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Title: Hazelnut *milk* fermentation using probiotic *Lactobacillus rhamnosus* GG and inulin

Running title: Development of a new synbiotic hazelnut *milk*

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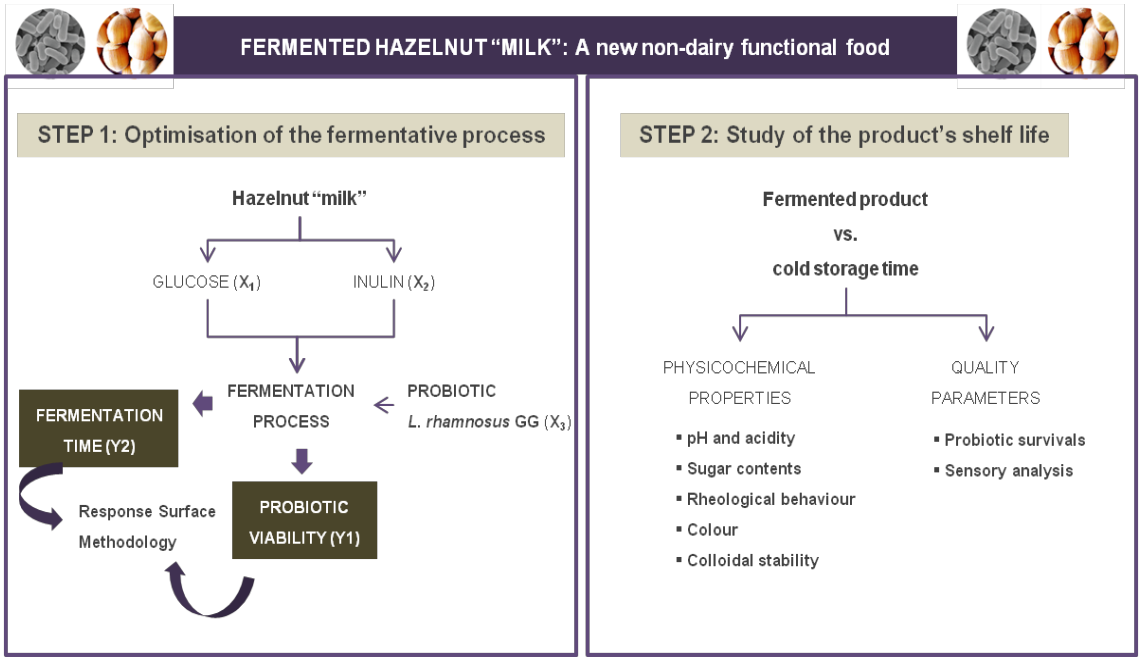
ABSTRACT

Following the consumer demand of healthy vegetable products due to their interesting nutritional profiles and potential functionalities, the fermentation process of hazelnut *milk* with *L. rhamnosus* GG and *S. thermophilus* was studied. The effect of different factors (glucose, inulin and inoculum contents) was analysed to ensure sufficient probiotic survivals in a minimum time. The shelf life of the optimised product was characterised in terms of its main physicochemical and quality parameters (probiotic survivals and sensory analysis). Results showed that the defined formulation allowed high probiotic survivals (≈10^8cfu/mL) throughout cold storage and >60% survived to the *in vitro* digestion process (≈10^5cfu/mL). *L. rhamnosus* GG was no able to degrade inulin, which remained to exert health benefits in the host. The product was highly appreciated by the sensory panel during its shelf life despite the formation of a weak gel, which presented syneresis at the last storage time.

Key words: Hazelnut *milk*, probiotic, prebiotic, response surface methodology, fermentation.

GRAPHICAL ABSTRACT



1. INTRODUCTION

The use of probiotics and prebiotics, both defined as elements that exert health benefits on the host (Ferreira *et al*., 2011), in food product development has recently been the aim of numerous scientific studies in which therapeutic effectiveness was demonstrated (Saad *et al.,* 2013). Among the nutritional health benefits, the reduction of hypercholesterolemia, the alleviation of constipation, protection against diarrhoea or some chronic diseases (i.e. inflammatory and irritable bowel diseases) and the prevention of food allergies can be found (Buddington, 2009).

Products containing probiotic microorganisms have been commonly produced by using animal milk, drinkable yoghurt being the best known. Nonetheless, new food matrices have been investigated, such as meat, baby food, ice-creams, juices and cereals (Granato *et al.,* 2010). In this sense, the so-called vegetable *milks* would have huge market potential due to the growing awareness of cow milk allergy and/or intolerance and the demand of health-promoting non-dairy products. Moreover, some of these vegetable *milks* contain prebiotics or can be easily added (i.e. inulin), which also provides the fermentation process with technological benefits, such as a viscosity increase in the food matrices, and might have a synergic effect on probiotic survival during processing and storage (de Souza-Oliveira *et al.,* 2009).

The most noteworthy of the vegetable *milks* available on the market are the ones derived from nuts, such as hazelnut *milk*. Nuts provide good sources of phytochemicals, dietary fibres and carbohydrates with low glycemic index (suitable for diabetics) (Bradley *et al*., 2011; Lovejoy, 2005). Moreover, the hazelnut's lipid profile, mainly based on oleic acid, together with the high content in vitamin E (potential antioxidant) are seen to be effective at reducing cholesterol and, thus, the risk of suffering from cardiovascular diseases (Tey *et al.,* 2011b). Besides the nutrient benefits, hazelnut is rich in taste active compounds (aminoacids, organic acids, among others), which makes this nut well accepted and widely consumed (Tey *et al*., 2011a).

In spite of the potential represented by developing new probiotic products with added nutritional value, there is little information about the criteria for fermentation and probiotic survival in non-dairy matrices (Kedia *et al.*, 2007), which represents a challenge. Shah (2007) reported the importance of the new formulation as a means of maintaining the activity and viability of the probiotic for extended periods of time.

The aim of this study is to analyse the fermentative process of hazelnut *milk* with the use of *L. rhamnosus* ATCC 53103 (usually known as GG), which is a well-documented probiotic strain (Doron *et*

66 *al.*, 2005), combined with *S. thermophilus* CECT 986. To this end, the effect of different factors (glucose,
67 inulin and inoculum content) on both the fermentation process and the probiotic survival in the final product
68 was analysed to ensure the development of a new functional food in the minimum processing time. The
69 most adequate fermented formulation would then be characterised as to its main physicochemical properties
70 and quality parameters, as well as the product shelf life.
71
72 **2. MATERIALS AND METHODS**
73
74 **2.1 Preparation of hazelnut *milk***
75 Hazelnut *milk* was produced by soaking and grinding hazelnuts (*Corylus avellana L.* cv. comuna),
76 supplied by Frutos Secos 3G S.L. (Valencia, Spain). The extraction was carried out using Sojamatic 1.5
77 (Sojamatic®; Barcelona, Spain) with a nut:water ratio of 8:100. The manufacturing process took 30 minutes
78 at room temperature. The milky liquid obtained was homogenised at 33 MPa (15M-8TA-SMD model,
79 Manton Gaulin, UK) and then pasteurised at 85 ºC-30 min.
80 To promote the colloidal stability of the *milk*, 0.05 g/100 mL of xanthan gum (ROKOgel, Asturias,
81 Spain), was added as thickener agent prior to the heat treatment. The compounds pre-selected as factors,
82 glucose (Sosa Ingredients S.L., Barcelona, Spain) and inulin (Beneo-Orafti, Tienen, Belgium), were also
83 added prior to the heat treatment.
84
85 **2.2 Preparation of fermented products**
86
87 **2.2.1 Inoculum preparation**
88 *Lactobacillus rhamnosus* ATCC 53103 (from now on GG) (LGC Standards S.L.U., Barcelona, Spain)
89 and *Streptococcus thermophilus* CECT 986 (from now on T) (CECT, Valencia, Spain) were activated from
90 their frozen forms (stored in 40 g/100 mL glycerol at -80 ºC), by transferring each one to its selective broth
91 until optimal bacterial growth is ensured. The selective broths were MRS (Scharlab; Barcelona, Spain) for
92 GG and M17 (DifcoTM; New Jersey; USA) for T. The incubation conditions were 37 ºC/24h/anaerobically
93 for GG, in which anaerobiosis was created by using anaerobic jars and a CO_2-generator system
94 (AnareroGen™; Oxoid Ltd, Basingstoke, England), and 42 ºC/24h/aerobically for T.

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, the 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)/mL, by measuring the turbidity (absorbance at 600 nm) by means of a spectrophotometer (Helios Zeta UV-vis, Thermo Scientific, USA).

2.2.2 Experimental design for the fermentation process.

Amounts of glucose, inulin and starter inoculum added to the *milk* were selected as factors (3 independent variables) to obtain fermented hazelnut *milks*. Central Composite Design (CCD) with randomised Response Surface methodology was used to analyse the effect of the different factor combinations on the fermentation processing time and on the survival of GG after 28 storage days at 4 ºC. The fermentation process was optimised in such a way that, even after the shortest fermentation time, minimum recommended amounts of probiotic were ensured at the end of 28 storage days. A statistical analysis of the data was carried out by using Statgraphics® Centurion XVI with an orthogonal 2^3 + star, which analysed the effects of the 3 factors in 18 runs. Levels of inulin, glucose and inoculum were 2 to 4 g/100 mL, 1.5 to 3 g/100 mL and 5 to 7 mL/100 mL, respectively. These parameters were chosen taking previous studies of fermentation with probiotics into account (Angelov *et al.*, 2006; Brennan and Tudorica, 2008). The response variables were the time (h) needed to develop the fermented product and the probiotic survival (log cfu/mL) after 28 storage days at 4 ºC.

The fermentation process in the 18 runs was carried out by adding the corresponding amount of starter culture (prepared by mixing GG:T buffer suspensions in a 1:1 volume ratio) to the formulated and pasteurised hazelnut *milks* and then incubating them at 40 ºC (optimal growth temperature of the mixed culture). When the pH of samples reached ≈4.6 the process was stopped by cooling the samples to 4 ºC.

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

2.3 Product characterisation

Both raw hazelnut *milk* and optimal fermented product stored for different times were characterised as to their content in different sugars, pH, acidity, rheological behaviour and colour. In hazelnut *milk*, the

chemical composition of major components (dry matter, protein, lipid, sugars and ashes) was obtained. Moreover, the fermented product was analysed throughout the storage time (0, 1, 7, 14, 21 and 28 days) at 4 ºC in terms of probiotic survival before and after having submitted the samples to a simulated gastrointestinal digestion (SGID), colloidal stability and sensory attributes. All the analyses were done in triplicate.

2.3.1 Chemical analyses

AOAC official methods of analysis were used to determine moisture (AOAC 16.006), total nitrogen (AOAC 958.48) and fat contents (AOAC 945.16) (Horwitz, 2000). Ashes were obtained following the protocol reported by Matissek *et al.* (1998).

Sugar profiles were analysed and the different sugars were quantified using a high-performance anion-exchange chromatograph (Metrohm 838-IC 861) with pulsed amperometric detector (Bioscan 817) (Metrohm® Ltd., Herisau, Switzerland). Prior to the analysis, samples were diluted 1:100 with nanopure water. Sample proteins were removed by precipitation with glacial acetic acid and the pH was then reconstituted at the initial values. Before injecting samples into the equipment, they were filtered through nylon membranes (0.45 μm). Metrosep CARB guard (5 x 4.0 mm) and CARB 1 analysis (250x4.6 mm) columns (Metrohm®) were used. 20 μL of sample was injected and eluted (1 mL/min) with 0.1 mol/L NaOH at 32 ºC. 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 ICNet 2.3 (Metrohm®) was used for data collection and processing. The concentration of each sugar was determined from their respective calibration curves, obtained from standard solutions of glucose, fructose and sucrose (Sigma-Adrich®, St. Louis, MO, USA), which were obtained in triplicate.

2.3.2 pH and titratable acidity (TA).

Measurements of pH were carried out at 25 ºC using a pH-meter (GLP 21+, Crison Instruments S.A., Spain). AOAC standard method was used to determine TA of samples (AOAC 947.05), expressing results as grams of lactic acid per 100 mL (Horwitz, 2000).

2.3.3 Probiotic survival before and after simulated gastro-intestinal digestion

Fermented hazelnut *milk* samples were submitted to a simulated gastro-intestinal digestion (SGID) and the viability of probiotic bacteria was then developed by carrying out bacterial counts of both non-

digested and digested samples. 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).

The pour plate technique was employed to quantify GG survivals (IDF standards, 1997). The selective medium was acidified MRS agar (Scharlab; Barcelona, Spain) and incubation conditions were 37 ºC for 48 h in anaerobic atmospheres.

2.3.4 Rheological behaviour

The rheological behaviour was characterised in a rotational rheometer (HAAKE Rheostress1, Thermo Electric Corporation; Germany) with a sensor system of coaxial cylinder (Z34DIN Ti). The shear stress (σ) was measured as a function of shear rate ($\dot{\gamma}$) from 0 to 512 s^{-1}, taking 5 minutes to reach the maximum shear rate and another 5 to fall. The Herschel-Bulkey model (Eq. 1) was fitted to the experimental points 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}, since the shear rates generated in mouth when food is being chewed and swallowed are between 10 and 100 s^{-1} (McClements, 2004).

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

2.3.5 Colloidal stability of fermented hazelnut *milk*

The colloidal stability of the obtained fermented product was determined by means of a phase separation analysis throughout the storage time (1, 7, 14, 21 and 28 days) at 4 ºC. To this end, 15 g of fermented hazelnut *milk* was poured into glass tubes of 16 mm diameter and the height of the separate phases was quantified.

2.3.6 Colour parameters

The colour coordinates were measured in a spectrocolorimeter (CM-3600d, MINOLTA Co; Japan). A 20 mm depth cell was used. CIE L^*a^*b coordinates were obtained using illuminant D65/10º observer. The colour of hazelnut *milk* samples was characterised as to Lightness (L^*), chrome (C^*_{ab}) and hue (h^*_{ab}), as defined in equations (2) and (3). The colour differences (ΔE) between fermented and non-fermented samples were also calculated by using equation (4).

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

$$h^*_{ab} = \arctan\left(b^*/a^*\right) \quad (3)$$

$$\Delta E = \sqrt{\left(\Delta L^*\right)^2 + \left(\Delta a^*\right)^2 + \left(\Delta b^*\right)^2} \quad (5)$$

183

184 **2.3.7 Sensory analysis**

185 A 16 member trained panel evaluated fermented hazelnut products after different storage times (1, 14,
186 and 28 days) at 4 ºC. Members were selected on the basis of their availability, lack of food allergies and
187 their threshold to basic flavours. Training was based on the method described by Mårtensson *et al*. (2001)
188 with some modifications. They were trained to score attributes of sweetness, acidity, hazelnut flavour,
189 consistency and mouthfeel and overall acceptability using interval scales that varied from 1 (slightly) to 5
190 (extremely).

191 Reference samples to set the interval scales for panel training were the following: for the acidity, 1
192 and 2 g/100 mL of sucrose were added to commercial milk yoghurt, corresponding to 3 and 1 on the scale,
193 respectively, and with 0.2 g/100 mL of citric acid corresponding to 5. Commercial milk yoghurt with added
194 sucrose at 2, 5 and 14 g/100 mL levels was used for the sweetness evaluation, corresponding to 1, 3 and 5
195 on the scale, respectively. For consistency and mouthfeel, drinkable yoghurt, commercial soy dessert and
196 Danone original® yoghurt were used, corresponding to 1, 3 and 5 respectively on the scale. For the hazelnut
197 flavour, the reference was the hazelnut *milk* used in the study, which corresponded to 5 on the scale.

198 Each panelist tested 3 samples (cold stored for 1, 14 and 28 days) containing 6 g/100 mL of sucrose,
199 to quantify the attributes in which each one was trained. The samples were randomly presented with a three-
200 digits code. The evaluation was conducted in a normalised tasting room at room temperature.

201

202 **2.4 Statistical Analysis**

203 The results were analysed by means of a multifactor analysis of variance using
204 Statgraphics® Centurion XVI. Multiple comparisons were performed through 95% LSD intervals.

205

206 **3. RESULTS AND DISCUSSION**

207

208 **3.1 Effect of factors on fermentation process**

Table 1 shows the experimental responses for the fermentation time (Y_1) and GG counts (Y_2) obtained for each formulation of the CCD. All the formulations were suitable as a means of developing a probiotic hazelnut fermented *milk*, since the probiotic survival was over 7 log cfu/mL in every case, which is the recommended minimum amount in order to ensure health effects (Sanz and Dalmau, 2008). Moreover, the duration of the fermentation process was also appropriate, since standard cow milk fermentations are generally developed in 3-4 h (Alais, 1998). Other authors observed longer fermentation times (≈6 h) in dairy yoghurt processing when GG and standard yoghurt bacteria were used as starters (Hekmat *et al.*, 2009).

Prior to the modelling, the experimental data (Y_1 and Y_2) were statistically analysed in order to remove possible outliers and to assess the likelihood that the given data sets conform to a normal distribution (ND). As Granato *et al.* (2014) stated, if the assumption of normality is not confirmed, the interpretation and inference from any statistical test may not be reliable or valid. To this aim, the normality of the experimental data was assessed through the determination of both standardized kurtosis and skewness parameters. Results were within the range of -2 to +2 (in Y_1 and Y_2), thus indicating that both data follow a normal distribution (Pérez, 2001).

Experimental fermentation time responses (Y_1) 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^2 adj), the term was included in the model. Due to the poor fitting of the probiotic counts data, variable Y_2 was not modelled.

Before going further with the evaluation of the model obtained, the basic assumptions of the residuals were checked, as they are supposed to be distributed normally and independently with mean zero and a constant variance (principles of normality and homoscedasticity). With regards to the normality, residuals were assessed by using Saphiro-Wilk (S/W) test and reinforced with the Kolmogorov-Smirnov (K/S) test, since they are considered powerful and accurate methodologies to check this principle (Granato *et al.*, 2014). The p-value of both tests were not statistically significant (0.747 for S/W and 0.884 for K/S), thus meaning that the hypothesis of normality is fulfilled. With regards to the homocedasticity, the square of residuals was submitted to multifactorial analysis of variance and results allowed to discard any case of heterocedasticity (data not shown).

Once the statistical assumptions were confirmed, the next step was to evaluate the goodness the fitted model. This step was performed throughout the analysis of variance, mainly based on the F-test, which

provides 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). The model obtained appeared to be adequate for predicting fermentation time response (Y_1), since the p-value of the lack-of-fit parameter was greater than 0.05. Table 2 summarises the fitted results and the regression coefficients of the fitted model are also included. In addition, the Durbin-Watson statistic was not significant ($p > 0.05$) (Table 2), meaning that there is no indication of serial autocorrelation in the residuals, thus supporting the proper prediction of the model.

As can be seen in the coefficients and F-ratios (Table 2), glucose affected the duration of the fermentation process positively, which was expected since it is a basic nutrient for GG (Corcoran *et al*., 2005). Inulin had a quite significant negative impact on the duration of this process (coefficient sign is positive and F-ratio is high). Despite being a prebiotic, inulin is also industrially used as a thickener (Franck, 2002), so it might reduce the mobility and availability of nutrients for the fermentation process. De Souza-Oliveira *et al*. (2009) also observed an increase in the duration on the fermentation of milk when it contained inulin. Inoculum addition also had a negative impact, which could be explained by considering the limiting effect of the availability of nutrients within the matrix, discussed above. Moreover, the interaction between inulin and the added starters had a synergic effect on the fermentation time, probably due to the known prebiotic property of inulin that positively affects the growth of lactobacilli (Kolida *et al.*, 2002)., The fitting coefficients of the model (R^2 and R^2 adj) were low, although it is difficult to obtain greater R^2 values because the variation of the experimental responses is very low (most fermentation times were very close to 3.5 h) (Table 1). Consequently, the model obtained can only provide rough predictions.

The health benefits of probiotic products are believed to be dependent on the bacterial viability within the matrix, recommending a survival of $\geq 10^7$ cfu/mL (Sanz and Dalmau, 2008). Furthermore, fermentation is a critical process and has to be done as quickly as possible to prevent non-desirable bacteria. Hence, despite the lack of fit in probiotic responses, experimental GG survivals (Y_2 data) together with the quantified fermentation times (Y_1 data), were used to optimise a hazelnut formulation. The optimal values of the factors (% inoculum, glucose, fructose content) were obtained by minimising the fermentation time (Y_1) and maintaining GG counts (Y_2) at 8 log cfu/mL for 28 days, via the least squares method, which minimizes the square's sum of the residuals (Statgraphics Centurion XV). This optimum corresponded to the addition of 3 g/100 mL of glucose, 2.75 g/100 mL of inulin and 6 mL/100 mL of mixed culture inoculum

to the hazelnut *milk*. With this formulation, the fermentation took 3.6 h and, after being cold stored for 28 days, GG survival in the fermented product was 8 log cfu/mL.

The obtained optimal formulation was submitted to fermentation and the resulting product was analysed in order to validate the model prediction and to characterise several relevant product properties. The results showed that the fermented product reached a pH value of 4.803 ± 0.015 in 3.5 h at 40 ºC with a GG survival of 8.350 ± 0.015 log cfu/mL after 28 storage days at 4 ºC, as predicted by the model.

3.2 Chemical composition of the hazelnut *milk*

The chemical composition of pure hazelnut *milk* (without added factors), expressed in average weight percentage was 5.3 ± 0.4 of dry matter, 4.021 ± 0.004 of fats, 0.65 ± 0.05 of proteins, 0.20 ± 0.04 of ashes, and 0.206 ± 0.019 of sugars of which sucrose was the only sugar present, as can be seen in Figure 1. As far as the nut:water ratio of the *milk* is concerned, these compositional values were almost in the same proportion as in the raw nuts (Köksal *et al.*, 2006).

Figure 1 shows the sugar profiles of both pure and optimal formulated hazelnut *milk*. Besides the expected glucose and sucrose peaks (1 and 3), 2 other peaks appeared in the formulated *milk*, which came from little degradations of the added inulin probably caused by either the pasteurisation treatment or impurities from the inulin extraction process (Böhm *et al*., 2005). One of the new peaks (peak 2) could be identified as fructose, and the other (peak 4) was classified as Fructan, which is a term that includes both inulin and its derivatives (Roberfroid, 2005). In addition, higher amounts of sucrose in formulated hazelnut *milk* were identified, which came from the added inulin. Sugar contents in formulated *milk* were 3.05 ± 0.25 g/100 mL of glucose, 0.030 ± 0.003 of fructose and 0.37 ± 0.03 of sucrose.

3.3 Properties of the fermented hazelnut product

3.3.1 Probiotic counts and acid production.

Average values of pH and Titratable Acidity (TA) in fermented hazelnut *milk* vs. storage time are summarised in Table 3. This table also includes GG count data throughout storage time before and after having the samples submitted to *in vitro* digestions. *S. thermophilus* counts were not obtained due to the inability of these bacteria to survive through the gut; hence, they do not play a role in the human gut (del Campo, 2005).

As it was expected, the physicochemical properties of hazelnut *milk* were modified by the fermentation process (Table 3). Once fermentation finished, the acidity values were around 0.1 g/100 mL of lactic acid, which were much lower than in standard yoghurt (0.8-1 g/100 mL) (Tamime and Robinson, 2000). This means that hazelnut *milk* has a lower buffering capacity than cow milk.

However, until the day 14 of analysis both pH and TA were gradually modified ($p < 0.05$) to levels that might not be desirable for consumers. These changes were expected due to the high viability of GG over storage time, which might still be generating acidic compounds. From 14 days of storage on, both physicochemical parameters were stabilised ($p < 0.05$) coherent with the GG survival trend (no growth was observed from 14 storage days onwards).

As regards the probiotic survivals, food substrate is considered as one of the major factors in regulating colonisation, since it might help to buffer the bacteria through the stomach or might contain other functional ingredients (such as inulin) that could interact with them (Ranadheera *et al.*, 2010). As can be seen from GG counts (Table 3), the hazelnut *milk* formula is an appropriate matrix with which to develop functional non-dairy products, since the probiotic bacteria still grew once fermentation was finished ($p < 0.05$). The low storage temperature slowed the GG growth down over time, which even stopped after 21 storage days. Nevertheless, GG was maintained in the product above the levels recommended ($\geq 10^7$ cfu/mL) in order to ensure health benefits until the last control day. The fact that the GG in the fermented product remained highly concentrated might be due to the prebiotic effect of the added inulin. Indeed, Donkor *et al.* (2007) also observed high probiotic viability in yoghurt through cold storage time when inulin was added.

The success of a probiotic, however, is dependent on the ability to survive within the gastrointestinal tract and to interact with other components in a manner that fosters improved health (Buddington, 2009). Hence, fermented products stored at different times were also submitted to a SGID and GG survivals are shown in Table 3. In all the samples tested, more than half (60-65%) of the initial bacteria survived to SGID, thus leading to a bacteria counts of around 5 log cfu/mL after SGID. Usually, lower probiotic survivals (around 20-40%) have been reported for fermented cow milk products (Bezkorovainy *et al.*, 2001). Generally, GG bacteria are seen to be highly resistant to acid and bile and have high adhesion ability in *in vitro* enterocytes (Hekmat *et al.*, 2009), although survival in acidic conditions might occur as long as easily metabolisable sugars were present within the matrix (Corcoran *et al*., 2005).

The results obtained point to the fact that GG might be able to colonise the human colon and, thus, exert health benefits, such as competing with non-desirable microbiota to obtain nutrients; this last

assumption is believed to be one of the probiotics' mechanisms of action (Saad *et al.*, 2013). Nevertheless, this should be reinforced with *in vitro* and *in vivo* assays.

3.3.2 Sugar contents

Knowing the sugars profiles in fermented products can provide interesting information about the fermentation process and bacterial activity during the product shelf life. Table 4 summarises the concentrations of the different sugars identified in both non-fermented and fermented hazelnut *milks* throughout storage time (0, 1, 7, 14, 21 and 21 days).

As can be seen, the glucose content dropped significantly after the fermentation process and completely disappeared after two storage weeks ($p < 0.05$). This was expected, since GG was viable throughout the 28 storage days (Table 3) and glucose is the basic nutrient of this bacterium (Corcoran *et al.*, 2005). The small amount of fructose present in non-fermented *milk* (peak 2) was also consumed. Moreover, the initial sucrose present decreased after the fermentation process ($p < 0.05$) (Table 4), although its content in fermented samples was not affected by the storage time ($p > 0.05$). GG is seen to be incapable of hydrolysing sucrose (Corcoran *et al*., 2005) but S. *thermophilus,* also used as starter inoculum, is able to use sucrose as nutrient (Tamime and Robinson, 2000).

A qualitative analysis of chromatograms shows that area of fructan (peak 4) was not modified by the fermentation process ($p < 0.05$), but it slightly increased from 7 storage days on, especially on the last day of analysis ($p < 0.05$) (Table 4). This trend suggested the starters had sufficient energy sources in the form of mono- or disaccharides and inulin was not consumed. Nevertheless, Corcoran *et al.* (2005) observed that GG was able to grow in a medium until glucose levels reached 0.018 g/100 mL. Therefore, not having sufficient glucose in hazelnut *milk* after 7 storage days, GG might start to hydrolyse this prebiotic so as to obtain the energy required to grow, thus generating higher amounts of inulin derivatives. This assumption was consistent with the high survivals of GG observed until the last day controlled (Table 3). Therefore, the hazelnut *milk* formulation is highly suitable for developing new non-dairy probiotic products.

To sum up, both the GG survivals and the sugar content results have reinforced the belief that inulin can enhance probiotic survivals (Frank, 2002, Kolida *et al.*, 2002).

3.3.3 Physical properties

Rheological behaviour plays a key role in the perceptions of a product's texture and sensory features. Both fermented and non-fermented hazelnut *milks* were shear thinning ($n < 1$) and time-dependent (hysteresis was observed), as are a large number of hydrocolloidal dispersions (Marcotte *et al*., 2001). Table 5 summarises the rheological parameters obtained from fitting Eq. 1 by means of a non-linear procedure, as well the thixotropic areas. The apparent viscosities of samples at a shear rate of 50 s^{-1} were also shown.

As can be seen, the fermentation process modified the rheological behaviour of hazelnut *milk*, although apparent viscosity was not significantly affected ($p < 0.05$). Nevertheless, the storage time did significantly increase the apparent viscosity and both the consistency index (K) and the flow behaviour index (n) changed. The maximum viscosity was reached on the 21st storage day ($p < 0.05$).

All the samples showed yield stress and a hysteresis area which was, in part, attributed to the gelling effect of adding xanthan gum as a stabiliser, since inulin solutions are not seen to provide this effect (Arcia *et al*., 2010). The fermentation process greatly increased the yield stress and hysteresis area ($p < 0.05$), which indicates that flocculation occurs in the system mainly due to a change in the pH and the effect of the solvent on the macromolecules and particles present. The rheological properties of xanthan gum are dependent on the temperature, salt concentrations and pH (García-Ochoa *et al.*, 2000). From the obtained rheological parameters, the progress of the degree of flocculation can be deduced. Data from 28 days onwards did not follow the above mentioned trend due to the significant phase separation in the system, discussed above, and shown in Figure 2, which is coherent with the gel matrix contraction and its subsequent loss of serum retention capacity.

Figure 2 shows pictures of fermented hazelnut *milk* stored for 1 (2.A), 14 (2.B) and 28 (2.C) days at 4 ºC. As can be seen, the fermentation process provoked serum separation in hazelnut *milk* due to the physicochemical changes discussed above. This phenomenon was evaluated through the percentage of serum separation, observed in Figure 2. After 1 storage day, 11 ± 2% of serum separation was observed which only significantly increased after 21 storage days ($p < 0.05$). After 28 storage days, 25.1 ± 0.9% serum separation was observed.

Previous studies have also shown stability problems in vegetable *milks* mainly due to the low content in proteins, which act as emulsifiers in water-oil emulsions (Walstra *et al.*, 1983). These problems are usually overcome by adding hydrocolloids, such as xanthan gum, which in this case lead to a gel formation by increasing the hydrogen bonds when the solvent properties of the aqueous phase change due to a

386 modification of the pH (Song *et al*., 2006). The gel structure is dynamic, increasing the bond formation
387 over time and giving rise the phenomenon of syneresis.
388 With regards to the colour analysis, the fermentation process slightly decreased the hue (from 93.8 ±
389 0.5 to 91.7± 0.2) and increased both lightness (from 84.98 ± 0.19 to 85.59·±0.14) and chrome (from 8.37
390 ± 0.05 to 8.78 ± 0.03) ($p < 0.05$), being these changes very mild. Few differences were also observed
391 between the colour parameters of the fermented samples cold stored for different times; these ranged over
392 an interval of less than one unit. The total colour difference between fermented and non-fermented hazelnut
393 *milks* (ΔE) was low and undetectable by the human eye since, according to Francis (1983), values lower
394 than 3 units cannot be easily detected.
395
396 **3.3.4 Sensory properties**
397 Figure 3 shows the scores of the attributes of appearance, sweetness, acidity, consistency, hazelnut
398 flavour and overall acceptability in the three fermented hazelnut samples analysed by the panel (1, 14 and
399 28 days stored at 4 ºC); statistical differences between storage times were also included.
400 Before tasting the three samples, the panelists evaluated the fermented hazelnut *milk* as having a very
401 good appearance with the exception of the sample stored for 28 days ($p < 0.05$). As these samples were
402 presented in transparent glasses, the panelists were able to notice the sample syneresis and serum separation
403 at the bottom; this separation was negatively evaluated.
404 With regards to sweetness, in spite of the fact that all the samples were equally sweetened with
405 sucrose, the panelists detected differences between samples stored for 1 day and the other ones ($p < 0.05$).
406 This appreciation could be due to the impact of acidity on this attribute's evaluation: the higher the acidity
407 level, the lower the sweetness perception (Ott *et al.*, 2000). The panelists did not appreciate differences
408 between samples stored for 14 and 28 days ($p < 0.05$), which is coherent with both the pH and TA values
409 (Table 3).
410 The consistency of the fermented product was quantified as low, which was expected, considering the
411 similarity of the tested product with the well-known drinkable yoghurts. The members of the panel detected
412 lower consistency in samples stored for 28 days ($p < 0.05$), probably due to the partial destabilisation of the
413 gel structure in the fermented product and phase separation, discussed above. This lower consistency is
414 negatively appreciated in terms of consumer acceptance, since they prefer drinkable yoghurts with a high
415 level of viscosity (Allgeyer *et al*., 2010).

Although non-fermented hazelnut *milk* flavour was well accepted (data not shown), the fermentation process modified this attribute ($p< 0.05$), owing to the synthesis of aromatic compounds brought about by starter bacteria. The panelists considered samples stored for 14 and 28 days to have less original hazelnut flavour, finding no differences between them ($p< 0.05$).

To sum up, the members of the panel accepted the fermented hazelnut *milk* (scoring the products 3 or over) but the early fermented product was better accepted. Moreover, the overall acceptability of the product after being stored for 28 days at 4 ºC is remarkable, which leads to the conclusion, in terms of sensory attributes, the product shelf life might be standardised as it is for conventional yoghurts.

4. CONCLUSIONS

Hazelnut *milk* containing 3 g/100 mL of glucose, 2.75 g/100 mL of inulin and 6 mL/100 mL of mixed culture inoculum allowed us to obtain a fermented product in 3.5 hours, which ensures high probiotic survivals above the level recommended as being the minimum in order to ensure health benefits and, thus, it may be considered as a functional food. The metabolic activity of the starters was maintained both throughout the 28 storage days and also after a simulated digestion in which the GG viability was only reduced by around 35%. Moreover, the non-degraded inulin (prebiotic) present would provide an added value, obtaining thus a non-dairy fermented product with synbiotic features. Although sensory evaluation showed a greater preference for samples stored for shorter times, the panel members also showed a good acceptability of the product after 28 storage days.

Hence, owing to the positive results in both physicochemical and microbiological analyses, as well as the sensory attribute evaluations, the obtained product might be considered a new functional food with potential health benefits, suitable for many different targeted groups, such as vegetarians, the lactose-intolerant or people allergic to animal proteins.

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Table 1. Fermentation time (Y_1) and total counts of *L. rhamnosus* GG (Y_2) after 28 storage days at 4 ºC, obtained in the different fermented products corresponding to the experimental design, as a function of the factors' levels.

	Factors (X)			Experimental responses (Y)	
Run order	**X_1**	**X_2**	**X_3**	**Y_1**	**Y_2**
1	0	0	-α	3	8.12
2	-1	-1	+1	4.5	8.52
3	+1	-1	+1	5	8.48
4	+1	-1	-1	3.5	8.35
5	0	- α	0	3.5	8.30
6	+ α	0	0	3.5	7.33
7	-1	+1	+1	3	8.42
8	0	0	0	3	8.24
9	-1	-1	-1	4	8.39
10	0	0	+ α	3	8.22
11	+1	+1	-1	5	8.40
12	0	+ α	0	3	8.00
13	0	0	0	3.5	8.35
14	+1	+1	+1	3	8.44
15	0	0	0	3.5	8.17
16	- α	0	0	3	8.33
17	+1	+1	-1	4	8.32
18	0	0	0	3.5	8.36

*Factors X_1, X_2, X_3, Y_1 and Y_2 stand for Glucose: 1.5-3 g/100 mL, Inulin: 2-4 g/100 mL, Inoculum: 5-7 mL/100 mL, fermentation time (h) and probiotic counts (log cfu/mL), respectively

Table 2. Regression coefficients and analysis of variance for fermentation time (hours) obtained from the fitted model.

Source	Coefficient/Value	F-Ratio	p-value
Constant	-1.608	-	-
Glucose	-0.33	4	0.139
Inulin	3.18	7.6	0.070
Inoculum	0.44	3.1	0.112
Glucose x inulin	0.17	2	0.252
Inulin x inoculum	-0.625	50	0.006
Inoculum x inoculum	0.10	2.20	0.234
Lack-of-fit	-	5.47	0.094
Standard error of est.	0.25	-	-
Mean absolute error	0.35	-	-
Durbin-Watson statistic	2.73	-	0.925

Table 3. Values (mean and (standard deviation)) of pH, Titratable Acidity (TA) and probiotic (GG) counts before and after a simulated human gastrointestinal digestion (SGID) of fermented hazelnut *milk* (FHM) throughout storage time at 4 ºC (0, 1, 7, 14, 21 and 28 days). Data of non-fermented hazelnut *milk* (HM) are included for comparisons.

Sample	pH	TA (g/100 mL of lactic acid)	GG counts before SGID (log cfu/mL)	GG counts after SGID (log cfu/mL)
HM	6.50 (0.02)	0.026 (0.003)	-	-
FHM 0 d	4.803 (0.015) [a]	0.104 (0.005) [a]	7.97 (0.05) [a]	4.91 (0.03) [a]
FHM 1 d	4.01 (0.05) [b]	0.226 (0.005) [b]	8.38 (0.03) [b]	5.58 (0.06) [b]
FHM 7 d	3.63 (0.05) [c]	0.322 (0.007) [c]	8.44 (0.06) [c]	5.48 (0.63) [bc]
FHM 14 d	4.027 (0.06) [b]	0.337 (0.007) [d]	8.46 (0.04) [c]	5.04 (0.05) [ca]
FHM 21 d	3.70 (0.07) [d]	0.337 (0.003) [d]	8.35 (0.03) [b]	4.94 (0.02) [a]
FHM 28 d	3.70 (0.05) [d]	0.338 (0.000) [d]	8.350 (0.015) [b]	4.904 (0.017) [a]

[a-d] Different letters in same column indicate significant differences between measurement times ($p < 0.05$)

Table 4. Concentrations (mean values and (standard deviation)) of the different sugars identified in fermented hazelnut *milk* (FHM) throughout storage time at 4 ºC. Sugars identified in non-fermented hazelnut *milk* (HM) are also included for comparisons. Peak areas throughout storage time of the oligosaccharide, named as fructan, are also included.

Sample	Glucose (g/100mL)	Fructose (g/100mL)	Sucrose (g/100mL)	Fructan (Area (μA·min))
HM	3.05 (0.25)	0.030 (0.003)	0.37 (0.03)	2014 (211)
FHM 0 d	1.24 (0.08) [a]	0 (0)	0.309 (0.009)	1943 (204) [a]
FHM 1 d	1.11 (0.09) [b]	0 (0)	0.330 (0.005)	1939 (179) [a]
FHM 7 d	0.08 (0.02) [c]	0 (0)	0.306 (0.011)	2433 (615) [a]
FHM 14 d	0 (0) [c]	0 (0)	0.292 (0.009)	2614 (95) [a]
FHM 21 d	0 (0) [c]	0 (0)	0.32 (0.04)	2705 (706) [a]
FHM 28 d	0 (0) [c]	0 (0)	0.34 (0.05)	3643 (817) [b]

[a, b, c] Different letters in same column indicate significant differences between measurement times (95% confidence level)

Table 5. Mean values and (standard deviation) of the consistency index (K), flow behaviour index (n) and yield stress (σy) of fermented hazelnut *milks* (FHM) throughout storage time (d). Non-linear correlation coefficient R^2 is included). Apparent viscosity (η) was calculated at a shear rate of 50 s^{-1}. Hazelnut *milk* data are included for comparisons. The hysteresis area quantified in flow curves is also presented

Sample	K ($Pa \cdot s^n$)	n	σ_y (Pa)	R^2	η_{50} ($Pa \cdot s$)	Hysteresis (ΔA (Pa/s))
HM	0.029 (0.002)	0.80 (0.00)	0.084 (0.014)	1	0.67 (0.05)	56 (19)
FHM 0 d	0.044 (0.013) [a]	0.71 (0.05) [a]	0.23 (0.04) [a]	0.995	0.69 (0.08) [a]	175 (29) [a]
FHM 1 d	0.04 (0.02) [a]	0.69 (0.06) [a]	0.239 (0.012) [a]	0.954	0.61 (0.13) [a]	200 (21) [a]
FHM 7 d	0.16 (0.0.06) [b]	0.53 (0.06) [b]	0.37 (0.18) [a]	0.998	1.2 (0. 3) [b]	369 (79) [b]
FHM 14 d	0.36 (0.08) [c]	0.42 (0.03) [c]	0.360 (0.113) [a]	0.997	1.8 (0.2) [c]	481 (72) [c]
FHM 21 d	0.50 (0.04) [d]	0.40 (0.00) [c]	0.720 (0.014) [b]	0.997	2.4 (0.2) [d]	646 (9) [d]
FHM 28 d	0.36 (0.098) [c]	0.42 (0.02)[c]	0.60 (0.00) [b]	0.996	1.8 (0.2) [c]	542 (30) [cd]

[a,b,c,d] Different letters in same column indicates significant differences between measurement times ($p < 0.05$)

FIGURE CAPTIONS

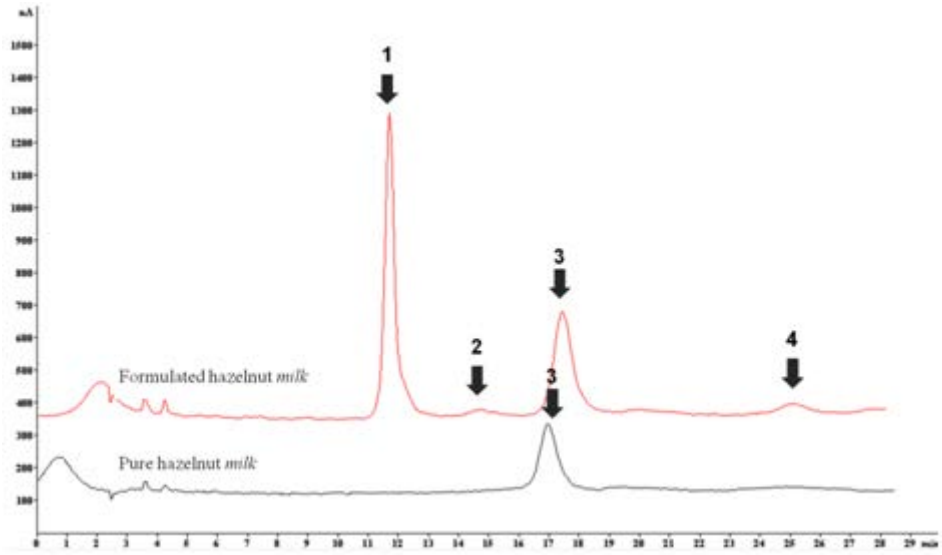

Figure 1 Chromatograms of sugar peaks obtained in HPAC-PAD assays from both pure and formulated hazelnut *milk*. Peaks identified were glucose (1), fructose (2), sucrose (3) and an oligosaccharide, residual from inulin, which was classified as Fructan (4).

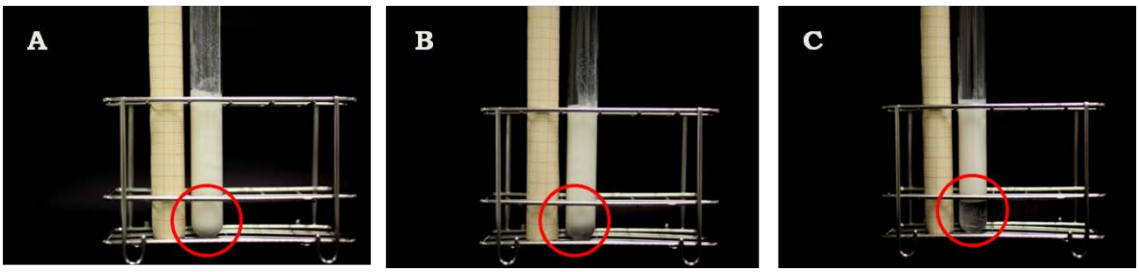

Figure 2. Pictures of fermented hazelnut *milk* stored for 1 (A), 14 (B) and 28 (C) days at 4 ºC. Circumference marks the separated serum phase.

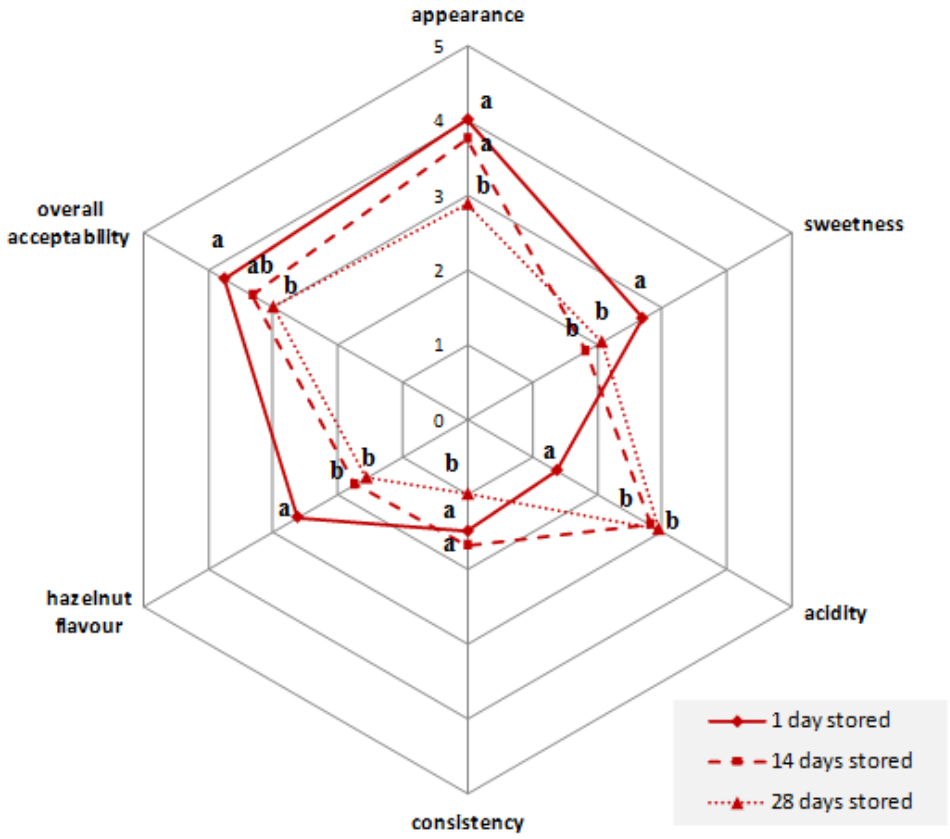

Figure 3. Panelists' scores for appearance, sweetness, acidity, consistency, hazelnut flavour and overall acceptability in the fermented hazelnut samples stored for 1, 14 and 28 days at 4 ºC

a, b Different letters in same attribute axis indicates significant differences between storage times ($p < 0.05$)