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Tittle: Development of a non-dairy probiotic fermented product based on almond *milk* and inulin

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Abstract

A new fermented almond “milk” that combined the properties of both almonds and probiotics was considered to cover the current versatile health-promoting foods’ demand. Almond *milk* fermentation with probiotic *Lactobacillus reuteri* and *Streptococcus thermophilus* was studied by using a Central Composite design with response surface methodology and different factors (glucose, fructose, inulin and starters) were optimised to assure high probiotic survivals in the final product. The optimal formulation was physicochemically characterised throughout cold storage (28 days) and both probiotic survivals to *in vitro*

digestion and proteolysis were quantified. Results showed that a high probiotic population (>10^7 cfu/mL) was obtained in the previously optimised almond *milk* throughout storage time, which correspond to the addition of 0.75 g of glucose/100mL, 0.75 g of fructose/100mL, 2 g/100mL inulin and 6 mL/100mL inoculum. Glucose was used as main nutrient and the production of mannitol by *L.reuteri* was detected. The fermentation process increased the viscosity values, forming a weak gel structure, whose physical properties hardly changed. Probiotic bacteria notably survived (51%) to the *in-vitro* digestion, surely related to the inulin presence, which would add value to the developed product by enhancing the potential health benefits of its consumption.

Keywords

L. reuteri, prebiotic, fermentation, response surface methodology, survivals.

1. INTRODUCTION

The term of "probiotics" was defined as live microorganisms that when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2001), being the lactobacilli and bifidobacteria genera the most widely recognised (Rivera-Espinoza and Gallardo-Navarro, 2010).

The use of probiotics, as a whole concept (live microorganisms), in fermented food product manufacturing is not new; although the aims have moved from food preservation and organoleptic improvements (Kopp-Hoolihan, 2001) towards benefits for health, such as reduction of hypercholesterolemia and host immune system modulation, among others (Saad *et al.*, 2013). Nevertheless, in order to effectively provide health functionalities, the minimum recommended number of viable probiotic bacteria is 10^7 colony forming per unit (cfu)/g or mL of a product at the time of consumption (Sanz and Dalmau, 2008). *L. reuteri* ATCC 55730 is a well-established probiotic strain (Casas and Mollstam, 1997) which could be used to develop beneficial products for targeted groups such as the

paediatric population, since these probiotic bacteria have been reported to improve symptoms of infantile colic (Savino *et al*., 2007), feeding tolerance and gut functions in pre-term infants (Indrio *et al*., 2008), reduce constipation (Coccorullo *et al.*, 2010) and modulate cytokine patterns involved in atopic diseases (Miniello *et al.*, 2010).

Although the dairy industry is the major sector involved in developing probiotic products, other food areas have recently become involved such as nut, cereal or other vegetable *milk* industries. The so-called vegetable *milks* have special relevance since, besides their nutritional and health benefits, they contain prebiotic compounds or can be easily added (i.e. inulin) which make them interesting and useful to produce synbiotic (combination of probiotic and prebiotic) products. In addition to the health benefits, prebiotics such as inulin are reported to provide fermented products technological benefits, since they can increase the viscosity of the final product and have a synergic effect on probiotic survival during processing and storage (de Souza-Oliveira *et al.*, 2009).

There is a wide range of commercial vegetable *milks*, although the ones derived from almond nuts have been the subject of interest due to the known impact of their compounds on some current chronic diseases such as cardiovascular diseases, type 2 Diabetes mellitus, obesity and some cancers (Kamil and Chen, 2012). Almond nuts are rich in mono- and polyunsaturated fatty acids (mainly oleic and linoleic acids), vegetable proteins, dietary fibre, phytosterols, polyphenols, vitamins and minerals (Yada *et al.* 2011); most of those compounds have antioxidant properties and have a proven beneficial effect on plasma lipid profile, low-density lipoprotein oxidation and inflammatory processes, among others (Liu, 2012; Egert *et al.*, 2011; Jones *et al.*, 2011). Moreover, almond nuts have a high K/Na ratio and the carbohydrates present have a low glycemic index (suitable for diabetics) (Li *et al.*, 2009). Therefore, almond *milks* could be very useful in the industrial production of new non-dairy fermented products with functional features in which the nutritional and health benefits of almonds and probiotic bacteria are included.

Previous fermentation studies carried out with almond *milk* and different lactic bacteria showed us that a non-formulated almond *milk* has a low carbohydrate content (around 0.3 g/100 mL *of milk*), in comparison with cow milk (4-5 g/100 mL) or soy beverages (1.7 g/100 mL) (Champagne *et al.*, 2009). This low carbohydrate content affects directly the acidification level that can be attained (Chang and Stone, 1990), which remained above 5 after 24 hours (Bernat, 2013). Thus, carbohydrate supplementation of almond *milk* was needed to improve the growth and acidification of the mixed culture used.

The aim of this study was to evaluate the fermentative process of almond *milk* with the mixed culture *L. reuteri* ATCC 55730 and *S. thermophilus* CECT 986 (ratio 1:1). To this end, the effect of different, previously chosen factors (glucose, fructose and inulin and starters contents) were analysed and optimised to define the most suitable almond *milk* formulation in which sufficient probiotic bacteria survivals is ensured in the final product. The fermented almond *milk* with the optimum factor values was characterised as to its physicochemical and probiotic survival properties throughout storage time at 4 ºC with the aim of determining the shelf life of the developed product.

2. MATERIALS AND METHODS

2.1 Almond *milk* processing

Almond *milk* was produced by soaking and grinding almonds (*Prunus amygdalus L.* cv. dulcis) supplied by Frutos Secos 3G S.L. (Valencia, Spain). The extraction was carried out in the Sojamatic 1.5 (Sojamatic®; Barcelona, Spain), a piece of equipment specifically designed for the production of vegetable *milks*, with a nut:water ratio of 8:100. The milky liquid obtained was then microfluidised in a high pressure homogeniser (M-110P model; Microfluidics Int. Corp., Westwood, MA, USA) by applying 172 MPa and further on pasteurised (85 ºC/30 min). The use of high pressures of homogenisation (HPH) contributed to the *milk* being of better quality in terms of its physical stability, since this innovative technology is able to reduce

the size of fat globule particles such a way that flocculation and coagulation phenomena are delayed (Pereda *et al*., 2007). Moreover, HPH may contribute to a better probiotic fermentation response, reducing coagulation times, acquiring sufficient probiotic survival, improving texture and mouthfeel and/or preventing syneresis (Cruz *et al.*, 2009; Patrignani *et al.*, 2007).

The compounds pre-selected as factors, glucose, fructose and inulin were added prior to the heat treatment to prevent further re-contaminations. The monosaccharides were purchased from Sosa Ingredients S.L. (Barcelona, Spain), while the inulin came from Beneo-Orafti (Tienen, Belgium).

2.2 Preparation of fermented almond *milk*

2.2.1 Inoculum preparation

Lactobacillus reuteri ATCC 55730 (Biogaia, Stockholm, Sweden) and *Streptococcus thermophilus* CECT 986 (CECT, Valencia, Spain) were activated from their frozen forms (stored in 40g/100 mL glycerol at -80 ºC), by transferring them to their selective broths until optimal bacterial growth is obtained. The selective broths were MRS (Scharlab, Barcelona, Spain) for the probiotic *Lactobacillus* and M17 (Difco™, New Jersey, USA) for *S. thermophilus*. Incubation conditions were 37 ºC/24h/anaerobically for *L. reuteri*, in which anaerobiosis was created by using anaerobic jars and a CO_2-generator system (AnareroGen™, Oxoid Ltd, Basingstoke, England) and 42 ºC/24h/aerobically for *S. thermophilus*.

As regards the starter inoculum, strains were independently incubated in their broths for 24 h and then centrifuged at 8,600 xg-10 min at 4 ºC; the supernatant was discarded. Immediately afterwards, bacteria were resuspended in PBS-1x buffer (10 mmol/L phosphate, 137 mmol/L NaCl, 2.7 mmol/L KCl, pH 7.4) until they reached concentrations of 10^8 colony forming units (cfu) per mL.

2.2.2 Experimental design for the almond *milk* fermentation process

Amounts of glucose, fructose, inulin and starter inoculum were selected as factors to obtain fermented almond *milk*. Central Composite Design (CCD) with randomised Response Surface methodology (RSM) was used to study how different combinations of these factors affect almond *milk* fermentation. Other authors also used RSM in the development of probiotic products (Cruz *et al.,* 2010; Yaakob *et al.,* 2012). Statistical analysis of the data was carried out by using an orthogonal CCD 2^4 + star, which studied the effects of 4 factors in 31 runs. Levels of glucose, fructose, inulin and starter inoculum are shown in Table 2. These parameters were established by taking previous fermentation studies with probiotics into account (Angelov *et al.,* 2006; De Souza-Oliveira *et al*., 2009; Franck, 2002). The variable response was defined as the probiotic survival (cfu/mL) after fermentation process.

Fermentation process of the 31 runs obtained in the design was carried out by adding the corresponding starters (prepared by mixing in a 1:1volume ratio *L. reuteri:S. thermophilus* buffer suspensions) to the formulated and pasteurised almond *milk* and incubating them at the optimal temperature of the mixed culture (40 ºC). When the pH of the samples reached ≈4.6, fermentation was stopped by cooling them to 4 ºC, which was the storage temperature until the analyses were performed.

A step-wise second grade polynomial fitting was used to model the response variable as a function of the factors. The optimal formulation of the fermented product was established on the basis of the obtained results for the response variable.

2.3 Fermented product characterisation

Both the formulated almond *milk* and the optimal fermented product stored for different times were characterised as to their content in different sugars, pH, acidity, particle size distribution and ζ-potential, rheological behaviour and colloidal stability. In almond *milk*, the chemical composition of major components (dry matter, protein, lipids, total sugars and ashes) was obtained. The fermented product was also analysed in terms of starters' viability throughout storage time (1, 7, 14, 21 and 28 days) at 4 ºC

143 Moreover the initial starters' proteolytic activity and probiotic survivals to a simulated gastrointestinal
144 digestion were studied. All the analyses were performed in triplicate.
145
146 **2.3.1 Chemical analyses**
147 AOAC official methods of analysis were used to determine moisture (AOAC 16.006), total nitrogen
148 (AOAC 958.48) and fat contents (AOAC 945.16) (Horwitz, 2000). Ashes were obtained following the
149 protocol reported by Matissek *et al.* (1998).
150 Sugar profiles were analysed and the different sugars were quantified using the following equipment:
151 A Metrohm 838 high-performance anion-exchange chromatograph (IC 861) equipped with a pulsed
152 amperometric detector (Bioscan 817) to monitor the separation (Metrohm® Ltd., Herisau, Switzerland).
153 Prior to the analysis, samples were diluted 1:100 with nanopure water. Sample proteins were removed by
154 precipitation with glacial acetic acid and the pH was then reconstituted at initial values. Before injecting
155 samples into the equipment, they were filtered through nylon membranes (0.45 μm). A Metrosep CARB
156 guard (5x4.0 mm) and CARB 1 (250 x 4.6 mm) analysis columns (Metrohm®) were used. 20 μL of sample
157 was injected and eluted (1 mL/min) with 0.1 mol/L NaOH, at 32 ºC. An Au working electrode was used
158 and applied potentials were +0.05 V (0-0.40 s) +0.75 V (0.41-0.60 s) and +0.15 V (0.61-1 s). Software
159 ICNet 2.3 (Metrohm®) was used for data collection and processing. The concentration of each sugar was
160 determined from their respective calibration curves, obtained from standard solutions of glucose, fructose
161 and sucrose (Sigma-Aldrich Corp., St. Louis, MO, USA), which were obtained in triplicate.
162 Fibre content was obtained by difference to 100 of the sum of rest of analysed components.
163
164 **2.3.2 Proteolytic activity analyses**
165 The extent of proteolysis in fermented almond *milk* was evaluated by measuring the free amino acids
166 and small peptides using the *o*-phtaldialdehyde method described by Church *et al.* (1983). The absorbance

of the solutions was measured at 340 nm in a quartz cuvette by using a UV-visible spectrophotometer (Helios Zeta UV-vis, Thermo Scientific, USA). The starters' proteolytic activity is quantified as the difference in absorbance measured between fermented and non-fermented almond *milks*.

Moreover, non-fermented and fermented almond *milk* samples were analysed by size-exclusion chromatography (SEC-HPLC) in order to obtain their unique peptide profiles. *In vitro*-digest inoculated samples were also characterised by SEC-HPLC; these samples were first subjected to a heat treatment (80 ºC/20 min) in order to suppress any possible residual enzymatic activities. All HPLC analyses were performed in duplicate on Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA). SEC-HPLC screening of samples was performed on a BioSep-SEC-S2000 (300 mm x 7.8 mm) column with a gel-filtration chromatography guard column (4 x 3 mm) (Phenomenex, Cheshire, UK). Before injecting samples they were filtered through nylon membranes (0.45 μm). A SEC-HPLC screening method that has great affinity for smaller Mw peptides (up to 20,000 Da) was used. The standards thyroglobulin, aprotinin, cyctochrome C, insulin, angiotensin I, angiotensin II, uridine and sodium azide (All Sigma–Aldrich Corp., St. Louis, MO, USA) were used to prepare a calibration curve for this method. The separations were performed at 30 °C by isocratic elution at a flow rate of 1 mL/min. The injection volume was 10 μL. Detection was at 214 nm. The mobile phase was acetonitrile-H_2O (ratio 45:100) containing 0.1 mL/100 mL trifluoroacetic acid.

2.3.3 Probiotic survivals to a simulated gastrointestinal digestion (SGID)

Optimised fermented almond *milk* underwent a SGID and the survival of probiotic bacteria was examined. This was also assessed in non-fermented *milk*. SGID was performed as described by Glahn *et al.* (1998) but no demineralization was carried out. Porcine pepsin (800-2500 units/mg protein), pancreatin (activity, 4 1 USP specifications) and bile extract were purchased from Sigma-Aldrich® (St. Louis, MO, USA).

After the SGID, *L. reuteri* survivals were quantified by using the pour plate technique, which is further on described.

2.3.4 Viability of starters

Survival of both *L. reuteri* and *S. thermophilus* in fermented almond *milks* were quantified using the pour plate technique, according to the method described by the International Dairy Federation (International IDF standards, 1997). The selective media used were MRS agar (Scharlab; Barcelona, Spain) for the probiotic strain, which was acidified to pH 5.4 with acetic acid to prevent growths of streptococcus strain, and M17 agar (DifcoTM; New Jersey; USA) for S. *thermophilus*. Incubation conditions were 37 ºC /48 h/aerobically for *S. thermophilus* and 37 ºC/24 h/anaerobically for *L. reuteri*; Anaerobiosis was created by using anaerobic jars and a CO_2-generator system (AnareroGen™; Oxoid Ltd, Basingstoke, England). Counts were reported as log cfu/mL.

2.3.5 pH and titratable acidity (TA)

Measurements of pH in non-fermented and fermented almond samples were carried out at 25 ºC using a pH-meter (GLP 21+, Crison Instruments S.A.; Spain). AOAC standard method was chosen to determine TA in samples (AOAC 947.05), which consisted of a titration with 0.1 mol/L NaOH solution, expressing the results as grams of lactic acid per L (Horwitz, 2000).

2.3.6 Particle size distribution and ζ-potential

Almond fat globule size distributions in both fermented and non-fermented *milks* were analysed with a laser diffractometer (Mastersizer 2000, Malvern Instruments Ltd, UK). The Mie theory was applied by considering a refractive index of 1.33 and absorption of 0.1. Samples were diluted in de-ionised water at 2,000 rpm until an obscuration rate of 10% was attained. Surface weighted mean diameter ($D_{3,2}$) and

volume weighted mean diameter ($D_{4,3}$) parameters were quantified and analysed. $D_{4,3}$ is sensitive to the presence of large particles, whereas $D_{3,2}$ is more sensitive to the presence of small particles (Couvreur and Hurtaud, 2007).

ζ-potential was determined at 25 ºC by using a Zetasizer nano-Z (Malvern Instruments Ltd; UK). Samples were diluted to a fat droplet concentration of 4 g/L using a phosphate buffer solution. The Smoluchowsky mathematical model (Sze *et al*., 2003) was used to convert the electrophoretic mobility measurements into ζ -potential values.

2.3.7 Serum retention capacity (SRC)

SRC of both non-fermented and fermented *milks* was analysed by sample centrifugation (Medifriger-BL, JP-Selecta; Spain). Conditions were 2,500 xg/45 min/20 ºC and the amount of serum separation was used to quantify sample stability.

2.3.8 Rheological behaviour

The rheological behaviour was characterised in a rotational rheometer (HAAKE Rheostress 1, Thermo Electric Corporation; Germany) with a sensor system of coaxial cylinders, type Z34DIN Ti. The shear stress (σ) was measured as a function of shear rate ($\dot{\gamma}$) from 0 to 512 s^{-1}, using 5 minutes to reach the maximum shear rate and another 5 to fall (up and down curves). The Herschel-Bulkey model (Eq. 1) was fitted to the experimental points of the up curve to determine the flow behaviour index (n), consistency index (K) and yield stress (σ_y) by using a non-linear procedure. Apparent viscosities were calculated at 50 s^{-1} (Eq. 2), since shear rates generated in mouth when food is being chewed and swallowed are between 10 and 100 s^{-1} (McClements, 2004).

$$\sigma = \sigma_y + K\,\dot{\gamma}^{\,n} \quad (1)$$

$$\eta = K \cdot \dot{\gamma}^{\,n-1} \quad (2)$$

238

239 **2.3.9 Colour parameters**

240 The colour coordinates were measured from the infinite reflection spectrum in a spectrocolorimeter
241 (CM-3600 d, MINOLTA Co; Japan). A 20 mm depth cell was used. The CIE L^*a^*b coordinates were
242 obtained using illuminant D65/10º observer. The colour of almond *milk* samples was characterised as to
243 Lightness (L^*), chrome (C_{ab}^*), hue (h_{ab}^*) and Whiteness Index (WI), as defined in equations (3) to (5). The
244 colour differences (ΔE) between fermented and non-fermented samples were also calculated by using
245 equation (6).

$$C^*_{ab} = \sqrt{a^{*2} + b^{*2}} \quad (3)$$

$$h^*_{ab} = \arctan\left(b^*/a^*\right) \quad (4)$$

$$WI = 100 - \sqrt{\left(100 - L^*\right)^2 + a^{*2} + b^{*2}} \quad (5)$$

$$\Delta E = \sqrt{\left(\Delta L^*\right)^2 + \left(\Delta a^*\right)^2 + \left(\Delta b^*\right)^2} \quad (6)$$

246

247 **2.4 Statistical Analysis**

248 Results were submitted to analysis of variance with 95% significance level using
249 Statgraphics® Centurion XV. Multiple comparisons were performed through 95% LSD intervals.

250

251 **3. RESULTS AND DISCUSSION**

252

253 **3.1 Chemical composition of almond *milk***

Values of both peeled almond nut and the derivative *milk* compositions are summarised in Table 1. Results (mean values and standard deviation) are expressed per 100 grams or mL.

Results obtained were consistent with those from Yada *et al.* (2011) for sweet almonds (*Prunus amygdalus L.* cv. dulcis). With the exception of the sugar content, the almond *milk* composition obtained was what was expected, considering the nut:water ratio (8:100) during extraction. Differences in *milk* sugar content (around 0.3 g/100 mL were expected in the *milk*) are probably due to the heat treatment that the almond *milk* received, which might have caused sugar losses due to caramelisation phenomena (Kroh, 1994). As commented on above, this low carbohydrate content affected the acidification level which can be attained and was insufficient for fermentation (data not shown). Thus, carbohydrate supplementation of almond *milk* was needed to improve the growth and acidification of the mixed culture used.

3.2 Optimisation of fermentation process

Table 2 shows the experimental response (probiotic survival (log cfu/mL)) obtained for all the formulations of the CCD. As can be seen, all the formulations were suitable for developing a probiotic almond fermented *milk*, since their variable responses were above 7 log cfu/mL, which is the minimum recommended probiotic amount to be present within the food matrix to ensure health effects in consumers (Sanz and Dalmau, 2008).

Results from the 31 runs were fitted to a second order polynomial equation and the removal of non-significant terms ($p > 0.05$) was applied when necessary. However, when the exclusion of such terms decreased the explained variance (R^2adj), the term was included in the model. The goodness of the fitted model was evaluated by ANOVA, based on the F-test and on the R^2adj, which provide a measurement of how much of the variability in the observed response values could be explained by the experimental factors and their interactions (Cruz *et al.,* 2010). Table 3 summarises the estimated regression coefficients of the second order model obtained, in which fit parameters from the analysis of variance are included.

As can be seen in Table 3, the coefficients for glucose and fructose factors seemed to negatively affect the probiotic survival (values are negative), although the coefficients corresponding to the interactions (second order terms) were positive and explained the overall positive impact of those growth factors on the probiotic counts. This result indicated that neither glucose nor fructose were truly independent, which is statistically known as "multicolinearity" and represents a common problem in regression analyses (Bender *et al*., 1989). When multicolinearity occurs, the elimination of non-significant explanatory variables in the model is not recommended (Bender *et al*., 1989). As regards the inulin and inoculum factors, both had a positive effect on the probiotic survival, being the inoculum concentration the factor which most positively influenced ($p< 0.05$).

With regards to the model fit, the lack-of-fit parameter was not significant ($p> 0.05$), which indicated that the obtained model is adequate for predicting probiotic *L. reuteri* survival in almond *milk*. In practice, a model is considered appropriate to describe the influence of the dependent variable(s) when the coefficient of determination (R^2) is at least 80% (Yaakob *et al.*, 2012) or values of R^2 adj (variation in the experimental data) over 70% (Cruz *et al*., 2010). R^2 and R^2adj of the model did not reach the recommended minimums (Table 3), probably due to the narrow range of experimental response obtained (less than one log cfu/mL). Nevertheless, this model could be a useful tool to make rough predictions.

The CCD model was then statistically optimised in order to maximise the viability of the *L. reuteri* (variable response) and the optimum formulation obtained corresponded to the addition of 0.75 g/100 mL of glucose, 0.75 g/100 mL of fructose, 2 g/100 mL of inulin and 6 mL/100 mL of starter inoculum (10^8 cfu/mL) to the almond *milk*. With this formulation, it would be expected that probiotic counts in the resulting fermented product would be 7.7 log cfu/mL.

The optimal formulation was then submitted to a fermentation process and it reached a pH of 4.83 ± 0.03 in 8 h at 40 ºC with a *L. reuteri* survival of ≈ 8 log cfu/mL, as the model predicted. Despite the pH, the final acidity of this fermented almond *milk* averaged 1.78 ± 0.05 g of lactic acid per L. This value is lower

than standard yoghurt, which has a lactic acid content of around 8-10 g/L (Tamime and Robinson, 2000). This acidity could be explained by considering that almond *milk* has a lower buffering capacity than cow milk (Al-Dabbas *et al*., 2010).

3.3 Initial characterisation of fermented samples

At the end of the process, a pH of 4.83 ± 0.03 at 40 ºC and a population of *L. reuteri* of 7.93 ± 0.02 log cfu/mL was obtained after 8 h, in agreement with the regression model. *S. thermophilus* reached similar log counts to *L. reuteri* (7.90 ± 0.01 log cfu/mL). The use of these mixed cultures resulted in faster acidifying rates (data not shown), suggesting some type of acidifying synbiosis. This has frequently been observed with traditional cow milk yogurt starter cultures and sometimes pointed out by others author working with others vegetable matrixes such as soy (Champagne *et al.*, 2009), but until now, has not been reported between *L. reuteri* and *S. thermophilus*.

The Titrable Acidity (TA) at the end of the fermentation process was 1.78 ± 0.05 g of lactic acid per L. This value is lower than standard yoghurt, which has a lactic acid content of around 8-10 g/L (Tamime and Robinson, 2000), surely due to the much lower protein content of almond milk protein than that of cow milk and to its lower buffering capacity.

3.3.1 Proteolytic activity analyses

During industrial processes, starter bacteria are repeatedly exposed to stress conditions, which induce the bacterial proteases synthesis in order to obtain nutrients for their growth (Aguirre *et al*., 2008); these proteases, besides the almond protein hydrolysis, contributed in flavour and texture of the resulted fermented products (Savijoki, Ingmer and Varmanen, 2006; Tamime and Robinson, 2000).

The proteolytic activity was evaluated through the difference in the absorbance values between fermented and non-fermented almond *milk*, this being around 0.080 ± 0.005, value much below that the activity observed in others probiotic cow-milk's yoghurts (ΔA_{340nm} = 0.1-0.2) (Vasiljevic *et al*., 2007).

This result allowed us to conclude that the enzymatic system of this starter bacteria contain enzymes which were able to hydrolyse almond proteins in some extent, since the absorbance values in fermented samples were higher than that obtained in non-fermented *milk*. This is also reflected in the peptide chromatogram profile of fermented samples, which is shifted to lower molecular weights, as Figure 1 shows. Donkor *et al.* (2005), when working with different probiotic fermented products from vegetable (soy) *milk*, also quantified low proteolytic activities (ΔA_{340nm}< 0.02). This could be related with the different probiotic strain and different concentration used, which often make difficult the comparison of the data.

3.3.2 Simulated gastrointestinal digestion (SGID)

Initially fermented samples were submitted to a SGID to analyse the effect of the digestion process on the almond proteins and on the viability of probiotic bacterial.

Figure 1 shows the peptide chromatogram profiles obtained from both non-fermented and fermented *milks* before and after the SGID. As can be observed, the main soluble peptides in the fermented *milks* were constituted by peptides with Mw lower than 400 Da, together with the highest Mw fraction (from 8 to 15 kDa). The *in vitro* digestion process of fermented samples led to the disappearance of the major part of the high Mw peptide fraction (from 8 to 15 kDa) and to the generation of greater amount of low Mw peptides (< 2.5 kDa). Hence, fermentation process together with the human digestion might have improved, on the one hand, the bioavailability of almond *milk*'s peptides and, on the other hand, the immune response, as has been observed in soy-based products (Wilson *et al*., 2005).

With regards the viability of the probiotic bacteria, results showed that 51 ±7% of *L. reuteri* survived to a SGID, this value being notably higher than the values reported by other authors working with probiotics (20-40%) (Bezkorovainy, 2001). The higher survival might be attributable to the presence of inulin, which is believed to improve probiotic viability (Capela *et al*., 2006; Kolida *et al*., 2002; Franck, 2002).

3.3.3 Sugar contents

The characterisation of sugar profiles in the products stored for different times is essential in order to know the metabolic activity of the starter bacteria within the almond matrix. Figure 2 shows the chromatograms of both non-fermented and fermented almond *milks* stored for different times (1, 14 and 28 days). As can be seen, prior to the fermentation process, our almond *milk* contained in order of importance sucrose, glucose and fructose, besides two other peaks (peaks 4 and 5). These latter were not present in almond nut (data not shown) and so, they must come from the added inulin and, thus, were classified as fructans, which is a term that includes both inulin and their derivatives (Roberfroid, 2005).

Moreover, a new peak was identified in fermented products as mannitol (peak 0), due to the fact that its retention time was the same as that of pure mannitol. The appearance of this compound is attributed to the heterofermentative metabolic pathway of the *L. reuteri* strain used, in which fructose is used as an e^- acceptor to regenerate NAD resulting in mannitol as the end product (Årsköld *et al.,* 2008). Mannitol yield (mol mannitol produced per mol fructose consumed) was 0.81 ±0.09 which is in agreement with other reported values (Ortiz *et al*., 2012). The presence of mannitol might be an added value in the product, since it is a non-metabolic sweetener with antioxidant properties (Wisselink *et al*., 2002).

As can be seen in Table 4, immediately after the fermentation process, a significant reduction in the glucose, fructose and sucrose contents occurred, being glucose the major substrate used (13% of the total), followed by the fructose (6%). Champagne *et al.* (2009) also observed the decrease in these sugars in soy beverage when it was fermented using a mixed culture of *L. helveticus* or *B. longum* and *S. thermophilus.*

3.4 Fermented samples characterisation throughout storage time

3.4.1 Starter survival and acid production

Table 5 shows the average values of pH and Titratable Acidity (TA) in fermented almond *milk* vs. storage time. This table also includes the bacterial count data of *L. reuteri* and *S. thermophilus* (log cfu/mL) in fermented almond *milk* throughout storage time.

As it has been reported, probiotic health benefits are seen to be dependent on the matrix in which they are present and their efficacy is linked with their ability to survive in the gastrointestinal tract (Buddington, 2009). Hence, in order to effectively provide such health functionalities, the minimum number of viable probiotic bacteria is suggested as 10^7 cfu/mL by the time of consumption (Sanz and Dalmau, 2008). The viability of both strains decreased throughout storage time ($p< 0.05$), especially for *S. thermophilus*, but the probiotic *L. reuteri* counts were above the minimum recommended level (10^7 cfu/mL) in the whole period analysed.

The pH values were almost maintained throughout the time period analysed, the mean value being 4.65. TA increased over storage time, although from the 7^{th} day onwards differences among values were non-significant ($p< 0.05$), being around 2.2 g/L. This low TA value might have a positive effect on the overall sensory acceptance of the final product, since it has a direct impact on sweetness attribute.

3.4.2 Sugar contents

Table 4 shows the amount of the different sugars identified in both fermented throughout storage time and non-fermented almond *milks*. Contrary to standard cow milk yoghurts, in which lactose is the only C-source available, in our almond *milk*, the starter bacteria had a variety of options. This strain could use simultaneously glucose or sucrose as energy source and fructose as alternative electron acceptor (Ortiz *et*

al., 2012). The assessment of the use of these C-sources is critical to understand the bacterial growth and survivals throughout storage. To enhance mannitol production by *L. reuteri*, fructose should be available at either the lag or log growth phase of cultures grown (Ortiz *et al*., 2012).

As can be observed, both glucose and sucrose gradually decreased ($p < 0.05$) throughout the storage time. These results were predictable, since starter bacteria were viable during the entire storage time (Table 5) and, therefore, they consumed these sugars as nutrients. Fructose did not follow the same tendency, since no differences in fructose concentrations were observed in fermented products stored either 1 day or 14 days (Table 4). The small amount of fructose available after the fermentation process remained constant throughout storage, which stopped the mannitol production, as can be observed in Table 4. Grobben *et al.* (2001) showed that when there is not enough fructose available in the medium, the fructose to mannitol conversion efficiency decreased.

In Figure 2, the chromatograms of sugar peaks obtained in HPAC-PAD assays from almond *milk* (AM) and its fermented products at the different storage times are shown. As can be observed, no changes in the long-chain fructan (peak 5) throughout time is detected, which suggests that *L. reuteri* was either not able to degrade this oligosaccharide or did not have to do it due to the fact that there was sufficient nutrient availability within the almond *milk*. Regarding the other fructan (peak 4), a trend towards a reduction seems to be observed but it was not large enough to quantify it. Makras *et al.* (2005) observed that, among the lactobacilli assessed, few of them are able to use inulin-type fructans as a C-source. Hence, most of the added inulin remains preserved in the product, thus, the targeted consumers of the fermented product developed can take advantage of the health benefits that this prebiotic may exert.

3.4.3 Colloidal stability parameters: Particle size, ζ-potential and SRC

The measurements of particle size distributions and ζ-potential are directly related to the colloidal stability of almond *milk* emulsions. Table 6 shows the mean particle diameters $D_{4,3}$ and $D_{3,2}$. As was

419 expected, the particle size distributions of fermented samples shifted to bigger sizes (both $D_{4,3}$ and $D_{3,2}$
420 values increased) (Table 6), probably due to the phenomenon of particle flocculation associated to the
421 acidification of the system. Both mean particle diameters reached a maximum value on the 7th storage day
422 after the fermentation process, when the ζ-potential reached the minimum value (Table 6).

423 Table 6 also shows ζ-potential value in both fermented and non-fermented *milks*. Fermentation
424 provoked a lower negative charge of the dispersed particles ($p< 0.05$), which means that the neutralisation
425 of some ionisable groups occurs as a consequence of the change in the pH of the product. The almond
426 protein charge will decrease, thus promoting a reduction in the ζ-potential and repulsive forces among the
427 dispersed particles. This effect will lead to the phenomenon of flocculation in the system, which can give
428 rise to a weak gel structure taking the volume fraction of the dispersed phase into account. Particle
429 flocculation will be responsible for the increase in particle size after fermentation. This result was coherent
430 with the isoelectric point (IP) range of amandin (4.55-6.3) reported by Albillos *et al.* (2009). The wide IP
431 range of amandin shows the high conformational complexity of this almond protein and explains the slight
432 negative values of ζ-potential obtained in fermented samples, since the pH of these samples (Table 5) were
433 above the minimum IP range reported. Usually ζ-potential values lower than ± 25 mV do not ensure the
434 stability of dispersed systems (Roland *et al.*, 2003), although changes in protein conformation did allow us
435 to obtain a stable matrix through the development of a weak gel structure, as commented on below.

436 Table 6 also shows the SRC obtained by sample centrifugation (expressed as percentage of precipitate
437 after centrifugation) in both fermented and non-fermented samples. A greater serum separation occurred in
438 non-fermented samples, while very few differences were observed in the case of fermented samples stored
439 for different lengths of time. These results confirm the formation of a weak gel in the fermented product as
440 a result of the flocculation of dispersed particles due to the action of proteins, which was able to retain part
441 of the serum present in the almond *milk*. Taking into account that neither the fermentation process nor the

storage time seems to notably affect inulin, it also contributed to the network formation due to its thickening and gelling capacity (Franck, 2002).

3.4.4 Rheological behaviour

Rheological parameters play a key role in the definition of the textural and sensory perception of a new product. Table 7 shows these parameters obtained by using a non-linear regression procedure to fit Eq. 1 to the flow curves of fermented and non-fermented almond *milks*. The apparent viscosity of samples at 50 s^{-1} shear rate and the non-linear correlation coefficient of the fitted model (R^2) are also shown.

The rheological analyses of all samples showed that both upward and downward shear rate curves demonstrated progressive structural degradation with repeated shearing, thus reflecting their thixotropic nature.

The upward shear-rate flow behaviour of the samples could be described by a Herschel-Bulkley model (parameters showed in Table 7), thus showing a shear-thinning behaviour ($n<1$) in all cases. These parameters were not significantly ($p> 0.05$) affected either by the fermentation process or the storage time. In Table 7, the values of the hysteresis area at the different storage times can be also observed. Both the apparent viscosity as well as the thixotropic character of the samples increased slightly after the fermentation step ($p< 0.05$) in line with the formation of a weak gel structure, as was commented on above. On the other hand, *L. reuteri* is also able to synthesise exopolysaccharides and, thus, it might also contribute to the gel formation and to the increase in the viscosity values (Årsköld *et al.*, 2007).

Statistical differences in the thixotropic nature of fermented products and in the apparent viscosity due to storage time at 4 ºC were non-significant ($p> 0.05$).

3.4.5 Colour measurements

Table 8 shows the colour parameters of both almond *milk* and fermented products cold stored at different times. As can be seen, L^*, h^*_{ab} and WI increased after the fermentation process, while C^*_{ab} decreased ($p< 0.05$). These changes in colour coordinates can be attributed to the different level of opacity (Hutchings, 1999), which is related to the aggregation level of particles. The higher the luminosity values, the higher the opacity and the lower the chrome, in line with a higher whiteness index. These parameters were barely affected by the storage time at 4 ºC until 21 storage days, at which point C^*_{ab} and h^*_{ab} slightly decreased ($p < 0.05$). Nonetheless, lightness was not affected by cold storage, while the whiteness (WI) only slightly increased on the last day of assays ($p< 0.05$).

The total colour difference (ΔE) values between non-fermented and fermented almond *milks* were not affected by the storage time ($p> 0.05$); the mean value being 2.69 ± 0.03. According to Francis (1983), values lower than 3 units cannot be easily detected by the human eye.

4. CONCLUSIONS

The optimal combination of growth factors which ensured a rapid fermentation of almond *milk* while maintaining significant probiotic yields was 0.75 g/100 mL of glucose, 0.75 g/100 mL of fructose, 2 g/100 mL of inulin and 6 mL/100 mL of inoculum. This fermented product showed a pH = 4.6 after fermentation and TA values of 2.3 g/L lactic acid. Fermentation process promoted the major changes in the physical properties of the almond *milk* due to the formation of a weak gel, which induces an increase in the viscosity, luminosity and whiteness index values. On the contrary, the storage time did not induce significant changes in these physical properties. High probiotic survivals were also observed in the fermented almond *milk* after submitting the product to an *in vitro* digestion, thus enhancing the product features to be considered as a functional food. The viability of the probiotic bacteria was maintained within the minimum suggested ($\geq 10^7$ cfu/mL) throughout the entire storage time. During this period, consumption of monosaccharides and sucrose, and mannitol production were observed, meanwhile long-chain fructan compounds remained

stable. Owing to both the probiotic survivals and the prebiotic compounds present, the fermented developed product can be considered as a synbiotic. Nevertheless, possible functional properties of the developed product have to be assessed both *in vitro* and *in vivo* assays in further studies in order to state the possible health benefits.

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References

Aguirre L., Garro M.S. and Savoy de Giori G. (2008). Enzymatic hydrolysis of soybean protein using lactic acid bacteria. *Food Chemistry* 111(4): 976-982.

Albillos S.M, Menhart N. and Fu T.J. (2009). Structural stability of amandin, a major allergen from almond (*Prunus dulcis*) and its acidic and basic polypeptides. *Journal of Agriculture and Food Chemistry* 57(11): 4698-4705.

Al-Dabbas M.M., Al-Ismail K., Taleb R.A. and Ibrahim S. (2010). Acid-base buffering properties of five legumes and selected food in vitro. *American Journal of Agricultural and Biological Sciences* 5(2): 154-160.

Angelov A., Gotcheva V., Kuncheva R. and Hristozova T. (2006). Development of a new oat-based probiotic drink. *International Journal of Food Microbiology* 112(1): 75-80.

511 Årsköld E., Lohmeier-Vogel E., Cao R., Roos S., Rådström P. and van Niel E.W.J. (2008). Phosphoketolase
512 pathway dominates in *Lactobacillus reuteri* ATCC 55730 containing dual pathways for glycolysis. *Journal*
513 *of Bacteriology* 190(1): 206-212.

514 Årsköld E., Svensson M., Grage H., Roos S., Rådström P. and van Niel E.W.J. (2007). Environmental
515 influences on exopolysaccharide formation in *Lactobacillus reuteri* ATCC 55730. *International Journal of*
516 *Food and Microbiology* 116(1): 159–167.

517 Bender F.E., Douglas L.W. and Kramer D.S. (1989). Statistical methods for food and agriculture. Westport:
518 Avi Publishing Co Inc. pp. 191-221.

519 Bernat N. (2013). Desarrollo, caracterización y optimización de productos fermentados a base de licuados
520 vegetales como alternativa a los yogures convencionales [Doctoral Thesis]. Universitat Politècnica de
521 València.

522 Bezkorovainy A. (2001). Probiotics: determinants of survival and growth in the gut. *American Journal of*
523 *Clinical Nutrition* 73(2): 399S-405S.

524 Buddington R. (2009). Using probiotics and prebiotics to manage the gastrointestinal tract ecosystem. In:
525 Charalampopoulos D. and Rastall R.A. (eds.), *Prebiotics and Probiotics Science and Technology*. New
526 York: Springer Science+Business Media. pp 1-32.

527 Capela P., Hay T.K.C. and Shah N.P. (2006). Effect of cryoprotectants, prebiotics and microencapsulation
528 on survival of probiotic organisms in yoghurt and freeze-dried yoghurt. *Food Research International* 39(2):
529 203-211.

530 Casas I.A. and Mollstam B. (1997) Treatment of diarrhea. Int. Patent WO/1997046104 A1.

Champagne C.P., Green-Johnson J., Raymond Y., Barrette J. and Buckley N. (2009). Selection of probiotic bacteria for the fermentation of a soy beverage in combination with *Streptococcus thermophilus*. *Food Research International* 42(5–6): 612-621

Chang C.Y. and Stone M.B. (1990). Effect of total soymilk solids on acid production by selected lactobacilli. *Journal of Food Science* 55(6): 1643-1646.

Church F.C., Swaisgood H.E., Porter D.H. and Catignani G.L. (1983). Spectrophotometric assay using o-phtaldialdehyde for determination of proteolysis in milk and isolated milk proteins. *Journal of Dairy Science* 66(6): 1219-1227.

Coccorullo P., Strisciuglio C., Martinelli M., Miele E., Greco L. and Staiano A. (2010) *Lactobacillus reuteri* (DSM 17938) in infants with functional chronic constipation: a double-blind, randomized, placebo-controlled study. *Journal of Pediatrics* 157(4): 598-602.

Couvreur S. and Hurtaud C. (2007). Globule milk fat: secretion, composition, function and variation factors. Productions Animales, 20 (5): 369-382.

Cruz A.G., Faria J.A.F.,Walter E.H.M., Andrade R.R., Cavalcanti R.N., Oliveira C.A.F. and Granato D. (2010). Processing optimization of probiotic yogurt containing glucose oxidasa using response surface methodology. *Journal of Dairy Science* 93(11): 5059-5068.

Cruz N.S., Capellas M., Jaramillo D.P., Trujillo A.J., Guamis B. and Ferragut V. (2009). Soymilk treated by ultra high-pressure homogenization: Acid coagulation properties and characteristics of a soy-yogurt product. *Food Hydrocolloids* 23(2): 490-496.

De Souza-Oliveira R.P., Perego P., Converti A. and de Oiliveira M.N. (2009). The effect of inulin as prebiotic on the production of probiotic fibre-enriched fermented milk. *International Journal of Dairy Technology* 62(2): 195-203.

553 Donkor O.N., Henriksson A., Vasiljevic T. and Shah N.P. (2005). Probiotic strains as starter cultures
554 improve angiotensin-converting enzyme inhibitory activity in soy yogurt. *Journal of Food Science* 70(8):
555 m375–m381.

556 Egert S., Kratz M., Kannenberg F., Fobker M. and Wahrburg U. (2011). Effects of high-fat and low-fat
557 diets rich in monounsaturated fatty acids on serum lipids, LDL size and indices of lipid peroxidation in
558 healthy non-obese men and women when consumed under controlled conditions. *Journal of Nutrition*
559 50(1): 71-79.

560 FAO/WHO (2001). Report on Joint FAO/WHO Expert consultation on evaluation of health and nutritional
561 properties of probiotics in food including powder milk with live lactic acid bacteria. Retrieved from:
562 http:// ftp.fao.org/es/esn/food/probio_report_en.pdf (Last access 28/02/14)

563 Francis F.J. (1983). Colorimetry of foods. In: Pelef M., Baglet E.B. (eds.) *Physical properties of foods*.
564 Westport: AVI Publishing. pp. 105-124.

565 Franck A. (2002). Technological functionality of inulin and oligofructose. *British Journal of Nutrition*
566 87(2), S287-S291.

567 Glahn R.P., Lee O.A., Yeung A., Goldman M.I. and Miller D.D. (1998). Caco-2 cell ferritin formation
568 predicts non-radiolabeled food iron availability in an in vitro digestion/Caco-2 cell culture model. *Journal*
569 *of Nutrition* 128(9): 1555-1561.

570 Grobben G.J., Peters S.W.P.G., Wisselink H.W., Weusthuis R.A., Hoefnagel M.H.N., Hugenholtz J. and
571 Eggink G. (2001). Spontaneous formation of a mannitol-producing variant of *Leuconostoc*
572 *pseudomesenteroides* grown in the presence of fructose. *Applied and Environmental Microbiology* 67(6):
573 2867–2870

574 Horwitz W. (2000). Official Methods of Analysis of AOAC International. Association of Official
575 Analytical Chemists (eds.). Gaithersburg. pp. 40.1-40.3.

576 Hutchings J.B. (1999). *Food Colour and Appearance*. Dordrecht: Springer-Science+Business Media. pp.
577 238-326.

578 Indrio F., Riezzo G., Raimondi F., Bisceglia M., Cavallo L. and Francavilla R. (2008) The effects of
579 probiotics on feeding tolerance, bowel habits, and gastrointestinal motility in preterm newborns. *Journal of*
580 *Pediatrics* 152(6): 801-806.

581 International IDF Standard (1997). Dairy starter cultures of Lactic acid Bacteria (LAB): Standard of
582 identity. Brussels: International Dairy Federation.

583 Jones J.L., Fernandez M.L., Mclntosh M.S., Najm W., Calle M.C., Kalynych C., Vulkich C., Barona J.,
584 Ackermann D., Kim J.E., Kumar V., Lott M., Volek J.S., Lerman R.H. (2011). A Mediterranean-style low-
585 glycemic-load diet improves variables of metabolic syndrome in women, and addition of aphytochemical-
586 rich medical food enhances benefits on lipoprotein metabolism. *Journal of Clinical Lipidology* 5(3): 188-
587 196.

588 Kamil A. and Chen C.Y. (2012). Health benefits of almonds beyond cholesterol reduction. *Journal of*
589 *Agriculture and Food Chemistry* 60(27): 6694-6702.

590 Kolida S., Tuohya K. and Gibsona G. R. (2002). Prebiotic effects of inulin and oligofructose. *British*
591 *Journal of Nutrition* 87(S2): S193-S197

592 Kopp-Hoolihan L. (2001). Prophylactic and therapeutic uses of probiotics: A review. *Journal of the*
593 *American Dietetic Association* 101(2): 229-241.

594 Kroh L.W. (1994). Caramelisation in food and milks. *Food Chem*isty 51(4): 373-379.

595 Li T.Y., Brennan A.M., Wedick N.M., Mantzoros C., Rifai N. and Hu F.B. (2009). Regular consumption
596 of nuts is associated with a lower risk of cardiovascular disease in women with type 2 diabetes. *Journal of*
597 *Nutrition* 139(7): 1333-1338.

598 Liu R.H. (2012). Health benefits of phytochemicals in whole foods. In: Temple N.J., Wilson T. and Jacobs
599 D.R. Jr (eds.), *Nutrition and health. Strategies for disease prevention.* New York: Springer
600 Science+Business Media. pp. 293-310.

601 Makras L., Van Acker G. and De Vuyst L. (2005) *Lactobacillus paracasei* subsp. *paracasei* 8700:2
602 degrades inulin-type fructans exhibiting different degrees of polymerization. *Applied and Environmental*
603 *Microbiology* 71(11): 6531-6537

604 Matissek R., Schnepel F.M., Steiner G. (1998). Análisis de los Alimentos: Fundamentos, Métodos y
605 Aplicaciones. Zaragoza: Acribia S.A. publishings. pp. 123-132.

606 McClements D.J. (2004). Food emulsions: principles, practices and techniques. Boca Raton: CRC Press.pp.
607 341-386.

608 Miniello V.L., Brunetti L., Tesse R., Natile M., Armenio L. and Francavilla R. (2010). *Lactobacillus reuteri*
609 modulates cytokines production in exhaled breath condensate of children with atopic dermatitis. *Journal of*
610 *Pediatric Gastroenterology and Nutrition* 50(5): 573-576.

611 Ortiz M.E.; Fornaguera M.J., Raya R.R. and Mozzi F. (2012). *Lactobacillus reuteri* CRL 1101 highly
612 produces mannitol from sugarcane molasses as carbon source. *Applied Microbiology and Biotechnology*
613 95(4). 991-999.

614 Patrignani F., Iucci L., Lanciotti R.,Vallicelli M., Mathara J.M., Holzapfel W.H. and Guerzoni M.E. (2007).
615 Effect of high-pressure homogenization, nonfat milk solids, and milkfat on the technological performance

of a functional strain for the production of probiotic fermented milks. *Journal of Diary Science* 90(10): 4513-4523.

Pereda J., Ferragut V., Quevedo J.M., Guamis B. and Trujillo A.J. (2007). Effects of ultra-high pressure homogenization on microbial and physicochemical shelf life of milk. *Journal of Diary Science* 90(3): 1081-1093.

Rivera-Espinoza Y. and Gallardo-Navarro Y. (2010). Non- dairy probiotic products. *Food Microbiology* 27(1): 1-11.

Roberfroid M.B. (2005). Introducing inulin-type fructans. *British Journal of Nutrition* 93(1): S13-S25.

Roland I., Piel G., Delattre L. and Evrard B. (2003). Systematic characterization of oil-in-water emulsions for formulation design. *International Journal of Pharmaceutics* 263(1-2): 85-94.

Saad N., Delattre C., Urdaci M., Schmitter J.M. and Bressollier P. (2013). An overview of the last advances in probiotic and prebiotic field. *Food Science and Technology* 50(1): 1-16.

Sanz Y. and Dalmau J. (2008). Los probióticos en el marco de la nueva normativa europea que regula los alimentos funcionales. *Acta Pediátrica Española* 66, 27-31.

Savijoki K., Ingmer H. and Varmanen P. (2006). Proteolytic systems of lactic acid bacteria. *Applied Microbiology and Biotechnology* 71(4): 394-406.

Savino F., Pelle E., Palumeri E., Oggero R. and Miniero R. (2007). *Lactobacillus reuteri* (American Type Culture Collection Strain 55730) versus simethicone in the treatment of infantile colic: a prospective randomized study. *Pediatrics* 119(1): e124-e130.

Sze A., Erickson D., Ren L. and Li D. (2003). Zeta-potential measurement using the Smoluchowski equation and the slope of the current–time relationship in electroosmotic flow. *Journal of Colloid and Interface Science* 261 (2): 402–410

638 Tamime A.Y. and Robinson R.K. (2000). Yoghurt. Science and Technology. Boca Raton: CRC Press. pp.
639 535-588.

640 Vasiljevic T., Kealy T. and Mishra V.K. (2007). Effects of β-Glucan Addition to a Probiotic Containing
641 Yogurt. *Journal of Food Science* 72(7):C405–C411.

642 Wilson S., Blaschek K. and de Mejia E.G. (2005). Allergenic proteins in soybean: processing and reduction
643 of P34 allergenicity. *Nutrition Reviews* 63(2): 47–58.

644 Wisselink H.W., Weusthuis R.A., Eggink G., Hugenholtz J., Grobben G.J. (2002). Mannitol production by
645 lactic acid bacteria: a review. *International Dairy Journal* 12(2–3): 151-161.

646 Yaakob H., Ahmed N.R., Daud S.K., Malek R.A. and Rahman R.A. (2012). Optimization of ingredient and
647 processing levels for the production of coconut yogurt using response surface methodology. *Food Science*
648 *and Biotechnology* 21(4): 933-940.

649 Yada S., Lapsley K. and Huang G. (2011). A review of composition studies of cultivated almonds:
650 Macronutrients and micronutrients. *Journal of Food Composition and Analysis* 24(4-5): 469-480.

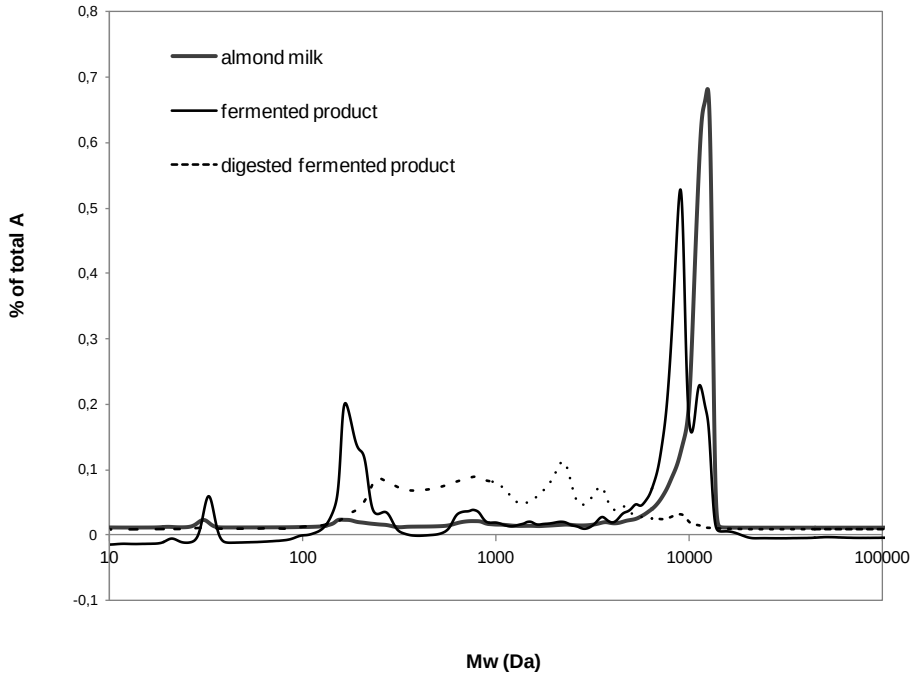

Figure 1 Peptide profile chromatograms of non-fermented (doubled line) and fermented (solid line) almond *milks* and the in vitro digested fermented products (dashed line).

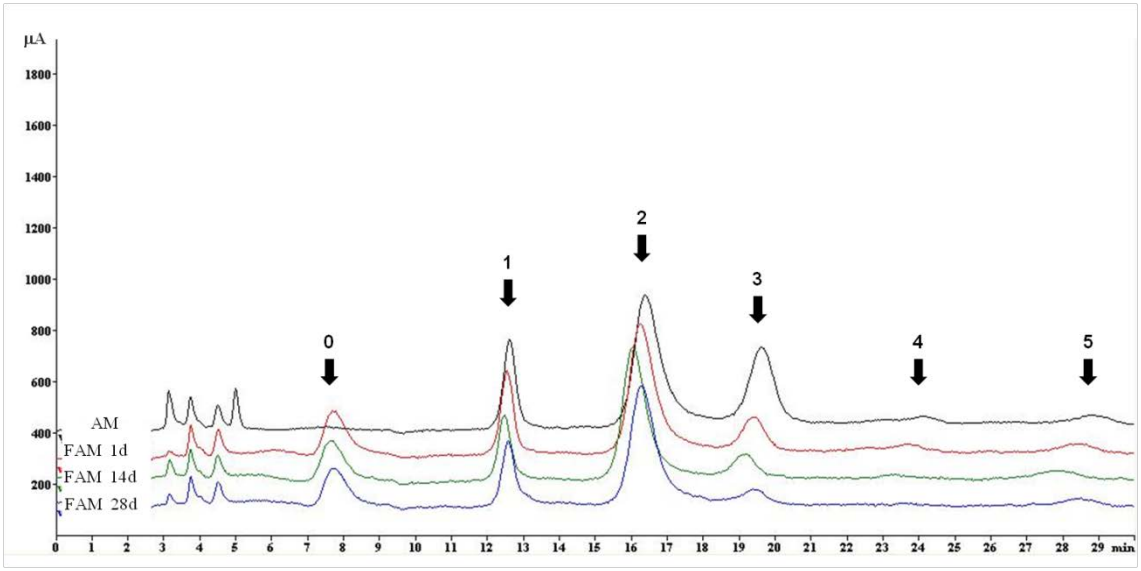

Figure 2 Chromatograms of sugar peaks obtained in HPAC-PAD assays from formulated almond *milk* (AM) and its fermented products after 1 (FAM 1d), 14 (FAM 14d) and 28 (FAM 28d) days of storage at 4 ºC. Peaks identified were mannitol (0), glucose (1), fructose (2), sucrose (3) and inulin-type fructans (4 and 5).

Table 1. Chemical composition (mean value and (standard deviation)) of peeled almond nut and the derivative *milk* used.

Composition	Peeled almond nut (g/100 g)	Almond *milk* (g/100 mL)
Moisture	3.06 (0.05)	93.4 (0.5)
Lipid	55.77 (0.29)	3.96 (0.2)
Protein	22.55 (0.12)	1.37 (0.03)
Ashes	3.86 (0.06)	0.325 (0.012)
Sugars	4.9 (0.4)	0.1285 (0.0003)
Fibre	6.82	0.58

Table 2. Experimental design and probiotic survival after 28 days of storage (log cfu/mL) for fermented almond *milk* formulations of the Central Composite Design.

	FACTORS				RESPONSE
Run order	X_1	X_2	X_3	X_4	**Y** (log cfu/mL)
1	0	0	0	0	7.683
2	0	0	0	0	7.392
3	-1	-1	-1	-1	7.601
4	-1	-1	+1	-1	7.596
5	0	0	0	+α	7.498
6	-1	-1	-1	+1	7.843
7	0	0	0	0	7.790
8	0	0	0	0	7.815
9	-1	-1	+1	+1	7.728
10	+1	-1	-1	-1	7.445
11	-α	0	0	0	7.615
12	-1	+1	-1	+1	7.656
13	+1	+1	+1	+1	7.705
14	+1	+1	-1	+1	7.653
15	0	0	0	0	7.783
16	+1	-1	-1	+1	7.388
17	0	0	0	0	7.292
18	+1	+1	-1	-1	7.278
19	0	0	0	0	7.804
20	-1	+1	+1	+1	7.503
21	+1	-1	+1	-1	7.577
22	0	0	-α	0	7.603
23	-1	+1	-1	-1	7.204
24	0	-α	0	0	7.684
25	0	+α	0	0	7.479
26	+1	+1	+1	-1	7.392
27	+1	-1	+1	+1	7.513
28	0	0	0	-α	7.225
29	0	0	0	0	7.797

30	-1	+1	+1	-1	7.204
31	+α	0	0	0	8.006

*Factors X_1, X_2, X_3, X_4 and Y stand for glucose (**-α** = 0.75, **-1** = 1.5, **0** = 2.25, **1** = 3 and **+ α** = 3.75 g/100 mL), fructose: (**-α** = 0.75, **-1** = 1.5, **0** = 2.25, **1** = 3 and **+ α** = 3.75 g/100 mL), inulin (**-α** = 1, **-1** = 2, **0** = 3, **1** = 4 and **+ α** = 5 g/100 mL), inoculum (**-α** = 4, **-1** = 5, **0** = 6, **1** = 7 and **+ α** = 8 mL/100 mL) and probiotic survivals after fermentation process (log cfu/mL).

Table 3. ANOVA results from the CCD with RSM used in the study adjusted to a second order equation.

Source	Regression coefficient/Value
Constant	4.864
Glucose	-0.304
Fructose	-0.662*
Inulin	0.437
Inoculum	1.042**
Glucose x Fructose	0.145*
Fructose x Fructose	0.076*
Fructose x Inoculum	0.099*
Inulin x Inulin	-0.076**
Inoculum x Inoculum	-0.098**
p-value of lack-of-fit	0.793
R^2	0.73
R^2-adj	0.61
Standard error of est.	0.153
Mean absolute error	0.079

R^2 = coefficient of determination **R^2-adj** = explained variance

***: statistically significant at 90% of confidence level**

****: statistically significant at 95% of confidence level**

Table 4. Concentrations of the different sugars identified in fermented almond *milk* (FAM) and mannitol yield (mean values and (s.d.)) throughout storage time. Concentrations of sugars identified in non-fermented almond *milk* (AM) are included for comparisons.

Sample	Mannitol (g/L)	Glucose (g/L)	Fructose (g/L)	Sucrose (g/L)
AM	-	11.8 (1.6)	7.2 (0.4)	1.64 (0.08)
FAM 1d	1.05 (0.06) [a]	9.1 (0.5) [a]	5.96 (0.13) [a]	0.58 (0.02) [a]
FAM 14d	0.97 (0.06) [a]	8.4 (0.4) [b]	6.01 (0.09) [a]	0.43 (0.03) [b]
FAM 28d	0.89 (0.03) [b]	8.0 (0.7) [b]	5.53 (0.13) [b]	0.243 (0.014) [c]

[a, b, c] Different letters in same column indicates significant differences between measurement times at 95% of confidence level.

Table 5. Mean values (and standard deviation) of pH, Titratable Acidity (TA) and bacterial counts of non-fermented (AM) and fermented almond *milks* (FAM) throughout storage time (d) at 4 ºC.

Sample	pH	TA (g/L of lactic acid)	*L. reuteri* (log cfu/mL)	*S. thermophilus* (log cfu/mL)
AM	6.567 (0.006)	0.39 (0.03)	-	-
FAM 1d	4.657 (0.012) [a]	1.90 (0.12) [a]	7.59 (0.04) [a]	7.54 (0.14) [a]
FAM 7d	4.63 (0.02) [b]	2.23 (0.09) [b]	7.30 (0.02) [b]	7.19 (0.14) [bc]
FAM 14d	4.657 (0.006) [a]	2.23 (0.0) [b]	7.26 (0.11) [b]	7.33 (0.10) [bd]
FAM 21d	4.633 (0.012) [b]	2.19 (0.07) [b]	7.00 (0.16) [c]	6.89 (0.21) [ce]
FAM 28d	4.650 (0.019) [ab]	2.26 (1.0) [b]	7.06 (0.06) [c]	6.57 (0.24) [e]

[a-e] Different letters in same column indicates significant differences between different times at 95% of confidence level.

Table 6. Mean particle size $D_{4,3}$ and $D_{3,2}$, ζ-Potential values and serum retention capacity (SRC) after centrifugation of fermented almond *milks* (FAM) throughout time stored at 4 ºC. Mean values (and standard deviation). Values of non-fermented *milk* (AM) are included for comparison.

Sample	$D_{4,3}$ (μm)	$D_{3,2}$ (μm)	ζ-Potential (mV)	SRC (volume % of precipitate)
AM	23 (3)	8.7 (0.3)	-16.7 (1.3)	36 (2)
FAM 1d	42.3 (1.7) [a]	16.6 (0.4) [a]	-12.8 (1.0) [a]	43 (2) [abc]
FAM 7d	56.9 (1.6) [b]	18.4 (0.9) [b]	-11.9 (1.2) [b]	42 (3) [bc]
FAM 14d	41 (3) [a]	16.7 (0.6) [a]	-13.9 (0.8) [c]	39 (0.7) [c]
FAM 21d	39.8 (1.4) [a]	14.8 (0.9) [c]	-13.0 (0.5) [a]	45 (3) [ab]
FAM 28d	39 (2) [a]	13.8 (1.3) [c]	-14.1 (1.5) [c]	48 (3) [a]

[a- d] Different letters in same column indicates significant differences between samples analysed at 95% of confidence levels.

Table 7. Mean values (and standard deviation) of yield stress (σ_y), flow behaviour index (n), consistency index (K), apparent viscosity (η)at a shear rate of 50 s^{-1} and hysteresis area in both non-fermented (AM) and fermented almond *milks* (FAM) throughout storage time (d). R^2 : non-linear correlation coefficient.

Sample	σ_y (Pa)	n	K (Pa·s^n)	R^2	$\eta_{50}\cdot10^3$ (Pa·s)	Hysteresis (ΔA (Pa/s))
AM	0.317 (0.002)	0.77 (0.07)	0.0239 (0.0014)	1	9.3 (1.4)	108 (24)
FAM 1d	0.300 (0.016)	0.78 (0.05)	0.0247 (0.0011)	0.999	10.04 (1.15)	144 (17)
FAM 7d	0.314 (0.010)	0.769 (0.013)	0.0269 (0.0005)	1	10.9 (0.5)	170 (10)
FAM 14d	0.30 (0.03)	0.749 (0.014)	0.0315 (0.0007)	0.999	11.8 (0.7)	155 (27)
FAM 21d	0.35 (0.02)	0.84 (0.02)	0.0185 (0.0005)	1	9.8 (0.5)	147 (12)
FAM 28d	0.328 (0.014)	0.77 (0.06)	0.0291 (0.0018)	1	11.5 (1.8)	183 (28)