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Milk fat globule membrane isolated from buttermilk or whey cream and their lipid components inhibit infectivity of rotavirus in vitro

K. L. Fuller,* T. B. Kuhlenschmidt,† M. S. Kuhlenschmidt,*† R. Jiménez-Flores,‡ and S. M. Donovan*[1]
*Division of Nutritional Sciences, 905 S. Goodwin Avenue, University of Illinois, Urbana 61801
†Department of Pathobiology, 2001 S. Lincoln Avenue, University of Illinois, Urbana 61802
‡Dairy Science Department and Dairy Products Technology Center, California Polytechnic State University, San Luis Obispo 93407-0257

ABSTRACT

Milk fat is encapsulated in a milk fat globule membrane (MFGM) that contains bioactive glycoproteins and glycolipids. The MFGM inhibits infectivity of rotavirus (RV), activity that has been attributed to its glycoprotein and carbohydrate components. However, previous studies of proteins and oligosaccharides in the MGFM have not accounted for all the bioactivity associated with the complete MFGM. The lipid fraction of the MFGM accounts for half of its composition by weight, and we postulate that this fraction should be tested by itself to determine if it plays a role in antiviral activity. Herein, the anti-RV activity of an organic extract of MFGM was tested. Natural and whey buttermilk powders containing bovine MFGM enriched in polar lipids were prepared by microfiltration and supercritical fluid extraction treatment to reduce the triglyceride content of the powders. Lipid fractions were then extracted from the MFGM using both single- and dual-phase extraction methods. Whole MFGM and organic extracts were screened in MA-104 cells for anti-infective activity against a neuraminidase-sensitive rotavirus using a focus-forming unit assay. Dose-dependent inhibition was observed for whole buttermilk and cheese whey MFGM against the rotavirus. In general, buttermilk MFGM exhibited greater RV percentage inhibition than cheese whey MFGM. Organic-soluble anti-RV compounds were identified in bovine MFGM. The most active fraction, isolated by dual-phase extraction and iatrobead chromatography, was free of proteins and highly nonpolar. Further separation of this fraction in a less polar solvent (30:1 chloroform:methanol) resolved at least 5 lipid-containing compounds, which likely contribute to the anti-RV activity associated with bovine MFGM. In summary, lipid components associated with MFGM appear to contribute in large part to the anti-RV activity associated with the bovine MFGM.

Key words: bovine milk, rotavirus, focus-forming unit assay

INTRODUCTION

Within milk, high-melting-point triglycerides, phospholipids, and glycosphingolipids are encased in the milk fat globule membrane (**MFGM**; Keenan and Patton, 1995). The glycosphingolipids and phospholipids account for half of the weight percentage of the membrane and function as intracellular signaling molecules in a variety of biological processes, including regulating cell growth, development, adhesion, and cross membrane trafficking (Astaire et al., 2003). In addition, a variety of bioactive proteins are embedded in the MFGM, including mucin 1 (**MUC1**), xanthine dehydrogenase/oxidase (**XDH/XO**), periodic acid Schiff III (**PAS III**), cluster of differentiation 36 (**CD36**), butyrophilin (**BTN**), adipophilin, and periodic acid Schiff 6/7 (Mather, 2000). There is insufficient information on these proteins to characterize their interaction with the lipids that surround them, making it difficult to ascribe specific functions without the potential interference of lipids.

Over the past 2 decades, evidence has accumulated from both in vivo and in vitro studies to suggest numerous potential health benefits associated with MFGM and its associated proteins and fats, including inhibition of gastrointestinal pathogens (Sprong et al., 2002; Spitsberg, 2005), such as *Helicobacter pylori* (Hirmo et al., 1998; Wang et al., 2001) and rotavirus (**RV**; Newburg et al., 1998; Kvistgaard et al., 2004).

Worldwide, RV infection is the most common cause of severe dehydrating gastroenteritis (Ramig, 2004; Widdowson et al., 2005). Rotavirus infections are also a concern for agricultural production because of their high incidence in many species of animals, particularly in weanling calves and piglets (Kuhlenschmidt et al., 1999), resulting in an estimated economic loss of $7 million each year for producers (House, 1978). Most previous studies have focused on the bioactivities of lactadherin and MUC1, 2 glycoproteins that are associated with the MFGM (Kvistgaard et al., 2004). These

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[1]Corresponding author: sdonovan@illinois.edu

proteins are thought to bind to pathogens and help remove them from the body via the ciliary action of the gut (Yolken et al., 1992; Kvistgaard et al., 2004). The concentration of lactadherin in human milk varies depending on the stage of lactation; concentrations in colostrum and mature milk average 139 and 66 μg/mL, respectively (Peterson et al., 1998). Lactadherin is resistant to digestion in the stomach because of its high degree of glycosylation (Cavaletto et al., 2004). Therefore, it passes into the intestine intact, where it can serve as a binding site for bacterial and viral pathogens (Cavaletto et al., 2004). An epidemiological study of infants infected with human RV showed that infants consuming human milk with a mean lactadherin concentration of 48.4 μg/mL did not exhibit symptoms of RV infection, whereas infants ingesting human milk with a mean concentration of 29 μg/mL were symptomatic for RV infection (Newburg et al., 1998).

Using an in vitro focus-forming unit (**FFU**) assay, Kvistgaard and colleagues (2004) found that 20 μg of human lactadherin/mL inhibited RV infectivity by 50% in Caco-2 cells infected with the WA strain of human RV. In addition, they quantified and isolated bovine milk lactadherin. Bovine milk lactadherin concentrations were comparable to those in human milk; however, isolated bovine lactadherin did not inhibit infectivity of neuraminidase-sensitive or neuraminidase-insensitive strains of RV (Kvistgaard et al., 2004). This led the authors to propose that other components of the bovine MFGM could be responsible for its anti-RV activity. Indeed, anti-RV components were identified in whole bovine MFGM and macromolecular whey proteins (**MMWP**). Bovine mucin, MUC1, is among the major components of MFGM and MMWP. It is a high-molecular-weight, heavily glycosylated protein that exists in a viscous gel in vivo (Mather, 2000). As a result, MUC1 binds to and sequesters pathogenic microorganisms, such as RV and *Escherichia coli*, thus inhibiting their ability to damage the gut epithelium (Schroten et al., 1992; Yolken et al., 1992). When MUC1 isolated from bovine MFGM was tested for anti-RV activity in an FFU assay, a concentration of 6.3 μg of MUC1/mL reduced RV infectivity by 62.5% (Kvistgaard et al., 2004). In human milk, the oligosaccharides contained on MUC-1 include fucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose, and sialic acid. The *N*-linked carbohydrate moieties that contain sialic acid at their nonreducing end appear to be responsible for the virus-inhibitory activity (Yolken et al., 1992; Newburg et al., 1998). A subsequent study identified immunoglobulin as the primary anti-RV factor in the MMWP (Bojsen et al., 2007).

In the current study, the potential for MFGM to inhibit RV infection was investigated, because components of bovine (Kvistgaard et al., 2004) and human (Newburg et al., 1998) MFGM have been shown to exert anti-RV activity. We compared MFGM isolated from buttermilk or cheese whey buttermilk by microfiltration and supercritical fluid extraction (**SFE**). This process selectively extracts triglycerides, which leads to enrichment of polar and more complex lipids in the MFGM (Astaire et al., 2003). This extraction method also results in the selective fractionation of various lipids without the use of conventional toxic solvents (Astaire et al., 2003). Using buttermilk as a source for these lipids as viral inhibitors is a sound strategy considering its unique properties as a functional food ingredient and its low cost and availability (Walstra et al., 1999; USDA-NASS, 2001). We hypothesized that MFGM isolated by SFE would inhibit RV infectivity and that this effect would be due, in part, to the enriched lipid components.

MATERIALS AND METHODS

Concentration of MFGM in Natural and Whey Cream Buttermilks

Milk (500 L) for each batch of buttermilk was obtained from the Cal Poly Dairy Farm (San Luis Obispo, CA). The skim milk was separated from the cream by a pilot-plant cream separator (DeLaval, Kansas City, MO). Whey cream for this experiment was produced and donated by Hilmar Co. (Hilmar, CA) and 3 batches of cream (120 L) were used. The cream was treated in the pilot plant at Cal Poly and subjected to regular pasteurization: 77.5°C (172°F) for 15 s. Buttermilk was pasteurized before storage at 75°C (169°F) for 15 s. The cream was processed into butter at 12°C using a continuous churn (Egli Co., Gumligen, Switzerland), and buttermilk and whey buttermilk were collected. Buttermilk for each sample was recovered in milk cans, small fat grains or aggregates were removed by filtration through cheese cloth, and the resulting buttermilk was passed through the cream separator again. The buttermilks were stored overnight at 4°C until membrane filtration was performed.

A pilot-plant scale system (R-12 model, GEA-Niro Filtration, Hudson, WI) with 2 spiral-wound polymeric membranes fitted in parallel on the module (10 kDa molecular mass cutoff, 11.33 m^2 total surface area) was used to remove most of the mineral salts, lactose, and free oligosaccharides. The process was carried out at 25°C; the trans-membrane pressure was 600 kPa, and the feed pump, attached to a frequency drive, was operated at 35 Hz. Microfiltration was conducted until a 10-fold volumetric concentration factor was reached. Diafiltration was done while continuously adding purified