Purified galactooligosaccharide, derived from a mixture produced by the enzymic activity of *Bifidobacterium bifidum*, reduces *Salmonella enterica* serovar Typhimurium adhesion and invasion *in vitro* and *in vivo*

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The prebiotic Bimuno® is a mixture containing galactooligosaccharides (GOSs), produced by the galactosyltransferase activity of *Bifidobacterium bifidum* NCIMB 41171 using lactose as the substrate. Previous *in vivo* and *in vitro* studies demonstrating the efficacy of Bimuno® in reducing *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) colonization did not ascertain whether or not the protective effects could be attributed to the prebiotic component GOS. Here we wished to test the hypothesis that GOS, derived from Bimuno®, may confer the direct anti-invasive and protective effects of Bimuno®. In this study the efficacy of Bimuno® basal solution of Bimuno® without GOS [which contained glucose, galactose, lactose, maltodextrin and gum arabic in the same relative proportions (w/w) as they are found in Bimuno®] and purified GOS to reduce *S. Typhimurium* adhesion and invasion was assessed using a series of *in vitro* and *in vivo* models. The novel use of three dimensionally cultured HT-29-16E cells to study prebiotics *in vitro* demonstrated that the presence of ~5 mg Bimuno® ml⁻¹ or ~2.5 mg GOS ml⁻¹ significantly reduced the invasion of *S. Typhimurium* (SL1344nal r)(*P*, 0.0001). Furthermore, ~2.5 mg GOS ml⁻¹ significantly reduced the adherence of *S. Typhimurium* (SL1344nal r) (*P*, 0.0001). It was demonstrated that cells produced using this system formed multi-layered aggregates of cells that displayed excellent formation of brush borders and tight junctions. In the murine ligated ileal gut loops, the presence of Bimuno® or GOS prevented the adherence or invasion of *S. Typhimurium* to enterocytes, and thus reduced its associated pathology. This protection appeared to correlate with significant reductions in the neutral and acidic mucins detected in goblet cells, possibly as a consequence of stimulating the cells to secrete the mucin into the lumen. In all assays, Bimuno® without GOS conferred no such protection, indicating that the basal solution confers no protective effects against *S. Typhimurium*. Collectively, the studies presented here clearly indicate that the protective effects conferred by Bimuno® can be attributed to GOS.

**Abbreviations:** 2D, two dimensional; 3D, three dimensional; DAPI, 4′,6′-diamidino-2-phenylindole; DP, degree of polymerization; FITC, fluorescein isothiocyanate; GOS, galactooligosaccharide; PAS, periodic-acid Schiff; RWV, rotating wall vessel; SEM, scanning electron microscopy; TEM, transmission electron microscopy; VLA, Veterinary Laboratories Agency.

Microscopy images of the treated human colonic cells and the treated murine ligated ileal gut loops are available as supplementary material with the online version of this paper.
**INTRODUCTION**

*Salmonella enterica* serovar Typhimurium (S. Typhimurium) is an important zoonotic enteropathogen that was responsible for 12,091 laboratory-confirmed human salmonellosis cases in the UK in 2008 and is contracted through contact with infected animals and/or their products (DEFRA, 2008). Historically, antibiotics have been used widely to control *Salmonella* infection in humans and animals. However, due to the rise in awareness of antibiotic resistance (Castanon, 2004) and the potential for the rise in awareness of antibiotic resistance (Castanon, 2004), alternative control strategies are being sought to reduce the burden of infection. Prebiotics may offer such an alternative control to the prophylactic use of antibiotics and are defined as ‘a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health’ (Gibson et al., 2004). Specifically, it has been documented that dietary β-1,4-mannobiose, mannan-oligosaccharides and fructooligosaccharide reduce *Salmonella* colonization in poultry (Bailey et al., 1991; Spring et al., 2000; Fernandez et al., 2002; Agunos et al., 2007). Moreover, it has been demonstrated that some, although not all (Petersen et al., 2009; Naughton et al., 2001), galactooligosaccharide (GOS) formulations have the potential to reduce the adherence of enteropathogens such as enteropathogenic Escherichia coli and S. Typhimurium *in vitro* and *in vivo* (Tzortzis et al., 2005a; Shoaf et al., 2006; Searle et al., 2009). Our previous studies demonstrated that Bimuno®, a mixture containing GOS, reduced the colonization and pathology associated with *S. Typhimurium* in the murine model, possibly due to reducing invasion (Searle et al., 2009). To determine whether the protective effect conferred by Bimuno® (Searle et al., 2009) could be attributed specifically to the prebiotic GOS we tested the efficacy of Bimuno®W, a basal solution of Bimuno® without GOS and the purified prebiotic GOS on *S. Typhimurium* using a range of techniques, including adhesion and invasion assays using three dimensionally cultured cells [cells grown in a three dimensional (3D) environment] and murine ligated ileal gut loop studies. It is probable that GOS confers the anti-invasive property of Bimuno®; however, to demonstrate this the involvement of the other components had to be excluded. Culturing cells in three dimensions using a rotating wall vessel (RWV) offers an attractive alternative to conventional methods and has been widely used to investigate host–pathogen interactions, for example, the effect of *S. Typhimurium* on HT-29 (human colonic) and Int-407 (human intestinal embryonic) cells, and the effect of enteropathogenic and enterohaemorrhagic *E. coli* on HCT-8 (human enterocyte) cells (Carvalho et al., 2005; Nickerson et al., 2001; Höner zu Bentrup et al., 2006).

The murine ligated ileal gut loop model is a well established model for studying host–microbe interactions, providing superior tissue quality and allowing intimate interactions to be monitored over a time-course (Searle et al., 2009; Smith & Hall, 1967; Meyerholz et al., 2002; Wales et al., 2002; Girard et al., 2008). Here we present our findings.

**METHODS**

**Bacterial strains and inocula.** A nalidixic-acid-resistant derivative of *S. Typhimurium* SL1344, SL1344nal [from the Veterinary Laboratories Agency (VLA), Weybridge, UK, culture collection] was used throughout these studies. *E. coli* DH5α, K-12 (Gibco) was used as a control for bacterial invasion in adhesion and invasion assays. All bacterial isolates were maintained in heart infusion broth + 30% glycerol (Oxoid) at −80°C. Both strains were used in previous studies (Searle et al., 2009).

For all studies, bacterial isolates were cultured for 16 h aerobically at 37°C with agitation (225 r.p.m.) in Luria–Bertani broth (20 ml) (Oxoid). Subsequently, they were centrifuged at 4000 r.p.m. for 10 min to pellet the bacteria and resuspended in tissue culture inocula following the methods of Searle et al. (2009) to give a final concentration of 5 × 10^7 c.f.u. ml⁻¹ for cell culture studies and in 0.1 M PBS pH 7.2 to give a final concentration of 1 × 10^7 c.f.u. in a 100 μl volume per loop for murine ligated ileal gut loop experiments.

**Test substances used**

The test substances, Bimuno®W, Bimuno® without GOS and GOS, were prepared and resuspended in PBS as described below. Briefly, GOS is a complex mixture of disaccharide–tetrasaccharide GOSs (DP2, DP3 and DP ≥ 4, with each fraction representing an increasing degree of complex polymerization). Bimuno®W is a combination of GOS with various additives and processing aids necessary for its production (including glucose, galactose, lactose, and the stabilizers maltodextrin and gum arabic). A basal solution of Bimuno®W without GOS contained, with the exception of GOS, all the components of Bimuno® in the same relative proportions. In all assays referring to filtered test substances, solutions were prepared and subsequently filter sterilized using a 0.22 μm filter (Sartorius Stedim), in order to eliminate any bacteria present due to the manufacturing process of Bimuno®W.

Bimuno®. The GOS mixture (Bimuno®; Clasado) used in this study was produced from the activity of galactosyltransferases from *Bifidobacterium bifidum* NCIMB 41171 using lactose as substrate (*Tzortzis et al., 2005a, b*). It has been shown to consist of GOSs in mainly the β 1–3, β 1–4, and β 1–6 linkages, as well as a disaccharide fraction of α 1–6 galactobiose (*Tzortzis et al., 2005a; Depeint et al., 2008*). GOS accounts for 50% of Bimuno®W. Additionally, Bimuno®W contains glucose, galactose and lactose (which are involved in the manufacturing process), and the stabilizers maltodextrin and gum arabic. In all assays, whole Bimuno®W was used at −5 mg ml⁻¹.

Bimuno® without GOS. Bimuno® without GOS was prepared to contain all of the sugars in the commercially available Bimuno® product with the exception of GOS. Thus, the solution contained glucose (0.26 mg ml⁻¹), galactose (0.25 mg ml⁻¹), lactose (1.3 mg ml⁻¹) and the processing aids (maltodextrin and gum arabic) (0.7 mg ml⁻¹) in the same relative proportions as they are present in Bimuno®W. This was determined by isocratic HPLC using an Aminex HPX-87C Ca²⁺-resin-based column (Bio-Rad Laboratories) and high performance anion-exchange chromatography coupled with pulsed amperometric detection using a pellicular anion-exchange resin based column CarboPac PA1 (Dionex Chromatography). In all assays Bimuno®W without GOS was used at −2.5 mg ml⁻¹ as these sugars make up 50% of Bimuno®W.

**GOS.** The various oligosaccharide fractions of GOS were purified from Bimuno® by gel filtration on a Biogel P2 (Pharmacia) column eluted at 3 ml min⁻¹ with water (*Tzortzis et al., 2005a*). This column elutes the elution of compounds of 100–1800 Da molecular mass. The individual fractions were combined to create the GOS complex.
equivalent to that in Bimuno® and it was used at a concentration of ~2.5 mg ml⁻¹ in all assays as GOS makes up 50% of Bimuno®.

**Tissue culture assays**

**Adhesion and invasion assays.** For conventional adherence and invasion assays human colonic, HT-29-19A (non-mucus-secreting) and HT-29-16E (mucus-secreting), cells (Augeron & Laboisse, 1984; Lesuffleur et al., 1995) were seeded and cultured as previously described (Searle et al., 2009). Adhesion and invasion assays were conducted essentially as described previously (Searle et al., 2009) with minor modifications. Specifically, bacteria were resuspended in tissue culture media to yield a final concentration of 5 x 10⁷ c.f.u. ml⁻¹. Inocula were prepared with and without ~5 mg Bimuno® ml⁻¹, ~2.5 mg Bimuno® without GOS ml⁻¹ or ~2.5 mg GOS ml⁻¹, and in some instances the inocula were filter-sterilized using a 0.22 mm filter (Sartorius Stedim) prior to the addition of bacteria. All experiments were conducted in triplicate.

For studies where adhesion and invasion assays were conducted on 3D human colonic HT-29-16E (mucus-secreting) cells, culture was essentially as described by Hönér zu Bentrup et al. (2006) with minor modifications. Specifically, cells were cultured in the RWBV at 37 °C, in the presence of 5% CO₂, for 21–23 days. Initially, cells were cultured for 2 days without changing the culture medium to allow adherence of the cells to the beads, subsequently 90% of the medium was changed daily and the rotation increased to ensure cells remained in suspension. Following the 21–23 days incubation cells were removed from the RWBV, resuspended to yield a final concentration of 5 x 10⁶ cells ml⁻¹ and seeded into non-tissue culture treated 24-well plates (BD Falcon) as described by Hönér zu Bentrup et al. (2006). For adhesion and invasion assays bacterial inoculum was resuspended in MEM-x media with or without ~5 mg Bimuno® ml⁻¹, ~2.5 mg Bimuno® without GOS ml⁻¹ and ~2.5 mg GOS ml⁻¹ (in their filtered or unfiltered states) and delivered in 1 ml volumes to each well (5 x 10⁷ c.f.u. ml⁻¹).

The plates were incubated at 37 °C, in the presence of 5% CO₂, for 2 h. To count the number of bacteria associated with the cells and invaded into them, the cells were treated essentially as described for two dimensional (2D) cells (those conventionally cultured using flasks and plates, and without use of a RWBV) by Searle et al. (2009). All assays were conducted in triplicate.

**Scanning electron microscopy (SEM).** 3D HT-29-16E cells, cultured and incubated with the test substances as described above, were fixed in 3% glutaraldehyde for 24 h prior to analysis by SEM. Briefly, aliquots of 3D cells were washed in 0.1M PBS, post-fixed in 1% osmium tetroxide (Agar Scientific), dehydrated through a gradual series of alcohol steps (increasing to 100% ethanol) and then treated with hexamethyldisilazane (Sigma-Aldrich) for 5 min. The air-dried cells were allowed to settle onto poly-l-lysine coated glass coverslips (BD Biosciences) and were subsequently attached to aluminium stubs, sputter-coated with gold and examined using a Stereoscan S250 mark III (Cambridge Instruments) or a Carl Zeiss EVO 10 LS (Zeiss) SEM at 10–20 kV.

As a comparison, conventional monolayers of HT-29-16E cells were cultured using standard procedures on glass coverslips (13 mm; VWR International) for 5 days at 37 °C in the presence of 5% CO₂. Cells were then fixed in 3% glutaraldehyde and one sample from each replicate was processed for SEM essentially as described above.

**Transmission electron microscopy (TEM).** 3D-cultured and conventionally cultured HT-29-16E cells were fixed in 3% glutaraldehyde for at least 24 h prior to being processed for TEM. In brief, cell pellets were washed in 0.1 M PBS, post-fixed in 1% osmium tetroxide (Agar Scientific), dehydrated through a gradual series of alcohol steps (increasing to 100% ethanol) and placed in propylene oxide (Agar Scientific) prior to being embedded in araldite resin (Agar Scientific). The resin was polymerized at 60 °C for 48 h and then 1 µm sections, stained with toluidine blue (VWR International) were prepared for light microscopy examination to select areas for ultrastructural examination. Ultra thin sections of 70–90 nm thickness were then prepared on copper grids using a diamond knife and contrasted with uranyl acetate (Agar Scientific) and lead citrate (Agar Scientific) prior to examination using a CM10 TEM microscope (Philips). One sample from each replicate was examined.

**Confocal microscopy.** 3D HT-29-16E cells, cultured and incubated with test substances as described above, were fixed for 24 h in 4% paraformaldehyde prior to analysis by confocal microscopy. Specifically, in studies assaying for Salmonella, cells were washed twice with PBS, permeabilized for 15 min with 0.1% Triton X-100 (Sigma Aldrich) and washed a further two times prior to incubating cells with goat anti-Salmonella antibody against common structural antigen (CSA-1, 1:200) (KPL) for 60 min. Cells were then washed a further two times, incubated with anti-goat IgG fluorescein isothiocyanate (FITC)-labelled antibody (1:100) (Sigma) for 60 min, and washed a further two times prior to incubating the cells for 45 min with phalloidin–tetramethyl rhodamine isothiocyanate (TRITC) (1:100) (Sigma). Cells were then mounted on glass coverslips with Vectashield (containing DAPI) (Vector). In studies where differential staining was conducted cells were processed essentially as described above; however, they were treated with CSA-1 coupled with either FITC or Alexa Fluor 546 (Invitrogen) before and after permeabilization of the cells.

In studies where 3D-cultured and conventionally cultured HT-29-16E cells were compared, cells were cultured as described previously and fixed in 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 and incubated for 1 h with one of the following primary antibodies and a FITC-labelled secondary antibody: the tight junction markers E-cadherin (Abcam; 1:50), β-catenin (Abcam; 1:100), ZO-1 (Abcam; 1:50); the brush border differentiation marker villin (Abcam; 1:50); the extracellular matrix marker collagen IV (Abcam; 1:100); and the epithelial cell marker cytokeratin 18 (Abcam; 1:100). Phalloidin–TRITC (1:100) was also used for actin staining. Cells were visualized by confocal laser scanning microscopy using a Leica TCS SP2 AOBs confocal system attached to a Leica DMLIRE2 microscope equipped with Ar Kr laser excitation (488 nm), He Ne laser excitation (546 nm) and a diode laser (405 nm) (Leica Microsystems). Oil-immersion objective lenses (×40 and ×63) were used, and imaging parameters were selected to optimize resolution. One sample from each replicate and at least 20 fields of view were examined, and representative images taken.

**Murine ligated ileal gut loop studies**

Murine ligated ileal gut loop studies were performed essentially as described previously (Searle et al., 2009), with minor modifications. Eight 8-week-old female SLC:ICR mice (VLA colony) were used for these studies. The ileal gut loops of terminally anaesthetized mice were inoculated with 100 µl (1 x 10⁶ c.f.u.) S. Typhimurium SL1344(Δλf; with or without Bimuno® (~5 mg), filtered Bimuno® (~5 mg), filtered Bimuno® without GOS (~2.5 mg) or filtered GOS (~2.5 mg) by transmural injection. Control loops were inoculated with 100 µl sterile PBS or Bimuno®, filtered Bimuno® , filtered Bimuno® without GOS or filtered GOS alone. Following an incubation period of 30 min the loops were harvested, and immediately fixed for electron microscopy and histopathology analysis as described previously (Searle et al., 2009). All animal studies were approved by the VLA (Weybridge) local ethics committee and conducted under the jurisdiction of the UK Home Office, licence 70/6103, in accordance with the Animals (Scientific Procedures) Act 1986.
Light microscopy. For light microscopy, murine ligated ileal gut loops were fixed in 10% neutral buffered formalin at ambient temperature for a minimum of 24 h. Trimmed tissues were processed routinely in paraaffin wax, and 4 μm sections were stained with haematoxylin and eosin. Samples were then examined using a DMLB light microscope (Leica Microsystems) and photographed using a DFC320 digital camera (Leica Microsystems). To confirm that the bacteria present in the loops were Salmonella, immunohistochemistry, using goat anti-Salmonella CSA-1 (1 : 100 for 60 min) (KPL) labelled with FITC-conjugated AffiniPure Fab fragment rabbit anti-goat (H + L) (1 : 200 for 30 min) (Jackson ImmunoResearch) and counterstained with Vectashield (containing DAPI) (Vector), was conducted. Samples were examined using a Nikon Eclipse E400 microscope (coupled to a Nikon YFL laser) and photographed using a DXM1200 digital camera (Nikon).

Electron microscopy. Glutaraldehyde-fixed sections of murine ligated ileal gut loops were sectioned to 1–2 mm in thickness, processed for TEM and analysed using a Philips CM10 TEM (Philips) as previously described (La Ragione et al., 2006; Searle et al., 2009).

Mucus detection. To assay for mucus subtypes, trimmed tissues were processed routinely in paraaffin wax, 4 μm sections were stained with periodic-acid Schiff (PAS) stain (VWR International) and alcian blue 8Gx (GCC Diagnostics). Samples were analysed microscopically using an Eclipse E400 microscope (Nikon). Morphometric image analysis was conducted using a Nikon Eclipse E400 and Lucia image analysis software (Laboratory Imaging) to quantify the amount of neutral and acidic mucins. A mask editor was applied to define the total area measured. Representative areas of positive staining (PAS and alcian blue) were selected within the mask editor to define the threshold. Any staining within this threshold was transformed into binary to yield a result as a percentage of the total area measured. Twelve independent fields of view were measured for each sample and the means calculated for semiquantitative comparisons.

Statistical analysis. Statistical analysis was carried out on complete adhesion and invasion datasets by transforming the data onto the log2 scale and using Dunnett’s pairwise statistical tests. All comparisons were based on 95% confidence intervals on the log2 scale and back-transformed onto the raw scale with their associated P value calculated (given to 4 decimal places) and standard deviation (SD) calculated. The analysis allowed for all the different parameters to be compared simultaneously and was conducted in line with previous experiments (Searle et al., 2009). The parameters included the treatment (Salmonella, test substance before Salmonella and test substance with Salmonella), test substance (Bimuno®, Bimuno® without GOS and GOS) and mucus (+/−) cell type. Student’s t-test was conducted on data generated from morphometric analysis of neutral and acidic mucins detected in goblet cells in murine ligated ileal gut loops, with all comparisons being based on 95% confidence intervals, and their SD and P value calculated (given to 4 decimal places). This allowed for an individual test condition to be compared to its negative or positive control.

RESULTS

**In vitro tissue culture assays**

Adhesion and invasion of *S. Typhimurium* to 2D and 3D HT-29 cell lines exposed to Bimuno®, Bimuno® without GOS and GOS. 2D human colonic cell lines were infected with *S. Typhimurium* in the presence or absence of ~5 mg Bimuno® ml⁻¹, ~2.5 mg Bimuno® without GOS ml⁻¹ or ~2.5 mg GOS ml⁻¹. The adherence and invasion of *S. Typhimurium* to either cell line was significantly reduced by the addition of GOS prior to or in combination with the bacterial isolate (P<0.0001) (Fig. 1). The invasion of *S. Typhimurium* into HT-29-16E cells was significantly reduced when ~5 mg Bimuno® ml⁻¹ was added prior to or at the same time as the bacteria (P<0.0001) (Fig. 1). Additional experiments on 2D cells infected with *S. Typhimurium* in the presence or absence of filtered test substances (filtered to remove bacterial debris in Bimuno® present due to the manufacturing process) similarly demonstrated that filtered GOS significantly reduced the adherence of *S. Typhimurium* to HT-29-16E cells (P<0.0001) and significantly reduced its invasion into either cell type (P<0.0001) (Fig. 2). The invasion of *S. Typhimurium* into HT-29-16E cells was also significantly reduced in the presence of filtered Bimuno® (P<0.0001) (Fig. 2). Conversely, filtered or unfiltered Bimuno® without GOS had no effect on either cell line.

In line with the 2D cell data, the adherence and invasion of *S. Typhimurium* was significantly reduced by the addition of GOS in its filtered or unfiltered states to 3D HT-29-16E cells (P<0.0009) (Fig. 3). Furthermore, the invasion of *S. Typhimurium* into 3D HT-29-16E cells was significantly reduced by the addition of Bimuno® in either its filtered or its unfiltered states (P<0.0001) (Fig. 3). Conversely, the adhesion or invasion of *S. Typhimurium* was unaffected by the addition of Bimuno® without GOS in its filtered or unfiltered states (Fig. 3).

**Comparison of 2D and 3D cultured HT-29-16E cells.** The results here are in agreement with reports by Höner zu Bentrup et al. (2006) who demonstrated that cells grown in three dimensions displayed superiorly organized adherens and tight junctional proteins; however, some differences are observed. Whereas Höner zu Bentrup et al. (2006) demonstrated that HT-29 cells grown in the 3D cell culture system grew predominantly as monolayers, the HT-29-16E cells grown here formed multilayered aggregates of cells in the 3D cell culture system, some measuring 350 μm in diameter, with excellent cellular architecture such as superiorly differentiated brush borders and tight junctions between cells (Supplementary Fig. S1b and Supplementary Fig. S2b available with the online journal) when compared to monolayers (Supplementary Fig. S1a and Supplementary Fig. S2a available with the online journal). Moreover, the 3D cells displayed more relevant distribution of junctional proteins (β-catenin, E-cadherin, ZO-1) and brush border markers (villin) (Supplementary Fig. S3f, h, j, d available with the online journal) as compared to monolayers (Supplementary Fig. S3e, g, i, c available with the online journal).

**SEM and confocal analysis of *S. Typhimurium* infected 3D HT-29-16E cells exposed to Bimuno®, Bimuno® without GOS and GOS.** SEM analysis of 3D cells infected with *S. Typhimurium* or (unfiltered or filtered) Bimuno® without GOS + *S. Typhimurium* showed marked pathology
Fig. 1. Histograms of the adherence and invasion of *S. Typhimurium* to human colonic HT-29-16E (mucus-secreting) and HT-29-19A (non-mucus-secreting) cells in the presence or absence of 5 mg Bimuno<sup>®</sup> ml<sup>−1</sup>, 2.5 mg Bimuno<sup>®</sup> without GOS ml<sup>−1</sup> and 2.5 mg GOS ml<sup>−1</sup>. The asterisks indicate significant differences induced by the delivery of test substances in combination with SL1344nal<sup>r</sup> as compared to SL1344nal<sup>r</sup> alone (*P*, 0.0001). Cells incubated with GOS before or at the same time as SL1344nal<sup>r</sup> showed significantly reduced adherence and invasion to HT-29-16E and HT-29-19A cells. Additionally, delivery of Bimuno<sup>®</sup> before or at the same time as SL1344nal<sup>r</sup> significantly reduced the invasion of *S. Typhimurium* into HT-29-16E cells. Error bars indicate SD (95 % confidence intervals). Black bars, test substance before SL1344nal<sup>r</sup>; hatched bars, test substance + SL1344nal<sup>r</sup>; white bars, SL1344nal<sup>r</sup>.

Fig. 2. Histograms of the adherence and invasion of *S. Typhimurium* to human colonic HT-29-16E (mucus-secreting) and HT-29-19A (non-mucus-secreting) cells in the presence or absence of 5 mg filtered Bimuno<sup>®</sup> ml<sup>−1</sup>, 2.5 mg filtered Bimuno<sup>®</sup> without GOS ml<sup>−1</sup> and 2.5 mg filtered GOS ml<sup>−1</sup>. The asterisks indicate significant differences induced by the delivery of test substances in combination with SL1344nal<sup>r</sup> as compared to SL1344nal<sup>r</sup> alone (*P*, 0.0001). Cells incubated with filtered GOS at the same time as SL1344nal<sup>r</sup> showed significantly reduced adherence to HT-29-16E cells. Additionally, delivery of filtered Bimuno<sup>®</sup> or filtered GOS at the same time as SL1344nal<sup>r</sup> significantly reduced the invasion of *S. Typhimurium* into HT-29-16E cells. Moreover, delivery of filtered GOS at the same time as SL1344nal<sup>r</sup> significantly reduced the invasion of *S. Typhimurium* to HT-29-19A cells. Error bars indicate SD (95 % confidence intervals). Hatched bars, filtered test substance + SL1344nal<sup>r</sup>; white bars, SL1344nal<sup>r</sup>. 

*Fig. 1.* Adherence of *S. Typhimurium* to HT-29-16E cells.

*Fig. 2.* Adherence of *S. Typhimurium* to HT-29-19A cells.
loops were of normal diameter and no differences were observed in the PBS- and test-substance-inoculated loops with respect to wall colour, thickness and the appearance of the intestinal contents. Furthermore, no differences were observed in either (filtered or unfiltered) Bimuno<sup>®</sup> alone or filtered GOS + S. Typhimurium inoculated loops. However, loops inoculated with S. Typhimurium alone or filtered Bimuno<sup>®</sup> without GOS + S. Typhimurium were hyperaemic, mildly oedematous on the serosal aspect, and the contents were mucoid and blood stained.

**Electron microscopy studies.** All gut loop tissues sampled were processed for TEM and those infected with either S. Typhimurium, or filtered Bimuno<sup>®</sup> without GOS + S. Typhimurium showed marked pathology, indicative of necrosis (Fig. 5b, e). Bacteria were observed adhered to the epithelium and invading the enterocytes. Conversely, gut loops infected with Bimuno<sup>®</sup> + S. Typhimurium, filtered Bimuno<sup>®</sup> + S. Typhimurium or filtered GOS + S. Typhimurium showed no pathology and no evidence of bacterial adherence or invasion (Fig. 5c, d, f). Tissues examined from all other loops (including the PBS control, Bimuno<sup>®</sup>, filtered Bimuno<sup>®</sup>, filtered Bimuno<sup>®</sup> without GOS and filtered GOS inoculated loops) showed no pathology (Supplementary Fig. S6 available with the online journal).

**Histopathology.** Transmural inoculation of Bimuno<sup>®</sup>, filtered Bimuno<sup>®</sup>, filtered Bimuno<sup>®</sup> without GOS or filtered GOS into the ligated ileal intestinal loops did not induce any significant histopathological changes in the intestinal mucosa (when compared with control PBS inoculated loops). Specifically, tissues appeared within normal limits, with intact epithelium and the presence of a normal brush border. However, inoculation of S. Typhimurium into loops resulted in moderate specific changes such as vacuolar degeneration, focally extensive lesions and lytic necrosis of the enterocytes at the tip of villi associated with bacterial attachment and invasion resulting in enterocyte sloughing. In contrast, no morphological

**In vivo murine ligated ileal gut loop studies**

**Macroscopic findings.** Following removal from the animal, loops were of normal diameter and no differences were observed in the PBS- and test-substance-inoculated loops with respect to wall colour, thickness and the appearance of the intestinal contents. Furthermore, no differences were observed in either (filtered or unfiltered) Bimuno<sup>®</sup> alone or filtered GOS + S. Typhimurium inoculated loops. However, loops inoculated with S. Typhimurium alone or filtered Bimuno<sup>®</sup> without GOS + S. Typhimurium were hyperaemic, mildly oedematous on the serosal aspect, and the contents were mucoid and blood stained.

**Electron microscopy studies.** All gut loop tissues sampled were processed for TEM and those infected with either S. Typhimurium, or filtered Bimuno<sup>®</sup> without GOS + S. Typhimurium showed marked pathology, indicative of necrosis (Fig. 5b, e). Bacteria were observed adhered to the epithelium and invading the enterocytes. Conversely, gut loops infected with Bimuno<sup>®</sup> + S. Typhimurium, filtered Bimuno<sup>®</sup> + S. Typhimurium or filtered GOS + S. Typhimurium showed no pathology and no evidence of bacterial adherence or invasion (Fig. 5c, d, f). Tissues examined from all other loops (including the PBS control, Bimuno<sup>®</sup>, filtered Bimuno<sup>®</sup>, filtered Bimuno<sup>®</sup> without GOS and filtered GOS inoculated loops) showed no pathology (Supplementary Fig. S6 available with the online journal).

**Histopathology.** Transmural inoculation of Bimuno<sup>®</sup>, filtered Bimuno<sup>®</sup>, filtered Bimuno<sup>®</sup> without GOS or filtered GOS into the ligated ileal intestinal loops did not induce any significant histopathological changes in the intestinal mucosa (when compared with control PBS inoculated loops). Specifically, tissues appeared within normal limits, with intact epithelium and the presence of a normal brush border. However, inoculation of S. Typhimurium into loops resulted in moderate specific changes such as vacuolar degeneration, focally extensive lesions and lytic necrosis of the enterocytes at the tip of villi associated with bacterial attachment and invasion resulting in enterocyte sloughing. In contrast, no morphological

![Fig. 3. Histograms of the adherence and invasion of S. Typhimurium to 3D human colonic HT-29-16E (mucus-secreting) cells in the presence or absence of 5 mg Bimuno<sup>®</sup> ml<sup>-1</sup>, 2.5 mg Bimuno<sup>®</sup> without GOS ml<sup>-1</sup> and 2.5 mg GOS ml<sup>-1</sup> in their filtered and unfiltered states. The asterisks indicate significant differences induced by the delivery of test substances in combination with SL1344nal<sup>®</sup> as compared to SL1344nal<sup>®</sup> alone (P<0.0001). Cells incubated with GOS before or at the same time as SL1344nal<sup>®</sup> showed significantly reduced adherence and invasion to 3D cells. Additionally, delivery of Bimuno<sup>®</sup> before or at the same time as SL1344nal<sup>®</sup> significantly reduced the invasion of S. Typhimurium into 3D cells. Error bars indicate 95% confidence intervals. Black bars, test substance before SL1344nal<sup>®</sup>; hatched bars, filtered test substance + SL1344nal<sup>®</sup>; white bars, SL1344nal<sup>®</sup>.](image-url)
changes in the epithelium were observed after the inoculation of the test substances in the presence of S. Typhimurium despite abundant numbers of bacteria being present in the intestinal lumen and occasional attachment to enterocytes. Furthermore, immunohistochemistry of PBS and S. Typhimurium loops confirmed that the bacteria detected in the loops were Salmonella (Supplementary Fig. S7 available with the online journal).

**Mucus subtype detection.** Transmural inoculation of test substances into the ligated ileal gut loops reduced the amount of neutral and acidic mucins detected by PAS and alcian

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**Fig. 4.** SEM of 3D cultured HT-29-16E cells incubated with PBS (a), S. Typhimurium SL1344nalr (b), GOS + SL1344nalr (c) and filtered GOS + SL1344nalr (d). Arrow (i) indicates the presence of brush borders, arrow (ii) indicates cellular damage and arrow (iii) highlights the presence of bacteria. Inset images show lower magnification images of the 3D cells. No pathology was observed in the PBS control incubated cells (a) as evidenced by excellent brush border differentiation and cellular architecture. Cells incubated with S. Typhimurium alone (b) showed bacterial adherence and invasion into the epithelial cells and signs of membrane ruffling and cellular damage. Conversely, incubation of cells with GOS (unfiltered or filtered) appeared to protect cells from S. Typhimurium invasion and pathology (c, d). Specifically, better preservation of brush borders and markedly less cellular damage was observed despite bacteria being observed close to and associated with the epithelial cells.

**Fig. 5.** TEM of murine ligated ileal gut loops inoculated with PBS (a), S. Typhimurium SL1344nalr (b), Bimuno1+ SL1344nalr (c), filtered Bimuno1+ SL1344nalr (d), filtered BimunoLgalr + SL1344nalr (e) and filtered GOS + SL1344nalr (f). Arrow (i) indicates the presence of brush borders, arrow (ii) indicates the presence of S. Typhimurium and arrow (iii) indicates the presence of a damaged cell, with bacterial invasion and loss of cell architecture. (a) Illustrates no cytopathic effects induced by transmural inoculation of PBS into the loops. Loops inoculated with S. Typhimurium alone or in combination with filtered BimunoLgalr without GOS (b, e) showed bacterial adherence and invasion into the enterocytes and marked signs of pathology. Loops inoculated with unfiltered or filtered BimunoLgalr or filtered GOS + S. Typhimurium (c, d, f) showed no signs of pathology, indicating that GOS in BimunoLgalr may protect the enterocytes from S. Typhimurium pathogenicity and invasion.
Anti-invasive properties of GOS
blue staining, respectively (when compared to PBS controls). Specifically, filtered Bimuno<sup>®</sup> and filtered GOS significantly reduced levels of PAS detected using morphometric analysis (P<0.0001) (Table 1) and inoculation of loops with all of the test substances resulted in significant reductions in the detection of alcian blue (P≤0.0029) (Table 1). Inoculation of Bimuno<sup>®</sup> in combination with <i>S. Typhimurium</i> to loops similarly significantly reduced the levels of neutral and acidic mucins (when compared to <i>S. Typhimurium</i> controls) (P<0.0353) whereas Bimuno<sup>®</sup> without GOS + <i>S. Typhimurium</i> significantly increased levels of neutral mucins (P=0.0121) (Table 1).

**DISCUSSION**

The studies reported here aimed to evaluate whether the protective effect of Bimuno<sup>®</sup> as previously reported (Searle <i>et al.</i>, 2009) could be attributed specifically to GOS and to components of the commercial formulation. Results from adherence and invasion assays on conventional and 3D cultured HT-29 cells and microscopy of murine ligated ileal gut loop models collectively demonstrate that GOS is, at least in part, responsible for the protective effects conferred by Bimuno<sup>®</sup>.

In previous studies (Searle <i>et al.</i>, 2009) it was demonstrated that Bimuno<sup>®</sup>, when applied as the complete commercial formulation, significantly reduced the colonization of <i>S. Typhimurium</i> in a murine model with concomitant reduction of associated pathology and clinical symptoms (Searle <i>et al.</i>, 2009). Moreover, evidence from adherence and invasion assays, and murine ligated ileal gut loop studies, showed that protection may, at least in part, be a consequence of Bimuno<sup>®</sup> reducing the invasion of the enteropathogen (Searle <i>et al.</i>, 2009). These studies did not address whether the prebiotic component, GOS, which makes up 50 % of Bimuno<sup>®</sup>, accounted for the protective effects. Thus, here we have tested the efficacy of Bimuno<sup>®</sup>, a basal solution of Bimuno<sup>®</sup> without GOS (containing, with the exception of GOS, all the components of Bimuno<sup>®</sup> in the same relative proportions) and purified GOS [equivalent amount (w/w) to that in Bimuno<sup>®</sup>] on <i>S. Typhimurium</i> adherence and invasion. Efficacy was assessed using a series of <i>in vitro</i> tissue culture assays and a murine ligated ileal gut loop study to reduce the number of animals used in studies and to support the validity of the findings. Specifically, the murine ligated ileal gut loop model was utilized to allow multiple test conditions to be monitored in a single animal.

**Table 1.** PAS and alcian blue staining of murine ligated ileal gut loops to detect neutral and acidic mucins in loops inoculated with 5 mg Bimuno<sup>®</sup> ml<sup>−1</sup>, 5 mg filtered Bimuno<sup>®</sup> ml<sup>−1</sup>, 2.5 mg filtered Bimuno<sup>®</sup> without GOS ml<sup>−1</sup> and 2.5 mg filtered GOS ml<sup>−1</sup> in the presence or absence of <i>S. Typhimurium</i>

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<th>Mucin stained area (%)</th>
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<th>P value (comparison to SL1344nal&lt;sup&gt;®&lt;/sup&gt;)</th>
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SD, SD at 95% confidence intervals.
Adherence and invasion assays using conventionally cultured monolayers demonstrate that the anti-invasive effects conferred by Bimuno® can be attributed to GOS and moreover that GOS has anti-adhesive properties, a finding supporting previous findings by Tzortzis et al. (2005a). Specifically, GOS significantly reduced the adherence of S. Typhimurium to HT-29-16E human colonic mucus-secreting cells ($P<0.0001$) (Figs 1 and 2). It is interesting to note that, unlike GOS, Bimuno® did not have anti-adhesive effects for Salmonella on either HT-29 cell types, consistent with previous observations by Searle et al. (2009). It is possible that the discrepancies may be due to some of the other constituents of Bimuno® masking the anti-adhesive effect of purified GOS. However, the involvement of the basal solution, Bimuno® without GOS, can be disregarded since Bimuno® without GOS conferred no such anti-adhesive or anti-invasive properties. Thus, it is plausible that the viscosity of Bimuno® or the presence of bacteria in the formulation may be masking the anti-adhesive properties of GOS. Bimuno® and GOS significantly reduced the invasion of the enteropathogen to the mucus-secreting derivative of HT-29 cells ($P<0.0001$) (Figs 1 and 2). The anti-invasive effect of Bimuno® documented here is in line with previous studies by Searle et al. (2009) and extends this to demonstrate that GOS confers the anti-invasive property. Conversely, Bimuno® without GOS conferred no such protective effects, indicating that the basal solution does not account for the anti-invasive properties of Bimuno®.

Of greater biological significance is the finding that GOS significantly reduced the adherence of S. Typhimurium to 3D cultured HT-29-16E cells ($P<0.0001$), and Bimuno® and GOS significantly reduced the invasion of S. Typhimurium into these cells ($P<0.0009$) (Fig. 3). These results confirm the findings from conventional assays in a system that produces cells more representative of native tissues, by enabling cells to associate with each other, based on their natural affinities with minimal turbulence and mechanical interference (Carvalho et al., 2005; Nickerson et al., 2001; Höner zu Bentrup et al., 2006). In line with findings documented by Höner zu Bentrup et al. (2006) the HT-29-16E cells in this study, cultured for 21–23 days, exhibited superior differentiation and relevant distribution of junction proteins and villin markers (Supplementary Figs S1, S2 and S3 available with the online journal). Compared to monolayers, which appeared flat and less differentiated, 3D cultured HT-29 cells formed multilayer aggregates of cells with superiorly differentiated brush borders and tight junctions between cells (Supplementary Figs S1 and S2 available with the online journal). Moreover, the junction proteins ($\beta$-catenin, E-cadherin and ZO-1) were more apparent in 3D cultured cells, concentrated primarily at the borders between adjacent cells as might be anticipated (Supplementary Fig. S3 available with the online journal). Furthermore, SEM and confocal analysis confirmed that Bimuno® and GOS, to some extent, protected 3D HT-29 cells from the pathology associated with S. Typhimurium. Specifically, they preserved cell brush borders, reduced cellular damage despite bacteria being observed close to and associated with the epithelium (Supplementary Fig. S4 available with the online journal) and reduced the number of bacteria associated with the epithelium (Supplementary Fig. S5 available with the online journal). Similar levels of pathology were observed in instances where Bimuno® without GOS was delivered in combination with S. Typhimurium (as compared to S. Typhimurium alone) (Supplementary Fig. S4 available with the online journal), with bacteria being observed attaching and invading the epithelial cells, resulting in the formation of membrane ruffling and cellular damage (Guiney & Lesnick, 2005).

This is, to the best of our knowledge, the first report of 3D cells being used to test the efficacy of GOS in directly mitigating against an enteropathogen. As a consequence of cells, cultured in three dimensions, being more representative of in vivo tissue this model could be utilized in efficacy experiments in the future to test a panel of prebiotics and enteropathogens. This would be similar to how it is being developed for drug toxicity studies where conventional tissue culture assays and animal experiments miss some toxic effects (Dash et al., 2009).

The murine ligated ileal gut loop model clearly indicated that the protective effects conferred by Bimuno® could be attributed to GOS. Specifically, Bimuno® and GOS suppressed the attachment, invasion and associated pathology of S. Typhimurium despite abundant numbers of bacteria being present in the lumen (Fig. 5). This level of protection, however, was not afforded by the basal solution of Bimuno® without GOS, where extensive pathology, similar to levels in S. Typhimurium controls, was observed. Such pathology included degradation of brush borders and necrosis of the enterocytes (Fig. 5).

An interesting observation was that in the presence of test substances alone there appeared to be significantly less neutral and acidic mucins in the goblet cells in the loops (Table 1). It is feasible that the test substances may have triggered the release of mucins into the lumen and thus agglutinated the Salmonella to prevent its adherence and invasion into the enterocytes. Moreover, as evidenced by TEM there appears to be a protective barrier preventing Salmonella from accessing the enterocytes (Fig. 5). It would be of interest to quantify the levels of mucin in the lumen; however, current methodologies used here utilizing PAS/alcan blue staining would need to be adapted to ensure the intestinal content was not stained by the PAS/alcan blue and steps would have to be taken to ensure that the size of the loops and intestinal content was heterogeneous. An alternative method that could be used is conducting a mucosal scrape and quantifying the amount of mucin present using an ELISA (Satchithanandam et al., 1990). Interestingly, Satchithanandam et al. (1990) demonstrated that supplementing dietary fibre, such as 5% citrus-fibre, to the rat feed significantly increased luminal mucin detected in the small intestine and stomach ($P<0.05$).
Collectively, the studies presented here clearly indicate that the protective effects conferred by Bimuno\textsuperscript{R} can be attributed to GOS. It is plausible that this could be attributed to GOS acting as a receptor mimic or modulating mucus production by host cells; however, further studies are required to elucidate the exact mechanisms of its action. Such future experiments could involve visualizing the interaction of GOS with host cells and \textit{Salmonella} by manufacturing and utilizing radiolabelled GOS and quantifying the amount of mucus secreted into the lumen by ELISA.

**ACKNOWLEDGEMENTS**

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