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Short communication

Fat-soluble vitamin intestinal absorption: Absorption sites in the intestine and interactions for absorption

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ABSTRACT

The interactions occurring at the intestinal level between the fat-soluble vitamins A, D, E and K (FSVs) are poorly documented. We first determined each FSV absorption profile along the duodenal–colonic axis of mouse intestine to clarify their respective absorption sites. We then investigated the interactions between FSVs during their uptake by Caco-2 cells. Our data show that vitamin A was mostly absorbed in the mouse proximal intestine, while vitamin D was absorbed in the median intestine, and vitamin E and K in the distal intestine. Significant competitive interactions for uptake were then elucidated among vitamin D, E and K, supporting the hypothesis of common absorption pathways. Vitamin A also significantly decreased the uptake of the other FSVs but, conversely, its uptake was not impaired by vitamins D and K and even promoted by vitamin E. These results should be taken into account, especially for supplement formulation, to optimise FSV absorption.

1. Introduction

Vitamins are essential nutrients that ensure optimal growth, reproduction and function. They have been categorised based on solubility as water- (Bs and C) and fat-soluble (FSVs), which are, in turn, classed as four groups of compounds, specifically A, D, E and K (Table 1). As the body cannot synthesise vitamins, or at least insufficient vitamin D, adequate amounts have to be provided by the diet. However, the fundamental mechanisms involved in the absorption of FSVs are still unclear. Initially, Hollander et al. (Hollander, 1981) provided most of the evidence concerning FSV absorption. They suggested that vitamins E and D were absorbed by passive diffusion (Hollander, Rim, & Muralidhara, 1975; Hollander & Truscott, 1976) while vitamins A and K were absorbed via carrier-dependent proteins (Hollander, 1973; Hollander & Muralidhara, 1977). However, recent studies have reconsidered these assumptions, and have shown that absorption mechanisms are more complex than previously described: passive diffusion occurs at high concentrations of these compounds, while protein-mediated transport occurs at dietary doses (Reboul & Borel, 2011). Our recent work shows that vitamin D intestinal uptake is not just passive, but also involves cholesterol transporters, such as SR-BI (Scavenger Receptor class B type I), CD36 (Cluster Determinant 36) and NPC1-L1 (Niemann–Pick C1-Like 1) (Reboul et al., 2011). In the same way, SR-BI was shown to mediate tocopherol uptake in both Caco-2 and mouse models (Reboul et al., 2006), as well as NPC1-L1 (Narushima, Takada, Yamanashi, & Suzuki, 2008). The proteins involved in vitamin A and K membrane transport into the enterocyte remain unidentified.

Interestingly, several food component-vitamin interactions have been previously reported. We first showed that phytosterols inhibited vitamin D incorporation into micelles (Goncalves et al., 2011). Inhibition of α-tocopherol uptake was also observed in Caco-2 cells in the presence of different compounds, such as the phenolic naringenin, carotenoids and γ-tocopherol (Reboul et al., 2007), and vitamin D uptake was impaired by α-tocopherol (Reboul et al., 2011) and phytosterols (Goncalves et al., 2011). Another study has shown that FSVs could have antagonist

Abbreviations: FSVs, fat-soluble vitamins; FBS, fetal bovine serum; wt mice, wild-type mice; PDA, photodiode array detector; SR-BI, Scavenger Receptor class B type I; CD36, Cluster Determinant 36; NPC1-L1, Niemann–Pick C1-Like 1.

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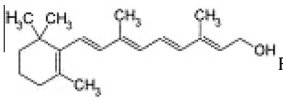
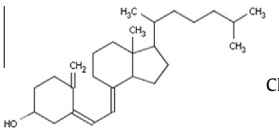
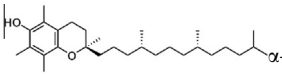
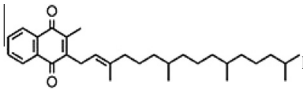
Table 1.
Fat soluble vitamins.

Vitamins	Structure of the molecules used	Other dietary forms
Vitamin A	 Retinol	Retinyl palmitate
Vitamin D	 Cholecalciferol (D_3)	Ergocalciferol (vitamin D_2)
Vitamin E	 α-Tocopherol	γ-Tocopherol
Vitamin K	 Phylloquinone (K_1)	Menaquinone-4 (vitamin K_2)

interactions affecting their intestinal absorption *in vivo*. Indeed, in chicks fed with high dietary levels of vitamin A (retinyl palmitate) during 24 days, plasma α-tocopherol levels were significantly depressed compared to chicks fed control vitamin A (Sklan & Donoghue, 1982).

After investigating FSV absorption in mouse intestine, our aim was to identify the competitive or synergistic interactions between vitamins A, D, E and K at a key step of intestinal absorption, i.e. during their uptake by the enterocyte.

2. Materials and methods

2.1. Chemicals

Retinol, retinyl acetate, retinyl palmitate, cholecalciferol, tocopherol acetate, phylloquinone, 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (phosphatidylcholine), 1-palmitoyl-sn-glycero-3-phosphocholine (lysophosphatidylcholine), monoolein, free cholesterol, oleic acid, sodium taurocholate were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France). RRR-α-tocopherol and echinenone were generous gifts from DSM Nutritional Products Ltd. (Basel, Switzerland). RRR-γ-tocopherol and apo-8′-carotenal were purchased from Fluka (Vaulx-en-Velin, France). Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and trypsin–EDTA (500 mg/L and 200 mg/L, respectively), non-essential amino acids, penicillin/streptomycin and PBS was purchased from Life Technologies (Illkirch, France), and fetal bovine serum (FBS) came from PAA (Vélizy-Villacoublay, France).

2.2. Preparation of FSV-enriched vehicles for cell and mouse experiments

2.2.1. Preparation of FSV-rich emulsions

To deliver FSV to mice, emulsions were prepared as previously described (Reboul et al., 2011). Depending on the experiment, mice received either retinyl palmitate (250 μg), cholecalciferol (100 μg), γ-tocopherol (500 μg) or phylloquinone (170 μg).

2.2.2. FSV-rich micelles

For delivery of vitamins to Caco-2 cells, mixed micelles were prepared as previously described (Reboul et al., 2011). FSVs were added at different concentrations depending on the conditions (see Table 2). Concentrations of FSV in the micellar solutions were checked before each experiment.

2.3. Characterisation of vitamin D uptake in mouse intestine

2.3.1. Animals

Six-week-old wild-type male C57BL/6 Rj mice were purchased from Janvier (Janvier, Le-Genest-St-Isle, France). The mice were housed in a temperature-, humidity- and light-controlled room. They were given a standard chow diet and water *ad libitum*. Mice were fasted overnight before each experiment. The protocol was approved by the ethics committee of Marseilles (Agreement #4-5032010).

2.3.2. FSV uptake in wild-type mice in the intestine

On the day of the experiment, the mice were force-fed with a FSV-enriched emulsion. After 4.5 h of digestion, intestine, caecum and colon of each animal was quickly harvested after euthanasia by cervical dislocation. The intestine, caecum and colon were carefully rinsed with PBS. The small intestine was cut into 6 cm segments along a total length of 30 cm. The part of the colon taken represented the first 6 cm. All the samples were suspended in 500 μL PBS, homogenised and rapidly stored at −80 °C until analysis.

2.4. Cell culture

2.4.1. Caco-2 cell culture

Caco-2 clone TC-7 cells were cultured as previously described (Reboul et al., 2005, 2006). For each experiment, cells were seeded and grown on transwells for 21 days as previously described (Reboul et al., 2005, 2006) to obtain confluent and highly differentiated cell monolayers. Twelve hours prior to each experiment, the media in apical and basolateral chambers were replaced with serum-free complete medium.

2.4.2. Characterisation of FSV apical transport and competition in cells

Apical uptake of FSV incorporated in mixed micelles were determined after a 1 h-incubation as previously described

Table 2
Fat-soluble vitamin concentrations in mixed-micelles delivered to Caco-2 cells.

	Concentration (μM)		
	Dietary	Supplementation	Pharmacological
Retinol (A)	5	10	100
Cholecalciferol (D_3)	0.5	10	100
α-Tocopherol (E)	10	50	100
Phylloquinone (K_1)	2.5	10	100

(Goncalves et al., 2013; Reboul et al., 2011). Absorbed FSV was estimated based on FSV present in harvested cells.

All the samples (harvested cells and culture medium after incubation) were sealed under nitrogen and stored at −80 °C until FSV analysis. Aliquots of cell samples were used to assess protein concentrations using a bicinchoninic acid kit (Pierce, Montluçon, France).

2.5. Vitamin extraction

FSVs were extracted from 500 μL aqueous samples using the method previously described (Reboul et al., 2011). The internal standards were echinenone, retinyl acetate, tocopherol acetate and apo-8′-carotenal for vitamin A, D, E and K, respectively. After lipid extraction with hexane, dried residues were dissolved in 200 μL of mobile phase (70% acetonitrile – 20% dichloromethane – 10% methanol, 60% acetonitrile – 38% methanol – 2% water, 100% methanol, 80% methanol – 19.45% ethanol – 0.55% water; for vitamin A, D, E and K, respectively). A volume of 180 μL was used for HPLC analysis.

2.6. HPLC analysis

The HPLC systems and methods were set up according to previous studies: vitamin A and E (Reboul et al., 2009), vitamin D (Goncalves et al., 2013; Reboul et al., 2011) and vitamin K (Oostende, Widhalm, & Basset, 2008). All the vitamins were identified by retention time compared with pure standards. Retinyl linoleate, retinyl oleate, and retinyl stearate produced in mouse intestinal mucosa were identified by their retention times and spectral analyses, and quantified on the basis of their molecular extinction coefficient ratio compared with retinyl palmitate.

2.7. Statistical analysis

Results are expressed as means ± SEM. Differences between two groups of unpaired data were tested using the nonparametric Mann–Whitney U test. Values of $p < 0.05$ were considered significant. All statistical analyses were performed using Statview software, version 5.0 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Intestinal FSV uptake in mice

As shown in Fig. 1, the intestinal profile of absorption was characteristic for each vitamin. Vitamin A (as retinol and retinyl ester) uptake by intestinal mucosa was preferentially localised in the proximal intestine (Fig. 1A). Vitamin D (as cholecalciferol) absorption was optimal in the median intestine (Fig. 1B). Finally, vitamins E (as γ-tocopherol) and K (as phylloquinone) were essentially absorbed in the distal part of the intestine with a significant uptake in the caecum. For example, there was 11-fold greater absorption of vitamin K in the ileum than duodenum.

3.2. Vitamin uptake efficiency by Caco-2 cells in presence of other FSVs

Neither vitamin D nor K impacted on vitamin A uptake. Interestingly, vitamin E significantly improved vitamin A uptake at medium and high concentrations (upto 40%, Fig. 2A). In contrast, Fig. 2B shows that vitamin D uptake was significantly reduced by vitamin E at medium and high concentrations (−15% and −17% respectively), as well as by vitamin A at high concentration (−30%). Vitamins A and D significantly reduced vitamin E uptake in a dose-dependent manner, while vitamin K had a negative effect only at the highest concentration (Fig. 2C). Finally, all the FSVs significantly decreased vitamin K uptake (from −34% to −58%, Fig. 2D).

4. Discussion

During the competition experiments, micellar solutions were delivered to Caco-2 cell monolayers to evaluate the uptake efficiency of a given FSV in presence of other FSVs. The human Caco-2 TC-7 cell model has frequently been employed to evaluate the intestinal transport of FSVs (Goncalves et al., 2013; Reboul et al., 2006, 2011), and three doses of other FSVs representing dietary, supplementation or pharmacological concentrations, were used. Our results elucidated mostly antagonist interactions.

The presence of retinol reduced the uptake of the three other FSVs. Interestingly, a study conducted in chicken reported that high vitamin A intake (100-fold the usual dose) interacted with vitamin E absorption (Sklan & Donoghue, 1982). This competition was confirmed in other studies where vitamin A reduced tocopherol plasma concentrations in chicken (Abawi & Sullivan, 1989; Aburto & Britton, 1998). To explain this observation, it was hypothesised that vitamin E, when given concomitantly with vitamin A, acts as an antioxidant in the proximal part of the intestine and preserves vitamin A. As a result, a part of the vitamin E present in the bolus is degraded and consequently a reduced absorption occurs in the distal part of the intestine, leading to a diminished tocopherol plasma concentration. This is in agreement with the fact that vitamin E improved retinol uptake upto 40% in our experiment, likely by protecting the retinol present in the mixed micelles from degradation. Moore and collaborators have shown that the presence of vitamin E in rat diets led to an increase in vitamin A absorption and storage (Moore, 1940), but the opposite effect has also been reported (Aburto & Britton, 1998), suggesting a possible recycling antioxidant protection. However, such an explanation cannot be applied to vitamin D, which is not an antioxidant. Consequently, the reason for decreased uptake of vitamin D in the presence of vitamin A remains unclear, although this antagonist interaction between vitamins A and D has been described elsewhere (Metz, Walser, & Olson, 1985). This was also observed in chicken where vitamin A negatively affected the utilisation of dietary vitamin D_3, but not of vitamin D_3 produced by sun exposure (Aburto & Britton, 1998). Moreover, dietary vitamin A was shown to antagonise calcium response to vitamin D in humans (Johansson & Melhus, 2001). The mechanisms underlying the negative effect of dietary vitamin A on the absorption of vitamins E and D remain unknown and should be investigated further. Finally, no data were available to explain the observed interaction between vitamins A and K.

Vitamin E showed a negative effect on vitamin D uptake at dose mimicking supplementation and pharmacological intake (−15% and −17% respectively), and vice versa vitamin D pharmacological dose reduces vitamin E uptake upto −35%. This is in agreement with previous data in Caco-2 cells (Reboul et al., 2011) and in chickens (Aburto & Britton, 1998) showing that vitamin E had an inhibitory effect on dietary vitamin D uptake. Indeed, these two vitamins share common transport proteins such as NPC1-L1 and SR-BI (Reboul & Borel, 2011; Reboul et al., 2011).

Vitamin K uptake by Caco-2 cells was impaired (−45%) by vitamin E while vitamin E uptake was impaired at high vitamin K concentration. Interestingly, our results could be relevant in regard to another study showing that a long-term vitamin E treatment at pharmacological doses was associated with hemorrhages, which were eliminated following vitamin K supplementation (Wheldon, Bhatt, Keller, & Hummler, 1983). Later, vitamin E supplementation was associated with a significant decrease of the incidence of

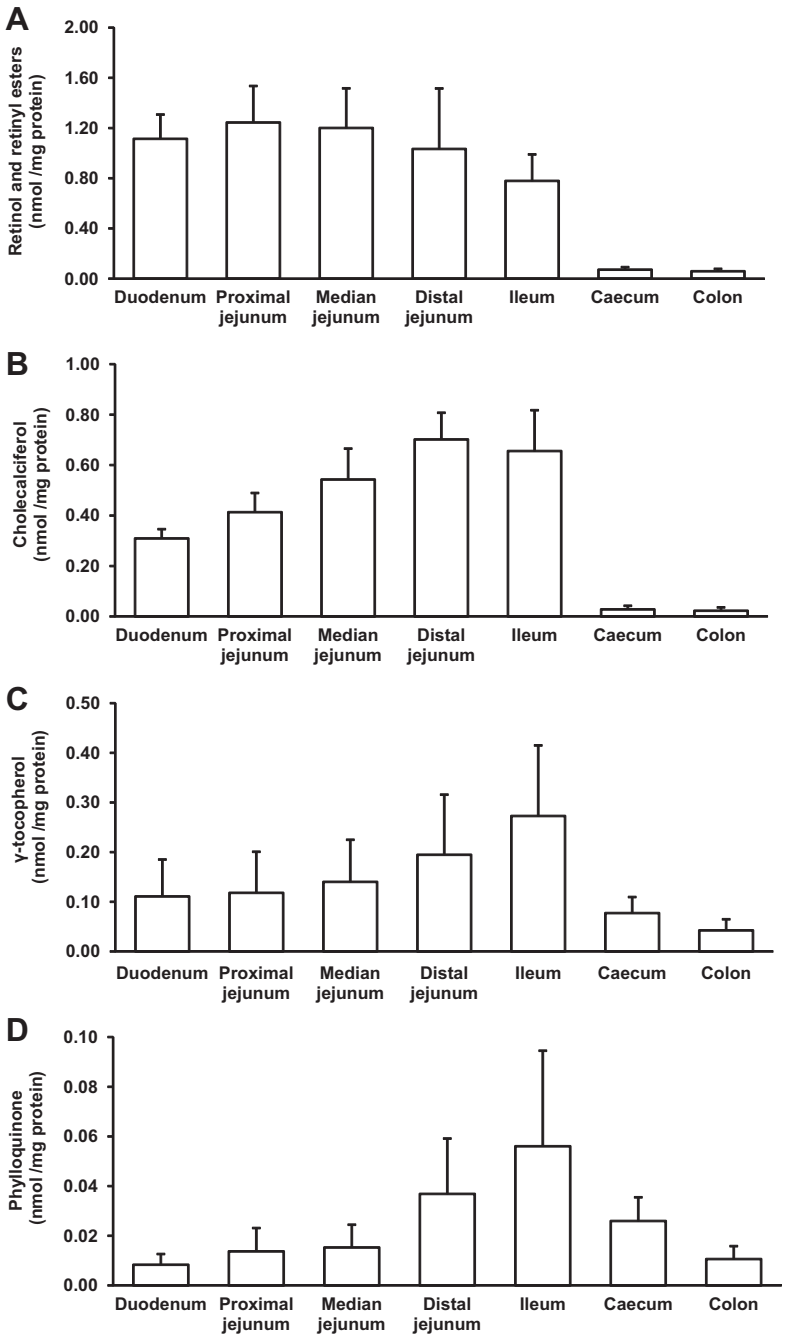


Fig. 1. Fat soluble vitamin content of mouse intestinal fragments after force-feeding with different FSV-enriched emulsions. (A) Retinol and retinyl esters intestinal profile. (B) Cholecalciferol intestinal profile. (C) γ-Tocopherol intestinal profile. (D) Phylloquinone intestinal profile. Mouse intestines were harvested and cut in 6 fragments plus the caecum, 4.5 h after force-feeding with FSV-enriched emulsions (i.e. duodenum represented the 6 first intestine cm) Data are means ± SEM, $n = 4$.

thrombosis (Booth et al., 2004). Vitamin E may have anticoagulant properties, acting via a range of mechanisms, such as the inhibition of the enzyme allowing phylloquinone conversion to menadione, which is in turn alkylated into menaquinone-4 (MK-4), the main storage form in animals (Shearer, Fu, & Booth, 2012; Tovar et al., 2006). Vitamin E may also stimulate production of enzymes involved in vitamin K excretion (Booth et al., 2004). Thus, it is generally acknowledged that vitamin E may interfere with vitamin K activity, leading to bleeding in supplemented patients (Traber, 2008). Additionally, we suggest vitamin E may affect vitamin K through competition for absorption via (a) transporter(s) localised in the distal intestine, where both vitamins are predominantly absorbed – atleast in mice and perhaps in humans based on results from Caco-2 experiments.

Similarly, vitamin D significantly reduced vitamin K uptake in a dose-dependent manner up to −58%. No data in the literature can explain this result but we suppose that vitamins D and K also share common uptake pathways.

Altogether, our results show for the first time that the four FSVs may have different absorption sites in the intestine, suggesting different uptake mechanisms at the enterocyte level. We hypothesise common absorption pathways for vitamins D, E and K, which

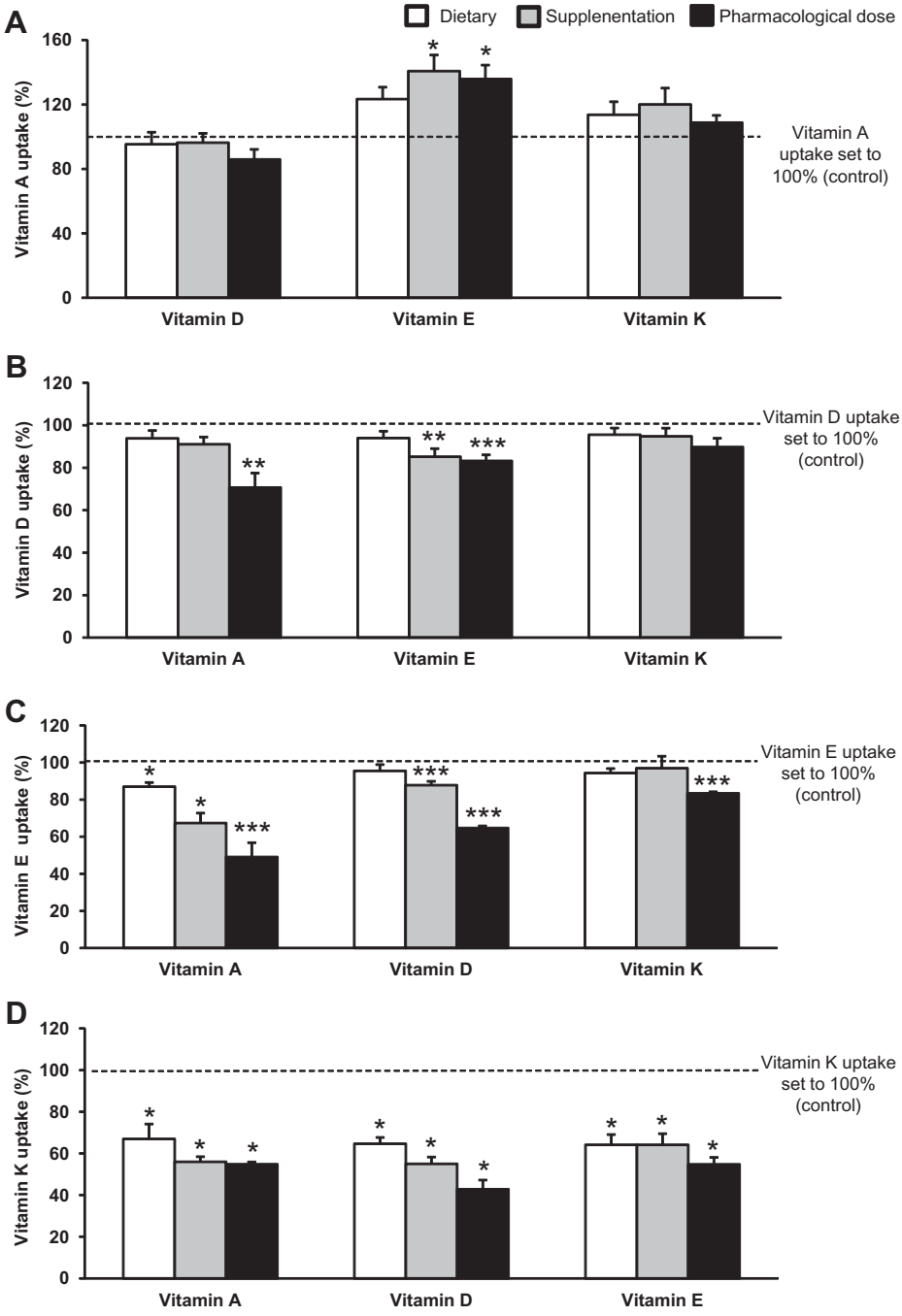


Fig. 2. Effect of fat soluble vitamins at different doses, on vitamin A, D, E or K at nutritional dose by differentiated Caco-2 TC-7 monolayers. (A) Effect of vitamin D, E and K on retinol uptake. (B) Effect of vitamin A, E and K on cholecalciferol uptake. (C) Effect of vitamin A, D, and K on α-tocopherol uptake. (D) Effect of vitamin A, D and E on phylloquinone uptake. The basolateral sides received FBS-free medium. The apical sides of the cell monolayers were incubated for 60 min with FSV-enriched mixed micelles containing on one hand the vitamin at nutritional dose (i.e. retinol 5 μM, cholecalciferol 0.5 μM, α-tocopherol 10 μM or phylloquinone 2.5 μM) and on the other hand either no other vitamin (control set up at 100%) or different doses of FSVs as described in Table 2. Data are means ± SEM of 6–9 assays. An asterisk indicates a significant difference with the control (assay performed with the vitamin alone).

would explain the competition observed *in vitro*. Further investigations are needed to understand the negative effect of vitamin A on the uptake of other FSVs. A better understanding of the mechanisms of FSV absorption is a priority to avoid deleterious interactions, especially during long-term supplementation strategies.

Conflict of interest

We declare no conflicts of interest or financial interest.

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