Glycoconj J (2010) 27:89–97
DOI 10.1007/s10719-009-9269-2

Bovine Muc1 inhibits binding of enteric bacteria to Caco-2 cells

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Received: 4 August 2009 / Revised: 28 October 2009 / Accepted: 4 November 2009 / Published online: 21 November 2009

Abstract Inhibition of bacterial adhesion to intestinal epithelial receptors by the consumption of natural food components is an attractive strategy for the prevention of microbial related gastrointestinal illness. We hypothesised that Muc1, a highly glycosylated mucin present in cows' milk, may be one such food component. Purified bovine Muc1 was tested for its ability to inhibit binding of common enteric bacterial pathogens to Caco-2 cells grown *in vitro*. Muc1 caused dose-dependent binding inhibition of *Escherichia coli*, *Salmonella enterica* serovar Typhimurium (S. Typhimurium), *Staphylococcus aureus* and *Bacillus subtilis*. This inhibition was more pronounced for the Gram negative compared with Gram positive bacteria. It was also demonstrated that Muc1, immobilised on a membrane, bound all these bacterial species in a dose-dependent manner, although there was greater interaction with the Gram negative bacteria. A range of monosaccharides, representative of the Muc1 oligosaccharide composition, were tested for their ability to prevent binding of *E. coli* and S. Typhimurium to Caco-2 cells. Inhibition was structure dependent with sialic acid, L(-) fucose and D(+) mannose significantly inhibiting binding of both Gram negative species. *N*-acetylglucosamine and *N*-acetylgalactosamine significantly inhibited binding of *E. coli* whilst galactose, one of the most abundant Muc1 monosaccharides, showed the strongest inhibition against S. Typhimurium. Treatment with sialidase significantly decreased the inhibitory properties of Muc1, demonstrating the importance of sialic acid in adhesion inhibition. It is concluded that bovine Muc1 prevents binding of bacteria to human intestinal cells and may have a role in preventing the binding of common enteropathogenic bacteria to human intestinal epithelial surfaces.

Keywords Milk mucins · Muc1 · Enteric pathogen · Adhesion inhibition · Detachment

Introduction

Enteric food-borne pathogenic bacteria represent a major world-wide health problem. The World Health Organization reported that in 2002 an estimated 2.1 million people, predominantly from developing countries, died from diarrhoeal diseases caused by a variety of enteric pathogens [1]. Food borne disease is also a concern throughout developed countries as there is now increasing risk of bacterial infection associated with extended time periods between food production, preparation and consumption.

The initial step in intestinal colonisation is the attachment of ingested pathogenic bacteria to intestinal epithelial cells. This is facilitated by bacterial adhesins which bind to an array of highly conserved structures, such as specific cell surface receptors expressed on human epithelial cell surfaces and extracellular matrix proteins [2–4]. Mucus, which contains mucin glycoproteins, is secreted by epithelial cells and is thought to act as a physical barrier preventing access of intestinal bacteria to host epithelial cell receptors whilst also providing lubrication to mucosal cell surfaces [5–7]. Mucins may also present epithelial

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receptor-like motifs in the form of specific oligosaccharide structures, which bind and immobilise bacteria, thus acting as decoys for epithelial cell receptors [7–9]. The subsequent shedding of the mucus layer into the gastrointestinal tract eliminates bound bacteria. Anti-adhesion therapy seeks to exploit this natural mucin-based decoy mechanism by introducing soluble oligosaccharides or mimetopes with structures analogous to those of the bacterial binding targets [10–12]. Glycoproteins, such as the mucins expressed in bovine milk, have a demonstrated capacity to act as anti-adhesive agents [13–15]. Indeed, the milk fat globule membrane (MFGM) and Muc1, a highly glycosylated mucin associated with the MFGM, have both been shown to inhibit binding of certain intestinal pathogens to cells cultured *in vitro* [15, 16]. More recently Muc1 has been implicated in the protection of the murine gastric mucosa from *Helicobacter pylori* colonisation and *Campylobacter jejuni* infection [17, 18]. Bovine Muc1 is very different in its amino acid sequence and size compared with human and murine Muc1 [19] and thus it is not known whether it has similar functions. We hypothesised that Muc1, present in bovine milk, binds to potential pathogenic bacteria, thereby inhibiting their subsequent adhesion to intestinal epithelia. In this study, the bacterial anti-adhesive properties of purified bovine Muc1 were tested, using the CaCo-2 cell line as a model of the human intestinal epithelia, against four human enteric bacterial pathogens; *E. coli*, S. Typhimurium, *S. aureus* and *B. subtilis*.

Materials and methods

Bacterial strains and culture

Bacteria used included: a piliated clinical isolate of *E. coli* (O-, H48); a piliated, non-invasive, aro-A mutant of *Salmonella enterica* serovar Typhimurium (1, 4, [5], 12 : 1 : 1, 2); coagulase positive *S. aureus* and a pathogenic, clinical isolate of *B. subtilis*. These bacteria were obtained from the reference culture collection of known bacteria at the School of Exercise and Nutrition Sciences, Deakin University, Burwood, Victoria, Australia. All cultures were used within two passages. Log-phase cultures were prepared by inoculating an aliquot of overnight culture into Brain Heart Infusion broth (BHI; Oxoid) (1×10^9 cfu/ml) followed by incubation for 3 h at 37°C.

Caco-2 cell culture

All cell culture media were purchased from Invitrogen (Australia). Caco-2 human adenocarcinoma cells (kindly provided by Associate Professor Leigh Ackland, Deakin University, Burwood, Victoria, Australia) were routinely cultured at 37°C in humidified air containing 5% CO_2 and Advanced Dulbecco's modified Eagles Media (A-DMEM) containing 4.5 g/l glucose, 2% foetal bovine serum, 2 mM L-glutamine and 10 mM HEPES (pH 7.2). The cells were used between passages 69–79. All seeding was performed at a density of 6×10^4 cells/cm^2 of culture surface [20] and monolayers were used within 48 h of reaching confluency [21]. Bacterial binding inhibition and detachment assays were performed using bacterial cultures in log phase growth and a multiplicity of infection (MOI) of 100.

Isolation, purification and SDS-PAGE analysis of Muc1

Bovine milk cream was kindly provided by Parmalat Australia Ltd, Brisbane, Australia. The purification and characterisation of bovine Muc1 has been described elsewhere [19]. Briefly, MFGM were isolated from full cream using centrifugation. The supernatant derived from a tryptic digestion of MFGM was collected and fractionated by anion exchange chromatography followed by hydrophobic interaction chromatography. The extensive glycosylation and lack of trypsin cleavage sites within the Muc1 extracellular domain largely protected it against tryptic digestion, which selectively released this domain from the MFGM. Hence, the purified protein represents the large trypsin-resistant and highly glycosylated extracellular domain of the full length Muc1 protein. Purified Muc1 was analysed under reducing conditions on 6–18% gradient SDS-PAGE gels stained by a combination of silver and Alcian blue [19].

Immobilised Muc1 bacterial binding assay

A variation of the method of Takahashi *et al.* [22] was used to assess the direct binding of bacteria to immobilised Muc1. Serial quantities of purified Muc1 (1 ng–1 μg) were adsorbed onto Westran S® PVDF membrane (Schleicher & Schuell) in triplicate using a Bio-Dot® SF microfiltration apparatus (BioRad). As a negative control, matching quantities of BSA in PBS were adsorbed onto the same membrane. The membrane was blocked for 1 h at room temperature with PBS containing 5% BSA. Labelling of bacteria with Sulfosuccinimidyl-6-(biotinamido) hexanoate (EZ-Link Sulfo-NHS-LC-Biotin, Pierce) was performed following the manufacturer's instructions for labelling cell surface proteins. Briefly, a 1 ml aliquot containing 1×10^9 cfu bacteria in PBS was incubated for 30 min at room temperature with 1 mg of EZ-Link Sulfo-NHS-LC-Biotin. Excess label was removed by three washes in 1 ml PBS containing 100 mM glycine. Biotinylated bacteria were resuspended in 40 ml of PBS containing 5% BSA (2.5×10^7 cfu/ml final concentration). An aliquot of this suspension was overlayed onto the PVDF membrane (0.3 ml/cm^2 of membrane) and incubated overnight at

4°C. Unbound bacteria were then removed with three 5 min washes in PBS containing 0.1% Tween 20. Adherent bacteria were detected using horseradish peroxidase-conjugated streptavidin complex (Pierce) and standard chemiluminescence techniques. Images for densitometric quantification of bacterial binding were captured on a Kodak 440CF imaging station (Kodak Eastman Co) and analysed using Kodak 1D imaging software.

Bacterial inhibition assay

The ability of purified Muc1 to inhibit binding of bacteria to Caco-2 cells was quantified using a variation of the microplate assay described by Simon *et al*. [23]. Each inhibitor dilution was analysed in triplicate and each experiment was replicated twice. Caco-2 cells were seeded in 96-well, black-sided tissue culture plates (Greiner-bio) with 100 μl of supplemented A-DMEM. The culture media was changed daily and the cells grown to confluency. Bacteria (1×10^9 cfu in 1 ml of PBS) were incubated with 100 μg of fluorescein-5-isothiocyanate (FITC; Molecular Probes) for 30 min at room temperature [24] followed by dilution to 1×10^8 cfu/ml in A-DMEM. A 25 μl aliquot of fluorescently labelled bacterial suspension was pre-incubated with 25 μl of the test inhibitor for 20 min with gentle shaking. When examining the Muc1 dose-dependent inhibition of bacterial binding, serial dilutions of Muc1 ranging from 1.67 ng/ml–167 ng/ml final concentrations were used. Monolayer's of the Caco-2 cells containing 100 μl of fresh A-DMEM were incubated with the bacterial/inhibitor suspension for 1 h at 37°C in humidified air containing 5% CO_2. Non-adherent bacteria were removed by gentle washing of the cells with 100 μl of PBS. The wells were examined with a light microscope between each wash to ensure there was no detachment of the Caco-2 monolayer. The fluorescence intensity within each well, representing adherent FITC-labelled bacteria and background fluorescence, was measured using a Fusion™ universal microplate analyser (Perkin-Elmer Life and Analytical Sciences) with excitation and emission filters set at 490 nm and 530 nm, respectively. Binding of bacteria was determined using the following formula (Eq. 1):

$$\text{Percent binding} = \left[\left(I_{\text{experimental}} - I_{\text{negative}}\right)/\left(I_{\text{positive}} - I_{\text{negative}}\right)\right] \times 100; \quad (1)$$

where $I_{\text{experimental}}$ is the fluorescence intensity measured in wells incubated with FITC-labelled bacteria, test inhibitor and Caco-2 cells; I_{negative} is the fluorescence intensity in wells containing only Caco-2 cells (accounting for background fluorescence signal); I_{positive} is the fluorescence intensity in wells incubated with FITC-labelled bacteria and Caco-2 cells in the absence of any test agent. Bacteria were also incubated with BSA in PBS matching the final concentrations of the test inhibitor. No inhibitory effect of BSA was observed (data not shown).

Bacterial detachment assay

The ability of purified Muc1 to detach bacteria bound to Caco-2 cells was examined using a method similar to the bacterial inhibition assay. An aliquot of FITC-labelled bacteria (25 μl of 1×10^8 cfu/ml) was incubated for 1 h (37°C in humidified air, 5% CO_2) with Caco-2 cells grown to confluency in a 96-well plate. A 25 μl aliquot of Muc1 (16.7 ng/ml final concentration) was added to test wells and the incubation continued for 20 min. Non-adherent bacteria were removed with three washes in PBS as described above and levels of residual FITC-labelled bacteria were determined using Eq. 1.

Enzymatic desialylation

Purified bovine Muc1 was treated with *Clostridium perfringens* sialidase (Roche Molecular Biochemicals) (1 mU/μg glycoprotein) in 50 mM sodium acetate buffer pH 5.0, containing 150 mM NaCl, at 37°C for 24 h. Desialylation was verified by monosaccharide composition analysis, changed mobility on SDS-PAGE and altered staining properties of Muc1 with Alcian blue after separation by SDS-PAGE.

Monosaccharide inhibition assay

A bacterial binding inhibition assay, adapted from Ryan *et al.* [25], was performed using commercially available monosaccharides. The monosaccharides represent those residues present on Muc1, as determined by compositional analysis [19], and were employed to elucidate the oligosaccharide structures that might be active in the inhibition of *E. coli* and S. Typhimurium binding to the Caco-2 cells grown *in vitro*. L(-)fucose (6-deoxy-L-galactose, Fuc), D(+)galactose (Gal), *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc) and D(+)mannose (Man) (Sigma-Aldrich Chemical Co) were prepared as 50 mM solutions in PBS. Sialic acid (*N*-acetylneuraminic acid) (Vector Laboratories) was prepared in 10 mM Tris–HCl (Tris buffer), pH 6.9 [25]. Aliquots of FITC-labelled bacteria (25 μl, 1×10^8 cfu/ml) in PBS or Tris buffer were incubated with the same volume of monosaccharide solution (25 mM final concentration) for 20 min at 37°C. After three washes in the same buffer, the bacterial pellet was resuspended to its original volume. Bacteria were then incubated for 1 h (37°C, 5% CO_2) in a 96 well plate containing the Caco-2 cells. The wells were washed three times, their fluorescence intensities measured and percent binding determined using Eq. 1. Prior to these

experiments being performed, 25 mM monosaccharide solutions were shown to have no affect on the viability of the bacterial colonies using a spread plate assay (data not shown).

Statistical analyses

Results are presented as the mean ± standard error of the mean (SEM). The data were analysed using Student's *t*-test. $P \leq 0.05$ was considered statistically significant.

Results

SDS-PAGE analysis of Muc1

Figure 1 shows the SDS-PAGE profile of the purified extracellular domain of bovine Muc1. The Muc1 protein band stained poorly with silver and Coomassie Blue but was more readily visualised with Alcian blue. The SDS-PAGE band was also quite diffuse. These properties are typical of a highly glycosylated protein containing significant quantities of sialic acid [26, 27]. The purified Muc1 was originally released from the MFGM fraction by trypsin treatment and was largely intact with a size range of 110–180 kDa. Tryptic cleavage presumably occurred only in the juxtamembrane region of this type 1 membrane protein thereby releasing intact the highly glycosylated extracellular domain from the MFGM. This domain was resistant to tryptic cleavage as a consequence of the lack of tryptic cleavage sites and probably also due to extensive glycosylation.

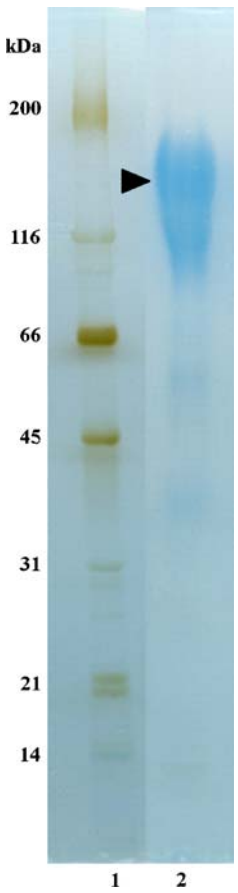

Fig. 1 SDS PAGE analysis of Muc1 purified from bovine MFGM. Bovine Muc1 was purified as described in the "Experimental procedures". *Lane 1*, silver-stained protein size standards; *lane 2*, Muc1 stained with silver and counter-stained with Alcian blue. The *arrow*-head denotes the position of Muc1 which has a size range of 110–180 kDa

Binding of bacteria to Muc1 immobilised on PVDF membrane

To determine whether Muc1 directly binds bacteria, serially diluted quantities of purified Muc1 (1–1,000 ng) were adsorbed onto a PVDF membrane using a vacuum manifold. The same amounts of BSA were used as negative controls. Specific binding was defined as the signal for each bacterium minus the signal from BSA at each protein concentration, and then this was expressed as a percentage of the maximal binding. Both the Gram negative and Gram positive bacteria bound immobilised Muc1 in a dose dependent manner (Fig. 2).

Muc1-mediated inhibition of bacterial binding to Caco-2 cells

Figure 3 shows the effect of 167 μg/ml of MFGM (Fig. 3a) or 167 ng/ml Muc1 (Fig. 3b) on bacterial binding to Caco-2 cells grown in culture. Muc1 or MFGM were pre-incubated with *E. coli,* S. Typhimurium, *S. aureus* or *B. subtilis*. The MFGM caused 82±17% and 100±1.0% inhibition of binding of *E. coli* and S. Typhimurium, respectively. In contrast, MFGM inhibited binding of *S. aureus* to the Caco-2 cells by 26±6.0% and there was no apparent effect on *B. subtilis* binding. Muc1 (Fig. 3b) showed activity similar to that of MFGM, decreasing binding of *E. coli* and S. Typhimurium to Caco-2 cells by 100±14% and 100±5.0%, respectively while the binding of *S. aureus* and *B. subtilis* were decreased by 10±4.0% and 3.0±11%, respectively.

Analysis of the inhibitory activity of bovine Muc1 demonstrated dose-dependent inhibition of Gram negative bacterial binding to the Caco-2 cells (Fig. 4a, b). At the highest Muc1 concentration (167 ng/ml final concentration) binding of *E. coli* was reduced by 97 ± 3.0% and that of S. Typhimurium by 82±3.0% ($p \leq 0.05$). The effects of Muc1 on the Gram positive bacterial binding were not as pronounced (Fig. 3c, d). At the highest Muc1 concentration adherences of *S. aureus* and *B. subtilis* to Caco-2 cells were reduced by 43±1.0% and 28±4.0%, respectively.

Muc1-mediated detachment of bacteria from Caco-2 cells

Figure 5 shows the ability of Muc1 to detach pathogenic bacteria that have been allowed to bind Caco-2 cells in

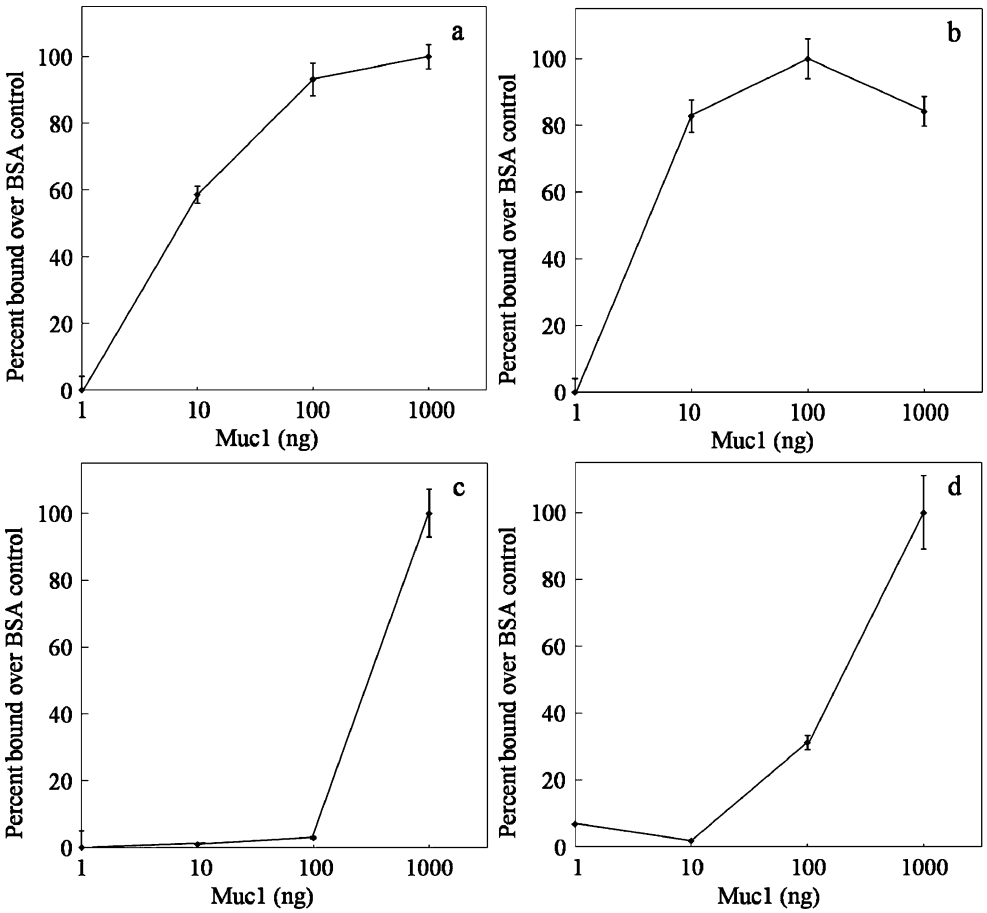

Fig. 2 Biotinylated bacteria bind Muc1 in a dose-dependent manner. Serially diluted Muc1 (1 ng–1 μg total protein) was adsorbed onto a PVDF membrane and overlayed with biotinylated *E. coli* (**a**), S. Typhimurium (**b**), *S. aureus* (**c**) or *B. subtilis* (**d**) for an overnight incubation. The same quantities of BSA were adsorbed onto the membrane as a negative control. Non-adherent bacteria were removed by washing in PBS. Adherent bacteria were detected using HRP-conjugated streptavidin and chemiluminescence. Densitometry was used to quantify the extent of binding. Specific binding was defined as the signal for each bacterium minus the signal from BSA at each protein concentration, and then this was expressed as the percentage of maximal binding. Each dilution was analysed in triplicate and the experiment was replicated twice. *Error bars* represent the SEM

culture. At 167 ng/ml, Muc1 caused some displacement of the bound Gram negative bacteria, 28±1.0% for *E. coli* and 27±4.0% for S. Typhimurium. Muc1 was unable to significantly detach either *S. aureus* or *B. subtilis* (8±7.0% and 7±6.0% displacement, respectively).

Effect of desialylation on the ability of Muc1 to inhibit binding of bacteria to Caco-2 cells

The importance of sialic acid residues present on Muc1 for inhibition of bacterial binding was examined by enzymatic

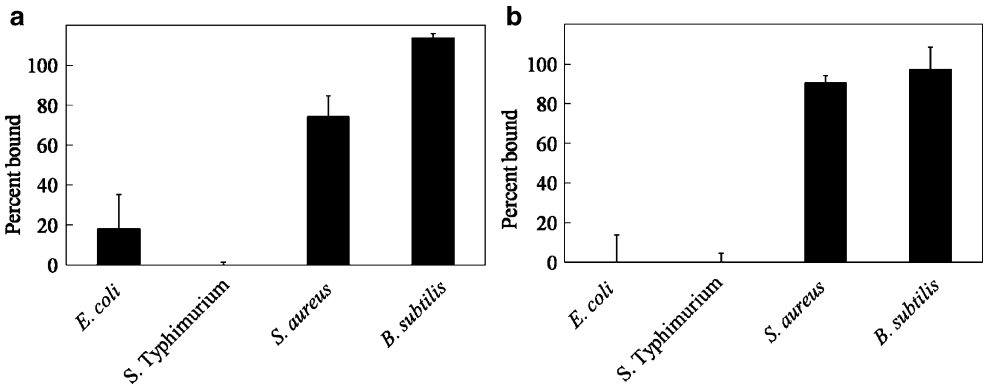

Fig. 3 MFGM and Muc1 inhibit bacterial binding to Caco-2 cells. FITC-labelled bacteria were pre-incubated for 20 min with 167 μg/ml of MFGM (**a**) or 167 ng/ml concentration of purified Muc1 (**b**). The suspension was then incubated with Caco-2 cells for 1 h at 37°C. Non-adherent bacteria were removed with three washes in PBS and adherent bacteria detected by measuring the resultant fluorescence. The percent binding was calculated from Eq. 1, which represents the percentage of bacteria binding to Caco-2 cells relative to the control *i.e.* bacteria binding to Caco-2 cells in the absence of Muc1. Binding inhibition of each bacterial species was analysed in triplicate and the experiment was replicated twice. *Error bars* represent the SEM across each assay

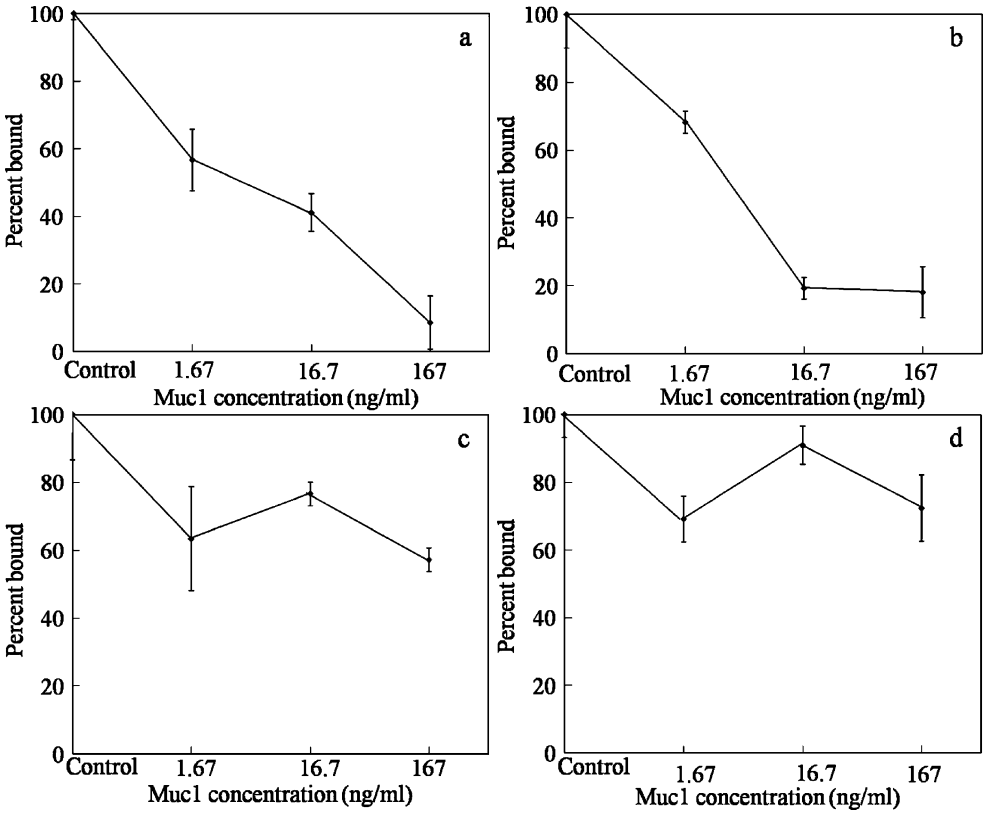

Fig. 4 Purified Muc1 inhibits binding of bacteria to Caco-2 cells in a dose-dependent manner. FITC-labelled bacteria were pre-incubated for 20 min with Muc1 (1.67–167 ng/ml final concentration) and then incubated for 1 h with the Caco-2 cells. Non-adherent bacteria were removed by washing in PBS and adherent bacteria detected by measuring fluorescence. Data points represent the percentage of *E. coli* (**a**), S. Typhimurium (**b**), *S. aureus* (**c**) and *B. subtilis* (**d**) binding to Caco-2 cells relative to the control. Each inhibitor dilution was analysed in triplicate and the experiment was replicated twice. *Error bars* represent the SEM

desialylation of Muc1. Treatment of Muc1 with *Clostridium perfringens* sialidase, which selectively releases sialic acid residues from glycoproteins, substantially reduced the electrophoretic mobility of Muc1 in SDS-PAGE, caused loss of Alcian blue staining with a concomitant increase in intensity of staining with silver and quantitatively removed greater than 99% of the sialic acid attached to Muc1 (data not shown). The ability of 16.7 ng/ml (final concentration) of Muc1 to inhibit binding of the Gram negative bacteria *E. coli* and S. Typhimurium to Caco-2 cells was greatly reduced ($p \leq 0.01$) after sialidase treatment (Table 1).

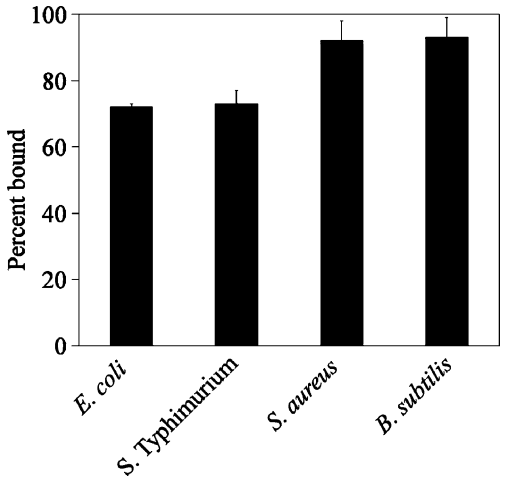

Fig. 5 Muc1 detaches bacteria from intestinal epithelia *in vitro*. FITC-labelled bacteria were allowed to adhere to Caco-2 cells for 1 h at 37°C. This was followed by the addition of 167 ng/ml of Muc1 and further incubation for 20 min. Detached bacteria were removed by washing and adherent FITC-labelled bacteria detected by measuring fluorescence. The graph shows the percentage of residual bacteria bound to Caco-2 cells relative to the control. Detachment of each bacterial species was analysed in triplicate and the experiment was replicated twice. *Error bars* represent the SEM

Monosaccharide-mediated inhibition of bacterial binding to Caco-2 cells

Muc1 is a heavily glycosylated protein with extensively sialylated O-linked oligosaccharides [19] and it preferen-

Table 1 The effect of sialidase treatment on the Muc1-mediated inhibition of binding of bacteria to Caco-2 cells

	E. coli % binding[a]	S. Typhimurium % binding[a]
Muc1	36±2	0±2
nMuc1[b]	70±1**	38±1**

[a] values represent the percentage of bacteria bound to Caco-2 cells relative to the control (*i.e.* bacteria and Caco-2 cells in the absence of Muc1 or nMuc1). 16.7 ng/ml (final concentration) of glycoprotein was used for both the Muc1 and nMuc1samples

[b] sialidase-treated Muc1

**$p \leq 0.01$

Table 2 Effect of monosaccharides on the binding of *E. coli* and S. Typhimurium to Caco-2 cells grown *in vitro*

Monosaccharide[b]	*E. coli*[a] % bound	S. Typhimurium[a] % bound
Fuc	68±3*	29±3*
Gal	82±5	0±2**
GalNAc	72±4*	94±2
GlcNAc	48±4**	122±4
Sialic Acid	74±3*	11±2*
Man	46±4**	15±1*

[a] values represent the percentage of bacteria bound to Caco-2 cells relative to the control (*i.e.* bacteria and Caco-2 cells in the absence of inhibitor)

[b] Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Man, mannose

*$p \leq 0.05$, **$p \leq 0.01$

tially binds the Gram negative bacteria. Hence, the nature of the monosaccharides attached to Muc1 may be key determinants of the inhibition of bacterial binding to epithelial cells. Table 2 shows the ability of a range of monosaccharides, characteristic of the oligosaccharide composition of bovine Muc1 [19], to inhibit the binding of *E. coli* and S. Typhimurium to Caco-2 cells. Sialic acid, Man, and Fuc, were able to significantly ($p \leq 0.05$) inhibit both Gram negative bacteria from binding the Caco-2 cells. A species-specific effect among the Gram-negative enteric bacteria was also observed, with Gal strongly inhibiting binding of S. Typhimurium ($p \leq 0.01$). However, binding of *E. coli* was not significantly affected by this monosaccharide. GlcNAc and GalNAc both significantly ($p \leq 0.01$ and $p \leq 0.05$, respectively) reduced *E. coli* binding to Caco-2 cells without significantly affecting the binding of S. Typhimurium.

Discussion

The ability of bacteria to colonise the intestinal tract and initiate pathogenesis is intimately associated with their ability to bind receptors displayed on the surface of intestinal epithelial cells. Interfering with binding has been proposed as a strategy for preventing colonisation of the human intestinal tract by potentially pathogenic microbes [11, 28, 29]. This non-bactericidal anti-microbial strategy has the added benefit of reducing selection pressure on enteric pathogens as opposed to bactericidal antibiotic treatments, which increase the rate of emergence of antibiotic-resistant strains [10, 11]. Several studies have examined the capacity of soluble milk oligosaccharides to inhibit binding of potential pathogens to intestinal epithelia [12]. However, little is known about the ability of milk glycoproteins and the oligosaccharides expressed on these glycoproteins to inhibit bacterial binding to intestinal epithelial cells. This is particularly relevant to components within bovine milk, such as Muc1, which are heavily glycosylated.

Bovine MFGM, which is rich in membrane-bound Muc1, and purified Muc1 strongly inhibited the binding of the Gram negative bacteria *E. coli* and S. Typhimurium to human intestinal Caco-2 cells *in vitro*. In contrast, there was a relatively small inhibition of the binding of the Gram positive bacteria *S. aureus* and *B. subtilis*. This pattern of preferential interaction with Gram negative bacteria was a recurring theme throughout this study. The inhibitory effects of Muc1 on the binding of the Gram negative bacterial species did not reflect a simple binding model. Thus, it is likely that Muc1 interacts with the bacteria in a manner which could involve multi-site attachments and potentially cross-linking and network formation. Muc1 directly and preferentially binds the Gram negative bacteria (Fig. 2), and therefore, it is likely that this direct interaction mediates the inhibitory effect of Muc1 on bacterial binding to the Caco-2 cells. Recently, similar conclusions have been reached when investigating the role of Muc1 in murine gastrointestinal infections [17, 18].

The dynamic nature of adhesion between the bacteria and the intestinal epithelial Caco-2 cells implies that a competitor molecule, such as Muc1, should be able to displace bacteria from the cells after microbial binding has been established. Indeed, this detachment has been observed previously in *in vitro* experiments during which *H. pylori* was detached from the human gastrointestinal HuTu-80 cell line by 3′-sialyllactose [23]. Surprisingly, bovine Muc1 was only able to partially detach adherent Gram negative bacteria from the Caco-2 cells. This result suggests that bacterial detachment is not simply the reverse of attachment and that after bacteria bind to the cells further non-reversible interactions occur, such as bacterial cell wall changes associated with pathogenesis or changes in the repertoire of bacterial adhesins. Therefore, Muc1 may have potential as a prophylactic, rather than therapeutic, agent.

Various forms of sialic acid have been implicated as receptors for the binding of a range of pathogens, including *E. coli* [16], human influenza virus [30, 31], rotavirus [15, 32], *Helicobacter pylori* [23] and *Pseudomonas aeruginosa* [9]. Further, Coppa *et al.* [33] recently demonstrated that 3′-sialyllactose inhibited binding of three bacterial species (*E. coli*, *Salmonella fyris* and *Vibrio cholerae*) to Caco-2 cells *in vitro*. Indeed, sialic acid residues on human Muc1 have been implicated as mediators of *P. aeruginosa* and rotavirus infections in cystic fibrosis and childhood diarrhoea, respectively [8, 9, 32].

In this study we report that sialic acid is a potential receptor component as there is a reduction of *E. coli* and S. Typhimurium binding to Caco-2 cells following pre-incubation of the bacteria with sialic acid. In addition,

treatment of Muc1 with sialidase greatly reduced, but did not abolish, the capacity of Muc1 to inhibit bacterial binding to Caco-2 cells. The residual binding activity may indicate that sialic acid is one component of a larger oligosaccharide structure involved in bacterial binding. This binding would likely entail a large number of weak interactions mediated by hydrogen bonding, electrostatic forces and hydrophobic bonds that ultimately generate a strong but low specificity interaction [34–36]. It is also possible that the bacteria were able to bind the small quantity of sialic acid not removed from Muc1 by sialidase treatment, the protein itself or additional monosaccharide elements on Muc1 such as galactosyl residues [37]. Indeed, the monosaccharide-based inhibition assays described here showed that Gal reduced *E. coli* binding to Caco-2 cells by 18% and abolished binding of S. Typhimurium. However, there were also major differences in the binding specificities of these Gram negative bacteria that require further investigation. For example, GlcNAc was able to strongly inhibit *E. coli* binding while S. Typhimurium binding to the Caco-2 increased.

In conclusion, bovine Muc1 has the capacity to inhibit binding of Gram negative bacteria to human intestinal cells *in vitro*, and a component of this activity is mediated by sialic acid residues attached to this extensively glycosylated protein. Ingested bovine Muc1 may be exploited as a functional food component to prevent illness caused by enteric pathogens such as S. Typhimurium and *E. coli* by inhibiting their ability to adhere to the gut epithelia. Further studies are required to demonstrate efficacy *in vivo*.

Acknowledgements This research was supported by the Geoffrey Gardiner Dairy Foundation and the Co-operative Research Centre for Innovative Dairy Products. Access to the Australian Proteome Analysis Facility was aided by the Australian Government's Major National Research Facilities Program. The authors thank Parmalat Australia Ltd. for their donation of cream for isolation of Muc1.

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Author's contributions

PP and LS contributed equally to this research. PP carried out all the direct binding and inhibition assays and monosaccharide binding studies. LS and RP carried out the protein purification, SDS-PAGE analysis and sialidase treatment. RT, SS and KK participated in the conception, design and overall coordination of the study and provided advice on the various assays. All authors have read and approved the final manuscript.