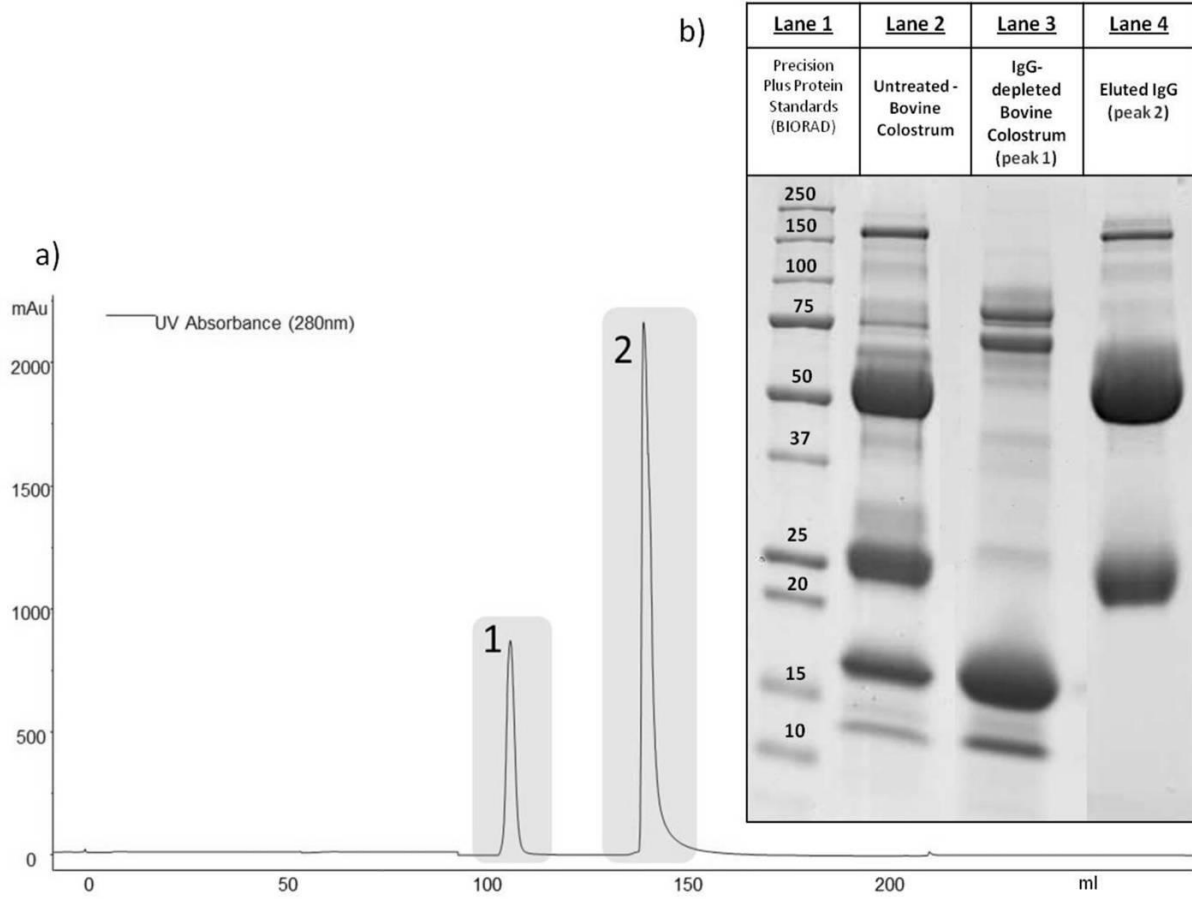
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618 **Figure 1.**

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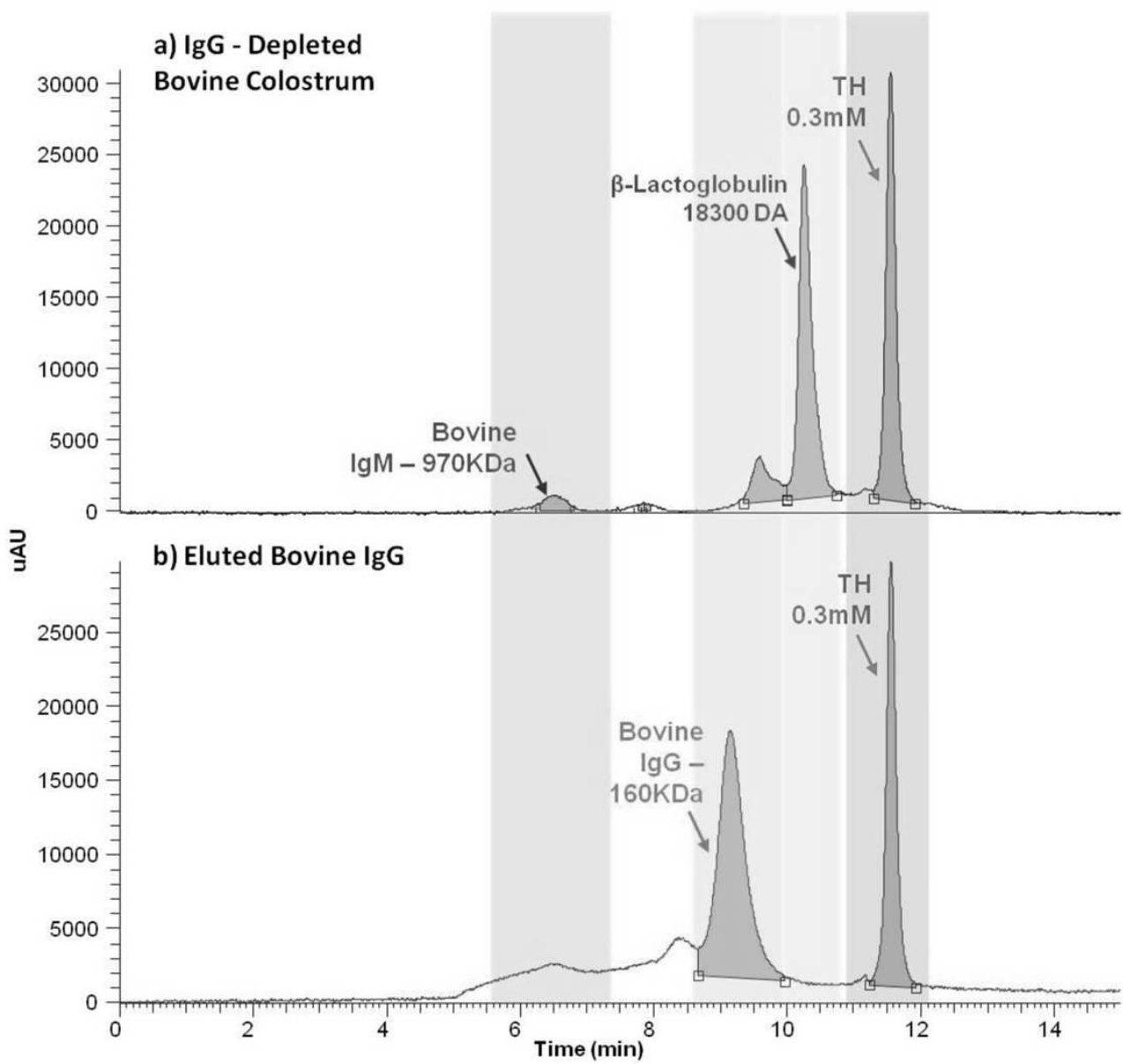


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621 **Figure 2.**

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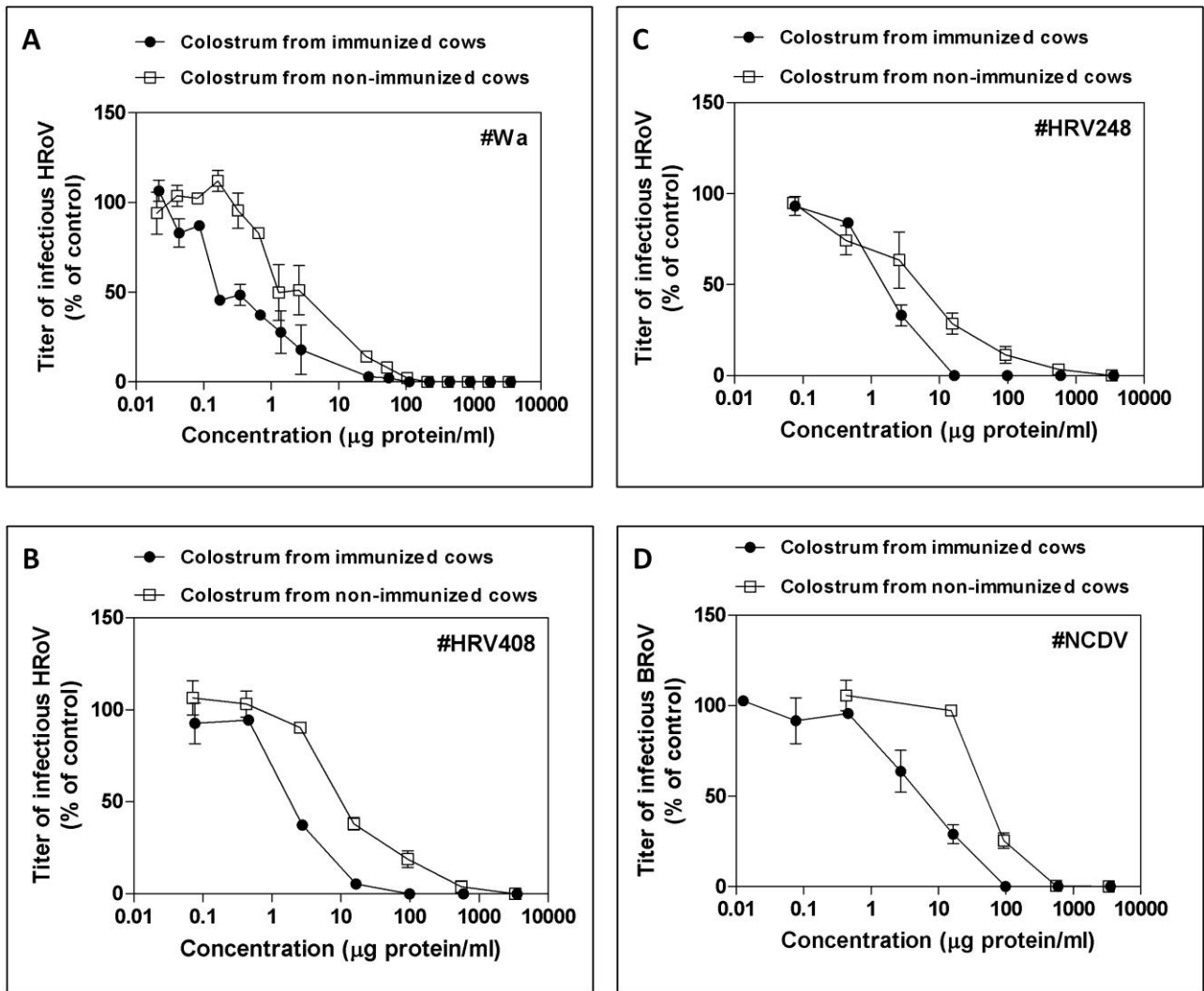


624

625 **Figure 3.**

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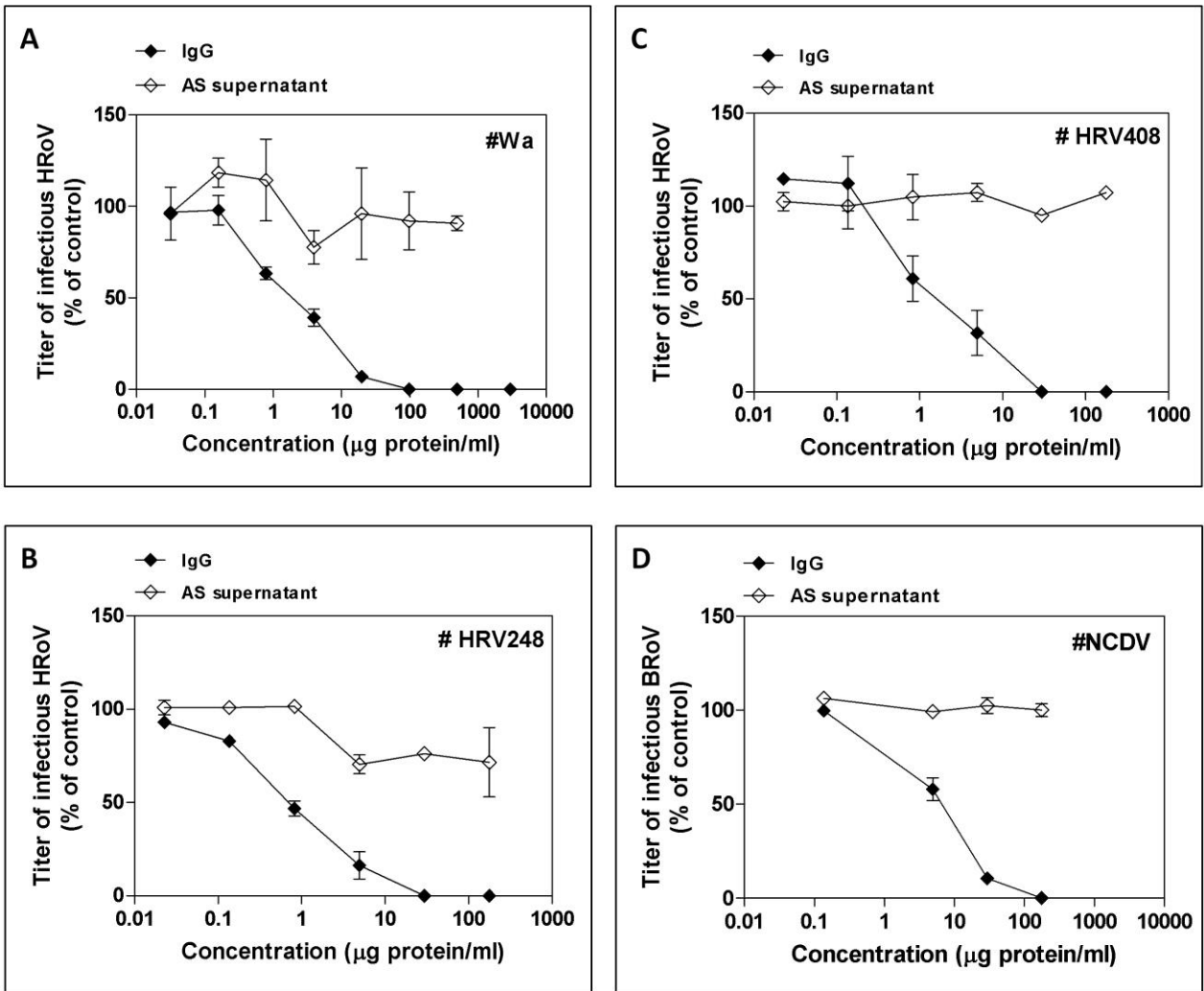
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629 **Figure 4.**

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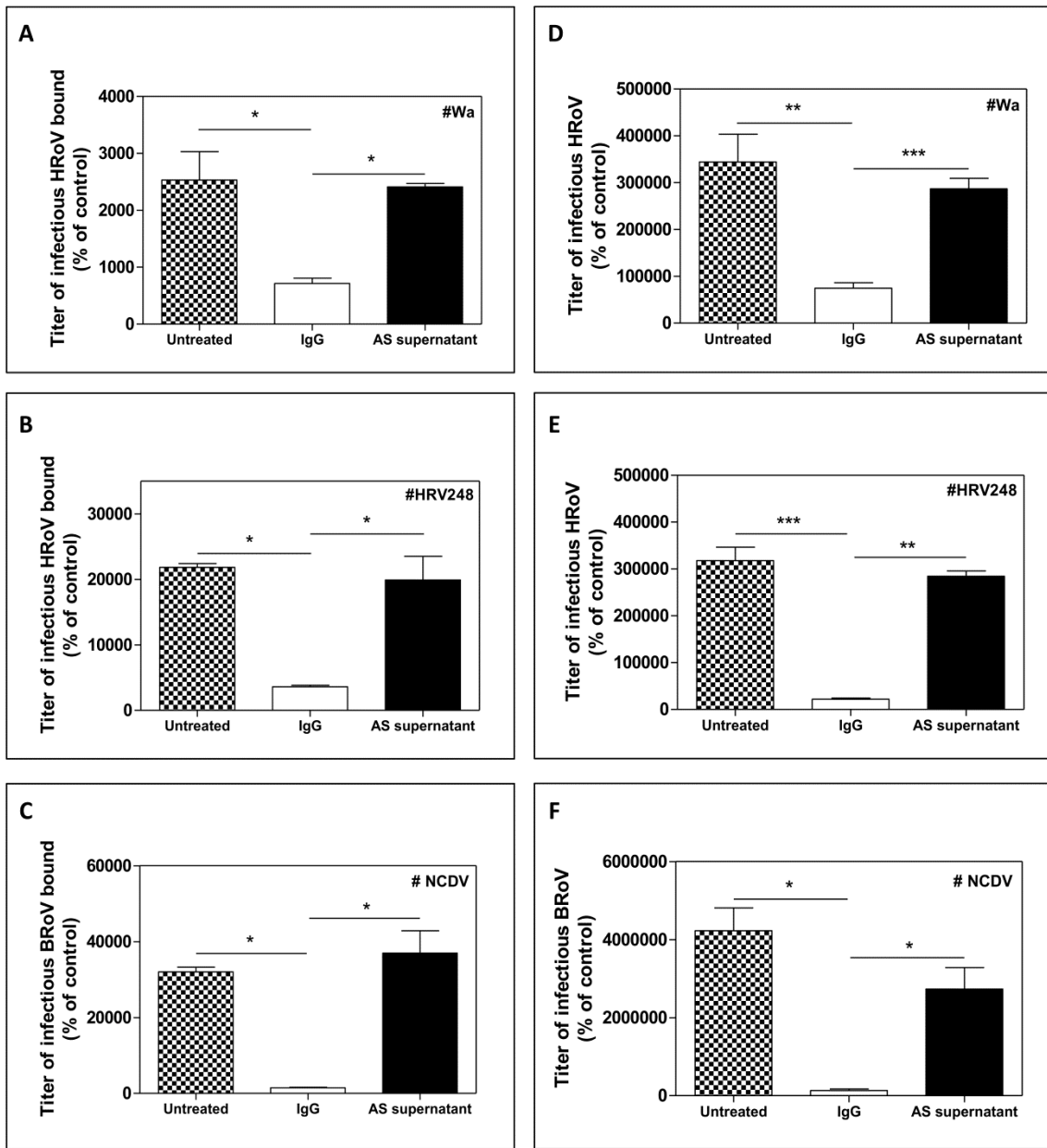


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634 **Figure 5.**

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639 **Figure 6.**

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641 **Legends**

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

646 **Figure 2.** IgG purification by affinity chromatography. Panel a) FPLC-UV chromatogram of the
647 untreated-Bovine Colostrum (casein-depleted and defatted bovine colostrum) obtained by using
648 protein G as affinity stationary phase; peaks 1 and 2 are attributed to the IgG-depleted and IgG
649 fractions as demonstrated by the SDS-gel electrophoresis patterns. Panels b) reports the gel
650 electrophoretic pattern of peak 1 (lane 3) and 2 (lane 4) compared to the untreated bovine colostrum
651 pattern, run in reducing conditions. The characteristic bands at 25 and 50 Da are well evident in peak
652 2 and only in negligible amounts in peak 1.

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

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

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

667 **Figure 6.** Mechanism of action of HBC-derived IgGs. Panels A, B, and C show the effect of HBC-
668 derived IgGs respectively on Wa, HRV248, and NCDV binding to the MA104 cell surface. Panels
669 D, E, and F show the results of virus inactivation assays on infectious particles respectively of Wa,
670 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
672 bound to control untreated MA104 cells. Error bars represent the SEM of independent experiments.
673 * $p_{ANOVA} < 0.05$ ** $p_{ANOVA} < 0.001$ *** $p_{ANOVA} < 0.0001$