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Could the beneficial effects of dietary calcium on obesity and diabetes control be mediated by changes in intestinal microbiota and integrity?

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Abstract

Evidence from animal and human studies has associated gut microbiota, increased translocation of lipopolysaccharide (LPS) and reduced intestinal integrity (II) with the inflammatory state that occurs in obesity and type 2 diabetes mellitus (T2DM). Consumption of Ca may favour body weight reduction and glycaemic control, but its influence on II and gut microbiota is not well understood. Considering the impact of metabolic diseases on public health and the role of Ca on the pathophysiology of these diseases, this review critically discusses possible mechanisms by which high-Ca diets could affect gut microbiota and II. Published studies from 1993 to 2015 about this topic were searched and selected from Medline/PubMed, Scielo and Lilacs databases. High-Ca diets seem to favour the growth of lactobacilli, maintain II (especially in the colon), reduce translocation of LPS and regulate tight-junction gene expression. We conclude that dietary Ca might interfere with gut microbiota and II modulations and it can partly explain the effect of Ca on obesity and T2DM control. However, further research is required to define the supplementation period, the dose and the type of Ca supplement (milk or salt) required for more effective results. As Ca interacts with other components of the diet, these interactions must also be considered in future studies. We believe that more complex mechanisms involving extraintestinal disorders (hormones, cytokines and other biomarkers) also need to be studied.

Key words: **Calcium: Gut microbiota: Intestinal permeability: Endotoxin: Lipopolysaccharide**

Ca is the major mineral component of the skeletal system, and it is also an essential nutrient required for blood clotting, nerve conduction and muscle contraction, besides being essential for endocrine and hormone secretion[1]. In adults, adequate Ca intake, as recommended in the dietary reference intakes, seems to prevent obesity[2] and type 2 diabetes mellitus (T2DM)[3]. Possible mechanisms involving Ca that may favour weight and glycaemic control are still not well understood. The results of *in vitro* and animal studies suggest that low-Ca diets increase calcitriol (1,25-dihydroxyvitamin D) and parathormone concentrations, resulting in Ca influx into adipocytes. Increased intracellular Ca^{2+} activates lipogenesis (mediated by fatty acid (FA) synthase) and suppresses lipolysis (hormone-sensitive lipase), increasing body fat and inducing insulin resistance (IR)[4]. Calcitriol also inhibits the expression of adipocyte uncoupling protein 2, reducing mitochondrial FA transport and lipid oxidation[4]. Another possible mechanism is the interaction between dietary Ca and FA in the gut, forming insoluble Ca FA soaps, which in turn increases faecal fat excretion and reduces dietary energy[5].

Influx of Ca^{2+} into muscle cells promotes GLUT4 translocation and, hence, increases glucose uptake and insulin sensitivity in skeletal muscle[6]. A moderate influx of Ca into pancreatic β-cells is essential for converting proinsulin to insulin and promoting insulin release[7]. In healthy adults, Ca supplementation increased the concentrations of gastrointestinal insulinotropic hormones, especially glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 (GLP-1)[8], and hence increased insulin sensitivity indirectly.

The beneficial effects of Ca ingestion could be associated with intestinal microbiota modulation and with increased integrity of the intestinal mucosa, as the results of human and animals' studies indicate the importance of these intestinal parameters on obesity and diabetes development[9–14]. Gut microbiota constitutes an important factor that affects nutrient absorption, energy homoeostasis, body weight control and IR[13]. Intestinal permeability can be defined as the property that allows solute and fluid exchange between the intestinal lumen and the tissues[15]. Gut barrier is a functional unit that prevents

Abbreviations: BA, bile acid; FA, fatty acid; FW, faecal water; GLP, glucagon-like peptide; II, intestinal integrity; LPS; lipopolysaccharide; T2DM, type 2 diabetes mellitus.

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this exchange, composed of gut microbiota, mucus, polarised epithelial cell membrane, tight junctions and the innate and adaptive immune cells forming the gut-associated lymphoid tissue[15–18]. Integrity of these barrier structures is essential to maintain normal intestinal permeability[18]. When the intestinal barrier is unimpaired, permeability is highly selective, avoiding the entrance of undesirable solutes, pathogenic microorganisms and toxins to the blood stream[16–18]. Integrity breakdown and increased intestinal permeability have been associated with obesity and diabetes aetiopathogenic mechanisms through the activation of proinflammatory pathways[9,12,19].

In animal studies, increased intestinal permeability caused metabolic endotoxaemia (measured by the translocation of bacterial lipopolysaccharide (LPS) derived from gram-negative intestinal microbiota into the peripheral circulation), low-grade inflammation and glucose intolerance[9,20]. Studies have shown differences in gut microbiota composition and higher concentrations of circulating endotoxins when obese and/or diabetic subjects are compared with lean and normoglycaemic ones[16,21–23].

Considering the low-Ca consumption by industrialised populations[24] and the increase in the worldwide prevalence of obesity and T2DM, this review aims to discuss the influence of dietary Ca on gut microbiota composition and intestinal integrity (II) in order to elucidate a possible therapeutic strategy for the prevention and/or treatment of obesity and T2DM.

Methods

Medline/PubMed, Scielo and Lilacs were searched using the following terms: calcium, dairy food, gut or intestinal or gastrointestinal microbiota, gut or intestinal or gastrointestinal barrier, gut or intestinal or gastrointestinal permeability, lipopolysaccharide, endotoxins, metabolic endotoxemia, tight junction. For data searches, the terms in English, Spanish or Portuguese were used either alone or in association. Review and original studies published from 1993 to 2015 were selected according to their titles and abstracts. *In vitro* studies were excluded. Each selected manuscript was critically analysed.

Gut microbiota and intestinal integrity: how might dietary calcium work?

Human gut microbiome may be affected by short-term (about a few days) dietary Ca intake[25]. Dietary Ca may affect gut microbiota and II through mechanisms involving gastric acid secretion, bile acid (BA) and FA precipitation, competition among intestinal bacterial communities and changes on fermentation products in the colon and on intestinal mucosal integrity (Fig. 1). Online Supplementary Table S1 briefly describes studies that evaluated the effects of Ca supplementation on II in animals and humans.

Antimicrobial effect of gastric acid secretion. High-Ca diets (30 mmol Ca/l[26] and 180 mmol Ca/kg diet[27]) are associated with reduced number of viable bacteria in the stomach. A high intraluminal concentration of Ca^{2+} stimulates the release of gastrin and, consequently, increases acid secretion[28]. Several bacterial species (e.g. *Salmonella*) are destroyed by gastric

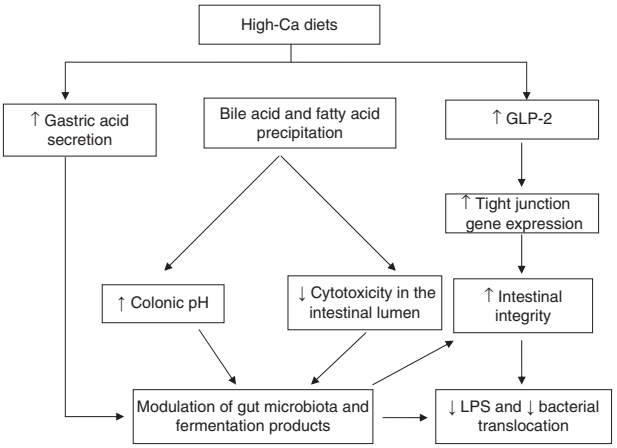

Fig. 1. Possible mechanisms explaining the effects of high-calcium diets on intestinal integrity and gut microbiota. High-calcium diets seem to change the intestinal environment through the following mechanisms: (1) increasing gastric secretion leading to increased gastric pH and reduced number of viable bacteria; (2) causing bile acid and fatty acid precipitation, increasing colonic pH and reducing cytotoxic components (especially NEFA and ionised secondary bile acids) that damage the epithelial cells; and (3) increasing glucagon-like peptide-2 (GLP-2) secretion, which has a trophic effect on intestinal mucosa and reduces gene expression of tight junctions (occludin and zonula occludens-1). These mechanisms may reduce bacterial and lipopolysaccharide (LPS) translocation, by bacterial fermentation and intestinal microbiota modulation, leading to a highly selective and controlled intestinal permeability.

acid[29]. However, some factors, such as the buffering effect of food, gastric emptying rate and mechanisms of bacterial resistance, interfere with the interaction of gastric acid and ingested bacteria[30]. Wistar rats (*n* 9 per group) were fed *ad libitum* for 12 d a low-Ca diet (control: lactose-free low-Ca milk, 3·8 % fat, 6 mmol Ca/l), regular lactose-free milk (3·7 % fat, 30 mmol Ca/l), acidified milk or yoghurt (both prepared with regular lactose-free milk)[26]. These rats were orally infected with *Salmonella enteritidis* just after food consumption. Animals fed yoghurt had lower faecal excretion of bacteria than those in the other groups. The authors suggest that the lower gastric emptying rate after yoghurt consumption could have prolonged the exposure to gastric acid and, thus, reduced the effectiveness of the inoculation. Thereafter, faecal *Salmonella* excretion declined rapidly in all high-Ca groups compared with the control group[26]. Another study conducted by the same authors showed similar results[27]. Mice infected with *S. enteritidis* were fed high-, medium- or low-Ca diets (*n* 8 per group; Ca in the form of $CaHPO_4$: 180, 60 and 20 mmol/kg diet, respectively). Compared with the low-Ca diet, the medium- and high-Ca diets favoured bacterial colonisation[27]. High-Ca intake (30 mmol Ca/l[26] and 180 mmol Ca/kg diet[27]) probably increased the gastric acidity and, hence, reduced viable counts of *S. enteritidis*[26,27]. Other studies also showed the antimicrobial effect of gastric acid[29,31], which potentially modified the endogenous microbiota by reducing viable bacteria in the gut. In general, few microorganisms, such as *Helicobacter pylori*, some streptococci, lactobacilli and probiotics, can survive extremely acidic conditions within the stomach[32,33]. However, in some studies[26,27], gastrin release and/or acid secretion were not measured. Therefore, we cannot conclude that the results of

those studies[26,27] were mediated by changes in gastric secretion. High protein content and the liquid state of the diets facilitated bacterial survival within the stomach[29]. Furthermore, some dairy product components, such as Ig, peptides, lactoferrin, lactoperoxidase and lysozyme, have antimicrobial effects. Other dairy components such as lactose, peptides and probiotics stimulate potentially beneficial bacteria that compete with pathogens for nutrients and attachment, and enhance the mucosal immune response to pathogens[34,35].

Bile acid and fatty acid precipitation: reducing luminal cytotoxicity. Because of the low pH, dietary Ca in the stomach exists in dissociated form, whereas in the small intestine there is an equilibrium between its dissociated and nondissociated forms. In the distal ileum and the colon, where pH > 6, Ca interacts with dietary phosphate, forming an insoluble complex that precipitates intestinal BA and FA. Hence, Ca increases their faecal excretion in animals[27,36–38] and humans[39–44]. BA precipitation increases the *de novo* synthesis of BA from cholesterol in the liver and, hence, reduces serum cholesterol. FA precipitation reduces fat absorption, increasing faecal energy loss[5].

Primary BA (cholic acid and chenodeoxycholic acid) are synthesised in the liver from cholesterol and then conjugated with either glycine or taurine, often called bile salts. About 5 % of BA are deconjugated and dehydroxylated by bacterial enzymes in the intestine to form secondary BA (deoxycholic acid and lithocholic acid), which are more cytotoxic. The dehydroxylation process involves the removal of the OH group at the 7-position of the steroid nucleus (also termed 7-dehydroxylation). Deconjugation results in amino acid side chain cleavage. Among intestinal bacteria, 7-dehydroxylase was detected in the *Eubacterium* and *Clostridium* genera but not in lactobacilli and bifidobacteria[45,46]. BA hydrolysis is mediated by several gut microbiota genera, including *Clostridium*, Bacteroides, *Lactobacillus*, *Bifidobacterium* and *Enterococcus*[45]. Approximately 95 % of BA are reabsorbed in the distal ileum and return to the liver (enterohepatic circulation of BA). About 400–800 mg BA/d elude enterohepatic circulation and are subjected to extensive modifications by the endogenous colonic microbiota. Secondary BA formed by colonic bacteria can be absorbed passively and, thus, may contribute to the BA pool[28].

A small amount of NEFA and ionised secondary BA that reaches the colon can damage the intestinal epithelium and thus increase colonic permeability[47]. Therefore, BA and FA precipitation caused by dietary Ca promotes cytoprotective effects by reducing the bacteria's formation of cytotoxic surfactants. BA and FA precipitation ultimately maintains the integrity of the colonic epithelium.

Measuring the BA and FA concentrations in the soluble portion of the faeces (so-called faecal water (FW)) is more reflective of luminal cytotoxicity than measuring the total faecal BA and FA concentration[48]. FW refers to the supernatant obtained after intense centrifugation of the faeces. FW contains aqueous soluble BA and FA that are not linked to other faecal compounds[49]. In some studies, a high-Ca diet reduced the BA and FA concentration in FW[26,27,36–38,40,41]. A high-Ca diet also reduced FW cytotoxicity by precipitating cytotoxic surfactants, resulting in lower colonic epithelium damage and higher resistance to infections[26,27,36–38,40,41,47,50–52]. High-Ca diets contained 30 mmol/l[26], 225 μmol/g diet[36,40], 120[47,51], 150[37] or 180[27,38] mmol/kg diet and 4·8 g/kg diet[52] in the rats studies, and 1200 mg/d in the human study[41]. Some effects of cytotoxic surfactants are cell membrane disruption, inflammatory reaction activation and epithelium hyperproliferation enhancement[24,32,53]. Guarner[54] criticised the use of erythrocytes instead of intestinal epithelial cells to analyse FW cytotoxicity, as done in some studies[26,27,36–38,40,41,47,50–52]. Erythrocytes are susceptible to changes in pH resulting from the production of SCFA after the fermentation of non-digestible carbohydrates. Therefore, haemolysis caused by changes in pH by SCFA may not reflect epitheliolysis[54]. On the other hand, intestinal cells normally use organic acids as an energy source[54]. However, the erythrocyte assay has previously been validated[49]. In a mouse study, the type of dietary fat influenced faecal FA excretion. Ca-PUFA soaps were more soluble and, therefore, better absorbed than Ca-saturated FA soaps[36]. The decreased absorption of intestinal fat (mainly saturated fat) caused by dietary Ca is of interest for the improvement of obesity control. By contrast, FA precipitation was independent of the source of Ca (milk, calcium carbonate or calcium phosphate)[37] and dietary phosphate content (75, 125 or 275 mmol/g diet)[40].

Increased faecal fat excretion after Ca supplementation seems to favour weight control. An average daily intake of 1200 mg of Ca results in the excretion of 5·2 g fat/d and a weight loss of 2·2 kg/year[55]. Therefore, it is possible that this mechanism contributes to obesity control but does not fully explain the effect of Ca on weight loss[5]. We believe that the impact of dietary Ca on weight loss is also related to dysbiosis attenuation. In this context, a high-Ca diet (>1100 mg/d) seems to modulate gut microbiota by reducing the number of BA and FA available for bacterial metabolism. It is possible that this effect increases *Lactobacillus* and reduces bile-tolerant bacteria, as discussed below.

Resistance to pathogens and changes in gut microbiota composition. In rodents, high-calcium phosphate diets increased faecal lactobacilli excretion[38,50,52], reduced faecal *Enterobacteriaceae* excretion[50,56] and increased resistance to *S. enteritidis* after 6–7 d of infection[38,50,52,56]. Ca concentrations of these diets were previously mentioned[38,52], except the concentration adopted in the study conducted by Ten Bruggencate *et al.*[50,56], which was 100 mmol Ca/kg diet. Rats were fed diets with 60 g/kg cellulose (control), fructo-oligosaccharides (FOS) or inulin with either a low (30 mmol/kg) or a high (100 mmol/kg) Ca concentration. After 2 weeks of adaptation, the animals were infected with *S. enteritidis*. During the following 6 d, FW cytotoxicity increased in the rats on inulin and FOS diets, but the high-Ca diet minimised this adverse effect[50]. In another study, rats infected with *S. enteritidis* were fed different sources of Ca supplements (calcium phosphate, milk Ca, calcium chloride or calcium carbonate (a total of 100 mmol Ca supplement/kg)) or a low-Ca diet (20 mmol calcium phosphate/kg). After an adaptation period of 2 weeks, all the Ca supplements reduced infection and increased

resistance to *Salmonella*[56]. Effects of Ca salts were similar to milk Ca[56], suggesting a strategy to increase Ca intake in cases of restricted consumption of dairy foods – for example, lactose intolerance.

The authors suggest that the resistance to pathogens was because of BA and FA precipitation and, consequently, because of reduced cytotoxic surfactants in FW after calcium phosphate supplementation[38,50,52,56]. However, only Bovee-Oudenhaven *et al.*[38] evaluated BA and FA faecal excretion. gram-negative bacteria (such as *Escherichia coli* and *Salmonella* spp.) are more bile-tolerant than gram-positive bacteria (such as some species of bifidobacteria, *Bacillus*, *Lactobacillus* and *Enterococcus*)[45]. Therefore, the reduction in cytotoxicity in the intestinal lumen (reduction in the lytic activity of luminal surfactants), induced by dietary calcium phosphate, could improve the growth of *Lactobacillus* and other gram-positive bacteria compared with that of gram-negative bacteria.

Another mechanism by which dietary calcium phosphate interferes with resistance to pathogens is by binding directly to *Salmonella* and, hence, increasing the excretion of faecal bacteria, which is also known as bacterial shedding. This reduces pathogen competition and thus enhances the growth of lactobacilli. *In vitro*,[38,56] but not *vivo*,[52] studies confirmed this mechanism. In an *in vivo* study[52], rats (*n* 8 per group) infected with *Salmonella* were treated with an antibiotic and were either fed the control diet (1·2 g/kg diet) or a high-Ca diet (4·8 g/kg diet). Both diets did not decrease *Salmonella* colonisation (measured by the excretion of faecal bacteria). The authors rejected the hypothesis that the binding of the calcium phosphate complex to *Salmonella* has a significant effect *in vivo* and, therefore, suggested that surfactant precipitation may increase endogenous microbiota.

Research on pigs showed different effects of calcium phosphate in intestinal lactobacilli colonisation. In general, high-calcium phosphate intake did not affect *Lactobacillus* spp. growth[57–59] in the pigs' stomachs, ilea or colons. Only in the study by Mann *et al.*[60] did high- *v.* adequate-calcium phosphate diets increase *Lactobacillus* spp. growth in the stomach. These studies were performed with growing[57] or weaned[58–60] pigs (*n* 8 per group) fed high-calcium phosphate diets: with an ileal pectin infusion[57], with a high or low *β*-glucan content[58], associated with a corn diet, or associated with a wheat–barley diet[59,60]. High-calcium phosphate diets contained 15 g Ca/kg diet[50], 10 g Ca/kg diet[51] and 14·8 g Ca/kg diet[59,60]. The main difference between these pig studies that explain the changes in gastrointestinal microbiota was the methodology used to quantify bacterial communities. Studies in which no effects on lactobacilli count were observed used quantitative PCR[57–59], whereas Mann *et al.*[60] used the pyrosequencing of 16S rRNA genes. Because of the diversity and complexity of bacterial communities between species, the latter technique has been recommended. It has the capacity to sequence multiple fragments simultaneously and, thus, achieves more rapid and accurate bacterial genome sequences[59,61]. Although some authors criticise pyrosequencing for the phylogenetic classification of sequences obtained at the species level[61], Mann *et al.*[60] did not use it for pyrosequencing the total genome; rather, they only used it for the 16S rRNA gene. Thus, they demonstrated increases on operational taxonomic units for *Lactobacillus*, indicating an increase in the number of micro-organisms of this genus. This is extremely beneficial because of the effects of these bacteria on intestinal health.

Calcium phosphate diet modulated gastrointestinal microbiota in all pig studies, but the results were very different. Because of that, these studies will not be described in detail (see online Supplementary Table S1). In general, calcium phosphate intake over 14 d increased *Clostridium* cluster XI and XVIa in the ilea, caeca and colons of weaned pigs[57–60]. Several species of *Clostridium* cluster IV and XIVa produce butyrate, which is an important energy source for colonocytes[62].

A double-blind, placebo-controlled, crossover study evaluated the composition of the gut microbiota after calcium phosphate and probiotic supplementation in humans[43]. Participants (thirty-two healthy men and women aged 25 (SD 5) years and BMI of 22 (SD 3) kg/m^2) consumed a probiotic drink containing 10^{10} colony-forming unit (CFU)/d *Lactobacillus paracasei* alone or in combination with bread containing calcium phosphate (1 g/d) for 4 weeks. Calcium phosphate supplementation decreased total cholesterol, LDL-cholesterol and the LDL:HDL ratio, and increased faecal pH and the faecal excretion of secondary BA compared with supplementation with probiotics or placebo alone. Probiotic supplementation, alone or with calcium phosphate, increased faecal *Lactobacillus* excretion. The authors explained these effects as resulting from BA precipitation by amorphous calcium phosphate, particularly when BA are deconjugated by probiotics, and from the increased faecal excretion of these components. A less cytotoxic intestinal lumen, which contains a low concentration of cytotoxic surfactants, might favour the growth of lactobacilli and reduce blood cholesterol concentration because of the increased conversion of cholesterol into BA[43].

The results of the studies with mice, pigs and humans are difficult to compare because of the diversity of micro-organism species in different hosts. Within the same species (mouse, pig or man), the intestinal maturation stage can also influence gut microbiota composition. For instance, *Enterococcus* spp. in the ileum were observed to decrease and increase in growing and weaned pigs, respectively, after calcium phosphate supplementation[57,59]. In humans, gut microbiota is dominated by bifidobacteria in the first 2–3 years of life (especially among breastfed children), remaining relatively stable in adults with 90 % of the bacteria from the Bacteroidetes and Firmicutes phyla. In the elderly, gut microbiota becomes less diverse again (higher Bacteroides:Firmicutes ratio, an increase in Proteobacteria and a decrease in *Bifidobacterium*)[63]. Human gut microbiota also varies according to genetic background, diet, antibiotic use and the health status of the host[64]. Therefore, the interaction between Ca and other dietary nutrients (such as lactose, dietary fibre and probiotics) probably influences its effect on microbiota composition and activity.

Furthermore, the different results observed may be because of the variety of procedures used on microbiological analyses. These range from simple techniques such as counting CFU in mouse studies[38,50,52] to sophisticated molecular biology techniques such as quantitative PCR and pyrosequencing 16S rRNA genes in pig and human studies[43,57–60]. The variety in materials or parts of the gastrointestinal tract used to evaluate microbiota composition may also make comparisons difficult. Mouse and human studies

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used faeces[38,43,50,52], whereas pig studies used different viscera (stomachs, small intestines, caeca and colons)[57–60].

Differences between BA in mice (predominantly taurine-conjugated) and those in pigs (glycine-conjugated) also partly explain the diversity in results. In general, unconjugated BA and glycine-conjugated BA are more strongly precipitated by calcium phosphate than taurine-conjugated BA[38]. The taurine-conjugated:glycine-conjugated BA ratio in human bile is usually 3:1, which is more similar to that in pigs than to that in rodents[45].

Obesity is associated with changes in gut microbiota composition in animal and human research[10,65]. Some studies show an increase in the Firmicutes:Bacteroidetes ratio[66], and others only show an overall decrease in Bacteroidetes and no change in Firmicutes[22]. There are also differences between the gut microbiota of obese and lean subjects[65]. However, it is not yet clear whether obesity leads to dysbiosis or vice versa. If the effects of dietary Ca on lactobacilli growth are confirmed in human clinical trials, Ca supplementation will be a useful strategy in obesity treatment.

Change in faecal pH and in fermentation products. Most mouse[26,50,67] and human[41,43] studies have found an increase in faecal pH after high-Ca diets (30 mmol Ca/l[26], 100[50] and 120[67] mmol Ca/kg diet in mouse studies, and 1000[43] or 1200 Ca mg/d[41] in human studies). Other mouse studies[36], as well as pig studies[58,59], did not reveal such differences, and only one study showed a decrease in faecal pH as a result of Ca supplementation[37] (225 μmol Ca/g diet[36], 10[58] or 14·8[59] g Ca/kg diet, and 1 g/d[37]). The decrease in faecal pH, caused by products of the colonic fermentation of non-digestible carbohydrates, supports the growth of beneficial bacteria (particularly bifidobacteria and lactobacilli)[68]. As previously mentioned, dietary Ca is soluble in acids, and it precipitates at alkaline pH. Gastric acidity (pH 1–3) is sufficient to release Ca complexed to salts or foods. Thus, the ionised Ca can be absorbed via transcellular active transport in the duodenum and proximal jejunum and via a passive paracellular process throughout the ileum. Less than 10 % of Ca absorption occurs in the colon, and it involves the paracellular and transcellular pathways[69]. About 25 to 35 % of ingested Ca is generally absorbed. As pH increases from the ileum to the colon, the intestinal phosphate concentration also increases, causing Ca precipitation and Ca absorption reduction[28]. Therefore, an increase in Ca intake may increase the buffering capacity of faeces because of the formation of an amorphous calcium phosphate complex. In the studies discussed in our review, the acidification caused by bacterial fermentation may have been buffered by the high amounts of calcium phosphate in the colonic lumen (quantities described in the first paragraph of this session), causing no change or increase in faecal pH[26,36,41,43,50,58,67]. The buffering effect is suggested by the increase in the faecal excretion of Ca and phosphate[36,37,41,43] and/or the decrease in BA and FA concentration in FW[26,36,37,41,43].

In the animal research that evaluated the effect of Ca supplementation on the products of bacterial fermentation, there was an increase in acetate in the ilea[57] and caproate in the colons[59] of pigs. There was also an increase in lactate in the caeca[47], and in propionate and butyrate in the faeces of mice[70]. Metzler-Zebeli *et al.*[58] observed a decrease in propionate in the caeca of pigs. The sources and quantities of fibre used to stimulate colonic fermentation varied among the studies (60 g pectin/d[57]; 60 g FOS/kg diet[47]; 36 g crude fibre/kg diet[59]; 6 % (w/w) galacto-oligosaccharides[70]). The use of probiotics in humans[71] and animals[72] increases Ca absorption, especially in the colon. However, even with this increase, it is possible that much of the ingested Ca transits through the colon without being absorbed[71].

In a crossover study, isoenergetic diets with similar macronutrient compositions, rich in semi-skimmed milk (1·7 g Ca/d) or cows' semihard cheese (1·7 g Ca/d), and ingested by fifteen healthy adult men over 14 d increased faecal SCFA (acetate, propionate and butyrate) in comparison with the control diet (which was rich in butter with approximately 360 mg Ca/d)[44]. In comparison with the control diet, both experimental diets increased faecal fat excretion, mainly in the milk group. Cheese intake resulted in higher faecal concentrations of propionate and butyrate, whereas milk intake promoted greater faecal excretion of acetate. These results indicate that milk and cheese stimulate bacterial activity differently. According to the authors, further studies are needed to explore the reasons for this difference. A possible interference factor is the protein profile of cheese (mainly casein, absorbed slowly) and of milk (20 % casein and 80 % whey protein, with the latter absorbed faster)[44].

Colonic fermentation of fibre mainly generates lactate and SCFA. The amount and type of these metabolites formed depends on gut microbiota composition, the substrate and the intestinal transit time[73]. Acetate is produced by several groups of bacteria and comprises about 60 to 75 % of total SCFA. In addition, acetate is metabolised in peripheral tissues (through the formation of acetyl-CoA) and/or used for butyrate synthesis[73]. The number of microorganisms that can produce propionate and butyrate is low. Propionate is mainly produced by Bacteroides spp., *Clostridium* cluster IX, *Mitsuokella* and *Veillonella* spp.[58]. Proprionate is metabolised in the liver via glycogenesis and is a lipolysis inhibitor and an inhibitor of the formation of acetyl-CoA from acetate[44]. Butyrate, an inhibitor of acetate synthesis, is the main energy source for colonocytes, followed by acetate and then propionate[44,74]. Some SCFA production, such as butyrate, is important not only as an energy source for colonocytes but it also prevents the accumulation of potentially toxic metabolites such as D-lactate. In addition, butyrate acts in visceral sensitivity and intestinal motility, regulates transcellular fluid transport, reinforces the gut barrier and reduces mucosal inflammation and oxidative stress[75]. *Eubacterium rectale*, *Clostridium coccoides* and *Roseburia*, which belong to the genus *Clostridium* cluster XIVa, are the largest butyrate producers[69]. Some species of *Clostridium* cluster XIVa can convert lactate to butyrate, whereas some cluster IX members can convert lactate to propionate[76].

Lactic acid is a primary metabolite of fermentation in the caecum[73]. The production of lactic acid and SCFA lowers the pH, inhibiting the activity of microorganisms that metabolise lactate, for example, propionate-producing bacteria[77]. Excessive lactate production culminates in its accumulation in the

colon as it has low intestinal absorption[76,77]. Overduin *et al.*[70] suggest that dietary calcium phosphate supplementation influences this fermentation as the amorphous complex formed acts as a buffer against caecal acidification by lactate, thereby accelerating lactate conversion to SCFA in the caecum. According to the authors, colonocytes' rapid uptake of butyrate may have masked SCFA production, which explains why they observed lower concentrations of butyrate in the colons of rats that had been fed prebiotics. Moreover, as lactate is less absorbable, it can accumulate in the colon in larger quantities[70].

Using the quantification of fermentation products to evaluate bacterial metabolic activity may be biased. Many of these products can act as intermediate substrates (i.e. lactate and acetate) and, therefore, may be associated with the metabolic activity of bacterial producers and/or bacterial users of these substrates. For example, an increase in lactate concentrations may indicate an increase in lactate producers or a decrease in lactate users. Therefore, it does not allow definitive conclusions. Moreover, about 95 % of the SCFA produced by bacterial fermentation are absorbed by colonocytes during the intestinal transit. Therefore, a lack in the alteration of these components may not represent real changes in the gut microbiota. Perhaps it represents the more effective use of the components, mediated by diet[70,76,77].

Obese subjects and animals have more SCFA in their caeca than lean ones. This seems to favour higher energy storage after the intake of non-digestible carbohydrate[66,78] and lower intestinal transit time induced by the hormone peptide YY[79]. Overall, this favours weight gain. Although the effects of Ca have not been confirmed, considering the results of the studies analysed, it is possible that the increased faecal fat excretion and the modulation of gut microbiota that resulted from high-Ca diets (approximately1100 mg Ca/d in the human studies[41,43]) counteracted these effects.

Effects on intestinal permeability and integrity. Paracellular permeability allows substance movement between adjacent cells, by a passive process. In contrast, through transcellular permeability, transport can occur across the cells, and it involves both active and passive processes[17]. Several high-Ca salts (calcium phosphate, milk, calcium carbonate and calcium chloride) decreased intestinal permeability in rats (100[56] or 120 mmol Ca/kg[47,51,67,80], and 1·5 % Ca[81]). Most of these studies used oral administration of EDTA chromium (Cr-EDTA) as a marker of intestinal paracellular permeability[47,51,67,80,81]. As Cr-EDTA is stable throughout the gastrointestinal tract, its excretion in the urine reflects total intestinal permeability[67]. Sugars, such as lactulose (reflecting paracellular permeability) and mannitol (transcellular permeability), are usually ingested to measure region-specific permeability. These sugars are readily degraded by colonic microbiota. Thus, the urinary excretion rate of these sugars (lactulose:mannitol ratio) is used to express the small intestine permeability[82].

Compared with the control diet, high-Ca diets (quantities described in the previous paragraph) decreased Cr-EDTA urine excretion, and increased the lactulose:mannitol ratio in rats[67]. However, there was no statistical difference when the urinary excretion of the lactulose and that of mannitol were analysed individually. On the basis of individual lactulose results, the authors concluded that dietary Ca did not affect the permeability of the small intestine. Consequently, they questioned the relevance of the lactulose:mannitol ratio and recommended measuring the excretion of each sugar individually[67]. This is relevant because, in some situations in which both sugars are excreted in large or small quantities, the ratio remains unchanged[17]. Obese women appear to have higher urinary excretion of lactulose and mannitol, whereas their lactulose: mannitol ratio does not vary from that of lean women[83]. The use of large probes such as lactulose is the best way to analyse macromolecule passage through the intestinal barrier, such as dietary antigens and other components derived from bacteria[17]. Another relevant example involves coeliac patients, who tend to have low mannitol excretion because of villous atrophy, whereas their lactulose excretion is high. When calculating the lactulose:mannitol ratio, this information does not lead to accurate results[84]. Therefore, it is suggested that Ca supplementation mainly affects colonic permeability[67], which is expected, considering the previous discussion about the effects of Ca on BA and FA precipitation in the colon. This effect reduces cell damage and, consequently, increases the integrity of the epithelial mucosa.

In a transgenic animal model of colitis induction, Ca supplementation prevented colitis-induced increase in the expression of extracellular matrix remodelling genes such as matrix metalloproteinases, procollagens and fibronectin. This suggests that Ca strengthened the integrity of the colonic mucosa[51]. Even in animal models, high dietary Ca (90 mmol/kg) prevented the FOS-induced increase in intestinal permeability (measured by Cr-EDTA) only when phosphate content was medium (70 mmol/kg diet) or high (160 mmol/kg diet). This was not the case with low-phosphate diets (5 mmol/kg diet). The effect was attributed to the buffering capacity of the colonic lumen because of the formation of a calcium phosphate complex, which could reduce luminal cytotoxicity. In this respect, the phosphate content of the diet is not decisive, but it is necessary for the effect of Ca on intestinal permeability[47]. Therefore, based on Schepens *et al.*[47], a ratio of about 1:1·3 of Ca:P can affect colon permeability.

Extracellular Ca (luminal Ca) was essential for intestinal tight-junction maintenance[85]. Tight junctions are apical intercellular joints, which contain transmembrane proteins, cytoskeleton components and cytoplasmic plaques[86]. These junctions act on cellular adhesion, intracellular signalling, protection against extracellular entrance and paracellular transportation of substances to the intestinal lumen[87]. Among the various tight-junction proteins, the transmembrane proteins (such as occludin and claudin) and cytoplasmic plaques (como a zonula occludens (ZO)) are important for paracellular transport[88]. Low-Ca and/or low-vitamin D diets reduce tight-junction gene expression in calbindin-null mice[88], suggesting the importance of this mineral for the synthesis of tight-junction proteins and, therefore, for paracellular permeability. The intact microbiome appears to be essential for normal gut–brain axis signalling and the expression of calbindin, restoring the intrinsic and extrinsic

enteric nerve function in germ-free mice, and causing changes to intracellular calbindin concentrations[89]. Thus, it is believed that the microbiome may contribute to improve dietary Ca absorption.

Calcium phosphate supplementation (1 g/d) during 3 weeks increased GLP-1 and GLP-2 secretion in healthy adult men (*n* 10) in a double-blind placebo-controlled crossover study[8]. Trautvetter and Jahreis[8] suggest that Ca supplementation stimulates the secretion of gastrointestinal hormones (GLP-1 and GLP-2) through the modulation of the intestinal environment. GLP-2 has trophic effects in the intestinal mucosa and influences the tight-junction gene expression (occludin and ZO-1)[90]. On the other hand, Metzler-Zebeli *et al.*[91] observed a substantial down-regulation of occludin and ZO-1 protein expression in the jejunum of weaned pigs (*n* 8 per group) fed high-Ca diets (14·8 g Ca/kg) as compared with adequate-Ca diets (8·2 g/kg), whereas gene expression in the colon was unaffected by dietary Ca concentration. The authors suggest that alterations in gene expression were not translated into functional protein, as they did not observe higher intestinal permeability, measured by an enhanced serum acute-phase response or intestinal translocation of LPS[59,91].

LPS or serum anti-endotoxin antibodies, gut barrier disintegration and endotoxaemia markers were lower after high-Ca diets than after the control diet in mice[51,52] (but not in pigs)[59] (120 mmol Ca/kg[51], 4·8[52] or 14·8 g/kg diet[52]). Moreover, Ca supplementation reduced bacterial translocation after *Salmonella* infection, indicating increased mucosal integrity[27,38,50,52]. However, we emphasise that differences in circulating endotoxin or bacteria may also reflect differences in detoxification or post-absorption clearance. Therefore, it is not related to intestinal translocation only[92,93].

Metabolic endotoxaemia, characterised by moderately elevated serum levels of LPS, is associated with obesity, T2DM and IR[10,20]. High-fat diets, which are generally associated with obesity, also seem to induce a reduction in II and low-grade endotoxaemia[94]. The factors involved in II breakdown in obese patients mainly consist of dysbiosis, adoption of dietary pattern characterised by foods rich in fat and fructose and deficiencies in the intake of nutrients[95]. In congruence with the mechanisms discussed in this review, we consider that the effects of Ca on II may involve gut microbiota and bacterial fermentation product modulation, in addition to direct action on tight junctions and a decrease in luminal cytotoxicity. For example, butyrate enhances the intestinal barrier because it facilitates the assembly of tight junctions[96]. It is possible that a high-Ca (>1100 mg/d in the human studies selected for this review[41,43]) intake contributes to the maintenance of II, especially in the obese. However, because no human clinical trials have been conducted to date, it is not possible to confirm this association yet.

Conclusions

Dietary Ca appears to positively affect gut microbiota composition and II, which may improve obesity and T2DM treatment. The mechanisms suggested involve BA and FA precipitation and, consequently, a decrease in luminal cytotoxicity, lactobacilli growth and intestinal mucosal damage reduction. Ca appears to affect colon integrity to a great degree, and the amount of phosphate in the diet or the source of the Ca supplement appears to have minimal effect. PUFA faecal excretion seems to be lower than SFA excretion.

To our knowledge, the contribution of this modulation to the control of obesity and diabetes mellitus is uncertain. Further human clinical trials are needed to explore the potential of dietary Ca or Ca salt supplementation in the modulation of microbiota and intestinal barrier integrity and to ultimately determine the applicability of relatively simple dietary interventions to the treatment of chronic diseases. Further research is required to define the supplementation period, the dose and the type of Ca supplement (milk or salt) that is more effective in healthy, obese and diabetic subjects. As Ca interacts with other components of the diet, these interactions should also be considered in future research. We believe that more complex mechanisms involving extraintestinal disorders (hormones, cytokines and other biomarkers) also need to be studied.

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Supplementary material

For supplementary material/s referred to in this article, please visit http://dx.doi.org/doi:10.1017/S0007114515003608

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