

Maillard neoglycans as inhibitors for *in vitro* adhesion of F4⁺ enterotoxigenic *Escherichia coli* to piglet intestinal cells

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Adhesion of enterotoxigenic (ETEC) *E. coli* to host intestinal cells is mediated by lectin-like fimbriae that bind to specific glycan moieties on the surfaces of enterocytes. To prevent *in vitro* binding of *E. coli* F4 fimbriae (F4 ETEC⁺) to piglet enterocytes, neoglycans were synthesized by the Maillard reaction conjugating lactose (Lac), galacto-oligosaccharides (GOS) or chitin oligosaccharides (Ochit) to porcine serum albumin (PSA). Neoglycans were characterized by SDS-PAGE, intrinsic tryptophan fluorescence and recognition by plant lectins, as well as by F4 ETEC variants. Electrophoretic patterns suggested the binding to PSA of 63, 13 and 2 molecules of Lac, GOS and Ochit, respectively. All neoglycans displayed quenching of tryptophan fluorescence consistent with the degree of glycation estimated by SDS-PAGE. Plant lectins recognized the neoglycans according to their specificity, whereas antigenic variants of F4 ETEC (ab, ac and ad) recognized PSA-Ochit and PSA-Lac with higher affinity than that for GOS. Neoglycans partially hindered the *in vitro* binding of F4⁺ ETEC to piglet enterocytes in a dose-dependent manner. The most effective blocking was observed with PSA-Lac that partially inhibited the adhesion of bacteria to enterocytes in a dose dependent manner, as quantified by flow cytometry. Increased production of the cytokines IL-6 and TNF- α was observed in response to F4⁺ ETEC infection of enterocytes and production was reduced in the presence of PSA-Ochit and PSA-GOS. These results suggest that neoglycans synthesized by the Maillard reaction could be useful in the prophylaxis of diarrhea in piglets.

Key words: Albumin glycation, biorecognition; anti-adhesion; *E. coli*

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Abbreviations: ATCC, American type culture collection; cfu, colony-forming unit; DMEM-F12, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12; ELLA, enzyme-linked lectin assay; ELISA, enzyme-linked immunosorbent assay; ETEC, enterotoxigenic *Escherichia coli*; FITC, fluorescein isothiocyanate; GOS, galacto-oligosaccharides; Lac, lactose; Ochit, chitin oligosaccharides; PBS, phosphate-buffer saline; PE, phycoerythrin; PSA, porcine serum albumin; PSA-GOS galactosylated porcine serum albumin; PSA-Lac, lactosylated porcine serum albumin; RCA I, *Ricinus communis* agglutinin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin

INTRODUCTION

Infections caused by enterotoxigenic *E. coli* (ETEC) are one of the leading causes of diarrhea in children and young animals (Qadri *et al.*, 2005). The pathogenic effect

of various ETEC strains on enterocytes depends on the activities of their toxins (LT and ST). However, in order to deliver the toxins, bacteria must first bind to the intestinal cells. ETEC binding to intestine cells is achieved by biological recognition between lectin-like adhesins, located at the tip of bacterial fimbriae or pili, and complex carbohydrates attached to lipids or proteins expressed on the cell surface of enterocytes (Sharon, 2006). ETEC strains expressing F4 (K88) fimbriae are the major etiological agents of diarrhea in neonates and newly weaned pigs (Dubreuil *et al.*, 2016). In North America, ETEC infections resulting in diarrhea in piglets causes important economic losses to the porcine industry. In addition, such infections increase feeding costs by 10–12%, raise mortality, escalate the medical treatment expenses and delay the time of sacrifice by 2 to 3 days per day (Nagy & Fekete, 2005; Dubreuil *et al.*, 2016).

One way to prevent porcine ETEC infection would be to block the adhesin-carbohydrate recognition, and in turn block the binding of the pathogen to the cell surface. Due to the specificity of adhesins, this blockage could be carried out with molecules that effectively mimic the binding site of cellular receptors (Sharon, 2006). Different exogenous molecules have been tested *in vitro* to hinder the adhesion of F4 ETEC to pig intestinal cell lines. In this respect, certain dietary ingredients, such as casein glycomacropeptide and some galactomannans obtained from locust bean gum, are notable for their ability to partially block the adhesion of F4 ETEC to the IP-EC-J2 cell line (Gonzalez-Ortiz *et al.*, 2013). More elaborate studies evaluating the blocking of F4 binding to cell surfaces include those based on the chemical synthesis of dendrimers, application of genetic engineering to create microorganisms expressing oligosaccharides that are recognized by pathogens, or synthesis of neoglycans as inhibitors (Watts *et al.*, 2012; Sattin & Bernardi, 2016).

Neoglycan synthesis by glycosylation is a costly and difficult process due to the limited availability of the enzymes required. Alternatively, neoglycans can also be obtained through non-enzymatic glycation by a controlled Maillard reaction that conjugates non-reducing sugars to the reactive amine groups of different proteins (Ledesma-Osuna *et al.*, 2008). Using this strategy, Sarabia-Sainz *et al.* (2011) conjugated lactose (Lac) to porcine serum albumin (PSA), obtaining PSA-Lac, and demonstrated that this neoglycan complex was recognized by the of F4 ETEC adhesins. The advantage of this type of synthesis is low cost, as the materials are readily available and are inexpensive, and thus, this process has potential in veterinary applications for preventing or reducing ETEC infections. Other carbohydrates that could potentially

be recognized by F4 ETEC are prebiotic galacto-oligosaccharides (GOS), as well as chitin oligosaccharides (Ochit), derived from the controlled hydrolysis of chitin (Ledesma-Osuna *et al.*, 2010; Sarabia-Sainz *et al.*, 2013). The purpose of this study was to test the effectiveness of different neoglycans synthesized by non-enzymatic glycation in preventing *in vitro* binding of F4⁺ ETEC to piglet enterocytes.

MATERIALS AND METHODS

Materials. Porcine albumin, Lac and shrimp shell chitin were purchased from Sigma-Aldrich (St. Louis, MO, USA). GOS (oligomate 55) were kindly donated by Yakult Pharmaceutical Inc. (Somerset, NJ, USA). Lectins from *Ricinus communis* (RCA1) and wheat germ agglutinin (WGA) were purchased from Vector (Burlingame, CA, USA). The anti-F4 monoclonal antibody was obtained from BIOTREND Chemikalien GmbH (Germany). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise.

Isolation of GOS and obtaining chitin hydrolysates. Oligomate 55 is a mixture of 50–55% GOS (mostly tri- and tetra-saccharides) and 45–50% mono- and disaccharides (galactose, glucose, and Lac) (Nishibata *et al.*, 2009). Mono- and disaccharides were removed from the mixture by adsorption chromatography using activated charcoal (Darco G60, 100 mesh; Sigma, St. Louis, MO), following the technique described by Sarabia-Sainz and coworkers (2013). The adsorption of Oligomate 55 to the activated carbon was enhanced using 15:85 (v/v) ethanol/water, while GOS elution was carried out using a 50:50 mixture. After removing the ethanol in a vacuum concentrator (Centrivap, Labconco, Kansas City, MO, USA), the isolated GOS were dissolved in deionized water, filtered through 0.22 µm filters (Millipore Corp., Bedford, MA, USA), lyophilized and stored at –80 °C until use.

Shrimp shell chitin oligosaccharides (Ochit) were prepared by controlled acid hydrolysis in 6 M HCl at 40°C for 110 min. To stop the reaction, the sample was placed in a salt and ice bath and slowly neutralized with 50% NaOH under continuous mixing. Samples were centrifuged at 10 000 × g for 25 min at 4°C, followed by ultrafiltration of the supernatant through 3 and 1 kDa membranes in tandem to obtain Ochit (in a tetramer to heptamer range). Filtrates were lyophilized and stored at –40°C until use (Ledesma-Osuna *et al.*, 2010).

Synthesis of neoglycans. Three types of neoglycans were synthesized: PSA-Lac, PSA-GOS and PSA-Ochit. The synthesis of PSA-Lac was carried out using the method described by Sarabia-Sainz and coworkers (2011). Briefly, PSA was mixed with Lac (1:200 molar ratio) in PBS (phosphate buffer saline solution, 50 mM phosphate buffer, 0.15 M NaCl, pH 7.2), lyophilized (Virtis Benchop 6.6, NY, USA), and subsequently incubated at 100°C for 30 min, followed by immediate cooling with 5°C deionized water. Extensive dialysis was then performed at 4°C with double-distilled water to remove free Lac. The obtained dialysate was lyophilized and stored at –40°C until use. PSA-GOS were synthesized by the same method using a pH of 9.0 at 90°C.

For the synthesis of PSA-Ochit the PSA:Ochit mixture (1:200 molar ratio) was incubated at 60°C at a pH of 8.0 for 48 h, lyophilized and heated at 100°C for 30 min following the procedure already described. Modifications in the synthesis of PSA-GOS and PSA-Ochit were conducted to increase the reactivity between PSA and the oligosaccharides.

Electrophoresis. The obtained neoglycans were characterized by SDS-PAGE using 8% polyacrylamide gels and loading 15 µg of protein in each well. Untreated PSA was included as a control. Gels were performed in triplicate, run at 100 V using a Powerpac 3000 power source (Bio-Rad, Hercules, CA, USA) and subsequently stained with Coomassie blue R-250. The protein migration patterns were compared with the broad range molecular weight standards from Bio-Rad and analyzed using the ImageLab program (Bio-Rad, Hercules, CA, USA) of the Molecular Imager Gel Doc XR+.

Intrinsic tryptophan fluorescence assay. The intrinsic tryptophan fluorescence of the synthesized neoglycans was determined following the methods described by Westwood and Thornalley (1995). A 5 µM solution of each neoglycan was prepared by dilution with 20 mM PBS, pH 7.2, and adjusted to 0.05 units of optical density at 280 nm. Tryptophan was excited at 295 nm using a Perkin-Elmer LS-50B fluorescence spectrophotometer (Waltham, MA, USA). Emission was recorded at 5 nm intervals and the emission spectrum in the 300 to 400 nm range was obtained. Analyses were performed in duplicate.

Lectin binding assays. The binding of neoglycans (PSA-Lac, PSA-GOS, PSA-Ochit) to *Ricinus communis* lectin 1 (RCA 1) and wheat germ agglutinin (WGA lectin) was measured through an enzyme-linked lectin sorbent assay (ELLA), as described by Sarabia-Sainz *et al.* (2013). Each neoglycan (500 ng) was diluted separately in 50 mM Na₂CO₃ buffer with a pH of 9.6 (100 µL), and adsorbed onto 96 well plates for 16 h at 4°C. After 4 washes with T-TBS (0.05% Tween 20 and 50 mM Tris-HCl buffered saline, pH 7.5), nonspecific interactions were blocked using 1.5% bovine serum albumin (BSA) in T-TBS for 2 h at 37°C. The plates were then washed four times with T-TBS and incubated for 1 h at 37°C with 100 µL (5 µg/mL) of biotinylated RCA 1 or biotinylated WGA diluted in TBS (50 mM Tris-HCl buffer, pH 7.5). Plates were washed again with T-TBS prior to the addition of 100 µL of streptavidin-peroxidase (1:2000 in TBS) for 1 h at 37°C. After 4 additional washes with T-TBS, plates were developed by adding 100 µL of Sigma FAST OPD per well, and binding interactions were quantified by measuring the absorbance at 450 nm using an ELISA detector (Anthos Zenyth 340ST microplate, Alcobendas, Spain). Untreated PSA was used as a control. Analyses were performed in duplicate for a total of 32 measurements for each treatment.

Recognition of the synthesized neoglycans by F4⁺ ETEC adhesins. Recognition of the neoglycans, PSA-Lac, PSA-GOS and PSA-Ochit, by F4⁺ ETEC bacteria isolated from piglets with diarrhea (kindly donated by Dr. Carlos Eslava from the Universidad Nacional Autónoma de México) and by F4ab ATCC 1373, F4ac ATCC 1374 and F4ad ATCC 1375 (all isolated from pigs) was tested according to Ledesma-Osuna and coworkers (2010). For these assays, F4⁺ ETEC cultures were grown overnight in trypticase soy broth (Difco, Becton, Dickinson and Co., USA) with constant shaking at 37°C, and then harvested by centrifugation. Bacterial pellets were washed twice with PBS, and adjusted to approximately 2 × 10⁸ cfu/mL (0.5 units of optical density at 660 nm in PBS). Bacteria solutions (100 µL per well) were immobilized (1 h at 25 °C) on microtiter plates using 1% glutaraldehyde. After four washes with T-PBS (0.05% Tween 20 in PBS), nonspecific interactions were blocked (16 h at 4°C) using 300 µL of 1.5% BSA in T-PBS, and the solutions were incubated for 2 h at 25°C with 10 µg/mL of biotin-labeled PSA-Lac, PSA-

GOS or PSA-Ochit in PBS. Following four washes with T-PBS, the plates were incubated for 1 h at 25°C with streptavidin-peroxidase (1:1000), and color development was processed as previously described for lectin assays. Untreated PSA and duodenal piglet mucins were used as negative and positive controls, respectively.

Inhibition of the F4⁺ ETEC adhesion to piglet enterocytes. The binding of F4⁺ ETEC to piglet enterocytes was measured by flow cytometry. To obtain the enterocytes, five healthy two-week-old piglets were sacrificed. Thirty centimeters of jejunum were removed from each piglet and immersed in PBSA (100 IU/mL penicillin, 100 µg/mL amphotericin, 50 mM phosphate buffer, 0.15 M NaCl, pH 7.2). The jejunum was cut into 4 cm long fragments and washed with sterile PBSA; coarse particles and pieces of mucosa were carefully removed. The cut fragments were placed in Petri dishes, and the cells were obtained by scraping with a sterile scalpel. After three washes with PBSA at 200×g, 7 min and 4°C, the cell pellets were suspended in 25 mL of DMEM-F12 culture medium (VMRD Inc. Pullman, WA, USA), supplemented with gentamicin (50 µg/mL), penicillin (100 IU/mL), amphotericin (100 µg/mL), 10% fetal bovine serum (FBS), insulin (5 µg/mL), transferrin (5 µg/mL), selenium (5 ng/mL) and epidermal growth factor (5 ng/mL). This suspension was filtered through sterile gauze using a 20 mL syringe to retain pieces of mucosa and tissue fragments (Lu *et al.*, 2002; Schierack *et al.*, 2006).

Harvested cells were maintained in culture flasks and incubated for 2 h at 37°C and 5% CO₂ in a humidified atmosphere. Following incubation, the cells were centrifuged at 200×g for 10 min and resuspended in PBS with 0.1% bovine albumin (PBA), adjusting the concentration to 1×10⁶ cells/mL (Lu *et al.*, 2002; Schierack *et al.*, 2006). Cells (200 µL) were then labeled with 10 µL (0.5 µg/100 µL) primary mouse anti-pig SLA I antibody (Invitrogen-Thermo Fisher Scientific, USA) and incubated at 25°C for 30 min. After two washes with 500 µL of PBA (centrifuged at 200×g for 7 min at 4°C), cells were labeled with 10 µL (5 µg/100 µL) of secondary rabbit anti-mouse IgG conjugated to phycoerythrin (PE), followed by incubation at 25°C for 30 min and washed twice with PBA.

Once labeled, the cells were incubated separately with 100 µL of F4⁺ ETEC (2.0×10⁸ cfu/mL) in PBS-M (4% mannose, 50 mM phosphate buffer, 0.15 M NaCl, pH=7.2) for 2 h at 4°C. Mannose was used to block possible interactions of the bacteria through type 1 fimbriae, which can be expressed by *E. coli*. Cells were then washed once with PBSA and 10 µL (0.1 µg/100 µL) of biotinylated anti-F4 antibody was added, followed by incubation for 30 min 25°C. After an additional wash, 10 µL of avidin conjugated to fluorescein isothiocyanate (FITC) was added (1:5000), and the samples were incubated for 30 min at 25°C. The interaction of the bacteria with the cells was determined by flow cytometry using a FACSCanto II instrument (Becton, Dickinson and Co., USA). The data were analyzed with the FACSDiva software version 6.1.1 (Becton, Dickinson and Co., USA). As a negative control, *E. coli* 10407 ATCC 35401 cells (isolated from humans with cholera-like diarrhea) were used, whose adhesins do not recognize galactose or N-acetylglucosamine.

For the binding sites competition assay, 100 µL aliquots of F4⁺ *E. coli* (bacterial cell numbers adjusted to 2.0×10⁸ cfu/mL) were individually incubated with 1, 3 or 6 µg of PSA-Lac, PSA-GOS or PSA-Ochit for 30 min at 25°C. Subsequently, bacteria-enterocyte interactions were detected by flow cytometry according

to the procedure already described. Untreated PSA was used as a negative control.

Infection of enterocytes with F4⁺ ETEC and their pro-inflammatory response in the presence of neoglycans. The infection of the cells was carried out in sterile 96 well culture plates under a laminar flow hood. To each well, 200 µL of cell suspension were added containing 0.5×10⁶ enterocytes in an antibiotic-free DMEM-F12 medium. Plates were incubated for 24 h at 37°C in a humidified atmosphere with 5% CO₂. Enterocytes were infected by adding 50 µL of F4⁺ *E. coli* (2.0×10⁸ cfu/mL suspended in PBS-M). Sterile PBS-M and uninfected cells were used as controls and to determine the baseline reading. Sixteen observations were made in two independent experiments. To determine the effects of the presence of the synthesized neoglycans, the cells were infected as previously described and individually treated with 9 µg/mL of each neoglycan (PSA-Lac, PSA-GOS or PSA-Ochit). Untreated PSA was used as a control. After incubation for 3 h at 25°C, the culture medium was decanted, and cells were washed three times with sterile PBS to remove unbound bacteria. Antibiotic-free DMEM-F12 was then added (200 µL/well), and the enterocytes were cultured for 3 days. At the end of this incubation period, the supernatant was recovered to determine the concentrations of IL-6 and TNF-α by ELISA assays, following the procedure provided by the manufacturer (Bethyl Laboratories Inc., Montgomery, TX, USA).

The proteins from the infected cells present in the supernatant (100 µL) were immobilized onto ELISA plates for 18 h at 25°C in the presence of 10 µL of 1M carbonate buffer (pH 9.0). Plates were then washed with the carbonate buffer and blocked with 1.5% BSA for 8 h at 25°C. After two washes, the immobilized proteins were incubated for 2 h at 25°C with biotinylated antibodies specific for pig pro-inflammatory cytokines IL-6 (0.2 µg/mL) or TNF-α (0.5 µg/mL) (Bethyl Laboratories Inc., Montgomery, TX, USA), accordingly. Following the corresponding washes, samples were incubated with avidin-peroxidase (1:1000) for 40 min at 25°C. After the final wash, the presence of cytokines was revealed by adding the OPD substrate and after 10 min measuring the absorbance at 450 nm with an ELISA plate reader. Experiments were performed in triplicate.

Statistical analysis. Statistical analysis was performed using the NCSS 2000 (Statistical Analysis and Graphics, Kaysville, UT, USA). The data were expressed as means ± standard deviation (S.D.) values. The results of ELLA and the cytokines assays were subjected to analysis of variance (ANOVA), and evaluated by the Tukey-Kramer's test. Differences of *p*<0.05 were considered to be significant.

RESULTS

Characterization of neoglycans

The synthesized neoglycans were characterized by electrophoresis, intrinsic tryptophan fluorescence, and binding to lectins, as well as to the adhesins of different F4 ETEC variants.

The electrophoretic profiles of the different neoglycans were compared with untreated PSA (Fig. 1, lane 2). The migration of Lac-conjugated PSA (lane 3) was the slowest when compared to PSA and the other treatments. This indicates a greater glycation for PSA-Lac than for PSA-GOS (lane 4) and PSA-Ochit (lane 5). Analysis of molec-

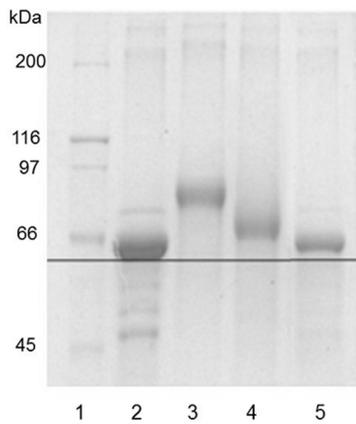


Figure 1. SDS-PAGE of PSA conjugated with various neoglycans. Samples were resolved in an 8% gel and stained with Coomassie Blue R-250. Lane 1, molecular weight standards; lane 2, untreated PSA; lane 3, PSA conjugated with lactose; lane 4, PSA conjugated with GOS; lane 5, PSA conjugated with Ochit.

Table 1. Estimation of mass values for the Molecular Imager Gel Doc XR+ obtained from untreated and conjugated PSA

Treatment	Molecular mass (kDa)
Untreated PSA	63.0±0.6
PSA-Lac, 100 °C, pH 7.0	86.0±5.0
PSA-GOS 90°C, pH, 9.0	71.0±2.7
PSA-Ochit 60 °C/100 °C, pH 8.0	64.0±0.4

PSA, Porcine serum albumin; Lac, Lactose; GOS, galactooligosaccharides Ochit, Chitin oligosaccharides. Gels were performed in triplicate.

ular weights by the ImageLab software (Table 1) indicated the greatest conjugation with Lac, followed by GOS and Ochit, respectively. The number of molecules added to PSA, as estimated from the ImageLab software, was 63 for Lac, 13 for GOS, and 2 for Ochit.

Intrinsic tryptophan fluorescence of PSA agreed with the electrophoresis results (Fig. 2). The greatest quenching of the fluorescence signal was observed when PSA was conjugated with Lac, followed by conjugations with GOS and Ochit, respectively.

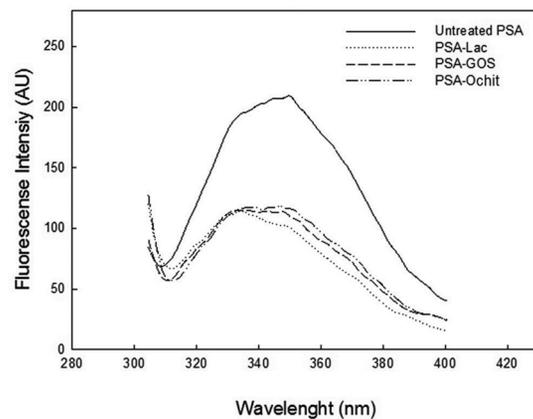


Figure 2. Intrinsic tryptophan fluorescence spectra of untreated and conjugated PSA.

The excitation wavelength was 295 nm, and emission was scanned from 300 to 400 nm. PSA samples were glycosylated with lactose (PSA-Lac; 100°C, 30 min, pH 7.2), galacto-oligosaccharides (PSA-GOS; 90°C; 90°C, 30 min, pH 9.0), or chitin oligosaccharides (PSA-Ochit; 60°C, 48 h, pH 8.0).

To complement the Maillard neoglycans characterization, biological recognition tests were carried out with plant lectins and F4⁺ *E. coli*. Lectin RCA 1 interaction was stronger for PSA-Lac than for PSA-GOS; in contrast, WGA lectin recognized neoglycans of PSA-Ochit (Fig. 3A). The recognition of the synthesized neoglycans by adhesins from F4⁺ ETEC (isolated from Mexican piglets) was compared with that of the three existing F4⁺ ETEC variants, ab, ac, and ad (Fig. 3B). All of the adhesins recognized the neoglycans, although the interaction differed ($p < 0.05$) depending on the type of carbohydrate conjugated to PSA. In general, the degree of interaction was PSA-Ochit > PSA-Lac > PSA-GOS.

Inhibition of adhesion of F4⁺ ETEC to piglet enterocytes

To evaluate F4⁺ ETEC-enterocytes inhibition by flow cytometry, enterocytes were labeled with a PE-conjugated anti-SLA 1 antibody, while bacteria were labeled with a FITC-conjugated antibody. Assays were conducted individually before and after incubation of bacteria with PSA-Lac, PSA-GOS, or PSA-Ochit.

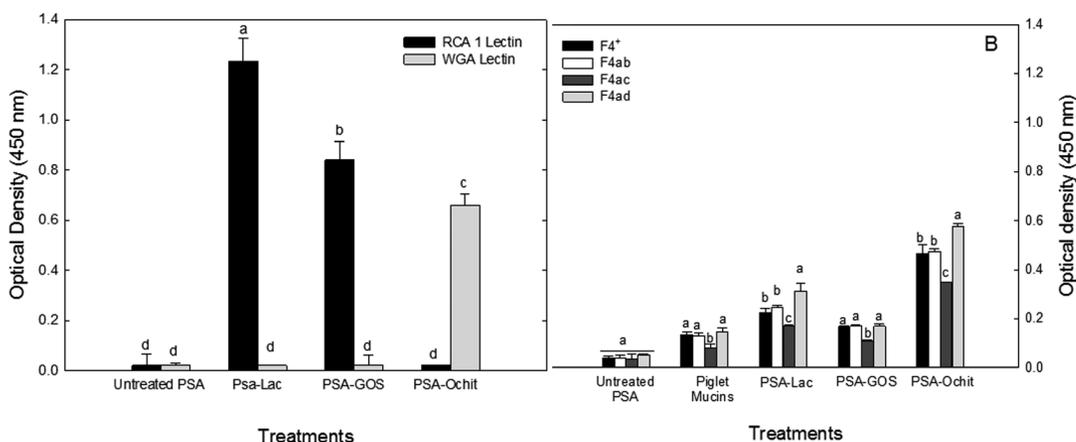


Figure 3. Enzyme-linked recognition assay for PSA neoglycans by lectins and F4 ETEC adhesins.

PSA samples were glycosylated with lactose (PSA-Lac; 100°C, 30 min, pH 7.2), galacto-oligosaccharides (PSA-GOS; 90°C, 30 min, pH 9.0), or chitin oligosaccharides (PSA-Ochit; 60°C, 48 h, pH 8.0). (A) *Ricinus communis* (RCA1) lectin and Wheat germ agglutinin (WGA) lectin; (B) F4 ETEC phenotypes. Different letters indicate significant differences ($p < 0.05$) within the same treatment group.

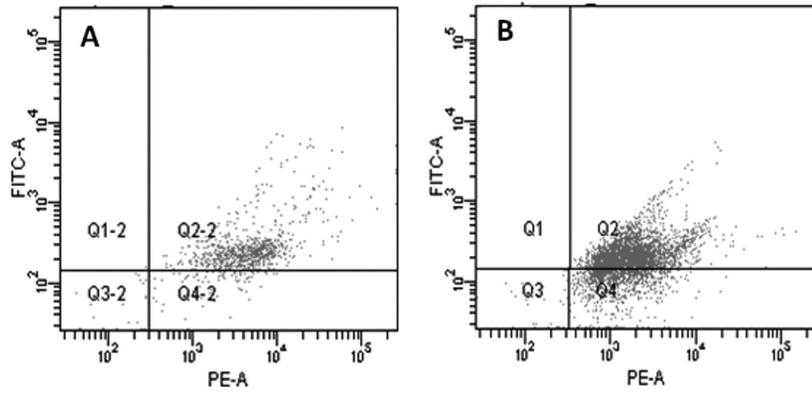


Figure 4. Representative flow cytometry plots of F4+ ETEC-piglet enterocyte adhesion; (A) SLA 1 labeled enterocytes recognized by F4+ ETEC; (B) SLA 1 labeled enterocytes recognized by F4+ ETEC in the presence of porcine albumin conjugated with lactose (PSA-Lac).

The binding of F4+ ETEC to enterocytes was characterized by gating cells that were positive for both PE and FITC. A representative example of piglet enterocyte recognition by bacteria for one experiment is shown in Fig. 4A. In the inhibition experiments, the population of enterocytes that interacted with F4+ *E. coli* was considered to be 100%. Figure 4B shows an example of the cytograms obtained.

Figure 5 shows the results of the bacterium-enterocyte interaction inhibition assays. The ability of neoglycans to compete with enterocyte receptors for recognition by F4+ ETEC depended on the animal from which the

cells were extracted and the type of neoglycan incubated with the bacteria. In general, the PSA-Lac and PSA-GOS conjugates were stronger inhibitors of the bacteria than PSA-Ochit, indicating they mimicked the receptors expressed on piglet enterocytes.

Infection of enterocytes with F4+ ETEC and their pro-inflammatory response in the presence of neoglycans

Infection of enterocytes with F4+ ETEC significantly induced the production of pro-inflammatory cytokines IL-6 and TNF- α (Fig. 6) as compared to non-infected

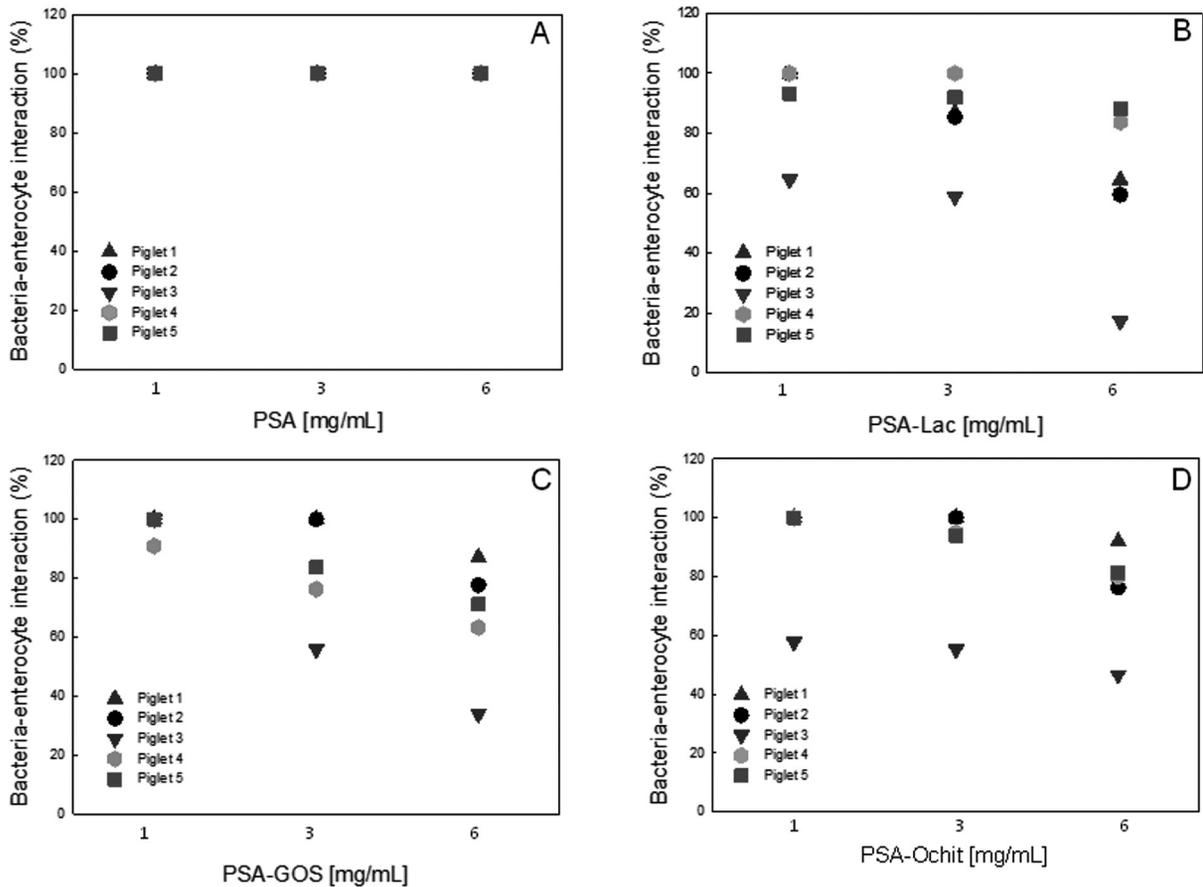


Figure 5. *In vitro* inhibition assay of the F4+ ETEC adhesion to piglet enterocytes by PSA-conjugated neoglycans. Different symbols represent enterocytes isolated from five healthy two-week-old piglets. Bacteria were individually pre-incubated with PSA (A); PSA-Lac (B); PSA-GOS (C) or PSA-Ochit (D), before the interaction with piglet enterocytes.

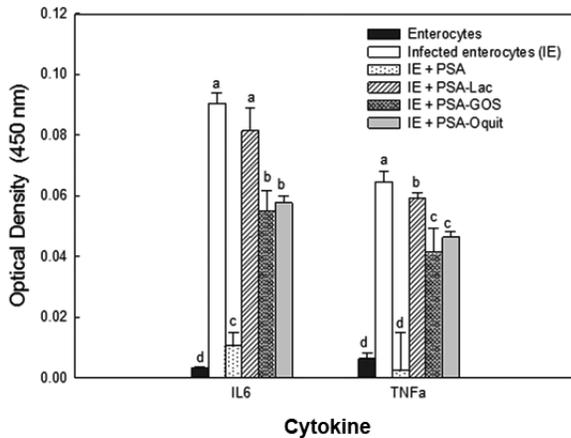


Figure 6. Effect of PSA conjugated neoglycans in the response of piglet enterocytes to a challenge with F4⁺ ETEC. (A) IL 6; (B) TNF- α . Different letters indicate significant differences ($p < 0.05$) within the same treatment group.

cells. Stimulation of IL-6 synthesis was diminished in the presence of all neoglycans. However, PSA-GOS and PSA-Ochit were more effective in reducing IL-6 production than PSA-Lac (Fig. 6). Significant reduction of TNF- α also was observed in the presence of PSA-GOS and PSA-Ochit, while no effect was detected when bacteria were in contact with PSA-Lac (Fig. 6).

DISCUSSION

Through evolution, F4 ETEC have developed the ability to target surface glycan structures on piglet enterocytes and thereby initiate infection. As such, glycans that mimic these structures and interactions present attractive drug alternatives for diarrhea treatments. In this study, neoglycans synthesized by a controlled Maillard reaction were examined for their capacity to prevent *in vitro* binding of enterotoxigenic F4 ETEC to piglet enterocytes. The extent on glycation of the synthesized neoglycans was estimated considering the migration patterns of glycated PSA with respect to untreated PSA. The number of glycan molecules coupled to PSA was projected considering the mass of lactose, or the masses obtained for GOS and Ochit under similar conditions (Ledesma-Osuna *et al.*, 2010; Sarabia-Sainz *et al.*, 2013). Analysis by Image Lab software suggested PSA binding of 63, 13 and 2 molecules of Lac, GOS and Ochit, respectively. These differences could be due to the higher reactivity of Lac (a disaccharide) compared with that of tri- or tetrasaccharides (Ledesma-Osuna *et al.*, 2010). All neoglycans showed quenching of tryptophan fluorescence consistent with the degree of glycation estimated by SDS-PAGE. PSA has three tryptophan residues (Trp) in its primary sequence that fluoresce when excited. A quenching of the fluorescence emitted by this amino acid is an indication that the protein has been modified (Wooster & Augustin., 2006).

The RCA 1 lectin interacted readily with PSA-Lac and less with PSA-GOS. This is consistent with the specificity of RCA 1 that shows stronger recognition for structures containing lactosamine (β -D-Gal-[1 \rightarrow 4]-D-GlcNAc) and lactose (β -D-Gal-[1 \rightarrow 4]-D-Glc) (Wu *et al.*, 2006), than those of GOS where 2 to 5 galactose residues are attached to glucose (for example, Gal [β 1 \rightarrow 4] Gal [β 1 \rightarrow 4] Glc or Gal [β 1 \rightarrow 6] Gal [β 1 \rightarrow 4] Glc) (Torres *et al.*, 2010). As expected, no interaction was ob-

served with the RCA 1 lectin and PSA-Ochit because its structure does not contain galactose. In contrast, the WGA lectin recognized neoglycans in PSA-Ochit which is indicative of the attachment of N-acetylglucosamine molecules to PSA.

Adhesion of ETEC to intestine cells results from interactions with different lectin-like adhesins that are located at the tip of the fimbriae of this bacterium. The F4 (K88) fimbria are the most common in ETEC strains that infect pigs (Grange *et al.*, 2002). There are three F4 fimbria variants (ab, ac and ad) that differ in the primary sequence of the major fimbrial subunit (FaeG) that has lectin-like activity (Moonens *et al.*, 2015). Due to these differences, the carbohydrate specificity varies somewhat among F4 ab, ac and ad (Zhang *et al.*, 2009). Several glycolipids and glycoproteins have been identified in piglet intestine (in mucus, intestinal mucosa or enterocytes) that could serve as possible receptors for FaeG F4.

In this study, the strongest recognition was observed for PSA-Ochit showing that the different F4 variants recognize N-acetylglucosamine (GlcNAc) in the terminal position. Grange and coworkers (2002) also proved that GlcNAc recognition occurs when this carbohydrate is located in the penultimate position in the lactosamine-type structures (β -D-Gal-[1 \rightarrow 4]-D-GlcNAc). Other researchers have also demonstrated that FaeG recognizes galactosylated structures. Particularly, greater affinity is observed when galactose is located in the terminal position and is attached to the rest of the molecule by a β bond (β -D-Gal) (Payne *et al.*, 1993; Grange *et al.*, 1999). These observations also could explain why PSA-Lac neoglycans were recognized by F4⁺ ETEC with greater affinity than PSA-GOS neoglycans. This is supported by work by Moonens and coworkers (2015) who showed that Lac strongly interacts with FaeG F4ad. The F4⁺ *E. coli* isolated from Mexican piglets exhibited a recognition pattern similar to F4ab *E. coli*, which could indicate that the FaeG subunits share similar sequences.

In vitro binding of enterotoxigenic F4⁺ *E. coli* (ETEC) to piglet enterocytes was partially blocked by neoglycans in a dose-dependent manner. The control levels of bacterial adhesion when pre-incubated with 6 mg/mL of neoglycans were 12–82%, 12–70% and 10–50% for PSA-Lac, PSA-GOS and PSA-Ochit, respectively (Fig. 5). However, a high heterogeneity was observed among enterocytes from different piglets. These observations suggest differences in susceptibility to F4⁺ ETEC infection in different animals (Moonens *et al.*, 2015). Piglets are classified into six different phenotypes (A to F) depending on the ability of the different F4 variants (ab, ad and ac) to bind their enterocytes. All three variants can bind to phenotype A enterocytes, while none can bind to phenotype E cells. With the remaining phenotypes, differences are seen in the binding of one or two variants (Zhang *et al.*, 2009).

In general, the PSA-Lac and PSA-GOS conjugates competed more successfully than PSA-Ochit for recognition by the bacterium, better mimicking the receptors expressed on piglet enterocytes. This result is contrary to what was observed in the adhesion assays by ELLA, where F4⁺ *E. coli* recognized structures with N-acetylglucosamine (PSA-Ochit) with a greater affinity than the galactosylated structures (PSA-Lac and PSA-GOS). Possibly, the greater effect shown by PSA-Lac and PSA-GOS could be due to greater glycation when compared with PSA-Ochit (as shown with the fluorescence and SDS-PAGE assays). The greater the number of exposed carbohydrate molecules in the PSA conjugates, the greater the possibility of interaction with various bacteria at the

same time, thus preventing their adherence to enterocytes (Sharon, 2006). As seen in Fig. 5, only a partial inhibition of adhesion was achieved with the neoglycans synthesized in this study. To enhance this effect, the amounts of neoglycans could be increased. Glycation could be also improved by varying the reaction conditions used in this study (pH, temperature, Aw, time of reaction) to optimize the number of carbohydrate molecules conjugated to PSA.

Infection of enterocytes stimulates the production of IL-6 and TNF- α (Fig. 6). The F4 fimbriae are very potent antigens that induce strong mucosal and systemic immune responses when orally administered to pigs (Verdonck *et al.*, 2004). Hermes and coworkers (2011), observed a statistically significant increase in the production of TLR-5, IL-1, IL-8 and TNF- α when F4 fimbriae interacted with intestinal cell lines. In contrast, Devriendt and coworkers (2010) observed an increase in the concentrations of IL-8 and IL-6 after orally immunizing pigs susceptible to F4+ ETEC. The strong immune response elicited by the F4 fimbriae is attributed to its high degree of polymerization that apparently influences binding of the FaeG F4 subunit to its receptors and confers resistance to the gastric pH and enzymatic attack (Verdonck *et al.*, 2008; Devriendt *et al.*, 2010). In fact, the immune response is significantly reduced when pigs are immunized with depolymerized F4 fimbriae (Verdonck *et al.*, 2008). It has been also observed that the increased production of pro-inflammatory cytokines could be mediated by TLR-5 expressed after detecting flagellin virulence factor present in F4+ ETEC.

Stimulation of IL-6 synthesis was diminished in the presence of all neoglycans (Fig. 6). These results could be related to the blocking of bacterial binding to enterocytes mediated by neoglycans via competitive exclusion (Hermes *et al.*, 2011). However, the flow cytometry inhibition assays showed that PSA-Lac and PSA-GOS were more effective in blocking the binding of the bacteria to the enterocytes, whereas PSA-GOS and PSA-Ochit were more effective in reducing IL-6 production. These differences could be also explained by the abilities of different glycans to modulate the immune response. GOS modulates the immune systems of humans and horses, decreasing the production of pro-inflammatory cytokines, such as IL-6 and TNF- α (Vulevic *et al.*, 2008; Vendrig *et al.*, 2014). In the case of N-acetylglucosamine, it has been observed that chitin and its oligosaccharides reduce the inflammation induced by the lipopolysaccharide (LPS) of Gram negative bacteria (Wagener *et al.*, 2014). In contrast, Chun *et al.* (2016), showed that Lac glyated to milk proteins induces the release of IL-6. Thus, more studies are needed to understand the immunomodulatory effects of these neoglycans on piglet intestinal cells.

CONCLUSIONS

Neoglycans synthesized by low cost controlled Maillard reaction are a promising alternative for the treatment and prophylaxis of diarrhea in piglets.

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REFERENCES

- Chun SH, Lee HA, Lee KB, Kim SH, Park KY, Lee KW (2016) Effects of glycated whey protein concentrate on proinflammatory cytokine expression and phagocytic activity in RAW264.7 macrophages. *Biol Pharm Bull* **39**: 199–206. <http://doi.org/10.1248/bpb.15-00596>
- Devriendt B, Stuyven E, Verdonck F, Goddeeris BM, Cox E (2010) Enterotoxigenic *Escherichia coli* (K88) induce proinflammatory responses in porcine intestinal epithelial cells. *Dev Comp Immunol* **34**: 1175–1182. <http://doi.org/10.1016/j.dci.2010.06.009>
- Dubreuil JD, Isaacson RE, Schifferli DM (2016) Animal enterotoxigenic *Escherichia coli*. *EcoSal Plus* **7**: 10.1128/ecosalplus.ESP-0006-2016. <http://doi.org/10.1128/ecosalplus.ESP-0006-2016>
- Gonzalez-Ortiz G, Hermes RG, Jimenez-Diaz R, Perez JF, Martin-Orue SM (2013) Screening of extracts from natural feed ingredients for their ability to reduce enterotoxigenic *Escherichia coli* (ETEC) K88 adhesion to porcine intestinal epithelial cell-line IPEC-J2. *Vet Microbiol* **167**: 494–499. <http://doi.org/10.1016/j.vetmic.2013.07.035>
- Grange PA, Erickson AK, Lavery SB, Francis DH (1999) Identification of an intestinal neutral glycosphingolipid as a phenotype-specific receptor for the K88ad fimbrial adhesin of *Escherichia coli*. *Infect Immun* **67**: 165–172
- Grange PA, Mouricout MA, Lavery SB, Francis DH, Erickson AK (2002) Evaluation of receptor binding specificity of *Escherichia coli* K88 (F4) fimbrial adhesin variants using porcine serum transferrin and glycosphingolipids as model receptors. *Infect Immun* **70**: 2336–2343
- Hermes RG, Manzanilla EG, Martin-Orue SM, Perez JF, Klasing KC (2011) Influence of dietary ingredients on *in vitro* inflammatory response of intestinal porcine epithelial cells challenged by an enterotoxigenic *Escherichia coli* (K88). *Comp Immunol Microbiol Infect Dis* **34**: 479–488. <http://doi.org/10.1016/j.cimid.2011.08.006>
- Ledesma-Osuna AI, Ramos-Clamont G, Vazquez-Moreno L (2008) Characterization of bovine serum albumin glycated with glucose, galactose, and lactose. *Acta Biochim Pol* **55**: 491–497
- Ledesma-Osuna AI, Ramos-Clamont G, Guzman-Partida AM, Vazquez-Moreno L (2010) Conjugates of bovine serum albumin with chitin oligosaccharides prepared through the Maillard reaction. *J Agric Food Chem* **58**: 12000–12005. <http://doi.org/10.1021/jf102869f>
- Lu S, Yao Y, Meng S, Cheng X, Black DD (2002) Overexpression of apolipoprotein A-IV enhances lipid transport in newborn swine intestinal epithelial cells. *J Biol Chem* **277**: 31929–31937. <http://doi.org/10.1074/jbc.M201418200>
- Moonens K, Van den Broeck I, De Kerpel M, Deboeck F, Raymaekers H, Remaut H, De Greve H (2015) Structural and functional insight into the carbohydrate receptor binding of F4 fimbriae-producing enterotoxigenic *Escherichia coli*. *J Biol Chem* **290**: 8409–8419. <http://doi.org/10.1074/jbc.M114.618595>
- Nagy B, Fekete PZ (2005) Enterotoxigenic *Escherichia coli* in veterinary medicine. *Int J Med Microbiol* **295**: 443–454. <http://doi.org/10.1016/j.ijmm.2005.07.003>
- Nishibata T, Tashiro K, Kanahori S, Hashizume C, Kitagawa M, Okuma K, Gordon DT (2009) Comprehensive measurement of total non-digestible carbohydrates in foods by enzymatic-gravimetric method and liquid chromatography. *J Agric Food Chem* **57**: 7659–7665. <http://doi.org/10.1021/jf9101056>
- Payne D, O'Reilly M, Williamson D (1993) The K88 fimbrial adhesin of enterotoxigenic *Escherichia coli* binds to beta 1-linked galactosyl residues in glycosphingolipids. *Infect Immun* **61**: 3673–3677
- Qadri F, Svennerholm AM, Faruque AS, Sack RB (2005) Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clin Microbiol Rev* **18**: 465–483. <http://doi.org/10.1128/CMR.18.3.465-483.2005>
- Sarabia-Sainz A-i, Ramos-Clamont G, Winzerling J, Vazquez-Moreno L (2011) Bacterial recognition of thermal glycation products derived from porcine serum albumin with lactose. *Acta Biochim Pol* **58**: 95–100
- Sarabia-Sainz HM, Armenta-Ruiz C, Sarabia-Sainz JA-i, Guzmán-Partida AM, Ledesma-Osuna AI, Vázquez-Moreno L, Ramos-Clamont Montfort G (2013) Adhesion of enterotoxigenic *Escherichia coli* strains to neoglycans synthesized with prebiotic galactooligosaccharides. *Food Chemistry* **141**: 2727–2734. <http://dx.doi.org/10.1016/j.foodchem.2013.05.040>
- Sattin S, Bernardi A (2016) Glycoconjugates and glycomimetics as microbial anti-adhesives. *Trends Biotechnol* **34**: 483–495. <http://doi.org/10.1016/j.tibtech.2016.01.004>
- Schierack P, Nordhoff M, Pollmann M, Weyrauch KD, Amasheh S, Lodemann U, Jores J, Tachu B, Kleta S, Blikslager A, Tedin K, Wieler LH (2006) Characterization of a porcine intestinal epithelial cell line for *in vitro* studies of microbial pathogenesis in swine. *Histochem Cell Biol* **125**: 293–305. <http://doi.org/10.1007/s00418-005-0067-z>

- Sharon N (2006) Carbohydrates as future anti-adhesion drugs for infectious diseases. *Biochim Biophys Acta* **1760**: 527–537. <http://doi.org/10.1016/j.bbagen.2005.12.008>
- Torres DPM, Gonçalves MdPF, Teixeira JA, Rodrigues LR (2010) Galacto-oligosaccharides: Production, properties, applications, and significance as prebiotics. *Comp Rev Food Sci Food Safety* **9**: 438–454. <http://doi.org/10.1111/j.1541-4337.2010.00119.x>
- Vendrig JC, Coffeng LE, Fink-Gremmels J (2014) Effects of orally administered galacto-oligosaccharides on immunological parameters in foals: a pilot study. *BMC Vet Res* **10**: 278. <http://doi.org/10.1186/s12917-014-0278-4>
- Verdonck F, Cox E, Vancaeneghem S, Goddeeris BM (2004) The interaction of F4 fimbriae with porcine enterocytes as analyzed by surface plasmon resonance. *FEMS Immunol Med Microbiol* **41**: 243–248. <http://doi.org/10.1016/j.femsim.2004.03.009>
- Verdonck F, Joensuu JJ, Stuyven E, De Meyer J, Muilu M, Pirhonen M, Goddeeris BM, Mast J, Niklander-Teeri V, Cox E (2008) The polymeric stability of the *Escherichia coli* F4 (K88) fimbriae enhances its mucosal immunogenicity following oral immunization. *Vaccine* **26**: 5728–5735. <http://doi.org/10.1016/j.vaccine.2008.08.017>
- Vulevic J, Drakoularakou A, Yaqoob P, Tzortzis G, Gibson GR (2008) Modulation of the fecal microflora profile and immune function by a novel trans-galactooligosaccharides mixture (B-GOS) in healthy elderly volunteers. *Am J Clin Nutr* **88**: 1438–1446
- Wagener J, Malireddi RK, Lenardon MD, Koberle M, Vautier S, MacCallum DM, Biedermann T, Schaller M, Netea MG, Kanneganti TD, Brown GD, Brown AJ, Gow NA (2014) Fungal chitin dampens inflammation through IL-10 induction mediated by NOD2 and TLR9 activation. *PLoS Pathog* **10**: e1004050. <http://doi.org/10.1371/journal.ppat.1004050>
- Watts RE, Tan CK, Ulett GC, Carey AJ, Totsika M, Idris A, Paton AW, Morona R, Paton JC, Schembri MA (2012) *Escherichia coli* 83972 expressing a P fimbriae oligosaccharide receptor mimic impairs adhesion of uropathogenic *E. coli*. *J Infect Dis* **206**: 1242–1249. <http://doi.org/10.1093/infdis/jis493>
- Westwood ME, Thornalley PJ (1995) Molecular characteristics of methylglyoxal-modified bovine and human serum albumins. Comparison with glucose-derived advanced glycation end product-modified serum albumins. *J Protein Chem* **14**: 359–372
- Wooster TJ, Augustin MA (2006) Beta-lactoglobulin-dextran Maillard conjugates: their effect on interfacial thickness and emulsion stability. *J Colloid Interface Sci* **303**: 564–572. <http://doi.org/10.1016/j.jcis.2006.07.081>
- Wu AM, Wu JH, Singh T, Lai LJ, Yang Z, Herp A (2006) Recognition factors of *Ricinus communis* agglutinin 1 (RCA1). *Mol Immunol* **43**: 1700–1715. <http://doi.org/10.1016/j.molimm.2005.09.008>
- Zhang W, Fang Y, Francis DH (2009) Characterization of the binding specificity of K88ac and K88ad fimbriae of enterotoxigenic *Escherichia coli* by constructing K88ac/K88ad chimeric FaeG major subunits. *Infect Immun* **77**: 699–706. <http://doi.org/10.1128/1AI.01165-08>