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Citation for published version:

Digital Object Identifier (DOI):
10.1371/journal.pone.0087658

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
PLoS One

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Dietary Supplementation with Soluble Plantain Non-Starch Polysaccharides Inhibits Intestinal Invasion of *Salmonella Typhimurium* in the Chicken

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Abstract

Soluble fibres (non-starch polysaccharides, NSP) from edible plants but particularly plantain banana (*Musa* spp.), have been shown *in vitro* and *ex vivo* to prevent various enteric pathogens from adhering to, or translocating across, the human intestinal epithelium, a property that we have termed contrabiotic. Here we report that dietary plantain fibre prevents invasion of the chicken intestinal mucosa by *Salmonella*. *In vivo* experiments were performed with chicks fed from hatch on a pellet diet containing soluble plantain NSP (0 to 200 mg/d) and orally infected with *S*. *Typhimurium* 4/74 at 8 d of age. Birds were sacrificed 3, 6 and 10 d post-infection. Bacteria were enumerated from liver, spleen and caecal contents. *In vitro* studies were performed using chicken caecal crypts and porcine intestinal epithelial cells infected with *Salmonella enterica* serovars following pre-treatment separately with soluble plantain NSP and acidic or neutral polysaccharide fractions of plantain NSP, each compared with saline vehicle. Bacterial adherence and invasion were assessed by gentamicin protection assay. *In vivo* dietary supplementation with plantain NSP 50 mg/d reduced invasion by *S*. *Typhimurium*, as reflected by viable bacterial counts from splenic tissue, by 98.9% (95% CI, 98.1–99.7; *P* < 0.0001). *In vitro* studies confirmed that plantain NSP (5–10 mg/ml) inhibited adhesion of *S*. *Typhimurium* 4/74 to a porcine epithelial cell-line (73% mean inhibition (95% CI, 64–81); *P* < 0.001) and to primary chick caecal crypts (82% mean inhibition (95% CI, 75–90); *P* < 0.001). Adherence inhibition was shown to be mediated via an effect on the epithelial cells and Ussing chamber experiments with *ex vivo* human ileal mucosa showed that this effect was associated with increased short circuit current but no change in electrical resistance. The inhibitory activity of plantain NSP lay mainly within the acidic/pectic (Homogalacturonan-rich) component. Supplementation of chick feed with plantain NSP was well tolerated and shows promise as a simple approach for reducing invasive salmonellosis.


Editor: Michael Hensel, University of Osnabrueck, Germany

Received: August 1, 2013; Accepted: December 28, 2013; Published: February 3, 2014

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Funding: BNP and CLR were supported by a BBSSC Link award (BB/G01969X/1) to AJMW, BJC, JMR and PW, with matched funding from Provexis plc. HLS was supported by a BBSRC award (BB/E013651/1). BJC acknowledges support of the European Science Foundation (ESF), in the framework of the Research Networking Programme, The European Network for Gastrointestinal Health Research. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: JMR is a member of advisory boards for Atlantic, Procter & Gamble and Falk, and has received speaking honoraria from Abbott, Falk, Ferring, Glaxo Smith Kline, Procter & Gamble and Schering Plough and, with the University of Liverpool and Provexis plc, holds a patent for use of a soluble fibre preparation as maintenance therapy for Crohn’s disease. JMR, BJC, PW and AJMW have received support from Provexis plc. NO is a current employee, and CLRa and RD were former employees, of Provexis plc. BJC has received a speaking honorarium from Amgen Inc. This does not alter the authors’ adherence to all PLOS ONE policies on sharing data and materials.

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Introduction

*Salmonella enterica* infection in humans is associated with self-limiting diarrhoea, fever, and abdominal pains [1,2]. In England and Wales, 9,685 human cases of *Salmonella* infection were confirmed in 2010, the most commonly isolated serovars *Salmonella enterica* Enteritidis and *Salmonella enterica* Typhimurium [3]. Poultry-related products are one of the major sources of *Salmonella* infection for humans [2,4,5]. *Salmonella* also causes considerable worldwide economic loss through chicken mortality, primarily caused by the avian-adapted serovars *S*. *Gallinarum* and *S*. *Pullorum* [6,7]. Pigs are also a frequent source of zoonotic infection [8]. The prevalence of *Salmonella* in pigs varies from 7.9 to 30% depending upon the country [8–10].
Vaccination has been successfully used to reduce Salmonella in laying hens, however the cost and practicalities make vaccines unsuitable for use in broilers. The use of therapeutic antimicrobials against Salmonella is increasingly limited in poultry production due to problems with the emergence of resistant epidemic isolates [11]. Since there are no vaccines to prevent salmonellosis, or indeed other food-borne bacteria in humans, there is a clear need for an alternative preventative approach.

Various substances have been investigated for their potentially inhibitory effects on Salmonella infection and faecal shedding, including butyrate [12], honey [13], acidification of feed using lactic, formic and acetic acid [14,15], glutamine [16], glycopeptides derived from soybeans [17], and partially digested whey protein [18]. Butyrate showed promising results for reducing Salmonella colonisation in chickens in vivo via up-regulation of host defence peptides [12]. Acidified feed also inhibited Salmonella shedding in pigs, [15], but other interventions showed limitations, such as possible cytotoxicity to cell monolayers at high concentrations [18], or attenuated effects in vivo [14,16].

We have previously demonstrated that soluble NSP from plantain banana (Musa spp.), inhibits the adhesion of Escherichia coli to, and invasion into, human intestinal epithelial cells [19] and translocation across specialised microfold (M)-cells of the follicle-associated epithelium (FAE) cultured in vitro [19,20]. Subsequently we recently described that soluble plantain NSP was also able to block adhesion of various enteric gut pathogens to the human intestinal epithelial cell-line Caco2, including S. Typhimurium, Shigella sonnei, Clostridium difficile and diarrheagenic enterotoxigenic E. coli (ETEC) [21], with the only exception being enteropathogenic E. coli, where plantain NSP did not block bacterial adherence [21]. In the same study, soluble plantain NSP was also shown to block translocation of S. Typhimurium across M-cells in culture and ex vivo human ileal FAE mounted in Ussing chambers [21]. Other soluble plant NSP preparations, such as broccoli NSP, have also showed significant ability to block pathogen-epithelium interaction [20].

We therefore speculated that soluble plantain NSP may also inhibit Salmonella in an un-manipulated animal model. To investigate this we performed additional in vitro experiments to assess the inhibitory action of plantain NSP on Salmonella interaction with porcine-derived intestinal epithelial cells (B10XI cell-line) and primary chicken caecal crypts. We also conducted an in vivo study to investigate the potential protective effect of dietary supplementation with soluble plantain NSP in a model of invasive salmonellosis in inbred White Leghorn Line 0 chicks.

**Results**

Supplementation of chick feed with soluble plantain NSP reduces S. Typhimurium 4/74 translocation to the spleen in vivo

In chicks ingesting a custom-made commercial pellet diet supplemented with soluble plantain NSP there was significant reduction observed in the translocation of S. Typhimurium 4/74 across the chick gut. The most profound effect observed, with all three doses of ingested soluble plantain NSP (12.5, 50 and 200 mg/d; all P<0.05 Kruskal-Wallis), was a significant reduction in Salmonella found in the splenic tissue (e.g. CFUs reduced in splenic tissue by 98.9% (95% CI, 98.1–99.7) 3 d post-infection, in birds on 50 mg/d plantain NSP (N=5 birds, n=2 replicates) compared to those birds on a non-supplemented NSP control diet (N=5, n=2; P<0.0001); **Figure 1A.**

Supplementation with soluble plantain NSP had little effect on presence of S. Typhimurium CFU cultured from the liver, excepting at 10 d post infection (P<0.05), however bacterial counts in the liver were orders of magnitude lower than spleen counts. There was no significant effect of plantain NSP supplementation on the total CFU observed within the caecal lumen at all doses of plantain NSP supplementation, with the exception of some reduction at day ten with one dose only, compared to birds fed a control diet (see **Figure 1B and 1C**).

Consistent with S. Typhimurium infection in birds of this age, mild inflammation of caecal tissue was seen in all infected birds receiving the standard commercial pellet feed (i.e. not receiving plantain NSP). Livers from infected birds exhibited mild perportal inflammation. However, livers from broilers fed plantain NSP containing diets exhibited no visible signs of inflammation or periportal infiltrate. *Figure 1B*

**Figure 1. Dietary supplementation with soluble plantain NSP reduces chick salmonellosis in vivo.** (A) Following infection of 8 day-old inbred specified pathogen-free White Leghorn Line 0 chicks with S. Typhimurium 4/74, soluble plantain NSP supplementation of a commercial pellet feed significantly reduced bacterial numbers found in the spleen 3 d post-infection. (B) Supplementation with soluble plantain NSP had little significant effect on presence of S. Typhimurium CFU cultured from the liver, excepting at 10 d post infection. (C) Total CFU observed within the caecal lumen were relatively unchanged at all doses of plantain NSP supplementation compared to birds fed a control diet. Significant differences from control (non-supplemented NSP) diet, * P<0.05; ** P<0.01; *** P<0.0001 Kruskal-Wallis (N=4–7 birds, n=2 replicates).

doi:10.1371/journal.pone.0087658.g001
and multifocal lymphoplasmacytic, histiocytic, and heterophilic infiltrates, with variable single cell hepatocellular necrosis. Focal necrosis was only seen in 3/36 livers examined, two of which were in control feed birds, whilst the other was in the group fed 50 mg/d plantain NSP. No significant abnormalities were observed in spleen and ileal tissue sections taken from all treatment groups.

Further experiments to clarify the mechanism of inhibition of *Salmonella* spp. interaction with intestinal epithelia: *in vitro* studies

Soluble plantain NSP, at 10 mg/mL, reduced adhesion of *S. Typhimurium* 4/74 to primary caecal crypts isolated from 14-day old Lohmann Brown Classic egg-layer chicks (mean reduction of 82% (95% CI, 75–90), N = 4 birds, n = 3 replicates; P<0.001 Mann Whitney U test). Likewise, plantain NSP reduced strain 4/74 adherence to caecal crypts from 33-day old Hubbard JA57 broiler chickens by 42% (95% CI, 19–64), N = 3, n = 2; P = 0.05 (see Figure 2). No significant effects on crypt viability were observed with either soluble plantain NSP pre-treatment nor during the 90 min infection with *S. Typhimurium* 4/74 as assessed by adénylate kinase release into the culture medium (with levels within 90–98% of vehicle-treated control cells).

Plantain NSP at 10 mg/mL also reduced adhesion of *S. Typhimurium* 4/74 (as used in the in vivo infection studies) to human Caco2 cells (50% (95% CI, 46–63) reduction in adhesion (N = 3, n = 4) albeit to a lesser extent than that seen for *S. Typhimurium* LT2-infected human Caco2 cells (81% (95% CI, 65–90)); both P<0.001 Kruskal-Wallis; see Figure S1).

Pre-treatment of the porcine B1OXI enterocyte-like cell-line with soluble plantain NSP also significantly inhibited adhesion and invasion of *S. Typhimurium* LT2 in a dose-dependent manner. Similar results were observed for plantain NSP in blockade of *S. Typhimurium* 4/74 to porcine enterocytes (Figure 3). Peak reduction in both adhesion and invasion of B1OXI cells was observed at concentrations of 10 mg/mL soluble plantain NSP; e.g. mean reductions in *S. Typhimurium* adherence compared to vehicle-treated control were 75% (95% CI, 66–84) and 73% (95% CI, 64–81) for strains LT2 and 4/74 respectively (N = 3, n = 4; both P<0.01 Kruskal-Wallis). In addition, soluble plantain NSP also significantly blocked adhesion of *S. Enteritidis*, another key *Salmonella enterica* serovar relevant to production animals, with an 80% reduction (95% CI, 73–87) seen using 10 mg/mL plantain NSP (N = 3, n = 4; P<0.001 Mann Whitney U test), see Figure S2.

The effects of soluble plantain NSP on epithelial adherence of *Salmonella* spp. are mediated via an effect on the epithelium that is associated with a marked increase in short-circuit current

In additional experiments using B1OXI cells, we were able to demonstrate that plantain NSP blockade of *Salmonella* adhesion to cell monolayers acts primarily through action on the epithelium. When plantain NSP (5 mg/mL) was added to monolayers 30 min prior to infection, then removed by three washes with sterile PBS (1 min each; at 37°C), levels of adherent *Salmonella* were observed to be significantly reduced (59.2±5.0% inhibition compared to untreated control; N = 1, n = 3; P<0.001 Kruskal-Wallis), albeit lower than that seen in experiments where plantain NSP was added to cells for 30 min without removal before infection (89±5.0% inhibition; N = 1, n = 3; P<0.001); see Figure 4. In contrast, plantain NSP added to bacteria for 30 min, and then removed by centrifugation before re-suspension of bacteria in antibiotic free media and inoculation of B1OXI cell monolayers, resulted in much less inhibition (22.3±10.5%) compared to untreated control (100%); Figure 4.

We have recently shown that plantain NSP (5 mg/mL) blocks translocation of EGFK-expressing *S. Typhimurium* LT2 across human ileal follicle-associated epithelium (FAE) mounted in Ussing chambers [21]. We now report that this is associated with a marked increase in transmucosal short circuit current (*Isc*) indicating increased ion efflux. Pre-treatment of tissue with 5 mg/mL plantain NSP for 20 min prior to infection, increased transmucosal *Isc* with the peak change seen at T<sub>m</sub> post-infection (*ΔIsc*, 3.86±1.89 μA·cm<sup>-2</sup>) N = 4, with n = 2 replicates; P<0.01 ANOVA vs. untreated control tissue (see Figure 5A). Concomitant changes in potential difference (PD) across ileal FAE were also observed in response to plantain NSP treatment; P<0.05 ANOVA (Figure 5B).

*S. enterica* infection alone resulted in no change in transmucosal *Isc* nor electrical PD in non-plantain ileal FAE (Figure 5A-B). Trans-epithelial electrical resistance (TEER) was maintained throughout the 2 h Ussing chamber experiments of both infected plantain NSP pre-treated ileal FAE and buffer-treated controls, although interestingly there was a trend to a more stable TEER following treatment with soluble plantain fibre (Figure 5C).

The inhibitory effect of plantain NSP on *Salmonella*-intestinal epithelial cell adherence is mediated primarily by the acid (pectic) polysaccharide fraction

At 5 mg/mL, the acidic polysaccharide fraction of plantain NSP isolated by Q-Sepharose anion-exchange fractionation inhibited adhesion of *S. Typhimurium* LT2 to the human intestinal Caco2 cell-line by 92% (95% CI, 85–100) and was at least as effective as the unfractionated soluble plantain fibre 86% (95% CI, 85–100), whereas the neutral polysaccharide fraction had lower inhibitory activity (47% inhibition of adhesion (95% CI, 21–72); both P<0.05 compared to vehicle-treated control cells (N = 2 experiments, n = 3 replicates, Kruskal-Wallis; see Figure 6). The acidic fraction was also shown to inhibit adhesion of *S. Enteritidis* to B1OXI cells was not however significantly reduced following pre-treatment with plantain NSP.

As per previous studies [20,21], treatment of epithelial cells with plantain NSP generated no significant release of adénylate kinase to the medium indicating a lack of cytotoxicity. This was also confirmed by Giemsa microscopy (see Figure 3). Likewise, as per previous studies [20,21], no bacteriocidal effect of plantain NSP was seen on *Salmonellae*.

### Figures

- **Figure 2.** Soluble plantain NSP reduces adherence of *S. Typhimurium* 4/74 to primary caecal crypts. Soluble plantain NSP (10 mg/mL) reduced adherence of *S. Typhimurium* 4/74 to primary chick caecal crypts isolated from both (A) 14-day old Lohmann Brown Classic egg-layer chicks (N = 4 experiments, n = 3 replicates; *** P<0.001 Mann Whitney U test) and (B) 33-day old Hubbard JA57 broiler chickens (N = 3, n = 2; * P = 0.05 Mann Whitney U).
- **Figure 4.** In contrast, plantain NSP added to bacteria for 30 min, and then removed by centrifugation before re-suspension of bacteria in antibiotic free media and inoculation of B1OXI cell monolayers, resulted in much less inhibition (22.3±10.5%) compared to untreated control (100%).
- **Figure 5A.** Concomitant changes in potential difference (PD) across ileal FAE were also observed in response to plantain NSP treatment; P<0.05 ANOVA (Figure 5B).
- **Figure 5B.** *Salmonella* infection alone resulted in no change in transmucosal *Isc* nor electrical PD in non-plantain ileal FAE (Figure 5A-B).
Typhimurium LT2 to porcine B1OXI enterocytes by 52% (95% CI, 27–76; \( P < 0.05 \)), with the neutral fraction having no inhibitory activity (N = 1, n = 4, \( P < 0.01 \)).

Composition analysis reveals the pectic fraction of plantain NSP to be mainly or only homogalacturonan. The whole plantain NSP preparation as tested in these studies contained substantial maltodextrin (added up to 40% by weight to facilitate resolubilisation after freeze-drying – see Methods) and 2.5% by weight of galacturonic acid, indicating the presence of pectic material (see File S3). Composition of the acidic plantain NSP fraction, obtained by Q-Sepharose anion-exchange fractionation contained approximately 15% (by weight total carbohydrate) of galacturonic acid. On acid hydrolysis and digestion with Driselase®, negligible rhamnose, galactose or arabinose was formed from the acidic fraction nor from the whole plantain NSP, indicating little or no rhamnogalacturonan-I and rhamnogalacturonan-II; File S3. Thus, the pectic material within the acidic fraction of plantain NSP is mainly or only homogalacturonan. The neutral plantain NSP fraction contained no detectable galacturonic acid indicating absence of pectic material.

**Discussion**

These studies show that supplementation of pelleted feed with soluble plantain NSP was well tolerated by chickens and reduced S. enterica serovar. Typhimurium 4/74 translocation as shown by reduction in splenic bacteria. Histopathological findings were consistent with S. Typhimurium infection in birds of this age as...
This is the first time that dietary supplementation with soluble plantain NSP has been shown to block bacterial invasion in an animal model. Soluble plantain NSP also reduced adherence of *S. Typhimurium* to caecal crypts. Figure 4. Soluble plantain NSP acts on the epithelium to block interaction of *S. Typhimurium* LT2. Plantain NSP (5 mg/mL) blockade of adhesion of *S. Typhimurium* LT2 to B1OXI cells under different pre-treatment conditions. (A) Standard pre-treatment of cell monolayers with soluble plantain NSP (30 min), followed by infection for 90 min. (B) Pre-treatment of cell monolayers with soluble plantain NSP (30 min), followed by removal from monolayers using three sterile PBS washes prior to infection for 90 min. (C) Pre-treatment of bacteria with plantain NSP for 30 min, followed by centrifugation, re-suspension of bacteria in antibiotic free media and infection for 90 min. Data (mean ± SEM) expressed relative to adherence of vehicle-treated control (100%); n = 3; **P < 0.01, ***P < 0.001, Kruskal-Wallis. doi:10.1371/journal.pone.0087658.g004

Previously described [22]. This is the first time that dietary supplementation with soluble plantain NSP has been shown to block bacterial invasion in an animal model. Soluble plantain NSP also reduced adherence of *S. Typhimurium* to caecal crypts.

Figure 5. Soluble plantain NSP increases the transmucosal short circuit current of *ex vivo* human ileal follicle-associated epithelium. Plantain NSP (5 mg/mL) significantly increased (A) transmucosal short circuit current ($I_{SC}$), with a concomitant decrease in (B) epithelial potential difference (PD, apical-side negative), during pre-treatment of, and blockade of translocation of *S. Typhimurium* across *ex vivo* human ileal follicle-associated epithelium (FAE) mounted in Ussing chambers. (C) Trans-epithelial electrical resistance (TEER) was maintained throughout the experiment. N = 4, with 2 tissue replicates in each case. *P < 0.05, **P < 0.01, ANOVA. For each tissue, $I_{SC}$ and PD responses were calculated and expressed as the increment change ($\Delta$) for each sampling period. Arrows at T0 min indicate addition of EGFP-expressing *S. Typhimurium* LT2 to the mucosal compartment ($1 \times 10^8$ CFU/mL). Overnight culture of Ussing chamber serosal medium following 2 h infection had already demonstrated soluble plantain fibre to block translocation of *Salmonella* across isolated human FAE in this experiment; see reference [21].

doi:10.1371/journal.pone.0087658.g005
Plantain NSP Blocks Salmonella Invasion

The inhibitory activity of soluble plantain NSP to block Salmonella-host intestinal epithelium interaction lies within an acidic polysaccharide component. At 5 mg/mL, the acidic polysaccharide fraction of plantain NSP significantly blocked adhesion of S. Typhimurium LT2 to human intestinal Caco2 cells, whereas the neutral fraction had a lesser effect compared to vehicle-treated control (N = 2 experiments, n = 3 replicates; * P < 0.05, ** P < 0.01 Kruskal-Wallis).

doi:10.1371/journal.pone.0087658.g006

Figure 6. The inhibitory activity of soluble plantain NSP to block Salmonella-host intestinal epithelium interaction lies within an acidic polysaccharide component. At 5 mg/mL, the acidic polysaccharide fraction of plantain NSP significantly blocked adhesion of S. Typhimurium LT2 to human intestinal Caco2 cells, whereas the neutral fraction had a lesser effect compared to vehicle-treated control (N = 2 experiments, n = 3 replicates; * P < 0.05, ** P < 0.01 Kruskal-Wallis).
Thus, soluble plantain NSP reduces adhesion and invasion of *Salmonella* spp. *in vitro*, in primary cell culture models, and *in vivo* in the chicken. This suggests that dietary supplementation with soluble plantain NSP has potential to achieve a useful protection against invasive salmonellosis in animals and man. The epithelial adhesion of other human pathogens such as enterotoxigenic *E. coli*, *Shigella sonnei*, and *Clostridium difficile* is also inhibited by soluble plantain NSP [20,21]. We have recently described this action of dietary soluble NSP in inhibiting bacteria-host epithelium interactions as a ‘contrabiotic’ effect [44]. *In vitro* studies suggest that the acidic (homogalacturonan-rich) fraction is particularly inhibitory and the Ussing chamber studies of *that the acidic (homogalacturonan-rich) fraction is particularly inhibitory and the Ussing chamber studies of human follicle-associated epithelium (FAE) mounted in Ussing chambers, as previously described [21].

Materials and Methods

Ethics statement

All work was conducted in accordance with UK legislation governing experimental animals under project licences PPL 40/3063 and PPL40/3652 and was approved by the University of Liverpool ethical review process prior to the award of the licence. Chicks were reared in the high-biosecurity poultry unit, University of Liverpool, in secure floor pens at a temperature of 30°C until 3 weeks of age, then at 20°C. Birds were allowed *ad libitum* access to water and vegetable protein-based laboratory poultry pelleted diets under test. All animals were checked a minimum of twice daily to ensure their health and welfare.

Studies described using human tissue specimens from macro- and microscopically normal terminal ileum were obtained from patients who underwent surgery for colon cancer and who had given their informed written consent as previously described [21]. The study was approved by the Regional Human Ethics Committee; Linköping, Sweden.

**Bacterial strains and growth conditions**

*Salmonella enterica* serovar Typhimurium LT2 and *S. Typhimurium* 4/74 were obtained from Professor Craig Winstanley (Institute of Infection & Global Health, University of Liverpool) and Professor Mark Stevens (Roslin Institute, University of Edinburgh) respectively. *S. Typhimurium* 4/74 was used for *in vivo* chick studies, due to its high virulence [45]. Serovar *S. Enteritidis* (P125109) was obtained from Professor Paul Barrow (Veterinary Medicine, University of Nottingham). Bacteria were grown from frozen stocks on solid Luria-Bertani (LB) agar at 37°C, for 24 h. Prior to infection of cultured epithelial cells, all strains of *Salmonella* were washed three times in sterile phosphate-buffered saline (PBS), pH 7.4 and re-suspended to an OD600 nm of 1.0 (*S. Typhimurium LT2 and *S. Enteritidis*) or 1.2 (*S. Typhimurium* 4/74), equating to ~1x10^8 CFU/mL.

*S. Typhimurium* LT2, transformed with plasmid pEGFP carrying the enhanced green fluorescent protein gene *egfp*, was used in experiments examining bacterial translocation across *ex vivo* human follicle-associated epithelium (FAE) mounted in Ussing chambers, as previously described [21].

**Soluble plantain fibre (non-starch polysaccharide) preparation**

Non-starch polysaccharide (NSP) preparations from Confoco plantain flour (Trobana Green Plantain flour; Confoco International Ltd; Ripley, UK) were prepared by Provenix Plc (Windsor, UK) at the Teagasc Food Research Centre (Moorepark, Ireland). In brief, dry plantain flour was homogenised in reverse-osmosis purified water (ratio 1:2), heated to between 90°C and 100°C for 10 min with continuous high-shear mixing to effect starch swelling and gelatinisation. Following cooling to 25°C, the homogenate was treated with fungal 3-amylose Fungamyl® (Novozymes; Bagsvaerd, Denmark) for 2 h at pH 6–7. The mixture was then heated to 72°C for 20 min to fully inactive the Fungamyl enzyme. Insoluble NSP was removed by centrifugation and subsequently, low molecular weight components (<300 Da), including starch degradation products, were removed from the soluble NSP by nanofiltration. The concentrated retentate (containing in addition up to 45% by weight plantain-derived maltodextrin carrier as part of the bulk manufacturing process to counter difficulties in freeze-drying/resolubilisation) was spray-dried to a fine dry powder with a particle size distribution of 50–100 μm and a bulk density of 175 g/L (see File S4).

Plantain NSP concentrations tested for the *in vitro* studies were selected to be within the range of effective luminal concentrations in the human distal colon that would be readily achievable with dietary supplementation [20] (around 5 mg/mL, observed to inhibit adhesion of adherent, invasive *E. coli*, *Salmonella* and *Shigella* to human intestinal epithelial cell-lines [19–21]. For the *in vivo* study, given that chickens each have two caeca with a typical volume of about 1 mL each, usually emptied twice per day, a minimum dietary intake of soluble plantain fibre to give a maximum inhibitory effect on bacterial adhesion (achieved with a final concentration of 5 mg/mL [21]) was calculated to be ~20 mg/d. A typical chick feed intake is 20 g/d of which 5% (i.e. 1 g/d) would usually be fibre. Plantain NSP supplementation was therefore evaluated in the range of 0–200 mg/d/chick.

**Preparation of purified acidic and neutral polysaccharide fractions from soluble plantain NSP**

Initial analytical fractionation of soluble plantain NSP (1.6 g dissolved in 50 mL 50 mM Tris-HCl, pH 7.4) using a HiPrep™ Q-Sepharose® FF 16/10 anion-exchange column on an AKTA-prime plus liquid chromatography system (GE Healthcare Life Sciences, Chalfont St Giles, UK) demonstrated that bound acidic polysaccharides (as determined by uronic acid content) could be eluted step-wise in 50 mM Tris-HCl buffer containing 0.1, 0.5 and 1 M NaCl at a flow rate of 5 mL/min (data not shown). Using this information, a bulk preparation of both neutral and acidic fractions of soluble plantain NSP was then conducted using preparative Q-Sepharose® (counter-ion Cl⁻) Fast Flow anion-exchange medium (GE Healthcare) in a 2.5 litre container [46]. Q-Sepharose (300 mL) was washed extensively with three 1 L volumes of sterile deionised water and then equilibrated twice with 1 L of sterile-filtered 50 mM Tris-HCl buffer, pH 7.4. Plantain NSP (25 g) was added to 750 mL sterile 50 mM Tris-HCl, pH 7.4, mixed thoroughly for 1 h at room temperature and left to settle overnight at 4°C. The majority upper, clear layer (~700 mL) was removed, filtered under vacuum through a sintered glass funnel and Whatman No.1 filter paper, and then added to a 2.5 L mixing vessel containing Q-Sepharose® and rotated for 1 h, at 4°C. Unbound neutral polysaccharide was collected and filtered again. Following two 15 min washes with equilibration buffer to remove any residual unbound material, Q-Sepharose®-bound acidic polysaccharides were eluted with 800 mL 1 M NaCl in 50 mM Tris-HCl, with rotating overnight at 4°C.

Neutral and acidic polysaccharide fractions were then desalted using multiple pre-packed PD MidiTrap G-10 gravity mini-columns (1 mL per column), eluted with sterile deionised water as per the manufacturer’s instructions (GE Healthcare). Elution...
profiles for the purified neutral and acidic plantain polysaccharides on the G-10 mini-columns were established [File S5]. Fractions were assayed for total carbohydrate content, and the void fraction (approximate M<sub>v</sub> > 700) was collected. All columns were calibrated using phenol red (354 Da) as a low molecular size marker. Desalted fractions were shelf-frozen in round-bottomed glass vacuum flasks by immersion and rapid rotation in 100% ethanol containing dry ice. Flasks were stored for a least 20 min at ~80°C before lyophilisation overnight under vacuum. The total yield of acidic material from 25 g plantain NSP was 1.16 g (4.64% by weight); the total yield of neutral material was 4.21 g (16.84% by weight).

### Assessment of total carbohydrate and uronic acid content of chromatography fractions

Total carbohydrate content of NSP fractions was assayed using a modified method of Dubois et al. [47]. Briefly, 10 μL fractions were added to 96-well microtitre plates (Corning/Costar) in triplicate, and 100 μL of 4% (wt/vol) phenol dissolved in deionised water was added at room temperature for 5 min. Concentrated sulphuric acid (150 μL) was then rapidly delivered to all wells (with great care) and vigorously aspirated to generate heat required for reaction colour development. Plates were left to cool for 20 min and then measured for A<sub>540</sub>. Carbohydrate content of samples was determined using a calibration curve of d-glucose (0–20 μg/mL). Hexuronic acid content (d-glucuronic acid and d-galacturonic acid) was measured using a commercial K-URONIC assay (Megazyme International; Bray, Ireland). Increase in absorbance at 340 nm was determined upon incubation of fractions or 0–150 μg of d-glucuronic acid with uronate dehydrogenase in the presence of nicotinamide adenine dinucleotide (NAD<sup>H</sup>) at 25°C for 10 min, as per manufacturer’s instructions.

### Analysis of the hydrolysis products of plantain NSP, and the neutral and acidic NSP anion-exchange fractions

Plantain NSP and Q-Sepharose derived neutral and acidic NSP fractions were each hydrolysed with either 0.5% Driselase<sup>®</sup> (a commercial enzyme mixture of hydrolitic enzymes capable of digesting homogalacturonan and rhamnogalacturonan-I (RG-I) efficiently to galacturonic acid and associated neutral monosaccharides) or 2 M trifluoroacetic acid (TFA) as per [48]. Thin-layer chromatography (TLC) was performed on Merck silica-gel plates and on plates pre-washed for in acidic acetone to enhance mobility of the uronic acids. Each loading was derived from 25 μg of plantain NSP or polysaccharide fraction (or contained an equivalent amount of Driselase<sup>®</sup> or TFA). Plates were run under two solvent conditions, ethyl acetate/pyridine/acetic acid/water (6:3:1:1) and butan-1-ol/acetic acid/water (2:1:1), each followed by staining using thymol/H<sub>2</sub>SO<sub>4</sub>. To better determine galacturonic acid yields, high-voltage paper electrophoresis (HVPE) of the neutral and acidic NSP anion-exchange fractions was performed using Whatman No. 1 paper in pH 2.0 buffer at 4.7 kV for 80 min, with monosaccharide and oligogalacturonic acid markers included for reference. Staining was with aniline hydrogen-phthalate [49].

### Salmonella adhesion and invasion assays in mammalian and avian epithelial cells

The enterocyte-like BIOXI cell-line (BioNutriTech; Montpellier, France) originally thought to be from dissected colonic tissue of 19-day old chicken embryos [50], but recently verified as porcine in origin [51] was seeded at 1×10<sup>6</sup> cells/well in 24-well tissue culture plates (Costar; High Wycombe, UK) and maintained in advanced Dulbecco’s-modified Eagle’s medium (DMEM), supplemented with 5% (vol/vol) fetal calf serum (FCS) (Invitrogen; Paisley, Scotland). Media was supplemented with 100 μ/mL penicillin, 100 μg/mL streptomycin and 8 mM glutamine (Sigma-Aldrich; Poole, UK). Cultures were maintained at 37°C in a humidified atmosphere of 5% (vol/vol) CO<sub>2</sub>, 95% air for 24 h.

Prior to infection with Salmonella strains, confluent cells were washed three times with sterile PBS and cultured overnight in DMEM without antibiotics. Following 30 min pre-treatment of cells with or without solubile plantain NSP (0 to 10 mg/mL in antibiotic-free DMEM), cells were infected at a multiplicity of infection (MOI) of 20, for 90 min. Each monolayer was then washed with sterile PBS to remove non-adherent bacteria and adherence to, and invasion of, epithelial cells assessed by gentamicin protection assay, as per [19,21]. Cells were lysed with sterile 1% (vol/vol) Triton X-100, serial dilutions performed and bacteria enumerated in triplicate, following overnight growth on LB agar.

Additional experiments were also performed to determine whether action of plantain NSP to block Salmonella adhesion was via an action on the epithelial monolayer or direct interaction with bacteria. To test the former, plantain NSP was added to BIOXI cells 30 min prior to infection as described above, but then removed by three washes with sterile PBS (1 min each; at 37°C). Monolayers were then provided fresh antibiotic-free DMEM, infected and levels of adherent Salmonella assessed. To test for direct interaction with bacteria, plantain NSP was pre-incubated with Salmonella for 30 min, followed by centrifugation, resuspension of bacteria in anti-biotic free media and inoculation of epithelial cell monolayers.

Plantain NSP blockade of S. Typhimurium 4/74 was also examined in vitro using the human colorectal adenocarcinoma cell-line Caco2, as this isolate was to used in the in vivo studies, and to compare to our previous studies using S. Typhimurium LT2 [21].

As per previous studies [20,21], epithelial cell viability during plantain NSP treatment and infection was carefully monitored by measurement of adenylate kinase released to the culture medium using a ToxiLight<sup>®</sup> bioassay kit (Lonza; Walkersville, USA), and confirmed in selected experiments by Giemsa microscopy.

### Primary chick caecal crypt culture and Salmonella infection

Cysts were isolated from the caeca of 14-day old Lohmann Brown Classic egg-layer chickens and, in separate experiments, from the caeca of 33-day old Hubbard JA57 broiler chickens using methods adapted from Van Deun et al [52]. In brief, caeca were transported on ice and washed in Hank’s balanced salt solution (HBSS) supplemented with 20 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL gentamicin and 2 mM L-glutamine. Tissue was cut into smaller pieces and digested in a shaking water bath (120 rpm) at 37°C for 50 min. Cysts were then washed through a 200 μm nylon filter membrane, and collected in 40 μm filters. Cysts were subsequently plated into 6-well tissue culture plates coated with bovine Type I collagen (Inamed BioMaterials; Fremont, USA) and maintained in DMEM containing 2.5% (vol/vol) FCS, 40 μg/mL dispase (Roche; Little Chalfont, UK) and 150 U/mL collagenase XI (Sigma), in a shaking water bath (120 rpm) at 37°C for 50 min.

Cysts were then cultured with a flood of bacteria, plantain NSP treatment and infection was carefully monitored by measurement of cytoplasmic and invasive bacteria using a ToxiLight<sup>®</sup> bioassay kit (Lonza; Walkersville, USA), and confirmed in selected experiments by Giemsa microscopy.
supplements were from Sigma. Crypts were incubated at 37°C in 5% CO₂, 95% air, and washed daily with sterile PBS, and media was replaced with supplemented media but with reduced FCS and chicken serum (both 0.5% vol/vol). Crypt viability was determined by measurement of adenylate kinase released to the culture medium using a ToxiLight™ bioassay kit (Lonza).

Infection assays were all performed 4 d following initial isolation of caecal crypts (seeded at ~7×10⁴ crypts per well), with crypts (absent of any contaminating fibroblasts) pre-treated for 30 min either with soluble plantain NSP (10 mg/mL, in DMEM without antibiotics) or vehicle. S. Typhimurium 4/74 (4.2×10⁶ bacteria) were added to each well, prior to a 90 min incubation. Crypts were lysed with sterile 1% (vol/vol) Triton-X, serial dilutions were added to each well, prior to a 90 min incubation. Crypts were sequentially removed from each bird. Splenic and liver tissue were homogenised diluted 1:10 (wt/vol) in sterile PBS in a Colworth 80 stomacher (AJ Seward & Co. Ltd.; London, UK), divisible into treatments were obtained on day of hatch from the Pirbright Institute (Compton Laboratory; Newbury, UK), divided into treatments and housed in secure floor pens at a temperature of 30°C, followed by five washes with sterile PBS before pre-incubation for 20 min with either 5 mg/mL plantain NSP or Kreb's buffer vehicle, were previously performed in Ussing chambers as a daily intake of 12.5, 50 and 200 mg NSP per bird. At 8 d of age, chicks were inoculated by gavage with 4×10⁷ bacteria. Typhimurium LT2 (N=3 experiments, n = 4 replicates; *P<0.05, ** P<0.01, *** P<0.001, Kruskal-Wallis). Data (mean ± SEM) expressed relative to adherance (or invasion) of vehicle-treated control (100%).

Supporting Information

**File S1 Contains:** Figure S1: Soluble plantain NSP inhibits adhesion of S. Typhimurium strains LT2 and 4/74 to the human intestinal Caco2 cell-line in vitro. Pre-treatment (30 min) with soluble plantain NSP dose-dependently blocked (A) adhesion and (B) invasion of S. Typhimurium 4/74 to human Caco2 cells, at similar levels to that observed for S. Typhimurium LT2 (N=3 experiments, n=4 replicates; *P<0.05, ** P<0.01, *** P<0.001, Kruskal-Wallis). Data (mean ± SEM) expressed relative to adherance (or invasion) of vehicle-treated control (100%).

**File S2 Contains:** Figure S2: Soluble plantain NSP blocks adhesion of S. Enteritidis to the porcine enterocyte cell-line B1OXI in vitro. Pre-treatment with soluble plantain NSP at 10 mg/mL blocked (A) adhesion to, and (B) invasion of S. Enteritidis to B1OXI cells (N=3, n=4; ***p<0.001 Mann Whitney U). Data (mean ± SEM) expressed relative to adherance (or invasion) of vehicle-treated control (100%).

**File S3 Contains:** Figure S3A: Thin-layer chromatography (TLC) of whole plantain NSP, preparative Q-Sepharose neutral and acidic polysaccharide fractions and their hydrolysis products. Samples were hydrolysed with either trilluoroacetic acid (TFA) or Driselase. Each loading was derived from 25 μg of plantain NSP or polysaccharide fraction (the acidic fraction subjected to TLC had been reconstituted in physiological saline and contained ~70% by weight salt, and thus its loading was ~7.5 μg carbohydrate; blanks contained an equivalent amount of Driselase or TFA). All samples contained in addition up to 45% plantain-derived maltodextrin carrier (responsible for the glucose content). Samples were loaded on to
Merck silica-gel plates pre-washed in acidified acetone to enhance the mobility of the uronic acids. The running solvent was ethyl acetate/pyridine/acetic acid/water (6:3:1:1). The stain was thymol/H₂SO₄. 

**Figure S3B: High-voltage paper electrophoresis (HVE)** of whole plantain NSP, preparative Q-Sepharose neutral and acidic polysaccharide fractions and their hydrolysis products. Samples were hydrolysed with either trifluoroacetic acid (TFA) or Driaselae. Each loading was derived from 200 μg of plantain NSP or polysaccharide fraction (the acidic fraction subjected to HVPE had been reconstituted in physiological saline and was ~70% by weight salt, thus its loading was ~60 μg carbohydrate; blanks contained an equivalent amount of Driaselae or TFA). As before all samples contained in addition up to 45% plantain-derived maltodextrin. Electrophoresis was performed on Whatman No. 1 paper in pH 2.0 buffer at 4.7 kV for 80 min. Staining was with aniline hydrogen-phthalate.

(PDF)

**File S4: Contains:** Table S4: Water soluble non-starch polysaccharide preparation derived from plantain (*Musa AAB* (Hort)), in powder format, containing in addition up to 45% plantain-derived maltodextrin carrier, and nature-equivalent colours and flavours. (PDF)

**References**


