Absorption of calcium from milks enriched with fructo-oligosaccharides, caseinophosphopeptides, tricalcium phosphate, and milk solids[1–3]

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ABSTRACT

Background: Adequate intakes of calcium are required for optimal bone health and protection against chronic disease. Dairy products are an excellent source of calcium.

Objective: The absorption of calcium from a range of fortified milks was measured in humans with the use of stable isotopes.

Design: Fifteen volunteers participated in a randomized, controlled, double-blind crossover study. Five types of semi-skimmed (1.9% fat) milk drinks were administered with a light breakfast: standard milk (control milk); milk enriched with calcium from milk solids and tricalcium phosphate [(TCP) MSS milk]; milk enriched with calcium from concentrated milk (CON milk); milk with added fructo-oligosaccharides [(FOSs) FOS milk]; and milk with added caseino-phosphopeptides [(CPPs) CPP milk]. All the milks were labeled with ^{42}Ca as $CaCl_2$. The MSS milk was also labeled with ^{44}Ca as TCP. The quantity of calcium in each drink was kept the same by varying the volume given.

Results: Calcium absorption did not differ significantly between the control milk and the calcium-fortified milks (MSS and CON milk) or the FOS and CPP milks. However, calcium absorption from the TCP added to the MSS milk was significantly higher than that from the control milk ($27.5 \pm 7.6\%$ and $24.5 \pm 7.3\%$, respectively; $P = 0.003$).

Conclusions: Calcium-enriched milks are a valuable source of well-absorbed calcium. Absorption of added calcium as TCP was higher than that of calcium from the control milk, but the addition of FOSs or CPPs did not significantly increase calcium absorption. Further research is needed to ascertain the cost-effectiveness and public health benefits of consuming fortified milks. *Am J Clin Nutr* 2006;83:310–6.

KEY WORDS Calcium, stable isotope, absorption, milk, fortification, tricalcium phosphate, fructo-oligosaccharides, caseino-phosphopeptides

INTRODUCTION

Calcium intake, particularly that during childhood, is a major determinant of bone mass in adults, and it also influences the rate of bone loss associated with aging (1–5). Osteoporosis, a disease affecting many millions of people around the world, is characterized by bone fragility that over time leads to bone fracture. In countries with a high incidence of osteoporotic fractures, a low calcium intake is associated with increased risk of fracture (6).

Low dietary intake of calcium also is associated with higher risks of colon cancer and hypertension and may affect normal growth in children (6, 7).

Dairy products are an excellent source of bioavailable calcium. Food intake surveys indicate that milk and dairy products generally are the main source of dietary calcium, constituting 66%, 72%, and 45% of the typical Spanish (8), US (9), and UK (10) diet, respectively. However, in some groups, the average daily calcium intake may be significantly less than the dietary reference intakes, ranging from 900 to 1500 mg/d for adults, and may not even reach 50% of the adequate intake (11).

Several strategies have been suggested to increase calcium intake or absorption (or both). The fortification of milk with milk calcium or calcium salts is among these strategies, but the availability of calcium salts in milk has not been well characterized. Likewise, several food ingredients, including fructo-oligo-saccharides (FOSs) and caseinophosphopeptides (CPPs), have been proposed as enhancers of the absorption of calcium from milk or other foods. FOSs belong to the group of nondigestible oligosaccharides (NDOs) that also includes inulin, oligofructose (fructans from the chicory root), and galacto-oligosaccharides. NDOs can be digested by the intestinal microflora, mainly in the colon, which produces short-chain fatty acids that decrease intestinal pH and increase calcium solubility and paracellular and transcellular calcium transport (12, 13). It has also been suggested that NDOs increase active calcium transport by the activation of calbindin-D9k (14, 15). Several human studies have shown an increase in calcium absorption when NDOs were included in the diet for a period of days or weeks (16–18). CPPs are phosphorus-rich peptide fragments of casein that are assumed to contribute to the high bioavailability of milk calcium by preventing the formation of insoluble calcium salts and possibly by

TABLE 1
Composition of the semi-skimmed (1.9% fat) milk drinks used in the study[1]

	Milk				
	Control	MSS	CON	FOS	CPP
Energy (kcal/100 mL)	46.5	53	56	46.5	46.5
Protein (g/100 mL)	3.1	3.9	4.3	3.1	3.1
Carbohydrates (g/100 mL)	4.7	5.8	6.3	4.7	4.7
Fat (g/100 mL)	1.9	1.6	1.6	1.9	1.9
Calcium (mg/100 mL)	120	160	160	120	120
Vitamin A (μg/100 mL)	120	120	120	120	120
Vitamin D (μg/100 mL)	0.75	0.75	0.75	0.75	0.75
FOS (g/100 mL)	—	—	—	0.5	—
CPP (g/100 mL)	—	—	—	—	0.2

[1] Control milk, standard semi-skimmed (1.9% fat) milk; MSS milk, milk enriched with calcium from milk solids and tricalcium phosphate; CON milk, milk enriched with calcium from concentrated milk; FOS milk, milk supplemented with fructo-oligosaccharides (FOSs); CPP milk, milk supplemented with caseinophosphopeptides (CPPs).

reducing interactions between calcium and other minerals (19). Although in vitro and animal studies showed a positive effect of CPPs on calcium absorption, the results from human studies were not conclusive (20–23). Because milk is one of the main sources of dietary calcium, we tested the possibility of using milk as a matrix for supplying additional calcium, through either enhanced bioavailability or higher calcium concentration.

This report details results from a stable-isotope study measuring the absorption of calcium in human volunteers from semi-skimmed milk and a range of modified milks that have a potential for delivering more calcium to the consumer: milk with added milk solids and TCP, concentrated milk, milk with added short-chain FOSs, and milk with added CPPs. The ingredients were added to the milks at low doses, and the aim was to produce affordable, functional dairy products that could be widely used.

SUBJECTS AND METHODS

Subjects

Fifteen subjects (8 men and 7 women) aged 25–36 y were recruited from among the staff of Puleva Biotech SA (Granada, Spain). The subjects were given a physical examination and their medical history was reviewed before they were included in the study. None of the subjects had ever had any disease known to influence calcium absorption or metabolism, and nor had they ever had malabsorption, diabetes, rheumatoid arthritis, diseases with disturbances in parathyroid hormone, or renal, intestinal, or liver disease. The participants took no medication throughout the study. All subjects were nonsmokers and were instructed not to change their physical activity or their usual diet; however, those who customarily consume alcohol were asked not to do so during the study period.

The volunteers were fully informed of the aims and purposes of the study and gave written informed consent. The study was conducted according to the Helsinki Declaration, and the protocol was approved by the Ethics Committee of the San Cecilio University Hospital (Granada, Spain).

Preparation of stable isotope solutions

A double-stable-isotope technique was used, which involves the simultaneous administration of 2 different stable isotopes, one given orally and one intravenously. True fractional absorption (FA) of calcium was calculated from the enrichment of both isotopes in urine samples by taking into account the amounts administered and the natural abundance of the stable isotopes (24, 25).

Stable isotopes were obtained from Chemotrade (Dusseldorf, Germany) as calcium carbonate: ^{42}Ca and ^{44}Ca were used in the milk drinks (ie, were given orally), and ^{43}Ca was administered intravenously. The abundances of the different isotopic labels were measured by thermal ionization mass spectrometry: enriched ^{42}Ca (24.3% ^{40}Ca, 73.4% ^{42}Ca, 0.4% ^{43}Ca, 1.8% ^{44}Ca, <0.01% ^{46}Ca, and 0.09% ^{48}Ca), enriched ^{43}Ca (39.2% ^{40}Ca, 0.65% ^{42}Ca, 49.5% ^{43}Ca, 10.5% ^{44}Ca, <0.01% ^{46}Ca, and 0.14% ^{48}Ca), and enriched ^{44}Ca (3.42% ^{40}Ca, 0.13% ^{42}Ca, 0.03% ^{43}Ca, 96.4% ^{44}Ca, and 0.02% ^{48}Ca). The ^{43}CaCO$_3$ salt was dissolved in ultrapure hydrochloric acid, sterile saline for injection was added, the pH WAS adjusted to 5.0 with the use of NaOH, and the solution was filtered. The solution was sterilized with the use of an autoclave, and aliquots were dispensed into sterile ampoules containing 1 mg ^{43}Ca each. The ^{42}CaCl solution was prepared by using the same basic procedure. For the preparation of the ^{44}Ca TCP salt, ^{44}CaCO$_3$ was first dissolved with concentrated HNO$_3$ to produce Ca(NO$_3$)^2 and then tested against KH$_2$PO$_4$ and NaOH at pH 9 as described elsewhere (26). The ^{44}Ca TCP precipitate was washed with deionized water and dried at 90 °C overnight. Aliquots containing 6 mg ^{42}Ca or 15 mg ^{44}Ca were prepared and stored at −80 °C until they were used.

Test drinks

Five different drinks were administered in randomized order to all of the volunteers during the 5 study periods (**Table 1**). The test drinks, all semi-skimmed (1.9% fat) milk, were as follows: standard milk extrinsically labeled with ^{42}Ca as CaCl$_2$ (control; 1); milk enriched with calcium by the addition of 12.6% calcium from milk solids and 15.5% calcium from TCP [Ca$_3$(PO$_4$)^2] and extrinsically labeled with ^{42}Ca as CaCl$_2$ and with ^{44}Ca from a TCP solution [(MSS milk) Puleva Calcio; Puleva Food, Granada, Spain]; milk enriched with calcium by the addition of 33% calcium from concentrated milk labeled with ^{42}Ca as CaCl$_2$ (CON milk); milk containing 5 g FOS/L (Ebro-Puleva, Spain; composition given in **Table 2**) extrinsically labeled with ^{42}Ca as CaCl$_2$ (FOS milk); and milk supplemented with 2 g phosphopeptide CE

TABLE 2
Composition of the fructo-oligosaccharides (FOSs) supplemented in the FOS milk used in the study[1]

	Value
Carbohydrate content (% of DM)	> 99.5
FOS (% of DM)	> 95.0
GF$_2$ (% of DM)	> 55.0
GF$_3$ (% of DM)	> 33.0
GF$_4$ (% of DM)	> 6.5
Glucose + fructose + sucrose (% of DM)	< 5.0
Dry matter (%)	72.0
Ash (% wt:wt)	< 0.1

[1] DM, dry matter; G, galactose; F, fructose.

TABLE 3
Subject characteristics[1]

	Value
Age (y)	28.7 ± 3.7 (24–26)
BMI (kg/m^2)	22.3 ± 1.7 (19.5–26)
Serum iPTH (pg/mL)	35.1 ± 8.2 (16.6–52.1)
Serum 25(OH)D (nmol/L)	56.3 ± 9.3 (32.9–71.4)
Energy intake (kcal/d)	2248 ± 344 (1522–2723)
Protein intake (g/d)	81.4 ± 14.2 (53–112)
Fat intake (g/d)	92.0 ± 15.0 (62–135)
Carbohydrate intake (g/d)	266.6 ± 41.7 (184–362)
Calcium intake (mg/d)	913.0 ± 189.0 (690–1287)

[1] All values are $\bar{x} \pm$ SD; range in parentheses. $n = 15$. iPTH, intact parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D. Values were calculated from food-intake diaries.

90 CPP/L (DMV International, Veghel, Netherlands) consisting of 89.1% protein (containing 22.5% CPP), 0.1% fat, 0.1% carbohydrates, 5.7% ash (60 mg Ca/100 g product), and 5% moisture, labeled with ^{42}Ca as CaCl$_2$ (CPP milk).

The milk drinks were labeled with 6 mg ^{42}Ca the day before the absorption test and left overnight at 4 °C to allow for equilibration between the calcium label and the native milk calcium. The MSS milk was also labeled with 15 mg ^{44}Ca to specifically measure the absorption from the added TCP. The solubility of FOSs and CPPs was checked before they were added to the test drinks, and they were completely dissolved in the milks before addition of the labels. The test drinks were volume adjusted to provide a total of 268 mg calcium, taking into account the calcium derived from the added ingredients and the isotope dose. The volume of the test drinks ranged from 165 to 224 mL.

Study design

The study was a controlled, randomized, double-blind crossover with 5 study periods that corresponded with the 5 drinks tested. The volunteers were advised to follow their habitual diet and to avoid unusual foods that they would not be able to obtain again during the rest of the study. They were asked to record all food and drink consumed in a diary provided by the investigator using household measures for 4 d before the first absorption test, on the absorption test day itself, and for 2 d (48 h) after dosing (7 d in total). Volunteers were then instructed to review their diaries and to consume the same foods and drinks in the same amounts (as far as possible) during the other 4 periods, excluding the 2-wk washout before the next absorption test. Dietary intake was assessed at weeks 1 and 11 by using the diaries completed by the volunteers. Intakes of nutrients and calcium were calculated according to the Spanish food-composition tables (27). On day 1, before the first study period, and at the end of the study, a blood sample was taken from each volunteer to determine 25-hydroxyvitamin D [25(OH)D] and intact parathyroid hormone (iPTH) concentrations. Subject characteristics are shown in **Table 3**.

On day 2, after an overnight fast, the volunteers ingested one of the dairy products indicated above in the middle of a light breakfast consisting of 2 slices of toasted white bread with butter and jam (59 mg total Ca, excluding the test drink). Between 30 min and 1 h after the oral administration of the dairy product, 1 mg ^{43}Ca (as CaCl$_2$) was infused into an antecubital vein over a 20-min period under medical supervision. During this time and for the subsequent 3 h, volunteers were not allowed to consume any food or drink except deionized water. Twenty-four-hour urine samples were collected in acid-washed containers from day 1 (24 h before the test day) to day 4. Then the subjects underwent a 2-wk wash-out phase before they began the next study period as described above. Aliquots of urine were kept at −80 °C until analysis was conducted.

Preparation of urine samples

Urine samples were thawed and thoroughly mixed by using a vortex mixer. Approximately 2 mL of 7.2 molar HNO$_3$ and 0.5 mL H$_2$O$_2$ (both: Ultrapure grade; Merck, Lutterworth, United Kingdom) were added to this mixture and left to react overnight at room temperature. To complete digestion, samples were irradiated at ≈90 °C in a closed ultraviolet digestion system (Model 707; Metrohm AG, Herisau, Switzerland) for 40 min. Clear digests were transferred into acid-washed Teflon vials and evaporated to dryness under a 1-kW lamp in a laminar flow cabinet.

Calcium, intact parathyroid hormone, and 25-hydroxyvitamin D measurements

Total calcium was measured in all the foods and ingredients by using atomic absorption spectrometry (AAS) as previously described (25). Urine samples were analyzed for total calcium by using flame AAS (model 3300; Perkin-Elmer, Norwalk, CT) after dilution (1:25 or 1:50 as required) with 0.5% La (as lanthanum chloride solution). Standards were prepared by using standard AAS solutions (1 g Ca/L; VWR International Ltd, Poole, United Kingdom; 1 g Na/L; Fluka; Sigma-Aldrich Company Ltd, Gillingham, United Kingdom) in a 1:3:5 ratio as described previously (25). All samples were analyzed in duplicate, and certified urine controls (Lyphochek Quantitative Urine Control, level 1–62161; Bio-Rad Laboratories Ltd, Hemel Hempstead, United Kingdom) were analyzed with each batch. The mean of all samples analyzed in duplicate (51.5 ± 1 mg/L) agreed well with the certified calcium concentration (48 mg/L; range: 43–53 mg/L). iPTH was measured in serum by chemoluminescence (DPC, Los Angeles, CA) by using 2 labeled antibodies raised against the *N*- and *C*-terminal fragments of the hormone. 25(OH)D in serum was measured by radioimmunoassay with the use of a commercial kit (Biosource International, Inc, Camarillo, CA).

Inductively coupled mass spectrometric analysis and calculation of fractional absorption

The isotope ratios were measured on a single-focus multicollector mass spectrometer (Isoprobe; Micromass, Manchester,

TABLE 4
Mean calcium absorption from the 5 test semi-skimmed (1.9% fat) milks measured in 24-h pooled urine samples collected on day 2 (24–48 h after dosing)[1]

		Milk				
		MSS				
	Control	Milk calcium (^{42}Ca)	TCP (^{44}Ca)	CON	FOS	CPP
	%	%		%	%	%
$\bar{x} \pm$ SD	24.5 ± 7.3	23.8 ± 7.4	27.5 ± 7.6[2]	24.1 ± 6.0	25.6 ± 5.5[3]	22.9 ± 5.8
CV	29.9	31.1	27.6	25.1	21.5	25.2
Range	14.9–37.4	16.3–37.9	13.5–40.4	15.4–34.8	16.3–35.6	12.9–32.7

[1] n = 15. Control, standard semi-skimmed milk; MSS milk, milk enriched with calcium from milk solids and tricalcium phosphate; CON, milk enriched with calcium from concentrated milk; FOS, milk supplemented with fructo-oligosaccharides; CPP, milk supplemented with caseinophosphopeptides (CPPs).
[2,3] Significantly different from the control milk (repeated-measures ANOVA and Tukey's honestly significant difference post hoc test): [2] $P < 0.01$, [3] $P = 0.055$.

United Kingdom) by using an Aridus desolvating sample-introduction system with a T1H microconcentric nebulizer (both: Cetac, Omaha, NE). NIST915, a calcium carbonate (clinical) standard, was used for bracketing each experimental sample, and measurement precision was optimized by normalizing experimental ratios to standard with known isotope ratios (28).

The FA of calcium was calculated from the ratio of the mass of the orally (^{42}Ca and ^{44}Ca) and intravenously (^{43}Ca) administered stable isotopes measured in urine, expressed as the fraction of the administered dose. This technique assumes that the oral tracer, once absorbed, follows the same kinetics as the intravenous tracer and natural calcium. A matrix inversion technique (29) was used to yield the mole fractions of each calcium source present in urine specimens. The use of a dual-isotope technique showed that the sources of calcium were oral (^{42}Ca and ^{44}Ca), intravenous (^{43}Ca), and natural abundance. By dividing the oral amount (dose corrected) by the intravenous amount (dose corrected) of the 24-h urine pools, the true FA of the oral dose was calculated according to the following equations (30):

$$TFA = {}^{42}Ca_{oral\ mass\ in\ urine} / {}^{43}Ca_{IV\ mass\ in\ urine}$$
$$\times\ {}^{43}Ca_{IV\ oral\ dose} / {}^{42}Ca_{oral\ dose} \quad (1)$$

and

$$TFA = {}^{44}Ca_{oral\ mass\ in\ urine} / {}^{43}Ca_{IV\ mass\ in\ urine}$$
$$\times\ {}^{43}Ca_{IV\ dose} / {}^{44}Ca_{oral\ dose} \quad (2)$$

where TFA = true FA, and IV = intravenous.

Although urine was collected in 24-h pools for 3 d after dosing, the calculation of FA was carried out on day 2 (24–48-h) samples because the percentage of enrichment in the oral isotopes ^{42}Ca and ^{44}Ca in the day 3 (48–72-h) pool was found, on average, to be below the acceptable detection limits. This approach also eliminated any potential error in the absorption estimate that may result from the time lag in the equilibration of the oral and intravenous dose kinetics during the first 24 h after dosing. All day 2 urine samples were analyzed in triplicate, and the level of enrichment necessary to provide acceptable limits of certainty in the calculated FA was ascertained on the basis of the previous studies (31).

Statistical analysis

A repeated-measures analysis of variance was applied to evaluate differences in the absorption of calcium from the 5 different drinks tested, followed by Tukey's honestly significant difference post hoc test. Paired t tests were used to evaluate differences in the iPTH, 25(OH)D, body mass index, and intake of nutrients. Data are expressed as means ± SEMs, and $P < 0.05$ was considered significant. SPSS software (version 11.5; SPSS Inc, Chicago, IL) was used for statistical analysis.

RESULTS

All participants completed the study, and compliance was good. Characteristics of the subjects, including serum concentrations of iPTH and 25(OH)D and the average daily intake of calcium and other nutrients, are shown in Table 3. There was no significant difference in serum concentrations of iPTH and 25(OH)D between the start and the end of study ($P > 0.05$), none of the subjects had 25(OH)D concentrations that indicated vitamin D deficiency (< 25nmol/L), and the range was relatively narrow (32.9–71.4 nmol/L). There was no significant change in the intake of nutrients or calcium during the study ($P > 0.05$). Data collected from food diaries at the beginning and the end of the intervention indicated a mean calcium intake of 913 mg/d (between-subject range: 690–1287 mg/d).

The within-run precision (%CV) of the isotope ratios in isotope-enriched samples, according to triplicate analysis of all day 2 urine samples, was 0.07 ± 0.07% for the ratio of ^{42}Ca to ^{40}Ca, 0.14 ± 0.14% for ^{43}Ca:^{40}Ca, and 0.19 ± 0.17% for ^{44}Ca:^{40}Ca. Enrichment of ^{43}Ca ranged from an average of 12.2% 24 h after dosing to 6.4% 72 h after dosing. The lowest mean enrichment of ^{42}Ca and ^{44}Ca measured in day 3 urine samples was 2.5%. Because of the low enrichment of day 3 urine samples, the results are based on data from the day 2 urine samples.

The calcium absorption values obtained from the 5 drinks tested are given in **Table 4**. The mean percentage of calcium absorption values from the 5 drinks tested was within the range of published values for milk—ie, 13.5–40.4%. Absorption of calcium from calcium-fortified milks (MSS and CON milks) was similar to that from the control milk. However, absorption of calcium from the TCP (labeled with ^{44}Ca) added to the MSS milk

was significantly higher ($27.5 \pm 7.6\%$; $P = 0.003$) than that from the control milk ($24.5 \pm 7.3\%$), as shown in Table 4. Absorption of calcium from the FOS milk ($25.6 \pm 5.5\%$) and the control milk was nearly significantly different but not statistically significantly different ($P = 0.055$). No differences in absorption of milk calcium were found between control milk and CPP milk.

DISCUSSION

Adequate calcium intake is critical to achieving optimal peak bone mass, and it influences the rate of bone loss associated with aging. Food sources of calcium are recommended for optimal calcium intake (7) because they prevent mineral imbalance and also supply other nutrients that play a role in maintaining bone health. Although dairy products, a naturally rich calcium source, are the major source of highly bioavailable calcium in the diet, fortification would provide significantly more calcium and therefore help consumers of these products achieve optimal calcium intake. However, the use of different calcium salts in fortified products should be evaluated in terms of calcium availability.

The aim of the current study was to evaluate the absorption of calcium from milk, milk with added milk calcium and calcium salts, concentrated milk, and milk supplemented with FOSs or CPPs. A dual-label stable-isotope technique was used to estimate accurately the FA of calcium from the urine samples collected 24–48 h after dosing (day 2 urine samples). Differences between the percentage of absorption calculated from the day 1 (0–24 h) urine samples and from the day 2 (24–48 h) urine samples were observed for some but not all volunteers (data not shown). This variation is most likely due to small initial differences in the rate of oral and intravenous isotope excretion (32). Gut motility will affect the time required for absorption to be complete, especially taking into account the fact that calcium can be absorbed in the more distal parts of the gastrointestinal tract such as the colon (33).

Mean absorption of calcium from the different milks used in the study ranged from $22.9 \pm 5.8\%$ to $27.5 \pm 7.6\%$, which agreed with values previously reported by our group (32) and other groups (34–37). Because calcium absorption depends on the total calcium load (38), the 5 drinks tested in our study were adjusted in volume to contain the same quantity of calcium. Extrinsically labeled calcium accounted for 2.2% of the calcium contained in all of the test drinks except the MSS milk, which contained an additional 5.6% as ^{44}Ca. It is generally accepted that the calcium extrinsic labeling approach is a valid tool for studying the absorption of calcium from milk. The physicochemical state of calcium in cow milk is such that the tracer exchanges with virtually all the calcium moieties in the milk, irrespective of their physical state and chemical ligands (39), so quantification in urine of the isotopic enrichment of the label added to the milk drink and the intravenous label represents true absorption of calcium from the drink.

The MSS milk, which included a combination of milk solids and an inexpensive calcium salt, contained one-third more calcium than did standard (control) milk. TCP is a common source of calcium for the fortification of foods (39), and 45% of milk calcium is in the form of TCP of the phosphocaseinate, which is insoluble and colloidal and is released during digestion (40). We added 15.5% calcium in the form of TCP to the MSS milk to reach 160 mg Ca/100 mL. Further addition of TCP yielded a dairy product with poor stability. The combination of calcium salt and milk solids used for calcium fortification, instead of milk solids only, also reduced the protein content of the MSS milk by 10%. Reducing the milk protein content of the diet may reduce urinary calcium loss in humans, as shown in a study in which a milk product with a higher calcium content and lower contents of protein, phosphorus, and energy was compared with standard milk (41). In our study, the percentage of calcium absorbed from standard milk or calcium-fortified milks, which contained 33% more calcium per volume unit, was the same. Although no advantage in terms of FA of calcium was observed, the intake of 30% less volume of the fortified milks with a meal led to absorption of the same percentage of calcium.

Previous reports showed that organic and inorganic calcium salts, including TCP, calcium carbonate, and calcium citrate, exhibit essentially the same FA when tested in humans, despite having very different solubility in aqueous solutions, which suggests that the absorbability of calcium from food sources is affected mainly by other food components (42).

The absorption of calcium from the TCP supplemented in the MSS milk was significantly higher ($P = 0.003$) than that from the milk itself. Most controlled trials in humans have shown no difference between FA of total calcium from milk and from calcium salts (35, 36, 43, 44), with a few exceptions such as a higher percentage of absorption of calcium from calcium sulfate–fortified bread (45) and a lower percentage from TCP-fortified soy imitation milk as compared with that from milk (39). These results highlight the influence of the food matrix on absorbability of calcium. Our study shows superior absorbability for the calcium salt in the fortified milk, but the difference was relatively small and may have little nutritional significance.

In our study, the intake of a single 1.1-g dose of FOSs administered in milk provoked a marginal increase in calcium absorption ($P = 0.055$) over that from the control milk. A few studies in humans, using stable isotopes, reported positive effects of lactulose, inulin, FOSs, and galactooligosaccharides on calcium absorption, but the substances being tested were given in larger amounts (5–20 g/d) and for an extended period of days or weeks (16–18). Short-chain FOSs, mainly those with 3 monomers such as GF2, are preferentially used by bifid bacteria and lactobacillus for short-chain fatty acid production (46, 47). The FOS mixture used in our study was especially rich in GF2 structures ($> 55\%$; 90% GF2+GF3), which could explain the trend we observed, despite the low amounts supplemented in the FOS milk. Although the production of short-chain fatty acids may lead to an increase in paracellular and transcellular pathways of calcium transport, another possible explanation for the increasing trend observed in our study is the induction of active calcium transport, because animal studies have shown that NDOs may induce calbindin-D9k in the intestine (14, 15). However, the current study was not specifically designed to investigate the calcium-enhancing effects of the FOSs tested but rather to test whether low amounts of short-chain FOSs supplemented in one glass of milk would make a difference in calcium absorption. A study design using different amounts of FOSs should be used to address this point.

CPPs seem to have a large capacity to form soluble complexes with calcium at luminal pH, a feature that seems to explain the promoting effect on passive calcium absorption (23). The few published studies of CPP-supplemented foods in humans suggest that the quantity of CPP in the milk drink (87 mg) was possibly

too low to produce a significant effect on the percentage of calcium absorption (CPP:calcium = 0.32). In other studies, much larger amounts were used, although the results are controversial: for example, the supplementation of dairy products or bread with 1 g CPPs did not produce any significant effects in calcium absorption (22, 23), whereas incorporation of the same quantity into rice-based cereals did have significant effects (21). Several factors, such as the food matrix, the nutritional and physiologic status of the subjects, and CPP:calcium (48), clearly have an influence on the effects of CPPs on calcium absorption, and more research is needed in this area.

This study supports the use of calcium-enriched milks as a good source of absorbable calcium. The absorption of the calcium salt from the supplemented milk compares favorably with that from standard milk. The relatively low-level addition of FOSs or CPPs did not enhance calcium absorption. To ascertain cost-effectiveness and potential public health benefits, further research is needed to evaluate the effects produced by the consumption of calcium-supplemented milks over different periods of time, in different target populations, and with various patterns of intake.

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