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PARA EL DESARROLLO

Desarrollo, caracterización y optimización de productos fermentados a base de licuados vegetales como alternativa a los yogures convencionales

TESIS DOCTORAL

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Per a la família Bernat i Pérez

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RESUMEN

La tesis doctoral tiene como objetivo desarrollar y caracterizar productos fermentados a partir de licuados vegetales (más conocidos como *leches* vegetales) de almendra, avellana y avena, seleccionados por su interés composicional y nutricional. Se utilizaron cepas potencialmente probióticas con el fin de obtener productos fermentados funcionales que aporten un efecto beneficioso para la salud, y que a la vez, representen una alternativa de consumo a los lácteos de origen animal.

En primer lugar, se analizaron y definieron unas condiciones de procesado de las *leches* que garantizaran una estabilidad física y seguridad microbiológica. Las *leches* procedentes de frutos secos tienen alto contenido en grasas, lo que las convierte en emulsiones con grandes problemas de estabilidad relacionados con fenómenos de separación de fases. En ese sentido, la aplicación de la tecnología emergente de las altas presiones de homogenización (utilización de presiones superiores a 100 MPa) en combinación con tratamientos térmicos mejoró notablemente la estabilidad tanto en el producto fermentado como en el licuado sin fermentar. Por otra parte, en la *leche* de avena, los β-glucanos presentes en el cereal le proporcionan una gran estabilidad tras el tratamiento térmico, gracias a la capacidad espesante y gelificante del mismo, no presentando problemas de estabilidad física. Además, las propiedades prebióticas de los β-glucanos (capacidad de estimular el crecimiento de las bacterias beneficiosas de nuestra microflora intestinal) suponen un valor añadido en el desarrollo de productos fermentados a partir de esta materia prima.

En el diseño y optimización del proceso de fermentación a partir de microorganismos probióticos, se estudió el efecto de distintos factores de crecimiento (glucosa, fructosa, inulina y cantidad de inóculo) sobre la supervivencia del probiótico en las diferentes matrices vegetales, pues se recomienda una cantidad mínima de 10^7 unidades formadoras de colonias por mL para que el producto a desarrollar pueda considerarse como funcional. Tras analizar el efecto individual e interacciones de los factores de crecimiento sobre la supervivencia de los microorganismos y/o tiempos de fermentación en las distintas matrices vegetales, se determinó una formulación óptima que hizo posible un proceso fermentativo rápido y una alta supervivencia de la bacteria probiótica. Cuando los niveles elegidos para los distintos factores de crecimiento dieron lugar a respuestas similares en cuanto a la supervivencia microbiana, se optó por buscar los niveles mínimos de dichos factores que favorecieran un menor coste productivo. El producto fermentado desarrollado se caracterizó a distintos tiempos de almacenamiento (1, 7, 14, 21 y 28 días) a 4 ºC para analizar la variación de los principales parámetros que afectan a su calidad fisicoquímica, sensorial y de supervivencia del probiótico en función del tiempo y, de esta forma, poder determinar un periodo óptimo de almacenamiento en el que el producto mantenga unas propiedades de excelencia. Los resultados mostraron que las *leches* fermentadas con los microorganismos potencialmente probióticos seleccionados permitieron mantener una buena viabilidad, estabilidad física y apreciación sensorial durante el almacenamiento en refrigeración, estimándose una vida útil similar a la de los yogures convencionales.

Dentro del amplio abanico de propiedades saludables que proporcionan los probióticos se encuentra la capacidad de influir positivamente en el sistema inmune, evitando la aparición de reacciones alérgicas, entre otros efectos. La almendra es un fruto muy consumido pero contiene alérgenos, por lo que el probiótico podría ser una buena herramienta para reducirlos. Por ello se realizaron estudios *in vitro* de las propiedades inflamatorias de los fermentados de almendra con distintas bacterias potencialmente probióticas. Estos estudios mostraron efectos positivos en algunas de las cepas utilizadas, las cuales fueron capaces de reducir la respuesta alérgica inicial asociada al producto sin fermentar. Los resultados obtenidos abren las puertas a continuar con la investigación y realizar más estudios tales como estudios *in vitro* e *in vivo* en grupos de población sensibles.

ABSTRACT

The aim of this doctoral thesis was the development and characterisation of fermented vegetable beverages (most known as vegetable *milks*) derived from almond, hazelnut and oat, which were selected owing to their compositional and nutritional values. Potentially probiotic strains were used in order to obtain functional fermented products, not only able to exert health benefits, but also as an alternative to dairy based products.

Firstly, the processing conditions to ensure the physical stability and microbiological safety of *milks* were analysed. The *milks* from tree nuts have a high fat content, which causes physical stability problems related to the phase separation phenomena. The application of high homogenisation pressures (around 100 MPa) together with heat treatments markedly improved the stability of both fermented and non-fermented nut *milks*. On the other side, in oat *milk*, the β-glucans present provides a great physical stability after the heat treatment due to its gelling and thickening capacity, not showing thus physical stability problems. Furthermore, the prebiotic properties of β-glucans (the ability to stimulate the growth of beneficial bacteria in our gut microflora) give the finished product significant added value.

For the design and optimisation of the fermented processing by using probiotic bacteria, the effect of several growth factors (glucose, fructose, inulin and inoculum additions) on the probiotic survivals within the vegetable matrices was studied, since a minimum concentration of 10^7

colonies forming units per mL is recommended to consider the product as a functional food. After this study, an optimal *milk* formulation was determined, where a fast fermentation time was attained and high probiotic survivals were ensured. When similar probiotic survival responses were obtained, minimum levels of each growth factor were chosen in order to favour a low-cost production. Afterwards, the fermented products were characterised at different storage times (1, 7, 14, 21 and 28 days) at 4 ºC to analyse how storage time affect their main physicochemical and sensory properties and probiotic survivals; hence, an optimal period of storage time was defined. Results showed that the *milks* fermented with the selected potentially probiotic microorganisms were able to maintain a high viability, physical stability and sensory appreciation throughout the cold storage time, being the shelf life similar to that of standard yoghurts.

One of the healthy properties that probiotics can provide is the ability to positively influence the immune system, thus preventing the occurrence of allergic reactions, among other effects. Almond is a nut highly consumed but it contains allergens; hence, probiotic bacteria might be a good tool to reduce its allergic response. Therefore, *in vitro* studies of the inflammatory properties of fermented almond *milk* with different potentially probiotic microorganisms were carried out. These studies showed positive effects in some of the strains used, which were able to decrease the initial allergic response associated to the non-fermented *milk*. These results offer new interesting expectations to continue with this research line and more *in vitro* and *in vivo* studies with sensitised populations are needed.

RESUM

La tesi doctoral té com objectiu desenvolupar i caracteritzar productes fermentats a partir de liquats vegetals (més coneguts com *llets* vegetals) d'ametlla, avellana i avena, seleccionats pel seu interès composicional i nutricional. S'utilitzaren ceps potencialment probiòtics amb la finalitat d'obtenir productes fermentats funcionals que aporten un efecte beneficiós per a la salut i, a la vegada, representen una alternativa de consum als lactis d'origen animal.

En primer lloc, s'analitzaren i es definiren unes condicions de processat de les *llets* que garantiren estabilitat física i seguretat microbiològica. Les *llets* procedents de fruits secs tenen alt contingut en greix, la qual cosa les converteix en emulsions amb grans problemes d'estabilitat relacionats amb fenòmens de separació de fases. En aquest sentit, l'aplicació de la tecnologia emergent de les altes pressions d'homogeneïtzació (ús de pressions superiors a 100 MPa) en combinació amb tractaments tèrmics millorà notablement l'estabilitat tant en el producte fermentat com en el liquat sense fermentar. D'altra banda, en la *llet* d'avena, els β-glucans presents al cereal li proporcionen una gran estabilitat després del tractament tèrmic, gràcies a la capacitat espessant i gelificant d'aquest, sense presentar problemes d'estabilitat física. A més, les propietats prebiòtiques dels β-glucans (capacitat d'estimular el creixement dels bacteris beneficiosos de la nostra microflora intestinal), suposen un valor afegit en el desenvolupament de productes fermentats a partir d'aquesta matèria primera.

En el disseny i optimització del processat de fermentats a partir de microorganismes probiòtics, s'estudià l'efecte de diversos factors de creixement (glucosa, fructosa, inulina i quantitat d'inòcul) sobre la supervivència del probiòtic dins les diferents matrius vegetals, ja que es recomana una quantitat mínima de 10^7 unitats formadores de colònies per mL per a que el producte a desenvolupar es puga considerar com funcional. Una vegada analitzat l'efecte individual i les interaccions dels factors de creixements sobre la supervivència dels microorganismes i/o temps de fermentació en les distintes matrius vegetals, es va determinar una formulació òptima que féu possible un procés fermentatiu ràpid i una alta supervivència del bacteri probiòtic. Quan els nivells escollits per als diferents factors de creixement donaren lloc a respostes similars pel que fa a la supervivència microbiana, s'optà per buscar els nivells mínims d'aquests factores que afavoriren un menor cost productiu. El producte fermentat desenvolupat es va caracteritzar a distints temps d'emmagatzematge (1, 7, 14, 21 i 28 dies) a 4 ºC per a analitzar la variació dels principals paràmetres que afecten a la qualitat fisicoquímica, sensorial i de supervivència del probiòtic en funció del temps i, d'aquesta manera, poder determinar un període òptim d'emmagatzematge amb el qual el producte mantinga unes propietat d'excel·lència. Els resultats mostraren que les llets fermentades amb els microorganismes potencialment probiòtics seleccionats permeteren mantenir una bona viabilitat, estabilitat física i apreciació sensorial durant l'emmagatzematge en refrigeració, per la qual cosa s'estima una vida útil similar a la dels iogurts convencionals.

Dins l'ampli ventall de propietats saludables que proporcionen els probiòtics es troba la capacitat d'influir positivament en el sistema immune, que evita l'aparició de reaccions al·lèrgiques, entre altres efectes. L'ametlla és un fruit molt consumit però conté al·lèrgens, per la qual cosa el probiòtic podria ser una bona ferramenta per a reduir-los. Per això es realitzaren estudis *in vitro* de les propietats inflamatòries dels fermentats d'ametlla amb diferents bacteris potencialment probiòtics. Aquestos estudis mostraren efectes positius en alguns dels ceps utilitzats, els quals van ser capaços de reduir la resposta al·lèrgica inicial associada al producte sense fermentar. Els resultats obtinguts obrin les portes a continuar amb la investigació i realitzar més estudis *in vitro* i *in vivo* en grups de població sensibles.

Justificación e interés del trabajo

El hecho de que la leche animal representa una fuente esencial, incluso irremplazable de proteínas, calcio y fósforo para una alimentación saludable, es un concepto indiscutible. Sin embargo, existen sectores de la población que no toleran la leche de origen animal. En este sentido, los principales problemas asociados al consumo de leche de vaca son la intolerancia a la lactosa de la leche y/o la alergia e intolerancia a las proteínas de la misma. Según la Asociación de Intolerantes a la Lactosa, ADILAC (2008) el 15% de la población española y el 20% de la Europea (García-Onieva, 2007) es intolerante a la lactosa. Además, la leche constituye uno de los más comunes alérgenos alimentarios (Ros-Berruezo, 2009). Si bien estos problemas pueden reducirse notablemente tomando leches fermentadas (yogures y demás), esto solo suele paliar los problemas de intolerancia a la lactosa pero no los de alergia a las proteínas de la leche. En el caso de niños alérgicos/intolerantes a la leche de vaca, se suele sustituir la leche de vaca por fórmulas hidrolizadas o leche de soja. El gran problema de las leches hidrolizadas es que aún contienen residuos de alérgenos (Fiocchi *et al.*, 2003; Wall, 2004) y por tanto, no garantizan la ausencia completa de reactividad y su mal sabor, que provoca el rechazo en los niños (García-Onieva, 2007).

En los últimos años, ha habido un incremento notable de la producción y del consumo de productos sustitutos de la leche y derivados, mal denominadas, pero comúnmente conocidas como “leches” de origen vegetal (Mårtensson *et al*., 2000), siendo la más extendida actualmente, la *leche* de

soja y otros productos derivados de la misma (sogur o yogur de soja, tofu, entre otros). No obstante, el consumo de este tipo de productos pueden conllevar otro tipo de problemas, en especial en la alimentación infantil: contienen fitatos que dificultan la absorción de zinc, calcio, magnesio, hierro y cobre (aunque se adicionen a las fórmulas) y altas concentraciones de manganeso, aluminio y fitoestrógenos, cuya repercusión a largo plazo se desconoce en este tipo de población (García-Onieva, 2007). Además, alrededor del 80% de los niños que presentan alergia a las proteínas de la leche, también la desarrollan frente a las proteínas de la *leche* de soja (García-Onieva, 2007). En este caso, la población infantil se queda con muy pocas opciones nutricionales en el mercado (Fiocchi *et al.*, 2003; Fiocchi *et al.*, 2006). A este mercado potencial se le suma un grupo poblacional muy amplio que son las personas con algún tipo de intolerancia y/o alergia a las leches de origen animal, o procedentes de hábitos alimentarios específicos (vegetarianos, ecológicos, etc.). En estos casos, sería necesario desarrollar nuevos productos fermentados, a base de *leches* vegetales (diferentes de la soja), que ofrezcan nuevas posibilidades al consumidor, dentro del sector de productos no-lácteos y siempre dentro de una dieta sana y equilibrada. Los fermentados resultantes deberán tener unas propiedades texturales, aromáticas y sensoriales adecuadas para una buena aceptación por parte de los consumidores y no presentar sinéresis o separaciones de fases a lo largo de su vida útil. Esto se puede conseguir mediante una correcta formulación del producto y mediante la aplicación de tratamientos tecnológicos adecuados (térmicos, homogeneización, ultra homogeneización, entre otros) que permitan conseguir los objetivos de calidad propuestos. Además, se

pretende que el producto fermentado pueda ser considerado como probiótico, y por tanto los microorganismos presentes deberán estar vivos y ser ingeridos en cantidades adecuadas para producir efectos beneficiosos (Guarner *et al*., 1998). En este sentido, se necesitará realizar estudios microbiológicos de viabilidad *in vitro* de los cultivos iniciadores.

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I. INTRODUCCIÓN

En la introducción se recopila la información encontrada respecto a las denominadas "leches" vegetales en el mercado actual, centrándose sobre todo en aquellas derivadas de cereales y frutos secos, así como en la obtención de productos fermentados a partir de los mismos. En esta recopilación se incluyó la *leche* de chufa por el interés local y, en cambio, no se incluyó la *leche* de soja por su amplio conocimiento.

En una primera parte, se analizan las propiedades nutricionales y/o beneficiosas de las *leches* vegetales mencionadas. De forma general se caracterizan por la ausencia de lactosa, proteína de origen animal y colesterol y por su perfil lipídico rico en ácidos grasos insaturados. Sin embargo, existen otras muchas propiedades de alto interés nutricional. Para profundizar en este ámbito, la información recopilada se presentó atendiendo a la siguiente clasificación: aquellas *leches* derivadas de cereales y las procedentes de frutos secos. Ambos tipos se encuentran en alza debido a la creciente concienciación del impacto que aportan nuestras dietas en la salud y el bienestar, unido a los numerosos estudios sobre el efecto de muchos de los nutrientes que proporcionan este grupo de alimentos en la prevención de determinadas enfermedades tales como diabetes, obesidad, enfermedades cardiovasculares o, incluso, algunos tipos de cáncer; ésta última ligada al alto contenido en antioxidantes de los mismos.

El proceso industrial utilizado para la obtención de las *leches* referidas se presenta en un segundo apartado, en el que se detalla los objetivos y condiciones de cada una de las etapas del procesado. Además, se indican los problemas habituales con los que se encuentran las industrias, entre las que

destaca la baja estabilidad física de sus productos, y las soluciones aplicadas y/o posibles mejoras aplicando nuevas tecnologías emergentes.

Por último, se presenta el gran potencial de las *leches* vegetales como materia prima para la obtención de productos fermentados, especialmente mediante el uso de bacterias probióticas. Las *leches* vegetales poseen de forma natural compuestos de carácter prebiótico (estimulan el crecimiento de microorganismos beneficiosos), con lo que, sumado a los beneficios para nuestra salud, mejorarían el crecimiento de las bacterias utilizadas como cultivos iniciadores. De este modo se estaría ofreciendo al mercado nuevos productos de valor añadido dentro del grupo de alimentos conocidos como "simbióticos" (combinación de prebióticos y probióticos), aptos para un gran número de grupos de población específicos tales como vegetarianos, intolerantes a la lactosa o alérgicos a la proteína de la leche de origen animal. Además, se está estudiando con éxito el uso de bacterias probióticas para el tratamiento preventivo y/o profiláctico de enfermedades cardiovasculares, dermatitis atópica y otros eczemas, así como algunos tipos de cáncer. En este sentido, y desde el punto de vista del paciente, el uso de *leches* vegetales sería una buena matriz para el posible desarrollo de estos tratamientos con probióticos, comparado con el uso de cápsulas o sobres. En este apartado de la tesis se especifica también el proceso industrial requerido para la obtención de estos fermentados, indicando las condiciones requeridas en cada etapa, así como los aditivos comúnmente utilizados y posibles mejoras en el desarrollo de los mismos.

Vegetable *milks* and fermented derived/derivative products

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ABSTRACT

The so-called vegetable *milks* are in the spotlight of the food and beverage industry thanks to the lactose-free, animal protein-free, cholesterol-free and low-calorie features linked to the current demand for healthy food products. Nevertheless, and with the exception of soya, little information is available about these types of *milks and* their derivatives. The aim of this review, therefore, is to highlight the main nutritional benefits of the nut and cereal vegetable *milks* available on the market, fermented or not, to describe the basic processing steps involved in their manufacturing process and to analyse the major problems affecting the overall quality together with the current possible, feasible solutions. On the basis of the information gathered, vegetable *milks* and their derivatives exert excellent nutritional properties which provide them a high potential and positive market expectation, in agreement with the current demand of healthy products.

Nevertheless, optimal processing conditions for each raw material or the application of new technologies have to be reviewed to provide better quality of the products. Hence, further studies need to be developed to ensure the physical stability of the products through their whole shelf life. These studies would also allow to decrease the amount of additives added (hydrocolloids and/or emulsifiers) and thus, to reduce the costs. In the particular case of fermented products, the use of proper starters able to both improve the quality (by synthesising proper flavors and providing optimal textures) and exert healthy benefits to consumers (i.e. probiotics) is the main challenge to be faced in future studies.

Key words: nut *milk*; cereal *milk*; processing; fermentation

1. INTRODUCTION

Nowadays, there is a global awareness of nutrition-related chronic diseases. In its 2009 annual report on global health risks, the World Health Organization (WHO) determined the distribution of deaths attributable to 19 leading risk factors worldwide. More than half of these factors were nutrition-related: blood pressure due to sodium consumption, cholesterol, obesity, deficiencies of iron and zinc, among others (Stuckler and Basu, 2011). Increasingly, consumers are more aware of the relationship between nutrition and health. Indeed, newly designed foods are not only intended to satisfy hunger and provide nutrients for humans, but also to prevent nutrition-related chronic diseases and to improve well-being, both physical and mental (Burdock and Carabin, 2008; Granato *et al*., 2010; Kaur and Das, 2011; Ozen *et al*., 2012). This trend is justified if several factors are considered, such as an increase in public health awareness (a consequence of a more highly educated population), an aging population and their desire for improving the quality of their later years, an increase in healthcare costs, advances in research and technology or changes in government regulations and accountability.

The food market reflects to an ever greater degree the consumer demand for healthy food products. A clear example of this tendency can be seen in the so-called vegetable *milks*, which are mainly made of nuts and cereals and have a long history in both Eastern and Western cultures. European sales of soya *milk* and other non-dairy *milks* are increasing by over 20% per year, Spain being the EU country in which the non-dairy drinks market grew the

most (Organic Monitor, 2006). Similarly, total USA retail sales of soy, almond, rice and other plant *milks* reached $1.3 billion in 2011 (Packaged Facts, 2012).

The best known and most popular vegetable *milk* derives from soy, although the demand for almond, rice, oat and coconut *milks* is on the increase. Wide ranges of nut and cereal vegetable *milks* are currently available on the market in a broad array of formulations: flavoured, sweetened/unsweetened, low-fat and/or fortified. Excluding Asia, non-dairy *milk* alternatives (vegetable *milks*) still represent a relatively small market overall; nonetheless, the growing awareness of allergy and intolerance issues and the lactose-free, cholesterol-free and low-calorie positioning of these products are bringing about a rise in purchase levels (Stone, 2011). In fact, marketing strategies of those products focus on comparing their health benefits with those of dairy products. Furthermore, experts are starting to consider possible relations between vegetable products and the prevention of cancer, atherosclerosis or inflammatory diseases, since free radicals play a key role in those pathologies and these types of food are an excellent source of antioxidants (Scalbert and Williamson, 2000). The lactose intolerant and/or those people allergic to cow milk are prime consumers of these types of *milks*, but this kind of products is in great demand even with people without health problems, such as vegans and vegetarians.

The development and further increment of the demand of such products would have an extra advantage or benefit, which could have an economic interest for many countries: the raw material they derive from (nuts and cereals) do not generally require specific soil nor climatic conditions, they are

able to adapt to different climates although, of course, the productivity might change (Osca, 2001; Coniglio, 2008). For example, almond tree farming is considered to be a dry cultivation with low soil fertility, low rainfall and minimum pruning and plant protection requirements (Navarro-Muñoz, 1996; Saura *et al.*, 1988). Oat is a temperate crop which grows well in damp, marginal upland areas (Welch and McConnell, 2001). These facts would benefit the rapid implementation of these raw materials in non-cultivated lands around the world and maybe, this could contribute to the rural development of developing countries and allowing these vegetable products to attain highly competitive prices within the world market.

Taking into account the positive trends of these products in the food market and bearing in mind that the literature contains little information about them, the aim of this work is to highlight the main nutritional benefits of these kind of *milks*, fermented or not, to describe the basic processing steps involved in their manufacturing process and to analyse the major problems affecting the overall quality together with the possible solutions currently available. Therefore, this review focuses on the study of nut and cereal vegetable *milks* available on the market and their fermented derivatives.

2. TYPES OF NUT AND CEREAL VEGETABLE *MILKS* AND THEIR NUTRITIONAL BENEFITS.

All the commercial vegetable *milks* share common features such as being lactose-free, animal protein-free or cholesterol-free. Taking into account the raw materials and their nutritional and health properties, vegetable *milks* can

be broadly classified in two large differentiated groups: nut and cereal *milks*. Both kinds of products are in the state of the art owing to the new-knowledge impact of their compounds on some current chronic diseases, such as cardiovascular diseases (CVD), type 2 Diabetes mellitus (DM-2), obesity and some cancers. These metabolic diseases are linked with our daily lifestyle, notably an unbalanced energy-rich diet, lacking in fibre and protective bioactive compounds, such as micronutrients and phytochemicals (Fardet, 2010). All these limited nutrients commented on above are readily available in both cereals and nuts.

Apart from nuts and cereals, other raw materials have been used industrially, such tubers (e.g. tigernuts) and plants (e.g. hemp, sunflower...). However, these milky based products are only well accepted in specific countries. Despite its local commerce, tigernut *milk* has also been explained in detail in this review due to its interesting composition and health properties.

2.1 Cereal grains and their *milks*

Cereals are known as a good source of the necessary daily energy, vitamins, several minerals, dietary fibre and phytochemicals, including phenolic compounds, carotenoides, vitamin E, lignans, inulin, starch, sterols and phytates (Okarter and Liu, 2010; Ward *et al*., 2008). The chemical composition of those cereals whose vegetable *milk* has been commercialised is summarised in Table 1. With respect to the supply of vitamins, cereals are considered an important source of group B vitamins, especially thiamin, riboflavin, folates and niacin (McKevith, 2004). Dietary fibre is present in

large quantities and this is rich in fructo-oligosaccharides, which are reportedly effective at stimulating the growth of *Bifidobacteria* and *Lactobacilli* in the human intestine (Kaur *et al.*, 2012). Besides this prebiotic effect, their phenolic compounds have also been reported to possess gastro-protective properties, in addition to their antioxidant, cholesterol-lowering, anti-atherogenic, anti-carcinogenic and anti-inflammatory effects (Chen *et al*., 2004; Dykes and Rooney, 2006; Prior and Gu, 2005). Indeed, epidemiological studies have shown an association between increased wholegrain consumption and reduced risks of various types of chronic diseases, such as CVD, obesity, DM-2 and some cancers (Chan *et al*., 2007; de Munter *et al*., 2007; Esmaillzadeh *et al*., 2005; Larsson *et al*., 2005; Mellen *et al*., 2008; Murtaugh *et al*., 2007; Schatzkin *et al*., 2008; van der Vijver *et al*., 2009). More specific properties and health benefits of each cereal grain whose vegetable *milk* has been commercialised are summarised in Table 2.

In order to gain the greatest benefit from the health properties of cereals, several aspects have to be considered. For instance, it is important to use and consume whole grain and not the refined, since most of the health components are located in the bran and germ. So, the use of the whole grain is highly recommended when producing the cereal *milks*. Another point to consider is the anti-nutrient content in some cereals, primarily phytic acid (mineral chelator) or saponins (toxic in high amounts and bitter tasting), although their presence can be reduced and/or eliminated by pre-treatment processes such as grinding, soaking, heat treatments, fermentations and germinations (Brady *et al*., 2007; Sharma and Kapoor, 1996; Zhu *et al.*,

2002). Despite the anti-nutritional components, so beneficial are wholegrain cereal's properties that important food associations, such as the U.S. Department of Agriculture (USDA), have strongly recommended 6-11 servings of grain products daily (Dewanto *et al*., 2002).

2.2 Nuts and nut *milks*

Due to their composition, nuts and nut-based products have recently attracted a great deal of attention from food nutrition and health specialists. Table 1 shows the chemical composition of those nuts whose vegetable *milks* have been commercially produced. Nuts are rich in mono- (MUFA) and polyunsaturated fatty acids (PUFA), vegetable proteins, dietary fibre, phytosterols, polyphenols, vitamins and minerals (Philips *et al*., 2005; Segura *et al*., 2006). Most of those compounds have antioxidant properties and are proven to provide a beneficial effect on plasma lipid profile, low-density lipoprotein (LDL) oxidation and inflammatory processes, among others (Carlson *et al*., 2011; Egert *et al*., 2011; Gillingham *et al*., 2011; Jones *et al*., 2011; Liu, 2012; Myers and Allen, 2012; Ward *et al*., 2012; Whent *et al*., 2012). Additionally, Vinson and Cai (2012) analysed the antioxidant efficacy in different nuts, obtaining the following order of importance: walnut > cashew > hazelnut ≈ almond. Epidemiological studies have linked frequent nut consumption to a reduced risk of CVC, DM-2 or death by all-cause mortality (Kelly Jr and Sabaté *et al*., 2006). Moreover, Li *et al*. (2009) observed that an increase in nut consumption was significantly associated with a more favourable plasma lipid profile, including lower LDL cholesterol, total cholesterol and apolipoprotein B-100 concentrations; but they did not

observe significant associations with non-high-density lipoprotein (HDL) cholesterol or inflammatory markers. In addition, nuts have a high K/Na ratio, which contributes to maintain well-balanced electrolytes in the human body, and, in addition to the prebiotic effect of their dietary fibre commented on above, the carbohydrates from nuts are complex (low Glycemic Index (GI)), which help to maintain blood sugar at healthy levels

In spite of the fact that around 50% of a nut is made up of lipids, regular nut consumption within a balanced diet has been shown to improve humans' lipid profile, increase endothelial function and reduce inflammation, without causing weight gain (Chen *et al*., 2006; Mattes, Kris-Etherton and Foster, 2007; Salas-Salvadó *et al*., 2008; Zambón *et al.,* 2000). Thus, in addition to providing both nutrients and bioactive antioxidants, nut *milks* may be a useful dietary tool for reducing risk factors that cause diseases with a major mortality rate in developed countries, such as metabolic syndrome, DM-2 or CVD. Indeed, the USDA approved a health claim between nuts and heart disease, suggesting that 42 g per day of most nuts as part of a low saturated-fat and cholesterol diet may reduce the risk of heart disease (FDA, 2003). The European Food Safety Authority (EFSA) also published a scientific opinion on the substantiation of health claims related to nuts and essential fatty acids (omega-3/omega-6) in nut oil, which is related to anti-inflammatory, heart health, weight management and healthy cardiovascular system effects (EFSA, 2011).

Although coconut is commonly classified as a nut, its composition does not follow the trend of this food group (Table 1), which means that not all the aforementioned are attached to it. As can be seen in the chemical composition

of coconut (Tables 1 and 2), the particularity of this traditional *milk* from the Asian, African and Pacific regions is its medium chain fatty acid (MCFA) lipid profile, which is similar to human milk (Chiewchan *et al*., 2006); the most predominant is lauric acid (45-53% of total coconut fats) and this MCFA was reported to be antibacterial, antiviral and antifungal (Raghavendra and Raghavaro, 2010). In spite of the lipid profile being mostly saturated, Enig (2004) reported that MCFA are absorbed directly from the intestine and sent straight to the liver to be rapidly metabolised for energy production and, thus, they do not participate in the biosynthesis and transport of cholesterol. Furthermore, the high amount of antioxidants determines the long shelf life of this vegetable *milk* and is good for the health. Other interesting health benefits are summarised in Table 2.

Tigernuts are another interesting source of raw material to be used for the production of vegetable *milks*. The major components (Table 1) of this tuber are complex carbohydrates, mainly starch and dietary fibre, which provide vegetable *milk* with low GI. Furthermore, the protein content is rich in arginine, which liberates hormones that produce insulin; thus being suitable for diabetics (Adejuyitan, 2011). Besides its antioxidant compounds, the lipid profile of tigernuts is similar to that found in olive oil; therefore, the derived *milk* has a positive effect on the cholesterol level. Other interesting health benefits are detailed in Table 2.

More specific properties and health benefits of each nut whose derived vegetable *milk* has been commercialised are summarised in Table 2.

Table 1. Raw materials composition commercially used for producing vegetable *milks*. Average values shown are expressed per 100 g of edible part. Nutrient Recommended Daily Allowances (RDA) corresponding to a healthy adult with standard physical activity and lifestyle are also included.

Name	Almond[A]	Chestnut[A]	Hazelnut[B]	Walnut[B]	Amaranth[B]	Barley[B]	Corn[B]	Kamut[B]	Millet[B]	Oat[A]	Quinoa[A]	Rice[A] (brown)	Sesame[D]	Spelt[B]	Coconut[B]	Tigernut[A]	RDA[X]
Lipids (g)	54.65	5.3	60.75	65.21	7.02	1.16	4,74	2.2	4.22	6.9	5.56	2.64	49.7	2.43	33.49	23.74	<70
MUFA (g)	*35.01*	*0.6*	*45.65*	*8.93*	*1.685*	*0.15*	*1,25*	*0.214*	*0.773*	*2.2*	*1.4*	*0.83*	*18.8*	*0.445*	*1.425*	*16.47*	
PUFA (g)	*12.28*	*1.3*	*7.92*	*47.17*	*2.778*	*0.56*	*2,16*	*0.616*	*2.134*	*2.5*	*2.1*	*0.89*	*21.8*	*1.258*	*0.366*	*2.21*	
SFA (g)	*4.93*	*3.2*	*4.46*	*6.13*	*1.459*	*0.25*	*0,67*	*0.192*	*0.723*	*1.2*	*0.5*	*0.52*	*7.6*	*0.406*	*29.698*	*4.02*	*<20*
Cholesterol(mg)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Protein (g)	19.13	4	14.95	15.23	13.56	9.91	9.42	14.7	11.02	16.9	13.8	7.5	17.7	14.57	3.33	6.13	50
moisture (g)	5.87	4.48	5.31	4.07	11.29	10.09	10.37	10.95	8.67	0	11.5	11.4	4.24	11.02	46.99	n.a.	
Carbohydrates(g)	6.2	39.7	16.70	13.71	65.25	77.7	74.26	70.38	72.85	66.3	49.2	81.3	9.28	70.19	15.23	42.54	270
sugars (g)	*5.3*	*7.9*	*4.34*	*2.61*	*1.69*	*0.8*	*0.64*	n.a.	n.a.	n.a.	*5.92*	n.a.	*0.45*	n.a.	*6.23*	n.a.	
starch (g)	*0.11*	*31.8*	n.a.	*2.1*	*57.27*	n.a.	*73.3*	n.a.	n.a.	n.a.	*43.27*	*72.7*	*0.4*	n.a.	n.a.	*29.15*	
Dietary fiber (g)	8.35	7.1	9.7	6.7	6.7	15.6	7.3	9.1	8.5	10.6	7.9	3	7.9	10.7	9	17.4	25
Vitamin A* (μg)	0	0	1	1	0	0	11	1	0	0	0	0	n.a.	0	0	0	800
Vitamin E** (mg)	24	1.4	15.03	0.7	1.19	0.2	0.49	0.6	0.05	0.7	0.45	0.6	n.a.	0.79	0.24	10	12
Vitamin C (mg)	n.a.	0	6.3	1.3	4.2	0	0	0	0	0	0	0	0	0	3.3	6	80
Thiamin (mg)	0.21	0.18	0.643	0.34	0.116	0.19	0.385	0.591	0.421	0.76	0.2	0.39	0.791	0.364	0.066	0.23	1.1
Riboflavin (mg)	0.78	0.1	0.113	0.15	0.2	0.114	0.20	0.178	0.29	0.14	0.4	0.08	0.247	0.113	0.02	0.1	1.4
Niacin (mg)	5.3	n.a.	1.8	1.125	0.923	4.61	3.64	6.35	4.72	0.96	n.a.	6.8	4.52	6.843	0.54	1.8	50
Vitamin B6 (mg)	0.11	0.3	0.563	0.537	0.591	0.26	0.62	0.255	0.384	n.a.	0.2	0.51	0.79	0.23	0.054	n.a.	200

Table 1 (cont.)

Biotin (µg)	64	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.5
Folate (µg)	70	85	113	98	82	23	19	n.a.	85		30	40	97	45	26	141	200
Vitamin B12 (µg)	0	0	0	0	n.a.	0	0	n.a.	0	0	0	0	0	n.a.	0	0	2.5
Pantothenic acid (mg)	0.44	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1.35	n.a.	1.5	0.05	n.a.	n.a.	n.a.	6
Sodium (mg)	10.36	10.6	11	2	4	9	35	6	5	2	61	6	2.31	8	20	37.63	<6
Potassium (mg)	767.25	591.6	680	441	508	280	287	446	195	429	780	223	468	388	356	519.2	2000
Calcium (mg)	248.25	47.3	114	98	159	29	7	24	8	54	79	21	96	27	14	69.54	800
Phosphorous(mg)	524.88	87.6	290	346	557	221	210	386	285	523	230	303	604	401	113	232.23	700
Magnesium (mg)	258.13	39	163	158	248	79	127	134	114	177	210	143	324	136	32	86.88	375
Iron (mg)	3.59	1.06	4.7	2.91	7.61	2.5	2.71	4.41	3.01	4.72	7.8	1.7	14.6	4.44	2.43	3.41	14
Zinc (mg)	3.6	0.6	2.45	3.09	2.87	2.13	2.21	3.68	1.68	3.97	3.3	1.8	5.74	3.28	1.1	4.19	10
Copper (mg)	1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.63	n.a.	0.85	1.58	n.a.	n.a.	n.a.	1
Selenium (µg)	4	n.a.	n.a.		n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2	<51.9	n.a.	n.a.	n.a.	55
Iodide (µg)	2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	6	n.a.	22	<5	n.a.	n.a.	n.a.	150
Manganese (µg)	2.54	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1.23	n.a.	n.a.	n.a.	2

***A**: Data extracted from Spanish Food Composition Database (RedBEDCA)*

***B**: Data extracted from United States Department of Agriculture Database (USDA)*

***C**: Data extracted from French agency for food environmental and occupational health and safety Database (Anses)*

[x] :Recommended Daily Allowances. Data extracted from European Directive 90/496/CEE.

***RDA**: Recommended Daily Allowances*

******: Retinol equivalents* *******: alfa-tocopherol equivalents*

***n.a.**: data not available*

***MUFA**: mono-unsaturated fatty acids* ***PUFA**: poly-unsaturated fatty acids* ***SFA**: saturated fatty acids*

Table 2. Main features and health benefits of the raw materials commercially used for producing vegetable *milks*.

Raw material	Product features	Health benefits	References
ALMOND **(nut)**	▪ Good source (arranged by importance) of E Vit., K, Mn, Mg, Cu, P, dietary fiber, riboflavin and protein. ▪ Good lipid profile, mainly MUFA ▪ Important source of phytonutrients, mainly flavonoids and proanthcyanidins. ▪ Lactose-free; low digestible carbohydrate ▪ Cholesterol-free ▪ High digestibility ▪ Low GI ▪ Low Na content	▪ Hypocholesterolemic benefits ▪ Protective effect against CVD ▪ Antioxidant properties ▪ Electrolite balance contribution ▪ Glucorregulation properties ▪ Prebiotic effect ▪ Anti-inflammatory properties ▪ Possible contribution in protection against some cancers such as colon cancer ▪ Suitable for celiacs and lactose intolerants	Chen *et al.* (2006); Hollis and Mattes (2007); Iacono *et al.* (2008); Li *et al.* (2009); Mandalari *et al.* (2008); Rajaram *et al.* (2010); Vinson and Cai (2012)
CHESTNUT **(nut)**	▪ Good source of K, Mg, Fe, Ca, Mn, Cu, dietary fiber, and protein rich in leucine and arginine. ▪ Low-fat content ▪ Good lipid profile, mainly PUFA and followed by MUFA. ▪ Important amounts folates, thiamin and riboflavin ▪ Important source of phenolic compounds ▪ Lactose-free; low digestible carbohydrate ▪ Cholesterol-free ▪ High digestibility ▪ Low GI ▪ Low Na content	▪ Hypocholesterolemic benefits ▪ Protective effect against CVD ▪ Antioxidant properties ▪ Electrolite balance contribution ▪ Glucorregulation properties ▪ Prebiotic effect ▪ Possible contribution in protection against some cancers such as colon cancer ▪ Suitable for celiacs and lactose intolerants	Borges *et al.* (2008); Borges *et al.* (2007); De Vasconcelos *et al.* (2010);
HAZELNUT **(nut)**	▪ Good source of E Vit., K, Fe, Ca, Mg, Zn, dietary fiber, and protein rich in arginine and leucine. ▪ Good lipid profile, mainly MUFA. ▪ Important amounts of niacin, B_1 Vit., B_2 Vit., B_6 Vit. and ascorbic acid. ▪ Important source of bioactives and phytochemicals and antioxidant phenolics (mainly caffeic acid) ▪ Lactose-free; low digestible carbohydrate ▪ Cholesterol-free ▪ High digestibility ▪ Low GI ▪ Low Na content	▪ Hypocholesterolemic benefits ▪ Protective effect against CVD ▪ Antioxidant properties ▪ Electrolite balance contribution ▪ Glucorregulation properties ▪ Prebiotic effect ▪ Possible contribution in protection against some cancers such as colon cancer ▪ Suitable for celiacs and lactose intolerants	Alasalvar *et al.* (2003); Durak *et al.* (1999); Köksal *et al.* (2006); Li *et al.* (2009); Mercanligil *et al.* (2007); Özdemir *et al.* (2001), Tey *et al.* (2011)

Table 2 (cont.)

WALNUTS **(nut)**	▪ Good source of K, P, Mg, Fe, Mn, Cu, Zn, dietary fiber, and protein rich in arginine. ▪ Good lipid profile, mainly PUFA (linoleic and α-linolenic acids). ▪ Appreciable amounts of E Vit., niacin, thiamin, riboflavin and folic acid. ▪ Important source of bioactives and phytochemicals ▪ Cholesterol-free ▪ High digestibility ▪ Low GI ▪ Low Na content	▪ Hypocholesterolemic benefits ▪ Protective effect against CVD ▪ Antioxidant properties ▪ Electrolite balance contribution ▪ Glucorregulation properties ▪ Prebiotic effect ▪ Anti-inflammatory properties ▪ Possible contribution in protection against some cancers such as colon cancer ▪ Suitable for celiacs and lactose intolerants	Almario *et al.* (2001); Banel and Hu (2009); Chisholm *et al.* (1998); Elaine and Feldman (2002); Li *et al.* (2009); Sze-Tao and Sathe (2000); Vinson and Cai (2012); Zambón *et al.* (2000)
AMARANTH **(cereal)**	▪ Good source of E Vit., Ca, Mg, K, P, Fe and Zn ▪ High content in both soluble and insoluble fiber ▪ Good lipid profile, mainly PUFA (linoleic acid), followed by MUFA (oleic acid) ▪ Good protein source rich in lysine and methionine ▪ High phytosterol content, mainly β-sitosterol ▪ High levels of tocotrienols and squalene (cholesterol-lowering comp.) ▪ Important amounts of C Vit., riboflavin and niacin. ▪ Important source flavonoids and phenolic compounds. ▪ Source of Lunasin (antitumoral peptide) ▪ Lactose-free; low digestible carbohydrate ▪ Gluten-free ▪ Cholesterol-free ▪ High digestibility ▪ Low GI	▪ Hypocholesterolemic benefits ▪ Protective effect against CVD ▪ Antioxidant properties ▪ Electrolite balance contribution ▪ Glucorregulation properties ▪ Prebiotic effect ▪ Possible contribution in protection against some cancers such as colon cancer ▪ Suitable for celiacs, diabetics and lactose intolerants ▪ Antitumor effects ▪ Anti-inflammatory properties ▪ Anti-anemic effects	Alvarez-Jubete *et al.* (2010); Caselato-Sousa and Amaya-Farfán (2012); Marcone *et al.* (2004); Sanz-Penella *et al.* (2012)
BARLEY **(cereal)**	▪ Good source of Ca, Mg, K, P, Fe and Zn ▪ High content in dietary fiber, rich in β-glucans ▪ Good lipid profile, mainly PUFA ▪ Good protein source rich in lysine ▪ Important source of tocopherols and tocotrienols (cholesterol-lowering comp.) ▪ Important amounts of group B Vit., mainly nicacin, riboflavin folate and thiamin. ▪ Good source of phenolic compounds ▪ Lactose-free; low digestible carbohydrate ▪ Gluten-free ▪ Cholesterol-free ▪ High digestibility ▪ Low GI	▪ Hypocholesterolemic benefits ▪ Protective effect against CVD ▪ Antioxidant properties ▪ Electrolite balance contribution ▪ Glucorregulation properties ▪ Prebiotic effect ▪ Possible contribution in protection against some cancers such as colon cancer ▪ Suitable for diabetics and lactose intolerants ▪ Anti-anemic effects	AbuMweis *et al.* (2010); Ames and Rhymer (2008); Baik and Ullrich (2008); Kaur *et al.* (2012); Thondre *et al.* (2011); Ward *et al.*, (2008).

Table 2 (cont.)

KAMUT **(cereal)**	▪ Good source of E Vit., Ca, Mg, K, P, Fe, Mn, Se and Zn ▪ High content in dietary fiber ▪ Good lipid profile, mainly PUFA ▪ Good protein source ▪ High levels of, tocopherols and tocotrienols ▪ Good source of β-carotene and group B Vit., mainly thiamin, riboflavin, nicacin, B_6 Vit. and folates. ▪ Important source of phenolic compounds. ▪ Lactose-free; low available carbohydrate ▪ Cholesterol-free ▪ High digestibility ▪ Low GI	▪ Hypocholesterolemic benefits ▪ Protective effect against CVD ▪ Antioxidant properties ▪ Glucorregulation properties ▪ Prebiotic effect ▪ Possible contribution in protection against some cancers such as colon cancer ▪ Suitable for diabetics and lactose intolerants ▪ Anti-anemic effects	Canavari *et al.* (2010); Dinelli *et al.* (2009); Marotti *et al.* (2012); Piergiovanni *et al.* (2009).
MAIZE **(cereal)**	▪ Good source of A Vit., E, thiamin, niacin and also K, P, Mg, Fe, Zn and dietary fiber. ▪ Good lipid profile, mainly PUFA (linoleic acid) followed by MUFA (oleic acid). ▪ Important source of pantothenic and folic acids ▪ Important source of phenolic compounds, mainly ferulic acid. ▪ Lactose-free; low digestible carbohydrate (they are in the form of starch, fiber and pentosan) ▪ Gluten-free ▪ High digestibility ▪ Low GI ▪ Cholesterol-free	▪ Hypocholesterolemic benefits ▪ Protective effect against CVD ▪ Antioxidant properties ▪ Suitable for celiacs, diabetics and lactose intolerants ▪ Possible contribution in protection against some cancers such as colon cancer	Del Pozo-Insfran *et al.* (2006); Kaur *et al.* (2012)
MILLET **(cereal)**	▪ Good source of Ca, Mg, K, P, Fe Zn ▪ High content in insoluble dietary fiber ▪ Good lipid profile, mainly PUFA (linoleic acid), followed by MUFA ▪ Important amounts of group B vitamins, mainly thiamin, riboflavin, folate and niacin. ▪ Good source of phenolic compounds ▪ Lactose-free; low digestible carbohydrate ▪ Gluten-free ▪ Cholesterol-free ▪ Low GI	▪ Hypocholesterolemic benefits ▪ Protective effect against CVD ▪ Antioxidant properties ▪ Glucorregulation properties ▪ Prebiotic effect ▪ Possible contribution in protection against some cancers such as colon cancer ▪ Suitable for celiacs, diabetics and lactose intolerants ▪ Anti-anemic effects	Chandrasekara and Shahidi (2011); Devi *et al.* (2011); Hegde *et al.* (2005); Irén Léder (2004); Kaur *et al.* (2012);
OAT **(cereal)**	▪ Good source of E Vit., Ca, Mg, K, P, Fe, Cu and Zn ▪ High content in dietary fiber, rich in β-glucans ▪ Good lipid profile, mainly PUFA ▪ Good protein source rich in sulfur aminoacids, such as methionine and cystine (essential aminoacids) ▪ Important amounts of group B vitamins, mainly nicacin, riboflavin and thiamin. ▪ Good source of tocopherols and phenolic compounds ▪ Lactose-free; low digestible carbohydrate ▪ Gluten-free ▪ Cholesterol-free ▪ Low Na content ▪ High digestibility ▪ Low GI	▪ Hypocholesterolemic benefits ▪ Protective effect against CVD ▪ Antioxidant properties ▪ Electrolite balance contribution ▪ Glucorregulation properties ▪ Prebiotic effect ▪ Possible contribution in protection against some cancers such as colon cancer ▪ Suitable for celiac, diabetics and lactose intolerants ▪ Antimicrobial and immune effects ▪ Anti-anemic effects	Biel *et al.* (2009); Daou and Zhang (2012); Jing and Hu (2012); Kaur *et al.* (2012); Kemppainen *et al.* (2009); Sadiq Butt *et al.*, (2008); Thompson (2003); Ward *et al.*, (2008);

Table 2 (cont.)

QUINOA **(cereal)**	▪ Good source of E Vit., Na, Ca, Mg, K, P, Fe Zn ▪ High content in dietary fiber ▪ Good lipid profile, mainly PUFA (linoleic acid), followed by MUFA (oleic acid) ▪ Good protein source rich in lysine and methionine ▪ High phytosterol content. ▪ High levels of squalene (cholesterol-lowering comp.) ▪ Important amounts of group B Vit., mainly riboflavin folate and thiamin. ▪ Source of bioactive peptides ▪ Lactose-free; low digestible carbohydrate ▪ Gluten-free ▪ Cholesterol-free ▪ High digestibility ▪ Low GI	▪ Hypocholesterolemic benefits ▪ Protective effect against CVD ▪ Antioxidant properties ▪ Glucorregulation properties ▪ Prebiotic effect ▪ Possible contribution in protection against some cancers such as colon cancer ▪ Suitable for celiacs, diabetics and lactose intolerants ▪ Antitumor effects ▪ Anti-inflammatory properties ▪ Anti-anemic effects	Abugoch-James (2009); Alvarez-Jubete *et al.* (2010); Brady *et al.* (2007); Chauhan *et al.* (1999); Zhu *et al.* (2002).
RICE **(cereal)**	▪ Good source of K, Ca, Mg, P, Fe, Zn, Se and Si. ▪ Low Na content ▪ Good lipid profile, mainly PUFA (linoleic and α-linolenic acids), followed by MUFA (oleic acid) ▪ Important source of phytosterols, especially β-sitosterol and γ-oryzanol (oxidation inhibitors) ▪ Important source of tocopherols (E Vit.) and tocotrienols (LDL-cholesterol lowering) ▪ Important amounts of group B Vit., mainly nicacin, riboflavin, folate, thiamin, B_6 Vit., and panthothenic ac. ▪ Good source of phenolic compounds ▪ Lactose-free ▪ Gluten-free ▪ Cholesterol-free ▪ High digestibility	▪ Hypocholesterolemic benefits ▪ Protective effect against CVD ▪ Antioxidant properties ▪ Electrolite balance contribution ▪ Glucorregulation properties ▪ Suitable for celiacs, and lactose intolerants ▪ Antitumor effects ▪ Anti-inflammatory properties.	Biswas *et al.* (2011); Cicero and Gaddi (2001); Faccin *et al.* (2009); Sierra *et al.* (2005).
Sesame **(cereal)**	▪ Good source of E Vit. Ca, Mg, K, P, Fe, Zn and Cu. ▪ High content in dietary fiber ▪ Good lipid profile, mainly PUFA (rich in linoleic) ▪ Good protein source ▪ High content in lignan (phytostrogen) ▪ Important amounts of group B Vit., mainly nicacin, riboflavin folate and thiamin. ▪ Good source of phenolic compounds ▪ Lactose-free; low digestible carbohydrate ▪ Gluten-free ▪ Cholesterol-free ▪ High digestibility ▪ Low GI	▪ Hypocholesterolemic benefits ▪ Protective effect against CVD ▪ Antioxidant properties ▪ Electrolite balance contribution ▪ Glucorregulation properties ▪ Prebiotic effect ▪ Possible contribution in protection against some cancers such as colon cancer ▪ Suitable for celiacs, diabetics and lactose intolerants	Cooney *et al.* (2001); Sirato-Yasumoto *et al.* (2001); Wu *et al.* (2006);

Table 2 (cont.)

Spelt **(cereal)**	▪ Good source of E Vit., Ca, Mg, K, P, Fe, Mn and Zn ▪ High content in dietary fiber ▪ Low Na content ▪ Good lipid profile, mainly PUFA (linoleic acid), followed by MUFA (oleic acid) ▪ Good protein source ▪ High levels of, tocopherols and tocotrienols ▪ Good source of group B Vit., mainly thiamin, riboflavin, nicacin, Vit. B_6 and folates. ▪ Important source of phenolic compounds. ▪ Lactose-free; low digestible carbohydrate ▪ Cholesterol-free ▪ High digestibility ▪ Low GI	▪ Hypocholesterolemic benefits ▪ Protective effect against CVD ▪ Antioxidant properties ▪ Electrolite balance contribution ▪ Glucorregulation properties ▪ Prebiotic effect ▪ Possible contribution in protection against some cancers such as colon cancer ▪ Suitable for diabetics and lactose intolerants ▪ Anti-anemic effects	Ward *et al*. (2008); Zieliński *et al.* (2008); Marques *et al.* (2007), Ruibal-Mendieta *et al*. (2005); Ranhotra *et al.* (1996).
Coconut **(fruit)**	▪ Good source of C Vit. and also K, P, Mg and Fe. ▪ Rich in MCFA, mainly lauric acid ▪ Important source of polyphenols ▪ Lactose-free ▪ Cholesterol-free ▪ High digestibility ▪ Low GI	▪ Hypocholesterolemic benefits ▪ Protective effect against CVD ▪ Antiviral, antibacterial and antifungical properties ▪ Antioxidant properties ▪ Antithrombotic and antiatheroclerotic effects ▪ Inmunostimulatory properties ▪ Suitable for celiacs and lactose intolerants	DebMandal and Mandal (2011); Enig (2004); Raghavendra and Raghavarao (2010); Sri Lanka Med. Assoc. (2006).
Tigernut **(tuber)**	▪ Good source of E Vit., Ca, Na, K, P, Cu and dietary fiber. ▪ Good lipid profile, mainly MUFA (oleic acid) and followed by linoleic acid. ▪ Important amounts of C Vit., folates, thiamin and riboflavin ▪ Important source of bioactives and phytochemicals and phenolic compounds. ▪ Lactose-free; low digestible carbohydrate ▪ Cholesterol-free ▪ High digestibility ▪ Low GI	▪ Hypocholesterolemic benefits ▪ Protective effect against CVD ▪ Antioxidant properties ▪ Glucorregulation properties ▪ Prebiotic effect ▪ Possible contribution in protection against some cancers such as colon cancer ▪ Suitable for celiacs and lactose intolerants	Adejuyitan (2011); Arafat *et al*. (2009); Sánchez-Zapata *et al.* (2012); Sanful (2009).

***MUFA**: mono-unsaturated fatty acids* ***PUFA**: poly-unsaturated fatty acids* ***CVD**: Cardiovascular diseases* ***GI**: Glycemic index*

3. VEGETABLE *MILK* PROCESSING

Industrial vegetable *milk* processing is based on five main steps: grinding, water extraction, filtration, homogenisation and pathogen removal treatment. Nevertheless, depending on the raw material and the desired final product characteristics, the process slightly differs. Thus, the processing is subsequently explained separately, taking into account the different groups of these abovementioned *milks* commented.

3.1 Cereal *milk* processing

A typical flow diagram of cereal *milk* processing is shown in Figure 1. Before going through the extraction procedure, cereals are conditioned: this mostly refers to husking, washing and grain classification. Pre-conditioning is a requisite for Quinoa grain due to its saponins contents (toxic in high amounts and imparts a bitter taste). Mechanical abrasion and/or washing are sufficient to remove this unwanted compound (Brady *et al.*, 2007).

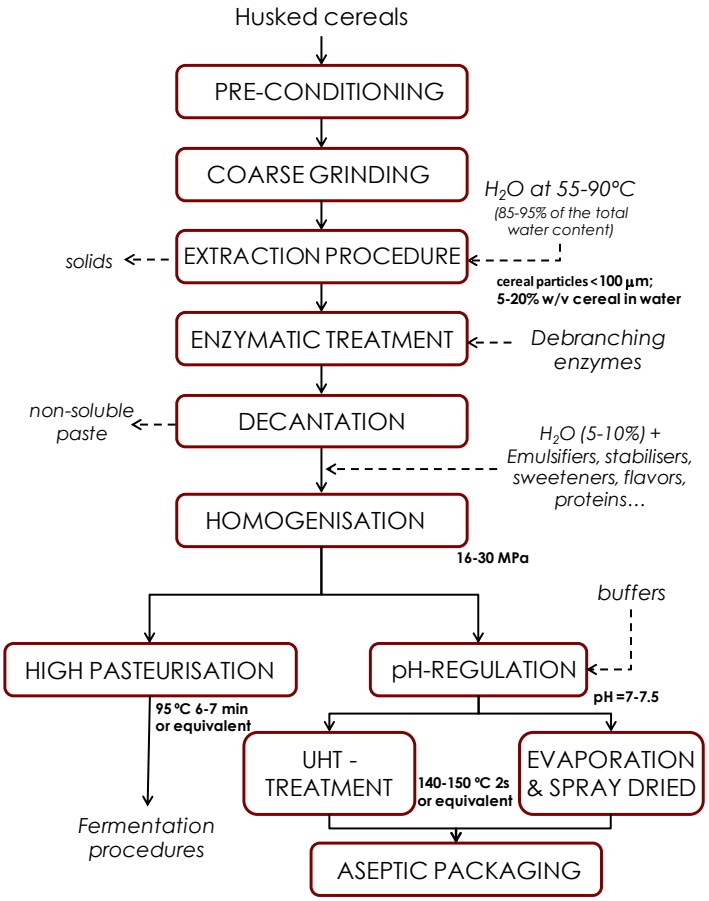

Figure 1. Typical cereal *milk* processing flow diagram.

Once the cereal grain is conditioned, it is submitted to a coarse dry grinding to facilitate the subsequent water extraction. This water extraction process is usually carried out in colloidal mills by adding at the same time hot water and the grinded cereal. The colloidal mills are used to reduce the particle size of the solid in aqueous suspension by applying high levels of hydraulic shear to the process liquid. This step is carried out in hot

conditions, on one hand to, ease the outlet of soluble compounds and, on the other hand, to provoke starch gelatinisation and improve the subsequent enzymatic treatment. The main difference observed between these and other types of vegetable *milks* is the enzymatic treatment they are submitted to after the extraction procedure, a step which becomes relevant in order to attain the low viscosity which the consumer demands of these products. This viscosity is provided mainly by starch and other polysaccharides after the thermal treatments.

After removing the non-extracted solids, the milky liquid obtained is then temperature and pH adjusted with appropriate buffers to the optimum level for the enzymatic treatment; pH values, temperature and the time-remaining would depend on the types of enzymes used and those would be chosen taking into account the final product's desired viscosity, sugar content and/or texture parameters. Generally, enzyme composition comprises mainly α-amylase and β-amylase activities and is devoid of β-glucanase and proteinase activities, since glucans and proteins have interesting nutrient value and their degradation is not desirable (Triantafyllou, 2004). When the desired textural properties are reached, a homogenisation process is applied to ensure physical stabilisation during the product's shelf life. The usual homogenisation pressures range between 16-30 MPa, although some researchers are studying the use of ultra-high pressures (UHPH, >150 MPa) in vegetable *milk* production (Bernat *et al.*, 2011; Cruz *et al.*, 2007; Valencia-Flores *et al.*, 2013). Food additives, such as emulsifiers (lecithin), stabilisers (hydrocolloids), sweeteners, either natural (sucrose, fructose or glucose syrups from agave, corn, rice or wheat) or synthetic (acesulfame K, aspartame

or sucralose) and, sometimes, flavouring agents (cocoa, soluble coffee, vanilla or cinnamon) are often introduced before the homogenisation step. The amounts of these additives incorporated ranged from 0.4-2.5 g/100mL in emulsifiers, 0.025-0.3 g/100mL in stabilisers, 5-8 g/100mL in sweeteners and 0.5-3 g/100mL in flavouring agents (Erra *et al.*, 2010; Triantafyllou, 2004). Further information on the ingredients used to formulate already commercialised cereal *milks* is shown in Table 3.

To ensure quality and safety, after having readjusted the pH to standard values of *milks* with buffers such as sodium carbonates, potassium carbonates, sodium hydroxides or potassium diphosphates (Erra, 2012), homogenised *milks* are either heat treated or spray dried and finally aseptically packaged. Heat parameters of temperature, time and pressure would be stipulated taking into account the type of product, the particle size, viscosity, initial microbial load and stability of components under thermal conditions. The inactivation conditions of the enzymes previously used is also a variable when choosing heat parameters, since this step is also used to eradicate the activities of residual enzymes. Ultra High Temperature (UHT) treatment is commonly chosen (140-150 ºC, 2 s) or high pasteurisation (95ºC > 6 min or equivalent treatment) might also be used when the *milk* product is to be fermented (Erra, 2012; Pereyra and Mutilangi, 2012; Pérez *et al.*, 2010; Triantafyllou, 2004).

Table 3. Chemical composition of commercial vegetable *milks*. Average values shown are expressed per 100 mL of liquid product.

Product	Ingredients	Proteins	Lipids				Carbohydrates		Dietary fiber	Sodium	Manufacturer
			TOTAL	MUFA	PUFA	SFA	TOTAL	SUGARS			
Almond	Water, almonds (7-8%), thickener (corn starch/maltodextrin), stabilizer (carrageenan/gellan gum), emulsifier (sunflower/soya lecithin), salt, almond flavor	**1.1-1.8**	**1.46-2.6**	0.8-4	0.42-1.4	0.3-0.6	**1-8**	0.1-1.6	**0.4-2**	**0.01-0.06**	*(1), (3), (6), (10), (12), (13), (14), (15)*
Chestnut	Water, chestnut (14%), sweetener (rice syrup/ agave syrup), thickener (maltodextrin), almond oil, natural flavor, salt	**0.05**	**3.5**	2.3	0.8	0.5	**6.2**	0.3	**2.4**	**0.03**	*(1)*
Hazelnut	Water, hazelnut (7%), agave syrup, thickener (corn maltodextrin)	**0.6-0.8**	**1.5-2.8**	2	0.1	0.7	**6.5-8**	3.2-6	**0.42-0.9**	**0.175**	*(1), (15)*
Walnut	Water, walnut (7%), sweetener (agave syrup), corn maltodextrin	**1.2**	**1**	0.3	0.6	0.1	**7.4**	1.7	**0.4**	**0.02**	*(1), (12)*
Amaranth	Water, amaranth (7%), sweetener (agave syrup), thickener (corn maltodextrin), high oleic sunflower oil	**0.6**	**1.9**	1.1	0.3	0.5	**8**	n.a.	**0.3**	**0.05**	*(1)*
Barley	Water, barley (17%), sunflower oil, salt	**0.5**	**1**	0.3	0.6	0.1	**11**	7	**0.5**	**0.09**	*(3)*
Corn	Water, white corn (17%), sunflower oil, salt	**0.52**	**1.4**	0.7	0.5	0.2	**9.5**	6.5	**0.6**	**0.03**	*(3)*
Kamut	Water, Kamut (12-14%), sunflower oil, salt	**0.65-0.7**	**0.8-1.45**	0.2-0.4	0.5-0.88	0.1-0.21	**7.5-20**	4.6-7.1	**0.4-0.5**	**0.04-0.09**	*(3), (4), (6), (12)*
Millet	Water, millet (15%), sunflower oil, salt	**0.7**	**1.2**	0.3	0.8	0.1	**9.8**	5.7	**0.4**	**0.09**	*(3)*

Table 3 (cont.)

Oat	Water, oat (8-14%), sunflower oil, salt	**0.5-1**	**0.7-1.6**	0.84-0.99	0.2-0.4	0.1-0.3	**4.75-11.8**	1.5-5.5	**0.44-0.9**	**0.04-0.1**	*(2), (3), (4), (5), (6), (7), (8), (9), (10), (11), (12), (15), (16)*
Quinoa	Water, quinoa (7-14%), sweetener (agave syrup), thickener (corn maltodextrin), stabilizer (pectin/carrageenan), sunflower oil, emulsifier (soya lecithin)	**1.5**	**2.8**	1.5	0.6	0.7	**3.7**	3.4	**0.6**	**0.2**	*(1), (2), (6)*
Rice	Water, rice (12-20%),stabilizer (carrageenan/xanthan gum), sunflower/rape seed oil, salt	**0.12-0.8**	**0.9-3**	0.33-1	0.11-1.7	0.1-0.37	**4.6-15.6**	4.3-7.1	**0.12-0.15**	**0.04-0.1**	*(2), (3), (4), (5), (6), (7), (8), (11), (12), (15), (16)*
Sesame	Water, sesame (7%), sweetener (agave syrup), thickener (corn maltodextrin), high oleic sunflower oil	**0.6**	**2.4**	1.4	0.5	0.5	**6.7**	n.a.	**0.2**	**0.01**	*(1)*
Spelt	Water, spelt (14-15%), sunflower oil, salt	**0.2-0.7**	**1-1.4**	0.3-0.37	0.8-0.87	0.1-0.16	**8.4-9.6**	4-7	**0.2-0.7**	**0.05-0.13**	*(2), (3), (5), (6)*
Coconut	Coconut cream(10%), sweetener (cane sugar), natural flavor, stabilizer **(**carrageenan/guar gum), salt	**0.2-0.7**	**1.9-2.4**	0.1	n.a.	1.4-2.3	**0.9-3.7**	0.4-1	**0.4-1.1**	**0.02-0.1**	*(13), (14)*
Tigernut	Water, tigernuts (10-14%), sweetener (agave syrup/sucralose), thickener (corn maltodextrin/tapioca starch), stabilizer (carrageenan/ carboxymethyl cellulose)	**0.4-0.7**	**2-2.4**	1.1-1.6	0.19-0.3	0.4-0.6	**5.3-8.3**	4.5-6.5	**0.67-1**	**0.02-0.06**	*(1), (3), (12)*

***(1)** Nutriops S.L. (Spain)* ***(2)** Soria Natural® (Spain)* ***(3)** Isola Bio®; Abafoods SL(Italy)* ***(4)** La finestra sul Cielo® (Italy)*

***(5)** LIMA® (France)* ***(6)** The Bridge S.L. (Italy)* ***(7)** Alinor® (Italy)* ***(8)** Naturgreen®; Lab. Almond S.L.(Spain)*

***(9)** OALTY® (Sweden)* ***(10)** Liquats vegetals S.L. (Spain)* ***(11)** Vegetalia® (Spain)* ***(12)** Costa Concent. Levant., S.A. (Spain)*

***(13)** So-delicious® dairy-free (USA)* ***(14)** Silk ® (USA)* ***(15)** Pacific® (USA)* ***16)** Vitasoy Int. Holdings Ltd. (Japan)*

***n.a.:** data not available* ***SFA**: saturated fatty acids* ***PUFA**: poly-unsaturated fatty acids* ***MUFA**: mono-unsaturated fatty acids*

The final milky product chemical composition of major components (protein, lipids and carbohydrates) in different types of cereals is summarised in Table 3. Considering these *milks* are to be used as substitutes for cow milk, it is remarkable that there is a high fibre content and a low lipid content with a better profile than standard milk.

3.2 Nut *milk* processing

The general industrial flow diagram of nut *milk* production is presented in Figure 2. The steps shown are mostly the same as in cereal *milk* processing but the enzymatic treatment is removed, since the low starch content of these nuts do not confer a negative effect on the viscosity of the final product.

Nut conditioning consists of washing and selection, plus a blanching treatment in order to facilitate both the further peeling of nuts and the initial microbial load reduction. In view of the fact that no enzymes are used, possible food additives such as emulsifiers, sweeteners, hydrocolloids and/or flavours can be introduced during the grinding step, and thereby facilitate the optimal dispersion of ingredients. The types of food additives chosen are the same as the ones mentioned in cereal *milk* processing.

The final milky product chemical composition of major components (proteins, lipids and carbohydrates) in different types of tree nuts is summarised in Table 3. In spite of the fact that the over 50% of a nut's content is made up of lipids (Table 1), final contents end up with lower values than non-defatted cow milk, which varies from 3 to 5 g/100 mL. Moreover, considering these vegetable *milks* are to be used for the same purposes as cow milk, the MUFA/SFA (Saturated Fatty Acids) ratio is much higher than other

animal milks and, hence, they are healthier. Almond *milk* stands out among other nut *milks* as being an appropriate alternative to cow milk, since, besides the lipid profile, it has a low ratio of Na/K and a balanced ratio of Ca/P (Luengo, 2009).

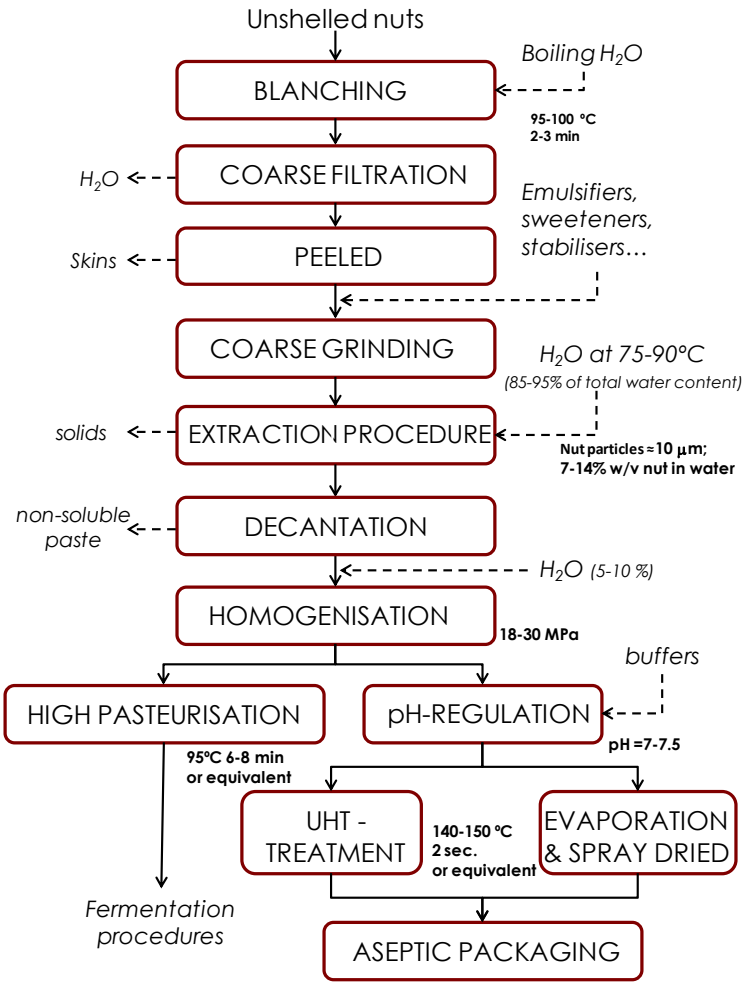

Figure 2 Typical nut *milk* processing flow diagram.

In the production of tigernut *milk*, the pre-conditioning step is more complex: in the extraction procedure, tigernuts need to be softened prior to the milling process (by rehydration with water for around 18 hours) and a preliminary germicidal treatment (active chlorine) is required to decrease the initial microbial load (it is a tuber) (CRDO, 2012; Sanful, 2009). Also, an enzymatic treatment is required due to the high starch content of this nut, as has been commented on above for the cereal *milk* manufacture.

In industrial coconut *milk* production, the coconut meal has to be obtained by shelling, paring and washing. After that, coconut meal is submitted to a blanching process reinforced with chemical agents, such as $NaHSO_3$, for different purposes: facilitate the removal of the brown testa, enhance oxidation stability due to inner enzyme denaturation and facilitate further grinding. Once coconut meat is ground and water extraction is developed, the milky liquid is finally filtrated using double layers of cheese cloth (Mepba *et al.*, 2009).

The major technological problem found during the processing or shelf life of these cereals and nut derived *milks* is related with the low physical stability of the liquid dispersion, usually with low viscosity, which promotes the phase separation of the unstable fat globules caused by flocculation and coagulation phenomena in a short period of time. Moreover, fibres and non-soluble material will also separate, either by sedimentation or floatation, thus contributing to the watery effect of the product. The employment of an optimal thermal treatment and homogenisation pressures during the *milk* processing, the addition of both amphiphilic compounds and hydrocolloids or the use of UHPH could contribute to the development of an excellent product

with great sensory attributes. Using UHPH allows for a longer shelf life of the product, since greater physical stability is achieved mainly due to a reduction in the size of the fat globule that prevents coalescence phenomena. Figure 3 shows pictures of hazelnut *milk* obtained by means of the Confocal laser scanning microscopy (CLSM) technique in which the effect of UHPH is proved; as can be seen, almost all the fat globules in untreated *milk* (Fig. 3A) are coalesced, while in that which has been UHPH-treated (Fig. 3B), not only are the fat globules non-coalesced but they are also distributed, forming a kind of network that enhances physical stability. Sometimes homogenisation pressures are also capable of reducing the microbial load of the product prior to the thermal treatment, if they are greater than 200 MPa (Cruz *et al.*, 2007; Pereda *et al.*, 2007; Valencia-Flores *et al.*, 2013).

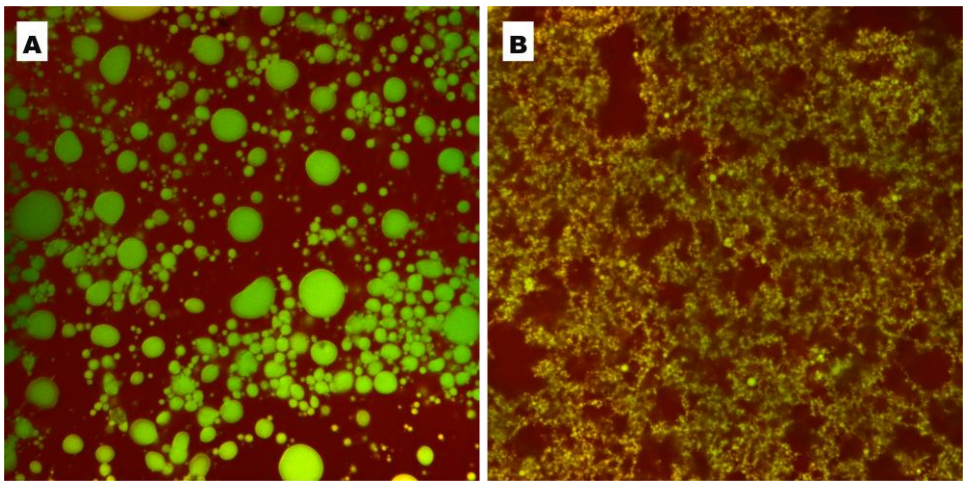

Figure 3. Confocal pictures of hazelnut *milk* non-treated (A) and homogenised at 172 MPa (B), where fat globules appeared green-yellow coloured.
***Source*: personal compilation.**

Despite the advantages, this emergent technology is being only used in a laboratory and pilot-scale due to the high investment costs. Hence, as can be seen on Table 3, vegetable *milk*'s processing industries are normally using hydrocolloids and emulsifiers to prevent phase-separation phenomena in developed products.

4. FERMENTATION OF VEGETABLE *MILKS*

Besides the direct consumption of vegetable *milks*, they might be also used as raw materials to develop yoghurt-type products, as has been done with soy *milk*. Hence, these newly fermented products would satisfy a market sector focused on the current consumers' demand of non-dairy products.

In this regards, nut and cereal *milks* are considered as good substrates for the growth of different strains, owing to the presence of non-digestible components with prebiotic properties in both vegetable matrices. Thus, starch and fibre materials are reported to enhance the physical stability of the fermented vegetable *milk* and to promote the survival of the starters used, not only due to their nutritional contribution but also, since fibres are resistant to gastric juices, they act as protective barriers within the human gastrointestinal tract, (Bosnea *et al*., 2009; Patel *et al*., 2004; Perrin *et al*., 2000; Wang *et al*., 1999). Nevertheless, the ability of the starter microorganism to grow in these vegetal raw materials varies largely with the strain. Therefore, studies into bacterial survival are required prior to processing the fermented product.

Most of these innovative fermented products found in the literature have been developed by using probiotic bacteria from bifidobacteria, lactobacilli

and Streptococcus genera. If probiotic bacteria are used as starter microorganisms, the newly product designed would have an added value, owing to the healthy benefits that these type of bacteria can exert. Although oat is often used as a raw material, other matrices have also been studied, such as almond, hazelnut or rice. A list of them has been summarised in Table 4.

Table 4. Main characteristics of vegetable non-soya fermented products found in the literature.

Raw material	Probiotic bacteria used	Growth enhancer (prebiotic) present and/or added	Reference
Malt, barley and wheat	*L. plantarum, L. acidophilus*	FOS, β-glucan, starch and other dietary fibres	Charalampopoulos *et al.*, 2002
	L. plantarum	FOS, β-glucan, starch and other dietary fibres	Charalampopoulos and Pandiella, 2010
Wheat, oat and barley	*L. plantarum*	FOS, β-glucan, starch and other dietary fibres	Salmeron *et al.*, 2009
Oat	*L. plantarum*	β -glucan and other dietary fibres	Angelov *et al.*, 2006; Gupta *et al.*, 2010
	L. reuteri, L. acidophilus, B. bifidum	β -glucan and other dietary fibres	Mårtensson *et al.*, 2002
	L. plantarum, L. paracasei ssp.casei, L. acidophilus	Inulin, β -glucan and other dietary fibres	Gokavi *et al.*, 2005
Rice-based	*L. acidophilus, L. casei ssp. rhamnosus*	Starch	Boonyaratanakornkit and Wongkhalaung, 2000
Maize	*B. lactis*	Starch, pentosan and other dietary fibres	McMaste *et al.*, 2005
	L. paracasei, L. casei, L.	Inulin, starch, pentosan	Nyanzi *et al.*, 2010

	rhamnosus, L. acidophilus	and other dietary fibres	
Maize and barley	*L. reuteri, L. acidophilus, L. rhamnosus*	Starch, pentosan and other dietary fibres	Helland *et al.*, 2004
Emmer wheat	*L. rhamnosus, other LAB*	FOS and other dietary fibres	Coda *et al.*, 2011
Coconut	*L. paracasei, B. lactis*	Starch and dietary fibre	Corrêa *et al.*, 2008
Chestnut	*L. rhamnosus*	Starch and other dietary fibres	Blaiotta *et al.*, 2012
Walnut	Kefir grain microorganisms with potentially probiotic effects	Starch and other dietary fibres	Cui *et al.*, 2013
Tigernut, almond or hazelnut	*B. lactis, S. thermophilus*	Starch, FOS and other dietary fibres	Pérez-Martínez *et al.*, 2005

B: Bifidobacterium **L:** Lactobacillus **S:** Streptococcus

4.1. Processing of fermented vegetable *milk*

General industrial processing used to develop nut and cereal fermented vegetable products is based on four main steps: the procedure to obtain vegetable *milk* previously commented on above (Figures 1 and 2), conditioning the *milk* until the optimal starters' growth temperature is reached, the inoculation and incubation procedures (fermentation) and cooling to 4 ºC. Nevertheless, depending on the raw material, the type of starters used and the final product features, the whole process may differ.

Some additives are frequently introduced into the vegetable matrix, mainly sugars and prebiotics (as growth enhancers), to promote the viability of bacteria and to reduce the length of the fermentation process (in order to avoid cross-contamination problems). Mono and oligosaccharides, some

prebiotics such as inulin, β-glucans and dietary fibres have been the growth enhancers most commonly used by different authors (Akalin *et al.*, 2007; Gokavi *et al.*, 2005; Özer, Akin and Özer, 2005; Rosburg *et al.*, 2010; Sendra *et al.*, 2008). Potentially, prebiotics are naturally present in both cereals and nuts (i.e. dietary fibre); nevertheless, prebiotic compounds are sometimes added in order to increase the product's health benefits or its technological properties, since the majority are able to increase the viscosity of the *milk*. On the other hand, hydrocolloids, such as carrageenan and xanthan gum, are often added to prevent syneresis and, thus, ensure the physical stability of the product during the stated shelf life. Nonetheless, if the raw material used naturally contains these types of compounds, it might not be necessary to add them during the industrial processing (i.e. β-glucans present in oat *milks*).

Prior to the addition of the starter inoculum, pathogen-free *milks* must be conditioned until fermentation temperature. This is usually around 37 ºC, but it depends on the optimal growth temperature of the starter bacteria used. With regards to the fermentation time, much longer is usually needed than during standard cow milk yoghurt production, since potential probiotic bacteria (type of bacteria currently chosen to develop these foodstuffs due to the added value on the final product) have more complex nutritional requirements (Severson, 1998), especially when growing in vegetable matrices. The reported fermentation times have been found to be around 16-24 h if not grow enhancers are used in the formulation (Charalampopoulos and Pandiella, 2010; Charalampopoulos *et al.*, 2002; Coda *et al.*, 2011; Corrêa *et al.*, 2008; Blaiotta *et al.*, 2012; Cui *et al.*, 2012; Gokavi *et al.*, 2005; Gupta *et al.*, 2010; Mårtensson *et al.*, 2002). The fermentation procedure

finishes when the pH of *milks* reaches 4.2-4.5 values. Immediately afterwards, the fermented products are sealed, cooled to 4 ºC and stored at refrigeration temperatures.

The major challenges affecting these fermented products are related with the sensory quality (appearance and texture) and the resistance of the probiotic microorganisms. Most of these fermented products might have physical stability problems caused by phase separation between components (usually proteins coagulate, forming a non-continuous weak gel, and serum separation occurs at the very beginning of storage time or during storage period). This structure can be observed in Figure 4, where the microstructure is shown of an oat fermented *milk* with probiotic *L. reuteri* and *S. thermophilus* obtained by using CLSM. As can be observed, the aqueous continuous phase is not completely entrapped in the non-continuous protein-fibre network.

The appearance of these products is often very similar to that observed in a low-fat stirred yoghurt type. To promote physical stability during the acidification process, hydrocolloids are generally used, as has been commented on above. The most common hydrocolloids used as thickening agents are xanthan gum, modified starches, pectin and cellulose derivatives, among others. Nevertheless, other tools have been used to promote better textural properties. For instance, Mårtensson *et al.* (2000) selected strains able to produce exopolysaccharides (EPS) in order to obtain oat-based fermented products. Yoghurts with EPS-producing bacterial strains showed higher viscosity and less phase separation in comparison with yoghurt made with strains not producing EPS. This structural property would give rise to a

new generation of in situ produced thickeners. This is of general interest, as there is an increasing demand from manufactures to decrease the addition of stabilisers in yoghurt products.

Cruz *et al.* (2009) instead, studied the effect of UHPH treatments (around 250-300 MPa) in soy *milk* on the fermentation processing: This technology is quite similar to the conventional high-pressure homogenization used in the food industry but considerably higher pressures are applied. Some benefits have been reported for its application in the food industry as it causes interesting changes in structural components and increases shelf life of liquid products. The results showed that UHPH soy-yoghurts displayed greater firmness and higher water holding capacity than gels produced from conventional homogenised samples.

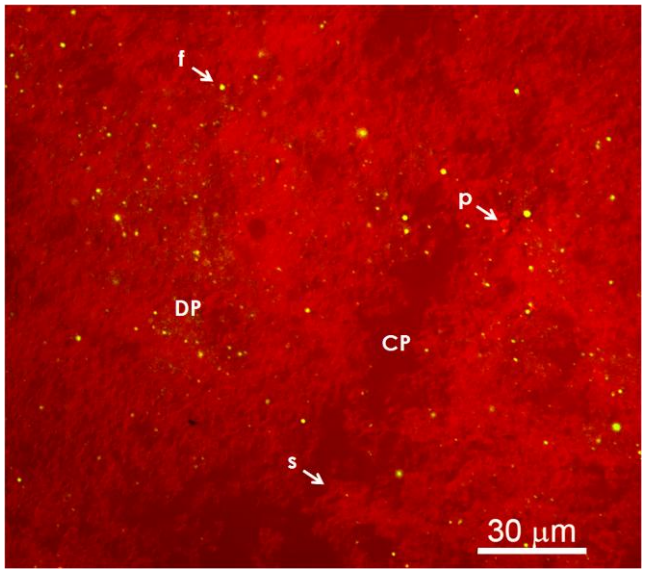

Figure 4. Microstructure obtained by CLSM of fermented oat *milk* with a mixed culture of L.*reuteri* and S.*thermophilus*. (CP: Continue phase; DP: Dispersed phase; f: fat globule; s: starters + other fibres). *Source: personal compilation.*

On the other hand, to enhance the resistance of probiotics during the product´s entire shelf life, new approaches are being taken by different researchers, such as the use of oxygen-impermeable containers, two-step fermentations and the incorporation of micronutrients into the matrices (peptides and/or amino-acids). Finally, the use of microencapsulation techniques has also been studied as a means of promoting the survival of the probiotic bacteria through the gastrointestinal tract (ability to resist gastric juices and bile) (Soccol *et al.*, 2010).

5. CONCLUSIONS

The development of nut and cereal products, fermented or not, by means of probiotic bacteria fully meets the current trends towards an increased consumer demand for healthier products, mainly because of the close relationship between the consumption of vegetable products and the prevention of cancer, atherosclerosis or inflammatory diseases, as has been claimed by some official American and European organisations. In this sense, the demand for and consumption of these products is expected to rise in the next few years, especially in that section of the population which is more aware of health issues. Moreover, and taking into account the low requirements for producing nuts and cereals, the high market potential of these products would be used in a non-far future to increase richness in developing countries by implementing nut and cereals crops such as almonds and oats in non-cultivated lands.

In the development of these products, some important technological deficiencies have been found, mainly related to the product´s physical stability during its entire shelf life. To this end, the optimisation of processing techniques must be encouraged and more studies focusing on the microstructure and arrangement of the different components of the products after processing are needed in order to clarify and understand how to improve the appearance and texture of the final product. These studies would also allow to decrease the amount of additives added (hydrocolloids and/or emulsifiers) and thus, to reduce the economic costs. In the particular case of fermented products, the use of proper starter microorganisms able to both improve the quality (by synthesising proper flavors and providing optimal textures) and exert healthy benefits to consumers (i.e. probiotics) is the main challenge to be faced in future studies.

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II. APORTE CIENTÍFICO DEL TRABAJO

La información aportada en la introducción muestra el gran interés nutricional y beneficios para la salud de las *leches* vegetales y productos derivados, obtenidos a partir de frutos secos y cereales, en especial para grupos de población específicos (lacto-intolerantes, alérgicos a la leche de vaca, vegetarianos…).

La presente tesis doctoral pretende contribuir a la mejora tecnológica del procesado tanto de las *leches* de cereales y frutos secos como de los productos fermentados derivados, en concreto en almendra, avena y avellana, debido principalmente a tres factores: la escasa información encontrada en la bibliografía por tratarse un de tema relativamente novedoso, las deficiencias encontradas en las *leches* vegetales comerciales disponibles en el mercado actual (la gran mayoría presenta separación de fase y baja calidad sensorial) y por la posibilidad de desarrollar nuevos productos (como los fermentados derivados) en un mercado altamente competitivo como es el de los productos alimentarios.

Además, y con el objetivo de satisfacer la demanda actual de productos saludables, se incluye el uso de bacterias probióticas incorporadas a estas matrices vegetales a través del desarrollo de productos fermentados. De este modo, se podrían desarrollar en un futuro próximo nuevos productos saludables aptos para grupos de población específicos, que podrían utilizarse como tratamiento preventivo de determinadas enfermedades crónicas, tales como enfermedades cardiovasculares, intolerancias, dermatitis atópica u otras alergias o, incluso, algunos tipos de cáncer.

Por último, el trabajo pretende conocer posibles beneficios del consumo de fermentados de *leche* de almendra, con distintas bacterias potencialmente

probióticas, sobre la respuesta inmune y mejora de la biodisponibilidad de hierro. Hay que tener en cuenta que las caseínas de la leche de vaca dificultan la absorción del hierro no-hemo, además de estar dentro del listado de componentes alérgenos. Los resultados derivados servirían, por tanto, para aumentar el conocimiento entorno a las propiedades nutricionales y saludables que éstos podrían ejercer en cada uno de los grupos de población específicos y en la población en general.

III. OBJETIVOS

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III.i Objetivo general

El objetivo de la presente tesis doctoral consiste en desarrollar y caracterizar licuados vegetales (más conocidos como *leches* vegetales) y sus productos fermentados derivados para contribuir a la mejora tecnológica de su procesado a escala industrial y que sirvan de alternativa de consumo a los lácteos de origen animal en grupos de población específicos como vegetarianos, intolerantes a la lactosa o alérgicos a las proteínas de la leche de origen animal. Para ello, se seleccionarán previamente tanto las *leches* vegetales con las mejores propiedades funcionales como las cepas de microorganismos con efecto probiótico, aportando así un valor añadido al producto final. Las *leches* seleccionadas se reformularán y optimizarán para favorecer un alto contenido en probióticos en el producto fermentado y unas características fisicoquímicas y sensoriales en su vida útil adecuadas para su consumo.

III.ii Objetivos específicos

- Evaluar y seleccionar *leches* vegetales y cepas probióticas que presenten buenas condiciones para procesos de fermentación en base a su calidad composicional, sensorial y sus propiedades nutricionales y/o saludables.
- Obtener las condiciones óptimas de procesado de las *leches* vegetales para su uso como materia prima para la elaboración de productos fermentados a través del estudio fisicoquímico del sistema,

microestructura (distribución de los distintos componentes en la matriz vegetal) y de la evaluación de la calidad. Para ello, se analizarán diferentes condiciones de procesado y reformularán las *leches* vegetales, previamente seleccionadas, para asegurar una buena estabilidad tanto física como microbiológica del producto y, además, que asegure un crecimiento óptimo de los microorganismos utilizados como cultivo iniciador en los procesos fermentativos.

- Optimizar los procesos fermentativos utilizando como matrices las *leches* vegetales caracterizadas y los microorganismos probióticos seleccionados.
- Caracterizar y determinar la vida útil de los productos fermentados optimizados a través de la evolución de las diferentes propiedades fisicoquímicas, viabilidad microbiológica y atributos sensoriales durante su almacenamiento en refrigeración.
- Analizar la viabilidad de las bacterias probióticas en la *leche* fermentada en procesos de digestión *in vitro* con el fin de asegurar el posible efecto funcional del producto desarrollado.
- Evaluar las posibles propiedades anti-inflamatorias de los fermentados finales mediante estudios *in vitro* sobre células que simulan el epitelio intestinal.

IV. RESULTADOS Y DISCUSIÓN

Se seleccionaron las *leches* de almendra y avellana para desarrollar productos fermentados, al tratarse de frutos secos de gran interés composicional. Además de sus propiedades nutricionales, entre las que destaca el alto contenido en vitamina E (antioxidante) y un perfil lipídico rico en ácidos grasos insaturados, se ha visto que ambos frutos son capaces de reducir el colesterol en sangre y prevenir enfermedades cardiovasculares, entre otros beneficios. A pesar de que estas *leches* no son tan conocidas y comercializadas como los frutos de las que derivan, en España siempre ha habido una tradición en el consumo de *leche* de almendra, especialmente en la costa mediterránea, y su consumo se recomienda en grupos de población específicos (embarazadas y lacto intolerantes) como sustitutivo de la leche de vaca por su similitud con respecto a la relación calcio/fósforo. La *leche* de avellana, por otra parte, es muy aromática y podría influir positivamente en la aceptación sensorial del fermentado a desarrollar.

Otro tipo de *leches* vegetales en auge son las procedentes de cereales, menos apreciadas sensorialmente pero con propiedades nutricionales muy demandadas en el mercado, como es la fuente de fibra. En especial, el β-glucano (fibra soluble de la avena clasificada como prebiótico) es efectivo en la reducción de colesterol en sangre, prevención de enfermedades cardiovasculares o estimulación de las bacterias beneficiosas de nuestra microflora intestinal. Por ello se ha seleccionado la *leche* de avena como buena matriz para el desarrollo de fermentados, teniendo en cuenta que la presencia de componentes prebióticos (β-glucano) podría estimular el crecimiento de las bacterias a utilizar en el proceso fermentativo.

Tanto la *leche* de almendra como la de avellana son emulsiones poco estables por lo que se estudiaron las condiciones de procesado óptimas para asegurar una vida útil comercial a través del estudio de tratamientos combinados de altas presiones de homogeneización y tratamientos térmicos, una vez asegurada la calidad microbiológica del producto. Para ello, se realizaron diferentes combinaciones de presiones de homogeneización e intensidad del tratamiento térmico y se analizaron las características fisicoquímicas y de estabilidad física del producto obtenido, así como su estructura a nivel microscópico. A partir de estos resultados se pudieron escoger las presiones de trabajo y tratamientos térmicos óptimos para los estudios posteriores. Este trabajo constituye el Capítulo 1 del presente apartado. La *leche* de avena, en cambio, gracias a los β-glucanos, es muy estable físicamente incluso tras someterse a tratamientos convencionales de esterilización.

Una vez optimizado el proceso de obtención de las *leches*, se pasó al diseño del proceso fermentativo, cuyos estudios vienen resumidos en el Capítulo 2. Teniendo en cuenta que el perfil del consumidor de *leches* vegetales se caracteriza por su preocupación por mantener dietas saludables, se optó por el uso de bacterias probióticas para la fermentación de las *leches* elegidas, lo que además supondría un valor añadido al nuevo producto. Sin embargo, estas bacterias suelen ser más sensibles a las condiciones ambientales en las que se encuentran y más exigentes nutricionalmente. Las *leches* vegetales por lo general tienen poco contenido en azúcares (principal fuente de carbono de las bacterias), por lo que se estudió y optimizó una formulación en la que se asegurase una adecuada supervivencia del

microorganismo con un tiempo de fermentación lo más corto posible para conseguir un proceso industrial viable. Una vez optimizado, los productos fermentados obtenidos se caracterizaron a lo largo del tiempo de almacenamiento en refrigeración en sus principales propiedades fisicoquímicas y sensoriales, así como de supervivencia del probiótico que permitiera así cumplir con las recomendaciones para productos probióticos.

Entre los nuevos productos fermentados diseñados, se seleccionó el de almendra para analizar los posibles efectos funcionales que esta nueva bebida podría aportar por tratarse de un producto probiótico. Según la bibliografía consultada, las bacterias probióticas utilizadas se ha demostrado que son capaces de modular nuestro sistema inmune e incluso hay estudios en los que se han utilizado con éxito para el tratamiento de dermatitis atópica o eczemas en población infantil. En este sentido, se pensó que la matriz vegetal en la que se encuentran podría influir positivamente; es decir, que hubiese un efecto sinérgico entre las propiedades de la almendra (rica en compuestos antioxidantes) y las del probiótico. Por otra parte los probióticos, al igual que otras bacterias ácido lácticas, presentan actividad proteolítica, pues necesitan el nitrógeno que aportan las proteínas y/o péptidos presentes en el medio en que se encuentran para su crecimiento. Algunas de las proteínas de las almendras son potenciales alérgenos, por lo que el proceso fermentativo podría reducir su efectividad y/o eliminarla, ya que la reactividad de las proteínas es dependiente de sus características bioquímicas y estructurales. En el tercer capítulo de este apartado se presentan los estudios *in vitro* realizados sobre los fermentados de *leche* de

almendra diseñados para analizar la hipótesis inicial sobre su efecto funcional.

Capítulo I

Elección de las "*leches*" vegetales a fermentar y definición de las condiciones de su procesado para asegurar estabilidad física, microbiológica y sensorial

Effect of high pressure homogenisation and heat treatment on physical properties and stability of almond and hazelnut *milks*

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ABSTRACT

The effect of high pressure homogenisation (HPH) and heat treatments on physicochemical properties and physical stability of almond and hazelnut *milks* was studied. Vegetable *milks* were obtained and homogenised by applying 62, 103 and 172 MPa (MF1, MF2 and MF3, respectively). Untreated and MF3 samples were also submitted to two different heat treatments (85 ºC/30 min (LH) or 121 ºC/15 min (HH)). Physical and structural properties of the products were greatly affected by heat treatments and HPH. In almond *milk*, homogenised samples showed a significant reduction in particle size, which turned from bimodal and polydisperse to monodisperse distributions. Particle surface charge, clarity and Whiteness Index were increased and physical stability of samples was improved, without affecting either viscosity or protein stability. Hazelnut beverages showed similar trends, but HPH notably increased their viscosity while change their rheological behaviour, which suggested changes in protein conformation. HH treatments caused an increment of particle size due to the formation oil droplet-protein body clusters, associated with protein denaturation. Samples submitted to the combined treatment MF3 and LH showed the greatest stability.

Key words: Particle size, DSC, viscosity, ζ-potential, confocal.

1. INTRODUCTION

In the last few years, the population ratio demanding vegetable-based products is growing, either because of the increasing problems related with the intolerances to cow milk (Fiocchi *et al.*, 2010) or because of changes in the food preferences. As a consequence of new consumer tendencies, food industries are currently producing new, high quality, nutritionally improved products with added value. Vegetable-based "milks" are included in these new products, which are available at any supermarket as an alternative to dairy products, with an increasing consumer acceptance.

There is a wide variety of vegetable-based *milks*, although most of the research activity has been focused on those obtained from soy. For soy *milk*, studies into the physicochemical characterisation, the effects of processing, the application of new technologies, such as electric pulses and ultra-high homogenisation pressures have been carried out (Cruz *et al.*, 2007; Li *et al.*, 2008).

Research dealing with the use of non-soy vegetable *milk* is still scarce and most of it is related with the nutritional quality of such products. In this sense, almond and hazelnut *milks* have been used as an alternative to cow milk in lacto-intolerant people, pregnant women and celiacs, due to their high levels of calcium, phosphorous and potassium (Eroski Foundation, 2007; Luengo, 2009). These nuts have low sodium content and an equilibrated mono-unsaturated fatty acid-polyunsaturated fatty acids ratio, which define the products which are healthy for people with heart disease (Mateos, 2007). They are also considered helpful for maintaining

cholesterol at healthy levels due to their high content of antioxidant compounds which contributes to heart disease prevention (Fraser *et al.,* 2002; Jenkins *et al.,* 2008; Kris-Etherton *et al.,* 2008; Tey *et al.,* 2011).

Vegetable based *milks* are emulsified products where the nut fat is dispersed in an aqueous phase and where the rest of the components play different roles in the product stability. The different process steps, such as homogenisation and heat treatments usually produce changes in the arrangement of components, thus leading to modifications in the particle size, colour, viscosity and physical stability of the product. These physicochemical modifications have to be known to efficiently control the process and to implement the necessary improvements in the production lines. The most commonly homogenisation pressures used in the food industry range between 20 and 50 MPa, although much higher pressures are used in high pressure homogenisation (HPH) processes with some advantages: the deflocculation of clusters of primary fat globules (Floury *et al.,* 2000) and uniform dispersion of agglomerates, the changes in protein conformation (Pereda *et al.,* 2009), the increase in emulsion viscosity (Desrumaux and Marcand, 2002) and stability and the microbial inactivation (Diels *et al.,* 2005; Pereda *et al.,* 2007; Cruz *et al.,* 2007).

The objective of the present study is to analyse the effect of heat treatments and high homogenisation pressures on the physical properties and stability of almond and hazelnut *milks* in order to define processing conditions which ensure the product quality and stability.

2. MATERIALS AND METHODS

2.1 Preparation of almond and hazelnut *milks*

Nut *milks* were produced by soaking and grinding almonds (*Prunus amygdalus L.* cv. dulcis) and hazelnuts (*Corylus avellana* cv. comuna*)*, supplied by Frutos Secos 3G S.L. (Valencia, Spain). The extraction was carried out in Sojamatic 1.5 (Sojamatic®; Barcelona, Spain), equipment specifically designed for the production of vegetable *milks*, with a nut-water ratio of 8:100. The manufacturing process takes 30 minutes at room temperature. The milky liquid obtained was used as control sample (untreated).

2.2 High pressure homogenisation and heat treatments

High pressure homogenisation (HPH) treatments were carried out in a high pressure homogeniser (M-110P model; Microfluidics International Corporation, USA) by applying 62, 103 and 172 MPa (samples MF1, MF2 and MF3 respectively). Some samples were submitted to a low temperature heat treatment (LH) at 85 ºC for 30 min and to a high temperature heat treatment (HH), 121 ºC for 15 min. The heat treatment conditions chosen were those in which the destruction of all vegetative cells and enzymes are ensured (Walstra *et al.,* 2006). Samples submitted to heat treatment were the control samples (LH and HH samples) and those homogenised at 172 MPa (MF3LH and MF3HH samples).

2.3 Characterisation of chemical composition

The quantification of moisture, ashes, fat content, proteins and sugars was carried out in the nut *milks*. Fibre content was estimated by means of the difference in terms of component percentages. Beverages were freeze-dried (ioalfa-6 freeze-dryer, TELSTAR; Terrassa, Spain) prior to the analysis. AOAC Official Methods were chosen to determine water, total fats and total nitrogen contents (AOAC 16.006, AOAC 945.16 and AOAC 958.48, respectively) (Horwitz, 2000). Total sugars and ashes were obtained following the protocols suggested by Matissek *et al.,* (1998). All the determinations were performed in triplicate.

2.4 Characterisation of physical and structural properties

2.4.1 pH and density

Measurements were carried out at 25 ºC using a pH-meter (GLP 21+, Crison Instruments S.A.; Barcelona, Spain) and a digital densitometer (DA-110 M, Mettler Toledo; Barcelona, Spain), respectively. These determinations and those described below were carried out in triplicate.

2.4.2 Particle size distribution and ζ-potential

Analysis of the particle size distribution was carried out using a laser diffractometer (Mastersizer 2000, Malvern Instruments Ltd; Worcestershire, 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 obtained. $D_{3,2}$ (surface weighted

mean diameter) and $D_{4,3}$ (volume weighted mean diameter) were obtained. The volume-weighted average diameter is sensitive to the presence of large particles, whereas the surface-weighted average diameter is more sensitive to the presence of small particles.

ζ-potential was determined at 20 ºC by using a Zetasizer nano-Z (Malvern Instruments Ltd; Worcestershire, UK). Samples were diluted to a fat droplet concentration of 0.4 g/100 mL using 0.02 mol/L phosphate buffer solution. The Smoluchowsky mathematical model was used to convert the electrophoretic mobility measurements into ζ-potential values.

2.4.3 Rheological behaviour

The rheological behaviour of nut *milks* were characterised by using 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 112 s^{-1}. The up and down curves were obtained, taking 1 minute to rise and 1 minute 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 100 s^{-1} (Eq. 2).

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

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

2.4.4. Optical properties

Colour coordinates were measured from the infinite reflection spectrum in a spectrocolorimeter (CM-3600 d, MINOLTA Co; Osaka, Japan). CIE $L^*a^*b^*$ coordinates were obtained using illuminant D65/10º observer. Colour of samples was characterised as to Lightness (L^*), Chrome (C^*_{ab}), hue (h^*_{ab}) and Whiteness Index (WI) as defined in Eq. (3) to (5). Colour difference (ΔE) between treated and untreated samples was also calculated by using Eq. (6).

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

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

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

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

2.4.5. Protein denaturation

The protein denaturation degree in each sample was analysed by Differential Scanning Calorimetry in DSC 220 calorimeter (CU-SSC5200, Seiko Instruments; USA). Prior to the analyses, samples were freeze-dried (ioalfa-6 free-dryer, TELSTAR; Terrassa, Spain) and afterwards rehydrated with 70 g/100 mL of water. 25 mg of rehydrated samples were introduced in hermetic aluminium capsules (P/N SSC000C008, Seiko Instruments, USA). An empty capsule was used as reference. Sample heating was carried out from 25 ºC to 120 ºC at 5 ºC/min. From the obtained thermograms (heat flux vs. temperature), the peak temperature and enthalpy for protein denaturation were obtained.

2.4.6 Confocal laser scanning microscopy (CLSM)

A Nikon confocal microscope C1 unit, which was fitted on a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan), was used. An Ar laser line (488 nm) was employed as light source to excite fluorescent dyes Rhodamine B and Nile Red. Rhodamine B (Fluka, Sigma-Aldrich, Missouri, USA) with λex max 488 nm and λem max 580 nm was dissolved in distilled water at 0.2 g/100 mL. This dye was used to stain proteins and carbohydrates. Nile Red (Fluka, Sigma-Aldrich, Missouri, USA) with λex max 488 nm and λem max 515 nm was dissolved in PEG 200 at 0.1 g/L. This dye was used to stain fat. An oil immersion objective lens (60x/1.40NA/Oil/ Plan Apo VC Nikon) was used.

For sample visualisation a microscopy slide was elaborated with two razor blades (platinum coated double edge blades with 0.1 mm thickness) stuck to a glass. 20 μL of the sample were placed on the microscope slide, within the central gap of the blades; 10 μL of Rhodamine B solution and 10 μL of Nile Red solution were added and the cover slide was carefully positioned. Observations were performed 10 min after diffusion of the dyes into the sample. Images were observed and stored with 1,024 x 1,024 pixel resolution, using the microscope software (EZ-C1 v.3.40, Nikon, Tokyo, Japan).

2.4.7 Colloidal stability of *milks*

Colloidal stability of the obtained products was determined through the phase separation analysis throughout storage time (28 days) at 4 ºC, in all samples. To this end, about 15 g of almond and hazelnut *milks* were poured

into glass tubes of 16 mm diameter and the height of the separate phases was quantified. 0.04 g/100 mL of sodium azide was added to samples, thus assuming no microbial growth took place during storage.

2.5 Statistical Analysis

Results were analysed by analysis of variance with 95% significance level using Statgraphics® Centurion XV. Multiple comparisons were performed through 95% LSD intervals.

3. RESULTS AND DISCUSSION

3.1 Chemical composition

Chemical composition of both types of nuts and their derivative *milks* has been summarised in Table 1.The obtained composition of both nuts was similar to those found by other authors for the same varieties of these products (Luengo, 2009; Saura *et al.*, 1988). Composition of both beverages was quite similar, although the protein content, and so the protein-fat ratio, were greater in almond, coherent with the higher content of this component in almonds. In comparison with cow milk (3.2 and 3.4 g/100 mL of fat and protein, respectively), these vegetable *milks* have a slightly higher fat content and a lower protein content. Nevertheless, they contain vegetable fibre and according to Gallier *et al.* (2012), 68 and 23 g/100 g of monounsaturated and polyunsaturated fatty acids respectively are present in the lipid fraction of almond *milk*.

Table 1. Chemical composition (g/100 g product) of almond and hazelnut nuts and derivative *milks*. Mean values and (standard deviation).

Composition (g/100 g)	Almond nut	Almond *milk*	Hazelnut	Hazelnut *milk*
Moisture	3.06 (0.05)	93.4 (0.5)	3 (1)	94.1(0.5)
Lipid	55.77 (0.29)	3.96 (0.2)	62.4 (0.4)	4.02 (0.00)
Ashes	3.86 (0.06)	0.325 (0.012)	3.14 (0.11)	0.20 (0.04)
Total sugars	4.9 (0.4)	0.030 (0.002)	4.13 (0.25)	0.03 (0.00)
Protein	25.55 (0.12)	1.37 (0.03)	13.43 (0.12)	0.65 (0.05)
Fibre	6.82	0.58	14.28	0.40
Dry matter	96.94 (0.05)	6.64 (0.5)	97 (1)	5.3 (0.4)

3.2 pH and density

In almond *milk*, no significant differences were found in pH and density values between the samples submitted to the different treatments ($p > 0.05$), the average values being 6.66 ± 0.08 and 1001.1 ± 0.1 kg/m^3, respectively. Regarding to hazelnut *milk*, non-treated samples showed a pH value of 6.66 ± 0.02 and a density of 1001.2 ± 0.4 kg/m^3, whereas in treated samples (regardless of the treatment) a slight increase in pH (6.81 ± 0.08) and a decrease in density (mean value for treated samples: 995.4 ± 0.6 kg/m^3) was observed. This might be explained by the fact that thermal or pressure effects could cause conformational changes in components (especially biopolymers) which may inhibit the ionisation of some acidic groups and induce small changes in density.

3.3 Particle size distribution and ζ-potential

Figure 1 shows the typical particle size distribution obtained for one of the *milks* (almond) as affected by the homogenisation and thermal treatments. Similar behaviour was found for hazelnut *milks*.

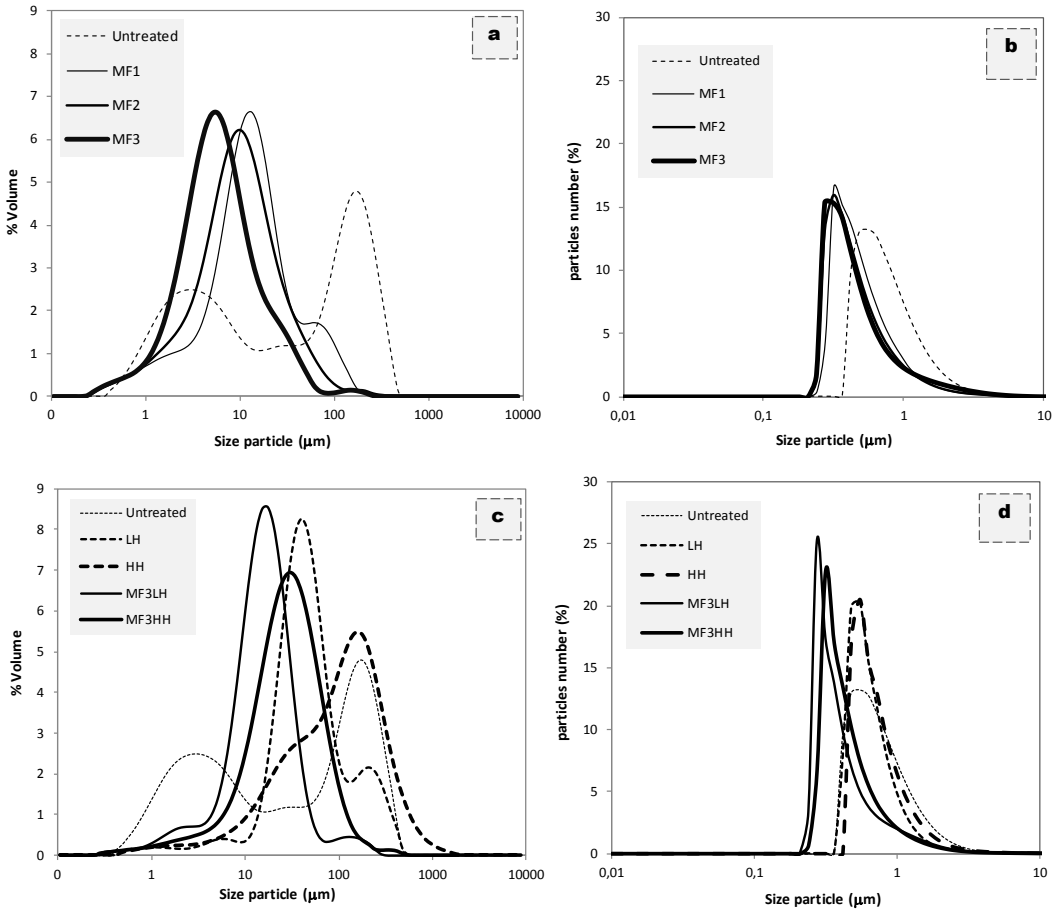

Figure 1. Typical particle size distribution curves for the untreated and treated almond *milks* in terms of percentage of volume (a,c) and percentage of number of particles (b,d). (MF = homogenised samples; HH = High Heat treated samples; LH = Low Heat treated samples; MF3HH and MF3LH= samples homogenised at 172 MPa and high and low heat treated, respectively).

As could be observed, both non-homogenised samples presented bimodal and polydisperse distributions in terms of volume percentage (Figure 1 a, c) but monomodal in terms of the number of particles (Figure 1 b, d), which indicates that there is a very small number of big particles. The finest particle fraction is probably mainly constituted by proteins, whereas fat droplets and remains of cellular tissue constitute the biggest particles. Nevertheless, particle aggregates could also be present in the biggest particle fraction. Particle size distribution became monomodal when samples were homogenised and the biggest particles of the initial product were greatly reduced in size. However, some finest particles evidenced in the first peak of the distribution of untreated samples seem to aggregate since they are not appear in the tail of the peak of HPH samples. A part of the protein bodies could be unfolded or aggregated by the high pressure effect. The increase in homogenisation pressure progressively reduced the mean particle diameter while distributions became narrower due to the reduction in size of oil droplets and plant cell remains. This can also be deduced from Table 2, where the overall decrease in both the mean particle diameters and the difference between $D_{4,3}$ and $D_{3,2}$ in MF samples can be seen. No significant differences ($p > 0.05$) in these parameters were found when applying 103 (MF2 treatment) or 172 MPa (MF3 treatment) pressures.

Table 2. Particle size parameters $D_{4,3}$ and $D_{3,2}$ and ζ-Potential values of untreated and treated samples. Mean values and (standard deviation).

Treatment	Almond *milk*		
	$D_{4,3}$ (μm)	**$D_{3,2}$ (μm)**	**ζ-Potential (mV)**
Untreated	92.9 (1.9) [ab]	5.2 (0.2) [ab]	-17.0 (1.4) [a]
MF1	35 (20) [cd]	5.7 (0.6) [a]	-21.2 (1.3) [b]
MF2	15.9 (1.7) [ce]	4.8 (0.3) [ab]	-19.41 (1.06) [c]
MF3	14 (7) [e]	3.91 (0.14) [b]	-19.16 (1.43) [c]
LH	78 (2) [b]	21.4 (0.6) [e]	-15.99 (1.18) [a]
HH	158 (20) [f]	24.5 (1.0) [c]	-17.01 (2.12) [a]
MF3LH	23 (3) [cde]	8.7 (0.3) [f]	-16.7 (1.3) [a]
MF3HH	40 (4) [d]	13.0 (1.3) [d]	-15.0 (1.0) [d]
Treatment	**Hazelnut *milk***		
	$D_{4,3}$ (μm)	**$D_{3,2}$ (μm)**	**ζ-Potential (mV)**
Untreated	101 (13) [a]	6.5 (0.5) [abc]	-23.8 (1.2) [a]
MF1	39 (2) [b]	7.94 (0.14) [b]	-21.6 (0.8) [bc]
MF2	26 (3) [c]	6.94 (0.09) [bc]	-21.2 (0.5) [c]
MF3	17.7 (0.9) [c]	5.6 (0.5) [cd]	-23.6 (0.8) [a]
LH	113 (4) [a]	6.0 (0.3) [cd]	-18.2 (1.2) [d]
HH	147 (15) [d]	17.9 (0.8) [e]	-22 (2) [bc]
MF3LH	15.7 (0.2) [c]	5.88 (0.06) [cd]	-22.4 (1.2) [bd]
MF3HH	62 (15) [e]	15 (3) [f]	-21 (2) [c]

[a, b, c, d] **Different letters in same column indicates significant differences between treatments at 95% of confidence level**

MF = homogenisation, **HH** = high temperature heating; **LH** = low temperature heating

The effect of the thermal treatment is shown in Figure 1c. The application of both thermal treatments led to the disappearance of the finest particles probably due to the change in the protein conformation (denaturation) and the promotion of particle aggregations, which increases their hydrodynamic volume, with the subsequent increase in the product viscosity (as observed in the rheological data). Thermal treatments can also promote the increase in the size of fat globules, due to flocculation and coalescence phenomena (Walstra, 2003), this effect being more intense in HH treated samples. When thermal treatments were applied to homogenised samples (treatments MF3LH, MF3HH), the thermal effects seem to be mitigated probably due to the greater stability of the smaller fat globules which are less sensitive to the flocculation and coalescence phenomena than the big ones of non-homogenised samples. Nevertheless, the wider distribution of particles in sample MF3HH is remarkable. This agrees with a greater progress of the aggregation phenomena in this case, in comparison with MF3LH samples, treated a lower temperature.

As far as ζ-potential values are concerned, particles showed negative charge as can be observed in Table 2. This can be explained taking into account the isoelectric point (IP) of the major proteins of almonds and hazelnuts (5 and 4.5, respectively) (Albillos *et al.*, 2009; Ma *et al.*, 2008). Thus, at the pH of the beverages (above their IP), proteins exhibited negative charge. Gallier *et al.* (2012) reported values of ζ-potential of -30 mV for particles of almond *milk*, which is higher than those found in this work. This can be due to a lower adsorption degree of proteins on the

surface of oil droplets in this case, which reduces their effective surface charge.

In almond *milks*, the homogenisation process led to a higher negative charge of the dispersed particles ($p < 0.05$), which indicates that a re-arrangement of components occurs in the dispersed phase. The interfacial adsorption of proteins with their ionisable groups could be promoted by high pressure, thus increasing the surface charge of the dispersed oil droplets and so, the overall net charge and the ζ-potential value. Changes in the protein conformation could also be promoted, increasing the ratio of the surface ionisable groups and so the water affinity of proteins. In general, this treatment led to smaller particles with a higher electrical charge, in comparison to untreated samples. On the contrary, heat treatments did not significantly affect the ζ-potential of dispersed particles ($p > 0.05$) with respect to the untreated samples.

In hazelnut *milks*, all treatments led to a slight decrease ($p < 0.05$) in the charge of the particles, especially thermal treatments. The denaturation of proteins and further aggregation processes could explain the lower particle electrical charge in the treated products.

3.4 Rheological behaviour

Table 3 shows the rheological parameters (K, n and σ_y) of both almond and hazelnut *milks* submitted to different treatments. Apparent viscosity (η) at a shear rate of 100 s^{-1} and the non-linear correlation coefficient (R^2) of the fitted model are also shown. Rheological parameters of HH samples were

anomalous due to the fast phase separation during the rheological measurements and have not been reported.

In almond *milks*, untreated samples showed a slight shear thickening behaviour (n = 1.18) which is typical in dispersions/emulsions when the ortokinetic flocculation occurs associated with the shear rate. Nevertheless, homogenised almond *milks* exhibited almost Newtonian behaviour ($n \approx 1$) probably due to the lowest sensitivity of the smaller particles to shear flocculation. Homogenisation treatment did not cause significant changes ($p > 0.05$) in the consistency index, or apparent viscosity of samples. However, heat treated samples behaved as a Bingham plastic fluid, the MF3HH samples showing the highest yield stress. Moreover, heated samples (submitted or not to homogenisation processes) showed a significant increase ($p < 0.05$) in the apparent viscosity. This behaviour indicates that a weak gelation effect was produced, due to the thermal treatment probably associated with the protein denaturation and subsequent cluster formation. Cluster formations have also been observed in heated and homogenised cow milk (Walstra, 2003). The soluble fibre fraction could also contribute to the increase in the product viscosity by the extension and hydration of the biopolymer chains induced by the temperature.

Table 3. Mean values and standard deviation of consistency index (K), flow behaviour index (n) and yield stress (σ_y) obtained from fitting experimental data to Ostwald-de-Waele model (non-linear correlation coefficient R^2 is included). Apparent viscosity (η) was calculated at shear rate of 100 s^{-1}.

Sample	K ($x10^3$) (Pa s^n)	n	σ_y (Pa)	R^2	η_{100} ($x10^3$) (Pa·s)
	Almond *milk*				
Untreated	0.62 (0.09) [a]	1.18 (0.03) [a]	0 [a]	0.990	1.44 (0.01) [a]
MF1	1.6 (0.2) [a]	1.039 (0.006) [abc]	0 [a]	0.999	1.9 (0.2) [a]
MF2	2.25 (1.05) [a]	0.925 (0.001) [b]	0 [a]	0.980	1.6 (0.7) [a]
MF3	1.55 (0.03) [a]	1.026 (0.006) [bc]	0 [a]	0.998	1.75 (0.02) [a]
MF3HH	15 (10) [b]	0.97 (0.12) [bc]	0.875 (0.007) [b]	0.990	12 (2) [b]
LH	4 (2) [a]	1.09 (0.09) [ac]	0.20 (0.04) [c]	0.997	5.5 (0.7) [c]
MF3LH	4.7 (0.5) [a]	1.084 (0.009) [ac]	0.44 (0.04) [d]	0.990	6.9 (0.5) [c]
	Hazelnut *milk*				
Sample	**K ($x10^3$) (Pa s^n)**	**n**	**σ_y (Pa)**	**R^2**	**η_{100} ($x10^3$) (Pa·s)**
Untreated	1.1 (0.2) [a]	1.08 (0.02) [a]	0 [a]	0.990	1.61 (0.03) [ab]
MF1	4.7 (0.7) [ab]	0.84 (0.02) [b]	0 [a]	0.999	2.21 (0.09) [bc]
MF2	8 (5) [b]	0.79 (0.08) [b]	0 [a]	0.980	3.0 (0.7) [de]
MF3	7.9 (0. 3) [b]	0.769 (0.005) [b]	0 [a]	0.998	2.72 (0.05) [cd]
MF3HH	2.59 (0) [ab]	1.08 (0.00) [a]	0.2 (0.0) [b]	0.990	3.8 (0.0) [e]
LH	0.91 (0.05) [a]	1.085 (0.007) [a]	0 [a]	0.980	1.35 (0.03) [a]
MF3LH	8.0 (0.2) [b]	0.796 (0.005) [b]	0 [a]	0.990	3.121 (0.002) [de]

[a, b, c, d] **Different letters in same column indicates significant differences between treatments at 95% of confidence level**

MF = homogenisation, **HH** = high temperature; **LH** = low temperature

As concerns hazelnut *milks*, untreated samples showed Newtonian behaviour. Nevertheless, the homogenisation process significantly affected the product rheological behaviour, leading to shear thinning behaviour ($n < 1$). Homogenised samples showed greater values of the consistency index and apparent viscosity than the untreated samples. These results reveal that some changes in the component conformation have been induced by high pressure which makes the system more flow resistant and sensitive to flow orientation. These components could be proteins which can be unfolded by pressure effect. Homogenised samples submitted to thermal treatments also exhibited greater viscosity, as commented on above for almond products, but they showed yield stress only when the highest temperature was applied. However, the LH treatments did not induce significant changes in rheological behaviour as compared to non-treated samples, which indicates that no significant changes in the component arrangement were induced by thermal treatment. This could indicate that hazelnut proteins are more sensitive to pressure than almond proteins and less sensitive to temperature. Their unfolding and denaturation was caused by the high pressure effect but not by the low temperature treatment. Thermal treatments of homogenised samples gave rise to an increase in the sample viscosity which may associate to protein aggregation. Nevertheless, the weak gel formation, reflected in a yield stress value, is only evidenced when the highest temperature was applied. This can be due to the low protein content of hazelnut, as compared to almond. With low protein content, gel formation requires a more intense thermal treatment to induce enough chain aggregation for the network formation. Likewise, it is remarkable that

viscosity of thermally treated almond products was higher than that of hazelnut *milks*, coherent with their higher protein content and the subsequent greater density of aggregates.

3.5 Protein denaturation

Figure 2 shows typical thermograms obtained by using DSC for almond *milk*. As can be observed, homogenisation treatments did not cause protein denaturation, since denaturation endotherms appeared with similar area and temperature peak as in untreated samples. Cruz *et al.* (2007) reported that denaturation of proteins occurs when applying pressures around 200 MPa (partial denaturation) or higher (total denaturation), but it depends on the protein nature. No differences ($p > 0.05$) were found between untreated and homogenised samples which showed endothermic peaks at around 98.0 ± 0.4 ºC, with a total enthalpy of around 10 ± 1 J/g protein. This denaturation temperature is relatively high, in agreement with the reported thermo-stability of the major almond protein (amandin), which represents up to 70 g/100 g of the total soluble proteins (Sathe *et al.,* 2002). On the contrary, both heat treatments provoked total protein denaturation as no endothermic peak was observed in the heated samples.

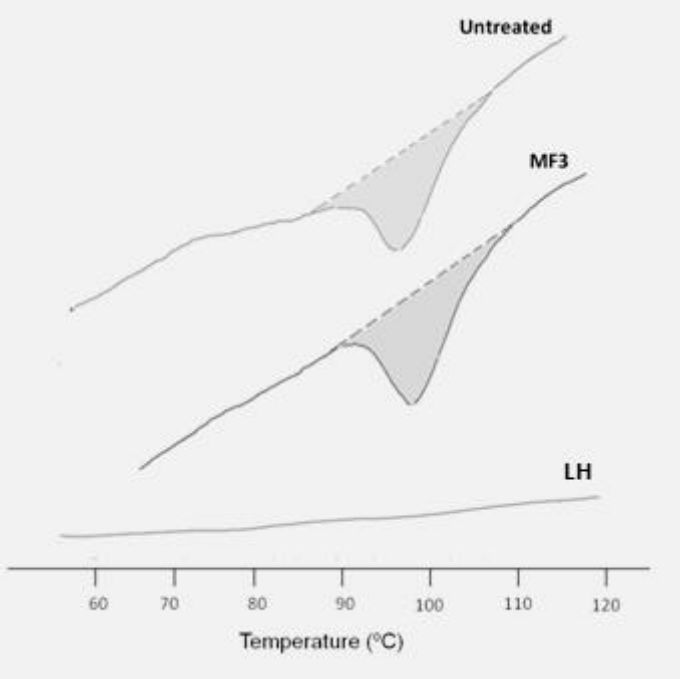

Figure 2. Typical DSC thermograms obtained for almond samples submitted to different treatments. (MF3 = homogenised samples at 172 MPa; LH = Low Heat treated samples).

In hazelnut samples, in no case were endothermic peaks observed. Since in non-treated samples protein will be in the native state, the non-detection of denaturation endotherm by DSC could be due to the low ratio of proteins of these samples and to the low denaturation enthalpy of these proteins. Therefore, the effect of pressure or temperature on hazelnut protein conformation has not been probed by this technique, although rheological behaviour of the different treated samples suggests changes in the protein conformation due to high pressure.

3.6. Sample microstructure

Figure 3 shows the CLSM images of almond *milk* untreated and submitted to different treatments. Oil droplets and protein bodies dispersed in the serum phase are clearly distinguished in Figures 3 A and B for the untreated *milk*. A certain degree of flocculation in protein bodies can be observed, which can be due to their hydrophobic character. Most of the almond proteins belong to the oleosin family with low-molecular-weight and poor water solubility, due to a long highly hydrophobic domain of about 70 amino acid residues (Beisson *et al.*, 2001). In some cases, protein bodies appear adsorbed on the oil droplet surface, forming bridges between them. The low affinity of proteins by the aqueous medium contributes to the low stability of the obtained emulsions where steric stability did not occur due to the poor solvent effect (McClements, 2005).

In LH treated samples (Figures 3 C and D), protein aggregates can be observed to be spread over big areas in the sample, whereas isolated protein bodies are not frequently present. In many cases, protein aggregates include oil droplets. This observation is coherent with described rheological behaviour where LH treatment gives rise to a plastic fluid with yield stress and higher apparent viscosity, which may be due to the formation of a weak gel, associated with a three-dimensional network of aggregated particles at relatively low concentration.

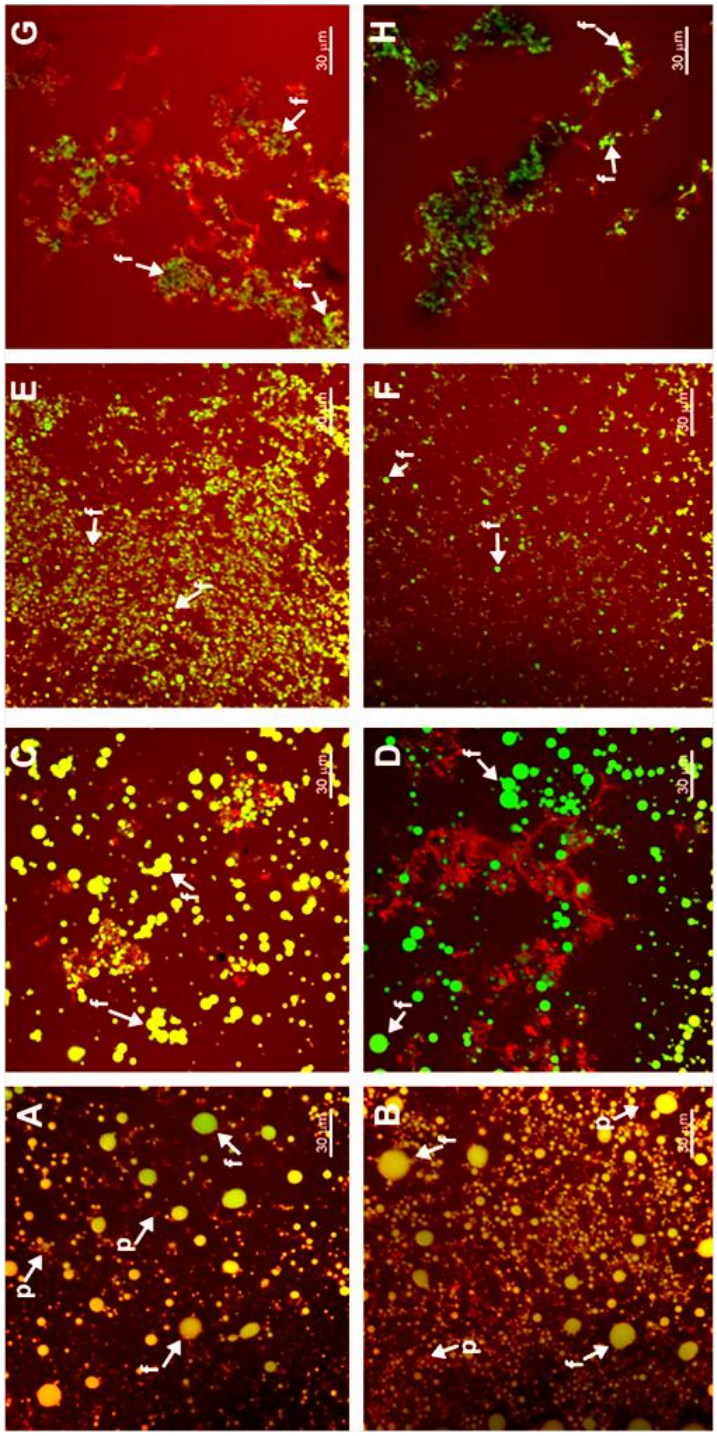

Figure 3. Confocal laser scanning microscopy (CLSM) images of almond *milks* stained with Rodamine B and Nile Red (proteins and carbohydrates in red, fat in green).A and B: untreated product, C and D: Low Heat treated milks, E and F: microfluidised treated *milks*, and G and H: microfluidised and Low Heat treated *milks*

The effect of the homogenisation pressure on the product microstructure can be observed in Figures 3 E and F. The great reduction in the particle sizes, detected by the light scattering diffraction, can be observed. Nevertheless, most of the small particles are flocculated through protein bridges, which explain the low stability of the emulsion despite the small particle sizes. The poor stabilising properties of the protein, associated to its high hydrophobicity and low water affinity, is the cause of the flocculation process and subsequent phase separation, as commented on below.

Combined MF3LH treatment provoked the formation of big oil droplet-protein aggregates which appear embedded in a continuous protein matrix. This new structure is the result of the combined effect of high pressure and temperature. HPH reduces droplet size and promotes partial protein solubilisation and thermal effect provokes soluble protein denaturation and aggregation, as in a gel, thus greatly modifying the product microstructure. Denaturation of the soluble protein gives rise to the formation of a three-dimensional network (evidenced by the yield stress exhibited by these samples in rheological analyses) which entraps big aggregates of the small protein-lipid particles.

So, microstructural observations of almond *milk* samples reveal that almond protein did not show good stabilising properties for oil droplets, probably due to their hydrophobic character that negatively affected the steric stabilisation effect expected for adsorbed proteins in a good solvent. These proteins were thermal sensitive and denatured during thermal treatments, thus inducing the formation of big aggregates which entrap both

oil and protein bodies. In the combined treatments, the big aggregates seemed to be embedded in a continuous protein network (weak gel) which could contribute to stabilise the emulsion.

Although the microstructure of hazelnut *milks* was not analysed, similar behaviour could be expected, taking into account the similar nature of product.

3.7 Sample colour

Lightness, hue and chrome values obtained in both *milks* are shown in Table 4, together with the whiteness index and the colour difference between untreated and treated samples (ΔE). Almond *milks* appeared whiter and with greater lightness than hazelnut *milk* due to the natural brownish colour of hazelnut.

Both *milks* showed the same trends in the colour parameters when treated, the changes being more intense in the whiter almond *milks*. Lightness and whiteness index significantly increased ($p < 0.05$) due to the homogenisation process, as the number and size of particles contribute to the light reflection. In heated samples and in samples submitted to the combined treatments, these parameters decreased ($p < 0.05$) in agreement with the observed increase in particle size. On the other hand, hue and chrome significantly decreased ($p < 0.05$) giving rise to a less saturated reddish colour in all treated samples, regardless of the treatment applied. This was more marked when using the highest temperature, to some extent probably due to the occurrence of Maillard reactions.

Table 4 Mean values (and standard deviation) of Lightness (L^*), hue (h^*_{ab}), chrome (C^*_{ab}) and White Index (WI) of almond and hazelnut *milks* and colour difference between untreated and treated samples.

Almond *milk*	**L^***	**C^*_{ab}**	**h^*_{ab}**	**ΔE**	**W.I.**
Untreated	86.1 (0.2)^{a}	7.15 (0.15)^{a}	96.1 (0.6)^{a}	-	84.3 (0.2)^{a}
MF1	87.4 (0.1)^{c}	6.66 (0.21)^{b}	95 (1)^{a}	1.9 (0.2)^{b}	86.1 (0.2)^{c}
MF2	90.5 (0.2)^{e}	5.80 (0.05)^{c}	96.6 (0.5)^{b}	4.81 (0.12)^{c}	89.1 (0.1)^{e}
MF3	88.5 (0.1)^{d}	5.22 (0.02)^{d}	94.7 (0.2)^{b}	2.93 (0.08)^{d}	87.2 (0.1)^{d}
HH	78.8 (0.5)^{f}	5.48 (0.16)^{e}	94.6 (0.5)^{b}	7.2 (0.4)^{e}	77.5 (0.4)^{f}
MF3HH	86.8 (0.1)^{b}	7.67 (0.08)^{f}	95.2 (0.3)^{c}	1.54 (0.11)^{f}	85.5 (0.16)^{b}
LH	86.0 (0·0)^{a}	6.00 (0.02)^{g}	90.3 (0.2)^{a}	0.43 (0.02)^{a}	84.3 (0.15)^{a}
MF3LH	87.8 (0.1)^{c}	6.73 (0.03)^{bf}	96.6 (0.3)^{a}	2.23 (0.01)^{b}	86.5 (0.1)^{c}
Hazelnut *milk*	**L^***	**C^*_{ab}**	**h^*_{ab}**	**ΔE**	**W.I.**
Untreated	83.4 (0.4)^{a}	9.9 (0.5)^{a}	90.2 (1.2)^{a}	-	80.6 (0.6)^{a}
MF1	83.0 (0.2)^{ab}	9.33 (0.11)^{b}	85.9 (0.7)^{bc}	1.01 (0.09)^{ab}	80.6 (0.2)^{ab}
MF2	83.9 (0.2)^{cd}	9.4 (0.4)^{b}	86.1 (1.4)^{bc}	1.11 (0.13)^{a}	81.4 (0.4)^{c}
MF3	84.38 (0.14)^{c}	8.24 (0.12)^{c}	86.2 (0.8)^{bc}	2.04 (0.02)^{a}	82.34 (0.07)^{d}
HH	77.1 (0.3)^{e}	11.5 (0.3)^{d}	89.4 (0.5)^{a}	6.5 (0.4)^{bc}	74.3 (0.4)^{e}
MF3HH	78.7 (0.8)^{f}	10.0 (0.2)^{ae}	82.2 (0.9)^{d}	4.9 (0.7)^{d}	76.4 (0.7)^{f}
LH	79.6 (0.3)^{g}	10.5 (0.4)^{e}	86.9 (0.3)^{b}	3.9 (0.3)^{cd}	77.1 (0.4)^{g}
MF3LH	83.88 (0.07)^{d}	7.90 (0.03)^{b}	85.29 (0.03)^{c}	2.19 (0.02)^{d}	82.05 (0.05)^{c}

$^{a, b, c, d}$ Different letters in same column indicates significant differences between treatments at 95% of confidence level

MF = homogenisation, **HH** = high temperature; **LH** = low temperature

Total colour difference values (ΔE) were low, taking into account that values lower than 3 units cannot be easily detected by the human eye (Francis, 1983). So, only samples submitted to the most intense heat treatment (HH) showed values considered as detectable.

3.8 Physical stability over storage time

All samples, except those MF3 submitted to LH (MF3LH treatment), showed phase separation after 1 storage day and no notable differences in the height of each of the separate phases were observed throughout time. Figure 4 shows the appearance of the samples at 28 storage days where the samples submitted to MF3LH treatments were the only ones which showed colloidal stability, for both almond and hazelnut *milks*. The combined effect of homogenisation and thermal treatment seems to promote a weak gel formation, mainly associated with the protein solubilisation and subsequent denaturation during thermal treatment, which contributed to stabilise the particle dispersion, thus avoiding phase separation during the product storage.

The observed behaviour indicates that nut proteins did not show adequate emulsifying properties to stabilise fat globules by interfacial protein adsorption, as commented on above, even with the particle size reduction induced by HPH. Only when homogenised samples were submitted to thermal treatment and the proteins were denatured, can these contribute to stabilise the emulsions, mainly due to a viscous effect. Capacity of nut proteins to stabilise colloidal systems has not been previously reported.

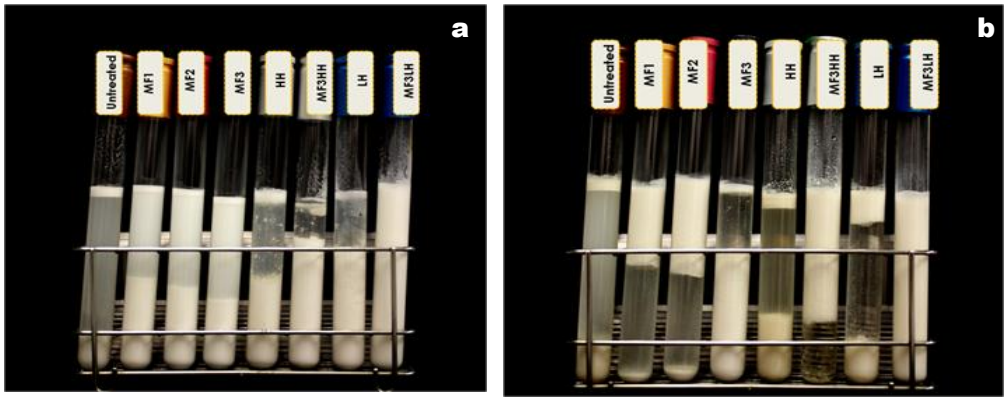

Figure 4. Phase separation observed in almond (a) and hazelnut (b) *milks* submitted to different treatments after 28 storage days at 4 ºC. (MF = homogenised samples; HH = High Heat treated samples; LH = Low Heat treated samples; MF3HH and MF3LH= samples homogenised at 172 MPa and high and low heat treated, respectively).

Phase separation occurs in a coherent way with the microestructural observations. A thin cream phase can be seen in almond *milks*, corresponding to an oil-rich phase, whereas thick sediment corresponding to the contraction of dispersed phase, entrapping protein-oil droplet aggregates, can also be observed. The ratio oil-protein in the clusters determines their mean density. In almond *milk*, the density of these clusters is higher than that of the serum phase due to the high protein-lipid ratio (0.35) and so, they sediment in the glass tube. In hazelnut *milks*, the protein-lipid ratio is much lower (0.16) and the proportion of both components in the protein-lipid aggregates is critical to determine the migration direction (up or down) in the tube. In some samples, creaming was predominantly observed, whereas in others sedimentation occurs. Nevertheless, in all cases,

the progressive aggregation of the protein-oil clusters will be responsible for this behaviour, regardless of the lipid-protein ratio present in the clusters. This progressive aggregation process was inhibited in MF3LH samples due to the viscous effect and yield stress induced by combined thermal and homogenisation treatments, probably due to the lower size of the lipid-protein aggregates. In MF3HH samples, with bigger oil-protein clusters, the viscous stabilisation is not enough to control the effect of gravitational forces.

4. CONCLUSIONS

Physical properties and stability of almond and hazelnut *milks* were affected by both homogenisation pressure and heat treatments. The homogenisation process greatly reduced particle size but the resulting emulsions were not stable and phase separation occurred in relatively few hours. Microestructural observations reveal that proteins did not contribute to stabilise the emulsions due to their hydrophobic character which did not favour the steric stabilisation in a good solvent. So, flocculation of protein bodies and oil droplets occurred, giving rise to the formation of oil-protein clusters. These clusters suffer progressive aggregation promoting phase separation process. Thermal treatment at the lowest temperature provoked protein denaturation, thus enhancing the aggregation process. Nevertheless, when samples were previously high pressure homogenised, denaturation and aggregation of the serum proteins seem to contribute to the formation of a three-dimensional network (reflected in the sample yield stress), which

exerts a stabilising viscous effect that inhibited phase separation during the product storage. So, the combination of low heat treatment with high homogenisation pressure greatly improved the physical stability and appearance of almond and hazelnut *milks*.

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Capítulo II

Diseño y optimización del proceso fermentativo de "*leches*" de avena, almendra y avellana. Estudio de la vida útil de los productos finales.

Oat *milk* fermentation using probiotic *Lactobacillus reuteri* microorganisms

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ABSTRACT

Functional advantages of probiotics combined with interesting composition of oat were considered an alternative to dairy products. In this study, fermentation of oat *milk* with *Lactobacillus reuteri* and *Streptococcus thermophilus* was analysed to develop a new probiotic product. Central Composite Design with Response Surface methodology was used to analyse the effect of different growth factors (glucose, fructose, inulin and starters) on the probiotic population in the product. Optimised formulation was characterised throughout storage time at 4 ºC in terms of pH, acidity, β-glucan and oligosaccharides contents, colour, rheological behaviour and sensory evaluation. All formulations studied were adequate to produce fermented foods and minimum dose of each factor was considered as optimum. The selected formulation allowed starters survival above 10^7 cfu/mL to be considered as a functional food and was maintained during the 28 days controlled. β-glucans remained in the final product with a positive effect on viscosity. Sensory evaluation showed good acceptability until 14 day storage, assuring good sweetness, acidity and consistency. Therefore, a new probiotic non-dairy *milk* was successfully developed with a shelf life, in terms of sensory acceptance, slightly shorter than that of standard yoghurts.

Key words: fermented oat *milk*, probiotic, prebiotic, Response surface methodology, formula optimisation.

1. INTRODUCTION

Probiotics are defined as "live microorganisms that when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). *Lactobacillus* and *Bifidobacterium* genus are mostly recognised within this group, although *Lactococcus*, *Enterococcus*, *Saccharomyces* and *Propianobacterium* genera are currently being investigated (Rivera-Espinoza and Gallardo-Navarro, 2010). However, strains are not classified as probiotic unless they accomplish several requirements, such as total safety for the host, resistance to gastric acidity and pancreatic secretions, adhesion to epithelial cells, antimicrobial activity, inhibition of adhesion of pathogenic bacteria, stimulation of immune system and metabolic activity, evaluation of resistance to antibiotics, tolerance to food additives, technological procedures and stability in the food matrix (Prado *et al.*, 2008).

The use of probiotics in food product manufacturing dates back to the ancient world, although the purposes have changed over time. Nowadays, not only are probiotic microorganisms used for food preserving and organoleptical improvements but also to enhance the nutritional and health benefits: reduction of hypercholesterolemia, host immune modulation, prevention of urogenital diseases, alleviation of constipation, protection against traveller's diarrhoea, protection against colon and bladder cancer, prevention of osteoporosis and food allergies, among other aspects (Lourens-Hattingh and Viljoen, 2001). Nevertheless, host benefits are subject to the strain type used in product manufacture (Sharareh *et al.*,

2009). Although there is no legal definition of the term "probiotic", different probiotic's dosage recommendations can be found. According to different authors, the minimum number of viable probiotic bacteria should be 10^7-10^9 colony forming units (cfu)/g or mL of a product at the time of consumption and, as to exert healthy effects, it should be consumed daily (Gomes and Malcata, 1999; Stanton *et al*., 2003; Van Niel *et al.,* 2002). These recommendations are in compliance with the minimum requirements for standard milk fermented products by the International Dairy Federation and the Japan and EU Associations of Fermented Milks, which is 10^7 cfu/g or mL of starter (Sanz and Dalmau, 2008).

Due to their health properties, there has been a notable increase in the consumption of food products containing probiotic microorganisms. As Granato *et al.* (2010) reported, the consumption of probiotic products increased by around 13% and 18% between 2002 and 2007 in Eastern and Western Europe, respectively. Those products have been traditionally produced by using animal milk, 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) to produce probiotic products. In this sense, several beverages obtained from soy, rice, wheat and maize would have huge market potential due to the current consumer demand for cow-milk substitute products (Mårtensson *et al.,* 2000). Those consumers are principally vegetarians, people allergic to animal proteins or lactose intolerant. In this sense, oat *milks* can be alternative matrices with which to elaborate probiotic products, adding the nutritional and functional characteristics of this cereal, such as the high content in soluble and non-

soluble fiber which makes oats a useful product to use in the prevention of different diseases, especially those affecting the colon (Sadiq-Butt *et al.*, 2008). Several studies have shown that β-glucans, the most prevalent oat soluble fiber, have probiotic activity, while they decrease the blood-cholesterol levels, cardiovascular disorders and improve the lipid and glucose metabolism. Prebiotics are non-digestible components of functional foods that stimulate the proliferation and activity of bacterial populations desirable in the colon and inhibit pathogen multiplication; hence acting beneficially on the host (Mattila-Sandholm *et al.*, 2002; Roberfroid, 2000). β-glucans are able to stimulate the intestinal microflora, with a particular effect on lactic acid bacteria and bifidobacteria genus (Angelov *et al.*, 2006).

Regardless of the health benefits of oat consumption, its sensory properties lead to low consumer acceptance. Nevertheless, both technological and fermentative processes are bound to improve sensory quality, as well as providing health and nutritional benefits due to the combination of both probiotic and prebiotic compounds, the so-called synbiotics. Previous studies demonstrated that the fermentation step with the mixed culture of *L. reuteri* and *S. thermophilus* required less than 6 hours and that the addition of *S. thermophilus* led to an improvement in organoleptic properties, mainly flavour. Mårtensson *et al.* (2001) also observed that the use of mixed culture containing *S. thermophilus*, *L. acidophilus* and *Bifidobacterium spp*. gave a balanced sour taste and a fresh aroma to oat *milks*, similar to the typical yoghurt flavour.

The aim of the present study was to evaluate the fermentative process of oat *milks* (*Avena Sativa L.*) with the mixed culture *L. reuteri* ATCC 55730 and *S. thermophilus* CECT 986 (1:1) and the quality of the fermented product. To this end, the effect of different factors, such as the added amount of glucose, fructose, inulin and inoculum, was analysed to ensure there was enough viable probiotic strain (*L. reuteri*) in the final product. The most adequate fermented formulation was characterised as to its main physicochemical properties and quality parameters (sensory analysis) in order to determine the product shelf life.

2. MATERIALS AND METHODS

2.1 Preparation of oat *milk*

Oat *milk* was produced by soaking and grinding peeled oat (*Avena Sativa L.*), supplied by Salud e Imaginación S.L. (Masquefa, Barcelona, Spain). The oat:water ratio was 8:100 (w/v), which ensures enough quantity of β-glucan (oat prebiotic compound) for the subsequent fermentative process (Angelov *et al*., 2006). The extraction was carried out in Starsoja (Farmanutrients Labs, S.L.; Barcelona, Spain), equipment specifically designed for the production of vegetable *milks*. To obtain the oat *milk*, three grinding cycles were used at 90 ºC for 20 minutes. The liquid obtained was then homogenised in a rotor-stator homogeniser (Ultraturrax T25, Janke and Kunkel, Germany) for 3 min at 13,500 rpm and, finally, sterilised at 121 ºC for 15 minutes (Presoclave II, JP-Selecta; Barcelona, Spain).

2.2 Preparation of fermented products

2.2.1 Inoculum preparation

Lactobacillus reuteri ATCC 55730 (Biogaia, Stockholm, Sweden) and *Streptococcus thermophilus* CECT 986 (CECT, Paterna (Valencia), Spain) were activated from their frozen forms (stored in 40g/100 mL glycerol at -80 ºC), by transferring each one to its selective broth until optimal bacterial growth is obtained. Selective broths were MRS (Scharlab; Barcelona, Spain) for the probiotic *Lactobacillus* and M17 (DifcoTM; New Jersey; USA) for *S. thermophilus*. Incubation conditions were 37 ºC/24h/anaerobically for *L. reuteri* and 42 ºC/24h/aerobically for *S. thermophilus*.

Likewise, strains were independently incubated in their broths for 24 h and then centrifuged at 10,000 rpm-10 min at 4 ºC; supernatant was discarded. Immediately after, 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 cfu/mL.

2.2.2 Experimental design for fermentation process

Amount of glucose, fructose, inulin and starter inoculum were selected as growth factors (4 independent variables) to obtain fermented oat *milks*. Central Composite Design (CCD) with randomised Response Surface methodology (RSM) was used to analyse the effect of the different growth factor combinations on the total count of probiotic bacteria (response variable) and then optimise fermentation process, such as described by other authors (Chen *et al*., 2004; Cruz *et al.*, 2010; Gupta *et al.*, 2010; Liew *et al.*,

2005; Stepheine *et al*., 2007; Yaakob *et al*., 2012). Statistical analysis of the data was carried out in Statgraphics® Centurion XVI by using a orthogonal 2^4 + star design, which studied the effects of the 4 factors in 31 runs. Factors and levels were chosen taking into account previous studies into oat fermentation studies (Angelov *et al.*, 2006; Sumangala *et al.*, 2005): Glucose: 1 to 2 g/100 mL, Fructose: 1 to 2 g/100 mL, Inulin: 0.7 to 1.3 g/100 mL and Inoculum: 3 to 4.5 mL/100 mL. The response variable was the probiotic population at the end of fermentation process.

Fermentation process in the 31 runs was carried out by adding the corresponding amount of starter culture (prepared by mixing in a 1:1volume ratio the *L. reuteri* and *S. thermophilus* PBS buffer suspensions) to the formulated and sterilised oat *milks* and then incubating at 40 ºC, which was the optimal growth temperature of the mixed culture, according to a preliminary study (data not shown). Fermentation process was stopped when pH of samples reached 4.4-4.6, by cooling the samples to 4 ºC, which was the storage temperature until the analyses were done.

A step-wise second grade polynomial fitting was used to model the response variable as a function of the growth factors.

Optimal formulation of the fermented product was established on the basis of the obtained results for the response variable.

2.3 Product characterisation

Newly obtained oat *milk* and optimal formulation of fermented product stored at different times, were characterised as to content in different sugars and β-glucan (prebiotic), pH, acidity, density, colour, rheological behaviour

and microstructure. In oat *milk*, dry matter, protein, lipid and ash contents were also analysed. In fermented product, the starter survival throughout storage time (1, 7, 14, 21 and 28 days) at 4 ºC was analysed, as well as the 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).

Total β-glucan content was determined enzymatically with a mixed-linkage β-glucan detection assay kit (Megazyme TM International Ltd., Wicklow, Ireland).

Sugar profiles were analysed and the different sugars were quantified using the following HPAC-PAD equipment: Metrohm 838 Advanced Sample Processor (Metrohm® Ltd.; Herisau, Switzerland) in an Advanced Compact IC 861 ion chromatograph (IC) equipped with a pulsed amperometric detector to monitor the separation (Bioscan 817). Prior to the analysis, samples were diluted 1:100 with nanopure water. Sample proteins were removed by precipitation with glacial acetic acid and centrifugation at 10,000 rpm for 10 min; pH was then reconstituted at initial values. Before injecting samples into the equipment, they were filtered through nylon membranes (0.45 μm). A Metrosep CARB guard column (5 x 4.0 mm Metrohm) and a Metrosep CARB 1 (250 x 4.6 mm Metrohm) analyses column were used. 20 μL of sample was injected and eluted (1 mL/min)

with 0.1 mol/L NaOH, at 32 ºC. An Au working electrode was used and applied potentials were +0.05 V (between 0 – 0.40 s) +0.75 V (between 0.41 – 0.60 s) and +0.15 V (between 0.61 – 1 s). Software ICNet 2.3 (Metrohm® Ltd.; Herisau, Switzerland) was used for data collection and processing. The concentration of each sugar was determined from their respective calibration curves, obtained from standard solutions of mannitol, glucose, fructose and sucrose (Sigma-Adrich®, Spain), obtained in triplicate.

2.3.2 pH, density (ρ) and tritratable acidity (TA)

Measurements of pH and ρ were carried out at 25 ºC using a pH-meter (GLP 21+, Crison Instruments S.A.; Barcelona, Spain) and a picnometer Gay-Lussac, respectively. AOAC standard method was used to determine TA of samples (AOAC 947.05), expressing results as g/100 mL of lactic acid (Horwitz, 2000).

2.3.3. 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 112 s^{-1}, using 1 minute to reach the maximum shear rate and another minute to attain zero shear rate. Non-linear model (Eq. 1) was applied to determine the flow behavior index (n), consistency index (K) and yield stress (σ_y). 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).

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

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

2.3.4 Colour parameters

Colour coordinates were measured from the reflection spectrum in a spectrocolorimeter CM-3600 d (MINOLTA Co; Osaka, Japan). A 20 mm depth cell was used. CIE L^*a^*b coordinates were obtained using illuminant D65/10º observer. Lightness (L^*), chrome (C^*_{ab}) and hue (h^*_{ab}) of the different samples as well as colour difference (ΔE) (equations 3 to 5) with respect to the non-fermented sample were obtained.

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

$$h^*_{ab} = arctg\,\frac{b^*}{a^*} \quad (4)$$

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

2.3.5 Confocal laser scanning microscopy (CLSM)

A Nikon confocal microscope C1 unit, which was fitted on a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan), was used. An Ar laser line (488 nm) was employed as light source to excite fluorescent dyes Rhodamine B and Nile Red. Rhodamine B (Fluka, Sigma-Aldrich, Missouri,

USA) with λex max 488 nm and λem max 580 nm was dissolved in distilled water at 0.2 g/100 mL. This dye was used to stain proteins and carbohydrates. Nile Red (Fluka, Sigma-Aldrich, Missouri, USA) with λex max 488 nm and λem max 515 nm was dissolved in PEG 200 at 0.1 g/L. This dye was used to stain fat. An oil immersion objective lens (60x/1.40NA/Oil/ Plan Apo VC Nikon) was used.

For sample visualisation a microscopy slide was elaborated with two razor blades (platinum coated double edge blades with 0.1 mm thickness) stuck to a glass. 20 µL of the sample were placed on the microscope slide, within the central gap of the blades; 10 µL of Rhodamine B solution and 10 µL of Nile Red solution were added and the cover slide was carefully positioned. Observations were performed 10 min after diffusion of the dyes into the sample. Images were observed and stored with 1,024 x 1,024 pixel resolution, using the microscope software (EZ-C1 v.3.40, Nikon, Tokyo, Japan).

2.3.6 Starter survival

Counts of *L. reuteri* and *S. thermophilus* were performed using pour plate technique, according to the method described by the International Dairy Federation (International IDF standards, 1997). Acidified MRS agar (Scharlab; Barcelona, Spain) selective media was used for *L. reuteri* and M17 agar (Difco™; New Jersey; USA) for *S. thermophilus*. Incubation conditions were 37 ºC for 48 h in aerobic conditions for *S. thermophilus* and 37 ºC for 24 h in anaerobic conditions for *L. reuteri*.

2.3.7 Sensory analysis

A 16 member's semi-trained panel evaluated oat fermented products with different storage times (0, 14, and 28 days) at 4 ºC. The panelists were selected on the basis of their interest, availability, lack of food allergies and their threshold to basic flavours. Panel members were trained following the method described by Mårtensson *et al*. (2001) with some modifications. They were trained to score attributes of sweetness, acidity, oat flavour, consistency and mouthfeel and overall acceptability using interval scales that varied from 1(slightly) to 5 (extremely).

Reference samples were used for setting the interval scales for panel training. For acidity reference, 1 and 2 g/100 mL of sucrose was added to commercial milk yoghurt, corresponding to 3 and 1 respectively on the scale, and with 0.2 g/100 mL of citric acid corresponding to 5. Commercial milk yoghurt with added sucrose at 2, 5 and 14 g/100 mL levels was used for sweetness evaluation, corresponding to 1, 3 and 5 respectively on the scale. For consistency and mouthfeel, liquid yoghurt, commercial soy dessert and Danone original® yoghurt were used as references, corresponding to 1, 3 and 5 respectively on the scale. For oat flavour, reference was oat *milk* used in the study, which corresponded to 5 on the scale.

Each panelist tested 3 samples (cold stored for 0, 14 and 28 days, respectively) containing 6 g/100 mL of sucrose, to quantify the attributes in which each one was trained. Samples were randomly presented with a code of three digits. Evaluation was conducted in a normalised tasting room at room temperature.

2.4 Statistical Analysis

Results were analysed by multifactor analysis of variance with 95% significance level using Statgraphics® Centurion XVI. Multiple comparisons were performed through 95% LSD intervals.

3. RESULTS AND DISCUSSION

3.1. Characterisation of the oat *milk*: chemical composition and microstructure

Results in oat *milk* chemical composition were: 6.5 ± 0.3 g/100 mL of dry matter, 0.65 ± 0.03 g/100 mL of proteins, 0.241 ± 0.004 g/100 mL of β-glucan, 0.094 ± 0.003 g/100 mL of fats, 0.099 ± 0.005 g/100 mL of ashes and 0.047 ± 0.007 g/100 mL of total sugars, the latter obtained from the sum of all the individual sugars analysed. These compositional values are in agreement with those reported by other authors (Sadiq-Butt *et al*., 2008). As was observed during the extraction process, the major losses of oat components occur in the fibre and lipid fractions, which remained in the waste by-product during the extraction process. This fact is coherent with that observed in the oat *milk* microstructure, by using the confocal technique (Figure 1), where the presence of a small amount of lipid droplets, yellow-green in colour, can be observed. Proteins and carbohydrates appear red-coloured, together with some cellular fragments. As confocal pictures show, the oat *milk*'s microstructure is organised as a polysaccharide network (PM) where fat and protein are embedded. This arrangement is associated to the gelling properties of β-glucans, once is heated (Lazaridou and Biliaderis,

2009). Moreover, almost all the lipid droplets are retained in the polysaccharide-protein matrix which is responsible for the physical stability of the oat *milk*, even after the sterilisation treatment. It was observed that some proteins were attached to fat globules, thus providing protection against the so-called Ostwald ripening or other destabilisation processes in emulsions.

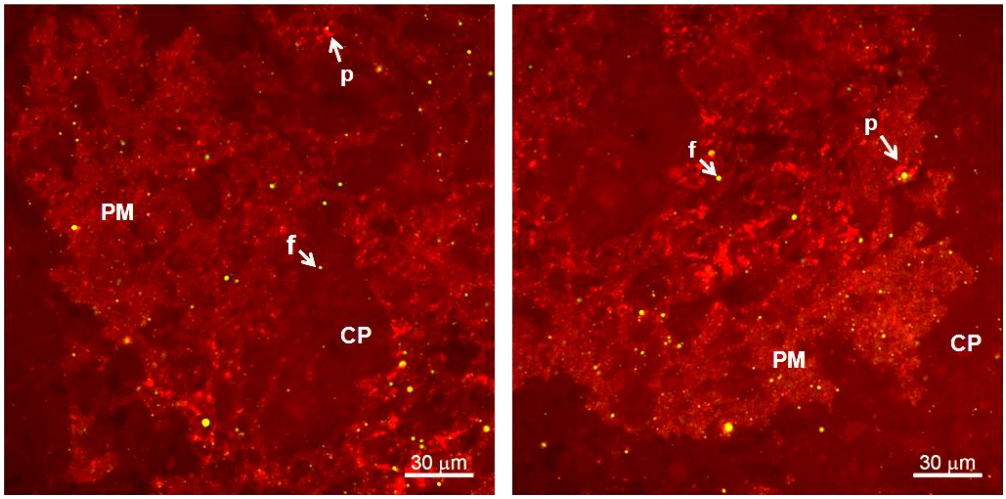

Figure 1. Confocal pictures of sterilised oat *milk*, where fat component appears yellow-green in color, proteins are vivid red in color and carbohydrates are dull red in color. PM: Polysaccharide-protein matrix, CP: Continuous phase, f: fat droplet, p: protein.

3.2. Effect of growth factors on fermentation process

Table 1 shows the experimental values of the *L. reuteri* counts (log cfu/mL) obtained for each run of the CCD. All the formulations permitted the development of probiotic oat fermented *milk*, since their response variables were over 7 log cfu/mL, which is within the probiotic amount

recommended to ensure health effects in consumers (Gomes and Malcata, 1999; Stanton *et al*., 2003; Van Niel *et al.*, 2002)

Table 1. Total counts of *L. reuteri* obtained in the different fermented products corresponding to the experimental design, as a function of the levels of the growth factors.

	GROWTH FACTORS				RESPONSE VARIABLE
Run order	**X_1**	**X_2**	**X_3**	**X_4**	**Y**
1	0	+α	0	0	8.04
2	0	0	+ α	0	8.23
3	-1	+1	-1	+1	8.04
4	0	0	0	0	8.26
5	+1	+1	-1	+1	9.00
6	0	0	0	0	8.08
7	0	0	0	0	8.36
8	- α	0	0	0	9.15
9	-1	+1	+1	+1	8.73
10	-1	+1	-1	-1	9.20
11	+1	-1	-1	+1	8.95
12	+1	-1	+1	-1	7.46
13	0	0	0	0	9.34
14	+ α	0	0	0	10.25
15	-1	-1	+1	-1	8.81
16	+1	+1	+1	-1	9.60
17	+1	-1	-1	-1	8.76
18	0	0	0	0	9.20
19	0	0	- α	0	9.34

20	+1	+1	+1	+1	9.28
21	0	0	0	+ α	8.67
22	-1	+1	+1	-1	9.11
23	0	- α	0	0	9.46
24	-1	-1	-1	+1	9.28
25	+1	-1	+1	+1	8.21
26	0	0	0	0	8.08
27	0	0	0	0	8.26
28	+1	+1	-1	-1	8.32
29	0	0	0	- α	8.54
30	-1	-1	-1	-1	9.83
31	-1	-1	+1	+1	8.15

*Factors X_1, X_2, X_3, X_4 and Y stand for Glucose: 1-2 g/100 mL; Fructose: 1-2 g/100 mL; Inulin: 0.7-1.3 g/100 mL; Inoculum: 3-4.5 mL/100 mL; Probiotic counts (log cfu/mL), respectively.

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, except when the elimination of such terms decreased the explained variance (R^2adj). The goodness of the fitted model was evaluated through an analysis of variance, mainly 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 (Granato *et al.*, 2010). Table 2 summarises the estimated regression coefficients of the second order model obtained, in which fit parameters from the analysis of variance are included.

Table 2. Regression coefficients and analysis of variance for probiotic counts (log cfu/mL) obtained from the fitted model.

Factor/ Parameter	Regression coefficient / Value
Constant	22.18
Glucose	-8.22
Fructose	-3.81
Inulin	-4.11
Inoculum	-1.14
Glucose x Glucose	1.25
Glucose x Fructose	0.95
Glucose x Inoculum	0.76
Fructose x Inulin	2.38
P-value Lack-of-fit	0.880
R^2	0.59
R^2-adj	0.51
Standard error of est.	0.53
Mean absolute error	0.32
Durbin-Watson statistic (P-value)	1.678 (0.218)

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

As can be seen in the coefficients (Table 2), when the growth factors appear as linear variables, they seem to negatively affect the total probiotic counts. Nevertheless, a more thorough examination of the fitted model

indicated that all the coefficients corresponding to factor interactions (second order terms) were positive in value, which explains the overall positive impact of the increasing levels of glucose, fructose, inulin and starter inoculums on the total probiotic counts. These results indicated that the individual factors were not truly independent of one another, 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 model fit, the lack-of-fit parameter was not significant ($p > 0.05$) and the p-value of the Durbin-Watson statistic was greater than 0.05, meaning that there is no indication of serial autocorrelation in the residuals at the 5% significance level. Both parameters indicated that the obtained model is adequate for predicting probiotic survival in oat *milk*. In practice, a model is considered adequate to describe the influence of the variable(s) when the coefficient of determination (R^2) is at least 80% (Yaakob *et al*., 2012) or the values of R^2adj over 70% (Cruz *et al*., 2010). The obtained model explained only 51% of the variation in the experimental data (R^2adj) (Table 2), which is partially explained by the narrow range of experimental response variable (≈ 2.5 log cfu/mL). The narrow variation in response variable made it difficult to obtain greater R^2adj values. Therefore, the obtained prediction model should only be used to make rough predictions.

Figure 2 shows the Response Surface plots for the *L. reuteri* counts. 4 different plots were obtained in each of which one of the factors was fixed at the smallest value. As deduced from Table 1, surfaces showed that many

formulations could be used for the production of probiotic fermented oat *milk*, by considering the minimum recommended strain survival ($\geq 10^7$ cfu/mL). Taking these results into account, a possible optimum formulation should be defined as one that has the minimum production costs. In this sense, the formulation considered as optimum was the one to which the smallest amount of ingredients was added. This optimum corresponds to the formulation where 0.65 g/100 mL of glucose, 0.65 g/100 mL of fructose, 0.4 g/100 mL of inulin and 3 mL/100 mL of mixed culture inoculum was incorporated into the oat *milk*.

This optimal formulation was submitted to fermentation process and data were analysed in order to validate the prediction model. Results showed that the fermented product reached 4.37 ± 0.02 value of pH in 3.5 h at 40 ºC with a *L. reuteri* survival of around 9 log cfu/mL. Other oat fermentation studies made with *L. reuteri* showed longer fermentation times (16 h) and lower probiotic survivals, which were 8 log cfu/mL when it was the only starter and one log less when it was combined with typical yoghurt starter bacteria (Mårtensson *et al.*, 2002).

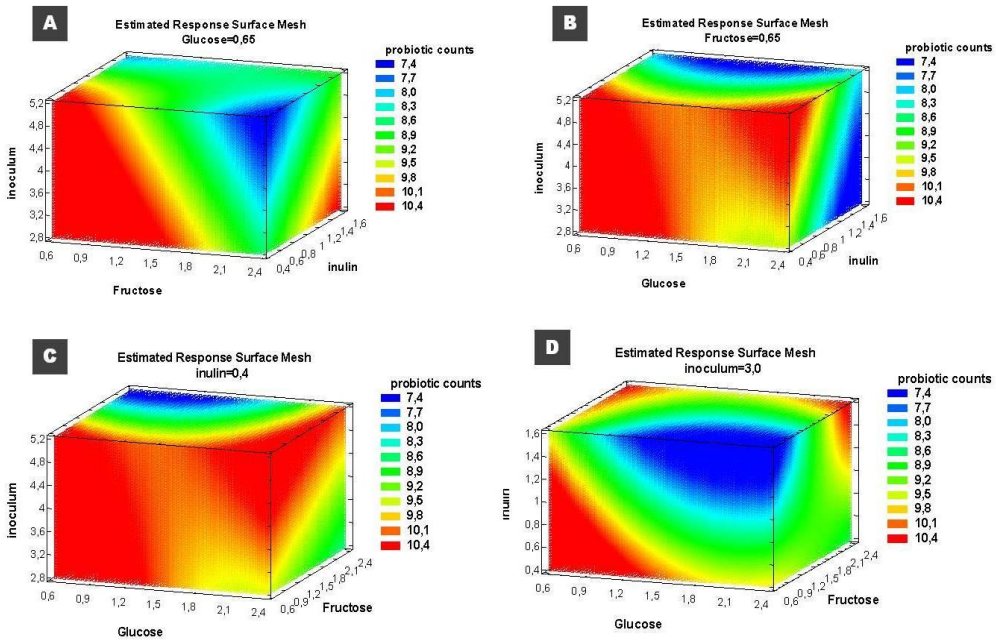

Figure 2. Response Surface plots of the effect of the different growth factors (glucose, fructose, inulin and starters inoculum) on the viability of *L. reuteri* (expressed in log cfu/mL). Plots were obtained by keeping the level of one factor constant.

3.3. Properties of oat fermented product

3.3.1 Bacterial counts and acid production

Average values of pH and Titratable Acidity (TA) in fermented oat *milk* vs. storage time are summarised in Table 3. This table also includes bacterial counts values of *L. reuteri* and *S. thermophilus* (log cfu/mL) throughout storage time.

Table 3. Values (mean and (standard deviation)) of pH, Titratable Acidity (TA) and bacterial counts of fermented oat *milk* throughout storage time at 4 ºC. Data of non-fermented oat *milk* are included for comparisons.

Sample	Storage time (d)	pH	TA (g/100mL lactic acid)	*L. reuteri* (log cfu/mL)	*S. thermophilus* (log cfu/mL)
Oat *milk*	**-**	6.41 (0.02)	0. 053 (0.003)	-	-
Fermented oat product	**0**	4.37 (0.02) [a]	0.167 (0.004) [a]	8.80 (0.03) [a]	8.01 (0.02) [a]
	1	4.08 (0.04) [b]	0.21 (0.02) [ab]	8.49 (0.11) [b]	7.89 (0.04) [b]
	7	3.79 (0.05) [c]	0.25 (0.02) [bc]	7.72 (0.05) [a]	7.75 (0.03) [c]
	14	3.65 (0.06) [d]	0.28 (0.03) [c]	7.48 (0.07) [c]	7.43 (0.02) [d]
	21	3.61 (0.07) [d]	0.37 (0.06) [d]	7.31 (0.14) [d]	7.28 (0.03) [e]
	28	3.30 (0.05) [e]	0.5 (0.04) [e]	7.43 (0.06) [c]	7.629 (0.015) [c]

[a, b, c, d, e] Different letters in same column indicates significant differences among samples at different control times (95% confidence level)

Initial pH value of the oat fermented *milk* was similar to that reported in standard yoghurts, although initial acidity (0.17 g/100 mL of lactic acid) was lower than in standard yoghurt, in which it is around 0.8-1 g/100 mL of lactic acid (Mistry and Hassan, 1992; Tamime and Robinson, 2000). This means that oat *milk* has a lower buffering capacity than cow milk. Moreover, besides the lactic acid synthesis, the *L. reuteri* strain has a heterofermentative metabolic pathway which ends in acetic acid synthesis, which acid might be contributing to increase the pH values (Årsköld *et al.*, 2008).

Physicochemical properties of oat *milk* were modified due to fermentation process. pH of the fermented product remained within the desired ranges (above 4) for the first day but, after 7 days, pH significantly

decreased reaching a unit below the initial value. These changes were expected considering that starters were viable over the entire storage time and, therefore, they were still generating acidic compounds.

Although the viability of starter bacteria decreased during the storage time, especially in *L. reuteri*, the minimum survival recommended (10^7 cfu/mL) was ensured in both strains until the end of the storage (Sanz and Dalmau, 2008). Results show that both microorganisms are highly resistant to an acidic environment and that the oat formulation contained sufficient nutrients for starter growth during the whole storage time. Previous works have also shown good fermentation results of *L. reuteri* by using oat as culture media (Johansson *et al.*, 1993; Mårtensson *et al*., 2002).

3.3.2. Sugar and β-glucan contents

The concentration values of these components can provide interesting information about bacterial activity during the product shelf life. Table 4 shows the values of each sugar identified in oat fermented product and their changes throughout the storage time. In Figure 3, typical chromatograms, with the sugar peaks obtained for both non-fermented *milk* and that fermented for 28 days at 4 ºC, are shown.

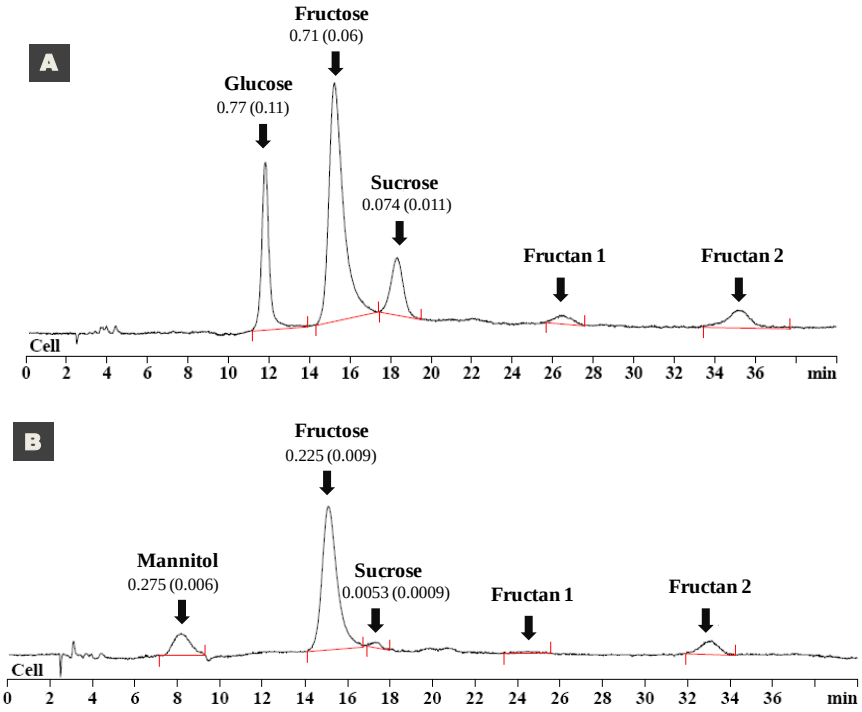

Figure 3. Chromatograms with sugar peaks obtained in HPAC-PAD analysis for oat *milk* (A) and fermented oat product after 28 days of storage at 4 ºC (B). Sugar concentrations are indicated (mean values in g/100 mL and (standard deviation)).

Prior to the fermentation process, sucrose (from oat), glucose and fructose (added), were present in oat *milk*. Other sugars (peaks), which could not be identified, could come from the added inulin, taking into account the thermal stability of β-glucans (Lazaridou and Biliaderis, 2007): They were classified as fructans, which is a term that includes both inulin and its derivatives (Roberfroid, 2005). After the fermentation process, a

huge reduction in the contents of initial monosaccharides and sucrose was observed and subsequently, a significant ($p < 0.05$) decrease, gradual throughout storage time, was observed (Table 4). These results are expected, since bacteria starters were viable during the whole storage period and they consumed sugars as nutrients (Table 3). On the other hand, a new peak appeared in fermented products that was not present in non-fermented *milk*, which was identified as mannitol. The appearance of this compound is attributed to the capacity of *L. reuteri* to synthesise this sugar (Årsköld *et al.,* 2008), and it could add value to the designed product, since it is seen to have antioxidant properties (Wisselink *et al.,* 2002).

Table 4. Concentrations (mean values and (standard deviation)) throughout storage time of the different sugars identified in fermented oat *milk*. Concentrations of sugars identified in non-fermented oat *milk* are also included for comparisons.

	Time stored (d)	**Mannitol** (g/100 mL)	**Glucose** (g/100 mL)	**Fructose** (g/100 mL)	**Sucrose** (g/100 mL)
Oat *milk*	**-**	-	0.77 (0.11)	0.71 (0.06)	0.074 (0.011)
Fermented oat product	**1**	0.322 (0.026) [a]	0.116 (0.006) [a]	0.345 (0.024) [a]	0.0126 (0.0013) [a]
	7	0.276 (0.019) [b]	0.013 (0.012) [b]	0.34 (0.04) [a]	0.0100 (0.0008) [b]
	14	0.314 (0.029) [a]	0 (0) [c]	0.30 (0.03) [b]	0.0081 (0.0007) [c]
	21	0.277 (0.022) [b]	0 (0) [c]	0.29 (0.06) [b]	0.0049 (0.0007) [d]
	28	0.275 (0.006) [b]	0 (0) [c]	0.225 (0.009) [c]	0.0053 (0.0009) [d]

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

Being a monosaccharide, glucose was a selective nutrient for starter bacteria, since it was the one which decreased most after fermentation. Indeed, this sugar was not present in 14-day fermented products. Surprisingly, fructose did not have the same tendency as glucose, which could be explained by considering that starters might have hydrolysed inulin (unions of fructose) for nutrition purposes. Inulin and its derivatives are seen to be able to stimulate the growth and/or metabolic activity of bacteria, mainly the genera of bifidobacteria and lactobacilli (Gibson *et al*., 2004). This assumption is reinforced by the qualitative analysis of chromatograms, since the concentration of fructan decreased at the end of the storage (Figure 3).

Regarding β-glucan content (data not shown in figure), oat *milk* initially contained 0.241 ± 0.004 g/100 g. Once the fermentation process ended, the initial concentration significantly ($p < 0.05$) decreased ≈ 17%, despite the thermal and acidic stability of β-glucans (Lazaridou and Biliaderis, 2007; Velasco *et al.,* 2009). Therefore, starter bacteria might have hydrolysed this compound in order to obtain nutrients for their growth. Nevertheless, β-glucan content in fermented products did not change significantly throughout storage time, reaching an average value of 0.199 ± 0.008 g/100g. Results reflected that the starter bacteria did not have preferences in this polysaccharide and, from the development of the concentration of analysed sugars, they could also use the added inulin for their survival. Nevertheless, this fact is positive for the final product, since β-glucan prebiotic properties are still available in the product and are beneficial for consumer health.

3.3.3. Microstructure

Figure 4 shows pictures of fermented oat *milk* microstructure obtained in confocal microscope. The main difference initial oat *milk* microstructure (Figure 1) was the presence of a cloudy red area (S) that could be the starter bacteria. Moreover, much smaller amounts of proteins were observed; the starters might have hydrolysed them so as to obtain aminoacids for their nutrition. Nonetheless, and in spite of having observed some coalesced fat droplets, the major fat component was still integrated in the structure in PM network, which was positive for the product's physical stability.

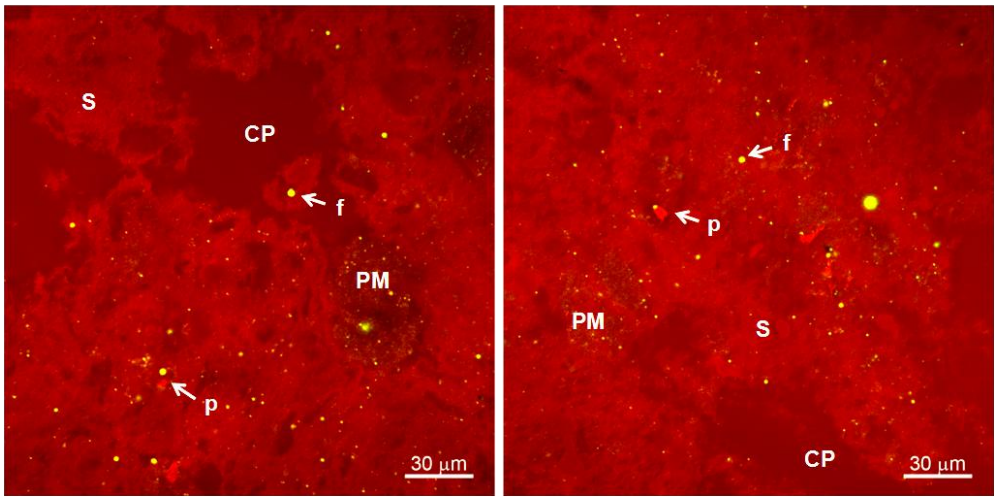

Figure 4 Confocal pictures of fermented oat *milk*, where the fat component appears yellow-green in colour, proteins appear vivid red in colour and carbohydrates and starter bacteria dull red in colour. PM: Polysaccharide matrix, CP: Continuous phase, S: starter bacteria, f: fat droplet, p: protein

3.3.4. Physical properties

No statistical differences were found between the density parameters (ρ) of fermented samples after different storage times, the mean value being 1043 ± 3 kg/m^3. Nevertheless, fermentation causes a slight increase in ρ, since values for non-fermented *milk* were 1019 ± 9 kg/m^3. Starters could modify the inner structure of oat *milks*, probably due to their proteolytic activity, inferred both from the analysis of their microstructure (Figure 4) and from possible changes in charge within the product matrix.

Rheological parameters play a key role in the definition of textural and sensory perception of a new product. These parameters were obtained by using a non-linear regression procedure to fit Eq. 1 to the flow curves of fermented and non-fermented oat *milks* and are summarised in Table 5. The apparent viscosity of samples at 50 s^{-1}shear rate was also shown.

Both fermented and non-fermented oat *milks* were classified as plastic, since samples showed yield stress and flow behaviour index (n) values < 1. Other authors also observed shear thinning behaviour in cereal β-glucan aqueous dispersions (Lazaridou and Biliaderis, 2009; Vasiljevic *et al.,* 2007; Velasco *et al*., 2009). The fermentation process modified the original rheological behaviour of oat *milk* ($p < 0.05$), increasing the apparent viscosity of the samples. This parameter did not show significant changes throughout the storage time ($p < 0.05$). Although proteins seem to be hydrolysed by the starter bacteria (Figure 4) and the fermented samples had a lower β-glucan concentration than the non-fermented (≈ 17% less), the remaining oat β-glucans showed a thickening and gelling capacity, which means they have the ability to increase the viscosity of aqueous solutions.

Several authors (Lizaridou and Biliaderis, 2009) reported that the lower the molecular weight of the β-glucan, the greater is its gelling capacity, which can be attributed to the higher mobility of the shorter chains that enhances diffusion and lateral interchain associations. Indeed, Piotrowska *et al.* (2009) and Sahan *et al.* (2008) observed that β-glucan additions to yoghurt production improved sensory properties and physical stability at concentrations of 0.3 and 0.5 g/100 mL. Besides the effect of β-glucans on the final viscosities of fermented products, *L. reuteri* is able to synthesise exopolysaccharides which might contribute to the observed increase in viscosity (Årsköld *et al.*, 2007).

Table 5. Mean values and (standard deviation) of consistency index (K), flow behaviour index (n) and yield stress (σ_y) of fermented oat *milk*, obtained from fitting experimental data to non-linear model (non-linear correlation coefficient R^2 is included). Apparent viscosity (η) was calculated at shear rate of 50 s^{-1}. Data of oat *milk* are included for comparisons

	Storage time (d)	**K** ($Pa \cdot s^n$)	**n**	**σ_y** (Pa)	**R^2**	**η_{50}** (Pa·s)
Oat *milk*		0.425 (0.013)	0.806 (0.006)	4.0 (0.2)	0.991	0.43 (0.006)
Fermented oat product	**1**	0.70 (0.04) [a]	0.75 (0.03) [a]	8.8 (0.7) [a]	0.987	0.47 (0.02) [a]
	7	1.042 (0.004) [b]	0.72 (0.0) [abc]	11 (0.0) [b]	0.997	0.55 (0.03) [b]
	14	0.74 (0.08) [a]	0.721 (0.022) [ab]	11.3 (1.4) [b]	0.986	0.487 (0.013) [a]
	21	0.85 (0.02) [c]	0.688 (0.007) [c]	11.7 (0.6) [b]	0.979	0.494 (0.008) [a]
	28	0.876 (0.098) [d]	0.699 (0.022) [bc]	11.2 (1.4) [b]	0.979	0.50 (0.02) [a]

[a, b, c, d] Different letters in same column indicates significant differences between samples analysed (95% confidence level)

Although storage time was not observed to have a significant effect on rheological parameters, a little syneresis was observed after 28 storage days, coherent with the progressive aggregation of the particles forming the dispersed phase.

The colour parameters of the samples are shown in Table 6, in which the mean values and standard deviation of colour coordinates of fermented and non-fermented samples are shown. The different values in fermented samples were not observed to differ significantly ($p < 0.05$) throughout the storage time, and so the average values of all fermented samples were included in the table.

Table 6 Mean values and (standard deviation) of Lightness (L^*), colour coordinates a^* and b^*, hue (h^*_{ab}), chrome (C^*_{ab}) and colour difference (ΔE) between non-fermented and fermented oat *milks*.

Sample	L^*	a^*	b^*	C^*_{ab}	h^*_{ab}	ΔE
Oat *milk*	68.8 (0. 4)	-0.4 (0.2)	14.1 (0.4)	14.1 (0.4)	91.5 (1.0)	-
Fermented product	69.9 (0.3)	-0.67 (0.05)	13.43 (0.25)	13.44 (0.25)	92.9 (0.3)	1.4 (0.2)

The structural changes caused by fermentation were reflected in the optical properties of fermented oat *milk*, since significant differences were observed in colour parameters between non-fermented and fermented products. Lightness and hue parameters increased after the fermentation process, while chrome decreased ($p < 0.05$). Nevertheless, the total colour difference between fermented and non-fermented oat *milks* (ΔE) was very

small since, according to Francis (1983), values lower than 3 units cannot be easily detected by the human eye.

3.3.5. Sensory properties

Figure 5 shows the scores of the attributes of appearance, sweetness, acidity, consistency and overall acceptability in the three oat fermented samples analysed by the members of the panel (1, 14 and 28 days' storage at 4 ºC). Significant differences between samples corresponding to different storage times were also included, by indicating the homogeneous groups (same letter in the plot), by means of an LSD test.

Before tasting the three samples, the panelists considered the fermented oat *milk* to have a good appearance with the exception of the sample stored for 28 days. This might be due to the phenomenon of syneresis, which is negatively evaluated in these types of products.

As regards sweetness, in spite of the fact that all the samples were equally sweetened with sucrose, the panelists detected differences between the samples stored for 1 and 28 days ($p < 0.05$). This could be due to the low pH and greater acidity of samples at the end of the storage (Table 3), which has a direct impact on the sweetness evaluation. Although samples stored for 14 days were evaluated as less sweet than those stored for 1 day, the differences in the response were not significant.

The results given by the evaluation of the attribute of acidity coincide with what was observed for sweetness. The panelists did not appreciate any differences between samples stored for 14 and 28 days ($p < 0.05$), which exhibited significant differences in both pH and TA (Table 3).

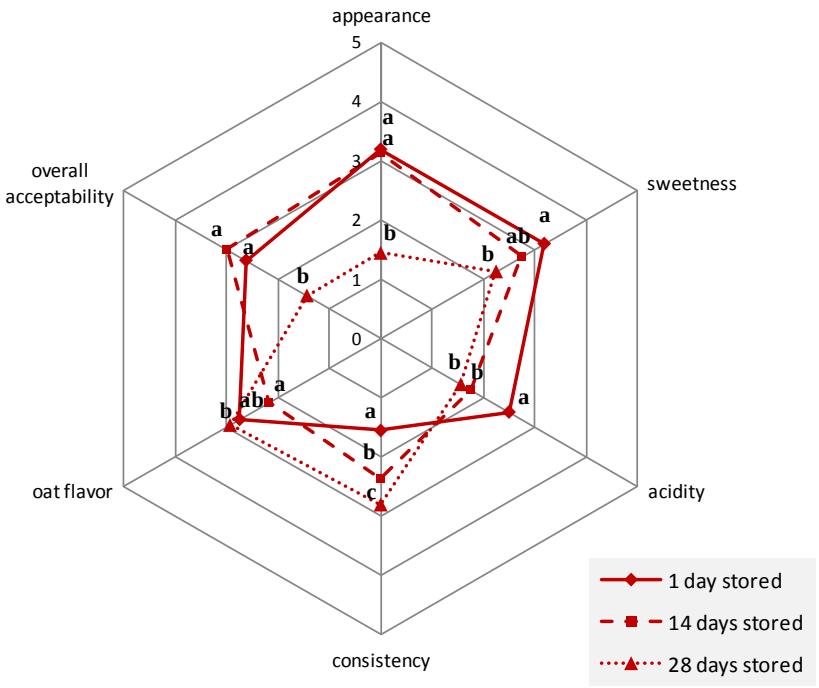

Figure 5. Panelists' evaluation of the attributes of appearance, sweetness, acidity, consistency and overall acceptability in the oat fermented samples after 1, 14 and 28 days' storage at 4 ºC

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

The members of the panel found significant differences in sample consistency depending on the storage time, despite the similar values of the apparent viscosities (Table 5); the longer the storage time, the greater the perceived consistency. This could be attributed to the progressive aggregation of the dispersed particles and β-glucan chains, finally giving rise to the appearance of syneresis, since a gel is not a static structure (Tosh

et al., 2003). Although an oat fermented product is not a strong gel, the observed network in the sample micrographs (Figure 4) can be associated to a weak gel whose aggregation level increased with time, promoting the effective volume reduction of the polysaccharide network. This effect increased the mouthfeel perception of the oat fermented *milk*, since a smaller amount of continuous phase was trapped in the network, leading to less free movement of the overall molecules. This sensation is positive in terms of consumer acceptance, since they prefer highly viscous drinkable yoghurts, which is what this oat fermented product would be (Allgeyer *et al.*, 2010).

The flavour of all the fermented samples was better accepted than original oat *milk* (data not shown), which means that the fermentation process positively modified the flavour attribute, probably due to the synthesis of aromatic compounds by the starter bacteria. The results obtained in this residual oat flavour showed no tendency, which would be attributable to progressive changes in the volatile compounds. Cheng (2010) reported that, during storage, the volatile constituents in yoghurt may change depending on the starters, milk formulation and storage conditions.

From a holistic point of view, around 60% of the panelists accepted oat fermented *milk* (scored the products ≈ 3 or more) with the exception of samples stored for 28 days. Therefore, despite the viability of starters (Table 3), the shelf life of the designed fermented oat *milk* should not be the same as standard yoghurts (28 days).

4. CONCLUSIONS

The Response Surface Methodology was used to identify the levels of the different growth factors which permit an optimal oat *milk* formulation for the fermentation process to be obtained with starter bacteria, *L. reuteri* ATCC 55730 and *S. thermophilus* CECT 986. The defined oat formulation achieved a starter survival above the minimum level suggested for ensuring health benefits (10^7 cfu/mL) and so it can be considered as a functional food. The starter viability was maintained during the whole shelf life typical of this kind of product. The metabolic activity of the starters remained during cold storage due to the availability of nutrients, of which, as expected, monosaccharides are mainly consumed. Thanks to their high stability, a reasonable ratio of oat β-glucans remained in the final fermented products which have a positive effect on the product matrix structure, due to their thickening and gelling properties. Besides the technological advantages, these compounds, together with the added inulin, make the product one of great nutritional interest, since both of them are stated as prebiotics. Thus, consumers might benefit from both the nutritional and health properties of these functional food ingredients: probiotics and prebiotics. Sensory evaluation showed there was a good acceptance of the developed oat fermented product until 14 days of cold storage, ensuring good sweetness, acidity and consistency. Nevertheless, despite the probiotic viability, its shelf life would be shorter than that of the standard yoghurts from the point of view of its sensory acceptance.

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

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ABSTRACT

Functional advantages of probiotic bacteria combined with the nutritional interest of almond (*Prunus amygdalus L.* cv. dulcis) compounds has been considered to obtain a fermented product, which potentially covers the current demand of versatile health-promoting foods. Fermentation process of almond *milk* with a mixed starter culture *Lactobacillus reuteri* ATCC 55730 and *Streptococcus thermophilus* CECT 986 was studied by using a Central Composite Design with Response Surface methodology (RSM) to optimise the values of different growth factors (glucose, fructose, inulin and starter culture addition). The response variable was the final viable cell population in the fermented product. The optimal formulation obtained was characterised throughout cold storage time (28 days) as to pH, acidity, particle size distribution, ζ-potential, serum retention capacity, rheological behaviour, colour properties and probiotic viability. The product was also submitted to sensory analysis to determine the optimal product shelf life. The formulated product allowed starter survival above the minimum suggested to ensure health benefits and be able to be considered as a functional food. This viability was maintained during the whole typical shelf life of this kind of products (28 days). No differences in the sensory acceptability of the product were found between 1 and 28 storage days.

Key words: *L. reuteri*, prebiotic, response surface methodology, survivals, sensory analysis.

1. INTRODUCTION

Probiotics are defined as "live microorganisms that when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). Within this group, *Lactobacillus* and *Bifidobacterium* genera are the most widely recognised, although *Lactococcus*, *Enterococcus*, *Saccharomyces* and *Propianobacterium* genera have been investigated (Rivera-Espinoza and Gallardo-Navarro, 2010). They should meet several requirements, such as total safety for the host, resistance to gastric acidity and pancreatic secretions, adhesion to epithelial cells, antimicrobial activity, inhibition of adhesion of pathogenic bacteria and immune system and metabolic activity stimulation, among others (Prado *et al.*, 2008).

The use of probiotics in food product manufacturing dates back to the ancient world, although the purposes have been changed. Nowadays, not only are these microorganisms used for food preservation and organoleptic improvements but also for their nutritional and health benefits: reduction of hypercholesterolemia, host immune modulation, prevention of urogenital diseases, alleviation of constipation, protection against traveller's diarrhoea, protection against colon and bladder cancer, prevention of osteoporosis and food allergies, among others (Ferreira *et al.*, 2011; Oelschlaeger, 2010; Saad *et al.*, 2013). Host benefits, however, are subject to strain type used in product manufacture (Sharareh *et al.*, 2009). In order to effectively provide health functionalities, the minimum recommended number of viable probiotic bacteria is 10^7-10^8 colony forming per unit (cfu)/g or mL of a

product at the time of consumption (Gomes and Malcata, 1999; Stanton *et al*., 2003; Van Niel *et al*., 2002).

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 which make them interesting and useful to produce synbiotic (combination of probiotic and prebiotic) products. Prebiotics were defined by the Food Agriculture Organization of the United Nations (FAO) as "non-viable food components that confer health benefits on the host associated with modulation of the microbiota".

There is a wide range of commercial vegetable *milks*, although the ones derived from nuts have recently been the subject of interest due to the known impact of their compounds on some current chronic diseases such as cardiovascular diseases (CVD), type 2 Diabetes mellitus (DM-2), obesity and some cancers (Fardet, 2010). Tree nuts are rich in mono- and polyunsaturated fatty acids, vegetable proteins, dietary fibre, phytosterols, polyphenols, vitamins and minerals (Philips *et al.*, 2005; Segura *et al*., 2006); most of those compounds have antioxidant properties and have a proven beneficial effect on plasma lipid profile, low-density lipoprotein (LDL) oxidation and inflammatory processes, among others (Liu, 2012; Carlson *et al*., 2011; Egert *et al.*, 2011; Gillingham *et al*., 2011; Jones *et al.*, 2011). Indeed, epidemiological studies have linked frequent nut consumption to a reduced risk of CVC, DM-2 or death by all-cause mortality (Kelly Jr and Sabaté, 2006). Li *et al.* (2009) observed that an

increase in nut consumption was significantly associated with a more favourable plasma lipid profile, including lower LDL cholesterol, total cholesterol and apolipoprotein B-100 concentrations without any significant associations with high-density lipoprotein cholesterol or inflammatory markers. Moreover, nuts have a high K/Na ratio and the carbohydrates present have a low Glycemic Index (suitable for diabetics). In terms of the world production of tree nuts, almonds ranked third in 2010, the USA and Spain being the two major producers of almonds (FAO ProdStat database). The increasing consumption of almonds is a consequence of their stated potential health benefits. Nevertheless, this nut is also classified as a potential allergenic seed known to be responsible for triggering several immune reactions in sensitised and allergic individuals (Costa *et al*., 2012). On the other hand, probiotic bacteria are seen to influence the human immune system positively and, hence, they might be able to reduce almond allergenicity. Therefore, almond nut *milks* are very useful in the industrial production of new non-dairy products with synbiotic features in two senses; on the one hand, to cover the consumer demand for food with versatile health-promoting properties and, on the other, to reduce the allergenicity of almonds by offering a product in which the nutritional and health benefits of almonds are included and which is safe for the targeted population.

The aim of this study was to evaluate the fermentative process of almond (*Prunus amygdalus L.* cv. dulcis) *milk* with the probiotic *L. reuteri* ATCC 55730. *S. thermophilus* CECT 986 was also introduced in the starter inoculum (ratio 1:1), since it was able to improve fermentation times in previous studies (data not shown). To this end, the effect of different,

previously chosen factors (glucose, fructose and inulin and inoculum additions) were analysed and optimised to define the most suitable almond *milk* formulation in which sufficient probiotic bacteria survivals would be ensured in the final product. The fermented almond *milk* with the optimum growth factor values was characterised as to its physicochemical, microbiological and sensory 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 International Corporation, USA) by applying 172 MPa and further on high 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 initial microbiological load and physical stability, since this innovative technology is able to reduce and/or eliminate non-desirable microorganisms and reduce the size of fat globule particles such a way that flocculation and coagulation phenomena are delayed (Capra *et al.*, 2009; 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 growth factors, glucose, fructose and inulin were added prior to the heat treatment. 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, Paterna (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 37ºC/24h/anaerobically for *L. reuteri* and 42ºC/24h/aerobically for *S. thermophilus*.

As regards the starter inoculum, strains were independently incubated in their broths for 24 h and then centrifuged at 10,000 rpm/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 growth factor variables to obtain fermented almond *milks*. Central Composite Design (CCD) with randomised Response Surface methodology (RSM) was used to study how different combinations of growth factors affect almond *milk* fermentation. Other authors also used RSM in the development of probiotic products (Cruz *et al.,* 2010; Gupta *et al.,* 2010; Liew *et al.,* 2005; Shuhaimi *et al.,* 2009; Stepheine *et al*., 2007; 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 were 1.5 to 3 g/100 mL for both glucose and fructose, 2 to 4 g/100 mL for inulin and 5 to 7 mL/100 mL for starter inoculum. 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 4.6-4.8, 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 growth 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 raw 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 colour. In almond *milk*, the chemical composition of major components (dry matter, protein, lipids, total sugars and ashes) was obtained. Moreover, the fermented product was analysed throughout storage time (1, 7, 14, 21 and 28 days) at 4 ºC in terms of starter survival, proteolytic activity, colloidal stability and sensory attributes. All the analyses were performed 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 the following HPAC-PAD equipment: Metrohm 838 Advanced Sample Processor (Metrohm® Ltd., Herisau, Switzerland) in an Advanced Compact IC 861 ion chromatograph (IC) equipped with a pulsed amperometric detector to monitor the separation (Bioscan 817). 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 initial values. Before injecting samples into the equipment, they were filtered through nylon membranes (0.45 μm). A Metrosep CARB guard column (5x4.0 mm Metrohm) and a Metrosep CARB 1 (250 x 4.6 mm Metrohm) analyses column were used. 20 μL of sample was injected and eluted (1 mL/min) with 0.1 mol/L NaOH, at 32 ºC. An Au working electrode was used and applied potentials were +0.05 V (between 0 – 0.40 s) +0.75 V (between 0.41 – 0.60 s) and +0.15 V (between 0.61 – 1 s). Software ICNet 2.3 (Metrohm® Ltd., Herisau, Switzerland) 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® , Spain), which were obtained in triplicate.

2.3.2 Bacterial counts

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 acidified MRS agar

(Scharlab; Barcelona, Spain) for the probiotic strain and M17 agar (DifcoTM; New Jersey; USA) for S. *thermophilus*. Incubation conditions were 37 ºC/24 h/anaerobically for *L. reuteri* and 37 ºC /48 h/aerobically for *S. thermophilus*. Counts were reported as log cfu/mL.

2.3.3 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 100 mL (Horwitz, 2000).

2.3.4 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 0.4 g/100 mL using a phosphate buffer solution. The Smoluchowsky mathematical model was used to convert the electrophoretic mobility measurements into ζ -potential values.

2.3.5 Colloidal stability

Serum retention capacity (SRC) of both non-fermented and fermented *milks* was analysed by sample centrifugation (Medifriger-BL, JP-Selecta; Spain). Conditions were 3,000 rpm/45 min/20 ºC and the amount of serum separation was used to quantify sample stability.

2.3.6 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).

$$\sigma = \sigma_y + K\,\dot{\gamma}^{\,n} \tag{1}$$

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

2.3.7 Colour parameters

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

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

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

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

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

2.3.8 Sensory analysis

A 16-member semi-trained panel evaluated fermented almond products with two different storage times (1 and 28 days) at 4 ºC. The panelists were selected on the basis of their interest, availability, lack of food allergies and their threshold to basic flavours.The members of the panel were trained following the method described by Mårtensson *et al*. (2001) with some modifications. They were trained to score attributes of sweetness, acidity, almond flavour, consistency and mouthfeel and overall acceptability using interval scales that varied from 1(slightly) to 5 (extremely).

Reference samples were used for setting the interval scales for panel training. For acidity reference, 1 and 2 g/100 mL of sucrose was added to commercial milk yoghurt, corresponding to 3 and 1 on the scale, respectively; 0.2 g/100 mL of citric acid corresponded to 5. Commercial milk yoghurt with 2, 5 and 14 g/100 mL added sucrose was used for sweetness evaluation, corresponding to 1, 3 and 5 on the scale, respectively. For consistency and mouthfeel, drinkable yoghurt, commercial soy dessert and Danone original® yoghurt were used as references, corresponding to 1, 3 and 5 on the scale, respectively. For almond flavour, the reference was the almond *milk* used in the study, which corresponded to 5 on the scale.

Each panelist tested 2 fermented samples (cold stored for 1 and 28 days, respectively) containing 6 g/100 mL of sucrose, to quantify the attributes in which each one was trained. Samples were randomly presented with a code of three digits. Evaluation was conducted in a normalised tasting room at room temperature.

2.4 Statistical Analysis

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

3. RESULTS AND DISCUSSION

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. Fibre content was obtained by difference to 100 of the sum of rest of analysed components.

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

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)
Fiber	6.82	0.58

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 sugars /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).

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 ensure health effects in consumers (Gomes and Malcata, 1999; Stanton *et al*., 2003; Van Niel *et al.,* 2002).

Table 2. Probiotic survival after 28 days of storage (log cfu/mL) for fermented almond *milk* formulations of the CCD.

	FACTORS				RESPONSE
Run order	$\mathbf{X_1}$	$\mathbf{X_2}$	$\mathbf{X_3}$	$\mathbf{X_4}$	**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	$+\alpha$	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: 1.5-3 g/100 mL; Fructose: 1.5-3 g/100 mL; Inulin: 2-4 g/100 mL; Inoculum: 5-7 mL/100 mL.

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 (Granato *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.

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**

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 adequate 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^2adj (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). Hence, this model should only be used to make rough predictions.

The CCD model was then statistically optimised in order to maximise the viability of the *L. reuteri* (variable response) after 28 days of storage 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 0.178 ± 0.005 g of lactic acid per 100 mL. This value is lower than standard yoghurt, which has a lactic acid content of around 0.8-1 g/100 mL (Mistry and Hassan, 1992; Tamime and Robinson, 2000). This acidity could be explained by considering that, on the one hand, almond *milk* has a lower buffering capacity than cow milk and, on the other, the *L. reuteri* used is a heterofermentative microorganism and synthesises acetic acid (Årsköld *et al.*, 2008).

3.3 Fermented samples characterisation

3.3.1 Bacterial counts and acid production

Table 4 shows the average values of pH and Titratable Acidity (TA) in non-fermented and 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. The viability of both strains decreased throughout storage time ($p < 0.05$), especially for *S. thermophilus*. Despite the final *S. thermophilus* viability, the probiotic

L. reuteri survival was above the minimum recommended level (10^7 cfu/mL) in the whole period analysed. Therefore, taking into account the typical shelf life of fermented milk products, the obtained fermented almond *milk* might be considered as a new functional food within the drinkable yoghurt-like products. Nevertheless, the final product should be *in-vitro* and *in-vivo* tested, since, among other variables, probiotic health benefits are seen to be dependent on the matrix in which they are present (Buddington, 2009).

Table 4. 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/100 mL of lactic acid)	*L. reuteri* (log cfu/mL)	*S. thermophilus* (log cfu/mL)
AM	6.567 (0.006)	0.039 (0.003)	-	-
FAM 1d	4.657 (0.012) ^{a}	0.190 (0.012) ^{a}	7.59 (0.04) ^{a}	7.54 (0.14) ^{a}
FAM 7d	4.63 (0.02) ^{b}	0.223 (0.009) ^{b}	7.30 (0.02) ^{b}	7.19 (0.14) ^{bc}
FAM 14d	4.657 (0.006) ^{a}	0.223 (0.00) ^{b}	7.26 (0.11) ^{b}	7.33 (0.10) ^{bd}
FAM 21d	4.633 (0.012) ^{b}	0.219 (0.007) ^{b}	7.00 (0.16) ^{c}	6.89 (0.21) ^{ce}
FAM 28d	4.650 (0.019) ^{ab}	0.226 (0.10) ^{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.

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 7th day onwards differences among values were non-significant ($p < 0.05$). These results were in accordance with starter survival, since bacterial growth was not exponential and, hence, synthesis of acidic compounds was bound to stabilise. Standard yoghurts have a TA of 0.8-1 g/100 mL of lactic acid (Mistry and Hassan, 1992; Tamime and Robinson, 2000) and the obtained fermented almond *milks* had a TA of around 0.216 g/100 mL. This lower TA might have a positive effect on the overall acceptance of the final product, since it has a direct impact on sweetness attribute.

3.3.2 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 1 shows the chromatograms of both non-fermented (AM) and fermented (FAM) almond *milks* stored for different times (1, 14 and 28 days). As can be seen, prior to the fermentation process, sucrose, glucose and fructose were present in almond *milk*, besides two other peaks which could not be identified. The latter were not present in pure almond *milk* (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).

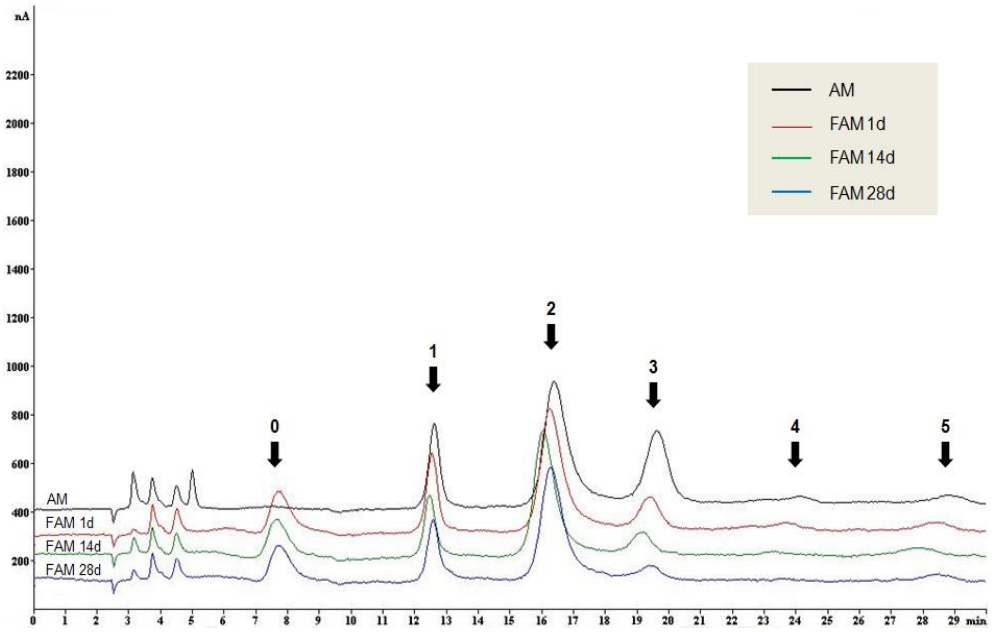

Figure 1 Chromatograms of sugar peaks obtained in HPAC-PAD assays from formulated almond *milk* (AM) and its fermented products after 1 (FAM 1d), 14 (FAM 14d) and 28 (FAM 28d) days of storage at 4 ºC. Peaks identified were mannitol (0), glucose (1), fructose (2), sucrose (3) and oligosaccharides becoming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

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 *L. reuteri* strain is able to synthesise this compound (Årsköld *et al.*, 2008). 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). Table 5 shows the amount of the different sugars identified in both fermented throughout storage time and non-fermented almond *milks*. As can be seen, after the fermentation process, a significant

reduction in the monosaccharide and sucrose contents occurs, while afterwards, they gradually decreased ($p < 0.05$) throughout the storage time. These results were predictable, since starter bacteria were viable during the entire storage time (Table 4) and, therefore, they consumed these sugars as nutrients.

Table 5. Mean values (and standard deviation) of concentrations of the different sugars throughout storage time (days) at 4 ºC, in fermented almond *milk* (FAM). Values for non-fermented formulated almond *milk* (AM) are also included for comparisons.

Sample	Mannitol (g/100 mL)	Glucose (g/100 mL)	Fructose (g/100 mL)	Sucrose (g/100 mL)
AM	-	1.43 (0.17)	0.69 (0.04)	0.164 (0.008)
FAM 1d	0.89 (0.03) [a]	1.14 (0.07) [a]	0.596 (0.013) [a]	0.058 (0.002) [a]
FAM 14d	0.869 (0.011) [a]	1.05 (0.05) [b]	0.601 (0.009) [a]	0.043 (0.003) [b]
FAM 28d	0.79 (0.03) [b]	1.00 (0.09) [b]	0.55 (0.02) [b]	0.0243 (0.0014) [c]

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

As observed in Table 5, the amount of mannitol within the fermented product decreased over the storage time, although this was only significant in the last storage day. In case of fructan peaks (Figure 1), it seems that the longer-chain fructan (peak 5) did not decrease during the storage time, which suggests that the bacteria 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 matrix. Hence, most of the added inulin might be preserved in the product, thus, the targeted consumers of the fermented product can take advantage of its prebiotic activity.

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

Table 6 also shows ζ-potential value in both fermented and non-fermented *milks*. Fermentation provoked a lower negative charge of the dispersed particles ($p < 0.05$), which means that the neutralisation of some ionisable groups occurs as a consequence of the change in the pH of the product. The almond protein charge will decrease, thus promoting a reduction in the ζ-potential and repulsive forces among the dispersed particles. This effect will lead to the phenomenon of flocculation in the system, which can give rise to a weak gel structure taking the volume fraction of the dispersed phase into account. Particle flocculation will be responsible for the increase in particle size after fermentation. This result

was coherent with the isoelectric point (IP) range of amandin (4.55-6.3) reported by Albillos *et al.* (2009) and Sathe *et al.* (2002). Despite the fact that the value of the sample pH was close to the IP of the major almond protein, all the values of ζ-potential in fermented samples were negative, possibly due to the high conformational complexity of the almond protein. Although changes in protein conformation could allow a stable matrix in a network distribution, as occurred in standard yoghurts, the low protein content does not ensure the stability of fermented almond samples. Indeed, ζ-potential values lower than ± 25 mV do not ensure the stability of dispersed systems (Roland *et al.,* 2003).

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

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 6 also shows the SRC obtained by sample centrifugation (expressed as percentage of precipitate after centrifugation) in both fermented and non-fermented samples. A greater serum separation occurred in non-fermented samples, while very few differences were observed in the case of fermented samples stored for different lengths of time. These results confirm the formation of a weak gel in the fermented product as a result of the flocculation of dispersed particles due to the action of proteins, which was able to retain part of the serum present in the almond *milk*. Taking into account that neither the fermentation process nor the storage time might affect inulin, it could also contribute to the network formation due to its thickening and gelling capacity (Frank, 2002).

3.3.4 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 was also shown.

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

Sample	σ_y (Pa)	n	K ($Pa \cdot s^n$)	R^2	$\eta_{50} \cdot 10^3$ ($Pa \cdot s$)	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)

Results showed that the inner structure of the almond *milk* were not significantly ($p > 0.05$) modified due to either fermentation procedure or storage time at 4 ºC.

Both the apparent viscosity as well as the thixotropic character of the samples increased slightly after the fermentation step ($p < 0.05$). Figure 3 shows the up flow curves of the different samples and Table 7 shows the values of the hysteresis area which reflects the thixotropic nature of the products. This character increased after fermentation in line with the formation of a weak gel structure, as commented on above. *L. reuteri* is able to synthesise exopolysaccharides and, thus, it might also contribute to the

gel formation and the increase in viscosity (Årsköld *et al.*, 2007). Differences in the thixotropic nature of fermented products due to storage time at 4 ºC were non-significant, surely due to the high variability of data (Table 7).

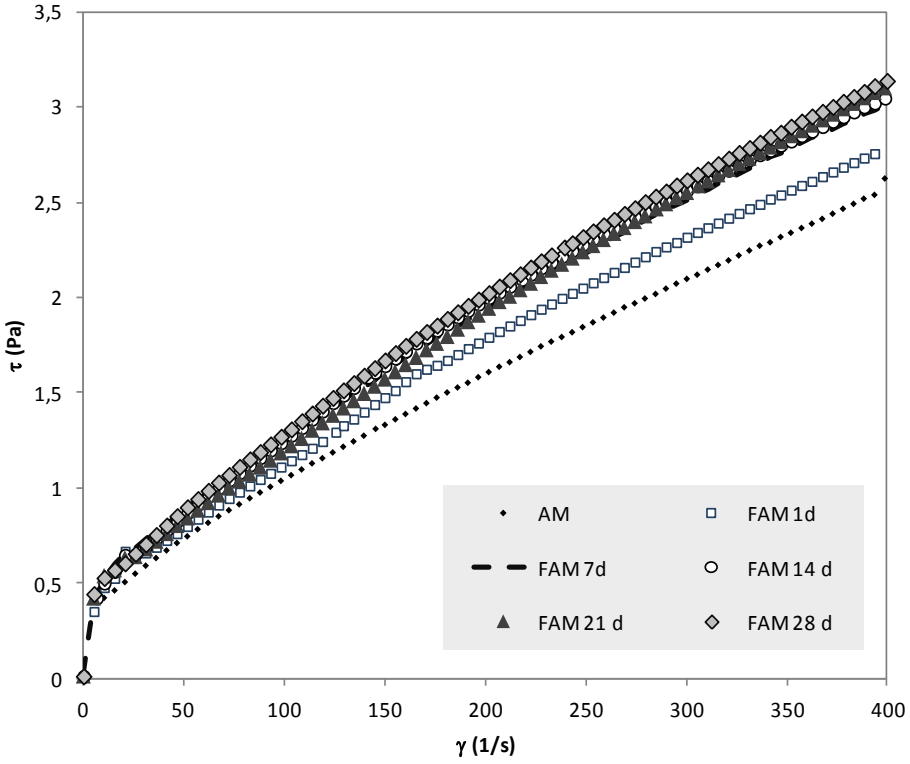

Figure 3. Up-flow curves of fermented almond *milks* (FAM) at different times (1, 7, 14, 21 and 28 days) stored. Non fermented almond *milk* (AM) curve is also presented for comparison.

3.3.5 Colour measurements

Table 8 shows the values of lightness (L^*), chrome (C^*_{ab}), hue (h^*_{ab}) and whiteness index (WI) of both non-fermented almond *milk* (AM) and fermented products (FAM) at different storage times (1, 7, 14, 21 and 28 days). Structural changes occasioned by the fermentation process were reflected in the optical properties of almond *milk* ($p < 0.05$).

Table 8 Mean values and standard deviation of lightness (L^*), chrome (C^*_{ab}) and hue (h^*_{ab}) of non-fermented (AM) and fermented (FAM) almond *milks* throughout storage time at 4ºC (days).

Sample	L^*	C^*_{ab}	h^*_{ab}	W.I.
AM	87.83 (0.02)	5.80 (0.03)	97.0 (0.3)	86.516 (0.002)
FAM 1d	90.48 (0.05) ^{a}	5.47 (0.02) ^{a}	100.56 (0.24) ^{a}	89.02 (0.06 ^{a}
FAM 7d	90.43 (0.03) ^{a}	5.45 (0.07) ^{a}	99.89 (0.09) ^{bc}	88. 99 (0.02) ^{a}
FAM 14d	90.47 (0.06) ^{a}	5.49 (0.03) ^{a}	100.2 (0.5) ^{ab}	89.00 (0.06) ^{a}
FAM 21d	90.46 (0.01) ^{a}	5.39 (0.04) ^{b}	99.44 (0.07) ^{c}	89.04 (0.03) ^{ab}
FAM 28d	90.51 (0.07) ^{a}	5.33 (0.03) ^{b}	99.79 (0.22) ^{bc}	89.11 (0.05) ^{b}

$^{a-c}$ Different letters in same column indicates significant differences between samples analysed at 95% of confidence levels.

L^*, h^*_{ab} and WI increased after the fermentation process, while C^*_{ab} decreased ($p < 0.05$). 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 ($p < 0.05$). Moreover, 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.

3.3.6 Sensory analysis

Figure 4 shows the scores of the attributes of appearance, sweetness, acidity, consistency and overall acceptability in the fermented almond samples tested by the members of the panel (after 1 and 28 storage days at 4ºC). Statistical differences between storage times were also included, showing the homogenous groups according to a LSD test (95% of confidence level).

Before tasting the fermented products, the panelists considered their appearance was good and this attribute was unaffected by the storage time ($p < 0.05$). Nor were any significant differences appreciated in sample consistency. Once the samples were tasted, in spite of the fact that the same amount of sucrose was added to both samples, the panelists appreciated more sweetness in the sample stored for 28 days ($p < 0.05$). Coherent with the sensory perception acidity-sweetness relationship, samples stored for 1 day were appreciated as being significantly more acid in taste than those stored for 28 days. However, the longer the storage time, the greater the TA (Table 4). This controversial result could be explained by considering the synthesis of the volatile acetic acid brought about by *L. reuteri*, which is seen to transfer detectable vinegary, pungent and acidic odours into

fermented products (Cheng, 2010). This can be formed mainly at the beginning of fermentation and, after 28 storage days, the negative effect of acetic acid might have disappeared due to its volatilisation.

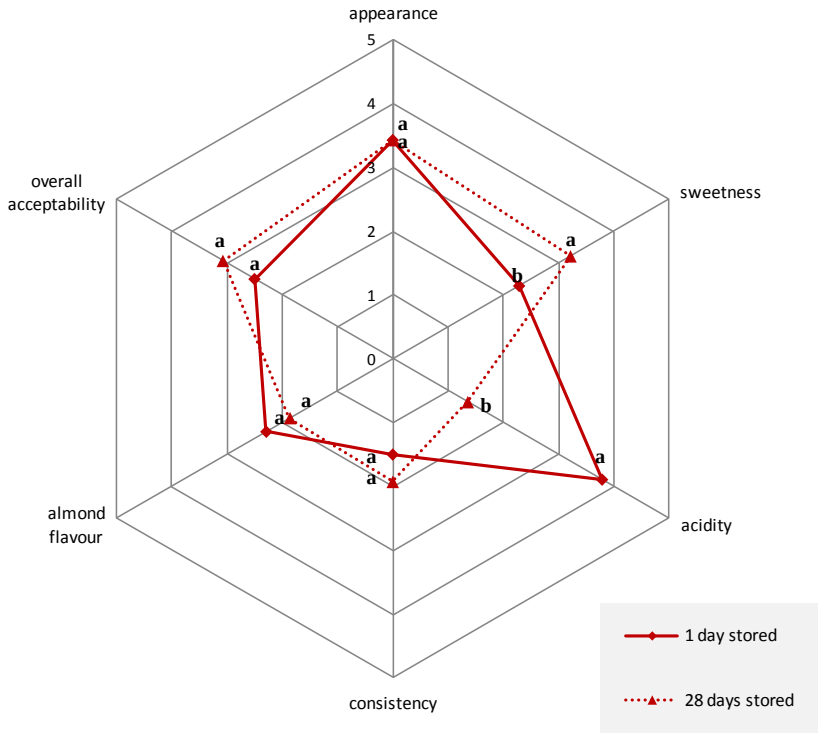

Figure 4. Panelists' scores for sweetness, acidity, consistency and overall acceptability of the fermented almond samples stored for 1 and 28 days at 4 ºC

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

As far as the almond flavour is concerned, the fermentation process modified this attribute, probably due to the aromatic compounds synthesised by the starter bacteria. This result might negatively affect the market success

of the product, since the almond flavour would hardly be detected. Therefore, the success of this product in the food market would depend on a firm marketing strategy focusing on the product's beneficial health properties. From a holistic point of view, it is remarkable that fermented almond *milks* were approved without distinction due to the storage time effect ($p < 0.05$), albeit with low marks. Therefore, some modifications in mouthfeel and/or flavour should be studied in more detail in order to ensure the developed product enjoys wide acceptance.

4. CONCLUSIONS

The optimal combination of growth factors which would ensure the minimum recommended cell population ($\geq 10^7$ cfu/mL) in the functional fermented product 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, with no changes taking place during storage time, while the TA increased from 0.19 to 0.23 g/100 mL lactic acid. The viability of bacteria ($\geq 10^7$ cfu/mL) was maintained throughout the entire storage time and their consumption of monosaccharides and sucrose was observed, while they released mannitol into the medium and longer-chain fructan did not decrease during the storage time. The particle size increased during fermentation, while the negative ζ- potential decreased, in line with the partial neutralisation of the protein ionisation groups and the flocculation induced by the poor quality of the solvent caused by the fall in pH. This promoted the formation of a weak

gel which retained serum to a greater extent than non-fermented *milk*. Gel formation implied an increase in the apparent viscosity and in the thixotropic nature of the product, which exhibited a plastic behaviour with yield stress. No notable changes occurred in the product rheology or colour parameters during storage time. Likewise, the sensory evaluation of the product at 1 and 28 storage days did not reveal any changes in its appearance, overall acceptability, almond flavour or consistency. Nevertheless, storage led to a sweeter product with lower acidity levels, which is not coherent with the TA obtained. So, the product's shelf life is within the range of that given for this kind of fermented functional products. Nevertheless, some modifications in mouthfeel and/or flavour should be studied in order to increase its sensory scores and ensure that it enjoys a wide market acceptance.

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

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ABSTRACT

The fermentative process of hazelnut *milk* with probiotic *L. rhamnosus* GG and *S. thermophilus* was studied in order to develop a new non-dairy probiotic product. To this end, the effect of different factors (the addition of glucose, inulin and inoculum), was analysed to ensure sufficient probiotic survival to exert health benefits in a shorter processing time. The optimised fermented product was characterised throughout storage time (0, 1, 7, 14, 21 and 28 days) at 4 ºC as to its main physicochemical properties (pH, acidity, sugar content, proteolytic activity, rheological behaviour, colour and colloidal stability) and quality parameters (probiotic survival before and after *in vitro* digestion and sensory analysis) and determine, thus, its shelf life. The defined formulation allowed probiotic survival above the recommended minimum (10^7 cfu/mL) and more than 60% survived the *in vitro* digestion. This viability was maintained for 28 storage days. The metabolic activity of the starters had an expected preference for glucose, while inulin remained in the product and was able to exert health benefits. Fermentation modified the rheological behaviour of hazelnut *milk* giving rise to the flocculation of macromolecules and dispersed particles forming a weak gel which generates syneresis on the last storage time controlled. Nevertheless, sensory evaluation showed well acceptance of the fermented product until the end of the cold storage (28 days).

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

1. INTRODUCTION

The use of probiotics and prebiotics, both concepts defined as elements that exert health benefit on the host (FAO/WHO, 2001; 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 can be found the reduction of hypercholesterolemia, host immune system modulation, the prevention of urogenital diseases, the alleviation of constipation, protection against traveller's diarrhoea, protection against colon and bladder cancer and the prevention of osteoporosis and food allergies (Lourens-Hattingh and Viljoen, 2001).

Products containing probiotic microorganisms have been commonly produced by using animal milk, drinkable yoghurt being the best known. Nonetheless, new food matrices are being 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 allergy and intolerance issues and the fact that these products are lactose-free, cholesterol-free and low-calorie (Stone, 2011). Furthermore, experts are starting to consider possible relationships between vegetable products and the prevention of cancer, atherosclerosis or inflammatory diseases, since free radicals play a key role in those pathologies and this type of food is an excellent source of antioxidants (Scalbert and Williamson, 2000). Moreover, some of these vegetable products contain prebiotics (i.e. inulin and the derivative

fructooligosaccharides) which, on top of the health benefits to consumers, provide the fermentation process with technological benefits, such as a viscosity increase in the food matrices, and are seen to have a synergic effect on probiotic survival during processing and storage (Capela, Hay and Shah, 2006; Franck, 2002; 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 *milks*. Indeed, hazelnut has recently been used in non-traditional foods due to the fact that is has acknowledged nutritional and nutraceutical properties (Alasalvar *et al.*, 2003). This nut provides a good source of dietary fibre, antioxidants, phytosterols and carbohydrates with low glycemic index (suitable for diabetics). Moreover, the 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 (Mercanligil *et al.*, 2007; Tey *et al.*, 2011). 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 (Alasalvar *et al.*, 2003; Tey *et al*., 2011).

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 probiotic *L. rhamnosus* ATCC 53103 (usually known as GG) combined with *S. thermophilus* CECT 986. To this end, the effect of different growth factors (the addition of glucose, inulin and inoculum) was analysed to ensure sufficient probiotic survival able to exert health benefits. The most adequate fermented formulation would then be characterised as to its main physicochemical properties and quality parameters (including sensory analysis), as well as the product shelf life.

2. MATERIALS AND METHODS

2.1 Preparation of hazelnut *milk*

Hazelnut *milk* was produced by soaking and grinding hazelnuts (*Corylus avellana L.* cv. comuna), supplied by Frutos Secos 3G S.L. (Valencia, Spain). The extraction was carried out using Sojamatic 1.5 (Sojamatic®; Barcelona, Spain) equipment specifically designed for the production of vegetable *milks*, with a nut:water ratio of 8:100. The manufacturing process takes 30 minutes at room temperature. The milky liquid obtained was homogenised at 33 MPa (15M-8TA-SMD model, Manton Gaulin, UK) and then pasteurised at 85 ºC - 30 min.

To promote the colloidal stability of the *milk*, 0.05 g/100 mL of xanthan gum, supplied by ROKOgel (Asturias, Spain), was added as thickener agent prior to the heat treatment.

2.2 Preparation of fermented products

2.2.1 Inoculum preparation

Lactobacillus rhamnosus ATCC 53103 (from now on GG) (LGC Standards S.L.U., Barcelona, Spain) and *Streptococcus thermophilus* CECT 986 (from now on T) (CECT, Paterna (Valencia), Spain) were activated from their frozen forms (stored in 40 g/100 mL glycerol at – 80 ºC), by transferring each one to its selective broth until optimal bacterial growth is ensured. The selective broths were MRS (Scharlab; Barcelona, Spain) for GG and M17 (DifcoTM; New Jersey; USA) for T. The incubation conditions were 37 ºC/24h/anaerobically for GG 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 10,000 rpm - 10 min at 4 ºC; the supernatant was subsequently 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.

2.2.2 Experimental design for the fermentation process.

Amounts of glucose, inulin and starter inoculum added to the *milk* were selected as growth factors (3 independent variables) to obtain fermented hazelnut *milks*. Central Composite Design (CCD) with randomised Response Surface methodology (RSM) was used to analyse the effect of the different growth 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. Other authors have also recently used this methodology to optimise the fermentation process of vegetable *milks* (Hassani, Zarnkow and Becker, 2013; Khoshauand *et al.*, 2011; Yaakob *et al*., 2012). 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, Fávaro Trindade *et al*., 2001; Yang and Li, 2010; Paseephol and Sherkat, 2009; 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, which was the optimal growth temperature of the mixed culture. When the pH of samples reached 4.6-4.8 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 growth factors. The optimal

formulation of the fermented product 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, total sugars and ashes) was obtained. Moreover, the fermented product was analysed throughout the storage time (0, 1, 7, 14, 21 and 28 days) at 4ºC in terms of probiotic survival before and after having submitted the samples to a simulated gastrointestinal digestion (SGID), proteolytic activity, 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 the following HPAC-PAD equipment: Metrohm 838 Advanced Sample Processor (Metrohm® Ltd., Herisau, Switzerland) in an Advanced Compact IC 861 ion chromatograph (IC) equipped with a pulsed amperometric detector to monitor the separation (Bioscan 817). 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). A Metrosep CARB guard column (5 x 4.0 mm Metrohm) and a Metrosep CARB 1 (250x4.6 mm Metrohm) analyses column were used. 20 μL of sample was injected and eluted (1 mL/min) with 0.1 mol/L NaOH, at 32 ºC. An Au working electrode was used and applied potentials were + 0.05 V (between 0 – 0.40 s) + 0.75 V (between 0.41 – 0.60 s) and +0.15 V (between 0.61 – 1 s). Software ICNet 2.3 (Metrohm® Ltd., Herisau, Switzerland) 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®, Spain), 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 SGID

Fermented hazelnut *milk* samples were submitted to 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) with some modifications. Porcine pepsin

(800 – 2500 units/mg protein), pancreatin (activity, 4 1 USP specifications) and bile extract were purchased from Sigma Chemical (All Sigma–Aldrich Corp., St. Louis, MO, USA). To start the peptic digestion, the pH of the samples was adjusted to 2 with 5 mol/L HCl and 0.5 mL of pepsin solution (0.2 g pepsin dissolved in 10 mL of 0.1 mol/L HCl) was added per 10 mL of sample; then they were incubated for 60 min stirring constantly. Once the peptic digestion is finished, 2.5 mL of pancreatin-bile extract mixture (0.05 g pancreatin and 0.3 g bile extract dissolved in 35 mL of 0.1 mol/L $NaHCO_3$) was added per 10 mL of the original sample, after having raised the pH of samples to 6 pH by drip addition of 1 mol/L $NaHCO_3$; after that, the pH was readjusted to 7 with 1 mol/L NaOH and the final volume was brought to 15 mL with 12 mmol/L NaCl and 5 mmol/L KCl. The samples were finally incubated at 37 ºC for 120 min, stirring constantly, to proceed with the intestinal digestion.

The pour plate technique was employed to quantify GG survivals, according to the method described by the International Dairy Federation (International 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 Proteolytic activity

The extent of proteolysis in fermented hazelnut *milk* samples stored for different times (0, 1, 7, 14, 21 and 28 days) was evaluated by measuring the free amino acids and small peptides using the *o*-phtaldialdehyde (OPA) method described by Church *et al.* (1983). The fermented samples were

diluted 1:1 (w/w) in deionised water and 30 μL were removed and added to 3 mL of OPA reagent. The absorbance of the solutions was measured spectrophotometrically at 340 nm in a quartz cuvette by using a UV-visible spectrophotometer (Helios Zeta UV-vis, Thermo Scientific, USA) after 2 min incubation at room temperature. The starters' proteolytic activity is quantified as the difference in absorbance measured between fermented and non-fermented hazelnut *milks*.

2.3.5 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}, 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 (η) were calculated at 50 s^{-1} (Eq. 2), since the shear rates generated in mouth when food is being chewed and swallowed are between 10 and 100 s^{-1} (McClements, 2004).

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

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

2.3.6 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.

2.3.7 Colour parameters

The colour coordinates were measured from the infinite reflection spectrum in a spectrocolorimeter (CM-3600 d, 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}), hue (h^*_{ab}) and Whiteness Index (WI), as defined in equations (3) to (5). The colour differences (ΔE) between fermented and non-fermented samples were also calculated by using equation (6).

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

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

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

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

2.3.8 Sensory analysis

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

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

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

2.4 Statistical Analysis

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

3. RESULTS AND DISCUSSION

3.1 Effect of growth 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 minimum recommended probiotic amount in order to ensure health effects in consumers (Gomes and Malcata, 1999; Stanton *et al*., 2003; Van Niel *et al.*, 2002). 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 and Reid, 2006).

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 levels of the growth factors.

Run order	Growth factors			Responses	
	X_1	X_2	X_3	Y_1 (h)	Y_2 (log CFU/mL)
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: 5-7mL/100 mL, fermentation time (h) and probiotic counts (log cfu/mL), respectively.

Results from the 18 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^2 adj), the term was included in the model. The quality of the fitted model was evaluated by means of an analysis of variance, mainly based on the F-test and on the R^2 adj, 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 (Granato *et al.*, 2010). As regards the GG response, the model fitted the experimental results poorly (data is not shown). On the contrary, the model obtained for the fermentation time appeared to be adequate for predicting this response (Y_2), since the p-value of the lack-of-fit parameter was greater than 0.05. Table 2 summarises the fitted results with the corresponding R^2adj and F-ratio values; 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 and, thus supporting the proper prediction of the model.

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
R^2	0.56	-	-
R^2-adj	0.32	-	-
Standard error of est.	0.25	-	-
Mean absolute error	0.35	-	-
Durbin-Watson statistic	2.73	-	0.925

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 all bacteria, especially in 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, commented on 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 Lactobacillus genus (Kolida, Tuohy and Gibson, 2002). As

shown in Table 2, the fitting coefficients of the model were very low. The determination coefficient (R^2) was 0.56 and only 32% of the variation in the experimental data (R^2adj) was explained by the predictive model. A model is considered adequate to describe the influence of the dependent variable(s) when R^2 is at least 80% (Yaakob *et al*., 2012) or values of R^2adj over 70% (Cruz *et al.,* 2010). In this case, it is difficult to obtain greater values because the variation of the experimental responses is very low (most fermentation times were around 3.5 h) (Table 1), and 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 minimum survival of over 10^7 cfu/mL (Gomes and Malcata, 1999; Stanton *et al*., 2003; Van Niel *et al.,* 2002). Furthermore, fermentation is a critical process within the product development 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 fermentation time was minimised and GG counts for 28 days were maintained at 8 log cfu/mL. 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 vegetable *milk* fermented in 3.6 h and, after being stored for 28 days at 4 ºC, GG survival in the fermented product was 8 log cfu/mL. Figure 1 shows the overlay of the RSM contour plots for the two variable responses and the location of the optimised formulation.

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.

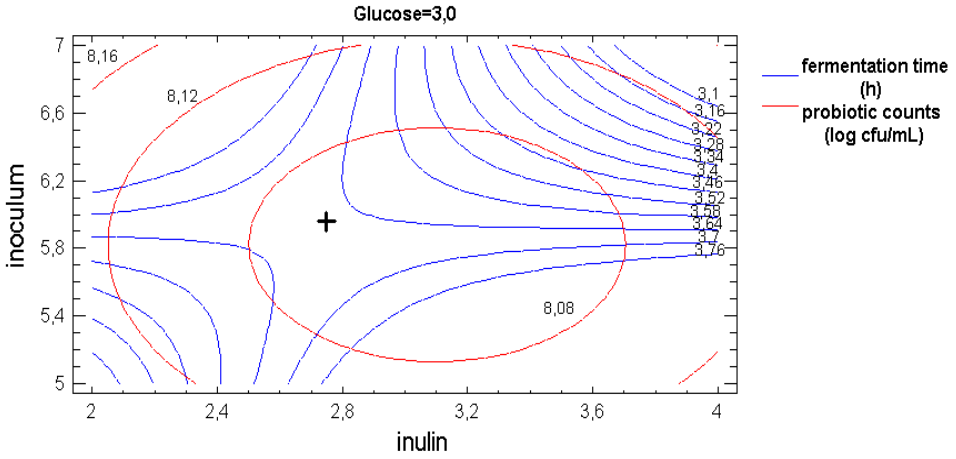

Figure 1. Overlay of the contour plots for both fermentation time and probiotic count responses from the CCD design. Optimum formulation, in which fermentation time was minimised and probiotic counts after 28 storage days were maintained at 8 log cfu/mL is represented (+). This plot was obtained by keeping the level of glucose factor at a constant 3 g/100 mL.

3.2 Chemical composition of the hazelnut *milk*

The chemical composition of pure hazelnut *milk* (hazelnut without added growth 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 total sugars of which sucrose was the only sugar present, as can be seen in Figure 2. 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; Venkatachalam and Sathe, 2006); the few differences observed might be a consequence of the losses during the extraction process and/or thermal treatment.

Figure 2 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.

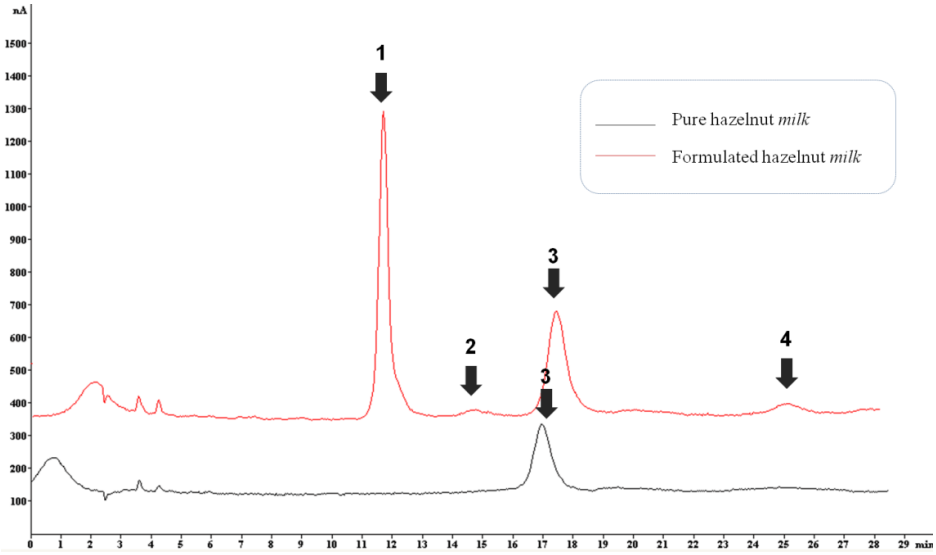

Figure 2 Chromatograms of sugar peaks obtained in HPAC-PAD assays from both pure and formulated hazelnut *milk*. Peaks identified were glucose (1), fructose (2), sucrose (3) and an oligosaccharide, residual from inulin, which was classified as Fructan (4).

3.3 Properties of the fermented hazelnut product

3.3.1 Probiotic counts, acid production and protein hydrolysis.

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 gastrointestinal tract (GIT): hence, they do not play a role in the human gut (Gilliland, 1979).

Table 3. Values (mean and (standard deviation)) of pH, Titratable Acidity (TA) and bacterial counts before and after a simulated human gastrointestinal digestion (SGID) of fermented hazelnut *milk* (FHM) and absorbance at 340 nm, 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)	Absorbance at 340 nm
HM	6.50 (0.02)	0.026 (0.003)	-	-	0.870 (0.005)
FHM 0 d	4.803 (0.015) [a]	0.104 (0.005) [a]	7.97 (0.05) [a]	4.91 (0.03) [a]	0.69 (0.04) [a]
FHM 1 d	4.01 (0.05) [b]	0.226 (0.005) [b]	8.38 (0.03) [b]	5.58 (0.06) [b]	0.53 (0.03) [b]
FHM 7 d	3.63 (0.05) [c]	0.322 (0.007) [c]	8.44 (0.06) [c]	5.48 (0.63) [bc]	0.472 (0.019) [b]
FHM 14 d	4.027 (0.06) [b]	0.337 (0.007) [d]	8.46 (0.04) [c]	5.04 (0.05) [ca]	0.40 (0.03) [c]
FHM 21 d	3.70 (0.07) [d]	0.337 (0.003) [d]	8.35 (0.03) [b]	4.94 (0.02) [a]	0.38 (0.07) [c]
FHM 28 d	3.70 (0.05) [d]	0.338 (0.000) [d]	8.350 (0.015) [b]	4.904 (0.017) [a]	0.369 (0.014) [c]

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

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 higher than in standard yoghurt, in which it is around 0.8-1 g/100 mL of lactic acid (Mistry and Hassan, 1992; 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, Baines and Adams, 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 as being minimum (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 a high retention of 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

simulated human gastrointestinal digestion (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, which was higher than that those values (20 - 40%) reported by other authors (Bezkorovainy, 2001). Generally, GG bacteria are seen to be highly resistant to acid and bile and have high adhesion ability in *in vitro* enterocytes (Deepika *et al*., 2011; 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 (Buddington, 2009; Saad *et al*., 2013). Nevertheless, this should be reinforced with *in vitro* and *in vivo* assays.

As concerns proteolytic action, the OPA-based spectrophotometric assay detects α-amino groups released from peptides. Hence, information regarding starter proteolysis can be measured by obtaining the differences between the absorbance values of non-fermented and fermented hazelnut *milks*. Proteolysis has a great influence on the final flavour of the fermented product (Tamime and Robinson, 2000). Nevertheless, the obtained results (Table 3) showed that the absorbance of the pasteurised *milk* was higher than that of fermented products. This suggests that hazelnut *milk* processing might have already hydrolysed the small amount of peptides present and generated free amino acids that were directly consumed by the starter bacteria to obtain the necessary nitrogen (Heller, 2001). Therefore, the heat

conditions involved in hazelnut processing might provoke the breakage of protein bonds, as confirmed by the analyses after and before pasteurisation. The absorbance value prior to the heat treatment was lower (0.502 ± 0.008) than that of pasteurised *milk* (0.870 ± 0.005). Other authors also reported that high heating can cause some proteins to break down in cow milk (Douglas *et al*., 1981; Walstra, 2003).

Amino acid consumption by bacteria provokes the elimination of the amino group that reacts with the OPA reagent (Heller 2001). In fact, the longer the storage period, the lower the absorbance values (Table 3), this concurs with the fact that bacteria need nitrogen sources for their metabolism, despite the low storage temperature (Tamime and Robinson, 2000). Nevertheless, the observed decrease was not significant after 14 storage days ($p < 0.05$), which could be explained by considering that GG will be in a stationary growth phase.

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. Figure 3 shows typical chromatograms of sugar peaks obtained from non-fermented (HM) and fermented hazelnut *milk* (FHM) samples after 1, 14 and 28 storage days at 4 ºC. Table 4 summarises the concentrations of the different sugar concentrations present in fermented hazelnut *milks* throughout storage time (0, 1, 7, 14, 21 and 21 days).

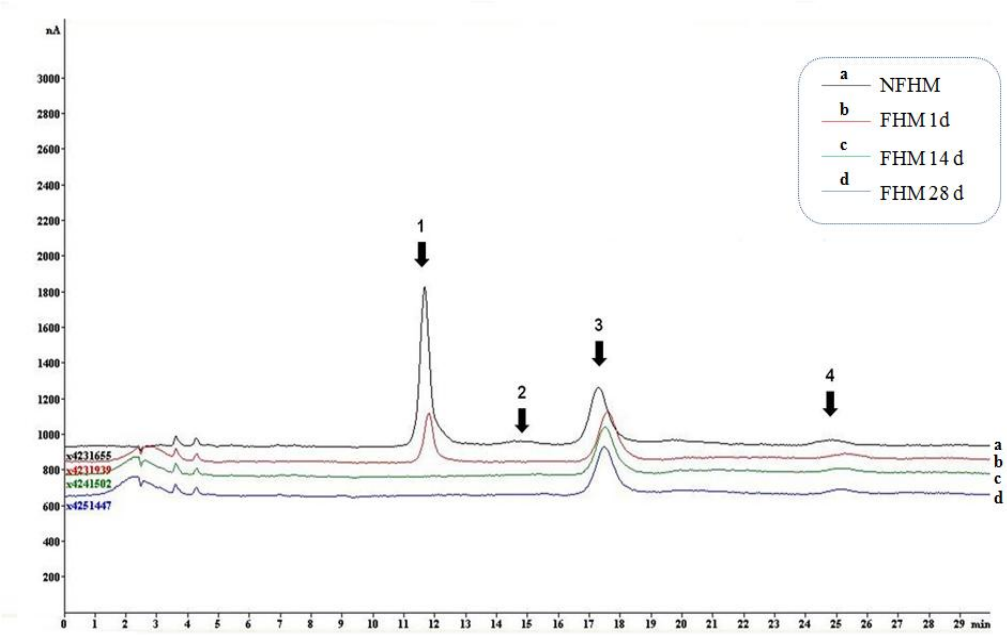

Figure 3. Chromatograms of sugar peaks obtained in HPAC-PAD assays from non-fermented hazelnut *milk* (HM) and its fermented products (FHM) after 1, 14 and 28 storage days at 4 ºC. Peaks identified were glucose (1), fructose (2), sucrose (3) and an oligosaccharide, residual from inulin, which that was classified as fructan (4)

As can be seen, the glucose content dropped significantly after the fermentation process, falling from 3.05 ± 0.25 to 1.11 ± 0.09 g/100 mL after 1 storage day at 4 ºC 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 (T), is able to use sucrose as nutrient (Garro *et al.*, 1998).

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 ($g \cdot 100mL^{-1}$)	Fructose ($g \cdot 100mL^{-1}$)	Sucrose ($g \cdot 100mL^{-1}$)	Fructan (Area ($\mu A \cdot min$))
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)

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 (Capela, Hay and Shah, 2006; 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. Figure 4 shows the up-flow curves obtained in the different samples. The flow curve of samples stored for 28 days was not shown since product phase separation occurred at this time (Figure 5). 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, Taherian-Hoshahili and Ramaswamy, 2001). Table 4 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.

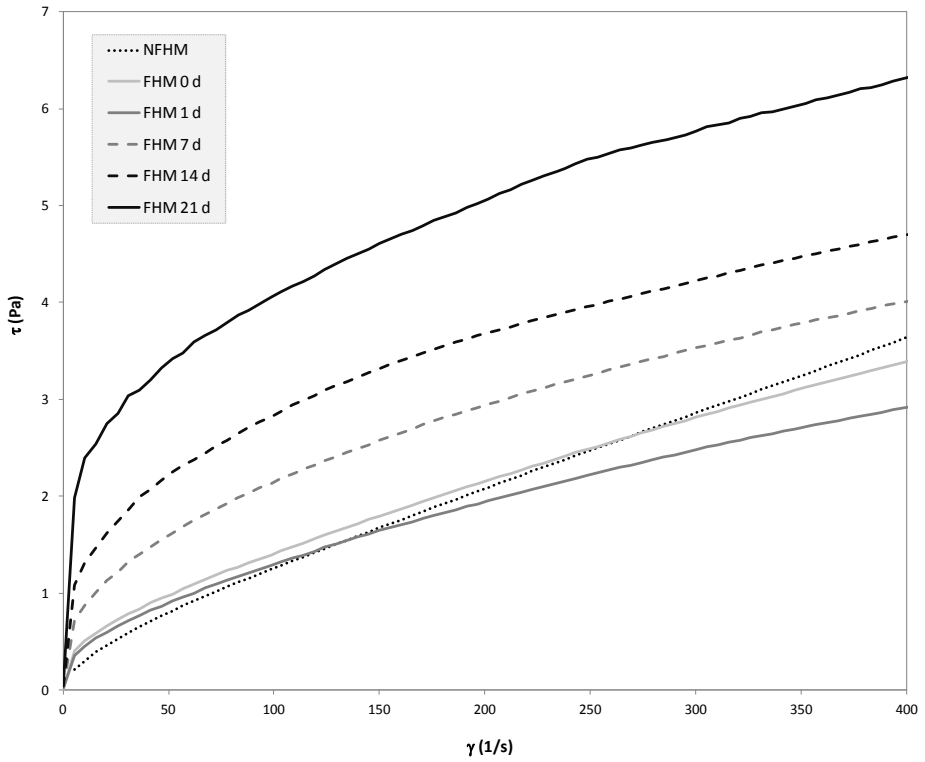

Figure 4. Up-flow curves of fermented hazelnut *milks* (FHM) at different storage times (1, 7, 14 and 21 days at 4 ºC). Non-fermented hazelnut *milk* (HM) curve was also shown for comparisons.

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 ($p < 0.05$).

Table 4. Mean values and (standard deviation) of the consistency index (K), flow behaviour index (n) and yield stress (σ_y) 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 ($p < 0.05$)

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, Costell and Tárrega, 2010; Villegas and Costell, 2007). 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, commented on above, and shown in Figure 5, which is coherent with the gel matrix contraction and its subsequent loss of serum retention capacity.

Figure 5 shows pictures of fermented hazelnut *milk* stored for 1 (5.A), 14 (5.B) and 28 (5.C) days at 4 ºC. As can be seen, the fermentation process provoked serum separation in hazelnut *milk* due to the physicochemical changes commented on above. This phenomenon was evaluated through the percentage of serum separation, observed in Figure 5. 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.

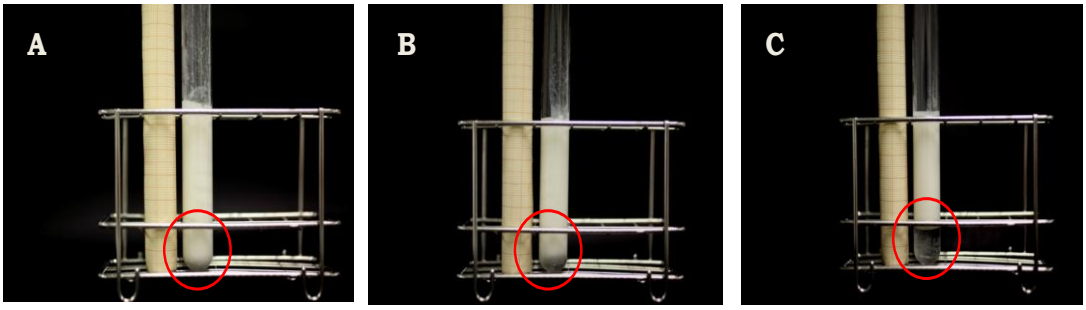

Figure 5. Pictures of fermented hazelnut *milk* stored for 1 (A), 14 (B) and 28 (C) days at 4 ºC. Red circumference marks the separated serum phase.

Previous studies have also shown there are 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 modification of the pH (Song, Kin and Chang, 2006). The gel structure is dynamic, increasing the bond formation over time and giving rise the phenomenon of syneresis.

Table 5 shows the colour parameters of non-fermented and fermented products at different storage times. The fermentation process modified the optical properties of hazelnut *milk*, increasing both lightness and chrome and decreasing the hue ($p < 0.05$). Moreover, the *milk* was whiter in fermented samples, which was considered positive, since consumers associate this type of products with the colour white.

Nevertheless, very few differences were observed between the colour parameters of the fermented samples cold stored for different times; these ranged over an interval of less than one unit. The total colour difference between fermented and non-fermented hazelnut *milks* (ΔE) was low and undetectable by the human eye since, according to Francis (1983), values lower than 3 units cannot be easily detected.

Table 5. Mean values and (standard deviation) of Lightness (L^*), hue (h^*_{ab}), chrome (C^*_{ab}) and White Index (WI) of fermented hazelnut products stored for different times at 4 ºC. Values of hazelnut *milk* are shown for comparison. Colour difference (ΔE) between non-fermented and fermented hazelnut *milks* is also presented.

Sample	L^*	C^*_{ab}	h^*_{ab}	WI	ΔE
HM	84.98 (0.19)	8.37 (0.05)	93.8 (0.5)	82.81 (0.16)	-
FHM 0 d	85.59·(0.14) ^{ab}	8.78 (0.03) ^{ab}	91.7·(0.2) ^{ab}	83.13·(0.10) ^{a}	0.80· (0.15) ^{ab}
FHM 1 d	85.5 (0.3) ^{a}	8.48 (0.16) ^{c}	92.5 (0.7) ^{c}	83.2 (0.3) ^{a}	0.55 (0.26) ^{a}
FHM 7 d	85.3 (0.4) ^{a}	8.61 (0.20) ^{bc}	92.4 (0.4) ^{bc}	82.9 (0.4) ^{a}	0.53· (0.19) ^{a}
FHM 14 d	85.98 (0.20) ^{bc}	8.76 (0.15) ^{ab}	91.4 (0.3) ^{a}	83.5 (0.2) ^{c}	1.14· (0.17) ^{c}
FHM 21 d	86.25 (0.06) ^{c}	8.86 (0.04) ^{b}	91.0 (0.3) ^{a}	83.64 (0.04) ^{c}	1.12· (0.07) ^{c}
FHM 28 d	85.96 (0.05) ^{bc}	8.75 (0.05) ^{ab}	91.4 (0.3) ^{a}	83.45 (0.07) ^{c}	0.50· (0.02) ^{bc}

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

3.3.4 Sensory properties

Figure 6 shows the scores of the attributes of appearance, sweetness, acidity, consistency, hazelnut flavour and overall acceptability in the three fermented hazelnut samples analysed by the members of the panel (1, 14 and 28 days stored at 4 ºC); statistical differences between storage time were also included.

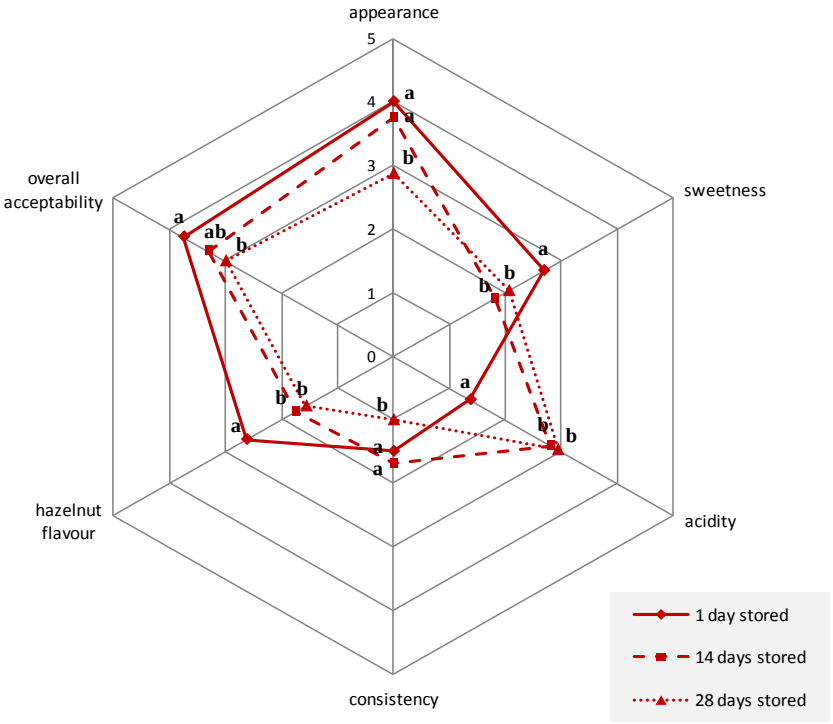

Figure 6. Panelists' scores for appearance, sweetness, acidity, consistency, hazelnut flavour and overall acceptability in the fermented hazelnut samples stored for 1, 14 and 28 days at 4 ºC

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

Before tasting the three samples, the panelists evaluated the fermented hazelnut *milk* as having a very good appearance with the exception of the sample stored for 28 days ($p < 0.05$). As these samples were presented in transparent glasses, the panelists were able to notice the sample syneresis and serum separation at the bottom; this separation was negatively evaluated.

With regards to sweetness, in spite of the fact that all the samples were equally sweetened with sucrose, the panelists detected differences between samples stored for 1 day and the other ones ($p < 0.05$). This appreciation could be a consequence of the impact of acidity on this attribute's evaluation: the higher the acidity level, the lower the sweetness perception (Ott *et al.*, 2000). The panellists did not appreciate differences between samples stored for 14 and 28 days ($p < 0.05$), which is coherent with both the pH and TA values (Table 3).

The consistency of the fermented product was quantified as low, which was expected, considering the similarity of the tested product with the well-known drinkable yoghurts which are more consistent. The members of the panel detected lower consistency in samples stored for 28 days ($p < 0.05$), probably due to the partial destabilisation of the gel structure in the fermented product and phase separation, commented on above. This lower consistency is negatively appreciated in terms of consumer acceptance, since they prefer drinkable yoghurts with a high level of viscosity (Allgeyer, Miller and Lee, 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 after 3.5 h of fermentation time, which ensures high probiotic survivals. Indeed, at 28 storage days, GG viability was maintained above the level recommended as being the minimum in order to ensure health benefits (10^7 cfu/mL) 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%. These results point to the adequate selection of growth factors and a wide availability of nutrients, including nitrogen sources. Although sensory evaluation showed a greater preference for samples stored for short times, the panel members also accepted the product after 28 storage days.

Hence, thanks 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 suitable for many different targeted groups, such as vegetarians, the lactose-intolerant or

people allergic to animal proteins. Moreover, the inulin (prebiotic compound) present would provide an added nutritional value to the product.

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Conclusiones Capitulo II

La tabla que se presenta a continuación (TABLA 1) resume los resultados más relevantes obtenidos en la caracterización de los fermentados probióticos de *leche* de avena, almendra y avellana desarrollados. A partir de esta tabla se puede concluir que:

1. Las dos cepas probióticas estudiadas, *L. reuteri* ATCC 55730 (R) y *L. rhamnosus* ATCC 53103 (GG), crecen adecuadamente en las *leches* vegetales obtenidas, aunque es necesario enriquecerlas con azúcares y/o prebióticos (nutrientes) para asegurar un buen crecimiento del probiótico, en especial en la *leche* de avellana.

2. A la vista de los resultados, parece ser que el probiótico GG sobrevive mejor que R ya que, en las matrices ensayadas, se mantiene viable a concentraciones mayores a los 8 log cfu/mL durante el almacenamiento y, además, es capaz de sobrevivir a digestiones *in vitro* en más de un 58%.

3. En el caso concreto del probiótico R, desde el punto de vista funcional y, teniendo en cuenta las matrices ensayadas, se recomienda el uso de *leche* de avena frente a la de almendra para el desarrollo de productos fermentados puesto que, tal y como se observa en la TABLA 1, la viabilidad del microorganismo probiótico (log cfu/mL) es mayor a lo largo de los 28 días de almacenamiento del producto controlados y se encuentra en fase de crecimiento (el pH del fermentado de avena desciende mientras que el de almendra se mantiene constante). Desde el punto de vista

sensorial, en cambio se valoraron mejor las *leches* fermentadas de almendra.

4. En el caso del probiótico GG, a pesar de que la viabilidad del mismo en las dos *leches* de frutos secos elegidas fue similar, sensorialmente fue mejor valorado el producto fermentado con *leche* de avellana.

5. La viscosidad de los productos fermentados fue muy superior en el caso de las *leches* de avena y avellana, debido a la presencia de hidrocoloides en la materia prima incial (β-glucano en avena) o de su adición (goma xantana en avellana), componentes necesarios para asegurar y/o mejorar la estabilidad física de los mismos. En el caso de la *leche* de almendra, la estabilidad física se mantuvo gracias a la utilización de las altas presiones de homogeneización combinada con tratamiento de pasteurización y, por tanto, no fue necesario añadir espesantes en la formulación de la misma.

TABLA 1. Tabla-resumen de los datos más relevantes obtenidos en los estudios que engloban el capítulo II.

Materia prima	Cepa probiótica	Formulación	Viabilidad del probiótico (log ufc/mL)		Supervivencia del probiótico al tracto gastrointestinal (%)	pH		η_{50} (x 10^2)	Aceptabilidad global (1:muy baja– 5: muy alta)	
			1 día a 4 °C	28 días a 4 °C		1 día a 4 °C	28 días a 4 °C	1 día a 4 °C	1 día a 4 °C	28 días a 4 °C
AVENA	R	**g:** 0.65 g/100 mL **f:** 0.65 g/100 mL **i**:0.4 g/100 mL **s**: 3 mL/100 mL	8.49(0.11)	7.43(0.06)	-	4.08(0.04)	3.30(0.05)	47(2)	≈3	1-2
ALMENDRA	R	**g:** 0.75 g/100 mL **f:** 0.75 g/100 mL **i**:2 g/100 mL **s**: 6 mL/100 mL	7.59(0.04)	7.06(0.06)	≈ 51%*	4.66(0.01)	4.65(0.02)	0.10(0.02)	3	2-3
	GG		8.57(0.18)*	-	≈ 59%*	-	-	-	-	-
AVELLANA	GG	**g:** 3 g/100 mL **i**:2.75 g/100 mL **x:** 0.05 g/100 mL **s**: 6 mL/100 mL	8.38(0.03)	8.35(0.02)	≈ 67%	4.01(0.05)	3.70(0.05)	61(13)	≈4	3

R: *L.reuteri* ATCC 55730; **GG:** *L.rhamnosus* ATCC 53103 **g:** glucosa; **f:** fructosa; **i:** inulina; **x:** goma xantana; **s:** inóculo (cepa probiótica:*S. thermophilus* 1:1)

* Datos procedentes del **Capítulo III** ("Almond *milks* as probiotic carrier food; bacterial survival and anti-inflammatory response")

Capítulo III

Posibles efectos funcionales de la "*leche*" de almendra fermentada con bacterias potencialmente probióticas

Almond *milk* as probiotic carrier food: bacteria survival and anti-inflammatory response

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ABSTRACT

Functional advantages of probiotic bacteria combined with interesting composition of almonds has been considered as an alternative to traditional dairy products. The aim of this study was to evaluate the viability of different probiotic bacteria (*Lactobacillus reuteri* or *Lactobacillus rhamnosus,* combined or not with *Streptococcus thermophilus*) in the fermented almond *milk* after *in vitro* digestion and the bioactivity of these developed products as affected by the type of almond *milk* used (commercial and laboratory-made) and bacteria strains. Samples were characterised in terms of probiotic survivals after *in vitro* digestions, proteolytic activity and anti-inflammatory properties. Results showed that around 40-50% of probiotic bacteria survived after digestion process, being greater when the mixed cultures were used. Both probiotic strains produced similar peptide profiles, but those samples incubated with *L. reuteri* generated a greater ($p < 0.05$) amount of peptides with low molecular weight, both in the digested and non-digested samples. The type almond *milk* did not significantly affect the proteolytic activity of starter bacteria. Fermented samples did not show an anti-inflammatory bioactivity *in vitro*, except those samples fermented using commercial *milk* and *L. reuteri* and *S. thermophilus,* which exhibited an IL-8 inhibition to a certain degree. Almond *milks* are seen to be appropriate raw materials for producing dairy-free probiotic products with interesting nutritional and healthy properties.

Key words: inulin, *L. rhamnosus* GG, *L. reuteri*, proteolytic activity.

1. INTRODUCTION

At present there is a global awareness of nutrition-related chronic metabolic diseases. The World Health Organization (WHO) determined in its 2009 report on global health risk that more than half of the distribution of deaths attributable to 19 leading risk factors worldwide were nutrition-related (Stuckler and Basu, 2011). This trend is increasingly acknowledged by consumers and food manufacturers, with newly designed foods available not only to help satisfy hunger and provide nutrients, but also to prevent nutrition-related chronic diseases and improve physical and well-being of consumers (Menrad, 2010; Roberfroid, 2000).

The emergence of the Functional Foods (FF) (foods that beyond the nutritional effects provide health benefits to humans) market is testament to consumers demanding more healthy nutritious foods with added health benefits. The EU FF generated € 10,000 million during 2011 (Mercasa, 2011) and within the different categories available, "probiotics and/or prebiotics" is the one sector that is experiencing the fastest growth and acceptance.

The WHO defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). Among the health benefits aimed with the use of probiotics are reduction of hypercholesterolemia, host immune system modulation, prevention of urogenital diseases, alleviation of constipation, protection against traveler's diarrhea, protection against colon and bladder

cancers, prevention of osteoporosis and food allergies (Lourens-Hattingh and Viljoen, 2001).

Although dairy industry is the major sector in developing probiotics products, others such as nut and cereal beverages (known as vegetable "milks") industries have been currently involved. These vegetable *milks* have especial relevance since, besides their nutritional and health benefits, they may contain prebiotic compounds which make them interesting and useful to produce synbiotic products (combination of probiotics and prebiotics) and, thus, benefit consumers from these cutting-edge functional elements. Prebiotics were defined by Food Agriculture Organization of the United Nations (FAO) as "non-viable food components that confer health benefits on the host associated with modulation of the microbiota" (FAO, 2008).

Vegetable *milk*s have a long history in both Eastern and Western cultures and, although so far they had not been well established in the current European market, many are the purchasers who choose them. Typically vegetarians, individuals with lactose intolerance, or cholesterol-concerned individuals are the targeted consumers for these *milks*. Moreover, experts on food nutrition and health are starting to consider possible relation between vegetable products and cancer, atherosclerosis or inflammatory diseases prevention, since free radicals play a key role in those pathologies and these foodstuffs are an excellent source of antioxidants (Scalbert and Williamson, 2000).

There is a wide range of commercial vegetable *milks* and currently nut *milks* are in the state-of-the-art due to the new-knowledge impact of their

compounds on some current chronic diseases such as cardiovascular diseases (CVD), type 2 Diabetes mellitus (DM-2), obesity and some cancers (Fardet, 2010). Tree nuts are rich in mono- and polyunsaturated fatty acids, vegetable proteins, dietary fibre, phytosterols, polyphenols, vitamins and minerals (Philips *et al.,* 2005; Segura *et al.,* 2006); and most of those compounds have antioxidant properties and are proved to have a beneficial effect in plasma lipid profile, low-density lipoprotein (LDL) oxidation and inflammatory processes, among others (Liu, 2012; Myers and Allen, 2012; Ward *et al.,* 2012; Whent *et al*., 2012; Carlson *et al.,* 2011; Egert *et al.,* 2011; Gillingham *et al*., 2011; Jones *et al.,* 2011). Indeed, epidemiological studies have linked frequent nut consumption to reduced risk of coronary heart disease and DM-2 (Kelly Jr and Sabaté, 2006).

Among nuts, almonds have experienced great increase in consumption, probably due to the consumers-acknowledged potential health benefits mentioned above coupled with high K/Na ratio and low glycemic index (Casas-Agustench *et al*., 2011, Sing-Chung *et al*., 2011). These healthy properties, hence, made almonds and their derivatives to be suitable for sensitised population such as diabetics or those who suffer from either hypertension or hypercholesterolemia. Nevertheless, this nut has been also classified as a potential allergenic seed known to be responsible for triggering several immune reactions in allergic individuals (Costa *et al*., 2012). On the other hand, probiotic bacteria are seen to influence human immune system positively, so they could reduce almonds' allergenicity. Therefore, almond nut *milks* might be very useful in order to industrially produce new non-dairy products with synbiotic features and with reduced

allergenic effects. Since the probiotic health benefits are dependent on the final amount of bacteria present within the human microbiota, data regarding the bacterial survival should be also analysed in the final product (Buddington, 2009).

The aim of the present study was, hence, to evaluate the viability of different probiotic bacteria in the fermented almond *milk* after *in vitro* digestion and the bioactivity of these developed products (in terms of anti-inflammatory properties) as affected by the type of almond *milk* used (commercial and laboratory-made) and bacteria strains. Probiotics assessed were either *Lactobacillus reuteri* ATCC 55730 or *Lactobacillus rhamnosus* ATCC 53103 (or most commonly known as *L. rhamnosus* GG) used as pure starters or in combination with *Streptococcus thermophilus* CECT 986. Both lactobacilli strains were reported to have a positively effect on prevention or treatment of allergies (Shida and Nanno, 2008). Anti-inflammatory properties were analysed using a Caco-2 cell monolayer model, since these cells are able to reproduce the human intestinal epithelium.

2. MATERIALS AND METHODS

2.1 Almond *milk* processing

Laboratory 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 Sojamatic 1.5® equipment (Barcelona, Spain), which is specifically designed for vegetable beverages production, using an almond:water ratio of 8:100. The milky

liquid obtained was then homogenised in a high pressure homogeniser (M-110P model; Microfluidics International Corporation, USA) at 172 MPa and then pasteurised at 85 ºC/30 min. Prior to the heat treatment, 0.75 g/100 mL of glucose, 0.75 g/100 mL of fructose and 2 g/100 mL of inulin (as prebiotic) were added, as data from other studies (not published) suggest the addition of these ingredients help otimise the fermentation conditions. The monosaccharides were purchased from Sosa Ingredients S.L. (Barcelona, Spain), while inulin from Beneo-Orafti (Tienen, Belgium).

Commercial almond (*Prunus amygdalus L.* cv. dulcis) *milk* was supplied by Nutriops S.L. (DieMilk®; Murcia, Spain), which contains around 2.2 g/100 mL lipids, 3.6 g/100 mL sugar, 1.1 g/100 mL proteins and 0.4 g/100 mL fibres. The industrial extraction procedure was carried out by using an almond:water ratio of around 7:100, and the milky product was later on sweetened with cane sugar, homogenised and UHT treated. This commercial product was also enriched with 2g/100 mL of inulin prior to inoculation.

2.2 Chemical composition of almond *milk*

Quantification of moisture, ashes, fats, proteins and total sugars were carried out in pure laboratory almond *milk* (beverage prior glucose, fructose and inulin additions); the fibre content was estimated by means of the difference in terms of component percentages. Laboratory-made *milks* were freeze-dried (ioalfa-6 freeze-dryer, TELSTAR; Terrassa, Spain) prior to compositional analyses. AOAC Official Methods were chosen to determine water, total fats and total nitrogen contents (AOAC 16.006, AOAC 945.16

and AOAC 958.48, respectively) (Horwitz, 2000). Sugars and ashes contents were obtained following the protocols suggested by Matissek *et al*. (1998). All the determinations were done in triplicate.

2.3 Preparation of fermented products

2.3.1 Inoculum preparation

Two different probiotic bacteria, *Lactobacillus reuteri* ATCC 55730 (further on R) (Biogaia, Stockholm, Sweeden) and *Lactobacillus rhamnosus* ATCC 53103, most known as *Lactobacillus rhamnosus* GG (further on G) (LGC Standards S.L.U., Barcelona, Spain) were used to obtain almond fermented *milks* either alone or as a mixed starter culture with *Streptococcus thermophilus* CECT 986 (further on T) (CECT, Paterna (Valencia), Spain) by using a ratio 1:1.

Bacteria were activated and propagated from their frozen forms (stored in 40 g/100 mL glycerol at -80 ºC), by transferring each one to its selective broth until optimal bacterial growth was obtained. The selective broths were MRS (Scharlab; Barcelona, Spain) for both probiotic lactobacilli and M17 (DifcoTM; New Jersey; USA) for T. Incubation conditions were 37 ºC/24 h/ anaerobically for both R and G and 42 ºC /24 h/aerobically for T.

For the starter inoculumm, strains were independently incubated in their broths for 24 h and then centrifuged at 10,000 rpm-10 min at 4 ºC; 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 cfu/mL.

2.3.2 Fermentation procedure

Four different fermented samples were obtained by inoculating the almond *milks* with a 3 mL/100 mL of pure culture (R or G) and 6 mL/100 mL of mixed starter culture (named as RT and GT, for the *L. reuteri* and *L. rhamnosus* combined with T, respectively) in PBS buffer suspensions to the formulated *milks*. Inoculated *milks* were then incubated at 37 ºC for the pure culture and, at 40 ºC for the mixed culture until reaching pH values of 4.4 - 4.6 and then, cooled to 4 ºC prior to the analyses.

Fermented samples submitted to proteolytic and anti-inflammatory analyses were freeze-dried (ioalfa-6 freeze-dryer, TELSTAR; Terrassa, Spain) and then reconstituted prior assays.

2.4 Determination of probiotic bacteria viability after Simulated Gastrointestinal Digestion (SGID)

Fermented almond *milk* samples underwent a SGID and the viability of probiotic bacteria was examined. This was also assessed in non-digested samples. SGID was performed as described by Glahn *et al.* (1998) with some modifications. Porcine pepsin (800–2500 units/mg protein), pancreatin (activity, 4 1 USP specifications) and bile extract were purchased from Sigma Chemical (All Sigma–Aldrich Corp., St. Louis, MO, USA). To start the peptic digestion, the pH of the samples was adjusted to 2 with 5 mol/L HCl and 0.5 mL of pepsin solution (0.2 g pepsin dissolved in 10 mL of 0.1 mol/L HCl) was added per 10 mL of sample; then they were incubated for 60 min stirring constantly. Once the peptic digestion was finished, 2.5 mL of pancreatin-bile extract mixture (0.05 g pancreatin and 0.3 g bile extract

dissolved in 35 mL of 0.1 mol/L $NaHCO_3$) was added per 10 mL of the original sample, after having raised the pH of samples to 6 pH by drip addition of 1 mol/L $NaHCO_3$; after that, the pH was readjusted to 7 with 1 mol/L NaOH and the final volume was brought to 15 mL with 12 mmol/L NaCl and 5 mmol/L KCl. The samples were finally incubated at 37 ºC for 120 min, stirring constantly, to finally complete the intestinal digestion. The viability analyses were carried out in triplicate.

2.5 Bacterial counts

Probiotic counts were performed by triplicate using pour plate technique, according to the method described by the International Dairy Federation (1997). The selective media used was acidified MRS agar (Scharlab; Barcelona, Spain). Samples' decimal dilutions were made in sterile 0.1 g/100 mL peptone water (Scharlab; Barcelona, Spain). Incubation conditions of both R and G were 37 ºC /24 h/anaerobically. Counts were reported as log cfu/mL of fermented sample.

T counts were not obtained due to the lack of ability of this bacterium to survive through the gastrointestinal tract (GIT) and, hence, not playing a role in the human gut (Gilliland, 1979).

2.6 Determination of proteolytic activity

Proteolytic activity of starters in fermented almond *milk* samples were assessed by measuring liberated amino acids and peptides using the *o*-phtaldialdehyde (OPA) method described by Church *et al*. (1983). Fermented samples were diluted 1:1 (v/v) in deionised water and 10 μL was removed and added to 1 mL of OPA reagent. The absorbance of the

solutions was measured spectrophotometrically using a UV-visible spectrophotometer UVmini-1240 (SHIMADZU Europe Corporation, Germany) at 340 nm after 2 min incubation at room temperature. The proteolytic activity was expressed as the difference in absorbance of free amino groups measured at 340 nm between fermented and non-fermented almond samples.

2.7 Size-exclusion high-performance liquid chromatography analysis (SEC-HPLC)

Fermented almond *milk* samples were analysed by SEC-HPLC in order to obtain their unique peptide profiles. Digest inoculated samples were also characterised on the 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, U.S.A.). SEC screening of samples was performed on a BioSep-SEC-S2000 (300 mm x 7.8 mm i.d.) column with a Gel Filtration Chromatography guard column 4 x 3 mm (Phenomenex, Cheshire, UK). Before injecting samples to the equipment, fermented and non-fermented almond *milk* samples were filtered through nylon membranes (0.45 μm).

Two methods of analysis by SEC-HPLC were examined. The first is specific for larger Mw peptides (roughly 17,000 - 700,000 Da). The standards thyroglobulin, γ-globulin, myoglobin, BSA, ovalbumin, aprotinin, uridine and sodium azide 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 100 mmol/L phosphate buffer at pH 6.8.

The second SEC-HPLC screening method has a greater affinity for smaller Mw peptides (roughly 700 - 17,000 Da). The standards thyroglobulin, aprotinin, cyctochrome C, insulin, angiotensin I, angiotensin II, uridine and sodium azide 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 (ACN)-H_2O (ratio 45:100) containing 0.1 mL/100 mL trifluoroacetic acid (TFA).

2.8 Evaluation of the anti-inflammatory bioactivity of fermented almond *milks*

The anti-inflammatory bioactivity of fermented and non-fermented almond *milks* were evaluated in human intestinal epithelial-like (Caco-2) cells. Caco-2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen Corp., San Diego, CA, USA) supplemented with 10 mL/100 mL fetal bovine serum (Invitrogen Co., Carlsbad, USA), 1 mL/100 mL non-essential amino acids, 1g/100 mL sodium pyruvate and penicillin (100 U)/streptomycin (100 g/mL) (all lasts purchased from Sigma–Aldrich Corp., St. Louis, MO, USA). Cells were maintained in vented 75 cm^2 flasks in a humidified cell culture incubator with 5% CO_2 at 37 ºC.

Caco-2 cells (10^5 cells/mL) were seeded in 24 well cell culture plates and culture media changed in every 48 h and grown for 14 days. On the day of the experiment, cells were washed with DMEM and incubated for 3 h in serum and antibiotic free media. Then, cells were treated with pro-inflammatory inducer Tumour Necrosis Factor-α (TNF-α) (10 ng/mL) in absence or presence of fermented and non-fermented almond *milk* samples (1 mg/mL). Following 24 h incubation with TNF-α and the almond samples, supernatant was collected and stored at -80 ºC.

The concentration of Inerleukin-8 (IL-8), a marker of pro-inflammatory response, in the supernatants was quantified using a human IL-8 sandwich ELISA according to the manufacturer's instructions (R&D Systems Europe Ltd., Abingdon, UK). Signal detection was performed in a microtiter plate reader at an absorbance of 450 nm against 570 nm. Each measurement was performed in triplicate.

2.9 Statistical Analysis

Results were analysed by using a multifactor analysis of variance with 95% significance level using Statgraphics® Centurion XV. Multiple comparisons were performed through 95% LSD intervals.

3. RESULTS AND DISCUSSION

3.1 Chemical composition of almond *milks*.

The chemical composition of the laboratory (lab) *milk* was 3.96 ± 0.2 g/100 mL lipids, 1.37 ± 0.03 g/100 mL proteins, 0.135 ± 0.002 g/100 mL

initial sugars, 0.325 ± 0.012 g/100 mL ashes and 0.58 g/100 mL fibre. This lab-made almond *milk* was richer in lipids than the commercial one (2.2 g/100 mL lipids), probably due to the different extraction method used. Not only these compositional differences could affect the fermentation process, as lipid content is seen to play an important role in milk fermentations (Saint-Eve *et al*., 2006; Sandoval-Castilla *et al*., 2004; Trachoo, 2003; Harte *et al.*, 2002), but also the different heat treatment received.

The sugar content was higher in the commercial almond *milk* (3.6 g/100 mL) as a consequence of the cane sugar addition. Nevertheless, lab *milk* was enriched with glucose and fructose, which increased total sugars percentage up to 1.6 g/100 mL.

3.2 Proteolytic activity in fermented almond *milks*

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.

Table 1 shows the proteolytic activity obtained for each of the fermented samples, expressed as the difference in absorbance measured at 340 nm between fermented and non-fermented almond *milk*. The statistical results showed that the type of almond *milk* used as a raw material did not statistically affect ($p > 0.05$) the proteolytic activity of the different starter bacteria.

Table 1. Proteolytic activity of fermented samples. Average value and (standard deviation).

Sample	**Proteolytic activity** (ΔA_{340nm})	
	Lab-made almond *milk*	Commercial almond *milk*
R	0.09 (0.007) [a]	0.105 (0.020) [a]
RT	0.08 (0.005) [b]	0.056 (0.019) [b]
G	0.051 (0.006) [bc]	0.060 (0.019) [bc]
GT	0.044 (0.014) [c]	0.052 (0.015) [c]

[a, b, c] Different letters indicates significant differences between fermented samples inoculated in different starters (95% confidence level).

R = *L. reuteri*, **RT** = L. *reuteri* + *S. thermophilus*; **G** = *L .rhamnosus*, **GT** = *L. rhamnosus* + *S. thermophilus*

As was expected, all starters contain enzymes which were able to hydrolyse almond proteins (higher absorbance values than AM were obtained) since, among other requirements, they are known to need amino-acids and peptides to grow (Savijoki, Ingmen and Varmanen, 2006). The greatest proteolytic activity was observed in samples inoculated with R strains ($p < 0.05$), both in pure and in the mixed culture (RT). This could contribute to a major extent to the flavour and texture of these fermented products (Savijoki, Ingmer and Varmanen, 2006; Tamime and Robinson, 2000) and to modify the allergic effects associated to proteins (Clemente, 2000; De Angelis, 2007).

3.3 Peptide profiles of *in vitro* digested and non-digest fermented almond *milks*

Figure 1 shows the typical peptide chromatogram profiles obtained from one of the fermented *milks* (lab-made) and one given strain (R) before and after the SGID. These profiles were obtained by using ACN/TFA as a mobile phase. Poor reproducibility of peptide profiles was obtained by using phosphate buffer as most of peptides found had a molecular mass lower than 10 kDa, below the recommended detection limit of this buffer.

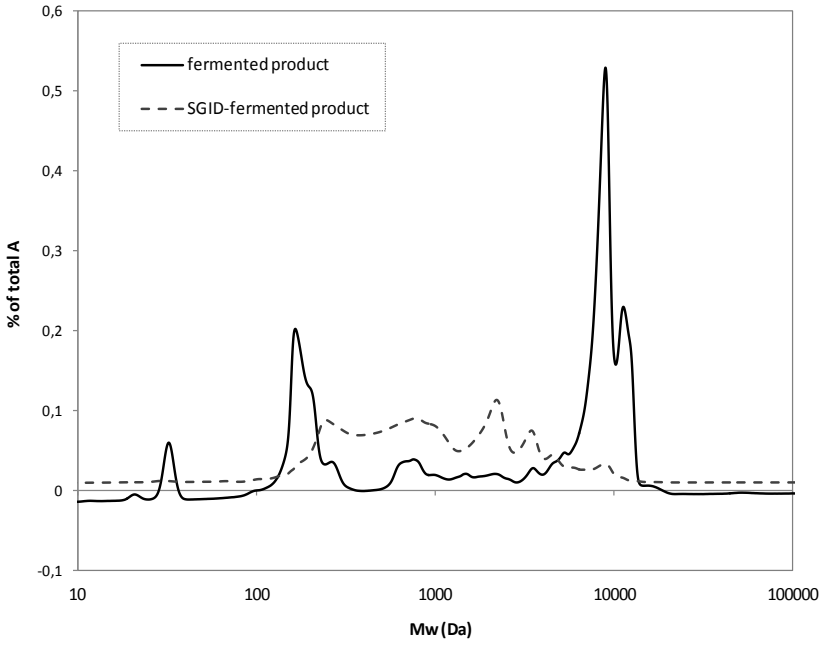

Figure 1. Peptide profile chromatograms of lab-made almond *milk* fermented with *L. reuteri* before (continuous line) and after SGID (dashed line)

The percentage of water-soluble peptides found in the fermented almond *milks* (before SGID) as a function of their molecular weight (Mw) is

presented in Table 2. As can be observed, the main soluble peptides in the fermented *milks* were constituted by peptides with Mw lower than 400 Da (Table 1) together with the highest Mw fraction (from 15 to 8 kDa).

Scarce differences were found between the peptide profiles of the lab-made and commercial samples. Nevertheless, the amount of peptides with Mw < 400 Da was significantly greater ($p < 0.05$) in the almond commercial *milks* (Table 2). All fermented samples presented similar proteolytic patterns as reported by Papadimitriou *et al*. (2007), when working with goat yoghurts obtained with different starters. However, the use of the different strains in the fermentation process led to the generation of quantitative differences in the almond protein fractions as can be seen in Table 2; major differences were found when using the R strain, which produced a greater release of small peptides (Mw < 400 Da), but only when the commercial *milk* was used as a fermentative matrix. These results suggest that R strain is able to produce a larger amount of peptides with Mw lower than 400 Da than G strain, which is coherent with their more intense proteolytic activity (Table 1).

The percentage of water-soluble peptides found in the fermented almond *milks* after the *in vitro* SGID as a function of their molecular weight (Mw) is presented in Table 3. For comparison purposes, the SGID non-fermented almond *milk* (AM) was also analysed. The *in vitro* digestion process of fermented samples led to the disappearance of the major part of the high Mw peptide fraction (from 15 to 8 kDa) and to the generation of greater amount of low Mw peptides (< 2.5 kDa), which capability to exert inflammatory effect has never been evaluated.

Table 2. Percentage of water-soluble peptides as a function of their molecular weight (Mw) for the different fermented samples. Average value and (standard deviation).

Sample	15,000-8,000 Da		8,000-2,500 Da		2,500-400 Da		<400 Da	
	Lab *milk*	Commercial	Lab *milk*	Commercial	Lab *milk*	Commercial	Lab *milk*	Commercial
R	39 (4)^{a}	48.4 (8.7)^{a}	26.4 (0.4)^{a}	18.7 (0.4)^{a}	11.7 (0.4)$^{x\,a}$	4.6 (0.5)$^{y\,a}$	27.5 (2.1)$^{x\,a}$	50 (5)$^{y\,a}$
RT	37 (2)^{a}	22.5 (1.9)^{b}	27.5 (2.0)^{a}	35 (4)^{c}	12.0 (0.5)$^{x\,a}$	8 (2)$^{y\,ab}$	28.3 (2.3)$^{x\,a}$	51 (7)$^{y\,a}$
G	36.50 (0.13)^{a}	34.1 (6.5)^{ab}	29 (3)^{a}	27.9 (1.6)^{b}	12.1 (0.6)$^{x\,a}$	9.1 (0.5)$^{y\,b}$	28.0 (0.7)$^{x\,a}$	32 (4)$^{y\,b}$
GT	35 (2)^{a}	42.5 (11.2)^{ab}	29 (3)^{a}	35 (2)^{c}	12.2 (1.2)$^{x\,a}$	5.2 (0.4)$^{y\,a}$	28.42 (0.02)$^{x\,a}$	37 (5)$^{y\,ab}$

$^{x, y}$ different letters in the same row indicates significant differences between lab and commercial almond *milks* ($p<0.05$)

$^{a, b, c}$ different letters in same column indicates significant differences between fermented samples due to starters ($p<0.05$)

R = L. *reuteri*, RT = L. *reuteri* + S. *thermophilus*; G = L .*rhamnosus*, GT = L. *rhamnosus* + S. *thermophilus*

Table 3. Percentage of water-soluble peptides as a function of their molecular weight (Mw) for the different digest fermented samples. Average value and (standard deviation).

Sample	15,000-8,000 Da		8,000-2,500 Da		2,500-400 Da		<400 Da	
	Lab *milk*	Commercial	Lab *milk*	Commercial	Lab *milk*	Commercial	Lab *milk*	Commercial
AM	4.5 (1.4)	3.2 (0.2)	23.7 (0.6)$^{x\,a}$	22.5 (0.5)$^{y\,a}$	44.6 (1.7)^{a}	42.1 (0.6)^{a}	24.8 (1.6)$^{x\,a}$	32.1 (1.1)$^{y\,a}$
R	3.5 (0.4)	3.83 (0.02)	16.6 (0.7)$^{x\,b}$	17 (3)$^{y\,b}$	47.2 (0.4)^{b}	46 (2)^{b}	30.6 (1.2)$^{x\,b}$	34 (2)$^{y\,b}$
RT	3.5 (0.3)	3.6 (0.5)	20.6 (0.4)$^{x\,c}$	19.9 (0.7)$^{y\,ab}$	47.1 (0.5)^{b}	45.9 (1.2)^{b}	26.6 (0.5)$^{x\,a}$	30.6 (1.5)$^{y\,a}$
G	3.4 (0.3)	3.5 (0.1)	22.4 (0.4)$^{x\,d}$	21.1 (0.4)$^{y\,a}$	46.8 (0.6)^{ab}	44.4 (0.1)^{ab}	25.5 (0.2)$^{x\,a}$	31.0 (0.5)$^{y\,a}$
GT	3.5 (0.2)	3.8 (0.2)	27.0 (0.2)$^{x\,e}$	20.3 (0.3)$^{y\,a}$	42.2 (2.1)^{a}	45.5 (0.1)^{a}	26 (3)$^{x\,a}$	30.4 (0.4)$^{y\,a}$

^{x,y} different superscripts within a raw indicate significant differences between almond *milks* ($p < 0.05$).

^{a,b} different superscripts within a column indicate significant differences among samples due to bacteria ($p < 0.05$).

AM = non-fermented almond *milk* R = *L. reuteri*, RT = *L. reuteri* + *S. thermophilus*; G = *L .rhamnosus*, GT = *L. rhamnosus* + *S. thermophilus*

As commented on above for the non-SGID samples, scarce differences were found between the peptide profiles of the lab-made and commercial *milks*, but again, the amount of peptides with Mw < 400 Da was higher in the commercial almond *milk*. The most intense treatment of these *milks* could make easier the breakdown of some proteins and generate free amino acids; this effect has been previously observed in cow milk proteins under high heat treatments (Douglas *et al*., 1981; Walstra *et al*., 2003).

As regard the different starters used, the peptides generated in samples inoculated with R strains (both pure and mixed) showed significantly lower molecular weights (Mw < 2500) than those generated by G strains after the SGID, in both lab and commercial *milks*.

To sum up, both fermentation and SGID processes modified the initial protein profile of almond *milks*. This effect might have a positive impact as regards the inflammatory response of the newly designed products on the epithelial cells, since the allergenicity of almond proteins is related to their biochemical properties and conformation (Albillos *et al.*, 2009).

3.4. Viability of probiotics in almond matrices

The amount of probiotics survived after having gone through gastrointestinal tract (GIT) is critical. The efficacy and success of probiotics is dependent on the ability to survive in the GIT ecosystem and to interact with other components in a manner that fosters improved health (Buddington, 2009). Hence, in order to effectively provide such health functionalities, the minimum number of viable probiotic bacteria is

suggested as 10^7-10^8 cfu/mL of a product by the time of consumption (Gomes and Malcata, 1999; Stanton *et al.*, 2003; Van Niel *et al*., 2002).

Table 4 shows the probiotic strains (R and G) counts before and after having submitted fermented samples through SGID. As can be observed, initial probiotic counts of all samples (before SGID) accomplished the minimum survivals suggested and were established around 7-8 log cfu/mL.

Table 4. Initial probiotic strains (R and G) counts, expressed as log cfu/mL in fermented almond *milks* and the probiotic viability after having submitted them to a simulated gastrointestinal digestion. Mean values and (standard deviation).

Sample	**Initial probiotic counts** (log cfu/mL)		**Probiotic viability** (% of survival)	
	Lab-made almond *milk*	Commercial almond *milk*	Lab-made almond *milk*	Commercial almond *milk*
R	7.95 (0.22) $^{x\,a}$	7.62 (0.24) $^{y\,a}$	56 (5) $^{x\,a}$	43.8 (2.9) $^{y\,ab}$
RT	7.47 (0.14) $^{x\,a}$	8.46 (0.08) $^{y\,ab}$	51 (7) $^{x\,ab}$	46.8 (0.5) $^{y\,a}$
G	8.1 (0.2) $^{x\,a}$	8.17 (0.17) $^{y\,a}$	48.7 (0.5 $^{x\,b}$	42.2 (1.6) $^{y\,b}$
GT	8.57 (0.18) $^{x\,b}$	8.24 (0.15) $^{y\,b}$	59.42 (1.03) $^{x\,a}$	51.0 (2.2) $^{y\,c}$

^{x,y} different superscripts within a raw indicate significant differences between almond *milks* ($p<0.05$).

^{a,b} different superscripts within a column indicate significant differences between samples due to bacteria strain effect ($p < 0.05$).

R = *L. reuteri*, **RT** = *L. reuteri* + *S. thermophilus*; **G** = *L .rhamnosus*, **GT** = *L. rhamnosus* + *S. thermophilus*

The statistical results showed that all factors (digestion process, type of almond *milk*, and starter used) affect significantly ($p < 0.05$) the probiotic

counts. Survival values reached were around 40-50% (Table 4), which were higher than those reported by others authors in studies of probiotic survivals to GIT (20-40%) (Bezkorovainy, 2001; Marteau *et al*., 1997). This might be attributable to the presence of inulin which, as a prebiotic, is believed to enhance probiotic viability (Capela, Hay and Shah, 2006; Kolida *et al.*, 2002, Frank, 2002). Therefore, the combination of almond *milk* and inulin appears to be a suitable matrix to develop probiotic or, more specifically, synbiotic products.

In general, greater viability ($p < 0.05$) was reached when using the lab-made almond *milk* as fermentative matrix and the mixed culture in the fermentative process. This result could be consequence of, on one hand, the capability of T to hydrolyse sucrose, to produce stimulating factors which enhances the growth of lactobacilli (such as formic acid) and, on the other hand, the oxygen consumption and CO_2 formation, leading to a more anaerobic environment that is beneficial for the probiotics survival (as they are anaerobic facultative bacteria) (Tamime and Robinson, 2000). The highest viability ($p < 0.05$) was reached in samples fermented using GT as inoculum bacteria.

The lab-made almond *milk* seems to be a more suitable matrix than commercial one for the probiotic growth and further viability. This effect could be due to their different lipid content or/and their different industrial treatments (heat treatment was more intense in the commercial samples) which could affect negatively to the bacterial growth. In this sense, Mandalari *et al.* (2000a) concluded that after the digestion process, the lipid component of almonds is relevant in the alteration of bacterial growth and

metabolism. On the other hand, the availability of limiting amino acids for starters growth could be lower in the commercial almond *milks* (submitted to the UHT treatment) due to the further development of Maillard reactions (Markowicz *et al*., 2012), especially in a low protein environment. UHT treatment destroys around 2% of available lysine of cow milk and sterilisation process, around 10-15% (Korhonen *et al*., 1998).

3.5 Anti-inflammatory bioactivity of non-fermented and fermented almond *milks*

The fermentation process and also the *in-vitro* gastrointestinal digestion of proteins led to the generation of peptides with different Mw, which could exert inflammatory effects on intestinal epithelial cells (Shan *et al*., 2005; Laparra *et al*., 2010; Costa *et al*., 2012).

Figure 2 shows the results of the evaluation of anti-inflammatory properties of non-fermented and fermented almond *milks*, once submitted to SGID, which was measured throughout the IL-8 production in Caco-2 cells once treated with TNF-α agent. TNF-α is a pro-inflammatory factor produced by macrophages that exerts important effects in the systemic inflammation (Hue *et al*., 1997) and induces the production of other inflammatory cytokines such as IL-6 and IL-8. A modulation of the pro-inflammatory TNF-α induced IL-8 expression in Caco-2 cells by probiotic fermented products was expected, since both G and R strains have been reported to exert anti-inflammatory positive results (Ma, Forsythe and Bienenstock, 2004; Zhang *et al*., 2005; Pelto *et al*., 1998).

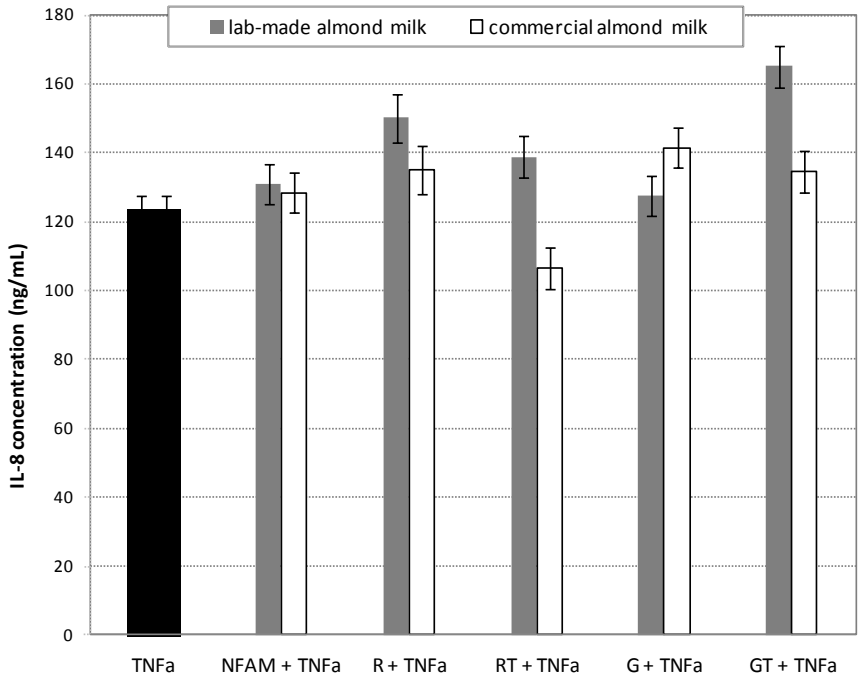

Figure 2. Interleukin (IL)-8 productions in Caco-2 cells exposed to digests of non-fermented (AM) and the different fermented almond *milk*s. Intervals LSD are included.

TNFa = Tumour Necrosis Factor-α, **R** = *L. reuteri*, **RT** = *L. reuteri* + *S. thermophilus*, **G** = *L. rhamnosus*, **GT** = *L. rhamnosus* + *S. thermophilus*

Nevertheless, none of the samples exhibited an anti-inflammatory effect *in vitro*, except fermented samples using the commercial almond *milk* and RT culture. These samples presented an inhibition of the IL-8 production of 17% in comparison with the ones exposed to the pro-inflammatory TNF-α agent (black bars) and to the AM (Figure 2). The latter observations are well correlated with the higher proteolytic activity and the smaller molecular

masses of peptides generated during *in vitro* digestion of the RT fermented samples. It seems that the use of the mixed culture RT could have also modified qualitatively the aforementioned peptide fraction, leading to a different peptide sequence and thus, modifying the allergic response, as has been previously observed by Laparra *et al.* (2010) working with different bifidobacteria strains. Nevertheless, further analysis should be done to clarify the obtained results.

Immunomodulatory effects of fermented soy- or dairy-milks and casein hydrolysates by lactic acid bacteria have been widely reported (Laffineur *et al*., 1996; Sutas *et al*., 1996; Wagar *et al*., 2009). However, there is lack of data concerning "milks" from vegetal origin. Only recent scientific studies have been suggested their potential prebiotic (Mandalari *et al*., 2008b) and anti-inflammatory activities (Rajaram *et al*., 2010; Bernat *et al*., 2011).

In general, digests from lab-made almond fermented *milks* provoked a greater production of IL-8 than the commercial ones (Figure 3). It has been postulated by several authors (Korhonen *et al*., 1998; Kilshaw *et al*., 1982; Poulsen *et al*., 1987; Høst and Samuelsson, 1988) that intense heat treatment on cow milk increase the ability of milk proteins to elicit allergic reactions in sensitised population. Further studies have, however, not been carried out to substantiate this hypothesis (Korhonen *et al*., 1998). Moreover, no related information has been found based on almond *milks*.

4. CONCLUSIONS

Almond *milk* was able to be used as a raw material to produce non-dairy probiotic products with interesting nutritional properties and health benefits. The assayed bacteria can cleave almond peptides during intestinal digestion, originating different peptide patterns that would reach the intestinal epithelia. Considering the overall results of fermentation processing, probiotics' survival and inflammatory response, commercial almond *milk* was considered the best option to produce fermented products, especially when probiotic *L. reuteri* combined with *S. thermophilus* were used as starters. These samples presented a reduction of the IL-8 production of around 17% in comparison with the ones exposed to the pro-inflammatory TNF-α marker, as a result of the proteolytic capacity of this mixed culture on almond peptides.

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Almond *milk* fermented with different potentially probiotic bacteria improves iron uptake by intestinal epithelial (Caco-2) cells

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ABSTRACT

A fermented almond (*Prunus amygdalus* cv. dulcis) *milk* was developed by using different potentially probiotic bacteria. An *in vitro* digestion / Caco-2 cell model was used to evaluate the effect of both non-fermented and fermented almond *milks* on the mitochondrial enzymatic activities of enterocytes. Moreover, macrophages were challenged with the digests and the production of pro-inflammatory biomarkers TNF-α and IL-6 was quantified. Enzymatic activities increased in cell cultures exposed to almond *milks* either fermented or not. Both biomarkers decreased ($p < 0.05$) in fermented almond *milks* with either *B. bifidum* or *B. longum*. Therefore, fermented almond *milk* favoured the energetic metabolism of enterocytes and had a lower inflammatory response, suggesting it is beneficial for the management of allergies/intolerances. Moreover, the fermentation process enhanced the iron uptake by Caco-2 cells, especially with *L. rhamnosus* and either *B. bifidum* or *B. longum* as starters, thus improving the product bioactivity.

***Keywords*:** Almond *milk*, probiotics, iron availability, inflammation.

1. INTRODUCTION

The current alarming increase in the incidence of allergic diseases in both children and adults in developed countries has been attributed to the so-called "hygiene hypothesis"; this theory suggests that the increased level of hygiene in both the environment and the food supply leads to a reduced exposure to a variety of microbes, which early in life is a crucial factor in the development of allergies (Björkstén, 2009). Indeed, several prospective follow-up studies have found that alterations in gut microbiota precede allergy development (Kalliomäki, 2010). Hence, recent clinical allergy studies have principally focused their attention on the manipulation of gut microbiota composition (Kalliomäki *et al.,* 2010). Nowadays, probiotics, prebiotics and synbiotics (combination of pre- and probiotics) are considered as good tools which to elicit changes in the gut biomass composition, since they can improve and stimulate beneficial gut microflora, among other effects that are beneficial for the health. Since the late 1990s, over 30 randomised clinical trials have been published, in which probiotics have been used either in the treatment or prevention of allergies (mainly atopic diseases) (Kalliomäki *et al.,* 2010). Hence, considering the high prevalence of atopic disease in childhood in the industrialised countries (Anandan *et al*., 2010), the use of yoghurt-type foods as carriers of probiotics and/or synbiotics would be helpful as a means of attaining the either preventive or prophylactic treatment in this targeted population. Moreover, despite the little known and untrustworthy concept of functional food, consumers are seen to be familiar with yoghurt-type products and

considered them as healthy foods (Annunziata and Vecchio, 2011), which would facilitate the inclusion of these functional fermented products in their diets.

The immunomodulatory effects of fermented dairy-milks, casein hydrolysates and soy-beverage caused by lactic acid bacteria have been widely reported (Sutas *et al*., 1996; Wagar *et al*., 2009). However, in addition to the allergenic proteins, both matrices might provoke iron deficiencies in infants and toddlers. On the one hand, the calcium together with the casein provided by cow milk are seen to inhibit the absorption of dietary non-heme iron, in addition to the intestinal blood loss observed in approximately 40% observed in infants during feeding with cow milk and/or its derivatives (Agostoni and Turck, 2011). On the other hand, soya-based products contain phytates, which negatively interfere in the absorption of iron, among other minerals (García-Onieva, 2007).

By contrast, almond *milk* is not seen to interfere negatively in iron absorption and is considered an appropriate alternative to cow milk, since, besides the healthy lipid profile, it has a low Na/K ratio and a balanced Ca/P ratio (Luengo, 2009). Nevertheless, there is lack of data concerning beverages of vegetable origin, such as almond *milk*. Only recently have data appeared concerning almond seeds (Rajaram *et al.*, 2010), in which the authors studied whether, in addition to the lowering blood lipids, monounsaturated fat-rich almonds influenced other coronary heart disease risk factors, such as inflammation. The study concluded that incorporating about 68 g almonds in a 2000 kcal cholesterol-lowering diet decreased serum E-selectin (molecules which are indicative of endothelial

dysfunction) and C-reactive protein (sensitive marker of inflammation that, in high concentrations, is strongly linked with coronary events, stroke and peripheral vascular disease) in healthy men and women.

On the other hand, almonds have been defined by the Food and Drug Administration (FDA) as an excellent source of vitamin E, manganese, magnesium, copper, phosphorous, fiber, riboflavin and arginine-rich protein. Although around 50% of almond composition is fat, intakes of 7 g per day of this nut reduce low-density lipoprotein cholesterol concentration by 1% (Sabaté *et al.,* 2003) and up to 84 g per day can be consumed without there being any weight gain (Chen *et al.*, 2006). In fact, the FDA promulgated almonds a qualified B-level health claim: eating 42 g daily as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease. Moreover, these nuts have a low glycemic index and do not adversely impact insulin sensitivity (Chen *et al.*, 2006). Almonds have been found to possess antioxidant activity thanks to the bioavailable α-tocopherol and polyphenolic constituents (Chen *et al.,* 2006 and 2007; Milbury *et al.,* 2006) and prebiotic effects, since they stimulated the growth of gut bifidobacteria genus and *Eubacterium rectale* (Mandalari *et al.,* 2008). Hence, taking into account the health benefits of almond intake, almond *milk* might be considered as a good food matrix with which to obtain healthy fermented products. Moreover, if the fermentation process is carried out by potentially probiotic bacteria, the developed fermented product could be useful as a means of preventing some inmunomodulatory diseases, such as allergies.

The Caco-2 cell line, commonly used in conjunction with *in vitro* digestion techniques, is a useful model for studying intestinal human iron uptake which it allows to occur simultaneously with food digestion and is generally regarded as the best available intestinal cell model for studying the mechanisms associated with vectorial iron transport (Glan *et al*., 2002). In addition, the macrophage-derived raw 264.7 cell line expresses key genes and proteins of principal pathways for the production of regulatory cytokines (Novak *et al*., 2003) and constitutes a cell model used worldwide and a useful tool with which to study the inflammatory response(s) and metabolic activity promoted by food-derived components (Barrington *et al*., 2009; Deepika *et al*., 2011; Kabeerdoss *et al*., 2011; O'Sullivan *et al*., 2013) while still maintaining a rapid and inexpensive system.

The aim of this study, therefore, was to evaluate whether almond *milk* fermented with different potential probiotic bacteria affects the energetic metabolism in intestinal cells and the production of pro-inflammatory biomarkers in order to gain insights into the potential benefits of the designed products for the consumer's gut health.

2. MATERIAL AND METHODS

2.1 Preparation and fermentation of almond *milk*

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 (Barcelona, Spain), equipment specifically designed for the production of vegetable

milks, using a nut : water ratio of 8:100. The milky liquid obtained was then microfluidised in a high pressure homogeniser (M-110P model; Microfluidics International Corporation, Westwood, MA, USA) by applying 172 MPa, sterilised (121 ºC/15 min) and subsequently cooled down to 37 ºC (fermentation temperature). The use of high pressures of homogenisation (HPH) contributed to a better stability of the *milk*, since this emergent technology is able to reduce the size of fat globule particles, greatly delaying the flocculation and coagulation phenomena (Capra *et al.*, 2009; 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 the prevention of syneresis (Cruz *et al.*, 2009; Patrignani *et al.*, 2007).

Lactobacillus rhamnosus CECT 278, *Lactobacillus plantarum* 3O9, *Bifidobacterium bifidum* CECT 870, *Bifidobacterium longum* CECT 4551, *Streptococcus thermophilus* CECT 986, *Lactobacillus delbrueckii* subs. *bulgaricus* (CECT 4005) were used as starters pure or mixed, as can be observed in Table 1. All the bacteria were purchased from CECT (Paterna (Valencia), Spain), with the exception of the *L. plantarum* 3O9 strain, which was isolated from Guirra sheep milk and selected as probiotic in previous studies (Amorocho, 2011).

Table 1. Microbial strains used to produce the different fermented almond *milks*.

Formulation	Starters inoculum		
F1	-	-	*S. thermophilus + L. delbrueckii*
F2	*L. rhamnosus*	-	-
F3	*L. plantarum*	-	-
F4	*L. rhamnosus*	-	*S. thermophilus + L. delbrueckii*
F5	*L. plantarum*	-	*S. thermophilus + L. delbrueckii*
F6	-	*B. bifidum*	*S. thermophilus + L. delbrueckii*
F7	-	*B. longum*	*S. thermophilus + L. delbrueckii*
F8	*L. rhamnosus*	*B. bifidum*	-
F9	*L. rhamnosus*	*B. longum*	-
F10	*L. plantarum,*	*B. bifidum*	-
F11	*L. plantarum*	*B. longum*	-

For the preparation of starters inoculum, the strains were independently incubated for 24 h in their selective broths and then centrifuged at 100 g (Medigriger-BL-S, JP-Selecta; Barcelona, Spain) for 10 min at 4 ºC to re-suspend the pellet in PBS-1x buffer (10 mmol/L phosphate, 137 mmol/L NaCl, 2.7 mmol/L KCl, pH 7.4) until reaching strain concentrations of 10^8 cfu/mL.

For each formulation, 1 mL/100 mL of starter suspension was added to the almond *milk* and subsequently incubated at 37 ºC until pH values of 4.4 -4.6 were reached, which was controlled by using a GLP 21+ pH-meter (Crison Instruments S.A.; Barcelona, Spain). The fermented samples were

frozen and stored at -22 ºC prior to analysis. A non-fermented almond sample was used as a control (C).

2.2 Simulated gastrointestinal digestion

The human gastrointestinal digestion process was simulated by using porcine pepsin (800–2,500 units/mg protein), pancreatin (activity, 4_ USP specifications) and bile, as previously described by Laparra *et al.* (2009). All reagents were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Prior to the *in vitro* digestion, 1.5 mL aliquot of each assayed sample was diluted in 5mL of a saline solution (140 mmol/L NaCl, 5 mmol/L KCl adjusted to pH 2). For gastric digestion (pepsin in 0.1 mol/L HCl adjusted to pH 2; 1 h), samples were placed on a rocking platform shaker in an incubator (37 ºC; 5% CO_2; 95% relative humidity). The intestinal digestion (pancreatin-bile extract in 0.1 mol/L $NaHCO_3$ adjusted to pH 6.9–7; 2 h) was carried out in the upper chamber of a two-chamber system in 6-well plates.

The upper chamber was formed by fitting the bottom of an appropriately sised Transwell insert ring (Corning B.V. Life Sciences, Amsterdam, The Netherlands) with a 15,000 molecular mass cut-off dialysis membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA, USA). Aliquots (1.5 mL) of the gastrointestinal digest were loaded into the upper chambers and incubated for 2 h. Afterwards, the inserts were removed and the dialysates were diluted (1:4, v/v) with culture media and incubated with intestinal epithelial (Caco-2) or macrophage (RAW 264.7) cells.

2.3 Ferritin analysis in intestinal epithelial cell monolayer

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 17 and used in experiments at passages 33 to 38. Cells were maintained with Dulbecco's modified Eagle's medium (DMEM) (Gibco®, Madrid, Spain) under conditions previously described by Glahn *et al.* (1998).

For the assays, Caco-2 cells were seeded at 50,000 cell/cm^2 in collagen-treated 6-well culture plates (Costar, Cambridge, MA, USA), and were grown with DMEM for 12 days. On the day prior to the experiments, the DMEM medium was replaced by 2 mL of minimum essential medium (MEM) (Gibco®, Madrid, Spain) and then the cells were returned to the incubator. 50 μmol/L of $FeCl_3$ were added to the digested almond *milk* samples and the ferritin formation by Caco-2 cells over a 24 h period was proportional to the cell iron uptake. A latex-enhanced turbidimetric immunoassay (Ferritin-turbilatex, Spinreact, Girona, Spain) was used to measure the Caco-2 cell ferritin content. The concentrations of ferritin were normalised by the determination of the total protein content in cell cultures. The control cells (basal), exposed to *in vitro* digestions of control solutions containing digestive enzymes but not sample, were monitored throughout. Base-line cell ferritin in cultures grown in MEM averaged 4.2 ng/mg cell protein. The samples were analysed in triplicate.

2.4 Mitochondria enzyme activities

For the assays, Caco-2 cells were seeded at 50,000 cell/cm^2 in 24-well culture plates (Costar, Cambridge, MA, USA), and were grown with

DMEM for 12 days. These activities were evaluated in Caco-2 cell cultures by monitoring MTT (3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyl tetrazolium bromide) conversion on exposed cultures after an incubation period of 3 h. This colorimetric method is based on the reduction of the tetrazolium ring of MTT by mitochondria dehydrogenases yielding a blue formazan product, which can be measured spectrophotometrically. Control cells (basal) exposed to digests containing enzymes but not samples were used throughout each assay. Four replicates were analysed.

2.5 Analysis of pro-inflammatory markers

For the assays, raw 264.7 cells were seeded at 50,000 cell/cm^2 and were grown with RPMI medium for 24 hours (Novak *et al*., 2003). Tumour Necrosis Factor-α (TNF-α) and Interleukin 6 (IL-6) (eBioscience Ltd., Hatfield, UK) were determined by ELISAs, following the manufacturers' instructions, on exposed RAW 264.7 cell cultures after an incubation period of 3 h. Results were expressed as picograms per mL of media. Four replicates were analysed.

2.6 Statistical analysis

Each of the experiments was conducted on four independent replicates. Aone-way analysis of variance (ANOVA) and the Tukey *post hoc* test were applied. Statistical significance was established at a confidence level of 95% for all the comparisons. SPSS v.15 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

3. RESULTS AND DISCUSSION

3.1 Fermented almond-based *milks*

The chemical composition of the almond *milk* per 100 mL (mean value ± standard deviation) is summarised in Table 2. Taking into account the nut:water ratio used, these values are similar to those found in the literature (Yada *et al.*, 2011).

Table 2. Chemical compositionof almond *milk* used in the study. Values (mean ± standard deviation) are expressed as grams per 100 mL of beverage.

Compound	Concentration (g/100 mL)
Dry matter	6.64 ± 0.5
Lipids	3.96 ± 0.2
Proteins	1.37 ± 0.03
Total sugars	0.41 ± 0.002
Ashes	0.325 ± 0.012
Fiber	0.58*

*** Fibre concentration was obtained by substracting the dry matter content from the sum of the rest of compounds shown.**

The initial pH of the almond *milk* was 6.6. A significant decline in pH, from 6.6 to 4.6 after 20 h at 37 ºC of incubation, took place in the developed product along with a corresponding increase in acidity caused by fermentation.

3.2 Bacterial fermentation effects on TNFα and IL-6 production

Figure 1 shows the Tumour necrosis factor (TNF)-α and interleukin (IL)-6 production in macrophage (raw 264.7 cells) cultures exposed to digests of fermented *milks*. A non-fermented almond *milk* was used as a control (C).

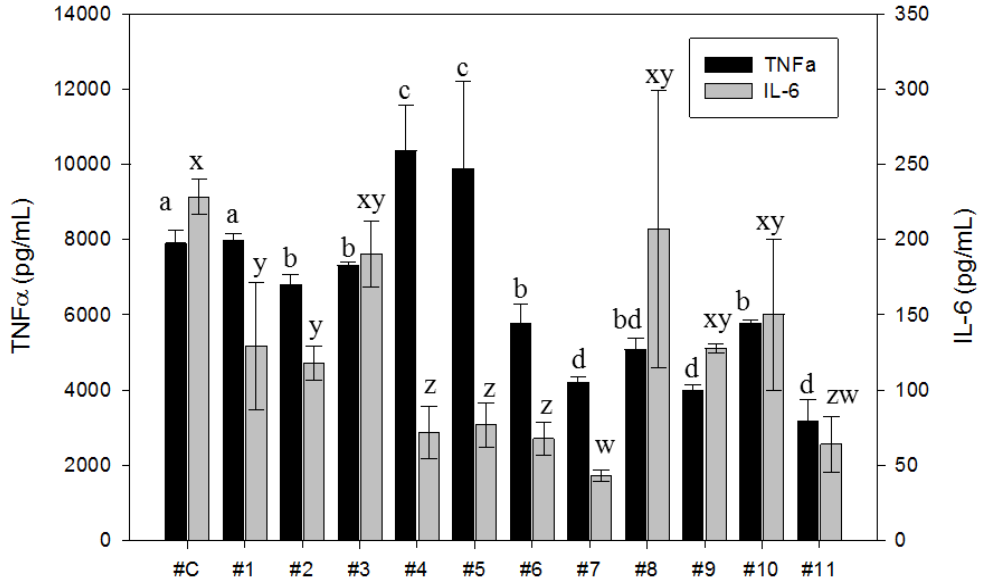

Figure 1. Tumour necrosis factor (TNF)-α and interleukin (IL)-6 productions in macrophages (raw 264.7 cells) cultures exposed to digests of fermented *milks*. C: non-fermented almond *milk*

[a-e] different superscript letters indicate significant differences between samples in TNF-α production ($p < 0.05$)

[w-z] different superscript letters indicate significant differences between samples in IL-6 production. ($p < 0.05$)

Focusing on the cells treated with dialysable fractions samples not fermented by using bifidobacteria (F1 to F5) (Table 1), the fermentation with either *L. rhamnosus* (F2) or *L. plantarum* (F3) decreased the TNF-α production ($p < 0.05$) at the same level with respect to the control (C), meanwhile similar concentrations were observed when the standard yoghurt bacteria was used (F1). Opposite effects were observed when the fermentation was developed by combining those lactobacilli with standard yoghurt bacteria (F4 and F5, respectively), since the TNF- α production increased ($p<0.05$) compared to the control (Figure 1). As regards the IL-6, and contrary to that observed in TNF- α, all samples had a positive effect in the production of this pro-inflammatory marker with respect to the control, especially when the almond *milk* fermentation was done by using mixed-cultures (F4 and F5). These samples exhibited the lowest IL-6 concentrations ($p < 0.05$), showing an inhibition of the initial IL-6 production induced by non-fermented almond *milk* (C) greater than 50% ($p < 0.05$).

With respect to the samples fermented using the bifidobacteria (F6 to F11), all the cells exposed to those dialysed samples exhibited very low TNF- α productions, which were up to 50% less than the control ($p < 0.05$). When considering IL-6 production, different patterns were observed, depending on the type of bifidobacteria present during the fermentation process. In general, the fractions from fermented samples with *B. bifidum* (F6, F8 and F10) induced cells to produce IL-6 amounts similar to that obtained in fermented samples with standard yoghurt bacteria (F1). However, fermenting the samples with *B. longum* (F7, F9 and F11) led to

lower ($p < 0.05$) IL-6 concentrations, especially when it was combined with either standard yoghurt bacteria (F7) or *L. plantarum* (F11).

The early inflammatory response(s) is mediated predominantly by macrophages of the immune system and is characterised by an increased production of pro-inflammatory cytokines (Baeuerle and Baltimore, 1996). TNF-α is a pro-inflammatory factor produced by macrophages that exerts important effects on systemic inflammation (Hue *et al.*, 1997) and induces the production of other inflammatory cytokines such as IL-6 and IL-8. The observed bacterial fermentation effects could have important consequences on the intestinal barrier function because TNF-α plays a crucial role increasing paracellular permeability and impairing tight junction functionality (Ma *et al*., 2004) and leukocyte infiltration in intestinal wall (Hoffman, 2000). In addition, the reduction in TNF-α production might also have important physiological consequences preventing allergic inflammatory processes. This is because IL-6 is one of the most important mediators of a multifunctional pro-inflammatory cytokines, and actually the major cytokine produced by activated mast cells (Burd *et al.*, 1989).

The immunomodulatory effects of fermented soy- or dairy-milks and casein hydrolysates produced by lactic acid bacteria have been widely reported (Laffineur *et al*., 1996; Sutas *et al*., 1996; Wagar *et al*., 2009). However, there is a lack of data concerning "milks" from vegetal origin, and only recently have data appeared on almond seeds (Rajaram *et al*., 2010; Wu *et al*., 2010). Almonds are known to have several nutritional benefits, including that of lowering cholesterol and protection against diabetes. Furthermore, scientific studies have pointed out their potential prebiotic

(Mandalari *et al*., 2008) and anti-inflammatory activities (Rajaram *et al*., 2010). Almond lipids available for fermentation have been associated with increasing numbers of bifidobacteria, but not lactobacilli, and with different profiles in the short fatty acid content with high butyrate concentrations in the ferments (Mandalari *et al.*, 2008). These results are coherent with the marked positive effects in the inflammatory response(s) induced by dialysates from samples inoculated with bifidobacteria; nevetheless, the active components and underlying mechanism(s) responsible for these effects are still unknown. In this sense, a recent randomised, controlled, crossover study reported the anti-inflammatory effect levels of an almond-enriched high-monounsaturated (MUFA) fat diet with decreased circulating levels of E-selectin (Rajaram *et al*., 2010). These aspects have not been investigated since they are beyond the objective of this study.

The results point out that the potential immunomodulatory effects of fermented almond *milk* with potentially probiotic bacteria might contribute to preventing and/or controlling the severity of pathological manifestations of allergies and/or food intolerances caused by proteins from cow milk, especially when using *B. longum* in the starter inoculum.

3.3 Bacterial fermentation effects on energetic metabolism of intestinal cells

The results of the cytotoxicity of fermented samples in intestinal epithelial (Caco-2) cells, which was quantified by monitoring the mitochondrial enzyme (MTT test) activities, are shown in Figure 2. This assay showed that none of the dialysates exposed to cell cultures caused

toxic effects, as concluded from the fact that MTT values (%) were similar to ($p > 0.05$) or higher ($p < 0.05$) than those calculated for the control. The only differences detected concerned the stimulation effects in mitochondrial enzyme activities coupled to the cellular energetic metabolism.

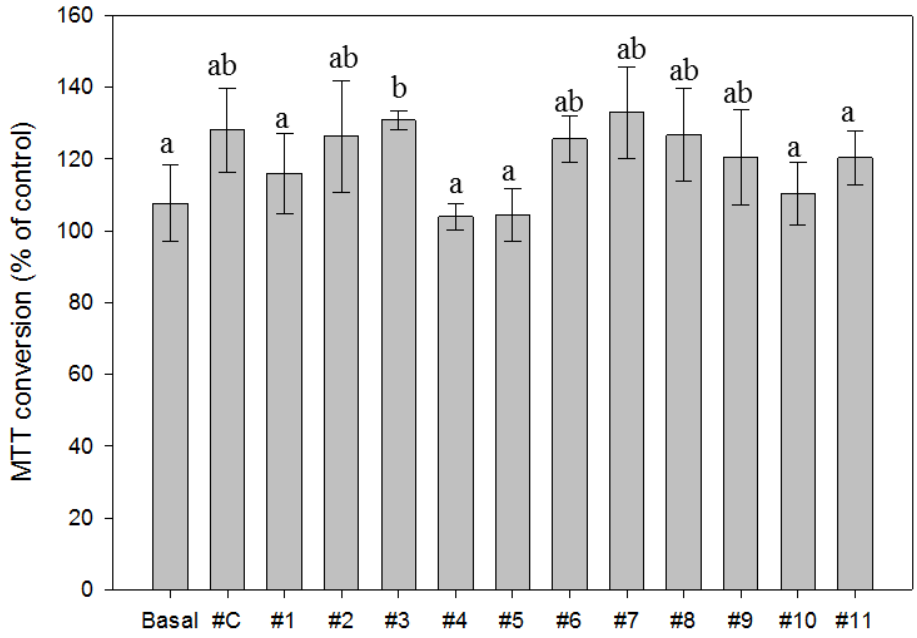

Figure 2. MTT conversion and neutral red uptake precentages in Caco-2 cell cultures exposed to digested almond *milk* (C) and digest fermented products (1-11).

[a,b] different superscript letters indicate significant differences between samples ($p < 0.05$)

As regards the samples not fermented with bifidobacteria (F1 to F5) (Table 1), the MTT values showed that dialysates from samples fermented with either *L. rhamnosus* CECT 278 (F2) or *L. plantarum* 3O9 (F3) had a

positive effect on energetic cell metabolism, similar to that of non-fermented almond *milk* (C). However, when these bacteria were used in combination with standard yoghurt bacteria, the resulted dialysates (F4 and F5) did not have a stimulatory effect on cell metabolism. This effect cannot be interpreted as cytotoxic because cell cultures exhibited MTT conversion values similar to those of cell cultures exposed only to culture media (Basal) (Figure 2).

Cell cultures exposed to dialysates from samples fermented with bifidobacteria (F6 to F9) exhibited similar MTT conversion percentages to the control, also suggesting positive effects on the energetic cell metabolism (Figure 2). This effect was not observed in samples using *L. plantarum* in the mixed culture (F10 and F11).

The intestinal epithelium constitutes the first physiological barrier to exogenous compounds and nutrient absorption. Mitochondria and endo-lysosomal enzyme activities were proven to be as sensitive biomarkers of changes in cellular metabolism in response to internalised food-derived components (Laparra *et al*., 2009; Wu *et al.*, 2010). The positive results obtained in both the energetic cell metabolic response (Figure 2) and the production of pro-inflammatory cytokines by immune cells (Figure 1) indicated that almond fermented products might exert beneficial effects on human gut health, especially when using standard yoghurt bacteria with *B. longum* (F7) and *L. rhamnosus* with *B. longum* (F9) as starters. Increases in MTT conversion values have been associated with a greater ferritin formation by Caco-2 cells in response to both an enhanced dietary iron absorption (Laparra *et al*., 2009; Mandalari *et al.*, 2008) and also to the use

of food-derived components as a means of producing reducing equivalents thus preserving alterations in the cellular redox status (Cilla *et al*., 2008). In this context, it has been reported that almond extracts exhibited excellent metal ion chelation efficacies (ability to maintain oligoelements such as iron in the reduced form needed to be absorbed by the epithelial cells), owing to its source of bioactive polyphenols with antioxidant activity (Garrido *et al*., 2008; Wijeratne *et al*., 2006). These almond-derived components with functional characteristics may also be present in the fermented samples and might explain, at least in part, the effects observed.

3.4 Iron uptake in the presence of fermented almond *milk*

The ferritin concentrations in cell cultures exposed to the different dialysed fermented almond *milks* are shown in Figure 3. Apparently, the fermentation process improved the bioavailability of iron, since in every fermented formulation the iron uptake resulted higher than that obtained in the cells exposed to non-fermented almond *milk* dialysates (C) ($p < 0.05$). In particular, samples fermented with *L. rhamnosus* with either *B. bifidum* (F8) or *B. longum* (F9) were the ones which induced most the iron uptake ($p < 0.05$).

Previous studies have shown the *in vitro* enhancing effect of probiotic bacteria on iron uptake in fermented vegetable matrices, such as carrot juice (Bergqvist *et al*., 2006), maize (Proulx and Reddy, 2007) or beans (Laparra *et al*., 2008). The present study extends the bacterial-mediated positive effects on iron uptake from vegetable products, particularly those derived from the genus of bifidobacteria. The involvement of *B. bifidum* and *B.*

longum strains in the fermentation processes of food products developed to reduce inflammatory and/or allergic intestinal processes may be of interest owing to, on the one hand, the results obtained commented on above and, on the other hand, the results of previous *in vitro* and *in vivo* studies carried out by using these bacteria species, in which both the beneficial effects reducing inflammatory milieu and positive immunomodulatory responses were observed (Laparra and Sanz, 2010; Laparra *et al*., 2012).

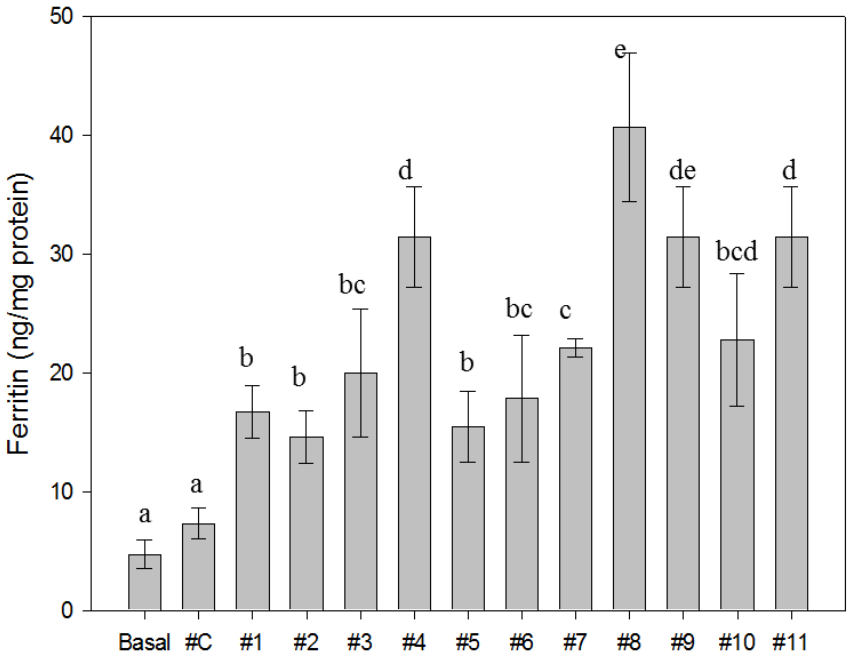

Figure 3. Ferritin concentration in Caco-2 cultures exposed to digests of fermented *milks* with added $FeCl_3$ (50 µmol/L). (MEM: Minimum Essential Medium; C: non-fermented almond *milk*)

[a-e] Different superscript letters indicate significant differences between samples ($p < 0.05$).

Therefore, the results obtained (Figure 3) indicate the positive effects of fermented almond *milk.* It increased iron uptake, which could have important consequences as it preserves the nutritional iron status in the pedriatic community which appears to be the most susceptible population to the negative effects of cow milks. Furthermore, the intake of this type of products could reduce allergies and intolerances derived from the consumption of cow milk by this population (Agostoni and Turk, 2011).

4. CONCLUSIONS

This study has shown that almond *milk* fermented with potentially probiotic bacteria exerted positive immunomodulatory effects on macrophages and did not impair, or even improved, the energetic metabolism of intestinal epithelial cells, especially when this vegetable *milk* was fermented with either standard yoghurt bacteria and *B. longum* CECT 4551 or *L. rhamnosus* CECT 278 and *B. longum*. Moreover, some combinations of specific strains had markedly significant positive effects on the iron uptake by intestinal epithelial cells that could help to improve the nutritional status of targeted consumers. In particular, samples inoculated with *L. rhamnosus* CECT 278 and either *B. bifidum* CECT 870 or *B. longum* CECT 4551 exhibited the highest ferritin concentrations in Caco-2 cultures. The obtained results also suggest an improvement in the bioactivity of almond *milk* due to fermentation; nevertheless, the identification of biologically active components is needed and will provide further insights into the potential nutritional and health benefits of fermented almond-based

products. To sum up, the results suggest that almond *milk* fermented with potentially probiotic bacteria may be beneficial for human gut health and, hence, might be helpful in managing cow-milk allergies and/or intolerances.

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V. CONCLUSIONES

Conclusiones

- Las altas presiones de homogenización y los tratamientos térmicos utilizados en el procesamiento de las *leches* de almendra y avellana estudiadas afectaron a las propiedades físicas y la estabilidad de las mismas. El uso de altas presiones de homogeneización de hasta 100 MPa redujo significativamente el tamaño de los glóbulos de grasa en estas *leches* pero no fue suficiente para asegurar la estabilización de las emulsiones con el tiempo. A partir de las observaciones de la microestructura de las muestras, se vio que las proteínas no contribuyeron a estabilizar las emulsiones debido a su carácter hidrófobo, que no favoreció una estabilización estérica. Sin embargo, la combinación de las altas presiones de homogeneización con tratamientos de pasteurización dio lugar a la desnaturalización y agregación de las proteínas del suero, que favoreció la formación de una red tridimensional. Esta red ejerció un efecto viscoso estabilizante que inhibió los fenómenos de separación de fase del producto durante su almacenamiento.

- En general, los ajustes de los modelos matemáticos empleados para analizar el efecto de los distintos factores de crecimiento (glucosa, fructosa, inulina y cantidad de inóculo) sobre la supervivencia del microorganismo probiótico, proporcionaron bajos coeficientes de correlación (R^2) debido al estrecho margen de variación de la variable respuesta. A pesar de ello, los resultados de la modelización fueron una

buena herramienta para obtener información cualitativa dentro de los niveles establecidos, y permitieron optimizar la formulación, asegurando la cantidad mínima de probiótico para poder considerar el producto fermentado como funcional, con el menor coste posible.

- Las tres *leches* vegetales, avena, almendra y avellana, fueron excelentes matrices para el desarrollo de productos fermentados con bacterias probióticas, cuya viabilidad se mantuvo por encima de los valores recomendados. La adición de inulina a las *leches* formuladas, permitió el desarrollo de nuevos productos calificados como simbióticos (con probióticos y prebióticos), lo que aumenta sus cualidades en cuanto a beneficios para la salud. Además, estos productos son potencialmente aptos para grupos de población específicos tales como vegetarianos, lacto-intolerantes o alérgicos a las proteínas de la leche de origen animal.
- El proceso fermentativo modificó las propiedades reológicas, estructurales y fisicoquímicas de las *leches* vegetales estudiadas, en mayor o menor medida. En general, las diferencias de color entre los productos antes y después de fermentar no fueron notables. El proceso de fermentación dio lugar a la formación de una red tridimensional, que presentó cierto grado de sinéresis en los últimos días del almacenamiento en refrigeración al ser incapaz de retener la totalidad de la fase continua. Esta sinéresis, junto con el progresivo aumento de acidez, hizo que la vida útil de estos productos fuera algo inferior a la

de los yogures convencionales, especialmente en el caso de la *leche* de avena.

- En el estudio del proceso fermentativo de la *leche* de avena, la presencia de β-glucanos impartió una gran estabilidad física tanto durante la fermentación como durante el almacenamiento en refrigeración, gracias a sus propiedades espesantes y gelificantes. Además, este componente junto con la inulina le dieron un valor añadido al producto fermentado, al ser compuestos prebióticos.

- En los estudios realizados tras la digestión *in vitro* de los productos fermentados a base de almendra y avellana, se observó que la supervivencia de las bacterias probióticas fue muy elevada (40-65%), especialmente en el caso de *L. rhamnosus* GG. Estos datos apuntan a que las concentraciones de probiótico serían suficientes para colonizar el intestino del hospedador y, consecuentemente, ejercer posibles efectos saludables.

- En la digestión de los fermentados derivados de la *leche* almendra se observó la hidrólisis de una gran parte de los péptidos de alto peso molecular presentes, generando ciertas diferencias en los perfiles de péptidos que alcanzan el epitelio intestinal. La *leche* de almendra comercial presentó las mejores características para el desarrollo de fermentados, especialmente cuando se utilizan *L. reuteri* y *S. thermophilus* como cultivos iniciadores. Estas muestras redujeron la

producción *in vitro* del biomarcador pro-inflamatorio IL-8, en comparación con las células control expuestas al agente pro-inflamatorio TNF-α.

- En el estudio de las propiedades inflamatorias *in vitro* de *leches* de almendra fermentadas, realizado en paralelo con el anterior y utilizando otras bacterias potencialmente probióticas, se observó que la producción de los biomarcadores pro-inflamatorios TNF-α y IL-6 fue mínima en los fermentados inoculados con *B. longum*. Estos fermentados, por tanto, podrían ser útiles para la prevención y/o tratamiento de alergias, aunque se necesitan más estudios para determinar el potencial funcional de estos productos. Además, las muestras inoculadas con *L. rhamnosus* CECT 278 y *B. bifidum* CECT 870 mejoraron notablemente la absorción *in vitro* de hierro por parte de células intestinales, con lo que estos productos fermentados supondrían un valor añadido al ser capaces mejorar la biodisponiblidad de este micronutriente.

En resumen, podemos decir que los resultados de esta tesis han permitido obtener unas condiciones óptimas de procesado de las *leches* vegetales estudiadas (avena, almendra y avellana) y productos fermentados derivados, que permiten asegurar una adecuada calidad, estabilidad y funcionalidad durante su vida útil. Además, en algunos de los fermentados obtenidos de *leche* de almendra se ha puesto de manifiesto cierta actividad inmunológica y de mejora de la biodisponiblidad de nutrientes esenciales de interés para determinados grupos de población específicos (intolerantes a la

leche de vaca, vegetarianos…). Desde el punto de vista sensorial, se obtuvieron productos fermentados con una buena aceptación por parte del panel de catadores, a pesar de no ser comparables con los fermentados lácteos existentes en el mercado, sobre todo en cuanto al flavor. Consequentemente, se debería, por una parte, ampliar el conocimiento entorno a los posibles beneficios para la salud y, por otra, optimizar las propiedades sensoriales de los mismos en futuros estudios. Estas nuevas líneas de investigación vendrían encaminadas a afianzar los nuevos fermentados obtenidos en el mercado actual.