Dietary non-digestible carbohydrates and the resistance to intestinal infections

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Dit onderzoek is uitgevoerd binnen de onderzoeksschool VLAG

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Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op vrijdag 19 november 2004
des namiddags om vier uur in de Aula.

Ten Bruggencate, S.J.M.

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Thesis Wageningen University, The Netherlands, 2004 - with summary in Dutch

ISBN: 90-8504-102-3

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Abstract

Background Non-digestible carbohydrates (NDC) stimulate the protective gut microflora resulting in increased production of organic acids. This may result in increased luminal killing of acid-sensitive pathogens and hence reduce intestinal colonisation and translocation of a pathogen. However, host defence against invasive pathogens like salmonella also depends on the barrier function of the intestinal mucosa. High concentrations of organic acids may induce injury to the intestinal mucosa and impair the barrier function. The aim of this work was to determine the effects of NDC's on the intestinal barrier in rats and humans.

Results In rats, well fermentable NDC (resistant starch, inulin, fructo-oligosaccharides and lactulose) stimulated the protective endogenous gut microflora and subsequently increased lactate concentrations and lowered intestinal pH. However, in contrast to cellulose, wheat fibre and resistant starch, the rapidly fermentable NDC inulin, fructo-oligosaccharides (FOS) and lactulose stimulated mucosal translocation of invasive salmonella. In addition, FOS significantly increased infection-induced growth impairment, gut inflammation and diarrhoea. Before infection, inulin, FOS and lactulose increased luminal cytotoxicity and mucus secretion. This likely reflects irritation of the intestinal mucosa by fermentation metabolites. Moreover, FOS increased intestinal permeability. Dietary calcium phosphate counteracted most of these adverse effects of rapidly fermentable NDC. In humans, FOS also stimulated the endogenous gut microflora and lactate concentrations. Additionally, FOS increased mucus secretion, indicating mucosal irritation. However, FOS consumption did not significantly affect luminal cytotoxicity and intestinal permeability.

Conclusions Stimulating the endogenous microflora by intestinal NDC fermentation is often assumed to be beneficial for intestinal health and resistance to infections. However, the work presented in this thesis does not support this concept.

Keywords prebiotics, fructo-oligosaccharides, inulin, calcium, fermentation, mucin, intestinal permeability, salmonella, infection, rat, human

Abbreviations

CFU	colony forming units
CrEDTA	chromium ethylenediamine-tetraacetic acid
DP	degree of polymerisation
DSS	dextran sodium sulphate
ETEC	*enterotoxigenic Escherichia coli*
FOS	fructo-oligosaccharides
GALT	gut-associated lymphoid tissue
GOS	galacto-oligosaccharides
IBD	inflammatory bowel disease
ICP-AES	inductively coupled plasma-atomic emission spectrophotometry
M-cells	microfold cells
MHC	major histocompatibility complex
MPO	myeloperoxidase
NDC	non-digestible carbohydrates
NEC	necrotising enterocolitis
NO	nitric oxide
NO_x	sum of nitrate and nitrite
PCR	polymerase chain reaction
SCFA	short-chain fatty acids
S.enteritidis	*Salmonella enterica serovar enteritidis*
SEM	standard error of the mean
SIP	secreted invasion protein
SPI	salmonella pathogenicity island
TNBS	trinitrobenzene sulphonic acid
TOS	transgalacto-oligosaccharides

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Introduction

There is a current commercial and scientific interest in functional food ingredients, like dietary prebiotics. Prebiotics are non-digestible food ingredients, usually non-digestible carbohydrates, which are assumed to be beneficial for the host health because they stimulate the protective intestinal microflora.[1] Among the numerous claimed beneficial health effects of prebiotics are: a reduction in the risk for constipation, intestinal infections, colon cancer, and osteoporosis.[2] Because of these proposed health benefits, prebiotics have been added to a variety of products, such as dairy products and infant formulas. Indeed, many studies showed that dietary non-digestible carbohydrates affect the intestinal microflora and the intestinal epithelium. Though these parameters are important for host resistance, their subsequent effects on intestinal infections were hardly ever subject of further study. Therefore, this thesis focuses on the effects of dietary non-digestible carbohydrates on the resistance to intestinal bacterial infections.

1.1 Dietary non-digestible carbohydrates

Carbohydrates are usually classified according to their molecular size (degree of polymerisation ; DP) **(Table 1.1)**. One group of non-digestible carbohydrates are the fructo-oligosaccharides (FOS). FOS is composed of linear chains of monosaccharide units, linked by ß(2-1) bonds and often terminated by a glucose unit **(Fig 1.1)**. The number of monosaccharide units ranges from 3-10. FOS is found in a variety of plants including onions, asparagus, Jerusalem artichokes and wheat.[3,4] The average daily consumption of FOS is estimated to be between 3-11 g in Europe.[4] Assuming a daily dry food intake of about 500 g, the total concentration of FOS in an average Western human diet is about 2%. This estimation does not take into account consumption of specific meals and products supplemented with FOS, typically 3-10 g per portion.[5] Non-digestible carbohydrates (NDC) are resistant to salivary amylases, gastric hydrolysis and are not hydrolysed by pancreatic enzymes or human small intestinal ß-galactosidase.[6] Thus, they pass virtually intact into the large intestine,[6] where they can be degraded by the intestinal microflora.[7,8]

Table 1.1. Classification of dietary carbohydrates according to their degree of polymerisation (DP). Dietary non-digestible carbohydrates, which are used in the work of this thesis, are printed italic.

carbohydrate	DP	subgroups	examples
monosaccharide	1	monosaccharide	glucose, galactose, fructose
disaccharide	2	disaccharide	sucrose, lactose, *lactulose*
		sugar alcohol	sorbitol, lactitol
oligosaccharide	3-10	oligosaccharide	maltodextrins, raffinose, stachyose, *fructo-oligosaccharide*, galacto-oligosaccharide
polysaccharide	10+	starch	amylose, amylopectin, *resistant starch*
		non-starch polysaccharide	*cellulose*, hemicellulose, *inulin*, pectin

1.2 The intestinal microflora

Non-digestible carbohydrates and the intestinal microflora

The intestinal microflora is characterised by a great diversity of bacterial populations, with at least 500 different species, of which 30-40 species comprise up to 99% of the total population. While the upper intestinal tract contains relatively few bacteria, numbers increase in the colon with bacterial concentrations up to 10^{12} bacteria per gram content. Within the large intestine, micro-organisms have a strictly anaerobic metabolism. Numerically important anaerobes are gram-negative rods belonging to the Bacteroides genus. Other important groups are bifidobacteria, clostridia, eubacteria, lactobacilli, gram-positive cocci, coliforms, methanogens and dissimilatory sulphate-reducing bacteria.[9]

Several dietary NDC can be extensively fermented by the intestinal microflora, which subsequently stimulates bacterial growth.[7,8] In general, short-chain carbohydrates are fermented faster than long-chain carbohydrates. Similarly linear chains are fermented faster than branched chains and soluble carbohydrates are fermented faster than insoluble carbohydrates.[10] Many human[8,11-14] and animal[15,16] studies have shown that fermentable NDC stimulate the growth of bifidobacteria and lactobacilli. However, the effect of fermentable NDC is fairly non-specific. Possibly both the growth of potentially beneficial bacterial species as well as potentially detrimental species is increased.[11,16] Indeed many

different species of intestinal bacteria are capable of fermenting NDC, for example bifidobacteria, lactobacilli, clostridia and enterobacteria.[17,18] Predominant end products of NDC fermentation are short-chain fatty acids (SCFA) (primarily acetate, propionate and butyrate).[19-21] Fermentation intermediates that can be further metabolised to SCFA include lactic acid, pyruvate, ethanol and succinate. In addition to NDC, sloughed epithelial cells can also provide growth substances for the intestinal microflora.[22] Fermentation of prebiotics increases gas production[23,24] and induces abdominal pain and excessive flatus.[23,24]

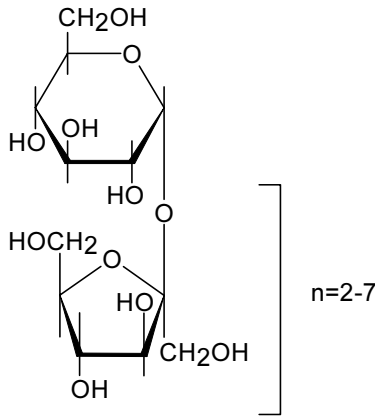

Fig 1.1 Chemical structure of the non-digestible carbohydrate fructo-oligosaccharides (FOS); linear chains of fructose units, linked by ß(2-1) bonds and often terminated by a glucose unit.

The concept of probiotics

Probiotics are classically defined as a viable microbial dietary supplement that beneficially affects the host through its effects in the intestinal tract.[2] From studies with germ-free animals and antibiotics-treated animals it is evident that the intestinal microflora is an important factor of host defence that protects animals and humans against intestinal colonisation by pathogens.[25-28] The endogenous microflora produces bactericidal organic acids (lactic acid and short-chain fatty acids)[19-21] and possibly other inhibitory compounds (e.g. hydrogen peroxide and bacteriocins), which suppress growth of pathogens at least *in-vitro.*[29] Many *in- vitro*[29-35] and *in-vivo*[36-39] studies have been done in the field of probiotics. Promising beneficial health effects of probiotics are in the field of rota virus infection, antibiotic-associated diarrhoea and inflammatory bowel disease (IBD).[40] However, compelling evidence that probiotics really enhance resistance to intestinal bacterial infections in animals or humans is lacking.

1.3 Intestinal bacterial infections

Incidence of intestinal bacterial infections

Gastro-intestinal infections are still a major health problem, not only in developing countries.[41,42] While the efficiency of treating diarrhoea in developing countries has improved, WHO still estimates that globally 2.2 million die annually from diarrhoea caused mainly by food- and waterborne pathogens.[43] In western countries, like the Netherlands, the problems are also significant, although morbidity and mortality are much lower than in developing countries.[43] Outbreaks of salmonella have been reported for decades, but within the past 25 years the disease has increased in incidence. In Europe, *Salmonella enterica serovar enteritidis* has become the predominant strain.[44,45] Salmonella infection ranges from mild, self-limiting diarrhoea to life-threatening systemic infection.[46] campylobacter is also an important pathogen and in some countries, the reported number of cases is higher than the incidence of salmonellosis.[41,43] Acute effects of campylobacteriosis include severe abdominal pain, fever, nausea and diarrhoea. Another important pathogen, especially among young children, is shigella.[41,43] Infection with shigella is confined to the colonic mucosa where it causes abscesses and ulceration, leading to severe tissue damage. Finally, infections due to *enterohaemorrhagic E. coli* and listeriosis are important foodborne diseases.[41,43] Although their incidence is relatively low, their severe and sometimes fatal health consequences, particularly among infants, children and the elderly, make them among the most serious foodborne infections.

Host defence against intestinal bacterial infections

Infection occurs when the balance between the virulence characteristics of the pathogen and the defence mechanisms of the host is impaired. The oropharynx, oesophagus, stomach and small intestine have a number of non-specific host defence mechanisms **(Table 1.2)**.[47] In the large intestine the abundant intestinal microflora competes for nutrients and adhesion sites. They produce several fermentation metabolites, which lower intestinal pH and oxidation-reduction potential. This can potentially inhibit the growth of acid-sensitive pathogens, at least *in-vitro*.[48] Moreover, epithelial shedding and the mucus layer (viscous gel of hydrated mucin glycoprotein) inhibit pathogen adhesion.[49,50] Mucins are produced by goblet cells within the intestinal epithelium and consist of oligosaccharide

chains linked to serine and threonine residues on a peptide backbone. They are classified according to their subgroups as sulphated, non-sulphated, acidic or neutral mucins. Within the small intestine immature goblet cells within the crypts produce neutral mucins, while the mature goblet cells at the villus tip contain sialic acid and are called acidic mucins.[51] Within the large intestine, goblet cells within the crypt contain sulphomucins, while the mature goblet cells at the colonic surface contain neutral mucins.[51] The sialic acid and sulphate group could be important as this may protect mucins from bacterial degradation.[22] The intestinal goblet cells also produce peptides called intestinal trefoil factors (ITF).[52] ITF are closely associated with mucins and are important for stabilisation of the mucus layer. They are resistant to acid and enzymes and could protect the mucus layer from bacterial degradation.[53] Therefore these peptides could strengthen the intestinal barrier.[54]

Table 1.2 The non-specific defence mechanisms of the gastro-intestinal tract[47]

Site	Host defence
oropharynx	lysozyme, microflora, proteolytic enzymes
oesophagus	peristalsis
stomach	low pH, proteolytic enzymes
small intestine	peristalsis, bile acids, proteolytic enzymes, lactoferrin, epithelial shedding, mucus, microflora
large intestine	microflora, epithelial shedding, mucus production

Besides lumen-associated host defence, the intestine contains gut-associated lymphoid tissue (GALT) **(Fig 1.2)**. This includes the Peyer's patches in the small intestine, which are separated from the intestinal lumen by a single layer of columnar epithelium including microfold (M) cells. M-cells function as antigen-sampling cells and transport antigens to the underlying lymphoid tissues, where protective immune responses are initiated. Antigen-presenting cells, which reside in the Peyer's patches like macrophages and dendritic cells couple the antigen with MHC class II receptors and activate T-helper cells (CD4), which then produce various cytokines. These subsequently stimulate B-cells within the Peyer's patches to produce IgA.[55] IgA seems able to inhibit pathogen motility.[56,57] Following antigen uptake by antigen-presenting cells, myeloperoxidase (MPO) is discharged into the phagosome. MPO reacts with H_2O_2, formed by the respiratory burst, to form powerful

oxidants, which are effective against a variety of pathogens.[58] However, these oxidants can induce tissue damage and subsequently contribute to the progression of disease.[58] Moreover, activated antigen-presenting cells produce high amounts of nitric oxide ($NO^{\bullet}$) from L-arginine, which can kill pathogens.[59] Released into the bloodstream most NO is quickly bound to haemoglobin and converted to nitrate, which is excreted in urine.[60,61] Therefore, during bacterial translocation[62] and systemic infectious disease[63-65] NO metabolites accumulate in urine.

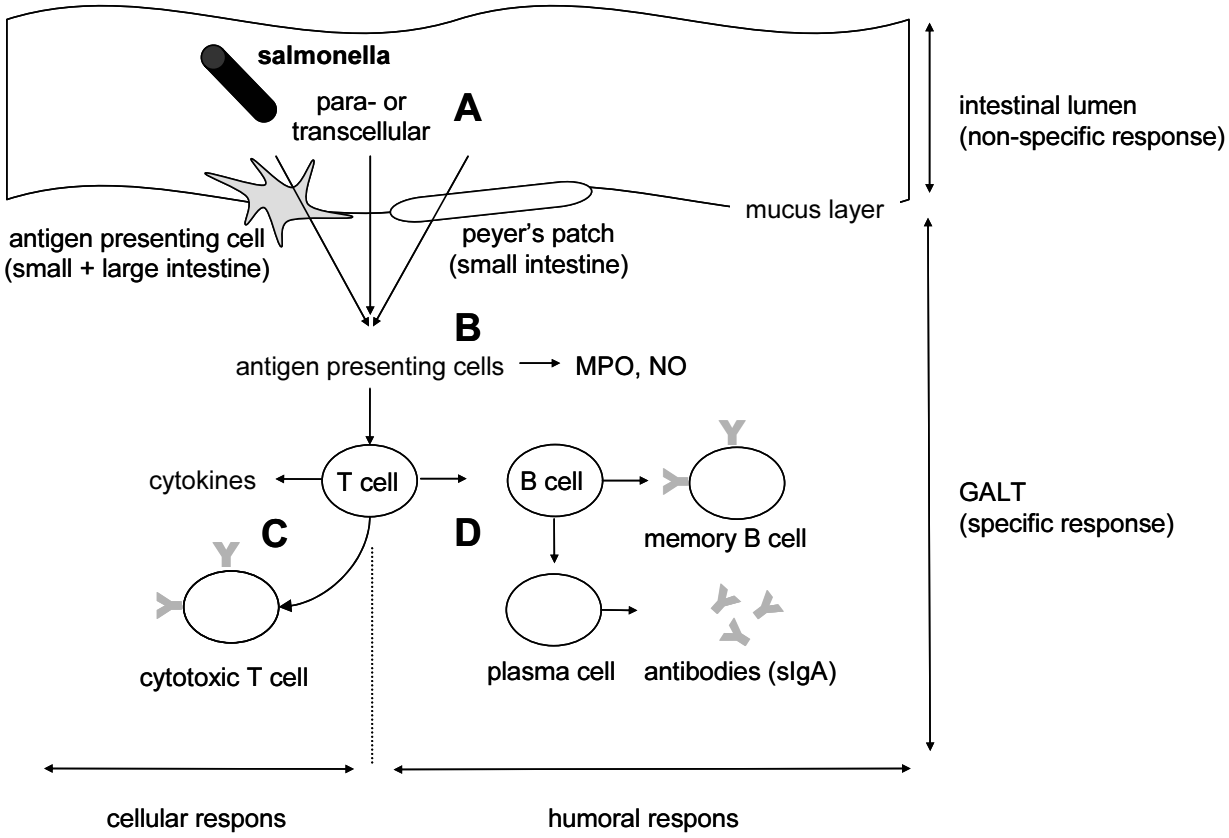

Fig 1.2 The non-specific (innate response) and specific (acquired response) defence mechanisms against pathogens like salmonella.[66] A, salmonella translocation across the epithelial barrier through antigen presenting cells, the M-cells of the Peyer's patches or para- or transcellular; B, The antigen presenting cells activate T-cells; C, T-cells produce cytokines; D, The activated T-cells stimulate B-cells to produce antibodies.

Course of intestinal bacterial infections

Following host entry, pathogens must withstand the above mentioned host defence mechanisms and successfully compete with the endogenous bacteria. Therefore, they have evolved a variety of strategies to invade the host, avoid or resist the innate immune

response, damage the cells, and multiply in specific and normally sterile regions.[67] Many pathogens adhere to the intestinal epithelium by flexible glycoprotein structures extending from the bacterial surface, called pili or fimbriae.[68] Penetration of the mucosal barrier by invasive pathogens, like salmonella, is supposed to occur through M-cells within ileal Peyer's patches[69], but this issue is still debated.[70] Salmonella can also translocate to extra-intestinal sites via antigen-presenting cells, present in the mucosa throughout the entire intestine.[71,72] In addition, salmonella is able to cross epithelial cells para- and transcellularly.[73] (Fig 1.2) As epithelial cells and antigen-presenting cells vastly outnumber intestinal M-cells, they may be the main port of entry for salmonella.[70] An essential step in salmonella pathogenicity is its ability to enter intestinal epithelial cells. This requires expression of invasion genes, encoded by Salmonella pathogenicity island 1 (SPI-1).[74] The expression is regulated by environmental factors (low oxygen, high osmolarity and acidic pH) present in the intestine.[75] Efficient entry of salmonella into epithelial cells requires production of secreted invasion proteins (Sips). These Sips are injected into the host cell by a type III contact-dependent protein secretion system. Subsequently, Sips remodel the cell membrane into ruffles, leading to macropinocytosis of the bacteria.[74] In addition, salmonella is able to survive and replicate within macrophages. This requires the expression of invasion genes, encoded by Salmonella pathogenicity island 2 (SPI-2).[76]

1.4 Non-digestible carbohydrates, microflora and infections

Fermentable NDC may potentially enhance resistance to intestinal pathogens, by stimulating the protective intestinal microflora[15] and increasing bactericidal organic acid concentrations[14,21] within the lower gut **(Fig 1.3)**.

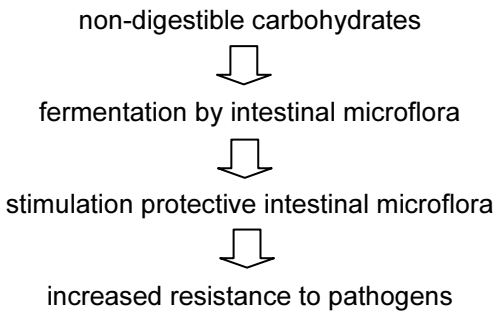

Fig 1.3 Hypothetical mechanism by which dietary non-digestible carbohydrates may increase the resistance to pathogens

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,[77] especially when invasive pathogens are involved. Besides being bactericidal, high concentrations of organic acids may induce injury to the intestinal mucosa.[78,79] Moreover, alterations in organic acid concentrations might also increase translocation of salmonella to extra-intestinal sites by increasing pathogen virulence.[75,80] In a previous study, fermentable NDC increased the percentage of acetate at the expense of other SCFA, while total SCFA concentrations remained unchanged due to their efficient absorption.[81] This NDC-induced alteration in SCFA might increase pathogen virulence by providing a signal for expression of invasion genes.[75] Thus, stimulation of the protective endogenous microflora is not directly a functional effect nor a direct health advantage. Irrespective of their prebiotic effect, the contribution of NDC in increasing resistance to intestinal infections can only be verified in strictly controlled *in-vivo* experiments including a challenge with a pathogen. The previous studies that focused on the *in-vivo* effects of NDC on intestinal bacterial infections **(Table 1.3a)** and gut inflammation **(Table 1.3b)** show beneficial, inconsistent or adverse effects. However, many studies have an improper study design or are irrelevant for human foodborne infectious disease. For instance, several studies have been done in poultry (chicken or quail).[82-85] In these animals, fermentation already takes place within the crop.[86] In addition, many studies show inconsistent data. For example, Lettelier *et al*[87] showed that 1% fructo-oligosaccharides (FOS) in water decreased colonisation of salmonella, while FOS in feed increased colonisation. Chambers *et al*[83] showed that 5% refined FOS decreased colonisation of salmonella, while artichoke flour (containing 5% FOS) increased colonisation. Moreover, many studies use a high dose of NDC, which is irrelevant for human consumption,[84,88-90] or score outcome parameters subjectively .[91-93]

Table 1.3a The effects of dietary non-digestible carbohydrates (NDC) on resistance to pathogens: overview of previous *in-vivo* studies

Author	Organism	NDC and dose	Model	Effect of NDC on main outcome	Remarks
Positive effect of NDC					
Bailey[82]	Chicken	FOS 0.8 g/kg diet	*S.typhimurium*	Decrease in caecal colonisation	Very low dose of FOS
Buddington[90]	Rat	FOS/inulin 100g/kg diet	*C.albicans, S.typhimurium, L.monocytogenes*	Increased survival of rats infected with salmonella or listeria Decreased colonisation *C.albicans*	Salmonella and listeria given intraperitoneally High dose of FOS
Chambers[83]	Chicken	FOS 50 g/kg diet (refined)	*S.typhimurium*	Decrease in caecal colonisation	No effect of 50 g/kg crude FOS
Correa-Matos[91]	Pig	FOS 7,5g/kg diet	*S.typhimurium*	Decrease in diarrhoea	Only subjective assessments; Not blinded
Lettelier[87]	Pig	FOS 10 g/kg (in water)	*S.typhimurium*	Decrease in colonisation	No effect of 10 g/kg in food; More FOS-induced translocation
No or negative effect of NDC					
Asahara[94]	Mice	TOS 2-50 mg/day	*S.typhimurium*	No additional effect on bifidobacteria-induced decreased colonisation and translocation	Mice were treated daily with streptomycin; streptomycin-resistant bifidobacteria decreased colonisation and translocation
Cummings[92]	Human	FOS 10 g/kg diet	Travellers diarrhoea	No significant effect on diarrhoea	Many different pathogens; subjective scoring
Duggan[95]	Human	FOS 0.7 g/day	Infectious diarrhoea	No effect on diarrhoea prevalence	
McDonald[96]	Pig	Guar gum 70 g/kg diet	ETEC	Increased ileal ETEC colonisation	High dose of guar gum
Oli[97]	Pig	FOS 50g/kg diet	Cholera-toxin induced diarrhoea	No effect on diarrhoea	
Oyarzabal[84]	Chicken	FOS 100 g/kg diet	*S.typhimurium*	No effect on colonisation	No dietary intervention before infection High dose of FOS

Table 1.3b The effects of dietary non-digestible oligosaccharides (NDC) on gut inflammation: overview of previous *in-vivo* studies

Author	Organism	NDC and dose	Model	Effect of NDC on main outcome	Remarks
Positive effect of NDC					
Butel[85]	Quails	FOS 30 g/kg diet	NEC	Decrease in NEC lesions	Strange model: gnotobiotic quails associated with human NEC flora
Cherbut[98]	Rat	FOS 1 g/day	TNBS-induced colitis	Decreased inflammation score	FOS administered before colitis induction
Spapen[99]	Human	Guar gum>17 g/day	Post-operative sepsis	Reduced incidence diarrhoea	Dietary intervention only after operation
Videla[100]	Rat	Inulin 1% in water	DSS-induced colitis	Lower lesion scores and MPO	
Welters[101]	Human	Inulin 24 g/day	Ileal pouch	Decreased pouchitis disease score	Well-controlled study
No or negative effect of NDC					
Anderson [102]	Human	FOS 32 g/day	Post-operative sepsis	No effect on translocation and inflammation	High dose of FOS
Holma[16]	Rat	GOS 4g/kg body weight	TNBS-induced colitis	No effect on colonic damage scores or MPO	
Hunter[103]	Human	FOS 6g/day	IBD	No effect on IBD scores	Well-controlled study
Kien[89]	Pig	Lactulose 74 g/kg diet	healthy animals	Increased colonic inflammation scores	High dose of lactulose
Moreau[104]	Rat	FOS 60 g/kg diet	DSS-induced colitis	No effect on histological scores	
Olesen[105]	Human	FOS 20g/day	IBD	No effect on IBD scores	Well controlled study
Ozaslan[88]	Rat	Lactulose 2 g/day	obstructive jaundice	No sign decrease in translocation of *E-coli*	High dose of lactulose
Saavedra[106]	Human	FOS 0.55g/day	Healthy infants	No effect on frequency of diarrhoea or infections	Subjective scoring by mother of infant. Decrease in regurgitation
Szilagyi[107]	Human	Lactulose 20g/day	IBD	No effect on IBD scores	Not blinded

NB DSS, dextran sodium sulphate; ETEC, enterotoxic e-coli; FOS, fructo-oligosaccharides; GOS; galacto-oligosaccharides; IBD, inflammatory bowel disease; MPO, myeloperoxidase; NEC, necrotising enterocolitis; TNBS, trinitrobenzene sulphonic acid; TOS, transgalacto-oligosaccharides

1.5 Rationale and outline of this thesis

Several non-digestible carbohydrates (NDC) stimulate the protective gut microflora, resulting in an increased production of organic acids. This may result in increased luminal killing of acid-sensitive pathogens. However, this assumption has largely been based upon *in-vitro* studies. Moreover, host defence against invasive pathogens, like salmonella, also depends on the barrier function of the intestinal mucosa. Rapid fermentation of NDC, leading to high concentrations of organic acids, may impair the barrier function. Thus, stimulation of the protective endogenous microflora is not directly a functional effect or a direct health advantage. The contribution of NDC in increasing resistance to intestinal infections can only be verified in strictly controlled *in-vivo* experiments including a challenge with a pathogen. Therefore, we studied the effect of several non-digestible carbohydrates on the resistance of rats to salmonella infection.

Chapter 2 in this thesis describes the effect of non-digestible carbohydrates with different chain length and fermentation properties on colonisation and translocation of salmonella. More specifically, rats were fed diets containing 4% lactulose, fructo-oligosaccharides, resistant starch, wheat fibre, or cellulose.

The main purpose of the study in **Chapter 3** was to see whether the effects of fructo-oligosaccharides on the resistance to infection, observed in the first study, are dose-dependent. For this study, rats were fed diets supplemented with 0%, 3% or 6% FOS.

Chapter 4 addresses the question whether inulin, which is more slowly fermented than fructo-oligosaccharides, has similar effects on the resistance to infection. Moreover, this study was conducted to see whether supplemental calcium phosphate, as a luminal buffering component, is able to inhibit the adverse effects of rapid fermentation of non-digestible oligosaccharides. In order to answer these questions rats were fed diets with 6% cellulose, FOS or inulin and with either a low (30 mmol/kg) or a high (100 mmol/kg) calcium concentration.

The main purpose of the study described in **Chapter 5** was to see whether fermentation of fructo-oligosaccharides increases intestinal permeability and hence impairs the intestinal barrier. In order to answer these questions rats were fed diets supplemented with 6% cellulose or FOS and the intestinal permeability marker chromium ethylenediamine-tetraacetic acid (CrEDTA).

The study in **Chapter 6** was conducted to determine the relevance of our results obtained in rats for humans. A double-blind placebo-controlled cross-over study of 2x2 weeks, with a wash-out period of 2 weeks, was performed with healthy men. Subjects consumed a low-calcium diet and consumed either 20 g FOS or placebo and 150 µmol of CrEDTA per day.

Chapter 7 of this thesis provides a general discussion and conclusions from the studies described.

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Chapter 2

Dietary fructo-oligosaccharides and lactulose inhibit intestinal colonisation but stimulate translocation of salmonella in rats

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Gut 2003;52:1572-1578

Abstract

Background

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 enterica serovar 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.

2.1 Introduction

Gastro-intestinal infections are still a major health problem, not only in developing countries. Even 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.[1] Dietary modulation of host resistance to intestinal infections might be an attractive alternative approach. By influencing the composition of gastro-intestinal contents, diet affects the gastro-intestinal survival of pathogens,[2,3] the autochthonous intestinal microflora,[4,5] and the epithelial barrier function.[6] These primary non-immunological host defences of the gastro-intestinal tract are particularly important in withstanding the first encounter with a pathogen. Of all dietary components, the non-digestible and non-absorbable ones seem to have the best potential to modulate resistance to intestinal infections. First, because most bacterial pathogens that cause human infectious diarrhoea mainly produce their noxious effects in the lower gut (ileum and colon). Second, because critical steps in the establishment of intestinal infections, like pathogen multiplication and adhesion to the gut mucosa, take place at the interface between the gut lumen and the intestinal epithelium.

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[5,7] and animal[8,9] 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. Especially, 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.[7,10] 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 (e.g. hydrogen peroxide and bacteriocins) which suppress growth of pathogens like salmonella in co-cultures.[11] 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,[6] 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 enterica serovar enteritidis*. Considering that the efficacy of non-digestible carbohydrates to improve resistance might depend on their fermentability, non- 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[12] and we have shown earlier that this model is sensitive to dietary modulation.[2]

2.2 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 ^{0}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),[13] 10 g vitamin mix,[13] 3.44 g $CaHPO_4.2H_2O$ (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%; Arbocel, Minerals & Chemicals Assistance, Zutphen, The Netherlands), wheat fibre (purity 97%; Vitacel, International B.V. Zutphen, The Netherlands), resistant starch (purity 88%; Hylon VII, National Starch and Chemical Company, Bridgewater, New Jersey, USA), fructo-oligosaccharides (abbreviated as FOS, purity 93%; Raftilose $^{®}$P95, Orafti, Tienen,

Belgium), or lactulose (crystalline powder, purity 98%; Solvay Pharmaceuticals B.V., Weesp, The Netherlands). The diets were administered to the animals as a porridge (demineralised water was added and mixed with the dry diets to obtain a dry weight of 68%), to prevent spilling and subsequent contamination of the faeces and urine by food. Food and demineralised drinking water were supplied ad libitum. 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.[14,15] All rats received 1 ml of saline containing 3% (wt/vol) sodium bicarbonate and 1×10^9 viable *S. enteritidis*, as determined by plate count on Brilliant Green Agar (Oxoid, Basingstoke, England). The virulence of this strain is sustained by routine oral passage in Wistar rats.

Microbiological analyses of faeces

Immediately before and on 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 sulphamandelate (Oxoid) and incubating overnight at 37 ^{0}C, as described earlier.[4] Fresh faecal samples collected just before infection and on day 4 after infection were also analysed for the number of lactobacilli, bifidobacteria, and enterobacteria. Faecal enterobacteria were determined as described above for salmonella, except that Levine EMB Agar (Difco Laboratories, Detroit, Michigan, USA) was used. Lactobacilli were quantified by plating on Rogosa Agar (Oxoid) and incubating the plates in an anaerobic cabinet (Coy Laboratory products Inc., Ann Arbor, Michigan, USA) at 37 ^{0}C for 3 days, as described previously.[4] Faecal bifidobacteria were specifically determined by real-time quantitative PCR targeting a 110-bp transaldolase gene sequence as described and validated elsewhere.[16] 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 the slope of the standard curve was not altered when these pure bifidobacteria cultures were added to rat faeces indicating that recovery was good.

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.[14] Faecal L- and D-lactic acid were measured in freeze-dried faeces with a colourimetric enzymatic kit (Boehringer Mannheim, Germany), as described elsewhere.[14] Mucins were extracted from freeze-dried faeces and quantified fluorimetrically, as described earlier.[14] 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 µmol 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 5 after infection, were immediately frozen at -20^0C until determination of short-chain fatty acids by gas chromatography. Faeces (200 mg) was mixed with double-distilled water (450 µl) and (50 µl of) the internal standard 2-ethylbutyric acid (150 mM dissolved in 100% formic acid; Fluka Chemie, Zwijndrecht, The Netherlands). After thorough mixing in an ultrasonic water bath (5 minutes), samples were centrifuged (5 minutes at 500 *g*; Eppendorf 5415). Aspirated supernatants were diluted with acetone (Mallinckrodt Baker, Deventer, The Netherlands) (1:50 vol/vol), vigorously mixed, and centrifuged (5 minutes at

500 *g*; Eppendorf 5415) again. A fraction (0.7 µl) of the aspirated supernatants was injected (cold on-column) to a gas chromatograph (Carlo Erba, model HRGC5160, Milan, Italy), equipped with a capillary fused silica column (length 15 m, internal diameter 0.53 mm) coated with DB-FFAP (film-thickness: 1.0 µm) (J&W Scientific, Folsom, Ca), and a Flame Ionisation Detector. Initial pressure of the carrier gas (hydrogen) was 20 kPa. The temperature program was: 60^0C (1 minute), 7^0C/minute 130^0C (0 minutes), 30^0C/minute 240^0C (15 minutes). The coefficient of variation of this method was <4% and the recovery of short-chain fatty acids (acetate, propionate, butyrate, valerate, isobutyrate, and isovalerate; all Fluka Chemie) added to faeces was >90%.

pH and cytotoxicity of faecal water

Freeze-dried faeces was reconstituted to prepare faecal water of physiological osmolarity (300 mOsmol/l) as described elsewhere,[2] except that the second centrifugation step was extended to 1 hour at 14,000 *g* (Eppendorf 5415). Probably due to the high viscosity of faeces in the FOS and lactulose groups, extended centrifugation was necessary to achieve clear precipitation of sediment. After determination of pH at 37 ^{0}C, the cytotoxicity of faecal water was determined with an erythrocyte assay as previously described[17] and validated earlier with intestinal epithelial cells.[18] 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

Complete 24 h urine samples were collected during almost 2 weeks, starting 1 day before infection. Oxytetracycline (1 mg; Sigma) was added to the urine collection vessels of the metabolic cages to prevent bacterial deterioration. Urine samples were stored at -20^0C until analyses. The concentration of NO_x (sum of nitrate and nitrite) was determined by automated flow injection analyses. Briefly, diluted urine was passed over a cadmium column to reduce nitrate to nitrite, followed by reaction of nitrite with Griess reagent.[19] The purple-coloured product was measured spectrophotometrically at 538 nm. Recovery of nitrate added to rat urines always exceeded 95%.

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 as described above for the first infection experiment. The infective dose in the second infection experiment was 1×10^8 colony-forming units of *S. enteritidis*. Two days after oral infection, rats were killed by carbon dioxide inhalation. The spleen, mesenteric lymph nodes, and the liver were aseptically excised, homogenised in sterile saline, and plated on Modified Brilliant Green Agar (Oxoid) to quantify viable salmonella as described previously.[15] 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 ^{0}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*.[20] 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^{0}C in the final reaction volume containing sodium acetate. The recovery of purified MPO isolated from human leukocytes (Calbiochem, Darmstadt, Germany) added to the mucosal scrapings always exceeded 85%. Protein content of the mucosal scrapings was determined spectrophotometrically using the BCA Protein Assay Kit (Pierce, Rockford, Illinois, USA) with bovine serum albumin (Sigma) as standard.

Statistical analysis

Results of the first infection experiment are expressed as 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). Student *t* test was used to determine whether the FOS and lactulose groups significantly differed from the cellulose control group (one-sided, $P<0.05$).

2.3 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 2.1)**. After infection, 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/day) and FOS (14.3 ± 0.3 g/d) was significantly less than that of rats fed resistant starch (16.7 ± 0.6 g/day), 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/day in all groups. Thereafter, mean daily food intake increased to pre-infection levels mentioned above.

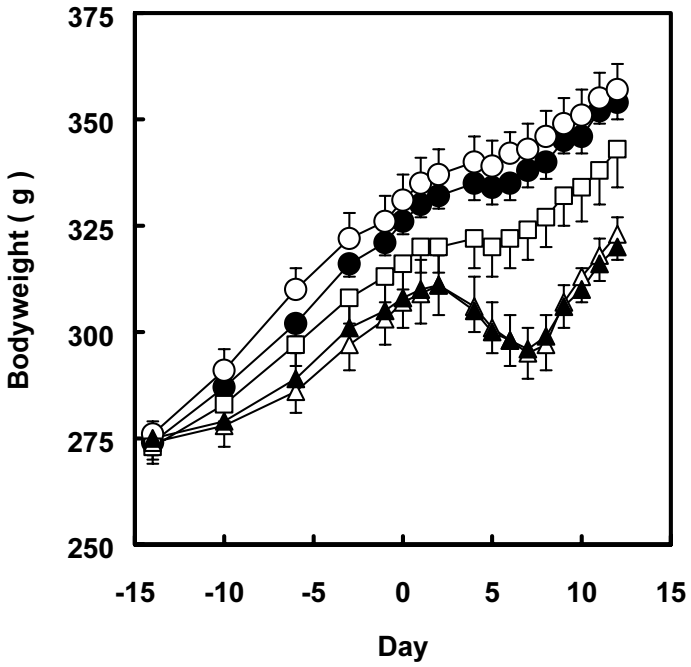

Fig 2.1 Effect of dietary cellulose (●), wheat fibre (O), resistant starch (❑), fructo-oligosaccharides (△), and lactulose (▲) on growth of the rats before and after oral administration of 1.10^9 colony-forming units of *S. enteritidis* on day 0. Results are expressed as means ± SEM (n=7 in the wheat fibre group and n=8 in the other groups).

Effects of non-digestible carbohydrates on faecal parameters

Resistant starch, and especially FOS and lactulose, decreased the daily excretion of faecal dry matter **(Table 2.1)**.

Table 2.1 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^{a}	1.04 ± 0.04^{a}	0.45 ± 0.03^{b}	0.28 ± 0.04^{c}	0.24 ± 0.08^{c}
Relative wet weight (%)	46 ± 3^{a}	49 ± 3^{a}	69 ± 2^{b}	73 ± 1^{b}	67 ± 1^{b}
pH of faecal water	7.10 ± 0.13^{a}	7.13 ± 0.08^{a}	6.89 ± 0.15^{a}	6.34 ± 0.05^{b}	6.34 ± 0.10^{b}
Total SCFA (µmol/g wet faeces)	38.8 ± 5.7^{ab}	38.1 ± 4.7^{ab}	49.2 ± 8.2^{b}	31.1 ± 3.4^{a}	27.8 ± 3.4^{a}
Acetate (% of total)	80 ± 2^{a}	82 ± 1^{a}	80 ± 4^{a}	72 ± 1^{b}	78 ± 2^{a}
Propionate (% of total)	17 ± 1^{a}	16 ± 1^{a}	19 ± 3^{a}	26 ± 1^{b}	20 ± 2^{a}
Butyrate (% of total)	2 ± 1^{a}	1 ± 1^{a}	1 ± 1^{a}	1 ± 1^{a}	1 ± 1^{a}
Lactic acid (µmol/g wet faeces)	0.57 ± 0.24^{a}	0.45 ± 0.09^{a}	1.38 ± 0.34^{b}	1.52 ± 0.50^{b}	6.96 ± 2.46^{c}

NB Results are expressed as means ± SEM (n=7 in the wheat fibre group and n=8 in the other groups).
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. Values in the same row not sharing the same superscript are significantly different (P<0.05).

The 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 2.1). Addition of FOS and lactulose to the diets decreased pH of faecal water when compared with the other non-digestible carbohydrates (Table 2.1). No major differences between dietary groups were observed in the total short-chain fatty acid concentration of faeces. The total concentration of short-chain fatty acids was highest in faeces of rats fed resistant starch, though significance was only reached when compared with faeces of the FOS and lactulose groups (Table 2.1). In all groups, the main short-chain fatty acid present in faeces was acetate (± 75%), followed by propionate (± 20%). The relative amount of faecal butyrate was less than 2% in all groups (data not shown).

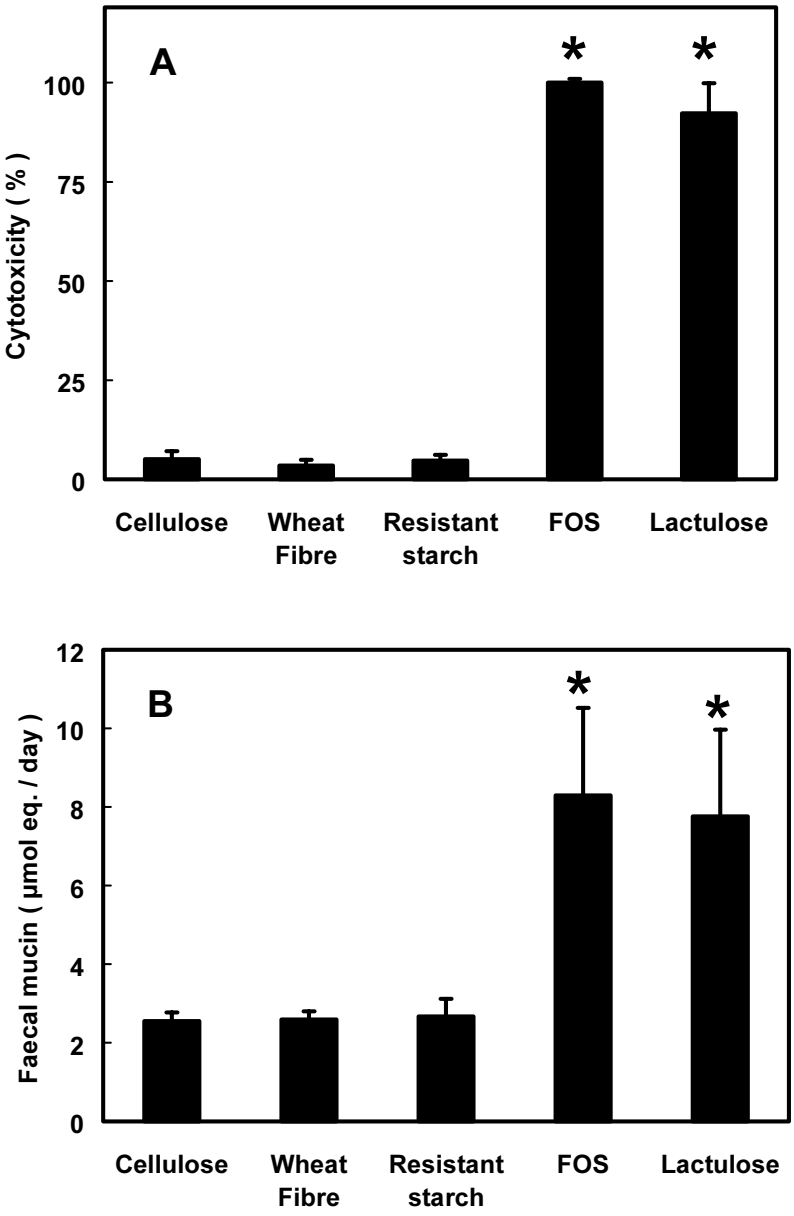

Fig 2.2 Effect of non-digestible carbohydrates on cytotoxicity of faecal water (panel A) and daily faecal mucin excretion (panel B) before infection of the rats. Cytotoxicity was determined with an haemolysis assay and mucins were measured fluorimetrically. Results are expressed as means ± SEM (n=7 in the wheat fibre group and n=8 in the other groups). An asterisk denotes that these faecal parameters were significantly increased compared to the control group (P<0.05). Abbreviations: FOS fructo-oligosaccharides.

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 wheat fibre- and cellulose-fed rats (Table 2.1). The type of non-digestible carbohydrate added to the diets affected

the ratio of L-lactic acid to total faecal lactic acid: cellulose 82 ± 8, wheat fibre 79 ± 8, resistant starch 57 ± 7, FOS 34 ± 7, and lactulose 43 ± 8%.

Faecal mucin excretion was significantly stimulated by FOS and lactulose supplementation **(Fig 2.2)**. These oligosaccharides also greatly increased the cytotoxicity of faecal water (Fig 2.2).

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 2.3)**. FOS and especially lactulose increased the number of bifidobacteria (Fig 2.3). Lactulose, FOS, and resistant starch also increased the number of enterobacteria in faeces (Fig 2.3). The levels of these bacterial genera were not affected by *S. enteritidis*, as determined in faecal samples collected 4 days after infection (data not shown).

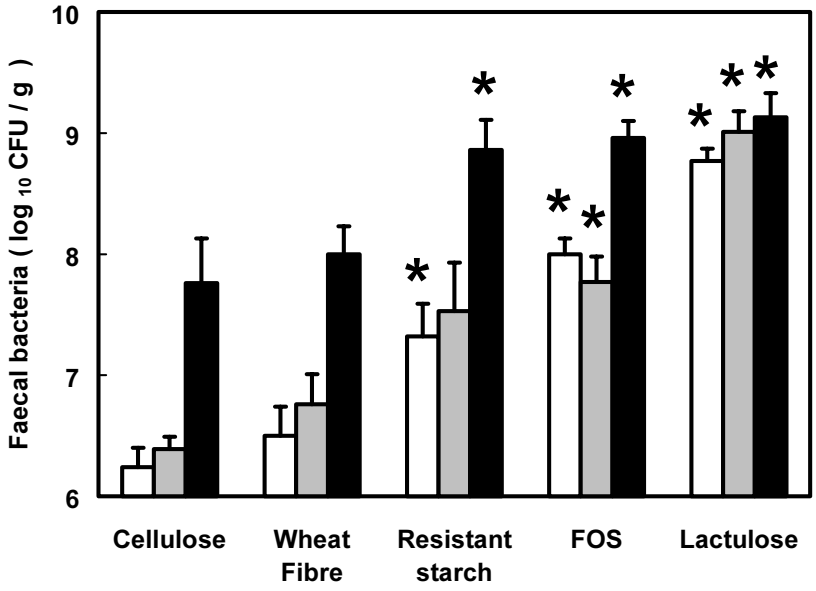

Fig 2.3 Effect of non-digestible carbohydrates on lactobacilli (white bars), bifidobacteria (grey bars), and enterobacteria (black bars) in faecal samples collected before infection of the rats. Lactobacilli were cultured anaerobically on Rogosa Agar and enterobacteria were cultured aerobically on Levine EMB Agar. Bifidobacteria were determined by specific real-time quantitative PCR. Results are expressed as means ± SEM (n=7 in the wheat fibre group and n=8 in the other groups). An asterisk denotes that these faecal bacteria were significantly increased compared to the control group (P<0.05). Abbreviations: FOS fructo-oligosaccharides, CFU colony-forming units.

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 2.4)**. Already the first day after oral infection with *S. enteritidis*, the number of viable salmonella excreted by the FOS-fed animals was significantly less, a phenomenon maintained afterwards. 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 4 post-infection. No significant differences in faecal salmonella output were observed between the cellulose, wheat fibre, and resistant starch groups at any time point after infection (Fig 2.4).

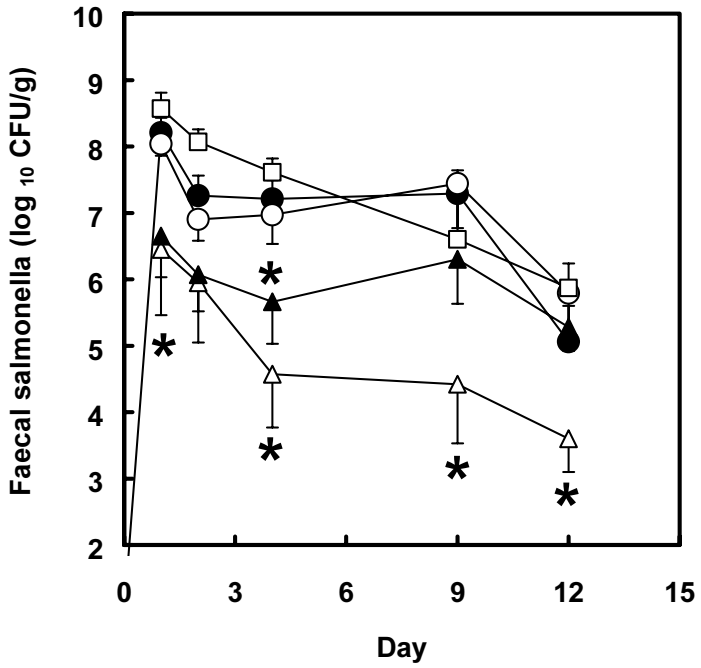

Fig 2.4 Effect of dietary cellulose (●), wheat fibre (**O**), resistant starch (❑), fructo-oligosaccharides (△), and lactulose (▲) on faecal salmonella excretion of rats after oral administration of 1.10^9 colony-forming units of *S. enteritidis* on day 0. Results are expressed as means ± SEM (n=7 in the wheat fibre group and n=8 in the other groups). An asterisk denotes that the indicated group is significantly different from all other groups at that time point (P<0.05). Abbreviation: CFU colony-forming units.

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

NO_x excretion over time **(Fig 2.5)**. After a latency period of 2 days, urinary NO_x excretion of lactulose- and FOS-fed rats increased strongly to 180 µmol/day, a maximum reached at day 7 after infection. Within the next week, urinary NO_x output decreased gradually to baseline levels again. Peak urinary NO_x excretion of the cellulose, wheat fibre, and resistant starch groups was approximately half that of the lactulose and FOS groups (Fig 2.5). Nevertheless, the kinetics of urinary NO_x excretion largely paralleled those of the lactulose and FOS groups. The total infection-induced urinary NO_x excretion (corrected for baseline output) was 412 ± 49, 370 ± 46, 530 ± 74, 834 ± 81, and 835 ± 106 µmol/12 days for the cellulose, wheat fibre, resistant starch, FOS, and lactulose group, respectively (the latter two groups were significantly different from the other dietary groups).

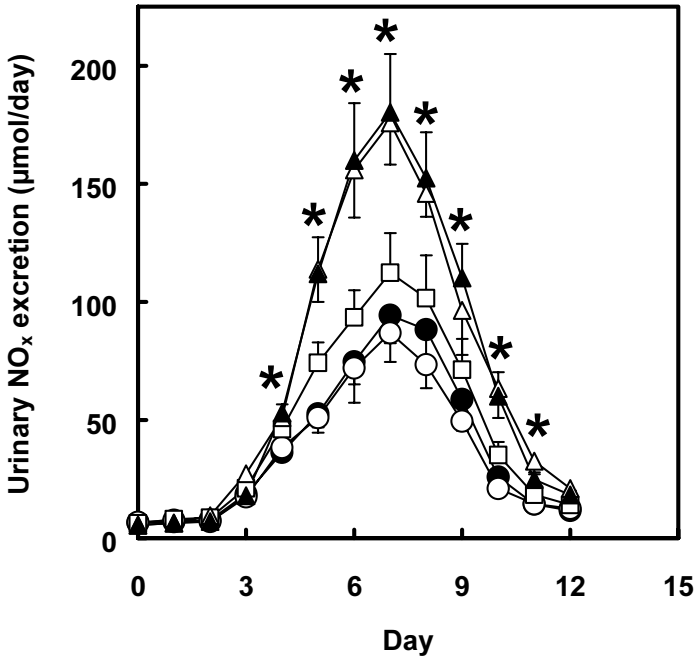

Fig 2.5 Effect of dietary cellulose (●), wheat fibre (**O**), resistant starch (❑), fructo-oligosaccharides (△), and lactulose (▲) on the kinetics of urinary NO_x excretion of rats after an oral challenge with 1.10^9 colony-forming units of *S. enteritidis* on day 0. Results are expressed as means ± SEM (n=7 in the wheat fibre group and n=8 in the other groups). An asterisk denotes that the FOS and lactulose groups are significantly different from all other groups at that time point (P<0.05).

To validate that the strong infection-induced increase in urinary 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 2.2)**.

Table 2.2 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 *

NB Results are expressed as means ± SEM (n=8 per group). An asterisk denotes that the indicated group is significantly different from the cellulose group (P<0.05). Abbreviation: CFU colony-forming units.

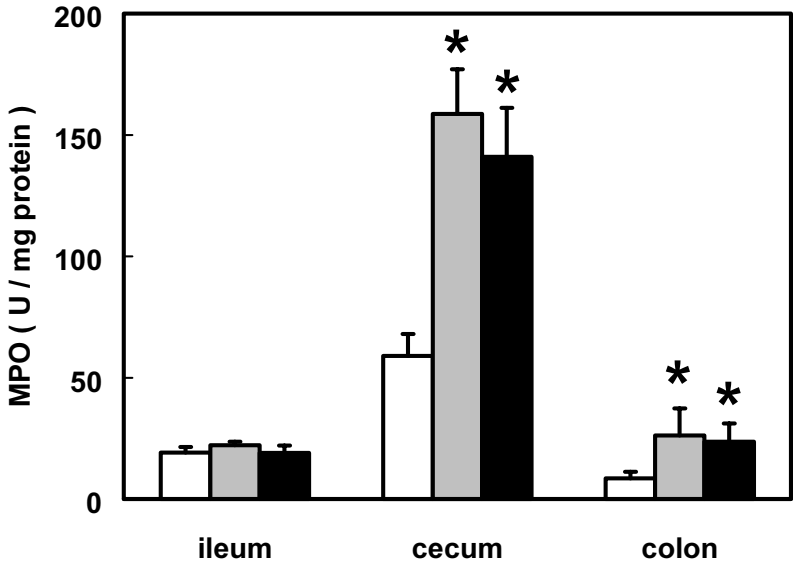

Fig 2.6 Effect of dietary cellulose (white bars), fructo-oligosaccharides (FOS) (grey bars), and lactulose (black bars) on the specific activity of myeloperoxidase (MPO) in the ileal, caecal, and colonic mucosa of rats two days after oral infection of the animals with 1.10^8 colony-forming units of *S. enteritidis*. Results are expressed as means ± SEM (n=8 per group). An asterisk denotes that the FOS and lactulose group are significantly different from the cellulose group (P<0.05).

In addition, the specific 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 2.6)**. Remarkably, dietary FOS and lactulose did not affect this marker of inflammation in the ileal mucosa (Fig 2.6).

2.4 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 with time.[21] 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. 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[22] and lactulose[23] 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 autochthonous gut flora is stimulated and intestinal contents are acidified due to the production of lactic acid and short-chain fatty acids.[24] Consequently, the colonisation of foodborne pathogens might be inhibited through enhanced competition for nutrients and mucosal adhesion sites and increased luminal killing of acid-sensitive pathogens, like salmonella.[14] Indeed, the present study showed that FOS and lactulose significantly lowered faecal pH and increased the number of lactobacilli and bifidobacteria. These lactic acid bacteria may contribute to host resistance as clinical investigations have shown that orally administered probiotic lactobacilli alleviate antibiotic-associated diarrhoea.[25] Besides a trophic effect of resistant starch, FOS, and lactulose on the endogenous lactobacilli and bifidobacteria, faecal enterobacteria were stimulated as well. 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.[7] The general opinion is that enterobacteria are not potentially beneficial because members of that bacterial family are involved in foodborne infectious disease[26] and gut-derived septicemia.[27]

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_x output and by higher viable salmonella counts in extra-intestinal organs. 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. Classically, bacterial translocation is quantified by blood or organ cultures. However, organ cultures notably underestimate total bacterial translocation because the majority of translocated pathogens is rapidly killed by the innate immune system upon translocation.[28] We have shown earlier that urinary NO_x excretion correlates with organ cultures, but is a more sensitive and quantitative biomarker of intestinal bacterial translocation.[2,15] Recently, several other rodent[29,30] and human[31,32] studies have been reported, showing that serum or urinary NO_x 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.

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 septicaemia. 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 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 also points to irritation of the gut mucosa. The mucus layer (viscous gel of hydrated mucin glycoprotein) 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[33] and short-chain fatty acids.[34] High concentrations of short-chain fatty acids can damage the intestinal epithelium, resulting in an increased permeability and epithelial cell proliferation.[35,36] Under normal circumstances, the major site of salmonella translocation seems to be the ileal Peyer's patches,[37] but this issue is still debated.[38] Considering that fermentation of FOS and lactulose in the human[22] and rat ileum[39] 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. 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.[40] 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 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[41] and rat[42] gut microflora, as indicated by its absence in faeces. However, the fermentation rates of polymers are generally less than that of oligomers.[43,44] Thus resistant starch feeding leads to a more gradual and distributed intestinal fermentation and concomitant production of organic acids.[45] 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.[46] 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.[46] 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*.[47] 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.

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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Chapter 3

Dietary fructo-oligosaccharides dose-dependently increase translocation of salmonella in rats

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J Nutr 2003;133:2313-2318

Abstract

Background

Prebiotics, such as fructo-oligosaccharides (FOS), stimulate the protective gut microflora, resulting in an increased production of organic acids. This may result in increased luminal killing of acid-sensitive pathogens. However, host defence against invasive pathogens, like salmonella, also depends on the barrier function of the intestinal mucosa. Rapid fermentation of prebiotics leading to high concentrations of organic acids may impair the barrier function. Therefore, we determined the dose-dependent effect of dietary FOS on the resistance of rats to *Salmonella enterica serovar enteritidis*.

Methods

Male Wistar rats were fed restricted quantities of a 'humanised' purified diet supplemented with 0%, 3% or 6% FOS (n=7 in the 6% FOS group and n=8 in the other diet groups). After an adaptation period of 2 weeks, rats were orally infected with 1.7×10^{10} colony-forming units of *S. enteritidis*. Supplement-induced changes in the intestinal microflora and faecal cation excretion were determined before and after infection. Cytotoxicity of faecal water was determined with an *in-vitro* bioassay, and faecal mucins were quantified fluorimetrically. Colonisation of *S. enteritidis* was determined by quantification of salmonella in caecal contents and mucosa. Translocation of *S. enteritidis* was quantified by analysis of urinary nitric oxide metabolites with time.

Results

Before infection, FOS decreased caecal and faecal pH, increased faecal lactic acid concentration and increased bifidobacteria and enterobacteria. FOS also increased cytotoxicity of faecal water and faecal mucin excretion, indicating mucosal irritation. Remarkably, FOS dose-dependently increased salmonella numbers in caecal contents and mucosa and caused a major increase in infection-induced diarrhoea. In addition, FOS enhanced translocation of salmonella.

Conclusions

Thus, in contrast to most expectations, FOS dose-dependently impairs the resistance to salmonella infection in rats. These results await verification by other controlled animal and human studies.

3.1 Introduction

Intestinal infections are still a major cause of disease in Western countries.[1] Due to the large-scale use of antibiotics and hence the development of resistant bacterial strains, research strategy is now focusing on prevention.[2] By influencing the composition of gastrointestinal contents, dietary non-digestible carbohydrates, i.e. oligosaccharides, could potentially modulate intestinal infections. Oligosaccharides affect the intestinal microflora.[3,4] and the gastro-intestinal survival of foodborne pathogens.[5] Some studies have shown that oligosaccharides stimulate the growth of bifidobacteria and lactobacilli.[3,4] These bacteria may reduce the survival of a pathogen by enhancing competition for nutrients and adhesion sites and production of organic acids.[6] Organic acids, i.e. lactic acid and short-chain fatty acids (SCFA), can inhibit growth of acid-sensitive pathogens like salmonella.[5] Despite these potential beneficial effects, few studies show the effects of oligosaccharides on the gastro-intestinal survival of foodborne pathogens.[7-10] We have shown earlier that lactulose and fructo-oligosaccharides reduce the intestinal colonisation of salmonella in rats.[5,11] However, oligosaccharides had inconsistent effects on colonisation of salmonella in studies with broiler chicks, [7,8] mice [9] and swine.[10]

Besides resistance to colonisation, host defence against invasive pathogens, like salmonella, also depends on the mucosal barrier. Bacterial translocation can occur when the ecological balance of the normal endogenous microflora is disrupted, when host immune defences are impaired, or during physical loss of the mucosal barrier. [12] High concentrations of organic acids, due to rapid fermentation of oligosaccharides, may potentially inhibit the colonisation of acid-sensitive pathogens, but can also induce injury to the intestinal mucosa and hence impair its barrier function.[13,14] Thus, inhibiting the intestinal colonisation of an invasive pathogen under conditions of increased intestinal fermentation may not necessarily result in an overall resistance enhancing effect.

Therefore, the aim of the present study was to determine the effect of increasing concentrations of dietary fructo-oligosaccharides (FOS) on the resistance of rats to intestinal colonisation and translocation of *Salmonella enterica serovar enteritidis. S. enteritidis* is a major cause of human foodborne infectious diarrhoea in industrialised countries [1] and is sensitive to dietary modulation.[5]

3.2 Materials and methods

Diets, infection and dissection of the rats

The animal welfare committee of Wageningen University (Wageningen, The Netherlands) approved the experimental protocol. Specific pathogen-free male Wistar rats (WU, Harlan, Horst, The Netherlands), 8 weeks old, with a mean body weight of 239 $\pm$ 2 g, were housed individually in metabolic cages. All rats were kept in a temperature (22-24 ^{0}C) and humidity (50-60%) controlled environment in a 12 h light-dark cycle. Rats (n=8 per diet group) were fed restricted quantities (13 g/day; 266 kJ/day) of a purified diet. Restricted food intake was necessary to prevent differences in food consumption and hence differences in vitamin and mineral intake. The experimental diets were supplemented with 3% or 6% (purity 93%; Raftilose ®P95, Orafti, Tienen, Belgium). FOS was added at the expense of glucose. The exact composition of the diets is provided in **Table 3.1**.

Table 3.1 Composition of the experimental diets

	Control	**3% FOS**	**6% FOS**
Ingredients, g/kg			
Dextrose	513	483	453
Acid casein	200	200	200
Palm oil	160	160	160
Corn oil	40	40	40
Cellulose	20	20	20
Fructo-oligosaccharide	0	30	60
Mineral mix [1]	48	48	48
$CaHPO_4.2H_2O$	5.16	5.16	5.16
Vitamin mix [1]	14	14	14

NB The composition of the vitamin and mineral mixtures is according to the recommendation of the American Institute of Nutrition 1993[15], except that calcium was omitted. In addition, tri-potassium citrate was added instead of KH_2PO_4 and choline chloride was added instead of choline tartrate. The concentrations of vitamins and minerals mixtures were increased to ensure adequate intake during restricted feeding.

Compared with the AIN-93 diet,[15] diets were low in calcium (30 mmol $CaHPO_4.2H_2O$/kg) and had a high fat content (200 g fat/kg), to mimic the composition of a Western human diet. Demineralised drinking water was supplied ad libitum. Rats were acclimatised to the housing and dietary conditions for 2 weeks. Subsequently, the rats were orally infected by gastric gavage of 1.7×10^{10} colony-forming units (CFU) *S. enteritidis* (clinical isolate, phage type 4 according to international standards; B1241 culture of NIZO food research, Ede, the Netherlands) suspended in 1 mL of saline containing 30 g sodium bicarbonate/L. *S. enteritidis* was cultured and stored, as described earlier.[5] At day 9 after infection, the rats were killed by carbon dioxide inhalation.

Microbiological analyses

Before infection, faeces were quantitatively collected for 5 days. Faeces were freeze-dried and subsequently ground to obtain homogeneous powdered samples. Real-time quantitative PCR targeting a 110-bp transaldolase gene sequence was used to specifically quantify bifidobacteria in faecal samples, as described and validated earlier.[16] DNA was isolated from freeze-dried faeces using the QIAamp DNA stool mini kit (QIAgen, Westburg, Leusden, The Netherlands). Real-time PCR was performed using the ABI Prism 7700® Sequence Detection System (PE Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). 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 synthesized by PE Applied Biosytems Custom Oligonucleotide Synthesis Services (Nieuwerkerk a/d Ijssel, The Netherlands). Universal Taqman® PCR Master Mix was purchased from PE Applied Biosystems. The cycle threshold values generated by real-time PCR from dilutions of DNA extracted from *Bifidobacterium infantis* and *Bifidobacterium breve* (cultures B651 and B655 of NIZO food research, respectively) were used to plot a standard curve from which the number of bifidobacteria in rat faeces could be calculated.

Before infection, fresh faecal samples were collected directly from the anus of the rats and analysed for the number of lactobacilli and enterobacteria. Lactobacilli were quantified by plating 10-fold dilutions in saline on Rogosa Agar (Oxoid, Basingstoke, U.K) and incubating in an anaerobic jar (MART microbiology, Lichtenvoorde, The Netherlands) at 37 ^{0}C for 3 days. To determine faecal enterobacteria, 10-fold dilutions in saline were plated

on Levine EMB Agar (Difco Laboratories, Detroit) and incubated aerobically overnight at 37°C. The detection limit of this method was 10^2 CFU/ g wet weight.

To determine salmonella numbers in caecal contents and mucosa, the caecum was excised and caecal contents was taken out 9 days after infection. After extensive washing of the caecum in sterile saline, the caecal mucosa was scraped off using a sterile spatula. Mucosa was suspended in 1 mL sterile saline and homogenised (Ultrathurrax Pro200, Pro Scientific Inc., Monroe, CT). 10-Fold dilutions in sterile saline were plated on Modified Brilliant Green Agar (Oxoid, Basingstoke, U.K), and incubated aerobically overnight at 37 ^{0}C. Sulphamandelate (Oxoid, Basingstoke, U.K) was added to the agar plates to suppress swarming bacteria, like proteus species. The detection limit of this method was 10^2 CFU/ g wet weight.

Chemical analyses of caecal contents and faeces

The pH of caecal contents was measured at 37°C. Total lactic acid was measured in caecal contents, collected on day 9 after infection, using a colourimetric enzymatic kit (Boehringer Mannheim, Germany), as described elsewhere.[5] SCFA (acetate, propionate and butyrate) in caecal contents were determined by gas chromatography, as described elsewhere.[17] Faeces were quantitatively collected and pooled per animal for 5 days before *S. enteritidis* infection and also on days 1-5 after infection for chemical analyses. Faeces were freeze-dried and subsequently ground to obtain homogeneous powdered samples. To measure sodium and potassium, faeces were treated with 50 g/L trichloroacetic acid for 1 h at room temperature and centrifuged for 2 min at 14.000 g. The supernatants were diluted with 0.5 g/L CsCl, and sodium and potassium were analysed by Inductive Coupled Plasma-Atomic Emission Spectrophotometry (ICP-AES; Varian, Mulgrave, Australia). Faecal ammonia was determined using a colourimetric kit (Procedure No. 640, Sigma Chemical Co., St. Louis, USA), as described earlier.[5] Faecal mucin was extracted from freeze-dried faeces and quantified fluorimetrically, as described earlier.[5] Standard solutions of N-acetylgalactosamine (Sigma) were used to calculate the amount of oligosaccharide side-chains liberated from mucins. Interfering oligosaccharides of dietary origin were removed by filtration. Faecal mucins are expressed as µmol oligosaccharide equivalents.

Analyses of faecal water

Faecal water was prepared by reconstituting freeze-dried faeces with appropriate amounts of double-distilled water to obtain the physiological osmolarity of 300 mOsmol/L. Samples were homogenised, incubated for 1 h at 37 °C and subsequently centrifuged for 1 h at 14,000 g (Hettich, Micro-rapid 1306, Tuttlingen, Germany). Supernatants (faecal water) were stored at -20 ^{0}C until further analyses. The pH of faecal water was measured at 37°C. Cytotoxicity of faecal water was determined with an erythrocyte assay, as previously described[18], and validated earlier with intestinal epithelial cells.[19] The incubations were of physiological ionic strength (300 mOsmol/L) and buffered at pH 7.0 (final 100 mmol/L 3-N-morpholino-propanesulfonic acid, Sigma) to prevent acid-induced haemolysis.

Analysis of urine samples

Complete 24h urine samples were collected starting 1 day before infection until 9 days after infection. Oxytetracycline (1 mg; Sigma) was added to the urine collection vessels of the metabolic cages to prevent bacterial deterioration. The concentration of NO_x (sum of nitrate and nitrite) was determined by automated flow injection analyses. Briefly, diluted urine was passed over a cadmium column to reduce nitrate to nitrite, followed by reaction of nitrite with Griess reagent.[20] The red azo-dye formed was measured spectrophotometrically at 538 nm.

Statistical analysis

Results are expressed as mean ± SEM (n=7 in the 6% FOS group and n=8 in the other diet groups). A commercially available package (Statistica 5.5, StatSoft Inc., Tulsa, OK) was used for all statistics. Not all data were normally distributed as indicated by the Shapiro Wilk's test. Therefore, the diet-induced differences within each period, either before or after infection were tested for their significance using a Kruskall-Wallis ANOVA. In case of significant treatment effects, the non-parametric Mann-Whitney U test (two sided), was used to test for differences between each treatment group and the control group. In all cases, Bonferroni correction was made for the number of comparisons (n=3). Differences were considered statistically significant when $p<0.05$.

3.3 Results

Animal growth and food intake

One animal in the 6% FOS group was excluded from the study results because of oropharyngeal reflux of the salmonella suspension, resulting in pneumonia. Before infection, no significant differences in growth between the diet groups were observed; mean growth was 2.5 g/day. However, the first week after infection FOS decreased growth; 3.1 ± 0.3 g/day in the control group, 2.4 ± 0.6 g/d in the 3% FOS group and 0.7 ± 1.4 g/day in the 6% FOS group ($p<0.05$). Before infection, all rats consumed the provided 13 g/day. During the first week after infection, food intake in the control group was still 13 g/day. However, food intake in the 6% FOS group was less; 13.0 ± 0.05 g/day in the control group, 12.4 ± 0.3 g/day in the 3% FOS group and 11.6 ± 0.5 g/day in the 6% FOS group ($p<0.05$). Thereafter, mean daily food intake was 13 g/day in all diet groups.

Intestinal microflora

Compared with the control group, 6% FOS increased the number of bifidobacteria in faeces **(Fig 3.1)**. There were no significant differences in faecal lactobacilli numbers between the diet groups. Both FOS groups increased the number of enterobacteria in faeces by a 100-fold (Fig 3.1). Neither the bifidobacteria nor the lactobacilli and enterobacteria levels were affected by *S. enteritidis* infection, as determined in faecal samples collected after infection (data not shown).

Caecal and faecal parameters

Dietary FOS dose-dependently decreased caecal pH **(Table 3.2)**. Moreover, FOS decreased pH of faecal water. Faecal pH was 6.9 ± 0.1 in the control group, 6.5 ± 0.1 in the 3% FOS group and 6.3 ± 0.1 in the 6% FOS group ($p<0.05$). No significant infection-induced change in caecal and faecal water pH was found for the diet groups (data not shown).

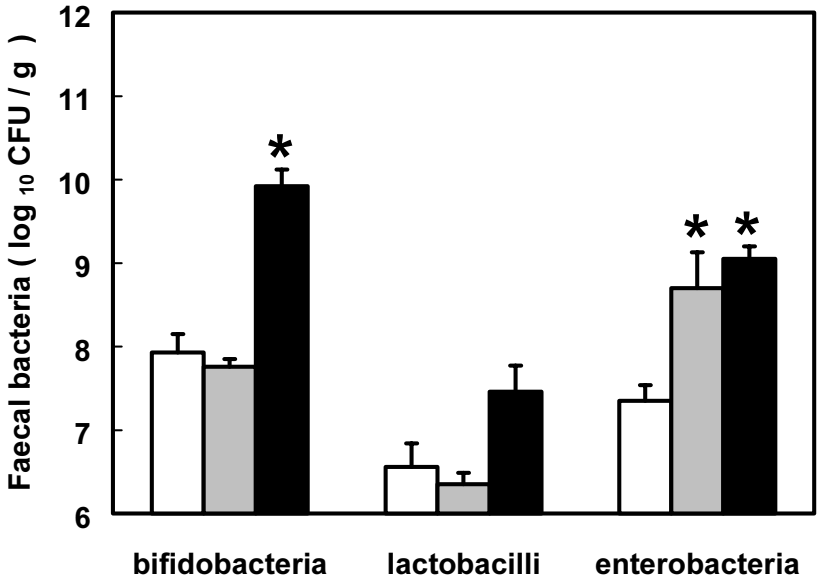

Fig 3.1 Effects of 0% (white bars), 3% (grey bars) and 6% (black bars) dietary fructo-oligosaccharides (FOS) on the number of bifidobacteria, lactobacilli and enterobacteria in faecal samples of rats, before infection. Bifidobacteria were specifically quantified by real-time quantitative PCR. Lactobacilli and enterobacteria were quantified by standard culturing on appropriate agar plates. Results are expressed as mean ± SEM (n=7 in the 6% FOS group and n=8 in the other groups). *, the indicated group is significantly different from the control group ($p<0.05$).

In addition, 6% FOS resulted in higher lactic acid concentrations in caecal contents compared to the control group (Table 3.2). However, there were no differences in caecal SCFA concentration or caecal SCFA pool, expressed as µmol/caecum, between the diet groups (Table 3.2). Before infection, faeces of both FOS groups were softened. After infection, especially faeces of the 6% FOS group was watery while the control group still had normal faeces. In the FOS groups, no proper assessment of faecal wet weight excretion could be made because of drying up of the pellets during collection in the metabolic cages. Therefore, faecal wet weight excretion was determined by the summed concentration of the faecal cations sodium, potassium and ammonia.[21] Before infection, FOS increased the daily faecal cation excretion. This was largely due to a 3-fold increase in faecal sodium and a 2-fold increase in faecal ammonia. No changes in faecal potassium were observed. After infection, daily faecal cation excretion increased in all diet groups, especially in the 6% FOS group **(Fig. 3.2)**.

Table 3.2 Effects of dietary fructo-oligosaccharides (FOS) on pH, short-chain fatty acids (SCFA) and lactic acid in caecal contents of rats

	Control	3% FOS	6% FOS
pH	7.6 ± 0.3^{a}	6.7 ± 0.1^{b}	5.8 ± 0.1^{c}
SCFA, µmol/g	35.9 ± 5.5^{a}	31.9 ± 4.3^{a}	32.5 ± 5.9^{a}
SCFA, µmol/caecum	33.4 ± 6.9^{a}	53.0 ± 14.7^{a}	82.8 ± 20.8^{a}
Lactic acid, µmol/g	3.2 ± 0.2^{a}	5.7 ± 1.4^{a}	30.1 ± 5.6^{b}
Lactic acid, µmol/caecum	2.7 ± 0.4^{a}	7.7 ± 2.2^{a}	76.4 ± 18.4^{b}

NB Results are expressed as mean ± SEM (n=7 in the 6% FOS group and n=8 in the other groups). Means in the same row without a common letter differ ($p<0.05$).

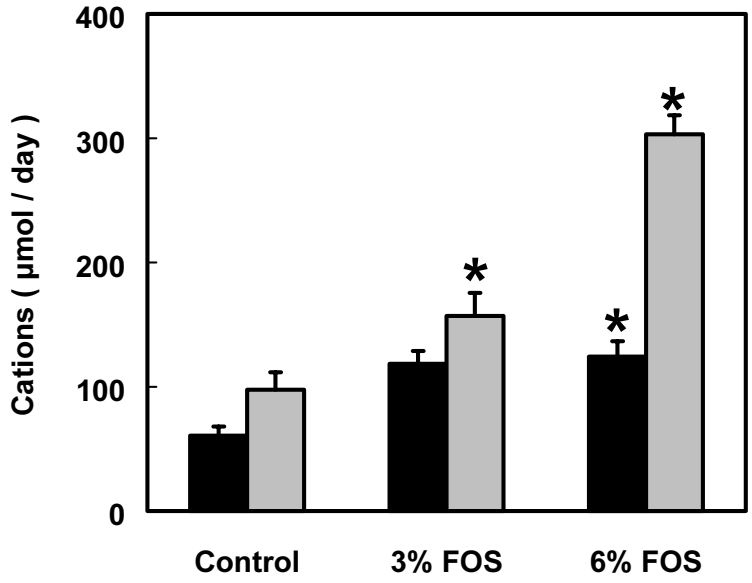

Fig 3.2 Effects of dietary fructo-oligosaccharides (FOS) on daily faecal cation excretion of rats, before (black bars) and after (grey bars) infection. Sodium, potassium and ammonia were measured as the major faecal cations. Results are expressed as mean ± SEM (n=7 in the 6% FOS group and n=8 in the other groups). *, the indicated group is significantly different from the control group, before or after infection ($p<0.05$).

This was due to a 6-fold increase in faecal sodium, a 3-fold increase in faecal ammonia and a 2-fold increase in faecal potassium, in the 6% FOS group compared to the control group. Both FOS groups strongly increased the cytotoxicity of faecal water, before infection **(Fig. 3.3)**. Cytotoxicity of faecal water was not affected by *S. enteritidis* infection.

Moreover, 6% FOS stimulated daily faecal mucin excretion before infection **(Fig. 3.4)**. Faecal mucin excretion increased even further after infection in the 6% FOS group

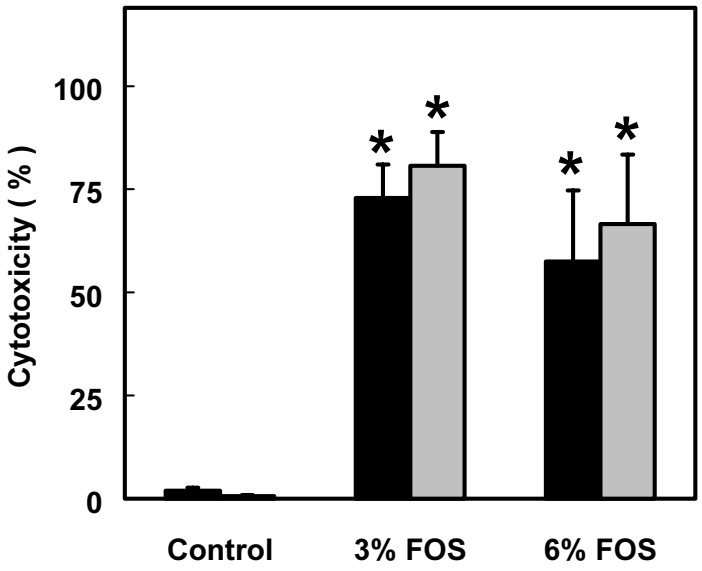

Fig 3.3 Effects of dietary fructo-oligosaccharides (FOS) on the cytotoxicity of faecal water of rats, before and after infection. Cytotoxicity was determined with a haemolysis assay. Results are expressed as mean ± SEM (n=7 in the 6% FOS group and n=8 in the other groups). *, the indicated group is significantly different from the control group, before or after infection ($p<0.05$).

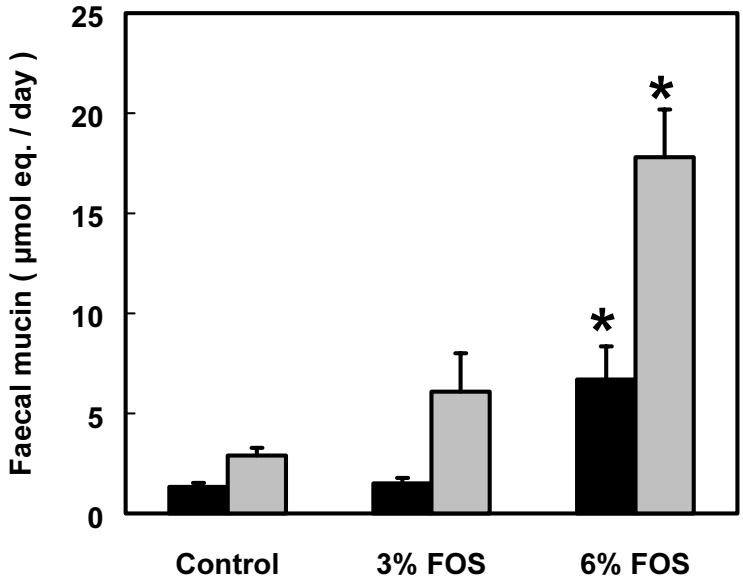

Fig 3.4 Effects of dietary fructo-oligosaccharides (FOS) on daily faecal mucin excretion of rats, before and after infection. Mucins were measured fluorimetrically and expressed as µmol oligosaccharide equivalents. Results are expressed as mean ± SEM (n=7 in the 6% FOS group and n=8 in the other groups). *, the indicated group is significantly different from the control group, before or after infection ($p<0.05$).

Colonisation and translocation of salmonella

FOS dose-dependently increased salmonella numbers in caecal contents, 9 days after infection **(Fig. 3.5)**. Moreover, 6% FOS increased salmonella numbers in caecal mucosa. In addition, major differences were observed in the effects of FOS on translocation of *S. enteritidis*, as measured by the infection-induced urinary NO_x excretion with time **(Fig. 3.6)**. After infection, urinary NO_x excretion of the 6% FOS group strongly increased to 202 µmol/d, a maximum reached at day 7. After day 7, urinary NO_x output gradually decreased to baseline levels again. Peak urinary NO_x excretion of the control and 3% FOS group was approximately half that of the 6% FOS group. However, the kinetics of urinary NO_x excretion was similar in all diet groups. The total infection-induced urinary NO_x excretion was higher in the 6% FOS group. The area under the curve corrected for baseline output was 332 ± 71, 452 ± 64, and 826 ± 51 µmol/9 days for the control, 3% and 6% FOS group, respectively.

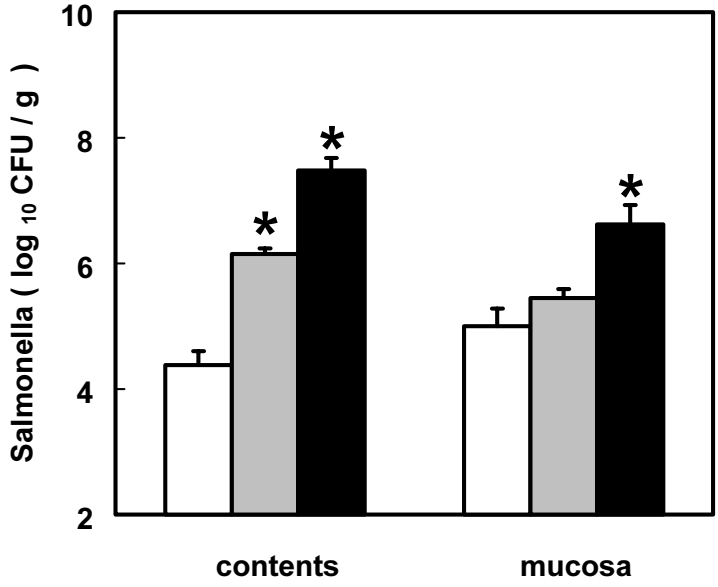

Fig 3.5 Effects of 0% (white bars), 3% (grey bars) and 6% (black bars) dietary fructo-oligosaccharides (FOS) on salmonella numbers in caecal contents and mucosa of rats, 9 days after oral administration of 1.7×10^{10} colony-forming units (CFU) of *S. enteritidis*. Salmonella was cultured aerobically on Modified Brilliant Green Agar. Results are expressed as mean ± SEM (n=7 in the 6% FOS group and n=8 in the other groups). *, the indicated group is significantly different from the control group, before or after infection ($p<0.05$).

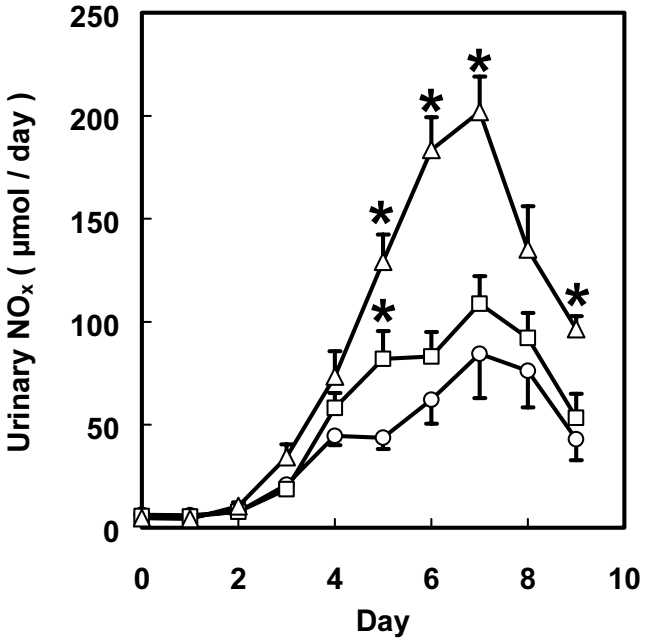

Fig 3.6 Effects of 0% (**O**), 3% (❑) and 6% (△) dietary fructo-oligosaccharides (FOS) on the kinetics of urinary NO_x excretion of rats after an oral challenge with 1.7×10^{10} colony-forming units of *S. enteritidis* on day 0. Results are expressed as mean ± SEM (n=7 in the 6% FOS group and n=8 in the other groups). An asterisk denotes that the indicated group is significantly different from the control group at that time point ($p<0.05$).

3.4 Discussion

In contrast to most expectations, dietary FOS impaired the resistance to intestinal infections, as it dose-dependently increased colonisation and translocation of *S. enteritidis* in the present rat study.

FOS is resistant to degradation by host digestive enzymes in the proximal small intestine as it is almost completely recovered from human[22] and rat ileum.[23] Subsequently, FOS is rapidly fermented by the microflora of the lower gut. Fermentation results in the production of lactic acid and short-chain fatty acids. This results in a lower intestinal pH, which can inhibit growth of acid-sensitive pathogens like salmonella.[5] Indeed, FOS dose-dependently decreased caecal and faecal pH in the present study.

The intestinal microflora increases the resistance to pathogen colonisation by competing for nutrients and mucosal adhesion sites and possibly by producing anti-microbial substances.[6] Previous human and rat studies showed that FOS increases densities of bifidobacteria and lactobacilli, both of which are considered beneficial for gut health.[3,24]

Indeed, in the present study, FOS stimulated the growth of bifidobacteria and enterobacteria. This suggests that the effect of FOS is fairly non-specific, as it stimulates the growth of potentially beneficial bacterial species but also that of potentially noxious species. The general opinion is that enterobacteria are not beneficial, because they are linked to gut-derived sepsis.[25] Previous animal and human studies showed that FOS either stimulated growth of enterobacteria[26,27] or had no effect.[3,28]

Although FOS decreased intestinal pH and stimulated the endogenous bifidobacteria, it increased colonisation of *S. enteritidis*, as measured by increased salmonella numbers in caecal contents and mucosa. Obviously, FOS facilitates adhesion and multiplication of salmonella in the intestinal tract. Moreover, our *in-vitro* study showed that salmonella can use FOS as a substrate for growth (data not shown). In accordance, FOS increased the infection-induced diarrhoea, as measured by the increased faecal cation excretion. The few infection studies present in literature showed inconsistent effects of oligosaccharides on salmonella colonisation in broiler chicks,[7,8] mice[9] and swine.[10] For example, Lettelier *et al*[10] showed that 1% FOS in water decreased colonisation of salmonella, while FOS in feed increased colonisation. Moreover, Chambers *et al.*[8] showed that 5% refined FOS decreased colonisation, while artichoke flour (containing 5% FOS) increased colonisation. Other studies showed no effect of oligosaccharides on colonisation of salmonella.[7,9] In addition, in a study with human volunteers FOS had no significant effect on travellers' diarrhoea, which is mainly caused by the non-invasive enterotoxigenic *E.coli.*[29]

Besides multiplication in the intestine and adhesion to the intestinal mucosa (colonisation), salmonella is able to penetrate the intestinal mucosa and reach extra-intestinal sites (translocation). Strikingly, we also found that FOS stimulated salmonella translocation, considering the strong increase in infection-induced urinary NO_x excretion. In contrast to organ cultures, which notably underestimate translocation due to rapid immune-mediated killing of translocated salmonella, urinary NO_x is a sensitive and quantitative biomarker of intestinal bacterial translocation.[30] We have shown earlier that urinary NO_x correlates with organ cultures.[31,32] Several other animal[31] and human[33,34] studies have shown that serum or urinary NO_x correlates with severity of systemic infectious disease. In summary, the FOS-induced increase in caecal colonisation and intestinal translocation of salmonella, the dramatic increase in infection-induced diarrhoea and the inhibition of animal growth indicates that the infection was worse in FOS supplemented rats. The FOS-induced stimulation of translocation could be due to its rapid fermentation, and hence production of

organic acids. High concentrations of organic acids can induce colonic mucosal damage or inflammation.[14] The intestinal mucosa responds to these irritants (and other irritants like bile acids and bacterial toxins) by increasing mucin excretion.[35] In agreement, FOS strongly increased cytotoxicity of faecal water and faecal mucin excretion, in the present study. Considering that the major fermentation effects of FOS are seen within the distal gut,[36] the mucosal barrier would be impaired at this site.[37] Under normal circumstances, penetration of the mucosal barrier by salmonella is supposed to occur through specialized epithelial cells (called M-cells) that are located in ileal Peyer's patches,[38] but this issue is still debated.[39] Salmonella can also translocate to extra-intestinal sites via dendritic cells, present in the mucosa throughout the entire intestine.[40] In addition, salmonella is able to cross enterocytes para- and transcellularly.[41] Thus, it is tempting to speculate that rapid fermentation of FOS expands the possibilities for salmonella to translocate even in the distal gut.

The dose of FOS used in the present study is realistic for the human situation. Daily intake of fermentable fibres for the U.S. and Europe has been estimated up to 10 g.[42] This estimation does not take into account consumption of specific meals and products supplemented with inulin or FOS, typically 3-10 g per portion.[43] Assuming a food intake of 400-500 g dry weight/d, this corresponds to 3%-5% in the diet.

In summary, we found that FOS dose-dependently increased the colonisation and translocation of *S. enteritidis* in rats. The effect may be due to the rapid production of acidic fermentation metabolites and hence impairment of the mucosal barrier. The results of the present study await verification by other controlled animal and human studies.

Acknowledgements

The authors wish to thank the biotechnicians at the Small Animal Center of Wageningen University (Wageningen, The Netherlands) for expert biotechnical assistance and Martijn Katan (Wageningen Center for Food Sciences) for stimulating discussions.

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Chapter 4

Dietary fructo-oligosaccharides and inulin decrease resistance of rats to salmonella: protective role of calcium

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Gut 2004; 53: 530-535

Abstract

Background

We have shown recently that rapidly fermentable fructo-oligosaccharides (FOS) decreased resistance of rats towards salmonella. It is not known whether inulin (which is fermented more gradually) has similar effects and whether buffering nutrients are able to counteract the adverse effects of rapid fermentation. Therefore we determined the effects of dietary inulin and FOS on the resistance of rats to *Salmonella enterica serovar enteritidis* and we determined whether calcium phosphate counteracts the effects of fermentation.

Methods

Male Wistar rats (n=8 per group) were fed a human "Western style diet". Diets with 60 g/kg cellulose (control), FOS or inulin had either a low (30 mmol/kg) or a high (100 mmol/kg) calcium concentration. After an adaptation period of 2 weeks the animals were orally infected with 2×10^9 colony-forming units of *Salmonella enterica serovar enteritidis*. Colonisation of salmonella was determined by quantification of salmonella in caecal contents. Translocation of salmonella was quantified by analysis of urinary nitric oxide metabolites with time.

Results

Inulin and FOS decreased intestinal pH and increased faecal lactobacilli and enterobacteria. Moreover, both prebiotics increased cytotoxicity of faecal water and faecal mucin excretion. Both prebiotics increased colonisation of salmonella in caecal contents and enhanced translocation of salmonella. Dietary calcium phosphate counteracted most adverse effects of inulin and FOS.

Conclusions

Both inulin and FOS impair the resistance to intestinal infections in rats. This impairment is partially prevented by dietary calcium phosphate. The results of the present study await verification by other controlled animal and human studies.

4.1 Introduction

Gastro-intestinal infections induced by food-borne pathogens are a major clinical problem.[1] Survival of food-borne pathogens within the intestinal tract can potentially be modulated by dietary prebiotics. Prebiotics, which include fructo-oligosaccharides (FOS) and inulin, are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve the host health.[2] Stimulation of the endogenous microflora by dietary prebiotics[3,4] may inhibit colonisation of intestinal pathogens by production of organic acids and by competing for nutrients and mucosal adhesion sites.[5] However, we showed earlier that FOS decreases the resistance towards the invasive food-borne pathogen *Salmonella enterica serovar enteritidis.*[6,7] Rapid fermentation of FOS by the intestinal microflora may lead to high luminal concentrations of organic acids. These organic acids may induce damage to the mucosal barrier[8,9] and hence decrease the resistance to intestinal pathogens.

Both inulin and FOS are composed of linear chains of fructose units, linked by ß(2-1) bonds and often terminated by a glucose unit. The number of fructosyl moieties ranges from 2-60 for inulin and 2-7 for FOS. *In-vitro* fermentation experiments revealed that molecules with a degree of polymerisation (DP) > 10 are fermented on average half as quickly as molecules with a DP < 10.[10] Thus, the possible adverse consequences of rapid fermentation might be more pronounced with FOS than with inulin.

The consequences of excessive fermentation can potentially be inhibited by dietary calcium phosphate. Calcium forms an insoluble complex with phosphate in the upper small intestine.[11] This complex increases the buffering capacity throughout the intestinal lumen.[9,12] Thus, it can be speculated that dietary calcium phosphate counteracts the effects of rapid fermentation of inulin and FOS.

The aim of the present study was to compare the effects of FOS and inulin on the resistance of rats to the invasive pathogen *Salmonella enterica serovar enteritidis*. In addition, we determined whether dietary calcium phosphate is able to counteract the effects of rapid fermentation.

4.2 Materials and methods

Diets, infection and dissection of the animals

The animal welfare committee of Wageningen University and Research Centre (Wageningen, The Netherlands) approved the experimental protocol. Specific pathogen-free male Wistar rats (WU, Harlan, Horst, The Netherlands), 8 weeks old, with a mean body weight of 276 ± 2 g, were housed individually in metabolic cages. All animals were kept in a temperature (22-24 ^{0}C) and humidity (50-60%) controlled environment in a 12 hour light-dark cycle. Animals (n=8 per diet group) were fed restricted quantities (13 g/day; 266kJ/day) of a purified diet. Restricted food intake was necessary to prevent differences in food consumption and hence differences in vitamin and mineral (especially calcium) intake. The control diet contained (per kg) 200 g acid casein, 552 g glucose, 160 g palm oil, 40 g corn oil, 20 g cellulose, 49 g mineral mix (without calcium) and 14 g vitamin mix according to AIN93 recommendation.[13] The experimental diets were supplemented with 60 g/kg cellulose (Arbocel type B800, JRS, Zutphen, The Netherlands), inulin (purity 99%, Frutafit TEX! ®; Sensus, the Netherlands) or FOS (purity 93%, Raftilose P95®; Orafti, Tienen, Belgium). Calcium (30 or 100 mmol $CaHPO_4.2H_2O$ / kg diet; Merck, Darmstadt, Germany) was added at the expense of glucose. The final calcium content of the diets was measured by Inductively Coupled Plasma-Atomic Emission Spectrophotometry (ICP-AES; Varian, Mulgrave, Australia) and was 38, 34 and 34 mmol/kg for the low-calcium/control, inulin and FOS diet, respectively. Calcium content of the high-calcium/control, inulin and FOS diet was 111, 114 and 107 mmol/kg, respectively. Demineralised drinking water was consumed ad libitum. Animals were acclimatised to the housing and dietary conditions for 2 weeks. Subsequently, the animals were orally infected by gastric gavage with 1 ml of saline containing 30 g/L sodium bicarbonate and 2×10^9 colony-forming units (CFU) of *Salmonella enterica serovar enteritidis* (clinical isolate, phage type 4 according to international standards; B1241 culture of NIZO food research, Ede, The Netherlands). The virulence of this strain is sustained by routine oral passage in Wistar rats, followed by isolation of translocated salmonella from liver and spleen. Salmonella was cultured and stored, as described earlier.[12] At day 7 after infection, the rats were killed by carbon dioxide inhalation.

Microbiological analyses of faeces

Faeces were quantitatively collected and pooled per animal for 5 days before salmonella infection. Faeces were freeze-dried and subsequently ground to obtain homogeneous powdered samples. Real-time quantitative polymerase chain reaction (PCR) targeting a 110-bp transaldolase gene sequence was used to specifically quantify bifidobacteria in faecal samples, as described and validated earlier.[7,14] The cycle threshold values generated by real-time PCR from DNA extracts of dilutions of suspensions of *Bifidobacterium infantis* and *Bifidobacterium breve* (cultures B651 and B655 of NIZO food research, respectively) were used to plot a standard curve from which the number of bifidobacteria in rat faeces could be calculated.

Before infection, fresh faecal samples were collected directly from the anus of the animals and analysed for the number of enterobacteria and lactobacilli. Faecal enterobacteria were quantified by plating tenfold dilutions in saline on Levine EMB Agar (Difco Laboratories, Detroit, Michigan, USA) and incubating aerobically overnight at 37°C. Lactobacilli were quantified by plating on Rogosa Agar (Oxoid, Basingstoke, UK) and incubating in anaerobic jars (MART microbiology, Lichtenvoorde, The Netherlands) at 37^0C for 3 days. The detection limit of this method was 10^2 CFU /g faecal wet weight.

To determine salmonella numbers in caecal contents 7 days after infection, the caecum was excised and caecal contents were removed. The pH of caecal contents was measured at 37°C and caecal tissue was weighed. Tenfold dilutions of caecal contents in saline were plated on Modified Brilliant Green Agar (Oxoid), and incubated aerobically overnight at 37ºC. The agar plates contained sulphamandelate (Oxoid) to suppress swarming bacteria, like proteus species. The detection limit of this method was 10^2 CFU / g faecal wet weight.

Biochemical analyses of faeces

Total faecal lactic acid was measured using a colourimetric enzymatic kit (Boehringer Mannheim, Germany), as described elsewhere.[12] To measure calcium faeces were treated with 50 g/L trichloroacetic acid for 1 h at room temperature and centrifuged for 2 min at 14,000 *g*. The supernatants were diluted with 0.5 g/L CsCl and analysed by ICP-AES (Varian). To measure phosphate, dry-ashed faeces was destructed (15 minutes at 180ºC) with a perchloric acid (70%) / hydrogen peroxide (30%) mixture (5:1 vol/vol, total volume

600 µL). Subsequently, samples were centrifuged for 2 min at 14,000 *g*. The supernatants were diluted with 0.5 g/L CsCl and analysed by ICP-AES (Varian).

Mucin was extracted from freeze-dried faeces and quantified fluorimetrically, as described earlier.[12] Interfering oligosaccharides of dietary origin were removed by filtration. Standard solutions of N-acetylgalactosamine (Sigma) were used to calculate the amount of oligosaccharide side-chains liberated from mucins. Therefore, faecal mucins are expressed as µmol oligosaccharide equivalents.

Analyses of faecal water

Fresh faeces were freeze-dried and reconstituted with double-distilled water to obtain faecal water with a physiological osmolarity of 300 mOsmol/L. Samples were mixed, incubated for 1h at 37°C and subsequently centrifuged for 1 hour at 14,000 *g* (Hettich, Micro-rapid 1306, Tuttlingen, Germany). Cytotoxicity of faecal water was determined with an erythrocyte assay, as previously described,[15] and validated earlier with intestinal epithelial cells.[16] The incubations were of physiological ionic strength (300 mOsmol/L) and buffered at pH 7.0 (final 100 mmol/L 3-N-morpholino-propanesulfonic acid, Sigma) to prevent acid-induced haemolysis.

Analyses of urine samples

Complete 24h urine samples were collected starting 1 day before infection until 6 days after infection. Oxytetracycline (1 mg; Sigma) was added to the urine collection vessels of the metabolic cages to prevent bacterial deterioration. The concentration of NO_x (sum of nitrate and nitrite) was determined using a colourimetric enzymatic kit (Nr. 1746081, Roche diagnostics, Mannheim, Germany). Briefly, urinary nitrate is reduced to nitrite by nitrate reductase. Subsequently, nitrite reacts with sulphanilamide and N-(1-naphthyl)-ethylene-diamine dihydrochloride resulting in a red diazo dye, which was measured spectrophotometrically at 540 nm.

Statistical analysis

Results are expressed as means ± SEM. A commercially available package (Statistica 6.1, StatSoft Inc., Tulsa, OK, USA) was used for all statistics. We tested for differences between the low-calcium/control, FOS and inulin groups. In addition, we tested for differences between the high-calcium groups and their low-calcium counterparts. In case

of normally distributed data, differences between means were tested for their significance using a one-way ANOVA, followed by the Fisher's protected least significant difference test (two sided). Differences were considered statistically significant when $p<0.05$. When data were not normally distributed, differences between means were tested for their significance using a Kruskall-Wallis ANOVA, followed by the non-parametric Mann-Whitney U test (two sided). Bonferroni correction was made for the number of comparisons (n=6). Differences were considered statistically significant when $p<0.05$.

4.3 Results

Animal growth and food intake

One animal in the high-calcium/FOS group was excluded from all study results because of oropharyngeal reflux of the salmonella suspension, resulting in pneumonia. Before infection, there were no differences in animal growth between the diet groups. Mean animal growth was 1.26 ± 0.07 g/day. However, after infection animal growth was significantly reduced in the low-calcium/FOS group **(Table 4.1)**. Calcium counteracted the infection-induced impaired growth of the FOS groups. Before infection all animals consumed the provided 13 g/day, as intended. During the first week after infection, the low-calcium/inulin and FOS groups consumed less than the control group although this was not significant (data not shown).

Caecal and faecal parameters

Both low-calcium/inulin and FOS lowered caecal pH (Table 4.1). Calcium counteracted this effect in the inulin group. Moreover, both low-calcium/inulin and FOS increased caecal weight (Table 4.1). Dietary calcium did not affect caecal weight.

Table 4.1 Effects of dietary fructo-oligosaccharides (FOS), inulin and calcium on animal growth, caecal pH and caecum weight after infection

	Growth (g/day)	Caecal pH	Caecal weight (g)
Low-calcium			
control	1.4 ± 0.2	7.2 ± 0.2	1.2 ± 0.1
inulin	0.5 ± 0.2	6.0 ± 0.1 *	1.9 ± 0.1 *
FOS	-0.6 ± 0.5 *	6.5 ± 0.3 *	2.1 ± 0.1 *
High-calcium			
control	1.4 ± 0.2	7.5 ± 0.2	1.2 ± 0.1
inulin	0.7 ± 0.5	7.0 ± 0.3 #	1.8 ± 0.1
FOS	0.6 ± 0.3 #	6.8 ± 0.2	1.7 ± 0.1

NB Results are expressed as mean ± SEM (n=7 in the high-calcium FOS group and n=8 in the other diet groups). *, the indicated group is significantly different from the low-calcium/control group ($p<0.05$). #, the indicated group is significantly different from its low-calcium counterpart ($p<0.05$).

Dietary prebiotics or calcium did not affect faecal bifidobacteria counts. However, low-calcium/inulin and FOS did increase the number of lactobacilli in faeces **(Table 4.2)**. Dietary calcium increased faecal lactobacilli in the inulin and control group, only. In addition, low-calcium/inulin and FOS increased the number of enterobacteria in faeces (Table 4.2). Dietary calcium decreased enterobacteria numbers in the inulin and FOS group. Salmonella infection did not affect the level of these bacterial genera in faecal samples collected after infection (data not shown). Before infection, low calcium/inulin and FOS tended to increase faecal lactic acid excretion, although this was not statistically significant **(Table 4.3)**. Calcium strongly increased daily faecal lactic acid excretion in all dietary groups. Both low-calcium/inulin and FOS decreased daily faecal excretion of calcium and phosphate (Table 4.3). As expected, daily faecal output of these minerals was substantially higher in the high-calcium groups. In addition, low-calcium/inulin and FOS increased the cytotoxicity of faecal water **(Fig 4.1)** and stimulated daily faecal mucin excretion in the low-calcium groups, before infection **(Fig 4.2)**. Both cytotoxicity and faecal mucin excretion were inhibited by dietary calcium.

Table 4.2 Effects of dietary fructo-oligosaccharides (FOS), inulin and calcium on faecal bifidobacteria, lactobacilli and enterobacteria before infection

	Bifidobacteria	**Lactobacilli**	**Enterobacteria**
	number (log_{10} / g wet faeces)	CFU (log_{10} / g wet faeces)	
Low-calcium			
control	9.5 ± 0.1	5.1 ± 0.4	7.6 ± 0.1
inulin	9.8 ± 0.1	6.6 ± 0.4 *	8.8 ± 0.1 *
FOS	9.9 ± 0.2	7.1 ± 0.4 *	9.0 ± 0.1 *
High-calcium			
control	8.9 ± 0.1 #	6.7 ± 0.2 #	7.4 ± 0.2
inulin	10.1 ± 0.1	7.5 ± 0.5 #	7.7 ± 0.3 #
FOS	10.2 ± 0.1	7.9 ± 0.2	7.7 ± 0.2 #

Table 4.3 Effects of dietary fructo-oligosaccharides (FOS), inulin and calcium on faecal lactate, calcium and phosphate excretion before infection

	Lactate	**Calcium**	**Phosphate**
Low-calcium		μmol/day	
control	2.4 ± 0.3	109 ± 13	113 ± 8
inulin	6.9 ± 1.6	28 ± 3 *	64 ± 6 *
FOS	6.4 ± 2.1	30 ± 6 *	60 ± 5 *
High-calcium			
control	9.4 ± 2.6 #	766 ± 30 #	468 ± 40 #
inulin	21.3 ± 4.3 #	619 ± 39 #	374 ± 29 #
FOS	26.9 ± 7.9 #	615 ± 35 #	367 ± 42 #

NB Bifidobacteria were specifically quantified by real-time quantitative PCR. Lactobacilli and enterobacteria were quantified by standard culturing on appropriate agar plates. Lactate was measured using a colourimetric enzymatic kit. Calcium and phosphate were analysed by ICP-AES. Results are expressed as mean ± SEM (n=7 in the high-calcium FOS group and n=8 in the other diet groups). *, the indicated group is significantly different from the low-calcium/control group ($p<0.05$). #, the indicated group is significantly different from its low-calcium counterpart ($p<0.05$). Abbreviations: CFU, colony-forming units

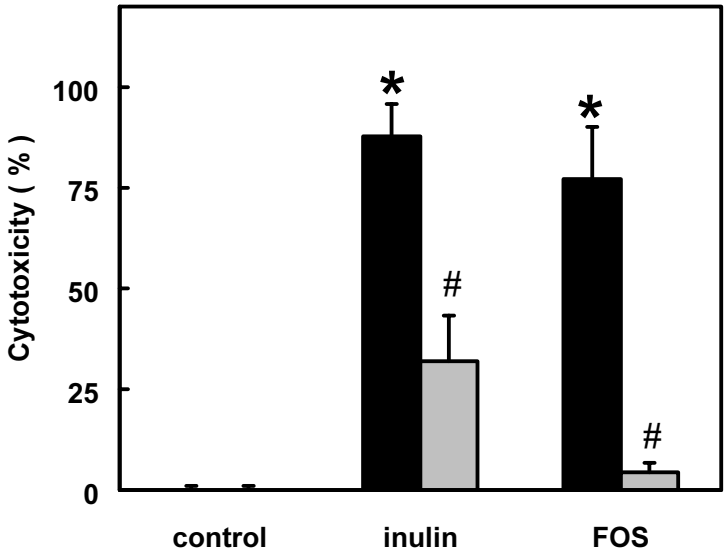

Fig 4.1 Effects of dietary inulin, fructo-oligosaccharides (FOS) and calcium on the cytotoxicity of faecal water of rats, before infection. Cytotoxicity was determined with a haemolysis assay. Results are expressed as mean ± SEM (n=7 in the high-calcium FOS group and n=8 in the other diet groups). Black bars indicate low-calcium groups. Grey bars indicate high-calcium groups. *, the indicated group is significantly different from the low-calcium/control group ($p<0.05$). #, the indicated group is significantly different from its low-calcium counterpart ($p<0.05$).

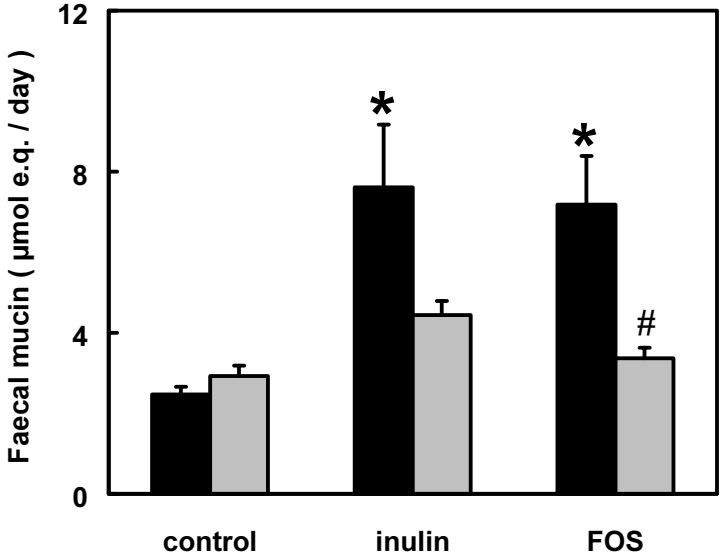

Fig 4.2 Effects of dietary inulin, fructo-oligosaccharides (FOS) and calcium on daily faecal mucin excretion of rats, before infection. Mucins were measured fluorimetrically and expressed as µmol oligosaccharide equivalents. Results are expressed as mean ± SEM (n=7 in the high-calcium FOS group and n=8 in the other diet groups). Black bars indicate low-calcium groups. Grey bars indicate high-calcium groups. *, the indicated group is significantly different from the low-calcium/control group ($p<0.05$). #, the indicated group is significantly different from its low-calcium counterpart ($p<0.05$).

Colonisation and translocation of salmonella

Both inulin and FOS increased salmonella numbers in caecal contents, 6 days after infection **(Fig 4.3)**. Dietary calcium did not affect the colonisation of salmonella in the caecum. Major differences were observed in the effects of low-calcium/inulin and FOS on translocation of salmonella, as measured by the infection-induced urinary NO_x excretion with time **(Fig 4.4)**. Both low-calcium/inulin and FOS increased urinary NO_x excretion with time. At day 6 after infection, urinary NO_x excretion was threefold higher in the low-calcium/inulin group and fourfold higher in the low-calcium/FOS group, compared with the low-calcium/control group. Dietary calcium inhibited translocation of salmonella to a large extend in the FOS group. The total infection-induced urinary NO_x excretion (corrected for baseline output) for the low-calcium groups was 122 ± 12 µmol/6 days in the control group, 290 ± 59 µmol/6 days in the inulin group and 448 ± 66 µmol/6 days in the FOS group. Total NO_x excretion for the high-calcium counterparts was 104 ± 13 µmol /6 days in the control group, 174 ± 40 µmol /6 days in the inulin group and 217 ± 41 µmol /6 days in the FOS group.

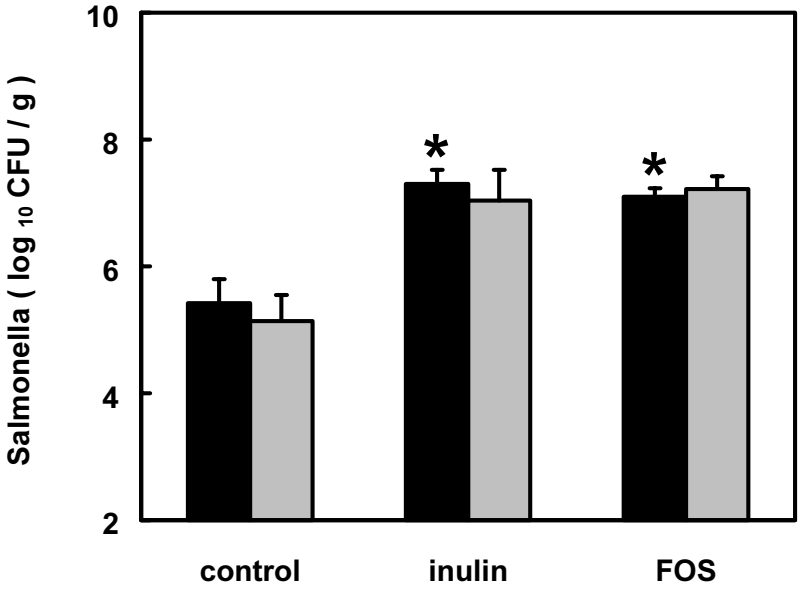

Fig 4.3 Effects of dietary inulin, fructo-oligosaccharides (FOS) and calcium on salmonella numbers in caecal contents of rats 6 days after oral administration of 2×10^9 colony-forming units of *Salmonella enterica serovar enteritidis*. Results are expressed as mean ± SEM (n=7 in the high-calcium FOS group and n=8 in the other diet groups). Black bars indicate low-calcium groups. Grey bars indicate high-calcium groups. *, the indicated group is significantly different from the low-calcium/control group ($p<0.05$).

4.4 Discussion

This study showed that inulin and FOS significantly stimulated intestinal colonisation and translocation of salmonella to extra-intestinal sites. These adverse effects were largely inhibited by dietary calcium phosphate.

The general opinion is that inulin and FOS may increase resistance to intestinal infections by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon.[2] Indeed, previous studies showed that inulin and FOS stimulated intestinal bifidobacteria and lactobacilli in rat and human.[3,4,17] However, in the present study, no effect of inulin and FOS on faecal bifidobacteria was observed. An explanation could be that when initial bifidobacteria counts are high to start with, then a further increase in their numbers might not be demonstrable.[10,18]

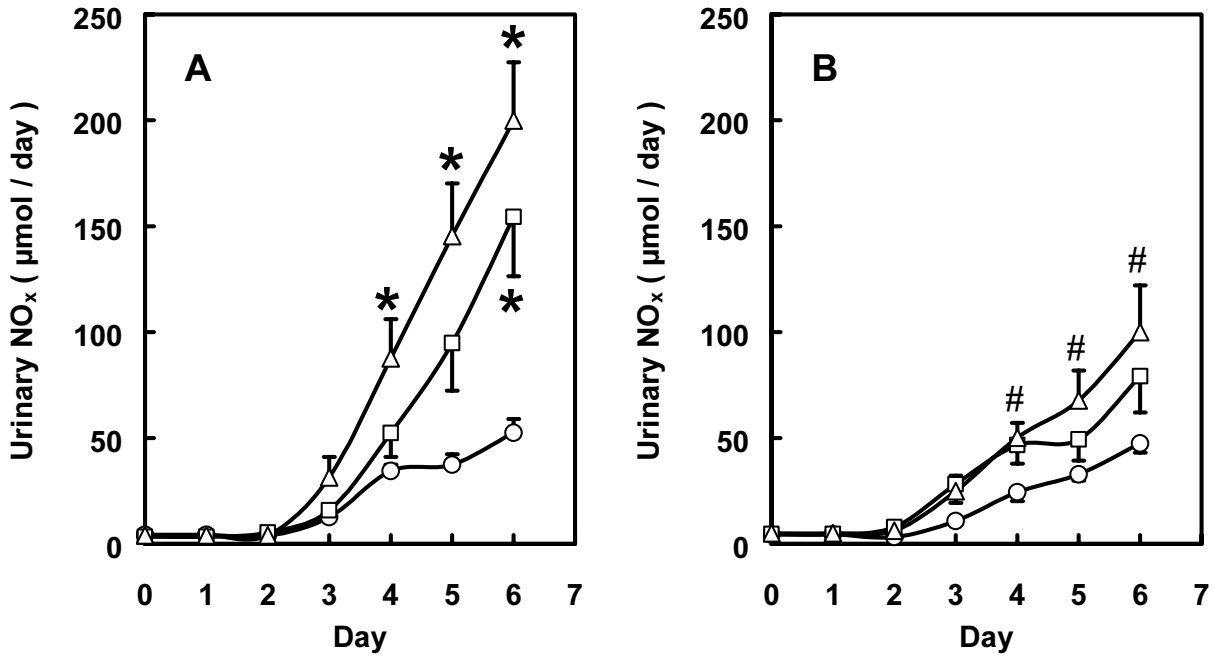

Fig 4.4 Urinary sum of nitrate and nitrite (NO_x) excretion in the control (**O**), inulin (❑), and fructo-oligosaccharide (FOS) (△) groups after an oral challenge with 2×10^9 colony-forming units of *Salmonella enterica serovar enteritidis* on day 0. *Panel A*, low-calcium groups. *Panel B*, high calcium groups. Results are expressed as mean ± SEM (n=7 in the high-calcium FOS group and n=8 in the other diet groups). *, the indicated group is significantly different from the low-calcium/control group at that time point ($p<0.05$). #, the indicated group is significantly different from its low-calcium counterpart at that time point ($p<0.05$).

Although lactobacilli were stimulated in the present study by inulin and FOS, our results as well as those of others do not support the concept of selective stimulation because

intestinal enterobacteria, which are associated with gut-derived septicaemia[19], were stimulated as well.[20,21] The endogenous microflora is known to be important for host resistance. However, the effects of inulin and FOS on the resistance to intestinal bacterial infections were hardly ever subject of *in-vivo* studies. The few, previously published studies showed inconsistent effects of oligosaccharides on colonisation of salmonella.[22-25] In the present study, inulin and FOS increased salmonella colonisation in caecum, despite stimulation of the intestinal microflora. These results do not confirm the results obtained in a previous study of our group. However, there is one important difference: in the previous study[6] salmonella was administered to the animals in a fed state, while in the present study the salmonella suspension was given after an overnight fast, so on an acidic empty stomach. This may have provoked an acid tolerance response in salmonella,[26,27] which may have increased its virulence and subsequent survival within the caecum and colon.[26,27] Besides the increase in colonisation, both prebiotics drastically stimulated salmonella translocation to extra-intestinal sites, as measured by the urinary excretion of nitric oxide (NO_x) metabolites. Urinary NO_x is a sensitive and quantitative biomarker of intestinal bacterial translocation,[28] which correlates with organ cultures[29] and severity of systemic infectious disease in rats and humans.[30-32] The increased colonisation and translocation of salmonella in the present study, concomitant with decreased animal growth after infection, indicates that the infection was worse in the low-calcium/inulin and FOS groups. These data confirm the results of our previous studies in which FOS increased translocation of salmonella.[6,7]

Why do inulin and FOS increase translocation of salmonella? Fermentation of both inulin and FOS results in the production of organic acids in the distal gut,[33] indicated by the high faecal lactate concentration and the low caecal pH. Accumulation of organic acids and other fermentation metabolites may lead to irritation and impairment of the mucosal barrier.[8,9,34] The intestinal mucosa responds to these irritating components by increasing mucus excretion.[35,36] Indeed, inulin and FOS increased faecal mucin excretion in the present study. In addition, previous studies showed that fermentable fibres increase faecal mucin excretion.[37,38] Thus, rapid fermentation of FOS and inulin may impair the mucosal barrier in the distal gut. Translocation of salmonella is believed to occur through ileal Peyer's patches, but this issue is still debated.[39] Impairment of the barrier by rapid fermentation of inulin and FOS might expand the possibilities for salmonella to translocate in the distal gut, para- or transcellularly.[40] Surprisingly, the effects of inulin and FOS were

similar. Probably both prebiotics were equally rapid fermented in the distal gut resulting in damage to the mucosal barrier. In addition, although organic acids inhibit growth of salmonella *in-vitro*, prebiotic-induced alterations in organic acid concentrations may increase salmonella virulence[41,42] and hence increase translocation.

Most adverse effects of inulin and FOS were inhibited by dietary calcium. Calcium forms an insoluble complex with phosphate in the upper small intestine.[11] The fermentation of inulin and FOS results in a considerable production of organic acids and hence acidification of gut contents. This can subsequently be counteracted by solubilisation of the calcium phosphate complex. However, when the dietary calcium phosphate supply is limited, the amounts of insoluble calcium phosphate may be insufficient to counteract acidification.[9] Moreover, the present study and previous studies[43,44] show that both inulin and FOS stimulate calcium absorption. Subsequently, less calcium phosphate will be available within the intestinal lumen to counteract the adverse effects of acidic fermentation.

Another mechanism by which calcium might improve the mucosal barrier is precipitation of cytotoxic components within the intestinal lumen. The amorphous calcium phosphate complex precipitates cytotoxic components in the intestinal lumen, which reduces epithelial cell damage[45] and increases resistance towards salmonella translocation. Indeed, dietary calcium inhibited the cytotoxicity of faecal water, induced by inulin and FOS. The cytotoxicity assay was performed in a buffer of neutral pH, precluding simple acid-induced lysis. Thus, calcium phosphate increases the buffering capacity of the intestinal lumen and has cytoprotective effects, which may preserve the mucosal barrier.[12]

In conclusion, the present study shows that FOS and inulin impair the resistance to salmonella infections in rats. This effect is likely due to the rapid production of fermentation metabolites and subsequent impairment of the mucosal barrier. Dietary calcium phosphate inhibited the adverse effects of acidic fermentation and largely prevented damage to the intestinal barrier. The results of the present study await verification by other controlled animal and human studies. However, considering the current interest in health foods supplemented with fermentable fibres, concern is warranted.

Acknowledgements

The authors wish to thank Wim van Doesburg for analytical analysis and the biotechnicians working at the Small Animal Centre of Wageningen University for expert biotechnical assistance.

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Chapter 5

Dietary fructo-oligosaccharides increase intestinal permeability in rats

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Submitted for publication

Abstract

Background

We have shown previously that fructo-oligosaccharides (FOS) decrease the resistance to salmonella infection in rats. However, the mechanism responsible for this effect is unclear.

Aim

We examined whether dietary FOS affects intestinal permeability before and after infection with *Salmonella enterica serovar enteritidis*.

Methods

Male Wistar rats were fed restricted quantities of a 'humanised' purified diet supplemented with 60 g/kg cellulose (control) or 60 g/kg FOS and with 4 mmol/kg chromium ethylenediamine-tetraacetic acid (CrEDTA) (n=8 or 10 per diet group). After an adaptation period of 2 weeks, animals were orally infected with 10^8 colony-forming units (CFU) of *S. enteritidis*. Cytotoxicity of faecal water was determined by a haemolysis assay and mucin concentrations in intestinal contents and mucosa were measured fluorimetrically, as markers of mucosal irritation. Intestinal permeability was determined by measuring urinary CrEDTA excretion. Translocation of salmonella was quantified by analysis of urinary nitric oxide metabolites with time.

Results

Before infection, FOS increased mucosal lactobacilli and enterobacteria in caecum and colon, but not in the ileum. However, FOS increased cytotoxicity of faecal water and intestinal permeability. Moreover, FOS increased faecal mucin excretion, mucin concentrations in caecal and colonic contents, and in caecal mucosa before infection. After infection, mucin excretion and intestinal permeability in the FOS groups increased even further in contrast to the control group. In addition, FOS increased translocation of salmonella to extra-intestinal sites.

Conclusion

FOS impairs the intestinal barrier in rats, as indicated by higher intestinal permeability. Whether these results can be extrapolated to humans requires further investigation.

5.1 Introduction

The enteric pathogen *Salmonella enterica serovar enteritidis* is one of the leading causes of gastro-intestinal infections ranging from mild, self-limiting inflammation of the intestinal mucosa to life-threatening systemic infection.[1] To successfully colonise the host, salmonella has to overcome the acidity of the stomach, the endogenous microflora and its bactericidal fermentation metabolites, and the intestinal barrier.

The epithelial barrier of the host can potentially be affected by dietary prebiotics. Prebiotics, which include fructo-oligosaccharides (FOS), are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that can improve host health.[2] By stimulating the protective endogenous microflora[3] and increasing bactericidal organic acid concentrations[4,5] within the lower gut, FOS may potentially enhance resistance towards intestinal pathogens. However, the effects of FOS on the resistance to intestinal bacterial infections were hardly ever subject of *in-vivo* studies. Unexpectedly, we consistently found that FOS increased translocation of salmonella towards extra-intestinal sites in several strictly-controlled rat infection studies.[6-8] Moreover, FOS resulted in significant infection-induced growth impairment,[6-8] gut inflammation[8] and diarrhoea.[6] At present, the mechanism responsible for these adverse effects of FOS is still unclear.

One possible explanation could be that rapid fermentation of FOS by the endogenous microflora results in overproduction of organic acids.[5,6] This may subsequently result in epithelial injury and increased intestinal permeability.[9-11] Therefore, the aim of the present rat experiment was to study the effect of FOS on intestinal permeability before and after infection with the invasive pathogen *S. enteritidis.*

5.2 Materials and methods

First experiment: animals and infection

The animal welfare committee of Wageningen University (Wageningen, The Netherlands) approved the experimental protocol. Specific pathogen-free male Wistar rats, 8 weeks old and with a mean body weight of 226 g (WU, Harlan, Horst, The Netherlands), were housed individually in metabolic cages. All animals (n=10 per diet group) were kept in a temperature (22-24^{0}C) and humidity (50-60%) controlled environment and in a 12 h light-

dark cycle. After adaptation to the housing and dietary conditions (see below) for 2 weeks, animals were orally infected by gastric gavage of 2×10^8 colony-forming units (CFU) *S. enteritidis* (clinical isolate, phage type 4 according to international standards; B1241 culture of NIZO food research, Ede, the Netherlands) suspended in 1 mL of saline containing 30 g sodium bicarbonate/L. Salmonella was cultured and stored, as described earlier.[12] On day 10 after infection, animals were killed by carbon dioxide inhalation.

Diets and CrEDTA

Animals were fed restricted quantities (14 g/day; 286 kJ/day) of a purified diet. Restricted food intake was necessary to prevent FOS-induced differences in food consumption as observed earlier,[8] and hence differences in vitamin, mineral, and CrEDTA intake. Demineralised drinking water was supplied ad libitum. The experimental diets were supplemented with 60 g/kg of cellulose or FOS (purity 93 g/100 g; Raftilose P95, Orafti, Tienen, Belgium). Compared with the AIN-93 recommendation for rat diets,[13] diets were low in calcium (30 mmol $CaHPO_4.2H_2O$/kg) and had a high fat content (200 g fat/kg), to mimic the composition of a Western human diet. The exact composition of the diets has been described earlier.[6] In addition, the inert intestinal permeability marker chromium ethylenediamine-tetraacetic acid (CrEDTA) was added to the diets.[14,15] The CrEDTA solution added to the diet was prepared as described elsewhere.[16] The complete formation and stability of the CrEDTA complex was checked by passing the prepared CrEDTA solution through a cation exchange resin column (Chelex® 100 Resin; Bio-Rad, Hercules, CA, USA). No uncomplexed Cr^{3+} ions were present in the final solution, which was added to the diets (final concentration CrEDTA as analysed was 3.9 mmol/kg diet). Moreover, no uncomplexed Cr^{3+} ions were present in faeces and urine samples (data not shown).

Microbiological analyses of faeces

Total 24 h faeces were collected on the last 4 days before and on 10 consecutive days after infection. Faeces were freeze-dried, pooled per animal per 2 consecutive days, and subsequently ground to obtain homogeneous powdered samples. DNA was isolated from faeces collected before infection using the QIAamp DNA stool mini kit (QIAgen, Westburg, Leusden, The Netherlands) according to the instructions of the manufacturer. However, after addition of the lysis buffer, the samples were shaken 3x1.5 min at 5000 rpm together with glass beads in a Bead Beater. Real-time quantitative polymerase chain reaction

(PCR), targeting a 110-bp transaldolase gene sequence, was used to specifically quantify bifidobacteria in faecal samples collected before infection, as described and validated earlier.[6,17] For lactobacilli determination, primers and probe targeted the 16S rDNA sequences. Forward primer LAB1 (5'-GGCAGCAGTAGGGAATCTTCCA-3') targets positions 350-371 of the Lactobacillus 16S rDNA molecule and is selective at the 3' end of the molecule. The LAB probe (5'-TGGAGCAACGCCGCGTGAGTGA-3') recognises positions 390-411 and is selective at the 5' end. Reverse primer LAB2 (5'-GTATTACCGCGGCTGCTGGCAC-3') is a semi-universal primer targeting 16S rDNA positions 504-524 of most bacteria. The fluorogenic oligonucleotide probe was labelled at the 5' end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3' end with the quencher dye 6-carboxytetramethylrhodamine (TAMRA). Universal Taqman® PCR Master Mix was purchased from PE Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands). Real-time PCR was performed using the ABI Prism 7700® Sequence Detection System (PE Applied Biosystems) under the following conditions: 2 min at 50ºC and 10 min at 95ºC to activate the AmpliTaq Gold DNA polymerase, and 50 cycles of 15s at 95ºC and 60s at 65ºC for amplification. To quantify *E-coli*, primers and probe targeting the *E-coli* specific 16S rDNA gene were designed as described and validated earlier.[18] The cycle threshold values generated by real-time PCR from DNA extracts of dilutions of a suspension of *Lactobacillus acidophilus* (culture B228 and B1836 of NIZO food research, the Netherlands) and *E-coli* (culture JM109 of ATCC, USA) were used to plot a standard curve from which the number of bacteria in faeces could be calculated.

Chemical analyses of faeces

Before infection, total faecal lactic acid was measured using a colourimetric enzymatic kit (Boehringer Mannheim, Germany), as described elsewhere.[12] Before and after infection, faecal mucin was extracted from freeze-dried faeces and quantified fluorimetrically, as described earlier.[12] Standard solutions of N-acetylgalactosamine (Sigma) were used to calculate the amount of oligosaccharide side-chains liberated from mucins. Control experiments showed that, interfering oligosaccharides of dietary origin were completely removed by the molecular filtration step in this procedure (data not shown).

Analyses of faecal water

Before infection, faecal water was prepared by reconstituting freeze-dried faeces with appropriate amounts of double-distilled water to obtain the physiological osmolarity of 300 mOsmol/L. Samples were thoroughly mixed, incubated for 1 h at 37°C and subsequently centrifuged for 1 h at 14,000 *g* (Hettich, Micro-rapid 1306, Tuttlingen, Germany). Supernatants (faecal water) were stored at -20°C until further analyses. The pH of faecal water was measured at 37°C. Cytotoxicity of faecal water was determined with an erythrocyte assay, as described previously[19] and validated earlier with intestinal epithelial cells[20]. The incubations were of physiological ionic strength (300 mOsmol/L) and buffered at pH 7.0 (final 100 mmol/L 3-N-morpholino-propanesulfonic acid, Sigma) to prevent acid-induced haemolysis.

Analyses of urine samples

Total 24 h urine samples were collected on the last 4 days before and on 10 consecutive days after infection. Oxytetracycline (1 mg) was added to the urine collection vessels of the metabolic cages to prevent bacterial deterioration. To measure CrEDTA, urine was treated with 50 g/L of trichloroacetic acid (1:1 vol/vol) and centrifuged for 2 min at 14,000 *g*. The supernatants were diluted with 0.5 g/L of CsCl solution, and chromium was analysed by Inductively Coupled Plasma-Atomic Emission Spectrophotometry (ICP-AES; Varian, Mulgrave, Australia). The concentration of NO_x (sum of nitrate and nitrite) was determined using a colourimetric enzymatic kit (Nr. 1746081; Roche diagnostics, Mannheim, Germany). Briefly, urinary nitrate is reduced to nitrite by nitrate reductase. Subsequently, nitrite reacts with sulphanilamide and N-(1-naphthyl)-ethylene-diamine dihydrochloride resulting in a red diazo dye, which was measured spectrophotometrically at 540 nm.[21]

Second experiment: animals, diets and biochemical analyses

A second experiment was performed to study the effect of FOS on the luminal and mucosal microflora and mucin concentration throughout the intestinal tract. Specific pathogen-free male Wistar rats (8 weeks old, mean body weight of 261 g, n=8 per diet group) were housed and fed the cellulose- or FOS-supplemented diets as described above for the first experiment. After an adaptation period of 14 days, the distal ileum (last 12 cm proximal to the ileocaecal valve), caecum, and the first 8 cm of the proximal colon were excised. Ileal, caecal and colonic contents were sampled. Ileal contents were pooled per diet group to obtain enough sample for measurements. Subsequently, the intestinal segments were longitudinally excised, and washed in sterile saline. The mucosa was scraped off using a spatula and suspended in sterile saline. Mucosal enterobacteria were quantified by plating tenfold dilutions in saline on Levine EMB Agar (Difco Laboratories, Detroit, Michigan, USA) and incubating aerobically overnight at 37°C. In addition, lactobacilli in intestinal mucosa were quantified by plating on Rogosa Agar (Oxoid, Basingstoke, UK) and incubating in anaerobic jars (MART microbiology, Lichtenvoorde, The Netherlands) at 37^0C for 3 days. The detection limit of this method was 10^2 CFU /g mucosal wet weight. Subsequently, ileal, caecal and colonic mucosa were freeze dried and sonicated for 1 min in phosphate buffered saline to liberate intracellular mucins. Mucins in ileal, caecal and colonic contents and mucosa were isolated and quantified fluorimetrically, as described above.

Statistical analysis

Results are expressed as means ± SEM (n=8 or 10 per diet group). A commercially available package (Statistica 6.1, StatSoft Inc., Tulsa, OK, USA) was used for all statistics. Differences between the FOS group and the control group were tested for their significance using a Kruskall-Wallis ANOVA, followed by the non-parametric Mann-Whitney U test (two-sided), as several parameters were not distributed normally. Differences were considered statistically significant when $P<0.05$.

5.3 Results

Animal growth, food intake and faecal parameters

Before and after infection, no significant differences in growth or food intake between the diet groups were observed. Before and after infection all animals consumed the provided 14 g/d, as intended. Before infection, FOS clearly stimulated faecal bifidobacteria, lactobacilli and enterobacteria **(Table 5.1)**. In addition, faecal lactate concentration was higher and pH of faecal water was lower in the FOS group (Table 5.1). FOS strongly increased the cytotoxicity of faecal water (Table 5.1). There were no infection-induced changes in faecal bacteria, pH and cytotoxicity of faecal water (data not shown).

Table 5.1 Effect of dietary fructo-oligosaccharides (FOS) on bacteria and lactate in faeces, and pH and cytotoxicity of faecal water of rats, before infection

	Control	**FOS**
Faecal bacteria		
Bifidobacteria (log_{10}/g wet)	6.92 ± 0.07	9.45 ± 0.08 *
Lactobacilli (log_{10}/g wet)	8.90 ± 0.08	10.57 ± 0.11 *
E.coli (log_{10}/g wet)	8.92 ± 0.16	9.44 ± 0.13 *
Other faecal parameters		
pH faecal water	6.63 ± 0.05	6.37 ± 0.09 *
Lactate (µmol/g dry)	2.93 ± 0.57	17.46 ± 5.20 *
Cytotoxicity faecal water (% lysis)	0.6 ± 0.1	71.2 ± 10.1 *

NB Results are expressed as means ± SEM (n=10 per diet group). Bifidobacteria, lactobacilli and *E.coli* were specifically quantified by real-time quantitative PCR. Cytotoxicity was determined with a haemolysis assay. *, FOS is significantly different from control (P<0.05).

FOS stimulated faecal mucin concentration, before infection **(Fig 5.1)**. After infection, FOS drastically increased faecal mucin concentration, while the control group still excreted pre-infection levels. Total daily mucin excretion before infection was 3.2 ± 0.4 µmol / day in the control group and 7.1 ± 0.8 µmol oligosaccharide equivalents / day in the FOS group (P<0.05). Peak mucin excretion after infection (day 8) in the control and FOS group was 4.0 ± 0.6 and 29.3 ± 4.1 µmol eq./day, respectively (P<0.05).

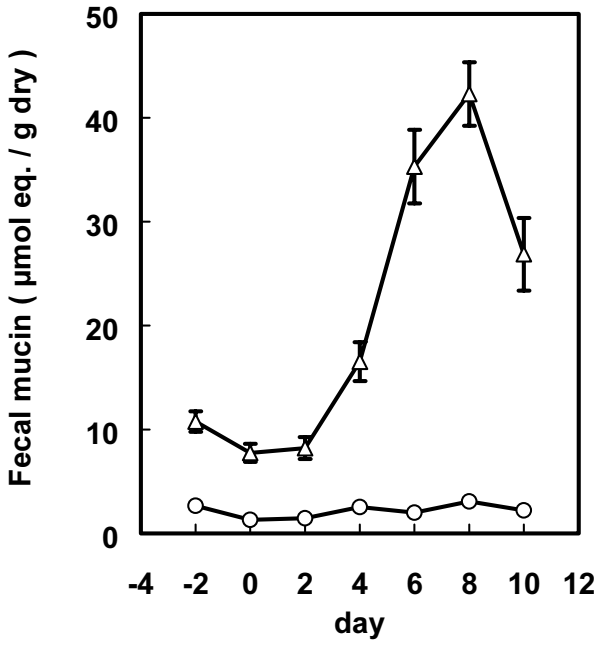

Fig 5.1 Mucin concentrations in rat faeces in the control (**O**) and fructo-oligosaccharide (FOS) (△) group, before and after an oral challenge with 2×10^8 colony-forming units of *Salmonella enterica serovar enteritidis* on day 0. Mucins were measured fluorimetrically and expressed as µmol oligosaccharide equivalents. Results are expressed as means ± SEM (n=10 per diet group). The FOS group is significantly different from the control group at all time points ($P<0.05$).

Microflora and mucin concentration in intestinal segments

FOS stimulated the growth of both lactobacilli and enterobacteria on caecal and colonic mucosa **(Table 5.2)**. However, no enhanced growth of these bacterial genera was seen in the ileal mucosa. Mucin concentration in ileal, caecal and colonic contents and the mucosa was determined **(Table 5.3)**. FOS increased mucin concentrations in caecal and colonic contents, but not in ileal contents. Moreover, FOS increased mucin concentration within the caecal mucosa. However, no FOS-induced change in mucin concentration was observed within the ileal and colonic mucosa.

Table 5.2 Effect of dietary fructo-oligosaccharides (FOS) on lactobacilli and enterobacteria on ileal, caecal and colonic mucosa of non-infected rats

	Control	FOS
Lactobacilli (log_{10} CFU/g wet)		
ileum	3.75 ± 0.26	3.82 ± 0.42
caecum	4.00 ± 0.27	4.99 ± 0.32 *
colon	3.70 ± 0.29	4.69 ± 0.40 *
Enterobacteria (log_{10} CFU/g wet)		
ileum	4.48 ± 0.19	4.21 ± 0.17
caecum	4.90 ± 0.28	6.08 ± 0.14 *
colon	4.42 ± 0.23	5.68 ± 0.26 *

NB Results are expressed as means ± SEM (n=8 per diet group). Lactobacilli and enterobacteria were quantified by plating on appropriate agar plates. *, FOS is significantly different from control (P<0.05).

Table 5.3 Effect of dietary fructo-oligosaccharides (FOS) on mucin concentrations in ileal, caecal and colonic contents and mucosa of non-infected rats

	Control	FOS
Contents (µmol eq. /g dry)		
ileum	31.2	32.0
caecum	6.6 ± 1.3	31.3 ± 6.1 *
colon	1.9 ± 0.2	20.0 ± 5.6 *
Mucosa (µmol eq. /g dry)		
ileum	15.9 ± 1.4	15.5 ± 1.0
caecum	6.3 ± 0.66	10.5 ± 0.5 *
colon	15.3 ± 1.9	14.2 ± 1.6

NB Results are expressed as means ± SEM (n=8 per diet group). Mucins were measured fluorimetrically and expressed as µmol oligosaccharide equivalents. Ileal contents were pooled per diet group to obtain enough sample for mucin measurement. *, FOS is significantly different from control (P<0.05).

Intestinal permeability

Due to restricted feeding, daily dietary intake of CrEDTA was similar in both diet groups (54 µmol CrEDTA/day). However, FOS increased urinary CrEDTA excretion before infection compared to the control group **(Fig 5.2)**. Before infection, the control group excreted 3.4 ± 0.2 % of the ingested CrEDTA in urine, while the FOS group excreted 6.0 ± 0.5 % ($P<0.05$). After infection, urinary CrEDTA excretion was increased even further, especially in the FOS group. The total infection-induced CrEDTA excretion (corrected for pre-infection baseline output) was significantly higher in the FOS group compared to the control group; 12.6 ± 2.3 versus 3.6 ± 1.0 µmol/10 days, respectively ($P<0.05$). In addition, similar results were obtained in control and FOS-fed animals, which received a single oral dose of CrEDTA pre-infection and on day 2 and day 6 post-infection (data not shown).

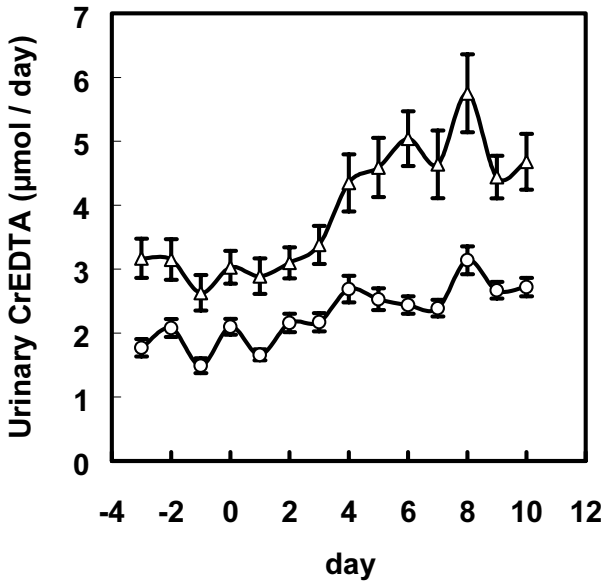

Fig 5.2 Urinary chromium ethylenediaminetetraacetic acid (CrEDTA) excretion in the control (**O**) and fructo-oligosaccharide (FOS) (△) group, before and after an oral challenge with 2×10^8 colony-forming units of *Salmonella enterica serovar enteritidis* on day 0. Daily dietary intake was 54 µmol CrEDTA. CrEDTA was analysed in urine by Inductively Coupled Plasma-Atomic Emission Spectrophotometry. Results are expressed as means ± SEM (n=10 per diet group). The FOS group is significantly different from the control group at all time points ($P<0.05$).

Translocation of salmonella

Major differences were observed in the effects of FOS on intestinal translocation of salmonella, as measured by the infection-induced urinary NO_x excretion with time **(Fig 5.3)**. After infection, urinary NO_x excretion of the FOS group strongly increased to 76 µmol/day, a maximum reached at day 8. After day 8, urinary NO_x output gradually decreased towards baseline levels again. The total infection-induced urinary NO_x excretion was significantly higher in the FOS group. The area under the curve corrected for baseline output was 202 ± 36 for the control group and 471 ± 69 µmol/10 days for the FOS group ($P<0.05$).

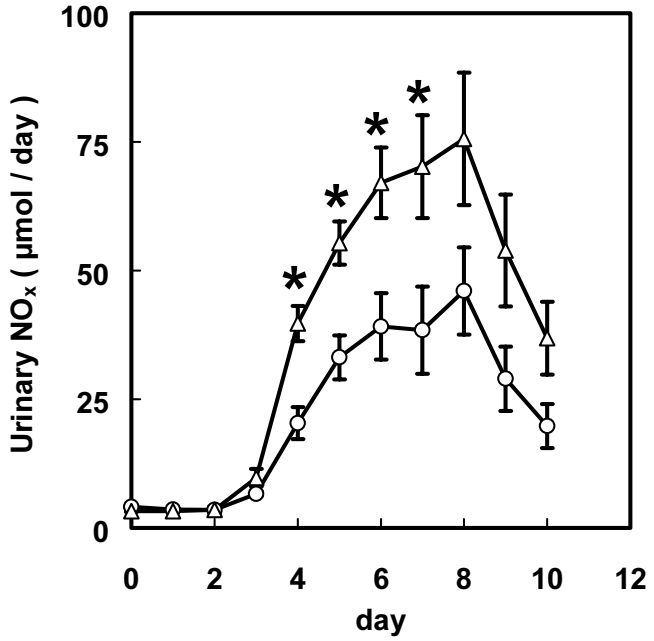

Fig 5.3 The kinetics of urinary NO_x excretion of rats in the control (**O**) and fructo-oligosaccharide (FOS) (△) group after an oral challenge with 2×10^8 colony-forming units of *Salmonella enterica serovar enteritidis* on day 0. Results are expressed as means ± SEM (n=10 per diet group). An asterisk denotes that the FOS group is significantly different from the control group at that time point ($P<0.05$).

5.4 Discussion

This study showed that FOS increased intestinal permeability and subsequently increased translocation of salmonella. Most previous studies focused on the effect of dietary prebiotics on the composition of the intestinal microflora and many of them suppose that improvements of host resistance to intestinal infections are likely. In our opinion,

irrespective of their prebiotic effect, the contribution of prebiotics in decreasing intestinal infections can only be verified in strictly controlled *in-vivo* studies including a challenge with a pathogen. The few previous studies that focused on the *in-vivo* effects of fermentable fibres on pathogen survival in healthy animals show either beneficial,[22-25] inconsistent[26-28] or adverse effects.[6,8,29] However, many of these studies have been performed in broiler chicks. In these animals, a large part of the fermentation of non-digestible carbohydrates already takes place within the crop. Moreover, many studies show data on pathogen colonisation, whereas translocation is not studied. However, in one study consumption of FOS seemed to increase salmonella numbers in liver and spleen of pigs, though it was not statistically significant.[28]

The mechanism behind the FOS-induced increase in intestinal permeability and salmonella translocation is not clear. We speculate that the FOS-induced accumulation of organic acids and other fermentation metabolites in the distal gut may result in irritation of the mucosal barrier.[9-11,30] To protect the intestinal mucosa, goblet cells respond to irritating components by increasing mucus excretion.[31-33] Indeed, before infection FOS increased faecal mucin excretion in the present study. Moreover, FOS increased mucin concentrations within caecal and colonic contents and within the caecal mucosa, while no effect was observed in the ileal contents and mucosa. In accordance with these results, Kleesen *et al*[34] found a thicker mucus layer and more goblet cells per crypt in response to FOS. Moreover, a study of Tsukuhara *et al*[35] showed that consumption of a high dose of FOS (100 g/kg) resulted in a very high mucus concentration in caecal contents. Mucins can be degraded by the intestinal microflora.[36] Thus, one could speculate that an increase in fermentable FOS in the large intestine reduces fermentation of gastro-intestinal mucins. This could subsequently result in a potential increase in the recovery of mucins in faeces. However, we have shown earlier that dietary resistant starch does not increase mucin secretion in rats,[8] although it is also completely fermented by the endogenous microflora of rats and humans.[37] Therefore, the increase in faecal mucin excretion induced by FOS indicates increased secretion and not decreased mucin degradation. In the present study, mucin excretion after infection was drastically increased in the FOS group, while the control group still excreted pre-infection levels. We do not dispute that mucin secretion itself is an important and beneficial host defence response. However, mucin production and secretion is enhanced by irritating components like bacterial pathogens,[38] endotoxins,[39] bile acids[40] or short-chain fatty acids.[31,32] Thus, the stimulated mucin

secretion indicates the necessity of the epithelial layer to protect itself against harmful substances.

In the present study, FOS clearly increased urinary CrEDTA excretion, even before infection. CrEDTA is a stable and inert molecule, which has a very low permeability through intact intestinal epithelium due to its size and hydrophilicity. Intestinal permeability to CrEDTA occurs passively by the paracellular route via tight junctions.[41] Enhanced intestinal fermentation and production of organic acids may result in mucosal injury as measured by the increased intestinal permeability of CrEDTA. In accordance, other studies showed that organic acids can increase intestinal permeability.[10] In the present study, the FOS-induced increase in intestinal permeability was more pronounced after infection. Indeed, several other studies have shown that intestinal inflammation increases intestinal absorption of CrEDTA.[42,43] Apparently, salmonella infection and the subsequent intestinal inflammation resulted in further impairment of the mucosal barrier in FOS-fed rats in contrast to the controls.

The enhanced translocation of salmonella in the FOS groups might also be the result of increased virulence of salmonella. Salmonella requires a large number of general stress response systems as well as specific virulence factors to successfully colonise the host, to translocate, and to avoid clearance by the host immune system. Virulence gene expression in salmonella is regulated by environmental factors. For example, changes in nutrient availability, osmolarity, pH, and organic acid concentrations can alter virulence of salmonella.[44-46] Although rapid fermentation of FOS results in production of organic acids,[4,5,11] which inhibit the growth of salmonella *in-vitro*, alterations in organic acid concentrations and profile might also increase translocation to extra-intestinal sites by affecting salmonella virulence.[44,45] The FOS- induced alterations in SCFA might increase salmonella virulence by providing a signal for expression of invasion genes.[44,45] We are currently investigating whether fermentation of FOS affects salmonella virulence and thus infection risk *in-vivo*.

In conclusion, the present study shows that rapid fermentation of FOS by the endogenous microflora impairs the gut mucosal barrier, indicated by the increased intestinal permeability before infection. This subsequently resulted in increased translocation of salmonella in the distal gut. However, caution has to be exercised when extrapolating findings from these animal experiments to the human situation as intestinal permeability of rats is higher than that of humans.[47] Therefore, we are now performing a human

intervention study to verify the effects of FOS on mucosal irritation and intestinal permeability. These data and that of other controlled *in-vivo* studies are needed to evaluate the effects of prebiotics on intestinal host resistance.

Acknowledgements

The authors wish to thank the biotechnicians at the Small Animal Center of Wageningen University (Wageningen, The Netherlands) for expert assistance and Hans Snel (NIZO food research, Ede, The Netherlands) for developing lactobacilli primers and probes.

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Chapter 6

Dietary fructo-oligosaccharides and the intestinal barrier in humans

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To be submitted

Abstract

Background

In contrast to most expectations we have shown in several rat infection experiments that dietary fructo-oligosaccharides (FOS) stimulate intestinal colonization and translocation of invasive *Salmonella enteritidis*. Moreover, FOS increased faecal lactic acid, cytotoxicity of the intestinal contents, faecal mucin excretion and intestinal permeability, before infection. The aim of the present study was to determine the human relevance. For obvious reasons, subjects can not deliberately be infected with a virulent invasive pathogen. Therefore, we studied whether FOS increases the above mentioned adverse effects, which were observed before infection.

Methods

A double-blind, placebo-controlled, cross-over study of 2x2 weeks, with a wash-out period of 2 weeks, was performed with 34 healthy men. Subjects consumed lemonade containing either 20 g FOS or placebo and 150 µmol of the intestinal permeability marker chromium ethylenediamine-tetraacetic acid (CrEDTA) per day. On the two last days of each supplement period subjects scored their gastro-intestinal complaints on a visual analogue scale and collected 24h faeces and urine. Faecal lactic acid was measured using a colourimetric enzymatic kit. Cytotoxicity of faecal water was determined with an in-vitro bioassay, faecal mucins were quantified fluorimetrically and intestinal permeability was determined by measuring urinary CrEDTA excretion.

Results

In agreement with our animal studies, consumption of FOS increased faecal wet weight, bifidobacteria, lactobacilli and lactic acid. FOS increased flatulence and intestinal bloating. Moreover, FOS consumption doubled faecal mucin excretion indicating mucosal irritation. However, FOS did not affect cytotoxicity of faecal water and intestinal permeability.

Conclusions

The FOS-induced increase in mucin excretion in our human study points in the direction of mucosal irritation in humans, but the overall effects are more moderate than those observed in rats.

6.1 Introduction

Fructo-oligosaccharides (FOS) are non-digestible carbohydrates, which are assumed to be beneficial for the host health because they stimulate the protective intestinal microflora.[1] FOS is found in varying concentrations in many foods such as wheat, banana, asparagus and garlic[2] and supplemented to several products, such as diary products and infant formulas. The average daily intake of non-digestible oligosaccharides for the U.S. and Europe has been estimated up to 10 g.[3] This estimation does not take into account consumption of specific meals and products supplemented with non-digestible oligosaccharides, typically 3-10 g per portion.[2] FOS is composed of linear chains of fructose units, linked by ß(2-1) bonds and often terminated by a glucose unit. The number of fructose units ranges from 2-7. FOS is not hydrolyzed by human small intestinal glycosidases and reaches the colon intact.[4] In the colon, FOS may specifically stimulate growth of the protective endogenous bifidobacteria and lactobacilli.[5,6] The subsequent production of organic acid may increase host resistance against acid-sensitive pathogens. However, this assumption has largely been based upon *in-vitro* studies. Host defence against invasive pathogens, like salmonella, also depends on the barrier function of the intestinal mucosa. High concentrations of organic acids may induce injury to the intestinal mucosa[7] and subsequently impair the barrier function.[8] Indeed, in several strictly-controlled rat infection experiments, we have shown that dietary FOS increases intestinal translocation of the invasive pathogen *S. enteritidis* to extra-intestinal organs.[9-11] Moreover, FOS significantly increased infection-induced growth impairment,[9-11] gut inflammation[11] and diarrhoea.[10] Before infection with salmonella, we observed that FOS increases cytotoxicity of the intestinal contents, mucin excretion and intestinal permeability, in rats. (Chapter 5) [9-11] We speculate that rapid fermentation of FOS results in accumulation of fermentation metabolites, i.e. lactic acid, which subsequently impair the intestinal barrier.

In view of the significant adverse effects of dietary FOS on the resistance to salmonella in rats and the fact that FOS has been added to a variety of products, such as dairy products and infant formulas, our aim was to show proof-of-principle in humans. For obvious reasons, subjects can not deliberately be infected with a virulent invasive pathogen. Therefore, we determined whether we could reproduce the adverse effects of FOS we observed before infection in rats. Thus, we studied the effects of FOS on cytotoxicity of faecal water, mucin excretion and intestinal permeability in healthy human volunteers.

6.2 Subjects and Methods

Subjects

Thirty-four healthy men aged 18-55 years participated in the study. Men were recruited by advertisements in regional newspapers and posters mounted in public buildings. Women were excluded because of the possible influence of the menstrual cycle on intestinal fermentation.[12] Moreover, it is more difficult for women to collect urine and faeces separately. Subjects had no history of gastro-intestinal disease, surgical operations of the small and large intestine or lactose intolerance. In addition, subjects did not use immunosuppressive drugs, antibiotics, anti-diarrhoeal drugs, laxatives and pre- or probiotics in the last 3 months prior to the study. The study was fully explained to the subjects and they gave their written informed consent before participating. The study protocol was approved by the Ethical Committee of Wageningen University, the Netherlands. After successfully completing the study, the subjects received a small financial reward to compensate for the inconvenience.

Design, diet and supplements

A double-blind, placebo-controlled cross-over design with two supplement periods of two weeks separated by one wash-out period of two weeks was used. Participants were stratified according to age. Throughout the two supplement periods, subjects were instructed to maintain their usual pattern of physical activity and maintain their habitual diet but to abstain from all dairy products and other foods with high calcium content. A low-calcium background diet was necessary because the FOS-induced adverse effects in the animal study were inhibited by high dietary calcium intake.[9] Moreover, foods containing large amounts of fermentable non-digestible carbohydrates (e.g. beans, leeks, onions) or pro- or prebiotics were forbidden.[2] In addition, alcohol consumption was restricted to a maximum of 4 glasses a day and a maximum of 20 glasses a week. Participants received a list of the forbidden foods and drinks. Subjects consumed either lemonade with 20 g of FOS (purity 93%, Raftilose P95, Orafti, Tienen, Belgium) or 6 g of sucrose (placebo) per day. The placebo lemonade was supplemented with sucrose to assure equal taste, viscosity and colour of both lemonades. This dose was divided into 3 cups of lemonade per day, which had to be consumed in the morning, afternoon and evening. The intestinal permeability marker, chromium ethylenediamine-tetraacetic acid (CrEDTA) was added to

the lemonade. The CrEDTA solution was prepared as described elsewhere.[13] We did not use ^{51}CrEDTA to exclude chronic exposure to γ-rays. The complete formation and the stability of the CrEDTA complex was checked by passing the final CrEDTA solution through a cation exchange resin column (Chelex 100 Resin; Bio-Rad, Hercules, CA, USA). No uncomplexed Cr^{3+} ions were present in the lemonade or in faeces and urine samples of the subjects in the supplement periods (data not shown). Based on analysis of the lemonade by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES; Varian, Mulgrave, Australia), CrEDTA intake in the FOS and placebo group was 150 µmol per day. Subjects and researchers were unaware who received the placebo or FOS lemonade.

Nutrition diary

On the last two consecutive days in both supplement periods, subjects quantitatively reported all consumed foods and drinks in a nutrition diary. Mean daily energy and nutrient intake in each period was calculated by using a computerized food consumption table (Dutch food composition database 2001, NEVO Foundation, Zeist, The Netherlands). In addition, subjects were asked to rate frequency and severity of gastro-intestinal complaints such as flatulence, bloating and abdominal pains or cramps on a visual analogue scale from 0 (none) to 5 (severe).

Microbiological analyses of faeces

On the last day in both supplement periods, 24 h faecal samples were collected for microbiological and biochemical analyses. Faeces was frozen on dry ice immediately after defecation, transported to the lab and stored at -40ºC. Thereafter, the whole sample was weighed, freeze-dried and subsequently ground to obtain homogeneous powdered samples. DNA was isolated from faeces collected before infection using the QIAamp DNA stool mini kit (QIAgen, Westburg, Leusden, The Netherlands) according to the instructions of the manufacturer. However, after addition of the lysis buffer, the samples were shaken 3x1.5 min at 5000 rpm together with glass beads in a Bead Beater. Real-time quantitative polymerase chain reaction (PCR), targeting a 110-bp transaldolase gene sequence, was used to specifically quantify bifidobacteria in faecal samples collected before infection, as described and validated earlier.[10,14]

For lactobacilli determination, primers and probe targeted the 16S rDNA sequences. Forward primer LAB1 (5'-GGCAGCAGTAGGGAATCTTCCA-3') targets positions 350-371 of the Lactobacillus 16S rDNA molecule and is selective at the 3' end of the molecule. The LAB probe (5'-TGGAGCAACGCCGCGTGAGTGA-3') recognizes positions 390-411 and is selective at the 5' end. Reverse primer LAB2 (5'-GTATTACCGCGGCTGCTGGCAC-3') is a semi-universal primer targeting 16S rDNA positions 504-524 of most bacteria. The fluorogenic oligonucleotide probe was labelled at the 5' end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3' end with the quencher dye 6-carboxytetramethylrhodamine (TAMRA). Universal Taqman® PCR Master Mix was purchased from PE Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands). Real-time PCR was performed using the ABI Prism 7700® Sequence Detection System (PE Applied Biosystems) under the following conditions: 2 min at 50ºC and 10 min at 95ºC to activate the AmpliTaq Gold DNA polymerase, and 50 cycles of 15s at 95ºC and 60s at 65ºC for amplification. To quantify *e-coli*, primers and probe targeting the *e-coli* specific 16S rDNA gene were designed as described and validated earlier.[15] The cycle threshold values generated by real-time PCR from DNA extracts of dilutions of a suspension of *Lactobacillus acidophilus* (culture B228 and B1836 of NIZO food research, the Netherlands) and *e-coli* (culture JM109 of ATCC, USA) were used to plot a standard curve from which the number of bacteria in faeces could be calculated.

Biochemical analyses of faeces

Total faecal lactic acid was measured using a colourimetric enzymatic kit (Boehringer Mannheim, Germany), as described elsewhere.[16] Freeze-dried faeces was reconstituted with double-distilled water to obtain faecal water with a similar dry weight percentage as the original sample (as determined by freeze-drying). Samples were mixed, incubated for 1h at 37°C and subsequently centrifuged for 1 hour at 14,000 *g* (Hettich, Micro-rapid 1306, Tuttlingen, Germany). pH of faecal water was measured at 37°C and cytotoxicity of faecal water was determined with an erythrocyte assay, as previously described, and validated earlier with intestinal epithelial cells.[17] Briefly, faecal water (5, 10, 20 and 40 µL) was buffered with 100 mmol/L 3-N-morpholino-propanesulfonic acid (Sigma) to a volume of 80 µL. After pre-incubation for 5 min at 37ºC, 20 µL of a washed human erythrocyte suspension was added and incubated for 2 hours at 37ºC. The incubations were of

physiological ionic strength (300 mOsmol/L) and buffered at pH 7.0 to prevent acid-induced haemolysis. The area under the curve (AUC) of the potassium release from erythrocytes, which were incubated with different concentrations of faecal water, was calculated.

Mucins were extracted from freeze-dried faeces and quantified fluorimetrically, as described earlier.[16] Standard solutions of *N*-acetylgalactosamine (Sigma) were used to calculate the amount of oligosaccharide side-chains liberated from mucins. Faecal mucin is therefore expressed as µmol oligosaccharide equivalents. Control experiments showed that interfering oligosaccharides of dietary origin were completely removed by the molecular filtration step (data not shown).

To measure faecal CrEDTA, calcium, sodium and potassium, freeze-dried faeces was treated with 50 g/L of trichloroacetic acid and centrifuged 2 min at 14,000 *g*. The supernatants were diluted with 0.5 g/L of CsCl and chromium and calcium were analyzed by ICP-AES (Varian).

Biochemical analyses of urine

On the two last consecutive days in both supplement periods, complete 24 h urine samples were collected in plastic bottles (volume 2 L) containing 10 mL 6 mol/L hydrochloric acid to prevent bacterial deterioration. To check whether 24h urine collection was complete, urinary creatinine was measured by an enzymatic colourimetric method (CRE2U,0-512; Roche, Germany). Urinary CrEDTA and calcium were analyzed by ICP-AES, as described above.

Statistical analysis

The randomisation code of the human intervention study was broken after finishing all laboratory analyses. All results are expressed as mean ± SEM in both supplement periods (n=34 per group). Because we used a cross-over design, a paired Student's *t* test (two-sided) was used to examine whether statistically significant differences existed between the placebo and FOS supplementation period ($p<0.05$)

6.3 Results

Participant characteristics and diet

Characteristics of the participants are given in **Table 6.1**. All participants completed the study successfully. However, five subjects did not collect faeces in one supplement period and were therefore excluded from all faecal analyses. Moreover, the urinary chromium results of one subject were excluded because of a very high urinary chromium concentration (approximately 10 fold higher than average). The mean daily intake of energy and nutrients, as determined by analysis of the nutrition diaries, is shown in **Table 6.2**. As intended, no significant differences between the placebo and FOS group were observed.

Table 6.1 Baseline characteristics of the subjects

	n=34
Sex	male
Age (y)	27.7 ± 1.7
Weight (kg)	76.9 ± 1.5
Height (m)	1.82 ± 0.01
BMI (kg/m^2)	23.2 ± 0.5

NB Characteristics are expressed as means ± SEM (n=34). BMI, body mass index

Table 6.2 Daily energy and nutrient intake of the subjects during the study

	Placebo	**FOS**	**Difference**
Energy (MJ)	10.5 ± 0.5	10.7 ± 0.5	0.2 ± 0.4
Carbohydrate (energy%)	49.1 ± 1.1	49.8 ± 1.5	0.7 ± 1.2
Fat (energy%)	32.6 ± 1.0	32.7 ± 1.3	0.1 ± 1.2
Protein (energy%)	14.6 ± 0.5	13.7 ± 0.4	0.9 ± 0.4
Calcium (mg)	298 ± 11	315 ± 14	16.7 ± 12.9
Dietary fibre (g)	29.8 ± 1.6	30.6 ± 1.6	0.8 ± 1.1
FOS (g)	0	20	20

NB Results are expressed as means ± SEM (n=34 per group). Energy and nutrient intake was determined by analysis of the nutrition diary (2×24 h) and assuming that the supplied supplements were consumed as instructed.

Compliance to the study protocol

Compliance of the subjects to the prescribed low-calcium diet was good, as indicated by the results of calcium excretion in urine and faeces. Normal calcium intake is approximately 1000 mg/day in the Netherlands. Calcium intake determined by the nutrition diaries was approximately 300 mg/day (Table 6.2). The calcium intake determined by faecal and urinary calcium excretion was approximately 400 mg/day. The daily urinary calcium excretion was 101 ± 11 mg/day in the placebo group and 100 ± 10 mg/day in the FOS group. The daily faecal calcium excretion was 326 ± 30 mg/day in the placebo group and 288 ± 25 mg/day in the FOS group. Total CrEDTA intake in the present study was 150 µmol/day. Considering the urinary and faecal excretion values of CrEDTA, compliance to supplement intake was good (Table 6.4). Finally, compliance to collecting the 24h urine samples was checked by measuring urinary creatinine excretion. Urinary creatinine values did not differ between FOS and placebo and were within the normal range of 0.13-0.22 mmol/kg body weight (data not shown).

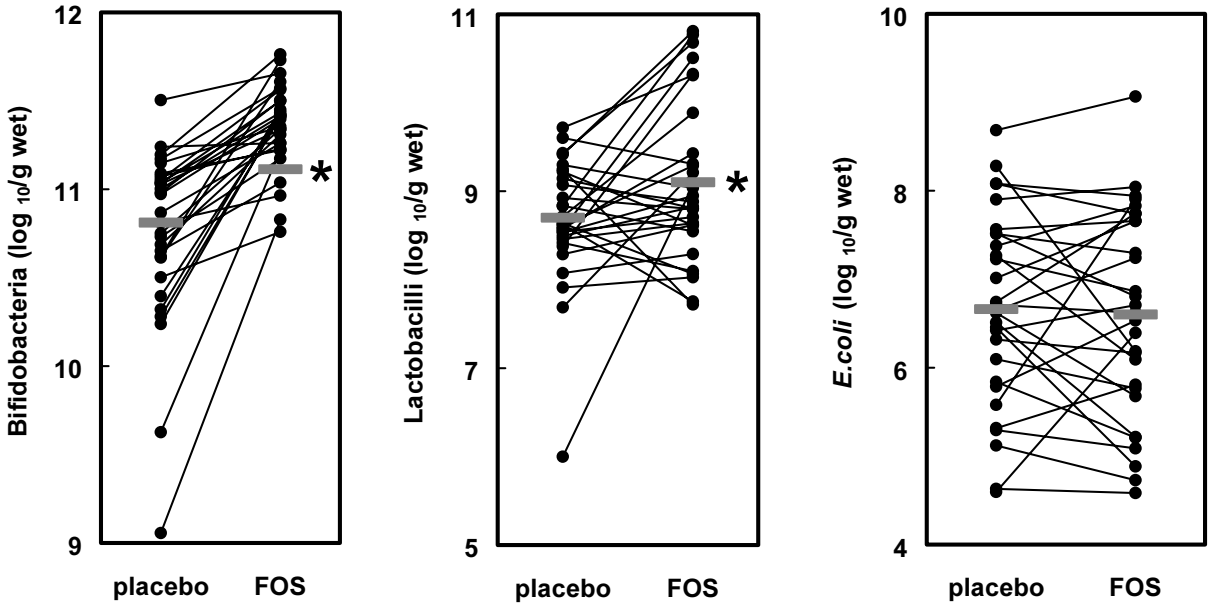

Fig 6.1 Effects of fructo-oligosaccharides (FOS) on faecal bifidobacteria (A), lactobacilli (B) and *E-coli* (C). Bacteria were specifically quantified by real-time quantitative PCR. Individual (•) and average values (—) are indicated. *, FOS is significantly different from placebo ($p<0.05$).

Intestinal microflora

Dietary FOS consumption resulted in a significant 4-fold increase in faecal bifidobacteria **(Fig 6.1A, $p<0.0001$)**. Except for one subject all subjects had higher faecal bifidobacteria

during the FOS period. Moreover, FOS also resulted in a 3-fold increase in faecal lactobacilli **(Fig 6.1B, p<0.05)**. However, no significant differences were found in faecal *E-coli* **(Fig 6.1C)**.

Gastro-intestinal complaints and stool characteristics

Subjects more frequently reported flatulence (p<0.0001) and bloating (p<0.005) in the FOS period than in the placebo period **(Fig 6.2)**. Scores for abdominal pain and cramps were slightly higher in the FOS group, though not statistically significant (p=0.05). The effects of dietary FOS on faecal characteristics were determined objectively. FOS increased daily faecal wet weight (p<0.05), but no significant effect was seen on the percentage of faecal dry weight. This was confirmed by measuring faecal cation excretion (data not shown). Faecal calcium and inorganic phosphate concentration was significantly lower in the FOS group (p<0.0005). In addition, FOS consumption resulted in a small and insignificant decrease in faecal pH **(Table 6.3; p=0.06)**. However, FOS consumption did result in a significant increase in total faecal lactic acid excretion **(Fig 6.3, p<0.05)**. Faecal lactic acid concentrations were 3.3 ± 0.7 µmol/g dry faeces in the control group and 22.0 ± 8.0 µmol/g dry faeces in the FOS group (p<0.05).

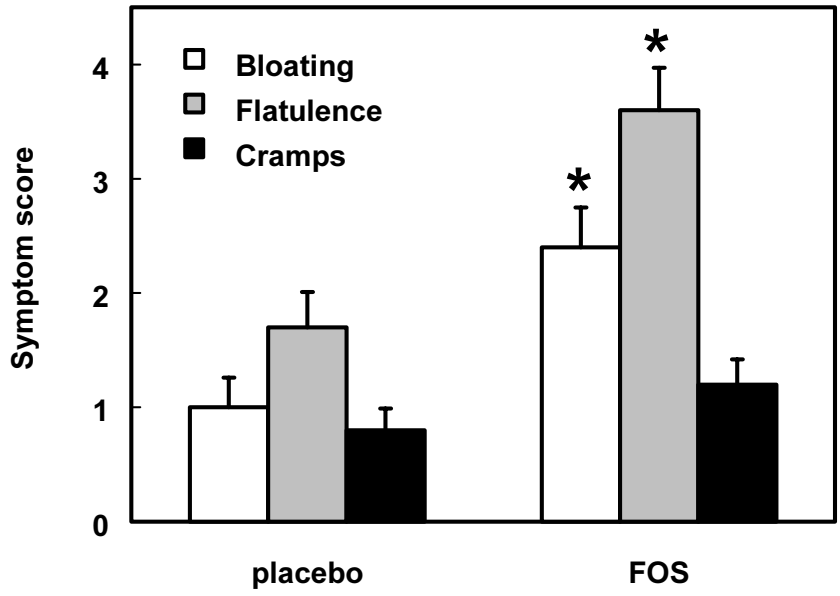

Fig 6.2 Effects of fructo-oligosaccharides (FOS) on flatulence, intestinal bloating and cramps. Subjects scored symptoms on a visual analogue scale from 0 (none) to 5 (severe). Results are expressed as mean ± SEM (n=34). *, FOS is significantly different from placebo (p<0.05).

Table 6.3 Effect of dietary supplementation with FOS on stool characteristics

	Placebo	FOS	Difference
Number of stools/day	1.2 ± 0.1	1.4 ± 0.1	0.2 ± 0.1
Wet weight (g/day)	203.7 ± 28.3	268.0 ± 29.4	64.3 ± 28.9 *
Dry weight (g/day)	44.2 ± 4.9	54.0 ± 5.1	9.8 ± 5.4
pH faecal water	6.1 ± 0.03	5.9 ± 0.06	0.1 ± 0.1
Calcium (µmol/g dry)	176.1 ± 9.5	147.2 ± 11.1	28.9 ± 7.4 *
Inorganic phosphate (µmol/g dry)	270.3 ± 15.2	208.5 ± 17.7	61.8 ± 15.5 *

NB Results are expressed as means ± SEM (n=34 per group). An asterisk indicates a significant difference between treatments ($p<0.05$).

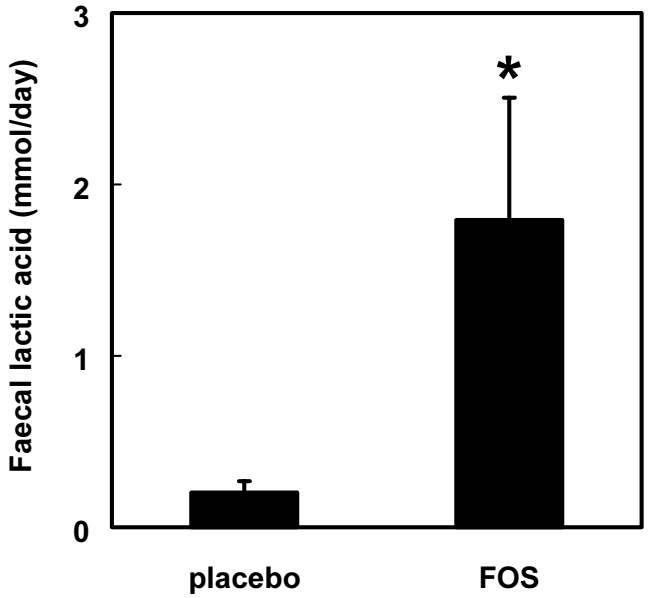

Fig 6.3 Effect of fructo-oligosaccharides (FOS) on daily faecal lactic acid excretion. Lactic acid was measured using a colourimetric enzymatic kit. Results are expressed as mean ± SEM (n=34). *, FOS is significantly different from placebo ($p<0.05$).

Cytotoxicity of faecal water, faecal mucin excretion and intestinal permeability

FOS consumption did not increase cytotoxicity of faecal water (**Table 6.4)**. However, FOS significantly increased faecal mucin excretion compared to the placebo group by approximately two-fold **(Fig 6.4, $p<0.05$)**. Daily intake of CrEDTA was similar in both supplement periods (150 µmol CrEDTA/day). There were no differences in urinary CrEDTA excretion between the placebo period and the FOS period (Table 6.4). Subjects

excreted 2.72 ± 0.28 % of the ingested CrEDTA in urine in the placebo period and 2.76 ± 0.44 % in the FOS period. These urinary CrEDTA values are within the normal range for healthy subjects.[18,19] Faecal CrEDTA excretion was similar in both dietary groups (Table 6.4).

Table 6.4 Effect of dietary supplementation with FOS on cytotoxicity of faecal water and urinary and faecal CrEDTA

	Placebo	**FOS**	**Difference**
Cytotoxicity (% of max lysis)	43.4 ± 7.5	45.8 ± 7.3	2.4 ± 9.0
Urinary CrEDTA (µmol/day)	3.3 ± 0.2	3.0 ± 0.3	0.3 ± 0.2
Faecal CrEDTA (µmol/day)	146.7 ± 13.8	136.8 ± 11.1	9.9 ± 17.3

NB Results are expressed as means ± SEM (n=34 per group). Cytotoxicity was determined with a haemolysis assay.

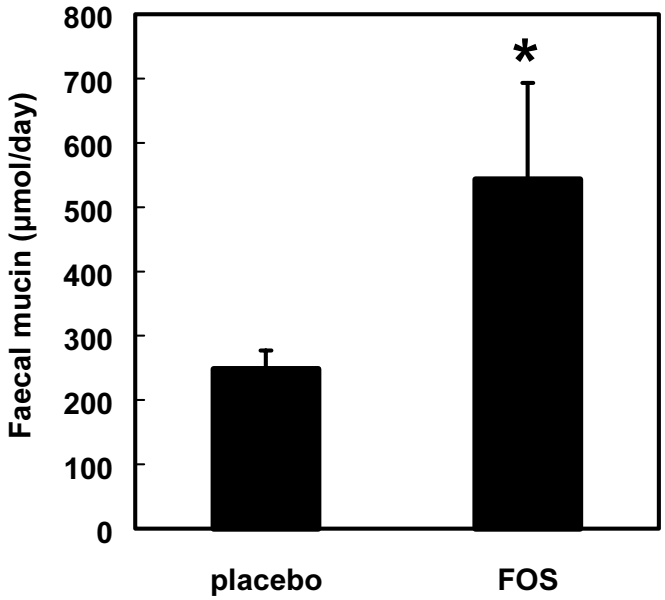

Fig 6.4 Effect of fructo-oligosaccharides (FOS) on daily faecal mucin excretion. Mucins were measured fluorimetrically and expressed as µmol oligosaccharide equivalents. Results are expressed as mean ± SEM (n=34). *, FOS is significantly different from placebo ($p<0.05$).

6.4 Discussion

In the present human study, intestinal fermentation of FOS stimulated the growth of faecal bifidobacteria and lactobacilli. Previous human[5] and animal[6] studies have also shown that highly fermentable non-digestible carbohydrates, like FOS, stimulate the growth of these bacteria. In agreement with our previous rat studies,[9-11] intestinal fermentation of FOS in humans resulted in high concentrations of faecal lactate. Lactate is a bacterial fermentation intermediate, which is normally metabolised to short-chain fatty acids (SCFA) by a range of acid-utilizing bacteria.[20] Therefore, lactate is either absent or present in low concentrations in faeces from healthy subjects.[21] However, lactate will accumulate during rapid carbohydrate breakdown because, unlike SCFA, it is not well absorbed by the epithelial cells of the large intestine.[20,22] In this relatively acidic environment SCFA are protonated and passively cross the enterocyte membrane. The subsequent reduction in intracellular pH, is compensated by the extrusion of H^+ in exchange for Na^+ via the ATP-using Na^+/H^+ antiporter in both the apical and basolateral membrane.[23,24] When the capacity of the enterocyte to cope with the influx of protons is exceeded it may result in exhaustion or even damage to the cell.[7,8]

We speculate that the rapid production of organic acids in the proximal colon induces the mucin excretion as observed in the present study.[25,26] This may subsequently impair intestinal barrier function, like we observed in our earlier animal studies.[9-11] We do not dispute that mucin secretion itself is an important and beneficial host defence response. However, mucin production and secretion is enhanced by irritating components like bacterial pathogens,[27] endotoxins,[28] bile acids[29] or short-chain fatty acids.[25,26] Thus, the stimulated mucin secretion indicates the necessity of the epithelial layer to protect itself against harmful substances. Considering that mucins can be degraded by the intestinal microflora,[30] one could speculate that the abundance of FOS as a substrate for bacterial degradation reduces fermentation of gastro-intestinal mucins. This could result in a potential increase in the recovery of mucin in faeces. However, resistant starch is also completely fermented by the endogenous microflora in rats and humans,[31,32] but does not increase mucin secretion in rats.[11] Therefore, the FOS-induced increase in faecal mucin excretion indicates increased production and secretion and not decreased mucin degradation.

In our earlier rat studies as well as in the present human study, FOS resulted in increased faecal bifidobacteria and lactobacilli, increased faecal wet weight, accumulation of faecal

lactate and increased intestinal mucin secretion. Thus, based on these physiological parameters, rats are a relevant model for humans. However, in contrast to our animal studies, FOS did not increase cytotoxicity of faecal water. This may indicate that the FOS-induced cytotoxic components as observed in the animal studies might not have been formed or are metabolised and/or absorbed in the human large intestine due to the longer transit time.[33,34]

The adverse effects of FOS on intestinal infections in our previous rat studies [9] were partly inhibited by dietary calcium phosphate. Moreover, Rémésy *et al* showed that the adverse effects of inulin fermentation were also inhibited by dietary calcium phosphate.[35] Calcium forms an insoluble complex with phosphate in the upper small intestine,[36] which subsequently reduces its absorption and that of phosphate. Phosphate increases the buffering capacity of the intestinal lumen. This may subsequently counteract the adverse effects of rapid fermentation of FOS and hence preserve the mucosal barrier.[16] Moreover, the amorphous calcium phosphate complex may precipitate cytotoxic components in the intestinal lumen, which reduces epithelial cell damage.[37] We therefore speculate that the discrepancy in cytotoxicity and intestinal permeability between our human and rat studies can largely be explained by differences in intestinal calcium concentration. Both the dietary FOS and the dietary calcium concentration was comparable between our human and rat studies; 30 µmol Ca/g diet in the rat diet and 20-25 µmol Ca/g diet in the human diet. Nevertheless, the intestinal calcium concentration was lower in rats than in the subjects of the present study. This is largely due to the higher calcium absorption in growing rats than in adult humans. Faecal calcium concentration in subjects of the present study was 176 and 147 µmol Ca/g faeces in the control and FOS group, respectively. However, faecal calcium concentration in rats was only 100 and 55 µmol Ca/g faeces in control and FOS-fed rats.[9] It should be noted that the intestinal calcium concentration in both rats and humans was lower due to FOS consumption. The relatively high intestinal calcium concentration in humans in comparison with rats might have precipitated FOS-induced cytotoxic components,[38] if present, and hence have preserved the epithelial barrier in humans.

Although the recommended daily intake of calcium for adults in Western countries is approximately 1000 mg, the calcium intake of 400-450 mg/day in the present study is comparable to the daily calcium intake of several large population groups. Especially infants, elderly, lactose-intolerant subjects, and African-Americans in industrialized

societies,[39-41] and many people living in developing countries[42] have comparable low-calcium intakes. Moreover, calcium absorption of infants and children is high, resulting in even lower intestinal and faecal calcium concentrations of approximately 80 µmol Ca/g faeces.[43] These calcium levels are comparable to the observed faecal calcium concentration in our animal studies.

Thus in our opinion, the observed adverse effects of FOS on the resistance to intestinal infections in our animal studies[9-11] and the present human intervention study do no support the concept that stimulating the endogenous microflora and intestinal organic acid production, by rapid fermentation of non-digestible carbohydrates, is beneficial for intestinal health in humans. Although the overall observed effects in humans are more moderate than those observed in rats, concern is warranted since several population groups in both industrialised and developing countries have low calcium intakes.

Acknowledgments

We thank the volunteers for their participation, Denise Jonker-Termont for biochemical analysis and Petra Vissink for dietary analysis.

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Chapter 7

General discussion and concluding remarks

7.1 Non-digestible carbohydrates: selectivity and metabolic consequences of fermentation

7.2 Non-digestible carbohydrates and pathogen colonisation

7.3 Non-digestible carbohydrates and the intestinal barrier

7.4 Non-digestible carbohydrates and pathogen translocation

7.5 Protective role of dietary calcium

7.6 Rats versus humans

7.7 Conclusions

7.1 Non-digestible carbohydrates; Selectivity and metabolic consequences of fermentation

Non-digestible carbohydrates (NDC) are resistant to degradation by host digestive enzymes in the proximal small intestine[1,2] and are subsequently fermented by the microflora within the caecum and colon. *In-vitro* fermentation experiments showed that short-chain carbohydrates are fermented faster than long-chain carbohydrates. Similarly linear chains are fermented faster than branched chains and soluble carbohydrates are fermented faster than insoluble carbohydrates.[3] Thus, the fermentation rate of different NDC depends on their molecular structure; polysaccharides like cellulose and wheat fibre are non- or low-fermentable NDC, resistant starch is well-fermentable and inulin, fructo-oligosaccharides (FOS) and lactulose are rapidly fermentable.

As a result of fermentation, the intestinal microflora is stimulated. Indeed, studies of other research groups[4-6] and the work presented in this thesis showed that fermentable NDC increased the number of lactobacilli and bifidobacteria in faeces of rats and humans. Several NDC are assumed to be beneficial for the host health because they selectively stimulate the protective gut microflora. However, FOS stimulated the growth of both lactobacilli and enterobacteria in faeces and on caecal and colonic mucosa, while no enhanced growth of these bacterial genera was seen on the ileal mucosa in rats (Chapter 5). In contrast to lactobacilli and bifidobacteria, enterobacteria are not potentially beneficial because members of that bacterial family are involved in foodborne infectious disease[7] and gut-derived septicaemia.[8] Others have also shown in rat and human studies that fermentable NDC can stimulate growth of enterobacteria[9,10] or have no effect.[11,12] In addition, *in-vitro* studies have shown that enterobacteria can use FOS as a growth substrate.[13] Thus, rapidly fermentable NDC can not be classified as prebiotics, since one of the current criteria that allow such classification is selective stimulation of potentially beneficial intestinal bacteria.[14] As a result of rapid fermentation of NDC by the intestinal microflora intestinal contents are acidified due to the production of lactic acid and short-chain fatty acids (SCFA).[15] Indeed, the rapidly fermentable NDC lowered caecal and faecal pH and increased faecal lactic acid concentrations in previous studies[16,17] and in the present thesis. Moreover, the total caecal SCFA pool increased (Chapter 3). However, faecal SCFA concentrations decreased[16] (Chapter 2). We speculate that these SCFA concentrations can be explained by lactic acid accumulation and the subsequent acidic pH, which inhibit the growth or activity of the intestinal bacteria that produce SCFA from

lactic acid.[18] However, another explanation could be the more efficient absorption of SCFA by the intestinal epithelium. Indeed, due to the acidic pH, more SCFA are present in the well-absorbed protonated form and the caecal blood flow is increased.[16]

7.2 Non-digestible carbohydrates and pathogen colonisation

Rapidly fermentable inulin and FOS, increased salmonella colonisation of caecal mucosa and contents in rats (Chapter 4). This was also reflected by increased faecal excretion of salmonella with time **(Fig 7.1; panel B-D)**. Obviously, these NDC facilitate adhesion and/or multiplication of salmonella in the intestinal tract. This finding is in contrast to the general hypothesis. It is assumed that fermentation of NDC and the subsequent stimulation of the protective endogenous microflora and acidification of gut contents will increase resistance towards an acid-sensitive intestinal pathogen, like salmonella. However, pH of intestinal contents during NDC fermentation might not be acidic enough to sufficiently reduce pathogen growth (Chapter 3). Moreover, like other enterobacteria, salmonella can use FOS as a substrate for growth (data not shown).

In contrast, the first study of this thesis (Chapter 2) showed that rapidly fermentable NDC decreased faecal excretion of salmonella with time, indicating decreased intestinal colonisation of salmonella **(Fig 7.1; panel A)**. At present, it remains unclear why the colonisation of salmonella decreased in that particular study, while our other studies show the opposite. There were no significant differences in diet composition, oral gavage with salmonella and study design between these studies. However, the majority of our studies indicate that rapidly fermentable NDC increase colonisation of salmonella in rats, based on the caecal and faecal salmonella numbers. The studies of other groups that focused on the *in-vivo* effects of fermentable fibres on pathogen survival in healthy animals show either beneficial,[19-22] inconsistent[23-25] or adverse effects[26-28] (Table 1.3).

7.3 Non-digestible carbohydrates and the intestinal barrier

In previous studies[16,29] and in the present thesis, rapid NDC fermentation results in lactic acid accumulation and acidic intestinal pH. In this acidic environment, more SCFA (produced from lactic acid) are in the protonated form and therefore passively cross the enterocyte membrane. Indeed, rapidly fermentable NDC increased faecal lactate and decreased faecal SCFA concentrations in rats (Chapter 2). In agreement, faecal lactate also increased in humans during FOS consumption (Chapter 6). A study by Remesy *et al*[16]

showed similar effects of inulin in rats. The SCFA-induced epithelial influx of protons decreases intracellular pH of the enterocyte. This is compensated by the extrusion of H^+ in exchange for Na^+ via the ATP-using Na^+/H^+ pump in the apical and basolateral membrane.[30] When the capacity of the enterocyte to cope with the influx of protons is exceeded it may result in exhaustion or even damage to the cell.[31-33] Thus, accumulation of organic acids and other fermentation metabolites within the intestinal lumen may lead to impairment of the mucosal barrier.[17,33-35]

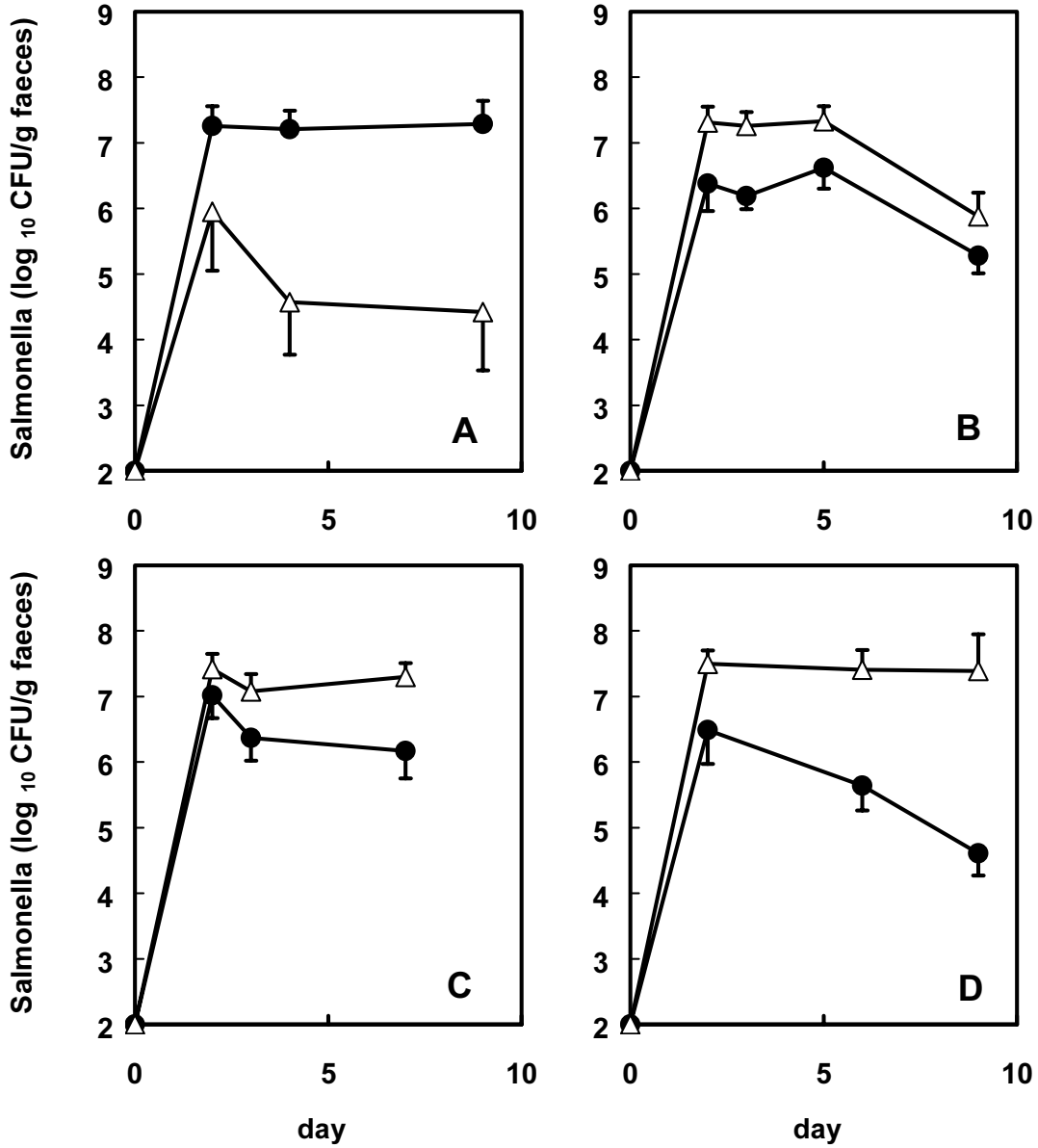

Fig 7.1 Effect of dietary cellulose (●) and fructo-oligosaccharides (FOS) (△) on faecal salmonella excretion of rats after oral administration of *S. enteritidis* on day 0 in our studies. Panel A, study described in Chapter 2; Panel B, Chapter 3; Panel C, Chapter 4; Panel D, study not described in this thesis. Results are expressed as means ± SEM (n=8 per diet group). Abbreviation: CFU colony-forming units.

Besides organic acids, the cytotoxic components, which resulted from NDC fermentation in rats, can irritate epithelial cells resulting in impairment of the mucosal barrier. However, in humans FOS did not result in an increase in cytotoxicity of faecal water (Chapter 6). This discrepancy between our animal and human studies will be discussed in paragraph 7.6. The intestinal mucosa responds to irritating and cytotoxic components by increasing mucus (viscous gel of hydrated mucin glycoprotein) production. Indeed, in this thesis and in other studies,[36,37] rapid fermentation of NDC increased faecal mucin excretion in rats and humans. FOS increased mucin concentrations within the contents and mucosa of the distal gut but not in ileal contents and mucosa (Chapter 5). Other studies also showed that fermentable fibres and their fermentation metabolites modulate the number of mucin-producing goblet cells and the production of mucin.[35,38-42] We do not dispute that mucin secretion itself is an important and beneficial host defence response. However, mucin production and secretion is enhanced by irritating components like bacterial pathogens,[43] endotoxins,[44] bile acids[45] or short-chain fatty acids.[35,39] Thus, the stimulated mucin secretion indicates the necessity of the epithelial layer to protect itself against harmful substances. Upon secretion, mucin can be degraded by the intestinal microflora.[46] Thus, one could speculate that the presence of fermentable substrates like inulin, FOS and lactulose in the large intestine reduces the need of bacteria to ferment gastro-intestinal mucin. This would also result in an increased recovery of mucin in faeces. However, resistant starch which is also completely fermented by the endogenous microflora of rats and humans[47] does not increase mucin secretion in rats, nor does it have the same adverse effects as inulin, FOS and lactulose (Chapter 2). Therefore, the increase in faecal mucin excretion induced by fermentation of inulin, FOS and lactulose likely reflects increased secretion and not decreased mucin degradation. Moreover, the increased mucin concentration in the caecal mucosa (Chapter 5) also points in this direction.

Besides the increased mucin secretion, fermentation of FOS clearly increased intestinal paracellular permeability in rats (Chapter 5). This might be due to the production of organic acids, since several other studies showed that organic acids can increase intestinal permeability.[33,48] However, FOS did not increase intestinal permeability in humans (Chapter 6). This discrepancy between our animal and human studies will be discussed in paragraph 7.6. Thus, the stimulated mucin secretion in rats and humans and the increased intestinal paracellular permeability in rats indicate that inulin, FOS and lactulose, but not resistant starch and wheat fibre can impair intestinal barrier function.

7.4 Non-digestible carbohydrates and pathogen translocation

The rapidly fermentable NDC inulin, FOS and lactulose but not cellulose, wheat fibre and resistant starch markedly stimulated salmonella translocation to extra-intestinal sites. This is indicated by the large increase in urinary nitric oxide metabolites (NO_x) output and the higher viable salmonella counts in spleen and liver of rats (Chapter 2). Recently, several other rodent[49,50] and human[51,52] studies showed that serum or urinary NO_x correlates with extra-intestinal pathogen load and the severity of systemic infection. The infection-induced NO_x response was dependent on the dietary concentration of NDC (Chapter 3).

Concordant with the stimulated translocation of salmonella in the rapidly fermentable NDC groups, the infection-induced inflammatory response in the caecal and colonic mucosa was also higher (Chapter 2). In addition, the severity of the infection in the rapidly fermentable NDC groups was also reflected by the significant growth impairment (Chapter 2-4), worsening of diarrhoea (Chapter 3), further increased infection-induced mucin secretion (Chapter 3 and 5) and higher intestinal permeability (Chapter 5). Obviously, both salmonella and the subsequent inflammatory response resulted in epithelial damage and increased intestinal permeability,[53,54] especially in the rats fed fermentable NDC. Under normal circumstances, penetration of the mucosal barrier by salmonella is supposed to occur through M-cells within ileal Peyer's patches,[55] but this issue is subject of recent debate.[56-58] Indeed, salmonella could also translocate to extra-intestinal sites via dendritic cells present in the mucosa throughout the entire intestine[56] and para- and transcellularly through enterocytes.[59] Considering that the major effects of fermentable NDC are seen within the distal gut (Chapter 5),[60] it can be speculated that the mucosal barrier is impaired at this site. Rapid fermentation of NDC might thus expand the possibilities for salmonella to translocate in the distal gut. The significantly increased mucosal inflammation of the caecum and colon, but not the ileum, of salmonella-infected rats fed rapidly fermentable NDC, strengthens this hypothesis (Chapter 2).

The occurrence of bacterial infections depends on both host defence and virulence characteristics of the intestinal pathogen. Besides impairment of the intestinal barrier, the enhanced translocation of salmonella in the rapidly fermentable NDC groups could also be due to increased virulence of salmonella. Expression of salmonella virulence genes is regulated by environmental factors. For example, changes in nutrient availability, osmolarity, pH, and SCFA concentrations can alter virulence of salmonella.[61-63] The alterations in SCFA induced by rapid fermentation might increase salmonella virulence by

providing a signal for stimulated expression of virulence genes including those involved in translocation.[61,62] However, the effect of the intestinal luminal components on virulence gene expression of luminal pathogens has never been studied *in-vivo*. Therefore, we are currently investigating the effect of dietary FOS on *in-vivo* virulence gene expression of salmonella.

Thus, the consistent observation of impaired animal growth, infection-induced diarrhoea, mucosal inflammation, increased intestinal permeability and enhanced translocation of salmonella to extra-intestinal sites all indicate that the rapidly fermentable NDC inulin, FOS and lactulose impair intestinal barrier function and the resistance to invasive pathogens. Cellulose, wheat fibre and resistant starch did not have these adverse effects **(Fig 7.2)**.

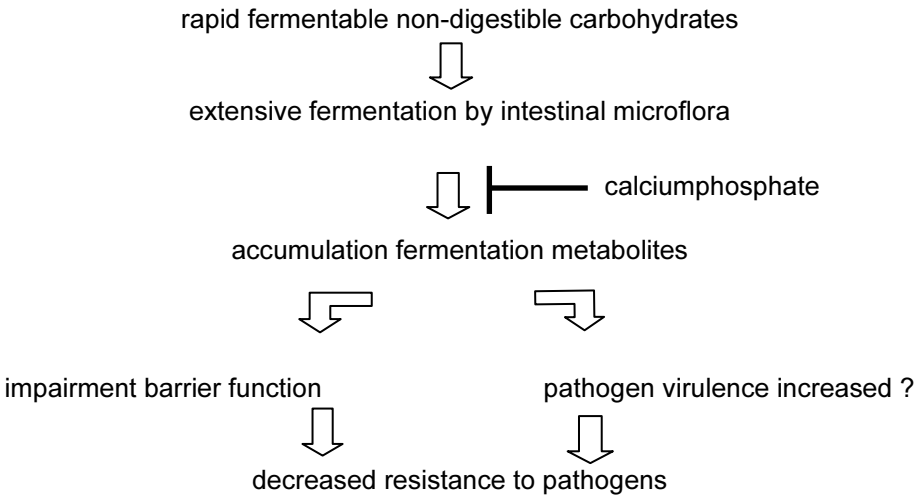

Fig 7.2 Adjusted hypothetical mechanism, based on work presented in this thesis, by which dietary non-digestible carbohydrates and calcium may affect the resistance against pathogens.

7.5 Protective role of dietary calcium

Dietary calcium has been known to increase resistance to intestinal pathogens both in rats[64,65] and humans.[66] The mechanism behind this resistance-enhancing effect of calcium could be stimulation of the protective endogenous microflora.[65] In the present thesis, calcium also stimulated the potentially beneficial endogenous microflora. It increased faecal lactobacilli and bifidobacteria, while decreasing enterobacteria numbers (Chapter 4). Moreover, most adverse effects of rapidly fermentable NDC on salmonella infection were inhibited by dietary calcium (Chapter 4).[67] Dietary calcium counteracted the acidic luminal pH, the increased cytotoxicity, and the stimulated mucin secretion. However,

calcium supplementation did not completely inhibit the enhanced translocation of salmonella induced by FOS and inulin.

One mechanism by which calcium phosphate might improve the mucosal barrier is by precipitating cytotoxic components within the intestinal lumen.[68] This reduces epithelial cell damage[69] and increases resistance to salmonella translocation. Indeed, dietary calcium inhibited the cytotoxicity of faecal water, induced by rapidly fermentable NDC. The protective role of dietary calcium could also be contributed to the buffering capacity of the intestinal calcium phosphate complex. Calcium forms an insoluble complex with phosphate in the upper small intestine.[70] The fermentation of inulin, FOS and lactulose results in considerable production of organic acids and hence acidification of the intestinal lumen. This can subsequently be counteracted by solubilisation of calcium phosphate. However, when the dietary calcium phosphate supply is limited, the amount of intestinal calcium phosphate may be insufficient to counteract acidification.[34] Moreover, the present thesis and other studies[71,72] show that fermentable NDC stimulate calcium absorption and thus phosphate absorption as well.[70] Subsequently, less phosphate will be available within the distal gut to counteract the adverse effects of acidic fermentation (Chapter 4). Thus, calcium phosphate increases the buffering capacity of the intestinal lumen and has cytoprotective effects, which at least partly preserves the mucosal barrier during rapid fermentation of NDC (Fig 7.2).

7.6 Rats versus humans

Non-digestible carbohydrates

Are the dietary concentrations of NDC's used in our animal studies, relevant for the human diet? Daily intake of inulin and FOS in Europe and the USA has been estimated up to10 g.[73] Assuming a daily dry food intake of about 500 g, the total concentration of these particular NDC's in an average Western human diet is about 2%. This estimation does not take into account consumption of specific meals and products supplemented with fermentable NDC's, typically 3-10 g per portion.[73] Many infant formulas are currently supplemented with relatively high amounts of fermentable NDC (5%). Moreover, lactulose is given for its laxative properties in doses of up to 10 g per day.[74] In addition, the human diet still contains other NDC's, which have similar fermentation properties as inulin and FOS. Therefore, supplementation of our animal diets with 3-6% inulin, FOS or lactulose is

realistic for the human situation. Moreover, many other animal and human studies applied similar dietary concentrations (Table 1.3). When we relate the dose of fermentable NDC to intestinal surface area, instead of comparing dietary concentrations as discussed above, the NDC dose is slightly higher in our rat studies than in our human study. The total surface area of the caecum and colon of rats and humans (calculated by π x diameter x length) is approximately 26 cm^2 and 2356 cm^2, respectively[75] **(Table 7.1)**. While the rats in most of our animal studies consumed 0.5 g FOS per day, the daily intake in our human study (Chapter 6) was 20 g FOS per day. Thus, the NDC dose expressed as ratio between luminal food contents and large intestinal mucosal area (gram of FOS per cm^2 intestinal surface) is 2-fold higher in rats than in humans. However, the intestinal surface area is also affected by differences in crypt depth between rats and humans.

Table 7.1 Average length, diameter and surface area of the caecum and colon in rats and humans

		Rat	Human
caecum	length (cm)	3	--
	diameter (cm)	1	--
	surface area (cm^2)	9	--
colon	length (cm)	11	150
	diameter (cm)	0.5	5
	surface area (cm^2)	17	2356

NB Considering that humans have a poorly defined caecal region continuous with the colon, only values of the colon are presented.

Calcium

Are the dietary concentrations of calcium used in our animal studies, relevant for the human diet? The dietary calcium concentration was comparable between our rat and human study **(Table 7.2)**. While the recommended intake of calcium for adults is approximately 1000 mg per day (25 mmol/day), several large population groups in industrialised societies and especially in developing countries have low calcium intakes comparable to our animal and human study. Populations with low calcium intakes include infants, elderly, lactose-intolerant subjects, and African-Americans.[76-78] Thus, the dietary concentrations of NDC and calcium in our animal and human intervention are relevant for

the human situation. However, calcium absorption in young adult rats is much higher than in adult humans (Table 7.2). Most of the absorbed calcium in rats was retained as indicated by the low urinary calcium excretion of approximately 0.005 mmol calcium/day (data not shown). We speculate that the discrepancy in cytotoxicity and intestinal permeability we observed between rats and humans (Chapter 6) might be explained by the above-mentioned differences in calcium absorption, and hence differences in luminal calcium concentration. The higher intestinal calcium in humans might have precipitated the FOS-induced cytotoxic components,[68] if formed, and hence have preserved the epithelial barrier. This may explain the lack of effect of FOS on intestinal permeability in the human study. Although luminal calcium concentration in the FOS-fed rats was very low, it does not solely explain the adverse effects of FOS on translocation of salmonella. A very low calcium diet (no FOS added), resulting in equally low luminal calcium concentrations as in FOS-fed rats, did not increase translocation of salmonella **(Fig 7.3)**.

Table 7.2 Daily intake and faecal excretion of calcium in our rat and human studies

		Rat		Human	
		control	**FOS**	**control**	**FOS**
Dietary intake	µmol Ca / g dry	30	30	20	20
	mmol Ca / day	0.4	0.4	10	10
Faecal excretion	µmol Ca / g dry	100	55	173	141
	mmol Ca / day	0.11	0.03	8.15	7.20

The gastro-intestinal tract

The infection studies described in this thesis were performed in rats. Thus, it is important to consider the differences in gastro-intestinal physiology and host defences between rats and humans. The quantity of bacteria within the stomach and proximal small intestine of rats and humans is different.[75] While the upper gastro-intestinal tract of humans contains few organisms, the rat stomach and proximal intestine contains 10^6 -10^8 CFU per gram contents. Nevertheless, we did not observe a FOS-induced stimulation of the lactobacilli and enterobacteria within the ileum, but only in caecum and colon (Chapter 5). Therefore, NDC's are fermented in the distal gut of the rats because it harbours most of the endogenous flora, like in humans. In addition, rats have a large caecum, while humans have a poorly defined caecal region continuous with the colon.[75] In rats, the caecum

accounts for approximately 26% of the length of the large intestine, while it is about 5% in humans.[79] Most adverse effects of fermentable NDC's were observed within the caecum and colon of the rat. In humans, most of the fermentation of NDC's will occur in the proximal colon. Despite these physiological or microbiological differences between rats and humans, FOS increased faecal bifidobacteria and lactobacilli, faecal wet weight, faecal lactate and mucin secretion, in both species. However, in contrast to our rat studies FOS did not increase cytotoxicity of faecal water and we did not observe a FOS-induced increase in intestinal permeability (Chapter 6).

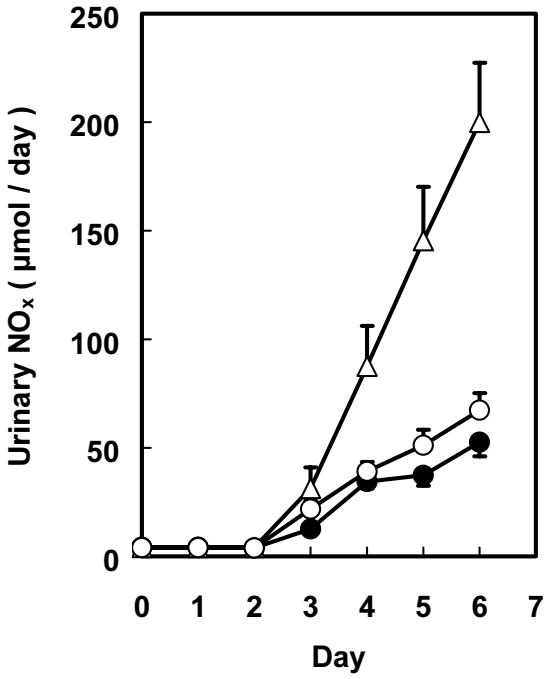

Fig 7.3 Sum of urinary nitrate and nitrite (NO_x) excretion in the normal low-calcium control group (30 µmol Ca/g) (●), the very low calcium control group (5 mmol Ca/kg) (**O**), and the fructo-oligosaccharide group (30 µmol Ca/g) (△) after an oral challenge with 2×10^9 colony-forming units of *Salmonella enteritidis* on day 0. NO_x is a marker of translocation of salmonella to extra-intestinal sites. Results are expressed as mean ± SEM (n=8 per diet group).

7.7 Conclusions and implications

Several non-digestible carbohydrates (NDC) have been defined as prebiotics. Prebiotics are claimed to exert beneficial health effects, i.e. protect against intestinal pathogens, by selectively stimulating the protective gut microflora. Because of the proposed health effects, NDC have been added to a number of products, including infant nutrition and dairy

products. We studied whether NDC protect against the invasive intestinal pathogen salmonella *in-vivo*. In contrast to the general expectation, we found that rapidly fermentable NDC decreased the resistance to salmonella in rats. Moreover, some adverse effects of NDC were already present before infection with salmonella. We were able to verify part of these effects in human volunteers.

In conclusion, we find no support for the concept that stimulating the protective gut microflora by fermentation of NDC protects against bacterial pathogens. However, it should be realised that the occurrence of the adverse effects of NDC described in this thesis depend on two conditions; low calcium intake and a simultaneous infection by an invasive bacterial pathogen. Although intestinal infections occur frequently even in Western countries, they only result in a systemic infection in susceptible groups. Thus, in our opinion, concern is warranted in specific groups, like infants and elderly, which have low calcium intakes and are susceptible for systemic infections by invasive pathogens.

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Summary

Introduction

Gastro-intestinal infections are still a major health problem, not only in developing countries. Due to the large-scale use of antibiotics and hence the development of resistant bacterial strains, research strategy is increasingly focusing on prevention of intestinal infections. Dietary modulation of host resistance to intestinal infections might be an attractive approach. By influencing the composition of gastro-intestinal contents, diet affects the intestinal microflora and the epithelial barrier function. These primary non-immunological host defences of the gastro-intestinal tract are particularly important in withstanding the first encounter with an invasive pathogen.

Dietary non-digestible carbohydrates (NDC), which are present in a variety of vegetables, can potentially increase the resistance to intestinal infections. NDC are resistant to degradation by host digestive enzymes in the small intestine and are subsequently fermented by the intestinal microflora. Fermentation of NDC stimulates the protective colonic microflora, resulting in an increased production of lactic acid and short-chain fatty acids. The subsequent decrease in pH may result in increased killing of acid-sensitive bacterial pathogens. Because of these proposed health effects several NDC's have been defined as prebiotics and a variety of products are supplemented with NDC's, like dairy products and infant formulas.

However, irrespective of their prebiotic effect, the contribution of NDC's in decreasing intestinal infections can only be verified in strictly controlled *in-vivo* studies including a challenge with a pathogen. Most previous studies focused on the effect of dietary non-digestible carbohydrates on the composition of the intestinal microflora and many of them suppose that improvements of host resistance to intestinal infections are likely. Moreover, host defences against intestinal infections do not solely depend on the intestinal microflora but also on the barrier function of the intestinal mucosa, especially when invasive pathogens are involved. Rapid fermentation of NDC by the intestinal microflora leading to high concentrations of organic acids may impair the barrier function. Therefore, the aim of the work in this thesis was to study the effect of NDC on the resistance to the invasive pathogen salmonella.

This thesis

In order to study the effect of NDC on salmonella infection, rats were fed a diet containing either non-fermentable NDC (cellulose and wheat fibre), well-fermentable NDC (resistant starch) or rapid-fermentable NDC (inulin, fructo-oligosaccharides or lactulose). The dietary concentrations of NDC used in these animal studies are relevant for the human situation. After adaptation to these diets, rats were orally infected with salmonella to mimic a foodborne infection. Fermentable NDC stimulated the protective intestinal microflora and subsequently increased microbial fermentation metabolite concentrations, like lactic acid. However, in contrast to most expectations, rapid-fermentable NDC decreased the resistance towards the invasive pathogen salmonella. Rapid-fermentable NDC stimulated mucosal translocation of invasive salmonella to extra-intestinal sites, like liver and spleen. Moreover, these NDC increased the subsequent mucosal inflammation. These adverse effects were not observed with cellulose, wheat fibre and resistant starch. Before infection with salmonella, rapid-fermentable NDC increased luminal cytotoxicity, mucin secretion and intestinal permeability.

We speculate that the rapid fermentation of NDC results in accumulation of organic acids and cytotoxic fermentation metabolites in the large intestine. These may irritate the distal gut epithelium and subsequently impair the mucosal barrier. To protect itself, the intestinal mucosa responds to organic acids and cytotoxic components by increasing mucin excretion. Considering that the major fermentation effects of NDC take place in the large intestine, the barrier would be impaired at this site.

Dietary calcium phosphate counteracted most adverse effects of rapid fermentation of NDC. It partly inhibited acidification of intestinal contents and prevented the NDC-induced increase in cytotoxicity, mucin secretion and salmonella translocation. The protective role of dietary calcium phosphate may be contributed to the buffering capacity of the intestinal calciumphosphate complex and/or the precipitation of cytotoxic components.

For obvious reasons, subjects can not deliberately be infected with a virulent invasive pathogen. Therefore, to determine the relevance of these findings for the human situation, we studied whether rapid-fermentable fructo-oligosaccharides (FOS) increase luminal cytotoxicity, mucin secretion and intestinal permeability, which was also observed before infection in the rat studies. In contrast to our rat studies, FOS did not increase cytotoxicity of faecal water and we did not observe a FOS-induced increase in intestinal permeability. However, in both our rat studies and our human study, FOS resulted in stimulation of the

endogenous microflora, accumulation of faecal lactic acid and increased mucin secretion. We speculate that the discrepancy in cytotoxicity and intestinal permeability we observed between rats and humans can be explained by differences in calcium absorption. While the dietary calcium concentration was comparable between our rat and human studies, calcium absorption in young rats is much higher than in adult humans. The subsequent higher luminal calcium concentration in humans might have precipitated the FOS-induced cytotoxic components, if present, and hence have preserved the epithelial barrier.

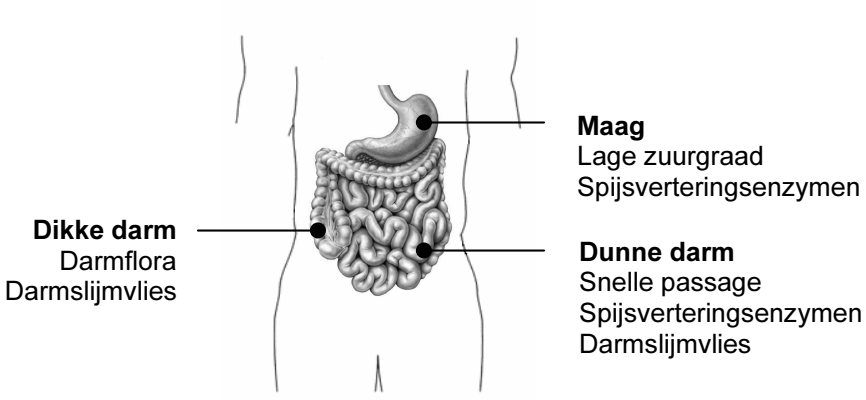
Conclusion

Our rat and human intervention studies do no support the concept that stimulating the endogenous microflora and intestinal organic acid production by rapid fermentation of non-digestible carbohydrates is beneficial for intestinal health in rats and humans.

Samenvatting

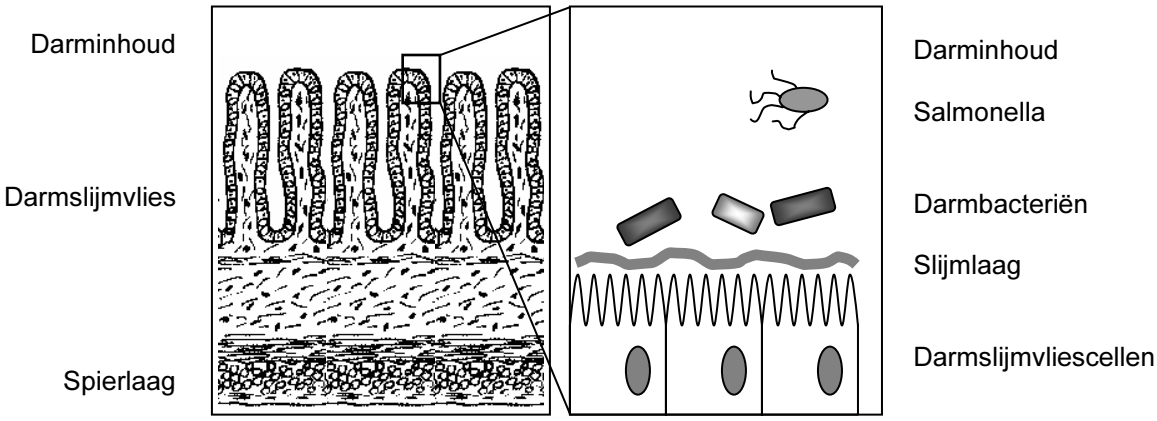
Inleiding

Darminfecties zijn nog steeds een groot gezondheidsprobleem, niet alleen in ontwikkelingslanden. Door het veelvuldig gebruik van antibiotica en de daarmee gepaard gaande ontwikkeling van antibiotica resistente bacterie stammen, richt de wetenschap zich nu op het voorkomen van darminfecties. Voeding kan de weerstand tegen darminfecties mogelijk verbeteren doordat het de samenstelling van de darminhoud, de darmflora en het darmslijmvlies beïnvloedt **(Figuur I)**. Deze niet-specifieke afweer is belangrijk voor de eerste bescherming tegen een ziekteverwekker **(Figuur II)**.



Figuur I Niet-specifieke afweer van het lichaam tegen ziekteverwekkers.

Voedingsvezels, die aanwezig zijn in verschillende groenten, kunnen mogelijk de weerstand tegen darminfecties verbeteren. Ze kunnen niet worden afgebroken door spijsverteringsenzymen in de dunne darm maar kunnen wel worden afgebroken door de darmflora (fermentatie). Hierdoor wordt de darmflora gestimuleerd en wordt er melkzuur en korte-keten vetzuren geproduceerd. Deze organische zuren verlagen de zuurgraad van de darminhoud waardoor zuur-gevoelige ziekteverwekkers kunnen worden afgedood. Vanwege deze potentiële gezondheidseffecten worden sommige voedingsvezels gedefinieerd als prebiotica en worden deze voedingsvezels toegevoegd aan verschillende producten zoals zuivelproducten en zuigelingenvoeding.



Figuur II Barrière functie van de darm. Het gebied in het vierkantje is rechts vergroot weergegeven.

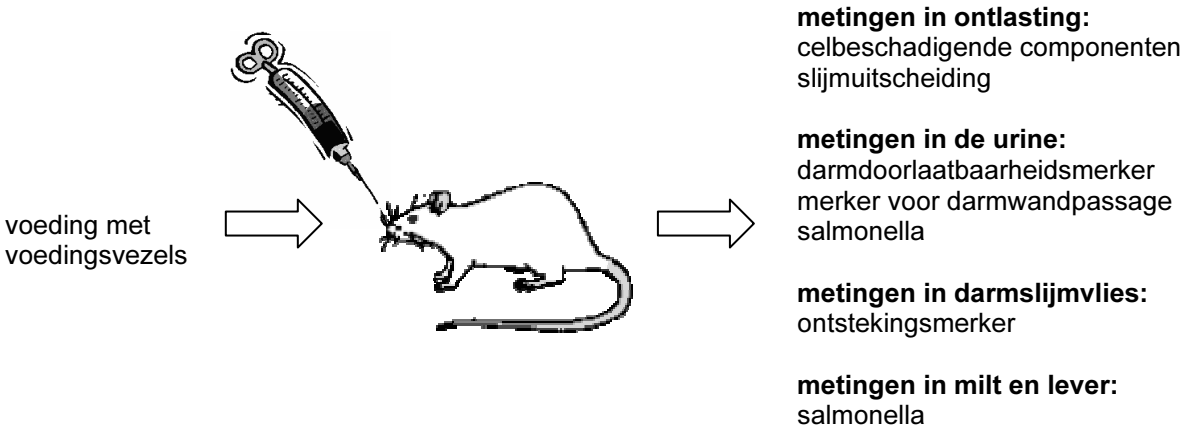
Er is echter nog onvoldoende bewijs voor deze gezondheidseffecten. Voorgaande studies tonen aan dat voedingsvezels de beschermende darmflora stimuleren en men neemt aan dat dit de weerstand tegen darminfecties verhoogt. De gezondheidseffecten kunnen echter alleen daadwerkelijk worden aangetoond in (dier)studies waarin direct gekeken wordt naar de effecten van voedingsvezels op een toegediende ziekteverwekker. Bovendien is niet alleen de darmflora belangrijk voor de weerstand tegen darminfecties maar ook het darmslijmvlies. Snelle fermentatie van voedingsvezels kan leiden tot hoge concentraties van organische zuren in de darminhoud die het darmslijmvlies kunnen beschadigen. Het doel van het werk in dit proefschrift is het bestuderen van het effect van voedingsvezels op de weerstand tegen de invasieve en veel vóórkomende ziekteverwekker salmonella.

Dit proefschrift

Om de effecten van vezels op een infectie met salmonella te bestuderen werden ratten gevoed met een dieet met niet-fermenteerbare (cellulose of tarwevezel), goed-fermenteerbare (onverteerbaar zetmeel) of snel-fermenteerbare (inuline, fructo-oligosacchariden of lactulose) vezels. De concentraties van deze vezels in het dieet zijn relevant voor de humane situatie. Na adaptatie aan de diëten kregen de ratten een orale dosis van salmonella om een voedselinfectie na te bootsen **(Figuur III)**.

Consumptie van fermenteerbare vezels stimuleerde de beschermende darmflora en resulteerde in verhoging van bacteriële producten zoals melkzuur. Echter, in tegenstelling tot de algemene verwachting resulteerden de snel-fermenteerbare vezels in vermindering van de weerstand tegen de ziekteverwekker salmonella. Deze vezels stimuleerden de passage (invasie) van salmonella door de darmwand naar de lever en milt. Bovendien

verhoogden ze de ontsteking van het darmslijmvlies. Cellulose, tarwevezel en onverteerbaar zetmeel hadden deze negatieve effecten niet. Voor infectie met salmonella resulteerden snel-fermenteerbare vezels in een verhoging van celbeschadigende componenten in de darminhoud, meer slijmuitscheiding en verhoogde darmdoorlaatbaarheid.

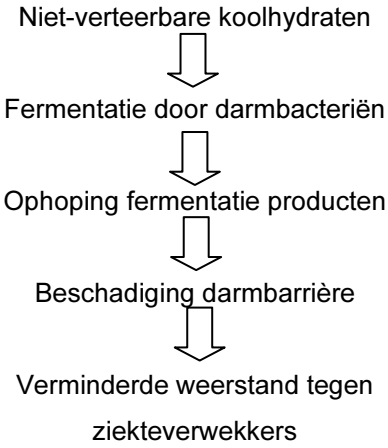


Figuur III Schematische weergave van een experimentele voedingsproef met ratten

We denken dat snelle fermentatie van vezels resulteert in ophoping van organische zuren en celbeschadigende componenten in de dikke darm. Deze kunnen vervolgens het darmslijmvlies irriteren en de darmbarrière beschadigen **(Figuur IV)**. Het darmslijmvlies beschermt zichzelf tegen organische zuren en celbeschadigende componenten door een slijmlaag te vormen. De negatieve effecten van snel-fermenteerbare voedingsvezels werden grotendeels geremd door calciumfosfaat in de voeding. Calciumfosfaat verminderde vooral de verzuring van de darminhoud, de celbeschadigende componenten, de slijmproductie en de passage van salmonella door de darmbarrière. De beschermende rol van calciumfosfaat is waarschijnlijk te danken aan de bufferende werking van fosfaat en/of het binden van celbeschadigende componenten in de darminhoud.

Vervolgens hebben we de relevantie, van bovengenoemde effecten van snel-fermenteerbare voedingsvezels, voor mensen bestudeerd. Om begrijpelijke redenen kunnen mensen niet worden blootgesteld aan ziekteverwekkers. Daarom hebben wij onderzocht of consumptie van de snel-fermenteerbare fructo-oligosacchariden (FOS) aanleiding geeft tot: vorming van celbeschadigende componenten in de darminhoud, verhoogde slijmproductie in de darm en verhoogde doorlaatbaarheid van de darm. Als deze factoren in mensen veranderen door het eten van FOS, gelijk aan de waargenomen

effecten in de dierstudies, dan is een negatief effect van FOS op de weerstand tegen ziekteverwekkers bij mensen niet uitgesloten. In tegenstelling tot onze rattenstudies, resulteerde FOS niet in een verhoging van celbeschadigende componenten in de darminhoud en niet in een verhoging van de darmdoorlaatbaarheid. Echter, zowel in onze ratten studies als humane studie resulteerde consumptie van FOS in de groei van de darmflora, een ophoping van melkzuur in de ontlasting en een verhoging van de slijmproductie van de darm.



Figuur IV Niet-verteerbare koolhydraten verlagen de weerstand tegen ziekteverwekkers in ratten; het mogelijke mechanisme.

We denken dat het waargenomen verschil tussen dier- en humane studies te wijten is aan verschil in calciumabsorptie. Ondanks dat de calciumconcentratie in de voeding vergelijkbaar was in dier en humane studie zorgt de hoge calciumabsorptie in de darm van jonge ratten ervoor dat de calciumconcentratie in de ratten darm lager was dan in die van mensen. Deze relatief hoge calciumconcentratie in de darm van mensen heeft vervolgens de celbeschadigende componenten in de darminhoud kunnen binden en de darmbarrière kunnen beschermen.

Conclusie

Onze rat en humane interventie studies geven geen ondersteuning voor het concept dat stimulering van de darmflora en organische zuurproductie door snelle fermentatie van voedingsvezels de darmgezondheid verbetert.

Dankwoord

Gedurende de afgelopen vier jaar heb ik het onzettend naar mijn zin gehad en ik werd er nog voor betaald ook! Eigenlijk is promotieonderzoek helemaal niet zo'n bevalling…
Nu de kans om al die mensen te bedanken die op een of andere manier een steentje hebben bijgedragen aan dit proefschrift.

Allereerst natuurlijk mijn (co)promotoren: Ingeborg, jij bent degene die de grootste stempel op dit proefschrift heeft gedrukt. Je maakte altijd tijd voor mijn vele vragen en kon mijn ingeleverde stukken altijd heerlijk volkladderen met goed commentaar. Ik vond het geweldig om met je samen te werken en kijk uit naar nog een jaar! Roelof, jouw enthousiasme voor het onderzoek is aanstekelijk. Met je ongekende kennis van alle biochemische routes (ken je nou echt heel Stryer uit je hoofd?) kon je altijd een mooie positieve draai geven aan vreemde resultaten. Mijn rug heeft trouwens aardig de schouderkloppen overleefd. Martijn, bedankt voor al je steun en hulp tijdens mijn promotieonderzoek. Ondanks je overvolle agenda maakte je altijd tijd vrij voor mijn manuscripten.

Ook wil ik mijn A257-lab collega's bedanken: Mischa, jij was onmisbaar bij het labwerk! Hoe jij al dansend en zingend op "Normale" muziek toch steeds tot de meest geweldige standaardaddities komt is mij nog steeds een raadsel! Ik ben blij dat ik straks de vragen kan doorspelen als je naast me staat als paranimf! Wanneer ga je trouwens zelf promoveren? Denise, bikkel, ongeloofelijk hoe jij je enthousiast door grootschalige cytotox experimenten slaat. Vooral bij de FOSTER studie was je onmisbaar. Wanneer is het volgende parochiefeestje? Het is alweer een tijdje geleden maar ik wil ook het duo Wim en Marco bedanken. Bedankt voor de ontgroening in mijn eerste lab-jaar! De strijd tussen analisten en AIO's leverden toch veel grappen op. Dan natuurlijk niet te vergeten, de CKP'ers Gerrit, Wilma, Judith en Suzanne. Door de goede dierverzorging liepen de experimenten altijd gesmeerd! De lange sectie dagen met ruis-radio (Oh, nee niet weer Queen?) zijn goed bevallen. Ook alle ratjes, die hun leven onvrijwillig voor de wetenschap hebben gegeven, bedankt! Proefdieren, ik wou dat ik iets beters kon verzinnen.

Daarnaast wil ik de vrijwilligers van de FOSTER studie bedanken voor het leveren van een berg monsters. Ik heb toch zo'n beetje 30 kg verwerkt! Wat lijkt het trouwens na vriesdrogen verdacht veel op koffiepoeder. "Het aroma komt je tegemoet zodra je 't potje open doet" krijgt een geheel andere betekenis.

Johan, mijn buurman en paranimf. Ook al heb ik drie jaar tegen je ontzettende niet cleandesk-verantwoorde bureau moeten kijken, de eindeloze discussies en berekeningen over factor X en Y waren geweldig. Hopelijk blijven we de filmavondjes nog een tijdje vol houden. De Nuts Arjan, Chantal, Corinne, Hans en Leontine: jullie inzet bij vragen, secties en lange plaatdagen op het achterste lab was onmisbaar. Mijn enige afstudeervak student Hante, bedankt voor de mooie bijdrage in hoofdstuk 4 van dit proefschrift. Rob en Fadel bedankt voor alle hulp bij weigeringen van de ICP. Maria en Marke, bedankt voor alle virulentie experimenten met mijn bulkmonsters. Hopelijk komt er wat uit! Mijn kantoor-tuin-collega's Arjen, Gabrielle, Jan en Johan. We zijn bijelkaar net een hoofdstuk uit "Het bureau". Ik heb genoten van de gezelligheid dus ik blijf nog een jaartje. Bestel die schutting alvast maar!

De Toffe Miepjes Annelies, Nanda, Rosemarijn en Sonja: samen afgestudeerd en nog steeds contact. Hopelijk komen er nog vele TM-uitjes. Rosemarijn, het theeleuten en het "hard" lopen waren zeer geslaagd. De Australië "Can we see Victoria-square again?" voedingsaio's. Ik heb een zeer geslaagde vakantie gehad down-under.

Niet te vergeten mijn (schoon)familie. De feest- en familiedagen waren erg gezellig en een goede onderbreking van het promoveren! Pap en mam zonder jullie zou dit proefschrift hier zeker niet liggen! Wat heb ik het toch getroffen met jullie. De volledig verzorgde weekendjes BOL zijn geslaagd!

Koen, na 1 jaar NIZO kwam jij om de hoek kijken bij het kopieerapparaat. Ik werd meteen spelersvrouw van het NIZO voetbalteam en Nicolas Maillard. Bedankt voor je hulp bij het afronden van dit boekje en de vele schitterende reizen. Jij bent het mooiste wat me is overkomen.

Sandra

Curriculum vitae

Sandra Johanna Maria ten Bruggencate werd geboren op 25 december, 1975 in Alkmaar. Zij behaalde het VWO diploma in 1995 aan het Han Fortmann College te Heerhugowaard. In hetzelfde jaar begon zij de studie “Voeding en Gezondheid” aan de Wageningen Universiteit. In het kader van deze studie heeft ze drie afstudeerprojecten uitgevoerd. Tijdens het eerste afstudeerproject bij de vakgroep Humane Voeding en Epidemiologie van de Wageningen Universiteit bestudeerde zij het metabolisme van polyfenolen uit de voeding. Bij de vakgroep Toxicologie van Wageningen Universiteit, onderzocht zij vervolgens het effect van PCB’s op hersenontwikkeling. In het laatse afstudeerproject bij de vakgroep Humane Biologie van de Universiteit van Guelph in Canada bestudeerde zij het effect van energie- en eiwitondervoeding op immuunparameters. Het ingenieursdiploma werd behaald in september 2000. Van augustus 2000 tot augustus 2004 deed zij promotieonderzoek in opdracht van Wageningen Centre for Food Sciences (WCFS) en NIZO food research te Ede onder begeleiding van Dr. ir. I.M.J. Bovee-Oudenhoven, Dr. R. van der Meer en Prof. dr. M.B. Katan. Momenteel werkt zij als post-doc bij het WCFS.

Publications

Abstracts

1. Ten Bruggencate SJM, Bovee-Oudenhoven IMJ, Van der Meer R. Dietary oligofructose dose-dependently increases translocation of salmonella in rats. Digestive Disease Week, American Gastroenterological Association, San Francisco, USA, 19-22 May 2002. Gastroenterology 2002;122:A38.

2. Bovee-Oudenhoven IMJ, Ten Bruggencate SJM, Lettink-Wissink MLG, Van der Meer R. Non-digestible carbohydrates inhibit intestinal colonization but stimulate translocation of salmonella in rats. European Intestinal Transport Group Meeting, Egmond aan Zee, The Netherlands, 28 September – 1 October 2002. J Physiol Biochem 2002;58(4):302.

3. Ten Bruggencate SJM, Bovee-Oudenhoven IMJ, Lettink-Wissink MLG, Van der Meer R. Effects of dietary fructo-oligosaccharides on the intestinal barrier in rats and humans. European Intestinal Transport Group Meeting, Guildford, UK, 17-20 april, 2004. Submitted to J Physiol Biochem.

Peer reviewed papers

1. Ten Bruggencate SJM, Hillyer LM, Woodward BD. The proportion of $CD45RA^{+}CD62L^{+}$ (quiescent-phenotype) T cells within the $CD8^{+}$ subset increases only in advanced weight loss in the protein- or energy-deficient weanling mouse. J Nutr 2001;131:3266-9.

2. Bovee-Oudenhoven IMJ, Ten Bruggencate SJM, Lettink-Wissink MLG, Van der Meer R. Dietary fructo-oligosaccharides and lactulose inhibit intestinal colonisation but stimulate translocation of salmonella in rats. Gut 2003;52:1572-8

3. Ten Bruggencate SJM, Bovee-Oudenhoven IMJ, Lettink-Wissink MLG, Van der Meer R. Dietary fructo-oligosaccharides dose-dependently increase translocation of salmonella in rats. J Nutr 2003;133:2313-8

4. Ten Bruggencate SJM, Bovee-Oudenhoven IMJ, Lettink-Wissink MLG, Katan MB, Van der Meer R. Dietary fructo-oligosaccharides and inulin decrease resistance of rats to salmonella: protective role of calcium. Gut 2004; 53: 530-5.

5. Ten Bruggencate SJM, Bovee-Oudenhoven IMJ, Lettink-Wissink MLG, Van der Meer R. Dietary fructo-oligosaccharides increase intestinal permeability in rats. Submitted for publication.

6. Ten Bruggencate SJM, Bovee-Oudenhoven IMJ, Lettink-Wissink MLG, Katan MB, Van der Meer R. Dietary fructo-oligosaccharides and the intestinal barrier in humans. To be submitted.

Training and supervision plan

Courses		
PhD week	VLAG	2000
Proefdierkunde	WUR	2000
English Scientific Writing	CENTA	2002
Zuivelcursus	NIZO	2002
Ecophysiology of the GI-tract	VLAG	2003
Conferences		
NWO-voeding Papendal	Werkgemeenschap Voeding	2000-2004
ORAFTI conference, UK	ORAFTI	2001
Najaarsymposium melkkundegenootschap	Melkkunde genootschap	2001
Darmendag	RIVM / ID Lelystad	2001-2002
Digestive Disease Week San Francisco, USA	AGA	2002
EITG symposium, Guildford, UK	EITG	2004
Gut flora in health and disease	KNAW	2004
Other		
Journal club	NIZO	2000-2004
WCFS seminars	WCFS	2000-2004
PhD excursion to Switzerland, Italy and Germany	Human Nutrition WUR	2001
Workshop diet and gut health	WCFS	2003
PhD excursion to Australia	Human Nutrition WUR	2003
Nutrition and infectious diseases in elderly people	VLAG	2003