Glycomacropeptide from Camel Milk Inhibits the Adhesion of Enterotoxigenic *Escherichia coli* K88to Porcine Cells

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) are a major cause of childhood diarrhea and of diarrhea in piglets and calves. This study compared the anti-adhesive activities against ETEC of glycomacropeptide (GMP) from Bactrian camels and dromedaries to bovine GMP. GMP was purified by ion exchange chromatography and ultra-filtration. The monosaccharide content of GMP from Bactrian camels and dromedaries was about twice as high when compared to bovine GMP. Glycans from camels included fucose and N-acetylglucosamine, which were absent in bovine GMP. GMP from both camel species prevented ETEC adhesion to porcine blood cells at a concentration of 0.24 g L^{-1} to 0.28 g L^{-1} respectively, a concentration that is about 20-fold lower when compared to bovine GMP. This increased activity likely relates to the increased glycosylation and the density of glycan spacing, and / or to differences in the glycan composition.

KEYWORDS: Bactrian camel, dromedary, glycomacropeptide, enterotoxigenic *Escherichia coli,* antiadhesive activity, glycosylation, glycan density spacing, glycan structure.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) are a major cause of childhood diarrhea in developing countries, and cause traveler's diarrhea. ETEC also cause watery diarrhea in newborn and post-weaning piglets and calves (DuPont, 1995; Qadri, Svennerholm, Faruque, & Sack, 2005). ETEC produce two enterotoxins, the heat stable enterotoxin (ST) and the heat labile enterotoxin (LT). ETEC adhere to the small intestinal epithelial cells and to the mucosal tissue through glycoprotein receptors of the host cells using specific fimbriae. Colonization of the microvilli and the production of enterotoxins lead to electrolyte imbalance and water loss (Chen, Woodward, Zijlstra, & Gänzle, 2014; Nagy & Fekete, 2005). *E. coli* expressing K88 fimbriae are among the most prevalent strains of ETEC that cause diarrhea in swine (Chen et al., 2014; Hermes, Manzanilla, Martín-Orúe, Pérez, & Klasing, 2011).

The mortality rate in farm animals due to bacterial infections is increasing especially at the weaning stage (Nagy & Fekete, 2005). The use of antibiotics to control ETEC in pig production increases costs, supports the emergence of antibiotics resistant pathogens in animals as well as the transmission of antibiotic resistance to human pathogens (Docic & Bilkei, 2003). These problems led to search for alternative approaches to control ETEC (Docic & Bilkei, 2003). Anti-adhesive biomolecules that act as glycan receptor analogues are a promising alternative to antibiotics. Glycan receptors bind to glycolipids or glycoproteins on the surface of host tissues and thus mediate adhesion of pathogens and toxins including the adherence of ETEC fimbriae to the epithelial cells (Kulkarni, Weiss, & Iyer, 2010; Shoaf-Sweeney & Hutkins, 2008). Glycan receptor analogues bind to these glycan receptors and thus inhibit the initial stages of infection and bacterial colonization (Kulkarni et al., 2010; Shoaf-Sweeney & Hutkins, 2008). Anti-adhesive agents do

not have bactericidal or bacteriostatic activity and therefore do not result in development of antimicrobial resistance (Krachler & Orth, 2013; Shoaf-Sweeney & Hutkins, 2008).

Anti-adhesive agents that inhibit the adhesion of ETEC include human milk oligosaccharides (HMOs) as well as oligosaccharides in bovine colostrum which prevent ETEC adhesion in calves (Fong, Ma, & McJarrow, 2011). Major HMOs are composed of fucose, galactose, glucose, N-acetyl-glucosamine, and N-acetyl-neuraminic acid or sialic acid (Bode et al., 2016). However, human milk is not commercially available and HMO analogs that are purified from bovine colostrum or produced with microbial cell factories are relatively expensive (Ackerman, Craft, & Townsend, 2017; Bode et al., 2016). Alternative oligosaccharides or glycopeptides known to inhibit ETEC adhesion include glycans formed by *Limosilactobacillus reuteri*, glycopeptides obtained from ovomucin hydrolysis, and galactosylated chitosan oligosaccharides (Sun, Gänzle, & Wu, 2017; Wang, Gänzle, & Schwab, 2010; Yan, Hu, Simpson, & Gänzle, 2017). Ovomucin-derived glycopeptides prevent adhesion of porcine ETEC K88 fimbriae at minimum inhibitory concentration (MIC) of 2.5 g L^{-1} while β-galactosylated chitosan-oligosaccharides inhibit ETEC K88 adhesion at MIC of 0.22 g L^{-1} (Sun et al., 2017; Yan et al., 2017).

Bovine glycomacropeptide (GMP) constitutes about 15-20 % of the total whey protein and represents the C-terminus of κ-casein obtained by specific hydrolysis of κ-casein with rennet. GMP contains a high portion of sialic acid, which constitutes 7-9 % of its the total weight (Li & Mine, 2004; Nakano, Ozimek, & Betti, 2018). Bovine GMP also blocks the adhesion of diverse enteric pathogens to the intestinal mucosa including enteroheamorrhagic *E. coli* (EHEC) O157 (Nakajima et al., 2005) and ETEC K88 adhesion in swine (Hermes et al., 2013). Bovine GMP prevents adhesion of porcine ETEC K88 fimbriae at MIC of 2.5 g L^{-1} (Hermes et al., 2013).

In North Africa and in the Middle East, the production of camel milk cheese has increased; this increase in camel milk cheese production also increased the amount of whey as a by-product (El-Agamy, 2009). Comparable to other domestic animals, ETEC causes diarrhea in camel calves (Al-Ruwaili, Khalil, & Selim, 2012). Camel milk is not as well studied as bovine milk and the chemical composition and the biological activities of camel GMP are not described. Therefore, this study aimed to purify GMP from camel milk, to characterize its glycan composition, and to assess its activity in preventing adhesion of ETEC K88 adhesion to porcine erythrocytes.

Materials and methods

Purification of GMP. Bovine GMP was purchased from Davisco Foods International (Eden Prairie, MN, USA). GMP from Bactian camels or dromedaries was purified from cheese whey that was prepared by treatment of reconstituted lyophilized milk from Bactrian camels (Inner Mongolia Agricultural University, China) or of reconstituted spray dried milk from dromedary camels (Al Ain Farms, Al Ain, UAE) with camel chymosin (Chr. Hansen, Bayswater, Australia), followed by separation of whey by centrifugation (Figure 1). GMP was purified as previously described with some modifications (Nakano et al., 2018), as illustrated in Figure 1. Briefly, about 25 mL whey with a pH of 6.2 was ultra-filtrated, dialyzed with 5 kDa membranes and then incubated in boiling water for 10-12 min to denature whey proteins. After cooling to room temperature (22 ˚C), precipitated protein was removed by centrifugation at 5, 000 ×g for 40 min at 20 ˚C. The pH of the supernatant was adjusted with 2M HCl to 4.3, the pI of camel milk casein, and precipitates were removed by centrifugation. The pH of the supernatant was adjusted to pH 3.0 with 2 M HCl and the solution was loaded on a 1.5 cm × 20 cm column of diethylaminoethyl (DEAE)-Sephadex A-25 (GE Healthcare, Chicago, IL). GMP was eluted with a linear gradient of 0 to 1 M NaCl. GMP eluting from the column was detected by measuring the absorbance at 549 nm after

derivatization with thiobarbituric acid (Warren, 1959). Camel milk oligosaccharides were prepared with the same protocol from acidic whey that was not treated with chymosin.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out with 4-20 % acrylamide ready to use gels (Mini-PROTEAN TGX Precast Protein Gels, Bio-Rad Laboratories, Hercules, CA, USA). GMP was dissolved in distilled water containing 2.5 % (v/v) mercaptoethanol to a concentration of 5 g L^{1}, diluted 1 : 4 (v/v) with SDS loading buffer, heated at 90°C for 5 min, and loaded on the gel. Proteins were separated for 50 min at 150 V and protein bands were stained with Coomassie Blue. Thermo Scientific PageRuler™ Prestained Protein Ladder (10-250 kDa), and Spectra™ Multicolor Low Range Protein Ladder (1.7- 40kDa) (Fisher Scientific) were used as molecular markers.

Reverse phase high performance liquid chromatography coupled to mass spectrometry (LC-MS). LC-MS analyses of GMP were by the Mass Spectrometry Laboratory of the Department of Chemistry at the University of Alberta. LC-MS was performed with an Agilent 1200 SL HPLC System and a Phenomenex Aeris 3.6 µm, WIDEPORE XB-C8, 200 Å, 2.1 x 50 mm guard column. The column was eluted at 0.5 ml min^{-1} and 40 °C with 0.1% (v/v) formic acid in water (A) and 0.1% formic acid in acetonitrile (B) with the following linear gradient: 0 min, 5 % B; 0.5 min, 5% B; 5.5 min, 60 % B; 7 min, 98 % B, followed by washing for 2.8 min and re-equilibration. Mass spectra were acquired in positive mode of ionization using an Agilent 6220 Accurate-Mass TOF LC/MS system (Santa Clara, CA, USA) equipped with a dual sprayer electrospray ionization source. Mass correction was performed for every individual spectrum using peaks at m/z 121.0509 and 922.0098 from the reference solution. Mass spectrometric conditions were drying gas 10 L min^{-1} at 325 °C, nebulizer 20 psi, mass range 100-3000 Da, acquisition rate of ~1.03 spectra/sec, fragmentor 225 V, skimmer 65 V, capillary 4000 V, instrument state 4 GHz High Resolution. Data

122 analysis was performed using the Agilent Mass Hunter Qualitative Analysis software package
123 version B.03.01 SP3.

124 **Determination of glycan composition.** To analyze monosaccharide content and distribution of
125 GMPs from cows, Bactrian camels, and dromedaries, 20 µL of a 5 g L^{-1} solution of GMP were
126 hydrolyzed by using 2 M trifluoroacetic acid (TFA). Briefly, samples were first dried with an
127 evaporator (Eppendorf Concentrator, Hamburg, Germany) at 45 °C, followed by addition of 1 mL
128 of 2 M TFA and incubation 1 h at 121 °C. For the determination of N-acetylneuraminic acid, a
129 reaction temperature of 70 °C was used for hydrolysis with 2 M TFA. After incubation, samples
130 were evaporated and subsequently washed twice with 200 µL ethanol. The dried hydrolysates were
131 finally dissolved in 200 µL of ultrapure water and analyzed by high performance anion exchange
132 chromatography with pulsed amperometric detection (HPAEC-PAD) on an ICS6000 system
133 (Thermo Fisher Scientific, Waltham, MA.) equipped with a Dionex™ CarboPac™ PA20 column
134 (150 mm x 3 mm i.d., 6.5 µm particle size, Thermo Fisher Scientific, Waltham, MA.). The eluents
135 used for the gradient were A) ultrapure water, B) 10 mM sodium hydroxide, C) 200 mM sodium
136 hydroxide and D) 200 mM sodium hydroxide with 200 mM sodium acetate. The flow rate was of
137 0.4 mL min^{-1} at 30 °C. Before every run, the column was rinsed with 100 % B for 10 min and then
138 conditioned with 30 % A and 70 % B for an additional 10 min. After injection, samples were eluted
139 with the following gradient: 0 - 27.5 min isocratic with 30 % A and 70 % B; 27.5-35 min linear to
140 100 % C; 35 - 45 min linear to 100 % D, 45 - 50 min isocratic 100 % D, 50 - 60 min isocratic with
141 100% C to remove the acetate from the column.

142 **Bacterial strains and growth conditions.** A porcine ETEC expressing K88 fimbriae, *E. coli* ECL
143 13795, was used to determine the anti-adhesion activity. ETEC K88 was cultivated on Minca agar
144 aerobically at 37 °C for 6 - 8 h. Cells were washed from the plates with 3 mL of phosphate buffered

saline (PBS, 137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4, pH 7.2). The cell density of the suspension was determined by measuring the optical density (OD) at 600 nm and adjusted to approximately 10^9 CFU mL^{-1} as described (Yan et al., 2017).

Hemagglutination assay to detect the impact of GMP on ETEC K88 adhesion to piglet erythrocytes. Hemagglutination was performed in V-bottom 96-well polystyrene microtiter plates (Corning) as previously described (Yan et al., 2017). Briefly, porcine whole blood cells (Innovative Research Inc., USA) were washed three times in PBS and erythrocytes were resuspended in PBS to a final density 5 % (v/v). ETEC K88 suspension (25 µL with about 10^9 CFU mL^{-1}) was added to the first-column of the microtitre plates and diluted horizontally in ten two-fold serial dilutions. Samples or controls were dissolved at 10 g L^{-1} and diluted in 8 serial twofold dilutions in PBS. Different concentrations of the same sample or control (25 µL each) were added to the same column of the microtitre plate. The plates were incubated at room temperature (23 °C) for 5 min prior to addition of 25 µL of erythrocyte suspension. Plates were incubated overnight (16 h) at 4 °C before visual scoring of agglutination of erythrocytes as described (Wang et al., 2010). Anti-adhesive activity was recorded if the sample or control solution increased the number of ETEC K88 cells that agglutinate erythrocytes at least four-fold. The lowest concentration of GMP with anti-adhesive activity was recorded as minimum anti-adhesive concentration (MAC). Addition of PBS, bovine serum albumin (BSA), lactose, and oligosaccharides from acidic whey served as negative controls. Bovine GMP was used as a positive control.

Enzyme-Linked Immunosorbent Assay (ELISA) to test the ability of GMP to prevent ETEC K88 adhesion to porcine erythrocytes. The ELISA assay was conducted in 96-well high bind

167 microtiter plates (Corning) as previously described (Yan et al., 2017) with minor modifications to
168 confirm the impact of GMP on ETEC K88 adhesion to piglet erythrocytes.

169 Briefly, 100 µL of 5 % porcine erythrocytes were added to coat high bind 96-well plate for 16 h,
170 and plates were then blocked by addition of 200 µL of 3 % BSA, followed by incubation for 60
171 min at 4 °C. GMP and controls were dissolved in PBS to 10 g L^{-1} and diluted in PBS in 8 serial
172 twofold dilutions. ETEC suspensions were mixed with GMP or control solutions 1:1 (v/v) before
173 100 µL of the mixtures were added to the plate and incubated for 60 min at 4 °C. Then 100 µL of
174 1:2000 diluted mouse anti *E. coli* K88A antibody (Bio-Rad Laboratories, Hercules, CA, USA) was
175 added and incubated at 4 °C for 60 min. Then 100 µL of 1:1000 diluted goat antimouse IgG (H+L)
176 secondary antibody (Invitrogen, Fisher Scientific, CA, USA) was added, followed by incubation
177 for 60 min at 4 °C. TMB substrate (50 µL) was then added to each well. The reaction was stopped
178 after 30 min by adding 50 µL of 2M sulfuric acid, and the absorbance at 450 nm was determined
179 with a Varioscan Flash Microplate reader (Thermo Scientific, CA, USA). Between each step of
180 the above protocol, three washing steps with 200 µL of PBS were performed. Erythrocytes without
181 ETEC suspension, erythrocytes with ETEC suspension but without samples, and ETEC suspension
182 without erythrocytes were used as controls in addition to the same negative and positive controls
183 that were also used in the hemagglutination assay.

184 **Deglycosylation of GMP.** To remove the glycans (free oligosaccharides) from GMP, the
185 *O*-glycosidase kit (P0733S, 40,000,000-units mL^{-1}, New England BioLabs, ON, Canada) was used
186 with and without neuraminidase (sialidase) (11585886001, 5 U, Sigma, Mannheim, Germany).
187 Briefly, 20 µg of GMP was mixed with 1 µL of 10X glycoprotein denaturing buffer in 10 µL H_2O.
188 After denaturation of GMP denaturation at 100 °C for 10 min, 2 µL of 10X GlycoBuffer (2), 2 µL
189 of 10 % NP40, 2 µL of sialidase, and 3 µL of *O*-glycosidase were added. The mixture was

incubated for 3 h at 37 °C. The enzyme kit that was used for protein deglycosylation hydrolyses O-glycosidic bonds of the disaccharide Gal-β-1-3GalNAc as well as larger oligosaccharides (Ishii-Karakasa, Iwase, Hotta, Tanaka, & Omura, 1992).

Statistical Analysis.

Bioassays were performed in triplicate biological repeats with three technical repeats each and results are presented as means ± standard deviation. Statistical differences were determined with one way ANOVA and Least Significant Difference (LSD) post hoc analysis in Minitab 19. Differences between values were significant with an error probability of 5% (P<0.05).

Results

GMP Purification. GMP from Bactrian camels and dromedaries were purified with a protocol that was developed for bovine GMP and employs rennet- and heat induced precipitation of casein and whey proteins, respectively, and ultra-filtration. Negatively charged GMP was then separated on an anion exchange column (Fig. 2 A, B). GMP is glycosylated and phosphorylated (Fig. 3). The predicted molecular weights (Mw) (https://peptidenexus.com/peptide) of non-glycosylated GMPs from Bactrian camel and cows were 6.774 kDa and 6.707 kDa respectively. The mass spectra obtained by ESI-LC-MS included the predicted ion species of non-glycosylated GMP for Bactrian camels and cows at 6.777 kDa and 6.787 kDa respectively; additional peaks were observed that likely represent the peptides with different levels of glycosylation and phosphorylation (Fig. S1). The purity of purified Bactrian camel GMP, dromedary GMP, and bovine GMP were assessed by SDS-PAGE (Figure 4). The pattern of all GMPs samples presented visible regular thin band located at about ~14 kDa, corresponding to the dimeric GMP form composed of 2 GMP monomers. The separation of bovine GMP and GMP from dromedary milk produced additional bands with an apparent Mw of 14 – 30 kDa.

Composition of the glycans in GMP from cattle, Bactrian camels, and dromedaries. To determine the glycan composition of the GMPs, glycans were hydrolyzed with 2 M TFA, and the concentration of resulted monosaccharides was determined (Table 1). Hydrolysis of glycans with 2 M TFA not only hydrolyses the glycosidic bonds but also partially or completely deacetylates N-acetylglucosamine and N-acetylgalactosamine; these are therefore detected as the corresponding amino sugars. The amount of total monosaccharides from bovine GMP was less than 50 % of the amount of monosaccharides from Bactrian camel and dromedary GMPs. Fucose and glucosamine were detected in GMP from Bactrian camels and dromedaries but absent (fucose) or only present in a low concentration (glucosamine) in bovine GMP. Galacturonic acid and glucuronic acid were detected in GMP from Bactrian camels only (Table 1). The monosaccharide composition after hydrolysis of GMP from Bactrian camels and dromedaries also differed qualitatively and quantitatively, e.g. GMP from dromedaries contained more glucose than GMP from Bactrian camels.

Impact of GMP on ETEC K88 adhesion to porcine erythrocytes. The hemagglutination assay was performed for GMP from Bactrian camel and dromedary milk, using bovine GMP, oligosaccharides from camel acidic whey, bovine serum albumin (BSA) and lactose served as controls. The strongest anti-adhesive activities against ETEC K88 were observed for Bactrian camel GMP (Table 2). BSA and lactose had no anti-adhesive activities against ETEC K88. However, oligosaccharides from Bactrian camel acidic whey that was prepared without chymosin treatment, had a lower minimum anti-adhesive concentration than the positive control (Table 2).

ELISA was used to confirm the activity of GMP from milk of Bactrian camels and controls in preventing ETEC K88 adhesion to porcine erythrocytes. Comparable to the hemagglutination assay, the highest anti-adhesive activity was observed for Bactrian GMP, followed by

236 oligosaccharides from Bactrian camel acidic whey, bovine GMP, lactose, and BSA respectively
237 (Figure 5). At a concentration of 10 g L^{-1}, Bactrian camel GMP reduced ETEC adhesion by about
238 75 %: at 0.125 g L^{-1}, Bactrian camel GMP still significantly reduced ETEC adhesion (Figure 5).
239 The results from both assays, hemagglutination and ELISA, thus demonstrate that GMP from
240 Bactrian camels and dromedaries as well as oligosaccharides from Bactrian camel acidic whey had
241 higher anti-adhesive activities when compared to bovine GMP.

242 **Deglycosylation of GMP.** To determine whether the effect on ETEC adhesion requires that
243 oligosaccharides are bound to the peptide backbone, the activity of GMPs was compared to the
244 activity remaining after enzymatic deglycosylation with sialidase and *O*-glycosidase. The resulting
245 free sugars and deglycosylated GMPs were redissolved in PBS at 10 g L^{-1} and diluted to determine
246 their biological activities (Table 3). The activity of the glycosylated GMPs was consistent with
247 results shown in Table 2; glycan hydrolysis with sialidase and *O*-glycosidase (Table 3) or with
248 *O*-glycosidase only (data not shown) eliminated anti-adhesive activity.

249 **Discussion**

250 Camel is an important source of milk in many countries including some of the developing countries
251 where childhood diarrhea caused by ETEC is very common (Jafar, Kamal, Mudgil, Hassan, &
252 Maqsood, 2018; Jrad et al., 2014). Whey is a byproduct of camel milk cheese production (Jafar et
253 al., 2018) and is thus an inexpensive source of bioactive compounds including GMP. In the present
254 study, purification of camel and dromedary GMP was achieved with a protocol that was developed
255 for purification of bovine GMP (Nakano et al., 2018). The experimental Mw of non-glycosylated
256 bovine GMP that was observed by LC-MS/MS, 6.787 kDa, matches prior observations (Mollé &
257 Léonil, 1995, 2005), LC-MS data for the GMP from Bactrian camels is not available. The average
258 molecular weight (Mw) of glycosylated bovine GMP is 7.5 kDa (Kreuss, Krause, & Kulozik,

2008). The SDS-PAGE analysis indicates that the GMP from Bactrian camels and dromedaries form dimers as was previously shown for bovine and goat GMP as a result of self-assembly (Farías, Martinez, & Pilosof, 2010; Nakano & Ozimek, 2000; Silva-Hernandez, Nakano, & Ozimek, 2002). The additional bands present in dromedary GMP and bovine GMP correspond to trimeric and tetrameric GMP. The observation of multimeric aggregated GMP was reported previously after separation of bovine GMP, which migrates on SDS-PAGE as a mixture of polymers (Farías et al., 2010; Mikkelsen et al., 2005; Nakano, Noriaki, & Ozimek, 2007). It was suggested that hydrophobic interactions stabilize the GMP dimers while electrostatic bonds additionally stabilize the multimeric aggregates of GMP (Farías et al., 2010; Mikkelsen et al., 2005; Nakano et al., 2007). The multimeric aggregates of GMP were least abundant in the sample prepared from milk of Bactrian camels; this was also the only sample that was not pasteurized prior to preparation of the GMP, indicating that pasteurization contributes to the aggregation of bovine and camel GMP.

The monosaccharide composition of bovine GMP matches prior reports on its glycan composition (Holland, Deeth, & Alewood, 2006; Hua et al., 2011). Bovine GMP is glycosylated with a disaccharide composed of galactose and N-acetylgalactosamine (GalNAc), which is decorated with one or two N-acetylneuraminic acid (NeuNAc) moieties. Glycosylation with oligosaccharides that additionally include fucose and N-acetylglucosamine (GlcNAc) were reported in GMP from bovine colostrum (O'Riordan, Kane, Joshi, & Hickey, 2014). GalNAc and GlcNAc were detected as the deacetylated amino sugars galactosamine (GalN) and glucosamine (GlcN). This is the result of extensive deacetylation during TFA hydrolysis (Dolgopyatova, Novikov, Konovalova, & Putintsev, 2013; Gizatulina, Chebotok, Novikov, & Konovalova, 2005; Prechtl, Wefers, Jakob, & Vogel, 2018). Falsification of the results by naturally occurring GalN and GlcN is unlikely because they are highly reactive in the Maillard reaction (Hrynets, Ndagijimana, & Betti, 2015) and were

not identified in glycoproteins in milk (O'Riordan et al., 2014). NeuNAc is also degraded when high temperatures are applied during acid treatment (Zhu, Chen, Yuan, Wu, & Yao, 2020). Therefore, the reaction temperature during TFA hydrolysis was reduced to 70 °C which allowed the quantification of NeuNAc in the different GMPs.

The monosaccharide content of GMPs from Bactrian camels and dromedaries was about twice as high as bovine GMP, corresponding to the higher number of potential glycosylation sites in these two species (Fig. 3). The difference is more significant for galactose, GalNAc, NeuNAc and GlcNAc. For instance, the content of GlcNAc in Bactrian camel and dromedary is 6 - 8 fold higher than in bovine GMP (Table 1). Many of the biological activities of GMP are mediated by the glycan structure (O'Riordan et al., 2014), therefore, the increased glycan content of GMP from *Camelus* species may also impact their biological activity.

ETEC K88 infect young piglets and calves (Moonens et al., 2015), and K88 fimbriae mediate the binding of *E. coli* ECL13795 to glycan receptors (Jin & Zhao, 2000). Porcine aminopeptidase N is a receptor for F4 (K88) fimbriae; in addition, surface glycan oligosaccharides composed of GalNAc, GlcNAc, GalN, and N-acetylmannosamine were proposed as receptors for ETEC K88 adhesion (Jin & Zhao, 2000; Moonens et al., 2015). GMP from both Bactrian camels and dromedaries showed potent anti-adhesive activity at concentrations of about 0.25 g L^{-1}, which is about 20-fold lower than the effective concentration of bovine GMP (Hermes et al., 2013; Sun, Gänzle, & Wu, 2019). This increased activity *in vitro* likely relates to the increased glycosylation and / or differences in the glycan composition and may also translate to an increased activity *in vivo*. Bovine GMP also reduced the attachment of ETEC K88 *in vivo* (Hermes et al., 2013; Rong et al., 2015) and improved growth performance of *E. coli* K88-challenged piglets (Hermes et al., 2013; Rong et al., 2015).

This relationship of glycan structure to biological activity was confirmed by comparison of the activity of glycosylated GMPs to the activity of free oligosaccharides and GMPs after enzymatic deglycosylation (Table 3). Free oligosaccharides from camel milk were less active than GMP and deglycosylation of GMP strongly reduced the prevention of ETEC adhesion. The higher activity of the more densely glycosylated camel GMP, and the strong decrease of activity after deglycosylation suggests that not only the structure but also the density spacing of glycans on the peptide backbone are important for anti-adhesive activity. The topological spacing of glycans is recognized as an important factor affecting the anti-adhesive activity of glycopeptides (Lewallen, Siler, & Iyer, 2009; Nakajima et al., 2005; Oyelaran & Gildersleeve, 2009; Oyelaran, Li, Farnsworth, & Gildersleeve, 2009).

In conclusion, hemagglutination and ELISA assays indicate that the anti-adhesive activity of GMP from Bactrian camels and dromedaries was substantially higher than the activity of bovine GMP. Free oligosaccharides from camel milk that were prepared as a control were also active but at a higher concentration when compared to GMP. Deglycosylation of GMP suggested that the spatial arrangement of glycans on the peptide backbone contributes to anti-adhesive activity. The *in vitro* anti-adhesive activity of bovine GMP was confirmed to also reduce ETC K88 adhesion *in vivo* (Hermes et al., 2013). Therefore, it is likely that the *in vitro* activity of GMP from *Camelus* species (this study) also translates to *in vivo* activity in swine. ETEC that infect humans, however, use different fimbriae with different binding specificity when compared to porcine ETEC (Eigel et al., 1984; Nagy & Fekete, 2005), therefore, the use of GMP from camels and dromedaries remains subject to future investigations.

Acknowledgements

We acknowledge the Natural Sciences and Engineering Research Council of Canada (NSERC), Canada Research Chairs (CRC), and Mutah University, Jordan, respectively, for their funding. We are grateful for Chr. Hansen, Australia for providing the camel chymosin enzyme.

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Figure legends

Figure 1. Flow diagram for the purification of the glycomacropeptide (GMP) from camel milk

Figure 2. Separation of the GMP from milk of Bactrian camels (**Panel A)** and dromedaries (**Panel B)** on a diethylaminoethyl (DEAE)- Sephadex A-25 column. Sialylated oligosaccharides were eluted with 0.5 M NaCl and detected at 549 nm after derivatization. Camel milk oligosaccharides were prepared as negative control from milk that was not treated with rennet.

Figure 3. Amino acid sequence of the glycomacropeptide (C-terminus of κ-casein) from Bactrian camel (L0P304), dromedary (P79139), and cows (P02668). Green colored amino acids indicate the glycomacropeptide (GMP); bold residues indicate potential sites for glycosylation or phosphorylation. Bold blue-colored letters correspond to amino acids that were shown to be glycosylated in variant A of bovine GMP; red-colored letters correspond to amino acids that were shown to be phosphorylated in variant A of bovine GMP.(Eigel et al., 1984) The red arrow indicates the cleave sites of chymosin.

Figure 4. Separation of purified GMP from Bactrian camel and dromedary, and of commercial bovine GMP by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Figure 5. Quantification of *E. coli* K88 ECL13795 binding to porcine erythrocytes with ELISA targeting K88 antibodies. ETEC were incubated with erythrocytes without addition of GMP (no GMP control) or with addition of different concentrations of GMPs. Lactose, bovine serum albumin, and acidic oligosaccharides from milk from Bactrian camels served as controls. Based on the yield of GMP and acidic oligosaccharides from the milk of Bactrian camels, 50 and 5 mg / L, respectively, the GMP preparation from Bactrian camels included about 10% of acidic oligosaccharides in addition to the GMP. Values obtained with different compounds or

preparations at the same concentration differ significantly ($P<0.05$) if they do not share a common letter. Results are reported as means ± standard deviation of three independent assays.

Table 1. Composition of monosaccharides after hydrolysis of bovine, Bactrian camel, or dromedary GMP with 2 M TFA. The error was calculated from the Residual Standard Error of the linear or linear quadratic regression of the standard curves.

amount mg sugar g^{-1} GMP (molar ratio)[a)]	**Bovine**	**Bactrian camel**	**Dromedary**
Gal	22.13 ± 0.35 (1)	58.63 ± 0.76 (1)	53.15 ± 0.23 (1) (0.295)
GalN	20.54 ± 0.18 (1)	38.53 ± 0.17 (0.7)	33.23 ± 0.23 (0.6)
NeuNAc[b)]	39.06 ± 0.27 (1)	52.98 ± 0.25 (0.5)	64.68 ± 0.28 (0.7)
GlcN	1.24 ± 0.16 (0.1)	19.75 ± 0.22 (0.3)	15.10 ± 0.05 (0.3)
Glc	1.48 ± 0.35 (0.1)	6.81 ± 0.31 (0.1)	12.03 ± 0.37 (0.2)
GalA	n.d[c)]	0.73 ± 0.16 (<0.1)	n.d.
Fuc	n.d.	0.43 ± 0.10 (<0.1)	0.60 ± 0.05 (<0.1)
GlcA	n.d.	traces[d)]	n.d.
GlcNAc, GalNAc and Neu5GC	n.d.	n.d.	n.d.
Sum of all sugars	84.4	178.0	178.2

[a)] The molar ratio was calculated relative to galactose and rounded to one significant digit.

Gal, galactose; GalN, galactosamine; NeuNAc, N-acetylneuraminic acid; GlcN, glucosamine, Glc, glucose, GalA, galacturonic acid; Fuc, fucose, GlcA, glucosamine, GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; Neu5GC, N-glycoylneuraminic acid.

[b)] The NeuNAc concentration was determined after hydrolysis at 70 °C.

[c)] n.d., not detected.

[d)] Concentration between limit of detection and limit of quantitation.

Table 2. Inhibition of erythrocyte agglutination by ETEC K88 strain with Bactrian camel GMP, dromedary camel GMP, bovine GMP, oligosaccharides from camel acidic whey, bovine serum albumin (BSA), and lactose. Data for bovine and camel GMPs and acidic oligosaccharides are shown as means ± standard deviation of three independent preparations from dry milk. Values obtained with different compounds or preparations at the same concentration differ significantly (P<0.05) if they do not share common letter.

sample	**minimum concentration for erythrocyte agglutination with ETEC (g L^{-1})**
Bactrian camel GMP[A]	0.24^{b} ± 0.02
Dromedary camel GMP	0.28^{b} ± 0.03
Bovine GMP	5.52^{a} ± 1.06
Oligosaccharides from milk of Bactrian camel	0.87^{b} ± 0.15
Lactose	> 10
BSA	> 10

[A] The preparations of camel GMP included about 10 % acidic oligosaccharides

Table 3. Inhibition of erythrocyte agglutination by ETEC K88 strain with Bactrian camel GMP, dromedary camel GMP, and bovine GMP before and after enzymatic deglycosylation with sialidase and O-glycosidase. Data for camel GMPs and bovine GMP are shown as means ± standard deviation of three independent assays for the same preparations. Values obtained with different compounds or preparations differ significantly ($P<0.05$) if they do not share a common superscript.

sample	**minimum concentration for erythrocyte agglutination with ETEC (g L^{-1})**	
	Before deglycosylation	**After deglycosylation**
Bactrian camel GMP	0.25^{b}± 0.00	> 10
Dromedary camel GMP	0.25^{b}± 0.00	> 10
Bovine GMP	5.00^{a} ± 0.00	> 10
Control (just enzyme/ no GMP)	> 10	> 10

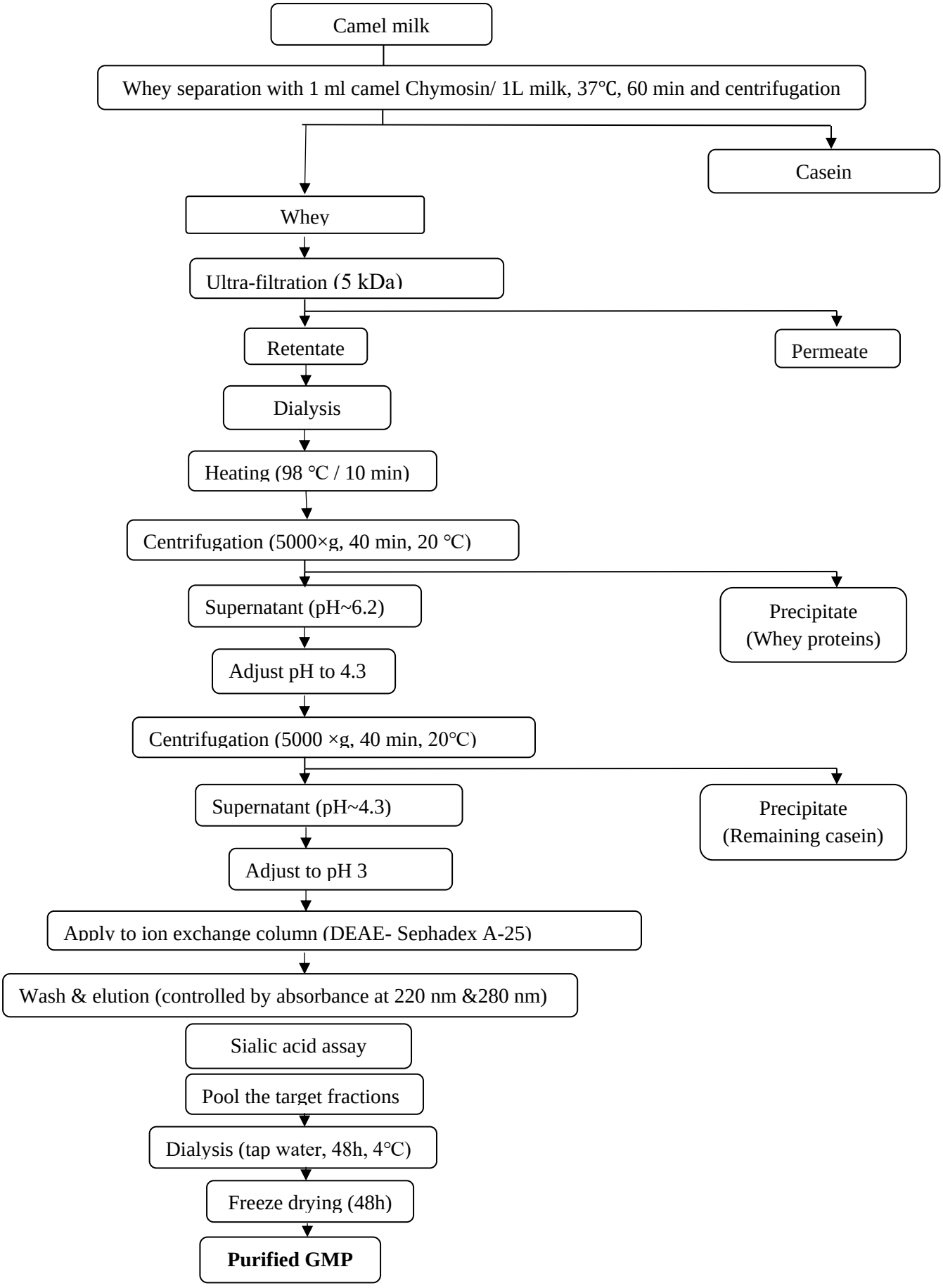
Camel milk
Whey separation with 1 ml camel Chymosin/ 1L milk, 37℃, 60 min and centrifugation
Casein
Whey
Ultra-filtration (5 kDa)
Retentate
Permeate
Dialysis
Heating (98 ℃ / 10 min)
Centrifugation (5000×g, 40 min, 20 ℃)
Supernatant (pH~6.2)
Precipitate
(Whey proteins)
Adjust pH to 4.3
Centrifugation (5000 ×g, 40 min, 20℃)
Supernatant (pH~4.3)
Precipitate
(Remaining casein)
Adjust to pH 3
Apply to ion exchange column (DEAE- Sephadex A-25)
Wash & elution (controlled by absorbance at 220 nm &280 nm)
Sialic acid assay
Pool the target fractions
Dialysis (tap water, 48h, 4℃)
Freeze drying (48h)
Purified GMP

Figure 1:

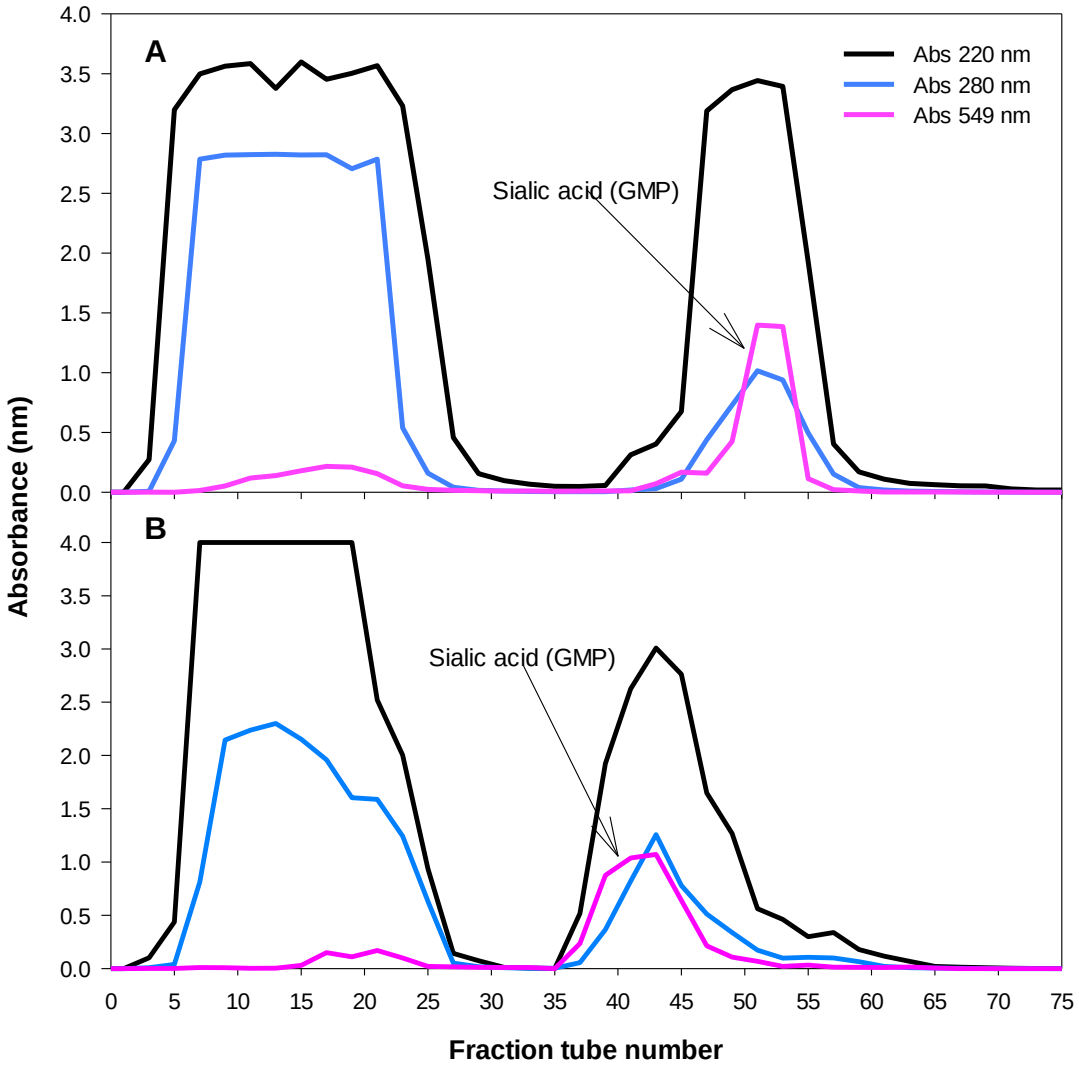
A
B
Abs 220 nm
Abs 280 nm
Abs 549 nm
Sialic acid (GMP)
Sialic acid (GMP)
Absorbance (nm)
Fraction tube number
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75

Figure 2.

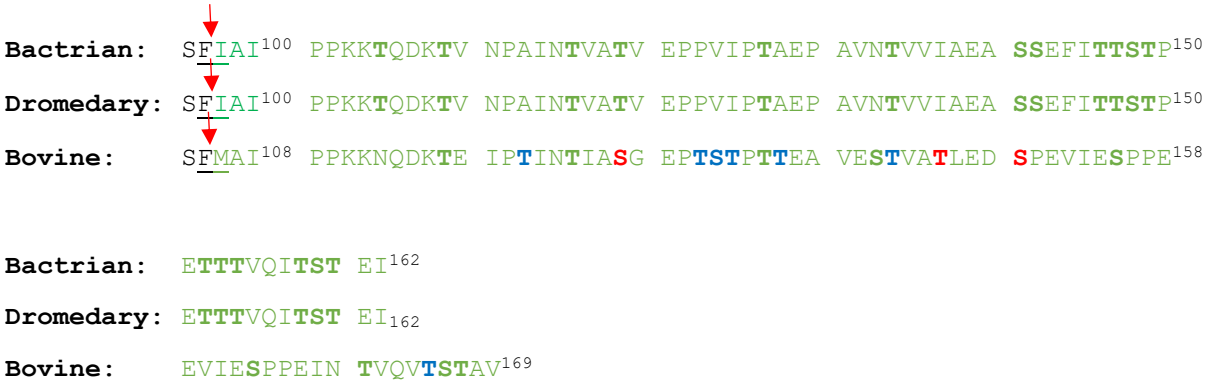
Bactrian: SFIAI100 PPKKTQDKTV NPAINTVATV EPPVIPTAEP AVNTVVIAEA SSEFITTSTP150
Dromedary: SFIAI100 PPKKTQDKTV NPAINTVATV EPPVIPTAEP AVNTVVIAEA SSEFITTSTP150
Bovine: SFMAI108 PPKKNQDKTE IPTINTIASG EPTSTPTTEA VESTVATLED SPEVIESPPE158
Bactrian: ETTTVQITST EI162
Dromedary: ETTTVQITST EI162
Bovine: EVIESPPEIN TVQVTSTAV169

Figure 3.

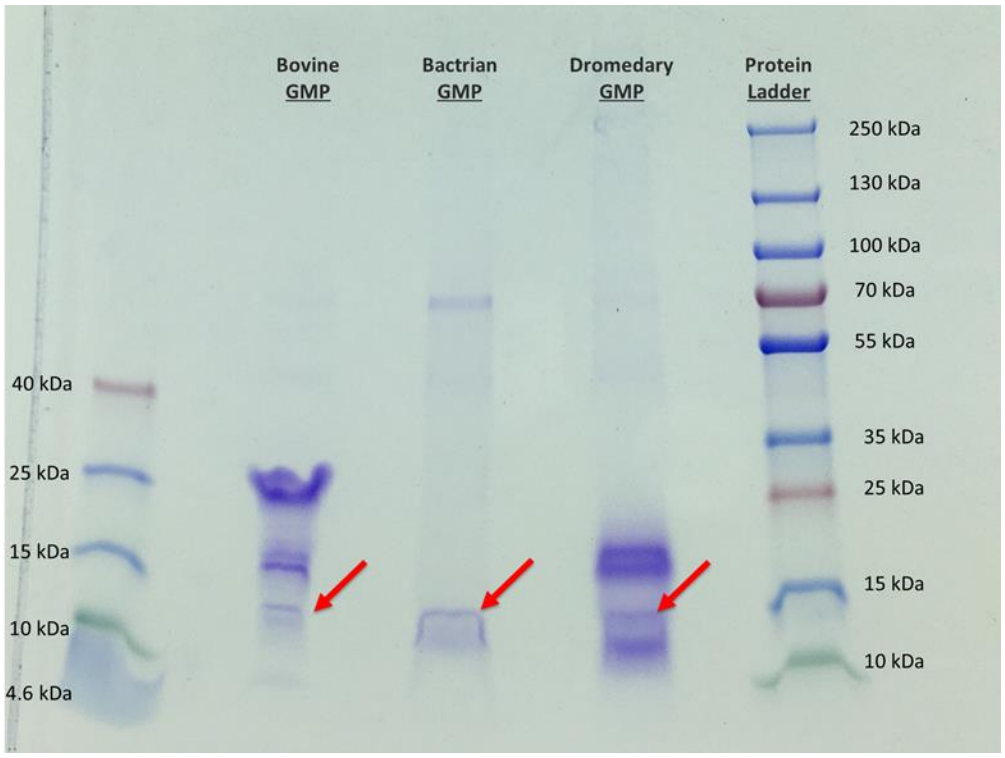
Bovine
GMP
Bactrian
GMP
Dromedary
GMP
Protein
Ladder
250 kDa
130 kDa
100 kDa
70 kDa
55 kDa
35 kDa
25 kDa
15 kDa
10 kDa
40 kDa
25 kDa
15 kDa
10 kDa
4.6 kDa

Figure 4.

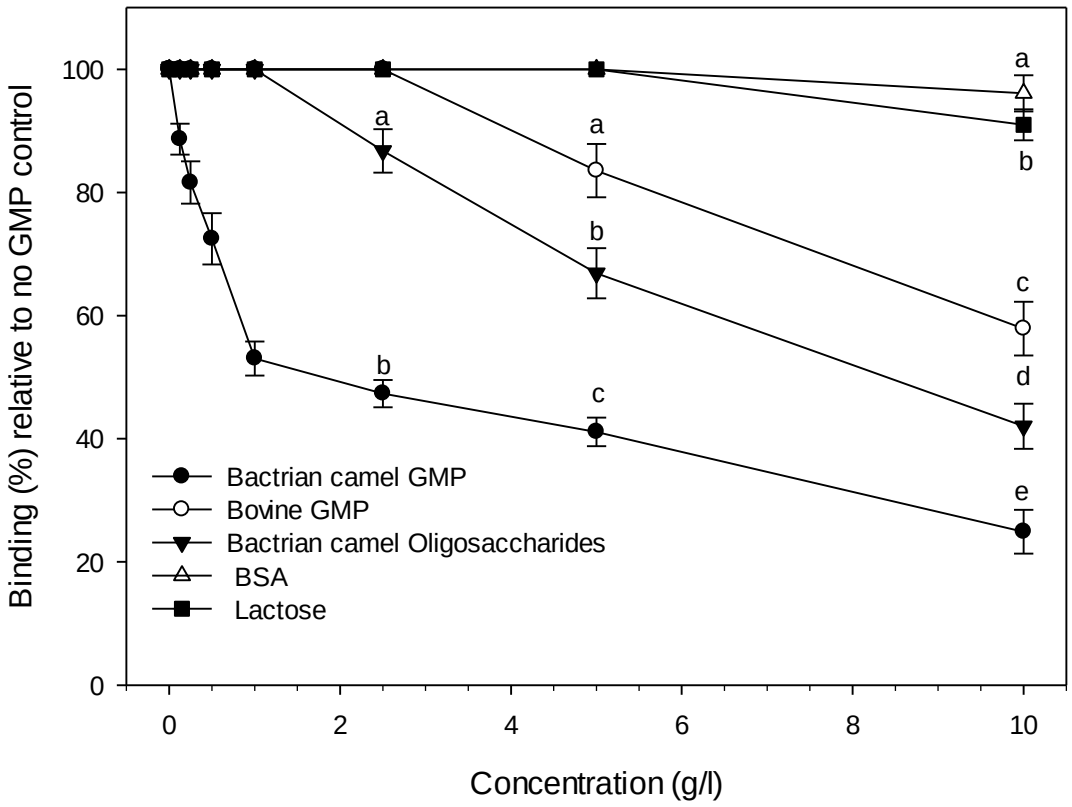
Binding (%) relative to no GMP control
Concentration (g/l)
Bactrian camel GMP
Bovine GMP
Bactrian camel Oligosaccharides
BSA
Lactose
0
20
40
60
80
100
0
2
4
6
8
10
a
a
a
b
b
b
c
c
c
d
e

Figure 5.

Graphic abstract: Anti-adhesion activity of different GMP against ETEC

Table of Content Graph