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Efficacy of various dietary calcium salts to improve intestinal resistance to *Salmonella* infection in rats

Sandra J. M. ten Bruggencate[1], Johannes Snel[1]*, Margriet H. C. Schoterman[2], Ellen Ertmann[2], Esther van der Meulen[1], Arjan Schonewille[1] and Ingeborg M. J. Bovee-Oudenhoven[1,3]

[1]*NIZO Food Research, Ede, The Netherlands*
[2]*FrieslandCampina Domo, Zwolle, The Netherlands*
[3]*TI Food and Nutrition, Nutrition and Health Program, Wageningen, The Netherlands*

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Abstract

Previous animal and human studies have shown protective effects of Ca on the resistance to enteropathogenic infections. Most interventions were performed with calcium phosphate and little is known about the protective effect of other dietary sources of Ca. Therefore, we investigated the efficacy of several Ca salts to enhance intestinal resistance to *Salmonella enteritidis* infection. Rats (n 7–8 per group) were fed a high-fat, Western human-style, purified diet with a low Ca content (20 mmol calcium phosphate/kg; negative control group) or the same diet supplemented with either (extra) calcium phosphate, milk Ca, calcium chloride or calcium carbonate (total of 100 mmol Ca supplement/kg). Diets contained Cr-EDTA for assessment of incremental changes in intestinal permeability. After an adaptation period of 2 weeks, animals were orally infected with *S. enteritidis* to mimic a human-relevant foodborne infection. Ca supplement-induced changes on faecal lactobacilli and enterobacteria were studied before infection. Changes in intestinal permeability were determined by measuring urinary Cr with time. Persistence of *Salmonella* was determined by studying faecal excretion of this pathogen in time. Overall, all Ca salts increased resistance towards *Salmonella*. After infection, body weight gain and food intake were higher in the calcium phosphate group. Calcium phosphate and milk Ca decreased faecal enterobacteria before infection. All Ca salts decreased infection-induced intestinal permeability and persistence of *Salmonella*. Calcium phosphate, milk Ca, calcium carbonate and calcium chloride are able to enhance the intestinal resistance to *Salmonella* in rats.

Key words: ***Salmonella*: Rats: Infection: Intestinal permeability: Calcium salts: Dairy products: Enterobacteria**

Gastrointestinal infections are still a major health problem, not only in developing countries[1,2]. The enteric pathogen *Salmonella enteritidis* is one of the leading causes of gastrointestinal infections in humans, ranging from mild, self-limiting diarrhoea and/or inflammation of the intestinal mucosa to life-threatening systemic infection[3,4]. The growing resistance of pathogenic bacteria to antibiotic drugs[4] makes it important to develop ways to prevent and treat intestinal infections by other means[5]. Besides hygienic measures, dietary modulation of host resistance to intestinal infections might be an attractive approach. By influencing the composition of gastrointestinal contents, diet affects the gastrointestinal survival of pathogens[6,7], the composition of autochthonous intestinal microbiota[8,9] and the epithelial barrier function[10]. These primary non-immunological host defence mechanisms of the gastrointestinal tract are particularly important in withstanding the first encounter with a pathogen.

Dietary Ca has been recognised to increase resistance by decreasing colonisation and translocation of common intestinal Gram-negative pathogens, both in rats[8,11] and in humans[12]. We hypothesise that this resistance-enhancing effect of calcium phosphate might be explained by three potential mechanisms. First, it is known that intake of Ca and phosphate results in the formation of an amorphous calcium phosphate complex that adsorbs and precipitates luminal cytotoxic components, such as bile acids and fatty acids. This can subsequently stimulate growth of protective members of the endogenous microbiota[8], which exerts antagonistic activity towards foodborne pathogens. Second, *Salmonella* is a pathogenic microorganism that can translocate to extra-intestinal organs such as the liver and spleen. Amorphous calcium phosphate-induced precipitation of cytotoxic components reduces epithelial cell damage[13] which might strengthen gut barrier function and reduce translocation of

* **Corresponding author:** J. Snel, fax +31 318 650 400, email hans.snel@NIZO.nl

Salmonella. Finally, amorphous calcium phosphate might bind *Salmonella* in the intestinal lumen, thereby preventing *Salmonella* adherence to the gut mucosa.

Until now, dietary intervention studies have focused on calcium phosphate. From the above-mentioned studies, it can be argued that the combination and luminal interaction of dietary Ca with phosphate is necessary for the resistance-enhancing effect as the adsorbing (binding) capacity of amorphous calcium phosphate is much larger than that of other Ca salts[14]. Little is known about the *in vivo* effects of other Ca salts on resistance to *Salmonella*. Considering the high phosphate content of human[15] and rodent[16] diets, the amorphous calcium phosphate could also be formed with other dietary Ca salts. Therefore, we tested the efficacy of several dietary Ca salts to increase the resistance of rats to *Salmonella* infection. In addition, *in vitro* experiments on pH-dependent solubility of the various Ca salts and their capacity to bind *Salmonella* were determined to gain further mechanistic insight.

Materials and methods

In vitro *experiments*

Solubility of calcium salts. Only ionic Ca can bind to phosphate and form the amorphous calcium phosphate complex in the gut lumen. Therefore, we studied the solubility of the various Ca salts in the gut lumen. Solubility of the following Ca salts was investigated: calcium phosphate ($CaHPO_4$; purity > 98 %; Merck, Darmstadt, Germany), whey product rich in milk Ca (29 % Ca, other minerals and milk salts 66 %; Vivinal® MCA FrieslandCampina Domo, Zwolle, The Netherlands) further referred to as milk Ca, calcium carbonate (purity > 99 %; Calcitec V/40S, Mineraria, Italy) or calcium chloride (purity > 99 %; Merck). These were tested at a final Ca concentration of 40 mM and at different pH levels representing the pH range of the gastrointestinal tract.The different pH levels ranging from 2 to 7 were achieved by either adding a glycine buffer (250 mM, pH 2 and 3), an acetate buffer (250 mM, pH 4 and 5) or a 3-(*N*-morpholino) propanesulphonic acid buffer (250 mM, pH 6 and 7). After 1 h incubation at 37°C, samples were centrifuged at 18 000 ***g*** for 5 min and supernatants were diluted in 0·5 g/l CsCl. Ca concentrations were determined in the diluted supernatants by inductive coupled plasma-atomic emission spectrophotometry (Varian, Mulgrave, VIC, Australia).

Binding of Salmonella. In order to test whether the amorphous calcium phosphate complex is able to precipitate *Salmonella* in the gut lumen (in contrast to crystalline calcium phosphate or ionic Ca), we adapted a method that has been previously described to study precipitation of bile salts[14]. Increasing concentrations (final 0–40 mM) of amorphous calcium phosphate, crystalline calcium phosphate or ionic Ca in HEPES buffer (500 mM) were added to a *Salmonella* suspension in saline (10^9 colony-forming units/ml). Amorphous calcium phosphate was freshly formed as described elsewhere[14]. In short, equimolar amounts of dissolved calcium chloride and sodium hydrogen phosphate were mixed to form the amorphous calcium phosphate complex. After 15 min incubation at 37°C, samples were centrifuged for 2 min at 500 ***g***. The supernatants were sonicated for 1 min using the Sonicator Ultrasonic Processor XL (Heat Systems, Farmingdale, NY, USA) to disintegrate bacterial cell walls. Total protein, a sensitive way to quantify bacterial content, was determined using the Bradford protein assay (Interchim, Montlucon, France).

In vivo *experiment*

Animals and diet. The experimental protocol was approved by the animal welfare committee of Wageningen University (Wageningen, The Netherlands). Specific pathogen-free, male, young adult (8 weeks old) Wistar rats with a mean body weight of 276 g (WU, Harlan, Horst, The Netherlands) were housed individually in metabolism cages. All the rats were kept in a temperature-(22–24°C) and humidity-controlled environment (50–60 %) with a 12 h light–dark cycle. To study the effects of the various dietary Ca salts on the main effect variables intestinal permeability and *Salmonella* persistence, the rats were randomly assigned to either an experimental diet with a total of 20 mmol/kg calcium phosphate (low-Ca negative-control group) or diets supplemented to 100 mmol Ca/kg with calcium phosphate ($CaHPO_4$; purity > 98 %; Merck), milk Ca (a whey-derived product; 29 % Ca, other minerals and milk salts 66 %; Vivinal® MCA FrieslandCampina Domo), calcium carbonate (purity > 99 %; Calcitec V/40S, Mineraria) or calcium chloride (purity > 99 %; Merck). Diets and demineralised drinking water were supplied *ad libitum*. Compared with the AIN-93 recommendation for rat diets[16], the diets had a high fat content (200 g fat/kg provided by 80 g/kg maize oil and 120 g/kg palm oil) to mimic the composition of a Western human diet. The composition of the diets has been described earlier[17]. Food intake and body weight were measured every 2–3 d before infection and daily after infection. After infection, the average of daily food intake (kJ/d) and growth was calculated per animal.

Calcium excretion. Ca was determined in freeze-dried faeces after dry-ashing and destruction. Faeces were treated with 50 g/l of TCA (1:1, v/v) and centrifuged for 2 min at 14 000 ***g***. The supernatants were diluted with 0·5 g/l CsCl and analysed by inductive coupled plasma-atomic emission spectrophotometry (Varian).

Urine was treated with 50 g/l of TCA (1:1, v/v) and centrifuged for 2 min at 14 000 ***g***. The supernatants were analysed as described for faeces. Since daily feed intake differed between animals and between dietary groups, faecal and urinary Ca excretion was calculated as percentage of intake.

British Journal of Nutrition

Composition of the intestinal microbiota. Fresh faecal samples collected 1 d before infection were analysed for the number of lactobacilli and enterobacteria. Faecal lactobacilli were quantified by plating appropriate 10-fold dilutions on Rogosa agar (Oxoid, prepared according to instructions of the manufacturer) and incubating the plates in an anaerobic cabinet (Coy Laboratory Products, Inc., Ann Arbor, MI, USA) under an anaerobic gas mixture (85 % N_2, 10 % CO_2 and 5 % H_2) at 37°C for 3 d as described previously[18]. Enterobacteria were determined by plating 10-fold dilutions on Levine EMB agar (Difco Laboratories, Detroit, MI, USA) and incubating overnight at 37°C[18].

Intestinal permeability. In order to measure intestinal permeability, the marker Cr-EDTA was added to all the diets. Cr-EDTA, an inert complex that is not actively taken up by the mucosa[19,20], was prepared as described elsewhere[21]. Total 24 h urine samples were collected on the last day before and on 7 consecutive days after oral infection of the rats. Oxytetracycline (1 mg) was added to the urine collection vessels of the metabolic cages daily to prevent bacterial deterioration. To measure Cr-EDTA, urine was treated with 50 g/l of TCA (1:1, v/v) and centrifuged for 2 min at 14 000 ***g***. The supernatants were diluted with 0·5 g/l CsCl and Cr was analysed by inductive coupled plasma-atomic emission spectrophotometry. Urinary Cr-EDTA excretion was calculated as a percentage of daily dietary Cr-EDTA intake.

Oral infection with Salmonella. After adaptation to the housing and dietary conditions for 2 weeks, the rats were orally infected by gastric gavage of 10^9 colony-forming units of *S. enteritidis* (clinical isolate, phage type 4 according to international standards; B1241 culture of NIZO food research, Ede, the Netherlands) suspended in 1 ml of saline. Stability of the infection stock solution was determined by plating serial dilutions on Modified Brilliant Green Agar (Oxoid) containing sulphmandelate (Oxoid) immediately before and after the oral dosing. *Salmonella* was cultured and stored as described earlier[22]. On day 10 after oral infection, the rats were killed by CO_2 inhalation.

Persistence of Salmonella. Immediately before and on days 1, 2, 5 and 7 after *S. enteritidis* infection, fresh faecal samples were collected directly from the anus of the animals and analysed for viable *Salmonella* by plating 10-fold dilutions on Modified Brilliant Green Agar (Oxoid) containing sulphmandelate (Oxoid) and incubating overnight at 37°C.

Statistical analysis. Results are expressed as means with their standard errors, n 7 in the calcium phosphate group and n 8 in the other groups. One animal from the calcium phosphate group was excluded from all the results because of oral–pharyngeal reflux of the *Salmonella* suspension resulting in pneumonia. A commercially available package (Statistica 6.1; StatSoft, Inc., Tulsa, OK, USA) was used for all statistics.

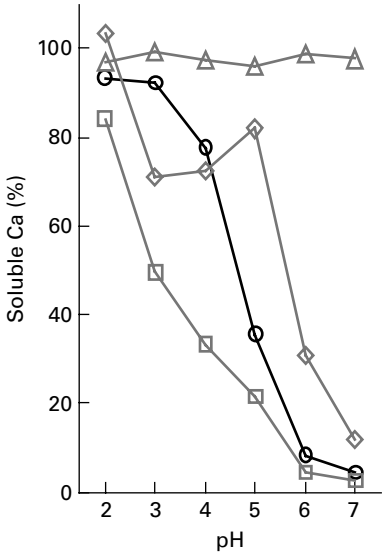

Fig. 1. Solubility of different Ca salts as a function of pH. Values are exemplary for a few studies with different Ca salts. ○, Calcium phosphate; □, milk Ca; ◇, Ca carbonate; △, calcium chloride.

All dietary Ca groups were only compared to the low-Ca control group. In case of normally distributed data (as indicated by the Shapiro–Wilk test), differences between means were tested for their significance using a one-way ANOVA, followed by the Student t test (two sided). When data were not normally distributed, differences were tested for their significance using a Kruskall–Wallis ANOVA, followed by the non-parametric Mann–Whitney U test (two sided). Repeated-measures ANOVA was used for intestinal permeability and *Salmonella* colonisation. Bonferroni correction was used for multiple testing (four comparisons). Differences were considered statistically significant when $P<0{\cdot}05$.

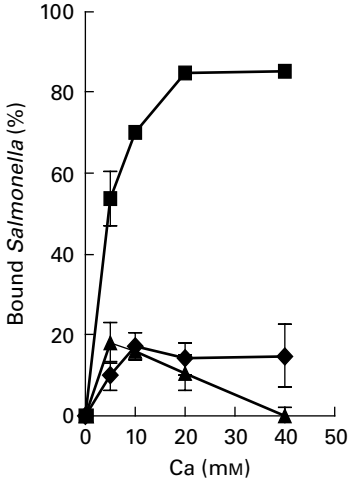

Fig. 2. Percentage of *Salmonella* bound to amorphous or crystalline Calcium phosphate or ionic Ca. Results are expressed as means and standard deviations. Values are means of triplicate incubations. Standard deviations are either shown or smaller than symbols. ■, Amorphous calcium phosphate; ◆, ionic Ca; ▲, crystalline calcium phosphate.

Results

In vitro *experiments*

Solubility of calcium salts and binding of Salmonella. Solubility of the different Ca salts was determined at pH 2–7 (Fig. 1), representing the pH range in the gastrointestinal tract. All the Ca salts were soluble at lower (gastric) pH. Calcium chloride was completely soluble at all pH levels tested. The other Ca salts were most soluble at pH 5–7.

Freshly formed amorphous calcium phosphate was capable of binding *Salmonella*. In contrast, crystalline calcium phosphate and ionic Ca showed almost no binding capacity (Fig. 2).

In vivo *experiment*

Calcium excretion and composition of the intestinal microbiota. Before infection, faecal Ca excretion (as a percentage of total intake) was higher in all Ca-supplemented groups compared to the low-Ca control group. Urinary Ca excretion was lower in the calcium phosphate and milk Ca group compared to the low-Ca control group (Fig. 3).

Before infection with *Salmonella*, no differences in faecal lactobacilli were observed between the diet groups. Calcium phosphate and milk Ca reduced the number of enterobacteria compared to the low-Ca control group (Fig. 4).

Food intake and body weight. Food consumption before infection was approximately 22 g and did not differ between the diet groups. During the first week after infection, mean food intake was significantly higher in the calcium phosphate group compared to the low-Ca group (Fig. 5(a)).

Body weight gain before infection was similar in all the diet groups. However, after infection average body weight gain was significantly higher in the calcium phosphate-supplemented group (Fig. 5(b)).

Intestinal permeability. Due to *ad libitum* feeding of the animals, daily dietary intake of Cr-EDTA (marker for intestinal permeability) differed between days and animals. Therefore, urinary Cr-EDTA excretion was calculated as a percentage of total daily dietary intakes. Urinary Cr-EDTA in the low-Ca control group strongly increased after infection, reaching a maximum at day 5 (Fig. 6). Infection-induced intestinal permeability was lower in all Ca-supplemented groups compared to the low-Ca control group. However, the kinetics of urinary Cr-EDTA was similar in all the diet groups and illustrate *Salmonella*-induced damage to the intestinal barrier.

Persistence of Salmonella. All the Ca-supplemented groups decreased persistence of *Salmonella*, as indicated by decreased faecal excretion of *Salmonella* with time (Fig. 7). In contrast, rats fed the low-Ca diet continued to excrete high *Salmonella* numbers in their faeces.

Discussion

The present study shows that besides calcium phosphate, also milk Ca, calcium carbonate and calcium chloride are able to enhance the resistance to infection, as indicated by the reduction of infection-induced intestinal permeability and the decreased persistence of *Salmonella* in

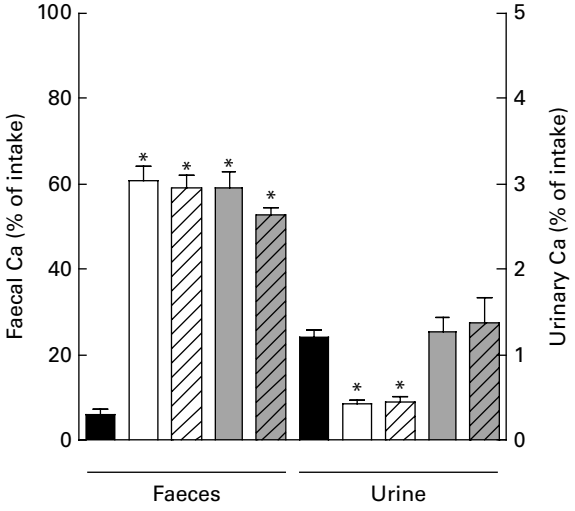

Fig. 3. Faecal and urinary Ca excretion (% of intake) before infection of the rats. Ca was determined by inductively coupled plasma-atomic emission spectrophotometry. Results are expressed as means with their standard errors (n 7 in the calcium phosphate group and n 8 in the other groups). ■, Low-Ca control; □, calcium phosphate; ▨, milk Ca; ▩, calcium carbonate; ▨, calcium chloride. *The indicated group is significantly different from the low-Ca control group ($P<0{\cdot}05$).

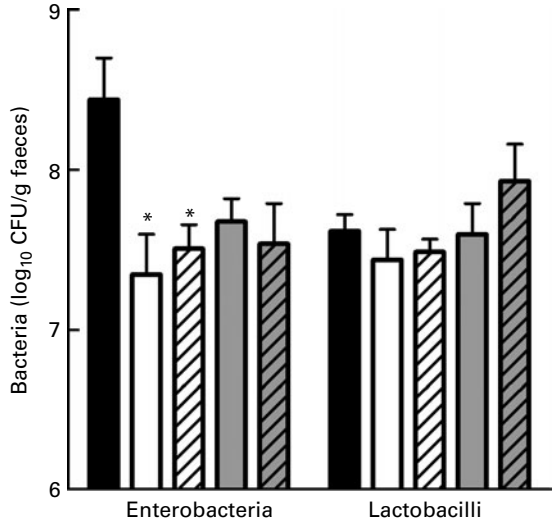

Fig. 4. Effect of dietary Ca supplementation on enterobacteria and lactobacilli in faecal samples collected before *Salmonella* infection of the rats. Lactobacilli were cultured anaerobically on Rogosa agar and enterobacteria were cultured aerobically on Levine EMB agar. Results are expressed as means with their standard errors (n 7 in the calcium phosphate group and n 8 in the other groups). ■, Low-Ca control; □, calcium phosphate; ▨, milk Ca; ▩, calcium carbonate; ▨, calcium chloride. *The indicated group is significantly different from the low-Ca control group ($P<0{\cdot}05$). CFU, colony-forming units.

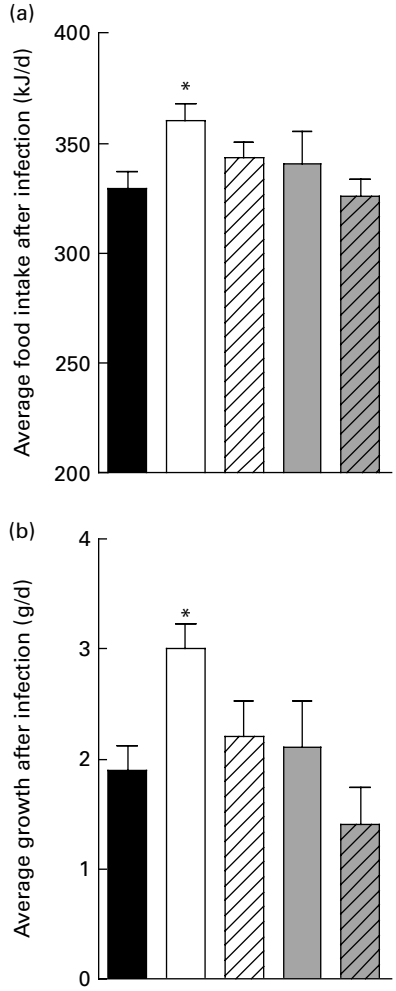

Fig. 5. Effect of dietary Ca supplementation on mean food intake (a) and growth of the rats (b) after oral administration of 1×10^9 colony-forming units of *Salmonella enteritidis*. Results are expressed as means (*n* 7 in the calcium phosphate group and *n* 8 in the other groups). ■, Low-Ca control; □, calcium phosphate; ▨, milk Ca; ▩, calcium carbonate; ▨, calcium chloride. * The indicated group is significantly different from the low-Ca control group ($P<0{\cdot}05$).

time. The calcium phosphate-induced improvement of intestinal resistance to *Salmonella* is in agreement with earlier studies[12,23,24].

Protection against *Salmonella* by direct binding of *Salmonella* to the amorphous calcium phosphate complex seems a likely explanation for the observed protective effect. The amorphous calcium phosphate complex is formed in the human[25,26] and rat[27] proximal small intestine from soluble ionic Ca together with phosphate originating from the diet. The amorphous calcium phosphate complex could be formed *in vivo* with all tested Ca salts; soluble ionic Ca was present after gastric passage *in vitro,* Ca was abundantly present in the intestinal lumen (as indicated by the faecal Ca excretion), and all the diets contained phosphate (originating from acid casein). The *in vitro* experiments also showed that amorphous calcium phosphate, in contrast to crystalline calcium phosphate and ionic Ca, was able to bind *Salmonella*.

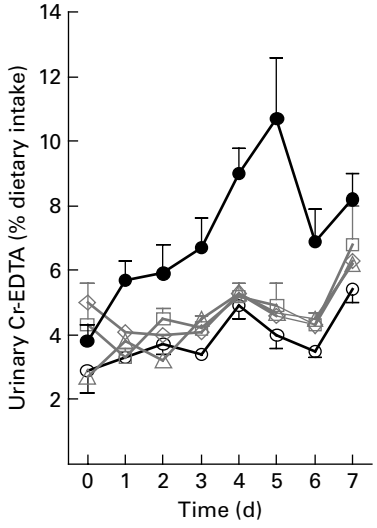

Fig. 6. Effects of dietary Ca supplementation on urinary Cr-EDTA excretion in rats. Daily urinary Cr-EDTA excretion is expressed as % of dietary intake. Rats were challenged with 1×10^9 colony-forming units of *Salmonella enteritidis* on day 0. Cr-EDTA was analysed by inductively coupled plasma-atomic emission spectrophotometry. Results are expressed as means with their standard errors (*n* 7 in the calcium phosphate group and *n* 8 in the other groups). ●, Low-Ca control; ○, calcium phosphate; □, milk Ca; ◇, calcium carbonate; △, calcium chloride. All the Ca-supplemented groups were significantly different from the low-Ca control group ($P<0{\cdot}05$).

Binding of *Salmonella* to the amorphous calcium phosphate complex in the intestinal lumen may subsequently prevent mucosal attachment of these pathogens and inhibit infection-induced pathology. This interaction may also

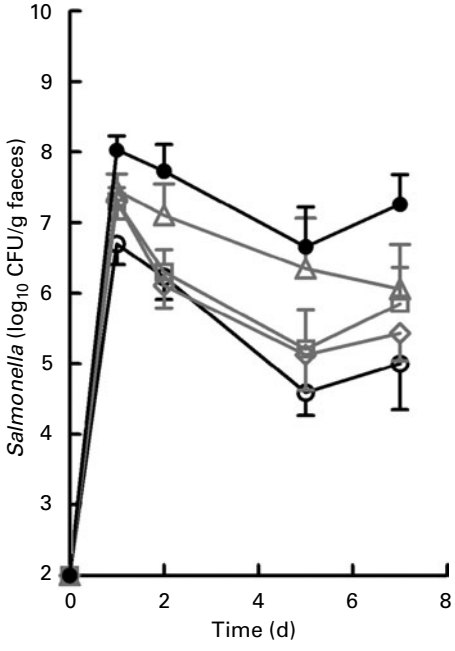

Fig. 7. Effect of dietary Ca supplementation on faecal *Salmonella* excretion with time. Rats orally received 1×10^9 colony-forming units (CFU) of *Salmonella enteritidis* on day 0. Results are expressed as means with their standard errors (*n* 7 in the calcium phosphate group and *n* 8 in the other groups). ●, Low-Ca control; ○, calcium phosphate; □, milk Ca; ◇, calcium carbonate; △, calcium chloride. All the Ca-supplemented groups were significantly different from the low-Ca control group ($P<0{\cdot}05$).

play a role in the observed dietary Ca-induced reduction in faecal enterobacteria in the present study.

Besides direct binding of *Salmonella* during gut transit, amorphous calcium phosphate can also precipitate cytotoxic luminal components, such as bile acids and fatty acids, within the intestinal lumen[25–27]. Binding of these surfactants can subsequently prevent epithelial cell damage and compensatory epithelial hyperproliferation[26,28], and may protect the intestinal barrier[25,26,29]. In fact, in the present study, all the tested dietary Ca salts prevented the infection-related increase in intestinal permeability.

The protective effects of dietary Ca are not restricted to *Salmonella*. We have previously shown that dairy Ca strongly improves the resistance to enterotoxigenic *Escherichia coli* in both rats and human subjects. To follow-up, a large intervention trial is currently being performed to determine whether milk Ca can protect against acute infectious diarrhoea in Indonesian children.

Are the dietary concentrations of Ca in our animal studies relevant for the human diet? In general, dietary Ca intake in the Western world ranges from 600 to 1100 mg/d[30,31]. Assuming a daily food intake of 500 g dry weight per d, the average concentration in the food would be 1·2–2·2 g/kg. The animal diets in this mechanistic study contained Ca levels of 0·8 g/kg (20 mmol/kg diet) and 4·0 g/kg (diets supplemented to 100 mmol/kg), and therefore represent conditions slightly lower and higher than normal human intake.

Overall, the present study showed that several dietary Ca salts (calcium phosphate, milk Ca, calcium carbonate, calcium chloride) are protective in improving resistance to *Salmonella* infection. This protective effect might be explained by binding of *Salmonella* (thereby preventing mucosal attachment of *Salmonella*) and/or binding of cytotoxic luminal components (thereby protecting the intestinal barrier). In view of the growing resistance of pathogenic bacteria to antibiotic drugs, modulation of host resistance to intestinal infections by dietary Ca might be an attractive approach.

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British Journal of Nutrition