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Anti-zika virus and anti-usutu virus activity of human milk and its components

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(Article begins on next page)

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1 **Anti-Zika virus and anti-Usutu virus activity of human milk and its components**

2 **Human milk and emerging flaviviruses**

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21 glycosaminoglycans

22 **Abstract**

23 The benefits of human milk are mediated by multiple nutritional, trophic, and immunological
24 components, able to promote infant's growth, maturation of its immature gut, and to confer
25 protection against infections. Despite these widely recognized properties, breast-feeding represents
26 an important mother-to-child transmission route of some viral infections. Different studies show
27 that some flaviviruses can occasionally be detected in breast milk, but their transmission to the
28 newborn is still controversial. The aim of this study is to investigate the antiviral activity of human
29 milk (HM) in its different stages of maturation against two emerging flaviviruses, namely zika virus
30 (ZIKV) and Usutu virus (USUV) and to verify whether HM-derived extracellular vesicles (EVs)
31 and glycosaminoglycans (GAGs) contribute to the milk protective effect.
32 Colostrum, transitional and mature milk samples were collected from 39 healthy donors. The
33 aqueous fractions were tested *in vitro* with specific antiviral assays and EVs and GAGs were
34 derived and characterized. HM showed antiviral activity against ZIKV and USUV at all the stages
35 of lactation with no significant differences in the activity of colostrum, transitional or mature milk.
36 Mechanism of action studies demonstrated that colostrum does not inactivate viral particles, but it
37 hampers the binding of both flaviviruses to cells. We also demonstrated that HM-EVs and HM-
38 GAGs contribute, at least in part, to the anti-ZIKV and anti-USUV action of HM.
39 This study discloses the intrinsic antiviral activity of HM against ZIKV and USUV and
40 demonstrates the contribution of two bioactive components in mediating its protective effect. Since
41 the potential infectivity of HM during ZIKV and USUV infection is still unclear, these data support
42 the World Health Organization recommendations about breast-feeding during ZIKV infection and
43 could contribute to producing new guidelines for a possible USUV epidemic.
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45
46

Author Summary

ZIKV and USUV are emerging flaviviruses that cause conditions ranging from mild febrile diseases to more sever outcomes. ZIKV is associated with microcephaly in newborns and USUV neurotropism represents a growing concern for human health. We studied these viruses in the context of breast-feeding. Breast-milk is a complex biofluid to nourish infants, support their growth and to protect them from numerous diseases, but it also represents a transmission route of several infections. It has been reported that flaviviruses can occasionally be detected in breast-milk, with limited information existing about their possible transmission through breast-feeding. We therefore explored the intrinsic protective role of human milk against ZIKV and USUV infections *in vitro* and we also assessed the contribution of specific components in mediating this activity. We demonstrated that human milk is endowed with anti-ZIKV and anti-USUV activity at all maturation stages and that it acts by altering virus attachment to the host cell. This activity is mostly due to non-specific bioactive factors, including extracellular vesicles and glycosaminoglycans. Our findings support the use of fresh milk (or from donor banks) as the food of choice for nutrition and protection of newborns in a possible context of ZIKV or USUV epidemics.

Introduction

Zika virus (ZIKV) and Usutu virus (USUV) are two emerging flaviviruses mostly transmitted by mosquitos. *Aedes aegypti* and *Culex pipiens* mosquitos are their major vectors respectively [1–3]. Most ZIKV and USUV infections are asymptomatic, but in symptomatic cases, they may cause conditions ranging from a mild febrile disease to more severe outcomes with neurological involvement.

ZIKV infection has been associated with the Guillain-Barré syndrome in adults and with a variety of neurological impairments, including microcephaly, in infants born to infected mothers [4,5]. It has caused a series of epidemics in the Americas, Asia and the Pacific in the past decade and it is now considered an important public health concern [6]. To date, 84 countries and territories have reported autochthonous transmission of ZIKV [7].

The less-known USUV has recently attracted the attention of the scientific community due its potential for emergence and its extensive spread in Europe [8,9]. It is phylogenetically closely related to West Nile virus (WNV) and it is maintained through an enzootic cycle between migratory birds and ornithophilic mosquitos, with humans representing incidental hosts [9]. Seroprevalence studies suggested that USUV infections in humans may have been largely underestimated, and many of them may be asymptomatic. The full clinical presentation of human severe USUV infection is still partially unknown, but cases of meningoencephalitis and facial paralysis have been reported and its neurotropism represents a growing concern for human health [10–12].

No antivirals or vaccines are currently available against either virus and the only way to prevent these infections is to avoid mosquito bites.

We studied ZIKV and USUV in the context of breastfeeding. Previous reports have shown that some flaviviruses, such as Dengue virus (DENV), WNV and Yellow fever virus (YFV), can occasionally be present in breast milk [13]. In particular, ZIKV RNA has been reported in breast milk from 3 to 33 days after maternal onset of fever and ZIKV infectious particles were also

91 detected in this biofluid [14–18]. Nevertheless, this mode of transmission to the newborn is still
92 controversial [19–25]. Probably due to the small number of human cases, the presence of USUV in
93 breast milk is currently unknown, but its strong correlation with WNV suggests it could be possible.
94 Notwithstanding these evidences, the short and long-term health advantages of breastfeeding for
95 both neonate and lactating mother outweigh any potential risk of transmission [26]. The World
96 Health Organization (WHO) recommends indeed that mothers with possible or confirmed ZIKV
97 infection continue to breastfeed [27].
98 Breast milk composition is extremely complex, individual-specific and variable according to the
99 stage of lactation. It includes macro- and micronutrients and a wide variety of non-nutritional
100 bioactive components [28]. Amongst the latter, secretory IgA (sIgA), toll-like receptors (TLRs),
101 lactoferrin, lactadherin, oligosaccharides (HMOs) support the development of the immature
102 immune system of the neonate and confer intrinsic protection against infections [29]. Therefore,
103 HM is a possible source of viral infections, but these substances could directly affect viral
104 infectivity. In the case of flaviviruses, DENV and Japanese encephalitis virus (JEV) are neutralized
105 by the lipid fraction of breast milk and ZIKV and hepatitis C virus (HCV) are destroyed by free-
106 fatty acids released upon storage by milk lipases in a time-dependent manner [30–33]. Herein, we
107 aimed to explore the intrinsic anti-ZIKV and anti-USUV activity of human milk, according to its
108 maturation stage and regardless of the storage affected-lipid fraction.
109 We also investigated the antiviral contribution of human milk-derived extracellular vesicles (HM-
110 EVs) and human-milk glycosaminoglycans (HM-GAGs). Briefly, EVs are lipid enclosed vesicles,
111 ranging from 30 to 1000 nm in diameter, that are released by most tissues including breast epithelial
112 cells, macrophages and lymphocytes present in breast milk [34–36]. These vesicles can selectively
113 be taken up by other cells, in which they release their molecular cargo (e.g. DNA, RNAs, enzymes,
114 signalling proteins), playing a role in intercellular signalling, immune response, stem cell
115 differentiation, tissue regeneration and viral replication [37,38]. GAGs are other abundant
116 constituents of human milk defined as linear heteropolysaccharides composed of repeating

117 disaccharidic units [39–41]. Detailed analyses performed on HM-GAGs demonstrated the presence
118 of a complex mixture made up of chondroitin sulfate (CS)/dermatan sulfate (DS), heparan sulfate
119 (HS)/heparin (Hep) and a minor percentage of hyaluronic acid (HA), with the CS/DS fraction being
120 the most represented (~ 55%) followed by HS/Hep (~ 40%) [39].
121 HM-GAGs and HM-EVs have recently become the subject of increasing interest for their
122 implication for infants [42,43]. HM-EVs have been demonstrated to be active *in vitro* against
123 human immunodeficiency virus (HIV) [44] and HM-GAGs have also shown anti-bacterial and anti-
124 viral activity due to their ability to act as soluble receptors inhibiting the attachment of different
125 pathogens to the intestinal mucosa [45–47]. Both human milk constituents, have been poorly
126 investigated for their antiviral action so far, therefore their role needs to be clarified.
127 Here we report that human milk is endowed with anti-ZIKV and anti-USUV activity at all
128 maturation stages and that it acts by altering virus attachment to the host cell. This activity is mostly
129 due to non-specific bioactive factors, including HM-EVs and HM-GAGs.
130

131 **Methods**

132 **Ethic statement**

133 An ethical review process was not required for this study since it was not a clinical trial. Each milk
134 donor involved in this research signed a written consent form, where the mother's and infant's data
135 protection was assured. Moreover, the donors were informed about the study design.

136 **Human milk sample collection and clarification**

137 Thirty-nine healthy mothers were enrolled in the study: 18 mothers donated as many colostrum
138 samples (days 1-5 postpartum), 11 mothers donated colostrum, transitional (days 6-14 postpartum)
139 and mature milk samples (beyond day 15 postpartum), and 10 mothers each donated 15 ml of
140 mature milk that were added in a unique pool. All mothers were admitted to Sant'Anna Hospital
141 (Città della Salute e della Scienza of Turin, Italy). The donors cleaned their hands and breasts
142 according to the Italian HMB guidelines [48], and the milk samples were collected in sterile
143 bisphenol-free polypropylene bottles using a breast pump and immediately stored at -20 °C unless
144 otherwise stated. After thawing, the milk samples were centrifuged at a low speed (2000 x *g*) for 10
145 minutes at room temperature to remove the fat globule layer. The defatted milk was then transferred
146 to a new tube and centrifuged at 12000 x *g* for 30 minutes to obtain the aqueous fraction. The
147 supernatant was filtered through a syringe, equipped with a 0.45 μm pore size sterile filter (Sarstedt,
148 Verona, Italy), to further eliminate any cells and cellular debris.

149

150 **Cell lines**

151 African green monkey fibroblastoid kidney cells (Vero) (ATCC CCL-81) were cultured in Eagle's
152 minimal essential medium (MEM; Sigma, St. Louis, MO) supplemented with heat-inactivated, 10%
153 (v/v) fetal bovine serum (FBS) (Sigma). The embryonic human kidney cells (293T) (ATCC CRL-
154 3216) were grown as monolayer in Dulbecco's modified Eagle's medium (DMEM; Sigma)
155 supplemented with 10% FBS and 1% Glutamax-I (Invitrogen, Carlsbad, CA) and low-passage-

number (<30) human foreskin fibroblasts (HFF-1) (ATCC SCRC-1041) were grown as monolayers in DMEM supplemented with 15% FBS. The media were supplemented with 1% (v/v) antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs, Berlin, Germany) and cells were grown at 37 °C in an atmosphere of 5% of CO2.

The antiviral assays against ZIKV and USUV were performed on Vero cells using MEM supplemented with 2% of FBS, unless otherwise stated.

Viruses

Two strains of infectious Zika viruses (1947 Uganda MR766 and 2013 French Polynesia HPF2013) were generated by transfection of 293T cells with two plasmids (pCDNA6.2 Zika MR766 Intron3115 HDVr MEG 070916 5 and pCDNA6.2 Zika HPF2013 3864,9388Intron HDVr MEG091316 2) as previously described [49]. The viruses were then propagated in Vero cells and titrated by plaque assay. All the antiviral assays were performed with ZIKV HPF2013 strain, unless otherwise stated.

Usutu virus (Strain: 3345 Isolate: Arb276) was isolated and produced by APHA (Animal & Plant Health Agency – GOV. UK) and kindly provided by the European Viral Archive Global (EVAg). It was propagated in Vero cells and titrated by means of the indirect immunoperoxidase staining procedure, by using a mouse monoclonal antibody direct to flavivirus protein E (D1-4G2-4-15 (4G2), Novus Biological) and a secondary antibody peroxidase-conjugated AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories Inc., 872 W. Baltimore Pike, West Grove, PA 19390).

Extracellular vesicles (EVs) isolation and characterization

Extracellular vesicles were extracted from the aqueous fraction of three colostrum samples before their freezing. The aqueous fraction was obtained as described above and subsequently incubated

181 with 2:1 v/v ratio of colostrum:ExoQuick solution (System Biosciences, CA) overnight at 4°C. The
182 extracellular vesicles were then purified, according to the manufacturer's instructions. The EV
183 pellet was resuspended in 1 x PBS, quantified to establish the protein concentration using a protein
184 assay kit (Bio-Rad Laboratories, Munich), aliquoted and stored at -80°C until use.
185 The EV protein profile was analyzed by means of western blotting. HFF cell lysate was used to
186 verify the reactivity of the anti-calnexin primary antibody. These cells were chosen as control since
187 they are a reliable and highly standardized human cell line. A RIPA buffer, containing protease
188 inhibitors, was added to the EV pellet, or to the HFF cells, for 10 minutes at RT to allow complete
189 lysis. Soluble proteins were collected, by means of centrifugation at 15,000 *x g*, and were then
190 quantified using a protein assay kit. The western blot was performed as previously described [50].
191 Primary antibodies: anti-CD63, anti-CD9, anti-CD81, anti-calnexin, anti-Hsp70 and anti-caveolin1;
192 secondary antibodies: anti-rabbit and anti-mouse (System Biosciences).
193 A nanoparticle tracking analysis system (NTA) (NanoSight NS300, Malvern Instruments Ltd., UK)
194 was used to determine particle size and particle concentration per milliliter at the ideal particle per
195 frame value (63–65 particles/ frame).

196

197 **Human milk glycosaminoglycans (HM-GAGs) isolation and characterization**

198 50 mL of mature milk were defatted with acetone. After centrifugation at 10,000 g for 15 min and
199 drying at 60°C for 24 h, the pellet was solubilized in 200 mL of distilled water and treated with 100
200 mg of pancreatin (Sigma-Aldrich, code 1071301000, 350 FIP-U/g Protease, 6000 FIP-U/g Lipase,
201 7500 FIP-U/g Amylase) at 60°C for 24 h in a stirrer. After boiling for 10 min and centrifugation at
202 5,000 g for 20 min, three volumes of ethanol were added to the supernatant and the mixture stored
203 at 4°C for 24 h. After centrifugation at 10,000 g for 15 min and dried at 60°C for 6 h, the dried
204 powder was dissolved in 100 ml of 50 mM NaCl and centrifuged at 10,000 g for 10 min. The
205 supernatant was applied to a column (5 x 10 cm) packed with QAE Sephadex® A-25 anion-

206 exchange resin equilibrated with the same NaCl solution. GAGs were eluted with a linear gradient
207 of NaCl from 50 mM to 2.0 M from 0 to 200 min using low-pressure liquid chromatography
208 (Biologic LP chromatography system from BioRad) at a flow of 1 ml/min. Fractions positive to
209 uronic acid assay were collected [51]. Three volumes of ethanol were added to the pooled fractions
210 and stored at 4°C for 24 h. The precipitate was centrifuged and dried at 60°C. The dried purified
211 HM-GAGs were dissolved in distilled water and lyophilized for the virus inhibition assay or further
212 analysis.
213 For the antiviral assays, GAGs were dissolved in PBS and stocked at 4°C until use.
214 HM-GAGs composition was evaluated by electrophoresis on acetate of cellulose [52]. The purity of
215 the milk extract was evaluated by measuring the protein content by the Folin-Ciocalteu test (Sigma-
216 Aldrich, code MAK365-1KT) and the GAGs content by the uronic acid assay [51].
217 Structural characterization of the CS/DS and HS/Hep components of HM-GAGs was performed by
218 determining the corresponding constituent disaccharides. Briefly, HM-GAGs were treated with
219 chondroitinase ABC or chondroitinase AC for 10 h at 37°C in 50 mM Tris-Cl pH 8.0 to produce the
220 CS/DS constituent disaccharides. HM-GAGs were also incubated with a cocktail of heparinases
221 (heparinases I, II and III) in 0.1 M sodium acetate/calcium acetate pH 7.0 at 38°C overnight to
222 release the HS/Hep disaccharides. The unsaturated disaccharides produced were derivatized with 2-
223 Aminoacridinone (AMAC) as previously described [39] and the fluorotagged disaccharides
224 separated and analysed by capillary electrophoresis equipped with a Laser-Induced Fluorescence
225 (LIF) detector [53]. By this analytical approach we also determined the HA content besides the
226 structural composition and charge density of the sulphated heteropolysaccharides CS/DS and
227 HS/Hep.
228

229 **Virus inhibition assay**

230 The anti-ZIKV and anti-USUV activity of human milk (colostrum, transitional or mature milk) was
231 determined by means of plaque reduction assay or focus reduction assay respectively.

232 Vero cells were seeded at a density of $6,5x10^4$ cells /well in 24 well plate for ZIKV antiviral assays
233 or at a density of $1,3x10^4$/well in 96 well plate for USUV antiviral assays. The following day, cells
234 were pre-treated with serial dilutions of human milk aqueous fraction (from 1:3 to 1:6561 parts) for
235 1h at 37°C. The virus was pre-treated under the same experimental conditions simultaneously:
236 mixtures of serial dilutions of human milk and the same amount of virus were incubated for 1 h at
237 37°C at multiplicities of infection (MOIs) of 0.0005 PFU/cell for ZIKV and 0.02 FFU/cell for
238 USUV. After a gentle wash, these mixtures were added to cells for 2h at 37°C. Subsequently, the
239 ZIKV infected cells were washed twice with warm medium and overlaid with a 1.2%
240 methylcellulose medium for 72 h at 37°C. In the case of USUV, infected cells were washed twice
241 and overlaid with fresh medium for 24h at 37°C.
242 The number of ZIKV plaques were counted after cell fixation and staining with a solution of 0.1%
243 crystal violet in 20% ethanol. The USUV-infected cells were detected by means of indirect
244 immunostaining as described above. The inhibitive dilution that produced a 50% reduction of ZIKV
245 or USUV infection (ID_{50}) was determined by comparing the treated with the untreated wells.
246 GraphPAD Prism 8.0 software (San Diego, CA) was used to fit a variable slope-sigmoidal dose-
247 response curve and calculate the ID_{50} values.
248

249 **Viability assay**

250 Cell viability was measured using the MTS [3-(4,5-dimethylthia-zol-2-yl)-5-(3-
251 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetra-zolium] assay. Confluent Vero cells were
252 treated with serial dilutions of colostrum or HM-EVs or HM-GAGs under the same experimental
253 conditions of the virus inhibition assay. Cell viability was determined using the Cell Titer 96
254 Proliferation Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.
255 Absorbances were measured using a Microplate Reader (Model680, BIORAD) at 490 nm. The
256 effect on cell viability at different dilutions of colostrum was expressed as a percentage, by

257 comparing absorbances of treated cells with those of cells incubated with culture medium alone.
258 The 50% cytotoxic concentrations (CC_{50}) was determined using Prism software.
259

260 **Immunofluorescence assay**

261 Subconfluent Vero cells plated on coverslips in 24-well plates were treated with colostrum (ID_{90})
262 following the virus inhibition assay protocol. The infection was performed with MOI of 3 for both
263 viruses. After 30 h or 24 h for ZIKV or USUV infected cells respectively, cells were washed twice
264 with PBS and then fixed in 4% PAF for 15 min RT. Cells were permeabilized in PBS with Triton
265 0,1% for 20 minutes on ice and then blocked with 5% BSA for 30 minutes. Next the incubation
266 with the primary antibody (Anti-dsRNA mAb, SCICONS J5 or anti-flavivirus protein E mAb D1-
267 4G2-4-15 (4G2), Novus Biological) diluted in blocking buffer was performed for 1 h RT. After
268 three washes in PBS with 0.05% Tween 20, the secondary antibody (Goat Anti-Mouse IgG
269 Rhodamine conjugated, Santa Cruz Biotechnology) diluted in blocking buffer was added to cells for
270 1 h RT. Subsequently, three washes with PBS were performed and coverslips were mounted and
271 analyzed on a confocal fluorescence microscope (LSM510, Carl Zeiss, Jena, Germany).
272

273 **Virus inactivation assay**

274 Approximately 10^6 FFU of ZIKV or USUV were incubated with 100 µl of colostrum for 2h at 37°C.
275 As control, the same number of viral particles was incubated with fresh medium. After the
276 incubation, both treated and untreated viruses were titrated to the non-inhibitory dilution of
277 colostrum. The residual viral infectivity was determined by plaque assay (ZIKV) or by indirect
278 immunostaining (USUV). Statistical analysis was performed using Student's t-test. Significance
279 was reported for p-values <0.05.
280

281 **Pre-treatment assay**

282 Confluent Vero cell monolayers in 24 well plate (for ZIKV test) or in 96 well plate (for USUV test)
283 were pre-treated with serial dilutions of colostrum (from 1:3 to 1:6561) for 2 h at 37°C. After
284 washing, cells were infected with ZIKV (MOI = 0.0005) or USUV (MOI = 0.02) for 2h 37°C. The
285 viral inoculum was then removed and two gentle washes were performed. The ZIKV infected cells
286 were overlaid with 1.2% methylcellulose medium for 72 h at 37°C and the USUV infected cells
287 were incubated with fresh medium for 24 h at 37°C. At the end of the incubation cells were fixed
288 and stained with 0.1% crystal violet in 20% ethanol to count the number of ZIKV plaques or fixed
289 and stained with indirect immunostaining to evaluate the number of USUV infected cells.
290 Where possible, the ID_{50} values were calculated by means of a regression analysis, using dose–
291 response curves generated by GraphPad Prism version 8.0.
292

293 **Binding assay**

294 Vero cells were seeded in 24 well plate at a density of $1.1x10^5$ cells/well. The following day, cells
295 and viruses (ZIKV or USUV, MOI=3) were cooled to 4°C for 10 minutes. The viruses were then
296 allowed to attach to the cells in the presence of colostrum (ID_{90}). After an incubation of 2 h on ice,
297 the cells were washed with a cold medium to remove any unbound virus. The cells were then
298 subjected to three rounds of freeze-thawing to release any bound virus, and the lysate was clarified
299 by means of low speed centrifugation for 10 minutes. The cell-bound virus titers were determined
300 by means of plaque assay (ZIKV) or indirect immunostaining (USUV), as outlined above. The
301 presence of any significant differences was assessed by means of Student's t-test, using PRISM 8.0
302 GraphPad Software.
303

304 **Entry assay**

305 The Vero cells were cultured to confluence in 24-well or 96-well trays. ZIKV (MOI = 0.005) and
306 USUV (MOI = 0.2), which had been cooled to 4°C, were allowed to attach to pre-chilled cells on

307 ice for 2 h at 4 °C. Unbound viruses were then washed, serial dilutions of colostrum (from 1:3 to 1:
308 6561) were added to cells and the plates were incubated at 37 °C to allow virus entry. After the viral
309 entry, the treatment was aspirated and viral particles still present on the cell surface were inactivated
310 by a wash with citrate buffer (citric acid 40 mM, potassium chloride 10 mM, sodium chloride 135
311 mM, pH 3) for 1 minute at room temperature, as previously described [49]. Cells were then washed
312 with warm medium 3 times and overlaid with 1.2% methycellulose medium (ZIKV entry assay) for
313 72h or with fresh medium (USUV entry assay) for 24h. Cells were fixed and stained with 0.1%
314 crystal violet in 20% ethanol to count the number of ZIKV plaques or fixed and stained with the
315 indirect immunostaining procedure to evaluate the number of USUV infected cells. The viral entry
316 blockade was determined and expressed as the mean percentage of the untreated control ± SEM.
317 Where possible, the ID_{50} values were calculated by means of regression analysis using dose–
318 response curves (GraphPad Prism 8.0)
319

320 **Post-entry assay**

321 Vero cells were seeded in 96-well plates at a density of $1.3x10^4$ cells/well. The following day, the
322 viral inoculum (ZIKV at MOI = 0.005 or USUV at MOI = 0.2) was added to cells for 2 h at 37°C.
323 The unpenetrated viruses were inactivated with citrate buffer for 1 minute at room temperature and
324 cells were then washed with warm medium 3 times and incubated with serial dilutions of colostrum
325 (from 1:3 to 1:6561) for 3 h at 37°C. Finally, after 2 gentle washes, Vero cells were incubated with
326 warm MEM for 30 h (ZIKV) or 24 h (USUV) at 37 °C. The number of infected cells was
327 determined by indirect immunostaining and the viral inhibition expressed as the mean percentage of
328 the untreated control ± SEM. Where possible, the ID_{50} values were calculated by means of
329 regression analysis using dose–response curves (GraphPad Prism 8.0)
330

331 **Evaluation of HM-EVs and HM-GAGs antiviral activity: virus inhibition assay**

332 The antiviral activity of colostrum-derived EVs and of HM-GAGs was evaluated by means of the
333 same experimental protocol described under the "virus inhibition assay" subheading. The anti-
334 ZIKV and anti-USUV activity of HM-EVs and HM-GAGs was determined by means of plaque
335 reduction assay (ZIKV) or focus reduction assay (USUV). Vero cells were seeded at a density of
336 $6,5x10^4$ cells /well in 24 well plate for ZIKV antiviral assays or at a density of $1,3x10^4$/well in 96
337 well plate for USUV antiviral assays. The following day, cells were pre-treated with serial dilutions
338 of HM-EVs (from 423 µg/ml to 0.19 µg/ml) or with dilutions of HM-GAGs (from 10 mg/ml to 0.1
339 mg/ml) for 1 h at 37°C. The viruses were pre-treated under the same experimental conditions
340 simultaneously: mixtures of serial dilutions HM-EVs or HM-GAGs and the same amount of virus
341 (MOI of 0.0005 PFU/cell for ZIKV and 0.02 FFU/cell for USUV) were incubated for 1 h at 37°C.
342 After a gentle wash, these mixtures were added to cells for 2h at 37°C. Subsequently, the ZIKV
343 infected cells were washed twice with warm medium and overlaid with a 1.2% methylcellulose
344 medium for 72 h at 37°C. In the case of USUV, infected cells were washed twice and overlaid with
345 fresh medium for 24h at 37°C. The number of ZIKV plaques were counted after cell fixation and
346 staining with a solution of 0.1% crystal violet in 20% ethanol. The USUV-infected cells were
347 detected by means of indirect immunostaining. The effective concentration that produced a 50%
348 reduction of ZIKV or USUV infection (EC_{50}) was determined by comparing the treated with the
349 untreated wells. GraphPAD Prism 8.0 software was used to fit a variable slope-sigmoidal dose-
350 response curve and calculate the EC_{50} values.
351

352 **Data analysis**

353 All the results are presented as the mean values of two independent experiments. The ID_{50} values of
354 the inhibition curves were calculated from a regression analysis using GraphPad Prism software,
355 version 8.0 (GraphPad Software, San Diego, California, the U.S.A.) by fitting a variable slope-
356 sigmoidal dose-response curve. Statistical analysis was performed using Student's t-test, ANOVA
357 Analysis of variance or the F-test, as reported in the Figure legends.

Results

Human milk is endowed with an intrinsic anti-ZIKV and anti-USUV activity

The first set of experiments was performed in order to investigate the intrinsic antiviral activity of human colostrum against ZIKV and USUV. Colostrum samples were collected from healthy donor mothers admitted to Sant'Anna Hospital of Turin for term or preterm delivery (Table 1). The aqueous fraction of these samples was selected as preferred biological matrix, due to its previously described lower impact on cell viability [54], and tested *in vitro* against ZIKV (HPF2013) and USUV. Briefly, cells and viruses were treated with serial dilutions of human colostrum before and during the infection. As reported in Fig 1A and S1 Table, all colostrum samples exhibited antiviral activity against both viruses although to a different extent from mother to mother. The ID_{50s} were ranging from 0.0003 to 0.0026 for ZIKV and from 0.0022 to 0.0355 for USUV indicating that human colostrum is significantly more active against ZIKV. The ID_{50s} obtained with fresh colostra, i.e. with colostra that were clarified and tested *in vitro* within 1 hour after collection, are comparable to those obtained from frozen samples (S1 Fig). Since an incomplete gestational period can affect the maturity of the mammary gland and its ability to secrete milk with the proper composition for the newborn's condition, we stratified the results comparing the antiviral activity of human colostrum from term and preterm mothers, but no significant difference was observed for both viruses (S2 Fig). Furthermore, the antiviral activity of human colostrum against different ZIKV strains was verified by testing 3 colostrum samples against ZIKV MR766 belonging to the African lineage. As shown in Fig 1B, the human colostrum inhibits the MR766 infectivity too, with ID_{50}s comparable to those obtained with the microcephalic HPF2013 strain ($ID_{50\ Colostrum1} = 0.0012$; $ID_{50\ Colostrum2} = 0.0009$; $ID_{50\ Colostrum3} = 0.0023$). To further confirm the antiviral action of human colostrum against ZIKV and USUV, immunofluorescence experiments detecting the dsRNA (an intermediate in flavivirus replication) and the flavivirus protein E were performed. As shown in Fig 2A and 2B, the synthesis of the dsRNA and the production of the protein E are significantly

383 inhibited by colostrum for both viruses. The same results were obtained with the MR766 ZIKV
384 strain (S3 Fig). Moreover, in order to exclude the possibility that the observed antiviral action was
385 due to a cytotoxic effect, viability assays were performed by treating cells with human colostrum
386 under the same experimental conditions of the virus inhibition assay described above. As expected,
387 results indicated that colostrum aqueous fraction is not toxic for cells even at the lowest tested
388 dilution (0.33). Results obtained with 3 randomly selected colostrum samples are reported in Fig 3.
389 Altogether, these results demonstrated that the aqueous fraction of human colostrum is intrinsically
390 endowed with antiviral activity against two emerging flaviviruses without being toxic for cells *in*
391 *vitro*.
392 Subsequently, we investigated the variations in the anti-ZIKV and anti-USUV activity of human
393 milk according to the different stages of lactation. To this aim, eleven mothers (Table 2) donated
394 samples of colostrum, transitional and mature milk each. First, the absence of cytotoxicity was
395 verified for transitional and mature milk too (S4 Fig). The virus inhibition assays revealed that all
396 samples exhibit net anti-ZIKV (Fig 4A) and anti-USUV activity (Fig 4B). Within each stage of
397 lactation, milk samples exhibited a wide range of ID_{50}s against both viruses, with mature milk
398 showing the greatest variation. In the anti-ZIKV assays, the ID_{50} values ranged from 0.0004 to
399 0.004 in colostrum, from 0.0006 to 0.005 in transitional milk and from 0.0003 to 0.007 in mature
400 milk (S2 Table). In the case of USUV, the ID_{50} values ranged from 0.002 to 0.01 in colostrum, from
401 0.001 to 0.007 in transitional milk and from 0.002 to 0.02 in mature milk (S3 Table). The mean
402 antiviral activity of milk samples appeared to differ according to the stages of lactation with
403 colostrum and transitional milk showing lower mean ID_{50} values than mature milk, but the
404 difference did not reach statistical significance. These results also indicated that human milk is
405 overall more active against ZIKV, confirming what previously demonstrated with colostrum
406 samples and described above. In Fig S5 A and B, the results obtained from every single mother are
407 separately reported.
408

409 **Table 1. Main clinical characteristics of the first study group**

Sample n°	Gestational Age	Mother's Age	Parity	Type of delivery
1	35+0	39	2002	CS
2	32+1	33	0000	CS
3	29+1	32	0000	CS
4	39+4	33	1001	CS
5	29+5	38	0000	S
6	38+5	40	0000	CS
7	33+2	35	0000	CS
8	27+3	43	1001	CS
9	30+0	30	1001	CS
10	37+4	35	0000	CS
11	38+4	39	1021	S
12	38+2	39	1001	S
13	38+4	37	0000	S
14	34+1	30	1001	S
15	37+4	23	0000	S
16	38+2	42	0000	CS
F1	40+1	32	0000	S
F2	33+3	39	1001	S

410 S: spontaneous delivery; CS: cesarean section.

411

412 **Table 2. Main clinical characteristics of the second study group**

Sample n°	Gestational Age	Mother's Age	Parity	Type of delivery
17	41+2	28	0000	CS
18	39+4	26	0000	S
19	39+6	33	1011	CS
20	39+4	30	0010	S
21	40+3	40	1001+1 VTP	S
22	37+1	33	1001	S
23	39+4	34	0000	S
24	38+5	40	1001	S
25	38+2	43	1001	CS
26	40+0	33	2002	S
27	40+4	41	0000	CS

413 S: spontaneous delivery; CS: cesarean section; VTP: voluntary termination of pregnancy

414 **Fig 1. Anti-ZIKV and anti-USUV activities of defatted colostrum samples**. Cells and viruses
415 were treated before and during the infection with serial dilutions of human colostrum aqueous
416 fraction (from 1:3 to 1:6561 parts). (A) Anti-ZIKV and anti-USUV inhibitory dilution-50 values
417 obtained from three independent experiments are reported. Results are expressed as mean ± SEM of
418 inhibitory dilution-50 values (Student's t test; ** $p<0.01$). Results obtained with fresh colostra are
419 indicated with white triangles. (B) Panel B shows the anti-ZIKV activity of three colostrum samples
420 against the African MR766 strain. The dose-response curves are reported. Data are presented as %
421 of control. Values are means ± SEM of three independent experiments performed in duplicate.

422 **Fig 2. Evaluation of the anti-ZIKV (A) and anti-USUV (B) activity of human colostrum with**
423 **the immunofluorescence assay detecting the dsRNA intermediate of replication and the**
424 **flavivirus protein E.** Cells and viruses (MOI=3) were treated before and during the infection with
425 the dilution of colostrum corresponding the ID_{90} in the virus inhibition assay. After 30 h of
426 infection, cells were fixed and subjected to immunofluorescence.

427 **Fig 3. Evaluation of cell viability after colostrum treatment.** Cells were treated under the same
428 conditions of the ZIKV (A) and USUV (B) inhibition assay. Results obtained from 3 randomly
429 selected colostrum samples are reported and indicated as % of untreated control. Values are means
430 ± SEM of three independent experiments performed in duplicate.

431 **Fig 4. Anti-ZIKV (A) and anti-USUV (B) activity of defatted human milk samples at different**
432 **stages of maturation**. Cells and viruses were treated before and during the infection with serial
433 dilutions of human milk aqueous fraction (from 1:3 to 1:6561 parts). The inhibitory dilution-50
434 values for colostrum, transitional milk and mature milk from a cohort of eleven mothers are
435 reported. Results are expressed as mean ± SEM and analyzed by ANOVA followed by Bonferroni
436 post hoc test;

437

438 **Human colostrum alters the binding of ZIKV and USUV to cells**

439 The second goal of this study is to investigate which step of ZIKV and USUV replicative cycle is
440 inhibited by HM. Colostrum was selected for the execution of these tests due to the overall lower
441 mean ID_{50} values compared to transitional and mature milk. Three samples of colostrum were
442 randomly selected from the initial screening group for both viruses. First, we investigated whether
443 the aqueous fraction of colostrum is endowed with an intrinsic virucidal activity, i.e. whether
444 colostrum acts by directly inactivating the viral particle. As reported in Fig 5, we did not observe
445 any significant virucidal activity for both viruses. The pre-treatment of cells with serial dilutions of
446 colostrum for 2 h before infection did not alter ZIKV and USUV infectivity, thus indicating that
447 colostrum does not act directly on cells preventing viral infection (Fig 6). Subsequently, the early
448 steps of viral replication, i.e. the binding and the entry steps, were evaluated: the treatment with the
449 aqueous fraction of colostrum was performed during the attachment of the virus to cells or during
450 the cell-penetration processes. Results demonstrated that human colostrum does not alter the entry
451 of viruses into cells (Fig 7 C-D), but it acts by preventing the binding of both viruses to cells. In
452 fact, as reported in Fig 7A-B, the titer of bound ZIKV and USUV to cells is significantly reduced in
453 presence of colostrum. The reduction of ZIKV and USUV titer resulted in more than one order of
454 magnitude between the treated and the untreated samples and was confirmed for all the tested
455 colostrum samples (numerical results are reported in S4 Table). Lastly, we verified the absence of
456 any additional activity on the later steps of viral replication. When cells were treated with colostrum
457 dilutions after ZIKV or USUV infection for three hours, no reduction in the number of infected
458 cells was observed (Fig 8).

459

460 **Fig 5. Evaluation of ZIKV (A) and USUV (B) inactivation by human colostrum aqueous**
461 **fraction.** Viruses were incubated with colostrum for 2h at 37°C and subsequently the residual viral
462 infectivity was evaluated. On the y- axis, the infectious titers are expressed as plaque- forming

463 units per ml (PFU/ml) (A) or focus-forming unit per ml (B). Error bars represent standard error of

464 the mean of three independent experiments (Student's t test; ns: not significant)

465 **Fig 6. Pre-treatment assay.** Cells were pre-treated with serial dilutions of colostrum for two hours

466 before infection. After washing, cells were infected with ZIKV (A) or USUV (B) and the number of

467 ZIKV plaques or USUV foci was evaluated after 72 or 24 hours respectively. The dose-response

468 curves are reported. Data are presented as % of control. Values are means ± SEM of three

469 independent experiments performed in duplicate.

470 **Fig 7. Binding assay (A, B) and entry assay (C, D).** ZIKV (A) or USUV (B) (MOI=3) were

471 allowed to attach to the cells in presence of colostrum (ID_{90}) for 2h on ice. The cell-bound virus

472 titers were determined by means of plaque assay (ZIKV) or indirect immunostaining (USUV). On

473 the y- axis, the infectious titers are expressed as plaque- forming units per ml (PFU/ml) (A) or

474 focus-forming unit per ml (B). Error bars represent standard error of the mean of three independent

475 experiments (Student's t test; * $p < 0.05$; *** $p < 0.001$; ****$p < 0.0001$); For the entry assay,

476 ZIKV (C) or USUV (D) were absorbed for 2 h at 4 °C on pre-chilled Vero cells. After the removal

477 of the unbound virus, the temperature was shifted to 37 °C to allow the entry of pre-bound virus in

478 presence of serial dilutions of colostrum for 2h. Unpenetrated virus was inactivated with citrate

479 buffer and the number of ZIKV plaques or the number of USUV foci was evaluated after 72 h or 24

480 h respectively. The dose-response curves are reported. Data are presented as % of control. Values

481 are means ± SEM of three independent experiments performed in duplicate.

482 **Fig 8. Post-entry assay.** Cells were infected with ZIKV (A) or USUV (B) for 2 h at 37°C. After

483 washing with citrate buffer, cells were treated with serial dilutions of colostrum for 3 h. The number

484 of ZIKV plaques or USUV foci was evaluated after 72 or 24 hours respectively. The dose-response

485 curves are reported. Data are presented as % of control. Values are means ± SEM of three

486 independent experiments performed in duplicate.

487 **Anti-ZIKV and anti-USUV components of HM: the HM-EVs and the HM-GAGs**

488 Prompted by the above findings, we sought to identify new antiviral components of human milk
489 that could contribute to the antiviral potency of this biofluid. To this aim EVs and GAGs were
490 isolated and characterized.
491 Colostrum was selected as preferred lactation stage for EVs isolation, due to the previously reported
492 higher concentration of EV in colostrum than transitional and mature milk [34].
493 The colostrum-derived EVs were characterized according to the "Minimal Information for Studies
494 of Extracellular Vesicles guidelines" (MISEV2018) proposed by the International Society for
495 Extracellular Vesicles (ISEV) [55]. As reported in Fig 9A, the EV-lysate was positive for the three
496 tetraspanines CD63, CD9 and CD81, which are known to be enriched in EVs from multiple tissue
497 sources, and it was positive for two cytosolic proteins (caveolin-1 and HSP70) usually recovered in
498 EVs. Furthermore, the colostrum-derived EV lysate was negative for the contaminating
499 endoplasmatic reticulum-related protein calnexin. We analyzed EV size and concentration by means
500 of the nanoparticle tracking analysis. The NanoSight instrument showed that most EVs were
501 between 100-400 nm in diameter: EVs had a mean diameter of 250.0 +/- 8.0 nm (EVs 6), 191.7 +/-
502 1.5 nm (EVs 7) and 200.3 +/- 2.9 nm (EVs 8) (Mode: 199.4 +/- 16.0 nm, 130.5 +/- 7.9 nm, 137.5
503 +/- 14.6 nm for sample 6, 7 and 8 respectively). Particle concentration was 7.55 $x10^{12}$ (EVs 6), 7.02
504 $x10^{12}$ (EVs 7) and 9.33 x 10^{12} (EVs 8) particles/ml. A representative analysis is reported in Fig 9B.
505 After the characterization, EVs were tested *in vitro* against ZIKV and USUV. The results (Fig 9 C-
506 D) demonstrated that colostrum-derived EVs are endowed with a strong antiviral activity against
507 both viruses. Notably, the EC_{50} values of the 3 EV populations were 11.47, 7.04 and 16.22 μg
508 protein/ml for ZIKV and 11.61, 12.33 and 32.57 μg protein/ml for USUV.
509 Next, the antiviral potency of GAGs isolated from a pool of mature milk samples was evaluated *in*
510 *vitro.* Mature milk was selected as preferred biological matrix because is more commonly available
511 than colostrum. According to previous studies [39,40,56], the HM-GAGs fraction tested in this
512 study was mainly composed of CS/DS and HS/Hep as evident from the electrophoresis (Fig 10A).

513 Moreover, from the structural characterization analysis (confirmed by the electrophoresis), we
514 obtained a percentage of ~55% CS and 1-2% DS, ~40% HS/low-sulfated Hep (known as fast-
515 moving Hep) and ~2% high-sulfated Hep (known as slow-moving Hep), and trace amount (1-2%)
516 of HA. Additionally, the HM-CS was confirmed to have a very typical low charge density (~0.35)
517 compared to the other known CS [57]. Finally, the purified HM-GAGs were tested to have a purity
518 greater than 98%.
519 Results from the antiviral assays revealed that HM-GAGs are active against ZIKV and USUV in the
520 range of physiological concentrations detected in term and preterm human colostrum [42] (Fig
521 10B). The EC_{50} values were 5.8 mg/ml for ZIKV and 3.3 mg/ml for USUV with the higher
522 concentration tested (10 mg/ml) able to completely inhibit USUV infection and to inhibit the 70 %
523 of ZIKV infection.
524 Both EVs samples and HM-GAGs did not show any cytotoxicity (S7 Fig).
525 Altogether these results demonstrated that colostrum derived EVs and HM-GAGs contribute, at
526 least in part, to the anti-ZIKV and anti-USUV intrinsic action of human milk.
527

528 **Fig 9. Characterization of colostrum derived-EVs (A, B) and study of their anti-ZIKV (C) and**
529 **anti-USUV (D) activity.** The protein profile of the colostrum-derived EVs was analysed by means
530 of Western blotting using Abs against the endoplasmatic reticulum-related protein calnexin and
531 against the EVs marker proteins CD63, CD9, CD81, Hsp70 and Caveolin-1. HFF cell lysate was
532 used as control (A). In panel B, the Nanoparticle tracking analysis (NTA) is reported. In panels C
533 and D the results obtained from the antiviral assays are reported. Cells and viruses were treated
534 before and during the infection with serial dilutions of EVs. The dose response curves are reported.
535 Data are presented as % of control. Values are means ± SEM of three independent experiments
536 performed in duplicate.

537 **Fig 10. Electrophoresis separation of HM-GAGs (A) and evaluation of their anti-ZIKV and**
538 **anti-USUV action (B).** (A) Purified HM-GAGs were separated by means of acetate of cellulose
539 electrophoresis. CS: chondroitin sulfate; DS: dermatan sulfate; HS: heparan sulfate; FM: fast-
540 moving heparin; SM: slow-moving heparin; o: origin; ST: GAGs standard; HM: human milk; (B)
541 Cells and viruses were treated before and during the infection with serial dilutions of HM-GAGs.
542 Data are presented as % of control and the dose response curves are reported. Values are means ±
543 SEM of three independent experiments performed in duplicate.

544

545 **Discussion and conclusion**

546 While the antiviral activity of HM and its components against numerous viral pathogens have been
547 described in literature, its role in protecting against emerging arboviruses has been poorly
548 investigated so far [25,58,59]. In this study we addressed this issue focusing on two emerging
549 flaviviruses: we assessed the anti-ZIKV and the anti-USUV activity of human milk in its different
550 stages of maturation and we explored the antiviral contribution of specific components, namely the
551 HM-EVs and the HM-GAGs.

552 The first notable finding is that HM is endowed with antiviral activity against ZIKV and USUV in
553 all the stages of lactation (colostrum, transitional and mature milk) with no significant differences
554 between them. We previously reported a different pattern in the anti-CMV activity of human milk
555 [54]: we demonstrated that colostrum from CMV-IgG+ mothers was significantly more potent than
556 transitional and mature milk and this was due to the higher content of specific immune factors,
557 especially sIgA, in the early stages of lactation. On the contrary, the presence of specific
558 immunoglobulins in the milk samples of the present study is very unlikely, considering the absence
559 of a past travel history in ZIKV endemic areas of the donor mothers and the low seroprevalence of
560 USUV in Europe [11]. The anti-ZIKV and anti-USUV action is attributable to non-specific
561 bioactive factors that act independently from the mother serostatus. Which is the antiviral potency
562 of colostrum from ZIKV or USUV infected mothers remains an interesting open question.

563 We also demonstrated that human colostrum exerts an antiviral action against two different ZIKV
564 strains, the HPF2013 and the MR766, belonging to the Asian and African lineage respectively.
565 These results indicate that breast milk could play a protective role against either the microcephalic
566 Asian strains, that have caused the latest epidemics, and the African strains, that have shown to be
567 more infectious *in vitro* and *in vivo*, but have not caused any recently reported human case [60]. To
568 the best of our knowledge, this is the first study reporting the intrinsic anti-USUV activity of human
569 milk. On the contrary, Pang et al. recently indicated a potential anti-ZIKV action of HM in an
570 artificial feeding model mice [25] and other two reports demonstrated that breast milk is able to

571 inactivate ZIKV in a time-dependent manner. These latter studies attributed the antiviral potential to
572 the fat containing cream fraction of human milk, in which the free fatty acids, released upon storage
573 by milk lipases in a time-dependent manner, incorporate into the viral envelope thereby destroying
574 the viral particle [30,61]. In our experiments, we eliminated the storage affected lipid components
575 from the milk samples and we analyzed the aqueous fractions after having verified that our storage
576 method did not alter the antiviral properties. Our results therefore indicate the presence of an
577 intrinsic anti-ZIKV activity in the aqueous fraction of HM too.
578 We explored the anti-ZIKV and anti-USUV activity of human milk analyzing the putative step of
579 viral replication inhibited by this biofluid. We focused on the first stage of lactation, because
580 colostrum samples collectively showed the lowest ID_{50} values in the virus inhibition assays. We
581 demonstrated that the aqueous fraction of colostrum acts by altering the binding of ZIKV and
582 USUV to cells, thus preventing cellular infection. Despite the early steps of USUV and ZIKV
583 replicative cycles are not yet fully defined, it has been demonstrated that different molecules play a
584 role in flaviviruses attachment to cells. The most common attachment factors are negatively charged
585 glycosaminoglycans (GAGs), which can be utilized by several flaviviruses, including DENV,
586 WNV, JEV and tick-borne encephalitis virus (TBEV), as low-affinity attachment factors to
587 concentrate the virus on cell surface [62]. Contradictory results have emerged instead from studies
588 about the dependence of ZIKV on cellular GAGs [63–66]. In addition, phosphatidylserine (PS)
589 receptors' families, such as T-cell immunoglobulin (TIM) and TYRO3, AXL and MERTK (TAM),
590 as well as integrins and C-type lectin receptors (CLRs), have been described as key factors during
591 the initial steps of flavivirus cell invasion [62,67]. Given the extremely complex composition of
592 human milk and the results obtained from the study of its mechanism of action, we could
593 hypothesize the presence of one or more factors in HM able to limit USUV and ZIKV interaction
594 with the above mentioned cellular receptors. We can exclude a possible action of residual free fatty
595 acids, since the mechanism of action indicated by Conzelmann et al. and attributed to the lipid
596 components consisted of a virucidal activity.

597 To support our results, we investigated the antiviral activity of two HM components derived from
598 the aqueous fraction of human milk. HM-EVs showed a strong inhibition of ZIKV and USUV
599 infection *in vitro*, suggesting that they could contribute, at least in part, to the overall antiviral
600 action of human milk. Consistently with these findings, previous studies demonstrated the antiviral
601 potency of HM-EVs and indicated that they compete with viruses for the binding to cellular
602 receptors [44,68]. In particular, Näslund and colleagues showed that HM-exosomes compete with
603 HIV-1 for binding to DC-SIGN receptor on monocyte-derived dendritic cells. Even though our
604 results are preliminary and the mechanism of action has not been investigated yet, we speculate that
605 a similar mechanism could be possible against ZIKV and USUV too.
606 GAGs are other HM components that could contribute in inhibiting the binding of both flaviviruses
607 to cells. Since numerous viruses, including some flaviviruses, attach to cell exploiting cellular
608 GAGs, these complex carbohydrates from different origins have been largely investigated for their
609 ability to act as soluble receptors [69–74]. Notably, the first data available on the antiviral role of
610 GAGs isolated from HM are those reported by Newburg et al. demonstrating that HM-CS was able
611 to inhibit the binding of the HIV envelop glycoprotein gp120 to the cellular CD4 receptor [46]. In
612 this study, HM-GAGs were tested against ZIKV and USUV at concentrations that would be
613 relevant to breastfed infants [40]. In particular, highest GAGs values are present at 4th day after
614 partum (~ 9.3 mg/ml and ~ 3.8 mg/ml in preterm and term milk, respectively), followed by a
615 progressive decrease up to day 30th (~ 4.3 mg/ml and ~ 0.4 mg/ml). Given the possibility that
616 cellular GAGs are low-affinity attachment factors for ZIKV and USUV, the antiviral action of HM-
617 GAGs (potentially acting as decoy receptors) is not as strong as expected, but the EC_{50} values fall
618 within the range of GAG concentrations in preterm and term colostrum. Furthermore, the GAGs
619 concentration detected in preterm mature milk is still partially active against USUV. These results
620 indicate that HM-GAGs could only partially contribute to the overall antiviral action of human
621 milk, mostly acting during the first days after birth. This also highlights that the dependence of
622 ZIKV and USUV on cellular GAGs for their attachment to cells warrant further investigations.

In conclusion, the intrinsic anti-ZIKV activity of human milk here reported, along with the higher viscosity detected in colostrum from ZIKV-infected mothers and the storage-dependent virucidal activity of HM lipid components, are three different factors that hinder the spread of ZIKV through breastfeeding [30,75]. Furthermore, we can now add USUV to the list of viral pathogens inhibited by human milk. Altogether, our data support the WHO recommendations about breast-feeding during ZIKV infection and could contribute to producing new guidelines for a possible USUV epidemic.

632 **Bibliography**

633 1.Ashraf U, Ye J, Ruan X, Wan S, Zhu B, Cao S. Usutu virus: an emerging flavivirus in Europe. Viruses.
634 2015 Jan 19;7(1):219–38.

635 2.Paixão ES, Barreto F, Teixeira M da G, Costa M da CN, Rodrigues LC. History, Epidemiology, and
636 Clinical Manifestations of Zika: A Systematic Review. Am J Public Health. 2016 Apr;106(4):606–12.

637 3.Weaver SC, Costa F, Garcia-Blanco MA, Ko AI, Ribeiro GS, Saade G, et al. Zika Virus: History,
638 Emergence, Biology, and Prospects for Control. Antiviral Res. 2016 Jun;130:69–80.

639 4.Koppolu V, Shantha Raju T. Zika virus outbreak: a review of neurological complications, diagnosis,
640 and treatment options. J Neurovirol. 2018;24(3):255–72.

641 5.Mlakar J, Korva M, Tul N, Popović M, Poljšak-Prijatelj M, Mraz J, et al. Zika Virus Associated with
642 Microcephaly. N Engl J Med. 2016 Mar 10;374(10):951–8.

643 6.Pacheco LD, Weaver SC, Saade GR. Zika Virus Infection - After the Pandemic. N Engl J Med. 2020
644 09;382(2):e3.

645 7. WHO | Zika situation report [Internet]. WHO. [cited 2020 May 4]. Available from:
646 http://www.who.int/emergencies/zika-virus/situation-report/10-march-2017/en/

647 8.Barzon L. Ongoing and emerging arbovirus threats in Europe. J Clin Virol. 2018;107:38–47.

648 9.Clé M, Beck C, Salinas S, Lecollinet S, Gutierrez S, Van de Perre P, et al. Usutu virus: A new threat?
649 Epidemiol Infect. 2019;147:e232.

650 10. Clé M, Barthelemy J, Desmetz C, Foulongne V, Lapeyre L, Bolloré K, et al. Study of Usutu virus
651 neuropathogenicity in mice and human cellular models. PLoS Negl Trop Dis. 2020
652 Apr;14(4):e0008223.

653 11. Roesch F, Fajardo A, Moratorio G, Vignuzzi M. Usutu Virus: An Arbovirus on the Rise. Viruses. 2019
654 Jul 12;11(7).

655 12. Salinas S, Constant O, Desmetz C, Barthelemy J, Lemaitre J-M, Milhavet O, et al. Deleterious effect of
656 Usutu virus on human neural cells. PLoS Negl Trop Dis. 2017 Sep;11(9):e0005913.

657 13. Mann TZ, Haddad LB, Williams TR, Hills SL, Read JS, Dee DL, et al. Breast Milk Transmission of
658 Flaviviruses in the Context of Zika Virus: A Systematic Review. Paediatr Perinat Epidemiol. 2018
659 Jul;32(4):358–68.

660 14. Cavalcanti MG, Cabral-Castro MJ, Gonçalves JLS, Santana LS, Pimenta ES, Peralta JM. Zika virus
661 shedding in human milk during lactation: an unlikely source of infection? International Journal of
662 Infectious Diseases. 2017 Apr 1;57:70–2.

663 15. Colt S, Garcia-Casal MN, Peña-Rosas JP, Finkelstein JL, Rayco-Solon P, Weise Prinzo ZC, et al.
664 Transmission of Zika virus through breast milk and other breastfeeding-related bodily-fluids: A
665 systematic review. PLoS Negl Trop Dis. 2017 Apr 10;11(4):e0005528

666 16. Dupont-Rouzeyrol M, Biron A, O'Connor O, Huguon E, Descloux E. Infectious Zika viral particles in
667 breastmilk. The Lancet. 2016 Mar 12;387(10023):1051.

668 17. Giovanetti M, Goes de Jesus J, Lima de Maia M, Junior JX, Castro Amarante MF, Viana P, et al.
669 Genetic evidence of Zika virus in mother's breast milk and body fluids of a newborn with severe
670 congenital defects. Clinical Microbiology and Infection. 2018 Oct 1;24(10):1111–2.

671 18. Sotelo JR, Sotelo AB, Sotelo FJB, Doi AM, Pinho JRR, Oliveira R de C, et al. Persistence of Zika
672 Virus in Breast Milk after Infection in Late Stage of Pregnancy. Emerg Infect Dis. 2017
673 May;23(5):854–6.

674 19. Besnard M, Lastere S, Teissier A, Cao-Lormeau V, Musso D. Evidence of perinatal transmission of
675 Zika virus, French Polynesia, December 2013 and February 2014. Euro Surveill. 2014 Apr 3;19(13).

676 20. Blohm GM, Lednicky JA, Márquez M, White SK, Loeb JC, Pacheco CA, et al. Complete Genome
677 Sequences of Identical Zika virus Isolates in a Nursing Mother and Her Infant. Genome Announc. 2017
678 Apr 27;5(17).

679 21. Blohm GM, Lednicky JA, Márquez M, White SK, Loeb JC, Pacheco CA, et al. Evidence for Mother-
680 to-Child Transmission of Zika Virus Through Breast Milk. Clin Infect Dis. 2018 19;66(7):1120–1.

681 22. Hemachudha P, Wacharapluesadee S, Buathong R, Petcharat S, Bunprakob S, Ruchiseesarod C, et al.
682 Lack of Transmission of Zika Virus Infection to Breastfed Infant. Clin Med Insights Case Rep. 2019
683 Mar 12;12:1179547619835179

684 23. Mello AS, Pascalicchio Bertozzi APA, Rodrigues MMD, Gazeta RE, Moron AF, Soriano-Arandes A,
685 et al. Development of Secondary Microcephaly After Delivery: Possible Consequence of Mother-Baby
686 Transmission of Zika Virus in Breast Milk. Am J Case Rep. 2019 May 21;20:723–5.

687 24. Sampieri CL, Montero H. Breastfeeding in the time of Zika: a systematic literature review. PeerJ.
688 2019;7:e6452.

689 25. Pang W, Lin Y-L, Xin R, Chen X-X, Lu Y, Zheng C-B, et al. Zika virus transmission via breast milk in
690 suckling mice. Clinical Microbiology and Infection [Internet]. 2020 Apr 25 [cited 2020 May 7];
691 Available from: http://www.sciencedirect.com/science/article/pii/S1198743X20302299

692 26. Mosca F, Giannì ML. Human milk: composition and health benefits. Pediatr Med Chir. 2017 Jun
693 28;39(2):155.

694 27. WHO | Infant feeding in areas of Zika virus transmission [Internet]. WHO. [cited 2020 May 4].
695 Available from: http://www.who.int/elena/titles/zika_breastfeeding/en/

696 28. Ballard O, Morrow AL. Human milk composition: nutrients and bioactive factors. Pediatr Clin North
697 Am. 2013 Feb;60(1):49–74.

698 29. Andreas NJ, Kampmann B, Mehring Le-Doare K. Human breast milk: A review on its composition and
699 bioactivity. Early Hum Dev. 2015 Nov;91(11):629–35.

700 30. Conzelmann C, Zou M, Groß R, Harms M, Röcker A, Riedel CU, et al. Storage-Dependent Generation
701 of Potent Anti-ZIKV Activity in Human Breast Milk. Viruses. 2019 Jun 28;11(7).

702 31. Falkler WA, Diwan AR, Halstead SB. A lipid inhibitor of dengue virus in human colostrum and milk;
703 with a note on the absence of anti-dengue secretory antibody. Arch Virol. 1975;47(1):3–10.

704 32. Fieldsteel AH. Nonspecific antiviral substances in human milk active against arbovirus and murine
705 leukemia virus. Cancer Res. 1974 Apr;34(4):712–5.

706 33. Pfaender S, Heyden J, Friesland M, Ciesek S, Ejaz A, Steinmann J, et al. Inactivation of hepatitis C
707 virus infectivity by human breast milk. J Infect Dis. 2013 Dec 15;208(12):1943–52.

708 34. de la Torre Gomez C, Goreham RV, Bech Serra JJ, Nann T, Kussmann M. 'Exosomics'-A Review of
709 Biophysics, Biology and Biochemistry of Exosomes With a Focus on Human Breast Milk. Front
710 Genet. 2018;9:92.

711 35. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol.
712 2013 Feb 18;200(4):373–83.

713 36. Zonneveld MI, Brisson AR, van Herwijnen MJC, Tan S, van de Lest CHA, Redegeld FA, et al.
714 Recovery of extracellular vesicles from human breast milk is influenced by sample collection and
715 vesicle isolation procedures. J Extracell Vesicles. 2014;3.

716 37. Abels ER, Breakefield XO. Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection,
717 Content, Release, and Uptake. Cell Mol Neurobiol. 2016 Apr;36(3):301–12.

718 38. van der Pol E, Böing AN, Harrison P, Sturk A, Nieuwland R. Classification, functions, and clinical
719 relevance of extracellular vesicles. Pharmacol Rev. 2012 Jul;64(3):676–705.

720 39. Coppa GV, Gabrielli O, Buzzega D, Zampini L, Galeazzi T, Maccari F, et al. Composition and
721 structure elucidation of human milk glycosaminoglycans. Glycobiology. 2011 Mar;21(3):295–303.

722 40. Coppa GV, Gabrielli O, Zampini L, Galeazzi T, Maccari F, Buzzega D, et al. Glycosaminoglycan
723 content in term and preterm milk during the first month of lactation. Neonatology. 2012;101(1):74–6.

724 41. Shimizu M, Uryu N, Yamauchi K. Presence of Heparan Sulfate in the Fat Globule Membrane of
725 Bovine and Human Milk. Agricultural and Biological Chemistry. 1981 Mar 1;45(3):741–5.

726 42. Coppa GV, Gabrielli O, Bertino E, Zampini L, Galeazzi T, Padella L, et al. Human milk
727 glycosaminoglycans: the state of the art and future perspectives. Ital J Pediatr. 2013 Jan 15;39:2.

728 43. Zempleni J, Aguilar-Lozano A, Sadri M, Sukreet S, Manca S, Wu D, et al. Biological Activities of
729 Extracellular Vesicles and Their Cargos from Bovine and Human Milk in Humans and Implications for
730 Infants. J Nutr. 2017;147(1):3–10.

731 44. Näslund TI, Paquin-Proulx D, Paredes PT, Vallhov H, Sandberg JK, Gabrielsson S. Exosomes from
732 breast milk inhibit HIV-1 infection of dendritic cells and subsequent viral transfer to CD4+ T cells.
733 AIDS. 2014 Jan 14;28(2):171–80.

734 45. Coppa GV, Facinelli B, Magi G, Marini E, Zampini L, Mantovani V, et al. Human milk
735 glycosaminoglycans inhibit in vitro the adhesion of Escherichia coli and Salmonella fyris to human
736 intestinal cells. Pediatr Res. 2016 Apr;79(4):603–7.

737 46. Newburg DS, Linhardt RJ, Ampofo SA, Yolken RH. Human milk glycosaminoglycans inhibit HIV
738 glycoprotein gp120 binding to its host cell CD4 receptor. J Nutr. 1995 Mar;125(3):419–24.

739 47. Viveros-Rogel M, Soto-Ramirez L, Chaturvedi P, Newburg DS, Ruiz-Palacios GM. Inhibition of HIV-
740 1 infection in vitro by human milk sulfated glycolipids and glycosaminoglycans. Adv Exp Med Biol.
741 2004;554:481–7.

742 48. Arslanoglu S, Bertino E, Tonetto P, Nisi GD, Ambruzzi AM, Biasini A, et al. Guidelines for the
743 establishment and operation of a donor human milk bank. The Journal of Maternal-Fetal & Neonatal
744 Medicine. 2010 Sep 1;23(sup2):1–20.

745 49. Francese R, Civra A, Rittà M, Donalisio M, Argenziano M, Cavalli R, et al. Anti-zika virus activity of
746 polyoxometalates. Antiviral Research. 2019 Mar 1;163:29–33.

747 50. Civra A, Francese R, Gamba P, Testa G, Cagno V, Poli G, et al. 25-Hydroxycholesterol and 27-
748 hydroxycholesterol inhibit human rotavirus infection by sequestering viral particles into late
749 endosomes. Redox Biol. 2018;19:318–30.

750 51. Cesaretti M, Luppi E, Maccari F, Volpi N. A 96-well assay for uronic acid carbazole reaction.
751 Carbohydrate Polymers. 2003 Oct 1;54(1):59–61.

752 52. Volpi N, Maccari F. Electrophoretic approaches to the analysis of complex polysaccharides. J
753 Chromatogr B Analyt Technol Biomed Life Sci. 2006 Apr 13;834(1–2):1–13.

754 53. Mantovani V, Galeotti F, Maccari F, Volpi N. Recent advances in capillary electrophoresis separation
755 of monosaccharides, oligosaccharides, and polysaccharides. Electrophoresis. 2018;39(1):179–89.

756 54. Donalisio M, Rittà M, Tonetto P, Civra A, Coscia A, Giribaldi M, et al. Anti-Cytomegalovirus Activity
757 in Human Milk and Colostrum From Mothers of Preterm Infants. J Pediatr Gastroenterol Nutr.
758 2018;67(5):654–9.

759 55. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal
760 information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the
761 International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell
762 Vesicles. 2018;7(1):1535750.

763 56. Coscia A, Peila C, Bertino E, Coppa GV, Moro GE, Gabrielli O, et al. Effect of holder pasteurisation
764 on human milk glycosaminoglycans. J Pediatr Gastroenterol Nutr. 2015 Jan;60(1):127–30.

765 57. Volpi N. Chondroitin Sulfate Safety and Quality. Molecules. 2019 Apr 12;24(8).

766 58. Lawrence RM, Lawrence RA. Breast milk and infection. Clin Perinatol. 2004 Sep;31(3):501–28.

767 59. Chirico G, Marzollo R, Cortinovis S, Fonte C, Gasparoni A. Antiinfective Properties of Human Milk. J
768 Nutr. 2008 Sep 1;138(9):1801S-1806S.

769 60. Beaver JT, Lelutiu N, Habib R, Skountzou I. Evolution of Two Major Zika Virus Lineages:
770 Implications for Pathology, Immune Response, and Vaccine Development. Front Immunol.
771 2018;9:1640.

772 61. Pfaender S, Vielle NJ, Ebert N, Steinmann E, Alves MP, Thiel V. Inactivation of Zika virus in human
773 breast milk by prolonged storage or pasteurization. Virus Res. 2017 15;228:58–60.

774 62. Agrelli A, de Moura RR, Crovella S, Brandão LAC. ZIKA virus entry mechanisms in human cells.
775 Infect Genet Evol. 2019;69:22–9.

776 63. Kim SY, Zhao J, Liu X, Fraser K, Lin L, Zhang X, et al. Interaction of Zika Virus Envelope Protein
777 with Glycosaminoglycans. Biochemistry. 2017 28;56(8):1151–62.

778 64. Gao H, Lin Y, He J, Zhou S, Liang M, Huang C, et al. Role of heparan sulfate in the Zika virus entry,
779 replication, and cell death. Virology. 2019;529:91–100.

780 65. Cagno V, Tseligka ED, Jones ST, Tapparel C. Heparan Sulfate Proteoglycans and Viral Attachment:
781 True Receptors or Adaptation Bias? Viruses. 2019 Jul 1;11(7).

782 66. Ghezzi S, Cooper L, Rubio A, Pagani I, Capobianchi MR, Ippolito G, et al. Heparin prevents Zika
783 virus induced-cytopathic effects in human neural progenitor cells. Antiviral Res. 2017;140:13–7.

784 67. Oliveira LG, Peron JPS. Viral receptors for flaviviruses: Not only gatekeepers. J Leukoc Biol. 2019
785 Sep;106(3):695–701.

786 68. van Dongen HM, Masoumi N, Witwer KW, Pegtel DM. Extracellular Vesicles Exploit Viral Entry
787 Routes for Cargo Delivery. Microbiol Mol Biol Rev. 2016 Jun;80(2):369–86.

788 69. Olofsson S, Bergström T. Glycoconjugate glycans as viral receptors. Ann Med. 2005;37(3):154–72.

789 70. Shukla D, Spear PG. Herpesviruses and heparan sulfate: an intimate relationship in aid of viral entry. J
790 Clin Invest. 2001 Aug;108(4):503–10.

791 71. Spillmann D. Heparan sulfate: anchor for viral intruders? Biochimie. 2001 Aug;83(8):811–7.

792 72. Barth H, Schnober EK, Zhang F, Linhardt RJ, Depla E, Boson B, et al. Viral and cellular determinants
793 of the hepatitis C virus envelope-heparan sulfate interaction. J Virol. 2006 Nov;80(21):10579–90.

794 73. Cagno V, Andreozzi P, D'Alicarnasso M, Jacob Silva P, Mueller M, Galloux M, et al. Broad-spectrum
795 non-toxic antiviral nanoparticles with a virucidal inhibition mechanism. Nat Mater. 2018;17(2):195–
796 203.

797 74. Grice ID, Mariottini GL. Glycans with Antiviral Activity from Marine Organisms. Results Probl Cell
798 Differ. 2018;65:439–75.

799 75. de Quental OB, França EL, Honório-França AC, Morais TC, Daboin BEG, Bezerra IMP, et al. Zika
800 Virus Alters the Viscosity and Cytokines Profile in Human Colostrum. J Immunol Res.
801 2019;2019:9020519.

802

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810

811 **Supporting information:**

812 **Table S1. Anti-ZIKV and anti-USUV activities of defatted colostrum (numerical results of Fig**
813 **1A)**

814 **S2 Table. ID_{50} values of defatted human milk samples at different stages of maturation**
815 **against ZIKV (numerical results of Fig 5A)**

816 **S3 Table. ID_{50} values of defatted human milk samples at different stages of maturation**
817 **against USUV (numerical results of Fig 5B)**

818 **S4 Table. Numerical results of the binding assays reported in Fig 8A and 8B (the mean values**
819 **are reported)**

820 **S1 Fig. Anti-ZIKV (A) and anti-USUV (B) activities of two fresh colostrum samples.** Cells and
821 viruses were treated before and during the infection with serial dilutions of colostrum aqueous
822 fraction (from 1:3 to 1:6561 parts). The dose-response curves are reported. Data are presented as %
823 of control. Values are means ± SEM of three independent experiments performed in duplicate. The
824 ID_{50} values obtained from the ZIKV antiviral assays values were 0.0037 (colostrum F1) and

825 0.00097 (colostrum F2). In the case of USUV, the ID_{50} values were 0.018 (colostrum F1) and 0.02
826 (colostrum F2)

827 **S2 Fig. Comparison between the antiviral activity of colostrum from term and preterm**
828 **mothers.** Cells and viruses were treated before and during the infection with serial dilutions of
829 human colostrum aqueous fraction (from 1:3 to 1:6561 parts). Anti-ZIKV and anti-USUV
830 inhibitory dilution-50 values obtained from three independent experiments are reported and
831 stratified to compare term and preterm mothers. Panel A reports the results obtained by testing
832 colostra against ZIKV, while panel B shows the results for USUV. Results are expressed as mean ±
833 SEM of inhibitory dilution-50 values (Student's t test; ns: not significant).

834 **S3 Fig. Evaluation of the anti-ZIKV activity of human colostrum against the MR766 strain**
835 **with immunofluorescence assays detecting the dsRNA and the flavivirus protein E.** Cells and
836 viruses (MOI=3) were treated before and during the infection with the dilution of colostrum
837 corresponding the ID_{90} in the virus inhibition assay. After 30 h of infection, cells were fixed and
838 subjected to immunofluorescence.

839 **S4 Fig. Evaluation of cell viability after the treatment with transitional (A, C) or mature milk**
840 **(B, D).** Cells were treated under the same conditions of the ZIKV (A, B) and USUV (C, D)
841 inhibition assays. Results from 3 randomly selected samples are reported in each graph. Data are
842 indicated as % of untreated control. Values are means ± SEM of three independent experiments
843 performed in duplicate.

844 **S5 Fig. Anti-ZIKV (A) and anti-USUV (B) activity of defatted human milk samples at**
845 **different stages of maturation**. The inhibitory dilution-50 values of colostrum, transitional and
846 mature milk obtained from every single mother are separately reported indicating the sample
847 number.

848 **S6 Fig. Nanoparticle tracking analysis (NTA) of EVs 6 (A) and EVs 8 (B).**

849

850 **S7 Fig. Evaluation of cell viability after EVs (A) or GAGs treatment (B).** Cells were treated
851 under the same experimental conditions of the ZIKV and USUV inhibition assay, but without
852 infection. Cell viability was evaluated after 24 h or 72 h, respecting the same experimental timing of
853 USUV or ZIKV antiviral assay respectively. Results obtained with one representative EV
854 population and with the GAGs preparation are reported and indicated as % of untreated control.
855 Values are means ± SEM of three independent experiments performed in duplicate.

856

Figure 1

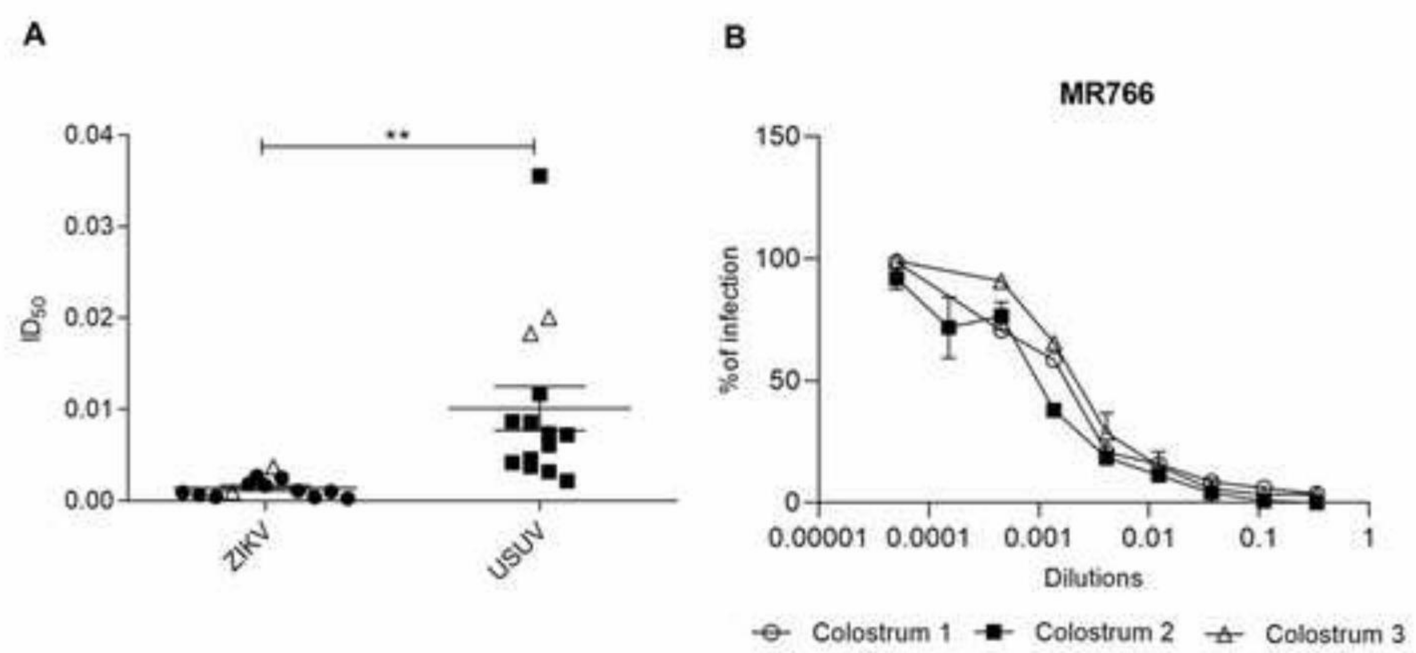
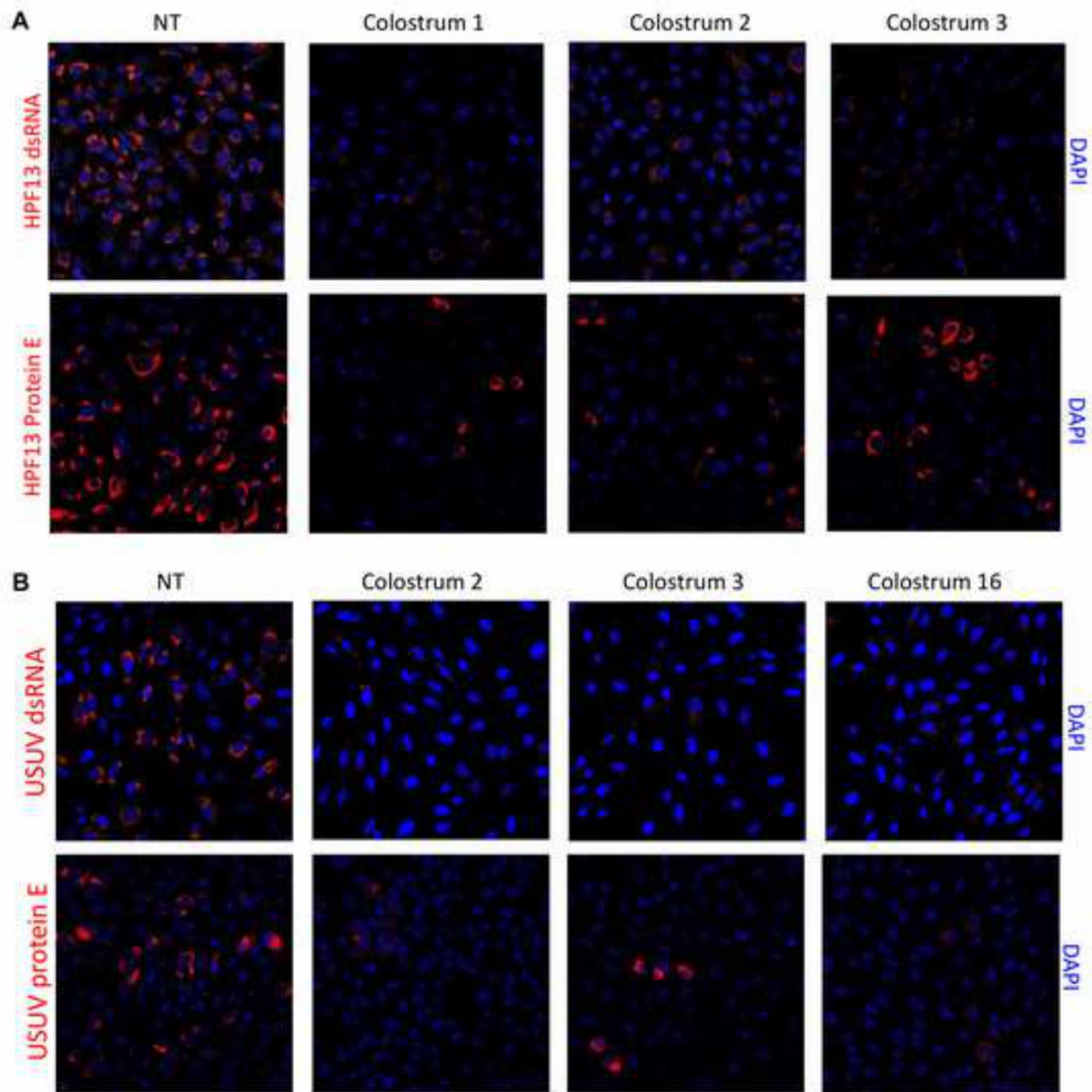


Figure 2



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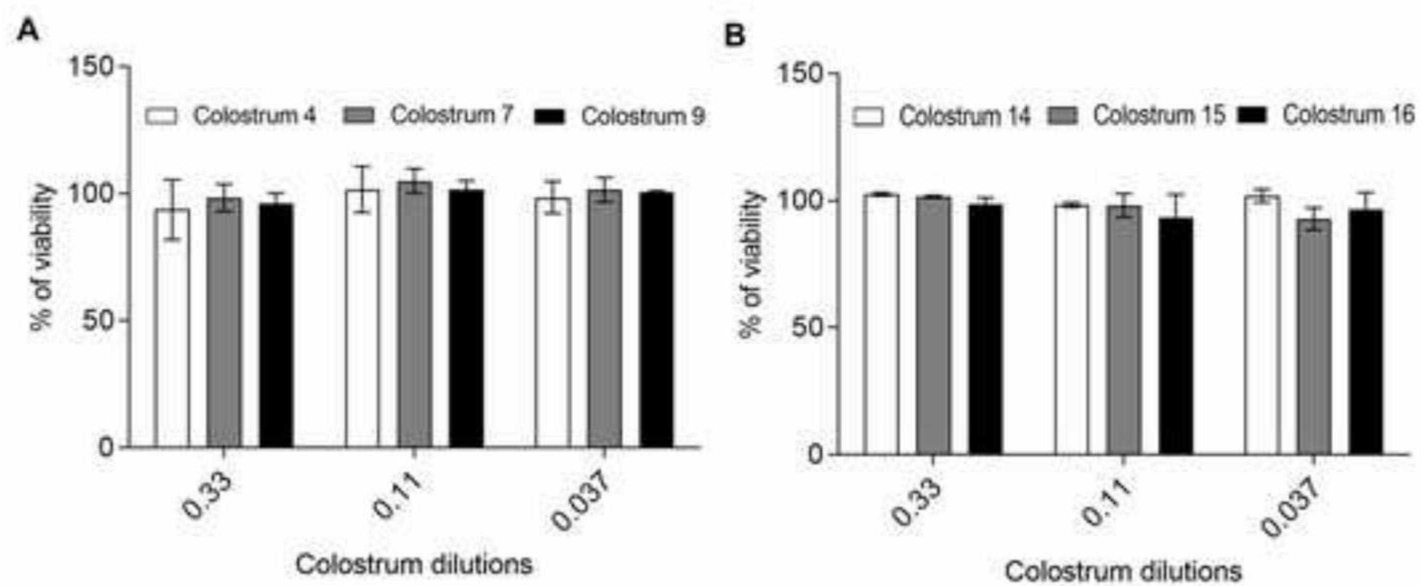

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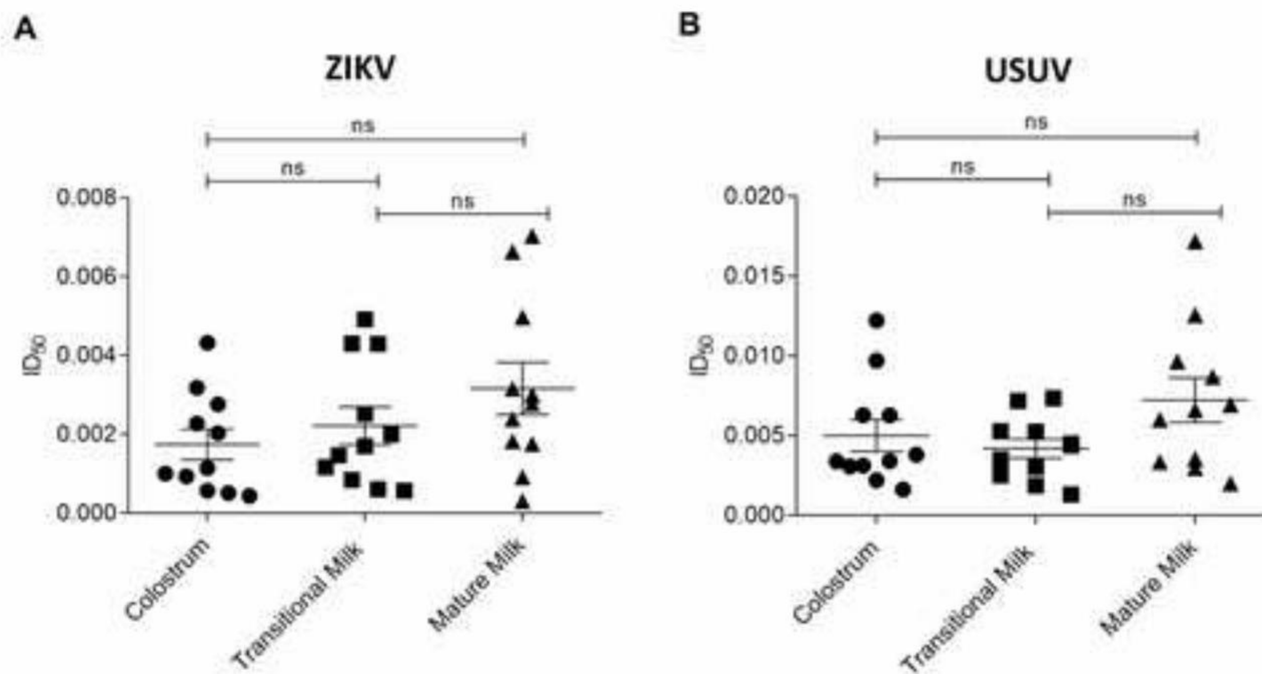

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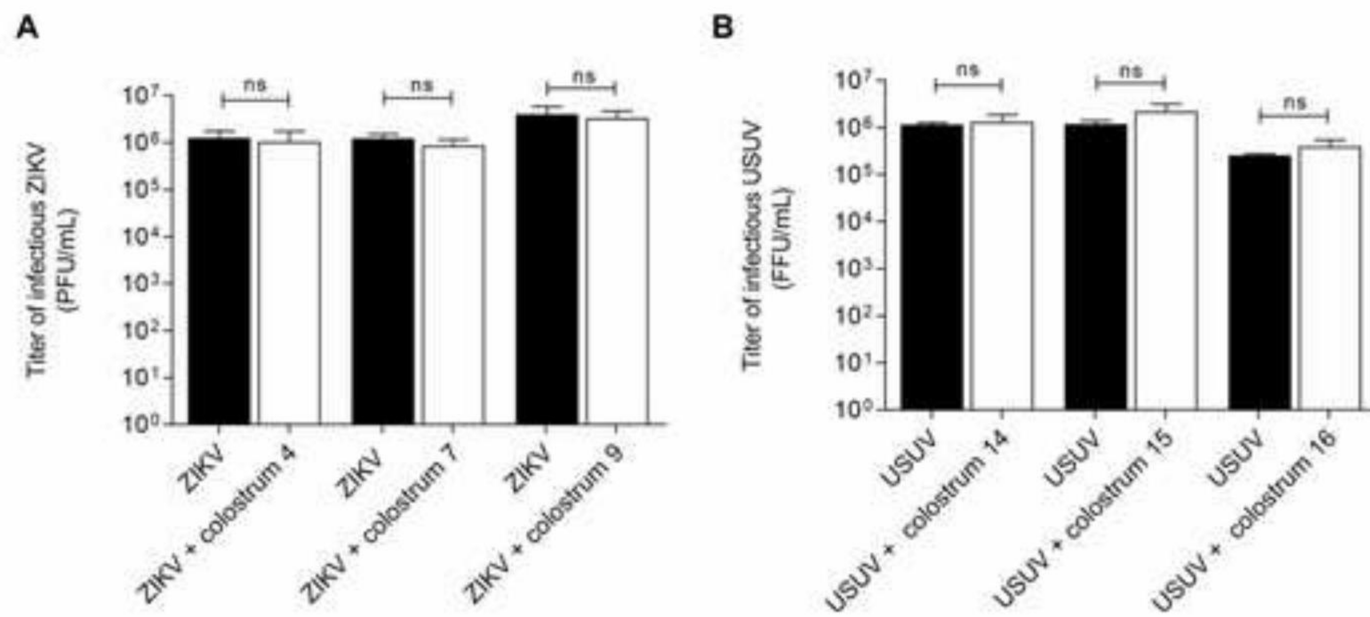

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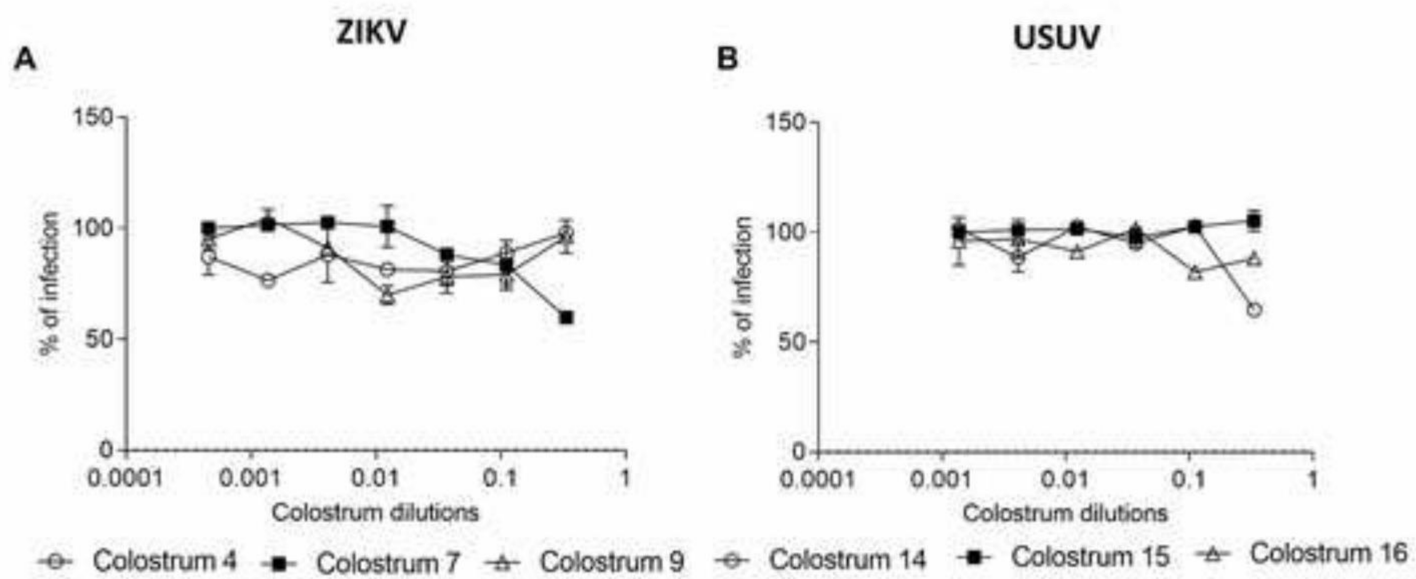

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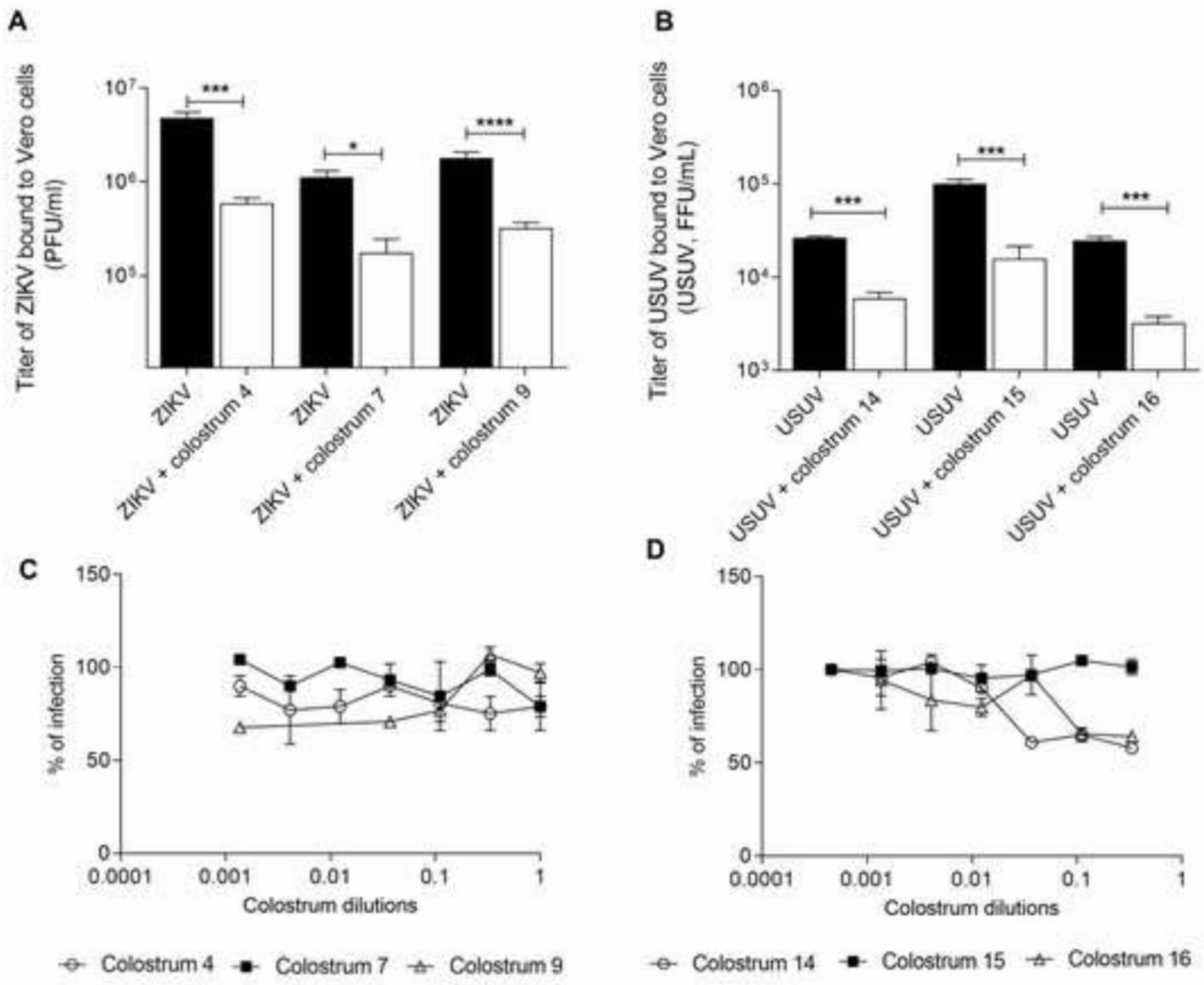

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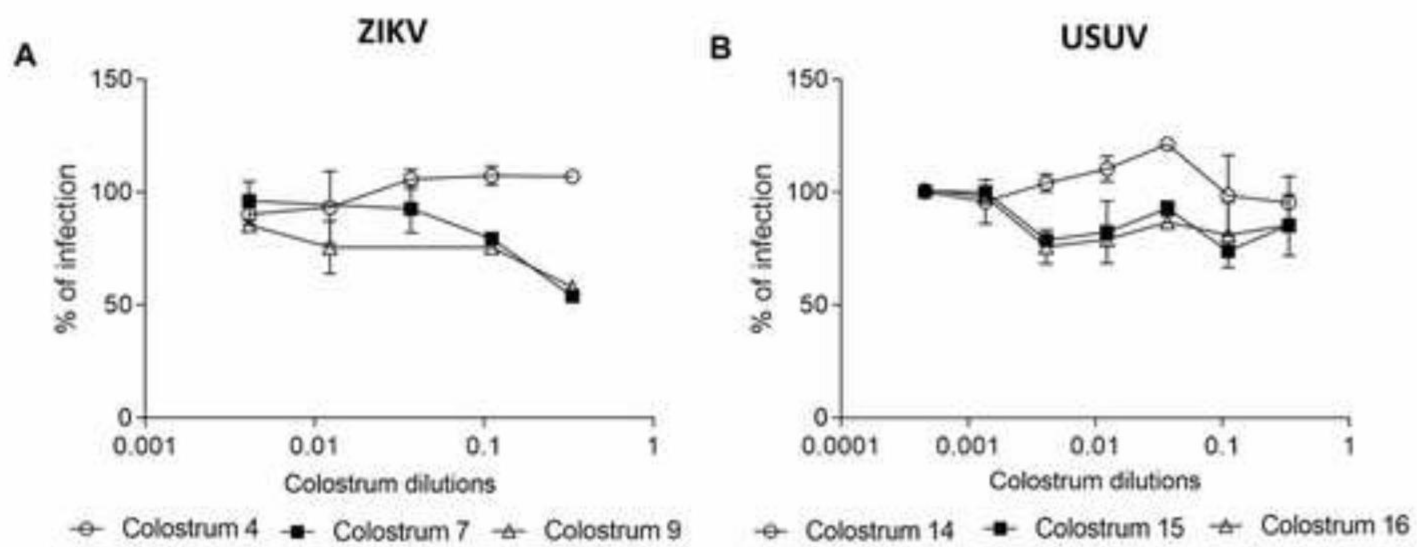

Figure 9

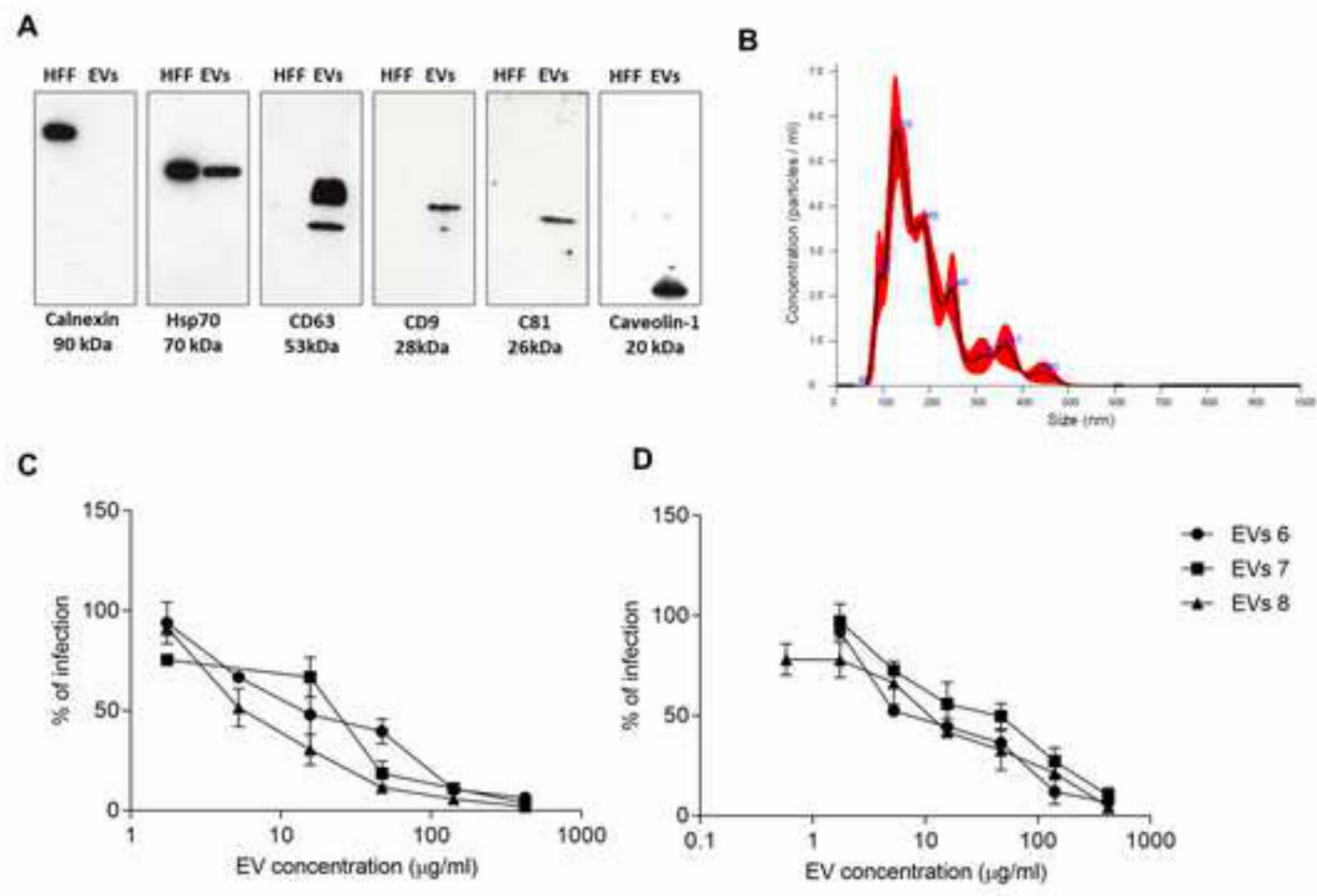

Figure 10