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Integrated in vitro approaches to assess the bioaccessibility and bioavailability of silicon-biofortified leafy vegetables and preliminary effects on bone

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Abstract Food industries are increasingly oriented toward new foods to improve nutritional status and/or to combat nutritional deficiency diseases. In this context, silicon biofortification could be an innovative tool for obtaining new foods with possible positive effects on bone mineralization. In this paper, an alternative and quick in vitro approach was applied in order to evaluate the potential health-promoting effects of five silicon-biofortified leafy vegetables (tatsoi, mizuna, purslane, Swiss chard and chicory) on bone mineralization compared with a commercial silicon supplement. The silicon bioaccessibility and bioavailability of the five leafy vegetables (biofortified or not) and of the supplement were assessed by applying a protocol consisting of in vitro gastrointestinal digestion coupled with a Caco-2 cell model. Silicon bioaccessibility ranged from 0.89 to 8.18 mg/L and bioavailability ranged from 111 to 206 μg/L of Si for both vegetables and supplement. Furthermore, the bioavailable fractions were tested on a human osteoblast cell model following the expression of type 1 collagen and alkaline phosphatase. The results obtained highlighted that the bioavailable fraction of biofortified purslane and Swiss chard improved the expression of both osteoblast markers compared with the supplement and other vegetables. These results underline the potentially beneficial effect of biofortified leafy vegetables and also indicate the usefulness of in vitro approaches for selecting the best vegetable with positive bone effects for further in vivo research.

Keywords In vitro models · Human intestinal cell line · Osteoblastic marker expression · In vitro digestion · Biofortification of vegetables

Introduction

Increasing evidence suggests that a healthy diet with increased consumption of plant-based foods plays an important role in preventing chronic illnesses, such as heart disease, cancer, strokes, diabetes, Alzheimer's disease, and age-related function decline (Liu 2013). Consumer requests are increasingly selective, as they demand food with more appropriate characteristics for specific situations, e.g. health status, age, sex, employment status, and cultural background, as reported by the European technology platform Food For Life (http://etp.fooddrinkeurope.eu/documents/2014/141201_SRIA_update.pdf). The food industry is challenged to provide new foods as part of balanced diets to improve the nutritional status of the target population and/or to combat deficiency diseases using different methods, such as supplementation and biofortification, as an alternative approach to pharmaceuticals (WHO report, 2006; Gomez-Galera et al. 2010; Joy et al. 2015).

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Supplementation is the short-term strategy for improving nutritional health and involves the distribution of capsules or mineral solutions for immediate consumption (Gomez-Galera et al. 2010). Chemical supplements, although they go into diet as a means of aiding the prevention of chronic diseases (diseases related to micronutrient deficiency), are less acceptable to consumers, who prefer to eat food rather than taking "chemical supplements". In this context, biofortification is a method for improving food quality and the nutritional status of the target population and can be achieved via different approaches, as reported by White and Broadley (2005) and Carvalho and Vasconcelos (2013). With regard to mineral components, in addition to iodide, calcium, and selenium, silicon (Si) is also considered a microelement important for health. Silicon is widely found in plant-based foods, drinking water, and some alcoholic beverages, notably beer (Powell et al. 2005; Jugdaohsingh et al. 2002), although its absorption depends on the food source (Sripanyakorn et al. 2009) and its chemical form (Calomme et al. 1998; Van Dyck et al. 1999). Silicon is also important for bone mineralization; Jugdaohsingh (2007) reported that a Si supplement (SiS) of 28 mg/d for 12 wk increased spinal column bone mineral density by 2.5% in six women with low bone mass. Several in vitro studies have reported beneficial effects, measured as an increase in bone differentiation markers (Carlisle 1970; Brady et al. 1991; Reffitt et al. 2003), but these studies were performed using different chemical forms of Si (Na_2SiO_3, K_2SiO_3, SiO_2, $Si(OH)_3CH_3$, and Zeolite) normally present in SiS composition, since Si bioavailability from foods is very low (Hodson et al. 2005). Given the difficulty of obtaining Si-biofortified vegetables via common farming techniques, one easy method of biofortification is the floating system approach (Malorgio et al. 2009; Voogt et al. 2010; Ferrarese et al. 2012). This closed soilless system is used to produce leafy vegetables and allows a rapid Si uptake by plants. A previous study by our research group used Si biofortification via a floating soilless system to produce six leafy vegetables (tatsoi, mizuna, purslane, basil, Swiss chard, and chicory) that are frequently used as fresh-cut products (D'Imperio et al. 2016). The effects of new foods are generally assessed via more expensive and time-consuming in vivo studies on laboratory animals. In keeping with the principles of Replacement, Reduction, and Refinement (3Rs) that were developed over 50 yr ago and are supported by the European Union, academic and industrial researchers have increasingly turned to alternative approaches, such as in vitro methods; these not only provide useful screening and help reduce costs and the number of animals needed but can also reproduce human physiological conditions and therefore aid our understanding about the mechanisms of action.

Considering these points, this work aimed to describe an integrated alternative approach to testing the potential effects on bone of these vegetables (with and without Si biofortification) compared with commercial SiS. The protocol included in vitro digestion and intestinal (differentiated human Caco-2 cell line) models to assess Si bioaccessibility and bioavailability of different leafy vegetables with (+Si) and without (−Si) Si biofortification and SiS, in order to reproduce their physiological uptake. Furthermore, an additional in vitro model, using human osteoblast cells, was used to evaluate Si's potential beneficial effect on bone mineralization. This model follows the expression of type 1 collagen (COLL-I) and alkaline phosphatase (ALP) markers for osteoblast activity, after exposure to the bioavailable fractions of vegetables and SiS.

Materials and Methods

Materials The following products were bought from Sigma (Milan, Italy): Dulbecco's phosphate-buffered saline (PBS), L-glutamine (GLN), Dulbecco's modified Eagle's medium (DMEM), antibiotic and antimycotic solution (AA), trypsin-EDTA, lipase from porcine pancreas type II (L3126), pancreatin from porcine (P1750), bile bovine dried unfractionated (B3883), fetal bovine serum (FBS), poly-L-lysine, ascorbic acid, beta-glycerophosphate, and *Clostridium histolyticum* neutral collagenase. Pepsin (0230.2) was purchased from Carl Roth Gmbh (Karlsruhe, Germany). Non-essential amino acid solution (NEAA) was supplied by EuroClone (Milan, Italy). Minimum essential medium (α-MEM), penicillin, streptomycin, and amphotericin B were purchased from Gibco Life Technology (Milan, Italy). Human intestinal Caco-2 (HTB-37) cell line was bought from the cell bank of the *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna* (IZSLER) (Brescia, Italy). Commercial SiS was purchased from a herbalist.

Vegetable samples and commercial silicon supplement The biofortified and non-biofortified leafy vegetables used in this work were obtained in the previous research study (D'Imperio et al. 2016). The experiments involved five different leafy vegetables (tatsoi, mizuna, purslane, Swiss chard, and chicory) grown in a soilless cultivation system, an innovative method for the biofortification of leafy vegetables. More information about plant production is reported in the Supplementary Information.

The SiS capsules were bought from a local herbalist and had a content of 90 mg Si/capsule. As observed in Table 1, the Si content of vegetables without biofortification (−Si) ranged from 0.711 to 1.12 mg/100 g fresh weight (FW) for purslane and chicory, respectively. The presence of Si in the nutrient solution significantly increased Si content in all vegetables, although accumulation was species-related. Of the +Si-biofortified samples, Swiss chard was one which accumulated the highest Si content (6.65 mg/100 g FW).

Table 1 Silicon content in non-biofortified and biofortified vegetables

Family name	Scientific name	Common name	Silicon content (mg Si/100 g fresh weight)	
			Non-biofortified (−Si)	Biofortified (+Si)
Portulacaceae	*Portulaca oleracea* L.	Purslane	0.71 ± 0.031a	3.95 ± 0.30b
Brassicaceae	*Brassica rapa* L., tatsoi group	Tatsoi	0.84 ± 0.024a	3.96 ± 0.012b
	Brassica rapa L., mizuna group	Mizuna	0.84 ± 0.13a	4.97 ± 0.61b
Asteraceae	*Cichorium intybus* L.	Chicory	1.12 ± 0.10a	3.34 ± 0.41b
Chenopodiaceae	*Beta vulgaris* L. ssp. *Vulgaris*	Swiss Chard	0.84 ± 0.14a	6.65 ± 1.43b

Silicon content in the studied vegetables as affected by silicon in the nutrient solution; Si−, and Si+, respectively for 0 and 100 mg/L of silicon. Significance: mizuna, $p \leq 0.001$; tatsoi, $p \leq 0.01$; purslane, $p \leq 0.01$; chicory, $p \leq 0.01$; Swiss chard, $p \leq 0.001$. Different *lowercase letters* within each vegetable indicate that mean values are significantly different, according to the SNK test ($p = 0.05$). Data are expressed as means ± SD

Assessment of Si bioaccessibility in leafy vegetables and Si supplement using the in vitro digestion process In order to simulate the physiological conditions of vegetable intake, the same amount of vegetables was used for in vitro digestion following the protocol described by Ferruzzi et al. (2001). Briefly, 3 g of +Si and −Si fresh vegetables and 0.3 g of SiS powder (the weight of capsule corresponding, on average, to the same amount of lyophilized leafy vegetables) were homogenized with 4.5 mL of 0.9% NaCl at the initial pH of 7. The gastric phase started with the addition of 0.9 mL of pepsin solution (40 mg/mL) in 0.1 N HCl and adjustment of pH to 2.5 (± 0.1) with 1 N HCl. Samples were flushed with N_2 and incubated at 37°C, then mixed (1×*g* at 37°C) for 1 h in a Rotator Type L2 (Labinco BV, Breda, Netherlands). Following gastric digestion, the small intestinal phase was started by adjusting the gastric digesta pH to 5.3 with a combination of 100 mM $NaHCO_3$ and 1 N NaOH followed by the addition of small intestinal enzyme solution (2.7 mL of porcine lipase [2 mg/mL], pancreatin [4 mg/mL], and bile [24 mg/mL] in 100 mM $NaHCO_3$). The pH of the final sample was adjusted to 6.5 ± 0.1 with 1 N NaOH; volume was standardized to 15 mL with 0.9% NaCl, and samples were blanketed with N_2, incubated at 37°C, and mixed for 2 h by Rotator Type L2 (1×*g* at 37°C). The samples were subsequently centrifuged at 10,000×*g* for 1 h at 4°C to separate the aqueous intestinal digested (AQ) from the residual solid. Aliquots of AQ were collected, filtered using a 0.2-μm PTFE filter, and stored at −80°C under a blanket of N_2 until spectroscopic analysis to determine their Si content.

Assessment of Si bioavailability using differentiated human intestinal cell line (Caco-2) Human intestinal cell line Caco-2 was cultured in a 75-cm^2 flask using DMEM with 10% FBS, 2 mM GLN, 1% NEAA, 1%, AA at 37°C under 5% CO_2, and humidified air. Cells were trypsinized at a confluence of 70% using trypsin-EDTA solution. The cell count and viability were assessed using a Scepter Cells Counter (Millipore, Milan, Italy). For the experiments, we used cells at passage numbers 18 to 22, and it resulted in their being free of DNA mycoplasma following the protocol reported in the Supplementary File (Appendix). To evaluate the potential bioavailability of Si, the cells were seeded onto polyethylene terephthalate (PET) track-etched membrane chamber inserts (24-mm diameter, 0.4-μm pore size; area 4.2 cm^2, Falcon, BD, Milan, Italy) pre-treated with 50 μg/mL of poly-L-lysine at a density of 12.5×10^4 cells/filter in medium (2 mL). The basolateral compartment was filled with 3 mL of DMEM. Cellular monolayers were cultured for 21 d in order to obtain fully differentiated cells with enzymatic and morphological characteristics similar to the human small intestinal tract. The integrity of the cell monolayer was evaluated by measuring transepithelial electrical resistance (TEER) with a volt-ohm meter (Millicell ERS-2, Millipore, Milan, Italy). TEER values were expressed as ohms per squared centimeter. Only Caco-2 monolayers with TEER values over 700 Ω/cm^2 were used for in vitro experiments. The uptake experiments were performed following the protocol described by D'Antuono et al. (2015). Briefly, monolayers were washed with 2 mL PBS (pH 5.5), and then 400 μL of the bioaccessible fraction from vegetables (+Si and −Si) and from SiS was placed in 1.6 mL of DMEM medium (dilution 1:5 to avoid monolayer cytotoxicity) and added to an apical chamber. Si levels ranged from 0.16 to 0.44 mg/L for −Si fractions and from 0.62 to 0.94 mg/L for +Si fractions, and it was 1.63 mg/L for the SiS fraction. Cell cultures were incubated at 37°C under 5% CO_2 with 95% relative humidity for 2 h. Following incubation, basolateral compartment media were aspirated and stored at −80°C until the Si analysis and evaluation.

Assessment of silicon content in bioaccessible and bioavailable fractions of vegetables and Si supplement The Si content in the bioaccessible and bioavailable fractions was determined using the spectroscopy method (ASTM D859-00 2000) reported in our previous study (D'Imperio et al. 2016). Briefly,

the solutions required for this procedure were $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (75 g/L), $HO_2C_2O_2H$ (10 g/L), and HCl/H_2O solution (1:1). The reducing solution consisted of 30 g of $NaHSO_3$, 1.5 g of Na_2SO_3, and 0.5 g of $H_2NC_{10}H_5(OH)SO_3H$ dissolved in 200 mL in H_2O. Then, in rapid succession, we added an aliquot of sample of variable quantity according to the Si concentration, 0.1 mL of the HCl solution, and 0.2 mL of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ solution. The solution was stirred for 5 min, then 0.15 mL of oxalic acid was added and the mixture was stirred for 1 min. After adding 200 μL of the reducing solution, it was mixed for 10 min. Concerning the bioavailable fractions, the growing medium of three wells was pooled, dried, and dissolved in 1 mL of medium to raise Si levels (>LOQ 352 μg/L). The absorbance of the samples was assessed at 815 nm. From a Si stock solution of 1.40 mg/L, Si was analyzed by using standard concentrations ranging from 1400 to 352 μg/L of Si. The quantification of Si in digested fluids was determined by interpolation of a calibration curve with an $R^2 = 0.9994$.

Isolation and characterization of human osteoblast cells Human osteoblasts (hOBs) were obtained from trabecular bone specimens from healthy subjects who underwent femur surgery following an accident. All subjects gave their informed consent.

The protocol described by Robey and Termine (1985) was used to isolate hOBs. Briefly, bone specimens were cleaned of soft tissues, reduced to small fragments, and digested with 0.5 mg/mL *Clostridium histolyticum* neutral collagenase in α-MEM gently agitated for 30 min at 37°C. Bone fragments were then washed three times with PBS and cultured in α-MEM supplemented with 10% FBS, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 2.5 mg/mL amphotericin B at 37°C in a water-saturated atmosphere containing 5% CO_2. Cells were fed by medium replacement every 3–4 d. In these conditions, the hOBs in the explants proliferated and migrated to the culture substrate, reaching confluence within 3–4 wk. Cells were then trypsinized and transferred to appropriate culture dishes for characterization and experiments.

The cells displayed an OB phenotype, as almost all were positive to histochemical staining for ALP, the osteoblast marker enzyme. Furthermore, flow cytometry procedures ascertained that OB contamination by CD45+ hematopoietic cells was <0.5% (data not shown). The experiments performed in this study used cell cultures at passages 2–3.

Expression of two osteoblast markers on human osteoblast cultures assessed after exposure to bioavailable fractions In order to assess the specific bone mineralization markers (COLL-I and ALP), hOBs were seeded at a density of 1×10^5 cells in six-well plates and cultured in α-MEM medium supplemented with 10% FBS. When they reached confluence, cells were grown using the same medium supplemented with osteogenic differentiating factors (50 μg/mL ascorbic acid and 10 mM β-glycerophosphate). In this condition, hOBs, independent of Si levels, were exposed to SiS, or −Si/+Si vegetable bioavailable fractions for 36 h in order to evaluate ALP and COLL-I messenger RNA (mRNA) levels. In particular, for −Si fractions, Si levels ranged from 114 to 179 μg/L and for +Si fractions from 111 to 206 μg/L, and for the SiS fraction it was 262 μg/L. As a parallel control, hOBs were incubated for 36 h in the presence of the bioavailable fraction with a Si level below LOQ. At the indicated times, the expression of COLL-I and ALP RNA was evaluated by extracting RNA from hOBs using spin columns (RNeasy, Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted RNA (1 μg) was reverse-transcribed using the iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. cDNA was amplified with the iTaq EVA Green Supermix ROX kit (Bio-Rad Laboratories), and PCR amplification was performed using the Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories). The following primer pairs were used for the real-time PCR amplification: ALP S, CGCACGGAACTCCTGACC; ALP AS, GCCACCAC CACCATCTCG (NM_000478.4); COLL-I S, CGTGGCAG TGATGGAAGTG; COLL-I AS, AGCAGGACCAGCGT TACC (NM_000089.3); GAPDH S, TCATCCCTGCCTCT ACTGGCG; AS, TGCTTCACCACCTTCTTG (NM_001256799.1). Running conditions were as follows: incubation at 95°C for 3 min and 40 cycles of incubation at 95°C for 15 s and 60°C for 30 s. After the last cycle, melting curve analysis was performed in the 55–95°C interval by increasing the temperature by 0.5°C. The changes in increase rates were calculated using the Pfaffl method (2001).

Statistical analysis SAS Version 9.1 software (SAS Institute, Cary, NC) was used for statistical analysis. Si content data for each species (+Si vs −Si) were analyzed separately by one-way analysis of variance using the GLM procedure. All means were compared using Student–Newman–Keuls (SNK). One-way analysis of variance according to Dunn's method was used to assess the significance between bioaccessible/bioavailable fractions of vegetables (+Si vs −Si) and SiS with digested liquid control. Concerning the biological effect on hOBs, the *t* test method was used in order to assess the significance of ALP and COLL-I fold changes. The results with a p value ≤0.05 were considered statistically significant.

Results and Discussion

Silicon bioaccessibility after in vitro digestion of silicon supplement and vegetables As shown in Fig. 1, after in vitro digestion, SiS had a significantly ($p \leq 0.001$) high release compared with all vegetables considered (biofortified

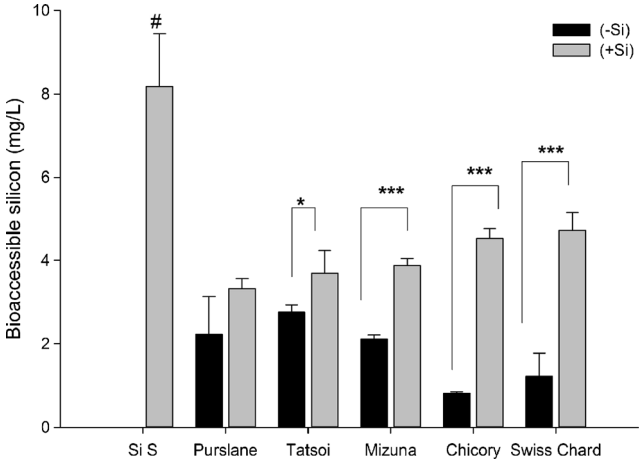

Figure 1. Silicon bioaccessibility after in vitro digestion of silicon supplement capsule (SiS) and non-biofortified and biofortified vegetables following the protocol of Ferruzzi et al. (2001) and determined using the ASTM Method (D859-00). The *number symbol* (#) indicates the difference between silicon supplement (SiS) and vegetables. SiS vs vegetables (+Si and −Si) significance ($p \leq 0.001$) was evaluated by Dunn's method. Concerning the bioaccessibility of Si in non-biofortified (−Si) and biofortified (+Si) vegetables, the *asterisk symbol* (*) indicates that mean values are significantly different, according to the Student–Newman–Keuls (SNK) ($p \leq 0.05$) significance: purslane, not significant (*ns*); tatsoi, $p \leq 0.05$; mizuna, $p \leq 0.001$; chicory, $p \leq 0.001$; Swiss chard, $p \leq 0.001$. Data are expressed as means ± SD of three independent experiments.

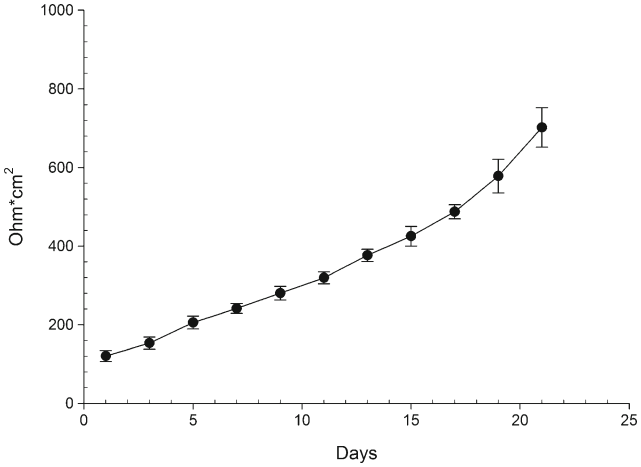

Figure 2. The transepithelial electrical resistance (TEER) was monitored during in vitro differentiation of Caco-2 cells on filter inserts from 3 to 21 d after seeding. Data represent means of representative experiments performed in triplicate.

and non-biofortified); the Si level in the SiS bioaccessibile fraction was 8.24 mg/L, compared with +Si (from 3.30 to 4.72 mg/L) and −Si (from 0.81 to 2.76 mg/L) vegetables. The greater bioaccessibility of SiS, due to the absence of a matrix, agrees with other authors (Lavu et al. 2014). With the exception of purslane, the Si bioaccessible levels were significantly ($p < 0.05$) higher in +Si vegetables compared to −Si ones. In fact, after digestion of +Si vegetables, it was seen that Swiss chard released the highest Si content in digestion liquids (4.7 mg/L), followed by chicory (4.5 mg/L), mizuna (3.9 mg/L), tatsoi (3.7 mg/L), and purslane (3.30 mg/L) as shown in Fig. 1. Moreover, in −Si vegetables, the level of bioaccessible Si was on average higher for tatsoi (2.76 mg/L), purslane (2.23 mg/L), mizuna (2.11 mg/L), and Swiss chard (1.21 mg/L) than for chicory, which gave a significantly ($p < 0.05$) lower amount (0.81 mg/L). These differences in Si bioaccessibility for different leafy vegetables were also reported by other authors for other foods (Robberecht et al. 2008). Similarly, high variability has already been demonstrated for other mineral elements of plant origin (Bhavyashree et al. 2009; Luo et al. 2010) and is probably attributable to antinutritional factors, such as phytate (Alkarawi and Zotz 2014). At present, it is not possible to compare our results with other publications because, with the exception of one previous study (D'Imperio et al. 2016), to our best knowledge there are no reports on the bioaccessibility of Si assessed using an in vitro digestion process for leafy vegetables.

Assessment of Si bioavailability on a differentiated human Caco-2 cell line Before the assessment of Si bioavailability, the integrity and differentiation of a Caco-2 cell monolayer were monitored by measuring TEER. As shown in Fig. 2, TEER values increased linearly during the 21 d, with a maximum increment in the last few days (15–21 d). The trend of increasing TEER values agreed with other authors (Ferruzza et al. 2012) and was related to the spontaneous differentiation of the cellular monolayer, which expressed the morphological and functional characteristics of normal enterocytes (Hidalgo et al. 1989). The bioaccessible fractions obtained after in vitro digestion of vegetables (+Si and −Si treatments) and SiS were added to the apical chamber in order to assess the bioavailability of Si on the differentiated Caco-2 cell line. The bioavailable Si level from SiS was significantly ($p \leq 0.001$) higher (262 μg/L) than all vegetables, whether biofortified (from 111 to 206 μg/L) or not (from 114 to 179 μg/L), as shown in Fig. 3. Regarding the vegetables, the bioavailable Si level in biofortified chicory was significantly higher than in the non-biofortified chicory. The opposite was true for Swiss chard. Biofortified Swiss chard presented significantly less bioavailable Si (111 μg/L) than did the non-biofortified sample (179 μg/L). No differences were recorded for all other vegetable species (Fig. 3). The obtained results suggest that the biofortification treatment of purslane, tatsoi, and mizuna did not significantly modify the bioavailable Si concentration, but did for chicory and Swiss chard. Si bioavailability is strongly dependent on endogenous and exogenous factors. It is possible that the amount of uptake could be enhanced or lowered not only by the type of food (meat, fish, fruit, vegetables, and drinks) but also by the matrix and food composition (such as dietary fiber content, valency state of the trace element, and antagonistic factors) (Robberecht et al. 2008). The different levels of bioavailable Si found for the tested

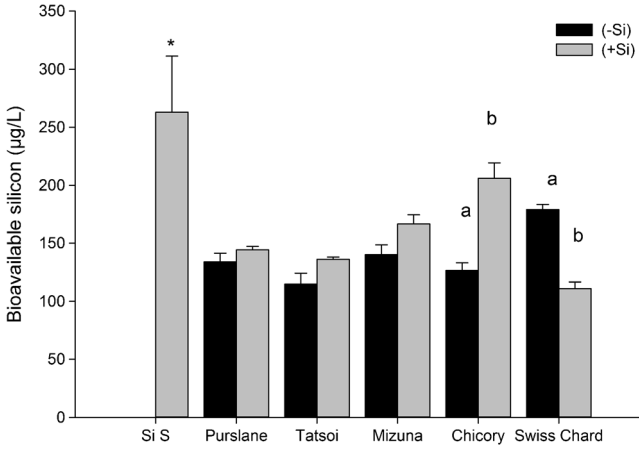

Figure 3. Silicon bioavailable levels after exposure (2 h) of human differentiated intestinal Caco-2 cells to samples (diluted 1:5) obtained by digestion of Si supplement (SiS) and non-biofortified and biofortified vegetables and quantified using the ASTM Method (D859-00). The *asterisk symbol* (*) indicates the difference between silicon supplement (SiS) and vegetables. SiS vs vegetables (+Si and −Si) significance ($p \leq 0.001$) was evaluated by Dunn's method. Concerning the Si bioavailability in non-biofortified (−Si) and biofortified (+Si) vegetables, *different letters* indicate that mean values are significantly different, according to Student–Newman–Keuls (SNK) ($p \leq 0.05$) significance: purslane, tatsoi and mizuna, non-significant; chicory $p \leq 0.05$; and Swiss chard, $p \leq 0.001$. Data are expressed as means ± SD of three independent experiments.

vegetable samples may be related to the following factors: (i) fibers, which reduce the bioavailability of different minerals, including Si, as reported by Kelsay et al. (1979); (ii) calcium content, which in low amounts enhances the uptake of Si for competition with the same absorption pathway, as reported by Male and Julson (2008); and (iii) the presence of phytate that can interfere with absorption in the gut (Alkarawi and Zotz 2014; Singh et al. 2015). Independent of the biofortification process, Si was bioavailable in all the vegetables studied, with levels ranging from 111 to 206 µg Si/L, similar to the serum levels reported from an in vivo study by Sripanyakorn et al. (2009) and Jugdaohsingh et al. (2013) [range 38–326 µg/L]). As far as we know, this is the first report on Si bioavailability in biofortified vegetables obtained with a soilless cultivation system and using a human intestinal cell model. Furthermore, the results obtained in this study cannot be compared with previous studies, which evaluated dietary Si intake of various foods or used an in vitro continuous flow dialysis method to estimate Si bioavailability (Robberecht et al. 2008, 2009). However, the release and solubilization of micronutrients as measured with an in vitro continuous flow dialysis method do not necessarily indicate the efficiency with which the micronutrient is translocated by the intestinal mucosa. On the contrary, the data obtained in this study using the simulated in vitro gastrointestinal digestion model with Caco-2 cells reproduced physiological conditions more closely. These models could be a useful preliminary tool for screening different food products by evaluating bioaccessibility and bioavailability, although these in vitro data cannot exactly replicate human in vivo conditions.

Effects of Si-biofortified vegetables on human osteoblast differentiation We analyzed the expression of ALP and COLL-I (two osteoblast markers for assessing the influence of osteoblast activity) in hOBs cultured for 36 h in the presence of the bioavailable fractions of +Si or −Si vegetables and SiS in order to evaluate their effect on bone differentiation. As shown in Fig. 4, mRNA levels of the two osteoblast markers were significantly higher in the cells treated with the bioavailable fractions of all +Si and −Si vegetables as well as SiS, compared to hOBs cultured in control conditions. Interestingly, the increase in ALP and COLL-I mRNA levels was much more marked in the hOBs cultured in the presence

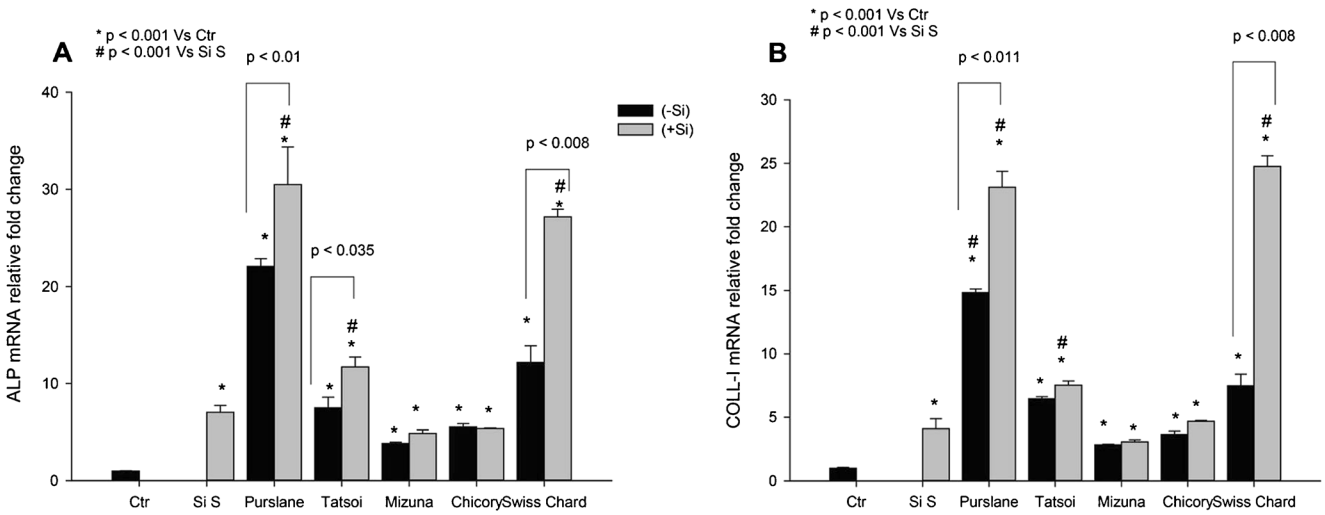

Figure 4. Effect of the bioavailable fraction of non-biofortified/biofortified vegetables and silicon supplement (SiS) on human osteoblast (hOB) differentiation. Real-time PCR of alkaline phosphatase (ALP) and collagen type 1 (COLL-I) expression in hOBs treated for 36 h with the bioavailable vegetable fractions and SiS. Data are expressed as means ± SD of three independent experiments.

of bioavailable +Si fractions of some vegetables if compared with the same −Si fractions and even more evident in comparison with SiS. In particular, treatment with the bioavailable fractions of +Si purslane, tatsoi, and Swiss chard enhanced the ALP mRNA levels by approximately 1.4-, 1.6-, and 2.3-fold, respectively, in comparison with the same −Si ones, and by approximately 4.3-, 1.7-, and 3.9-fold, respectively, vs SiS (Fig. 4A). At the same time, COLL-I gene expression was up-regulated by approximately 1.6- and 3.3-fold with +Si purslane and Swiss chard compared with the same −Si vegetables and by about 5.6- and 6-fold when compared to SiS. In addition, the +Si tatsoi caused a significant 1.83-fold increase of COLL-I mRNA levels compared only with SiS (Fig. 4B). The bioavailable fractions of mizuna, chicory, and SiS had a similar effect on the expression of both osteoblast markers. Since ALP and COLL-I are both regulated during the initial phase of OB differentiation and therefore represent the expression of the same functional moment of the cells, it is not surprising that they are modulated in the same way.

Consistent with our study, other authors (Reffitt et al. 2003; Kim et al. 2013) have reported an increase in COLL-I and ALP after exposure to orthosilicic acid or metasilicate, compounds normally used as supplements. In conclusion, we found a significant influence of Si biofortification on ALP marker in purslane, tatsoi, and Swiss chard. Concerning the COLL-I marker, the same results were found for purslane and for Swiss chard. A comparison between the results of Si bioavailability and the expression of osteoblast markers showed that the bioavailable Si levels of the tested vegetables could not be completely responsible for the effect on hOB differentiation, assessed as the modulation of ALP and COLL-I. Biofortification may have modified the levels of other compounds in the plant with effects on bone markers, as observed for Swiss chard, which is rich in phenolic acid (Young-Hee et al. 2004), and purslane, which contains high levels of omega-3 fatty acids (Simopoulos et al. 1992); both acids are known to positively affect hOB differentiation (Casado-Díaz et al. 2013; Chen et al. 2014). These results underline the usefulness of an in vitro approach for screening different vegetables to select those with the best effects on bone for future in vivo experiments. Our preliminary results would suggest that intake of biofortified vegetables enhances many more bone markers than SiS, due to the combined influence of Si with other bioactive components in vegetables.

Conclusion

In conclusion, this research explores a useful alternative in vitro approach to screening Si-biofortified leafy vegetables by assessing bioaccessibility, bioavailability, and effects on bone health, in order to select the vegetable with the best effect on bone markers. In addition, the biofortified vegetables studied in this work proved more effective on hOB differentiation than SiS, possibly due to the other components (e.g., polyphenols, carotenoids, vitamins, folate, and other minerals) present in the vegetables, which will be considered in a future work. These preliminary assessments on in vitro models should reduce the costs and number of animals involved in future in vivo experiments; they could also help to interpret the future in vivo studies to confirm the effect of purslane and Swiss chard on bone differentiation.

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References

Alkarawi HH, Zotz G (2014) Phytic acid in green leaves. Plant Biol 16: 697–701

ASTM D859-00 (2000) Standard test method for silica in water, ASTM International, West Conshohocken, PA.

Bhavyashree SH, Jamuna Prakash J, Kalpana Platel K, Srinivasan K (2009) Bioaccessibility of minerals from cereal-based composite meals and ready-to-eat foods. J Food Sci Technol 46:431–435

Brady MC, Dobson PRM, Thavarajah M, Kanis JA (1991) Zeolite A stimulates proliferation and protein synthesis in human osteoblast–like cells and osteosarcoma cell line MG-63. J Bone Miner Res S139

Calomme MR, Cos P, D'Haese PC, Vingerhoets R, Lamberts LV, De Broe ME, Van Hoorebeke C, Vanden Berghe DA (1998) Absorption of silicon in healthy subjects. In: Collery P, Bratter P, Negretti de Bratter V, Khassanova L, Etienne JC (eds) Metal ions in biology and medicine, vol. 5. John Libbey Eurotext, Paris, pp. 228–232

Carlisle EM (1970) Silicon: a possible factor in bone calcification. Science 167:279–280

Carvalho SMP, Vasconcelos MW (2013) Producing more with less: strategies and novel technologies for plant-based food biofortification. Food Res Int 54:961–971

Casado-Díaz A, Santiago-Mora R, Dorado G, Quesada-Gómez JM (2013) The omega-6 arachidonic fatty acid, but not the omega-3 fatty acids, inhibits osteoblastogenesis and induces adipogenesis of human mesenchymal stem cells: potential implication in osteoporosis. Osteoporos Int 24:1647–1661

Chen JR, Lazarenko OP, Zhang J, Blackburn ML, Ronis MJ, Badger TM (2014) Diet-derived phenolic acids regulate osteoblast and adipocyte lineage commitment and differentiation in young mice. J Bone Miner Res 29:1043–1053

D'Antuono I, Garbetta A, Linsalata V, Minervini F, Cardinali A (2015) Polyphenols from artichoke heads (*Cynara cardunculus* (L.) subsp. scolymus Hayek): in vitro bio-accessibility, intestinal uptake and bioavailability. Food Funct 6:1268–1277

D'Imperio M, Renna M, Cardinali A, Buttaro D, Santamaria P, Serio F (2016) Silicon biofortification of leafy vegetables and its bioaccessibility in the edible parts. J Sci Food Agr. doi:10.1002/jsfa.7142

Ferrarese M, Mahmoodi M, Sourestani M, Quattrini E, Schiavi M, Ferrante A (2012) Biofortification of spinach plants applying selenium in the nutrient solution of floating system. vegetable crops research bulletin 76:127–136

Ferruzza S, Rossi C, Scarino ML, Sambuy Y (2012) A protocol for differentiation of human intestinal Caco-2 cells in asymmetric serum-containing medium. Toxicol in Vitro 26:1252–1255

Ferruzzi MG, Failla ML, Schwartz SJ (2001) Assessment of degradation and intestinal cell uptake of carotenoids and chlorophyll derivatives from spinach puree using an in vitro digestion and Caco-2 human cell model. J Agric Food Chem 49:2082–2089

Gomez-Galera S, Rojas E, Sudhakar D, Zhu C, Pelacho AM, Capell T, Christou P (2010) Critical evaluation of strategies for mineral fortification of staple food crops. Transgenic Res 19:165–180

Hidalgo IJ, Raub TJ, Borchardt RT (1989) Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 96:736–749

Hodson MJ, White PJ, Mead A, Broadley MR (2005) Phylogenetic variation in the silicon composition of plants. Ann Bot 96:1027–1046

http://etp.fooddrinkeurope.eu/documents/2014/141201_SRIA_update.pdf

Joy EJM, Stein AJ, Young SD, Ander LE, Watts MJ, Broadley MR (2015) Zinc-enriched fertilisers as a potential public health intervention in Africa. Plant Soil 389:1–24

Jugdaohsingh R (2007) Silicon and bone health. J Nutr Health Aging 11: 99–110

Jugdaohsingh R, Anderson SH, Tucker KL, Elliott H, Kiel DP, Thompson RPH, Powell JJ (2002) Dietary silicon intake and absorption. Am J Clin Nutr 75:887–893

Jugdaohsingh R, Hui M, Anderson SHC, Kinrade SD, Powe JJ (2013) The silicon supplement 'Monomethylsilanetriol' is safe and increases the body pool of silicon in healthy pre-menopausal women. Nutr Metab 10:37

Kelsay JL, Behall KM, Prather ES (1979) Effect of fiber from fruits and vegetables on metabolic responses of human subjects. II. Calcium, magnesium, iron, and silicon balances. Am J Clin Nutr 32:1876–1880

Kim JE, Bu SY, Sung MK, Choi MK (2013) Effects of silicon on osteoblast activity and bone mineralization of MC3T3-E1 cells. Biol Trace Elem Res 152:105–112

Lavu RVS, Van de Wiele T, Pratti VL, Tack FMG, Du Laing G (2014) Bioaccessibility and transformation of selenium in the human intestine: selenium-enriched crops versus food supplements. In: Lin Z, Yin X (eds) Selenium in the environmental human health-Banuelos. Taylor and Francis group, London ISBN 978-1-138-00017-9

Liu RH (2013) Health-promoting components of fruits and vegetables in the diet. Adv Nutr 4:384S–392S

Luo Y, Xie W, Cui Q (2010) Effects of phytases and dehulling treatments on in vitro iron and zinc bioavailability in faba bean (*Vicia faba* L.) flour and legume fractions effects of phytase sand de hulling treatments on in vitro iron and zinc bioavailability in faba bean (*Vicia faba* L.) flour and legume fractions. J Food Sci 75:191–198

Male K, Julson JL (2008) Effects of high levels of dietary silicon on bone development of growing rats and turkeys fed semi-purified diets. Biol Trace Elem Res 123:191–201

Malorgio F, Diaz KE, Ferrante A, Mensuali A, Pezzarossa B (2009) Effects of selenium addition on minimally processed leafy vegetables grown in floating system. J Sci Food Agric 89:2243–2251

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:45

Powell JJ, McNaughton SA, Jugdaohsingh R, Anderson SH, Dear J (2005) A provisional database for the silicon content of foods in the United Kingdom. Br J Nutr 94:804–812

Reffitt DM, Ogston N, Jugdaohsingh R, Cheung HF, Evans BA, Thompson RP, Powell JJ, Hampson GN (2003) Orthosilicic acid stimulates collagen type I synthesis and osteoblastic differentiation in human osteoblast-like cells in vitro. Bone 32:127–135

Robberecht H, Van Cauwenbergh R, Van Vlaslaer V, Hermans N (2009) Dietary silicon intake in Belgium: sources, availability from foods, and human serum levels. Sci Total Environ 407(16):4777–4782

Robberecht H, Van Dyck K, Bosscher D, Van Cauwenbergh R (2008) Silicon in foods: content and bioavailability. Int J Food Properties 11:638–645

Robey PG, Termine JD (1985) Human bone cells in vitro. Calcif Tissue Int 37:453–460

Simopoulos AP, Norman HA, Gillaspy JE, Duke JA (1992) Common purslane: a source of omega-3 fatty acids and antioxidants. J Am Coll Nutr 11:374–382

Singh S, Swain S, Singh DR, Salim KM, Nayak D, Roy SD (2015) Changes in phytochemicals, anti-nutrients and antioxidant activity in leafy vegetables by microwave boiling with normal and 5% NaCl solution. Food Chem 176:244–253

Sripanyakorn S, Jugdaohsingh R, Dissayabutr W, Anderson SH, Thompson RP (2009) The comparative absorption of silicon from different foods and food supplements. Br J Nutr 102:825–834

Van Dyck K, Van Cauwenbergh R, Robberecht H, Deelstra H (1999) Bioavailability of silicon from food and food supplements. Fresenius J Anal Chem 363:541–544

Voogt W, Harmen T, Holwerda HT, Khodabaks R (2010) Biofortification of lettuce (*Lactuca sativa* L.) with iodine: the effect of iodine form and concentration in the nutrient solution on growth, development and iodine uptake of lettuce grown in water culture. J Sci Food Agric 90:906–913

White PJ, Broadley MR (2005) Biofortifying crops with essential mineral elements. Trends Plant Sci 10:586–593

WHO report (2006) Guidelines on food fortification with micronutrients. Allen L, de Benoist B, Dary O, Hurr R. http://www.who.int/nutrition/publications/micronutrients/GFF_Part_1_en.pdf

Young-Hee P, Tung-Ching L, Logendra L, Rosen RT (2004) Antioxidant activity and phenolic compounds of Swiss chard (*Beta vulgaris* subspecies cycla) extracts. Food Chem 85:19–26