

Prebiotics and Calcium Bioavailability

Kevin Cashman*

Departments of Food and Nutritional Sciences, and Medicine, University College Cork, Cork, Ireland

Abstract

A prebiotic substance has been defined as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon. Therefore, compared to probiotics, which introduce exogenous bacteria into the colonic microflora, a prebiotic aims at stimulating the growth of one or a limited number of the potentially health-promoting indigenous micro-organisms, thus modulating the composition of the natural ecosystem. In recent years, increasing attention has been focussed on the possible beneficial effects of prebiotics, such as enhanced resistance to invading pathogens, improved bowel function, anti-colon cancer properties, lipid lowering action, improved calcium bioavailability, amongst others. The objective of this review is to critically assess the available data on the effects of prebiotics on calcium bioavailability, and place it in the context of human physiology and, when possible, explain the underlying cellular and molecular mechanisms. The review will also try to highlight future areas of research that may help in the evaluation of prebiotics as potential ingredients for functional foods aimed at enhancing calcium bioavailability and protecting against osteoporosis.

Introduction

The maintenance of a community of bacteria which contains a predominance of beneficial species and minimal putrefactive (protein degrading) processes is believed to be important for maintaining intestinal health (Crittenden, 1999). Since specific components of the intestinal microflora have been associated with beneficial effects on the host, such as promotion of gut maturation and integrity, antagonisms against pathogens, and immune modulation, it would seem logical that the quantity of these components might be enhanced with dietary interventions (Brassart and Schriffin, 2000). Recently, Crittenden (1999) suggested that two separate approaches exist to increase the number of health-promoting organisms in the gastrointestinal tract. The first is the oral administration of live, beneficial microbes. This is the 'probiotic' approach, and is achieved most commonly by consumption of the probiotic bacteria, which have to date been selected mostly from lactic acid bacteria and bifidobacteria that form part of the normal intestinal microflora of humans, as milk-based products.

However, since these organisms are indigenous to the colon, a second strategy to increasing their numbers is to supply those already present in the intestine with a selective carbon and energy source that provides them with a competitive advantage over other bacteria in the ecosystem, that is, to selectively modify the composition of the microflora using dietary components, the 'prebiotics'. These two kinds of dietary components have become the focus of great interest in the general population, the food industry, and the scientific community because of their potential for positively modifying biological and physiological processes and, thereby, possibly enhancing human health and well-being.

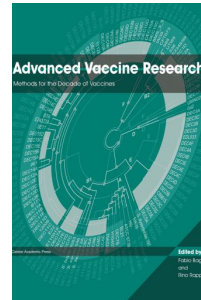
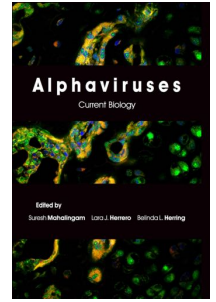
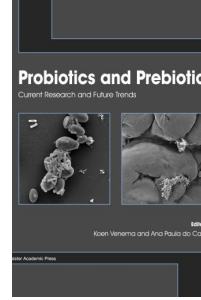
There is an impressive list of therapeutic and prophylactic attributes ascribed to the use of probiotics (see reviews by Tannock, 1999; 2002) and over the last two decades alone there has been a major international research effort to substantiate at least some of these health claims. In recent years, increasing attention has been focussed on the possible beneficial effects of prebiotics. The physiological importance and health benefits claimed for prebiotic substances are detailed in Table 1. Because of their putative beneficial effects, prebiotics (as well as probiotics) have been regarded as functional food ingredients. The working definition of a functional food is that "a food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way which is relevant to either the state of well-being and health or the reduction of the risk of a disease" (Roberfroid, 2001). It is not the aim of the present article to extensively review the scientific base of the various health benefits of prebiotics, other than their putative beneficial effects on calcium absorption and bone health; the other health benefits have recently been overviewed in several excellent articles that form part of the proceedings of two recent International Symposia which focussed on the influence of prebiotics and probiotics on human health (Supplement to the February edition of the *American Journal of Clinical Nutrition*, 2001; and Supplement to the *British Journal of Nutrition*, 2002). The objective of this review is to critically assess the available data on the effects of prebiotics on calcium bioavailability, and place it in the context of human physiology and, when possible, explain the underlying cellular and molecular mechanisms. The review will also try to highlight future areas of research that may help in the evaluation of prebiotics as potential ingredients for functional foods aimed at enhancing calcium bioavailability and protecting against osteoporosis. In the context of osteoporosis, the development of prebiotic-containing functional food products must be based on a detailed understanding of the influence of these dietary components on calcium bioavailability and bone health, and furthermore, must be supported by independent and appropriate scientific evidence to demonstrate efficacy with respect to the claimed health benefits. A good starting point before reviewing the putative beneficial effects of prebiotics

*For correspondence. Email k.cashman@ucc.ie.

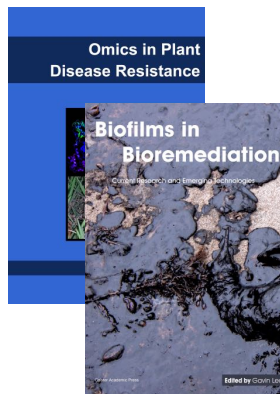
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Table 1. The physiological effects and putative health benefits claimed for prebiotic substances¹

Physiological Effects	Possible Health Benefit
Selection of probiotic bacterial growth in large intestine (colonization resistance)	Enhanced resistance to invading pathogens
Increased stool frequency and stool weight	Improved bowel function/Laxative effects
Non-specific stimulation of immune function	Resistance to infection
Not hydrolysed by oral micro-organisms	Anticariogenic effect
Not glycaemic	Potentially useful for diabetes
Modulation of carcinogen metabolism	Anti-colon cancer properties
Reduced synthesis of VLDL cholesterol and serum triglycerides	Cardioprotective
Increased absorption of calcium and magnesium	Protection against osteoporosis

Prepared using information from Macfarlane and Cummings (1999); Crittenden (1999) and Van Loo *et al.* (1999) and Roberfroid (2001).
¹Some of these benefits to health remain to be clearly established.

on calcium bioavailability, is to begin with a definition of a prebiotic substance.

Definition of a Prebiotic

A prebiotic substance has been defined as “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon” (Gibson and Roberfroid, 1995). Therefore, compared to probiotics, which introduce exogenous bacteria into the colonic microflora, a prebiotic aims at stimulating the growth of one or a limited number of the potentially health-promoting indigenous micro-organisms, thus modulating the composition of the natural ecosystem (Roberfroid, 2001). To be effective, prebiotics should escape digestion in the upper gut by pancreatic and brush-border enzymes, reach the large bowel (especially, the cecum), and be utilised selectively by a restricted group of micro-organisms that have clearly identified, health promoting properties, *i.e.*, probiotic bacteria (usually bifidobacteria and lactobacilli) (Macfarlane and Cummings, 1999; Cummings *et al.*, 2001). Recently, Roberfroid outlined the following criteria that can be used to classify a food component as a prebiotic: resistance to digestion, hydrolysis and fermentation by colonic microflora, and most importantly, selective stimulation of growth of one or a limited number of bacteria in the faeces (*in vivo* in humans) (Roberfroid, 2001).

In practise a range of dietary non-starch polysaccharides, resistant starches, undigested sugars, oligosaccharides and proteins are fermented by the microflora. Of these, it is the non-digested dietary carbohydrates that provide the principal substrates for colonic bacterial growth. Cummings *et al.* (2001) suggests that these are short-chain carbohydrates (SCCs) that are non-digestible by human enzymes. These range from small sugar alcohols and disaccharides, to oligosaccharides, and large polysaccharides (Table 2), all with a variety of sugar composition and glycosidic linkages. Analysis of these

substances indicates that although some are very pure, containing 86-87% oligosaccharides, *e.g.*, inulin and oligofructose, in others the oligosaccharides fraction is minor (about 20-30%), the rest being free monosaccharides, starch, and non-starch polysaccharides (Cummings *et al.*, 2001). Such substances have attracted considerable attention in the past decade for their physiological and health promoting properties and thus, for their potential as candidates for functional food ingredients (Fooks *et al.*, 1999). It has been suggested that of the various SCCs available, the non-digestible oligosaccharides (NDOs, oligosaccharides that resist hydrolysis and digestion in the upper gastrointestinal tract but are hydrolysed and fermented in the large bowel (Delzenne and Roberfroid, 1994)) are the only known components for which convincing evidence has been reported in favour of a prebiotic effect (Roberfroid, 2001). However, not all NDOs have prebiotic properties, and inulin, fructo-oligosaccharides, and (to a lesser degree) galacto-oligosaccharides dominate the published reports (Macfarlane and Cummings, 1999). The inulin/oligofructose-type products are the prebiotics that have been investigated most extensively for their nutritional properties (Roberfroid and Delzenne, 1998; Roberfroid, 1999). These low molecular weight carbohydrates occur naturally in artichokes, onions, chicory, garlic, leeks, and, to a lesser extent, in cereals. Other oligosaccharides such as raffinose and stachyose are the major carbohydrates in beans and peas. These simple molecules can also be produced industrially, and a number of new potential prebiotics are being commercially developed (see review by Cummings *et al.*, 2001). The evidence that such ingredients can positively influence calcium absorption, and possibly bone health, will be reviewed in the following sections. However, so that one can critically review the evidence, it is important firstly to briefly overview calcium absorption, calcium bioavailability and the various methodologies for assessing calcium bioavailability.

Table 2. Types of candidate prebiotic substances

Type of Short-chain Carbohydrates	Example(s) of Candidate Prebiotic Substances
Disaccharides	Lactose derivatives such as lactulose and lactitol
Oligosaccharides ¹ <i>e.g.</i> , <i>Fructo-oligosaccharides</i> <i>Galacto-oligosaccharides</i> <i>Soybean oligosaccharides</i>	<i>Raftilose</i> ® <i>Oligomate</i> ® Raffinose and stachyose
Other Non-digestible oligosaccharides	Xylo-oligosaccharides, isomalto-oligosaccharides, lactosucrose, palatinose polycondensates
Polysaccharides	Inulin ²
Resistant starch	
Type I	The physically inaccessible starch granules (such as whole and partially milled grains)
Type II	Native starch granules (<i>e.g.</i> , in potato, banana, high amylose maize)
Type III	Retrograded starch formed during starch processing
Type IV linking, esterification, or etherification	Chemically modified starches altered by cross-

Prepared using information from Macfarlane and Cummings (1999) and Crittenden (1999).

¹Oligosaccharides are usually defined as glycosides that contain between three and ten sugar moieties.

²Inulin extracted from chicory contains both oligosaccharides as well as polysaccharides.

Bioavailability of Dietary Calcium

The terms 'bioavailability' and 'absorption' of a nutrient are sometimes used interchangeably in the literature; however, there is an important difference between them. The absorption of a nutrient describes the process by which the nutrient is transported from the gastrointestinal lumen, across the intestinal mucosa, to the serosa (see the section below dealing specifically with intestinal absorption of calcium). The bioavailability of a nutrient, on the other hand, defines that fraction of the ingested nutrient that is utilised for normal physiological functions or storage. This definition recognises that one of the major determinants of bioavailability is that proportion which is absorbed from the gastrointestinal tract, but that this is not the only factor influencing bioavailability since tissue utilization (or lack of utilization) of the absorbed nutrient may vary dramatically (Jackson, 1997). Specifically in the case of calcium, bioavailability may be defined as the amount of calcium in foods that can be absorbed and utilised by the body for normal metabolic functions.

Intestinal Calcium Absorption

Calcium in food occurs as salts or associated with other dietary constituents in the form of complexes of calcium ions (Ca^{2+}). Calcium must be released in a soluble, and probably ionised, form before it can be absorbed (*i.e.*, its transfer from the intestinal lumen to the circulatory system). Once in a soluble form, calcium is absorbed by two routes, transcellular and paracellular transport (Bronner, 1987). The saturable, transcellular pathway is a multi-step process, involving the entry of luminal Ca^{2+} across the microvillar membrane into the enterocyte, then movement through the cytosol (*i.e.*, translocation to the basolateral

membrane), followed by active extrusion from the enterocyte into the lamina propria and, eventually, into the general circulation (see Figure 1). The intracellular Ca^{2+} diffusion is thought to be facilitated by a cytosolic calcium-binding protein, calbindin $\text{D}_{9\text{K}}$, whose biosynthesis is dependent on vitamin D. Calbindin $\text{D}_{9\text{K}}$ facilitates the diffusion of Ca^{2+} across the cell by acting as an intracellular calcium ferry or a chaperone. The active extrusion of Ca^{2+} at the basolateral membrane takes place against an electrochemical gradient and is mainly mediated by Ca-ATPase. The entry of Ca^{2+} across the apical membrane of the enterocyte is strongly favoured electrochemically because the concentration of Ca^{2+} within the cell (10^{-7} to 10^{-6} M) is considerably lower than that in the intestinal lumen (10^{-3} M), and the cell is electronegative relative to the intestinal lumen (Fullmer, 1992). Therefore, the movement of Ca^{2+} across the apical membrane does not require the expenditure of energy. It has been controversial as to whether a transporter or a channel is responsible for this process. It was widely believed that, because of the impermeability of lipid membranes to Ca^{2+} , a Ca^{2+} channel or integral membrane transporter must reside in the brush border membrane. Evidence would now suggest that the recently cloned calcium transport protein (CaT1) is a good candidate for this putative Ca^{2+} channel (Peng *et al.*, 1999). While each step in the transcellular movement of Ca^{2+} has a vitamin D-dependent component, calbindin $\text{D}_{9\text{K}}$ is believed to be the rate-limiting molecule in vitamin D-induced transcellular calcium transport.

The paracellular route of calcium absorption involves a passive calcium transport through the tight junctions between mucosal cells (see Figure 1); it is non-saturable, essentially independent of nutritional and physiological regulation, and is concentration dependent. Some debate still persists as to whether indeed the paracellular pathway

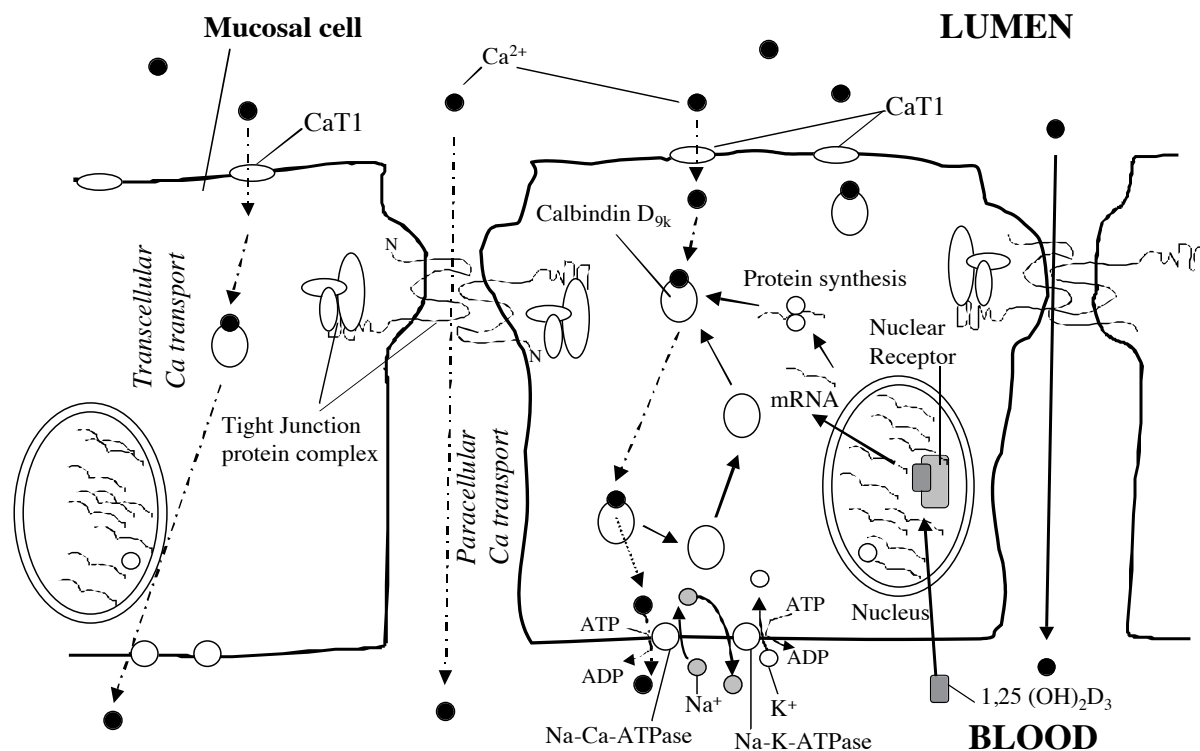


Figure 1. A schematic representation of transepithelial Ca^{2+} transport in the intestine. The central feature is that calcium absorption occurs by two independent processes, namely transcellular and paracellular transport of Ca^{2+} across an epithelium.

is vitamin D-dependent (Chirayath *et al.*, 1998; Fleet and Wood, 1999). Most calcium absorption in humans occurs in the small intestine, but there is some evidence (Barger-Lux *et al.*, 1989) for a small colonic component (typically believed to be no more than 10% of total calcium absorption). However, the large intestine may represent a site of increased importance for calcium absorption when acidic fermentation takes place (Younes *et al.*, 1996). This is important if one remembers that consumption of prebiotics will lead to acidic fermentation in the large intestine and this issue will be dealt with later in the section dealing with mechanisms of enhanced calcium absorption by prebiotics. When dietary calcium is abundant, the paracellular pathway is thought to be predominant. In contrast, when dietary calcium is limited, the active, vitamin D-dependent transcellular pathway plays a major role in calcium absorption. Transcellular calcium absorption responds to calcium needs, as reflected by changes in plasma Ca^{2+} concentration, by hormone-mediated up- or down-regulation of calbindin $\text{D}_{9\text{k}}$ in mucosal cells; for example, reduced plasma Ca^{2+} evokes a parathyroid hormone mediated increase in plasma $1,25$ -dihydroxyvitamin D_3 , which stimulates increased calbindin $\text{D}_{9\text{k}}$ synthesis in the intestinal mucosa (Bronner, 1987).

Methods for Measuring Calcium Bioavailability

Greger (1992) suggests that the bioavailability of a nutrient reflects the sum of the effects of various factors (dietary

and other) on the absorption, transport, cellular organisation, storage and excretion of the nutrient. Therefore, bioavailability is not definable by a single test or variable. Rather, the study of bioavailability is dependent on the strategic use of *in vitro*, subcellular and cellular systems, animal models and human subjects in an integrated manner.

Calcium status can be evaluated by measurements of the bone mass at different sites of the skeleton. Currently, dual energy X-ray absorptiometry (DEXA) is clearly the method of choice to assess bone mass, owing to its accuracy, precision and low radiation exposure. DEXA can be used to detect changes in the bone mass over time and thus to assess the effect of intervention measures aimed to prevent loss of bone mass or to increase this mass (Institute of Medicine, 1997). The measurement of bone mass is considered the best way to evaluate the long term effects on calcium status of factors which influence calcium metabolism or calcium absorption (Schaafsma, 1997). As already mentioned, calcium absorption is an important component of calcium bioavailability. The various methods of assessing intestinal calcium absorption have been reviewed by Schaafsma (1997). Assessment of intestinal absorption, based on measurements of the difference between calcium intake and calcium excretion (*i.e.*, metabolic balance technique) has major inherent shortcomings, including errors in estimating intake, incompleteness of faecal collections, and the inability to measure true calcium absorption. The latter point is

important, considering the magnitude of the endogenous faecal calcium excretion (*i.e.*, calcium secreted into the gastrointestinal tract which is not re-absorbed) and therefore the large difference between true- (accounting for endogenous loss) and apparent calcium absorption (which does not account for endogenous calcium loss). Measurement of true calcium absorption can be performed with radioactive tracers of calcium, such as ^{45}Ca and ^{47}Ca . These tracers are relatively cheap, can be measured accurately and can be used in very small (tracer) amounts. A disadvantage, however, is the ionizing radiation. Therefore, for ethical reasons, nutrition studies with these tracers in human volunteers are not preferred. Application of stable isotopes (^{42}Ca , ^{44}Ca , ^{46}Ca and ^{48}Ca) to measure calcium absorption has become common place in spite of the high isotope costs. Principles are similar to tracer studies with radioisotopes. Various methods of mass spectrometry analysis of stable calcium isotope enrichment can be used with this approach. True calcium absorption from a particular food can be measured after labelling this food extrinsically or intrinsically with a stable calcium isotope. If at the same time of administration of this food another stable calcium isotope is administered intravenously, true calcium absorption can easily be measured from stable calcium enriched values in samples of serum and urine obtained 24-48 hour after administration. The timing of the urine collection can be modified to take account of predominantly the small intestinal component of calcium absorption (*i.e.*, use of 24-hour urine collection) or to include the later colonic component (36-48 hour urine collection). A faecal collection method can also be used as a more laborious alternative for this dual labelling. It is also worth noting that dietary intervention trials in humans which employ such approaches, and which are aimed at assessing the influence of a dietary component on intestinal calcium absorption, are best carried out in randomized, double-blind, crossover design format, if possible. This type of study design, which is widely used in clinical, medical and pharmaceutical research, is considered a good approach for evaluating the efficacy of functional food ingredients.

While ideally calcium bioavailability should be measured by human studies, such studies are often very time consuming and expensive to run. As an alternative, experimental animal models, especially the laboratory rat, have been used quite extensively for assessing the impact of dietary and other factors on calcium absorption. The balance and isotope tracers approaches, mentioned above, can and have also been used in rats to determine calcium absorption. The adult rat, for example, has been shown to be a useful model for studies on calcium bioavailability since absorption mechanisms for calcium are similar in rats and in humans and a number of dietary and physiological factors affect calcium absorption similarly in the two species (Cashman and Flynn, 1996). However, while studies using laboratory animals are less expensive than studies in humans, they are somewhat limited by uncertainties with regard to differences in metabolism between animals and humans. More recently, intestinal cells in culture (particularly, the Caco-2 cells) have gained in popularity as an *in vitro* model of calcium absorption. When the human

colon carcinoma cell line, Caco-2, is grown on microporous membranes in bicarmel chambers the cells differentiate spontaneously into bipolar enterocytes that exhibit many of the characteristics of normal epithelial cells, such as microvilli of the brush border membrane, tight intercellular junctions and the excretion of border associated enzymes. As these cells differentiate within the chambers, the apical pole extends into the upper chamber while the basal lateral pole, in contact with the porous membrane, is exposed to the lower chamber. The design of the bicarmel chambers permits the study of calcium uptake from the apical chamber, transport into the cell and vectoral secretion into the basal chamber. In particular, these cells have a functional vitamin D receptor and have calcium transport kinetics that suggest the presence of both a saturable and nonsaturable calcium transport pathway, similar to observations in human and animal intestine (Fleet and Wood, 1999). Furthermore, it has been shown that in these cells, 1, 25 dihydroxyvitamin D₃ treatment induces the saturable, but not diffusional, component of calcium transport and induces accumulation of calbindin D_{9K} mRNA (Fleet and Wood, 1999). Therefore, this relatively simple *in vitro* method appears to be a good model for predicting calcium bioavailability in humans under certain conditions.

A Stimulatory Effect of Prebiotics on Calcium Bioavailability – What is the Evidence?

This section will review the various lines of evidence for putative beneficial effects of prebiotics on calcium bioavailability. As already mentioned, an effect on calcium bioavailability can be regarded as an effect on intestinal calcium absorption and/or on bone status. The scientific data which is currently available on the effect of prebiotics on calcium bioavailability comes from animal studies as well as from a limited number of human studies.

Evidence of a Stimulatory Effect of Prebiotics on Calcium Absorption in Animals

Numerous studies have repeatedly shown that prebiotics, such as oligofructose (also known as oligoifructose) and inulin, galacto-oligosaccharides, resistant starches or lactulose effectively stimulate calcium absorption in the rat and these have been reviewed in recent articles (Franck, 1998; Van Loo *et al.*, 1999; Scholz-Ahrens *et al.*, 2001a; Scholz-Ahrens and Schrezenmeir, 2002). Most experiments on prebiotics lasted 3-4 weeks and were carried out in young growing rats or in models of disease or altered physiological status, such as, gastrectomized, ovariectomized, cecectomized, and magnesium, calcium or iron deficient rats (see review by Scholz-Ahrens *et al.*, 2001a; Scholz-Ahrens and Schrezenmeir, 2002). It is widely believed that the effect of prebiotic substances on calcium absorption in these animal models occurs at the level of the large intestine (Ohta *et al.*, 1994; Baba *et al.*, 1996), although Brommage *et al.* (1993) reported that lactulose stimulated calcium absorption to the same extent in cecectomized rats as in sham-operated control rats. The mechanisms by which these prebiotic substances enhance calcium absorption in rats are discussed in a later section

in this review. Some interesting points arise from the findings of some of these studies and may, indeed, point the way in terms of future research that is needed, especially in human studies. For example, an interesting observation from some of these animal studies is that the effect of prebiotics on calcium absorption may be modulated by the amount of calcium in the diet. For example, Chonan and Watanuki (1996) reported a stimulatory effect of galacto-oligosaccharides on calcium absorption in intact (*i.e.*, not surgically altered) rats when the diets of these animals contained 5 g Ca/kg diet, but not when the diet contained only 0.5 g Ca/kg diet. Similarly, Scholz-Ahrens *et al.* (2001a) reported that the effect of oligofructose on metabolic calcium balance in ovariectomised rats became more prominent when dietary calcium was high (10 g Ca/kg diet) as compared to when it was at the recommended level (5 g Ca/kg diet). Lactulose has also been shown to significantly increase ($P<0.001$) calcium absorption in young growing intact rats, but only if the diet contained 5 g Ca/kg diet or more; no stimulatory effect of lactulose was observed with a dietary calcium level of 2 g Ca/kg diet (Brommage *et al.*, 1993).

There is some evidence of a dose-dependent effect of prebiotic substances on calcium absorption in rats. For example, Levrat *et al.* (1991) found that dietary inulin (in the range 0 – 200 g/kg diet) stimulated intestinal calcium absorption in a dose-dependent manner. This coincided with a dose-dependent decrease in cecal pH and a rise in cecum weight, cecal wall weight, and cecal pool of total short-chain fatty acids (SCFAs). Brommage *et al.* (1993) reported a near linear increase in intestinal calcium absorption in rats fed a diet containing 0, 50, and 100 g

lactulose per kg diet; there was no further increase when the diet contained 150 g lactulose/kg diet.

The majority of the animal studies which demonstrate a positive effect of prebiotics on calcium absorption were based on using the calcium balance method (Morohashi *et al.*, 1998). However, as already mentioned, a simple calcium balance study can examine only apparent calcium absorption, urinary calcium excretion and calcium balance, and cannot be used to evaluate true intestinal calcium absorption, the excretion of calcium into the intestine or the kinetics of calcium movement into or out of bone. Moreover, apparent calcium absorption cannot explain whether an increase is due to an enhancement of calcium absorption or a reduction of endogenous calcium excretion into the intestine. Morohashi *et al.* (1998) recently addressed this issue by carrying out a rat study in which animals were supplemented with fructo-oligosaccharides and then calcium balance and ^{45}Ca kinetics were determined (see Figure 2). This allowed them to investigate the effect of fructo-oligosaccharides on calcium metabolism at the level of the intestine, kidney and bone. They found that dietary fructo-oligosaccharides increased true intestinal calcium absorption and had no effect on endogenously excreted calcium relative to the control diet (see Figure 2). Urinary calcium excretion was significantly ($P<0.01$) higher in rats fed fructo-oligosaccharides than in those fed the control diet. However, despite this, calcium balance was still significantly higher ($P<0.01$) in the rats fed fructo-oligosaccharides. They reported that calcium flow into and out of bone (*i.e.*, bone formation and bone resorption, respectively) was unaffected by dietary fructo-oligosaccharides, despite the increased absorption and

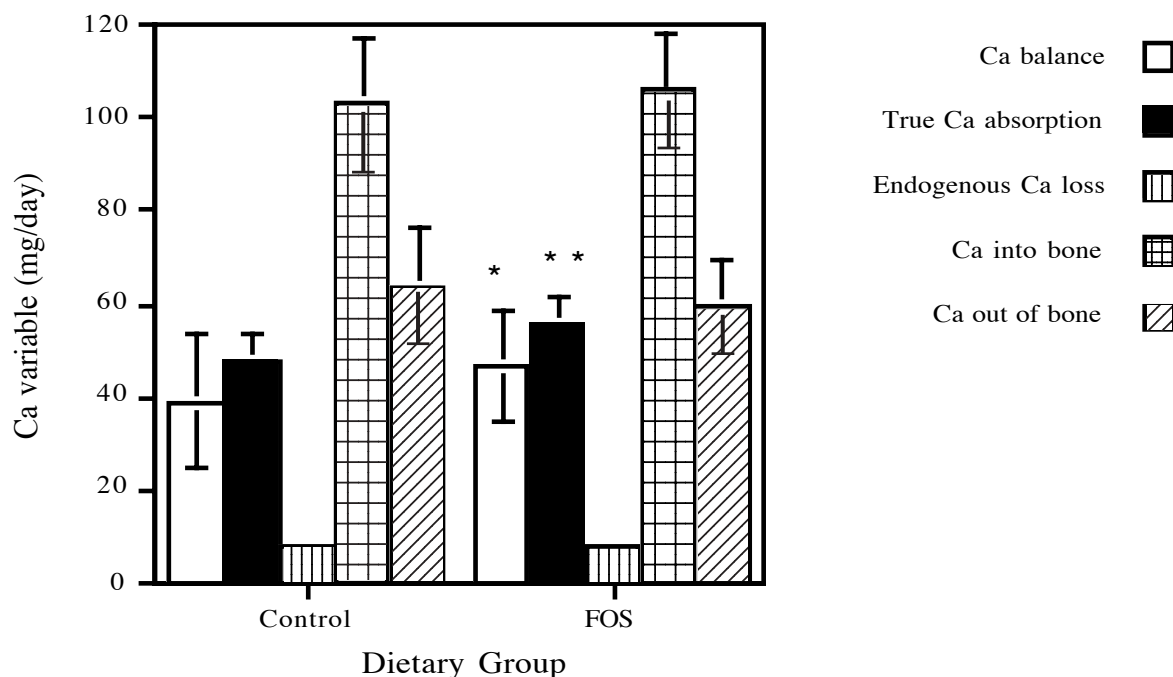


Figure 2. The effect of dietary fructo-oligosaccharides (FOS) (50 g/kg diet) on calcium balance, true calcium absorption, endogenous calcium loss, and calcium kinetics into and out of bone in young male rats. Values are means and standard deviations for 8 rats per group. ***Significantly different from control group (***) $P<0.05$, $P<0.01$). Graph constructed from data from Morohashi *et al.* (1998).

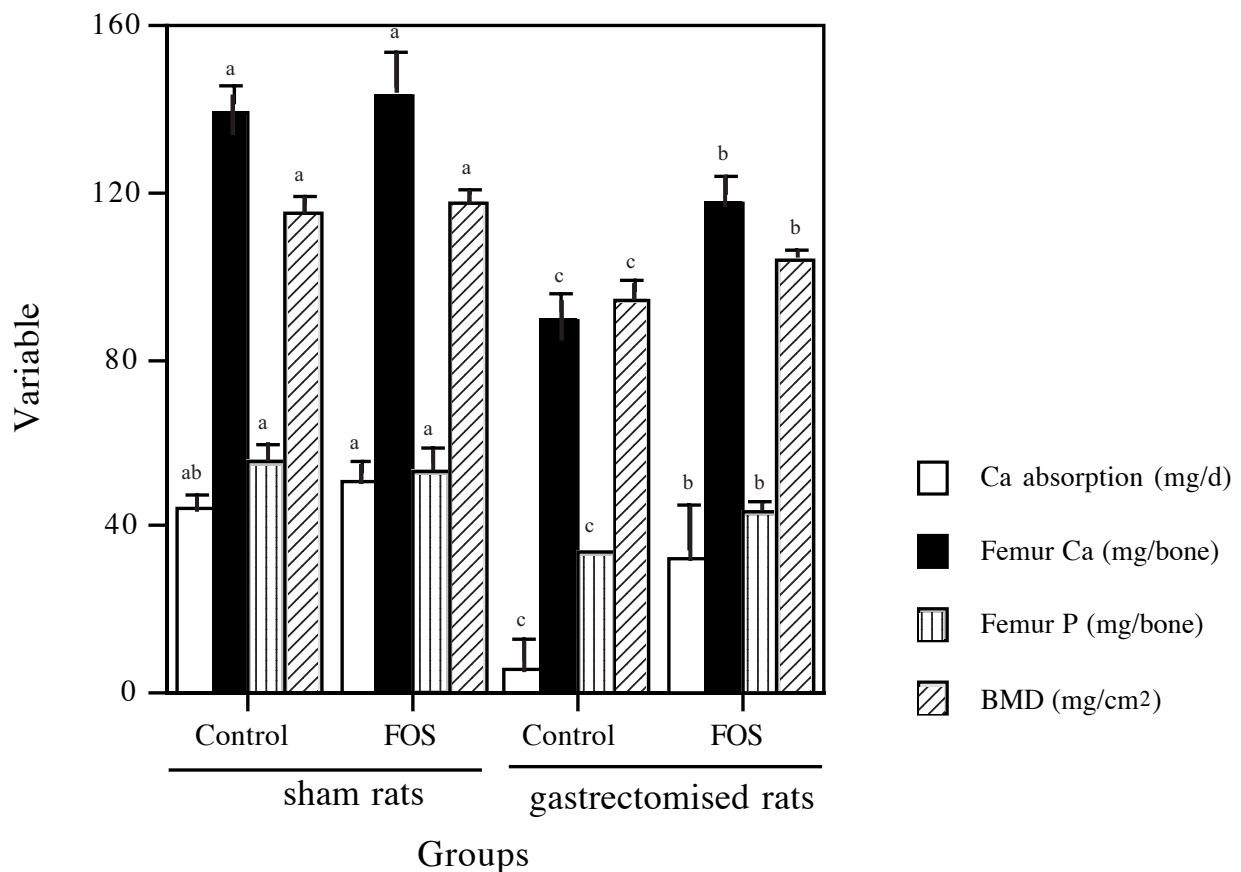


Figure 3. The effect of dietary fructo-oligosaccharides (FOS) (75 g/kg diet) on apparent calcium absorption, femur calcium content, femur phosphorus content, and femur BMD in male rats that underwent either a sham operation or a gastrectomy. Values are means and standard deviations for 7 rats per group. For a given parameter, values with different superscript letters are significantly different ($P < 0.05$). Graph constructed from data from Ohta *et al.* (1998a).

balance. This seems surprising considering the same group previously reported that fructo-oligosaccharides enhance bone mineral density and calcium content in rat bone (Ohta *et al.*, 1998a,b). An effect of prebiotics on bone arising from improved calcium absorption would presumably act through altered rates of bone turnover, which in turn would influence bone mass (Cashman and Flynn, 1999). However, Morohashi *et al.* (1998) suggest that because the effects on bone turnover were likely to be subtle, the techniques used in their study may not have been sensitive enough to detect such changes.

Another important consideration with respect to the stimulatory effect of prebiotics on calcium absorption is whether the enhanced calcium absorption is maintained over the longer term. The duration of many of the experiments with prebiotics was relatively short (14–28 d, see reviews by Scholz-Ahrens *et al.*, 2001a; Scholz-Ahrens and Schrezenmeir, 2002). The issue of adaptation of intestinal calcium absorption over time is highlighted by the findings of Brommage *et al.* (1993) which showed that dietary lactulose (50 g/kg diet), which stimulated calcium absorption in rats on the first day of the study, failed to enhance calcium absorption by the seventh day of the study. The authors hypothesized that this adaptive

response of intestinal calcium absorption over time occurred by a down-regulation of the active, transcellular route of calcium absorption which counter balanced the lactulose-induced increase in passive, paracellular calcium absorption. However, it should be noted that the capacity for this adaptation is limited, and Brommage *et al.* (1993) suggested that providing higher levels of lactulose in their study could possibly have resulted in continued elevation of intestinal calcium absorption. This raises the issue of defining the minimum effective dose of dietary prebiotics for the prolonged stimulation of calcium absorption. There is some evidence from repeated balance studies in gastrectomised and intact rats that the stimulating effect of fructo-oligosaccharides on calcium absorption was maintained over several weeks (Ohta *et al.*, 1994, 1998a). Chonan and Watanuki (1996) found that calcium absorption was stimulated in intact rats after 8–10 days and also after 18–20 days when galacto-oligosaccharides (50 g/kg diet) were included in the diet (containing 5 g Ca/kg diet).

It is also worth noting that certain prebiotic substances (such as, oligofructose, transgalacto-oligosaccharides, lactulose, resistant starch) have been shown to stimulate intestinal Mg absorption in various rat models (see reviews by Scholz-Ahrens *et al.*, 2001a; Scholz-Ahrens and

Schrezenmeir, 2002) and this may be important in terms of a beneficial effect of these compounds on bone mineral density.

Evidence of a Beneficial Effect of Prebiotics on Bone Health in Animals

There is some evidence that the stimulatory effect of prebiotic substances on calcium absorption in rats can be translated into functional benefits for bone health, such as increased bone mineralization, bone density and improved bone structure. This topic has been recently reviewed in two excellent articles by Scholz-Ahrens *et al.* (2001a) and Scholz-Ahrens and Schrezenmeir (2002).

In addition to the stimulatory effect of fructo-oligosaccharides on calcium absorption in gastrectomised rats, they also prevented the changes indicative of post-gastrectomy-induced osteopenia, such as reduced bone calcium content and bone mineral density (Ohta *et al.*, 1998a, see Figure 3). Using a rat model of postmenopausal bone loss, namely the ovariectomised rat, Chonan *et al.* (1995) showed that dietary galacto-oligosaccharides stimulated intestinal calcium absorption relative to a control diet, and importantly, the bone ash weight and bone calcium content of the ovariectomised rats fed the galacto-oligosaccharide-containing diet were significantly higher than those of the animals fed the control diet. The positive effect of dietary galacto-oligosaccharides and oligofructose on bone status has also been demonstrated in healthy intact rats. For example, Chonan and Watanuki (1996) showed that supplementation of the diet (containing 5 g Ca/kg diet) with galacto-oligosaccharides (50 g/kg diet) stimulated calcium absorption relative to the control diet, and furthermore, bone calcium content was significantly higher in the animals fed the galacto-oligosaccharides-containing diet than those of the animals fed control diet. Recently, Takahara *et al.* (2000) reported that fructo-oligosaccharides (50 g/kg diet) stimulated calcium absorption and enhanced femoral bone volume and mineral concentrations in young growing intact rats.

As was noted with their effect on calcium absorption, the effect of certain prebiotic substances on bone status may be modulated by the amount of calcium in the diet. For example, Scholz-Ahrens *et al.* (2001b,c) reported that the positive effect of oligofructose on calcium content of bone and on the prevention of ovariectomy-induced loss of trabecular structure became more prominent when dietary calcium was high (10 g Ca/kg diet) compared to when it contained the recommended level (5 g Ca/kg diet). Similarly, Chonan and Watanuki (1996) reported a positive effect of galacto-oligosaccharides on bone mineralisation in intact rats when the diets of these animals contained 5 g Ca/kg diet, but not when the diet contained only 0.5 g Ca/kg diet.

An important consideration in interpreting the data from the above animal studies of the effect of prebiotic substances on calcium bioavailability is whether prebiotic substances have similar effects on the large intestine of humans as they do in rats. It is possible that the rat is particularly sensitive to prebiotic substances, in terms of their stimulatory effect on cecal fermentation and cecal

enlargement. Therefore, a review of the evidence for a stimulatory effect of prebiotic substances on calcium absorption in human subjects is the most appropriate data when considering the efficacy of prebiotics for enhanced calcium bioavailability.

Evidence of a Beneficial Effect of Prebiotics on Calcium Absorption in Humans

According to the definition of a prebiotic, it must escape digestion in the small intestine in humans. While in healthy individuals with sufficient brush-border β -galactosidase activity, lactose is completely digested, in individuals with insufficient β -galactosidase activity, it may escape digestion, and thus, may be available for fermentation by the microflora in the colon. While numerous studies have shown a stimulatory effect of lactose on calcium absorption in the rat, studies on the effect of lactose on calcium absorption in humans have yielded inconsistent results. Miller, in a review of this area, concluded that it is likely that lactose enhances calcium absorption in human infants and in rats, while at levels normally present in milk, it does not have a significant effect on calcium absorption by healthy adults consuming normal diets (Miller, 1989). It is possible, however, that in subjects with low brush border β -galactosidase activity, lactose may stimulate calcium absorption because it reaches the terminal ileum and colon, where it can be fermented by the intestinal microflora (*i.e.*, it may behave as a prebiotic). In this regard, Griessen *et al.* (1989) reported that calcium absorption was similar from milk (21.4%) and lactose-free milk (lactose replaced by glucose) (26.8%) in healthy adult subjects, but lactose increased calcium absorption in β -galactosidase-deficient subjects.

Lactulose is a synthetic disaccharide which does not exist in nature. It can be made on a large scale from lactose by alkaline isomerization and is often used in the treatment of constipation and chronic hepatic encephalopathy. Lactulose is not digested in the stomach or small intestine, but is fermented in the colon by indigenous microflora. For this reason, it has been regarded as a potential prebiotic substance (Table 2). Recently, van den Heuvel *et al.* (1999a) investigated the effect of 2 doses of lactulose (5 and 10 g/d compared with 0 g/d) on calcium absorption in a randomized, double-blind, crossover design study with 12 healthy postmenopausal women. Lactulose was given at breakfast for 9 d. True intestinal calcium absorption was measured by using the dual-stable calcium isotope-labeling (^{44}Ca and ^{48}Ca) technique, which allowed the measurement of late colonic calcium absorption. Calcium absorption during the three dietary periods, namely control (without lactulose) and 5 g and 10 g lactulose daily was (mean \pm SD) 27.7 \pm 7.7%, 30.0 \pm 7.6%, and 32.2 \pm 7.0%, respectively, with the difference in calcium absorption between the control and the 10 g dose being significant ($P < 0.01$).

There is now increasing evidence that certain NDOs (such as, inulin, fructo- and galacto-oligosaccharides, see Table 2) can improve calcium absorption in adolescents and adults. For example, Coudray *et al.* (1997) investigated the effect of chicory inulin and sugar beet fibre on calcium

Table 3. Possible mechanisms by which prebiotics stimulate intestinal calcium absorption

1. Enhanced passive calcium absorption in the large intestine due to an increased solubility of calcium. The increased solubility occurs as a consequence of reduced luminal pH which, in turn, arises from the increased production of lactic acid and short chain fatty acids due to stimulated microbial growth and fermentation by the presence of prebiotics.
2. A direct effect of these short chain fatty acids on transcellular calcium absorption (by increasing the exchange of cellular H⁺ for luminal Ca²⁺).
3. Butyrate may (a) induce cell growth and thus increase the absorptive surface area of the gut, and/or (b) increase mucosal calbindin D_{9k} levels in the large intestine (and reduce levels in the small intestine).
4. The synthesis of polyamines, metabolites generated by several strains of microbes, may be increased by prebiotics, and these metabolites might, at least in part, (a) lead to cell growth and enlargement of the absorptive surface in the gut, and/or (b) stimulate gene expression, possibly including calbindin D_{9k}.

absorption in a crossover design study. They fed 9 healthy young adult males (mean age, 25.5 years) a control diet (containing 18 g dietary fibre per day) or the same diet supplemented with 40 g per day of either chicory inulin or sugar beet fibre for a period of 28 days (2 days of control diet followed by 14 d of progressive increase in test fibre amount and then 12 days at 40 g/d) and determined the apparent absorption of calcium by using the classic balance approach. They found that upon inulin ingestion, apparent calcium absorption increased significantly ($P < 0.01$) from 21.3% to 33.7% (an increase of 58%); ingestion of sugar beet fibre had no effect. In another randomized, double blind, crossover study, van den Heuvel *et al.* (1999b) fed 12 healthy male adolescents (aged 14-16 years) either orange juice supplemented with oligofructose (15 g/d) or sucrose (control treatment) three times daily for 9 days, after which time, they measured true fractional calcium absorption by a dual stable calcium isotope technique. An increase of 26% in true fractional calcium absorption (47.8% with placebo to 60.1% with oligofructose, $P < 0.05$) was observed upon ingestion of the daily 15 g supplement of oligofructose. In an earlier randomized crossover study by the same group, a daily supplement of 15 g of oligofructose, inulin, or galacto-oligosaccharides for 21 d had no effect on true calcium absorption (measured by a dual stable isotope technique) in healthy adult men (aged 20 to 30 years) (van den Heuvel *et al.*, 1998). However, in that study the colonic component of calcium absorption (a putative target for enhancement by prebiotics; see later) was not included because the urine collection was limited to 24-hours after isotope administration. This is in contrast with the later study in which the dual-labeling technique was slightly modified (*i.e.*, urine was collected for 36-hours after isotope administration) that allowed the measurement of late colonic effects on calcium absorption. In a third recent study by the same group, the effect of galacto-oligosaccharides, also referred to as transgalacto-oligosaccharides (TOS), on calcium absorption was re-evaluated, but in this case in postmenopausal women and importantly, using the modified dual-labeling technique (36-hour urine collection) for determining true calcium absorption (van den Heuvel *et al.*, 2000). This study was also a double-blind, randomized crossover study, consisting of two 9-day treatment periods in which the women drank yogurt drinks twice per day (at breakfast and lunch) containing either TOS (20 g/d) or a reference substance,

sucrose. TOS significantly ($P < 0.05$) increased true calcium absorption by 16% ($20.6 \pm 7.0\%$ to $23.9 \pm 6.9\%$) in these postmenopausal women.

In a very recent randomized, double-blind, crossover design study, 59 young adolescent girls (aged 11.0-13.9 years, consuming a relatively high calcium intake (1500 mg/d)) were randomized to receive either 8 g (as two 4 g servings) of a non-digestible oligosaccharide or placebo (sucrose) in a calcium-fortified orange juice daily for 3 weeks (Griffins *et al.*, 2002). Two similar protocols were carried out simultaneously and differed only in the non-digestible oligosaccharide used. In protocol I (n 30 girls), a chicory oligofructose (Raftilose[®]; see Table 2) was used as the test non-digestible oligosaccharide, whereas in protocol II (n 29 girls), the test non-digestible oligosaccharide was an inulin+oligofructose mixture (Raftilose[®] Synergy 1). True calcium absorption was measured using a stable isotope method at the end of each three week period. A 48-hour urine collection was carried out after the isotope administration so as to detect any modulatory effect of the non-digestible oligosaccharide on the colonic component of calcium absorption. In protocol I, there was no significant difference in true fractional calcium absorption on placebo (mean (SD) 31.8 (9.3) %) or on the Raftilose[®] (mean (SD) 31.8 (10.0) %; $P = 0.75$). In protocol II, consumption of the inulin+oligofructose mixture resulted in a 18% increase ($P = 0.007$) in true fractional calcium absorption and in an absolute increase in calcium absorption of 90 mg/day (Griffins *et al.*, 2002).

In another recently reported randomized, double-blind crossover study, Tahiri *et al.*, (2003) fed 12 healthy postmenopausal women (not receiving hormone replacement therapy; years since menopause, range 2-22 years) either 10 g of short-chain fructooligosaccharides or a placebo (sucrose) for 5 weeks, after which time, they measured fractional calcium absorption by a ⁴⁴Ca stable isotope fecal recovery technique. While mean calcium absorption during the short-chain fructooligosaccharide treatment period was not significantly ($P > 0.05$) different from that during the placebo period (mean (SD) 35.63 (9.40)% *v.* 36.44 (8.48)%), there was a tendency for calcium absorption to be enhanced by the short-chain fructooligosaccharide treatment in women (n 6) who were at least 6 years postmenopause (Tahiri *et al.*, 2003).

An important consideration in terms of the effects of these prebiotic substances on calcium bioavailability is

whether the additional calcium absorbed is retained within the body for use in the various physiological functions but particularly for use by skeletal tissue. Importantly, there was no significant ($P > 0.05$) difference in urinary calcium excretion during the prebiotic dietary periods compared to control periods in the above studies, suggesting that the additional calcium that was absorbed was retained within the body.

Mechanistic Aspects of the Stimulatory Effect of Prebiotics on Calcium Bioavailability

Several theories have been proposed to explain the stimulatory effect of prebiotic substances on intestinal calcium absorption. These theories, which are outlined in Table 3, refer to effects on the two routes of calcium absorption, namely transcellular and paracellular calcium transport, in the small and/or large intestine.

Prebiotic substances that escape digestion in the small intestine are substrates for the formation of short-chain fatty acids (SCFAs, essentially acetate, propionate, and butyrate) and other organic acids (*e.g.*, lactate) in the large intestine by the intestinal microflora. These SCFAs contribute to a reduced luminal pH in the large intestine, which is associated with an increased amount of soluble calcium, especially in the cecum. Moreover, this increased solubility may lead to an increased paracellular transport of calcium in the distal part of the small intestine and beginning of the large intestine (van den Heuvel *et al.*, 1999a). It is also possible that SCFAs directly influence the transcellular route of calcium absorption by modifying the exchange of intracellular H^+ for Ca^{2+} present in the distal colon. The protonated SCFA molecule diffuses across the apical membrane of the intestinal epithelial cells and once within the cell it dissociates, resulting in an increased intracellular H^+ , which is secreted from the cell in exchange for Ca^{2+} from the distal colon. Once outside the cell, H^+ becomes available to protonate another SCFA molecule. Therefore, there is an increased exchange of cellular H^+ for luminal Ca^{2+} (van den Heuvel *et al.*, 1999a). There is also some evidence from animal studies that certain prebiotics may influence transcellular calcium transport by altering the intracellular synthesis of the vitamin D receptor and calbindin D_{9K} . For example, in gastrectomised rats fed fructo-oligosaccharides, the amount of calbindin D_{9K} was increased in both cecal and colorectal segments and decreased in the proximal and distal small intestine. The overall effect, however, was an improved calcium absorption due to the prebiotic (Ohta *et al.*, 1998c). The mechanism(s) by which prebiotic substances modulate calbindin D_{9K} levels are not clear but may be due to increased synthesis of bioactive compounds such as butyrate and, possibly, certain polyamines. This issue has recently been extensively reviewed by Scholz-Ahrens and Schrezenmeir (2002).

Finally, in addition to possible effects of prebiotic substances on the paracellular and/or transcellular processes of calcium absorption, it may also be possible that such substances influence gut morphology and its absorptive surface, possibly via an increased production of butyrate and/or certain polyamines (see review by

Scholz-Ahrens and Schrezenmeir, 2002). For example, the villus crypt height, number of epithelial cells per crypt, and cecal vein flow have all been reported to be increased by prebiotics (see review by Scholz-Ahrens *et al.*, 2001a).

Conclusions and Suggested Further Research Areas

Upon critical review of the available data on the effect of certain prebiotics on calcium absorption, I think it is fair to conclude that, in general, prebiotic substances, such as inulin, oligofructose (fructo- oligosaccharide) and galacto-oligosaccharide, and lactulose have been found to stimulate calcium absorption and retention in rats. Furthermore, at least in some animal studies, the enhanced calcium absorption appeared to lead to improved bone status. Therefore, there is relatively good evidence of a beneficial effect of prebiotics on calcium bioavailability in rats. While there have only been a few studies on the effect of prebiotics (such as, lactulose, inulin, and fructo- and galacto-oligosaccharides) on calcium absorption in humans, so far, there would appear to be a stimulatory effect by these prebiotics on true intestinal calcium absorption, at least in subgroups of the population which have increased calcium requirements (*e.g.*, adolescents and postmenopausal women).

One of the difficulties in the communication of the benefits of functional foods is that the term 'health claim' is defined differently in different countries. A recent Consensus Document on scientific concepts in functional foods in Europe has proposed use of two types of health claims, Type A and Type B claims (Diplock *et al.*, 1999). Type A claims refer to 'enhanced function claims' while Type B claims refer to 'reduced risk of disease claims'. It was proposed that these claims should be based on evidence related to markers which are linked to clearly defined and measurable outcomes and are significantly and consistently modulated in rigorously controlled studies by the particular food component (Ashwell, 2001). Furthermore, enhanced function claims should be accompanied by evidence based on valid, reproducible, sensitive and specific markers relating to the target function or biological response, whereas reduction of disease risk claims would only be justified if the evidence is based on valid, reproducible, sensitive and specific markers relating to an intermediate endpoint of improved state of health and well-being and/or reduction of risk of disease (Diplock *et al.* 1999). Therefore, currently there is some, albeit limited, evidence to support a Type A health claim (*i.e.*, an enhanced function [improved calcium absorption in the present context]) for these prebiotic substances. There is, however, essentially no evidence to support a Type B claim (disease risk reduction) for these prebiotics. Therefore, there are still many out-standing research questions that would need to be answered before prebiotics could be marketed as functional food ingredients aimed at improving calcium bioavailability (*i.e.*, improved absorption and utilization by the body) and reducing risk of osteoporosis. The following are just some of the questions which would need to be addressed in future humans studies:

1. Human studies to show that the benefits of prebiotic

substances to true calcium absorption persist after their prolonged use (at least one year).

2. Human studies to investigate whether certain prebiotics have more efficacy (in terms of promoting true calcium absorption) than others, and to determine the minimum effective doses of these compounds.
 3. Human studies to investigate whether these prebiotics enhance true calcium absorption in population subgroups other than those who have increased calcium requirements namely, adolescents or postmenopausal women.
 4. Human studies to investigate whether the habitual dietary calcium content modulates the stimulatory effect of prebiotics on true calcium absorption.
 5. Human studies to investigate whether the beneficial effects of prebiotic substances on calcium absorption are translated into benefits to bone health. These may take the form of studies in which bone mineral density, bone mineral content, bone turnover and, indeed, bone structure and quality (all of which can act as surrogate markers of osteoporosis risk) are assessed in appropriately designed intervention trials.
- Needless to say, further research in experimental animals and possibly even in cells in culture may also help in better understanding the mechanistic aspects of the effects of prebiotics on calcium bioavailability. Future studies in model systems (and possibly in biopsy samples from human subjects) should consider using the newer molecular biology tools, such as transcriptomics and proteomics, to help gain new insights into the effect of prebiotics on calcium and bone metabolism. Such an integrated research approach (*i.e.*, human, animal and cellular research) to assess the functionality of new foods and food ingredients, including the prebiotics, would help address the growing concerns of regulatory bodies and health professionals, as well as consumer organization, with respect to the rapid appearance on the market place of more and more foods claiming to possess health-promoting properties, despite the fact that such products, to a large extent, having not being subjected to fundamental research to substantiate manufacturers' health claims.
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