

## intestinal colonisation but stimulate translocation of salmonella in rats

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*Gut* 2003;52:1572–1578

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Accepted for publication:  
30 May 2003

**Background and aims:** It is frequently assumed that dietary non-digestible carbohydrates improve host resistance to intestinal infections by stimulating the protective gut microflora. However, compelling scientific evidence from in vivo infection studies is lacking. Therefore, we studied the effect of several non-digestible carbohydrates on the resistance of rats to *Salmonella enteritidis* infection.

**Methods:** Rats (n=8 per group) were fed "humanised" purified diets containing 4% lactulose, fructo-oligosaccharides (FOS), resistant starch, wheat fibre, or cellulose. After an adaptation period of 2 weeks the animals were orally infected with *S enteritidis*. Supplement induced changes in faecal biochemical and microbiological parameters were studied before infection. Colonisation of salmonella was determined by studying the faecal excretion of this pathogen and translocation by analysis of urinary nitric oxide metabolites over time and classical organ cultures. Intestinal mucosal myeloperoxidase activity was determined to quantify intestinal inflammation after infection.

**Results:** Despite stimulation of intestinal lactobacilli and bifidobacteria and inhibition of salmonella colonisation, FOS and lactulose significantly enhanced translocation of this pathogen. These supplements also increased cytotoxicity of faecal water and faecal mucin excretion, which may reflect mucosal irritation. In addition, caecal and colonic, but not ileal, mucosal myeloperoxidase activity was increased in infected rats fed FOS and lactulose. In contrast, cellulose, wheat fibre, and resistant starch did not affect the resistance to salmonella.

**Conclusions:** In contrast to most expectations, FOS and lactulose impair the resistance of rats to intestinal salmonella infection. Obviously, stimulation of the endogenous lactobacilli and bifidobacteria is no guarantee of improved host defence against intestinal infections.

Gastrointestinal infections are still a major health problem and not only in developing countries. In Europe and the United States the annual incidence of (mostly foodborne) intestinal infections is more than 10%. The growing resistance of pathogenic bacteria to antibiotics makes it important to develop ways to prevent and treat intestinal infections by other means.<sup>1</sup> Dietary modulation of host resistance to intestinal infections might be an attractive alternative approach. By influencing the composition of gastrointestinal contents, diet affects the gastrointestinal survival of pathogens,<sup>2,3</sup> the endogenous intestinal microflora,<sup>4,5</sup> and the epithelial barrier function.<sup>6</sup> These primary non-immunological host defences of the gastrointestinal tract are particularly important in withstanding the first encounter with a pathogen.

Despite the current keen interest in the preventative and even therapeutic efficacy of so-called functional foods to improve gut health, compelling scientific evidence substantiating the role of dietary components in strengthening host resistance is scarce. Human<sup>7,8</sup> and animal<sup>9,10</sup> studies have shown that diet, and particularly non-digestible carbohydrates, affect the composition of the intestinal microflora and many of them suppose that consequences for host resistance are likely. Endogenous bifidobacteria and lactobacilli are claimed to be important for intestinal health, and increased numbers of these genera are assumed to improve the colonisation resistance to pathogens.<sup>7,10</sup> This assumption is largely an extrapolation of results obtained from in vitro experiments. Carbohydrate fermenting lactobacilli and bifidobacteria produce bactericidal organic acids (lactic acid and short chain fatty acids) and possibly other inhibitory

compounds (for example, hydrogen peroxide and bacteriocins) which suppress growth of pathogens like salmonella in co-cultures.<sup>11</sup> However, host defences against intestinal infections are not solely dependent on the composition and metabolic activity of the endogenous microflora but also depend on the barrier function of the intestinal mucosa,<sup>6</sup> especially when invasive pathogens are involved. Thus, stimulation of the intestinal lactobacilli and bifidobacteria, or inducing a change in gut flora composition as such, is not directly a functional effect nor a direct health advantage. In our opinion, irrespective of their prebiotic effect, the contribution of non-digestible carbohydrates in increasing resistance to intestinal infections can only be verified in strictly controlled in vivo experiments including a challenge with a pathogen.

Therefore, the aim of the present study was to determine the effect of dietary non-digestible carbohydrates on the resistance of rats to intestinal colonisation and translocation of *Salmonella enteritidis*. Considering that the efficacy of non-digestible carbohydrates to improve resistance might depend on their fermentability, non-fermentable or low fermentable carbohydrates (cellulose and wheat fibre), as well as highly fermentable carbohydrates (resistant starch, fructo-oligosaccharides, and lactulose) were studied. *S enteritidis* was chosen as infective agent because it is a major cause of human foodborne infectious diarrhoea in industrialised countries<sup>12</sup>

**Abbreviations:** FOS, fructo-oligosaccharides; MPO, myeloperoxidase

and we have shown earlier that this model is sensitive to dietary modulation.<sup>2</sup>

## MATERIALS AND METHODS

### First infection experiment: animals, diets, and oral pathogen administration

The experimental protocol was approved by the animal welfare committee of Wageningen University, The Netherlands.

Specific pathogen free male Wistar rats (WU, Harlan, Horst, The Netherlands), 8 weeks old and with a mean body weight of 274 g, were housed individually in metabolic cages in a room with controlled temperature (22–24°C), relative humidity (50–60%), and light/dark cycle (lights on from 6 am to 6 pm). Animals ( $n = 8$  per group) were fed a purified diet containing (per kg): 200 g acid casein, 502 g glucose, 160 g partly hydrogenated palm oil, 40 g corn oil, 10 g cellulose, 35 g mineral mix (without calcium),<sup>13</sup> 10 g vitamin mix,<sup>13</sup> 3.44 g  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  (corresponding to 20 mmol calcium/kg diet; Merck, Darmstadt, Germany), and 40 g non-digestible carbohydrates. The non-digestible carbohydrates added to the diet were either (extra) cellulose (purity 99%; Arboceel, Minerals & Chemicals Assistance, Zutphen, The Netherlands), wheat fibre (purity 97%; Vitacel, Internatio, Zutphen, The Netherlands), resistant starch (purity 88%; Hylon VII, National Starch and Chemical Company, Bridgewater, New Jersey, USA), fructo-oligosaccharides (FOS, purity 93%; Raftilose P95, Orafiti, Tienen, Belgium), or lactulose (crystalline powder, purity 98%; Solvay Pharmaceuticals BV, Weesp, The Netherlands). Food and demineralised drinking water were supplied freely on demand. Food intake was recorded at least once per 2 days and body weight at least once per 4 days.

Animals were acclimatised to the housing and dietary conditions for 2 weeks, after which they were orally infected with a single dose of *S enteritidis* (clinical isolate, phage type 4 according to international standards; B1241 culture of NIZO food research). *S enteritidis* was cultured and stored as described earlier.<sup>14–15</sup> All rats received 1 ml of saline containing 3% (wt/vol) sodium bicarbonate and  $1 \times 10^9$  viable *S enteritidis*, as determined by plate count on Brilliant Green Agar (Oxoid, Basingstoke, UK). The virulence of this strain is sustained by routine oral passage in Wistar rats.

### Microbiological analyses of faeces

Immediately before and several days after *S enteritidis* infection, fresh faecal samples were collected directly from the anus of the animals and analysed for viable salmonella by plating 10-fold dilutions on Modified Brilliant Green Agar (Oxoid) containing sulfamandelate (Oxoid) and incubating overnight at 37°C, as described earlier.<sup>4</sup> These fresh faecal samples were also cultured for lactobacilli and enterobacteria, as described previously.<sup>4</sup> Faecal bifidobacteria were specifically determined by real time quantitative polymerase chain reaction (PCR) targeting a 110-bp transaldolase gene sequence as described and validated elsewhere.<sup>16</sup> DNA was isolated from faeces using the QIAamp DNA stool mini kit (QIAGEN, Westburg, Leusden, The Netherlands) following the manufacturer's recommendations. PCR primers were purchased from Amersham Pharmacia Biotech Custom DNA Synthesis Service (Roosendaal, The Netherlands). Taqman probes, containing a 5' FAM fluorescent reporter dye and a 3' TAMRA quencher dye, were synthesised by PE Applied Biosystems Custom Oligonucleotide Synthesis Services (Nieuwerkerk a/d IJssel, The Netherlands). Real time PCR was performed using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The Taqman Exogenous Internal Positive Control (IPC) Reagent (VIC probe; PE Applied Biosystems)

was inserted in the PCR protocol in order to check for PCR efficiency and to rule out PCR inhibiting substances in the faecal DNA extracts. Universal Taqman PCR Master Mix was purchased from PE Applied Biosystems. DNA extracts of dilutions of suspensions of *Bifidobacterium breve* and *Bifidobacterium infantis* (cultures B655 and B651 of NIZO food research, respectively) were used to plot a mean standard curve from which the number of bifidobacteria in faeces, represented by the target transaldolase sequence in faecal DNA extracts, could be calculated. Control experiments showed that slope of the standard curve was not altered when these pure bifidobacterial cultures were added to rat faeces indicating that recovery was good. This real time PCR method for quantification of bifidobacteria was preferred over faecal culturing because the media described for cultivation of bifidobacteria do not equally support the growth of all of the bifidobacterium species encountered in faeces and therefore may bias results.<sup>17</sup>

### Biochemical analyses of faeces

Faeces were quantitatively collected during 4 days just before *S enteritidis* infection for chemical analyses. Faeces were freeze dried, weighed to determine daily faecal dry matter excretion, and subsequently ground to obtain homogeneous powdered samples. Relative faecal wet weight was determined as described earlier.<sup>14</sup> Total faecal lactic acid was measured in freeze dried faeces with a colourimetric enzymatic kit (Boehringer Mannheim, Germany), as described elsewhere.<sup>14</sup> Mucins were extracted from freeze dried faeces and quantified fluorimetrically, as described earlier.<sup>14</sup> Standard solutions of *N*-acetylgalactosamine (Sigma) were used to calculate the amount of oligosaccharide side chains liberated from mucins. Faecal mucins are therefore expressed as  $\mu\text{mol}$  oligosaccharide equivalents. The recovery of porcine stomach mucin (Sigma) added to freeze dried faeces was >90%. Fresh faecal samples, collected 2 days before infection and on day five after infection, were immediately frozen at  $-20^\circ\text{C}$  until determination of short chain fatty acids by gas chromatography, as described by Tangerman *et al.*<sup>18</sup>

### pH and cytotoxicity of faecal water

Freeze dried faeces was reconstituted to prepare faecal water of physiological osmolarity (300 mOsmol/l) as described elsewhere,<sup>2</sup> except that the second centrifugation step was extended to 1 h at 14 000 *g* (Eppendorf 5415) to achieve clear precipitation of sediment. After determination of pH at 37°C, the cytotoxicity of faecal water was determined with an erythrocyte assay as previously described<sup>19</sup> and validated earlier with intestinal epithelial cells.<sup>20</sup> The incubations were of physiological ionic strength (154 mM) and buffered at pH 7.0 (final 100 mM 3-*N*-morpholino-propanesulfonic acid; Sigma) to prevent acid induced haemolysis.

### Analysis of nitric oxide metabolites in urine

Starting the day before infection, complete 24 h urine samples were collected, preserved, and analysed for the nitric oxide metabolites nitrite and nitrate ( $\text{NO}_x$ ) by a colourimetric method,<sup>21</sup> as described earlier.<sup>4</sup>

### Second infection experiment: organ cultures and intestinal inflammation

A second infection experiment was performed with specific pathogen free male Wistar rats (WU, Harlan, Horst, The Netherlands), 8 weeks old, and with a mean body weight of 234 g. Animals ( $n = 8$  per group) were acclimatised, housed, fed the cellulose, FOS, or lactulose supplemented diets, and orally infected with *S enteritidis* (dose  $1 \times 10^8$  colony forming units) as described for the first infection experiment. Two days after oral infection, rats were killed by carbon dioxide

inhalation. The spleen, mesenteric lymph nodes, and the liver were excised, homogenised in sterile saline, and plated on Modified Brilliant Green Agar (Oxoid) to quantify viable salmonella, as described previously.<sup>15</sup> In addition, the ileum (last 12 cm proximal to the ileocaecal valve), caecum, and colon were pulled out, longitudinally excised, and freed of their contents by extensive washing in sterile saline. The mucosa of these intestinal segments was scraped off using a spatula. Scrapings were suspended in 1 ml sterile saline and stored at  $-20^{\circ}\text{C}$ . Intestinal inflammation was determined by analysis of the myeloperoxidase (MPO) activity in the mucosal scrapings using a colourimetric assay as described by Grisham *et al.*<sup>22</sup> One unit of activity was defined as the amount of enzyme present that produces a change in absorbance per min of 1.0 at  $37^{\circ}\text{C}$  in the final reaction volume containing sodium acetate. The recovery of purified MPO isolated from human leukocytes (Calbiochem, Darmstadt, Germany) added to the scrapings always exceeded 85%. Protein content of the scrapings was determined spectrophotometrically using the BCA Protein Assay Kit (Pierce, Rockford, Illinois, USA) with bovine serum albumin (Sigma) as standard.

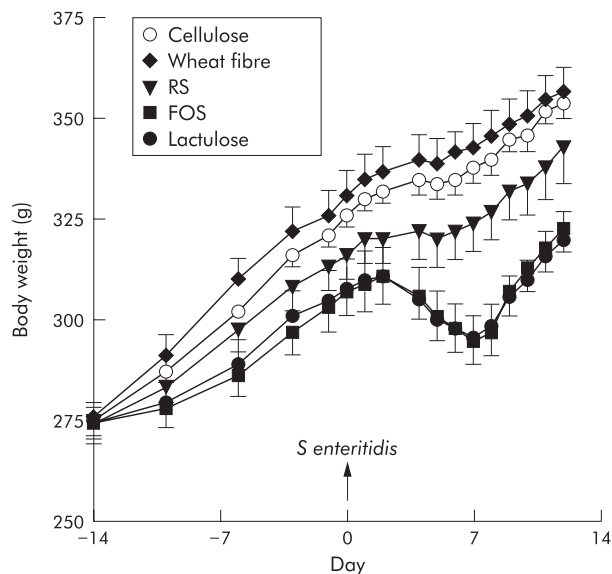
### Statistics

Results of the first infection experiment are expressed as standard error of the mean values (SEM) ( $n = 7$  in the wheat fibre group and  $n = 8$  in the other diet groups). Differences between the means of the diet groups were tested for significance by analysis of variance (ANOVA). Additionally, Fisher's protected least significance difference test, modified for multiple comparisons, was used to identify dietary groups that differed from each other (two sided,  $p < 0.05$ ; statistical package SPSS/PC + v2.0, SPSS Inc, Chicago, Michigan, USA). Results of the second infection experiment are expressed as mean values (SEM) ( $n = 8$  for all groups). Differences between the means of the diet groups were tested for significance by ANOVA. Student's *t* test was used to determine whether translocation of salmonella and mucosal inflammation of rats in the FOS and lactulose groups were significantly increased in comparison with the cellulose control group (one sided,  $p < 0.05$ ).

## RESULTS

### Growth and food intake of the animals

One rat in the wheat fibre group was excluded from the study because of immediate substantial wasting (5–10 g/d) for unknown reason. Animal growth was affected by the different diets, even before infection (fig 1). After infection,



**Figure 1** Effect of dietary cellulose, wheat fibre, resistant starch (RS), fructo-oligosaccharides (FOS), and lactulose on growth of the rats before and after oral administration of  $1 \times 10^9$  colony forming units (CFU) of *Salmonella enteritidis* on day 0. Results are expressed as means (SEM) ( $n = 7$  in the wheat fibre group and  $n = 8$  in the other groups).

significant weight loss was only observed in the FOS and lactulose groups. Before infection, food consumption of rats fed lactulose (14.7 (0.6) g dry weight/d) and FOS (14.3 (0.3) g/d) was significantly less than that of rats fed resistant starch (16.7 (0.6) g/d), wheat fibre (17.9 (0.4) g/d), and cellulose (17.7 (0.3) g/d). During the first week after infection, food consumption was reduced by approximately 3 g/d in all groups. Thereafter, mean daily food intake increased to pre-infection levels mentioned above. The reduced food intake of rats fed lactulose and FOS did not affect the faecal parameters and infection markers mentioned below, as the same results were obtained in an additional infection experiment with rats fed slightly restricted to achieve an equal food intake in the FOS and cellulose (control) groups (unpublished results).

### Effects of non-digestible carbohydrates on faecal parameters

Resistant starch, and especially FOS and lactulose, decreased the daily excretion of faecal dry matter (table 1;  $p < 0.05$ ). The

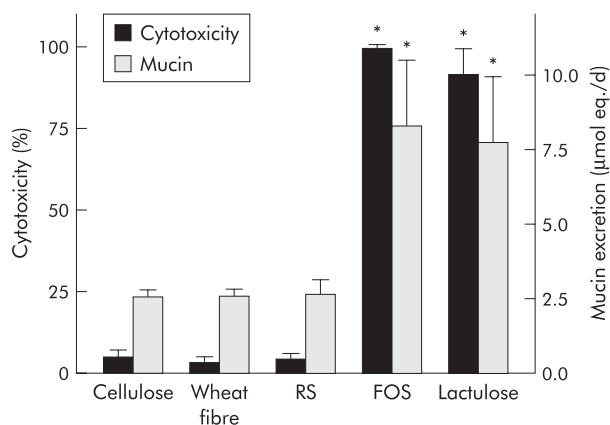
**Table 1** Effect of dietary non-digestible carbohydrates on faecal parameters of rats before infection

Faecal parameter	Non-digestible carbohydrate				
	Cellulose	Wheat fibre	Resistant starch	FOS	Lactulose
Dry weight excretion (g/d)	1.04 (0.03)*	1.04 (0.04)*	0.45 (0.03)†	0.28 (0.04)‡	0.24 (0.08)‡
Relative wet weight (%)	46 (3)*	49 (3)*	69 (2)†	73 (1)†	67 (1)†
pH of faecal water	7.10 (0.13)*	7.13 (0.08)*	6.89 (0.15)*	6.34 (0.05)†	6.34 (0.10)†
Total SCFA ( $\mu\text{mol/g}$ wet faeces)	38.8 (5.7)*†	38.1 (4.7)*†	49.2 (8.2)†	31.1 (3.4)*	27.8 (3.4)*
Acetate (% of total)§	80 (2)*	82 (1)*	80 (4)*	72 (1)†	78 (2)*
Propionate (% of total)	17 (1)*	16 (1)*	19 (3)*	26 (1)†	20 (2)*
Butyrate (% of total)	2 (1)*	1 (1)*	1 (1)*	1 (1)*	1 (1)*
Lactic acid ( $\mu\text{mol/g}$ wet faeces)	0.57 (0.24)*	0.45 (0.09)*	1.38 (0.34)†	1.52 (0.50)†	6.96 (2.46)‡

Results are expressed as means (SEM) ( $n = 7$  in the wheat fibre group and  $n = 8$  in the other groups).

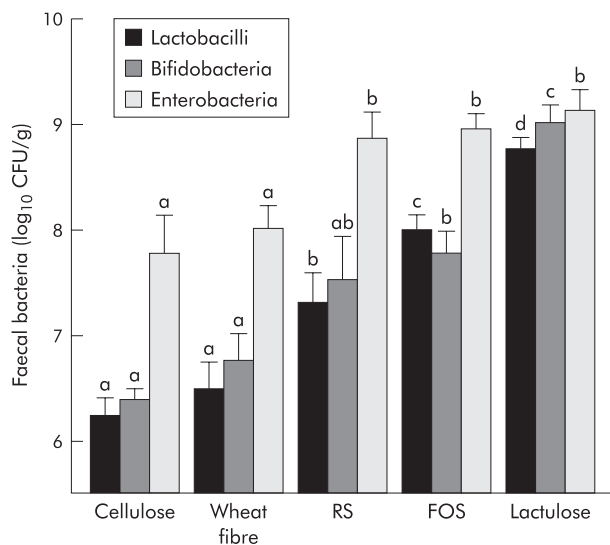
Values in the same row not sharing the same superscript are significantly different ( $p < 0.05$ )\*†‡.

§If the sum of the individual short chain fatty acids (SCFA) shown does not reach 100%, it is because of the presence of small amounts (<1% of total SCFA) of isobutyrate, valerate, and isovalerate. FOS, fructo-oligosaccharides.



**Figure 2** Effect of non-digestible carbohydrates on cytotoxicity of faecal water and daily faecal mucin excretion before infection of the rats. Cytotoxicity was determined with a haemolysis assay and mucins were measured fluorimetrically. Results are expressed as means (SEM) ( $n=7$  in the wheat fibre group and  $n=8$  in the other groups). \*These faecal parameters were significantly increased in the fructo-oligosaccharides (FOS) and lactulose group ( $p<0.05$ ). RS, resistant starch.

relative faecal wet weight of animals fed resistant starch, FOS, and lactulose was much higher than that of the cellulose and wheat fibre groups (table 1;  $p<0.05$ ). Addition of FOS and lactulose to the diets decreased pH of faecal water when compared with the other non-digestible carbohydrates (table 1;  $p<0.05$ ). No major differences between dietary groups were observed in the total short chain fatty acid concentration of faeces, except that FOS-fed rats had relatively less acetate and more propionate in their faeces (table 1;  $p<0.05$ ). The total lactic acid concentration was highest in faeces of lactulose fed rats, intermediate in the FOS and resistant starch groups, and lowest in faeces of



**Figure 3** Effect of non-digestible carbohydrates on lactobacilli, bifidobacteria, and enterobacteria in faecal samples collected before infection of the rats. Lactobacilli were cultured anaerobically on Rogosa Agar and enterobacteria were cultured aerobically on Levine EMB Agar. Bifidobacteria were determined by specific real time quantitative polymerase chain reaction (PCR). Results are expressed as means (SEM) ( $n=7$  in the wheat fibre group and  $n=8$  in the other groups). Bars indicated by different letters are significantly different ( $p<0.05$ ). RS, resistant starch; FOS, fructo-oligosaccharides; CFU, colony forming units.

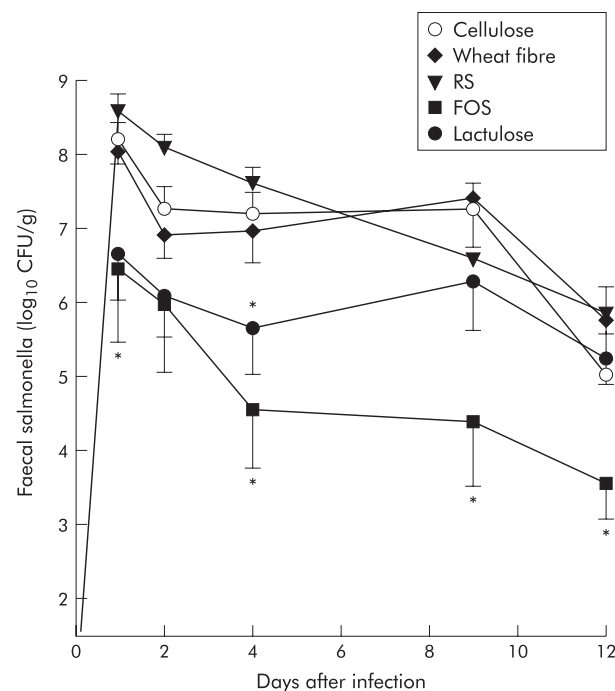
wheat fibre and cellulose fed rats (table 1;  $p<0.05$ ). Faecal mucin excretion was significantly stimulated by FOS and lactulose supplementation (fig 2). These oligosaccharides also greatly increased the cytotoxicity of faecal water (fig 2;  $p<0.05$ ).

### Non-digestible carbohydrates and the intestinal microflora

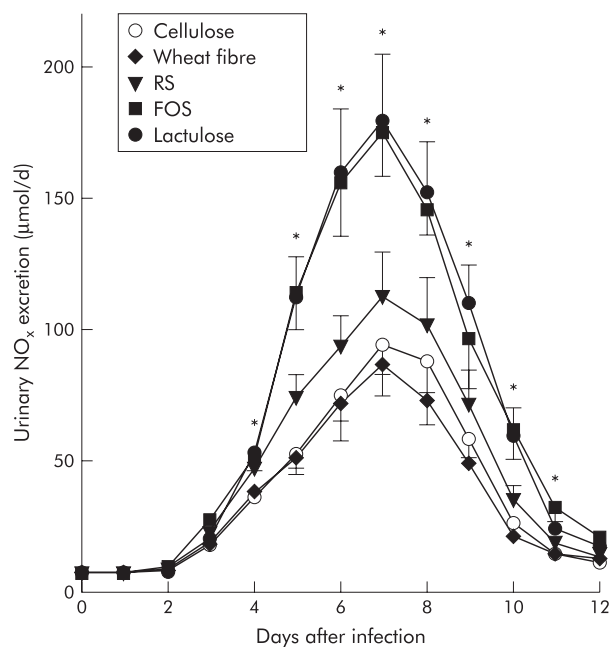
Compared with the cellulose and wheat fibre groups, a more than 100-fold increase in faecal lactobacilli was observed in rats fed the lactulose diet. These lactic acid bacteria were also stimulated by FOS and resistant starch, though to a lesser extent (fig 3;  $p<0.05$ ). FOS and especially lactulose increased the number of bifidobacteria (fig 3;  $p<0.05$ ). Lactulose, FOS, and resistant starch also increased the number of enterobacteria in faeces (fig 3;  $p<0.05$ ). The levels of these bacterial genera were not affected by *S enteritidis* infection (data not shown).

### Differential effects of non-digestible carbohydrates on colonisation and translocation of salmonella, and on intestinal inflammation

As expected, no salmonella could be detected in faeces collected before infection of the rats. Compared with the other diet groups, the FOS supplemented animals clearly had the best colonisation resistance to *S enteritidis*, considering the reduced faecal shedding of this pathogen over time (fig 4;  $p<0.05$ ). Though the lactulose group also tended to have lower faecal salmonella counts during the first days after infection, this was only significant from the cellulose, wheat fibre, and resistant starch groups on day four post-infection. Major differences were observed in the effects of non-digestible carbohydrates on the resistance to translocation of *S enteritidis*, as measured by the infection induced urinary  $\text{NO}_x$  excretion over time (fig 5). Peak urinary  $\text{NO}_x$  excretion of the



**Figure 4** Effect of dietary cellulose, wheat fibre, resistant starch (RS), fructo-oligosaccharides (FOS), and lactulose on faecal *Salmonella enteritidis* excretion of rats after oral administration of  $1 \times 10^2$  colony forming units (CFU) of *S enteritidis* on day 0. Results are expressed as means (SEM) ( $n=7$  in the wheat fibre group and  $n=8$  in the other groups). \*The indicated group is significantly different from all other groups at that time point ( $p<0.05$ ).



**Figure 5** Effect of dietary cellulose, wheat fibre, resistant starch (RS), fructo-oligosaccharides (FOS), and lactulose on the kinetics of urinary  $\text{NO}_x$  excretion of rats after an oral challenge with  $1 \times 10^9$  colony forming units of *Salmonella enteritidis* on day 0. Results are expressed as means (SEM) ( $n=7$  in the wheat fibre group and  $n=8$  in the other groups). \*Denotes that the FOS and lactulose groups are significantly different from all other groups at that time point ( $p<0.05$ ).

lactulose and FOS groups was twice as high as that of the cellulose, wheat fibre, and resistant starch groups (fig 5;  $p<0.05$ ). The total infection induced urinary  $\text{NO}_x$  excretion was 412 (49), 370 (46), 530 (74), 834 (81), and 835 (106)  $\mu\text{mol}/12$  d for the cellulose, wheat fibre, resistant starch, FOS, and lactulose group, respectively (the FOS and lactulose groups were significantly different from the other dietary groups). To validate that the strong infection induced increase in urinary  $\text{NO}_x$  excretion in the FOS and lactulose group reflected increased intestinal translocation of salmonella, the second infection experiment was performed. The significantly increased numbers of viable salmonella in extra intestinal organs confirmed that dietary FOS and lactulose stimulated intestinal bacterial translocation (table 2). In addition, the caecal and colonic mucosal MPO activity was 2–3 fold higher in FOS and lactulose fed infected rats in comparison with rats on a cellulose-supplemented diet (fig 6;  $p<0.05$ ). Remarkably, dietary FOS and lactulose did not affect this marker of inflammation in the ileal mucosa of infected rats (fig 6).

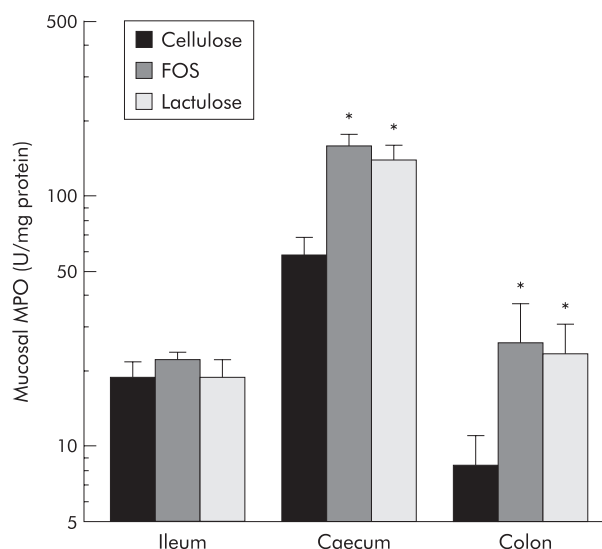
**Table 2** Effect of dietary non-digestible carbohydrates on viable salmonella counts in organs of rats two days after oral infection of the animals

Non-digestible carbohydrate	Salmonella counts in organ ( $\log_{10}$ CFU/g)		
	Mesenteric lymph nodes	Spleen	Liver
Cellulose	5.37 (0.56)	2.73 (0.15)	1.55 (0.27)
FOS	6.15 (0.18)	3.11 (0.09)*	2.32 (0.26)*
Lactulose	6.05 (0.18)	2.89 (0.32)	2.89 (0.38)*

Results are expressed as means (SEM) ( $n=8$  per group).

\*Denotes that the indicated group is significantly different from the cellulose group ( $p<0.05$ ).

CFU, colony forming units; FOS, fructo-oligosaccharides.



**Figure 6** Effect of dietary cellulose, fructo-oligosaccharides (FOS), and lactulose on the specific activity of myeloperoxidase (MPO) in the ileal, caecal, and colonic mucosa of rats two days after oral infection of the animals with  $1 \times 10^8$  colony forming units (CFU) of *Salmonella enteritidis*. Results are expressed as means (SEM) ( $n=8$  per group). Notice that the y axis is a log scale. \*Denotes that the FOS and lactulose group are significantly different from the cellulose group ( $p<0.05$ ).

## DISCUSSION

The most striking result of the present study is that dietary lactulose and FOS improve the colonisation resistance to *S enteritidis*, but concomitantly lower the resistance of rats to translocation of this invasive pathogen. No such effects were observed with resistant starch, wheat fibre, or cellulose supplementation of the diet. The colonisation resistance is inversely related to faecal pathogen excretion in time.<sup>23</sup> So, prolonged excretion of high numbers in faeces upon oral infection indicates adherence and multiplication of the pathogen in the intestinal tract, whereas a rapid decline in faecal pathogen excretion reflects inability of the pathogen to colonise. Supplementation of the diet with lactulose and especially FOS decreases colonisation of *S enteritidis*, as measured by the reduced faecal shedding of this pathogen over time (fig 4). At several time points after infection faecal excretion of *S enteritidis* was approximately 100-fold less in rats fed FOS in comparison to the cellulose, wheat fibre, and resistant starch groups. The oligosaccharides FOS<sup>24</sup> and lactulose<sup>25</sup> are not hydrolysed by host digestive enzymes in the proximal small intestine but are rapidly fermented by the microflora residing in the caecum and colon. As a result, the endogenous gut flora is stimulated and intestinal contents are acidified due to the production of lactic acid and short chain fatty acids.<sup>26</sup> Consequently, the colonisation of food-borne pathogens might be inhibited through enhanced competition for nutrients and mucosal adhesion sites<sup>27</sup> and increased luminal killing of acid sensitive pathogens, like salmonella.<sup>14</sup> Indeed, the present study showed that FOS and lactulose significantly lowered faecal pH (table 1) and increased the number of lactobacilli and bifidobacteria (fig 3). These lactic acid bacteria may contribute to host resistance as clinical investigations have shown that orally administered probiotic lactobacilli alleviate antibiotic associated diarrhoea<sup>28</sup> and rotavirus enteritis.<sup>29</sup> Besides a trophic effect of resistant starch, FOS, and lactulose on the endogenous lactobacilli and bifidobacteria, faecal enterobacteria were stimulated as well (fig 3). Accordingly, in our animal model we find no support for the claim that FOS (but also lactulose and resistant starch) can be classified as a

prebiotic, since one of the current criteria that allow such classification is selective stimulation of potentially beneficial intestinal bacteria.<sup>7</sup> The general opinion is that enterobacteria are not potentially beneficial because members of that bacterial family are involved in foodborne infectious disease<sup>30</sup> and gut derived septicemia.<sup>31</sup>

Although colonisation of *S. enteritidis* was inhibited by FOS and to a lesser extent by lactulose, these oligosaccharides remarkably stimulated bacterial translocation to extra intestinal sites, as indicated by the strongly increased infection induced urinary NO<sub>x</sub> output (fig 5) and by higher viable salmonella counts in extra intestinal organs (table 2). The evident infection induced loss of body weight of rats fed FOS and lactulose also confirms that the systemic infection was most severe in these groups (fig 1). Classically, bacterial translocation is quantified by blood or organ cultures. However, organ cultures notably underestimate total bacterial translocation because the majority of translocated pathogens are rapidly killed by the innate immune system upon translocation.<sup>32</sup> We have shown earlier that urinary NO<sub>x</sub> excretion correlates with organ cultures, but is a more sensitive and quantitative biomarker of intestinal bacterial translocation.<sup>2, 15</sup> Recently, several other rodent<sup>33, 34</sup> and human<sup>35, 36</sup> studies have been reported, showing that serum or urinary NO<sub>x</sub> correlates with extra intestinal pathogen load and the severity of systemic infectious disease. Concordant with the stimulated translocation of *S. enteritidis* in the FOS and lactulose groups in the present study, the inflammatory response in the caecal and colonic mucosa was also greater in these infected rats than in their cellulose fed counterparts (fig 6). We have recently performed an additional study in rats fed a FOS supplemented diet. It should be mentioned that intestinal mucosal histology showed that mucosal inflammation was absent in FOS fed rats before oral infection with salmonella. However, mucosal inflammation of the caecum and colon was notably more severe in the FOS group after salmonella challenge in comparison with the infected cellulose control group (Ten Bruggencate *et al*, unpublished data).

At first sight, the detrimental effect of FOS and lactulose on the translocation resistance seems contradictory to their beneficial effect on the colonisation resistance. However, intestinal mechanisms responsible for colonisation resistance might be different from those determining translocation resistance. For instance, optimal functioning of the mucosal barrier is essential to prevent translocation and gut derived septicemia. On the other hand, intestinal colonisation of a foodborne pathogen is likely to be more dependent on gut microbial ecology. The decreased faecal shedding of salmonella over time on the FOS and lactulose diets in the present study is probably a reflection of increased luminal killing of *S. enteritidis*. At the same time, these oligosaccharides obviously impair the gut mucosal barrier as indicated by the enhanced translocation of salmonella. The strongly increased cytotoxicity of faecal water of FOS and lactulose fed rats (fig 2) suggests that the intestinal mucosa was exposed to aggressive luminal contents, which might have led to epitheliolysis and impairment of the barrier function. The increased faecal output of mucin in these groups (fig 2) also points to irritation of the gut mucosa. The mucus layer (viscous gel of hydrated mucin glycoproteins) protects the intestinal epithelium from physical and chemical damage. As a defence mechanism, the intestinal mucosa is triggered to secrete more mucin in the presence of bacterial toxins<sup>37</sup> and short chain fatty acids.<sup>38</sup> High concentrations of short chain fatty acids can damage the intestinal epithelium, resulting in an increased permeability and epithelial cell proliferation.<sup>39, 40</sup> Under normal circumstances, the major site of salmonella translocation seems to be the ileal Peyer's patches,<sup>41</sup> but this

issue is still debated.<sup>42</sup> Considering that fermentation of FOS and lactulose in the human<sup>24, 25</sup> and rat ileum<sup>43</sup> is very limited, it is unlikely that these oligosaccharides facilitated translocation of *S. enteritidis* at that particular site. This is also indicated by the absence of FOS and lactulose induced ileal mucosal inflammation in infected rats of the present study (fig 6). Besides the ileum as entrance route, there is evidence from in vitro experiments that salmonella species can cross monolayers of epithelial cells by a transcellular and paracellular route.<sup>44</sup> We speculate that these migration pathways are explicitly exploited by salmonella in the caecum and colon in conditions of enhanced production of organic acids due to rapid fermentation of FOS and lactulose. The significantly increased mucosal inflammation of the caecum and colon of FOS and lactulose fed rats infected with salmonella (fig 6) strongly points in that direction. Identification of the cytolytic components in intestinal contents after FOS and lactulose consumption and their effect on mucosal barrier in different regions of the intestinal tract (ileum versus caecum and colon) will be the subject of future research.

Dietary resistant starch did not improve the colonisation resistance to *S. enteritidis* in the present study, nor did it share the detrimental effect of FOS and lactulose on the translocation resistance to this pathogen. In addition, no increase in faecal mucin and luminal cytotoxicity was observed during resistant starch feeding. Like FOS and lactulose, dietary resistant starch is well fermented by the human<sup>45</sup> and rat<sup>46</sup> gut microflora, as indicated by its absence in faeces. However, the fermentation rates of polymers are generally less than that of oligomers.<sup>47, 48</sup> Thus resistant starch feeding leads to a more gradual and distributed intestinal fermentation and concomitant production of organic acids.<sup>49</sup> This may have prevented damage to the caecal and colonic mucosal barrier as observed with FOS and the disaccharide lactulose.

Can these results be extrapolated to the human situation? Daily intake of fructose based non-digestible carbohydrates (mainly derived from wheat and onions) in Europe and the USA has been estimated at up to 10 g.<sup>50</sup> Assuming a daily dry food intake of about 500 g, the total concentration of these particular non-digestible carbohydrates in an average Western human diet is about 2%. This estimation does not take into account consumption of specific meals and products supplemented with inulin or FOS, typically 3–10 g per portion.<sup>50</sup> In addition, the human diet still contains other non-digestible carbohydrates. In view of this, supplementation of the animal diets with 4% non-digestible carbohydrates (as in the present study) is likely realistic to the human situation.

As far as we know, no controlled in vivo infection experiments are reported in literature, besides an interesting recent human study of Cummings *et al*.<sup>51</sup> Travellers to various high risk destinations for traveller's diarrhoea (frequently due to infection with non-invasive enterotoxigenic *Escherichia coli*) were either supplemented with FOS or placebo. Prevalence of self reported traveller's diarrhoea was 11% in the FOS group and 20% in the placebo group, which was borderline significant ( $p = 0.08$ ). The reduced diarrhoea of FOS supplemented travellers likely indicates inhibition of intestinal pathogen colonisation, which agrees with the inhibitory effects of FOS on salmonella colonisation in our rat study (fig 4).

In conclusion, the present study shows that dietary FOS and lactulose improve the colonisation resistance of rats to *S. enteritidis*, but concomitantly impair the intestinal resistance to translocation of this pathogen, in contrast to most expectations. The slowly fermentable non-digestible carbohydrates resistant starch, wheat fibre, and cellulose did

not have this detrimental effect. Although increasing the number of lactobacilli and bifidobacteria in the gut by stimulating intestinal carbohydrate fermentation and enhancing the production of short chain fatty acids is often assumed to be beneficial for intestinal health and resistance to infections, the results of the present study clearly warrant concern about this concept. The current keen scientific and commercial interest in enhancement of host defence by so-called prebiotics stresses the importance to verify this unexpected finding by additional well controlled intervention studies.

## ACKNOWLEDGEMENTS

The authors thank Maria Faassen-Peters and Wilma Blauw for expert biotechnical assistance and Martijn Katan for stimulating discussions.

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