Dietary modulation of the resistance to intestinal infections

Ingeborg MJ Bovee-Oudenhoven

Promotor:	Dr. J.T.M. Wouters Bijzonder hoogleraar Zuivelkunde
Co-promotor:	Dr. R. van der Meer Hoofd Sectie Nutrition & Health NIZO *food research*

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Proefschrift

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BIBLIOTHEEK
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Stellingen

1. Calciumfosfaat in de voeding remt de translocatie en kolonisatie van salmonella. *(dit proefschrift)*

2. Suppletie van de voeding met calciumfosfaat beschermt beter tegen een salmonella infectie dan suppletie met lactulose. *(dit proefschrift)*

3. In voedingsonderzoek naar de mogelijk positieve effecten van fermenteerbare vezels en oligosacchariden dient men rekening te houden met de interactie met calciumfosfaat. *(dit proefschrift)*

4. Precipitatie van surfactants in de darm door calciumfosfaat beschermt tegen een infectie met een Gram-negatief pathogeen zoals salmonella, maar verergert een darminfectie veroorzaakt door een Gram-positief pathogeen zoals listeria.

5. De recentelijke moleculair-biologische bevinding, dat ieder persoon een unieke mix van lactobacillen in zijn darmflora heeft, impliceert dat het prebiotica-concept kansrijker zal zijn dan het probiotica-concept. *(Kimura et al., Appl Environ Microbiol 1997; 63: 3394-3398)*

6. In de huidige definities van pre- en probiotica ontbreken eenduidige functionele effecten. *(Gibson et al., J Nutr 1995; 125: 1401-1412 en Tannock, Tibtech 1997; 15: 270-274)*

7. De veelgehoorde vergelijking van het schrijven van een proefschrift met een bevalling getuigt meestal van onervarenheid van promovendi op dit laatste gebied.

8. Beklimmingen van 's werelds hoogste toppen leveren bergen afval op.

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9. Gemeenten die eigen inwoners voorrang verlenen bij het kopen van nieuwbouw bevorderen het woon-werkverkeer en zijn dus weinig milieubewust.

10. Het succes van het nieuwe Nederlandse donorregister kan worden verhoogd door alleen aan geregistreerde donoren transplantaties aan te bieden.

11. Door de enorme aandacht in de media is Viagra in potentie eerder een financieel dan een sexueel succes.

12. Het aantal vrouwen in topfuncties zal sterker toenemen door maatschappelijke acceptatie van een 4-daagse werkweek voor mannen dan door uitbreiding van het aantal crècheplaatsen.

Stellingen behorend bij het proefschrift
"Dietery modulation of the resistance to intestinal infections"

Ingeborg Bovee-Oudenhoven, Wageningen, 25 november 1998

Aan pap en mam

Chapter 1

General introduction

Incidence of gastrointestinal infections

Gastrointestinal infections are still a major health problem. The worldwide incidence of acute infectious diarrheal disease is estimated to be 3-5 billion cases per year, resulting in 3-5 million deaths each year [1]. The increased transport of perishable foods, changes in consumer food preferences and food processing practices, and international travel may all add to an increased exposure to foodborne pathogens from all parts of the world. In addition, the use of immunosuppressive drugs, aging, and extension of life expectancy for the chronically ill through medical technology, have increased the population susceptible to severe illness after infection with foodborne or nosocomial pathogens [2]. Among foodborne disease outbreaks of known etiology the leading cause is bacterial pathogens (35%), followed by toxins, viruses and parasitic organisms other than bacteria and viruses. Cause of illness is determined in less than half of the outbreaks [2]. In the Western world, the bacterial pathogen most frequently isolated from patients suffering from acute gastro-enteritis is campylobacter spp. (50%), followed by salmonella spp. (25%) [2, 3]. Moreover, infections caused by these two pathogens have emerged importantly in the last two decades [2]. Salmonella epidemiology has shown that *Salmonella enteritidis* has now outnumbered *Salmonella typhimurium* as the most frequently isolated salmonella species [4].

While most intestinal infections result in a self-limiting gastroenteritis, the regular occurrence of gut-derived septicemia in people receiving immunosuppresive drugs, patients suffering from inflammatory bowel diseases and patients in intensive care units on (par)enteral nutrition regimen is a major complication [5-7]. Treatment of many foodborne infections by antibiotics is often discouraging, since it hardly ameliorates the severity of illness and often prolongs asymptomatic carriage [8, 9]. Another concern is the growing resistance of bacterial pathogens, including salmonella species [10, 11], to clinically important antibiotics [8]. It is even possible that some formerly treatable diseases may become incurable [12, 13]. The high frequency of antimicrobial resistance today is probably a consequence of the widespread use or the inappropriate therapy of infections in both humans and animals [8]. This stresses the importance of prevention to cope with the problem of emerging infections. Therefore, an important challenge of modern medicine is to understand what defenses are most important in preventing bacterial disease, how to identify people who lack the necessary defenses, and how to restore or replace these defenses [12].

Critical steps in the etiology of intestinal infections

Before discussing host defenses, it is useful to outline some important steps of the course of an intestinal infection. Although different (potential) pathogens use different tactics to resist host defenses in order to gain access to the intestinal mucosa [14, 15],some general strategies can be observed. Once a bacterium reaches the intestine, it must penetrate the mucus layer and adhere to host cells to colonize them (Figure 1, part A). Only bacteria that can adhere to the mucosa will be able to resist the constant flow of gut contents due to intestinal motility. Flexible glycoprotein structures extending from the bacterial surface,

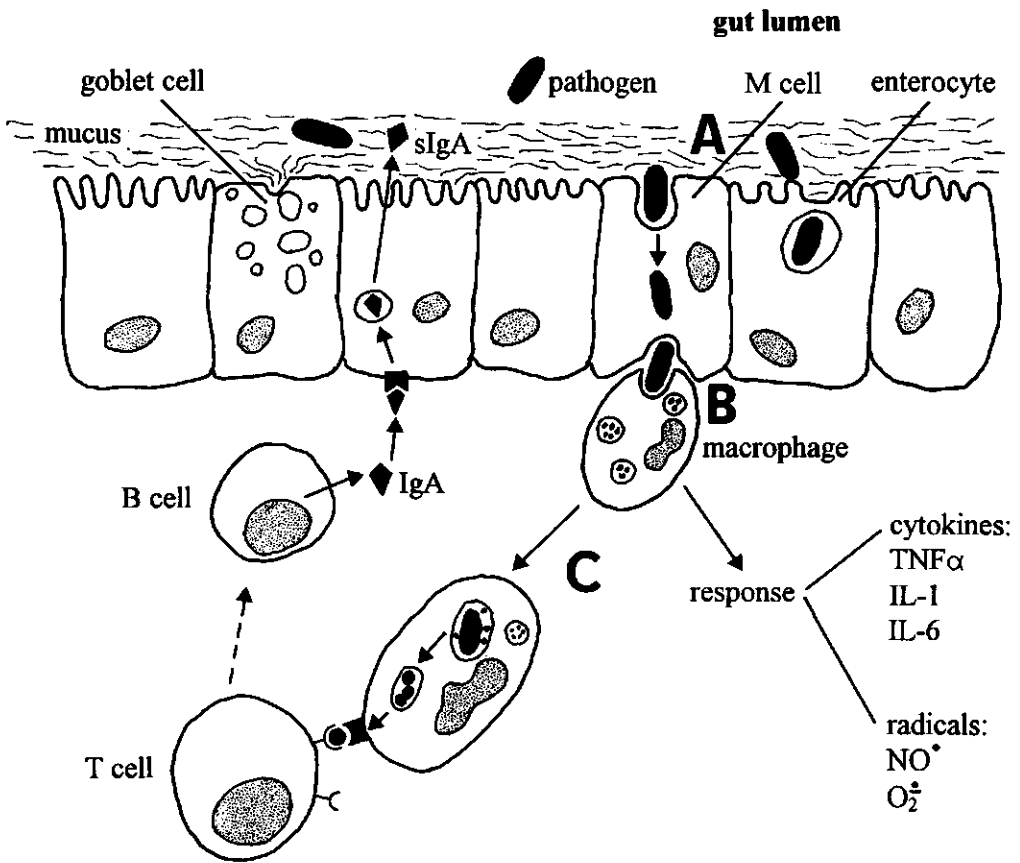

Figure 1 Schematic reproduction of the course of intestinal infections by invasive bacteria: **A** microbial adhesion to epithelial cells, **B** bacterial translocation across the epithelial barrier, **C** reaction of the immune system.

called pili or fimbriae, are sometimes involved in establishing contact between the bacterium and the apical cell membrane of enterocytes [16, 17]. Some pathogens, for instance yersinia [18] and salmonella [15, 19], also seem to use specialized antigen-sampling M cells to gain access to extra-intestinal tissues. After traversing the epithelial cell layer, the bacteria encounter the basement membrane, which acts as a filter and can to some extend hold up the infection, but its functional integrity is soon broken by epithelial cell damage or inflammation. At this site, the invaders are ingested by macrophages or other phagocytic cells (Figure 1, part B) and transported to the mesenteric lymph nodes [20, 21]. When massive translocation occurs, bacteria will spread to the blood stream and invade other organs, a life-threatening situation called bacteremia or septicemia [21].

Host defenses against bacterial pathogens

Of course, the most effective way of protecting the human body from pathogenic bacteria is to prevent contact with the bacterium in the first place. Fortunately, an array of host defenses is present to attack disease-causing bacteria, which succeeded in gaining access to the body by mouth. The first line of defense is non-immunological (Figure 2). The low pH of gastric juice (<3) has strong sterilizing properties, because many food-pathogens are acid-sensitive [22, 23]. Hypochlorhydria, in otherwise healthy elderly or in users of H_2 receptor antagonists and proton pump inhibitors, leads to increased intragastric bacterial counts and susceptibility to gastrointestinal infections [23-26], including salmonella [27]. Buffering of gastric content and physical protection of bacteria by food, in combination with the rate of gastric emptying, are additional factors influencing the amount of pathogens surviving the gastric barrier [23, 24].

The small intestine is protected to infective bacteria by a thick mucus layer covering the epithelium. Mucus not only acts as a lubricant to protect the relatively delicate epithelial cells from gastric acid, bile acids, chemical irritants and physical damage, but is also a trap for microbes to prevent their attachment to the mucosa. The mucus layer obtains its viscosity from mucins. Mucins are glycoproteins and its oligosaccharide units protect it from proteolytic enzymes. Furthermore, the sugar chains mimic epithelial receptors for bacteria and act like a physical sieve trapping microbes and bringing them in close contact with secreted antibodies (sIgA), lactoferrin, lysozyme and lactoperoxidase [26]. The small intestine has a relatively high motility, which prevents adhesion to the mucosal epithelium and subsequently bacterial overgrowth [24]. Duodenal secretions, as pancreatic enzymes and bile, have potent bactericidal activity. For instance, *Escherichia coli* and salmonella species are sensitive to pancreatic fluid in-vitro, as determined by Rubinstein et al. [28]. Considering that many bacterial toxins are proteins [29], the proteinases in pancreatic secretions might also protect against damage caused by intestinal infections. Although in-vitro studies demonstrated that surface-active bile acids exert a potential inhibitory effect on micro-organisms [30, 31], in-vivo studies in support of this observation are lacking. As a result of the combination of gastric acid, bile salts and rapid flow of contents, the small intestine is relatively sparsely populated by bacteria. Though not very numerous, the endogenous microflora colonizing the ileal mucosa might still be important in host defense, considering that many foodborne pathogens mainly elaborate their noxious effects in this region of the gastrointestinal tract [32].

In contrast to the small intestine, the most important defense mechanism of the colon is the presence of its luxuriant microflora [24]. Probably due to the slow flow of contents, the bacterial density is so great that nearly half of the volume of human colon contents is accounted for by bacteria [33]. Potential invaders have to compete with this extensive established bacterial population for nutrients and adhesion sites on the epithelium.

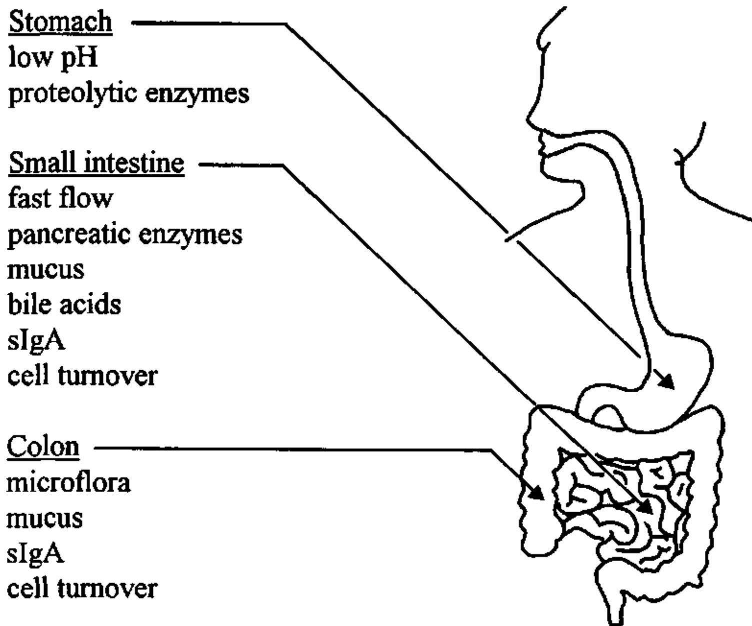

Figure 2 Host defenses of the gastrointestinal tract

The production of antibacterial substances by the colonic microflora, such as lactic acid, short-chain fatty acids and bacteriocins, may also inhibit growth of pathogenic bacteria [12, 24]. Several human [34, 35] and animal [36, 37] studies have shown that use of antibiotics, affecting the endogenous microflora, increases the susceptibility to contract intestinal infections.

All the above mentioned non-immunological defense mechanisms cooperate with the gut-associated lymphoid tissue (GALT) in eliminating microbial pathogens. The GALT is a combination of the Peyer's patches, lymphoid follicles, lymphocytes and mesenteric lymph node cells [26, 37]. The Peyer's patches in the small intestine are separated from the intestinal lumen by a single layer of columnar epithelium that includes microfold cells or M cells (Figure 1). M cells appear to be specialized in antigen uptake. Absorbed antigens are transported to the basolateral cell membrane and delivered to macrophages and other antigen-presenting cells. Antigen-presenting cells couple antigen with MHC class II receptors and activate T cells to secrete cytokines [26]. These cytokines in turn, stimulate B cells within the Peyer's patches to produce immunoglobulins, especially secretory IgA (sIgA, Figure 1 part C). sIgA plays an important role in antigen exclusion and bacterial agglutination, which prevents microbial contact with- and uptake by the intestinal epithelium [38, 39]. Translocation of bacterial pathogens (or components like endotoxin) strongly attracts and activates macrophages and neutrophils, which belong to the non-

specific immune system. These phagocytic cells produce a wide variety of cytotoxic metabolites (oxygen or nitrogen radicals and enzymes) in order to inhibit or kill the bacterial invader (Figure 1 part C) [40, 41].

Impairment or failure of these host defenses might result in an intestinal infection. In general, one or more of three basic pathophysiological conditions are necessary for bacterial translocation to occur. These are: (1) disruption of the ecological balance of the normal endogenous microflora, resulting in bacterial overgrowth of Gram-negative enteric bacteria; (2) physical loss of the intestinal mucosal barrier; and (3) impaired host immune defenses [7].

Composition of the intestinal microflora

The extensive differences that exist between the microfloras of various species, reported by various research groups, are in part obscured by the sampling and microbiological methods used and by the convention to describe the flora in terms of a relatively small number of bacterial genera or groups, neglecting bacterial species. Besides these shortcomings, some general trends can be observed. Bacterial counts in the proximal small intestine reflect the number of bacteria surviving the gastric barrier [42]. Probably due to the slower rate of transit and some bacterial multiplication, bacterial population levels in the ileum increase to $\pm$ 10^7/g contents in humans and $\pm$ 10^8/g in rats (Table 1). The human and rat colon harbours more than 400 different species in numbers up to 10^9/g contents. As a result of water resorption in the distal colon, viable counts in feces are even

Table 1. Differences in bacterial flora of the ileum, colon and feces of humans and rats ($\log_{10}$ bacteria per gram wet weight; Table adapted from Drasar BS [42]).

	Human			rat		
	ileum	colon	feces	ileum	colon	feces
Bacteroides	3-7	7-9	10-11	6-8	7-8	7-10
Eubacteria	5	-	9-10	-	-	9-10
Peptococci	-	-	10	-	-	9-10
Bifidobacteria	5-7	5-7	10	6-8	8-9	8-9
Lactobacilli	3-7	3-7	4-6	6-8	8-9	8-9
Clostridia	-	-	6-9	-	2-6	2-6
Fusobacteria	-	-	-	-	-	9
Enterobacteria	3-4	6-7	7-8	3-5	5-7	5-7
Streptococci	6	2-7	8	2-6	5-7	5-8

-: not measured

higher [42, 43]. It should be noted that the composition of the luminal flora (as measured in intestinal lavages or feces) may differ from the mucosal flora [44]. In contrast to large inter-individual variations in gut flora, the composition of major bacterial groups within an individual remains quite constant in time, even when dietary regimens change drastically [43]. Whether this is due to lack of sensitivity of the classical microbiological techniques used, will be elucidated in future experiments using specific molecular probes to identify and quantitate bacterial species.

Role of the gut flora in colonization resistance

The population of bacteria comprising the intestinal microflora continually exerts strong forces to maintain community stability. A practical effect of these activities is the exclusion of ingested exogenous bacteria, preventing their attempt to colonize the intestinal tract from time to time. Potentially pathogenic inhabitants of the gut are suppressed to harmless levels by the same forces [45]. This barrier formed was called "colonization resistance" by Van der Waay and coworkers [46]. Several studies are performed to unravel which components of the autochtonous intestinal microflora are responsible for this protection. Administration of more or less selective antibiotics, in order to eliminate particular bacterial groups of intestinal bacteria, showed that especially the anaerobic flora determines the colonization resistance to foodborne pathogens and potentially pathogenic indigenous bacteria [37, 46, 47]. Impairing the colonization resistance by administration of metronidazole, which eliminates obligate anaerobes, favored the translocation of the endogenous aerobic flora from the intestinal lumen to the mesenteric lymph nodes and beyond [37, 47]. However, facultative anaerobic, Gram-negative bacteria play at least some role in preventing the intestinal colonization and subsequent translocation of an exogenous pathogen (such as *Salmonella enteritidis*), as shown by oral streptomycin administration [37, 47, 48]. The consequences of reducing the colonization resistance by upsetting the autochtonous microflora are evident in clinical practice, considering the high incidence of antibiotic-associated diarrhea. Particularly, overgrowth of endogenous *Clostridium difficile* resulting in pseudomembranous colitis, is a regular occurring severe complication of recent exposure to antimicrobial agents [34].

Several mechanisms are proposed by which the resident microflora inhibits the establishment of invading bacteria in the intestine. Indigenous bacteria adhere to specific glycoprotein receptors on intestinal epithelial cells. The resulting barrier of microflora, which is firmly attached to the mucosa, prevents contact and colonization by pathogenic organisms. Mucosal attachment is a prerequisite for successful colonization and possible translocation of a pathogen. Without adhesion, bacterial multiplication is inadequate to compensate for the wash out of bacteria with the flow of intestinal contents [45]. Pathogens also have to compete with the resident flora for nutrients [45]. Metabolic products of fermentation, for instance lactic acid and short-chain fatty acids, and the concomitant decrease in pH and oxidation-reduction potential of intestinal contents are inhibitory for many intestinal pathogens [22, 45, 49]. Already in the ileum, conjugated bile acids are hydrolyzed by the indigenous microflora to their surface-active free counterparts [50]. The

subsequent flora-mediated dehydroxylation releases even more cytotoxic, secondary bile acids. Inhibitory effects of bile salts on bacterial growth in-vitro are documented [30, 31, 51]. On the other hand, experimental evidence for the relevance of bile acids for the resistance to intestinal infections in-vivo is lacking. Only a few human studies touch lightly on this subject. Tazume et al. [52] found that infectious diarrhea in humans coincided with a shift in fecal bile acid profile from the toxic unconjugated secondary bile acids to the less toxic primary and conjugated bile acids. However, no cause and effect relationship can be deduced from these results. Williams et al. [53] found that oral administration of a mixture of conjugated bile acids to human volunteers decreased the number of ileal anaerobes. However their results were inconsistent. The concentration of ileal bile salts did not significantly correlate with the number of ileal anaerobes. Moreover, administration of cholestyramine, which binds and precipitates bile acids, also decreased the anaerobes in the ileum. Likewise, bactericidal effects of bacteriocins are demonstrated in-vitro but not in-vivo [54].

The intestinal microflora and inflammation

Inflammatory bowel diseases (IBD), such as ulcerative colitis (involving the colon) and Crohn's disease (involving the ileum and colon), occur in regions of the intestine highly colonized by bacteria and their pathology resembles certain bacterial infections. Despite much effort, no convincing evidence incriminates a single bacterium as the cause. However, luminal bacteria and their products certainly play an important role in the exacerbation and recurrence of inflammation, considering that inflammation can hardly be induced in germfree animals [55, 56]. In addition, human studies showed that inflammation subsides in sections of the intestine that are operatively excluded from the fecal flow and that it deteriorates again when intestinal continuity is restored [57, 58]. Both commensal and pathogenic bacteria produce potent inflammatory molecules, including lipopolysaccharides (LPS or endotoxin), peptidoglycan polymers and N-formylmethionyl peptides [55]. Normally these strong activators of the immune system stay in the intestinal lumen. However, patients suffering from IBD have increased mucosal permeability, allowing uptake of bacteria and their immunogenic products, even during quiescent phases of the disease [59]. This implies that leakage is a primary abnormality rather than a secondary result of inflammation. Contact with the autochtonous microflora is necessary for normal development and maintenance of the intestinal immune system. Duchmann et al. [60] provided evidence that healthy individuals are tolerant to their own bacterial flora, but respond to the flora of others. In contrast, breakdown of tolerance to antigens (for instance LPS) of an individual's own intestinal flora occurs in IBD. Conversely to healthy subjects, high serum titers of antibodies to commensal bacterial species are observed in IBD patients [55,56].

Besides a genetic component, an interesting hypothesis regarding the cause of Crohn's disease is postulated by Demling [61]. The increased incidence of this inflammatory disease in the last 50 years parallels the increased use of antibiotics in human and veterinary medicine. Considerations that insufficient doses of antibiotics liberate LPS

from Gram-negative bacteria, induce bacteria to produce toxins or to invade host tissues and the observation that prior antibiotic therapy promotes relapses in Crohn's disease, formed the base for this hypothesis.

IBD is characterized by an overproduction of the pro-inflammatory cytokines IL-1, IL-6 and TNFα. Though therapeutic values remain to be established, experimental studies indicate that the diet can modulate the production of these cytokines and may be of help to regress the inflammation [62,63]. For instance, increasing the intake of fish oil or (n-3) fatty acids reduces inflammatory responses, probably by decreasing the production of the eicosanoids prostaglandin E_2 and leukotriene B_4. These eicosanoids are synthesized from arachidonic acid by cyclooxygenase and lipoxygenase respectively, and trigger leucocytes to release IL-1, IL-6 and TNFα. Replacement of arachidonic acid by (n-3) fatty acids in cell membrane phospholipids results in the production of the biologically less active prostaglandin E_3 and leukotriene B_5 [62]. Besides the type of dietary fat, dietary protein and antioxidants may also affect the activity of cytokines and the course of IBD [63].

Dietary modulation of the resistance to infection

Diet directly affects the non-immunological host defenses of the gastrointestinal tract, which are particularly important to withstand the first encounter with a pathogen (Figure 3). It is obvious that the composition of the diet determines the composition of gastrointestinal contents. The latter affects the survival of pathogenic bacteria in the gastrointestinal tract. In addition, intestinal contents may influence the composition and the activity of the protective endogenous microflora and subsequently their antagonistic activity towards pathogens. Besides an effect on bacteria, luminal contents also affect functioning of the intestinal epithelium and its barrier function against invading microbes. Diet also indirectly affects host defense. For instance, if the habitual diet is deficient in one or more essential nutrients, normal functioning of all cells, including these belonging to the immune system, is impaired [64].

Currently, there is a keen scientific and commercial interest in foods containing specific ingredients that modulate the intestinal microflora and subsequently the resistance to infection. The majority of these ingredients can be divided in two classes, the so-called probiotics and prebiotics. Besides these pro- and prebiotics, other dietary components (like minerals) may influence the course of an intestinal infection as well. These specific ingredients are discussed below in more detail.

Probiotics

By definition, probiotics are live micro-organisms which beneficially affect the host by improving its intestinal microbial balance [65]. This rather vague definition evokes several questions: what is microbial balance and which host functions are beneficially affected? Though the specific answers to these questions are certainly subject of debate, some general consensus does exist. Intestinal microbial balance is often associated with the suppression of putrefactive (protein degrading) and potentially pathogenic bacteria to harmless levels, and a stimulation of carbohydrate fermenting genera such as lactobacilli

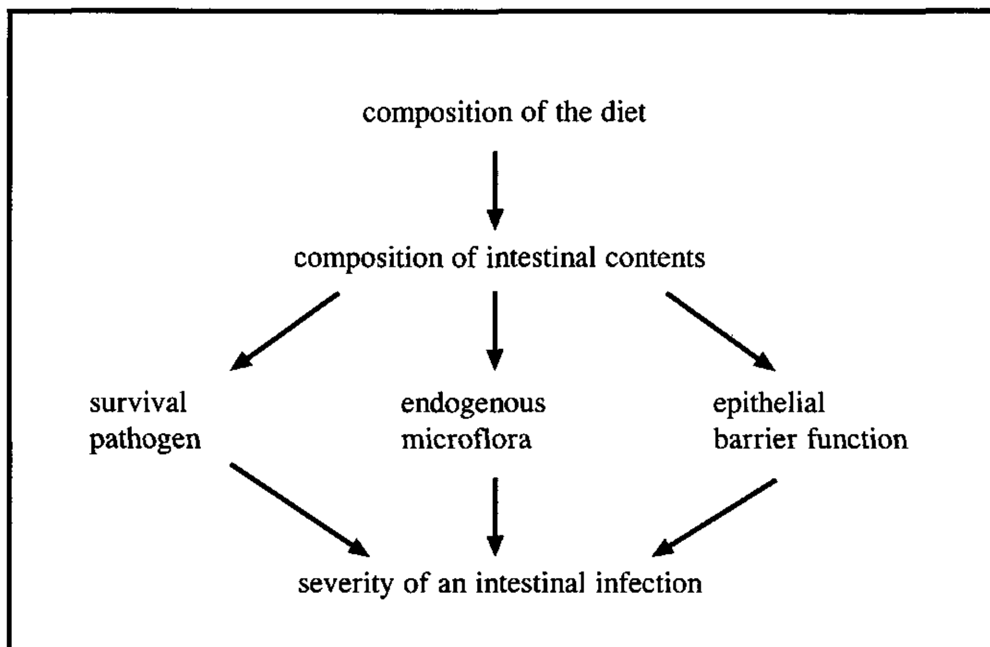

Figure 3 Dietary modulation of non-immunological host defenses of the intestinal tract, which determine the severity of an intestinal infection.

and bifidobacteria. Often mentioned benefits to the host upon ingestion of probiotic strains are reduction of symptoms of lactose intolerance, suppression of bacterial enzymes responsible for the production of toxic metabolites for the intestinal epithelium, amelioration of infectious diarrheal diseases and stimulation of the immune system [65-67]. Lactobacilli and bifidobacteria are the genera most frequently studied in probiotic research. However, these two genera are also normal residents of the intestinal tract of the majority of humans [42]. This implies that ingested probiotic strains have some health-promoting characteristics that the resident lactic acid bacteria lack and that they are able to compete sufficiently with the resident flora for the expression of these beneficial attributes [65].

Many investigations have shown that lactobacilli may exert antagonistic activity against intestinal and foodborne pathogens. In-vitro growth of pathogens, like *Escherichia coli*, salmonella, campylobacter, listeria, shigella and *Vibrio cholerae*, is inhibited in the presence of lactobacilli or bifidobacteria [68-70]. Administration of lactobacilli to rodents reduced the severity of a *Salmonella typhimurium* [68, 71] and shigella [72] infection, as measured by delayed or reduced mortality and fecal shedding of these pathogens. In a murine model, lactobacilli prevented *Helicobacter pylori* infection of the stomach [73]. A number of clinical studies showed that ingestion of lactobacilli resulted in shortening of the

duration of antibiotic-associated diarrhea [74], the prevention of recurrent relapses of *Clostridium difficile* colitis [75], amelioration of shigella infections [76] and acute rotavirus enteritis [77] in children. Consumption of formula supplemented with bifidobacteria also reduced the incidence of diarrhea and rotavirus shedding in hospitalized infants [78]. Though the protective effects observed in human intervention trials are often small and interpretation of results is sometimes hampered due to the inclusion of inappropriate control groups, they certainly justify the effort currently spend on probiotic research.

Several mechanisms are proposed for the improvement of the resistance to intestinal infections by lactobacilli or bifidobacteria. As shown in in-vitro experiments, the inhibitory action may result from competition for nutrients and adhesion sites on the intestinal epithelium [68, 79]. Lactic acid and acetic acid produced during bacterial metabolism inhibit the growth of many bacteria, including Gram-negative pathogens [80]. While lowering of the pH of intestinal contents is certainly a factor in such inhibitions, lower pH values also potentiate the bactericidal activity of these organic acids. Neutral, protonated organic acids traverse the bacterial cell membrane, accumulate intracellular due to dissociation, and subsequently disturb the bacterial cell membrane potential [81]. In addition to these organic acids, the production of hydrogen peroxide and bacteriocin-like compounds may also play a role in the antagonistic activity of lactobacilli [67], though their in-vivo relevance has not been demonstrated yet.

Lactobacilli are also reported to affect the immune response. First, consumption of lactic acid bacteria may stimulate the aspecific cell-mediated immune system, as measured by an enhanced proliferation and phagocytic acitivity of lymphocytes of rodents [71, 82] and humans [83]. Second, lactobacilli also stimulate the specific humoral immune response. For example, animal studies showed increased antibody titers against salmonella [71] and shigella [72] upon administration of lactobacilli. In addition, treatment of rotavirus-infected children with lactobacillus GG was associated with an enhancement of serum antibody-secreting cells and specific IgA against rotavirus at convalescence [84]. Furthermore, human volunteers consuming fermented milk containing *L. acidophilus* had significantly higher serum antibody titers against an orally administered attenuated *Salmonella typhi* strain [85].

It is not clear yet, how lactobacilli gain access to the host immune system. Lactobacilli may stimulate the immune system without leaving their luminal habitat, since it is now recognized that enterocytes are immunocompetent cells and produce cytokines involved in immuno-regulation [86]. It is also possible that lactobacilli (or their cell wall components) are sampled by M cells of the Peyer's patches of the distal small intestine [65, 87]. At present, intestinal inflammation or even translocation can not be excluded as the base for the observed immuno-stimulation by lactobacilli. Indeed, serum antibodies (IgM and IgG) against lactobacilli can be detected in healthy human subjects, whereas antibodies reactive with bifidobacteria are absent [87]. Additionally, the effects of lactobacilli on immune parameters in animal infection experiments do not always correlate with an indisputable functional parameter as mortality [71]. Therefore, at least some caution is warranted to interpret probiotic-induced immune stimulation as an always beneficial effect.

Prebiotics: dietary fibers and non-digestible oligosaccharides

Most of the predominant species of the intestinal microflora require a fermentable carbohydrate for growth. It is generally assumed that the carbon and energy needed to maintain bacterial mass are derived from host secretions or from dietary carbohydrates (or other components) that escape digestion in the small intestine. Mucins, extensively glycosylated proteins secreted by goblet cells, might be excellent endogenous bacterial growth substrates [88]. Dietary fibers (vegetable polysaccharides) and oligosaccharides are also more or less fermented in the intestinal tract, depending on their sugar composition, type of glycosidic linkages and degree of branching. Dietary fibers are essential for maintenance of intestinal mucosal integrity, which can be deduced from animal [89] and human [90] studies showing that oral administration of fiber-free elemental liquid diets induces spontaneous translocation of the gut microflora. Some say that the protective effects of fiber on the mucosal barrier function are independent of their fermentability and are mediated by stimulating the release of trophic gut hormones, preventing mucosal atrophy [91, 92]. By definition, prebiotics are fermented by the endogenous microflora in contrast to the non- or low-fermentable dietary fibers (for instance lignin and cellulose). Selective stimulation of the growth or activity of a limited number of resident bacteria and improvement of the host health are often mentioned criteria a prebiotic should meet [93, 94]. Polysaccharides like inulin, pectin, resistant starch and guar gum, and oligosaccharides based on galactose, fructose, and xylose are potential prebiotics [93].

A characteristic all the above mentioned potential prebiotics share is that they are fermented by the intestinal microflora in the lower gut to lactic acid and short-chain (C_2-C_6) fatty acids, resulting in a decreased pH of luminal contents [93, 95]. Consumption of prebiotics may change the composition of the intestinal microflora. For instance, dietary inulin and fructosyloligosaccharides may increase fecal bifidobacteria counts of rodents and humans [95-98]. Whether the in-vivo growth advantage of bifidobacteria is due to selective utilisation of these non-digestible sugars by this genera or due to their relative resistance to the organic acids formed is not established yet. The latter explanation seems more likely, considering that other genera (for instance bacteroides) can also degrade fructosyloligosaccharides in-vitro [93]. Moreover, increasing the number of bifodobacteria or inducing a change in gut flora composition as such, is not directly a functional effect nor a direct health advantage. The intestinal effects of oligosaccharides might depend on their composition and structure. In contrast to the above mentioned bifidogenic effect of inulin and fructosyloligosaccharides, no change in the composition of the fecal microflora was observed in a recently performed, placebo-controlled human study with galactosyl-oligosaccharides in doses up to 15 g/d (Alles MS, personal communication).

One of the benefits to be gained by the host upon consumption of non-digestible food ingredients is an improved resistance to intestinal infections. During fermentation of dietary fiber or non-digestible oligosaccharides, the organic acids generated and the concomitant drop in luminal pH may kill or inhibit the growth of acid-sensitive pathogens like salmonella, *Escherichia coli*, *Vibrio cholerae* and *Clostridium difficile*, as shown in-vitro [99, 100]. Surprisingly, data showing the efficacy of fermentable dietary fibers to

reduce the severity of intestinal infections in strictly controlled in-vivo experiments are rare. Lactulose therapy has been investigated in the management of salmonella and shigella infections in humans. This non-digestible disaccharide increased fecal clearance of salmonella and shigella in a significant proportion of the patients [101, 102]. Studies on inflammatory bowel diseases have shown that short-chain fatty acids are important for normal functioning of the intestinal epithelium and the maintenance of the mucosal barrier function [103]. Though the etiology and pathology of inflammatory bowel diseases are certainly different from an intestinal bacterial infection, gut bacteria play a crucial role in the mucosal inflammation [55, 56]. Increasing intestinal fermentation by supplementing the diet with lactulose or pectin reduced the severity of chemical-induced enterocolitis in rodents, with concomitantly decreased serum endotoxin levels and translocation of endogenous bacteria [104, 105]. Short-chain fatty acids or butyrate enemas also ameliorate the inflammatory process in patients suffering from ulcerative colitis and pouchitis [105, 106]. Short-chain fatty acids, and butyrate in particular, are normal substrates for energy production of intestinal epithelial cells. The prevailing hypothesis is that stimulation of intestinal fermentation replenishes the frequently observed low luminal concentrations of short-chain fatty acids or overcomes the impaired ß-oxidation of butyrate of enterocytes in inflammatory bowel disease patients [106]. Notwithstanding the beneficial effects of fermentable dietary fibers on intestinal physiology, some restraint should be applied in increasing the intake of non-digestible oligo- or polysaccharides. High concentrations of short-chain fatty acids may damage the intestinal epithelium, resulting in an increased permeability and epithelial cell proliferation [107-109].

Besides stimulation of intestinal fermentation, soluble oligosaccharides might also improve the colonization resistance by decreasing adhesion of pathogens to the gut mucosa. Many pathogenic bacteria use (glyco)proteins (called variously adhesins, fimbriae, lectins or hemaglutinins) to attach to carbohydrate structures expressed on epithelial cells. Competitive inhibition by oligosaccharides of this lock-and-key interaction may decrease attachment of pathogens to the mucosa and prevent the crucial step in the initiation of an infection [110]. For instance, α-methyl-D-mannoside injected into the urine bladder of mice significantly decreased the extent of bacteriuria caused by mannose-sensitive, type-1-fimbriated *Escherichia coli*. Glucoside derivatives were ineffective [111]. The addition of D-mannose to drinking water reduced the incidence and extent of *Salmonella typhimurium* colonization of young chickens [112]. Additionally, diarrhea-causing enterotoxins produced by many pathogenic bacteria also recognize carbohydrate structures on epithelial cells. As an example, cholera toxin B recognizes monosialoganglioside 1 on the cell surface. Sialyllactose could inhibit the cholera toxin-induced fluid accumulation in a rabbit intestinal loop model. Neither sialic acid nor lactose was effective, demonstrating the specificity of the interaction [113]. The membrane-associated lectins of bacteria differ per strain and the glycoproteins expressed on epithelial cells are highly species specific [114]. Based on this mechanism, the application of oligosaccharides to increase the resistance to infection is limited, unless a cocktail of inhibitors is used. In contrast, the above-mentioned stimulation

of intestinal fermentation by dietary oligo- or polysaccharides has a more general effect, because protection against several acid-sensitive pathogens might be expected.

Minerals

Very little is known about the effects of dietary minerals on the course of an infection. Only data about the role of iron and zinc are reported. Individuals with iron-overload, whether induced by excess dietary iron intake or due to diseases like ß-thalassaemia major and sickle cell disease, are more susceptible to infection [115]. Iron is essential for bacterial growth and may increase oxidative damage to host membranes or DNA during inflammation, as it is a catalyst in the production of hydroxyl radicals [116]. The observed drop in plasma iron levels during infectious diseases, the so-called anemia of infection, is a protective response of the body to combat the infection and to limit oxidative damage [117, 118]. On the other hand, there is also evidence that severe iron deficiency is associated with an increased incidence of infections [115]. Iron deficiency compromises host resistance by suppressing the cellular immune response [119].

Zinc is a cofactor of about 120 enzymes of mammals. Zinc deficiency has a pronounced effect on nucleic acid metabolism, thus influencing protein synthesis and cell growth. Furthermore, an inadequate zinc intake is associated with an impaired immune function [120]. Zinc supplementation can reduce the incidence and prevalence of acute infectious diarrhea [121] and reverse the increased intestinal permeability during shigellosis [122] in children with a marginal or deficient zinc status. Besides restoration of immune cell functioning, normalization of intestinal epithelial cell proliferation and strengthening of the mucosal barrier might be responsible for the observed beneficial effects of zinc supplementation.

The effects of calcium on the resistance to intestinal infections are unknown. At the same time, evidence accumulates showing the protective effects of dietary calcium on the development of colorectal cancer [123, 124]. A substantial amount of dietary calcium reaches the lower gut because the intestinal absorption of calcium is limited [125]. Calcium forms and insoluble complex with phosphate, which strongly adsorbs and precipitates intestinal surfactants, like bile acids and fatty acids [126]. Bile acids and fatty acids are damaging to the intestinal epithelium and stimulate epithelial cell proliferation [127], which may increase the risk of colon cancer [128]. It can be speculated that these cytoprotective effects of dietary calcium on the intestinal epithelium are not only relevant to colon carcinogenesis but also have major implications for the resistance to intestinal infections. Diminishing epitheliolysis, by decreasing the cytotoxicity of gut content, might strengthen the mucosal integrity. Conversely, increased epithelial cell damage provoked by dietary lectins [129], chemical irritants [130] or ischemia/reperfusion [131] leads to disruption of the physical barrier function of the intestinal mucosa and gut-derived septicemia.

Besides the above mentioned proposed effect of dietary calcium on the mucosal barrier function, calcium may directly affect pathogens or the endogenous microflora. For instance, the in-vitro expression of some virulence genes of pathogenic bacteria is decreased by extracellular calcium [132]. Additionally, calcium facilitates the adhesion of

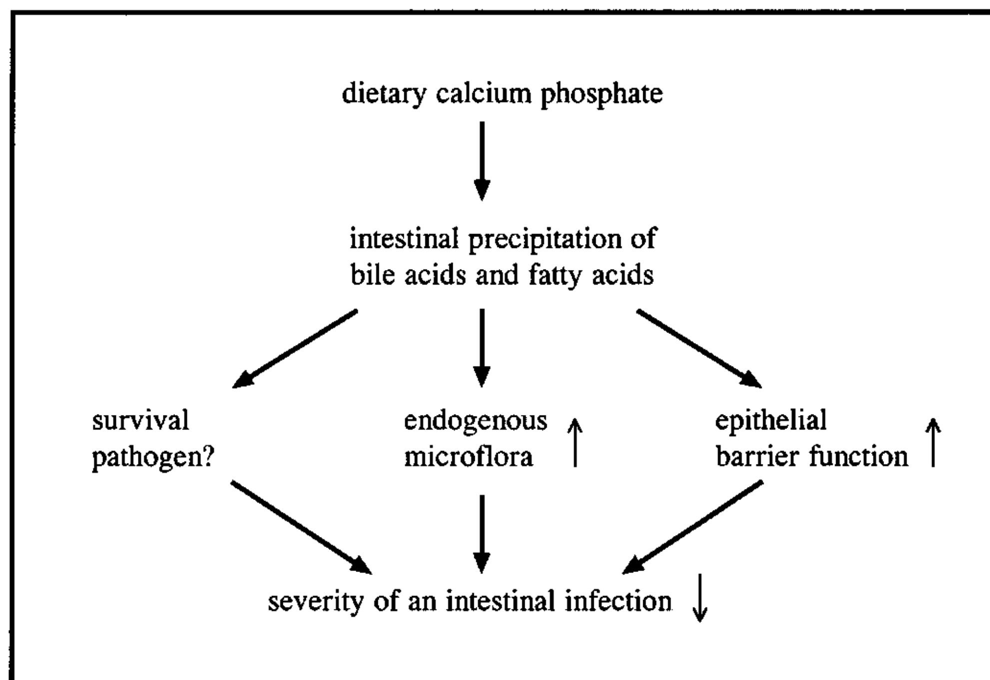

Figure 4 Hypothetical mechanism by which dietary calcium phosphate may decrease the severity of an intestinal infection.

lactobacilli to intestinal epithelial cell lines [133]. However, these results are not verified in-vivo and their relevance for the resistance to intestinal infections is yet unknown. By precipitating and thus inactivating intestinal bile acids, calcium may also indirectly affect the intestinal microflora. Bile is a classical supplement used in the preparation of several selective microbiological culture media to suppress contaminating flora. This indicates that bacterial species differ in their sensitivity to bile acids, at least in-vitro. In the seventies, Floch et al. [134, 135] reported that bile acids were especially inhibitory to anaerobes and Gram-positive aerobes, whereas Gram-negative bacteria were rather insensitive in-vitro. He also noticed that certain strains deconjugate bile acids, subsequently resulting in an inhibition of that particular bacterial strain. He postulated that bile acids may control bacterial populations in the intestinal tract and that bile acids are part of an autoregulatory system in which individual bacteria may control their own population. The restraining effect of bile in general on the intestinal microflora can be deduced from in-vivo studies on obstructive jaundice. A reduced or absent bile flow results in intestinal bacterial overgrowth and an increased risk for endotoxemia and septic complications. Oral administration of bile acids can normalize intestinal bacterial numbers and alleviate some of the symptoms in such cases [136]. In conclusion, only scattered data, mostly obtained from in-vitro experiments, indicate an effect of calcium or bile acids on bacteria. At present, no well-controlled studies

are available showing the effect of diet-induced changes in the intestinal bile acid concentration on intestinal ecology and its consequences for the resistance to invading pathogens.

Scope and outline of this thesis

As mentioned above and outlined in Figure 3, diet may modulate the resistance to intestinal infections by affecting the gastrointestinal killing of a pathogen, the protective endogenous microflora and the mucosal barrier function. Though, the importance of these three determinants of resistance is generally recognized, strictly controlled in-vivo experiments showing the impact of the diet on the severity or the course of a foodborne intestinal infection are lacking. This thesis mainly focuses on the possible protective effects of dietary calcium on the resistance to intestinal infections. The emphasis is laid upon the mechanism by which calcium may improve the resistance to intestinal infections. The hypothesis concerning this mechanism is outlined in Figure 4. Considering that only circumstantial evidence is present in literature, the performance of human studies is ethically not justified in this phase. Therefore, strictly controlled infection experiments were performed with rats. *Salmonella enteritidis* was chosen as the infective agent, because non-typhoidal salmonellosis is one of the most common, foodborne, bacterial infections in Europe and the United States [137]. Equally important, the development and pathology of salmonellosis in humans and rodents is largely similar and well-described in literature [138]. Because *S. enteritidis* has invasive properties, dietary modulation of the resistance to intestinal bacterial translocation could be studied in addition to modulation of the colonization resistance.

The first study described in this thesis concerns the development of a new method to determine intestinal bacterial translocation because classical organ cultures suffer from some major drawbacks. Bacteriological determination of pathogens in tissue samples is invasive, laborious, rather insensitive and only applicable as a one-point measurement. Therefore, it was evaluated whether daily nitric oxide-derived urinary nitrate excretion could be used to quantitate intestinal bacterial translocation (chapter 2). Second, the effect of different (fermented) milk products on the colonization resistance of rats to *S. enteritidis* was studied. Diet-induced as well as infection-induced changes in intestinal physiology were measured (chapter 3). Subsequently, a strictly controlled infection experiment was performed with rats consuming purified diets differing only in calcium phosphate. The effects of supplemental calcium phosphate on the translocation and colonization resistance were studied. In addition, several fecal bacterial mass markers were analyzed to verify the proposed stimulating effect of calcium phosphate on the endogenous microflora (chapter 4). The main purpose of the next study was to test whether supplemental calcium phosphate adds to the potential beneficial effect of lactulose fermentation on a salmonella infection in rats (chapter 5). The final study is an extension of the experiment described in chapter 4 and adresses the question whether a calcium phosphate-induced decrease in intestinal surfactants affects the intestinal lactobacilli and changes the resistance of rats to salmonella.

Special attention was given to the ileal flora, because this is the most relevant part of the intestinal tract in salmonellosis (chapter 6).

The thesis is closed with a summary and concluding remarks (chapter 7).

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Chapter 2

Nitric oxide-derived urinary nitrate as a marker of intestinal bacterial translocation in rats

Ingeborg MJ Oudenhoven, Henricus LBM Klaasen, John A Lapré,
Anton H. Weerkamp & Roelof van der Meer

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ABSTRACT

Bacterial translocation across the gut wall may lead to bacteremia and sepsis. Bacteriological analyses are laborious and time consuming, which precludes a rapid diagnosis of bacterial translocation. Synthesis of nitric oxide by macrophages is a primary response to bacterial infections. Therefore, the aim of this study was to examine whether NO-derived nitrite and nitrate (summed as NO_x) excretion can be used as a rapid and quantitative marker of intestinal bacterial translocation. The kinetics of urinary NO_x excretion was determined in rats intraperitoneally injected with increasing doses of *Salmonella enteritidis* lipopolysaccharide. Subsequently, the response to bacterial translocation was studied in rats infected orally with different doses of viable, invasive *S. enteritidis*. Increasing the lipopolysaccharide dose from 0.05 to 0.50 mg/kg resulted in a transient, dose-dependent, almost 10-fold increase in urinary NO_x excretion. Administration of the NO synthase inhibitor N^G-nitro-L-arginine methyl ester merely inhibited the increase in NO_x excretion after lipopolysaccharide injection. Increasing the infective dose of viable salmonella resulted in a time- and dose-dependent exponential increase in NO_x output. Translocation was a prerequisite for provoking a NO_x response. Total urinary NO_x excretion after infection and classical infection parameters, such as weight of the mesenteric lymph nodes and population levels of salmonella in feces, were highly correlated. In conclusion, urinary NO_x excretion is a quantitative, non-invasive biomarker of intestinal bacterial translocation, which can be used to follow the course of a systemic infection.

INTRODUCTION

Gut-derived bacteremia and sepsis can be a severe clinical complication in traumatized or immunocompromised patients [1] as well as in patients with cirrhosis and portal hypertension [2,3]. Unfortunately, bacteriological determination of pathogens in tissues or fecal samples is laborious, time consuming, and often a one-point measurement. Therefore, these analyses are less suitable for a rapid diagnosis and monitoring of bacterial translocation across the intestine. Because induction of nitric oxide synthesis is a primary reaction of macrophages to bacteria, fungi, and protozoa [4-14], we considered whether this parameter can be used as an alternative method to follow the course of systemic infections. In-vivo, the NO generated during infections is rapidly oxidized to nitrite and nitrate (summed as NO_x) and excreted in urine [14-16]. Direct proof that in such cases NO is the origin of NO_x in urine or blood is derived from in-vivo studies using competitive inhibitors of NO synthase, such as N^G-nitro-L-arginine and its methyl ester N^G-nitro-L-arginine methyl ester (L-NAME) or N^G-monomethyl-L-arginine [16-19]. These arginine analogs also exert a profound inhibition of NO synthesis in-vitro [20, 21]. When macrophages are activated in-vitro with increasing doses of lipopolysaccharide (LPS) or viable bacteria, the production of NO_x or citrulline increases dose-dependently [4-6]. Animal studies have shown that administration of a single dose of LPS [9, 10] or viable mycobacteria [16] results in enhanced urinary NO_x excretion. Patients with sepsis [8, 22] or acute diarrhea [7, 10] also show elevated blood or urinary NO_x levels. However, a dose-dependent

relationship between intestinal bacterial translocation and urinary NO_x excretion has hitherto never been shown.

Therefore, we studied whether urinary NO_x excretion can be used as a sensitive and non-invasive marker of intestinal bacterial translocation. Two kinds of strictly controlled experiments with rats were performed to address (1) if urinary NO_x excretion depends on the dose of intraperitoneally injected LPS and (2) what is the relationship between urinary NO_x excretion and increasing doses of orally administered, viable, invasive bacteria such as *Salmonella enteritidis*.

MATERIALS AND METHODS

Animals

In all experiments, specific pathogen-free, 8 weeks old, male Wistar rats (Cpb:WU derived from the Central Animal Laboratory, Catholic University, Nijmegen, The Netherlands) were housed individually in metabolic cages. Mean weight (± SE) of the animals was 256 ± 4 g in the LPS dose experiment (n=20), 233 ± 3 g in the LPS/L-NAME experiment (n=12), and 261 ± 3 g in the oral challenge experiment (n=24). Foods (purified diet consisting of 10% casein, 10% palm oil and 10% corn oil, 60% dextrose, 5% cellulose, vitamins, and minerals as described elsewhere [23]) and demineralized drinking water were supplied ad libitum. Food intake was recorded every 2-4 days. Complete 24 h urine samples were collected in plastic tubes containing oxytetracycline (twice the minimal inhibitory concentration; Pfizer Corp., Brussels, Belgium) to prevent bacterial deterioration and stored at -20 °C until analysis. Animals were acclimatized to housing and dietary conditions for at least 1 week before any experimentation. The experiments were approved by the Ethical Committee of Animal Experiments of the Catholic University, Nijmegen, The Netherlands.

S. enteritidis LPS experiment

After collection of urine for determination of basal NO_x excretion (3 days), rats were intraperitoneally injected with either 0,0.05, 0.10, 0.50, or 1.0 mg/kg body wt *S. enteritidis* LPS (lyophilized phenol extract; Sigma Chemical Co., St. Louis, MO, USA) in sterile saline. After LPS administration, urine samples were collected during 5 consecutive days. An additional control experiment was performed to determine whether the increased urinary NO_x excretion after LPS administration was caused by increased activity of NO synthase. Two groups of rats received a single intraperitoneal injection of 0.50 mg/kg body wt *S. enteritidis* LPS. L-NAME (Sigma Chemical Co.) in sterile saline was administered intravenously to the first group directly after the LPS injection and at the following 3 consecutive days. The second group was intravenously injected with saline daily.

Oral challenge with viable *S. enteritidis*

The strain of *S. enteritidis* (clinical isolate, phage type 1) used in this infection experiment is passaged routinely in Wistar rats to sustain its virulence and stored as a pure culture at -80°C in Brain Heart Infusion broth (Difco, Detroit, MI, USA) containing 7% (vol/vol) sheep blood and 20% (vol/vol) glycerol. Before use, the culture was streaked on Blood Agar Base (Oxoid, Basingstoke, England) supplemented with 5% (vol/vol) sheep blood and incubated overnight at 37 °C. Colonies were inoculated in 1 mL of brain heart infusion broth and incubated for 18 h at 37 °C. Four groups of six rats each were infected orally by gastric gavage with 1 mL of saline containing 3% (wt/vol) sodium bicarbonate with either $1.8x10^5$, $1.8x10^7$, or $1.8x10^9$ viable *S. enteritidis* or $1.8x10^9$ heat-killed *S. enteritidis* (incubation for 1 h at 56 °C). The exact number and viability of *S. enteritidis* in the inocula was determined by plating on Brilliant Green Agar (Oxoid). Complete 24 h urine samples were collected consecutively from 3 days before infection till 7 days after infection. On days 1 and 7 after infection, fresh fecal samples were collected. The number of salmonella in feces was quantified by a plating technique on Brilliant Green Agar as described and validated by Giaffer et al. [24]. To obtain representative cultures of the mesenteric lymph nodes (MLN), the experiment was terminated 7 days after infection to prevent progression to death [25]. That day, the rats were killed by carbon dioxide inhalation, and all MLN were excised aseptically, weighed, and homogenized (Ultraturrax TP 18.10; Janke & Kunkel, Staufen im Breisgau, Germany) in sterile saline. From several 10-fold dilutions, 10 μL was plated on Brilliant Green Agar and incubated at 37 °C for 24 h. Dilutions yielding 30-200 colonies of salmonella on plate were considered appropriate for scoring. Salmonella counts were expressed as the total $\log_{10}$ colony-forming units (CFU) in the MLN.

NO_x analyses in urine and food

The NO_x concentration was determined by automated flow injection analysis. Briefly, urine is passed over a cadmium column that reduces nitrate to nitrite, followed by reaction of nitrite with Griess reagent [26] to form a red azodye that is measured spectrophotometrically at 538 nm. Nitrite concentration in samples was measured with the same procedure, but without reduction of nitrate by cadmium. No nitrite or nitrate could be detected in watery extracts of the food. Recovery of a nitrate standard added to rat urine preserved with oxytetracycline was always $>96\%$. No nitrite was present in rat urine. Urinary NO_x concentration was multiplied by the urine volume produced in 24 h to obtain total urinary NO_x excretion per day. Creatinine excretion in urine was assayed chemically using a colorimetric kit (Creatinine procedure no. 555; Sigma Chemical Co.).

Statistics

Values are expressed as means $\pm$ SE. After analysis of variance, the differences between the means of the groups were evaluated using Student's *t* test for a single comparison or Bonferroni's test for multiple comparisons [27]. When variances were not

equal among groups, the nonparametric Kruskal-Wallis test was performed [27]. Differences were tested one-sided and regarded as significant at $P<0.05$.

RESULTS

Effect of increasing doses of intraperitoneal *S. enteritidis* LPS on kinetics of urinary NO_x excretion

Before LPS administration, food intake was 17.1 ± 0.9 g/d, which decreased 21 ± 4% after injection of either 0.10 or 0.50 mg/kg LPS and 39 ± 7% after administration of 1 mg/kg LPS ($P<0.05$). Mean basal urinary NO_x excretion before LPS administration was 4.28 ± 0.32 μmol/d (n=20) and did not differ between treatment groups. The kinetics of the NO_x response to different doses of LPS is shown in Figure 1. Administration of 0.05 mg/kg LPS had no effect on urinary NO_x excretion. Rats that received 0.10 mg/kg LPS showed a slight increase in urinary NO_x output to about 10 μmol during the first 24 h after LPS injection. NO_x excretion rapidly decreased to the basal level within the second day. Increasing the dose of LPS to 0.50 mg/kg resulted in a sharp increase in NO_x output to about 38 μmol during the first day. A slight elevation of NO_x excretion could still be noticed on the second day. The initial course of NO_x excretion of rats that received 1.0

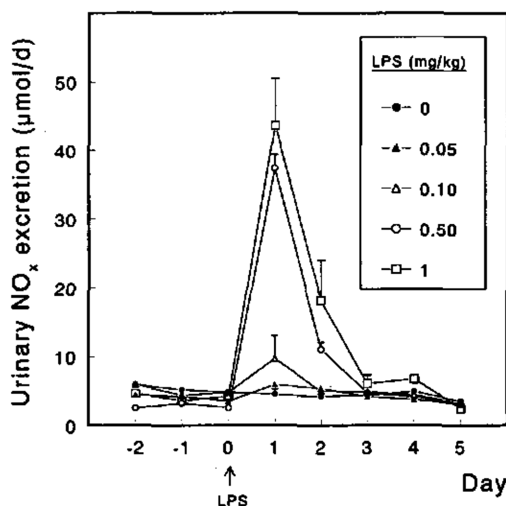

Figure 1. Urinary NO_x excretion of rats before and after intraperitoneal injection of different doses of *S. enteritidis* LPS. Results are expressed as means ± SE (n=4). The control group received saline only.

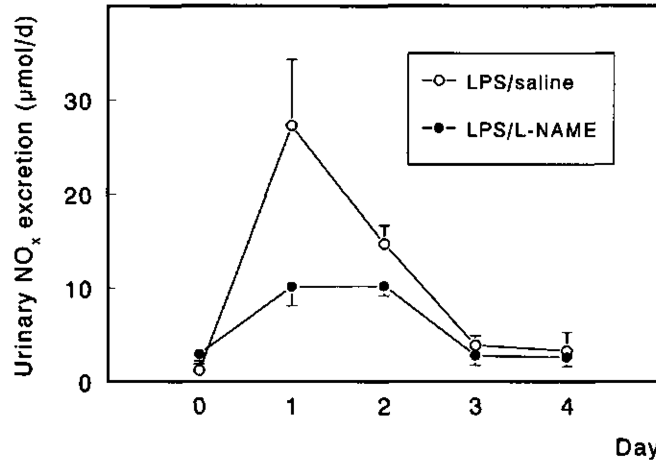

Figure 2 Urinary NO_x excretion of rats after a single intraperitoneal injection of *S. enteritidis* LPS (0.50 mg/kg) on day 0. Results are expressed as means ± SE (n=6). On day 0 up to and including day 4, L-NAME (3 mg/kg) or saline was injected intravenously.

mg/kg largely paralleled that of the 0.50 mg/kg treatment group. Total urinary NO_x excretion after injection of 0, 0.05, 0.10, 0.50, and 1.0 mg/kg LPS was 21.60 ± 1.38, 22.42 ± 1.48, 27.21 ± 3.85, 60.51 ± 3.31, and 77.17 ± 6.32 µmol/5 days, respectively. Mean values of the last three groups were significantly different from those of the control group and from each other ($P<0.05$).

Effect of L-NAME on urinary NO_x excretion after LPS administration

After LPS administration, food intake and body weight decreased equally in the L-NAME-supplemented group and the saline-treated control group. On the first day after LPS challenge, urinary NO_x excretion of rats treated with L-NAME was significantly less than that of rats receiving saline ($P<0.05$) (Figure 2). Total urinary NO_x excretion after LPS injection was 49.18 ± 9.08 µmol/4 days for saline-treated rats and 25.58 ± 1.93 for L-NAME-treated rats ($P<0.05$). Administration of 3 mg/kg body wt L-NAME did not completely prevent the NO_x response induced by LPS. A slight elevation could still be noticed. A previous tryout with a higher dose of LPS (1 mg/kg) and higher doses of L-NAME (5, 10, and 20 mg/kg) was lethal for some rats. Sections showed vascular complications, particularly in the lungs. Rats that survived showed complete prevention of the increase in urinary NO_x excretion (data not shown).

Table 1 Effect of oral *S. enteritidis* challenge on post-infection population levels of salmonella in feces and MLN.

	Salmonella counts		
	Feces ($\log_{10}$ CFU/g)		MLN ($\log_{10}$ CFU)
Challenge dose	Day 1	Day 7	Day 7
Control	n.d.	n.d.	n.d.
1.8×10^5	2.3 ± 0.8^a	3.3 ± 0.9^a	4.4 ± 0.1^a
1.8×10^7	4.3 ± 0.8^a	$5.7 \pm 0.9^{a,b}$	$4.7 \pm 0.1^{a,b}$
1.8×10^9	7.1 ± 0.1^b	7.1 ± 0.3^b	5.0 ± 0.2^b

The control group received 1.8×10^9 heat-killed *S. enteritidis*. Results are expressed as means ± SE (n=6). Different superscripts in the same column indicate a significant difference ($P<0.05$; Kruskal-Wallis test). Abbreviations: n.d. not detected, CFU colony-forming units.

Effect of oral administration of increasing doses of viable *S. enteritidis* on urinary NO_x excretion

During the course of the infection, the animals showed hardly any outward signs of illness. They were alert and did not have any diarrhea, and their food and drinking water consumption did not change. Only the rats that received the highest challenge dose consumed significantly less during the last 3 days of the experiment: food intake was 15.8 ± 0.6 g/d before challenge and decreased 35 ± 7% in the last 3 days. Table 1 summarizesthe effect of different challenge doses of *S. enteritidis* on salmonella counts in feces and MLN. As expected, no viable salmonella could be shown in the feces of rats receiving 1.8×10^9 heat-killed bacteria. Within treatment groups infected with viable *S. enteritidis*, the salmonella populations present in feces at day 1 and day 7 after infection were comparable. Cultures of the MLN of rats that received heat-killed *S. enteritidis* were negative. Rats infected with viable *S. enteritidis* had positive MLN cultures with absolute salmonella counts between 10^4 and 10^5. Increasing the infective dose had a dramatic effect on the weight of the MLN (Figure 3). The correlation coefficient between the salmonella counts in the MLN and weight of this organ was 0.80 ($P<0.05$). An interesting observation is that only in MLN cultures of rats infected with 1.8×10^9 viable *S. enteritidis* were translocated *Escherichia coli* also observed. Macroscopic observations during the section showed that the colon or cecum of all rats challenged with 1.8×10^7 or 1.8×10^9 of *S. enteritidis* had marked hypervascularization. In 3 of 6 rats infected with the highest dose, this phenomenon extended to the MLN. Despite the appearance of the feces as separate pellets, the cecum contents were usually watery in this group compared with the control group.

Figure 4 shows the kinetics of urinary NO_x excretion in response to the different challenge doses of *S. enteritidis*. Before induction of the infection, no differences in NO_x excretion were observed between treatment groups. Administration of heat-killed *S. enteritidis* had no effect on urinary NO_x output. As early as the second day after challenge, the rats inoculated with $1.8x10^9$ *S. enteritidis* showed an increase in urinary NO_x excretion. During the subsequent days, there was a continuous, marked increase in NO_x excretion. Rats infected with $1.8x10^7$ or $1.8x10^5$ *S. enteritidis* showed a post-infection latency period of 4 and 5 days, respectively, until NO_x excretion significantly increased. By adding up the NO_x excretion of days 1 to 7, total urinary NO_x excretion after infection can be calculated. The effect of different challenge doses of *S. enteritidis* on total urinary NO_x excretion after infection and weight of the MLN 7 days after infection is illustrated in Figure 3. The correlation coefficients between total NO_x excretion in urine and fecal salmonella or MLN weight were 0.75 and 0.85, respectively ($P<0.05$). At day 7 after infection, urinary creatinine excretion was 74 ± 4 μmol/d and did not differ between treatment groups. Thus, instead of total urinary NO_x excretion, the ratio of NO_x to creatinine in incomplete urine samples can also be used to monitor bacterial translocation.

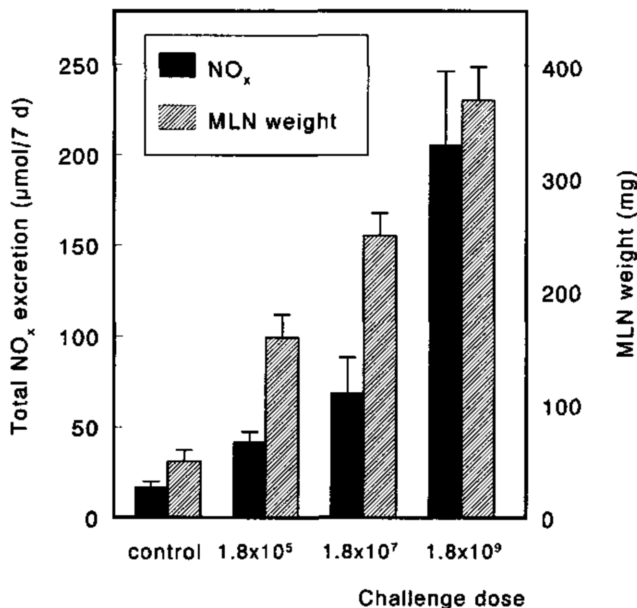

Figure 3 Effect of the oral challenge dose of *S. enteritidis* on total post-infection urinary NO_x excretion and weight of the MLN at day 7 after infection. Results are expressed as means ± SE (n=6). The control group received $1.8x10^9$ heat-killed bacteria.

DISCUSSION

Two recent in-vitro studies with rodent macrophages have shown a dose-dependent induction of nitrite production by various LPS samples. Bogle et al. [4] found a nearly linear relationship between the LPS concentration, which varied over a narrow range, and nitrite formation in the culture medium. Keller et al. [5] examined the effect of more diverse LPS concentrations and described a sigmoid-like relation between LPS and nitrite production. The results of our LPS experiment in-vivo partially resemble the results of the in-vitro study of Keller et al. [5]. Urinary NO_x excretion increased about fivefold in response to the dose of LPS injected from 0.10 to 0.50 mg/kg. LPS concentrations below 0.10 mg/kg failed to induce a NO_x response, whereas injection of 1 mg/kg compared with 0.50 mg/kg only slightly increased urinary NO_x excretion. Obviously, there is a threshold dose of LPS below which NO_x excretion does not respond. On the other hand, there also seems to be a maximum urinary NO_x excretion that LPS can induce the first day after administration.

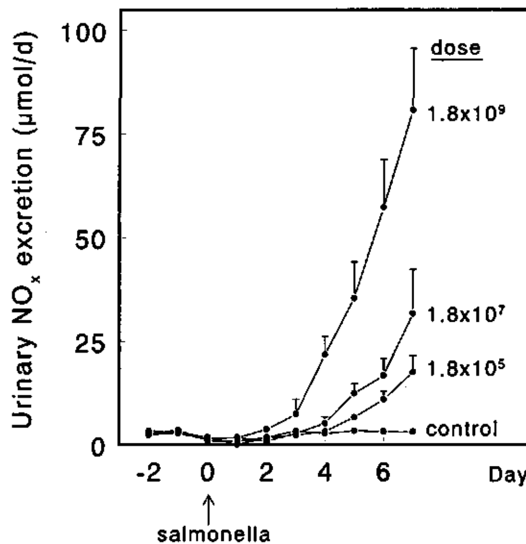

Figure 4 Urinary NO_x excretion of rats before and after oral challenge with different doses of viable *S. enteritidis*. Results ae expressed as means ± SE (n=6). The control group received $1.8x10^9$ heat-killed bacteria.

Intravenous administration of 3 mg/kg of the NO synthase inhibitor L-NAME merely prevented the increase in urinary NO_x excretion after LPS injection. This agrees with the results of several other studies. Jaeschke et al. [19] found that pretreatment of rats with nitro-L-arginine (10 mg/kg) inhibited LPS-induced urinary nitrate excretion by 97%. Billiar et al. [17] showed that N^G-monomethyl-L-arginine suppressed plasma nitrate levels in mice after LPS challenge. Studying the effect of L-NAME on urinary NO_x excetion in rats orally infected with invasive pathogens is in our opinion not very relevant, because L-NAME stimulates gastric and duodenal bicarbonate secretion [28] and increases intestinal epithelial permeability [29]. These effects promote the survival and translocation of invasive pathogens such as salmonella, thus precluding a straightforward interpretation of the effect of L-NAME on NO_x excretion. Therefore, Granger et al. [16] and Boockvar et al. [18] studied the inhibitory effect of N^G-monomethyl-L-arginine on urinary nitrate excretion of mice after intraperitoneal administration of *Mycobacterium bovis* and intravenous injection of *Listeria monocytogenes*, respectively. All these results indicate that the enhanced production and excretion of NO_x after LPS or live bacterial challenge is caused by an increased activity of NO synthase. In view of the observed differences in urinary NO_x excretion induced by different doses of LPS, the second infection experiment with orally administered viable *S. enteritidis* was performed. The population levels of salmonella in feces and MLN in the present study correspond with the observations in other infection studies [25, 30]. Weight of the MLN increased drastically with increasing challenge dose. This is probably caused by accumulation of increasing numbers of polymorphonuclear leukocytes and macrophages recruited from the blood [31, 32]. Despite this, relatively small differences in salmonella counts in the MLN were observed, especially when compared with the population levels of this pathogen in feces. This can be explained by the fact that the MLN do not inhibit the spread of *S. enteritidis* [31]. Rats infected with a higher dose of *S. enteritidis* could have had more severely infected livers or spleens [33]. Apparently, the severity of the infection is more accurately reflected by the observed enlargement of the MLN. Moreover, weight of the MLN was significantly correlated (r=0.85) with total urinary NO_x excretion after infection. Analogous to our results, Yamada et al. [12] reported significant correlations between colon and liver weights and plasma NO_x levels in mice after intramural injection of peptidoglycan-polysaccharide to initiate colonic inflammation.

Because of the invasive character of *S. enteritidis*, translocation from the gut lumen to the MLN takes place within 2 days. Increasing the challenge dose shortens the latency period necessary to infect spleen and liver [31, 34]. These literature data are remarkably consistent with our results of urinary NO_x excretion. As early as the second day after challenge with 1.8×10^9 *S. enteritidis*, a response in urinary NO_x excretion could be noticed. Decreasing the infective dose prolonged the time necessary to exceed basal urinary NO_x excretion. Regarding the inefficacy of heat-killed *S. enteritidis* and orally administered, live, enterotoxigenic *Escherichia coli* (data not shown) in stimulating urinary NO_x excretion, translocation is apparently a prerequisite for provoking a NO_x response. This makes urinary NO_x excetion a functional parameter for bacterial translocation. A major

advantage is that in contrast to tissue cultures, urinary NO_x excretion reflects the systemic pathogen load of the host. This makes urinary NO_x excretion a more accurate estimate of the severity of the infection.

Comparison of the urinary NO_x response to *S. enteritidis* LPS and viable *S. enteritidis* showed distinct kinetics. LPS induced a short-term, transient enhanced urinary NO_x excretion, whereas viable *S. enteritidis* resulted in a continuous increase in NO_x output. In contrast to LPS, the effect of viable pathogens on NO_x generation is determined by the resultant of bacterial multiplication and inactivation of the pathogen by the immune system. The exponentially increasing NO_x output suggests that multiplication of *S. enteritidis* exceeded killing of the pathogen during the first 7 days after infection . This suggestion is supported by the observations of Gregory et al. [11]. In mice surviving an intravenously administered dose of listeria, the increased serum NO_x levels declined to the uninfected control level. In mice injected with a lethal dose, the enhanced serum NO_x levels did not decrease before death occurred. Another striking difference between urinary NO_x excretion induced by LPS or viable bacteria concerns the extent of the NO_x response. Approximately 45 μmol/d is the maximum urinary NO_x excretion that *S. enteritidis* LPS can induce. In contrast, the results of the infection experiment with viable *S. enteritidis* do not indicate a maximum in response. At day 7 after infecction, urinary NO_x excretion amounted to 80 μmol in rats infected with 1.8×10^9 salmonella, and there were no signs that the exponential increase in NO_x output would cease. It is well known that the rate of formation of macrophages and other phagocytes from stem cells in the bone marrow is greatly increased during infection [32]. Therefore, it is possible that the observed continuous increase in urinary NO_x excretion in the experiment with viable *S. enteritidis* is caused by increasing numbers of circulating activated macrophages.

In this study, we used *S. enteritidis* as a model for invasive pathogens because human salmonellosis is one of the most common intestinal infections [35]. Application of urinary NO_x excretion as a parameter for bacterial translocation or systemic infections is certainly not restricted to salmonella infections only. The generation of NO is a nonspecific reaction of phagocytic cells to all kinds of microbial stimuli, such as a diversity of bacterial cell-wall fragments or intact bacteria, fungi, and protozoa [5, 6, 11-14, 16, 18].

Summarizing the present results, it can be concluded that a dose-dependent relationship exists between the degree of bacterial translocation and urinary NO_x excretion in-vivo. To our knowledge, this is the first study showing that urinary NO_x excretion can be used as a functional parameter to quantify the severity of a systemic infection and to follow its course. Regarding the rapid and easy determination of urinary NO_x, the present results suggests several applications in clinical studies. For instance, by monitoring the urinary NO_x to creatinine ratio (when a complete daily urine sample can not be obtained), sepsis can easily be recognized in patients in intensive care units who are likely to have bacterial translocation from the gut lumen into the systemic circulation [36, 37].

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Chapter 3

Calcium in milk and fermentation by yogurt bacteria increase the resistance of rats to salmonella infection

Ingeborg MJ Bovee-Oudenhoven, Denise SML Termont,
Pieter R Dekker & Roelof van der Meer

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ABSTRACT

Calcium in milk products stimulates gastric acid secretion and inhibits the cytolytic activity of intestinal contents. Based on these effects, it was hypothesized that calcium might lessen the severity of foodborne intestinal infections. The possible differential effects of a low-calcium milk and normal milk products (e.g. milk, acidified milk and pasteurized yogurt) on the resistance of rats to a salmonella infection was therefore studied. Rats were infected orally with *Salmonella enteritidis* just after food consumption. The first day after infection, fecal salmonella counts of yogurt-fed rats were significantly lower compared to the other groups. Thereafter, fecal salmonella excretion declined rapidly in all high-calcium groups, whereas rats fed low-calcium milk continued to excrete high numbers of salmonella. The reduced colonization resistance to salmonella of rats fed low-calcium milk might be caused by the high cytotoxicity of fecal water or the high iron concentration in fecal water already present before infection, or both. The reduced resistance of these rats corresponded with a large infection-induced increase in the cytotoxicity of fecal water, a marked reduction in apparent iron absorption and a large increase in fecal mucin and alkaline phosphatase excretion. In yogurt-fed rats, only minor infection-induced changes in luminal parameters were noticed. The rats fed milk and acidified milk always showed intermediate reactions. In conclusion, in addition to fermentation by yogurt bacteria, calcium in milk products strongly enhanced the resistance to salmonella infection by lowering luminal cytotoxicity or diminishing the availability of iron for pathogen growth, or both.

INTRODUCTION

The first defense mechanism of host resistance to gastrointestinal infections is the gastric barrier [1]. Pathogens, for instance salmonella species, introduced into the stomach are effectively destroyed by gastric acid [2-4]. The interaction of gastric acid and ingested bacteria is conditioned by other variables in addition to gastric acid secretion, namely physical protection of bacteria by food, buffering of gastric content and rate of gastric emptying. The effectiveness of the gastric barrier can be influenced by milk products. By stimulating gastrin release, calcium in milk products induces gastric acid secretion [5, 6]. In addition, the gastric emptying rate of fermented milk is half that of milk [7], so simultaneously ingested pathogens have to endure a prolonged exposure to gastric acid as well as to lactic acid in the fermented milk. Beyond the gastric barrier, a second line of non-immunological defense mechanisms is situated in the intestine. Antibacterial effects of bile acids and pancreatic enzymes, the motility of the intestine, normal epithelial cell turnover, the presence of an autochthonous microflora and intestinal mucin secretion, act together to eliminate harmful exogenous bacteria [1]. Many of these intestinal parameters are influenced by diet [8-11]. For instance, calcium in milk products inhibits the cell damaging activity of intestinal contents and decreases epithelial cell proliferation by precipitating bile acids among others [11-14].

Considering the above mentioned effects of calcium on gastric acid secretion and the intestinal epithelium, the question arises whether calcium in milk products increases resistance to foodborne infections. To address that question, we performed a strictly controlled infection experiment, to compare the resistance of rats to a salmonella infection when fed low-calcium

milk, regular milk, milk acidified with hydrochloric acid, or pasteurized yogurt. In addition, growth of salmonella in these milk products was studied in-vitro.

MATERIALS AND METHODS

In-vitro study: growth of salmonella in milk products

S. enteritidis (clinical isolate, phage type 1) was cultured and stored as described earlier [15]. *S. enteritidis* was precultured in Brain Heart Infusion broth (BHI; Difco, Detroit, MI, USA) for 1 day at 37 °C. Bacteria were collected by centrifugation (15 min at 5,000 g), washed once and resuspended in sterile saline to prepare a stock suspension containing approximately 10^9 bacteria/mL. Low-calcium milk (Calcinon, Nutricia, Zoetermeer, The Netherlands), milk (3.7% fat), milk acidified with (concentrated) hydrochloric acid to pH 4.2, and yogurt were inoculated with the stock suspension (final 1%), mixed, and subsequently incubated in a shaking waterbath at 37 °C. At t=0, 0.5, 1, 2, 3, and 6 h a small sample of the incubates was taken, serially diluted in saline, and plated on Brilliant Green Agar (BGA; Oxoid, Basingstoke, England). The plates were incubated 1 day at 37 °C.

Animals, diets and infection

The experimental protocol was approved by the animal welfare officer of the Agricultural University, Wageningen, The Netherlands. Specific pathogen-free male Wistar rats (WU, Harlan, Zeist, The Netherlands), 10 weeks old and with a mean body weight of about 330 g, were housed individually (n=9 per diet) in metabolic cages in a room with controlled temperature (22-24 °C), relative humidity (50-60%) and light/dark cycle (light, 6.00-18.00 h).

The experimental milk products were manufactured by the Technology department of our institute. Because rats are lactose-intolerant, the milk (3.7% fat, 3.0% protein, 30 mmol calcium per L) and low-calcium milk (Calcinon, Nutricia; 3.8% fat, 3.1% protein; $CaCO_3$ was added to obtain 6 mmol calcium per L) were first treated with lactase (Maxilact LX5000, Gist-brocades, Delft, The Netherlands). Sugar determination of the lactase-treated products by HPLC analysis [16] showed complete hydrolysis of lactose. Thereafter the low-calcium milk and part of the normal milk were sterilized (15 s at 140 °C) and bottled aseptically. Yogurt was prepared from the (lactase treated) regular milk by standard procedures, using cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. The yogurt was homogenized, pasteurized (15 s at 74 °C) and bottled aseptically. The milk products were stored at 4 °C until use. Acidified milk was prepared by acidifying (lactase-treated) regular milk with concentrated hydrochloric acid to pH 4.2, which was the pH of the yogurt. For animal food preparation 125 g of rice flour (Molenaar, Milupa B.V., Amersfoort, The Netherlands), 20 g cellulose, vitamins [17] and 0.1 mmol ferric citrate (BDH Chemicals Ltd, Poole, England) were added to 1 L of milk product. The dry weight percentage of these animal foods was 23.3%. Food was prepared freshly every 3 days and stored at 4 °C; the food racks of the animals were changed every day. Food and demineralized drinking water were supplied ad libitum. Food intake was recorded every day and body weight every 3 days.

Animals were acclimatized to the housing and dietary conditions for 9 days, after which they were infected with *S. enteritidis* (same strain as used in the in-vitro experiment), which is an increasingly important food pathogen [18]. Details of the kinetics of a salmonella

infection in rodents [3] and the properties and maintenance of this pathogen [15] are described elsewhere. Before infection, the rats were starved overnight. At 9.00 AM the next day, food was supplied ad libitum. At 11.00 AM all rats were infected orally by gastric gavage with 1 mL of saline containing 1.10^9 viable *S. enteritidis*. The exact viable counts of *S. enteritidis* in the inoculum were determined by plating on Brilliant Green Agar (Oxoid). Before, and on days 1, 5 and 12 after salmonella infection, fresh individual fecal samples were collected. The number of salmonella in feces was quantified by plating on Modified Brilliant Green Agar (Oxoid) supplemented with sulfamandelate (Oxoid) as described and validated by Giaffer et al. [19].

Total feces analyses

Feces were quantitatively collected 2 days before salmonella infection and on days 5 and 6 post-infection. Feces were freeze dried for dry weight determination. After dry ashing and destruction (15 minutes at 180 °C) with a perchloric acid (70%)/hydrogen peroxide (30%) mixture (3:1 vol/vol) of freeze dried feces, iron was measured using an atomic absorption spectrophotometer (Model 1100, Perkin Elmer Corp., Norwalk, USA). Inorganic phosphate was extracted from freeze dried feces with 5% (wt/vol) trichloroacetic acid and measured according to the method of Fiske and Subbarow [20]. Total bile acids were extracted from freeze-dried feces with a t-butanol/water mixture (1:1 vol/vol) as described previously [21]. Extracts were assayed for bile acids using a fluorimetric enzymatic assay [22]. For mucin determination, freeze dried feces was suspended in 20 volumes of phosphate-buffered saline, mixed, and immediately incubated in a shaking waterbath at 95 °C for 10 minutes to denature glycosidases. Control experiments showed no degradation of purified mucin (crude porcine stomach mucin, Sigma, St. Louis, USA) during this heat-treatment. Thereafter, mucins were solubilized by incubating for 90 min at 37 °C. After centrifugation for 1 min at 15,000 g (Eppendorf 5415), mucins were measured using a fluorimetric assay that discriminates O-linked glycoproteins (mucins) from N-linked glycoproteins [23]. Standard solutions of N-acetylgalactosamine (Sigma) were used to calculate the amount of oligosaccharides liberated from mucins during the procedure. Recovery of porcine stomach mucin added to dry feces varied between 95 and 105%. For alkaline phosphatase (ALP) determination, 30 mg of freeze dried feces was suspended in 5 mL saline. After homogenization, the suspensions were centrifuged for 5 min at 1,500 g (Heraeus, Boom B.V., Meppel, The Netherlands). ALP activity in the supernatants was determined spectrophotometrically as described elsewhere [13], except that inhibition with L-phenylalanine was omitted. In our earlier studies [11-14], inhibition of the intestinal isoenzyme with L-phenylalanine was used to quantify the contribution of bacterial ALP to total ALP activity. However, we now performed control experiments with feces from germfree and conventional rats, which showed that the remaining activity in the presence of L-phenylalanine was exactly the same for both feces (10% of total ALP activity, data not shown).

Fecal water analyses

Fecal water was prepared by adding double-distilled water to freeze dried feces to obtain 35% dry weight, which mimics the conditions in the colon. Individual deviations of the mean dry weight percentage were proportionally corrected for. After homogenizing, the samples were mixed regularly while incubated in a shaking waterbath (1 h at 37 °C), followed

by centrifugation for 20 min at 12,000 g (Hettich, Micro-rapid 1306, Tuttlingen, Germany). The aspirated supernatant was centrifuged for 2 min at 14,000 g (Eppendorf 5415). After determination of the pH of the supernatants at 37 °C, fecal water was stored at -20 °C until further use. For determination of cytotoxicity, human erythrocytes (20 μL of a 25% hematocrit suspension) were mixed with 80 μL of fecal water or 40 μL fecal water plus 40 μL saline. Before addition to the erythrocytes, the fecal waters were warmed to 37 °C. Mixtures were incubated for 2 h at 37 °C in a shaking waterbath. The intact erythrocytes in the pellet were washed three times with saline (centrifugation 1 min 1,500 g (Eppendorf 5415)). After lysing the erythrocytes in the pellet with double-distilled water, trichloroacetic acid (final 5% wt/vol) was added, followed by centrifugation for 1 min 14,000 g (Eppendorf 5415). The potassium content of the supernatant was measured by atomic emission spectrophotometry (Model 1100, Perkin Elmer). Simultaneously, erythrocytes were incubated in saline (0% hemolysis, corresponding to 100% potassium release) and in double-distilled water (100% hemolysis, corresponding to 0% potassium release). Percentage hemolysis was calculated from these 0% and 100% controls. The relevance and validation of this cytotoxicity assay is described elsewhere [24]. Iron was measured in saline-diluted fecal water, using an atomic absorption spectrophotometer (Model 1100, Perkin Elmer). Bile acid determination in samples taken before infection was performed directly in diluted fecal water as described elsewhere [22]. In contrast to pre-infection samples, the recovery of cholic acid added to post-infection samples was often poor, indicating that fecal water components interfered with the enzymatic assay. Therefore an alternative procedure was carried out as follows; post-infection samples were acidified (final 1 M HCl) and subsequently extracted three times with diethyl ether. After evaporation of diethyl ether, the residue was resolubilized in methanol. This solution was hydrolyzed in 80% (v/v) methanol and 1 M NaOH for 1 h at 80 °C. After evaporation of methanol and subsequent acidification with HCl (final 1 M), bile acids were again extracted with diethyl ether. After evaporation of diethyl ether, bile acids were resolubilized in methanol and measured as described above. Using this procedure, the recovery of a mixture of cholic acid/deoxycholic acid/lithocholic acid (4:3:1) added to fecal water always exceeded 90%. Control experiments showed that the bile acid concentration in fecal waters of the pre-infection period was identical when using the direct or the alternative procedure. Free fatty acids were analysed by gas chromatography as described earlier [14].

Statistics

Results are presented as means ± SE, with n=8 for the yogurt group and n=9 for the other groups. Within one dietary group, differences between the means of the pre-infection period and the post-infection period were tested by Student's *t* test for paired samples (two-sided). Within one period, differences between diet groups were tested on their significance, using analysis of variance (ANOVA). Additionally, Fisher's protected least significant difference test (two-sided), modified for multiple comparisons, was used to identify dietary groups that differed from each other. The level of significance was preset at $P<0.05$. A commercially available statistical package was used for all statistics (SPSS/PC+ v2.0, SPSS Inc. Chicago, USA).

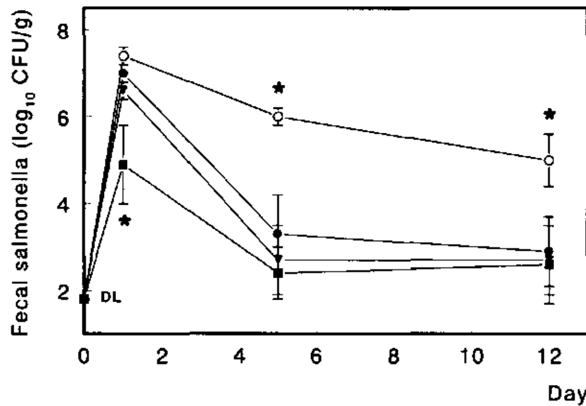

Figure 2 Fecal excretion of salmonella (means ± SE, n=8 or 9) after oral administration of 1.10^9 CFU of this pathogen on day 0. Symbols: ❍ low-calcium milk, ● milk, ▼ acidified milk and ■ yogurt. * Denotes that fecal salmonella excretion of the indicated group is significantly different from all other groups (P<0.05). Abbreviations: CFU colony-forming units, DL detection limit of the microbiological assay.

Colonization resistance

The first day after salmonella infection, rats fed yogurt had a significantly lower salmonella excretion in feces than rats fed the other diets (Figure 2). On day 5, population levels of salmonella in feces were decreased notably in the high-calcium diet groups. In contrast, rats fed the low-calcium milk continued to excrete high salmonella levels in their feces. This was also the case on day 12 post-infection. Thus, rats fed the low-calcium milk had a much lower colonization resistance to salmonella than rats fed the high-calcium diets.

Cytotoxicity of fecal water

The pH of fecal water of the yogurt group (6.54 ± 0.06) was higher (P<0.05) than values in the other groups (acidified milk 6.30 ± 0.03, milk 6.21 ± 0.01, and low-calcium milk 6.21 ± 0.05). Salmonella infection had no effect on the pH. Before infection, the cytotoxicity of fecal water from the high-calcium groups (milk, acidified milk and yogurt) did not differ from each other and varied between 10 and 22% (Figure 3). The cytotoxicity of fecal water of rats fed the low-calcium milk was 100% when the standard assay using 80 μL of fecal water was applied. To study whether salmonella infection increased the cytotoxicity any further in this group, erythrocytes were also incubated with a smaller volume (40 μL) of fecal water. After salmonella infection, the largest increase in cytotoxicity of fecal water occurred in the group fed the low-calcium milk. Rats fed regular milk showed a smaller, but significant, increase. Remarkably, salmonella infection had no significant effect on the cytotoxicity of fecal water of rats fed acidified milk or yogurt.

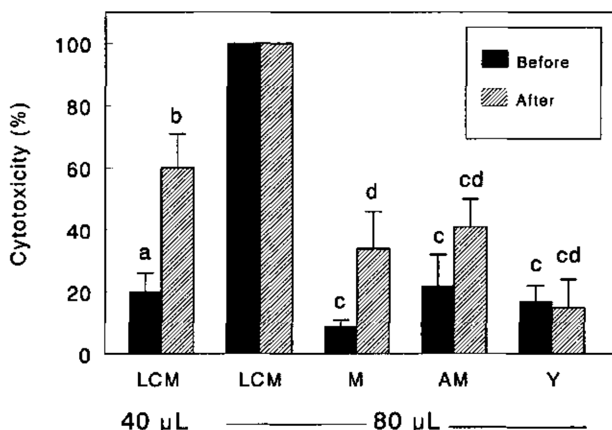

Figure 3 Cytotoxicity of fecal water (means ± SE, n=8 or 9) before and after infection. For all groups 80 μL of fecal water was used in the hemolyses assay. To detect the difference in cytotoxicity before and after infection in the low-calcium milk group, 40 μL of fecal water was also used in the assay for this group. Abbreviations: LCM low-calcium milk, M milk, AM acidified milk, Y yogurt. Bars not sharing the same letter are significantly different from each other ($P<0.05$).

Concentration of bile acids, fatty acids and iron in fecal water

Compared with the three high-calcium groups, the concentration of bile acids in fecal water of rats fed low-calcium milk was much higher (low-calcium milk 2.40 ± 0.17 mM, milk 0.77 ± 0.10 mM, acidified milk 1.09 ± 0.16 mM, yogurt 0.69 ± 0.05 mM ($P<0.05$)). Salmonella infection had no effect on the bile acid concentration in fecal water. Before infection, the mean free fatty acid concentration in fecal water was 0.46 ± 0.10 mM in the low-calcium milk group, 0.31 ± 0.03 mM in the milk group, 0.76 ± 0.16 mM in the acidified milk group, and 1.62 ± 0.52 mM in the yogurt group. The infection had no significant effect on the concentration of free fatty acids in fecal water. Despite an equal dietary iron intake of about 7.7 μmol/d, the iron concentration in fecal water of rats fed low-calcium milk was at least 5 times higher than that in fecal water of rats fed the high-calcium diets (Figure 4). Post-infection, soluble iron increased significantly only in the groups fed low-calcium milk and regular milk.

Apparent iron absorption and fecal excretion of mucin, ALP, and phosphate

The apparent iron absorption is calculated by subtracting the fecal iron excretion from the dietary iron intake. As mentioned above, dietary iron intake was about 7.7 μmol/d and was not affected by diet or by the infection. Before infection, all groups had an apparent iron absorption of about 2.7 μmol/d (Figure 5). After infection, the apparent iron absorption

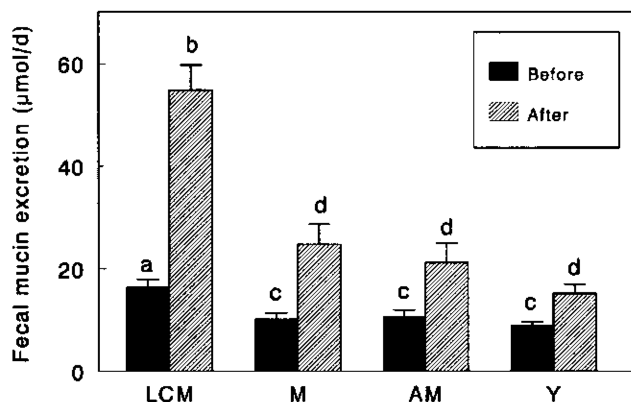

Figure 6 Daily mucin excretion in feces (means ± SE, n=8 or 9) before and after salmonella infection. Abbreviations: LCM low-calcium milk, M milk, AM acidified milk, and Y yogurt. Bars not sharing the same letter are significantly different from each other ($P<0.05$).

salmonella levels in feces of rats fed low-calcium milk corresponded with a significant reduction in the percentage dry weight of the feces in this group (Table 1).

Dietary calcium greatly reduced the cytotoxicity of fecal water in the pre-infection period (Figure 3). This agrees with previous work from our laboratory [11-14]. The high luminal cytotoxicity in the low-calcium milk group coincided with a reduced colonization resistance to salmonella. Considering these results, it is tempting to speculate that a high luminal cytolytic activity predisposes to salmonella infection. By lowering the cytolytic activity of luminal contents and reducing epithelial cell damage [11-14], the mucosal integrity and resistance to infection might be enhanced. In addition, we have now shown that salmonella infection has a striking effect on the cytotoxicity of fecal water. In accordance with high population levels of salmonella in feces of rats fed low-calcium milk, the cytotoxicity of fecal water of this group increased strongly after infection. No significant change could be noticed in rats fed acidified milk or yogurt. The concentrations of bile acids and fatty acids, important cytotoxic surfactants in fecal water [11-14], did not rise post-infection. This indicates that these surfactants were not responsible for the increase in cytotoxicity of fecal water after infection. Some salmonella strains seem to be able to produce enterotoxins, but none of these toxins has been shown to play a role in the virulence of this pathogen or the production of symptoms [25, 26]. The production of reactive oxygen metabolites or bactericidal proteinases by attracted inflammatory cells (like macrophages) may have contributed to the pathogenesis of infection. Investigations are now being carried out to identify the infection-related toxic substances in fecal water.

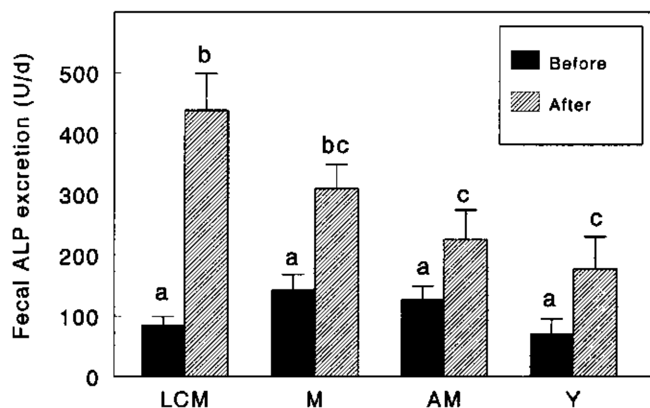

Figure 7 Fecal alkaline phosphatase (ALP) excretion (means ± SE, n=8 or 9) as a marker of intestinal epitheliolysis. Abbreviations: LCM low-calcium milk, M milk, AM acidified milk, and Y yogurt. Bars not sharing the same letter are significantly different from each other ($P<0.05$).

Soluble iron plays a critical role in inflammatory processes induced by bacterial infections. First, Fe^{2+}/Fe^{3+} is an important redox couple in the formation of reactive oxygen metabolites [27]. Second, the bactericidal action of lysosomal proteins of neutrophils is neutralized by excess iron [28]. Third, iron is an essential nutrient for bacterial growth [28]. To minimize epithelial cell damage and to maximize the resistance against pathogens, the soluble iron concentration in the intestinal lumen should be kept as low as possible. Our results showed that calcium in milk products strongly reduced the soluble iron concentration in fecal water (Figure 4). The phosphate content of the various diets was not different (mean 112 mmol/kg dry weight). However, in contrast to the high-calcium diets, the rats fed low-calcium milk almost completely absorbed the phosphate (Table 1) because hardly any precipitation with calcium could occur. These results suggest that luminal iron is coprecipitated by a calcium-phosphate complex. The relatively small, but significant increase in the iron concentration in fecal water after infection in the low-calcium milk and milk group might originate from iron in hemoglobin, due to salmonella-induced intestinal bleeding [29]. Because iron-withholding is a defense system of the host against bacterial infections [30], the apparent iron absorption, defined as dietary iron intake minus fecal iron excretion, was quantified. After salmonella infection, the apparent iron absorption had decreased towards zero in the low-calcium milk, milk and acidified milk group (Figure 5). Bezkorovainy also showed that iron absorption in the small intestine was decreased during infections [31]. Remarkably, in rats fed yogurt the infection had no significant effect on this parameter.

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Chapter 4

Dietary calcium inhibits the intestinal colonization and translocation of salmonella in rats

Ingeborg MJ Bovee-Oudenhoven, Denise SML Termont, Anton H Weerkamp,
Maria AW Faassen-Peters & Roelof van der Meer

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ABSTRACT

Dietary calcium decreases the cytotoxicity of intestinal contents and intestinal epitheliolysis by precipitating cytotoxic surfactants, like bile acids. A decreased luminal cytotoxicity might not only strengthen the barrier function of the gut mucosa, but also reinforce the protective, endogenous microflora. We hypothesized, therefore, that dietary calcium increases the resistance to intestinal infections. Rats on a low-, medium- or high-calcium purified diet were orally infected with a single dose of *Salmonella enteritidis*. The kinetics of fecal salmonella excretion was studied to determine the colonization resistance. Intestinal bacterial translocation was quantitated by measuring urinary NO_x excretion and culturing bacteria from tissues. Compared with the low-calcium group, the medium- and high-calcium fed rats had a substantially improved colonization resistance. Calcium supplementation also reduced translocation of salmonella, considering the diminished urinary NO_x excretion and viable salmonella counts in the ileal Peyer's patches and spleen. Dietary calcium decreased the bile acid concentration and cytotoxicity of fecal water. Several indicators of fecal bacterial mass were significantly increased by supplemental calcium. In conclusion, dietary calcium improves the colonization resistance and reduces the severity of gut-derived systemic infections, which is probably attributable to its luminal cytoprotective effects.

INTRODUCTION

Gastrointestinal bacterial infections are still a formidable health problem, mostly because of the increase in antibiotic resistance [1]. Of particular concern is the increased incidence of systemic infections [1]. Gut-derived septicemia occurs regularly in people receiving immunosuppressive drugs, patients suffering from inflammatory bowel diseases and patients in intensive care units on (par)enteral nutrition regimen [2, 3]. Non-typhoidal salmonellosis is one of the most common, foodborne, bacterial infections in the USA and Europe [4]. Most patients experience a self-limiting gastroenteritis, but 2-5% develop bacteremia and sepsis-associated complications [5]. Antibiotic treatment of gastroenteritis caused by salmonella species has been discouraging, because it does not reduce the duration or severity of illness and may prolong asymptomatic carriage [6]. Combined with the growing resistance of salmonella species to clinically important antibiotics [1], the prevention of salmonellosis becomes more important.

Many studies in the field of colon carcinogenesis have shown that supplemental dietary calcium precipitates intestinal cytotoxic surfactants and thus inhibits epithelial cell damage and epithelial proliferation, which may decrease the risk of colon cancer [7, 8]. It can be speculated that these cytoprotective effects of dietary calcium on the intestinal epithelium are not only relevant to colon carcinogenesis, but also have major implications for the resistance to intestinal infections. Diminishing epitheliolysis, by decreasing the cytotoxicity of gut contents, might strengthen the mucosal integrity. Conversely, increased epithelial cell damage provoked by administration of lectins [9], chemical inflammatory irritants [10] or ischemia/reperfusion [11] leads to disruption of the physical barrier function of the intestinal mucosa and translocation of potential pathogens to extra-intestinal organs. The calcium-dependent reduction in cytotoxicity of intestinal contents may not only protect the enterocytes,

but also favor growth of the endogenous microflora, thereby improving their antagonistic action towards (potential) pathogens. Recently, we showed that rats on a low-calcium milk diet had a significantly impaired colonization resistance to *S. enteritidis* compared with rats on high-calcium diets containing regular milk, acidified milk or yogurt [12].

In view of the above-mentioned protection of the intestinal epithelium by calcium-dependent precipitation of cytotoxic bile acids and the possible beneficial effects of calcium on the intestinal microflora, we performed strictly controlled infection experiments. After adaptation to purified diets differing only in calcium content, the resistance of rats to *S. enteritidis* was studied. The main purpose of the present experiments was to test the hypothesis that dietary calcium increases the colonization resistance to *S. enteritidis* and reduces translocation of this invasive pathogen to the systemic circulation.

MATERIALS AND METHODS

Animals, diets and infection

The experimental protocol was approved by the animal welfare officer of the Agricultural University, Wageningen, The Netherlands. Specific pathogen-free male Wistar rats (WU, Harlan, Zeist, The Netherlands), 9 weeks old and with a mean body weight of about 290 g, were housed individually in metabolic cages in a room with controlled temperature (22-24 °C), relative humidity (50-60%) and light/dark cycle (lights on from 6 AM to 6 PM). During the experimental period of almost 4 weeks, 3 groups of 8 rats each were fed a purified diet that differed only in $CaHPO_4$ content. The basal diet contained (per kg) 200 g acid casein, 505 g dextrose, 100 g palm oil, 100 g corn oil, 50 g cellulose and 35 g mineral mix (without calcium) and 10 g vitamin mix as previously described [13]. Varying amounts of $CaHPO_4.2H_2O$ (20, 60 and 180 mmol/kg; Merck, Darmstadt, Germany) were added to the basal diet in exchange for dextrose. After preparation, samples of the diets were dry ashed for mineral analyses. Calcium was measured using an atomic aborption spectrophotometer (Model 1100, Perkin Elmer Corp., Norwalk, CT, USA). The phosphate content of the diets was determined spectrophotometrically according to the method of Fiske and Subbarow [14]. The diets were administered to the animals as a porridge (demineralized water was added and mixed with the dry diets to obtain a dry weight of 68%). Food and demineralized drinking water were supplied ad libitum. Food intake was recorded every day and body weight every 3 days.

Animals were acclimatized to the housing and dietary conditions for 11 days, after which they were orally infected with *S. enteritidis* (clinical isolate, phage type 1). In the morning, all rats were infected orally by gastric gavage with 0.5 mL of saline containing 3% (wt/vol) sodium bicarbonate with 5.10^8 viable *S. enteritidis*. The exact viable count of *S. enteritidis* in the inoculum was determined by plating on Brilliant Green Agar (Oxoid, Basingstoke, England). Before, and on days 1, 3, 6 and 9 after salmonella infection, fresh fecal samples were collected directly from the anus of the animals by gently massaging the abdomen. The number of salmonella in feces was quantified by plating on Modified Brilliant Green Agar (Oxoid), with a bacteriological technique as described elsewhere [15]. Sulfamandelate (Oxoid) was added to the agar plates to suppress swarming bacteria, like proteus. The detection limit of the method used to quantify salmonella in feces was 10^2 colony-forming units/g feces. Feces were also collected 3 days before salmonella infection

and on days 3,4 and 5 after infection for biochemical analyses. Complete 24 h urine samples were collected for 2 successive weeks, starting 1 day before infection.

In a second infection study with rats, the effects of the low- and high-calcium diet on the initial translocation of *S. enteritidis* to the ileal Peyer's patches, mesenteric lymph nodes (MLN), and spleen were quantified by microbiological plating techniques at day 2 after oral infection. In addition, weight and sterility of these organs were also determined in non-infected animals consuming these diets. Characteristics of the rats, housing conditions, composition of the diets, and the administration and dose of salmonella were as described above. At the section day, the rats were killed by carbon dioxide inhalation. First, the spleen and all MLN were excised aseptically, weighed, homogenized (Ultraturrax Pro200, Pro Scientific Inc. Monroe, CT, USA) in sterile saline, 10-fold diluted and plated on Brilliant Green Agar (Oxoid) as previously described [16]. Second, the ileum (defined as the last 12 cm of the small intestine proximal to the cecum) was pulled out and freed of contents by flushing with 5 mL of sterile saline. Thereafter, all Peyer's patches of this intestinal segment were excised, weighed, homogenized (Ultraturrax Pro200) in sterile saline, serially diluted and plated on Modified Brilliant Green Agar (Oxoid) containing sulfamandelate (Oxoid). Viable salmonella counts were expressed as the total $\log_{10}$ colony-forming units in each organ.

In a third experiment, the effect of dietary calcium on the capacity of macrophages and other phagocytic cells to produce oxidation products of nitric oxide (NO_x) on stimulation was evaluated. Two groups of 6 rats each were intraperitoneally injected with *S. enteritidis* lipopolysaccharides (lyophilized phenol extract, 1 mg/kg dissolved in 1 mL sterile saline; Sigma Chemical Co., St. Louis, MO, USA) after adaptation to either the low- or high-calcium diet (characteristics of the rats, housing conditions and the composition of the diets as described above). Urinary NO_x excretion was determined in 24 h urine samples collected on successive days before and after the injection of lipopolysaccharides.

Total feces analyses

Feces were freeze dried for dry weight determination. For total nitrogen determination, feces were digested with a mixture of sulfuric acid, hydrogen peroxide, potassium sulfate and mercuric oxide, followed by colorimetric determination of the ammonia formed [17]. After acidification of freeze dried feces (1:50 wt/vol, final concentration 1 M HCl), phospholipids were extracted from feces according to the procedure described by Bligh and Dyer [18]. Briefly, a miscible system is obtained by adding a freshly prepared mixture of chloroform/methanol (1:2 vol/vol) to acidified feces (3.75:1 vol/vol). After mixing, the suspension was centrifuged for 5 minutes at 2,500 g (Heraeus, Sepatech GmbH, Osterode, Germany) to precipitate nonsoluble material. Chloroform and double-distilled water were added to the clear supernatant to obtain a final chloroform/methanol/watermixture of 10:10:9 vol/vol/vol. Samples were again thorougly mixed and subsequently centrifuged for 5 minutes at 2,500 g (Heraeus, Sepatech GmbH, Osterode, Germany). The chloroform layer was carefully removed. After evaporation of chloroform, the residue was destructed (15 minutes at 180 °C) with a perchloric acid (70%)/hydrogen peroxide (30%) mixture (5:1 vol/vol, total volume 120 μL). Liberated phosphate was determined with a sensitive, colorimetric assay [19]. Using this procedure, recoveries of phosphatidylcholine and lysophosphatidylcholine (egg yolk) were always >85%. After dry ashing and destruction (15 minutes at 180 °C) with a perchloric acid (70%)/hydrogen peroxide (30%) mixture (3:1 vol/vol, total volume 600 μL) of freeze dried feces, iron was measured using an atomic absorption spectrophotometer

(Model 1100, Perkin Elmer). In the same dry ashed and destructed samples, calcium and total phosphate were measured as described for the diets. Inorganic phosphate was extracted from freeze-dried feces with 5% (wt/vol) trichloroacetic acid and measured as described above. The amount of organic phosphate in feces was calculated by subtracting the inorganic phosphate concentration from the total phosphate concentration.

Fecal water analyses

Fecal water was prepared by reconstituting freeze dried samples with demineralized water to 30% dry weight, which reflects the dry weight percentage of colonic contents [20]. After homogenizing, the samples were incubated for 1 h at 37 °C in a shaking waterbath, followed by centrifugation for 20 minutes at 12,000 g (Hettich, Micro-rapid 1306, Tuttlingen, Germany). The aspirated supernatant was centrifuged for 2 minutes at 14,000 g (Eppendorf 5415). After determination of the pH of the supernatants at 37 °C, fecal water was stored at -20 °C until further use. Cytotoxicity of fecal water was determined using a human erythrocyte suspension as described previously [12]. Briefly, fecal water was added to washed human erythrocytes (final hematocrit 5%) and incubated for 2 h at 37 °C. Remaining intact erythrocytes were pelleted by centrifugation (1 minute at 1,500 g; Eppendorf 5415), washed, acidified with trichloroacetic acid (final 5% wt/vol) and subsequently centrifuged (1 minute at 10,000 g). Their potassium content was measured in the supernatant by atomic emission spectrophotometry (Perkin Elmer 1100). Simultaneously, erythrocytes were incubated in saline (0% potassium release) and in double-distilled water (100% potassium release). The cytotoxicity of fecal water was calculated and expressed as a percentage of maximal lysis. Bile acids in fecal water were determined using a fluorimetric enzymatic assay [21]. Iron was measured in demineralized water-diluted fecal water as described for total feces.

Analysis of NO_x in urine

The NO_x (nitrite and nitrate) concentration in urine was determined by automated flow injection analysis. Briefly, diluted urine is passed over a cadmium column that reduces nitrate to nitrite, followed by reaction of nitrite with Griess reagent [22]. The colored product was measured spectrophotometrically at 538 nm. Recovery of nitrate or nitrite added to rat urines always exceeded 96%. Urinary NO_x concentration was multiplied by the urine volume produced in 24 h to obtain total urinary NO_x excretion per day.

Statistics

Results are presented as means ± SE (n=6 or 8). Differences between the means of the pre-infection period and the post-infection period in the first study were tested by Student's *t* test for paired samples (two-sided). Within one period, differences between the three calcium groups were tested by analysis of variance, followed by Fisher's least significant difference test (one-sided). Differences between the low- and high-calcium groups in the other two experiments were tested by Student's *t* test (one-sided). Differences were regarded as significant if $P<0.05$. A commercial package was used for all statistics (SPSS/PC+ v2.0, SPSS Inc. Chicago, USA).

RESULTS

Animals, food intake, and fecal output

At the start of the experiment, mean body weight of the animals was 293 g. Food intake (mean 16 g dry wt/d) and body weight gain (mean 3 g/d) were not significantly affected by the different diets or the infection. Dietary calcium increased wet and dry daily fecal output in a dose-dependent manner (Table 1; $P<0.05$). After the infection there were no obvious signs of diarrhea. The daily output of wet and dry feces did not change significantly. Considering the unchanged food intake and body weight gain and absence of diarrhea, one may say that a relatively mild infection was inflicted on the animals.

Effect of dietary calcium on fecal excretion and intestinal translocation of salmonella

The first day after salmonella infection, rats fed the high-calcium diet had a significantly lower salmonella excretion in feces than rats fed the low- or medium-calcium diets (Figure 1; $P<0.05$). Already on day 3 after infection, the population levels of salmonella in feces had already decreased markedly in rats fed the medium- or high-calcium diets. In contrast, rats fed the low-calcium diet had a much lower colonization resistance: they continued to excrete high numbers of salmonella until day 6 after infection.

The impaired colonization resistance of rats fed the low-calcium diet coincided with a progressive increase in urinary NO_x excretion, reaching its maximum at day 6 after infection (Figure 2; $P<0.05$). However, rats fed the medium- and high-calcium diets had a significantly smaller infection-induced increase in urinary NO_x excretion. The medium- and high-calcium groups showed similar kinetics of urinary NO_x excretion. The inhibiting effect of dietary calcium on salmonella translocation as quantified by the reduced NO_x output in urine, was supported by the results of the tissue cultures in the second infection experiment.

Table 1 Effect of dietary calcium on fecal output, mineral, organic phosphate, phospholipid, and nitrogen excretion in feces before infection of the rats.

Fecal excretion	low-calcium	medium-calcium	high-calcium
Wet weight (g/d)	1.58 ± 0.12^a	1.92 ± 0.07^b	2.82 ± 0.11^c
Dry weight (g/d)	0.99 ± 0.06^a	1.20 ± 0.04^b	1.74 ± 0.07^c
Ca (μmol/d)	88 ± 8^a	493 ± 18^b	2442 ± 89^c
P_i (μmol/d)	55 ± 4^a	186 ± 10^b	1256 ± 55^c
Fe (μmol/d)	5.63 ± 0.35^a	6.43 ± 0.22^b	9.04 ± 0.63^c
P_o (μmol/d)	37 ± 7^a	58 ± 6^b	247 ± 59^c
PL (μmol/d)	3.48 ± 0.24^a	4.18 ± 0.19^b	6.59 ± 0.40^c
N (mmol/d)	1.08 ± 0.11^a	1.26 ± 0.10^a	2.03 ± 0.04^b

Results are expressed as means ± SE (n=8). Values in the same row not sharing the same superscript are significantly different ($P<0.05$). Abbreviations: Ca calcium, P_i inorganic phosphate, Fe iron, P_o organic phosphate, PL phospholipids, and N nitrogen.

Compared with the low-calcium group, the high-calcium group had significantly lower viable salmonella counts in the ileal Peyer's patches and spleen (Table 2). Equally important, only 4 of 8 animals fed the high-calcium diet had viable salmonella in their spleen, whereas the spleen of all animals fed the low-calcium diet was infected ($P < 0.05$). The mean viable counts in spleen of the high-calcium group was calculated using the detection limit (150 colony-forming units) for the four negative samples. The infection-induced increase in weight of the MLN was significantly less in the high-calcium group than in the low-calcium group. The high-calcium group also tended to have lower viable salmonella counts in the MLN, although this did not reach significance (Table 2).

Dietary calcium did not affect the capacity of the inducible NO_x-generating system to respond to bacterial stimuli. No significant difference in urinary NO_x exretion could be observed when rats were intraperitoneally injected with *S. enteritidis* lipopolysaccharides (Figure 3).

Effect of dietary calcium on mineral, organic phosphate, phospholipid and nitrogen excretion in feces

As expected, fecal calcium excretion increased with dietary calcium intake (Table 1, $P < 0.05$).Calcium supplementation also increased fecal iron excretion (Table 1, $P < 0.05$). This resulted in a significantly decreased apparent iron absorption in the high-calcium group

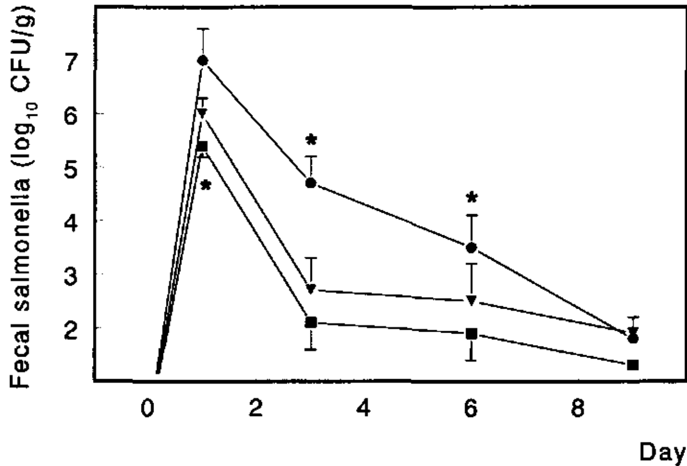

Figure 1 Effect of a low- (● 20 mmol/kg), medium- (▼ 60 mmol/kg), and high-calcium (■ 180 mmol/kg) diet on fecal salmonella excretion in rats orally infected with 5.10^8 viable *S. enteritidis* on day 0. Results are expressed as means ± SE (n=8). The asterisk denotes that the indicated group is significantly different from the other two groups ($P < 0.05$). SEs are either smaller than symbols or indicated by bars. Abbreviation: CFU colony-forming units.

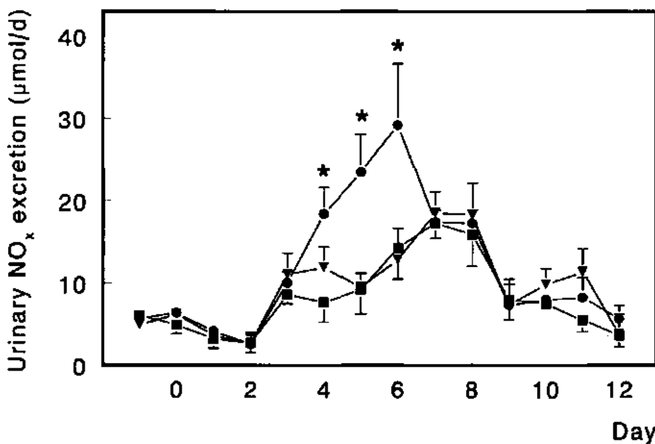

Figure 2 Effect of a low- (● 20 mmol/kg), medium- (▼ 60 mmol/kg), and high-calcium (■ 180 mmol/kg) diet on daily urinary NO_x excretion of rats before and after on oral challenge with 5.10^8 *S. enteritidis* on day 0. Results are expressed as means ± SE (n=8). The asterisk denotes that NO_x excretion of the low-calcium group is significantly different from the other two groups ($P<0.05$).

($P<0.05$), calculated by subtracting fecal iron excretion from dietary iron intake. Mean apparent iron absorption of the low-calcium, medium-calcium and high-calcium groups was 3.45 ± 0.23, 3.21 ± 0.23, and 1.81 ± 0.53 µmol/d, respectively. Fecal inorganic phosphate excretion increased with dietary calcium supplementation, because of the formation of insoluble calcium phosphate (Table 1, $P<0.05$). Dietary calcium also stimulated the excretion of organic phosphate, phospholipids and nitrogen in feces (Table 1, $P<0.05$).

Effect of dietary calcium on the composition and cytotoxicity of fecal water

Dietary calcium increased the pH of fecal water in a dose-dependent manner: the pH before infection was 6.7, 6.8, and 7.0 for the low-, medium-, and high-calcium group, respectively ($P<0.05$). After infection, the pH of fecal water of the low-calcium group was significantly increased to 7.3 ($P<0.05$), whereas no significant infection-induced change in pH was found in the other two groups. An inverse dose-dependent relationship existed between dietary calcium and the concentration of bile acids in fecal water (Figure 4A). The infection had no significant effect on this parameter. The fatty acid concentration in fecal water (mean 1.55 mM) was not affected by dietary calcium or the infection (data not shown). Calcium supplementation decreased the solubility of iron in feces. Animals fed the low-calcium diet had a significantly higher iron concentration in fecal water than their medium- and high-calcium-fed counterparts (Figure 4B, $P<0.05$). With the exception of the high-calcium group, a small but significant increase of the iron concentration was observed after infection. The cytotoxicity assay showed that the cytotoxic activity of fecal water was dose-

Table 2 Effect of dietary calcium and oral *S. enteritidis* infection on weight and viable salmonella counts of the ileal Peyer's Patches, MLN, and spleen of rats.

	Non-infected		2 Days after infection	
Organ	Low-calcium	High-calcium	Low-calcium	High-calcium
Peyer's patches				
Weight (mg)	38 ± 3[a]	44 ± 5[a]	70 ± 9[b]	59 ± 7[b]
Salmonella (log_{10})	n.d.	n.d.	4.93 ± 0.09[a]	4.31 ± 0.32[b]
MLN				
Weight (mg)	110 ± 10[a]	110 ± 10[a]	160 ± 10[b]	120 ± 10[a]
Salmonella (log_{10})	n.d.	n.d.	4.49 ± 0.08[a]	4.24 ± 0.26[a]
Spleen				
Weight (mg)	640 ± 40[a]	620 ± 40[a]	670 ± 20[a]	680 ± 20[a]
Salmonella (log_{10})	n.d.	n.d.	2.72 ± 0.10[a]	2.38 ± 0.11[b]

Results are expressed as means ± SE (n=8). Values in the same row not sharing the same superscript are significantly different ($P < 0.05$). Abbreviation: n.d. not detected.

dependently decreased by dietary calcium (Figure 5). Erythrocytes incubated with 80 µL of fecal water of the low-calcium group were lysed completely. Therefore, we also tested 40 µL of fecal water of this group, to reveal a possible difference in lytic activity between the pre- and post-infection period. However, no significant infection-induced difference could be detected in any group.

DISCUSSION

The present study clearly demonstrates that dietary calcium is very effective in reducing the severity of a salmonella infection. An important parameter in studies of the resistance to gastrointestinal infections is the colonization resistance [23]. The results of the fecal excretion of salmonella show that the colonization resistance to this pathogen was markedly impaired when the animals were fed the low-calcium diet (Figure 1). In contrast, rats fed the medium- and high-calcium diets had a significantly better colonization resistance as indicated by a rapid decline in fecal salmonella excretion. From these results it can be stated that dietary calcium inhibits colonization and thus shortens fecal shedding of the pathogen, which is supported by our study using various whole-milk products [12]. Probably even more important than reducing the duration of carriage are the significant inhibitory effects of calcium supplementation on the translocation of salmonella across the gut wall, as measured by the lower urinary NO_x excretion (Figure 2) and by the reduction in viable

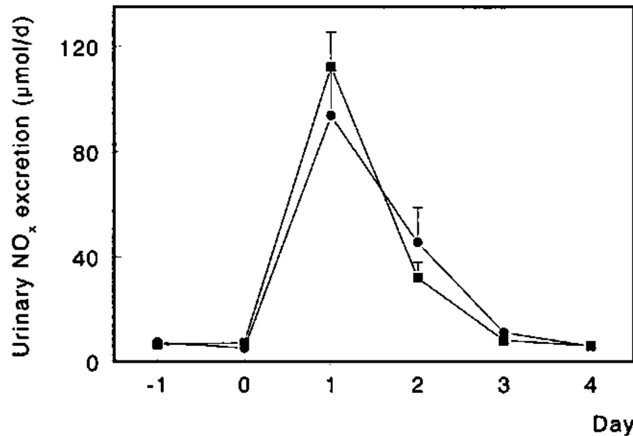

Figure 3 Effect of a low- (● 20 mmol/kg) and high-calcium (■ 180 mmol/kg) diet on urinary NO_x excretion of rats before and after an intraperitoneal injection with *S. enteritidis* lipopolysaccharides(1 mg/kg) on day 0. Results are expressed as means ± SE (n=6). SEs are either smaller than symbols or indicated by bars.

salmonella counts in the ileal Peyer's patches and spleen (Table 2). The infection-induced increase in MLN weight was also significanlty less in the calcium-supplemented group and viable salmonella counts tended to be lower. We have shown previously that, in contrast to classical tissue culture techniques, urinary excretion of NO_x is a more sensitive and quantitative biomarker for the severity of systemic infections [16]. Another advantage is that this method makes it possible to follow non-invasively the time course of the infection. Gianotti et al. [24] showed that organ cultures notably underestimate total bacterial translocation, mostly because this method only counts the still viable bacteria but not the bacteria already killed after invasion. In their experiments less than 0.01% of the total bacteria translocated to the mesenteric lymph nodes was still viable. In agreement, the results of our earlier study showed that large differences in oral salmonella dose (100-fold) do not give distinct counts of viable salmonella in the MLN, whereas highly significant differences in urinary NO_x excretion were observed [16]. However, despite the limitations of tissue cultures, in the present study the results obtained with this classical technique merely agree with the results obtained with our new NO_x method to quantitate the total bacterial translocation. Translocation of salmonella species from the gut lumen to the systemic circulation starts by penetration of the ileal Peyer's patches [25]. The subsequent infection of the mesenteric lymph nodes, liver and spleen takes about 2 days [26], which remarkably correlates with the onset of the rise in daily NO_x excretion. The reduced urinary NO_x output in the calcium-supplemented groups is due to an inhibitory effect of calcium on intestinal translocation processes because dietary calcium did not affect urinary NO_x excretion after intraperitoneal

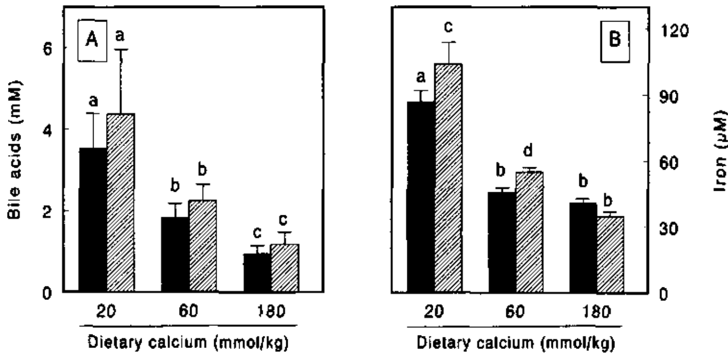

Figure 4 Effect of dietary calcium on the concentration of (A) bile acids and (B) iron in fecal water before (filled bars) and after (striped bars) oral infection with 5.10^8 *S. enteritidis*. Results are expressed as means ± SE (n=8). Bars not sharing the same letter are significantly different (P < 0.05).

injection of rats with lipopolysaccharides of *S. enteritidis* (Figure 3). As discussed earlier [16], the kinetics of urinary NO_x excretion after injection of lipopolysaccharides (Figure 3) is much faster than that observed after an oral infection (Figure 2).

For now, we can only speculate about the mechanism by which calcium exerts its protective effect against gastrointestinal infections. Dietary calcium induces gastric acid secretion, by stimulating gastrin release [27]. This might explain the significantly reduced fecal salmonella excretion of rats fed the high-calcium diet the first day after oral infection (Figure 2). In the intestine, dietary calcium exerts cytoprotective effects on epithelial cells. Earlier studies showed that in the upper small intestine calcium forms an insoluble complex with phosphate [20, 28]. Cytotoxic components in the intestinal lumen, such as bile acids and fatty acids, are strongly precipitated by amorphous calcium phosphate, thereby decreasing the cell-damaging potential and reducing epitheliolysis [29, 30]. Comparable results were obtained in the present study. Calcium decreased the cytotoxicity of fecal water (Figure 5), probably by reducing the bile acid concentration in fecal water (Figure 4A). By lowering the cytotoxicity of intestinal contents and reducing epithelial cell damage, the mucosal integrity and resistance to infection might have been enhanced by supplemental calcium.

The protective effects of calcium are probably not limited to the intestinal mucosa, but also have major consequences for the intestinal microflora. In that context, it is remarkable that dietary calcium phosphate stimulated the fecal excretion of organic phosphate, phospholipids, and nitrogen (Table 1). A similar effect of dietary calcium on the fecal output of organic phosphate has also been observed in humans [28]. Fecal nitrogen excretion is regarded as a marker of bacterial mass, despite the presence of endogenous and non-absorbed dietary nitrogen. Gestel et al. [31] showed that both fecal nitrogen and fecal output of

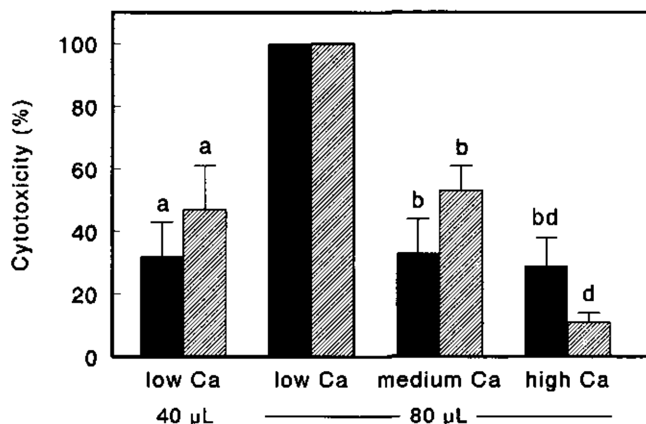

Figure 5 Effect of a low- (20 mmol/kg), medium- (60 mmol/kg), and high-calcium (180 mmol/kg) diet on the cytotoxicity of fecal water before (filled bars) and after (striped bars) oral infection with 5.10^8 *S. enteritidis*. For all groups, 80 µL of fecal water was used in the hemolysis assay. To detect a possible difference in cytotoxicity between the pre-infection and post-infection periods in the low-calcium group, 40 µL of fecal water was also used in the assay for this group. Results are expressed as means ± SE (n=8). Bars not sharing the same letter are significantly different ($P<0.05$).

diaminopimelic acid, another indicator of bacterial mass, increased when rats were fed diets containing fermentable fiber. In the present study fecal phospholipid and nitrogen excretion were highly correlated ($r=0.83$, $p<0.05$). Although these parameters also significantly correlated with fecal organic phosphate excretion, the calcium-induced increase in organic phosphate output notably exceeded the calcium-stimulated fecal excretion of phospholipids and nitrogen (Table 1). Many bacteria can accumulate excess phosphate in the form of polyphosphate granules, which are not hydrolyzed by 5% trichloroacetic acid treatment [32]. Thus, the more than proportional increase in organic phosphate probably reflects bacterial synthesis of polyphosphate. Taken together, these effects on fecal excretion of organic phosphate, phospholipids and nitrogen indicate that dietary calcium phosphate increases the fecal excretion of bacteria. Stimulation of bacterial growth may be of relevance because the endogenous intestinal microflora is an extremely important determinant of host defense mechanism [23, 33]. For instance, selective elimination of (facultative) anaerobic bacterial groups by treatment with antibiotics markedly impairs the colonization resistance and enables intestinal potential pathogens to reach extra-intestinal organs [34, 35]. An optimal resident flora adequately prevents pathogens colonizing by producing antimicrobial substances, like short-chain fatty acids, and by competing for nutrients and adhesion sites on the intestinal epithelium [33]. So, it is conceivable that an increase in the number of gut bacteria results in an enhanced resistance to salmonella. As mentioned above, the reduced cytotoxicity of

intestinal contents in the medium- and high-calcium supplemented groups (Figure 5) might not only protect the enterocytes, but also surround the endogenous flora with a less agressive environment. The proposed trophic effect of dietary calcium phosphate on the endogenous microflora and its relevance for the resistance to salmonella will be investigated in future experiments.

From in-vitro studies it is known that divalent cations, like calcium and magnesium, play a regulatory role in the expression of virulence genes of salmonella [36]. For instance, low concentrations of extracellular calcium induces salmonella to express the *pag* genes, which may increase its survival in the phagosomes of macrophages, as calcium is $< 50\,\mu M$ in this intracellular compartment. Vescovi et al. [36] also showed that these virulence genes are repressed when salmonella is present in extracellular fluids (i.e. at calcium levels of > 1 mM). In fact, several studies have shown that the concentration of calcium in the intestinal lumen is even substantially higher [20, 29, 37] and supplemental dietary calcium does not influence the systemic calcium concentration [38]. Therefore, the observed impaired resistance of rats on a low-calcium diet cannot be attributed to an increased expression of virulence genes of salmonella. Furthermore, we have shown earlier that growth of the salmonella strain used in the present infection studies did not depend on the environmental calcium concentration [12].

Soluble iron is an essential nutrient for growth of most bacteria, except some lactobacilli [39]. Considering that about 1 μM of available iron is already adequate to maintain maximal bacterial growth [39], it seems unlikely that the calcium-induced reduction in luminal iron solubility (Figure 4B) improved the resistance to infection by inhibiting pathogen growth in the intestine. On the other hand, in situations of systemic iron overload the susceptibility for infections, including salmonellose, is increased [40, 41]. Furthermore, iron is a catalyst in the generation of reactive oxygen species and plays a critical role in inflammatory processes, for instance induced by bacterial infections [42]. For these reasons, coprecipitation of iron by calcium phosphate in the intestinal lumen can be helpful to minimize epithelial cell damage and to maximize the resistance against pathogens.

In conclusion, in strictly controlled studies with rats we have shown for the first time that dietary calcium not only greatly improves the colonization resistance to *S. enteritidis*, but also reduces translocation of this pathogen across the intestine to the systemic circulation. Lowering the cytotoxicity of intestinal contents, thereby reducing the damage inflicted to epithelial cells as well as to the resident microflora, and/or precipitating iron might be important mechanisms by which calcium exerts its protective effect. An adequate supply of dietary calcium seems to be effective in reducing the severity of gastrointestinal infections. Especially for immunocompromised patients and patients in intensive care units receiving elemental liquid diets, who are particularly vulnerable to contract gut-derived bacterial infections [43], this may be of significant importance.

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Chapter 5

Increasing the intestinal resistance of rats to the invasive pathogen *Salmonella enteritidis*: additive effects of dietary lactulose and calcium

Ingeborg MJ Bovee-Oudenhoven, Denise SML Termont,
Peter J Heidt & Roelof van der Meer

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ABSTRACT

Lactulose fermentation by the intestinal microflora acidifies the gut contents, resulting in an increased colonization resistance to acid-sensitive pathogens. The extent of fermentation should be controlled to prevent acid-induced epithelial cell damage. Considering the buffering capacity of calcium phosphate and its intestinal cytoprotective effects, we studied whether supplemental calcium phosphate adds to the increased resistance to intestinal infections by lactulose fermentation. In a strictly controlled experiment, rats were fed a purified low-calcium control diet, a low-calcium/lactulose diet or a high-calcium/lactulose diet, and subsequently infected orally with *Salmonella enteritidis*. Lactulose fermentation lowered the pH and increased the lactic acid concentration of the intestinal contents, which significantly reduced excretion of this pathogen in feces; thus it improved the colonization resistance. This agreed with the high sensitivity of *S. enteritidis* to lactic acid (main metabolite of lactulose fermentation) in-vitro. Calcium phosphate decreased translocation of salmonella to the systemic circulation, an effect independent of lactulose. The unfavorable increased cytotoxicity of fecal water caused by lactulose fermentation was more than counteracted by supplemental calcium phosphate. Moreover, calcium phosphate stimulated lactulose fermentation, as judged by the reduced lactulose excretion in feces and increased lactic acid, ammonia, and fecal nitrogen excretion. In conclusion, extra calcium phosphate added to a lactulose diet improves the resistance to colonization and translocation of salmonella. This is probably mediated by a calcium-induced stimulation of lactulose fermentation by the intestinal microflora and reversion of the lactulose-mediated increased luminal cytotoxicity, which reduces damage inflicted on the intestinal mucosa.

INTRODUCTION

The disaccharide lactulose (4-O-ß-D-galactopyranosyl-α-D-fructofuranose) is a drug mainly used as a laxative and as treatment for portosystemic encephalopathy [1]. Following oral administration, lactulose reaches the lower gut unaltered because it is neither absorbed nor hydrolyzed by host digestive enzymes. Although its action is not fully understood, it is supposed to depend on extensive fermentation by the intestinal microflora, resulting in enhanced bacterial growth, acidification of the gut contents because of the production of lactic acid and short-chain fatty acids, trapping of ammonia, and stimulation of ammonia incorporation into bacterial protein [2]. Many Gram-negative bacteria like bacteroides, *Escherichia coli*, salmonella and proteus seem to be incapable of degrading lactulose, in contrast with many Gram-positive intestinal bacteria, such as lactobacilli and streptococci [3]. Lactulose therapy has also been used successfully in the management of foodborne intestinal infections and inflammatory bowel diseases [4-6]. Although they are probably one of the prerequisites for the above-mentioned beneficial effects of lactulose, the acidic metabolites generated during strong fermentation have to be at least partially neutralized, to prevent damage to the intestinal mucosa. High concentrations of short-chain fatty acids (for instance acetic acid) may induce injury to the colon which is characterized by degeneration and extrusion of enterocytes, increased permeability and epithelial cell proliferation [7-9]. Therefore, the extent of intestinal fermentation should certainly be controlled. That aim might

be achieved by calcium phosphate supplementation. Dietary calcium phosphate increases the buffering capacity, diminishes the cytotoxic activity of the gut contents and decreases epithelial cell proliferation [10-12]. In addition, we previously showed that dietary calcium strongly improves the colonization resistance to salmonella infection [11].

The main purpose of the present study was to test the hypothesis that supplemental calcium phosphate adds to the potential beneficial effect of lactulose fermentation on intestinal infections. Therefore, we first performed an in-vitro study to compare the bactericidal activity of lactic acid for *S. enteritidis* and *Lactobacillus acidophilus*. In addition, the resistance to colonization and translocation of *S. enteritidis* was studied in a strictly controlled experiment with rats on a purified low-calcium control diet, low-calcium/lactulose diet or high-calcium/lactulose diet. *S. enteritidis* was chosen as model pathogen because it is an increasingly important food-pathogen [13] and the course and pathology of a salmonella infection resembles that of other common human pathogens, like campylobacter, *Listeria monocytogenes* and some pathogenic *Escherichia coli* species [14].

MATERIALS AND METHODS

Bactericidal activity of lactic acid in-vitro

The bactericidal effect of lactic acid on *S. enteritidis* (clinical isolate, phage type 1) and *L. acidophilus* (NIZO B224, collection of our institute) were compared in an in-vitro experiment. *S. enteritidis* was cultured and stored as described earlier [15]. *L. acidophilus* was routinely stored at -80 °C in sterile skim milk containing 0.5% (wt/vol) yeast extract and 20% (vol/vol) glycerol. A few days before use, a vial containing the lactobacillus strain was quickly thawed, plated on agar plates according to De Man, Rogosa and Sharpe (MRS; Merck, Darmstadt, Germany) and incubated overnight at 37 °C in an anaerobic jar (Anoxomat, Mart bv Microbiology Automation, Lichtenvoorde, The Netherlands). Precultures of *S. enteritidis* and *L. acidophilus* were prepared by inoculating a few colonies from appropriate agar plates in Brain Heart Infusion broth (BHI; Difco, Detroit, MI, USA) and MRS broth (Merck) respectively, followed by overnight aerobic incubation at 37 °C. The next day, bacteria were collected by centrifugation (15 minutes at 5,000 g), washed once and resuspended in sterile saline to prepare stock suspensions. The bactericidal activity of increasing concentrations of L(+)-lactic acid (Acros Organics, Geel, Belgium) in a citric acid buffer (50 mM, pH 5.0, ionic strength set at 154 mM with sodium chloride, BDH Chemicals Ltd, Poole, England), was tested by inoculating the sterile test medium with the stock suspensions (final 2%), followed by a 2 hours aerobic incubation at 37 °C. Thereafter, several dilutions of the incubates in phosphate-buffered saline were plated on Brilliant Green Agar (BGA; Oxoid, Basingstoke, England) and MRS agar (Merck) to determine the viable counts of *S. enteritidis* and *L. acidophilus*, respectively. BGA plates were incubated aerobically for one day and MRS plates were incubated anaerobically for 2 days at 37 °C. The whole experiment was performed in triplicate.

Animals, diets, and infection

The experimental protocol was approved by the animal welfare officer of the Agricultural University, Wageningen, The Netherlands. Specific pathogen-free male Wistar rats (WU, Harlan, Winkelmann GmbH, Borchen, Germany), 8 weeks old and with a mean

body weight of about 225 g, were housed individually (n=8 per diet) in metabolic cages in a room with controlled temperature (22-24 °C), relative humidity (50-60%) and light/dark cycle (light, 6.00-18.00 h). During the experimental period of almost 3 weeks, the animals were fed purified diets. The first group (LCa) received a low-calcium control diet (calcium 20 mmol/kg) consisting of: 200 g acid casein, 502 g dextrose, 100 g palm oil, 100 g corn oil, 50 g cellulose, 35 g mineral mix and 10 g vitamin mix as described elsewhere [16], and 3,44 g $CaHPO_4.2H_2O$ (Merck). The second group (LCa/LACT) consumed a low-calcium diet (calcium 20 mmol/kg) supplemented with 10% (wt/wt) lactulose (purity 99%; Solvay Duphar, Weesp, The Netherlands). The third group (HCa/LACT) received a high-calcium diet (calcium 180 mmol/kg), also supplemented with 10% (wt/wt) lactulose. Lactulose and extra calcium phosphate were added at the expense of dextrose. After preparation, samples of the diets were dry ashed for mineral analyses. Calcium and iron were measured using an atomic absorption spectrophotometer (Model 1100, Perkin Elmer Corp, Norwalk, USA). Phosphate was determined spectrophotometrically [17]. The diets were supplied to the animals as a porridge (double-distilled water was mixed with the dry diets to obtain 68% dry weight). The rats had free access to food and demineralized drinking water. Food intake was recorded every 3 days and body weight every 4 days.

Before infection, non-fasted blood samples were collected by orbital-punction (after diethyl ether anesthesia). Plasma samples were prepared by low-speed centrifugation (10 minutes at 1,500 g; Eppendorf 5415) of the heparinized blood, and stored at -20 °C until analysis. Plasma urea concentrations were determined using a colorimetric kit (Procedure No. 640, Sigma Chemical Co., St. Louis, USA).

Animals were acclimatized to housing and dietary conditions for 10 days, after which they were orally infected by gastric gavage with 1 mL of saline containing 3% (wt/vol) sodium bicarbonate with *S. enteritidis* (same isolate as used in the in-vitro experiment). The inoculum contained 4.10^8 *S. enteritidis* per mL, as determined by plating on BGA. The virulence of the strain used is sustained by routine oral passage in Wistar rats, followed by isolation of translocated salmonella from liver or spleen. Before, and on days 1, 5, and 8 after salmonella infection, fresh individual fecal samples were collected. Feces were thoroughly mixed and homogenized in saline (1:3 wt/vol). Calibrated sterile loops were used to plate the fecal homogenates on Modified BGA (Oxoid) containing the detergent Sulfamandelate (Oxoid), to suppress swarming bacteria like proteus. Applying a rapid bacteriological technique, introduced and validated by Giaffer et al. [18], a set of partially overlapping streaks were made on the selective plates. Subsequently, the plates were cultured aerobically for 20 hours at 37 °C.

Analyses of nitric oxide oxidation products (NO_x) in urine

Complete 24 h urine samples were collected for 8 days, starting one day before infection. NO_x (nitrite and nitrate) concentration was determined by automated flow injection analysis. Briefly, diluted urine is passed over a cadmium column to reduce nitrate to nitrite, followed by reaction of nitrite with Griess reagent [19]. The red azo-dye formed was measured spectrophotometrically at 538 nm. Urinary NO_x concentration was multiplied by the urine volume produced in 24 h to obtain daily urinary NO_x excretion. Recovery of nitrite or nitrate added to rat urines always exceeded 90%.

Total feces analyses

Feces were quantitatively collected 3 days before salmonella infection and on days 2, 3 and 4 after infection. Feces were freeze dried for dry weight determination. For determination of unfermented lactulose, 13 volumes of distilled water were added per mg of freeze dried feces. After homogenization, samples were heated for 5 minutes at 95 °C to denature glycosidases, followed by centrifugation (5 minutes at 10,000 g; Eppendorf 5415). Supernatants were cleared from hydrophobic substances on octadecyl columns (100 mg; Varian, Harbor City, USA). Water-eluates were analyzed for lactulose by HPLC [20]. Recoveries of lactulose added to feces varied between 96 and 113%. For lactic acid determination, dry feces were acidified with 1.0 M perchloric acid (1:40 wt/vol), mixed and subsequently centrifuged (2 minutes at 10,000 g; Eppendorf 5415). The supernatant was neutralized to pH 7 by adding 2.0 M potassium bicarbonate (2:1 vol/vol), followed by centrifugation (2 minutes at 10,000 g; Eppendorf 5415). D- and L-lactic acid in the supernatants were separately determined using a colorimetric enzymatic kit (Nr. 1112821, Boehringer Mannheim, Germany). Recoveries of lactic acid added to feces were >90%. For ammonia determination, feces were acidified with 5% perchloric acid (1:20 wt/vol), incubated for 15 minutes at 37 °C, followed by centrifugation (2 minutes at 14,000 g; Eppendorf 5415). The aspirated supernatant was neutralized to pH 7 by adding 3.0 M KOH in 250 mM 3-N-morpholino-propanesulfonicacid (10:3 vol/vol). After mixing, samples were centrifuged (2 minutes at 14,000 g; Eppendorf 5415). Ammonia in the supernatants was determined using a colorimetric kit originally designed to measure urea (Procedure No. 640, Sigma Chemical Co.). However, by omitting the first incubation step with urease, ammonia can be determined directly. Recovery of ammonium chloride added to feces was >85%. Fecal nitrogen was determined as described elsewhere [21]. Briefly, feces were totally acid-digested, followed by colorimetric determination of the ammonia formed. Fecal mucins were extracted and determined as described earlier [11], with some modifications. Before analysis, interfering pigments were removed from watery fecal extracts by filtration (Ultrafree-MC NMWL, cut-off 30,000 D, Millipore, Bedford, USA) during centrifugation (30 minutes at 3,000 g; Eppendorf 5415). Subsequently, mucins on the filter membranes were washed once with an equal volume of methanol (100%) to remove remaining pigments (centrifugation for another 30 minutes at 3,000 g). After drying of the membrane-inserts by air, the mucin retentate was resolubilized in the original volume of phosphate-buffered saline. Mucins were measured using a specific fluorimetric assay [22]. Standard solutions of N-acetylgalactosamine (Sigma) were used to calculate the amount of oligosaccharide side-chains liberated from mucins. Fecal mucins are therefore expressed as μmol oligosaccharide equivalents. Using this procedure, recoveries of porcine stomach mucin (Sigma) added to freeze dried feces always exceeded 90%. After destruction (15 minutes at 180 °C) with a perchloric acid (70%)/hydrogen peroxide (30%) mixture (3:1 vol/vol) of freeze dried feces, calcium, total phosphate and iron were measured as described for the diets. Inorganic phosphate was extracted from freeze-dried feces with 5% (wt/vol) trichloroacetic acid and measured as described above. The amount of organic phosphate in feces was calculated by subtracting the inorganic phosphate concentration from the total phosphate concentration. In the trichloroacetic acid extracts sodium and potassium were analyzed by atomic emission spectrophotometry (Model 1100, Perkin Elmer). Total bile acids were extracted from freeze dried feces with a t-butanol/water mixture (1:1 vol/vol) as described previously [23]. Extracts

were assayed for bile acids using a fluorimetric, enzymatic assay [24]. Recovery of deoxycholic acid (Sigma) added to feces always exceeded 90%.

Fecal water analyses

Fecal water was prepared by reconstituting freeze dried feces with demineralized water to 30% dry weight, which reflects the dry weight percentage of colonic contents [10]. Samples were thorougly mixed and subsequently incubated (1 h at 37 °C) in a shaking waterbath. After centrifugation for 20 minutes at 12,000 g (Hettich, Micro-rapid 1306, Tuttlingen, Germany), the aspirated supernatant was centrifuged again for 2 minutes at 14,000 g (Eppendorf 5415). The pH of the supernatants was measured at 37 °C. Fecal waters were stored at -20 °C until further use. The cytotoxic activity was determined with an erythrocyte assay as described previously [11] and validated elsewhere [25]. The incubations were of physiological ionic strength (154 mM) and buffered at pH 7 (final 100 mM 3-N-morpholino-propanesulfonicacid; Sigma). After acidification with HCl (final 1 M), bile acids were extracted from fecal water three times with diethyl ether. After evaporation of diethyl ether from pooled fractions, the residue was resolubilized in methanol. Bile acids in the methanol extracts were quantified using the same method as described for total feces. Recoveries of a cholic acid/deoxycholic acid (Sigma) mixture, added to fecal waters, varied between 80 and 120%. Iron was measured in distilled-water-diluted fecal water as described for the diets.

Statistics

Results of the in-vitro experiment are presented as means $\pm$ SD (n=3). Results of the infection experiment are presented as means $\pm$ SE (n=8). Within one dietary group, infection-induced changes were tested by Student's *t* test for paired samples (one-sided). Within one period (before or after infection), differences between diet groups were tested for their significance, using analysis of variance (ANOVA). In addition, Fisher's protected least significant difference test (one-sided), modified for multiple comparisons, was used to identify dietary groups that differed from each other. The level of significance was preset at $p<0.05$. A commercially available statistical package was used for all statistics (*SPSS/PC+ version 2.0*, SPSS Inc, Chicago, USA).

RESULTS

Bactericidal activity of lactic acid in-vitro

S. enteritidis was much more sensitive to the bactericidal activity of lactic acid than *L. acidophilus* (Figure 1). Killing of *S. enteritidis* started at a lactic acid concentration of 20 mM and was complete at 40 mM lactic acid. In contrast, even a concentration as high as 50 mM lactic acid did not affect the viability of *L. acidophilus*. At 75 mM lactic acid, the highest concentration tested, the number of colony-forming units was reduced by a factor of 100 only.

Animals, food intake, and diarrhea markers

All data from one animal in the LCa/LACT group were excluded from the study results because of oropharyngeal reflux of the *S. enteritidis* suspension which resulted in

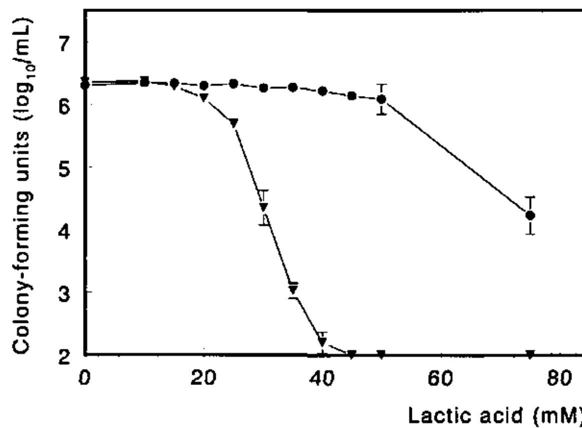

Figure 1 Bactericidal effect of lactic acid on *S. enteritidis* (▼) and *L. acidophilus* (●) in-vitro. Increasing concentrations of lactic acid were added to a citric acid buffer (50 mM, pH 5.0, physiological ionic strength). After 2 h incubation, survival of the bacteria was determined by plating techniques. Values are means ± SD of triplicate incubations. When SDs are not shown they are smaller than the size of the symbols.

pneumonia. At the start of the experiment mean body weight of the rats was 223 g. Weight gain of the LCa/LACT group was significantly less when compared with the other two groups, even before infection. At the end of the experiment (day 8 post-infection) body weights were 281 ± 7, 250 ± 7, and 273 ± 10 g for the LCa, LCa/LACT, and HCa/LACT groups, respectively. During the experiment, the LCa and HCa/LACT group consumed 14.5 g dry food per day, whereas the LCa/LACT group ate approximately 3 g less ($p<0.05$). Before infection, lactulose increased dry fecal output only in combination with high-calcium (Table 1, $p<0.05$). The infection stimulated fecal dry weight excretion in both lactulose groups (Table 1, $p<0.05$). Especially of the LCa/LACT group, no proper assessment of fecal wet weight excretion or the dry/wet weight ratio could be made because of drying up of fecal pellets during collection in the metabolic cages. For that reason, hydratation of feces was determined by the concentration of the electrolytes sodium and potassium. Before infection, the summed concentration of these electrolytes in feces increased in the order LCa < HCa/LACT < LCa/LACT (Table 1, all $p<0.05$). A significant infection-induced increase in fecal electrolyte concentration was observed only in the two low-calcium groups, in contrast to the calcium-supplemented group (Table 1). Lactulose supplementation significantly stimulated fecal mucin excretion, which was most pronounced in combination with low-calcium (Table 1, $p<0.05$). After infection, fecal mucin excretion increased significantly in both low-calcium groups, whereas no infection-induced stimulation was observed in the HCa/LACT group (Table 1, $p<0.05$). The infection had no defined effect on body weight gain or food intake.

Table 1 Effect of a low-calcium (LCa), low-calcium/lactulose (LCa/LACT), and high-calcium/lactulose (HCa/LACT) diet on fecal output, fecal electrolytes, and mucin excretion before and after oral infection with *S. enteritidis*.

Variable	Infection	LCa	LCa/LACT	HCa/LACT
Dry weight (g/d)	Before	0.79 ± 0.03^{a}	0.59 ± 0.14^{b}	1.39 ± 0.10^{c}
	After	0.81 ± 0.04^{a}	$1.01 \pm 0.20^{a!}$	$1.71 \pm 0.13^{b!}$
Sodium and potassium (μmol/g)	Before	34 ± 3^{a}	154 ± 14^{b}	75 ± 7^{c}
	After	$75 \pm 17^{a!}$	$175 \pm 15^{b!}$	88 ± 11^{a}
Mucins (μmol equivalents/d)	Before	1.7 ± 0.1^{a}	31.3 ± 5.7^{b}	13.6 ± 2.7^{c}
	After	$5.7 \pm 0.7^{a!}$	$51.7 \pm 9.9^{b!}$	14.3 ± 5.5^{c}

Values are means ± SE (n=8). Values in the same row not sharing the same letter are significantly different ($P<0.05$). The note of exclamation indicates a significant infection-induced change ($P<0.05$).

Resistance to colonization and translocation of salmonella

The lactulose fed rats had a significantly improved colonization resistance to salmonella. The first day after infection of the rats, lactulose feeding reduced fecal salmonella excretion approximately 1000-fold when compared with the low-calcium control group (Figure 2, $p<0.05$). Probably due to large inter-individual variation in the response to infection in the lactulose supplemented groups, no significant differences in fecal salmonella output were observed later in the experiment. Despite comparable fecal salmonella excretions of the LCa/LACT and HCa/LACT groups, these groups differed markedly in their resistance to translocation of salmonella, as measured by urinary NO_x excretion (Figure 3). Independent of dietary lactulose, both low-calcium groups showed a rapid, progressive increase in daily urinary NO_x output. In contrast, total infection-induced urinary NO_x excretion (expressed as the area under the NO_x curve after infection, corrected for baseline NO_x excretion) of the HCa/LACT group was significantly less ($p<0.05$).

Cytotoxicity and composition of fecal water

Dietary lactulose strongly reduced the pH of fecal water (Figure 4A). Fecal water of the HCa/LACT group was more acidic than that of the LCa/LACT group (Figure 4A, $p<0.05$). Considering the low pH of the fecal waters, the cytotoxicity assay was performed in a buffered (pH 7) system. Compared with the low-calcium control group, lactulose significantly increased the cytotoxic activity of fecal water. This adverse effect of lactulose was counteracted by supplemental calcium. In fact, fecal water of the HCa/LACT group had the lowest cytotoxic activity (Figure 4B, $p<0.05$). Lactulose did not significantly affect the concentration of bile acids in fecal water, whereas calcium decreased the concentration of these surfactants (Figure 4C, $p<0.05$). Lactulose greatly increased the solubility of iron in feces, an effect which was reversed by dietary calcium (Figure 4D, $p<0.05$). The infection did not significantly affect the cytotoxicity, pH or iron concentration of fecal water. Only in

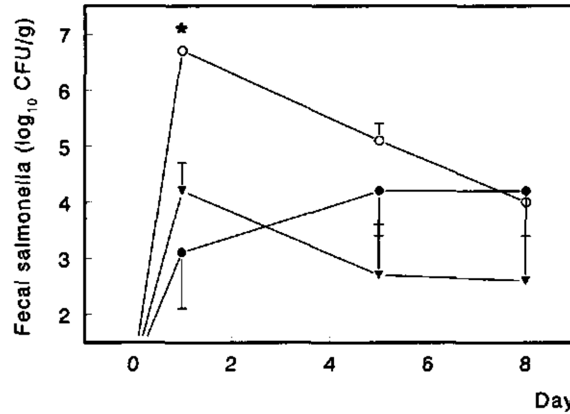

Figure 2 Effect of a low-calcium diet (○), low-calcium/lactulose diet (●), or a high-calcium/lactulose diet (▼) on fecal excretion of *S. enteritidis* after oral administration of 4.10^8 colony-forming units of this pathogen to rats on day 0. Results are expressed as means ± SE (n=8). SEs are either smaller than symbols or indicated by bars. The asterisk denotes that fecal salmonella excretion of the indicated group is significantly different from the other groups ($P < 0.05$). Abbreviation: CFU colony-forming units.

the LCa group was a significant infection-induced increase in the bile acid concentration of fecal water observed (+ 0.59 mM).

Composition of total feces

Lactulose decreased daily fecal excretion of calcium and inorganic phosphate when combined with low-calcium (Table 2, $P < 0.05$). As expected, daily fecal output of these minerals was substantially higher in the HCa/LACT group ($p < 0.05$). Fermentation of lactulose by the intestinal microflora was more complete in the high-calcium group. Daily lactulose excretion in feces was 3.9 μmol in the HCa/LACT group, whereas the LCa/LACT group excreted more than 20 times as much (Table 2, $p < 0.05$). Daily fecal excretion of lactic acid, a product of lactulose fermentation, was strongly increased by supplemental calcium (Table 2, $p < 0.05$). The contribution of D-lactic acid to total lactic acid excretion was 0%, 9% and 37% for the LCa, LCa/LACT and HCa/LACT groups, respectively. Lactulose consumption increased fecal ammonia excretion, which was further stimulated by supplemental calcium (Table 2, $p < 0.05$). The lactulose-induced stimulation of fecal nitrogen excretion was notably increased by supplemental calcium (Table 2, $p < 0.05$). The contribution of ammonia to total fecal nitrogen excretion did not exceed 3% in any group. The HCa/LACT group had an increased excretion of organic phosphate in feces (Table 2, $p < 0.05$). In combination with low calcium, lactulose decreased fecal bile acid excretion (Table 2, $p < 0.05$).

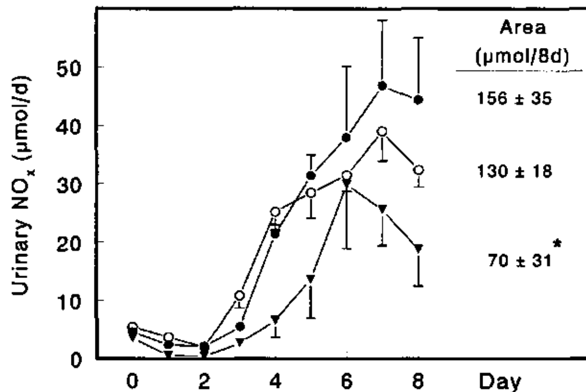

Figure 3 Effect of a low-calcium diet (○), low-calcium/lactulose diet (●), or a high-calcium/lactulose diet (▼) on the kinetics of urinary NO_x excretion of rats before and after an oral challenge with 4.10^8 viable *S. enteritidis* on day 0. In addition, the area under the NO_x time curve after infection is presented. Results are expressed as means $\pm$ SE (n=8). The asterisk denotes that the infection-induced NO_x excretion of the indicated group is significantly different from the other groups ($P<0.05$).

Plasma urea concentration

Before infection, lactulose consumption significantly decreased plasma urea concentration ($p<0.05$). Plasma urea concentrations were 4.49 $\pm$ 0.25, 3.38 $\pm$ 0.24, and 3.11 $\pm$ 0.25 mM for the LCa, LCa/LACT, and HCa/LACT groups respectively.

DISCUSSION

The present study clearly shows that dietary calcium phosphate substantially adds to the beneficial effect of supplemental lactulose on the resistance to *S. enteritidis*. Lactulose markedly enhanced the resistance to colonization of salmonella. The first day after infection of the rats, fecal excretion of viable salmonella was reduced more than 1000-fold when a lactulose diet was consumed (Figure 2). An important product of lactulose fermentation in the rat (Table 2) and human [3, 26] intestine is lactic acid, which appeared to be strongly bactericidal for *S. enteritidis* (Figure 1). Notwithstanding the improvement of the colonization resistance, lactulose did not affect translocation of salmonella, as measured by the kinetics of urinary NO_x excretion (Figure 3). We have previously shown that urinary excretion of

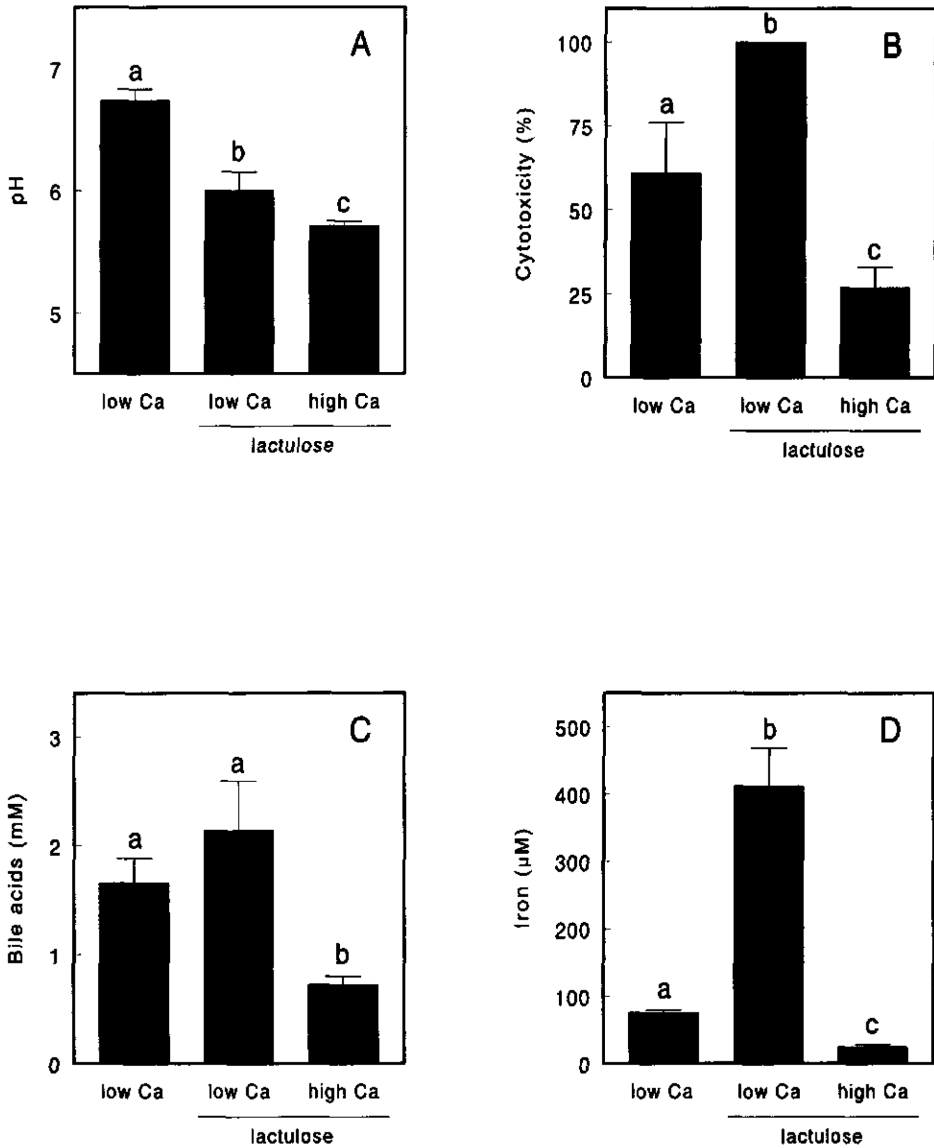

Figure 4 Effect of a low-calcium diet, low-calcium/lactulose diet, and high-calcium/lactulose diet on (A) the pH of fecal water, (B) cytotoxicity, (C) bile acid concentration, and (D) iron concentration of fecal water before infection of the rats. Results are expressed as means ± SE (n=8). Bars not sharing the same letter are significantly different ($P<0.05$).

Table 2 Effect of a low-calcium (LCa), low-calcium/lactulose (LCa/LACT), and high-calcium/lactulose (HCa/LACT) diet on several fecal excretion variables before infection of the rats.

Fecal excretion	LCa	LCa/LACT	HCa/LACT
Calcium (μmol/d)	53.2 ± 6.2[a]	9.6 ± 2.5[b]	1363 ± 105[c]
Inorganic phosphate (μmol/d)	30.2 ± 2.3[a]	10.9 ± 2.9[b]	650 ± 44[c]
Lactulose (μmol/d)	0[a]	90.8 ± 35.3[b]	3.9 ± 0.7[c]
Lactic acid (μmol/d)	1.6 ± 0.5[a]	26.3 ± 12.8[b]	122 ± 21[c]
Ammonia (μmol/d)	15.9 ± 1.6[a]	39.5 ± 13.2[b]	70.5 ± 8.7[c]
Nitrogen (mmol/d)	0.90 ± 0.04[a]	1.39 ± 0.28[b]	2.69 ± 0.22[c]
Organic phosphate (μmol/d)	32.3 ± 1.4[a]	30.6 ± 6.9[a]	145 ± 15[b]
Bile acids (μmol/d)	15.1 ± 1.1[a]	7.7 ± 1.5[b]	14.3 ± 1.3[a]

Values are means ± SE (n=8). Values in the same row not sharing the same letter are significantly different ($P<0.05$).

oxidation products of nitric oxide (nitrite and nitrate) is a sensitive biomarker to quantify bacterial translocation across the gut wall [15]. A major advantage is that in contrast to tissue cultures, urinary NO_x excretion reflects the systemic pathogen load of the host. Upon intestinal bacterial translocation and the subsequent systemic infection, nitric oxide is produced by many infected cells: for instance macrophages, Kupffer cells and neutrophils [27]. Moreover, tissue cultures do not estimate the total bacterial translocation because this method only counts the still viable bacteria and not the large majority of translocated bacteria that is killed after invasion [28]. The initial site of salmonella translocation seems to be the ileal Peyer's patches [29]. Particularly the specialized antigen-sampling M cells are exploited by this invasive pathogen to gain access to host tissues [30]. Fermentation of lactulose by the endogenous microflora, resulting in acidification of the intestinal contents (Figure 4A), is believed to be the basis for the therapeutic effect of this disaccharide on intestinal infections [3]. Considering that the majority of the endogenous microflora resides in the lower gut (cecum and colon), whereas the small intestine is relatively sparsely populated [31], it is not directly feasible that translocation processes can be influenced by lactulose. Indeed, the pH-lowering effect of lactulose fermentation in the human [2] and rat [32] ileum is limited: the pH of the soluble phase of ileal contents drops from 7.5 to 7.0 after lactulose supplementation. Although the small intestine does not have the luxuriant bacterial flora of the colon, lactobacilli are predominant species in this region of the gastrointestinal tract [33]. Several investigations have shown that these bacteria exert an antagonistic action towards food-borne pathogens (for review, see Mital and Garg [33]), including salmonella [34]. Moreover, the lactobacilli are well-capable of fermenting lactulose [35] and are rather insensitive to the lactic acid formed, certainly when compared with the salmonella species used in this study (Figure 1). If, in the present study, a growth advantage by lactulose

supplementation was achieved for these lactic acid bacteria in the ileum, it was apparently not enough to improve the competition with and resistance to translocation of salmonella. In contrast to the lack of protection of lactulose against translocation of salmonella, the present study clearly shows that supplemental calcium phosphate is well-capable of diminishing translocation (Figure 3). Thus, whereas the appreciated activity of lactulose is limited to the lower gut, calcium phosphate already exerts protection in the small intestine, reducing the severity of the systemic infection. That the HCa/LACT group had the best resistance to salmonella infection, was supported by the absence of significant infection-induced increases in fecal sodium and potassium concentration and mucin excretion (Table 1), which are diarrhea markers [36, 37]. In both low-calcium groups (with or without lactulose), fecal mucin output and the concentration of fecal electrolytes increased significantly after infection. Before infection, lactulose supplementation increased the fecal electrolyte concentration, the effect being most pronounced in combination with a low-calcium diet. Similar effects of lactulose on fecal sodium and potassium concentrations, in addition to softening of stools, have been reported in human studies [38, 39].

The observed lactulose fermentation-mediated increased colonization resistance to *S. enteritidis* in this study, might also be relevant for the resistance to other pathogenic bacteria. During lactulose therapy, the pH of human proximal colonic contents drops below 5 [2]. With the exception of shigella [40], common human pathogens like campylobacter, *Escherichia coli* and *Vibrio cholerae* share their acid-sensitivity with *S. enteritidis*. All these pathogens are largely killed in the acid stomach, considering that the viable oral inoculum necessary to cause intestinal disease in humans is very high ($>10^7$) [14].

A mechanism which might account for the dietary calcium-induced improvement of the barrier function of the intestinal mucosa is the formation of an amorphous calcium phosphate complex in the small intestine, which precipitates cytotoxic bile acids and fatty acids, thereby reducing epithelial cell damage [12, 41]. In the present study, the cytotoxicity of fecal water was highest in the LCa/LACT group, intermediate in the LCa group, and lowest in the HCa/LACT group (Figure 4B, $p<0.05$). Considering the lactulose fermentation-induced drop in the pH of fecal water (Figure 4A), the cytotoxicity assay was performed in a buffer of neutral pH, precluding simple acid-induced lysis. Excluding a direct effect of the low pH by these precautions, lactulose obviously increased the cytotoxicity of fecal water in combination with a low-calcium diet. This unfavorable stimulation of the cell damaging activity of intestinal contents by lactulose fermentation was more than reversed by supplemental calcium phosphate. In accordance with its lowest cytotoxicity, fecal water of the HCa/LACT group also had the lowest concentration of cytotoxic bile acids (Figure 4C, $p<0.05$). In the LCa/LACT group, the combination of a high bile acid concentration in fecal water and an enhanced protonation and absorption of these surfactants resulted in a decreased daily fecal excretion of bile acids (Table 2). When luminal calcium phosphate was limited, fermentation of lactulose greatly increased the intestinal solubility of iron (Figure 4D). Remarkably, coprecipitation of iron by calcium phosphate [11] was absolutely not counteracted by the acidic colonic environment. To minimize epithelial cell damage and to maximize the barrier function of the mucosa, the solubility of iron in the intestinal lumen should be low because iron is a catalyst in Fenton-like reactions, resulting in lipid peroxidation and depletion of antioxidant defenses [42]. The increased luminal cytotoxicity and potential iron-mediated peroxidative damage during extensive intestinal fermentations might account for the compensatory mucosal hyperproliferation in several studies [7, 9].

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Chapter 6

Dietary calcium phosphate stimulates intestinal lactobacilli and decreases the severity of a salmonella infection in rats

Ingeborg MJ Bovee-Oudenhoven, Mischa LG Wissink,
Jan TM Wouters & Roelof van der Meer

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ABSTRACT

We have shown recently that dietary calcium phosphate (CaP_i) has a trophic effect on the intestinal microflora and strongly protects against intestinal salmonella infection. It was speculated that precipitation of intestinal surfactants, like bile acids and fatty acids, by CaP_i reduced luminal cytotoxicity and favored growth of the microflora. Because lactobacilli may have antagonistic activity against pathogenic bacteria, the main purpose of the present study was to examine whether this dietary CaP_i-induced protection is mediated by a reinforcement of endogenous lactobacilli. In-vitro, *Salmonella enteritidis* appeared to be insensitive to bile acids and fatty acids, whereas *Lactobacillus acidophilus* was rapidly killed by physiologically relevant concentrations of these surfactants. Additionally, after adaptation to a purified diet differing only in CaP_i content (20 and 180 mmol $CaHPO_4.2H_2O$/kg), rats (n=8) were orally infected with *S. enteritidis*. Besides reducing the cytotoxicity and the concentration of bile acids and fatty acids of ileal contents and fecal water, CaP_i notably changed the composition of ileal bile acids into a less cell-damaging direction. Significantly increased numbers of ileal and fecal lactobacilli were detected in non-infected, CaP_i-supplemented animals. Moreover, dietary CaP_i significantly reduced translocation of salmonella to extra-intestinal organs and improved the colonization resistance, as judged by the decreased urinary NO_x and fecal salmonella excretion, respectively. In accordance less viable salmonella were detected in ileal contents and on the ileal mucosa in the CaP_i group. In conclusion, reducing the intestinal surfactant concentration by dietary CaP_i strengthens the endogenous lactobacilli and increases the resistance to salmonella.

INTRODUCTION

Serious concern about the wide-spread use of antibiotics and the subsequent growing antimicrobial resistance call for alternative approaches to prevent and handle intestinal infections. Dietary intervention might be important to strengthen host resistance to intestinal pathogenic bacteria. In strictly controlled studies with rats, we have recently shown that dietary CaP_i not only shortened fecal shedding of salmonella but also reduced translocation of this pathogen across the intestine to the systemic circulation [1, 2]. It was noticed that dietary CaP_i had a trophic effect on the intestinal microflora, considering the increased fecal excretion of several bacterial mass markers [2]. Stimulation of bacterial growth may be of relevance because the endogenous microflora is an extremely important determinant of host defense against intestinal pathogens [3-5]. Besides an appreciation of the protective endogenous gut flora in general, several investigations have shown that orally administered lactobacilli exert antagonistic action against intestinal and foodborne pathogens (for review see [6]). Competition for luminal nutrients and adhesion sites on the intestinal epithelium, and the production of antimicrobial compounds, like organic acids and hydrogen peroxide, may account for this inhibitory action [6]. Many important intestinal pathogens, for instance salmonella, campylobacter and *Escherichia coli* species, mainly elaborate their noxious effects in the small intestine [7]. The predominance of lactobacilli in this relatively sparsely populated region of the intestinal tract might explain their functionality in combating intruding microbes [8]. It is known that supplemental dietary CaP_i reduces the cytotoxicity of intestinal contents by precipitating luminal

surfactants, like bile acids and fatty acids [9, 10]. A less harsh environment might favor growth of the resident microflora and improve their antagonistic action towards pathogenic bacteria. It is conceivable that lactobacilli and other Gram-positive bacteria, lacking the outermembranous lipopolysaccharide screen, are particularly vulnerable to the intestinal surfactant concentration. Therefore, they might benefit most by the lowered luminal cytotoxicity induced by dietary CaP_i.

The main purpose of the present study was to test the hypothesis that the inhibitory effects of dietary CaP_i on salmonella colonization and translocation are mediated by a stimulation of the protective endogenous lactobacilli. Therefore, we first performed an in-vitro study to compare the bactericidal activity of bile acids and fatty acids to *S. enteritidis* and *L. acidophilus*. Additionally, the proposed stimulating effect of supplemental CaP_i on the endogenous lactobacilli and the subsequent resistance to salmonella were verified in a strictly controlled infection experiment with rats on either a low- or a high-CaP_i purified diet. Special attention was paid to the composition of the microflora in the ileum because this is the most relevant part of the intestinal tract in this infection model, as mentioned above.

MATERIALS AND METHODS

Bactericidal activity of bile acids and fatty acids in-vitro

S. enteritidis (clinical isolate, phage type 1) and *L. acidophilus* (NIZO B224, collection of our institute) were cultured and stored as described earlier [1, 11]. Precultures of *S. enteritidis* and *L. acidophilus* were prepared by inoculating a few colonies from appropriate agar plates in Brain Heart Infusion broth (BHI; Difco, Detroit, MI, USA) and De Man, Rogosa, and Sharpe broth (MRS; Merck, Darmstadt, Germany) respectively, followed by overnight aerobic incubation at 37 °C. The next day, bacteria were collected by centrifugation (20 min at 3,500 g; Heraeus, Sepatech GmbH, Osterode, Germany), washed once, and resuspended in sterile saline to prepare stock suspensions. The bactericidal activity of increasing concentrations (total 0-4 mM) of conjugated bile acids (taurodeoxycholic acid and taurochenodeoxycholic acid 1:2 mol/mol, sodium salts; Sigma Chemical Co., St. Louis, MO, USA), deconjugated bile acids (deoxycholic acid and chenodeoxycholic acid 1:2 mol/mol, sodium salts; Sigma) and the fatty acid C12:0 (lauric acid; Fluka Chemie AG, Buchs, Switzerland) in a buffer (250 mM 3-N-morpholinopropanesulfonic acid, adjusted to physiological pH and ionic strength) was determined by inoculating the sterilized testmedia with the stock suspensions (final 1% vol/vol, corresponding with approximately 10^7 viable bacteria/mL testmedium), followed by a 4 h aerobic incubation at 37 °C. Immediately after inoculation of the testmedia and after 4 h at 37 °C small samples were taken from the incubates, serially diluted in saline and plated on Brilliant Green Agar (BGA; Oxoid, Basingstoke, England) and MRS Agar (Merck) to determine the viable counts of *S. enteritidis* and *L. acidophilus*, respectively. The BGA plates were incubated aerobically for one day at 37 °C and the MRS plates for 2 days at 37 °C in an anaerobic cabinet (Coy Laboratory products Inc., Ann Arbor, MI, USA). The whole experiment was performed in triplicate.

Animals, diets and infection

The experimental protocol was approved by the animal welfare officer of the Agricultural University, Wageningen, The Netherlands. Four groups of specific pathogen-free, male Wistar rats (WU, Harlan, Zeist, The Netherlands; n=8 per group), 8 weeks old, 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). The animals were fed purified diets differing only in CaP_i content. The control diet contained 20 mmol and the supplemented diet 180 mmol $CaHPO_4.2H_2O$/kg diet. The exact composition of the diets is provided in Table 1. After preparation, the calcium content of the diets was checked in dry ashed samples using an atomic absorption spectrophotometer (model 1100; Perkin Elmer Corp., Norwalk, CT, USA). The animals had free access to daily fresh prepared porridges (demineralized water was added and mixed with the dry diets to obtain a final dry weight of 68%) and demineralized drinking water. Food intake was recorded every day and body weight every 2-3 days.

After acclimatizing to the housing and dietary conditions for 10 days, 1 control group and 1 CaP_i-supplemented group were orally infected with *S. enteritidis* (same strain as described for the in-vitro experiment), suspended in 0.5 mL of saline containing 3% (wt/vol) sodium bicarbonate, by gastric gavage. The exact administered dose was 6.10^8 viable salmonella as determined by plating on Brilliant Green Agar (Oxoid). The other 2 groups were gavaged with sterile 0.5 mL saline containing 3% (wt/vol) sodium bicarbonate only. The virulence of the salmonella strain used is sustained by routine oral passage in Wistar rats, followed by isolation of translocated salmonella from liver or spleen.

Microbiological analyses and dissection of the animals

Before and 1, 3 and 6 days after infection, fresh fecal samples were collected directly from the anus of the animals and analyzed for viable salmonella. For that purpose, 10-fold dilutions of the feces in saline were plated on Modified Brilliant Green Agar (Oxoid) containing sulfamandelate (Oxoid), and incubated overnight at 37 ^{0}C. The detection

Table 1 Composition of the purified diets.

Ingredient (g/kg diet)	Control	CaP_i
Acid casein	200	200
Dextrose	502	474
Palm oil	100	100
Corn oil	100	100
Cellulose	50	50
Mineral mix[1]	35	35
Vitamin mix[1]	10	10
$CaHPO_4.2H_2O$	3.44	30.98

[1] The vitamin mix and mineral mix (without calcium) were prepared as prescribed elsewhere [29].

limit of this method was 10^2 colony-forming units of salmonella/g feces. The numbers of lactobacilli and enterobacteria were also determined in the feces collected at day 6 after infection. Feces were handled as described for salmonella culture, except that Rogosa Agar (Oxoid) and Levine Agar (Oxoid) plates were used for the quantification of lactobacilli and enterobacteria, respectively. The Levine plates were incubated for 1 day at 37 °C and the Rogosa plates for 3 days at 37 °C in an anaerobic cabinet (Coy Laboratory products Inc.).

After collection of the fecal samples at day 6 after infection, the rats were killed by carbon dioxide inhalation. The distal ileum (defined as the last 12 cm of the small intestine proximal to the cecum) was pinched off and excised. Sterile saline (500 μL) was injected into the ileal lumen and mixed with the ileal contents by turning the ileum up side down 5 times. The total numbers of salmonella, lactobacilli, and enterobacteria in the ileal lavages were determined with the same microbiological technique as described for feces. Subsequently, the ilea were freed of remaining contents by rinsing with 5 mL of sterile saline, followed by longitudinal dissection. Using a sterile spatula, the ileal mucosa was scraped off to collect the bacteria adhering to the ileal epithelium. Scrapings were suspended in sterile saline, homogenized (Ultraturrax Pro200, Pro Scientific Inc., Monroe, CT, USA) and serially diluted in saline. Viable salmonella, lactobacilli, and enterobacteria were quantified as described for feces. However, to compare the number of bacteria in the ileal lavages with their number found in the ileal scrapings, results were expressed as total numbers instead of colony forming units per g ileal lavage or g ileal scraping.

Chemical analyses of feces and ileal contents

All feces produced by each animal at day 3, 4 and 5 after infection was quantitatively collected and pooled per animal for chemical analyses. At the same time, this was also done for the non-infected animals. Feces were freeze dried and subsequently grinded to obtain homogeneous samples. Sodium and potassium were extracted from freeze dried feces with 5% trichloroacetic acid (1:25 wt/vol) and analyzed by atomic emission spectrophotometry (Model 1100, Perkin Elmer). Fecal ammonia was determined using a colorimetric kit as described earlier [1]. The sodium, potassium, and ammonia concentrations were used to calculate the fecal wet weight %, assuming that these electrolytes are the main cations in feces and intestinal contents have an osmolarity of 300 mOsmol/L. Fecal L- and D-lactic acid were determined with a colorimetric enzymatic assay as described earlier [1].

Fecal water was prepared as described earlier [1] and stored at -20 °C until further use. To dispose insoluble material, ileal contents were centrifuged (5 min at 15,000 g; Eppendorf 5415, Merck, Darmstadt, Germany) and the supernatants were collected and stored at -20 °C. The cytotoxicity of fecal water (40 μL) was determined with an erythrocyte assay as described previously [12] and validated elsewhere [13]. The same bio-assay was used to determine the cytotoxicity of the ileal contents (10 μL). The total bile acid concentration of fecal water was determined with a fluorimetric assay as described earlier [1]. The composition of bile acids in ileal lavages was determined by HPLC as described earlier [14], but preceded by some purification. Before HPLC analyses, the ileal lavages were diluted with double-distilled water and cleared from hydrophilic substances on octadecyl columns (500 mg; IST, Mid-Glamorgan, England). The water eluates were discarded, whereas the methanol eluates were sampled and analyzed for its bile acid composition. The synthetic steroid 5ß-cholanic acid-7α,12α-diol (Steraloids Inc., Wilton,

NH, USA) was used as internal standard. Using this procedure, recoveries of taurodeoxycholic acid and cholic acid (both Sigma) added to the ileal lavages always exceeded 93%. The total concentration of ileal bile acids was calculated by adding up the concentrations of all individual bile acids appearing in the chromatogram. The conjugated or deconjugated nature of the unknown bile acids was determined using bile salt hydrolase as described elsewhere [15]. For extraction of fatty acids, fecal water and ileal contents were acidified with HCl (final concentration 1 and 4 M for fecal water and ileal contents, respectively). To improve the recovery, methanol (final 20%) was added to the acidified ileal contents, whereas this was not necessary for fecal water. Subsequently fatty acids were extracted 3 times with 10 volumes of diethyl ether. After evaporation of diethyl ether, fatty acids were resolubilized in ethanol and quantified using a colorimetric, enzymatic assay (NEFA-C, Wako Chemicals, Neuss, Germany). Addition of a mixture of lauric acid and palmitic acid to fecal water and ileal contents resulted in >93% and >85% recovery, respectively.

Analysis of NO_x in urine

Complete 24-hour urine samples were collected for 7 days, starting 1 day before infection. Oxytetracycline (approximately 100 times the minimal inhibitory concentration for most aerobes; Sigma) was added to the urine collection vessels of the metabolic cages to prevent bacterial deterioration. The concentration of NO_x (nitrite and nitrate) was determined with automated flow injection analysis. Briefly, diluted urine is passed over a cadmium column to reduce nitrate to nitrite, followed by reaction of nitrite with Griess reagent [16]. The purple colored product was measured spectrophotometrically at 538 nm. Recovery of nitrate added to rat urines always exceeded 95%. Daily urinary NO_x excretion was calculated by multiplication of the NO_x concentration with the urine volume produced in 24 hours.

Statistics

Results are expressed as means ± SE (n=8). Analysis of variance (ANOVA) and Student's *t* test (one-sided) were used to identify significant differences among the groups. A P value <0.05 was considered significant.

RESULTS

Bactericidal activity of bile acids and fatty acids in-vitro

In the absence of bile acids and fatty acids (control incubation), the viability of *S. enteritidis* and *L. acidophilus* did not change during the 4 h aerobic incubation. *S. enteritidis* was practically insensitive to bile acids, even at high concentrations (Figure 1A). In contrast, conjugated bile acids and especially unconjugated bile acids were strongly bactericidal for *L. acidophilus*. Similar effects were observed with lauric acid. *S. enteritidis* was completely unaffected, whereas 2 mM of this fatty acid was sufficient to kill all *L. acidophilus* present in the incubates (Figure 1B). The bactericidal activity of bile acids and fatty acids for the lactobacillus seemed to be an immediate effect, because almost similar effects of these surfactants on the viability of this strain were obtained when the incubation time was shorter (0 or 2 h; data not shown).

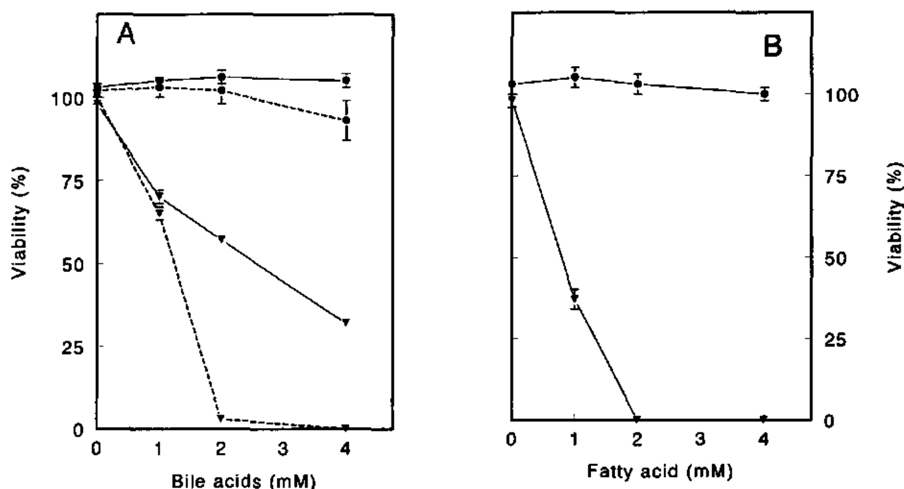

Figure 1 Bactericidal activity of (A) a mixture of conjugated bile acids (filled lines) and unconjugated bile acids (dotted lines), and (B) the fatty acid lauric acid for *S. enteritidis* (●) and *L. acidophilus* (▼) in-vitro. The viability of these bacterial strains was determined by standard microbiological plating techniques after a 4 h incubation at 37 °C. Results are expressed as % of the incubation without added bile acids or fatty acid. Values are means of triplicate incubations and SDs are smaller than symbols or indicated by bars.

Animals, food intake and fecal output

At the start of the experiment mean body weight of the animals was 252 g. Dietary CaP_i did not affect body weight gain of non-infected animals (mean 5.2 g/d), whereas salmonella infection significantly reduced growth of the rats in both diet groups (mean 4.3 g/d). Food intake (mean 18 g/d dry matter) was not significantly affected by dietary CaP_i or the infection. Non-infected animals had a mean calculated fecal wet weight percentage of 37%, which increased to 45% in the control group after infection ($P<0.05$) indicating slight diarrhea. No significant change in fecal wet weight % was observed in the CaP_i-supplemented group after infection (mean 39%). CaP_i supplementation significantly stimulated total fecal lactic acid output (control 1.91 ± 0.31 and CaP_i 12.19 ± 2.72 μmol/d). L-lactic acid comprised $53 \pm 2\%$ of total lactic acid in the control group and $67 \pm 2\%$ in the CaP_i group. The infection did not significantly change fecal lactic acid excretion (data not shown).

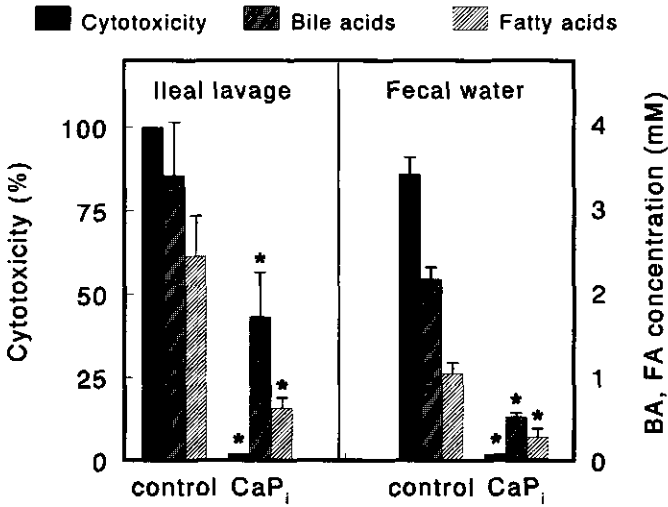

Figure 2 Effect of dietary calcium phosphate (CaP_i) on the cytotoxicity, bile acid (BA) and fatty acid (FA) concentration of ileal lavages and fecal water of non-infected rats. Results are expressed as means ± SE (n=8). The asterisk indicates a significant difference ($P<0.05$).

Cytotoxicity and chemical composition of ileal contents and fecal water

The cytotoxicity of ileal lavages was significantly decreased in the CaP_i-supplemented group (Figure 2). Dietary CaP_i approximately halved the total bile acid and fatty acid concentration of ileal lavages ($P<0.05$, Figure 2). In addition, major changes were observed in the composition of ileal bile acids due to CaP_i supplementation (Table 2). Dietary CaP_i significantly decreased the total concentration of unconjugated bile acids (control 1.43 ± 0.54 and CaP_i 0.34 ± 0.08 mM). The molar ratio of known secondary to primary bile acids was decreased 2-fold in the CaP_i group ($P<0.05$). Substantial amounts of 4 unknown bile acids were detected in the ileal lavages (Table 2). The in-vitro incubation experiments with bile salt hydrolase resulted in a complete disappearance of peak B, C and D in the chromatogram and a concomitant increase in free ß-muricholic acid and peak A. This indicates that peak B, C and D are taurine or glycine conjugates of ß-muricholic acid and the unidentified bile salt A. Additional analyses are currently performed to identify the exact nature of the unknown bile acids. Salmonella infection had no significant effect on the total concentration of bile acids and fatty acids in ileal lavages (data not shown). Animals fed the CaP_i-supplemented diet had a significantly reduced cytotoxicity of fecal water and concomitantly decreased concentration of bile acids and fatty acids in fecal water (Figure 2). The infection significantly increased the concentration of bile acids (146%) and fatty

Table 2 Effect of dietary calcium phosphate (CaP_i) on the bile acid composition of the soluble fraction of ileal lavages before infection of the rats.

Bile acid (μM)	Control	CaP_i
ω-Muricholic acid	24 ± 13	10 ± 7
α-Muricholic acid	77 ± 27	21 ± 8 *
ß-Muricholic acid	53 ± 35	24 ± 13
Cholic acid	974 ± 402	205 ± 62 *
Glycocholic acid	706 ± 274	306 ± 150
12-Ketolithocholic acid	14 ± 10	23 ± 12
Taurocholic acid	542 ± 227	686 ± 320
Chenodeoxycholic acid	20 ± 16	n.d. *
Deoxycholic acid	29 ± 16	n.d. *
Glycochenodeoxycholic acid	29 ± 17	3 ± 3
Glycodeoxycholic acid	32 ± 14	2 ± 2 *
Taurochenodeoxycholic acid	22 ± 8	9 ± 4
Taurodeoxycholic acid	52 ± 22	20 ± 8
Unknown bile acids		
A (unconjugated)	240 ± 110	53 ± 23 *
B (conjugated)	139 ± 80	73 ± 35
C (conjugated)	158 ± 68	168 ± 88
D (conjugated)	281 ± 74	109 ± 35 *

Results are expressed as means ± SE (n=8). Known bile acids are listed in order of appearance in the HPLC chromatogram. The concentrations of unknown bile acids is calculated using the response factor of ß-muricholic acid. *Abbreviation: n.d. not detected.* The asterisk denotes a significant difference from the control group ($P < 0.05$).

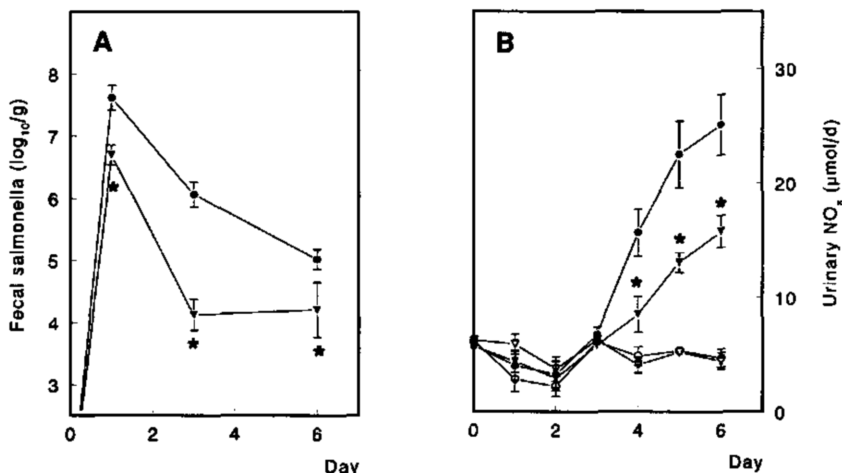

Figure 3 Effect of dietary calcium phosphate (CaP_i) on (A) fecal salmonella excretion after oral infection of the rats and (B) the infection-induced increase in urinary NO_x excretion. Symbols: ● infected control group, ▼ infected CaP_i-supplemented group, and the corresponding open symbols represent the non-infected groups. No salmonella was detected in feces of non-infected animals. Results are expressed as means ± SE (n=8). The asterisk indicates a significant difference from the control group ($P<0.05$).

acids (211%) in fecal water of the control group,and the cytotoxicity tended to be higher ($P=0.11$). In contrast, no significant infection-induced changes in these parameters were observed in the CaP_i-supplemented group (data not shown).

Resistance to colonization and translocation of salmonella

In comparison with the control group, fecal salmonella excretion of the CaP_i group was 10-100 times less after oral infection ($P<0.05$, Figure 3A). In accordance, significantly reduced viable salmonella were measured in ileal lavages and ileal scrapings of the CaP_i group (Table 3). The improved colonization resistance of rats fed the CaP_i-supplemented diet coincided with a significantly reduced translocation of *S. enteritidis*, as measured by the decreased infection-induced urinary NO_x excretion (Figure 3B).

Composition of the intestinal microflora

Dietary CaP_i stimulated the intestinal lactobacilli as judged by the significantly increased viable counts of these lactic acid bacteria in ileal contents, ileal scrapings, and feces of non-infected animals (Table 3). In addition, the ileal enterobacteria were also

Table 3 Effect of dietary calcium phosphate (CaP_i) and oral *S. enteritidis* infection on the number of lactobacilli, enterobacteria, and salmonella in ileal lavages, ileal scrapings and feces of rats.

	Non-infected		Infected	
	Control	CaP_i	Control	CaP_i
Ileal lavage (log_{10})				
Lactobacilli	5.46 ± 0.12^a	5.86 ± 0.10^b	5.34 ± 0.12^a	5.53 ± 0.18^a
Enterobacteria	3.57 ± 0.19^a	4.54 ± 0.27^b	4.73 ± 0.24^b	4.19 ± 0.44^{ab}
Salmonella	n.d.	n.d.	2.96 ± 0.24^a	2.07 ± 0.37^b
Ileal scraping (log_{10})				
Lactobacilli	3.44 ± 0.21^a	4.06 ± 0.24^b	4.14 ± 0.22^b	3.98 ± 0.35^{ab}
Enterobacteria	1.96 ± 0.11^a	2.36 ± 0.20^a	4.57 ± 0.09^b	3.49 ± 0.52^c
Salmonella	n.d.	n.d.	4.56 ± 0.09^a	3.34 ± 0.48^b
Feces (log_{10}/g)				
Lactobacilli	7.68 ± 0.15^a	7.96 ± 0.10^b	7.57 ± 0.11^a	7.77 ± 0.08^{ab}
Enterobacteria	7.16 ± 0.20^a	6.64 ± 0.17^b	7.24 ± 0.10^a	6.88 ± 0.20^{ab}
Salmonella	n.d.	n.d.	5.01 ± 0.16^a	4.20 ± 0.44^b

The intestinal flora was analyzed 6 days after oral infection of the rats as was the flora of their non-infected counterparts. Results are expressed as means $\pm$ SE (n=8). Abbreviation: n.d. not detected. Different superscripts in the same row indicate significant differences ($P<0.05$).

increased by CaP_i (Table 3). Most of these dietary CaP_i-mediated effects on the endogenous microflora were no longer present after infection of the rats with salmonella (Table 3).

DISCUSSION

The present study indicates for the first time that the protective effects of dietary CaP_i against *S. enteritidis* infection might be mediated by modulation of the endogenous microflora, and a stimulation of the intestinal lactobacilli in particular. In agreement with our earlier studies [2, 12], the colonization resistance to salmonella was greatly improved by dietary CaP_i, as fecal shedding of this pathogen was significantly reduced. We now show, that this coincides with a diminished colonization of the ileal epithelium by this pathogen. Compared with the control group, approximately 10 times less viable salmonella were detected in scrapings of the ileal mucosa and ileal lavages of the CaP_i group. The observation that salmonella was still present in the ileum 6 days after the oral introduction of this pathogen indicates that salmonella not simply passaged but truly colonized the ileal epithelium. It is known that the ileum is the initial intestinal region exploited by salmonella

to gain access to deeper host tissues, resulting in a systemic infection [17]. The CaP_i-mediated reduction in colonization of the ileal mucosa by salmonella resulted in a decreased translocation of this pathogen, considering the significantly reduced urinary NO_x excretion after infection. We have shown previously that urinary NO_x excretion is a more valid and quantitative biomarker to assess total intestinal bacterial translocation than classical microbiological organ cultures [2, 11]. Though obvious signs of diarrhea were absent, the more severe infection of the control group was also reflected in a significantly increased fecal wet weight %, as calculated by the concentration of fecal electrolytes. In contrast, the wet weight % of feces of the CaP_i-supplemented group after infection did not differ from that of non-infected animals.

The most interesting finding of the present study is the modulation of the intestinal microflora by dietary CaP_i, which might be the responsible mechanism for the observed protective effects of dietary CaP_i. The endogenous microflora is an extremely important determinant of host defense against intruding pathogenic bacteria [3, 4]. Elimination of parts of the protective intestinal microflora by use of antibiotics in man [18, 19] and animals [5] is frequently accompanied with unintended opportunistic infections. An optimal endogenous microflora outcompetes invading pathogens for adhesion sites on the intestinal mucosa and luminal nutrients. In addition, many products of the vivid bacterial metabolism in the intestine, for instance lactic acid and short-chain fatty acids, can damage allochtonous pathogens and prevent them from colonizing the epithelium [1, 3]. In a former study, we showed that dietary CaP_i has a trophic effect on the rat intestinal microflora in general. CaP_i-supplementation increased fecal excretion of dry weight, nitrogen, phospholipids and organic phosphate, which are supposed markers of bacterial mass [2]. A similar effect of dietary calcium was found in humans [10]. This phenomenon is now extended because the present study clearly shows that dietary CaP_i changed the composition of the endogenous microflora and stimulated the lactobacilli. Besides an increased excretion of these lactic acid bacteria in feces, significantly increased numbers were found in the ileal lumen and adhering to the ileal epithelium. Rather suggestive is the observation that the extent of the rise of intestinal lactobacilli and the increase of fecal lactic acid excretion due to CaP_i supplementation were roughly comparable. As mentioned above, translocation of salmonella mainly takes place in the ileum [17]. Because lactobacilli are predominant species in this region of the gastrointestinal tract of man and animals [8], they might play an important role in the resistance to colonization and translocation of food-borne pathogens. Though it is not proved yet that autochtonous lactobacilli are antagonistic towards invading bacteria, orally administered lactobacilli are reported to be protective. For instance, Hudault et al. [20] showed that *L. acidophilus* administration to mice improved their colonization and translocation resistance to *Salmonella typhimurium*. Additionally, in a number of clinical investigations ingestion of lactic acid bacteria bacteria resulted in shortening of the duration of antibiotic-associated diarrhea [21], the prevention of recurrent relapses of *Clostridium difficile* colitis [22] and amelioration of acute rotavirus enteritis in children [23]. In the present study, salmonella obviously disturbed the resident flora as the specific CaP_i-induced composition of the intestinal microflora was no longer present after infection.

An explanatory mechanism by which dietary CaP_i strengthens the intestinal lactobacillus flora might involve bile acids and fatty acids. The present in-vitro experiment showed that *L. acidophilus* was much more sensitive for the surface-active properties of

bile acids and fatty acids than *S. enteritidis*. In line with results reported earlier [24, 25], the more hydrophobic, unconjugated bile acids are stronger bactericidal than their conjugated counterparts. The range of surfactant concentrations used in the in-vitro experiment was physiologically relevant, because comparable concentrations were determined in ileal contents and fecal water. Moreover, to obtain sufficient material from the ileal lumen for analyses, the ileal contents were diluted (approximately 3-fold) with some saline. So, the reported bile acid and fatty acid concentration in ileal lavages even underestimate the in-vivo situation. In agreement with results reported earlier [9, 10, 25], bile acids and fatty acids are strongly adsorbed to and precipitated by intestinal CaP_i, resulting in a significantly decreased concentration of these surfactants in fecal water. CaP_i also reduced the bile acid and fatty acid concentration of the water phase of ileal contents. In the small intestine the majority of the bile acids is still conjugated with either taurine or glycine because extensive bacterial deconjugation of bile acids just takes place in the colon [26]. In general, unconjugated bile acids and glycine-conjugated bile acids were stronger precipitated by amorphous CaP_i than taurine-conjugated bile acids, in agreement with our earlier study [27]. The present study clearly shows that dietary CaP_i significantly changed the composition of ileal bile acids. The total concentration of unconjugated bile acids, as well as the ratio of secondary to primary bile acids was significantly decreased by CaP_i, indicating a CaP_i-mediated shift to a less cytotoxic bile acid pool. This might also have favored growth of the endogenous lactobacilli. Extrapolation to the human situation is probably permissible. As shown earlier in a human intervention trial, dietary calcium significantly decreased the ratio of dihydroxy bile acids to trihydroxy bile acids and thus decreased the cytotoxicity of the duodenal bile acid pool [28]. Though, no direct evidence is present yet demonstrating that dietary calcium stimulates the endogenous lactobacilli in humans.

Besides a trophic effect of dietary CaP_i on the endogenous lactobacilli, CaP_i also stimulated the enterobacteria in the ileum. Though one can consider this as an unwanted side-effect, one can also argue that these normal residents of the intestinal microflora contribute to the colonization resistance. In fact, due to homology, enterobacteria might be better competitors with salmonella for adhesion sites on the intestinal mucosa and nutrients for growth than lactobacilli.

In conclusion, by precipitating fatty acids and bile acids in the intestinal lumen and changing the composition of ileal bile acids into a less cytotoxic one, supplemental CaP_i creates a less aggressive environment for the endogenous lactobacilli and stimulates their growth. The increased numbers of intestinal lactobacilli might have improved the resistance to colonization and translocation of salmonella in rats. The growth-promoting activity of dietary CaP_i for the endogenous lactobacilli is probably also relevant for the functionality of probiotic strains used in foods. It is generally accepted that lactobacilli used as dietary adjunct must be able to survive the hostile environment in the gastro-intestinal tract and proliferate [6]. That aim might be achieved better when an adequate CaP_i intake is ensured.

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Chapter 7

Summary and concluding remarks

SUMMARY

Gastrointestinal infections are still a major health problem, not only in developing countries. Even in Europe and the United States about 10-15 % of the population contracts an intestinal infection each year, mostly of foodborne origin. The growing resistance of pathogens to antibiotics stresses the importance to prevent and treat intestinal infections by other means. Modulation of the diet to improve host resistance to foodborne infections might be an attractive, alternative approach. The diet determines the composition of intestinal contents, which in turn affects the gastrointestinal survival of pathogens, the protective endogenous microflora and the epithelial barrier function. These parameters ultimately determine the susceptibility of the host to intestinal infectious disease. Scientific interest in dietary modulation of the resistance to intestinal infections is just emerging. Notwithstanding the results of numerous in-vitro studies, strictly controlled infection studies showing the importance of the diet (supplemented with pre- or probiotics) to inhibit or ameliorate intestinal infections in-vivo are scarce. Even less is known about the potential protective effect of dietary calcium on the resistance to intestinal infections. At the same time, evidence accumulates showing that calcium is likely an antipromoter of colon carcinogenesis. In the intestine, calcium forms an insoluble complex with phosphate which strongly binds bile acids and fatty acids. In soluble form, these surfactants are highly irritating to the intestinal epithelium. Therefore, precipitation of bile acids and fatty acids by calcium phosphate decreases luminal cytotoxicity, resulting in diminished epithelial cell damage and reduced epithelial proliferation. This may also be relevant for host resistance to intestinal infections. A reduced epithelial cell damage may strengthen the mucosal barrier function. In addition, it can be speculated that the decreased cytotoxicity of intestinal contents by calcium phosphate may stimulate growth of the protective endogenous microflora and improve its antagonistic activity towards invading pathogens. Figure 1 summarizes the hypothetical mechanism by which dietary calcium phosphate may decrease the severity of an intestinal infection, for instance caused by salmonella.

The strictly-controlled experimental studies described in this thesis mainly focused on the proposed protective effect of dietary calcium phosphate on the resistance to intestinal infections. The rat was chosen as animal model and the invasive pathogen *Salmonella enteritidis* as infective agent. Salmonellosis is one of the most common foodborne, bacterial infections in the world and its pathology in humans and rodents is quite similar. The first study of this thesis investigated the application of urinary nitrate excretion as a marker of intestinal bacterial translocation (chapter 2). To study dietary modulation of host resistance to translocation of pathogens a non-invasive, sensitive and quantifiable marker is needed. Classical organ cultures do not meet those criteria. Nitric oxide (NO) is produced by inducible nitric oxide synthase of phagocytes upon contact with bacteria or cell wall components of bacteria, like lipopolysaccharides. To prevent damage to host cells, NO is rapidly oxidized to nitrite and nitrate (summed as NO_x) and these are quantitatively excreted in urine. It was shown that intraperitoneally injected *S. enteritidis* lipopolysaccharides

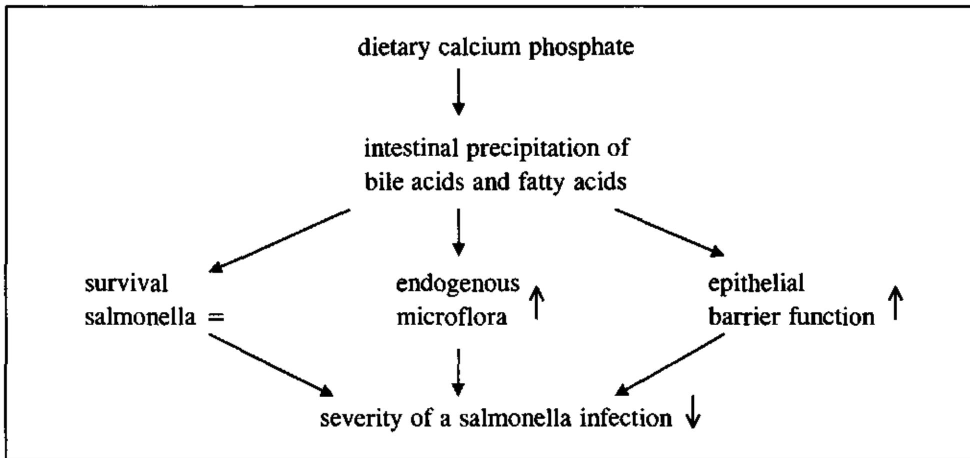

Figure 1 Mechanism by which dietary calcium phosphate may decrease the severity of an intestinal salmonella infection.

transiently increased the urinary NO_x output within a certain dose-range. Concomitant administration of a competitive inhibitor of nitric oxide synthase (N^G-nitro-L-arginine methyl ester) almost completely abolished the rise in NO_x excretion. Importantly, increasing the oral dose of viable *S. enteritidis* resulted in a time- and dose-dependent exponential increase in urinary NO_x excretion. Translocation was a prerequisite for provoking a NO_x response, because neither orally administered, heat-killed *S. enteritidis* nor non-invasive, enterotoxigenic *Escherichia coli* (data not shown) induced an increase in NO_x excretion above base-line level. Total urinary NO_x excretion after infection of the rats with viable *S. enteritidis* and weight of the mesenteric lymph nodes were highly correlated.

After validation of this new translocation marker, the effect of different milk products (low-calcium milk, milk, milk acidified with hydrochloric acid, and pasteurized yogurt) on the resistance of rats to *S. enteritidis* was studied (chapter 3). Compared with the low-calcium milk group, all high-calcium groups had an increased colonization resistance, as judged by the strongly reduced fecal salmonella excretion in time. The yogurt-fed rats had the best colonization resistance. Before infection, the bile acid concentration and cytotoxicity of fecal water of the low-calcium milk group were significantly higher than those of the high-calcium groups. The reduced resistance of the low-calcium milk group corresponded with strong infection-induced disturbances of normal intestinal physiology. For instance, the apparent iron absorption was reduced and considerable increases in cytotoxicity of fecal water, fecal mucin and alkaline phosphatase excretion were observed in this group. The least infection-induced changes in luminal parameters were noticed in the yogurt-fed rats. Surprisingly, no infection-induced increase in urinary NO_x excretion was observed in this study (data not shown).

As the milk-based diets differed in several respects, another strictly controlled infection study was performed with rats on purified diets differing only in calcium phosphate (20, 60 and 180 mmol/kg) content (chapter 4). Compared with the low-calcium group, the medium- and high-calcium group shedded 10-1000 times less salmonella in their feces and thus had a substantially improved colonization resistance. Calcium supplementation also reduced translocation of salmonella, considering the diminished urinary NO_x excretion and decreased viable salmonella counts in the ileal Peyer's patches and spleen. As shown earlier, the bile acid concentration and cytotoxicity of fecal water were decreased by dietary calcium phosphate. This resulted in an increased fecal output of several bacterial mass markers, indicating a stimulation of the endogenous microflora. Besides an enhanced fecal dry weight excretion, dietary calcium phosphate also increased fecal nitrogen, phospholipid and organic phosphate output.

The non-digestible disaccharide lactulose is well-fermented by the intestinal microflora and has been used successfully in the treatment of certain intestinal infections. The organic acids (e.g. lactic acid) formed during bacterial lactulose fermentation probably play an important role in this protection. Nevertheless, excess acid production may damage the intestinal epithelium and even impair the mucosal barrier function. Considering the above-mentioned resistance-enhancing effects of dietary calcium phosphate and its ability to increase the intestinal buffering capacity, the possible superiority of a combination of dietary lactulose and calcium phosphate to improve host resistance was studied (chapter 5). *S. enteritidis* appeared to be very sensitive to lactic acid in-vitro, whereas *Lactobacillus acidophilus* (as a representative of the protective endogenous microflora) was unaffected. The infection experiment showed that dietary lactulose decreased fecal shedding of salmonella, thus increased the colonization resistance. The protective effects of lactulose were limited to the cecum and colon because this disaccharide did not decrease translocation of salmonella, as measured by urinary NO_x excretion. In agreement with the study described above, calcium phosphate significantly inhibited translocation of salmonella. It is known that mucosal invasion of salmonella mainly takes place in the ileum, a region of the intestinal tract with a relatively less dense bacterial population. Obviously, the fermentation of lactulose in the ileum is limited and not sufficient to prevent translocation of salmonella. Supplementation of a lactulose diet with calcium phosphate reversed the unfavorable increased cytotoxicity of fecal water. In addition, calcium phosphate stimulated lactulose fermentation, as judged by the reduced lactulose excretion in feces and increased fecal lactic acid, ammonia, and nitrogen excretion.

Finally, it was investigated whether the calcium phosphate-induced protection against colonization and translocation of salmonella was mediated by a stimulation of the intestinal lactobacilli (chapter 6). In-vitro, *L. acidophilus* was rapidly killed by physiologically relevant concentrations of fatty acids and (un)conjugated bile acids. In contrast, even high concentrations of these surfactants did not affect the viability of *S. enteritidis*. Calcium phosphate-supplementation reduced the cytotoxicity and the concentration of bile acids and fatty acids in ileal contents and fecal water of rats. Moreover, calcium phosphate notably changed the composition of ileal bile acids into a less

cell-damaging direction. Consequently, significantly increased numbers of lactobacilli were detected in ileal contents, on the ileal mucosa and in feces of non-infected, calcium phosphate-supplemented animals. At the same time, the calcium phosphate group had less viable salmonella in ileal contents, on the ileal mucosa and in feces. In accordance, the infection-induced urinary NO_x excretion was diminished by calcium phosphate supplementation.

CONCLUDING REMARKS

The following conclusions can be drawn from the experimental studies described in this thesis:

1. The NO_x (sum of nitrite and nitrate) excretion in urine is a sensitive, non-invasive marker to quantify intestinal bacterial translocation and to follow the course of a systemic infection (chapter 2).
2. Milk or milk products, naturally rich in calcium, improve the colonization resistance of rats to salmonella. Compared with milk, pasteurized yogurt has an additional protective effect. The increased resistance to salmonella infection by these products is accompanied with reduced infection-induced disturbances of normal intestinal physiology (chapter 3).
3. Dietary calcium phosphate strongly increases the colonization and translocation resistance of rats to salmonella. Calcium phosphate precipitates intestinal bile acids, reduces the cytotoxicity of fecal water and increases fecal bacterial mass excretion (chapter 4). Therefore, the protective effect of calcium phosphate might be mediated by a stimulation of the endogenous microflora in general and enhancing its antagonistic activity towards pathogens and/or by strengthening the epithelial barrier function.
4. *S. enteritidis* is very sensitive to lactic acid, an important bacterial fermentation product of lactulose. Dietary lactulose improves the colonization resistance of rats to salmonella but does not protect against translocation of this pathogen in the ileum. Supplementation of a lactulose diet with calcium phosphate notably stimulates lactulose fermentation, probably by increasing the luminal buffering capacity. Morover, it reverses the unfavorable fermentation-mediated increase in luminal cytotoxicity and diminishes translocation of salmonella (chapter 5).
5. Lactobacilli are very sensitive to intestinal surfactants. By precipitating bile acids and fatty acids in the intestinal lumen and changing the composition of the ileal bile acids into a less cytotoxic one, supplemental calcium phosphate creates a less aggressive environment for the endogenous lactobacilli and stimulates their growth. The increased numbers of intestinal lactobacilli (especially in the ileum) might have improved the resistance to colonization and translocation of salmonella in rats (chapter 6).

ADDITIONAL REMARKS

Besides these relatively direct conclusions of the experiments described in this thesis, additional remarks should be made and some important questions need to be answered. For instance, the application of urinary NO_x excretion as a translocation marker in dietary intervention studies needs discussion. What are the implications of the results obtained with rats for the human situation and for the development of products containing probiotics? Finally, alternative mechanisms for the protective effect of dietary calcium phosphate and suggestions for further research are presented.

Application of urinary NO_x excretion as translocation marker

The application of urinary NO_x excretion as a marker of intestinal bacterial translocation is not limited to salmonella infections nor to rats only. The production of NO by phagocytes is a rapid, aspecific response to many bacteria and bacterial components. Furthermore, this non-invasive method is also applicable in humans. Patients suffering from gut-derived septicemia also have elevated serum and urinary NO_x levels. The measurement of urinary NO_x excetion as a translocation marker appeared to be very useful in the dietary intervention studies described in this thesis, except in the study testing several milk products (chapter 3). No increase in urinary NO_x excretion was observed after the oral challenge with *S. enteritidis* in that study (data not shown). There were no indications that the administered salmonella strain had lost its virulence. As usual, before use *S. enteritidis* was passaged in Wistar rats and subsequently isolated from liver or spleen. In addition, DNA analysis of this strain showed that the most important virulence plasmid was still present (data not shown). As the composition of the milk-based diets (chapter 3) was markedly different from the composition of the purified diets used in the other studies (chapter 2, 4-6), an effect of the diet on the NO_x production capacity of phagocytes could not be excluded. However, in a recently executed experiment, milk-fed rats and rats fed a purified diet had a similar high increase in urinary NO_x excretion after an intraperitoneal injection with lipopolysaccharides, showing that the milk diet did not impair the NO production capacity of phagocytes (data not shown). Therefore, the possibility remains that the absence of the NO_x response in rats fed milk-based diets and challenged with an oral dose of salmonella indicates that these diets completely prevented translocation of this invasive pathogen. This intriguing possibility certainly needs further study.

Extrapolation to the human situation

The infection experiments described in this thesis were conducted with rats. The complete absence of literature data about protective effects of calcium on intestinal infections did not justify the immediate performance of human intervention studies. What's more, well-controlled infection studies are difficult to perform in healthy subjects. However, the course and pathology of salmonellosis in rats is largely similar to that in humans. In both species the infective dose of *S. enteritidis* is of the same magnitude and salmonellosis can result in an extra-intestinal, systemic infection. For these reasons, the rat is a convenient and relevant model to study dietary effects on the resistance to salmonella

infection. Nevertheless, caution should be taken in directly extrapolating the observed beneficial effects of dietary calcium phosphate in rats to the human situation. The protective effect of calcium phosphate might be mediated by stimulation of the endogenous microflora and lactobacilli in particular (chapters 4 and 6). As mentioned in the General introduction section (Table 1), the intestinal microflora in humans and rats may be different, especially in quantitative respects. The total bacterial load of the small intestine is higher in rats than in man. However, as far as classical microbiological techniques can define, the differences on genera level seem to be small. In rats, as well as in humans lactobacilli are one of the predominant bacterial species in the ileum. Based on this, one of the first subjects of further study should be to verify the trophic effect of dietary calcium phosphate on intestinal lactobacilli in humans. In this regard it is important that the dose of calcium phosphate as administered to the rats is also feasible in the human diet. The main sources of dietary calcium are milk and dairy products, which account for approximately 70% of our daily calcium intake. A study described in this thesis (chapter 4) showed that a concentration of 60 mmol calcium per kg dry feed already gave significant protection against salmonella. Assuming that an average adult consumes about 500 g dry matter per day and considering that 1 liter of milk contains 30 mmol (or 1200 mg) calcium, this amount of milk (or dairy product) would be sufficient to improve the resistance to salmonella. As mentioned above, it is not easy to perform infection studies in humans. Nevertheless, possibilities do exist. For instance, dietary modulation of the colonization resistance to a safely-applicable, live, attenuated, oral vaccine can be studied. The hypothesis is that dietary calcium phosphate decreases the efficacy of a vaccine containing a Gram-negative pathogen.

Implications for the efficacy of probiotics

The final experiment described in this thesis shows that dietary calcium phosphate increases the numbers of autochtonous lactobacilli in the intestinal tract (chapter 6). By precipitating intestinal bile acids and fatty acids and decreasing the cytotoxicity of luminal contents, calcium phosphate improves the survival of lactobacilli and stimulates their growth. This may also have important implications for the efficacy of probiotics added to foodstuffs. At the moment, lactobacilli and bifidobacteria are the genera most frequently used as adjunct in probiotic products. To improve intestinal microbial balance and to increase the resistance of the flora to pathogens, the probiotic needs to be alive. It may be expected that, analogous to the autochtonous lactobacilli, the composition of the diet and subsequently the composition of gastrointestinal contents also determine the gastrointestinal survival and thus the functional effects of a probiotic strain. In other words, there is likely a strong interaction between the composition of the diet and the functionality of a probiotic. Up to now, this possible interaction is completely overlooked by probiotic researchers. What's more, the variable results reported about health effects of probiotics may well be due to differences in dietary regimen between studies. Fortunately, probiotics are often added to milk or dairy products, though non-dairy products containing probiotics are also on the market today. Milk and dairy products have a high calcium content and equimolar amounts of phosphate. This dairy matrix is a probably a good vehicle to deliver probiotics

to the intestinal tract and to achieve functional effects. However, further investigations will be necessary to find optimal formula to administer priobiotics to the consumer. For that purpose, more synergy between microbiologists and nutritionists is needed in probiotic research.

Alternative mechanisms for the protective effect of calcium phosphate

Without doubt, the endogenous microflora is very important for the resistance to intestinal infections. The colonization resistance is the result of complex interactions between bacteria and can not be attributed to a single genus of intestinal bacteria. Nevertheless, lactobacilli might play an important role in the resistance to intestinal infections. The observed stimulation of the endogenous lactobacilli by supplemental calcium phosphate (chapter 6), especially in the ileum, might have resulted in the decreased colonization and translocation of salmonella in rats. Calcium phosphate supplementation also increased the numbers of enterobacteria in the ileum (chapter 6). Though, one can say that this is an undesirable side-effect, one can also argue that these normal residents of the intestinal tract are valuable for the colonization resistance. Due to homology, enterobacteria might be stronger competitors with salmonella for adhesion sites on epithelial cells and nutrients for growth. In this regard, the results of a few non-controlled experiments described in literature were disappointing, showing no or even adverse effects of orally administered enterobacteria in the prevention or therapy of intestinal infections. However, stimulation of host-specific, endogenous enterobacteria is probably a different and safer approach than the administration of host-foreign enterobacteria. As discussed earlier, the improvement of the resistance to salmonella might also be due to strengthening of the mucosal barrier function. Calcium phosphate-mediated precipitation of intestinal surfactants decreases the cytotoxicity of luminal contents (chapters 4 and 6) and is known to diminish epithelial cell damage.

Besides an effect of dietary calcium on these determinants of host resistance, a direct effect of calcium phosphate on samonella in the intestine is also possible. Salmonella is a Gram-negative bacterium and its outermembrane is composed of lipopolysaccharides. Lipopolysaccharides are large, amphipathic molecules with a fatty acid-containing hydrophobic part and a saccharide-containing, hydrophilic part. Considering this structure, calcium phosphate might also have affinity for lipopolysaccharides, analogous to bile acids and fatty acids. Indeed, preliminary in-vitro experiments indicate that amorphous calcium phosphate binds and precipitates lipopolysaccharides. Moreover, calcium phosphate supplementation decreases the lipopolysaccharide concentration in rat and human fecal water (data not shown). Therefore, the observed protective effects might also be mediated by binding of salmonella to calcium phosphate in the intestinal tract and preventing the adhesion of this pathogen to the intestinal mucosa. Further research to verify this mechanism is in progress.

The studied and suggested mechanisms by which dietary calcium phosphate might increase the resistance to salmonella do not exclude each other. However, knowledge of

their importance is essential for the design of human intervention trials. For that purpose, combined biochemical and microbiological in-vitro experiments and animal studies are very useful and really the only accessible way. Ultimately, the protective effects of dietary calcium phosphate against intestinal infections need to be verified in humans. As mentioned above, it is possible to perform well-controlled infection studies in humans. For instance, the effect of dietary calcium phosphate on the colonization resistance can be studied by measuring the fecal excretion of a safely-applicable, live, attenuated, oral vaccine. If confirmative, it seems also worthwhile to study the possible protective effects of calcium phosphate supplementation in subjects with inflammatory bowel diseases, who are generally prone to gut-derived septicemia. Research on modulation of the resistance to intestinal infections by calcium phosphate or other dietary components is still in an early stage, but the results presented in this thesis certainly encourage the exploration of dietary intervention in the prevention or treatment of intestinal infectious disease.

Beïnvloeding van de weerstand tegen darminfecties door de voeding

Samenvatting

Het vóórkomen van darminfecties

Darminfecties zijn nog steeds een groot gezondheidsprobleem. Wereldwijd bezien wordt het aantal infecties waarbij acute diarree optreedt geschat op zo'n 3-5 miljard gevallen, resulterend in 3-5 miljoen doden per jaar. In tegenstelling tot wat vaak gedacht wordt zijn darminfecties niet alleen een probleem in ontwikkelingslanden. In Nederland loopt jaarlijks 1 op elke 8 personen een voedselinfectie op. Vaak wordt de oorzaak van de infectie niet achterhaald. Als dat wel het geval is, wordt van de bacteriële pathogenen (ziekteverwekkers) meestal campylobacter aangetroffen, gevolgd door salmonella. Hoewel men zich flink beroerd kan voelen als een voedselinfectie is opgelopen, blijft de schade meestal beperkt tot buikkrampen, diarree, misselijkheid en overgeven gedurende een aantal dagen. Toch kent ook iedereen de regelmatig opduikende kranteberichten dat inwoners van een verzorgings- of bejaardentehuis zijn overleden na het eten van bavarois of onvoldoende verhit voedsel. Bij ouderen en door ziekte verzwakte personen is blijkbaar de weerstand tegen een voedselinfectie verminderd. Hierdoor blijven de gevolgen soms niet beperkt tot een infectie van de darm, maar worden ook vitale organen zoals de lever geïnfecteerd. Deze levensgevaarlijke situatie wordt bloedvergiftiging of sepsis genoemd.

Het verloop van een darminfectie

Alhoewel verschillende pathogenen een verschillend ziektebeeld oproepen, zijn er toch een aantal gemeenschappelijke fasen te onderkennen in het verloop van een darminfectie. De meest relevante voedselpathogenen (campylobacter, salmonella, *Escherichia coli*) veroorzaken met name schade in de dunne darm. Om niet direct uitgespoeld te worden zullen ze zich moeten hechten aan de darmwand. De epitheelcellen van de darmwand hebben echter een korte levensduur. Na 4-6 dagen worden ze afgestoten naar het darmlumen en uitgescheiden met de ontlasting. Eventueel aangehechte bacteriën verdwijnen dan ook. Voor handhaving moeten bacteriën zich dus constant vermenigvuldigen om hechting aan nieuw gevormde epitheelcellen mogelijk te maken. Hechting van ziekteverwekkende bacteriën aan de darmwand of de door hen uitgescheiden toxines veroorzaken vaak schade aan epitheelcellen waardoor deze minder goed functioneren of zelfs stuk gaan. Sommige darminfecties kunnen zich uitbreiden naar andere delen van het lichaam. Salmonella bijvoorbeeld is in staat om, na hechting aan de darmwand, door de darmwand heen te dringen en via lymfe en bloed andere inwendige organen te infecteren. Het passeren van de darmwand door bacteriën wordt 'translocatie' genoemd en resulteert in een levensbedreigende infectie.

Verdedigingsmechanismen in het maagdarmkanaal

Aangezien vrijwel geen enkel voedingsmiddel vrij is van (ziekteverwekkende) bacteriën is het opmerkelijk dat we niet constant last hebben van darminfecties. Gelukkig beschikken we over een heel scala aan aspecifieke verdedigingsmechanismen in ons maagdarmkanaal. De eerste en zeer belangrijke barrière is de maag. De meeste ziekteverwekkende bacteriën die met het voedsel worden ingenomen kunnen slecht tegen de zure maaginhoud en worden hierdoor grotendeels afgedood. Bacteriën die de maagpassage overleven komen vervolgens in de dunne darm terecht. Hier is de zuurgraad weliswaar neutraal, maar is de

concentratie van toxische galzouten (uitgescheiden door de galblaas) en vetzuren (ontstaan door afbraak van vetten door spijsverteringsenzymen) dusdanig hoog dat voedselpathogenen het vaak niet overleven. Bovendien wordt in de gehele darm de darmwand van het darmlumen afgeschermd door een slijmlaag die moeilijk doordringbaar is voor bacteriën. Daarnaast is de passage van de spijsbrij in de dunne darm snel, zodat bacteriën gemakkelijk worden uitgespoeld. Het belangrijkste verdedigingsmechanisme van de dikke darm heeft te maken met zijn omvangrijke darmflora. Om precies te zijn: we hebben meer bacteriën in onze darmflora dan dat ons lichaam uit cellen bestaat! Voor handhaving moet een voedselpathogeen competitie aangaan met de darmflora voor voedingsstoffen en hechtingsplaatsen op de darmwand. Daarnaast produceert de darmflora zure metabolieten (melkzuur en korte-keten vetzuren) die pathogenen kunnen afdoden. Naast deze aspecifieke afweermechanismen worden er ook antilichamen aan het darmlumen afgegeven die heel specifiek gericht zijn tegen een bepaalde bacterie. De produktie van deze antilichamen vergt echter behoorlijk wat tijd en is pas 1 week na infectie functioneel. Het zorgt er wel voor dat bij herhaling van de infectie een pathogeen sneller onschadelijk gemaakt zal worden.

Bij gezonde personen zullen al deze verdedigingsmechanismen er in de meeste gevallen voor zorgen dat met het voedsel ingenomen ziekterverwekkers efficiënt worden uitgeschakeld. Er zijn echter ook groepen personen waarbij deze verdedigingsmechanismen niet goed werken. Bekend is bijvoorbeeld dat de maagzuurproduktie bij ouderen vaak sterk verminderd is. Hierdoor overleeft een groter percentage van de voedselpathogenen de maag. De statistieken laten dan ook zien dat ouderen vaker darminfecties hebben dan jong volwassenen. Een andere groep die gevoelig is voor darminfecties zijn personen die behandeld zijn met antibiotica. Verschillende antibiotica onderdrukken de eigen darmflora. Hierdoor is deze niet meer in staat om voldoende competitie te leveren, waardoor pathogenen meer kans krijgen om te overleven en te hechten aan de darmwand. Na een kuur met antibiotica kunnen bijvoorbeeld ernstige clostridium infecties optreden.

Beïnvloeding van de weerstand tegen darminfecties door de voeding

De behandeling van infecties wordt steeds moeilijker omdat verschillende pathogenen resistent dreigen te worden tegen antibiotica of het inmiddels zelfs al zijn. Onderzoek naar alternatieven om darminfecties te voorkómen of te behandelen is dus erg belangrijk. Eén aanpak zou kunnen zijn om met behulp van de voeding de weerstand tegen darminfecties te verhogen. Hoe kan voeding de weerstand tegen darminfecties beïnvloeden? De samenstelling van de voeding bepaalt de samenstelling van de inhoud van het maagdarmkanaal. Dit heeft op zijn beurt een effect op de overleving van voedselpathogenen tijdens hun passage door het maagdarmkanaal. Daarnaast wordt de compositie en activiteit van de beschermende darmflora en de barrièrefunctie van de darmwand beïnvloed door componenten of voedingsstoffen in het darmlumen. Interacties tussen de ziekteverwekkende bacteriën, de darmflora en de darmwand bepalen uiteindelijk de weerstand tegen pathogenen. Onderzoek naar de effecten van de voeding op de weerstand tegen darminfecties staat nog in de kinderschoenen. In de wetenschappelijke literatuur zijn weinig (correct opgezette) proeven beschreven. Toch is er op het moment, zowel in de wetenschappelijke wereld als in het bedrijfsleven, erg veel belangstelling voor 'functional foods'. Dit zijn voedingsmiddelen waaraan specifieke stoffen (prebiotica)

of bacteriën (probiotica) zijn toegevoegd met als doel de gezondheid van de consument te handhaven of te verbeteren. Gezondheid is natuurlijk een heel breed begrip, maar vaak streeft men naar het versterken of verbeteren van de darmflora waardoor uiteindelijk de weerstand tegen darminfecties kan toenemen.

Hoe wordt weerstand tegen darminfecties gemeten?

Voor het vaststellen van de effecten van de voeding op de weerstand tegen darminfecties zijn een tweetal parameters erg belangrijk. De eerste is het meten van de zogenaamde 'kolonisatieweerstand'. De kolonisatieweerstand is de weerstand die voedselpathogenen ondervinden bij hun pogingen om te hechten aan de darmwand en zich te vermenigvuldigen. Bij een lage kolonisatieweerstand kan een pathogeen zich goed handhaven en vermenigvuldigen en dit uit zich door een continue uitscheiding van grote aantallen van het pathogeen in de ontlasting. Bij een hoge kolonisatieweerstand kan een pathogeen zich slecht handhaven in de darm en is de uitscheiding in de ontlasting in de tijd gezien veel minder. Een tweede belangrijke weerstandsparameter is de 'translocatieweerstand'. Zoals boven vermeld, zijn sommige pathogenen (o.a. salmonella) in staat te transloceren, dus door de darmwand heen te dringen. De translocatieweerstand is de weerstand die een pathogeen ondervindt bij de invasie van inwendige organen vanuit de darm. De klassieke methode om de mate van translocatie te bepalen berust op de telling van het aantal pathogenen in bijvoorbeeld lever of milt met behulp van microbiologische kweektechnieken. Deze klassieke methode heeft jammer genoeg een groot aantal tekortkomingen. Het eerste nadeel is dat organen natuurlijk alleen van proefdieren verkregen kunnen worden en niet van mensen. Een ander nadeel is dat de microbiologische kweektechnieken nogal ongevoelig zijn en kleine veranderingen niet waarnemen. Het belangrijkste nadeel is misschien nog wel dat alleen de nog levende bacteriën in organen geteld worden, terwijl het grootste deel van de pathogenen na translocatie wordt afgedood door het aspecifieke immuunsysteem.

De experimenten: proefdieren versus mensen

Voor het uitvoeren van voedingsexperimenten met mensen heeft men toestemming nodig van een Medisch Ethische Commissie. Zo'n commissie van deskundigen beoordeelt of de geplande handelingen en metingen in een humane proef ethisch verantwoord zijn en bijdragen aan het beantwoorden van de onderzoeksvraag. Als reeds positieve resultaten gerapporteerd zijn in proefdieren, zal eerder toestemming worden gegeven voor het daadwerkelijk uitvoeren van een humane proef. Zoals hierboven vermeld, zijn er weinig goed gecontroleerde proeven beschreven in de literatuur die laten zien dat de voeding de weerstand tegen darminfecties beïnvloedt. We verkeren dus momenteel in een fase waarin vooral dierexperimenteel onderzoek nodig is om de effecten van voedingscomponenten vast te stellen en werkingsmechanismen te achterhalen. Als het werkingsmechanisme bekend is, heeft men namelijk beter inzicht in de noodzakelijke veranderingen in de voeding die nodig zijn voor het bereiken van het meest optimale effect. Kennis van het achterliggend mechanisme is vaak ook nodig om te bepalen welke metingen men moet verrichten in een latere humane proef om het effect van de voeding vast te stellen.

De experimenten beschreven in dit proefschrift gaan over de effecten van de voeding op het verloop van een salmonella infectie in ratten. Je kunt je afvragen in

hoeverre een rat lijkt op de mens? Wat betreft salmonella infecties is reeds het een en ander bekend. De orale dosis die nodig is om ratten en mensen te infecteren is ongeveer gelijk. Ook het (tijds)verloop van de darminfectie en de mogelijkheid dat de infectie zich uitbreidt naar andere organen buiten de darm is voor rat en mens gelijk. Daarom is de rat een relevant en handig model voor de mens om de voedingseffecten op weerstand tegen infecties te onderzoeken. Natuurlijk zullen de bevindingen van deze dierexperimenten nu geverifieerd moeten worden bij mensen.

Ontwikkeling van een nieuwe methode om bacteriële translocatie te meten

Zoals hierboven beschreven, heeft de klassieke bepaling van de mate van translocatie van pathogenen nogal wat nadelen. Daarom is eerst een nieuwe methode ontwikkeld waarbij de uitscheiding van nitriet en nitraat (opgeteld als NO_x) in de urine gebruikt wordt als maat voor translocatie. Urine is, zowel bij mensen als bij dieren, veel gemakkelijker en op verschillende tijdstippen te verzamelen. Stikstofmonoxide (NO) wordt o.a. geproduceerd door cellen van het aspecifieke immuunsysteem als afweerreactie op getransloceerde pathogenen. NO is een reactief molecuul en toxisch voor bacteriën. Het lichaam beschermt zichzelf tegen dit molecuul door het snel om te zetten in NO_x dat veel minder toxisch is. NO_x wordt vervolgens uitgescheiden in de urine. Uit proeven is nu gebleken dat er een dosis-afhankelijk verband is tussen de hoeveelheid oraal toegediende salmonella's, het aantal getransloceerde salmonella's en de uitscheiding van NO_x in de urine. Door de dagelijkse NO_x uitscheiding in de urine te meten kan dus het hele verloop van de translocatie tijdens een infectie gevolgd worden (hoofdstuk 2).

Calciumfosfaat in de voeding beschermt tegen een salmonella infectie

De laatste jaren staat calcium sterk in de belangstelling omdat het mogelijk het risico op het ontstaan van dikke darmkanker vermindert. In de darm vormt calcium een onoplosbaar complex met fosfaat afkomstig uit de voeding. Dit calciumfosfaat bindt en inactiveert toxische galzouten en vetzuren in de darm. Als de darminhoud minder toxisch is, is de schade aan het darmepitheel minder en wordt de celdelingssnelheid geremd. Die lagere celdelingssnelheid beschermt waarschijnlijk tegen het ontstaan van darmtumoren. Het lijkt op het eerste gezicht misschien wat vreemd, maar soortgelijke mechanismen beschermen mogelijk ook tegen darminfecties. Vermindering van de schade aan het darmepitheel door verlaging van de toxiciteit van de darminhoud, kan de barrièrefunctie van de darmwand verbeteren. Hierdoor zal de weerstand tegen darminfecties toenemen.

Deze hypothese over de beschermende effecten van calciumfosfaat in de voeding tegen salmonella is nu bevestigd in dierexperimenteel onderzoek. Toevoeging van (puur) calciumfosfaat aan de voeding beschermde tegen kolonisatie van salmonella. De dieren hadden minder salmonella in de inhoud van de dunne darm, minder salmonella gehecht aan de dunne darmwand en ook de uitscheiding van salmonella in de ontlasting was duidelijk minder. Nog belangrijker is dat calciumfosfaat ook beschermde tegen translocatie van salmonella in de dunne darm. Dit bleek zowel uit orgaankweken als uit een sterk verminderde stijging van de NO_x uitscheiding in de urine na orale infectie (hoofdstuk 4 en 6).

Normaal gesproken krijgen we geen puur calciumfosfaat binnen met de voeding. Fosfaat is ruim aanwezig in heel veel verschillende voedingsmiddelen. Calcium daarentegen wordt in onze voeding hoofdzakelijk geleverd door melk en

zuivelprodukten. De vraag is dus of consumptie van melkprodukten net zo goed beschermt tegen een salmonella infectie dan de toevoeging van puur calciumfosfaat aan de voeding. Dit bleek het geval te zijn. Consumptie van normale melk, aangezuurde melk en vooral yoghurt leidde tot een hoge kolonisatieweerstand tegen salmonella, terwijl consumptie van een een laag-calcium melk de weerstand sterk verminderde. De ernstige infectie in de laag-calcium melk groep ging gepaard met een sterke verstoring van de normale darmfysiologie. Zo was bijvoorbeeld de uitscheiding van slijm in de ontlasting sterk verhoogd en de absorptie van mineralen uit de voeding verminderd. Yoghurt bood de beste bescherming. Dit heeft te maken met het hoge melkzuurgehalte van yoghurt. Gebleken is dat salmonella gemakkelijk wordt afgedood door melkzuur (hoofdstuk 3 en 5).

Wat is het mechanisme achter het beschermend effect van calciumfosfaat?

Waarschijnlijk is niet een enkel mechanisme verantwoordelijk voor het positieve effect van calciumfosfaat, maar is het weerstands-verhogend effect het gevolg van een samenspel van meerdere mechanismen. In de darm vormen calcium en fosfaat een onoplosbaar complex. Celbeschadigende galzouten en vetzuren in de darm worden sterk gebonden aan calciumfosfaat, waardoor de toxiciteit van de darminhoud daalt. Als gevolg hiervan wordt er minder schade aan het darmepitheel toegebracht en verbetert de barrièrefunctie van de darmwand. Uit proeven is nu ook gebleken dat de verminderde toxiciteit van de darminhoud niet alleen het darmepitheel beschermt maar ook de darmflora versterkt (hoofdstuk 4). De lactobacillen (onderdeel van de beschermende darmflora) bleken heel gevoelig te zijn voor galzouten en vetzuren. Verlaging van de concentratie van galzouten en vetzuren in de darm door extra calciumfosfaat in de voeding bevorderde de groei van de lactobacillen in de darm (hoofdstuk 6). Mogelijk heeft salmonella hierdoor meer competitie ondervonden op het gebied van voedingsstoffen, nodig voor bacteriële groei, en hechtingsplaatsen op het darmepitheel. Voorlopige experimenten laten zien dat er ook een direct effect van calciumfosfaat op salmonella is. Salmonella en componenten van de buitenmembraan van salmonella (de zogenaamde lipopolysaccharides) binden ook aan calciumfosfaat. Het is niet onwaarschijnlijk dat de weerstand tegen kolonisatie en translocatie is verbeterd door binding van salmonella aan calciumfosfaat, met als gevolg een verminderde hechting van dit pathogeen aan de darmwand. Momenteel wordt dit mechanisme verder onderzocht.

Samenspel tussen calciumfosfaat en andere componenten in de voeding

Prebiotica, suikers die niet verteerd kunnen worden door spijsverteringssappen in het maagdarmkanaal maar die wel een goed substraat zijn voor de darmflora, staan momenteel volop in de belangstelling van de voedingsmiddelenindustrie. Zoals boven vermeld kan een stimulatie van de darmflora leiden tot een verbeterde weerstand tegen darminfecties. De darmflora zet prebiotica om in zure produkten (onder andere melkzuur). Prebiotica in de voeding maken de darminhoud dus zuurder. Veel pathogenen, waaronder salmonella, worden afgedood door zuur. Echter, te veel zuurvorming in de darm beschadigt het darmepitheel. Daarom moet een balans worden gevonden tussen enerzijds voldoende zuurvorming ter afdoding van pathogenen en anderzijds het voorkómen van schade aan de darmwand. Het is nu gebleken dat toevoeging van calciumfosfaat aan een voeding met een potentieel prebioticum (lactulose) zorgt voor een verbeterde

afdoding van salmonella in de darm en dat tegelijkertijd de toxiciteit van de darminhoud daalt waardoor het darmepitheel beter beschermd is (hoofdstuk 5).

Conclusie

Melkprodukten en calciumfosfaat in de voeding remmen de kolonisatie en translocatie van salmonella in proefdieren en verbeteren dus de weerstand tegen een salmonella infectie. Intermediair in het beschermend mechanisme is waarschijnlijk de binding van celbeschadigende galzouten en vetzuren aan calciumfosfaat in de darm waardoor de toxiciteit van de darminhoud daalt. Hierdoor vermindert de schade aan het darmepitheel en wordt de barrièrefunctie versterkt. De verlaagde toxiciteit van de darminhoud is niet alleen goed voor de darmwand maar stimuleert ook de beschermende darmflora, met name de lactobacillen. De volgende stap is om deze weerstands-verhogende effecten van melkprodukten en calciumfosfaat te onderzoeken in mensen.

CURRICULUM VITAE

Ingeborg Marie Jacqueline Bovee-Oudenhoven is geboren op 26 december 1967 te Eibergen. In 1986 slaagde zij voor het Atheneum ß aan het Elzendaalcollege te Boxmeer. Vanaf september 1986 studeerde ze Humane Voeding aan de Landbouwuniversiteit in Wageningen. Haar eerste afstudeervak is uitgevoerd op de afdeling Gastroenterologie van het Radboudziekenhuis in Nijmegen. Het verslag over dit onderzoek is bekroond met de Parke Davis prijs voor studenten in 1989. Daarna volgden een stage bij de afdeling Toxicologie van TNO Nutrition & Food Research in Zeist en een afstudeervak bij de vakgroep Toxicologie van de Landbouwuniversiteit in Wageningen. In augustus 1991 slaagde zij cum laude voor haar doctoraal. Vanaf september 1991 is zij werkzaam bij de sectie Nutrition & Health van NIZO *food research* in Ede. Daar houdt ze zich voornamelijk bezig met onderzoek naar Functional Foods en probiotica. In oktober 1996 ontving zij de 'Young Investigator Award' van de werkgemeenschap Voeding van NWO. Onder supervisie van Dr. R. van der Meer, heeft een deel van haar onderzoek geresulteerd in dit proefschrift (promotor Prof. Dr. J.T.M. Wouters). Momenteel is zij projectleider bij NIZO *food research* en zal ze ook gedetacheerd worden bij het Wageningen Centre for Food Sciences. Zij is getrouwd met Toine Bovee en ze hebben een zoon Nick.

Ingeborg Marie Jacqueline Bovee-Oudenhoven was born on December 26, 1967 in Eibergen, The Netherlands. In 1986, she finished secondary school at the Elzendaal college in Boxmeer (Athenaeum ß). Starting in September 1986, she studied Human Nutrition at the Agricultural University of Wageningen and graduated cum laude in August 1991. In this period, her first research subject was carried out at the Department of Gastroenterology of the Catholic University in Nijmegen. The manuscript concerning this research was awarded with the Parke Davis prize 1989 for students. Subsequently, other research subjects were carried out at the Department of Toxicology of the TNO Research Centre in Zeist and at the Department of Toxicology at the Agricultural University of Wageningen. From September 1991, she is working at the Section Nutrition & Health of NIZO *food research* in Ede. Her main research subjects are Functional Foods and probiotics. In 1996 she obtained the 'Young Investigator Award' during the October Meeting of the Nutrition Working Group of the Netherlands Organization for Scientific Research (NWO). Under supervision of Dr. R. Van der Meer, part of her work has resulted in this thesis (promotor Prof. Dr. J.T.M. Wouters). Currently, she is project leader at NIZO *food research* and will also be attached to the Wageningen Centre for Food Sciences. She is married to Toine Bovee and they have a son Nick.

LIST OF PUBLICATIONS (full papers on which this thesis is based)

1. Oudenhoven IMJ, Klaasen HLBM, Lapré JA, Weerkamp AH, Van der Meer R. Nitric oxide-derived urinary nitrate as a marker of intestinal bacterial translocation in rats. **Gastroenterology** 1994; 107: 47-53.

2. Bovee-Oudenhoven IMJ, Termont DSML, Dekker PR, Van der Meer R. Calcium in milk and fermentation by yogurt bacteria increase the resistance of rats to salmonella infection. **Gut** 1996; 38: 59-65.

3. Bovee-Oudenhoven IMJ, Termont DSML, Heidt PJ, Van der Meer. Increasing the intestinal resistance of rats to the invasive pathogen *Salmonella enteritidis*: additive effects of dietary lactulose and calcium. **Gut** 1997; 40: 497-504.

4. Bovee-Oudenhoven IMJ, Termont DSML, Weerkamp AH, Faassen-Peters MAW, Van der Meer R. Dietary calcium inhibits the intestinal colonization and translocation of salmonella in rats. **Gastroenterology** 1997; 113: 550-557.

5. Bovee-Oudenhoven IMJ, Van der Meer R. Protective effects of dietary lactulose and calcium phosphate against salmonella infection. **Scandinavian Journal of Gastroenterology** 1997; 32 (suppl. 222): 112-114.

6. Van der Meer R, Bovee-Oudenhoven IMJ, Sesink ALA, Kleibeuker JH. Milk products and intestinal health. **International Dairy Journal** 1998; 8: 163-170.

7. Van der Meer R, Bovee-Oudenhoven IMJ. Dietary modulation of intestinal bacterial infections. **International Dairy Journal** 1998; in press.

8. Bovee-Oudenhoven IMJ, Wissink MLG, Wouters JTM, Van der Meer R. Dietary calcium phosphate stimulates the intestinal lactobacilli and decreases the severity of a salmonella infection in rats. **Submitted for publication.**

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Ingeborg