Supplemental Information

METHODS

Human milk oligosaccharide (HMO) preparation

HMO were isolated from pooled human milk as previously described [1]. Milk was obtained and pooled from more than 40 different donors, recruited at the UCSD Medical Center, San Diego, CA (approved by the University's Institutional Review Board). The lipid layer was removed after centrifugation, and proteins were precipitated from the aqueous phase by addition of ice-cold ethanol and subsequent centrifugation. Afterwards, ethanol was removed by roto-evaporation. Lactose and salts were removed by gel filtration over a BioRad P2 column (100 cm x 16 mm, Bio-Rad, Hercules, CA) using semi-automated Fast Protein Liquid Chromatography (FPLC). Endotoxins were removed by affinity chromatography over polymyxin B gravity columns (Thermo Scientific, Rockford, IL). These pooled HMO were further separated into neural (non-sialylated) HMO and acidic (sialylated) HMO by anion-exchange chromatography over a QAE Sephadex A25 gravity column (Sigma) followed by desalting over the BioRad P2 column. Galactooligosaccharides (GOS, Vivinal®, dry matter 75%) were kindly provided by Friesland Campina Domo, The Netherlands, and subjected to gel filtration over the BioRad P2 column to remove monosaccharides, lactose and salts. 3'sialyllactose (3'SL) was purchased from Dextra, Reading, UK.

Adherence and Invasion Assay

HTB-9 bladder epithelial cells were seeded at ~1 x 10^5 cells/mL in 24-well plates two or three days prior to HMO pre-treatment. After 16 h of HMO incubation, confluent cells were infected with UPEC from a fresh overnight standing culture at a multiplicity of infection (MOI) = 20-50. Briefly, UPEC were diluted in LB media to OD_{600} of ~0.5 prior to infection. Plates were centrifuged at 600x*g* for 5 min to facilitate bacterial contact with the host cell monolayer. The bacteria were then allowed to establish attachment on monolayers for 2 h in the presence of HMO. To assay adherence, cells were washed 2 times with phosphate-buffered saline (PBS) to remove unbound bacteria. One mL of 0.5% Triton X-100 in PBS was added to each well for 5 min and cells lysed by trituration. Bacteria were immediately serially diluted and plated onto LB agar at 37ºC overnight to enumerate colony-forming units (CFU) representing total cell associate bacteria. For invasion, an antibiotic protection assay was employed. RPMI supplemented with 100 µg/mL of gentamicin (without HMO) was added to PBS-washed cells 2 h post-infection. Cells were incubated with gentamicin for an additional 2 h at 37°C prior to Triton X-100 treatment for CFU enumeration as above. The average percentage adherence and invasion were calculated by dividing bacteria at the 2 h adhesion or 2 h post-gentamicin time point(s) by the average of initial inoculum, respectively.

LIVE/DEAD Staining

Confluent HTB-9 monolayers were grown on sterile no. 1 round coverslips (Thermo Scientific) in 24-well or 6-well plates. Cells were infected with WT UPEC

at a MOI of 5-20 for 2 h followed by 2 h of gentamicin. Cells were washed with PBS three times before being treated with the viability assay mixture from the LIVE/DEAD Viability/Cytotoxicity Kit *for mammalian cells* (Molecular Probes, Invitrogen) for 30 minutes at 37ºC before being mounted on glass slides for visualization and imaging using an Olympus BX51 fluorescent microscope fitted with appropriate filters. The assay mixture consisted of 2 mM of an ethidium homodimer solution, a cell impermeable solution that stains the nuclei of dead or dying cells (visualized through a bright red fluorescence), and 2 mM calcein AM solution, a cell permeable solution that stains live cells (visualized through by uniform green fluorescence). Numbers of live (green) and dead (red) cells were enumerated in multiple frames (n=3-5) from each sample under 10X magnification. Experiments were performed in triplicate and repeated three times.

Cell Lysate Preparation and Western blotting

HTB-9 cells were grown in 24 well plates and pre-treated with HMO or different oligosaccharide derivatives in serum-free RPMI prior to infection as previously described. After 2 h of infection, cells were washed with PBS 2-3 times to remove any unbound bacteria followed by treatment with serum-free RPMI supplemented with 100μg/mL of gentamicin for additional 2 h. At the end of treatment, cells were washed twice with PBS and lysed using RIPA lysis buffer (150mM NaCl, 50mM Tris, pH 7.4, 5mM EDTA, 1% Nonidet P-40, 1% deoxcholic acid supplemented with protease inhibitor (Roche Applied Science). BCA assay

(Thermo scientific) was used to determine the protein concentration of each sample. Western blots were performed as previous described [2]. Briefly, equal amounts of total protein was loaded and separated on 10% or 6% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Bio-Rad Laboratories). Membranes were blocked with 5% non-fat milk and washed in Tris-buffered saline with 0.1% Tween-20 (TBST) 2 times for 5 min each. Primary antibodies used for immunoblotting were as follows: Mouse anti-phospho-p38 MAPK (Thr180/Tyr182), mouse anti-phospho-Erk1/2 (Thr202/Tyr204), mouse anti-phospho-p65 (Ser536) (93H1) were used at 1:1000 dilution in 5% BSA/TBST (Cell Signaling Technology). The following antibodies were diluted in TBST: Mouse anti-E-cadherin, used at 1:5000, mouse anti-β1-integrin (CD29) (BD Bioscience), used at 1:5000, mouse anti-desmocollin 2/3 (Invitrogen), used at 3μg/mL, mouse- anti-AIF (Cell Signaling Technology) used at 1:1000, mouse anti-cytochrome C (BD Biosciences), used at 1:1000, and mouse anti- caspase 3 (Cell Signaling Technology), used at 1:1000. β-actin (Sigma Aldrich) was used as a loading control (1:10,000). After overnight incubation at 4°C, membranes were washed and treated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Cell signaling Technology) for 1 hour at room temperature. Signals were detected by enhanced chemiluminescence (Perkin Elmer) and exposed on Kodak BioMax light film.

Cell Infections and Desmosome Immunolocalization

HTB-9 cells were grown to ~90% confluency on sterile 20 x 20 inch glass coverslips (Thermo Scientific) in 6-well plates. Untreated or treated cells were infected with UPEC at an MOI of ~1-3 as described above. After 2 h of infection, cells were washed with PBS 2-3 times to remove any unbound bacteria followed by treatment with serum-free RPMI supplemented with 100μg/mL of gentamicin for additional 2 h [3]. At the end of the treatment, cells were washed 3 times with PBS to remove any unbound bacteria, fixed for 15 min with 3% paraformaldehyde (150 mM NaCl, 5 mM KCl, 0.8 mM KH_2PO_4, 3.2 mM Na_2HPO_4, pH 7.3), washed three times with PBS, and incubated for 5 min at room temperature with 0.2% triton X-100 in PBS for permeabilization [2]. These cells were blocked with 5% normal goat serum (NGS) in TPBS/BSA for 20 min followed by treatment with mouse anti-Desmocollin 2/3 antibody (Invitrogen) at 50 μg/mL in 1% NGS in TPBS/BSA. Samples were visualized and imaged under 60x objective lens using an Olympus BX51 microscope fitted with the appropriate filter for detecting fluorescence images.

Proliferation Assay

Cell viability was detected using the cell proliferation reagent WST-1 (Roche) following the manufacturer's protocol. Briefly, 100μL of HTB-9 cells were cultured in serum-free RPMI in a 96 well plate at semi-confluency one day prior to HMO or GOS pre-treatment. Untreated and treated cells were infected with UPEC; at the indicated time point, 10 μL of WST-1 reagent was added to each well, gently shaken and incubated at 37°C for 15-30 min before reading on a

spectrophotometer at 450nm (Molecular Devices VERSA Max microplate reader). All experiments were performed in multiple replicates (n=3). Proliferation data are shown as a percentage of uninfected cells.

Caspase Activity Assay

Caspase-dependent apoptosis was determined using the APO-Caspase 3/7 activity assay (Promega) following the manufacturer's protocol. Briefly, 100μL of HTB-9 cells were cultured in a 96 well plate at semi-confluency one day prior to HMO treatment. After 16 h of HMO incubation, cells were infected with UPEC as described before. To quantify caspase activity, 100 μL of detecting reagent was added per well and gently shake for an hour. The plate was subsequently read at 520 nm using a fluorescent plate reader (Molecular Devices SpectraMax M3) to detect level of caspase 3/7.

Statistical Analysis

Each experiment was independently repeated at least three times. Statistical differences and standard error (S.E.M.) between two groups were analyzed using a student's two-tailed *t*-test. For comparison between multiple samples, statistical analysis was performed using ANOVA's one-way non-parametric test followed by Dunnet's multiple comparison post-test or Tukey's test. P values < 0.05 (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) were considered statistically significant (GraphPad Prism software; version 4.0B). All experiments were performed in triplicates and repeated at least three times unless stated otherwise.

FIGURE LEGEND

Figure S1. (**A**) Time and dosage-dependent effect of HMO on UPEC uptake into HTB-9 cells. To confirm HMO concentration to achieve the optimal effect, cells were pre-treated with 5mg, 10mg, 15mg HMO for 16 h. To assess the optimal duration for HMO pre-treatment, confluent HTB-9 cells were pre-treated with 15mg of HMO for 4, 8 or 16 h. Level of bacteria uptake is measured after 2h infection at MOI~20 followed by 2 h treatment with 100μg/mL gentamicin. Error bar= S.E.M. (n=3), N.S.= non-significant. **P<0.01, one- way ANOVA followed by Dunnet's post-test. (**B**) Level of apoptosis in HTB-9 cells treated with UPEC supernatant and heat-killed UPEC. Cells were treated for 4 hours with UPEC supernatant from overnight culture. Live cells (green) were detected by Calcein-AM and dead cells (red) were detected by Ethidium-homodimer from the LIVE/DEAD detection for mammalian cell kit using the manufacturer's protocol.

Figure S2. Level of viability in UPEC infected HTB-9 cells with or without prior HMO treatment. (**A**) Semi-confluent HTB-9 cells on coverslips were treated with HMO (or remain untreated) for 16 hours prior to infection with UPEC at MOI~15-20 for 2 hour (the adherence time point, top). Cells were washed twice and treated with 100 µg/mL of gentamicin for an additional 2 hours (the invasion time point, bottom). At the end of assay, cells were treated with Calcein-AM and ethidium homodimer to detect live and dead cells, respectively. Numbers of live (black bar), dead (blank bar) and total cells (gray bar) were counted per field of view. (**B**) HMO pre-treatment of HTB-9 cells at 5, 10 and 15 mg/mL prior to

UPEC infection shows HMO prevent cytotoxicity in a dose-dependent manner. Counts obtained from the post-gentamicin time point (2 h UPEC infection with 2 hour gentamicin treatment). The illustrated values (Y-axis) are obtained from the average of cell counts per field from multiple fields of view (n>3). The experiment was performed in duplicates and repeated 3 times. *P< 0.05, **P<0.01, ***P <0.001 as determined by one-way ANOVA non-parametric test followed by Dunnet's post-test.

Figure S3. Immunoblots illustrating the abundance of apoptosis-associated proteins and focal adhesion complex molecules in UPEC-infected cells. Whole cell lysates from HTB-9 cells that are untreated and uninfected (UT), untreated and HMO pre-treated followed by UPEC infection were collected and run on SDS-PAGE. Blots were probed for (**A**) focal adhesion kinase (FAK) (125kDa) and (**B**) vinculin (30kDa). Actin was used as loading control. (**C**) Caspase 3 protein abundance was detected by immunoblot using antibody detecting full-length caspase 3 (35kDa) and cleaved caspase 3 (15 and 17kDa). Caspase 3 cleavage represents increased caspase activation (**D**) Caspase 3 activity was assessed using the Capsase 3/7 detection assay as described in materials and methods. (**E, F**) Immunoblot analysis detecting the abundance of apoptotic inducing factor (AIF) (**E**) and cytochrome C (**F**) in untreated and UPEC-infected cells with or without HMO pre-treatment. Actin was used as loading control.

REFERENCE

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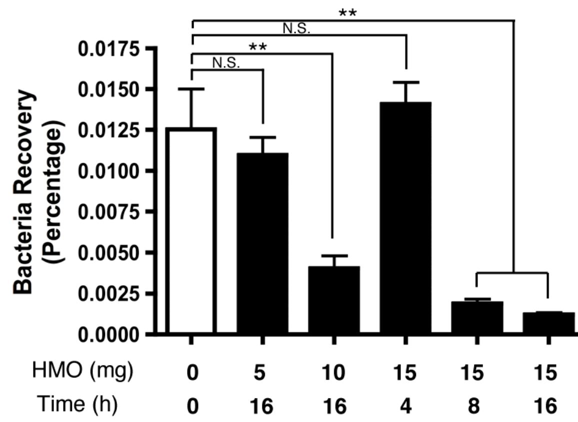
2. Lin AE, Benmerah A, Guttman JA. Eps15 and Epsin1 are crucial for enteropathogenic Escherichia coli pedestal formation despite the absence of adaptor protein 2. J Infect Dis **2011**; 204:695-703.
3. Martinez JJ, Mulvey MA, Schilling JD, Pinkner JS, Hultgren SJ. Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. EMBO J **2000**; 19:2803-12.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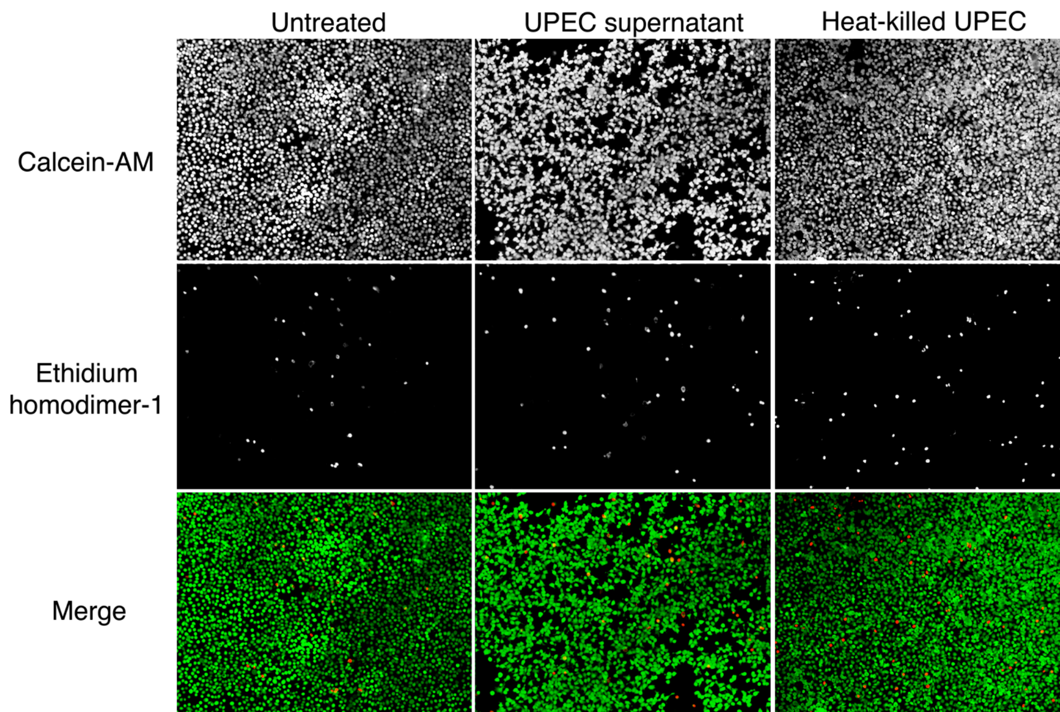
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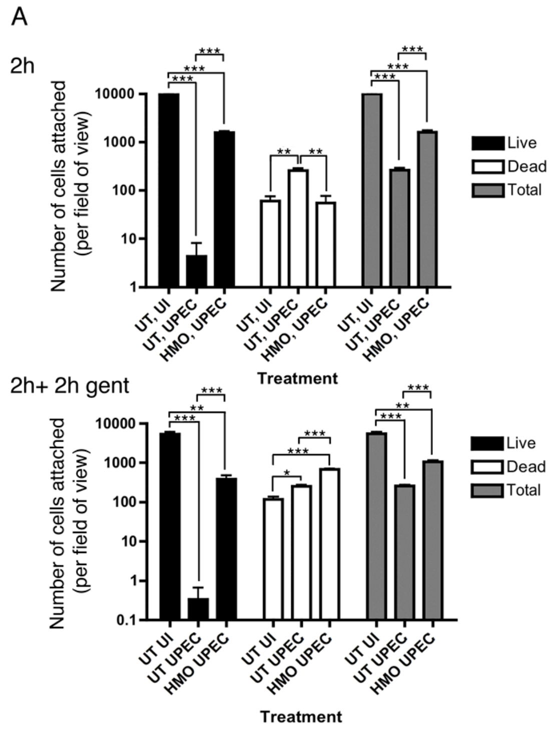
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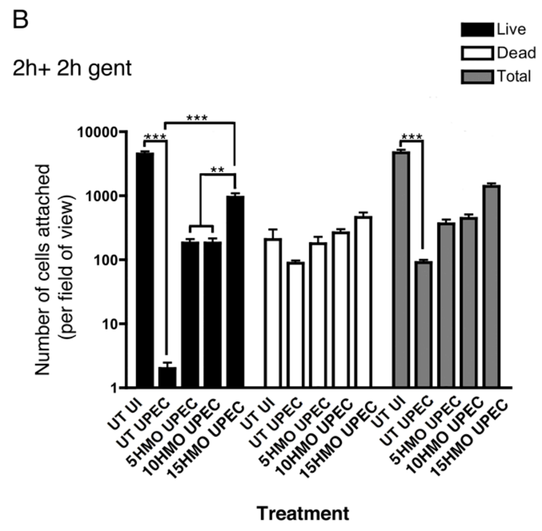
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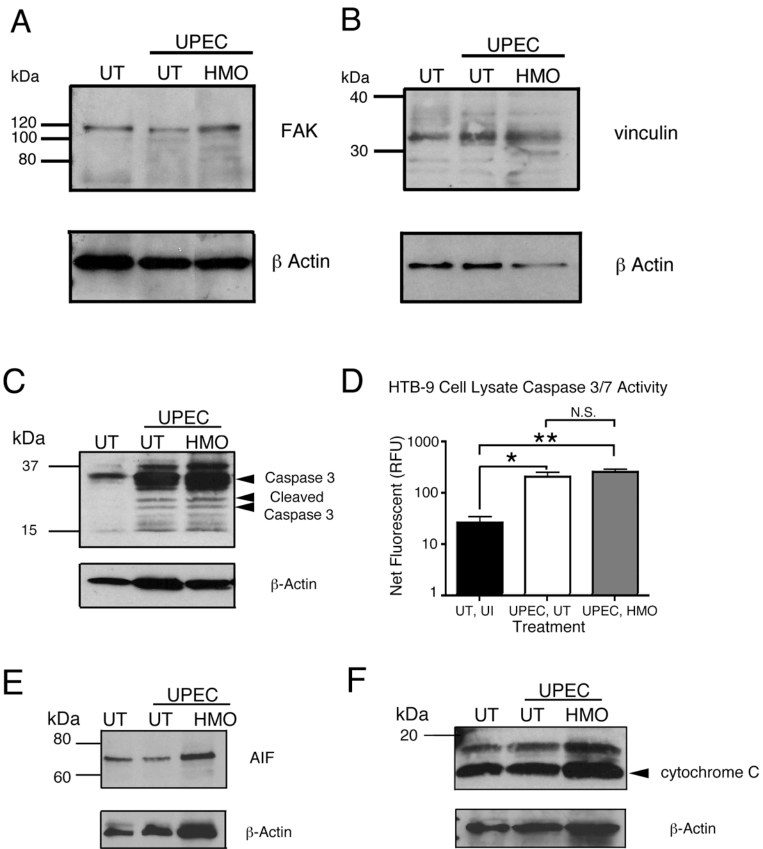
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