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Antirotaviral activity of bovine milk components: Extending the list of inhibitory proteins and seeking a better understanding of their neutralization mechanism

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ABSTRACT

The viral gastroenteritis mediated by rotavirus remains a major health problem, causing significant infant morbidity and mortality, and health care costs worldwide. Diverse studies have indicated that some milk-derived fractions exhibit antirotaviral activity, associated to proteins such as immunoglobulins, lactoferrin, mucins, and lactadherin. Nevertheless, further studies are needed to enable greater understanding of the interaction mechanisms involving rotaviruses, host cells, and neutralizing agents. Our results demonstrate that bovine milk is a source of antirotaviral compounds against a wide range of strains. The neutralizing activity of milk fractions was found to be directed to the spike protein VP4, while the purified milk proteins inhibited through interaction with either VP4 and/or VP7. The present results highlight the huge potential of bovine milk components for their inclusion in functional foods to control rotavirus diarrhea.

1. Introduction

The morbidity and mortality caused by viral gastroenteritis represent a major economic and public health burden, affecting mainly the poorest countries (Das, Salam, & Bhutta, 2014). Group A rotaviruses are the leading cause of severe diarrheal disease in infants and young children, being one of the top five causes of death worldwide across all age groups, and the second leading cause of death in children less than 5 years old (Marcotte & Hammarström, 2016).

The introduction of oral rotavirus vaccines has significantly reduced the incidence of the disease, especially in developed countries (Payne et al., 2013). However, these vaccines are scarcely used in lower income countries, leaving between a third to a half of children unprotected from severe rotavirus disease (Babji & Kang, 2012). In addition, they have shown a lower efficacy in countries with a high burden of diarrheal disease (Babji & Kang, 2012; Glass, Parashar, Patel, Gentsch, & Jiang, 2014), where they are needed most, so the impact of vaccine use on global estimates of rotavirus mortality has been limited (Tate, Burton, Boschi-Pinto, & Parashar, 2016). Furthermore, due to diverse hindrances regarding the availability of vaccines, contraindications in immunodeficient patients, and suboptimal use of therapeutic oral rehydration solutions (Binder, Brown, Ramakrishna, & Young, 2014; Gaspar, Hammarström, Mahlaoui, Borte, & Borte, 2014), the need for development of effective alternative approaches to prevent and control rotaviral gastroenteritis disease still remains.

Group A rotaviruses are non-enveloped viruses with a genome composed of 11 segments of dsRNA that are enclosed in a capsid consisting of three concentric protein layers. The outermost layer of the viral particle is composed of 260 trimers of the glycoprotein VP7 forming a smooth surface from which 60 trimeric spikes of VP4 protrude (Pesavento, Crawford, Estes, & Prasad, 2006). These viruses are classified into G- and P-types based on genetic and antigenic diversity of the two outer capsid proteins, VP7 (G-serotypes or genotypes) and VP4 (P-serotypes or genotypes), respectively. To date, 27 different G- and 37P-genotypes have been described in both humans and animals (Trojnar et al., 2013). The majority of the viruses infecting humans

Abbreviations: SA, sialic acid; NA, neuraminidase; TLPs, triple-layered particles; SC, serocolostrum; BM, buttermilk; BS, butter serum; MFGM, milk fat globule membrane; WPC, whey protein concentrate; IgG, immunoglobulin G; LF, lactoferrin; MUCs, mucins; LDH, lactadherin; XDH/XO, xanthine dehydrogenase/oxidase; β-LG, β-lactoglobulin; α-LA, α-lactalbumin; BSA, bovine serum albumin; CN, caseins; BTN, butyrophilin; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium

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belong to G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G12P[8] types (Bányai et al., 2012). With regard to bovine rotaviruses, at least nine G serotypes (G1-4, 6–8, 10, 11) and three P genotypes (P1, P5, and P11) have been found so far, but G6, G10, P[5], and P[11] have been reported to be the predominant G and P types, with G6P[11] being the most frequent combination (Alkan et al., 2010; Suocheng, Zhuandi, Tuanjie, Ayimu, & Fengling, 2013).

It has been shown that rotavirus cell entry is a multistep process involving the two virus surface proteins. The binding of the infectious particle to the cell surface is mediated by the initial interaction of the VP8 domain of the protein VP4 with a sialylated cellular receptor. In the post-binding steps, the VP5 domain of VP4 and the glycoprotein VP7 interact with several cell surface molecules, including integrins, the heat shock cognate 70 protein and gangliosides, which have been proposed to function as cellular co-receptors that allow the virus to gain access into the cell (Isa, Arias, & López, 2006).

A critical step in the rotavirus infection is the virus cell attachment. A variety of studies have shown that rotavirus strains differ in their binding preferences for host cell surface glycans, particularly for sialic acid (SA)-containing receptors (Arias, Silva-Ayala, Isa, Díaz-Salinas, & López, 2016; Prasad et al., 2014; Yu & Blanchard, 2014). The cell binding, and thus the infectivity, of some animal rotavirus strains is reduced after removing SA from the cell surface by neuraminidase (NA) treatment, leading to their classification as NA-sensitive strains. In contrast, other animal and most of human rotavirus strains are classified as NA-resistant, since their binding to cells and infectivity are not affected by the NA treatment. Despite these observations, the role of glycans in rotavirus infection is not fully understood. A better understanding of the binding of rotavirus to host cells is critical for the successful development of potential therapeutic strategies against rotavirus diarrhoea.

Several carbohydrate-based compounds have been identified as rotavirus inhibitors (Isa et al., 2006; Kiefel & Itzstein, 2003). Regarding food-derived components, *in vitro* and *in vivo* experiments have attributed antirotaviral activity to an oligosaccharide-enriched fraction and glycoproteins from egg (Koketsu et al., 1995), as well as to several natural extracts, essential oils, and juicy preparations from vegetal origin (Rajiv et al., 2016). Furthermore, diverse studies have demonstrated the antirotaviral activity of some milk-derived fractions, such as whey, buttermilk (BM), and milk fat globule membrane (MFGM), being a suitable source of free and conjugated glycans (O'Riordan, Kane, Joshi, & Hickey, 2014; Ross, Lane, Kilcoyne, Joshi, & Hickey, 2015). This activity of the milk fractions was mainly associated with defensive bioactive glycoproteins, such as immunoglobulins, lactoferrin, mucins, lactadherin and lactophorin (Parrón et al., 2016, 2017). Some lipidic components, such as sialyl-sphingolipids and triglycerides associated with MFGM, have also been involved in their antirotaviral activity (Fuller, Kuhlenschmidt, Kuhlenschmidt, Jiménez-Flores, & Donovan, 2013).

There are very few reports addressing the mechanism of neutralizing activity of food compounds against the infectivity of different rotavirus strains. In this work, we evaluated the potential of several bovine milk fractions, including dairy by-products and isolated proteins, for neutralizing a wide range of rotavirus strains from bovine (UK, WC3, B223, NCDV), monkey (RRV), and human origin (Wa), belonging to different P and G serotypes, and having different sensitivities to NA treatment. Furthermore, we investigated the mechanism through which the milk fractions and proteins interfere with the rotaviral infectious process by employing reassortant UK x RRV viruses.

2. Materials and methods

2.1. Cell culture and rotavirus strains

The rhesus monkey epithelial cell line MA104 (ATTC CRL-2378) was used to propagate the different rotavirus strains. Cells were grown in Dulbecco's modified Eagle's medium (DMEM)-reduced serum (Thermo Scientific HyClone, Logan, UT) supplemented with 5% fetal bovine serum (FBS) at 37 °C in a humidified 5% CO_2 incubator.

Table 1
Characteristics of the rotavirus strains used in this work.[a]

Rotavirus strain	Origin	G and P serotypes	NA sensitivity
RRV	Simian	G3P[3]	+
UK	Bovine	G6P[5]	−
WC3	Bovine	G6P[5]	−
B223	Bovine	G10P[11]	−
NCDV	Bovine	G6P[1]	+
Wa	Human	G1P[8]	−

[a] The characteristics of the rotavirus strains are from Isa et al. (2006).

Bovine rotavirus NCDV was obtained from D.R. Snodgrass (Moredun Research Institute, Edinburgh, UK); bovine rotavirus WC3 was purchased from ATCC (VR-2102), bovine rotaviruses B223 and UK, and the Rhesus RRV, as well as the UK × RRV reassortants used were donated by Y. Hoshino (National Institute of Health, Bethesda, MD); human Wa rotavirus strain was obtained from H.B. Greenberg (Stanford University, Stanford, CA). The characteristics of the rotavirus strains are listed in Table 1. All rotavirus strains were propagated as previously described (Pando, Isa, Arias, & López, 2002). Rotavirus cell lysates were activated with trypsin (Gibco Life Technologies, Carlsbad, CA) at 10 μg/mL for 30 min at 37 °C.

2.2. Antibodies, reagents and commercial dairy products

Rabbit anti-TLPs polyclonal serum was produced in our laboratory as described in Zárate et al. (2000). Horseradish peroxidase-conjugated goat anti-rabbit IgG polyclonal antibody was purchased from PerkinElmer Life Sciences (Waltham, MA).

Chymosin was provided by Chr. Hansen (Hørsholm, Denmark). Ammonium sulphate and trichloroacetic acid were from Panreac (Barcelona, Spain). Q-Sepharose, DEAE-Sepharose, CM-Sepharose, Sephacryl S300, Sephacryl S200 and Concanavalin A agarose were purchased from GE Healthcare (Uppsala, Sweden). Sephadex G-75 was purchased from Sigma-Aldrich (St. Louis, MO).

Three powdered commercial dairy co-products were studied; including whey protein concentrate (WPC), butter serum (BS) and buttermilk (BM) powders. WPC was produced by microfiltration (0.1–0.2 μm) of whey from commercial cheese production and spray-drying of retentate. BS was derived from the process of obtaining anhydrous milk fat. Briefly, raw bovine milk was pasteurized at 72 °C for 15 s, skimmed and cream concentrated by centrifugation giving a 75% fat fraction that was homogenized, subjected to phase inversion and concentrated to a 99% fat content, obtaining an aqueous fraction herein termed commercial BS. BM was obtained during butter manufacture as follows: raw bovine milk was pasteurized at 72 °C for 15 s, skimmed and the cream subjected to churning to obtain butter and BM. All co-products were spray-dried into powders. Bovine lactoferrin (LF) was kindly provided by Tatua Nutritionals Company (Morrinsville, New Zealand) with an iron-saturation below 10%.

2.3. Obtaining milk fractions

Colostrum from the first milking was obtained from healthy cows (Tauste Ganadera, Tauste, Spain) and skimmed by centrifugation at 2000*g* for 15 min at 4 °C. Serocolostrum (SC) was obtained after enzymatic coagulation of caseins with chymosin at 35 °C for 45 min and separation by centrifugation at 2000*g* for 15 min. Raw bovine milk was provided by the dairy industry Villacorona (El Burgo de Ebro, Spain), and skimmed as described above. Whey fraction was achieved by coagulation with chymosin and further recovered by decanting and filtering through glass wool.