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Salmonella induces prominent gene expression in the rat colon
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Abstract

Background: Salmonella enteritidis is suggested to translocate in the small intestine. In vivo it induces gene expression changes in the ileal mucosa and Peyer’s patches. Stimulation of Salmonella translocation by dietary prebiotics fermented in colon suggests involvement of the colon as well. However, effects of Salmonella on colonic gene expression in vivo are largely unknown. We aimed to characterize time dependent Salmonella-induced changes of colonic mucosal gene expression in rats using whole genome microarrays. For this, rats were orally infected with Salmonella enteritidis to mimic a foodborne infection and colonic gene expression was determined at days 1, 3 and 6 post-infection (n = 8 rats per time-point). As fructo-oligosaccharides (FOS) affect colonic physiology, we analyzed colonic mucosal gene expression of FOS-fed versus cellulose-fed rats infected with Salmonella in a separate experiment. Colonic mucosal samples were isolated at day 2 post-infection.

Results: Salmonella affected transport (e.g. Chloride channel calcium activated 6, H+/K+ transporting Atp-ase), antimicrobial defense (e.g. Lipopolysaccharide binding protein, Defensin 5 and phospholipase A2), inflammation (e.g. calprotectin), oxidative stress related genes (e.g. Dual oxidase 2 and Glutathione peroxidase 2) and Proteolysis (e.g. Ubiquitin D and Proteosome subunit beta type 9). Furthermore, Salmonella translocation increased serum IFNγ and many interferon-related genes in colonic mucosa. The gene most strongly induced by Salmonella infection was Pancreatitis Associated Protein (Pap), showing >100-fold induction at day 6 after oral infection. Results were confirmed by Q-PCR in individual rats. Stimulation of Salmonella translocation by dietary FOS was accompanied by enhancement of the Salmonella-induced mucosal processes, not by induction of other processes.

Conclusion: We conclude that the colon is a target tissue for Salmonella, considering the abundant changes in mucosal gene expression.
Background

Foodborne infections cause a major burden on public health services and represent significant costs in many countries. *Salmonella* infection is one of the most common and widely distributed foodborne diseases and can be severe in the young, the elderly and patients with weakened immunity. *Salmonella enteritidis* is the most frequently isolated serotype, causing gastroenteritis in most humans and systemic infection in a subpopulation [1,2]. The precise mechanisms of *Salmonella*-host interaction in vivo at early time points after infection are not well known. Insight in pathogen-induced host processes in vivo could help to design therapeutic or nutritional strategies for infection prevention. An approach to investigate the effects of a pathogen on host target cells is the use of microarrays that contain the whole genome of the host. This broad approach can reveal biological processes affected by the pathogen. The rat is a good model to study *Salmonella enteritidis*-induced host processes, since salmonellosis in the rat shares many features of human disease [3]. Besides gastroenteritis, a self-limiting systemic infection is observed in rats. The ileum is thought to be the main site of *Salmonella* invasion in both humans and rats [4]. For this reason we have previously studied *Salmonella*-induced gene expression in the ileum of rats. This study showed that *Salmonella* affects only a small number of genes at early time points post-infection [5]. Carbohydrate transport, antimicrobial defense and detoxification were the main affected biological processes. At later time points large numbers of inflammation genes were found to be up-regulated in the ileal mucosa. The colon mucosa is supposed to be protected from *Salmonella* colonization by the abundant intestinal microflora. Pathogens entering the colon have to compete for nutrients and binding places with the endogenous flora. However, biopsies taken from humans during an infection with nontyphoid *Salmonella* serotypes suggest that the colon is involved in *Salmonella* infections [6-8]. As most studies focus on the ileum, which is thought to be the most likely site of translocation, only little information is available on *Salmonella* translocation in the large intestine [9]. Besides indications from studies on biopsies, we have another reason to suspect colonic involvement in *Salmonella* infection pathology. We have shown earlier that diets supplemented with prebiotics such as fructo-oligosaccharides (FOS), lactulose and inulin consistently increased intestinal *Salmonella* translocation in rats [10-13]. As fermentation of FOS, and other prebiotics, occurs in cecum and colon and is very limited in the ileum of humans [14] and rats [15], it is unlikely that prebiotics facilitated translocation of *Salmonella* at that particular site. This is supported by the absence of ileal inflammation in FOS-fed and *Salmonella*-infected rats in contrast to profound cecal and colonic inflammation [11]. To extend the current limited evidence indicating colonic involvement in *Salmonella* infection, we used transcriptional profiling to investigate genes and biological processes in the rat colonic mucosa affected by *Salmonella*. We first studied colonic mucosal gene expression responses at days 1, 3 and 6 after oral *Salmonella* infection of rats using whole genome microarrays and Q-PCR. In a second infection experiment, we studied whether the increased translocation of *Salmonella* by dietary FOS was reflected by amplification of *Salmonella*-induced gene expression changes in the colon.

Results

**Time course infection study**

*General infection characteristics*

In agreement with previous studies, food consumption and growth of the Wistar rats were not affected by *Salmonella* infection [16]. *Salmonella* translocation to mesenteric lymph nodes was observed at days 1, 3 and 6 (table 1). This implies that at day 1, *Salmonella* has already crossed the intestinal barrier. In agreement with previous studies [16,17], *Salmonella* was detected in the spleen at days 3 and 6 and in the liver at day 6 (table 1). Urinary NOx excretion, a parameter of systemic infection, was found to be increased from day 3 onwards (figure 1a).

*Salmonella*-induced processes in colon mucosa

To identify *Salmonella*-regulated processes, microarray-based gene expression profiling of colonic mucosa at days 1, 3 and 6 days p.i. was performed. The arrays contained 44000 spots of which 32783 spots exceeded >2 times the

**Figure 1**

*Sum of urinary nitrate and nitrite (NOx) excretion in the non-infected (◇), infected (▼) groups of the time course infection study (A). And the urinary NOx excretion in the cellulose infected (■) and in the fructo-oligosaccharide (FOS) infected (▲) groups in the dietary infection study (B). Infected rats were orally challenged with *S. enteritidis* on day 0. Results are expressed as mean ± SEM (n = 8 in the time course infection study and n = 6 in the dietary infection study). * p < 0.05.*
biological processes affected by Salmonella, we focused on biological processes with at least three genes exceeding the cut-off FC > 2.0. Additionally, we observed that the genes within one biological process showed comparable patterns of expression (table 2), which strongly indicates that these processes are truly affected by Salmonella.

We focused on the early Salmonella-induced gene expression changes occurring at days 1 and 3 p.i. Presumably, these early modulated genes are more related to Salmonella-induced primary changes than gene expression at day 6 which is a secondary result of Salmonella-induced inflammation. Genes affected >2-fold on day 1 and/or day 3 p.i. that could be related to a biological process are shown in table 2. The biological processes that contained 3 or more modulated genes were transport, oxidative stress, immune response, antimicrobial defense, inflammatory response, interferon pathways and proteolysis. For more insight into these processes, genes that changed >2-fold at day 6 p.i. and also showing a >1.5-fold induction at day 1 or 3 p.i. were also added to this table. Genes that changed >2-fold on day 6 p.i. only are shown in Additional File 1. The gene most affected by Salmonella infection in the colon was pancreatitis associated protein (Pap), showing 11, 45 and 114 fold induction at days 1, 3 and 6 respectively. Seventy genes changed >2-fold at day 1 p.i., of these genes 7 encoded for transporters and 5 genes encoded for immune response proteins (table 2). At day 3 p.i., 57 genes showed FC > 2, again including genes encoding for immune response proteins. Induced expression of Interleukin 1β and 1α indicates activation of an inflammatory response. Induction of dual oxidase 2 and glutathione peroxidase 2 suggest oxidative stress in the colonic mucosa. At day 6 more than 200 genes were induced more than 2-fold in infected mucosa compared with non-infected mucosa (Additional File 1). Most of these genes were related to immune and inflammatory responses. Processes related to inflammation-induced damage and repair, such as connective tissue remodeling and chemo-attraction also showed clear induction at day 6 p.i. To exclude the possibility that the observed changes were due to cellular changes of the mucosa, we analyzed expression differences of cell-type specific genes [20,21] (Additional File 2). As transporters are most likely expressed by enterocytes [22], we examined expression of enterocyte specific genes (Fahp2, Vil2, Alpi2). These genes showed diverse regulation, indicating that the increased expression of transporters at day 1 is not due to altered enterocyte composition in the mucosal samples. A similar observation was found for Goblet cell specific genes (Muc2, Muc3, Tff1, Tff3) and Paneth cell specific genes (RD-5 and Pla2g2a). Expression of leukocyte specific genes was not altered at early timepoint, a mild increase was observed at day 6 p.i. Together this indicated that the observed Salmonella induced gene expression changes did

Table 1: Viable Salmonella counts in feces, mesenteric lymph nodes, spleen, and liver of rats 1, 3 and 6 days post infection

|          | Control     | Salmonella (logCFU/g)* |
|----------|-------------|------------------------|
|          | Day 1 p.i.  | Day 3 p.i.  | Day 6 p.i.  |
| Feces    | N.D.        | 7.22 ± 0.19 | 5.92 ± 0.24 | 6.04 ± 0.32 |
| MLN      | N.D.        | 3.38 ± 0.43 | 5.85 ± 0.13 | 5.44 ± 0.05 |
| Spleen   | N.D.        | 3.20 ± 0.33 | 3.49 ± 0.05 |
| Liver    | N.D.        | N.D.        | N.D.        | 2.45 ± 0.12 |

*a The rats were orally infected with 3 × 10^9 colony-forming units of S. enteriditis or control treated. Salmonella counts are expressed in log values as means ± SEM (n = 8). N.D. = not detected.
Table 2: Processes regulated in colon by Salmonella at days 1, 3 and 6 after oral Salmonella infection

| Gene Name                                      | Gene symbol | Sequence ID | Fold Change infected vs non-infected rats on different days p.i. |
|------------------------------------------------|-------------|-------------|------------------------------------------------------------------|
|                                                |             |             | Time course infection studya                                       |
| Transport                                      |             |             | Day 1   | Day 3   | Day 6   | Day 2   |
| Ion transport                                  |             |             |         |         |         |         |
| Chloride channel calcium activated 6           | Cka6        | NM_201419   | 2.3    | 2.2    | 3.7     | 2.3     |
| Calcium channel, voltage-dependent, alpha 1 subunit | Cacna1l     | NM_020084   | 2.2    | 1.5    | -       | 1.2     |
| Solute carrier family 4, member 1 (Slc4a1)     | Slc4a1      | NM_012651   | 2.0    | 1.7    | -       | 1.0     |
| Asp-ase, H+/K+ transporting, nongastric, alpha polypeptide | Atp1a2     | NM_133517   | 2.8    | -      | 2.0     | 1.1     |
| Solute carrier family 20 (phosphate transporter), member 1 | Slc20a1   | NM_031148   | 2.1    | -      | -       | -1.2    |
| Solute carrier family 15 (oligopeptide transporter), member 1 | Slc15a1   | NM_057121   | 2.0    | 1.5    | 1.6     | 0.9     |
| Transporter 1, ATP-binding cassette, sub-family B | Mrdr/Tap1  | NM_032055   | -      | 1.8    | 2.8     | 1.4     |
| Oxidative stress                               |             |             |         |         |         |         |
| Dual oxidase 2                                  | Duox2       | NM_024141   | 1.9    | 2.4    | 2.8     | 1.6     |
| Glutathione peroxidase 2                        | Gpx2        | NM_183403   | -      | 2.3    | 3.0     | 2.2     |
| Xanthine dehydrogenase                          | Xdh         | NM_017154   | -      | 1.8    | 2.5     | 1.4     |
| Immune response                                |             |             |         |         |         |         |
| Rat class III Fc-gamma receptor                | Fcgr3       | XM_64368    | 2.1    | 1.6    | -       | ND      |
| Immunoglobulin superfamily, member 4           | Igf4d       | XM_340958   | 2.1    | 1.6    | 1.4     | 1.1     |
| Rat MHC class I truncated cell surface antigen | RT1-Aw2     | M10094      | 2.0    | -      | 1.9     | 1.0     |
| Interleukin enhancer-binding factor 1           | Il1f        | XM_221212   | 2.0    | 1.5    | -       | 1.1     |
| Colony stimulating factor 2 (granulocyte-macrophage) | Gsf2      | XM_340799   | 2.0    | 1.6    | -       | 1.2     |
| Interleukin 1 alpha                             | Il1a        | NM_017019   | 1.8    | 2.0    | 2.3     | 1.4     |
| Interleukin 1 beta                              | Il1b        | NM_031512   | -      | 2.1    | 4.0     | 2.6     |
| TRAF2 binding protein                           | T2bp        | NM_0011404  | 1.8    | 2.8    | 4.4     | 2.4     |
| Toll-like receptor 2                            | Tlr2        | NM_198769   | -      | 1.5    | 2.3     | 1.3     |
| Antimicrobial defense                           |             |             |         |         |         |         |
| Lipopolysaccharide binding protein              | Lbp         | NM_017208   | 1.9    | 1.8    | 2.2     | 1.3     |
| Defensin 5 precursor (Enteric defensin)          | Rd-5        | XM_214386   | -1.9   | -1.6   | -1.6    | ND      |
| Phospholipase A2, group IIA (platelets, synovial fluid) | Pla2g2a    | NM_031598   | 3.4    | 5.2    | 10.5    | 7.3     |
| Inflammatory response                           |             |             |         |         |         |         |
| Pancreatitis-associated protein                 | Ptp         | NM_033889   | 11.4   | 44.6   | 114.2   | 17.7    |
| Tissue-type transglutaminase                    | Tgm2        | NM_019386   | -      | 2.3    | 4.9     | 1.8     |
| Regenerating islet-derived 3 gamma             | Reg3g       | NM_173097   | -      | 2.3    | 4.3     | 1.9     |
| Nitric oxide synthase 2, inducible             | Nos2        | NM_012611   | -      | 1.6    | 4.0     | ND      |
| S100 calcium binding protein A8 (calgranulin A) | S100a8      | NM_033822   | -      | 1.8    | 2.4     | 1.4     |
| S100 calcium binding protein A9 (calgranulin B) | S100a9      | NM_053587   | -      | 1.7    | 1.9     | 1.3     |
| Interferon                                     |             |             |         |         |         |         |
| Interferon-induced guanylate-binding protein 1  | Gbp1        | XM_221883   | 2.4    | 1.9    | 2.2     | 1.3     |
| Interferon gamma inducible protein              | Ifi47       | NM_172019   | 1.7    | 2.7    | 7.3     | 2.6     |
| Guanylate binding protein 2, interferon-inducible | Gbp2      | NM_133624   | 1.6    | 2.4    | 3.1     | 1.7     |
| Interferon-induced protein                     | Ifi2        | NM_00102475 | 1.5    | 1.7    | 3.4     | 1.3     |
| Interferon-stimulated protein                  | Gip2        | XM_216605   | -      | 1.6    | 4.1     | 1.5     |
| Immunity-related GTPase family, M              | Ifgm        | NM_00101200 | -      | 1.8    | 3.7     | 1.4     |
| Signal transducer and activator of transcription | Stat1      | NM_032612   | -      | 1.7    | 3.6     | 1.8     |
| Interferon regulatory factor 7                 | If7         | XM_215121   | -      | 1.5    | 2.6     | ND      |
| Alpha-interferon                               | Ifna        | XM_233145   | -      | 2.0    | -       | ND      |
| Proteolysis                                    |             |             |         |         |         |         |
| Ubiquitin D                                    | Ubd         | NM_053299   | 1.7    | 2.5    | 15.2    | 3.4     |
| Proteasome (prosome, macropain) subunit, beta type 9 | Pmb9     | NM_012708   | -      | 2.0    | 3.7     | 2.0     |
| Protease, serine, 22                           | Prss22      | XM_220222   | -      | 2.0    | 2.6     | 1.6     |
| Potential ubiquitin ligase                     | Herc6       | XM_342700   | -      | 1.7    | 3.3     | 1.5     |
| Proteasome (prosome, macropain) subunit, beta type 10 | Pmb10     | XM_214687   | -      | 1.5    | 2.1     | 1.5     |

*Values in bold exceed cut-off value FC > 2 or FC < -2. Values -1.5 < FC < 1.5 are indicated by (−).

*a Fold Change infected vs non-infected rats fed a cellulose diet at day 2 p.i (obtained from the dietary infection study). All fold changes are shown. Genes not detected in this independent study are indicated by ND.
not result from changes in cellular composition of the mucosa. This is in agreement with histology results from earlier Salmonella infection experiments, showing no or only minor deviations in intestinal mucosal architecture from healthy control slides (data not shown). This is further supported by the relatively constant expression of a group of well known housekeeping genes (Additional File 2). The largest group of related genes induced by Salmonella infection in colon mucosa is related to interferon pathways as more than 20 IFNγ-regulated genes showed increased expression at least one time point studied. The IFNγ-induced gene expression was most prominent at day 6 p.i. but already from day 1 onwards induction of several IFNγ-inducible GTPases (Gbp1, Gbp2, Ifi47, Ifit2) was seen (table 2). Furthermore at day 3 p.i. (table 2) induction of two members of the IFNγ-signaling pathway (Stat1 and Irf7) was observed. Despite induction of many interferon-related genes, increased expression of IFNγ mRNA itself could not be detected (changed 1.1-fold at days 1 and 3, and 1.3-fold at day 6 p.i.). IFNγ protein concentrations were measured in individual serum samples. IFNγ was not detected in serum of non-infected rats (all timepoints) and at the first day after Salmonella administration to rats. However, from day 3 p.i. serum IFNγ increased (figure 3). The serum IFNγ most probably originated from peripheral immune activation, as the increase in serum IFNγ followed the same trend as the increase in Salmonella CFU's in peripheral organs (table 1). In the time course infection study, the kinetics of urinary NOx excretion are reflected by Nos2 gene expression in colonic mucosal with a small 1.6-fold induction at day 3 p.i. and a 4 fold induction at day 6 p.i. (table 2).

Q-PCR confirmation of Salmonella-induced gene-expression
To determine inter-individual variation in gene expression within treatment groups, RNA from the colon of individual animals was analyzed by Q-PCR. We chose individual confirmation of Stat1 and Irf47 to gain insight in inter individual interferon response as we also focused on the individual protein levels of IFNγ. Confirmation of PAP was chosen to obtain insight in the individual kinetics of the most strongly induced gene in colon mucosa at all time points. Q-PCR analysis showed rather large inter-individual variation among the outbred rats. PAP expression levels in the non-infected colonic mucosa were near detection level, which made it difficult to determine precise fold changes. Nevertheless, the Q-PCR analysis of the three genes examined clearly confirmed the gene expression changes observed in the microarray analysis (figure 4). To further validate the array data of the time course infection study we compared the gene expression changes of day 1 and 3 p.i. with gene expression data obtained from the independent dietary infection study at day 2 p.i. (table 2). Similar biological processes were induced at early timepoints in both studies. At individual gene expression level several transporter genes (Cacna1i, Slc6a1, Slc15a1) and immune response genes (Igsf4d, Iif1, Csf2) showed no overlap possibly due to infection kinetics. However gene expression results of day 3 and day 2 p.i. largely overlapped (table 2).

Dietary infection study
General infection characteristics
Food consumption and growth of the Wistar rats on both cellulose and FOS diet were similar, before and after infection. The section was performed on day 2 p.i. because similar effects on gene expression at days 1 and 3 were observed in the time course infection study. Furthermore we were interested in the primary responses as we expect that diet will mainly influence early events such as attachment to the mucosa and translocation of the pathogen. These events occur for the most part luminal where direct interaction between dietary components, pathogens and mucosa take place, while later phases merely reflect secondary infection and inflammation responses. At day 3 p.i. the serum IFNγ concentration and the amount of translocated Salmonella in the spleen are already high, which indicates systemic infection. At day 1 p.i. no serum IFNγ or Salmonella translocation to the spleen was observed. We chose day 2 p.i. as this seems the appropriate time point to study the effects of FOS on early Salmonella-induced changes. Salmonella colonization was quantified by determination of colony-forming units (CFU/g) in fresh fecal samples with time. At day 1 Salmonella levels were not significantly different between cellulose and FOS-fed animals (7.23 ± 0.11 and 7.10 ± 0.22, respectively). At day 2 FOS-fed rats had more Salmonella in feces than their cellulose counterparts (7.25 ± 0.25 and

Figure 3
Serum IFNγ levels before and after infection (days 1, 3 and 6 p.i.). Each dot represents an individual rat. Group medians are presented by a black line. * p < 0.05, ***p < 0.001.
6.53 ± 0.25, respectively; p < 0.05). Salmonella translocation to mesenteric lymph nodes and spleen was not significantly different in the FOS group compared to the cellulose group when quantified by CFU. Viable Salmonella counts in MLN were 5.96 ± 0.08 in the cellulose group and 6.19 ± 0.10 log_{10} CFU/g in the FOS group. Numbers in spleen were 2.85 ± 0.14 (cellulose) and 2.98 ± 0.16 log_{10} CFU/g (FOS). Counts in liver were under the detection limit of 10^2 CFU/g tissue in the cellulose and FOS group. These numbers are comparable to those observed in the time course infection study at day 3 and highly similar to numbers observed in earlier studies which showed increased translocation in FOS-fed rats at later time points after infection [23]. To observe long term effects of FOS on Salmonella translocation in this study, urinary NOx excretion with time was determined in additional groups of rats. Urinary NOx excretion of FOS-fed rats increased to 132 µmol/d at day 6 p.i. and started to decline towards baseline levels thereafter (figure 1B). Peak urinary NOx excretion of infected rats fed the cellulose diet was just one third of the level reached by the infected rats fed the FOS diet, i.e. 41 µmol/day (figure 1B). The NOx values for the cellulose diet are similar to those obtained in the time course infection study (figure 1A). The kinetics of urinary NOx excretion were similar in both diet groups, but total infection-induced urinary NOx excretion was higher in the FOS group indicating enhanced Salmonella translocation.

Effect of dietary FOS on Salmonella-induced mucosal genes in colon mucosa

The rats of the time course infection study and the rats in the dietary infection study on cellulose diet showed a comparable urinary NOx excretion and thus Salmonella translocation response. Despite the fact that the two studies were separately performed and different time points were studied, the identified biological processes affected by Salmonella at day two p.i. were comparable to processes observed at days 1 and 3 p.i. Furthermore both studies showed that more genes were up-regulated than down-regulated by Salmonella. For detailed analysis, we focused on the most robust genes, i.e. genes that showed similar Salmonella induced regulation in the two independent studies. We choose a threshold of FC < 1.5 for both studies, which is less stringent than the threshold we choose for analysis within one study (FC < 2.0). We feel that this is legitimate, as genes with small but similar regulation in two completely independent studies are less likely to be selected by chance. Thirty-one genes fulfilled this criterion, 26 were up-regulated and 5 down-regulated. Eighteen of the up-regulated genes were categorized to the same processes found to be modulated by Salmonella in the colonic mucosa in the time course infection study, i.e. the transporter Clca6, the oxidative stress genes Gpx2 and Duox2, the immune response genes Il1b and T2bp, the

![Figure 4](image-url)

**Figure 4**

Individual expression of two interferon-related genes and Pap in the colon mucosa at different time points after Salmonella infection or control treatment. Genes expression is quantified by Q-PCR, using Rps26 as reference gene (using Arf1 as reference gene showed similar results; data not shown). Each dot represents an individual rat. Dotted line indicate lowest mRNA standard. Medians are presented by a black line. The median value of the uninfected group was set at 1.0. Y-axis is at log_{10} scale. * p < 0.05, **p < 0.01, ***p < 0.001.
antimicrobial defense gene Pla2g2a, the inflammatory response genes Pap, Tgm2 and Reg3g, the interferon related genes Ifi47, Gbp2, Iggp2, P47Iggp, Stat1, G1p2 and the proteasome related genes Psmb9, Prss22, Psmb10, Ubd (Table 2). The other 8 up-regulated genes which could not be grouped to a specific process were Palmitoyl-protein thioesterase, Schlafen 3 (Slfn3), Tripartite motif protein 15 (Trim15), Aquaporin 3 (Aqp3) and four unknown genes. The 5 down-regulated genes were Heat shock protein 70 kD 1A (Hspa1a), Resistin like alpha (Retnla), Resistin like gamma (Retnlg), Collectin sub-family member 10 (Colec 10) and Mammalian suppressor of Sec4 (Mss4). Not all processes that were identified in the time course infection study at both days 1 and 3 p.i. were confirmed in the dietary infection study at day 2 p.i. (table 2). This was the case for two processes, namely transport (Cacna1i, Slc4a1, Atp12a, Slc15a1) and immune response (Igsf4d, RT1, Ifi1, Csf2, I11a). Furthermore the antimicrobial defense gene Lbp and two interferon pathway genes (Gbp1, Ifit2) were not confirmed. To examine whether our choice for FC > 1.5 was legitimate, we studied whether application of threshold FC > 1.3 and FC > 1.7 resulted in identification of the same processes as identified with FC > 1.5. The general picture of processes affected was the same for FC > 1.5 and FC > 1.7. However, with FC > 1.3 more genes could be included in processes identified with FC > 1.5, such as the interferon response and proteolysis (data not shown). However, many other genes could not be grouped into (new) specific biological processes, indicating that a cut-off FC > 1.3 might be too flexible and results in introduction of false positive processes, probably not related to the treatment. Therefore, we choose FC > 1.5 for further analysis. To investigate the effects of FOS on Salmonella infection in the colon, we studied the expression of Salmonella-induced colonic mucosal genes in infected rats fed the cellulose diet versus infected rats fed the FOS supplemented diet. The five genes that were consistently downregulated by Salmonella in both studies (Hspa1a, Retnla, Retnlg, Colec 10 and Mss4) were not further influenced by FOS (equal gene expression in cellulose- and FOS-fed infected rats). For initiating early mucosal events after Salmonella infection (e.g. chemo attraction of inflammatory cells) increases in epithelial gene expression may be more important than decreases[24,25]. We focused on the 26 genes which showed a consistent increase in gene expression after Salmonella infection of FC > 1.5 in both studies. All 26 genes consistently induced by Salmonella infection in the colon mucosa showed a further up-regulation in colon mucosa of Salmonella infected rats fed FOS (figure 5). The effect of FOS on the cluster of Salmonella affected genes was statistically significant. To assess the inter-individual gene expression in the dietary infection study we selected genes from several Salmonella modulated process for individual Q-PCR confirmation: Clca6, Gpx2, Il1b, Pla2g2a, Pap, Tgm2, Stat1, Gbp2 and Ifi47. Q-PCR of the selected genes in individual samples showed high inter-individual variation but confirmed the fold changes of the microarray study using pooled samples (table 3). The confirmed Salmonella induced gene expression changes were significant (p < 0.05) for the 9 genes, except for Tgm2 (p = 0.09) and Stat1 (p = 0.08). Examination of FOS-fed versus cellulose-fed infected groups on individual gene level showed a significant increase of Clca6 and Pla2g2a. Expression of Gpx2, Il1b and Tgm2 was >1.5-fold increased by FOS feeding in comparison to cellulose feeding but this was not statistically significant. The genes Pap, Stat1, Ifi47 and Gbp2 showed non-significant and small increases of 1.1–1.4 fold. In t-testing each gene is tested independently, the FOS vs cellulose effect was not statistically significant for each independent gene. However, FOS significantly increased expression of the cluster of the 26 Salmonella induced genes (see figure 5 and Additional File 3). We also looked at overall gene expression differences between cellulose- and FOS-fed rats at day 2 after Salmonella infection (Additional File 3). This was done to determine whether the stimulated translocation in FOS-fed rats resulted in additionally affected genes or biological processes not induced by Salmonella in the cellulose groups. Twenty genes were induced by Salmonella >2-fold in cellulose-fed infected rats. In the FOS-fed infected rats 72 genes were induced by Salmonella >2-fold. Seventeen

Figure 5
Expression level of most consistent Salmonella-target genes in colon mucosa of rats fed a cellulose diet or a FOS diet. The gene expression is obtained from microarray analysis of pooled colonic mucosa samples collected at day 2 post-infection. Each dot represents a gene. The median value of each gene in the uninfected group is set to 1.0. Y-axis is at log2-scale. ***p < 0.001.
Table 3: Q-PCR analysis of Salmonella-induced colonic mucosal gene expression of rats on a cellulose or a FOS diet (day 2 p.i.)

| Gene symbol | Non-infected | Infected |
|-------------|-------------|----------|
|             | Cellulose   | FOS      |
| C1ca6       | 1 (0.9–1.3) | 1.6 (1.4–2.2) | 3.7 (2.8–4.1) |
| Plo2g2a     | 1 (0.8–1.3) | 4.6 (2.3–7.1) | 8.8 (6.1–21.1) |
| Gpx2        | 1 (0.8–1.2) | 2.1 (1.7–2.4) | 3.2 (1.3–4.8) |
| Ifi47       | 1 (0.6–1.1) | 2.0 (1.3–3.4) | 3.8 (0.6–5.4) |
| Tgm2        | 1 (0.5–1.2) | 1.6 (0.7–4.4) | 10.2 (0.8–20.6) |
| Pop         | 1 (0.4–2.4) | 236 (68–326) | 288 (13–1162) |
| Stat1       | 1 (0.7–1.2) | 1.2 (1.1–2.2) | 1.5 (0.7–4.0) |
| Relmα       | 1 (0.8–1.2) | 3.9 (2.1–6.4) | 5.6 (1.6–18.4) |
| Gbp2        | 1 (0.8–1.4) | 2.0 (1.1–3.2) | 2.1 (0.5–9.7) |

*The expression of genes is analyzed by QPCR, using Rps26 as reference gene (using Arf1 as reference gene gave similar results; data not shown). Data are represented as median (25% percentile–75% percentile).

**The median value of the non-infected group is set to 1.0.

Gene expression of rats on a cellulose or a FOS diet (day 2 p.i.)

- In the cellulose-fed group, gene expression was observed from day 5, no differences at NOx excretion between infected cellulose- and FOS-fed rats were observed from day 5, no differences at Nos2 gene expression were expected at day 2 p.i. Serum IFNγ was not detected at day 2 p.i., neither in infected cellulose-fed rats nor in infected FOS-fed rats.

**Discussion**

**Colon is an infection target**

This study shows quick and profound gene expression changes in the rat colon mucosa upon oral S. enteritidis infection, which implicates that not only the ileum, but also the colon, is a target for Salmonella infection. The earliest responses were noticed on mucosal transport and antimicrobial defense. The most responsive gene is Pap, which showed an 11-fold induction in colon mucosa on the first day after infection and increased to over 100 fold at day 6. At later timepoints, the most notable process affected is interferon-related. Colonic genes consistently induced by Salmonella infection in two independent studies, were all further enhanced by FOS supplementation, a known stimulus of colonic bacterial fermentation. Salmonella, ingested with contaminated foods or drinks, is thought to colonize the distal small intestine and to translocate through ileal Peyer’s patches to extra-intestinal organs [4,26]. Several observations suggest that other parts of the intestine are also involved in Salmonella infection. High numbers of Salmonella are found in the cecum and colon of orally infected rats [4,26] as well as pigs [27]. In humans Salmonella commonly affects the small intestine, but colonic involvement of S. enteritidis has been reported in humans [6,7,28] and may play an important role in induction of diarrhea [6]. Studies describing mucosal invasion via the paracellular and transcellular route [29,30] also suggest that translocation of Salmonella species to the systemic circulation is not restricted to the ileal Peyer’s patches. Our studies on the effects of prebiotics on resistance of the host to Salmonella infection also point to the colon as invasion site [11]. Together results from literature and those presented here indicate that the colon is one of the targets for Salmonella infection.

**Interferon-gamma response**

The increase of many IFNγ-regulated genes in the Salmonella-infected colon in the present in vivo study actually confirms the earlier suggested role of IFNγ in relation to host defense against Salmonella. Serum IFNγ levels increase in mice infected with Salmonella by oral or intraperitoneal route [31-34]. IFNγ is produced by natural killer cells, CD4 Th1 cells and CD8 cytotoxic lymphocytes [35-37]. IFNγ most likely exerts its function in host defense by activation of macrophages which can kill Salmonella [38]. In our Salmonella time course infection study, more than 20 IFNγ-related genes were up-regulated (table 2). This did not coincide with an increased IFNγ mRNA level at any of the time points studied. In addition, we could not detect IFNγ protein in Salmonella-infected colons (data not shown). Serum levels of this pro-inflammatory cytokine were undetectable at day 1 p.i. but rose steadily from day 3 p.i. with large inter-individual variation in the magnitude of response (figure 3). Despite the lack of detectable IFNγ protein in colonic mucosa and in serum at day 1, we did observe increased expression of genes in the INFγ induced pathway at that timepoint. These genes are most likely activated by INFγ [39]. We can not fully exclude that dilution of IFNγ-producing cells in the heterogeneous cell population of mucosal scrapings has lead to undetectable levels of this regulatory cytokine in the present study. At the later timepoints, serum IFNγ is strongly increased, whereas mucosal IFNγ remained below detection levels at all timepoints. This may suggest that systemic rather than colonic IFNγ seems to be the trigger for the later activation of IFNγ-related genes and processes in colonic mucosa upon Salmonella infection. However, dilution of IFNγ producing cells in colonic tis-
PAP might be used as a non-specific marker to follow and quantify intestinal infection or inflammation in humans.

Calprotectin
Calprotectin (S100a8/a9), a heterodimer of the two calcium-binding proteins S100A8 and S100A9, was up-regulated in the colonic mucosa by Salmonella (Table 2). Both subunits were increased in colon. Calprotectin is a 36 kDa calcium and zinc binding protein and constitutes approximately 60% of soluble cytosolic proteins in neutrophil granulocytes. Therefore, calprotectin is a marker of neutrophil influx and is elevated in a number of inflammatory conditions. In agreement with our results, Naughton et al (1996) also found increased levels of this marker in Salmonella-infected animals. Fecal calprotectin is emerging as a useful marker to quantify mucosal inflammation, not in the least because it appears to be stable in feces which can be obtained by non-invasive means [48].

Differences between colon and ileum
Ileum and colon are both targets for Salmonella. Remarkably, the number of genes showing increased expression is larger than the number of genes showing decreased expression upon Salmonella infection in both ileum and colon. However, this is more extreme in colon than in ileum mucosa [5]. Technical bias is unlikely as in a flavonoid intervention study with rats and using the same array system and data handling the number of down-regulated genes was similar to the number of up-regulated genes [49]. In an in vivo Salmonella infection study in pigs only up-regulated and no down-regulated genes were observed [42].

The extent of the early response to Salmonella is similar for both intestinal segments: From all genes expressed above background level on the arrays, 0.21% of the genes expressed in the colon and 0.26% of genes expressed in the ileum [5] were affected at day 1 p.i. The colonic response is less than the ileal response at day 3 p.i., as 0.15% of colonic mucosal genes were affected versus 0.67% of ileal mucosal genes. The smaller colonic response could be due to differences in crypt-villus architecture of the ileal and colonic mucosa. Furthermore, the colonic mucosa, which is constitutively exposed to bacteria, might be more efficient in repressing host- or more specifically immunological responses to bacteria, including pathogens [50-52].

Ileum and colon show overlapping as well as distinct processes affected upon oral infection [5]. At early time points after oral infection i.e. transport processes and antimicrobial defenses were regulated in both intestinal segments, but the process-related genes did not fully overlap. At day 1 p.i., glucose transporters were increased in the ileum, whereas in colon ion transporters were induced.
The role of ion transporters in water absorption support involvement of the distal part of the gut in diarrhea development during salmonellosis as reported earlier in humans [6]. The gene coding for antimicrobial defensin 5 was down-regulated by *Salmonella* in both ileum and colon. Other genes coding for antimicrobial proteins (*Pla2g2a* and lysozym) were clearly enhanced in the infected colon in contrast to ileal tissue [5]. At day 3 and 6 p.i. *Salmonella* reduced the expression of several phase I and II detoxification genes in the ileum, which was not observed in the colon. The downregulation of cytochrome P450 genes in ileum coincided with increased expression of inflammatory genes. It is known that inflammatory mediators can down-regulate cytochrome P450 genes [5,53,54]. This might suggest that the inflammatory response induced by *Salmonella* in colon, at later timepoints, is smaller than in ileum. Nevertheless, both tissues showed signs of an inflammatory response at later timepoints, but responsible genes were not the same. Mainly cytokines and chemokines were induced in the ileum, whereas in colon many interferon-related genes were up-regulated. No interferon response was observed in the ileum. Apparently, the immune response in the two intestinal segments is differentially regulated.

Finally, the *in vivo* transcriptional response of intact mucosa to infection by *Salmonella* is represented by a limited number of regulated genes compared to *in vitro* studies with HT-29 cells [24]. *In vitro* models provide insight in complex mechanisms of *Salmonella*-host interaction. However, results should be interpreted with caution as *in vitro* systems show massive cell death at 24 hours, whereas only minor inflammatory changes are observed in the intestine 24 hours after infection with *Salmonella in vivo* [55]. Several genes like Toll like receptors, Nf-kb or Il-8 that are regulated by *Salmonella in vivo*, were not found to be regulated by *Salmonella* infection in the present *in vivo* study. Possibly, transcription of these genes is highly specific for particular cell types in the colonic mucosa. Identification of cell type-specific responses of potential target cells could be addressed *in vivo* using laser microdissection.

**FOS and mucosal barrier function**

We consistently observed that diets supplemented with rapidly-fermentable prebiotics (such as FOS) increased translocation of *S. enteritidis* in rat infection studies despite stimulation of *Bifidobacteria* and *Lactobacilli* [23]. In other words, FOS decreases the resistance of the rat intestinal mucosa to intestinal pathogens. Because fermentation of FOS hardly occurs in the ileum of humans [14] and rats [15], it is unlikely that prebiotics facilitated translocation of *Salmonella* in the ileum. This is supported by the absence of ileal inflammation in FOS-fed and *Salmonella*-infected rats in contrast to profound cecal and colonic inflammation [11]. The precise mechanism underlying the effects of FOS on the colon mucosa is not known. FOS itself, the changed intestinal microflora or its fermentation products (e.g. SCFA) could play a role. Prebiotics, such as FOS resist enzymatic hydrolysis by digestive enzymes secreted in the small intestine and reach the colon intact. The resident colonic microflora ferments these carbohydrates to lactic acid and short-chain fatty acids (SCFA). This results in lowering of the pH of intestinal contents and stimulation of e.g. *Bifidobacteria* and *Lactobacilli* [23,56]. These lactic acid bacteria are assumed to enhance resistance [12] but we found opposite effects [11,23]. As shown earlier, dietary FOS increases intestinal permeability in non-infected rats and even more in infected rats [13]. At present it is unknown whether intestinal permeability is increased in ileum or colon, nor whether it is induced by the presence of FOS or by its fermentation metabolites. It has been shown that SCFA can induce colonic mucosal injury and increase permeability [10]. Furthermore, *in vitro* studies showed that SCFA can enhance expression of virulence (e.g. invasion) genes of *Salmonella* typhimurium [57,58], but data on *in vivo* consequences have not been reported. Preliminary experiments of our lab showed no evidence for increased expression of virulence genes of *Salmonella enteritidis* in infected rats fed a FOS-diet (unpublished results).

From two independent rat infection studies we identified 26 colonic mucosal genes consistently affected by *Salmonella*. These ‘robust’ *Salmonella* target genes were all further induced by the FOS diet. The pronounced effects of FOS on *Salmonella* translocation were reflected by a modest but highly consistent increase of all *Salmonella* target genes. Moreover, the total number of genes induced by *Salmonella* is nearly 3 times higher in FOS-fed rats than in cellulose-fed rats. However, biological processes identified to be affected by *Salmonella* in colon of FOS-fed rats were not different from those observed in cellulose-fed rats. So, the quality of the colonic response was the same, but clearly the magnitude of the response was increased by FOS feeding. Based on the physiological effects, larger gene expression differences might have been expected. The modest responses observed might be due to our focus on *Salmonella*-induced gene expression. It can not be totally excluded that FOS targets other genes and processes related to barrier function (in absence of infection) than *Salmonella*. However, the genes affected by *Salmonella* in FOS-fed rats did not show involvement of additional processes in comparison to their cellulose-fed counterparts. In our view, the enhanced expression of colonic *Salmonella* target genes in FOS-fed animals concomitant with stimulated translocation of this invasive pathogen indicates that infection and related inflammation is worsened by FOS supplementation. Histological analyses of intestinal samples from previous FOS intervention studies of
our lab did not show presence of intestinal mucosal inflammation in non-infected FOS-fed rats in contrast to post-infection samples (data not shown). Therefore, we feel that the observed aggravation of the intestinal response is due to interaction of FOS and Salmonella.

It should be stressed, that genes identified as Salmonella target genes in the present study are not necessarily Salmonella specific, but may well result from colonic inflammation in general and thus be similar in other enteric infections. Furthermore, effects of dietary FOS on gut barrier function may not be restricted to changes in mRNA expression, but exist on the translational or functional level of proteins. For instance, internalization of the tight-junction proteins occludin, claudin and junctional adhesion molecule-A, caused by IFNγ, results in profound mucosal barrier changes [59]. This cellular translocation can occur without concomitant changes in mRNA gene expression. Detection of such effects would require a different approach from transcriptomics. Many studies report on possible therapeutic effects of FOS on intestinal disease such as IBD and pathogenic infection. In addition to an increase in "beneficial" bacteria, the potential beneficial effects of FOS are based on the effects on surrogate markers, e.g. increase of mucin production [60], increase of the size and cytokine production of Peyer's patches and increased faecal or ileal IgA [61-63]. Changes in these markers are often presumed to reflect increased barrier function or resistance to pathogenic bacteria, but concomitant actual measurements of these functional effects are missing. In our study, genes involved in antimicrobial defense, immune response and inflammation were all induced by Salmonella infection and further enhanced by dietary FOS, but concomitantly translocation of Salmonella was evident and stimulated by FOS. Therefore, these surrogate markers should be interpreted with caution and always correlated with functional effects or clinical endpoints.

In this study we compared Salmonella-induced gene expression changes of two independent rat infection experiments at early time points after oral infection. The gene expression results were analyzed at two levels, at the level of gene expression itself and at the level of biological processes. Analysis at the level of gene expression showed some variation in the expression of individual genes between the two studies (table 2, Additional File 3). This variation between two studies can be due to the different time-points studied and the use of outbred rats showing inter-individual differences in infection kinetics. Rats did not all respond to Salmonella at the same time p.i. which is e.g. shown by individual gene expression levels of PAP (figure 4) and by serum IFNγ levels (figure 3). Variation in infection kinetics and inter-individual variation are expected features of infection studies in outbred species [64]. It can be argued that differences between studies, due to differences in time points measured or infection kinetics, will result in more pronounced variance at the level of individual genes than at the level of physiological processes [65,66]. Indeed, analysis of gene expression at the level of biological processes showed that both studies gave highly comparable Salmonella-induced effects at early time points.

In the dietary infection study, we were interested whether the FOS-stimulated Salmonella translocation was reflected in colonic gene expression changes. We observed an overlap in gene expression changes observed in the two experimental diets, and an additional set of 58 genes which were only significantly affected in the FOS-fed rats. Although the list of altered genes was different in FOS fed rats, this was not the case at the process level as exactly the same processes were observed for both dietary groups. This indicates involvement of similar underlying biological processes in cellulose and FOS-fed infected rats and no obvious role for other processes.

Comparison at the level of biological processes is a powerful tool to interpret microarray experiments and enables comparison of different microarray datasets [67]. Comparison at gene level has some drawbacks, one is redundancy in gene function, which means that different genes can provide the same physiological effect. In addition, the homeostatic condition as well as the precise nature of the stimulus will determine how individual genes within a process are controlled to provide the necessary physiological response. Differences in responses of individual genes are filtered out when they are analyzed at the level of pathways or processes. However, the translation of differentially expressed genes into biological processes also suffers from limitations [68]. The most important limitation is that annotations to pathways and processes are incomplete. Therefore it is important that results from pathway analysis are manually supplemented with the remaining significant genes using biological databases and scientific literature.

Conclusion

In conclusion, our results show that, in addition to the ileum, the colon mucosa is clearly a target for Salmonella infection. Early Salmonella-induced changes were observed in transport and oxidative stress, while at later stage, most likely secondary, infection and inflammation responses were observed. Some findings confirm expected results, such as induction of an immune and inflammatory response. However, the Salmonella-induced immune response in colon is clearly different from that in ileum. We newly identified that colonic transport processes and proteolysis are affected by Salmonella infection and that
pancreatitis associated protein was the most responsive gene in *Salmonella* infected rat colon.

An important observation is that FOS-stimulated *Salmonella* translocation (as measured by urinary NO$_x$), does not induce other processes than those observed in cellulose-fed and *Salmonella* infected rats. So, the quality or diversity of the colonic host response to *Salmonella* is not affected by colonic FOS fermentation in contrast to the magnitude of response. As far as we know, there are no literature data pointing to a functional effect of FOS in the ileum. Therefore, the FOS effects on *Salmonella* translocation are most likely due to colonic effects. Understanding the changes caused by FOS alone may provide insight in processes that ultimately result in the observed weakening of the barrier.

**Methods**

**Time course infection study**

**Animals, diet and infection**

The experimental protocols were approved by the animal welfare committee of Wageningen University (Wageningen, the Netherlands). Specific pathogen-free male outbred 9 weeks old Wister rats (WU, Harlan, Horst, the Netherlands, n = 48 in total), were housed individually in metabolic cages. All animals were kept in a temperature (22–24°C) and humidity (50–60%) controlled room with a 12 h light/dark cycle (lights on from 6 AM to 6 PM). Rats were fed a purified diet during the whole experimental period. The diet contained (per kg) 200 g acid casein, 502 g glucose, 160 g palm oil, 40 g corn oil, 50 g cellulose, 35 g mineral mix (without calcium) and 10 g vitamin mix according to AIN93 recommendations [69]. Diets were low in calcium content (20 mmol CaHPO$_4$·2H$_2$O/kg) [16] to mimic the com-

**RNA isolation**

Colon scrapings were homogenized in liquid N$_2$ using a mortar and pestle cooled with liquid N$_2$ (clinical isolate, phage type 4 according to international standards; B1214 culture of NIZO food research, Ede, the Netherlands). *Salmonella* infection was performed by gastric gavage with 1 mL of saline containing 3 × 10$^9$ colony forming units (CFU) of *S. enteritidis*. Non-infected rats received saline only (control). *S. enteritidis* was cultured and stored, as described earlier [23]. Fresh fecal samples were collected on days 1, 2, 3 and 6 post infection (p.i.) and analyzed for viable *Salmonella* by plat-

**Analysis of mRNA expression by Oligo Arrays**

For microarray hybridization, equal amounts of RNA of each animal were pooled per treatment group. Arrays were performed in duplicate. For this, RNA pools were split and separately reverse transcribed and labeled with Cy-5. A standard reference sample, consisting of a pool of all colonic RNA was labeled with Cy-3. For each oligo array, 35 µg of total RNA was used to make Cy-5 or Cy-3 labeled cDNA. Total RNA was mixed with 4 µg T21 primer, heated at 65°C for 3 min (RNA denaturation) followed by 25°C for 10 min (primer annealing). cDNA was synthesized by adding 5× first strand buffer (Invitrogen), 10 mM DTT, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, 0.04 mM dCTP, 0.04 mM Cy5-dCTP or Cy3-dCTP, 1.2 U RnaseOUT and 6 U SuperScript II Reverse Transcriptase to a total volume of 62.5 µL. The reaction was incubated at 42°C for 2 h. Puri-
fication, precipitation and denaturation of the labeled cDNA were performed as described earlier [70].

The 44 K rat whole genome Agilent array (G4131A, Agilent Technologies, Inc. Santa Clara, CA) used consists of 44290 60-mer rat oligonucleotides, including ~ 3000 control spots. The Cy5 labeled cDNAs of the Salmonella infected groups and the non-infected groups were mixed 1:1 with the Cy3 reference labeled cDNA, mixed with 2× hybridization buffer (Agilent Technologies) and 10× control targets (Agilent Technologies) and hybridized for 17 hours at 60°C in Agilent hybridization chambers in an Agilent hybridization oven rotating at 4 rpm (Agilent Technologies). After hybridization the arrays were washed with an SSPE wash procedure (Agilent Technologies) and scanned with an Agilent Microarray Scanner (Agilent Technologies).

Data analysis
Signal intensities for each spot were quantified using Feature Extraction 8.1 (Agilent Technologies). The data of the time course infections study are available in Additional File 4 and have been deposited in NCBI's Gene Expression Omnibus [71] and are accessible through GEO Series accession number GSE7496. Median density values and background values of each spot were extracted for both the experimental samples (Cy5) and the reference samples (Cy3). Quality check was performed for each microarray using both LimmaGUI package in R from Bioconductor [72] and Microsoft Excel. Data was exported into GeneMaths XT (Applied Maths, Sint-Martens-Latem, Belgium) for analysis. We discarded spots with an average intensity, over all arrays, of Cy5 lower than 2-fold above average background. Then, the Cy5 intensities were normalized against the Cy3 reference as described before [73]. The gene expressions of duplicate arrays were averaged. Array data of non-infected rats, killed on section day 1 and 6 were highly comparable and could therefore be considered as one group and were averaged. For unknown reason, arrays of non-infected rats killed on day 3 showed reduced expression of 14 mast cell protease genes when compared with non-infected rats of both days 1 and 6, which were highly comparable. Therefore, we decided not to include the non-infected rats of day 3. Cluster analysis and Principle component analysis were performed using GeneMaths XT. Infected/control ratio's between 0–1 were expressed as the negative inverse (1/value) for easier interpretation. Genes that changed more than 2-fold in comparison with controls at one of the time points studied were selected for pathway analysis. Pathway analysis was performed using two pathway programs, MetaCore (GeneGo Inc, St. Joseph, MI) [18] and ErmineJ [19], using Agilent gene annotation (Agilent Technologies, version 20060331). Processes were identified using statistical over-representation in both pathway programs. Since only 40% of the genes were annotated to GO processes in both pathway programs, processes with a p-value < 0.001 were manually supplemented with non-annotated genes with FC > 2 using biological databases (BIOCarta, SOURCE, GenMAPP, KEGG) and scientific literature.

Analysis of mRNA expression by Real-time Quantitative RT-PCR
Real-time Quantitative RT-PCR (Q-PCR) was performed on individual samples (n = 8 per group). 1 µg of RNA from all individual samples was used for the cDNA synthesis using the iScript cDNA synthesis kit of Bio-Rad Green Supermix of Bio-Rad using the MyQ single-color real-time PCR detection system (Bio-Rad). Each reaction (25 µl) contained 12.5 µl iQ SYBR green supermix, 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 8.5 µl RNase-free water and 2 µl diluted cDNA. The following cycles were performed 1× 3 min at 95°C, 40 amplification cycles (40× 10 s 95°C, 45 s 60°C), 1× 1 min 95°C, 1× 1 min 62°C and a melting curve (80× 10 s 55°C with an increase of 0.5°C per 10 s). A negative control without cDNA template was run with every assay. The optimal melting point of dsDNA (Tm) and the efficiency of the reaction were optimized beforehand. Data were normalized against the reference genes Ribosomal protein S29 (Rps29), ADP-Ribosylation Factor 1 (Arf1) and β-actin. Rps29 and Arf1 were chosen on the basis of microarray data which showed similar expression levels for all microarrays, β-actin was chosen as this is a well accepted reference gene. Primers were designed using Beacon designer 4 (Premier Biosoft International, Palo Alto, CA). For sequences see Additional File 5. A standard curve for all genes including reference genes was generated using serial dilutions of a pooled sample (cDNA from all reactions). mRNA levels were determined from the appropriate standard curve. Samples with mRNA levels below the lowest standard value, and thus below detection level, were given half the value of this lowest standard. Analysis of all individual samples was performed in duplicate.

Serum Interferon Gamma
The serum Interferon Gamma (IFNγ) concentration of individual rats was determined by an enzyme-linked immunosorbent assay (ELISA) specific for rats (Biosource International, Camarillo, CA) according to the manufacturer’s protocol.

Dietary infection study
Animals, diet and infection
A dietary intervention was performed to study the effect of FOS on S. enteritidis-induced gene expression. Specific pathogen-free male outbred Wister rats (8 weeks old, mean body weight of 253 g; n = 48 in total) were housed as described above (time course infection study). Rats...
were fed the same diet as described above. The experimental diets both contained 20 g/kg cellulose and were supplemented with either 60 g/kg FOS (purity 93%; Raftilose P95, Orafti, Tienen, Belgium) or additional 60 g/kg cellulose as described earlier [23]. Animals were fed restricted quantities (14 g/day) of the purified diet. Restricted food intake was necessary to prevent differences in food consumption and hence differences in vitamin and mineral intake as observed earlier in FOS interventions [11]. After an adaptation period of 14 days, rats were orally infected with $4 \times 10^8$ CFU of *S. enteritidis* or control-treated as described above. On day 2 p.i., 12 infected FOS-fed rats, 12 infected rats fed the cellulose diet, and 12 control-treated non-infected rats fed the cellulose diet were sacrificed to obtain colonic mucosal RNA. Two additional groups of rats fed either FOS (n = 6) or the cellulose diet (n = 6) and infected with *Salmonella* were kept until day 8 p.i. for determination of urinary NO$_x$ excretion in time as described above.

### Analysis of mRNA expression by Oligo Arrays and Real-time Quantitative RT-PCR

RNA isolation and analysis of mRNA expression by microarray (pooled samples) and Q-PCR (n = 12 per treatment group) were performed as described above. Arrays were scanned with a Scanarray Express HT scanner (Perkin Elmer). Signal intensities for each spot were quantified using ArrayVision 8.0 (GE Healthcare life sciences). Data analysis was performed as described above. The data of the dietary infection study available in Additional File 6 and have been deposited in NCBI's Gene Expression Omnibus [71] and are accessible through GEO Series accession number GSE7472.

### Statistical analysis

Results are expressed as median or mean depending on normality of distribution as indicated. We used Prism 4 for all statistics (Prism 4, GraphPad software Inc., San Diego, CA). Data was analyzed using the Student’s t-test (two-sided). Non-normally distributed data was analyzed using the non-parametric Mann-Whitney U test (two sided). Differences were considered statistically significant when p < 0.05.

### Authors’ contributions

WR, EK, CV and SR performed the experiments. WR performed the data analysis, interpreted the results and drafted the manuscript. JK and IB participated in the design of the study, in evaluation of the results and in revision of the manuscript. RvdM and MK discussed the results and critically read the manuscript. All authors read and approved the final manuscript.

### Additional material

#### Additional file 1

Salmonella affected colonic genes. Genes that were upregulated or downregulated at least 1.5-fold in rat colon mucosa by *Salmonella* at days 1, 3 and 6 after oral *Salmonella* infection compared to colon mucosa of non-infected rats. Genes are ordered based on function and within functional category based on absolute fold change.

Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2180-7-84-S1.doc](http://www.biomedcentral.com/content/supplementary/1471-2180-7-84-S1.doc)

#### Additional file 2

Housekeeping and cell type specific genes. The fold change in expression of housekeeping genes and cell-type specific genes in the colon mucosa at days 1, 3 and 6 after oral *Salmonella* infection.

Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2180-7-84-S2.doc](http://www.biomedcentral.com/content/supplementary/1471-2180-7-84-S2.doc)

#### Additional file 3

Dietary modulated genes. Processes regulated in colon by *Salmonella* at day 2 in cellulose fed and FOS fed rats.

Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2180-7-84-S3.doc](http://www.biomedcentral.com/content/supplementary/1471-2180-7-84-S3.doc)

#### Additional file 4

Time course infection study. Complete dataset of the time course infection study.

Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2180-7-84-S4.xls](http://www.biomedcentral.com/content/supplementary/1471-2180-7-84-S4.xls)

#### Additional file 5

Primer sequences. Sequences of the primers used for Q-PCR analysis.

Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2180-7-84-S5.doc](http://www.biomedcentral.com/content/supplementary/1471-2180-7-84-S5.doc)

#### Additional file 6

Dietary infection study. Complete dataset of the dietary infection study.

Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2180-7-84-S6.xls](http://www.biomedcentral.com/content/supplementary/1471-2180-7-84-S6.xls)

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