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Defatted bovine milk fat globule membrane inhibits association of enterohaemorrhagic *Escherichia coli* O157:H7 with human HT-29 cells

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ABSTRACT

The bovine milk fat globule membrane (MFGM) is a source of food-derived glycans that can offer an approach to prevent *Escherichia coli* O157:H7 infection by inhibiting attachment of the pathogen to host cells. Such glycans may decrease the need for antibiotic treatment by acting as prophylactics. In this study, we generated a defatted bovine MFGM fraction, rich in proteins and glycoproteins, and demonstrated its ability to prevent the association of several enterohaemorrhagic *E. coli* O157:H7 strains with human colonic adenocarcinoma, HT-29 cells. This defatted MFGM fraction reduced bacterial association with HT-29 cells in a concentration dependent and strain specific manner. This study may present a new approach to mitigate the adverse health effects caused by *E. coli* infections in humans.

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1. Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) serogroup O157:H7 is an enteric pathogen commonly associated with food borne illnesses. It has a very low infectious dose, which varies from 10 to a few hundred cells (Strachan, Doyle, Kasuga, Rotariu, & Ogden, 2005), and causes an estimated 73,480 illnesses and 2168 hospitalisations annually in the USA (Mead et al., 1999). The symptoms of infection include abdominal cramping, nausea, vomiting, abdominal distension and bloody diarrhoea (Symonds, 1988). Severe cases of infection can result in haemolytic uremic syndrome (HUS) (Wong et al., 2012), haemorrhagic colitis (Sang et al., 1996) and even death. Patients who develop HUS have a mortality rate of 3–5% (Feng, 2012). The incidences of death due to *E. coli* O157:H7 infection have reduced in recent years, reaching an average of 10 deaths annually and generally occurring in people aged ≥60 years (Gould et al., 2009; Scallan, Hoekstra, Mahon, Jones, & Griffin, 2015). However, large sporadic outbreaks due to contaminated food sources such as beef (Duffy et al., 2006), continue to emerge (CDC, 2008, 2013) which increases the risk of development of HUS. The costs associated with outbreaks in the USA were estimated to be US$405 million in 2003 (Frenzen, Drake, Angulo, & Group, 2005) highlighting the need for *E. coli* O157:H7 infection prevention and treatment.

In developing countries, enteropathogenic *E. coli* (EPEC) is a leading cause of diarrhoea in infants (Trabulsi, Keller, & Tardelli Gomes, 2002). The symptoms of EPEC infections most commonly include acute diarrhoea and may lead to persistent diarrhoea. EPEC infection is the cause of thousands of deaths annually, with the majority of these deaths occurring in children and infants (Ochoa, Barletta, Contreras, & Mercado, 2008). EPEC differs in its virulence to EHEC in that infection rates are higher in developing countries and infectious doses are also much higher (10^8–10^{10} bacteria to infect adults). Furthermore, EPEC colonise the small intestine as opposed to the colon – the area commonly targeted by EHEC (Mellies, Barron, & Carmona, 2007).

During infection, *E. coli* spp. overcome the harsh acidic environment of the gut to colonise the gastrointestinal tract (Foster, 2004). Adhesion is an important early step in *E. coli* pathogenicity. It enables the bacteria to gain access to essential nutrients, deliver bacterial toxins into the microenvironment of the host tissue, and triggers host cell invasion (Schlumberger & Hardt, 2006; Sharon & Ofek, 2000). It also increases the chance of bacterial survival as their resistance to cleansing mechanisms, immune factors and antibiotics is higher (Ofek, Hasty, & Sharon, 2003). *E. coli* adhesion mechanisms have been extensively reviewed in recent

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years (Croxen et al., 2013; Pereira & Giugliano, 2013) and some of the carbohydrate binding sites of EPEC and EHEC have been determined. These include intestinal glycosphingolipids (Teneberg, Ångström, & Ljungh, 2004), asialolactosamine (Vanmaele, Finlayson, & Armstrong, 1995), N-acetylgalactosamine on the surface of HeLa cells (Scaletsky, Milani, Trabulsi, & Travassos, 1988), fucosylated human milk oligosaccharides (Cravioto et al., 1991), monosialogangliosides (GM) (Idota & Kawakami, 1995) and mucin-type core 2 O-glycans (Ye et al., 2015).

As pathogen adhesion and colonisation is often a prelude to infection, intervention at the adhesion stage can reduce or prevent disease. Intervention can occur at birth via bioactive breast milk components such as free and bound glycans. These structurally mimic epithelial cell surface glycans and thus function as decoys that pathogens can bind to instead of the host and thereby prevent infection (Sharon & Ofek, 2000). Indeed, human milk oligosaccharides (HMO) have been shown to inhibit *E. coli*, *Vibrio cholerae* and *Salmonella fyris* adhesion to human epithelial colorectal adenocarcinoma Caco-2 cells in vitro (Coppa et al., 2006) and fucosylated HMO are thought to decrease *Campylobacter jejuni* associated diarrhoea in breast fed infants (Morrow et al., 2004). Human milk fat globule membrane (MFGM) glycoproteins have also been shown to possess anti-infective properties. For instance, mucins isolated from human MFGM prevented S-fimbriated *E. coli* adhesion to buccal epithelial cells (Schroten et al., 1992) and also decreased *Salmonella enterica* serovar Typhimurium invasion of human intestinal epithelial cells (Liu, Yu, Chen, Kling, & Newburg, 2012).

However, the limited availability of human milk makes it difficult to produce large quantities of these bioactives at a commercial scale for human consumption. As a result, scientists have begun to focus on domestic animal milks, particularly bovine milk, as a source of potential anti-infective agents such as glycans and glycoconjugates that are structurally similar to those found in human breast milk. For example, bovine milk oligosaccharides (BMO) were shown to prevent the cellular invasion of *C. jejuni* in vitro (Lane et al., 2012) and to display anti-infective activity against *Helicobacter pylori*, *Neisseria meningitides* and influenza virus (Hakkarainen et al., 2005; Matrosovich et al., 1993). Bovine MFGM may represent an alternative source of anti-infective glycans, which can be harvested from whole milk through conventional technologies. Indeed, MFGM is a rich source of glycoproteins that have complex glycan components (Ross, Lane, Kilcoyne, Joshi, & Hickey, 2015). Furthermore, recent studies have demonstrated the anti-infective potential of certain purified bovine MFGM glycoproteins and whey fractions against a range of pathogens including rotavirus and enteric bacteria (Inagaki et al., 2010; Kvistgaard et al., 2004; Parker et al., 2010). However, these studies used various forms of purified MFGM glycoproteins rather than a glycoprotein-enriched MFGM fraction.

In this study, a glycoprotein and protein enriched MFGM fraction from bovine milk was prepared and characterised and then assessed for its ability to prevent EHEC and EPEC association with human colonic intestinal epithelial cells. The main objectives of this study were to continue to explore bovine milk as a source of human health promoting bioactives and to potentially identify an alternative preventative therapy for *E. coli* infection.

2. Materials and methods

2.1. Preparation of a defatted MFGM-enriched fraction (dMFGM) from bovine milk

Milk was collected from the bulk tank of the Holstein-Friesian cattle milking parlour at the Teagasc Food Research Centre, Moorepark (Fermoy, Co. Cork, Ireland). Cream and milk fat globules were separated from the milk using a FT15 disc bowl centrifuge (Armfield Ltd., Ringwood, England) and the cream was stored at 4 °C for 12–24 h. After chilling, the cream was churned using a mixer to produce butter and buttermilk. The buttermilk was then passed through glass wool (Sigma–Aldrich, Dublin, Ireland) to remove minute butter granules. Washed cream was produced as described previously (Le, Van Camp, Rombaut, van Leeckwyck, & Dewettinck, 2009; Struijs et al., 2013). Briefly, warm deionised water (37 °C) was added to the cream at a ratio of 1:10 and the mixture was added to the FT15 disc bowl centrifuge. This process was repeated twice and the washed cream was then used to produce buttermilk as described above.

Modifications were made to the conventional Rose Gottlieb method (IDF, 2008) to delipidate the produced buttermilks. Briefly, 10 mL ethanol, 25 mL diethyl ether and 25 mL of petroleum spirit were added to 10 mL of buttermilk. The mixture was shaken for 30 s and then centrifuged (600 × g, 5 min) at 20 °C. The solvent layer was then removed and the aqueous layer was treated with 5 mL ethanol, 15 mL diethyl ether and 15 mL of petroleum ether prior to centrifugation as described above. The solvent was removed and the aqueous layer was treated as per the second extraction. After the third extraction, the aqueous layer was collected and all solvents were removed by rotary evaporation under reduced pressure (Buchi Rotavapor R-210, Mason Technology Ltd, Dublin, Ireland). The sample was then lyophilised and the dry powder stored in a desiccator at room temperature for long-term storage.

2.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis

Sample preparation and reduction for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed as per manufacturer's instructions (NuPAGE system, Life Technologies, Thermo Fisher Scientific Inc., Dublin, Ireland.). Briefly, 2.5 μL of sample buffer and 1 μL of reducing agent were added to 6.5 μL of sample 10 mg mL^{-1} (by mass of powder). The sample was then centrifuged and heated to 70 °C for 10 min. 10 μL of sample was then added to each well of a 4–12% bis-Tris gel (1.00 mm × 15 well, Life Technologies). A molecular mass standard solution (Invitrogen Mark12 Unstained Standard, Thermo Fisher Scientific Inc.), prepared as per manufacturer's instructions (diluted 1:10 with Invitrogen LDS Sample Buffer), was also loaded onto the gels. Electrophoresis was performed at 200 V for 50 min using MOPS buffer supplemented with 0.25% NuPAGE Antioxidant (Life Technologies) in the upper chamber. Protein bands were visualised on the gels using Coomassie blue stain (Invitrogen SimplyBlue SafeStain) following the manufacturer's procedure. MFGM proteins and contaminating proteins were identified through the use of bovine milk protein standards, β-lactoglobulin A, β-lactoglobulin B, α-lactalbumin, κ-casein, α-casein, β-casein, lactoferrin and immunoglobulin G (IgG), and by comparative analysis with previously published SDS-PAGE data (Rombaut, Dejonckheere, & Dewettinck, 2007; Ye, Singh, Taylor, & Anema, 2002).

2.3. Bacterial strains and culture conditions

EHEC serogroup O157:H7 strains (NCTC 12900*, DAF454†, DPC 6055†, P1432†, DPC 6054†, ATCC 43888‡) and EPEC O125:H19 (strain NCTC 8623*) and O111:H2 (strain NCTC 8007*) serogroups were obtained from the National Collection of Type Cultures* (NCTC; London, UK), the Dairy Products Research Centre culture collection at Teagasc Moorepark (DPC)† and the American Type Culture Collection (ATCC, Rockville, MD)‡. All strains were cultured directly from storage into brain heart infusion (BHI) (Oxoid® Ltd,

Basingstoke, England) broth and incubated under aerobic conditions at 37 °C. Strains were stocked in BHI broth containing 50% glycerol (v/v) and stored at −20 °C.

2.4. Mammalian cell culture

The human colonic adenocarcinoma cell line, HT-29, was purchased from the ATCC. HT-29 cells were routinely grown in McCoy's 5A modified medium (Sigma–Aldrich®) supplemented with 10% foetal bovine serum (FBS). All cells were routinely maintained in 75 cm^2 tissue culture flasks and incubated at 37 °C in 5% (v/v) CO_2 in a humidified atmosphere. Cells were passaged when the confluency of the flask was approximately 90% as previously described (Lane et al., 2012).

2.5. In vitro total association assays

A series of total association assays (adapted from Horemans et al., 2012; Salcedo, Barbera, Matencio, Alegria, & Lagarda, 2013), were performed with HT-29 cells and all EPEC and EHEC strains in the absence (control) and presence of the generated dMFGM fraction. HT-29 cells were seeded into 12 well PVDF plates (Corning, Sigma Aldrich) at a density of 1×10^5 cells $well^{-1}$. Cells were cultured for 48 h and the media was changed to McCoy's 5A modified medium supplemented with 2% FBS at least 24 h prior to total association assays. *E. coli* strains in the early stationary phase were harvested from BHI broth after overnight growth, washed three times in phosphate buffered saline, pH 7.2 (PBS) and re-suspended in McCoy's 5A modified medium supplemented with 2% FBS.

2.5.1. Standard competition assay

Prior to infecting the HT-29 cell line, the bacteria (1×10^8 cfu mL^{-1}) were pre-incubated for 1 h at 37 °C (5% CO_2) with the dMFGM fraction (5 mg mL^{-1}) in McCoy's 5A media (2% FBS). Confluent monolayers of HT-29 cells were washed twice in PBS and infected with 500 μL of the pre-incubated bacterial mix. HT-29 cells were then incubated for 1 h at 37 °C in a humidified atmosphere (5% CO_2). To determine the number of cell-associated bacteria, non-adherent bacteria were removed by washing the HT-29 cells five times with PBS and cells were lysed with 500 μL of 0.1% Triton X-100 solution in PBS. Cell lysates were then serially diluted in maximum recovery dilutant (Oxoid®) and plated onto BHI agar (Oxoid®). Plates were incubated at 37 °C overnight and cfu mL^{-1} was determined.

2.5.2. Concentration dependency assay

Prior to infecting the HT-29 cell line, the bacteria (1×10^8 cfu mL^{-1}) were pre-incubated for 1 h at 37 °C (5% CO_2) with various concentrations of the dMFGM fraction (5, 2.5, 1.25 and 0.625 mg mL^{-1}) in McCoy's 5A media (2% FBS). The total association assay was then performed as described previously.

2.5.3. Bacterial interaction assay

Prior to infecting the HT-29 cell line, the bacteria (1×10^8 cfu mL^{-1}) were pre-incubated for 1 h at 37 °C (5% CO_2) with the dMFGM fraction (5 mg mL^{-1}) in McCoy's 5A media (2% FBS). The bacterial mixture was then centrifuged at 3920 × *g* for 7 min to pellet the bacterial cells. The supernatant containing unbound dMFGM was removed and the bacterial pellet was then re-suspended in McCoy's 5A media (2% FBS). The total association assay was then performed as described previously.

2.5.4. Host cell interaction assay

The confluent monolayer of HT-29 cells was washed twice in PBS and 500 μL of the dMFGM fraction (5 mg mL^{-1}) in McCoy's 5A media (2% FBS) was added to the wells. Unbound dMFGM components were removed by washing the mammalian cells five times in PBS prior to bacterial infection. The HT-29 cells were then incubated for 1 h at 37 °C in a humidified atmosphere (5% CO_2). The total association assay was then performed as described previously.

2.5.5. No preincubation assay

Confluent monolayers of HT-29 cells were washed twice in PBS and infected with 500 μL of the non-pre-incubated bacterial mix. The total association assay was then performed as described previously.

2.6. Bacterial growth assays

To assess whether dMFGM had an effect on the growth of the *E. coli* strains used in this study, the bacteria were grown in optimum growth media BHI (Oxoid®) broth, and colonisation media, McCoy's 5A media modified with L-glutamine and $NaHCO_3$ (Sigma–Aldrich) (2% FBS) and supplemented with 5 mg mL^{-1} dMFGM. Unsupplemented media was used as a negative control. EPEC and EHEC strains harvested from BHI broth were used to inoculate (1%) the test media. Solutions were then incubated at 37 °C under aerobic conditions and bacterial growth was monitored by making serial dilutions of the inoculated media at the following time points: 0 h (T_0), 3 h (T_3), 6 h (T_6) and 24 h (T_{24}) post-inoculation. Colony forming units were determined by plating sample aliquots in triplicate on BHI agar (Oxoid®). Plates were incubated at 37 °C overnight and cfu mL^{-1} was determined.

2.7. Statistical analysis

All inhibition studies were carried out three times in triplicate. Data were graphed in Microsoft Excel and the unpaired student t-test was used to determine statistically significant results; $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Isolation and characterisation of the dMFGM fraction

Approximately 16 well separated protein bands were identified on the Coomassie-stained SDS gels (Fig. 1A). High intensity bands were observed for the MFGM glycoproteins XOR, CD36, butyrophilin (BTN), Pas 6 and Pas 7 (approximately 130, 68, 60, 50 and 46 kDa, respectively). High intensity bands were also observed for the milk glycoprotein κ-casein (approximately 26 kDa) and for the proteins α-casein, β-casein (approximately 34 kDa and 31 kDa, respectively) and the whey proteins α-lactalbumin and β-lactoglobulin (approximately 10 kDa and 19 kDa, respectively). Low intensity bands were observed for glycoproteins IgG (heavy chain 55 kDa and light chain 20 kDa, approximately) and proteose peptone 3 (PP3) (approximately 28 kDa) and for the protein adipophilin (approximately 40 kDa). These findings correlated well with previous publications (Rombaut et al., 2007; Ye et al., 2002) and suggested that MFGM was successfully isolated from whole milk but was contaminated with non-MFGM proteins such as caseins (Fig. 1A). Washing removed the majority of the contaminating proteins including α-lactalbumin, β-lactoglobulin, α-casein, β-casein and κ-casein, while enriching for MFGM proteins and glycoproteins (Fig. 1B). This approach was adapted from Le et al. (2009) where the use of deionised water to wash cream for the removal of contaminating non-MFGM proteins without the loss of

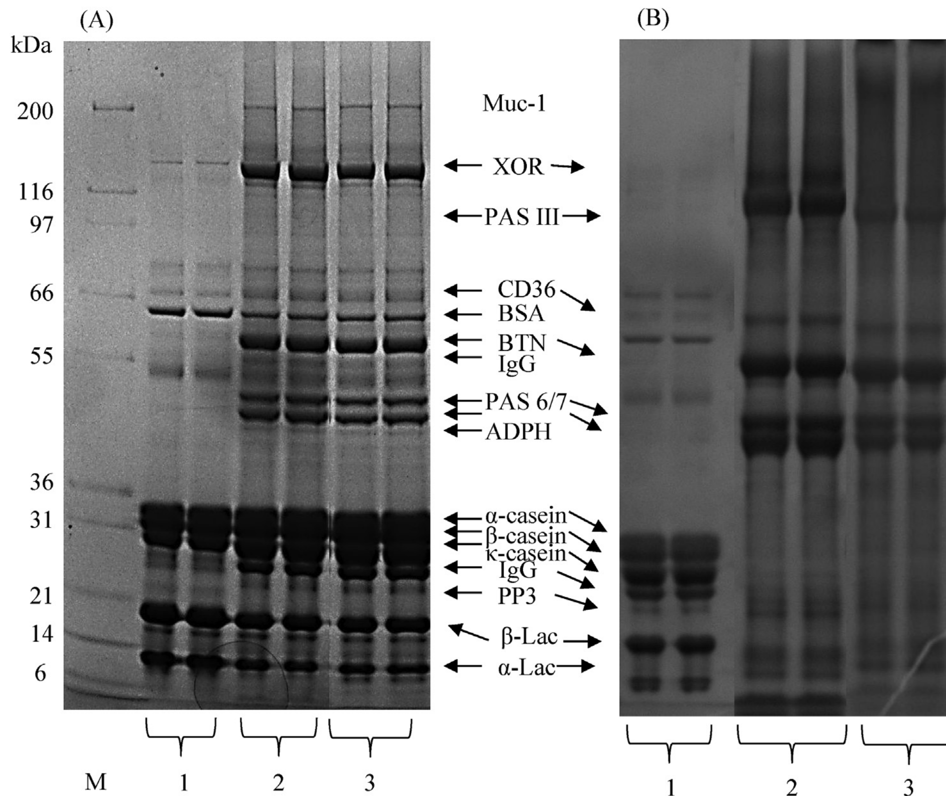

Fig. 1. Electrophoresis profile of bovine milk fractions: reducing 4–12% SDS-PAGE profiles of the dairy streams made from raw unpasteurised milk for unwashed total protein (A) and washed total protein (B). Lane 1, skim milk; lane 2, cream; lane 3, buttermilk; M, molecular mass marker. Gels were stained with Coomassie blue. Results are representative of duplicate experiments.

MFGM proteins was demonstrated. In this study, the anti-infective potential of defatted bovine MFGM-enriched fraction against *E. coli* was under investigation. Therefore, as the bovine MFGM is composed of membrane proteins and lipids, the MFGM-enriched fraction was delipidated and the subsequent dMFGM fraction was used for the inhibition assays.

3.2. dMFGM reduced the association of E. coli *O157:H7 with HT-29 cells*

Previously, Wang, Hirmo, Willen, and Wadstrom (2001) and Horemans et al. (2012) demonstrated that a concentration of $\geq$5 mg mL^{-1} of defatted MFGM was required to cause 50–80% inhibition of *H. pylori* adherence to HeLa S3 cell monolayers and NCI-N87 cells, respectively. Therefore, an initial concentration of 5 mg mL^{-1} dMFGM was selected for our study. Prior to mammalian cell infection, *E. coli* strains were pre-incubated with dMFGM. Subsequently, dMFGM was shown not to kill the bacteria, did not influence bacterial growth over the course of the assay and did not affect the viability of the HT-29 cells as confirmed by bacterial growth assays and real-time analysis of cell viability using an xCELLigence system (Roche), respectively (data not shown).

The standard competition assay demonstrated that the dMFGM inhibited the association of the EHEC strains only, namely *E. coli* NCTC 12900, *E. coli* DAF454 and *E. coli* DPC 6055, to HT-29 cells by 92%, 88% and 69%, respectively (P-value < 0.05) when compared with untreated controls (Fig. 2). No reduction in bacterial association with HT-29 cells was evident for *E. coli* P1432, *E. coli* DPC 6054, *E. coli* ATCC 43888, *E. coli* NCTC 8623 and *E. coli* NCTC 8007. Interestingly, bovine MFGM has been shown to influence the expression of genes involved in the motility of *E. coli* O157:H7 (Tellez et al., 2012). For instance, it was demonstrated that an increase in the expression of *fliC* gene, which encodes the major bacterial flagellar protein, occurs after 4 h treatment of *E. coli* with bovine MFGM. Thus, an increase in *fliC* expression could have occurred in some strains in this study, increasing their motility and aiding them in subverting the anti-adhesive activity of the dMFGM fraction. This may explain why no anti-infective activity was observed for some of the EHEC strains screened.

Previously, bacterial flagella were shown to impact greatly on bacterial ability to adhere and infect host cells (Mahajan et al., 2009). EHEC O157:H7 possess H7 type flagella, which have been shown to have adhesive properties and have demonstrated binding abilities to mucins. The EPEC strains, *E. coli* NCTC 8623 and *E. coli* NCTC 8007, possess H19 and H2 type flagella, respectively (Erdem, Avelino, Xicohtencatl-Cortes, & Girón, 2007). This difference in adhesive properties might explain the difference in cellular association observed for the EPEC strains when compared with the EHEC strains. It is also possible that since the EPEC strains bind to different ligands they may not interact with dMFGM in a similar manner to that of *E. coli* O157:H7. Moreover, EPEC and EHEC differ in several other ways. The main virulence property of EPEC is the production of attaching and effacing (A/E) lesions while the main EHEC virulence properties include production of A/E lesions and verotoxin (Bardiau, Szalo, & Mainil, 2010). Both serotypes contain the bacterial receptor Tir which is inserted into the host cell membrane prior to binding bacterial intimin protein which is necessary for pedestal formation. EPEC relies on tyrosine phosphorylation of its Tir protein for pedestal formation while EHEC does not (DeVinney, Puente, Gauthier, Goosney, & Finlay, 2001). Additionally it is known that attachment of EPEC to host cells relies on the presence of intimin-α and bundle forming pili which are also

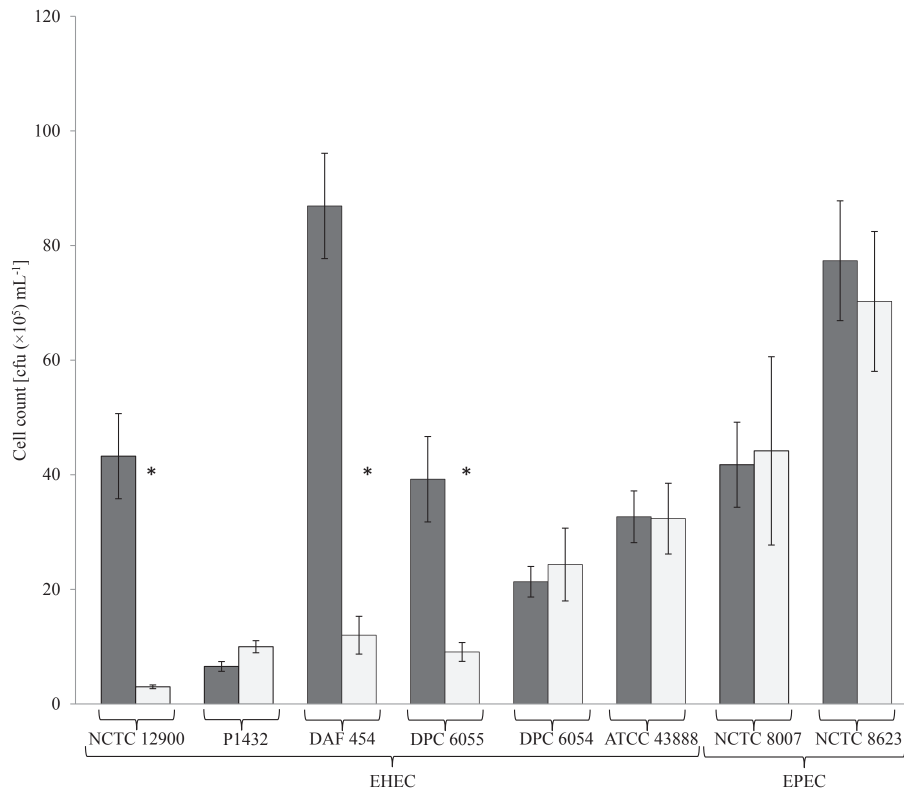

Fig. 2. The effect of the dMFGM fraction on association of *E. coli* strains with human HT-29 cells: ■, control (no dMFGM); □, dMFGM. Data are means ± standard deviation of 3 replicates; error bars represent standard deviation and an asterisk indicates values are significant ($P < 0.05$).

important in the formation of microcolonies. EHEC largely requires intimin-γ and long polar fimbriae for initial host cell adhesion (Croxen et al., 2013; Pereira & Giugliano, 2013). These differences may contribute to the differences seen in the anti-infective activity of the dMFGM fraction against the pathogens.

The anti-infective activity of the dMFGM fraction was demonstrated to be concentration dependent (Fig. 3A) with an 80%, 79%, 70% and 44% reduction of bacterial association with HT-29 cells, respectively. A plateau effect was observed where maximum inhibition was reached at 2.5 mg mL^{-1}. This would suggest that studies specifically targeting *E. coli* O157:H7 should use a dMFGM concentration of at least 2.5 mg mL^{-1}.

The mechanism by which the dMFGM fraction causes the anti-infective effect may have occurred in a number of ways: dMFGM may be interacting with the bacteria, preventing HT-29 cell association; dMFGM may be interacting with HT-29 cells, preventing bacterial interaction with the cell line; or a dual method which incorporates dMFGM interacting with both bacteria and HT-29 cells and reducing the association of bacteria with the cell line.

To investigate these possible modes of action(s), two additional assays were performed. Firstly, a bacterial interaction assay was performed. Inhibition of *E. coli* NCTC 12900, *E. coli* DAF454 and *E. coli* DPC 6055 association with the HT-29 cells was observed at 28%, 30% and 25% respectively compared with the control (Fig. 3B). This indicated that dMFGM likely interacted with the bacteria, thereby reducing the interaction of the bacteria with the HT-29 cells. However, the magnitude of inhibitory activity of dMFGM was reduced under these conditions. As the unbound material was removed in this assay, components of the dMFGM fraction binding directly to host cell surface receptors were potentially at least partially responsible for reducing the number of bacteria associating with the HT-29 cells. In addition, the removal of unbound dMFGM reduced the occurrence of competitive binding, thus increasing the likelihood of bacteria associating with HT-29 cells during natural association-dissociation.

To determine whether the dMFGM interacted with HT-29 cells, a host cell interaction assay was performed. No reduction in *E. coli* O157:H7 association with the HT-29 cells was observed (Fig. 3C). Similar findings were observed by other researchers when investigating the mode of action of their compounds of interest. For example, Manthey, Autran, Eckmann, and Bode (2014) pre-incubated HMO with HeLa, Hep-2 and T84 epithelial cell lines, resulting in a loss of anti-adhesive activity against EPEC. Inagaki et al. (2014) pre-incubated MA104 Rhesus monkey kidney cells with bovine κ-casein glycopeptides prior to rotaviral infection and demonstrated no viral inhibition, suggesting that bovine κ-casein glycopeptides do not bind host cell receptors. Furthermore, Marotta, Ryan, and Hickey (2014) demonstrated the loss of anti-infective activity of the milk oligosaccharide 3′-sialyllactose (3′SL) against *Pseudomonas aeruginosa* association with human pneumocytes when the human cell line was pre-incubated with 3′SL.

Overall, the results presented here suggest that dMFGM does not target host cell receptors for *E. coli* O157:H7 and instead a direct dMFGM-bacterial interaction is likely responsible for the anti-infective activity. Bacterial environmental sampling and competitive binding may be an important factor here and may explain why removal of unbound dMFGM led to a reduction in the anti-infective activity of the fraction. Bacterial association-dissociation which occurs in vivo may have led to the increased bacterial interactions with HT-29 cells due to the removal of unbound dMFGM.

Initially, screening studies were performed with the inclusion of a pre-incubation step that encouraged maximum exposure of the dMFGM to the bacteria. However, as this may not be a good

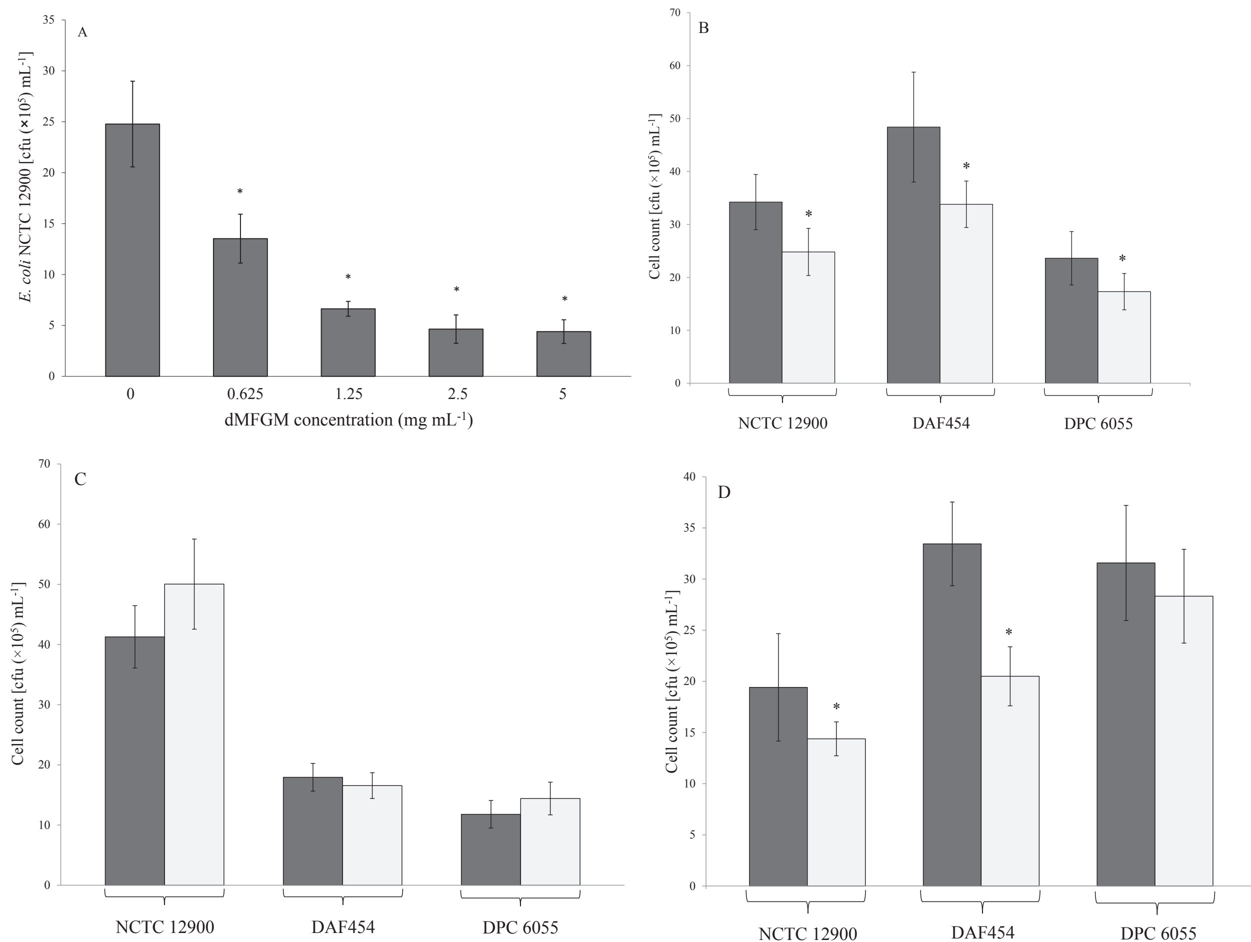

Fig. 3. The effect of the dMFGM fraction on association of *E. coli* O157:H7 with HT-29 cells: panel A, effect of dMFGM concentration on the anti-infective activity against *E. coli* NCTC 12900; panel B, effect of removing unbound dMFGM on *E. coli* O157:H7 association with HT-29 cells (■, control; □, dMFGM); panel C, effect of pre-incubating HT-29 cells with dMFGM prior to infection on the anti-infective activity (■, control; □, dMFGM); panel D, effect of no pre-incubation on the anti-infective activity against *E. coli* strains (■, control; □, dMFGM). Data are means ± standard deviation of 3 replicates; error bars represent standard deviation and an asterisk indicates values are significant ($P < 0.05$).

representation of an in vivo situation, HT-29 cells were also infected with *E. coli* O157:H7 in the presence and absence of dMFGM without a pre-incubation step (Fig. 3D). Overall, inhibition of *E. coli* adherence was still observed although at reduced magnitude compared with dMFGM pre-incubated with the bacteria. The inhibition observed was 19%, 47% and 5% for NCTC 12900, DAF454 and DPC 6055, respectively. These results suggest that the dMFGM fraction requires a certain period of time to exert its maximal inhibitory effect on *E. coli* cellular association. Indeed, a previous study indicated that optimum binding of a particular *E. coli* O157:H7 strain, CL-49, to mucins occurs at 37 °C for 2 h at pH 6.5 (Sajjan & Forstner, 1990). Thus, the result observed in this experiment is likely due to the reduced time period in which dMFGM and *E. coli* strains were in contact, compared with the standard competition assay. This would lead to reduced competitive binding of dMFGM and HT-29 cell adhesins for *E. coli* O157:H7 cell receptors. The lack of a pre-incubation step reduces the ability of the dMFGM fraction to inhibit bacterial binding to host cells. However, it is interesting that a reduction was still evident instantaneously.

It is likely that the anti-infective role of dMFGM is due to its glycans, as highlighted by many other studies demonstrating the anti-infective potential of MFGM glycoconjugates such as mucins, Pas6, Pas7 and acidic glycolipids, with much bioactivity being attributed to sialic acid in particular (Parker et al., 2010; Shida et al., 1994; Yolken et al., 1992). The dMFGM fraction had the ability to prevent the association of EHEC strains, but not EPEC strains, with HT-29 cells. This could reflect the different mechanisms and adhesins used by EHEC and EPEC strains in their colonisation of host cells. Further investigations are required to determine more information on the different glycan receptors used by each serotype which would allow the subsequent tailoring of anti-adhesives to target a wide variety of pathogens.

Ingestion of dMFGM fraction would lead to digestion of many of the dMFGM components prior to its interaction with intestinal epithelial cells that could alter its components. In fact, defatted bovine MFGM has previously been digested in vitro and SDS gels have been used to analyse the protein and glycoprotein composition post-digestion. Coomassie-stained gels demonstrated hydrolysis of MFGM proteins after 20 min of pepsin digestion; however, many bands corresponding to MFGM glycoproteins were observed on the PAS stained gel indicating incomplete hydrolysis and partial resistance to the action of pepsin (Le et al., 2012). In vitro intestinal digestion led to hydrolysis of all remaining MFGM glycoproteins with the exception of Muc1 (Le et al., 2012). Similarly, PAS staining of whole MFGM indicated resistance of CD36 and Muc1 to in vivo gastric digestion (Gallier et al., 2013a) and potential resistance of Muc1 to in vivo intestinal digestion (Gallier et al., 2013b). Thus, the high molecular weight Muc1 appears to be most resistant to digestion. The conformation of MFGM glycoproteins may result in

some glycans being unavailable to bind *E. coli* strains. Digestion of dMFGM fraction may release bioactive components, allowing access of bioactive glycans to bacterial receptors, contributing to further anti-infective activity of the fraction. Further studies are required to investigate the anti-infective potential of dMFGM after digestion has occurred.

In addition to the anti-infective activities evidenced in this study, MFGM glycoproteins have been shown to possess other health promoting activities. For example, such components have been shown to affect the metabolism of colonic microbiota, causing an increase in butyrate production, thus potentially reducing the risk of colon cancer (Struijs et al., 2013). Furthermore, Ito, Kamata, Hayashi, and Ushiyama (1993) demonstrated the ability of MFGM to inhibit β-glucuronidase intestinal activity in mice which could also contribute to the anti-cancer activities of MFGM. The anti-infective potential of glycans and MFGM fractions in vivo has been studied. For instance, bovine plasma glycans have demonstrated the ability to reduce *E. coli* adhesion to calf intestine in vivo (Mouricout, Petit, Carias, & Julien, 1990) and bovine milk 3′SL can reduce *H. pylori* infection in rhesus monkeys (Mysore et al., 1999). Furthermore, a HMO pool has been shown to reduce EPEC colonisation in mice (Manthey et al., 2014). Interestingly, a bovine MFGM fraction has been shown to reduce *Listeria monocytogenes* colonisation in a rat model (Sprong, Hulstein, Lambers, & van der Meer, 2012) and *H. pylori* colonisation in a mouse model (Wang et al., 2001). Although these studies indicate the anti-infective activity of the dMFGM fraction against EHEC could also be viable in vivo, further studies are required to validate this. This hypothesis requires further investigation.

4. Conclusions

Bovine MFGM is a source of glycoproteins with the potential for improving human health. Of the eight *E. coli* strains screened in this study, anti-infective activity of dMFGM was demonstrated against 3 strains of EHEC. The assays performed suggested that dMFGM was most likely associating with the infecting bacteria and thereby inhibiting bacteria from associating with HT-29 cells. This activity was also shown to be concentration dependent and required pre-incubation in order for the dMFGM fraction to exert its maximum protective activity. The inclusion of this bioactive fraction in functional foods may be of great benefit to the general population, as well as immune-compromised individuals, including infants and the elderly and may offer an alternative approach to the more technically challenging isolation of pure MFGM components. As demonstrated for the first time in this study, dMFGM may offer an alternative approach to reduce *E. coli* O157:H7 infection in humans.

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