Pérez-Esteve, É.; Ruiz Rico, M.; Fuentes López, A.; Marcos Martínez, MD.; Sancenón Galarza, F.; Martínez-Máñez, R.; Barat Baviera, JM. (2016). Enrichment of stirred yogurts with folic acid encapsulated in pH-responsive mesoporous silica particles: Bioaccessibility modulation and physico-chemical characterization. Food Science and Technology. (72):351-360. doi:10.1016/j.lwt.2016.04.061.

Enrichment of stirred yogurts with folic acid encapsulated in pH-responsive mesoporous silica particles: Bioaccessibility modulation and physico-chemical characterization

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Abstract

In this work, we have studied the ability of a mesoporous silica support loaded with folic acid and functionalized with amines (**S1)** to modulate the bioaccessibility of the vitamin after its incorporation in stirred yoghurts with different fat contents. Due to the novelty of using mesoporous silica supports in food matrixes, the influence of **S1** addition on the physicochemical, rheological and lactic acid bacteria viability of these yoghurts during 21 days of refrigerated storage at 4°C was also evaluated. The *in vitro* digestion procedure showed that **S1** was capable of inhibiting the release of folic acid in acidic solution at pH 2 (stomach) and controllably release their contents in neutral pH (intestine), thereby modulating the bioaccessibility. Moreover, the physicochemical and microbiological assays revealed that enrichment generally does not alter the physicochemical properties (pH, colour, syneresis and rheology) of either type of yoghurt and does not cause any effect on lactic acid bacteria survival.

Keywords: Folic acid, enrichment, yoghurt, smart delivery system, bioaccessibility.

1. Introduction

Folate deficiencies during pregnancy are the cause of birth defects, such as neural tube defects, all over the world. In other stages of life, folates are responsible for lowering the homocystein-level as well having an impact on preventig cardiovascular diseases (Lucock, 2000; Choi & Mason, 2002; Pitkin, 2007), Alzheimer (Clarke et al., 1998), arteriosclerotic disease (Hoag et al., 1997), or a disruption of the nucleotide biosynthesis which can result in colon and colorectal cancer (Choi & Mason, 2002; Stover, 2004).

In spite of the public-health campaigns in recent years, a sizeable proportion of women of reproductive age remain unaware of the need to take folic acid (FA) periconceptionally. The most vulnerable groups are women who have unplanned pregnancies, young people and less-educated people. The only way to reach this group of women might be through fortification or enrichment of foods with FA (Eichholzeret al., 2006). The incorporation of this vitamin into generally accepted and consumed products could lead to improvements in FA in vulnerable groups.

Although there are irrefutable evidences about the benefits of FA supplementation to prevent some diseases, recent studies suggest that massive exposure to high bioavailable FA is a double-edged sword. Humans have a reduced dihydrofolate reductase activity and a poor ability to reduce FA. Thus, oral doses of FA of about 260-280 μg (589-634 nmol) have been reported to lead to the direct appearance of untransformed FA in the systemic circulation. This is related to a possible role in certain cancer development (Lucock &Yates 2009).

In this context, as an alternative to traditional free FA supplementation, we recently presented a system based on the encapsulation of FA in a mesoporous silica support functionalized with amines to dose FA along a simulated digestion process (Pérez-Esteve et al., 2015). The FA loaded and amine functionalised support, not only was able to hinder the release of the vitamin during the passage across the stomach (simulated gastric fluid), but also was able to deliver progressively the vitamin when reaching the jejunum (simulated intestinal fluid), where it is completely absorbed (Sculthorpe et al., 2001; Younis et al., 2009), using a minimal quantity of the encapsulating support. Mesoporous silica supports are considered potential colloidal carriers with many unique features that make them excellent candidates for a broad range of biomedical applications, and have very good in vivo biocompatibility and biodegradability (Hamam & Al-Remawi, 2014).

Of all kinds of food, dairy products such as yoghurts and other fermented milks are great candidates to be enriched with FA. On the one hand, it is well known that dairy products and fermented milks are highly recommended during pregnancy, as they are a great source of vitamins A, B, D and E, protein and calcium (FAO, 2013). However, FA content in these products is negligible. On the other hand, dairy products have very good qualities to become enriched and/or fortified foods, since incorporation of nutrients is very simple, and hence the large number of functional dairy products have been developed to date (Estrada et al., 2011; Vélez-Ruiz et al., 2013; Perna et al., 2014).

Among dairy products, yoghurt, produced by symbiotic cultures of Streptococcus thermophilus and Lactobacillus delbrueckii ssp. bulgaricus, is the most popular and consumed due to its

association with good health (Ramírez-Sucre & Vélez-Ruiz, 2013; Wang et al., 2014; Tripathi & Giri, 2014). Moreover, stirred yoghurts, in which their structure is disintegrated by shearing processes before packing, facilitates the incorporation of new ingredients such us fruits, fibers and others relevant compounds. Finally, its pH close to 4, which makes it an appropriate matrix to incorporate porous matrices with molecular gates that respond to pH changes.

A significant body of research has focused on the evaluation of the influence of the addition different ingredients (minerals, dietary fiber, flavours, etc.) on the physicochemical properties of yoghurt (Achanta et al., 2006; Ramirez-Santiago et al., 2010; Ramirez-Sucre & Vélez-Ruiz, 2013). However, as far as we know, there are no studies dealing with the incorporation of smart delivery systems based on mesoporous silica supports on food matrixes. Thus, no data about the maintenance of controlled release efficiency after incorporation in real systems, as well as data related to the effect of MSPs on the physico-chemical properties of dairy products or on lactic acid bacteria viability are available.

The objective of the present work was to evaluate the ability of the smart delivery system (S1) to modulate the bioaccessibility of FA once incorporated in different fat containing yoghurts, as well as to study the influence of S1 addition on the physicochemical and microbial quality of yoghurts during cold storage.

2. Materials and methods

2.1 Materials

Tetraethylorthosilicate (TEOS), triethanolamine (TEAH3), *N*-cetyltrimethylammonium bromide (CTABr), sodium hydroxide (NaOH), the organosiloxane derivative N^1-(3-Trimethoxysilylpropyl)diethylenetriamine (N3), acetic acid, sodium phosphate monobasic, sodium phosphate dibasic, tetrabutylammonium hydrogen sulphate (TBAHS) and acetic acid were provided by Sigma-Aldrich (Poole, Dorset, UK). Folic acid (FA) was purchased from Schircks Laboratories (Jona, Switzerland). Acetonitrile HPLC grade was provided by Scharlau (Barcelona, Spain).

Two types of stirred yoghurts, low-fat (LF) and full-fat (FF) (Senoble, Ocaña, Spain) were used as raw material. Yoghurts were acquired in a local supermarket in 500 mL containers. The expiry date was superior to 21 days in all the cases. In FF and LF yoghurts the protein, carbohydrate and fat content were 4.7-4.1, 12.2-5.3, and 3.4-0.1 g/100g, respectively.

2.2 MSPs preparation and characterization

Microparticulated MCM-41 particles were synthesized following the so-called "atrane route", according to the method described by Pérez-Esteve *et al*. (2015). The route is based on the use of triethanolamine ligands as hydrolytic inorganic precursors and surfactants as porogen species. In a typical synthesis, a solution of $TEAH_3$ (52.4 g) containing 0.98 g of NaOH in 2 mL of water is heated at 118 °C. Afterward, TEOS (22 mL) is slowly added to prevent silica condensation and stirred until reaching 118 °C. In that moment, CTABr (9.36g) is carefully added and stirred. Finally, 180 mL of deionised water are added while being vigorously stirred at 70 °C. After 1h of stirring, the obtained gel is poured into a Teflon recipient hermetically closed and heated at 100 °C for 24 h. Using filtration, the resulting powder is collected and then it is washed with water and ethanol. The solid is dried at 70 °C. To prepare the final porous material **(S0)**, the as-synthesised solid is calcined at 550 °C in a muffle furnace for 5 h to remove the template phase.

FA was loaded in support **S0** by the impregnation method described by Pérez-Esteve *et al.* (2015). For this purpose, 15 mg of FA dissolved in 1.5 mL of phosphate buffer solution (PBS) were added to 100 mg of MCM-41 employing 3 cycles of addition (0.5 mL per cycle). After each addition cycle, solid was dried at 30 ºC to eliminate water content.

The loaded solid was functionalized with N3. In a typical synthesis, 100 mg of the solid were suspended in 10 mL of a solution of acetic acid (5 mL/100 mL), where an excess of N3 (0.43 mL, 0.015 mmol) was added. The mixture was stirred for 5.5 h at room temperature. The final

loadead and amine-gated solid (**S1**) was isolated by vacuum filtration, washed with 300 mL of acetic acid solution, and dried at 30 °C for 24 h.

The characterization of the different mesoporous solids (**S0** and **S1**) was made by powder X-ray diffraction (XRD), transmission electron microscopy (TEM), field emission scanning electron microscopy (FESEM), particle size distribution and zeta potential determinations. XRD was performed on a D8 Advance diffractometer (Bruker, Coventry, UK) using CuKα radiation. TEM images were obtained with a JEM-1010 (JEOL Europe SAS, Croissy-sur-Seine, France). FESEM images were acquired with a Zeiss Ultra 55 (Carl Zeiss NTS GmbH, Oberkochen, Germany) and observed in the secondary electron mode. The particle size distribution was determined using a Malvern Mastersizer 2000 (Malvern Instruments, Malvern, UK). For the measurements, samples were dispersed in distilled water. Data analysis was based on the Mie theory using refractive indices of 1.33 and 1.45 for the dispersant and particle, respectively. An adsorption value of 0.001 was used for all samples. Variation of this adsorption value did not significantly alter the obtained distributions. Measurements were performed in triplicate. A Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) was used to determine the zeta potential (ζ). Samples were dispersed in distilled water at concentration of 1 mg mL^{-1}. The zeta potential was calculated from the particle mobility values by applying the Smoluchowski model. The measurement was performed at 25°C. Measurements were performed in triplicate. The composition of **S1** was determined by thermogravimetrical analysis (TGA) and ^{1}H NMR. Thermogravimetric analyses were carried out on a TGA/SDTA 851e Mettler Toledo balance, using an oxidant atmosphere (air, 80 mL min^{-1}) with a heating program consisting of a heating ramp of 10 ºC per minute from 393 to 1273 K and an isothermal heating step at this temperature for 30 min. ^{1}H NMR spectra were recorded in at RT using a Bruker AV400 spectrometer after dissolving the sample in $NaOD/D_2O$ in the presence of tetraethyl ammonium bromide as internal standard.

2.3 Folic acid release study

To determine the effect of pH in FA release from the amine-gated mesoporous silica particles (**S1**), 10 mg of the corresponding solid were placed in 25 mL of water at pH 2 (adjusted with HCl), pH 4 (adjusted with HCl and with lactic acid) and pH 7.5 (PBS). At a certain times aliquots were separated, filtered and analysed by HPLC to quantify the amount of FA.

The maximum release capacity of the solid **S1** was determined by quantifying the amount of FA released after reaching the equilibrium (2h at pH 7.5). This released FA amount was used to quantify the amount of solid **S1** needed to provide the dietary reference intake (DRI) of FA in the population most in need of this nutrient (360 µg of synthetic FA per day in pregnant women) (Eichholzer *et al.*, 2006).

2.4 Sample preparation

Three different batches of samples were prepared for low-fat (LF) and full-fat (FF) yoghurts. Yoghurts without enrichment were considered control samples (C). Enriched yoghurts containing the 100% of the DRI of FA by addition of free FA were called E_F (enriched with free FA). Yoghurts containing the amount of **S1** needed to release 100% of the DRI of FA were called E_E (enriched with encapsulated FA in **S1**). After addition of free FA or **S1**, yoghurts were stirred for 1 min. In C yoghurts the same stirring procedure was performed to standardize conditions. Samples were then stored at 4 °C until they were analysed (0, 7 and 21 days). The sample preparation procedure is schematically shown in **Figure 1**.

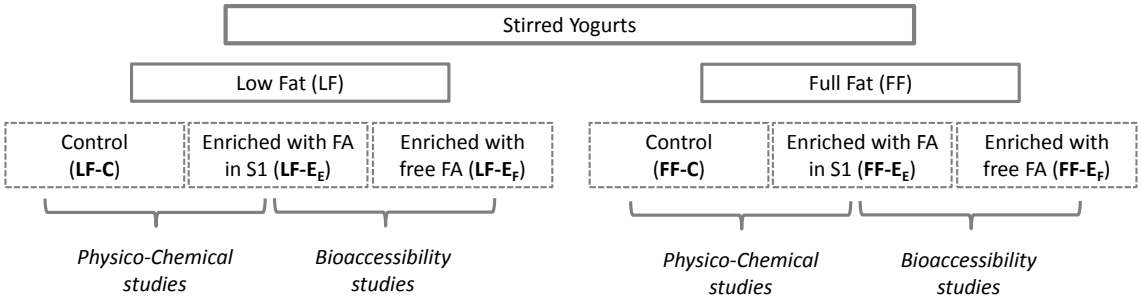

Figure 1. Scheme of sample preparation.

2.5 Yoghurts in vitro digestion and FA bioaccessibility determination

FA bioaccessibility from E_F and E_E yoghurts was determined by simulating a human digestion in mouth, stomach and small intestine adapting the procedure described by Versantvoort *et al.* (2005). The large intestinal track was not taken into account since *in vivo* FA absorption occurs throughout the jejunum (Younis *et al.*, 2009). In a typical experiment, 5 g of the corresponding yoghurt were suspended in 6 mL of saliva and incubated for 5 min at 37 °C. Then, 12 mL of gastric juice were added. The mixture was incubated for 2 h. Finally, 12 mL of duodenal juice, 6 mL of bile, and 2 mL of bicarbonate solution (1 mol/L) were added simultaneously. After the addition, the mixture was maintained under stirring at 37 ^{o}C for 2 h. All digestive juices were heated to 37 ^{o}C before being mixed. During this period, aliquots were taken, filtered using nylon filters with 0.45 μm pore size (Scharlab, Barcelona, Spain) and analysed by HPLC to determine the amount of FA (*vide infra*). All chemicals for the digestive fluids were provided by Sigma-Aldrich (Poole, Dorset, UK).

2.6 Folic acid quantification

FA was quantified by reversed-phase HPLC according to the method described by Pérez-Esteve *et al.* (2015). The HPLC instrument consisted of a Hitachi LaChrom Elite liquid chromatograph (Hitachi Ltd., Tokyo, Japan) equipped with an auto-sampler (modul L-2200) and UV detector (modul L-2400). A column Kromaphase 100 C18 (250 mm x 4.6 mm i.d., 5 μm particle size analytical column) (Scharlab, Barcelona, Spain) was used for the separations. Mobile phase consisted of (A) 0.125 mmol/L of NaH_2PO_4, 0.875 mmol/L of Na_2HPO_4 and 0.4 mmol/L of TBAHS in water and (B) acetonitrile-phase A 65:35 (mL/mL). The gradient program was as follows: the mobile phase was run isocraticaly for the first 5 min with 90% A and 10% B. The percentage of B was increased linearly to reach 36% at 15 min and 60% at 30 min. After that, percentage of B decreased linearly to the original composition in 5 min and remained in the initial conditions for 5 min. The wavelength of the UV detector was established at 280 nm. FA was quantified according to the external standard method using a calibration curve of the peak area against concentration of the compound.

2.7 Physicochemical analysis

2.7.1 pH, syneresis and colour determinations

The pH measurements were performed using a pH meter (Crison Basic 20 +, Crison Instruments SA, Barcelona, Spain) with puncture electrode. pH measurements were taken directly on yoghurt samples in triplicate.

For syneresis determinations, 20 g of yoghurt were centrifuged at 8000 rpm and 4 ^{o}C for 10 min in a 5804 Eppendorf centrifuge (Eppendorf, Hamburg, Germany). After centrifugation, the released serum was poured off, weighed and recorded as percentage of weight to the original weight of yoghurt in triplicate.

Colour values were obtained by measuring the reflection spectrum (Minolta, CM 3600D, Tokyo, Japan). CIE Lab uniform colour space was selected to calculate colour parameters L^* (brightness), a^* (red-green), b^*(yellow-blue) using a 10° observer and D65 illuminant. The total colour differences between C and E_E yoghurts were calculated using Eq 1. Five replicates were carried out for each sample.

$$\Delta E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \qquad (1)$$

2.7.2 Rheological determinations

The rheological analyses were carried out on a controlled stress rheometer (RheoStress, Thermo Haake, Karlsruhe, Germany). The flow curves and the oscillatory assays were performed using concentric cylinder (6 cm diameter; 1 mm gap). The yoghurts were loaded on

the inset plate and the temperature was maintained at 4 ± 0.1 °C during the tests. All trials were carried out in triplicate.

The flow curves were determined by performing shear rate sweeps from 0 to 300 s^{-1}. Three consecutive rising and falling cycles were made in order to evaluate and eliminate the effect of thixotropy. The thixotropic behaviour of the samples was evaluated by estimating the hysteresis loop area (A_{up} - A_{down}) between the upward (A_{up}) and downward (A_{down}) flow curves using the program RheoWin Data Manager (version 3.61, Thermo Haake). The experimental data obtained in the third down sweep were adjusted to the Herschel-Bulkley model for non-Newtonian fluids (Eq 2).

$$\sigma = \sigma_0 + K\gamma^n \qquad (2)$$

where σ_0 is yield shear stress (Pa), K the consistency index (Pa s^n), γ the deformation rate (s^{-1}), and n the flow behaviour index (dimensionless). The yield stress value used in Herschel-Bulkley's model was previously obtained by fitting the experimental data to the Casson model (Eq 3) (Tárrega & Costell, 2007). All measurements were carried out in triplicate.

$$\sigma^{0.5} = \sigma_0^{0.5} + K\gamma^{0.5} \qquad (3)$$

2.8 Determinations of microbial viability

To evaluate the effect of solid **S1** addition on the viability of lactic acid bacteria naturally present in the yoghurts (i.e. *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) viable cell counts were performed in control (LF-C and FF-C) and enriched with **S1** yoghurts (LF-E_E and FF-E_E). 1 mL of yoghurt was diluted in 9 mL of 0.15% sterile buffered peptone water (Scharlau, Barcelona, Spain). The mixtures were thoroughly stirred and serial dilutions were prepared and plated on selective agar in duplicate. These selective media were Man, Rogosa, and Sharpe agar (MRS) (Scharlau, Barcelona, Spain) and 0.5% lactose M-17 media (Sigma-Aldrich, Poole, Dorset, UK) for enumeration of *L. delbrueckii* and *S. thermophilus*, respectively. The inoculated plates were incubated at 30 °C for 48 h in anaerobic conditions for the growth of *L. delbrueckii* ssp. b*ulgaricus* by introducing the plates in anaerobic jars with gas pack AnaeroGen (Oxoid, Cambridge, UK). The plates with selective media for *S. thermophilus* were incubated in aerobic conditions at 37 °C for 48 h. Results were expressed as log colony-forming units (CFU) mL^{-1}.

2.9 Statistical analysis

The differences between the samples due to the type of yoghurt, the addition of particles and the time of storage were determined by analysis of variance (ANOVA multifactorial), with a confidence level of 95% LSD ($p<0.05$). The statistical program used was Statgraphics Centurion XV (Manugistics Inc., Rockville, MD, USA).

3. Results

3.1 Support S1. Synthesis and characterization

In a first step, MCM-41 support was synthesised following well-known procedures using *N*-cetyltrimetylammonium (CTABr) as a structure director agent and tetraethylorthosilicate (TEOS) as a silica source. After removal the surfactant by calcination the starting MCM-41 support was obtained. Then, the pores of the MCM-41 support were loaded with FA. Finally, the FA-loaded support was reacted with N^1-(3-Trimethoxysilylpropyl)diethylenetriamine resulting the capped mateial **S1**.

The prepared solids were characterized according to standard techniques. **Figure 2** shows the powder X-ray diffraction (XRD) patterns of the solids MCM-41 as synthesised, MCM-41 calcined (**S0**) and the final mesoporous solid loaded with FA and functionalized with N3 (**S1**). The XRD of MCM-41 as-synthesised (curve a) shows the typical low-angle reflections of a hexagonal ordered array indexed as (1 0 0), (1 1 0), (2 0 0) and (2 1 0) Brag peaks. In curve b, corresponding to the MCM-41 calcined sample, a significant shift of the reflections in the XRD is clearly observed. This displacement is consistent with a cell contraction of ca. 5.5 Å and attributed to the condensation of silanol groups during the calcination step. The reflections of the **S1** were practically lost due to the presence of FA in the pores. However, the permanence of the (1 0 0) peak indicate that the process of pore loading with FA, and the additional functionalization with the polyamine, did not modify the typical porosity of the mesoporous MCM-41 scaffold.

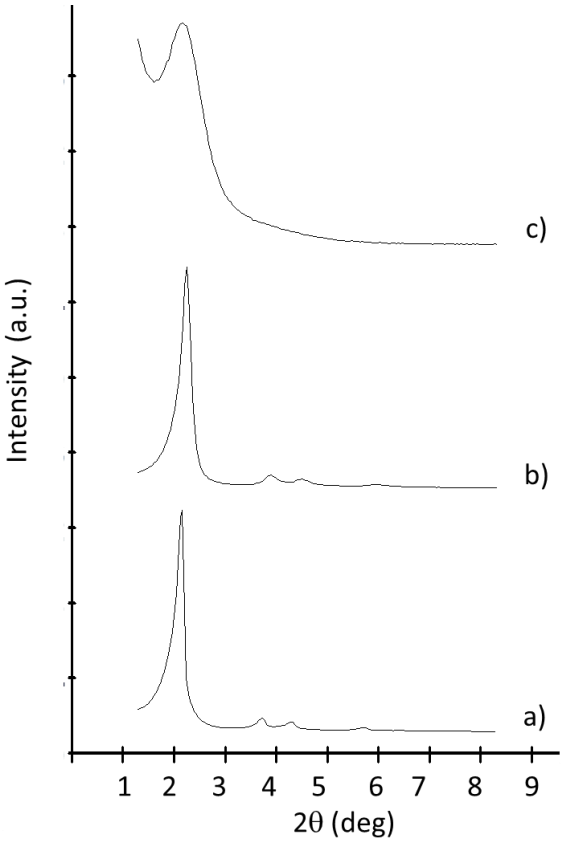

Figure 2. Powder X-ray patterns of the solids (a) MCM-41 as-synthesized, (b) MCM-41calcined **(S0)**, and (c) MCM-41 loaded with FA and functionalised with N3 (**S1**).

A morphologic analysis of both, **S0** and **S1**, was performed by TEM and FESEM studies. **Figure 3** shows that MCM-41 supports are irregular-shaped microparticles (**Fig 3a**) with porous in the range 2-3 nm (**Fig 3b**). **Figures 3c** and 3d revealed the preservation of morphology and the mesoporous structure in the final **S1** solid. The particle size was confirmed by particle size analysis. **S0** exhibited a particle size diameter (dv90) of 0.98 μm. An average particle size of 0.90 μm was observed for solid **S1**.

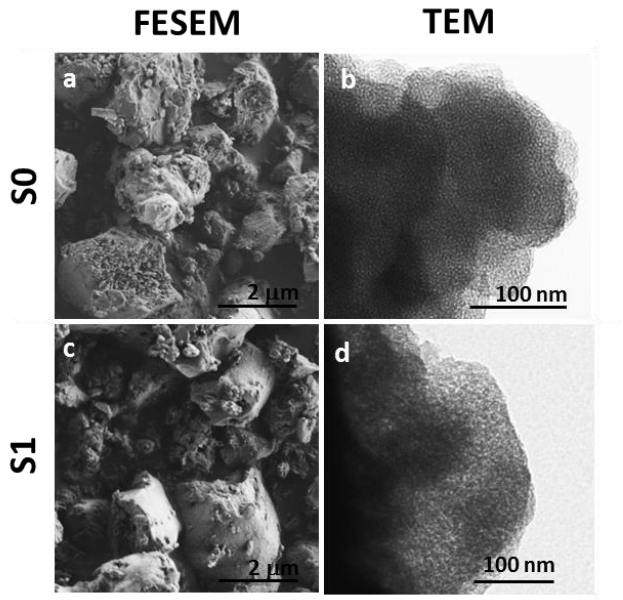

Figure 3. FESEM and TEM images of solids S0 (a,b) and S1 (c,d).

The differently prepared solids were also characterised by zeta potential determinations. **Figure 4** shows zeta potential values of **S0**, **S0** loaded with FA and the final **S1** solid. For both, **S0** and **S0** loaded with FA, zeta potential achieved negative values of ca. -40 mV. After functionalization with amines, zeta potential changed from negative to positive values (ca. 40 mv), confirming the effect of the functionalization on the surface charge. Content of organic matter obtained from elemental and thermogravimetric analysis revealed that 1 g of **S1** contained 97 mg of FA and 63 mg of N3, confirming the efficiency of FA loading as well as N3 functionalization.

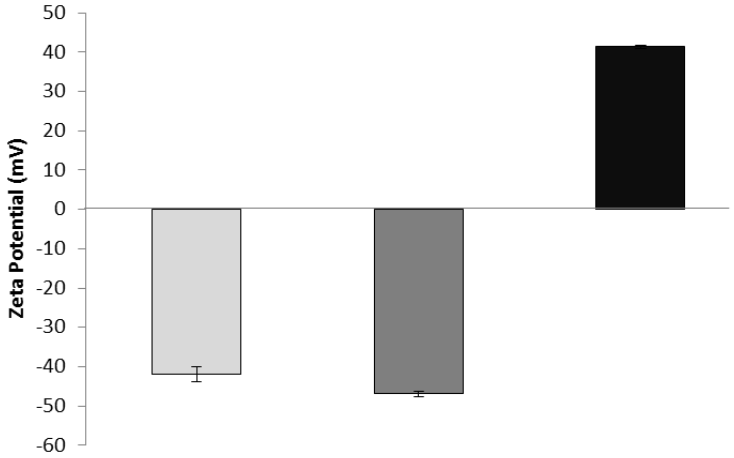

Figure 4. Zeta potential values (Mean±SD) of S0 (light grey), S0 loaded with FA (dark grey) and S1 (black).

3.2 S1 delivery profile at different pH and maximum FA delivery calculations

In order to confirm the feasibility of the FA-loaded N3-functionalised solid **S1** to modulate FA release as a function of the pH of the medium, several release experiments were carried out. FA release profiles from **S1** at different pH values (pH 2 and 4 adjusted with HCl, 4 adjusted with lactic acid and PBS at pH 7.5) are shown in **Figure 5**. At pH 2 (stomach pH) a maximum release of 1 μg mg^{-1} of FA (1.10 % of the maximum delivery) was reached at 4 h confirming that FA delivery was hindered by the effect of the low solubility of FA at acidic conditions and by the effect of the amines anchored to the surface of the silica support. At low pH, polyamines are transformed to polyammonium groups which favoured Coulombic repulsions among closely located polyammonium groups. These adopt a rigid-like conformation that block the pores and avoid the release of the vitamin (Bernardos *et al*., 2008).

When the release was performed in water adjusted to pH 4 with HCl a slight delivery of FA from the pores was observed. However it is well known that large anions are able to interact with the protonated polyamines forming strong complexes which increased pore blockage and enhanced the inhibition of the cargo release (Casasús *et al.*, 2008). Having this in mind a release assay from **S1** was also performed in water solution adjusted at pH 4 with lactic acid. In these conditions, delivery was almost zero (ca. 2%). This experiment confirmed that the gate-like superstructure based in polyamine/polyammonium groups was additionally closed by a cooperative effect of pH and the presence in the solution of bulky anions such as lactate (Bernardos *et al.*, 2008).

At pH 7.5 a sustained delivery of FA was achieved in the first 2 h. The amount released in these conditions (i.e. 94 μg mg^{-1}) was considered 100% of the FA that could be released from **S1**. The mechanism that allows this sustained release of FA from **S1** at neutral pH has been previously described by Pérez-Esteve *et al.* (2015). At pH 7.5 amines are less protonated and Coulombic repulsion between them and affinity for anions is significantly reduced. The overall effect results in a less effective pore blockage and in a FA delivery. On the other hand, it is known that at neutral pH, FA increases its solubility and this helps its delivery from the pores (Pérez-Esteve *et al.*, 2015).

This overall behaviour of FA release from **S1** as a function of pH would modulate the bioavailability of FA along the digestive tract. Under yoghurt and stomach conditions (acidic pH) amines are protonated preventing delivery of the vitamin. However when the yoghurt reach the intestine, molecular gates would open by effect of pH changes and FA would escape from **S1** in a sustained manner throughout the intestinal tract avoiding peaks of FA concentration. This mechanism would protect FA from the harsh conditions of the stomach, yet would favour a controlled release in the intestine.

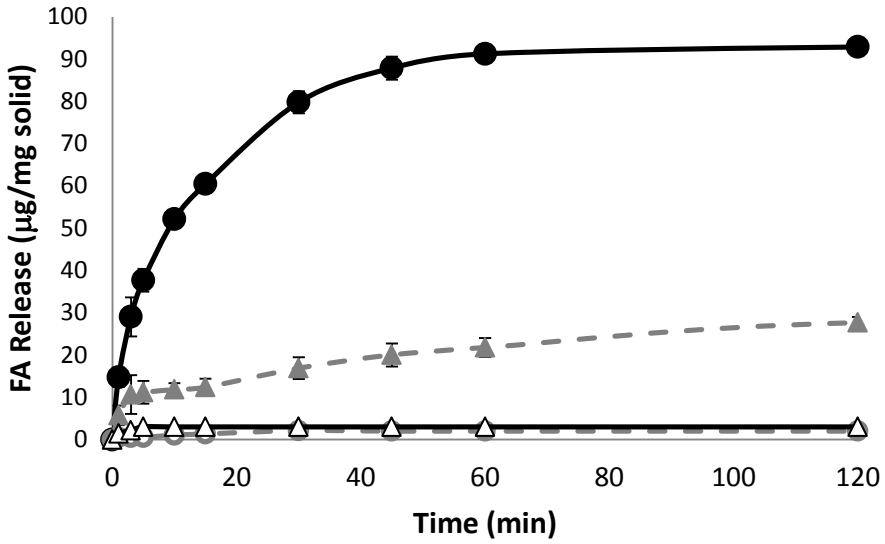

Figure 5. Release profiles of S1 in water adjusted at pH 2 with HCl (**- -o- -**), at pH 4 with HCl (- - ▲- -), at pH 4 with lactic acid (**—Δ—**) and PBS at pH 7.5 (**—●—**). Values are Means ± SD. n=3

The maximum amount of FA released from **S1** was calculated from the release profile at pH 7.5 (**Fig 5**) via the determination of FA concentration after reaching maximum delivery (4 h). At this time, 1 mg of solid **S1** releases 94 µg of FA. In USA, the DRI for FA is stablished in 400 µg folate day^{-1} in adults and 600 µg folate per day in pregnant women (USDA, 2010). In Europe, the European Food Safety Authority (EFSA) recommends a daily intake of 200-400 µg folate per day for adults and additional 400 µg folate per day in pregnant women (ESFA, 2009). Taking into account that the bioavailability of synthetic FA is approximately twice that of folate found in food, the dietary folate equivalent (DFE) establishes the relation between dietary folate and FA from supplements. One DFE is defined as 1 µg of dietary folate, 0.6 µg of FA supplement, or 0.5 µg of FA taken without food (Suitor & Bailey, 2000). Therefore to provide 360 µg FA (i.e. 600 µg of DFE) in only one standard portion of yoghurt (125 g), 3.8 mg of **S1** should be added to prepare enriched yoghurts with FA with controlled bioaccessiblity.

3.3 Bioaccessibility of FA during yoghurts in in vitro digestion

In order to evaluate the effect of FA encapsulation to control the bioaccessibility of the vitamin, the release behaviour of yoghurts enriched with free FA (LF-E_F and FF- E_F) were compared with yoghurts enriched with an equivalent amount of FA encapsulated in **S1** (LF-E_E and FF- E_E).

Figure 6 shows that in the four samples a very low amount of FA was detected during the first two hours of digestion, corresponding with the oral and stomach phase. The oral phase is too short to solubilize and extract FA entrapped in the yoghurt matrix. This explains why FA was no detected in saliva neither in yoghurts containing free nor encapsulated FA. Besides during the gastric phase FA remained mainly insoluble and thus bioinaccessible (Pérez-Esteve *et al.*, 2015).

After the addition of the intestinal juices (2 h), two different release behaviours were observed. Yoghurts containing free FA showed a fast increase of the bioaccessibility after the addition of intestinal juices that would provoke absorptions peaks. In contrast, samples containing encapsulated FA in **S1** exhibited a sustained release of the vitamin along the digestion time. This modulation of the bioaccessibility would control the bioavailability, favouring the metabolization of FA before reaching the bloodstream. This experiment confirms the suitability of the **S1** for modulating the bioaccessibility of FA in real food matrixes. The mechanism of action of the novel smart delivery system is schematically shown in **Figure 7**.

No differences on FA bioaccessibility between LF and FF yoghurts were observed. Moreover, no statistically significant differences were found in the release behaviour of yoghurts with different storage times, confirming the stability of solid **S1** in the yoghurt matrix.

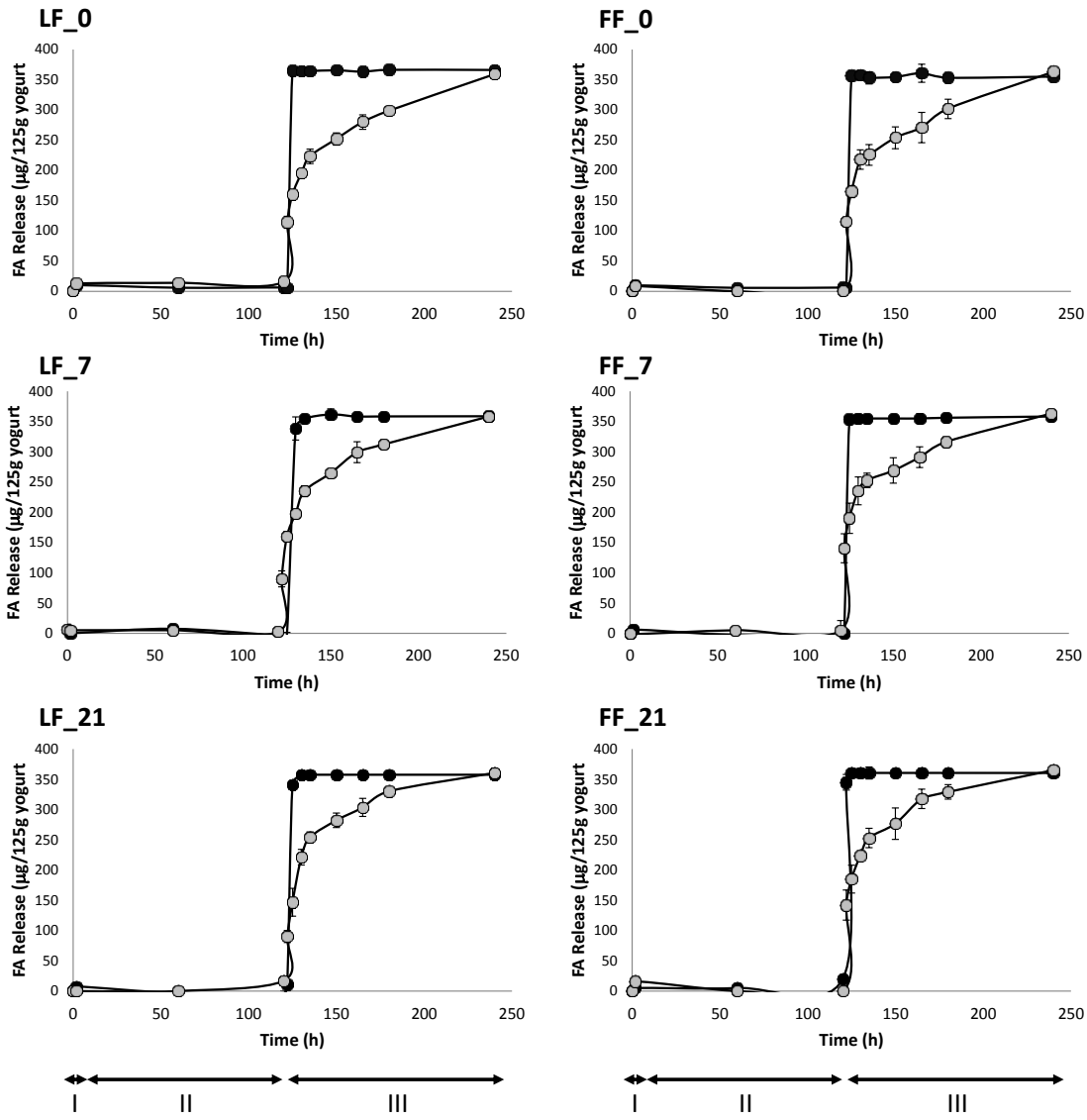

Figure 6. Bioaccessibility of FA during an *in vitro* digestion procedure in low-fat (LF) and full-fat yogurts (FF) at 0, 7 and 21 days of storage. E_F yogurts are represented with black dots; E_E yogurts with grey dots. I: oral phase; II: gastric phase; III: small intestine phase. n=3

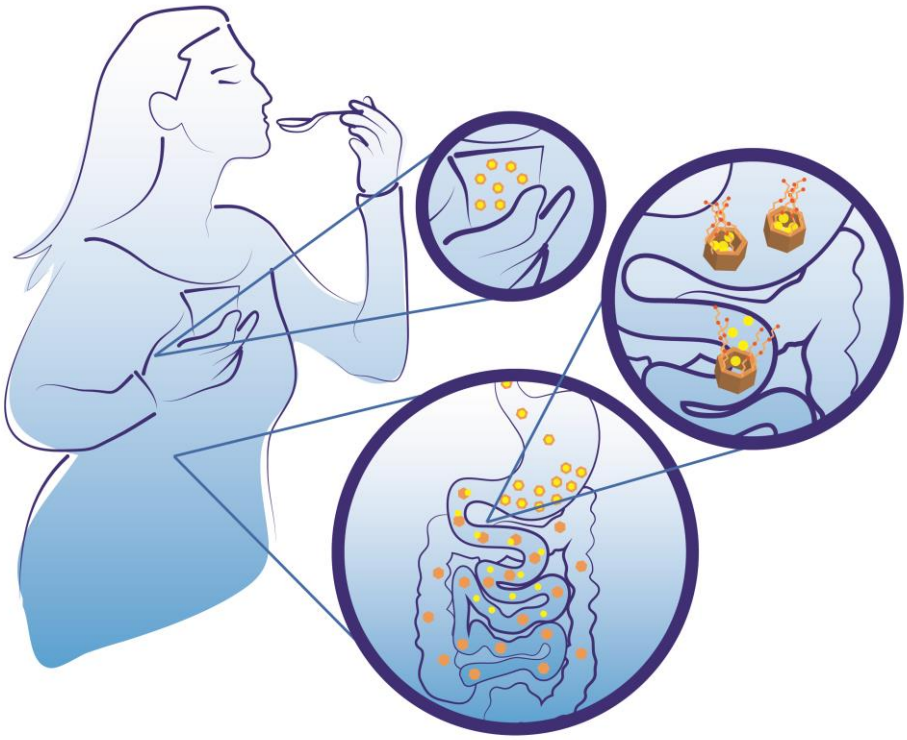

Figure 7. Illustration of the mechanism of action of the Smart Delivery System S1 when included in yogurts and ingested

3.4 Effect of S1 addition on the physicochemical properties of yoghurt during refrigerated storage

The effect of the incorporation of **S1** on the physicochemical properties of yoghurts was also studied. pH, syneresis, colour and rheology values of yoghurts containing **S1** (LF-E_E and FF-E_E) were compared with those of yoghurts without gated mesoporous particles (LF-C and FF-C).

3.4.1 pH, syneresis and colour

pH values ranged from 4.08 to 4.35 in LF yoghurts and from 4.2 to 4.5 in FF yoghurts along the 21 days of refrigerated storage. These values are similar to those reported by other authors for LF and FF yoghurts (Al-Sheraji *et al.*, 2012; Cruz *et al.*, 2013) and suggest a poor post-acidification process, a desirable characteristic in a modern yoghurt industry. This fact is related to the choice of the metabolism of lactic culture used (Cruz *et al.*, 2013). The addition of **S1** did not affect pH in neither of both LF or FF yoghurts.

Syneresis values of different yoghurts are shown in **Figure 8.** C samples exhibited syneresis values of ca. 47 and ca. 52 for LF and FF yoghurts, respectively. These values are similar to those reported by other authors (Brennan & Tudorica, 2008; Ramirez-Sucre & Vélez-Ruiz,

2013). For each type of yoghurt, the lowest syneresis values were found in samples analysed on the day 0 of storage and then increased throughout the storage process. It means that storage caused a loss of the three dimensional network of protein in both types of yoghurt. The increase of syneresis as a consequence of storage was lower in FF yoghurts. It was probably due to the presence of fat globules that difficult casein aggregation and prevents contraction and reorganization of the three dimensional network of protein. The result is a higher capacity of FF yoghurts to retain serum in the gel structure (Fox *et al*., 2000). No significant differences between C and E_E samples at each of the storage times were found, suggesting that the presence of **S1** support do not have any influence on synereris at the concentrations employed.

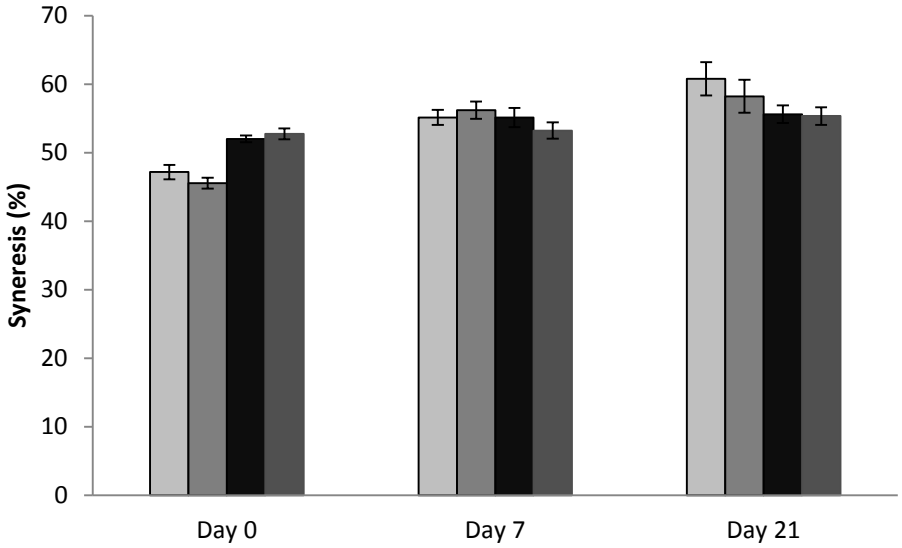

Figure 8. Syneresis values (mean ± SD) of low-fat control yogurts (light grey), low-fat yogurts enriched with FA encapsulated in S1 support (mild grey), full-fat control yogurts (black) and full-fat yogurts enriched with FA encapsulated in S1 support (dark grey).

The colour coordinates (L^*, a^*, b^*) of control and enriched yoghurts along a storage period of 21 days are shown in **Table 1**. Both control samples (LF-C and FF-C) showed high luminosity (L*≈92) and a yellowish hue (h°≈100), being these features characteristic of fermented milks (Ramirez-Sucre & Vélez-Ruiz, 2013). This similarity between the two types of yoghurts may be due to the trend in the industry to bring the whole products and those with reduced calories (low-fat) the same sensory properties. Despite the similarities, **Table 3** shows how FF yoghurts exhibited significantly higher values for all colour coordinates than LF yoghurts. **Table 1** also shows colour variations due to the FA enrichment through **S1** incorporation. As it can be seen, despite fortification and storage influenced significantly some colour parameters (**Table 3**), the total colour differences among C and E_E samples for the same type of yoghurt (LF or FF) and the same day of storage (0, 7 or 21) are lower than ΔE=0.5. Having in mind that ΔE values between 0-1 normally mean invisible differences for the human eye, it can be concluded that the enrichment of yoghurts with **S1** did not alter the visual perception of the colour.

Table 1. Colour coordinates and total colour differences of control and enriched yogurts at 0, 7 and 28 days of refrigerated storage.

Sample	L*			a*			b*			ΔE		
	0	7	21	0	7	21	0	7	21	0	7	21
LF-C	91.6±0.6	91.9±0.2	92.0±0.3	-2.48±0.06	-2.64±0.03	-2.64±0.02	9.9±0.2	10.9±0.1	11.0±0.1	-	-	-
LF-E_E	91.7±0.3	91.8±0.2	92.0±0.3	-2.46±0.04	-2.58±0.05	-2.61±0.02	10.4±0.4	11.0±0.1	11.2±0.1	0.40±0.08	0.11±0.06	0.14±0.07
FF-C	92.6±0.2	92.7±0.2	92.6±0.2	-1.67±0.02	1.61±0.05	-1.66±0.03	10.34±0.07	10.7±0.1	11.0±0.4	-	-	-
FF-E_E	92.5±0.2	92.6±0.2	92.5±0.1	-1.61±0.05	-1.61±0.04	-1.61±0.01	10.5±0.2	10.8±0.1	10.94±0.07	0.21±0.04	0.16±0.07	0.12±0.02

Mean values (± standard deviation). L^*=brightness. a^*=red-green. b^*=yellow-blue. ΔE=Total colour difference

Table 2. Rheological parameters obtained using the Herschel-Bulkley model for control and enriched yogurts at 0, 7 and 28 days of refrigerated storage.

Sample	σ_o (Pa)			K (Pa s^n)			n		
	0	7	21	0	7	21	0	7	21
LF-C	9.5±0.4	7.9±0.3	7.9±0.2	1.15±0.04	1.00±0.03	1.00±0.02	0.647±0.002	0.656±0.002	0.657±0.002
LF-E_E	8.9±0.3	8.22±0.06	8.1±0.8	1.09±0.04	1.022±0.006	1.07±0.08	0.651±0.001	0.655±0.001	0.654±0.001
FF-C	3.9±0.5	3.8±0.7	3.4±0.9	0.71±0.06	0.69±0.06	0.62±0.09	0.76±0.01	0.74±0.03	0.77±0.02
FF-E_E	4.2±0.1	3.6±0.1	3.5±0.9	0.71±0.01	0.66±0.05	0.642±0.009	0.758±0.003	0.75±0.03	0.75±0.02

Mean ± standard deviation. σ_o = yield stress, k = consistency index, n = flow behavior index.

Table 3. ANOVA F-ratio for each of the parameters and its interactions.

	Main effects			Binary interations			Tertiary interactions
Parameter	Y	E	S	Y*E	Y*S	E*S	Y*E*S
pH	396.0***	1.83ns	539.17***	0.4168ns	0.4783ns	0.1447ns	0.8813ns
Syneresis	3.53ns	1.91ns	9.21***	0.17ns	0.83ns	0.72ns	1.62ns
L*	139.43***	0.29ns	2.86 ns	0.71ns	2.47ns	0.39ns	0.21ns
a*	1043.49***	15.53***	21.79***	0.11ns	31.46***	0.40ns	3.15*
b*	0.26ns	8.33**	99.85***	1.80ns	12.56***	2.94***	1.05ns
C*	60.92***	16.50***	18.50***	0.71ns	29.51***	1.32ns	1.51ns
h*	11265.65***	64.63***	35.04***	0.67ns	1.53ns	5.48**	1.32ns
σ_0	779.69***	0.09ns	12.38***	0.01ns	2.87ns	0.21ns	1.08ns
K	728.24***	0.46ns	15.37***	0.68ns	3.97ns	1.90ns	1.70ns
n	406.00***	0.60ns	0.88ns	0.82ns	1.47ns	0.76ns	0.64ns
V.L	1.76ns	2.64ns	38.74***	0.02ns	3.87ns	0.87ns	2.27ns
V.S.	0.63ns	1.07ns	43.34***	0.87ns	2.32ns	1.91ns	0.72ns

Y = yogurt, E = enrichment, S = storage. σ_o = yield stress, k = consistency index, n = flow behavior index, V.L. = Viability of *Lactobacillus delbrueckii ssp. bulgaricus* , V.S. = Viability of *Streptococcus thermophilus.* Significance level (α): *** ($p<0.001$). ** ($p<0.01$). * ($p<0.1$). ns (no significant).

3.4.2 Rheological properties

Considering the rheological behaviour described by the flow curves, all the samples showed non-Newtonian behaviour and time dependence (thixotropy). Such behaviour is in agreement with previous studies by other authors in semisolid yoghurts and dairy desserts (Tárrega & Costell, 2007, Cruz *et al.*, 2013). Nevertheless, the rheograms of control yoghurts with different fat content (LF-C and FF-C) were different, indicating the importance of fat in the rheological behaviour of a sample (Ramirez-Sucre &Vélez-Ruiz, 2013).

All the analysed samples showed an hysteresis area between the curves up and down in one cycle. FF-C yoghurts showed greater hysteresis area than LF-C yoghurts (data not shown). This hysteresis area is related to the degree of breakdown of the structure occurred during shearing.

From the third up and down cycles the hysteresis area decreased dramatically and it was considered that from this moment rheological behaviour was independent of time. Thus, up curves of the third cycle were fitted to Herschel-Bulkley model. R^2 values were greater than 0.99 in all samples confirming the adequacy of the model to model the rheological behaviour.

Table 2 shows rheological parameters obtained using the Herschel-Bulkley model for control and enriched yoghurts at 0, 7 and 28 days of refrigerated storage. Values of the flow behaviour index (n) clearly indicates that both kind of control yoghurts (LF-C and FF-C) have a pseudoplastic behaviour ($0<n<1$). These results are in agreement with previous studies carried out by Ramirez-Sucre & Vélez Ruiz (2013). The flow index was statistically significantly higher in FF-C yoghurts than LF-C yoghurts. Neither the yoghurt fortification nor the storage period had an influence in this parameter (**Table 3**).

All the samples exhibited yield stress (σ_o), suggesting that all the samples had an initial resistance to flow (**Table 2**). Values of yield stress were higher in LF-C yoghurts. For both types of yoghurt, LF-C and FF-C, σ_o values decreased significantly along the storage period. However, no significant differences were observed between control and enriched yoghurts.

The consistency index was higher in case of LF-C yoghurts. Again, for all yoghurt types, k decreased significantly along the storage period and no significant differences were observed between control and enriched yoghurts.

According to these results, addition of **S1**, to low fat and full fat yoghurt, did not provoke any modification of the rheological properties.

3.5 Effect of S1 addition on viable counts on Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus during refrigerated storage

Initial count of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophiles* were 9.10 ± 0.03 and 9.07 ± 0.09 log CFU g^{-1} in LF-C yoghurt and 9.10 ± 0.03 and 9.11 ± 0.11 log CFU g^{-1} in FF-C yoghurt (**Fig 9**) with no significant variability between both types of samples. These values are in agreement with those reported by other authors (Ibrahim & Carr, 2006*;* Al-Sheraji *et al., 2012*). It is generally recommended that yoghurt or fermented milk should contain at least 10^8 CFU per serving (EFSA, 2010), which represents approximately one million viable cells per gram at the time of consumption. Initial microbial counts in all samples were in line with this recommendation. We did not find significant differences due to **S1** addition to samples indicating that **S1** do not affect microbial growth.

Recent studies suggest that biocompatibility of mesoporous silica with human cells and tissues is very high. Moreover it has been reported that toxicity of mesoporous silica is minimized or eliminated by using microparticles and by functionalizing them with certain organic molecules (He *et al.*, 2012; Tang, Li & Chen, 2012). Nevertheless, as far as we know, there are few studies evaluating the biocompatibility of mesoporous silica particles with acid lactic bacteria (LAB).

In yoghurt and fermented milks it is important not only to control initial LAB population but also to maintain these numbers. In this sense, it is essential to follow viability during yoghurt storage. To specifically analyse the effect of **S1** addition on the survival of LAB during storage process, the viability of both microorganisms, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophiles,* in **S1** free yoghurts (LF-C and FF-C) and in **S1** containing yoghurts (LF-E_E and FF-E_E) was followed during 21 of cold storage. As it can be observed, the number of CFU g^{-1} decreased significantly ($p<0.001$ **Table 3**) along the storage in both yoghurt types (**Figures 9a** and **9b**); however, this effect was independent of the presence or absence of **S1** (**Table 3**). Thus, **S1** support is biocompatible with the yoghurt microflora at the concentrations needed to provide the 100% of the DRI of FA.

These results confirm that the **S1** do not have any effect in the viable LAB population. Microbial growth continues during storage and the number of viable microorganisms is a critical factor in the final product in terms of the nutritional health benefits attributed to yoghurt starters as probiotics (EFSA, 2010). The yoghurt nutritional content, physical properties, appearance and texture are important aspects for consumer acceptability. Note that if the amount of **S1** to provide 100% of DRI did not provoke any change in viable counts, yoghurts prepared to cover 25, 50 or 75% of this DRI should not experience any affection.

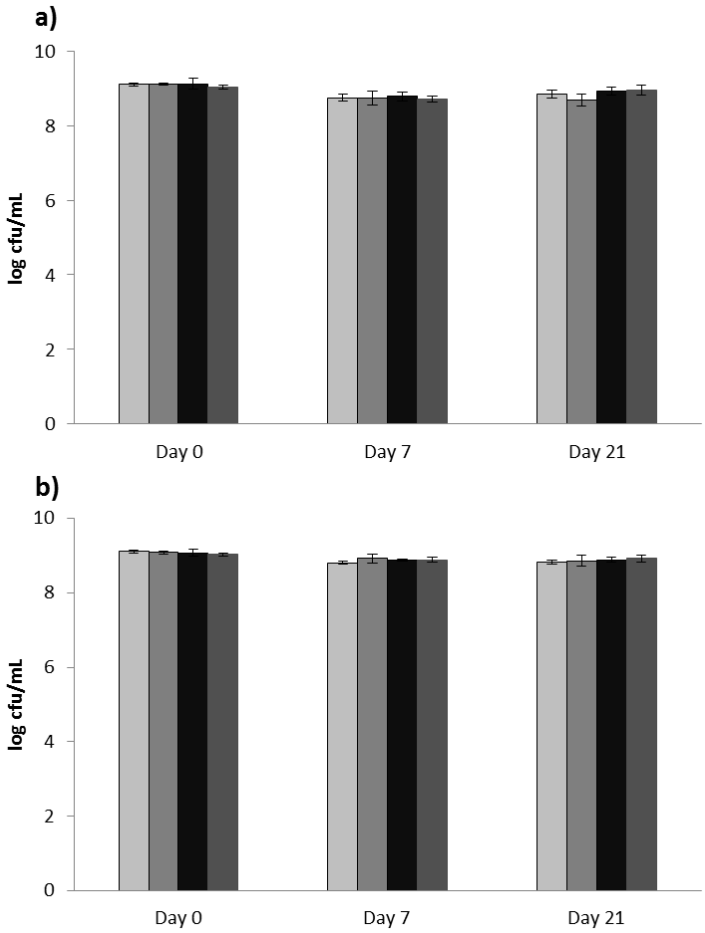

Figure 9. Viability of a) *L. delbrueckii spp.bulgaricus* and b) *S. thermophilus* in control (light grey) and enriched with FA encapsulated in S1 support (mild grey) low-fat yogurts and control (black) and enriched with FA encapsulated in S1 support (dark grey) full fat yogurts during 21 days of refrigerated storage at 4°C.

4.Conclusions

A novel preparation of FA-enriched yoghurts with controlled bioaccessibility is reported. pH-responsive mesoporous silica particles functionalised with polyamines and loaded with FA were able to modulate the release kinetics of FA during the *in vitro* digestion. Sensory analysis were not performed because mesoporous silica particles have not been yet declared as an authorised food ingredient or recognized as "Generally Recognized As Safe products" by the FDA. Nevertheless, physico-chemical characterization of enriched yoghurts with **S1** particles, suggest that **S1** addition did not have any impact neither in pH, syneresis, colour or rheology. Moreover, the addition of **S1** to yoghurts did not have any significant impact on the survival of starting cultures of yoghurts during refrigerated storage. We believe that the results obtained in this work should be easily extrapolated to other bioactive molecules and put forward that encapsulation of bioactive components in mesoporous silica carriers opens new opportunities for the development of novel functional dairy products with added value.

Acknowledgements

Authors gratefully acknowledge the financial support from the Ministerio de Economía y Competitividad (Projects AGL2012-39597-C02-01, AGL2012-39597-C02-02 and MAT2012-38429-C04-01) and the Generalitat Valenciana (project PROMETEO/2009/016). E.P.E. and M.R.R. are grateful to the Ministerio de Ciencia e Innovación for their grants (AP2008-00620, AP2010-4369). Electron Microscopy Service of the UPV is also acknowledged.

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