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Design and development of a fermented goat milk as functional food focusing on bioactive peptides

Diseño y desarrollo de un derivado fermentado de leche de cabra como alimento funcional. Péptidos bioactivos

Tesis Doctoral con Mención Internacional presentada por:

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Abbreviations

AAS: Atomic absorption spectrometry

ABTS: 2,2-azino-bis-(-3ethylbenzothiazoline-6-sulfonic acid) diammonium salt

ACE: Angiotensin-I-converting enzyme

ACEi: Angiotensin-I-converting enzyme inhibitory activity

ANOVA: Analysis of variance

BCA: Bicinconic acid assay (to measure total proteins)

CN: Casein

CFM: Commercial skimmed cow yoghurt fermented with *L. bulgaricus* plus *S. thermophilus*

CFU: Colony-forming units

D: Dialyzable fraction after the *in vitro* gastrointestinal digestion dialysis assay.

DPPH: 2,2-diphenyl-1-1-picrylhydrazyl

ET: Electron transfer

ETAAS: Electrothermal atomic absorption spectrometry

FAAS: Flame atomic absorption spectrometry

FRAP: Ferric reducing antioxidant power

GABA: γ-amino butyric acid

GFM: Probiotic commercial skimmed fermented goat milk

HAT: hydrogen atom transfer

(H)/$_H$: Sample where *L. plantarum* C4 was inoculated at 10^8 cfu/mL

IER: Inhybitory efficiency ratio

LA: Lactoalbumin

LAB: Lactic-acid bacteria

LG: Lactoglobulin

LPSM: *L.plantarum* selective medium

(L)/$_L$: Sample where *L. plantarum* C4 was inoculated at 10^6 cfu/mL

Milk (E): Milk sample after ion exchange at pH 2

MRS: Man-Rogosa-Sharpe agar, selective medium for lactic acid bacteria

NA: Nutrient Agar

NB: Nutrient Broth

NCN: Non-casein nitrogen

ND: Not dialyzable fraction after the *in vitro* gastrointestinal digestion dialysis assay

NPN: Non protein nitrogen

ORAC: Oxygen radical antioxidant capacity

P: Permeate fraction after WHEY ion exchange at pH 4.6

PBS: Phosphate Buffered Saline

PCA: Plate count agar

PFM: Fermented milk made with UFM fermented with *L. bulgaricus, S. thermophilus* plus *L. plantarum* C4 by the standardised procedure

PFM (E): PFM after ion exchange at pH 4.2

P<3: Permeate fraction after WHEY ion exchange at pH 4.6 < 3 kDa

P>3: Permeate fraction after WHEY ion exchange at pH 4.6 > 3 kDa

R: Retentate fraction after WHEY ion exchange

RM: Raw goat milk

RP-UPLC: Reverse phase ultra high performance liquid chromatography

S: Soluble fraction after *in vitro* gastrointestinal digestion solubility assay

SC4: Fermented milk made with SM fermented with *L. plantarum* C4

SD: Standard deviation

SFM: Fermented milk made with SM fermented with the *L. bulgaricus, S. thermophilus* plus *L. plantarum* C4

SM: Skimmed goat milk

SMP: Skimmed goat milk concentrated by powdered skimmed goat milk addition

St: Classical starter bacteria (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus)*

StC4: Classical starter bacteria and *L. plantarum* C4.

SY: Yoghurt made with SM fermented with the classical starter bacteria

SYP2: Yoghurt made with SM and 2% of commercial powdered SM fermented with the classical starter bacteria

SYP4: Yoghurt made with SM and 4% of commercial powdered SM fermented with the classical starter bacteria

TAC: Total antioxidant capacity

TSA: Tryptone Soy Agar

UC4: Fermented milk made with UFM fermented with *L. plantarum* C4

UFM: Skimmed goat milk concentrated by ultrafiltration

UPLC: Ultra high performance liquid chromatography

UV: Ultraviolet

UV-VIS: Ultraviolet-visible

UY: Yoghurt made with UFM fermented with the classical starter bacteria

UY (E): UY after ion exchange at pH 4.2

VRBL: Violet red bile glucose

WHEY: Fermented milk samples after ion exchange at pH 4.6

WHEY (E): Fermented milk samples after ion exchange at pH 2

SUMMARY / RESUMEN

Summary

1. Introduction

The benefits of goat milk on human health have been widely studied. Compared to cow milk, goat milk fat is more digestible and easily absorbed and its proteins are also more digestible, with higher biological value and less allergenic capacity, and additionally, some studies have also reported better mineral composition and bioavailability for goat milk (Barrionuevo et al., 2002; Haenlein, 2004; Lopez-Aliaga et al., 2003; Silanikove et al., 2010; Slačanac et al., 2010). Fermented milks, which have similar nutritional profile to the milk, have demonstrated being nutritionally better than other dairy products like cheese, cream, butter, etc. In recent years, an increased interest in foods with positive effect on health beyond their nutritional value has happened. Among them, much attention has been focused on probiotic in fermented milks due to the fact that this matrix is able to maintain viable probiotic bacteria, as well as the fermented milk intake has been daily recommended (Erdmann et al., 2008). The properties attributed to fermented milks, more when probiotics are present, are the following: improvement in lactose absorption, increasing of protein digestibility, antibacterial activity, immune system stimulation, preventive action against digestive system cancer and anticholesterolemic action (Mahaut et al., 2004). In this field, the *Lactobacillus* genus has a long and safe history in the manufacture of dairy products. For this reason, the putative probiotic strain *Lactobacillus plantarum* C4, isolated by our research group and with demonstrated antimicrobial, microbiota-modulating and immune-modulating properties, was chosen for the study (Bergillos-Meca et al., 2014; Bujalance, Moreno et al., 2007; Fuentes et al., 2008; Puertollano et al., 2008).

Goat set-yoghurts faces with some manufacturing problems like over-acidification and the formation of an almost semi-liquid gel (Martín-Diana et al., 2003). To obtain a good gel network able to retain the water and consequently obtain a good curd without tendency to syneresis is mandatory to control some manufacturing factors such as heat treatment of the milk, incubation temperature, proteins and total solids concentration (Abbasi et al., 2009; Espírito-Santo et al., 2013; Lucey, 2002).

During the fermentation, and mainly depending on the strains used, different bioactive peptides could be released from milk proteins (Gobbetti et al., 2002). Fermented milks are considered an excellent source of these peptides (Donkor et al., 2007). Biologically active peptides are food-derived peptides that exert, beyond their nutritional value, a physiological, hormone-like effect in humans (Erdmann et al., 2008). Some of these peptides have demonstrated antihypertensive, antioxidant, antibacterial, anticancer, immunomodulatory, mineral-binding, opioid and methabolic syndrome regulatory activities (Donkor et al., 2007; Korhonen, 2009; Minervini et al., 2003; Muguerza et al., 2006; Ricci-Cabello et al., 2012). Bioactive peptides are inactive within the sequence of the precursor protein, being released during gastrointestinal digestion or food processing as fermentation, heating, etc. (Hernández-Ledesma et al., 2004). Bioactive peptides usually contains between 2 and 20 amino acid residues and their activity is inherent to their amino-acid composition and sequence, which influences their physicochemical properties as charge or hydrophobicity (Erdmann et al., 2008). The most widely studied activity of peptides is the angiotensin-I-converting enzyme inhibition, which plays a crucial role in the blood pressure regulation. Despite most of publications on ACEi and antihypertensive peptides focused on peptides from cow milk, in recent years goat milk proteins have become an important alternative source for ACEi bioactive peptides (Espejo-Carpio et al., 2013; Haque et al., 2007; Park et al., 2007; Ricci et al., 2010). On the other hand, some researchers have stated that antioxidant peptides present in the food system play a vital role in the maintenance of antioxidant defense systems in the organism as well as protecting the food from oxidation (Gupta et al., 2009). Finally, also bioactive peptides with antibacterial activity useful for a further application in industry have been discovered (Benkerroum, 2010).

2. Rationale and aims of the study

Currently, the interest of consumers for their health through the food is increasing, doing so the market of the functional foods. In addition, Andalusia is on the top of goat milk production in Spain, being the manufacturing of goat cheese and other dairy products with certified quality, a basis for the development of underdeveloped regions. In this context, the Excellence Project AGR-4915 entitled “Goat milk as a basis for the

preparation of fermented milk. Proposal for a functional food") was granted to our research group by the Government of Andalusia. Its main objective was to develop a functional fermented goat milk. With this aim was also purposed this Doctoral Thesis, which includes the design and development of a novel fermented goat milk as well as the determination of its potential functional activity focused on activities that could be due to its biological peptides.

Specifically, the aim of this Thesis was to develop a novel fermented goat milk, its physicochemical, nutritional and organoleptic characterization as well as to determine its peptidic profile and to measure its functional properties.

The specific aims of this Thesis were:

- To establish an adequate milk concentration method for the fermented milk manufacturing.
- To study the physicochemical and nutritional benefits of the milk concentrated by the chosen concentration method in comparison to the skimmed milk and raw milk.
- To develop the novel fermented goat milk and to characterize it physicochemically, nutritionally and organoleptically.
- To measure the antioxidant, angiotensin-I-converting enzyme inhibitory and antibacterial activity of some fractions of the fermented milk in order to establish its potential benefits.
- To identify the peptides produced in the novel fermented goat milk during the fermentation and after its *in vitro* gastrointestinal digestion in order to find any biologically active peptide or sequence.

3. Methodology

3.1. Samples

3.1.1. Milk samples

Raw goat milk samples collected were produced by goats of Murciano-Granadina breed and were collected in a farm in the region of Granada over a period of one year to avoid seasonal variations. From this raw goat milk (RM) the subsequent milks were obtained,

namely skimmed goat milk (SM), skimmed and concentrated by ultrafiltration goat milk (UFM) and skimmed goat milk concentrated by powdered skimmed goat milk addition.

3.1.2. Fermented milk samples

For the different assays, different fermented milk samples were manufactured.

- To choose the milk concentration method: different fermented milks were elaborated with SM, UFM and skimmed milk with different concentration of added powdered skimmed milk, reaching different final pH values (4.2 and 4.7).
- To study the microorganisms viability: SM and UFM were fermented with (a) the classical starter bacteria (St: *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*), (b) the probiotic strain *Lactobacillus plantarum* C4 (at low and high concentration) and (c) St plus *Lactobacillus plantarum* C4 (at low and high concentration).
- Standardized fermented goat milks were manufactured with UFM and fermented until pH 4.2: Specifically, the first sample subgroup (UY: Ultrafiltered goat yoghurt) was fermented with St; the second sample subgroup (PFM: Probiotic fermented goat milk) was fermented with St plus *Lactobacillus plantarum* C4.

3.2. Milk and fermented milk characterization

- Analysis carried out in RM, SM and UFM were density, pH, acidity, dry extract, ashes, lactose, fat, total proteins, real proteins, caseins, whey proteins and levels of Ca, Zn, P, Cu, Mg and Fe in milk and milk fractions used to determine the protein contents.
- During the PFM standardisation, the evaluation of the viscosity and syneresis depending on the milk concentration method and the final pH, the interaction of the strains by the spot test, the election of temperature and fermentation time and the viability of the strains during the fermentation, storage and after *in vitro* digestion, were carried out.
- Analysis carried out in PFM were pH, acidity, D/L-lactic acid, syneresis, viscosity, dry extract, lactose, galactose, proteins, fat, levels of Ca, Zn, P, Cu, Mg and Fe and organoleptic characterization for which an answer sheet was created and used.

3.3. Biological activity of the fermented milks fractions

- Samples fractionation: UY and PFM were fractionated firstly (a) by centrifugation obtaining the water-soluble fraction (WHEY); (b) this WHEY was separated by ion exchange in the retentate (R) and permeate (P) fractions, finally the P was separated (c) by ultrafiltration through 3 kDa cut-off membrane in two fractions, lower than 3 kDa (P<3) and higher than 3 kDa (P>3).
- The analysis of total proteins (by BCA), antioxidant activity (measured by different assays: ABTS, DPPH, ORAC and FRAP) and angiotensin-I-converting enzyme inhibitory activity (ACEi) were performed.

3.4. Peptides identification in fermented milks and their *in vitro* digestion products

- Samples evaluated: the water-soluble compounds of the SM were separated by ultrafiltration through 50kDa and those from UY and PFM by centrifugation, obtaining their WHEY. Those samples were purified by cation exchange, obtaining the analytical samples of milk (Milk [E] and WSE [E] of each fermented milk sample). UY and PFM were subjected to two standardised procedures of *in vitro* gastrointestinal digestion (GID) obtaining the following: (1) Soluble fraction (S) and (2) Dialyzable (D) and Non dialyzable (ND) fractions.
- The chromatographic analysis of the samples was carried out by on-line reverse-phase ultra high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) and for the peptide identification, an informatic software was used. Total proteins were also measured by BCA in the samples.

4. Results

4.1. Physicochemical and nutritional characteristics of RM, SM and UFM.

The ultrafiltration method for milk concentration up to 12% of dry extract was chosen because fermented milks manufactured with this milk showed the best viscosity and syneresis parameters. The milk concentration by ultrafiltration significantly influenced the protein and mineral levels. UFM showed higher concentration of caseins, whey proteins, some minerals (Ca, P, Zn and Cu), ashes, density and acidity than RM and SM, whereas lactose concentration remained almost constant. In addition, mean level of

Mg and dry extract were significantly higher than in RM. Regarding the study of mineral distribution, significantly different mineral levels were found among the different protein fractions. Additionally, it was observed that ultrafiltration together with pasteurization process changed the Ca and Mg distribution in the goat milk, increasing their levels in the soluble portion.

4.2. Fermented goat milk development and characterization

Firstly, the milk concentration by ultrafiltration was chosen for the reason described above. The viability assays showed that the cultures used (St and *L.plantarum* C4) were viable at good concentration at the end of the fermentation, after 4 weeks of storage (>10^7 cfu/mL) and after *in vitro* gastrointestinal digestion of the fermented milk (St > 10^7 cfu/mL; *L. plantarum* C4 > 10^8 cfu/mL). The physicochemical and nutritional parameters were similar to those reported by some authors for fermented milks. With respect to texture results, in the organoleptic characterization the PFM had also good texture and syneresis as well as the best visual parameters in comparison to other commercial fermented milks analyzed and it was in overall as accepted as them.

4.3. Biological activity of fermented milk fractions

The smallest neutral and anionic peptides showed the highest antioxidant and ACEi activity. However, the cationic peptides had noteworthy activity against $ABTS^{\bullet +}$ and $DPPH^{\bullet}$ radicals in comparison with others. On the other hand, the direct supernatant of the fermented milks and its fraction with the anionic and neutral peptides had weak but significantly antibacterial activity against *E. coli* in both fermented milks. However, the fraction with cationic peptides showed this activity only in the milk fermented with *L. plantarum* C4. Despite we hypothesized that the small bioactive peptides could be responsible of most of the activities, the WHEY of the yoghurt had also an important bioactivity.

4.4. Peptides identification before and after *in vitro* gastrointestinal digestion

In overall, 121 peptides were identified, the majority were released from β-Casein (CN) (53%) followed by α_{s1}-CN (19%) and α_{s2}-CN (17%). Only 11 were released from κ-CN and 3 from β-Lactoglobulin (LG). Despite several peptides were identified in the WSE of milk and fermented milks, most of them were released after *in vitro* GID. Only few

peptides were found exclusively in PFM, but in spite of this, *L. plantarum* C4 could be the responsible for some peptides solubilization. Lastly, some of the identified peptides shared homology in all or part of their structure with previously reported bioactive peptides.

5. Discussion

In addition to the general highest mineral and protein concentration of UFM, milk concentration by ultrafiltration was reported as a good concentration method because it does not affect the nutritional value of the milk, in terms of components modification by heating (Spreer, 1991). Additionally, in a different way as when powdered skimmed goat milk was added, other compounds with molecular weight lower than 50kDa, such as lactose, remained constant.

The chosen fermentation conditions were 37 ºC for 6 h, time necessary to reach the pH 4.2, which is the isoelectric point of goat milk caseins (Rojas-Castro et al., 2007). After 4 weeks of storage all microorganisms remained viable in the fermented milks at higher concentration than the minimum required by the legislation (Real Decreto 179/2003). In addition, after *in vitro* GID, *L. plantarum* C4 was viable at the required concentration for a probiotic strain to exert its function and provide to the consumer the benefits attributable to it (Galdeano et al., 2004; Parvez et al., 2006; Shah, 2007).

Despite UFM demonstrated differences with the other milks, only the Zn concentration, which is linked to caseins, was higher than most of reported values for commercial fermented milks (De la Fuente et al., 2003; Güler et al., 2009). Finally, its good texture and visual parameters could be attributed to the low fermentation temperature together with the milk concentration in dry extract, especially in proteins (Domagala et al., 2012). The goat aroma and taste, usually rejected by the consumer, were not highly perceived, probably thanks to the skimming process, as this flavour is associated to the goat fat (Domagala, 2008).

Peptides released from milk proteins and their activity are influenced by different factors such as the animal species, breed, milk processing, fermenting bacteria and fermentation conditions (Aloğlu et al., 2011; Chobert et al., 2005; Li et al., 2013; Uluko et al., 2014). However, the highest proteolysis is not always associated with the highest

activity (Virtanen et al., 2007). The highest number of peptides found in GID fractions could be due to the no purification by IEX resin and to the highest proteolysis by digestive proteases (Kopf-Bolanz et al., 2014). Despite the lesser peptides identified in the water-soluble extract of fermented milks, they showed moderate TAC, higher than those reported for the milk and similar ACEi activity to those reported by others for fermented milk (Chobert et al., 2005; Donkor et al., 2007; Hernández-Ledesma et al., 2005; Papadimitriou et al., 2007).

Different authors associate those activities to the peptides present on it, giving less importance to other water-soluble compounds (Zulueta et al., 2009). Reported bioactive peptides usually have less than 20 amino acid residues and the smallest fractions usually present highest biological activities, which is in agreement with our results, where the smallest peptides showed the highest ACEi activity and TAC measured by ORAC (Chang et al., 2013; Contreras et al., 2009; Gómez-Ruiz et al., 2006; Quirós et al., 2007; Unal et al., 2012). On the other hand, the higher TAC of cationic peptides is supported by Ren et al. (2008), who stated that basic peptides had greater capacity to scavenge hydroxyl radical than acidic or neutral ones. In addition, the antibacterial activity of basic peptides against gram-negative bacteria is well reported (Demers-Mathieu et al., 2013).

Among the identified peptides, the following had homologous or identical sequences to those with bioactivity such as antihypertensive (β-CN f[191-205] YQEPVLGPVRGPFPI and β-CN f[197-207] GPVRGPFPIILV) (Silva et al., 2005), ACEi (α_{s1}-CN f[157-164] DAYPSGAW, α_{s2}-CN f[90-96] VQKFPQY and β-CN f[191-207] YQEPVLGPVRGPFPILV) (El-Salam et al. , 2013; Fitzgerald et al., 2006; Robert et al., 2004), antioxidant (β-CN f[197-206] GPVRGPFPIL) (Farvin et al., 2010), antibacterial against other non studied bacterial strains (α_{s1}-CN f[180-193] SDIPNPIGSENSGK and β-CN f[197-207] GPVRGPFPILV) (Benkerroum, 2010; Losito et al., 2006), immunomodulatory (β-CN f[191-207] YQEPVLGPVRGPFPILV) (Hernández-Ledesma et al., 2005) and opioid (β-CN f[106-119] HKEMPFPKYPVEPF) (Plaisancié et al., 2013). Most of identified peptides were present in D (bioaccesible peptides) and S (soluble peptides) fractions, and thus they were able to be absorbed and exert their probable activity in the organism. Those small peptides, together with others that share

only a part of the sequence with some already identified bioactive peptides, could be the responsible of the activities demonstrated by the fermented milk fractions.

6. Conclusion

The new probiotic fermented goat milk developed had adequate physicochemical, nutritional and organoleptic characteristics. It showed remarkable total antioxidant capacity and high angiotensin-I-converting enzyme inhibitory activity, which could be due to the peptides released during the fermentation, some of which show homologous sequences to peptides with already known biological activity. In addition, the release of high variety of potentially bioactive peptides with capacity to be absorbed after *in vitro* gastrointestinal digestion could imply their activity *in vivo*. Therefore, we could consider the developed fermented milk as a healthy alternative to the widely commercialized cow yoghurt.

Resumen

1. Introducción

Los beneficios de la leche de cabra sobre la salud humana han sido ampliamente estudiados. En comparación con la leche de vaca, su grasa es más digestible y fácilmente absorbible, sus proteínas además de más digestibles, tienen un mayor valor biológico y son menos alergénicas y por último, algunos estudios han referido una mejor composición y biodisponibilidad mineral (Barrionuevo et al., 2002; Haenlein, 2004; Lopez-Aliaga et al., 2003; Silanikove et al., 2010; Slačanac et al., 2010). Las leches fermentadas, con un perfil nutricional similar al de la leche, han demostrado ser nutricionalmente mejores que otros derivados lácteos como quesos, nata, mantequilla etc. En los últimos años ha aumentado el interés en los alimentos con un efecto positivo sobre la salud más allá de su valor nutricional. Entre ellos, en el campo de las leches fermentadas destacan los probióticos, debido a que esta matriz es capaz de mantener las bacterias viables y a que su consumo se recomienda diariamente (Erdmann et al., 2008). Las propiedades atribuidas a las leches fermentadas, más cuando están presentes los microorganismos probióticos, son las siguientes: Mejora de la absorción de lactosa, aumento de la digestibilidad de las proteínas, actividad antibacteriana, estimulación del sistema inmune, acción preventiva frente al cáncer del sistema digestivo y actividad anticolesterolémica (Mahaut et al., 2004) En este campo, el género *Lactobacillus* tiene una larga historia de uso y seguridad en la fabricación de productos lácteos y es por esa razón que se eligió la cepa posiblemente probiótica *Lactobacillus plantarum* C4, la cual fue aislada por nuestro grupo de investigación y que demostró actividades antimicrobiana y moduladora de la microbiota, así como propiedades inmuno moduladoras (Bergillos-Meca et al., 2014; Bujalance et al., 2007; Fuentes et al., 2008; Puertollano et al., 2008).

En la fabricación de yogures firmes de cabra existen varios problemas a los que hay que hacer frente, como son, la sobre acidificación y la formación de un gel semilíquido (Martín-Diana et al., 2003). Para obtener un gel con una buena red proteica capaz de retener agua para la obtención de un buen coágulo sin tendencia a sinéresis es necesario

el control de varios factores durante la fabricación como son el tratamiento térmico de la leche, la temperatura de incubación y la concentración en proteínas y sólidos totales (Abbasi et al., 2009; Espírito-Santo et al., 2013; Lucey, 2002)

Durante la fermentación, y principalmente en función de la cepa usada, pueden liberarse diferentes péptidos bioactivos (Gobbetti et al., 2002). Los péptidos biológicamente activos son péptidos derivados de alimentos que ejercen sobre el organismo, más allá de su valor nutricional, un efecto fisiológico similar al hormonal, siendo las leches fermentadas una excelente fuente de los mismos (Donkor et al., 2007; Erdmann et al., 2008). Algunos de estos peptidos han demostrado tener actividades antihipertensiva, antioxidante, antibacteriana, anticáncer, inmuno moduladora y opioide, así como capacidad de unir minerales y reguladora en el síndrome metabólico (Donkor et al., 2007; Korhonen, 2009; Minervini et al., 2003; Muguerza et al., 2006; Ricci-Cabello et al. 2012). Estos péptidos son inactivos en la secuencia proteica, liberándose durante la digestión gastrointestinal o durante el procesado de los alimentos, como es el caso de la fermentación, calentamiento, etc. (Hernández-Ledesma et al., 2004). Los péptidos bioactivos contienen normalmente entre 2 y 20 aminoácidos y su actividad es inherente a su composición y secuencia aminoacídica, de las cuales dependen sus propiedades fisicoquímicas como la carga o la hidrofobicidad (Erdmann et al., 2008). La actividad peptídica más estudiada es la de inhibición de la enzima convertidora de la angiogensina-I (ACEi), que juega un papel crucial en la regulación de la presión sanguínea. A pesar de que la mayor parte de las publicaciones sobre péptidos con actividad ACEi o antihipertensiva han sido realizadas con péptidos procedentes de leche de vaca, en los últimos años las proteínas de leche de cabra están ganando importancia como una fuente alternativa de péptidos con esta actividad (Espejo-Carpio et al., 2013; Haque et al., 2007; Park et al., 2007; Ricci et al., 2010). Por otro lado, algunos investigadores han señalado que los péptidos antioxidantes presentes en los alimentos juegan un papel vital en el mantenimiento de las defensas antioxidantes del organismo, así como en la protección del alimento frente a la oxidación. Finalmente, también se han descubierto péptidos con actividad antibacteriana, útiles para sobre todo para una posterior aplicación en la industria (Benkerroum, 2010).

2. Razón principal y objetivos del estudio

Actualmente está aumentando el interés de los consumidores por mejorar su salud a través de los alimentos, haciéndolo conjuntamente el mercado de los alimentos funcionales. Además, Andalucía está a la cabeza en la producción de leche de cabra, siendo la fabricación de queso de cabra y otros productos lácteos con calidad certificada la base del desarrollo de regiones subdesarrolladas. En este contexto fue concedido a nuestro grupo por la Junta de Andalucía el proyecto de Excelencia AGR-4195 "La leche de cabra como base para la elaboración de un fermentado lácteo. Propuesta de un alimento funcional", cuyo objetivo principal fue el desarrollo de un fermentado funcional de leche de cabra. Con este objetivo fue también propuesta la presente Tesis Doctoral, la cual incluye el diseño y desarrollo del nuevo fermentado de leche de cabra, así como el estudio de su potencial actividad funcional enfocada a las actividades que podrían ser atribuidas a los péptidos bioactivos presentes en ella.

En concreto, el objetivo general de esta Tesis fue desarrollar una nueva leche fermentada de cabra, su caracterización físico-química, nutricional y organoléptica, así como la determinación de su perfil peptídico y la medida de sus propiedades funcionales.

Los objetivos específicos de esta Tesis fueron:

- Establecer un adecuado método de concentración de la leche para la elaboración de la leche fermentada.
- Estudiar los beneficios físico-químicos y nutricionales de la leche concentrada por el método de concentración elegido en comparación con la leche desnatada y la leche cruda.
- Desarrollar una nueva leche fermentada de cabra y caracterizarla físico-química, nutricional y organolépticamente.
- Medir las actividades antioxidante, inhibidora de la enzima convertidora de la angiotensina-I y antibacteriana de ciertas fracciones de la leche fermentada desarrollada con el fin de establecer los posibles beneficios de su ingesta.

- Identificar los péptidos producidos en la nueva leche fermentada de cabra durante la fermentación y después de su digestión gastrointestinal *in vitro*, con la finalidad de encontrar algún péptido o secuencia biológicamente activos.

3. Metodología

3.1. Muestras

3.1.1. Muestras de leche

Las muestras de leche cruda de cabra fueron producidas por cabras de la raza Murciano-Granadina y se recogieron de una granja en la región de granada durante todo un año para evitar las variaciones estacionales. De esta leche cruda de cabra (RM) se obtuvieron las consiguientes leche desnatada de cabra (SM), leche desnatada concentrada mediante ultrafiltración (UFM) y leche desnatada de cabra concentrada mediante adición de leche desnatada en polvo de cabra (SMP).

3.1.2. Muestras de leches fermentadas

Se elaboraron distintas leches fermentadas en función de los ensayos:

- En la elección del método de concentración: se elaboraron diferentes leches fermentadas con la SM, UFM y SMP, llegando hasta dos pHs finales (4.2 y 4.7).
- Estudio de la viabilidad de los microorganismos: Las leches SM y UFM se fermentaron con (a) los microorganismos iniciadores clásicos (St: *Lactobacillus delbrueckii* subsp. *bulgaricus* y *Streptococcus thermophilus*), (b) la cepa probiótica *Lactobacillus plantarum* C4 (a baja y alta concentración) y (c) St y *Lactobacillus plantarum* C4 (a alta y baja concentración).
- Leches fermentadas de cabra estandarizadas: Se elaboraron con UFM fermentada durante 6 horas a 37 ºC: con St (obteniendo UY: yogur ultrafiltrado) y con St y *Lactobacillus plantarum* C4 (obteniendo PFM: leche fermentada probiótica).

3.2. Análisis de leche y leches fermentadas

- En RM, SM y UFM se analizaron: Densidad, pH, acidez, extracto seco, cenizas, lactosa, grasa, proteínas totales, proteínas reales, caseínas, proteínas séricas y niveles de Ca, Zn, P, Cu, Mg y Fe en leche y en sus fracciones proteicas.

- Durante la estandarización de la leche fermentada se llevaron a cabo los estudios de viscosidad y sinéresis en función del método de concentración y del pH final, la interacción de las cepas bacterianas mediante spot test, la selección de la temperatura y tiempo de fermentación y la viabilidad de las cepas durante la fermentación, almacenamiento y después de una digestión gastrointestinal *in vitro*.
- Los análisis llevados a cabo en PFM fueron: pH, acidez, ácido D/L-láctico, sinéresis, viscosidad, extracto seco, lactosa, galactosa, grasa, proteínas, niveles de Ca, Zn, P, Cu, Mg y Fe, así como la caracterización organoléptica de esta leche fermentada para la cual fue creada y usada una nueva hoja de cata.

3.3. Medida de la actividad biológica de las fracciones de las leches fermentadas

- Fraccionamiento de las muestras: UY y PFM fueron fraccionados primero (a) mediante centrifugación, obteniendo el suero (WHEY); (b) ese WHEY fue separado por intercambio iónico en las fracciones retenida (R) y permeada (P), siendo finalmente separada P (c) mediante ultrafiltración a través de una membrana de 3kDa de tamaño de poro en dos fracciones, menor de 3 kDa (P<3) y mayor de 3 kDa (P>3).
- En esas muestras se analizaron las proteínas totales (BCA), actividad antioxidante (mediante los ensayos ABTS, DPPH, ORAC y FRAP) y actividad inhibidora de la enzima convertidora de la angiotensina-I (ACEi).

3.4. Identificación de los péptidos en leches fermentadas y sus digeridos *in vitro*

- Muestras analizadas: los compuestos hidrosolubles de la leche desnatada fueron separados mediante ultrafiltración a través de una membrana de 50 kDa de tamaño de poro (UF) y aquellos procedentes de UY y PFM mediante centrifugación, obteniendo así su suero (WHEY). Esas muestras fueron purificadas mediante intercambio catiónico, obteniendo las muestras analíticas de la leche (Milk [E]) y leches fermentadas (WHEY [E]). Por otro lado, UY y PFM fueron sometidos a dos procesos estandarizados de digestión gastrointestinal *in vitro,* obteniendo: (1) Fracción soluble (S) y (2) Fracciones Dializada (D) y No Dializada (ND)
- El análisis cromatográfico de las muestras se llevó a cabo por cromatografía en fase reversa de ultra alta resolución acoplada a espectrometría de masas (UPLC-MS/MS)

y la identificación de los péptidos se realizó con un software informático. También se determinaron por BCA las proteínas totales en las muestras.

4. Resultados

4.1. Características físico-químicas y nutricionales de RM, SM y UFM.

Se eligió la concentración por ultrafiltración hasta un 12% de extracto seco como método de concentración ya que las leches fermentadas elaboradas con ella mostraron mejores parámetros de viscosidad y sinéresis. La concentración mediante ultrafiltración tuvo una influencia significativa sobre los niveles de proteínas y minerales. Esta leche mostró una mayor concentración de caseínas, proteínas séricas, algunos minerales (Ca, P, Zn y Cu), cenizas, densidad y acidez que RM y SM, mientras que la concentración en lactosa permaneció prácticamente constante. Además, los niveles medios de Mg y extracto seco fueron significativamente mayores que en la leche cruda (RM). En cuanto al estudio de la distribución mineral, los niveles de minerales fueron significativamente diferentes entre las fracciones proteicas. Además se observó que la ultrafiltración, junto con la pasteurización, modificó la distribución de Ca y Mg en la leche de cabra, aumentando sus niveles en la porción soluble.

4.2. Caracterización y desarrollo de la leche fermentada de cabra

En primer lugar, la ultrafiltración fue escogida como método de concentración, como se ha descrito anteriormente. Los ensayos de viabilidad mostraron que las tres cepas usadas (St y *L.plantarum* C4) se mantuvieron viables a una buena concentración al final de la fermentación, después de 4 semanas de almacenamiento (> 10^7ufc/mL) y tras una digestión gastrointestinal *in vitro* del producto fermentado (St > 10^7ufc/mL; *L. plantarum* C4 > 10^8ufc/mL). Los parámetros fisicoquímicos y nutricionales de la PFM fueron similares a los referidos por otros autores para leches fermentadas. De acuerdo con los resultados del análisis de textura, en la caracterización organoléptica la PFM mostró buena textura y sinéresis, así como los mejores parámetros visuales en comparación con otras leches fermentadas analizadas y fue en general aceptada como ellas.

4.3. Actividad biológica de las fracciones de la leche fermentada

Los compuestos aniónicos y neutros más pequeños mostraron la mayor actividad antioxidante y ACEi. Sin embargo, la actividad antioxidante frente a los radicales ABTS y DPPH de los péptidos catiónicos fue importante en relación a los otros. Por otro lado, el sobrenadante de las leches fermentadas y su fracción que con péptidos aniónicos y neutros mostraron una baja, aunque significativa, actividad antibacteriana frente a *E. coli.* En ambas leches fermentadas. Sin embargo, la fracción con péptidos catiónicos mostró esta actividad únicamente en la leche fermentada con *L. plantarum* C4. A pesar de la hipótesis de que los péptidos bioactivos de tamaño pequeño podrían ser los responsables de la mayor parte de las actividades, el WSE del yogur tuvo también una importante bioactividad.

4.4. Identificación de los peptidos antes y después de la digestión gastrointestinal *in vitro.*

En general se identificaron 121 péptidos, la mayoría liberados de la β-CN (53%), seguidos los liberados de la α_{s1}-Caseína (CN) (19%) y la α_{s2}-CN (17%). Sólo 11 fueron liberados de la κ-CN y 3 de la β-Lactoglobulina (LG). A pesar de que se identificaron algunos péptidos en la fracción soluble de la leche y de las leches fermentadas, la mayor parte fueron liberados después de la digestión gastrointestinal *in vitro.* Sólo unos pocos péptidos se encontraron exclusivamente en la PFM, pero a pesar de ello, *L. plantarum* C4 podría ser responsable de la solubilización de algunos péptidos. Por último, algunos de los péptidos identificaros tuvieron secuencia homóloga o comparten parte de su estructura con otros previamente referidos como bioactivos.

5. Discusión

Además del mayor contenido en minerales y proteínas de la UFM, la concentración mediante ultrafiltración ha sido referida como un buen método también porque que no afecta al valor nutricional de la leche en cuanto a la modificación de sus componentes por el calentamiento (Spreer, 1991). Además, a diferencia de cuando se adiciona leche en polvo, otros compuestos de peso molecular menor de 50kDa, como la lactosa, permanecen constantes.

Las condiciones de fermentación elegidas fueron 37 ºC durante aproximadamente 6 h, tiempo suficiente para alcanzar un pH de 4.2, al cual se encuentra el punto isoeléctrico de las caseínas de cabra (Rojas-Castro et al., 2007). En la leche fermentada, tras 4 semanas de almacenamiento, todos los microorganismos permanecieron viables a niveles superiores al mínimo necesario exigido por la legislación (Real Decreto 179/2003). Además, tras la digestión gastrointestinal *in vitro*, *L. plantarum* C4 se mantuvo viable a la concentración necesaria para que una cepa probiótica pueda ejercer sus funciones y proveer al consumidor de los beneficios que se le atribuyen (Galdeano et al., 2004; Parvez et al., 2006; Shah, 2007).

A pesar de que la leche concentrada por ultrafiltración demostró diferencias con las otras leches, solamente la concentración de Zn, mineral unido a caseínas, fue mayor que la mayoría de los valores referidos para leches fermentadas comerciales (De la Fuente et al., 2003; Güler et al., 2009). Finalmente, sus buenos parámetros visuales y de textura podrían deberse a la baja temperatura de fermentación junto con la concentración de la leche en extracto seco y especialmente en proteínas (Domagala et al., 2012). El aroma y sabor a cabra, normalmente rechazados por los consumidores, no fueron muy percibidos, probablemente gracias al proceso de desnatado ya que este flavor está asociado a la grasa de la leche de cabra. (Domagala, 2008).

La liberación de los péptidos de las proteínas de la leche así como su actividad están influenciados por distintos factores como: la especie animal e incluso la raza, el procesado de la leche, los fermentos bacterianos y las condiciones de fermentación (Aloğlu et al., 2011; Chobert et al., 2005; Li et al., 2013; Uluko et al., 2014). Sin embargo, una mayor proteólisis no está siempre asociada a una mayor actividad (Virtanen et al., 2007). El mayor número de péptidos encontrado tras la digestión gastrointestinal *in vitro* podría ser debido a la no purificaron con la resina de intercambio iónico y a la mayor proteólisis llevada a cabo por las proteasas digestivas (Kopf-Bolanz et al., 2014). A pesar de identificar menos péptidos en los extractos hidrosolubles de las leches fermentadas con respecto a sus digeridos, sus las fracciones del WHEY mostraron una moderada capacidad antioxidante total, mayor a la referida para la leche y una actividad ACEi similar a aquella referida por otros investigadores

para leches fermentadas (Chobert et al., 2005; Donkor et al., 2007; Hernández-Ledesma et al., 2005; Papadimitriou et al., 2007).

Diversos autores asocian esas actividades a los péptidos presentes en ellas, dando menor importancia a otros compuestos hidrosolubles (Zulueta et al., 2009). Los péptidos bioactivos normalmente tienen menos de 20 amino ácidos y las fracciones de menor tamaño normalmente presentan mayores actividades biológicas, lo que concuerda con nuestros resultados, donde los péptidos más pequeños mostraron mayor actividad ACEi y capacidad antioxidante total medida por ORAC (Chang et al., 2013; Contreras et al., 2009; Gómez-Ruiz et al., 2006; Quirós et al., 2007; Unal et al., 2012). Por otro lado, capacidad antioxidante de los péptidos catiónicos está sustentada por Ren et al. (2008), quien manifestó que los péptidos básicos tienen una mayor capacidad de captar los radicales hidroxilo que los péptidos ácidos o neutros. Además, la actividad antibacteriana de los péptidos básicos frente a bacterias gram negativas es bien conocida (Demers-Mathieu et al., 2013).

Entre los péptidos indentificados, los siguientes mostraron con secuencia homóloga o idéntica a péptidos referidos en bibliografía con actividades como antihipertensiva (β-CN f[191-205] YQEPVLGPVRGPFPI y β-CN f[197-207] GPVRGPFPILV) (Silva et al., 2005), ACEi (α_{s1}-CN f[157-164] DAYPSGAW, α_{s2}-CN f[90-96] VQKFPQY y β-CN f[191-207] YQEPVLGPVRGPFPILV) (El-Salam et al., 2013; Fitzgerald et al., 2006; Robert et al., 2004), antioxidante (and β-CN f[197-206] GPVRGPFPIL) (Farvin et al., 2010), antimicrobiana frente a otras bacterias no estudiadas en esta investigación (α_{s1}-CN f[180-193] SDIPNPIGSENSGK y β-CN f[197-207] GPVRGPFPILV) (Benkerroum, 2010; Losito et al., 2006), immunomoduladora (β-CN f[191-207] YQEPVLGPVRGPFPILV) (Hernández-Ledesma et al., 2005) y opioide (β-CN f[106-119] HKEMPFPKYPVEPF) (Plaisancié et al., 2013). La mayoría de éstos fueron identificados en la fracción D (péptidos bioaccesibles) y S (péptidos solubles), teniendo la capacidad de ser absorbidos y ejercer su actividad en el organismo. Estos péptidos pequeños, junto con otros que comparten sólo una parte de la secuencia de péptidos bioactivos referidos en bibliografía, podrían ser los responsables de las actividades demostradas por las fracciones de las leches fermentadas.

6. Conclusión

El nuevo fermentado probiótico de leche de cabra presentó unas características físico-químicas, nutricionales y organolépticas adecuadas. Éste mostró una destacable capacidad antioxidante total y una alta actividad inhibidora de la enzima convertidora de la angiotensina-I, que podrían ser debidas a los péptidos bioactivos liberados durante la fermentación, algunos de los cuales tuvieron secuencias homólogas a las de péptidos con conocida actividad biológica. Además, la liberación de gran variedad de péptidos potencialmente bioactivos con capacidad de ser absorbidos tras una digestión gastrointestinal *in vitro* podría implicar su posterior actividad *in vivo*. Por lo tanto, podríamos considerar la leche fermentada desarrollada como una alternativa saludable al ampliamente comercializado yogur de vaca.

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CHAPTER 1

Ultrafiltration of goat milk increases mineral and protein concentration

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Abstract

Goat milk has demonstrated nutritional and healthy interest. In order to obtain fermented products with good sensorial quality a previous concentration is required, and depending on the method used, the yoghurt characteristics will be different. The concentration by ultrafiltration mostly concentrates proteins and it is carried out without heating, which is supposed to be better to maintain the protein and mineral quality. In this research we have analysed physicochemical and nutritional parameters of raw goat milk (RM), skimmed goat milk (SM) and skimmed goat milk concentrated by ultrafiltration (UFM). Goat ultrafiltration significantly influenced protein and mineral levels. Specifically, in UFM higher values were found for all proteins, some minerals (Ca, P, Zn and Cu), ashes, density and acidity in comparison with RM and SM. Additionally, in UFM mean levels of Mg and dry extract were significantly higher than in RM. Mineral levels in different protein fractions were found significantly different. Additionally it was observed that ultrafiltration step process changed the Ca and Mg distribution in the goat milk, increasing their levels in the soluble portion. In conclusion, the UFM is a better source for manufacturing fermented goat milk than RM and SM mainly due to its higher concentrations in proteins and minerals, as well as its low fat content.

1. Introduction

Several studies have demonstrated the nutritional interest of the goat's milk in human health. Specifically, the small size of milk fat globules together with its high proportion in short and medium chain fatty acids (namely caproic, caprilic and capric) improve its digestion and absorption (Attaie et al., 2000; Chilliard et al., 2003; Silanikove et al., 2010). Currently, some studies have reported that goat milk has better nutritional quality than cows' milk due to its better mineral composition and bioavailability (Alférez et al., 2006; Barrionuevo et al., 2002; Lopez-Aliaga et al., 2003) Although the mineral composition of goat milk has been profusely studied, there is still little information about the distribution of these elements into the different protein fractions, which is an important issue because it is related to their bioavailability (De la Fuente et al., 1997).

Although protein concentration in goat milk is lower than in cow milk, its digestibility and biological value are higher (Bevilacqua et al., 2001; Haenlein, 2004; Ramos-Morales et al., 2005). Moreover, it has been demonstrated that a high percentage of people with allergy to cow milk, tolerate the goat milk well (Slačanac et al., 2010). For this reason, goat milk is an alternative choice to cow milk in malabsorption syndrome as well as in hyper sensibility to cow milk proteins in breastfeeding, children and adults (Lara-Villoslada et al., 2004)

On the other hand, it has been demonstrated that the consistency of goat yogurt is one of the critical points in its manufacturing (Farnsworth et al., 2006). In order to be used in fermented dairy products, goat milk needs a modification on its native composition and properties, or even a change in the yoghurt processing, so as to improve its texture and rheological properties with the final aim of reducing the yogurt syneresis (Jacek Domagała, 2009). In this sense, the strategies carried out to increase the sensory quality of goat fermented dairy products usually include an enhancement in non-fat solids or proteins. The milk raise in non-fat solid has traditionally been carried out by evaporation or adding powdered milk. It is known that heating-based methods diminish the nutritional value of the milk. Other methods have been also used such as: milk concentration with membranes, addition of whey or milk protein concentrates or isolates, as well as caseinates, stabilisers like pectins and inulin or even the addition of

lactic-acid bacteria (LAB) as producers of exhopolysacarydes (Martín-Diana et al., 2004; Herrero et al., 2006; Tratnik et al., 2006; Kearney et al., 2011; Prasanna et al., 2012; Karam et al., 2013).

Milk concentration by ultrafiltration has been reported as a good concentration method because it does not affect the nutritional value of the milk, in terms of components modification by heating (Spreer, 1991). Furthermore, other authors have demonstrated the benefits on sensorial and nutritional characteristics of the ultrafiltration method in comparison with other concentration methods (Marshall et al., 1986; Karademi et al., 2003; Magenis et al., 2006; Rinaldoni et al., 2009; Domagała, 2012). Considering the benefits described above, and taking into account that the composition of the concentrated milk will depend on the used cut-off size membrane, goat milk ultrafiltration could be considered as a suitable method for milk protein concentration for yoghurt manufacturing. Using a cut-off membrane that retains mainly caseins, the majoritary whey proteins, free minerals and lactose will be lost in the ultrafiltration permeate. These caseins are responsible for curd formation and the main source of Ca, P and Mg, and their concentration can lead to a final milk with better nutritional properties than others (Tratnik et al., 2006; Domagała et al., 2013).

In this context, the main objective of this research was to measure and compare the levels of several nutritional and physicochemical parameters during the milk processing until obtaining the milk concentrated by ultrafiltration. A secondary objective was to investigate how the ultrafiltration process influenced the different protein fractions of the milk as well as how mineral (Ca, P, Mg, Zn, Cu and Fe) distribution among these fractions could be modified, a fact that could be directly related to mineral bioaccessibility. These data would allow us to study how the goat milk processing affects its composition and its possible use for the goat yogurts manufacturing.

2. Materials and methods

2.1. Samples

Raw goat milk produced by goats of Murciano-Granadina breed was collected from a farm in the region of Granada over a period of one year to avoid seasonal variations. After that, all quality parameters included in Spanish legislation were checked such as

is described below, and if the milk did not meet the recommended quality criteria, it was discarded. After heating at 30-35ºC in a water bath, the raw goat milk (RM) was skimmed in a skimming centrifuge (Suministros químicos Arroyo, Santander, España). Then, the raw skimmed goat milk (SM) was ultrafiltrated through a 50 kDa membrane (Vivaflow 2000, Sartorius Stedim) using a peristaltic pump (Masterflex® L/S, Economy Drive, Cole Parmer®), at 2 bar pressure, up to a 12% ± 0.5 of dry extract (Concentration x 1.7) . Finally, this milk was pasteurised for 30 minutes at 81.65± 1.29ºC (Thermomix ®, Vorwek), obtaining the final skimmed goat milk concentrated by ultrafiltration (UFM). Therefore, the samples for our study were: RM (n = 12), SM (n = 12) and UFM (n = 12).

2.2. Parameters analysed in the goat milk samples

All analyses were carried out by triplicate and blanks were prepared with bidistilled water instead the sample when required.

2.2.1. ρ-lactams, sulfamides and tetracyclines test

This analysis was carried out only for RM in order to detect the presence of ρ-lactams, sulfamides and tetracyclines in which case the milk was discarded. For this test TriSensor milk (Unisensor, Liège, Belgium) was used together with a Heatsensor (40 ºC incubation; Unisensor, Liège, Belgium). The reaction mechanism involves two receptors and generic monoclonal antibodies. The procedure was followed according to the instructions of the tests manufacturer and when finished, the results were visually interpreted. For a valid test the upper red color line had to be visible after the second incubation. The other three capture lines (specific "test" lines) were placed below the control line (for ρ-lactam antibiotics (penicilins and cephalosporins), sulfamide and tetracyclines), and were coloured if the sample was free of antibiotics and coloured in the contrary case.

2.2.2. Total bacteria counts

Total bacteria of raw and pasteurized goat milk (cfu/mL) were counted according to the UNE-EN ISO 4833-1:2014 (Real Decreto 1728/2007) in plate count agar (PCA; Difco™, Becton, Dickinson and Company; Madrid, Spain) culture media and incubated

at 30 ºC for 72h. If a higher number was counted, the fermented milk made with this milk was discarded.

2.2.3. Enterobacteria

Enterobacteria in pasteurized goat milk (colony-forming units [cfu]/mL) were detected in violet red bile glucose (VRBL; Difco™, Becton, Dickinson and Company; Madrid, Spain) (EN-ISO 4832-2006). According to the law, the limits to discard the milk were: n5 c2 m1 M5 (Regulation (CE) 1441/2007). The confirmation of unclear colonies was carried out in brilliant green bile lactose media.

2.2.4. Heating tests

Test strips were used for the determination of alkaline phosphatase in milk whose inhibition indicates indicates that heat treatment was higher than 60 ºC (Phosphatesmo MI, Macherey-Nagel, Germany).

For the determination of lactoperoxidase in milk, test papers were used (Peroxtesmo MI, Macherey-Nagel, Germany). Its inhibition indicates that heat treatment was higher than 80 ºC.

2.2.5. Milk density determination

This analysis was carried out according to the official method proposed by the AOAC (2006), using a pycnometer. Density values were expressed as mg/mL.

2.2.6. pH measurement

A pH-Meter Crison Basic 20+ coupled to a Hamilton electrode was used to measure the pH at 20ºC for milk (AOAC, 2006).

2.2.7. Acidity determination

The total acidity of the milk was measured by potenciometric tritation with NaOH up to pH 8.4 (AOAC, 2006). The acidity was expressed as g lactic acid / 100 g of milk.

2.2.8. Dry extract content

Dry extract was measured according to the official method published (AOAC, 2006). In this sense, 5 g of sample were weighed and desiccated in a drying oven until constant weight was achieved. Dry extract was expressed as percentage of mass (% m/m).

2.2.9. Ashes weight

To obtain the ashes, 5 ml of the milk samples were incinerated at 550 ± 5 ºC in a muffle furnace (Nabertherm, LE 2/11/R6, Bremen, Germany) according to AOAC (2006). Ashes were expressed as percentage of mass (% m/m).

2.2.10. Lactose levels

Lactose in goat milk samples was analysed according to the standardized method for milk samples using chloramine T 3-hydrate (Panreac, Castellar del Vallès, Spain) and Tungstic acid (Sigma-Aldrich, Steinheim, Germany) (FIL-IDL 28:1974). Results were expressed as g of monohydrated lactose/100 g of milk.

2.2.11. Fat content

Fat determination was conducted in accordance to ISO 2446:2008. After milk digestion in a butyrometer (Funke-Gerber, Berlin, Germany) with sulphuric acid 90-91% (w/v; Panreac, Castellar del Vallès, Spain) and isoamilic alcohol (Panreac, Castellar del Vallès, Spain), a centrifugation in a centrifuge thermostated at 65ºC (Funke-Gerber, Berlin, Germany) was carried out to separate the fat. Finally, the direct lecture of fat content was performed in the butirometer and results were expressed as g of fat/100 g of milk.

2.2.12. Evaluation of different protein fractions

Non-casein nitrogen (NCN) and non protein nitrogen (NPN) fractions were separated as described elsewhere and nitrogen in these fractions and total nitrogen were determined by the Kjeldahl method (AOAC, 2006; Olalla et al., 2009). Results were expressed in g/100 ml of milk. A 93% of recovery using bovine serum albumin (Sigma-Aldrich, Steinheim, Germany) was obtained.

With those values, the following were calculated: Total protein (Total nitrogen x 6.34), real protein ([Total protein – NPN] x 6.34), casein ([Total protein – NCN] x 6.34) and whey proteins ([NCN – NPN] x 6.34).

2.2.13. Mineral (Ca, P, Mg, Zn, Cu and Fe) levels

Mineral determination was carried out according to procedures previously optimized (Aleixo et al., 2003; Bergillos-Meca et al., 2013; Velasco-Reynoldet al., 2008)

Mineral levels in NCN and NPN fractions were also measured. Taking into account that minerals in these fractions were soluble, no previous mineralization was needed for mineral determination.

Mineral levels measured in NCN and NPN fractions were used to calculate for each determined mineral: mineral bound to casein (total mineral – mineral in NCN fraction), mineral bound to all proteins (total mineral – mineral in NPN fraction) and mineral bound to whey proteins (mineral in NCN fraction – mineral in NPN fraction).

To diminish the risk of contamination, glassware was reduced to the minimum and polypropylene vessels and pipette tips were used. Besides, all the material was nitric acid-washed and rinsed several times with bidistilled deionoized water filtered through a Milli-Q purifier (Millipore, Waters, Mildford, MA, USA).

2.2.13.1. Chemicals for mineral determination

Standard solutions of Ca, P, Mg, Zn, Cu and Fe (1.000 ± 0.002 mg/l) (Tritisol, Merk, Damstadt, Germany) were used and diluted as necessary to obtain working standards. High quality concentrated HNO_3 (65%), $HClO_4$ (70%), HCl (37%) and V_2O_3 (Suprapur, Merck, Germany) were used for sample mineralization. A chemical modifier ($LaCL_3$) was used for Ca measurements.

2.2.13.2. Sample preparation and analysis for mineral determination

For raw and commercial goat milk samples a dry mineralization was carried out. The obtained ashes were weighted and diluted in 3 ml of 0.1 N HCl and filled up to 25 ml with bidistilled water to obtain the analytical solution. Minerals were also directly measured in the obtained filtrates for the determination of NCN and NPN.

2.2.13.3. ASS instrumentation and conditions

A Perkin-Elmer 1100B double beam atomic absorption spectrometer equipped with deuterium-arc-background correction (Perkin-Elmer, Norwalk, CT, USA) was used.

Measurements were performed at a wavelength of 213.9 nm for Zn, 422.7 nm for Ca, 324.8 nm for Cu, 285.2 nm for Mg and 248.3 nm for Fe using hollow cathode lamps (Perkin-Elmer). P was measured with a spectrophotometer settled at 750 nm.

The analytical procedures employed for the determination of Ca, Mg and Zn levels by flame atomic absorption spectrometry (FAAS) were similar to those previously optimized and published elsewhere (Jodral-Segado et al., 2003; Moreno-Torres et al., 2000 b; Navarro-Alarcon et al., 2007, 2011) Zn, Ca and Mg determinations were carried out by direct aspiration into the flame of the atomic absorption spectrophotometer. Ca levels were measured by the standard addition method to avoid matrix interferences (Bergillos-Meca et al., 2013; R. Moreno-Torres et al., 2000 b; Navarro-Alarcon et al., 2011) while Mg, Zn and Cu were determined by the linear calibration method.

For Cu and Fe measurements, previously optimized by electrothermal AAS (ETAAAS) procedures were used (Aleixo et al., 2003; Velasco-Reynold et al., 2008) The atomic absorption spectrometer Perkin-Elmer 5100 Zeeman AAS equipped with a HGA-5100 graphite furnace with pyrolytically coated graphite tubes (Perkin-Elmer, Germany) and AS-90 autosampler (Perkin-elmer, Germany) was used.

P levels were determined with a previously developed UV-VIS spectrophotometric method (Moreno-Torres et al., 2000a). The optical density of the developed color was measured spectrophotometrically at 750 nm using a Perkin-Elmer Lambda 25 UV-VIS spectrophotometer (Norwalk, CT, USA) with a band-pass setting of 1 nm. The optical density in each sample was correlated with the P concentration by the linear calibration method (Moreno-Torres et al., 2000 a; Navarro-Alarcon et al., 2011).

The analytical characteristics of the methods used to measure Ca, P, Mg, Zn, Cu and Fe were evaluated, establishing the detection limit and sensitivity (Table 1.1) for these elements. The inter-day repeatability and the percentage recovery of added Ca, P, Mg, Zn, Cu and Fe were adequate for the measurement of these elements in goat milk and its by-products (Table 1.1). The accuracy and precision of the Ca, P, Mg, Zn, Cu and Fe measurement procedures were also verified by testing two certified reference standards: CRM 063R skim milk powder (Community Bureau of Reference, Commission of

European Communities) and SRM 1572 citrus leaves (National Institute of Standards and Technology). Non-significant differences were found between the mean Ca, P, Mg, Zn, Cu and Fe concentrations determined in these materials and the certified concentrations (Table 1.2).

Table 1.1. Analytical parameters of methods used to determine Ca, P, Mg, Zn, Cu and Fe in goat milks

Element	Detection limit[a]	Characteristic mass[b] (ng)	Recovery[c] (%) (mean±SD)	Precision, RSD[d] (%)
Ca	110.0	46.0	100.49 ± 1.06	1.72
P	18	172	100.67 ± 2.09	4.03
Mg	5.0	58.0	100.09 ± 0.87	3.16
Zn	11.6	165	100.35 ± 0.75	5.36
Cu	57	10	100.07 ± 0.33	7.69
Fe	390	80	100.30 ± 0.75	6.20

[a]Detection limit units are expressed as ng/mL for Zn, Ca, Mg and P and as pg/mL for Cu and Fe; [b]Characteristic mass: in nanograms for Ca, Mg and P and in picograms for Zn, Cu and Fe, corresponding to 0.0044 milli-absorbance units; [c]Mean recovery obtained by analyte recovery assays in four fractions of the analyte in the same sample; [d]Mean relative standard deviation obtained by repeated measurements (*n*= 7) in four goat fermented milks (inter-day variability).

Table 1.2. Determination of Ca, Mg and Zn by FAAS, Cu and Fe by ETAAS and P by UV-VIS spectrophotometry in two certified reference materials (n = 10; data refer to dry weight)

Element	BCR - CRM 063R[a]		NIST. 1572[b]	
	Certified (mean ± SD)	Measured (mean ± SD)	Certified (mean ± SD)	Measured (mean ±S D)
Ca (mg/g)	13.49 ± 0.100	13.21 ± 0.150	-	-
P (mg/g)	11.10 ± 0.130	11.01 ± 0.210	-	-
Zn (µg/g)	49.0 ± 0.600	48.50 ± 2.32	138.6 ± 2.10	136.6 ± 2.90
Ca (mg/g)	13.49 ± 0.100	13.21 ± 0.150	-	-
Mg (mg/g)	1.262 ± 0.024	1.258 ± 0.042	5.80 ± 0.300	5.34 ± 0.250
Cu (µg/g	0.600 ± 0.020	0.570 ± 0.070	16.50 ± 1.00	16.38 ± 0.550
Fe (µg/g)	2.32 ± 0.23	2.62 ± 0.31	92.2 ± 9.00	84.5 ± 4.70

[ab] Reference of the certified reference materials.

2.3. Statistical analysis

Homogeneity of variance was first assessed using the Levene test at a significance level of 5% ($p < 0.05$). Statistical significance of data was then tested using the t-student test. The normal distribution of the samples was assayed with the Shapiro-Wilk test at a significance level of 5% ($p < 0.05$). Finally, evaluation of the relationship between different assays was carried out by computing the relevant correlation coefficient at the $p < 0.05$ confidence level by Pearson linear correlation (if normal distribution of the samples) or Spearman linear correlation (if not normal distribution of the samples). Analyses were performed using SPSS 15.0 (Windows version; SPSS Inc., Chicago, IL).

3. Results and discussion

The results of physicochemical analysis are shown in table 1.3, and in table 1.4 the results of the nutritional analysis.

3.1. Quality analysis of the milk and heating tests

No antibiotic nor sulphamide residues were found in RM and total germs were always in the range allowed by Spanish legislation for raw milk (< 1,500,000 cfu/mL; Regulation [CE] 853/2004). Mean total bacteria counts was $2.95 \cdot 10^4 \pm 0.41 \cdot 10^3$ cfu/mL, which means that RM samples were collected in good conditions from the farm.

Peroxidase and phosphatase enzymes were both inactivated with the pasteurization process, and then it was carried out at more than 80 ºC. This fact was confirmed with the total bacteria plate count, which was in the range allowed by European legislation (< 100,000 cfu/mL) established for milk (Regulation [CE] 853/2004) with a mean value of $1.70 \cdot 10^2 \pm 1.29 \cdot 10^2$ cfu/mL and absence of enterobacteria. Then, UFM was in good conditions to be used to manufacture the goat yogurt.

3.2. Dry extract percentages

Dry extract in RM was the significantly highest, but it was in the range reported (from 12.3 % to 15.9 %), because it depends mainly on the milk fat content (Güler-Akın et al., 2007; Quiles et al., 1994). We found that UFM had significantly higher dry extract than SM due to the loss of whey and concentration in compounds with molecular weight higher than 50 kDa, mainly proteins as the milk was previously skimmed. The

total solid content of UFM was chosen in the range 12 – 14% because it was described as the best solid content in the milk to improve the consistence of yoghurt (Tamine et al., 2007). When this dry extract was achieved by other authors by addition of whey protein concentrate, sodium caseinate or skimmed milk powder (obtaining 13.57, 12.14 and 13.89 % of dry extract respectively), less protein concentration than UFM was obtained (4.90, 4.93 and 4.92 g /100g) (Marafon et al., 2011).

3.3. Ashes weight

The significantly highest percentage of ashes was found in UFM. SM and RM, however, had levels for ashes in the range reported by others, from 0.76 to 0.86 (Güler, 2007; Güler-Akin et al., 2009; Park et al., 2007; Sanz Ceballos et al., 2009; Slačanac et al., 2010; Stelios et al., 2004). However, those values are lower than the ashes concentration of UFM.

3.4. Milk density

Milk density is not a constant physicochemical parameter because it depends on non-fatty solids as well as on fat (Baró-Rodríguez, et al., 2010). According to the results found in many studies, the density of analysed goat milk was in the range of bovine milk, which goes from 1.026 to 1.042 g/mL (Quiles et al., 1994). However, most reported values for goat milk density are slightly higher (Park et al., 2007). The density values measured in RM, SM and UFM were significantly different among them (Table 1.3). Specifically the density of RM was lower due to its fat content and the higher density of UFM could be a result of its increased concentration in non fat solids.

3.5. Acidity and pH values

Acidity measures the acid production in milk and pH represents the natural acidity of the milk from which casein stability depends on. For goat milk, pH reported values ranged from 6.3 to 6.8 (Güler, 2007; Quiles et al., 1994). Mean pH values found by us in analysed milks were slightly higher than the upper limit of this range, with non significantly differences among them (Table 1.3). However, the acidity behaviour was different. Acidity of UFM was significantly higher than that measured in RM and SM, in the range reported by Park et al. (2007), but higher than the reported by Güler-Akin et al. (2009).

Table 1.3. Physicochemical values of the three types of milk analysed

Sample	n	Dry extract (g/100g)	Ashes (g/100g)	Density (mg/mL)	pH	Acidity (g lactic acid /100g)
RM	12	14.86±0.84^{a**}	0.769±0.022^{a**}	1.030±0.002^{a**}	6.81±0.05	0.130±0.016^{a}
SM	12	9.99±0.23^{a**}	0.800±0.076^{b**}	1.035±0.002^{a**}	6.84±0.09	0.125±0.016^{b}
UFM	12	11.98±0.16^{a**}	0.961±0.093^{a**b**}	1.043±0.002^{a**}	6.84±0.10	0.143±0.016^{ab}

[a]RM: Raw goat milk; SM: Skimmed goat milk; UFM: Skimmed goat milk concentrated by ultrafiltration. Same letters means statitatistically significant differences among samples; p<0.05; *p<0.01; **p<0.001

Table 1.4. Nutritional values of the three types of milk analysed.

Sample	n	Lactose (g/100g)	Fat (g/100g)	Total proteins (g/100g)	Real proteins (g/100g) (% total proteins)	Caseins (g/100g) (% real proteins)	Whey proteins (g/100g) (% real proteins)
RM	12	4.71±0.18	5.31±1.06^{a**}	3.91±0.16^{a**}	3.62±0.19^{a**} (93%)	2.89±0.39^{a**} (80%)	0.73±0.08^{a} (20%)
SM	12	4.96±0.21	0.05±0.04^{a**}	4.40±0.34^{a**}	4.10±0.33^{a**} (93%)	3.36±0.32^{b**} (82%)	0.73±0.04^{b} (18%)
UFM	12	4.92±0.22	0.10±0.03^{a**}	6.07±0.30^{a**}	5.73±0.32^{a**} (94%)	4.88±0.36^{a**b**} (85%)	1.02±0.25^{ab} (18%)

RM: Raw milk; SM: Skimmed milk; UFM: Skimmed milk concentrated by ultrafiltration; (% total proteins): Percentage with respect to total proteins; (% real proteins): Percentage with respect to real proteins. Same letters means statistically significant differences among samples; p<0.05; *p<0.01; **p<0.001

3.6. Fat content

Fat content is the most quantitatively and qualitatively variable component of goat milk, depending on lactation stage, season, breed, genotype and feeding (Raynal-Ljutovac et al., 2008). Fat concentration of RM (Table 1.4) was in the range reported by other authors, namely from 3.1 % to 5.3 % (Güler-Akın et al., 2007; Sanz Ceballos et al., 2009). Although fat in UFM was significantly higher than in SM, both milks had a percentage lower than 0.3% (m/m), which is the maximum fat content allowed to be classified as skimmed milk by the current legislation (Baró-Rodríguez et al., 2010).

3.7. Lactose levels

According to Richardson (2004), lactose content in goat milk is lower than in other animal milks, and so it is less likely to cause digestion difficulties in people than cow milk. There were not significant differences among lactose values determined in analysed milks (Table 1.4). Despite this, a slightly higher lactose concentration was observed in SM, possibly due to its concentration in the hydrophilic portion after the skimming milk process. However, it was bit higher than those reported by other authors, values that ranged from 4.11 to 4.86 (Domagala et al., 2003; Güler-Akin et al., 2009; Richardson, 2004; Sanz Ceballos et al., 2009; Slačanac et al., 2010). Those results showed that ultrafiltration process did not affect to lactose concentration because the UFM had the same lactose levels than the SM. However, Domagala et al., (2003) reported a significant diminution of lactose concentration in ultrafiltration, which decreased concomitantly with the increasing of the pore size. Those different results could be owing to different ultrafiltration membranes and procedure as well as to the higher concentration rate performed by these authors.

3.8. Protein contents

Protein concentration was different depending on the type of milk (Table 1.4). RM had a concentration in proteins in the range found by other authors in the Murciano-Granadina breed, namely from 3.49 to 3.73 g/100g (Gonzalez-Crespoet al., 1995). In other goat breeds the range for total protein levels ranged from 3.1 to 4.6 g/100g (Domagala et al., 2003; Güler, 2007; Park et al., 2007; Richardson, 2004; Sanz-Ceballos et al., 2009; Tamime et al., 2011). The UFM protein concentration was higher

than other milks concentrated by ultrafiltration, which ranged from 5.22 to 5.49 g/100g, which means that it was concentrated to a greater extent (Domagala et al., 2003).

On the other hand, the UFM had the significantly highest percentage of casein, reaching the 85% of goat milk proteins, while for RM and SM caseins percentages were in the range reported by others (around 79% - 82%) (Baró-Rodríguez et al., 2010; Sanz-Ceballos et al., 2009). This increase was probably caused by the ultrafiltration process, which retains mainly caseins. Caseins are the most important proteins for the clot formation in the yoghurt fermentation, so, its higher concentration in UFM is an advantage when this milk is used for the yoghurt manufacturing (Baró-Rodríguez et al., 2005; Domagala et al., 2012).

The whey proteins concentration in RM was found a bit higher than values reported by other authors, which ranges from 0.52 to 0.62 g/100g (Olalla et al., 2009; Park et al., 2007; Sanz Ceballos et al., 2009). The UFM also had significantly higher concentration of whey proteins, in spite of the fact that its percentage was similar to those found for RM and SM (Table 1.4). This could be explained because the major whey proteins (β-lactoglobulin and α-lactoalbumin) have a molecular weight lower than the cut-off size, and therefore, they passed through the membrane during the ultrafiltration process. Nevertheless, other whey proteins like immunoglobulins (which represent around 15% of whey proteins), serum albumin or lactoferrin have higher molecular weights and they were retained in the retentate (Baró-Rodríguez et al., 2005). Moreover, unless the concentration had been completely finished, small whey proteins could have remained in the whey.

3.9. Mineral (Ca, P, Mg, Zn, Cu and Fe) levels

The UFM had the highest concentration for all measured minerals, with the exception of Fe. This finding was in accordance to its significantly highest percentage of ashes attributable to the ultrafiltration process. In table 1.5 total mineral concentrations present in analysed milks, mineral levels associated to non-casein nitrogen (NCN) and non-protein nitrogen (NPN) fractions, are collected.

The UFM presented mean Ca, P, Zn and Cu levels significantly higher than those for RM and SM. Additionally, UFM samples had statistically significant higher mean Mg

levels than RM ones. However, mean Fe concentrations were not significantly different among the three goat milk samples studied.In general, mineral concentration for RM and SM was in the range reported by others for goat milk, although Ca, P, Mg and Zn levels were higher in UFM than those reported values (Park et al., 2007; Sanz Ceballos et al., 2009; Slačanac et al., 2010). Nevertheless, for Cu and Fe, around the double concentration was reported. One possible explanation to this result is that Ca, P, Mg and Zn are partly bound to the casein structure and when this protein was concentrated, those minerals were also concentrated (De la Fuente et al., 1997).

Mineral levels in NCN and NPN fractions, obtained by acidification as described above, were measured to study the mineral distribution in the different protein fractions (Table 1.5). Table 1.6 shows the concentration of minerals in theory bound to proteins, which really are those that remained insoluble after the precipitation and filtration and table 1.7 shows the bounding percentage.

In UFM, mean Ca, P, Mg and Zn levels in the NCN and NPN fractions and Cu in NPN fraction were statistically higher than those measured in RM and SM (Table 1.5). Moreover, as it is observed in table 1.7, the percentage of Ca and Mg bound to proteins in UFM was lower than those corresponding to RM and SM. Other researchers reported that after filtration or coagulation, percentages of minerals bound to proteins were: 88% for Ca, 61% for P, 34% for Mg, 87% for Zn, 82 % for Cu and 56% for Fe and (Fransson et al., 1983; De la Fuente et al., 1996; De la Fuente et al., 1997). Our results were considerably different, especially for Ca bound to proteins (only 16%).

In addition, Ca and Mg levels bound to caseins were significantly lower in UFM when compared with those for RM and SM. Others reported previously that acid treatment modified mineral distribution in milk (Fransson et al., 1983), which could lead to a higher concentration for Ca and Mg levels in soluble fraction than that expected. Another reason could be that ultrafiltration process changed the distribution of these minerals in milk. It is worth pointing out that in order to be absorbed, all elements need to be in a soluble form in the intestinal tract or bound to compounds forming soluble complexes (Roig et al., 1999). Then, if this modification in mineral distribution happens also during the fermentation process, it could affect their bioavailability in fermented milks.

Table 1.5. Total mineral (Ca, P, Mg, Zn, Cu and Fe) levels and Ca/P ratio in analysed goat milk samples as well as levels associated to their NCN and NPN fractions (mean ± SD)

Sample		n	Ca (mg/100g)	Ca/P	P (mg/100g)	Mg (mg/100g)	Zn (µg/100g)	Cu (µg/100g)	Fe (µg/100g)
RM	Total	12	135.2 ± 10.0^{a**b}	1.36	101.2 ± 15.5^{a**}	14.97 ± 2.74^{a}	408.4 ± 32.8^{a**}	26.76 ± 4.40^{a*}	76.14 ± 12.10
	NCN	12	109.5 ± 6.4^{d**}	1.33	82.1 ± 7.7^{c*}	9.39 ± 10.09^{b**}	337.5 ± 93.1^{c**}	25.23 ± 8.43	66.52 ± 10.27^{a}
	NPN	12	94.2 ± 13.1^{f**}	1.91	49.4 ± 5.7^{e**}	8.23 ± 0.65^{d**}	288.7 ± 66.6^{e*}	20.23 ± 4.50^{b*}	42.68 ± 11.57
SM	Total	12	147.9 ± 10.1^{bc*}	1.39	106.6 ± 14.3^{b**}	15.74 ± 2.26	402.0 ± 31.1^{b**}	28.78 ± 8.57^{a*}	65.36 ± 18.12
	NCN	12	110.0 ± 10.1^{e**}	1.31	84.2 ± 13.3^{d*}	9.45 ± 1.32^{c**}	396.7 ± 99.3d**	26.98 ± 7.13	61.17 ± 6.76^{a}
	NPN	12	97.8 ± 10.9^{g**}	1.84	53.2 ± 4.0^{f**}	8.73 ± 0.758^{e**}	339.5 ± 77.2^{f}	20.79 ± 4.53^{c}	28.03 ± 5.22
UFM	Total	12	165.6 ± 15.1^{a**c*}	1.20	139.7 ± 10.4^{a**b**}	16.67 ± 1.32^{a}	616.4 ± 79.2^{a**b**}	36.45 ± 6.03^{a*}	61.58 ± 15.93
	NCN	12	152.0 ± 7.4^{d**e**}	1.34	113.6 ± 16.7^{c*d*}	12.33 ± 1.68^{b**c**}	548.2 ± 111.5^{c**d**}	28.76 ± 9.89	60.53 ± 11.73
	NPN	12	138.8 ± 11.7^{f**g**}	1.98	70.2 ± 7.1^{e**f**}	11.06 ± 1.69^{d**e**}	413.0 ± 101.0^{e*f}	27.18 ± 7.50^{b*c}	33.39 ± 12.41

RM: Raw milk; SM: Skimmed milk; UFM; Milk concentrated by ultrafiltration; NCN: Non casein nitrogen fraction; NPN: Non protein nitrogen fraction. Same letters means statistically significant differences; $p<0.05$; $*p<0.01$; $**p<0.001$

Table 1.6. Minerals (Ca, P, Mg, Zn, Cu and Fe) concentration in goat milk samples linked to milk proteins or soluble in the whey.

Sample		n	Ca (mg/100g)	P (mg/100g)	Mg (mg/100g)	Zn (µg/g/100g)	Cu (µg/g/100g)	Fe (µg/g/100g)
RM	Soluble	12	94.17	49.40	8.23	288.7	20.23	42.68
	Protein	12	41.03	51.77	6.74	119.7	6.53	33.46
	Casein	12	25.64	19.08	5.59	70.9	1.53	9.62
	Whey proteins	12	15.37	32.69	1.16	48.8	5.00	23.84
SM	Soluble	12	97.81	53.22	8.73	339.5	20.79	28.03
	Protein	12	50.08	53.40	7.00	62.5	7.99	37.33
	Casein	12	37.84	22.39	6.29	5.3	1.80	4.19
	Whey proteins	12	12.23	31.00	0.71	57.2	6.19	33.14
UFM	Soluble	12	138.83	70.15	11.06	413.0	27.18	33.39
	Protein	12	26.74	69.58	5.61	203.4	9.27	28.19
	Casein	12	13.62	26.17	4.34	68.2	7.69	1.05
	Whey proteins	12	13.12	43.41	1.27	135.2	1.58	27.14

RM: Raw milk; SM: Skimmed milk; UFM: Milk concentrated by ultrafiltration; Soluble: Minerals not linked to proteins, soluble in the whey. Protein: Minerals supposedly linked to proteins = Total minerals -minerals in non protein fraction. Casein: Mineral supposedly linked to caseins: Total minerals – minerals in non caseinic fraction; Whey proteins: Minerals supposedly linked to whey proteins = Minerals theorically linked to proteins – Minerals theorically linked to caseins.

Table 1.7. Percentage of minerals (Ca, P, Mg, Zn, Cu and Fe) in goat milk samples associated to their casein and whey protein fractions

Sample		n	Ca (%)	P (%)	Mg (%)	Zn (%)	Cu (%)	Fe (%)
RM	Soluble (% total)	12	70	49	65	71	76	66
	Protein (% total)	12	30	51	45	29	24	44
	Casein (% proteins)	12	63	37	83	59	23	29
	Whey prot. (% proteins)	12	37	63	17	41	77	71
SM	Soluble (% total)	12	66	50	56	84	72	43
	Protein (% total)	12	34	50	44	16	28	57
	Casein (% proteins)	12	76	42	90	8	23	11
	Whey prot. (% proteins)	12	24	58	10	92	77	89
UFM	Soluble (% total)	12	84	50	66	67	74	54
	Protein (% total)	12	16	50	34	33	26	46
	Casein (% proteins)	12	51	38	67	34	82	4
	Whey prot. (% proteins)	12	49	62	23	66	17	96

RM: Raw milk; SM: Skimmed milk; Milk concentrated by ultrafiltration; Soluble: Minerals not linked to proteins, soluble in the whey; Protein: Minerals supposedly linked to proteins = Total minerals -minerals in non protein fraction; (% Total): percentage of minerals linked to proteins in relation with total minerals; Casein: Mineral supposedly linked to caseins: Total minerals – minerals in non caseinic fraction; (% Prot.): Percentage of minerals linked in relation with minerals linked to proteins; Whey prot.: Minerals supposedly linked to whey proteins = Minerals theorically linked to proteins – Minerals theorically linked to caseins.

On the other hand, the increase in total Ca concentration of UFM is not proportional to the P increase, and then the Ca/P ratio decreased from 1.36 and 1.39 in RM and SM to 1.20 in UFM, which continues being placed in the recommended range for good Ca absorption and bioavailability (Baró-Rodríguez et al., 2010). But, in spite of lowering this ratio, because soluble fraction of Ca was increased, we think that this element would be more bioaccesible in UFM than in RM and SM.

For Mg, mean percentage in proteins was lower in UFM than in the RM and SM, as well as for Ca, soluble fraction of this mineral was increased with the ultrafiltration. Nonetheless, the percentage of Mg was also increased in whey proteins.

Despite the percentage of Cu bound to proteins was low and similar for the three goat milks (from 24 to 28%), its distribution in caseins and whey proteins (23 and 77 % respectively, for both RM and SM) was reversed after the ultrafiltration process (82 and 17% respectively, for UFM). This fact could increase the bioavailability of this mineral since the low bioavailability of the Cu was reported when it is bound to proteins (Ekmekcioglu, 2000). Finally, Fe and P distribution in goat milk was not modified by ultrafiltration treatment.

It should be noted that UFM was pasteurised, and although it has been reported that this process diminishes the mineral soluble fraction (de la Fuente et al., 1999), our results did not support this finding. On the other hand, milk protein digestion leads to the hydrolysis of casein structure, the release of minerals bound to it, and the formation of casein phosphopeptides, compounds that can form soluble complexes with minerals increasing their bioavailability.

Milk concentrated by ultrafiltration demonstrated its leading to a better curd formation after fermentation. Additionally, we have shown a change in the mineral distribution when this ultrafiltration process was used. According to Polychroniadou et al. (1986), the equilibrium of salts between the aqueous and the dispersed phase of milk affects its rennet coagulability and the physical properties of the curd. In this sense, the modification of mineral composition of the milk by ultrafiltration could be one of the reasons, together with the higher casein concentration, to obtain a better curd when

fermentation is carried out, which could finally influence the mineral bioavailability of yoghurt (Domagala et al., 2012).

4. Conclusions

In UFM obtained by the developed process the content of multiple physicochemical and nutritional parameters of the goat milks was modified in comparison with RM and SM, namely caseins, whey proteins, total and real proteins, minerals (Ca, P, Mg and Zn), ashes, dry extract, density, acidity and fat. Furthermore, the ultrafiltration process changed the mineral distribution in the milk for Ca and Mg, increasing their solubility. The observed dry extract enhancement by the ultrafiltration process was associated to an increase in milk caseins, which makes the UFM a good source to fermented milk manufacturing. Finally, the high mineral concentration measured in UFM, as well as its low fat content and high casein concentration, makes this milk more appropriate for goat yogurt manufacturing than RM and SM.

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CHAPTER 2

Development of a novel skimmed goat milk fermented with the probiotic strain *Lactobacillus plantarum* C4. Physicochemical, nutritional and organoleptic characterization

Development of a novel skimmed goat milk fermented with the Probiotic strain *Lactobacillus plantarum* C4. Physicochemical, nutritional and organoleptic characterization

Abstract

There have been well reported the benefits of goat milk, fermented milks as well as the probiotic microorganisms on the organism. In this research an appropriate process to manufacture a new probiotic skimmed fermented goat milk (PFM) with *Lactobacillus plantarum* C4 and good physicochemical, nutritional and organolecptic properties was developed. Firstly, it was necessary to choose and to standardise the milk concentration and manufacturing processes as well as to study the interactions among *L. plantarum* C4 and the yoghurt classical starter strains also used for the fermentation *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. As a result, goat milk was concentrated by ultrafiltration, which demonstrated best rheology parameters. *L. plantarum* C4 was inoculated at high concentration (~10^8cfu/mL) and the fermentation was carried out at 37 ºC for 6 h. The cultures used were viable at good concentration at the end of the fermentation, after 4 weeks of storage and after *in vitro* gastrointestinal digestion. An answer sheet has been developed to carry out sensorial analysis. The PFM had good viscosity and syneresis and it showed the best visual parameters and viscosity for the panellists in comparison with other commercial fermented milks. Despite nutritional and physicochemical values were in the range reported for commercial fermented milks, they were different to reported values for skimmed fermented goat milks. Finally, its low lactose and fat content, high protein proportion and good mineral concentration as well as the acceptability by the panellists, could lead us to consider this PFM such a good dairy product to be commercialised.

1. Introduction

Currently, an increased interest in foods with a positive effect on health beyond their nutritional value is happening and much attention has been focused on probiotic products. Many properties have been attributed to fermented milks and more when probiotics are present: lactose absorption improvement, protein digestibility increase, fat digestibility improvement, antibacterial activity, immune system stimulation, preventive action against digestive system cancer and anticolesterolemic action (Mahaut et al., 2004). In that sense, fermented milks has been recommended in: lactose intolerance, diarrohea, constipation, *Helicobacter pylory* treatment, prevention or improvement of infections, immunitary system improvement, atopic eczema, etc (Baró-Rodríguez et al., 2010; Khani et al., 2012; Sánchez et al., 2009). In addition, bioavailability of Ca from yoghurt is higher than from milk due to the acidic pH of yoghurt ionizes calcium and thus facilitates intestinal Ca absorption. The low pH of yoghurt may also reduce the inhibitory effect of dietary phytic acid on calcium bioavailability (Singh et al., 2008). Among fermenting bacteria, the *Lactobacillus* genera has a long and safe history in the manufacture of dairy products (Vaughan et al., 1999). In this context, the putative probiotic strain *Lactobacillus plantarum* C4 (*L. plantarum* C4) isolated by our research group, which fulfilled the *in vitro* criteria in order to be selected as potentially effective probiotic bacteria, was chosen for the study (Bujalance et al., 2007). This stain was selected to be used as milk starter concomitantly with the classical starter bacteria (St) *Lactobacillus delbruekii* sbsp. *bulgaricus* and *Streptococcus thermophilus.* Additionally, *L. plantarum* C4 demonstrated antimicrobial, microbiota-modulating, and immuno-modulating properties (Bergillos-Meca et al., 2014; Fuentes et al., 2008; Puertollano et al., 2008).

On the other hand, skimmed milk was previously found as appropriate vehicle for the intragastric administration of this probiotic strain in mice (Bujalance et al., 2007). Among different food matrix used to carry probiotic strains, fermented milks have been one of the most profusely used because the bacteria are maintained alive and for its recommended daily dietary intake (Erdmann et al., 2008). However, the manufacture of a goat fermented milk with probiotic properties requires compatibility among different bacteria involved in the fermentation process (Bergillos-Meca et al., 2013).

Among milks, goat milk reported better digestibility, less allergenic capacity, better mineral bioavailability and protein and fat profile when compared with cow milk (Barrionuevo et al., 2002; Haenlein, 2004; Lopez-Aliaga et al., 2003; Silanikove et al., 2010; Slačanac et al., 2010). Despite this, cow milk continues being the most consumed and commonly used to be fermented. This is mainly due to the higher milk production by cows and the problems in manufacturing and organoleptic acceptance by consumers of fermented goat milks. Additionally, goat set-yoghurts faces with some problems like over-acidification, due to the low buffering capacity of goat milk, and the formation of an almost semi-liquid gel because of the low α_{s1}-casein content and high degree of casein micelle dispersion in goat milk (Martín-Diana et al., 2003). The low firmness and the high whey separation on the surface of the yoghurt (syneresis process) are major defects in set yoghurts. Both could be reduced by monitoring some manufacturing parameters like heat treatment of the milk, incubation temperature as well as fat, protein and total solid concentration (Abbasi et al., 2009; Espírito-Santo et al., 2013; Lucey, 2002). Then, to obtain a satisfactory gel tension in fermented goat milk it is mandatory to control those factors.

Among the different methods used to increase milk concentration in non-fat solids we could remark: (a) the addition of powdered skimmed goat milk because it is one of the most widely employed methods in the industry, which concentrates all the milk solid compounds in the same proportion as they are in the milk; and (b) the ultrafiltration process, whose interest is based on it concentrates mainly caseins and some minerals bounded to those proteins, which are responsible of the curd formation, improving the final yoghurt properties, while their properties are maintained due to no additional heat treatment is carried out (Domagala et al., 2012). In terms of sensorial characteristics, the new developed products should be analysed to determine their sensorial profile and the acceptance or rejection by the consumers.

Among the multiple sensorial parameters it is important to distinguish: visual, aroma, taste and texture parameters. Whereas a good texture will lead to good visual and texture parameters, the kind of milk and other parameters will influence the taste and aroma. One of the most typical features of goat milk is the caprine flavour and aroma due to the presence of short and medium chain fatty acids, which is considered mostly

as a negative organoleptic attribute (Park et al., 2007; Slačanac et al., 2010). Therefore, to manufacture fermented goat milk it is required to avoid or diminish this flavuor.

In this context, the objective of this research was to develop an appropriate process to manufacture a novel probiotic fermented goat milk with good physicochemical, nutritional and organoleptic properties. With this aim were carried out: the selection and standardisation of milk concentration method; the research on the bacteria viability (*L. plantarum* C4, *L. bulcaricus* and *S. thermophilus*) and the development of an answer sheet to evaluate fermented milks.

2. Materials and methods

2.1. Samples

Different fermented milks were manufactured during the different steps of the developing process until obtaining the standardised probiotic fermented goat milk (PFM). With this aim were used combinations of different milks, fermenting bacteria as well as fermentation times and temperature.

2.1.1. Selection of the concentration method

With this aim different samples were manufactured, and those differences during the manufacturing process are shown in figure 2.1. Additionally, a commercial probiotic skimmed fermented goat milk (fermented with St and a probiotic *Bifidobacterium* strain) was purchased.

Three samples of each type were manufacturing and analysed by triplicate.

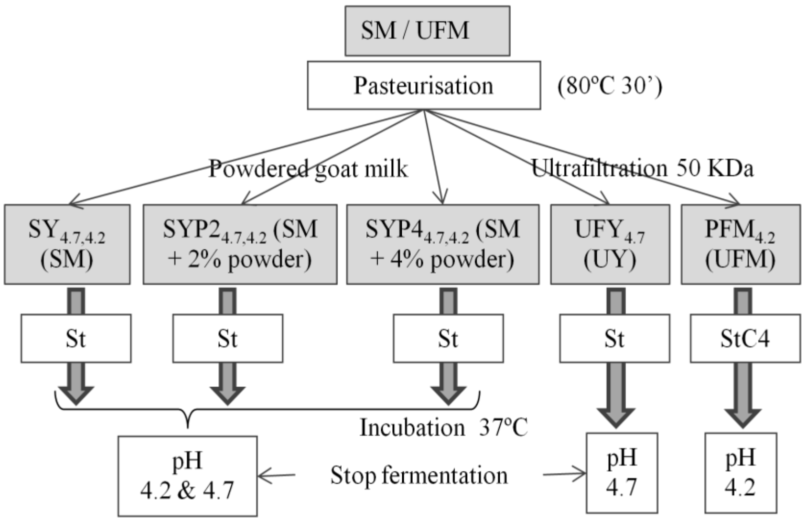

Figure 2.1. Diagram of fermented milks manufactured for the texture and syneresis study.

SM: Skimmed goat milk; UFM: Skimmed goat milk concentrated by ultrafiltration; St: Commercial classical starter bacteria (*L. bulgaricus* and *S. thermophilus*); StC4: Commercial classical starter bacteria and probiotic strain *L. plantarum* C4; SY: Yoghurt made with SM fermented St; SYP2: Yoghurt made with SM and 2% of commercial powdered SM fermented with St; SYP4: Yoghurt made with SM and 4% of commercial powdered SM fermented with St; UY: Yoghurt made with UFM fermented with St; PFM: Fermented milk made with UFM fermented with St and *Lactobacillus plantarum* C4 at high concentration during 6h, which lead to a pH = 4.2; $Sample_{4.7}$: The fermentation was stopped at pH 4.7 ; $Sample_{4.2}$: The fermentation was stopped at pH 4.2.

2.1.2. Viability of the fermenting bacteria

In the viability study of the fermenting bacteria the following fermented milks were manufactured in a 8 h fermenting process (Figure 2.2): (1) SY: Yoghurt made with SM fermented with St; (2) SFM_L: Fermented milk made with SM fermented with the classical starter bacteria and *L. plantarum* C4 at low concentration; (3) $SC4_L$: Fermented milk made with SM fermented with *L. plantarum* C4 at low concentration; (4) UY: Yoghurt made with UFM fermented with the classical starter bacteria; (5) UFM_L: Fermented milk made with UFM fermented with the classical starter bacteria and *L. plantarum* C4 at low concentration; (6) $UC4_L$: Fermented milk made with UFM fermented with *L. plantarum* C4 at low concentration; (7) PFM: Probiotic fermented milk by the standardised procedure described below. Three samples of each type were manufacturing and analysed by triplicate.

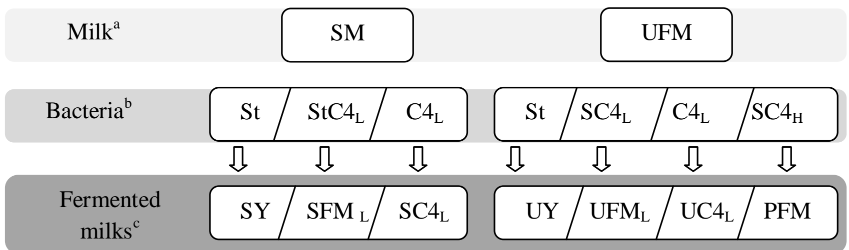

Figure 2.2. Scheme of the milks and fermenting bacteria combinations in the manufacturing of the samples used in the viability assay.

[a]Milks used as raw material for the fermentation: SM: Skimmed goat milk, UFM: Skimmed goat milk concentrated by ultrafiltration; [b]Bacteria used as starter: St: Classical starer bacteria (*L. bulgaricus* and *S. thermophilus)* that were always inoculated at the same concentration. $StC4_L$: St and *L. plantarum* C4 at low concentration (10^6cfu/mL); $C4_L$: *L. plantarum* C4 at low concentration (10^6cfu/mL); $StC4_H$: St and *L. plantarum* C4 at high concentration (10^9cfu/mL). [c]Final samples obtained after the fermentation of each combination milk-bacteria.

2.1.3. Physicochemical and nutritional characterisation of PFM

To carry out these assays 8 batches were manufactured in different weeks during one year and three samples of each batch were used to be analysed by triplicate.

2.1.4. Organoleptic characterisation

In this study the following samples were used: (1) PFM; (2) GFM: Commercial skimmed fermented goat milk fermented with St and a *Bifidobacterium* strain; (3) CFM: Commercial skimmed cow yoghurt fermented with St.

2.2. Strains inoculation

2.2.1. Classical starter bacteria

The inoculation with the commercial St (YO-MIX® 350, Dupont™ Danisco, Barcelona, Spain) was carried out according to the manufacturing instructions. The recommended amount of freeze-dried bacteria was weighted, re-suspended in 1% of the final volume of the milk (reaching 10^8 colony-forming units [cfu]/mL) and stirred until homogenisation. Finally, the inoculated milk was mixed with the remaining milk at approximately 42 ºC and homogenised in order to obtain an initial viable bacteria of 10^6 cfu/mL.

2.2.2. *L. plantarum* C4 at low concentration

In order to obtain the same number of bacteria as for the classical starter bacteria (10^6 cfu/mL), the *L. plantarum* C4 was recovered after overnight growth in Man Rogosa Sharpe agar (MRS; Difco™, Becton, Dickinson and Company; Madrid, Spain) and re-

suspended in sterile phosphate buffered saline (PBS; Sigma-Aldrich, Steinheim, Germany) at a concentration of 10^8 cfu/mL. Then, it was recovered by centrifugation at 3,500 rpm for 15 min and re-suspended in 1% of pasteurised milk under stirring until good homogenisation. Finally, the inoculated milk was added to the remaining milk at temperature of approximately 42 ºC.

2.2.3. *L. plantarum* C4 at high concentration

This inoculation was made in order to obtain an initial concentration of 10^9 cfu *L. plantarum* C4/mL, which is higher than the concentration required in the final product to be considered as probiotic. The *L. plantarum* C4 was recovered by centrifugation at 3,500 rpm for 15 min from MRS Broth (Difco™, Becton, Dickinson and Company; Madrid, Spain) after overnight incubation under stirring at 37 ºC. The pellet obtained was cleaned twice with sterile PBS. Finally, the *L. plantarum* C4 pellet was re-suspended in 1% of pasteurized milk and the inoculated goat milk aliquot was added to the remained goat pasteurised milk at approximately 42 ºC stirring to ensure good homogenization.

2.3. Standardisation of the probiotic fermented goat milk manufacturing

Firstly, the milk concentration method was chosen with the objective of establish a raw material for the fermentation. The different milks used to carry out this assays were fermented only with St. After that, were assayed the viability and interaction among the strains to establish the inocula concentration in the fermented milk, fermentation temperature and time. Finally, the standardised procedure of fermentation was described in the results.

All analyses were carried out by triplicate and blanks were prepared with bidistilled water instead the sample when required.

2.3.1. Selection of the concentration method

The influence of the milk concentration method on the fermented milk syneresis and viscosity was studied (Moreno-Montoro et al., 2013). With this aim were manufactured the fermented milks described in figure 2.1. After inoculation, the samples were shared out in sterile glass pots and incubated at 37 ºC. The incubation was stopped at pH 4.7 and 4.2, according to the specific isoelectric points of cow and goat milk proteins,

respectively. Then, samples were stored under refrigeration at 4 ºC. Afterwards, the viscosity and syneresis of fermented milks were analysed according to the procedures described in 2.4.1 and 2.4.2.

2.3.2. Viability and interactions of the fermenting bacteria

2.3.2.1. Spot test

The interactions between *L. bulgaricus, S. thermophilus* and and *L. plantarum* C4 were investigated using the spot test on Tryptone Soy Agar (TSA) (Difco™, Becton, Dickinson and Company; Madrid, Spain), MRS and LPSM (*L.plantarum* selective medium; Bujalance et al., 2006) agar plates, according to Jacobsen et al. (1999), with some modifications (Bergillos-Meca et al., 2013)**.**

2.3.2.2. Standardisation of starter inoculation, time and temperature of fermentation

This assay was carried out with the objective of standardise the fermentation, in terms of time, temperature and viable probiotic bacteria.

Firstly, the selection of the appropriate fermentation temperature was carried out. With this aim three UFM were fermented with a mixture of the three bacteria strains (St and *L. plantarum* C4 at low concentration) at 37 and 42 ºC and obtained results were compared. Once selected the temperature, with the objective of standardise the fermentation in terms of time and viable probiotic bacteria, 9 different fermented goat milk types were manufactured, as described above (samples paragraph 2.1.2. and figure 2.2) at the chosen temperature. The number of viable microorganisms was counted at different times during fermentation process by preparing serial dilutions of the fermented milks. Then these preparations were plated out onto TSA, MRS and LPSM agar and incubated for 24-48 h at 37 ºC. Then, results were expressed as cfu/ mL.

2.4. Physicochemical and nutritional characterization of the probiotic fermented goat milk

All analyses were carried out by triplicate and blanks were prepared with bidistilled water instead the sample when required.

2.4.1. Viscosity

For the viscosity determination a Brookfield DV-II + Viscosimeter (Brookfield, Harlow, UK) with a 21 spindle was used. The procedure used was previously reported elsewhere (Moreno-Montoro et al., 2013) The stress was measured at different rpm of the spindle and the sample was at 20 ºC. Results were expressed as stress (D/cm^2).

2.4.2. Spontaneous yogurt syneresis

When manufacturing the batches, fermented goat milk samples were also shared out by triplicate in 100 mL volumetric cylinders. The separated whey from the curd was measured weekly during a month period as described in Moreno-Montoro et al. (2013), following a modified method reported by others (Lucey et al., 1998).

2.4.3. Plate count of viable bacteria after fermentation and storage

Viable microorganisms in the final PFM were counted after culturing serial dilutions of the sample on TSA, MRS and LPSM agar plates and incubation for 24-48 h at 37 ºC. Microorganisms were counted at the beginning and the end of the fermentation and in order to assay their viability they were counted each week until 6 weeks of storage after manufacturing process. Results were expressed as cfu/mL.

2.4.4. Viability of fermenting microorganisms after *in vitro* digestion

The samples were subjected to a simulated gastrointestinal digestion (method described in chapter 3, paragraph 2.3.1.) and serial dilutions were plated out onto LPSM (*L. plantarum*) and MRS (St) as described above, in order to enumerate the viable microorganisms that remained viable after *in vitro* gastrointestinal digestion. Results were expressed as cfu/mL.

2.4.5. pH and acidity determination

The pH and acidity of the fermented goat milks were measured as previously explained for goat milk (Chapter 1, paragraphs 2.2.6 and 2.2.7). The acidity was expressed as g lactic acid / 100g of sample.

2.4.6. D/L-lactic acid test

The measurement of lactic acid isomers D and L was carried out with the D-Lactic/L-lactic test from Boeringer Mannheim (R-Biopharm, Darmstadt, Germany) following the manufacturer instructions for the kit. The validation was done using certified standard supplied with the kit, obtaining 99.7% percentage of recovery. Results were expressed as g /100 g of sample.

2.4.7. Dry extract content

The dry extract content was measured weighting 5 g of sample in a capsule with sea sand and desiccated until constant weight according to AOAC (2006). Results were expressed as g / 100 g of sample.

2.4.8. Lactose and galactose levels

Lactose and D-galactose levels were measured using an enzymatic kit fom Megazyme following the manufacturer instructions (Commission Regulation (EEC) N° 2676/90). The validation was done using certified standard supplied with the kit, obtaining a percentage of recovery of 92% (Megazyme International, Wicklow, Ireland). Results were expressed as g/100 g.

2.4.9. Protein concentration

Total protein concentrations were measured by the Kjeldahl method according to (Olalla et al., 2009), with the only modification that were weighted 3 g of fermented milk. A 93% of recovery using bovine serum albumin (Sigma-Aldrich, Steinheim, Germany) was obtained.

2.4.10. Fat concentration

Was carried out using the Gerber method described in chapter 1 (paragraph 2.2.11) but adapted to fermented milks according to Casado Cimiano (1991).

2.4.11. Mineral (Ca, P, Mg, Zn, Cu and Fe) levels

Mineral determination was carried out according to procedures previously optimized as was described in chapter 1 (paragraph 2.2.13) (Aleixo et al., 2003; Bergillos-Meca et al., 2013; Velasco-Reynold et al., 2008). A wet mineralization procedure was used for

the fermented milk samples. 1 g of samples were placed in Pyrex tubes with 5 mL of HNO_3-$HClO_4$ (4:1) and digested in the digestion block (Selecta SA, Barcelona, Spain) with the following temperature profile: 60 ºC during 45 min, 90 ºC during 30 min and 120 ºC for 90 min. After the sample was mineralized, cooled at room temperature and filled up to 10 ml with bidistilled water to obtaining the analytical sample.

2.5. Sensorial analysis

This analysis was carried out in fermented milk samples after 5 days of storage by 10 already-trained judges of the regular taste panel from our food science laboratory. They were more specifically trained in the recognition of goat milk descriptors and in the use of scales for evaluation. These judges had received previous training in test for recognising fundamental flavours, detecting and recognising smells recognising detection thresholds and texture profile, following the phases and procedures described in ISO. The interpretation of the screening test was conducted according to ASTM (UNE 87013, 1992; ASTM, 1981; *ISO 3972:* 1991; *ISO 8586:* 1993; *ISO 8586-2:*1993, *UNE 87025: 1996*.). For this analysis, four tablespoons of each sample were presented to the panellists in plastic randomly coded plates, using 3-digit numbers and 2 letters to name and differentiate them. At each session 3 samples were presented to the panel group, namely PFM, GFM and CFM (skimmed cow yoghurt).

An answer sheet (Anexe 1) was specifically designed for this analysis. The parameters analysed are represented in table 2.1. According to Tamine et al. (1991) they were divided in 4 parts (visual, textural, aroma and taste) and in addition they were scored on a descriptive scale of perception, as presence or absence or in a scale from undetected to detected at high intensity.

2.6. Statistical analysis

Homogeneity of variance was first assessed using the Levene test at a significance level of 5% ($p < 0.05$). Statistical significance of data was then tested using the t-student test. The normal distribution of the samples was assayed with the Shapiro-Wilk test at a significance level of 5% ($p < 0.05$). Finally, evaluation of the relationship between different assays was carried out by computing the relevant correlation coefficient at the $p < 0.05$ confidence level by Pearson linear correlation (if normal distribution of the

samples) or Spearman linear correlation (if no normal distribution of the samples). Analyses were performed using SPSS 15.0 program (Windows version; SPSS Inc., Chicago, IL).

Table 2.1. Parameters analysed in the answer sheet.

	Evaluation score		Parameter	Description
Visual	Scale of perception	0-5	Colour	From grey/yellow-white to pure white
			Syneresis	Amount of water on the sample surface
			Smoothness	Looks smooth and free of irregularities
	Pesence/absence		Curd homogeneity	
			Floury	Fermented milk looks like flour
			Lumps	
			Bubbles	
			Others	
Texture	Scale of perception	1-8	Oral viscosity	Yoghurt resistance to flow in mouth
		1-4	Stickiness	Degree to which the sample sticks or adheres to the teeth and palate
Aroma	Scale of perception	0-5	Aroma fineness	Natural yoghurt like aroma
			Aroma intensity	How strong the aroma is perceived
			Aroma persistency	How long is perceived the aroma
	Undetected Low intensity Medium intensity High intensity		Fermentation	Like bread fermentation
			Acetaldehyde	Yoghurt like aroma
			Dyacetil	Butter like aroma
			Goat, flower, fruit, Bitter, boiled milk, and others.	
Taste	Scale of perception	0-5	Taste fineness	Natural yoghurt like taste
			Taste intensity	How strong the taste is perceived
			Sweetness	Sweet taste
			Acidity	Acid taste
			Bitterness	Bitter taste
	Undetected Low intensity Medium intensity High intensity		Goat, fruit, astringent, spicy, metallic, salty, insipid, dirty, rancid, bitter, yeast like, boiled milk and humidity	
Overall acceptability				Final impression of the yoghurt

(Antunes et al., 2005; Ciron et al., 2011; Cruz et al., 2013; Domagała et al., 2013; Gonzalez et al., 2011; Janhoj et al., 2008; Le Jaouen, 1991; Mahaut et al., 2004b; Ranadheera et al., 2012; Sancho et al., 1999; Slačanac et al., 2010; Tamine et al., 1991; UNE 87025:1996.; Vargas et al., 2008)

3. Results

3.1. Standardisation procedure for PFM manufacturing

3.1.1. Selection of the concentration method

As far as SY, SYP2 and SYP4 fermented goat milks are concerned, the syneresis diminished concomitantly with the addition of powdered milk and with the pH diminution. The best whey retention, independently of its dry extract, was observed when UFM was used and no significant differences were observed between syneresis at pH 4.2 and 4.7, despite differences were observed in the other samples (Figure 2.4a).

As it can be observed in figure 2.4b, for the same samples when the final pH was 4.2, more time was needed to appreciate syneresis. The 6th day of storage only the fermented milks at pH 4.7 (With the exception of UY) showed syneresis and the $SYP4_{4.7}$ started to show syneresis the 12th day. The other fermented milks that presented after 21 days of storage it was appreciated after the 14th day.

On the other hand, the tyxotropic behaviour of the yogurts with less syneresis was compared with a commercial sample (GFM). The fermented goat milks manufactured with UFM and $SYP4_{4.2}$ showed similar viscosity to GFM and significantly different to $SYP2_{4.2}$ (Figure 2.3).

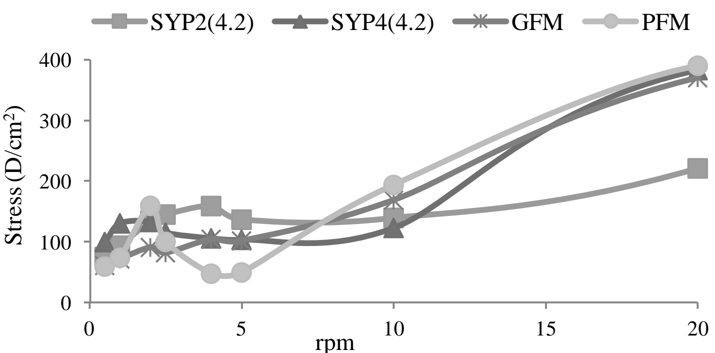

Figure 2.3. Viscosity representation of the goat milks samples fermented until pH 4.2 and a commercial one.

SYP2: Yoghurt made with skimmed milk (SM) and 2% of commercial powdered SM fermented with the classical starter bacteria (St); SYP4: Yoghurt made with SM and 4% of commercial powdered SM fermented with St; GFM: Commercial probiotic skimmed fermented goat milk (St and *Bifidobacterium*); PFM: Probiotic fermented milk made with UFM and fermented with St and *L. plantarum* C4 at high concentration during 6h, which lead to a pH ~ 4.2.

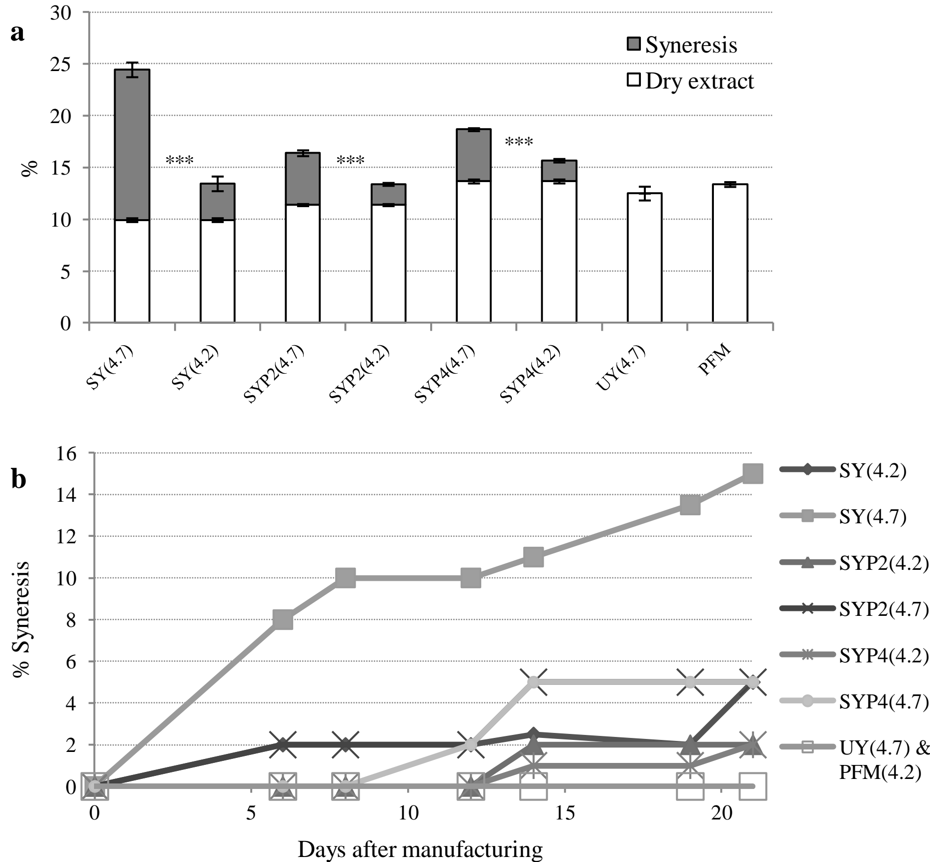

Figure 2.4. Representation of (a) the dry extract content and syneresis after 21 days of storage at 4ºC and (b) the syneresis evolution within the 21 days of storage, in fermented milk samples concentrated by different methods.

SY: Yoghurt made with skimmed milk (SM) fermented with the classical starter bacteria (St); SYP2: Yoghurt made with SM plus 2% of powdered SM fermented with St; SYP4: Yoghurt made with SM plus 4% of powdered SM fermented with St; UY: Yoghurt made with skimmed goat milk concentrated by ultrafiltration (UFM) fermented with St; PFM: Fermented milk made with UFM fermented with St plus *L.plantarum* C4; (4.7): The fermentation was stopped at pH 4.7; (4.2): The fermentation was stopped at pH 4.2. In figure (a), significant differences between the syneresis of the each sample at pH 4.7 and 4.2 were signalled as follows: * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

3.1.2. Viability and interactions of the fermenting bacteria

3.1.2.1. Spot test

When the antibiosis was investigated by the spot test in MRS agar (Selective LAB culture media with high dextrose content), the *L. plantarum* C4 inhibited the growth of both classical starter strains. Additionally, *L. bulgaricus* and *S. thermophilus* inhibited their growth to each other, although they did not inhibit that of the *L. plantarum* C4 (Table 2.2). However this inhibition did not occur in TSA (with low dextrose content),

with the only one exception of the inhibitory effect of *L. plantarum* C4 strain on the growth of *L. bulgaricus*.

Table 2.2. Inhibition among fermenting strains in spot test antibiosis assay expressed as the diameter of inhibition zone (mm)

Tested strain	n	Target strain					
		L. plantarum C4		*L. bulgaricus*		*S. thermophilus*	
		MRS	TSA	MRS	TSA	MRS	TSA
L. plantarum C4	3	12	0	27	18	23	0
L. bulgaricus	3	0	0	15	0	12	0
S. thermophilus	3	0	0	15	0	14	0

MRS: Man Rogosa Sharpe Agar (Lactic-acid bacteria selective culture media); TSA: Tryptone Soy Agar (Generic culture media).

3.1.2.2. Standardisation of starter inoculation, time and temperature of fermentation

As it is shown in table 2.3, whereas at 42 ºC the probiotic bacteria *L. plantarum* C4 hardly grew, at 37ºC it grew up almost one exponential unit. On the other hand, the growth of starter cultures was not affected by the fermentation temperature. Therefore, to obtain the maximum concentration of probiotic bacteria in the final fermented product, the fermentation temperature was established at 37ºC.

Regarding the growth of the fermenting bacteria in milk at 37ºC, when goat milk was fermented by the three strains (SFM and UFM samples), *L. plantarum* C4 did not inhibit the growth of St (Figure 2.5a). Additionally, this probiotic strain did not have any influence in the pH evolution when used lonely (Figure 2.5c). However, *L. plantarum* C4 grew better alone and differences between fermenting UFM and SM were observed, reaching up to 6.93 Log units when fermenting UFM. On the other hand, when used together with St, it grew at 6 h ~ 0.90 and it was no influenced by the milk used, reaching around 6.75 Log units (Figure 2.5b). Thereby, to obtain a load of viable probiotic high enough in the final fermented milk, it was necessary to enhance the initial probiotic concentration in the milk at around the final desired concentration (10^8 - 10^9 cfu/mL). When this enhancement in the number of viable *L. plantarum* C4 was carried out, despite it grew lesser, it was in the fermented product at concentration

high enough to exert its healthy properties and under these conditions, no negative interactions among the fermenting strains were found (Table 2.4).

Taking into account the assays performed, the optimum conditions for the fermentation process were: inoculation of the probiotic strain at 10^9 cfu/mL and fermentation at 37ºC for around 6 h, time that demonstrated to be enough to reach the maximum concentration of *L. plantarum,* checking that pH reached 4.2.

Table 2.3. Study of the viability and evolution of fermenting microorganisms when fermentation was carried out with all them at 37 and 42ºC.

Temperature (ºC)	n	Microorganism (*L. plantarum* C4 + St)	Viable bacteria (cfu/mL) Time = 0h	Time = 6h
42	3	*L. plantarum* C4	2.1×10^4	3.5×10^4
		St	1.6×10^6	9.5×10^8
37	3	*L. plantarum* C4	4.4×10^5	2.8×10^6
		St	2.9×10^6	9.2×10^8

St: Classical starter bacteria (*L. bulgaricus* plus *S. thermophilus)*

Table 2.4. Time evolution of the number of colony-forming units of St and *L. plantarum* C4 at high concentration inocula (10^9 cfu/mL)

Bacteria St	*L. plantarum* C4	n	Incubation time (h)	pH	Viable bacteria (cfu/mL) St	*L. plantarum* C4
Yes		3	0	6.51	7.41×10^6	
			6	4.44	1.00×10^9	
Yes	Yes	3	0	6.28	3.47×10^6	1.17×10^9
			6	4.37	3.47×10^8	$2.09\ \ 10^9$

St: Classical starter bacteria (*L.bulgaricus* plus *S. thermophilus*).

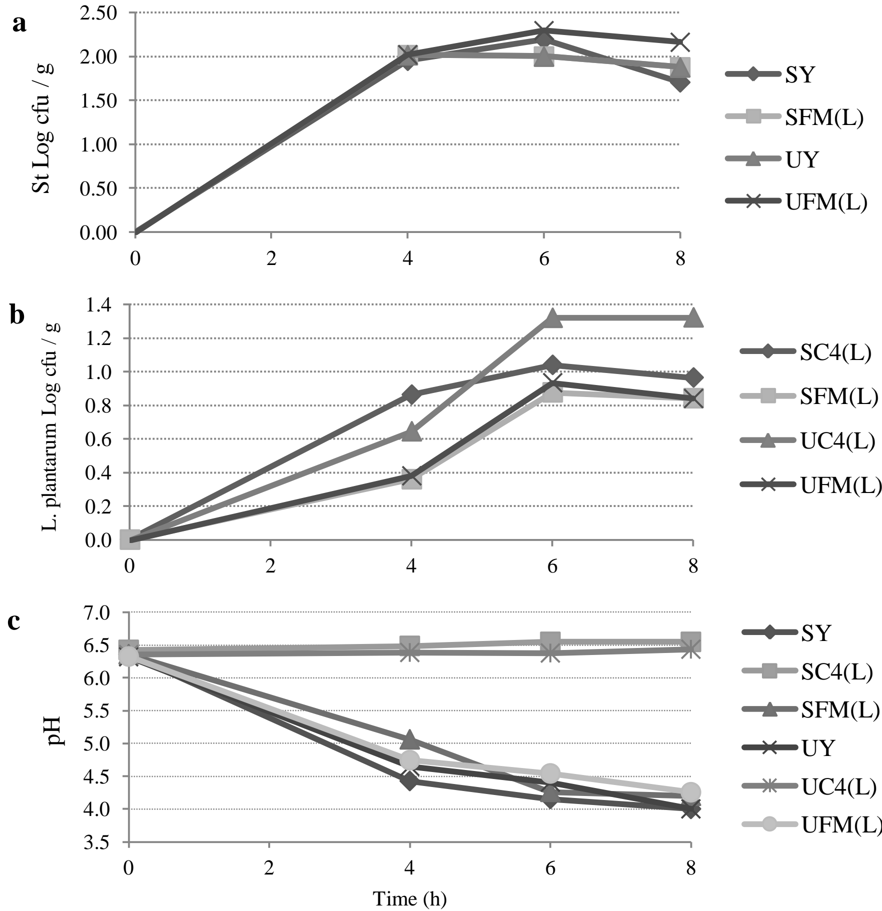

Figure 2.5. Time evolution of the number of viable (a) St (Classical starter bacteria: *L. bultaricus* plus *S. thermophilus)* and (b) *L. plantarum* C4 and (c) pH evolution in the different fermented milk samples during the fermentation.

SY: Skimmed milk (SM) fermented with the St; SFM_L: SM fermented with St plus *L.plantarum* C4 at low concentration level; UY: SM concentrated by ultrafiltration (UFM) fermented with St; UFM_L: UFM fermented with St plus *L.plantarum* C4 at low concentration level. $SC4_L$: SM fermented with *L.plantarum* C4 at low concentration; $UC4_L$: SM concentrated by ultrafiltration (UFM) fermented with *L.plantarum* C4 at low concentration; (L): Low concentration level (10^6cfu/mL); (H): High concentration level (10^9cfu/mL).

3.1.2.3. Final standardised procedure of the fermented milk manufacturing

Once optimized the fermentation conditions, the manufacturing procedure was carried out as follows: After UFM pasteurization (manufactured as described in chapter 1, paragraph 2.1.), it was quickly cooled down to around 45 ºC and inoculated with St, when obtaining UY, and St plus *L. plantarum* C4 at high concentration as described above (paragraphs 2.2.1.and 2.2.3.) when obtaining PFM. After milk homogenization, the inoculated milk was shared out in sterile glass pots of 200 mL and incubated at 37 ºC until pH 4.2 (approximately 6 h). Finally, the pots were quickly cooled down and kept at 4ºC for storage.

3.1.2.4. Viability of microorganisms after *in vitro* gastrointestinal digestion of the probiotic fermented goat milk

After *in vitro* gastric digestion the number of *L. plantarum* C4 dropped almost one Log unit whereas did it St more than two (Figure 2.5). However St and *L. plantarum* C4 showed similar resistance to the intestinal *in vitro* digestion.

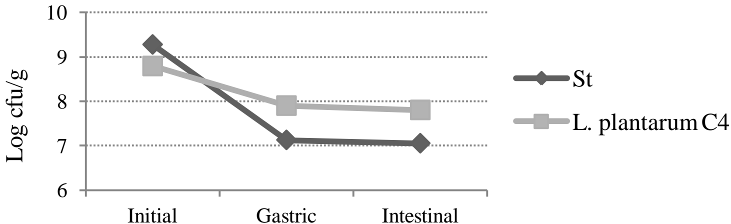

Figure 2.6. Number of viable bacteria in the PFM after *in vitro* gastrointestinal digestion.

St: classical starter bacteria (*L. bulgaricus* plus *S. thermophilus*); PFM: Probiotic fermented goat milk manufactured with skimmed milk concentrated by ultrafiltration and fermented with St and *L. plantarum* C4 by the standardised procedure.

3.2. Characterization of the probiotic fermented goat milk

Table 2.5 shows the average of physicochemical parameters in the characterisation of the PFM and table 2.6 collected the mean concentrations of nutritional parameters.

The mean viable *L. plantarum* C4 in PFM was 8.98 ± 0.32 Log cfu/mL and the mean viable St was 8.72 ± 0.31 Log cfu/mL. Within 4 weeks of cold storage at 4ºC, all the viable bacteria were even increased. After the fourth week, *L. plantarum* C4 began to decrease slowly, whereas St did it after the fifth week (Figure 2.7). Despite that, within 6 weeks of cold storage all the viable bacteria were higher than 10^7 cfu/g.

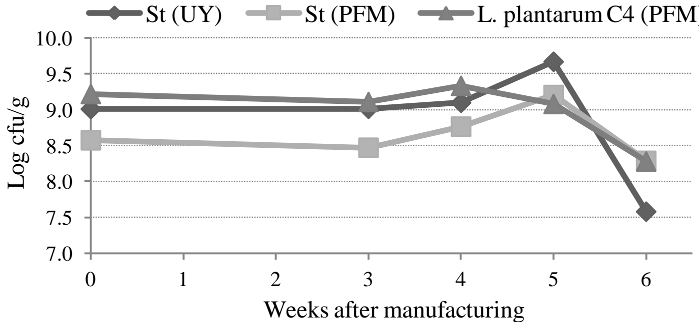

Figure 2.7. Evolution of the viable fermenting bacteria at the end of the fermentation and during the storage at 4ºC.

Starter: Classical starter bacteria (St) *L bulgaricus* and *S.*thermophilus; UY: Yoghurt made with skimmed milk concentrated by ultrafiltration (UFM) fermented with St; PFM: Probiotic fermented goat milk manufactured with UFM and fermented with St plus *L. plantarum* C4 by the standardised procedure.

Table 2.5. Physicochemical characteristics of the developed PFM

Parameter	n	PFM (mean ± SD)
pH	8	4.19 ± 0.23
Total acidity (g lactic acid/100g)	8	1.09 ± 0.18
D-Lactic acid (g/100g)	8	0.368 ± 0.113
L-Lactic acid (g/100g)	8	0.493 ± 0.154
Syneresis (g/100g)	8	0.20 ± 0.25
Dry extract (g/100g)	8	11.5 ± 0.3

PFM: Probiotic fermented goat milk manufactured with skimmed milk concentrated by ultrafiltration and fermented with *L. bulgaricus, S. thermophilus* plus *Lactobacillus plantarum* C4 by the standardised procedure

Table 2.6. Levels for nutritional parameters measured in the developed PFM

Parameter	n	PFM (mean ± SD)
Lactose	8	2.44 ± 0.60
Galactose	8	0.42 ± 0.14
Proteins (g/100g)	8	5.83 ± 0.13
Fat (g/100g)	8	< 0.1
Ca (mg/100g)	8	154 ± 19
P (mg/100g)	8	138 ± 9
Ca/P		1.12
Mg (mg/100g)	8	16.56 ± 3.35
Zn (mg/100g)	8	0.640 ± 0.047
Cu (µg/100g)	8	34.0 ± 3.3
Fe (µg/100g)	8	67.3 ± 14.8

PFM: Probiotic fermented goat milk manufactured with skimmed milk concentrated by ultrafiltration and fermented with *L. bulgaricus, S. thermophilus* plus *Lactobacillus plantarum* C4 by the standardised procedure

3.2.1. Sensorial analysis

In figure 2.8 the sensorial profile of analysed fermented milks is represented. Additionally, table 2.7 shows the parameters for which significant differences were found among fermented milks analysed.

In overview, fermented goat milks presented similar sensorial profile whereas the CFM profile was different. A significant correlation ($p<0.001$) was observed among the appearance parameters namely pure white, syneresis and smoothness (White-Syneresis: r^2 = -0.548; White-Smoothness: r^2 = 0.494; Syneresis-Smoothness: r^2 = -0.806). Consequently, if the sample had the purest white, it would have the least syneresis and

would be the smoothest. The purest white colour was found in the PFM, while the one for CFM was the least pure white. The least syneresis was found in PFM, and the most syneresis together with the least smoothness were observed in CFM. Therefore, the appearance of CFM milk (which includes the colour, syneresis and smoothness) was the worst.

The sample with the best texture parameters was the PFM, but only the viscosity was significantly different to the other samples. A weak and significant correlation ($p<0.01$) indicated that the highest acidity the least syneresis (r^2 = -0.290) and the highest viscosity (r^2 = 0.330). Taste fineness, which was almost the same in the three samples, was significantly correlated with the overall acceptability ($p<0.001$; r^2 = 0.747), aroma fineness ($p<0.05$; r^2 = 0.337), sweetness ($p<0.01$; r^2 = 0.409) and negatively with the acidity ($p<0.05$; r^2 = -0.399). In relation to aroma parameters, in spite of the significant correlation among some of them (Aroma fineness-intensity: $p<0.01$, r^2 = 0.406; Aroma fineness-persistency: $p<0.001$, r^2 =0.510; Aroma intensity-persistency: $p<0.001$, r^2 =0.735), only the aroma intensity was significantly lower in PFM than those in the other two yoghurts.

The sweetness and bitterness were hardly appreciated by the panellists, probably because the high acidity hided them. Finally, although fermented goat milks showed better overall acceptability than CFM, no significant differences were observed.

The parameters evaluated as presence/absence or in a scale from undetected to detected at high intensity were expressed as percentage of panellist who detected them (Figures 2.9, 2.10 and 2.11). The positive parameters found by more than 40% of panellists and the negative by more than 10% were chosen as representative positive or negative attributes of each fermented milk (Table 2.8). Positive attributes were expressed as highly representative, representative or non representative attribute and defects, depending on the percentage of perception, did that the analysed fermented milk were rejectable, possibly rejectable or non rejectable.

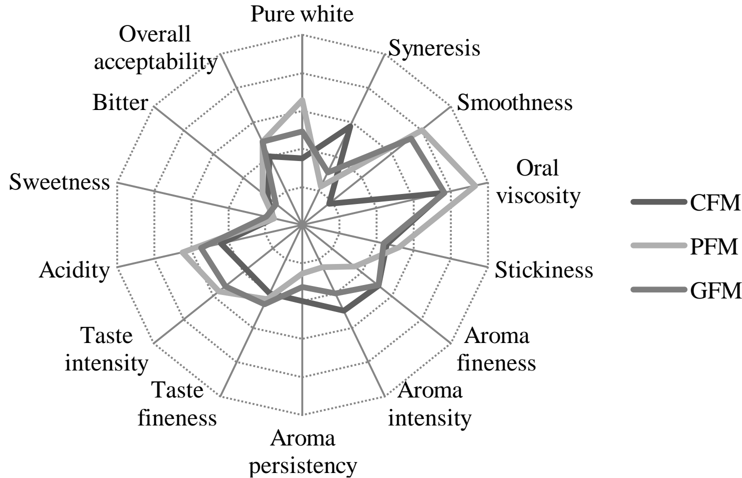

Figure 2.8. Representation of the quantitative sensorial parameters of analysed fermented milks.

CFM: Commercial skimmed cow yoghurt fermented with the classical starter bacteria (St); PFM: Probiotic fermented goat milk manufactured with skimmed milk concentrated by ultrafiltration and fermented with St plus *Lactobacillus plantarum* C4 by the standardised procedure; GFM: Commercial skimmed fermented goat milk fermented with St and an strain of *Bifidobacterium.*

Table 2.7. Parameters for which significant differences were observed among the three fermented milks[a].

Sample	Pure white	Syneresis	Smoothness	Oral viscosity	Aroma intensity	Aroma persistency	Taste intensity
CFM	a** c	a** c**	a** b**	a	a**	a	a* b
PFM	a** b*	a** b	a**	a b	a** b	a	a*
GFM	b* c	b c**	b**	b	b		b

CFM: Commercial skimmed cow yoghurt fermented with the classical starter bacteria (St); PFM: Probiotic fermented goat milk manufactured with skimmed milk concentrated by ultrafiltration and fermented with St plus *Lactobacillus plantarum* C4 by the standardised procedure; GFM: Commercial skimmed fermented goat milk fermented with St and an strain of *Bifidobacterium* .[a]No significantly differences were observed for: Stickiness, aroma fineness, taste fineness, acidity, sweetness, bitterness and overall acceptability. Same letters means statitatistically significant differences among samples; $^{letter}p$<0.05; ^{letter}*p<0.01; ^{letter}**p<0.001

Table 2.8. Organoleptic descriptors of the analysed fermented milks

		Parameter	CFM	PFM	GFM
Visual	+	Curd homogeneity			
	-	Floury			
		Lumps			
		Bubbles			
Aroma	+	Fermentation			
		Acetaldehide			
		Dyacetil			
		Goat			
		Flower			
		Fruit			
	-	Boiled milk			
		Other			
Taste	+	Goat			
		Fruit			
		Astringency			
		Spicy			
		Metallic			
		Salty			
	-	Insipid			
		Dirty			
		Rancid			
		Yeast			
		Boiled milk			
		Humidity			

+: Positive attribute:	<40%: No representative	>40%: Representative	>60% Very representative
-: Negative attribute:	<10%: No rejectable	>10% Possibly Rejectable	>20% Very rejectable

CFM: Commercial skimmed cow yoghurt fermented with the classical starter bacteria (St); PFM: Probiotic fermented goat milk manufactured with skimmed milk concentrated by ultrafiltration and fermented with St plus *Lactobacillus plantarum* C4 by the standardised procedure; GFM: Commercial skimmed fermented goat milk fermented with St plus an strain of *Bifidobacterium.*

As it is shown in table 2.8 (and figure 2.9), PFM was the best visually evaluated by the panelists, defined with a homogeneous curd and with the least negative visual parameters. In accordance to the results previously described, the CFM was the one with the worst visual parameters, where all negative attributes were perceived.

Regarding the aroma parameters, as it was previously described, in PFM they were hardly perceived. Although for the acetaldehyde the CFM and GFM had similar values, in CFM was perceived fermentation aroma and the boiled milk aroma defect. The "other aroma" perceived in PFM were different, for which we did not consider it as representative.

With respect to taste parameters, goat was very representative for both fermented goat milks, whereas astringency was perceived as representative only in GFM. All the analysed defects were perceived in some of the three fermented milks being noteworthy the insipid taste in CFM. The others were in the range to be acceptable.

To summarize, the PFM could be described as fermented milk with good appearance and texture, where only few lumps were appreciated. It did not have strong aroma, and despite some negative taste parameters were found, and the high acidity measured, it was in overall accepted in the range of the other analysed commercial fermented milks.

Finally, the positive descriptors of our fermented milk were: curd homogeneity and goat taste. On the other hand, the negative were: lumps and dirty, rancid, yeast and boiled milk taste.

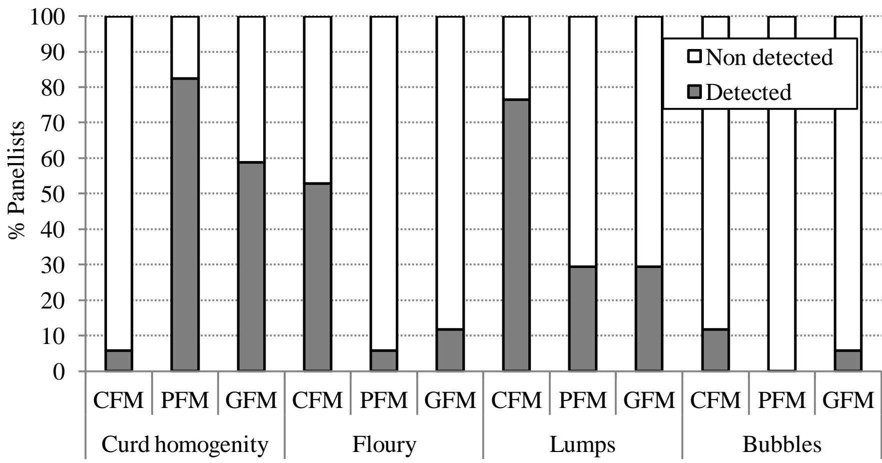

Figure 2.9. Percentage of panellists who detected or not the visual parameters on fermented milks analysed.

CFM: Commercial skimmed cow yoghurt fermented with the classical starter bacteria (St); PFM: Probiotic fermented goat milk manufactured with skimmed milk concentrated by ultrafiltration and fermented with St plus *Lactobacillus plantarum* C4 by the standardised procedure; GFM: Commercial skimmed fermented goat milk fermented with St and an strain of *Bifidobacterium.*

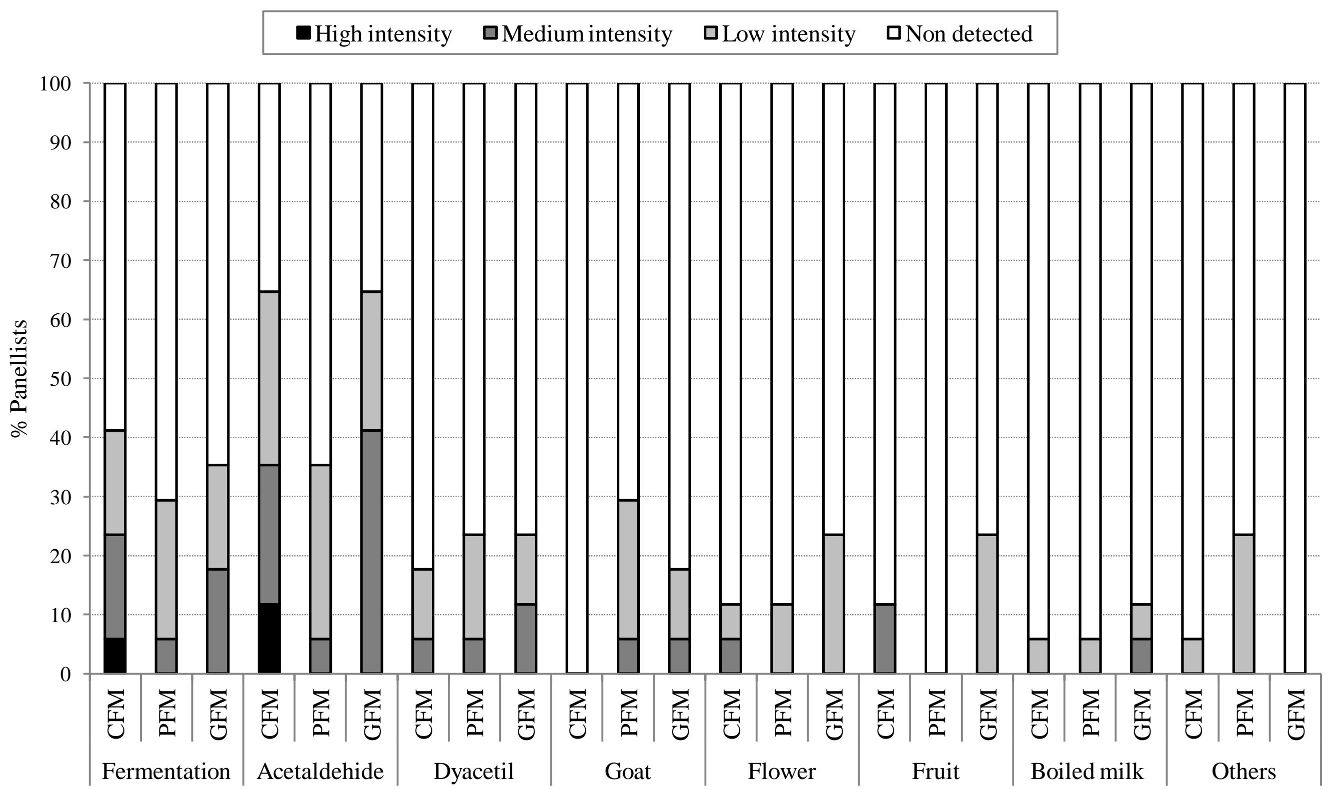

Figure 2.10. Percentage of panellists who detected each aroma parameter and its intensity on the different fermented milks analysed.

CFM: Commercial skimmed cow yoghurt fermented with the classical starter bacteria (St); PFM: Probiotic fermented goat milk manufactured with skimmed milk concentrated by ultrafiltration and fermented with St plus *Lactobacillus plantarum* C4 by the standardised procedure; GFM: Commercial skimmed fermented goat milk fermented with St and an strain of *Bifidobacterium.*

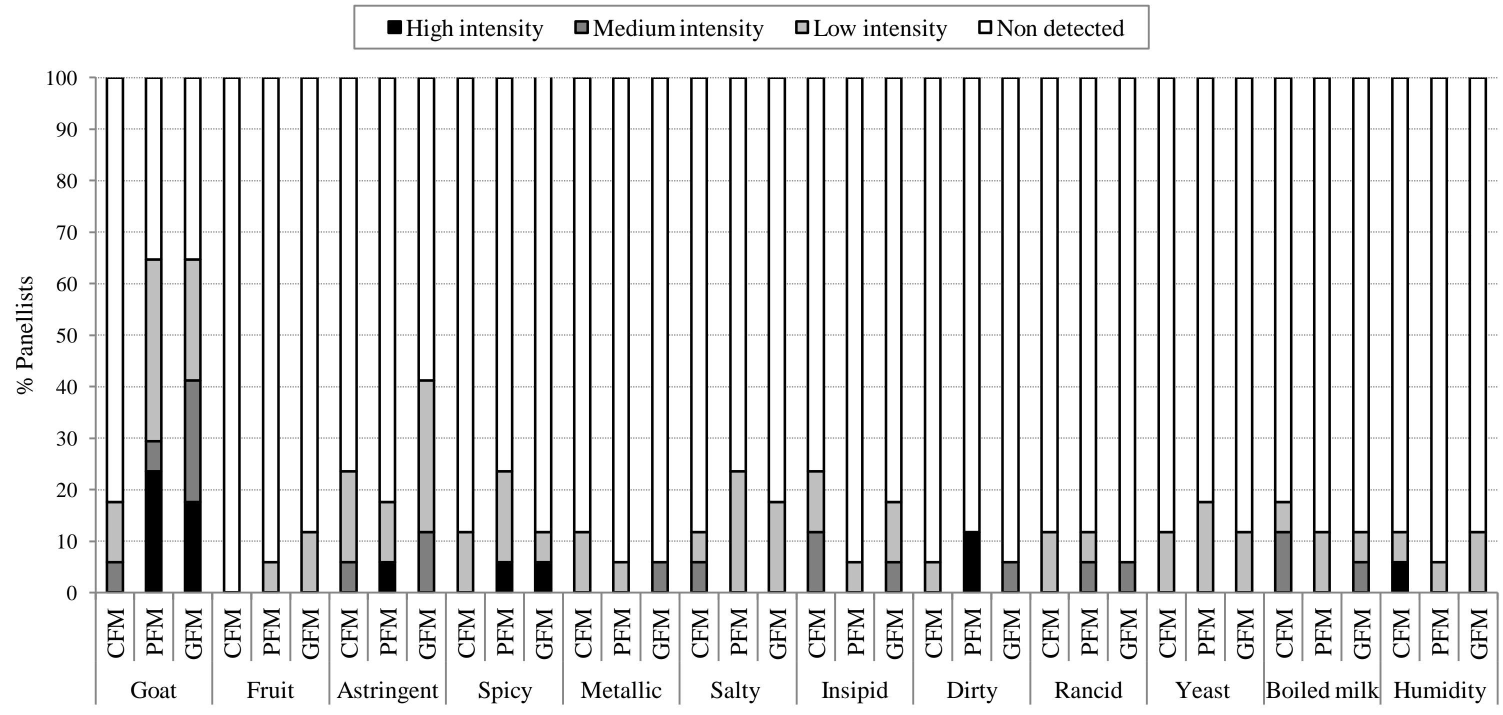

Figure 2.11. Percentage of panellists who detected each taste parameter and its intensity on the different fermented milks analysed.

CFM: Commercial skimmed cow yoghurt fermented with the classical starter bacteria (St); PFM: Probiotic fermented goat milk manufactured with skimmed milk concentrated by ultrafiltration and fermented with St plus *Lactobacillus plantarum* C4 by the standardised procedure; GFM: Commercial skimmed fermented goat milk fermented with St and a strain of *Bifidobacterium.*

4. Discussion

4.1. Viability and interaction between microorganisms

Currently, the importance of the bacterial strains study (viability and possible strains interactions) and selection for an efficient fermentation has been highlighted (Vinderola et al., 2002). The spot test results showed the growth inhibition of St by *L. plantarum* C4 in MRS but not in TSA. It suggested that it was due to the acid production from dextrose, which is in high concentration in MRS. The action of *L. plantarum* C4 on *L. bulgaricus* in TSA is perhaps attributable to bacteriocin-like produced by *L. plantarum* C4. Nevertheless, when the three strains were co-cultured in goat milk, the *L. plantarum* C4 did not inhibit the growth of both St. The main difference could be attributable to whereas in MRS microorganisms use dextrose, in milk they use lactose, which showed the importance of the environmental factors as the nature and concentration of sugars in each media. This observation has been widely reported by researches about the different growth and fermentation taxes of probiotics depending on the carbohydrates in the fermentation media (Champagne et al., 2009; Chervaux et al., 2000; Mlobeli et al., 1998; Perrin et al., 2001; Zhang et al., 2006).

Despite some interaction have been described between probiotic strains and the microorganisms of St, during the milk fermentation both microorganisms present in St grew in the same proportion alone and with the probiotic strain (Vinderola et al., 2002). The *L. plantarum* C4 grew less than St and when fermentation was carried out with the three strains it did not show significant changes, as it was demonstrated for the probiotic bacteria *L. helveticus* R0050 probiotic (Champagne et al., 2009). This is an example of the variability in relations and interactions between St and probiotics and it remarks the necessity to continue researching if they are due to the subtract competition, production of inhibitory compounds, proteolysis or just to the evolution of the pH during the fermentation (Wang et al., 2002).

In relation with the number of microorganisms, all them reached levels higher than 10^7cfu/g, which is the minimum required to manufacture fermented milk, and it is in the range reported by others for starter cultures (Beal et al., 1999; Real Decreto 179/2003). The probiotic inoculation at the same time as St was also described by other authors as

effective to this purpose (Champagne et al., 2009; Minervini et al., 2009). Furthermore, we found that *L. plantarum* C4 survived at around 10^8 cfu/g after *in vitro* gastrointestinal digestion, decreasing only a 10% (90% of resistance). This concentration is the minimum, supported by several studies, necessary for a probiotic strain to exert its function and provide the benefits attributable to it in the consumer (Galdeano et al., 2004; Parvez et al., 2006; Shah, 2007). Bujalance et al. (2007) demonstrated the *L. plantarum* C4 resistance to gastric acid (around 60% of resistance of bacteria in PBS) and bile (~100% resistance) as well as the buffering effect of the milk against acidification, choosing this matrix to administrate this probiotic bacteria to mice. Then, the observed resistance of the *L plantarum* C4 in yoghurt could be due, besides its own resistance to the acid environment, to a protective effect exerted by the yoghurt matrix. Regarding the starter bacteria, different behaviour against low pH (assayed with bacteria suspended PBS) has been reported depending on the strain, being described between 0 and 48% of resistance for *L. bulgaricus* and between 0 and 45% of resistance for *S. thermophilus* (Bokeet al., 2010). With regard to the intestinal stage, despite reported high resistance to bile salts for *L. plantarum* C4, medium resistance for *S. thermophilus* and high vulnerability for *L. bulgaricus* were reported, in our results all the bacteria showed high resistance (99%%) (Boke et al., 2010; Bujalance et al., 2007). In addition to the probable matrix protective effect, the differences found with reported viability could be owing to the differences among strains.

With this context, the viability of the fermenting bacteria was maintained at good concentration during the fermentation, and despite some interactions were described in culture media, they were no observed in milk fermentation. Additionally, they demonstrated to be resistant to *in vitro* gastrointestinal digestion, in part probably due to the yoghurt matrix protective effect.

4.2. Effects on texture of different concentration methods

The physical properties of set yoghurt are some of the quality parameters that play an important role in consumer acceptance. Spontaneous syneresis is the contraction of a gel without the application of any external force (e.g., centrifugation) and it is related to instability of the gel network resulting in the loss of the ability to entrap all the serum phase (Lucey, 2002). One of the difficulties to study the syneresis is the absence of a

standardised method to its quantification, making more difficult the comparison of the results. Among the different factors related with the viscosity and development of syneresis in fermented milks, some of them were chosen in the PFM manufacturing by other reasons like fat content, pasteurisation and incubation temperature. With these characteristics already chosen, the protein concentration and solid content were the factors on which we acted. The improvement in the rheological behaviour of the samples with the enhancement in the solid content was also previously described by other authors (Abbasi et al., 2009; Güler-Akin et al., 2009). However, in spite of the same solid content, the addition of powdered skimmed milk in comparison with the use of milk concentrated by ultrafiltration led to similar viscosity but more syneresis. On this sense, the differences in rheology properties of the fermented milks when concentrated by different methods with the same protein concentration were previously reported (Marafon et al., 2011). The differences found could be due to: not well dissolution of the powdered skimmed goat milk, the casein alteration by the spry-drying process or the increasing of all milk compounds in the same proportion which could lead to less concentration of casein in suspension able to retain water when coagulated than in milk concentrated by ultrafiltration. In addition, with the ultrafiltration, in spite of to lose part of the soluble compounds such as whey proteins, lactose or some soluble minerals in the filtrate, it is mainly concentrated in caseins that would improve the yoghurt rheology (González-Martinez et al., 2002). With regard to the different syneresis in fermented milks depending on the pH, it is well known that caseins start they aggregation at their isoelectric point, forming a fragile gel network (Espírito-Santo et al., 2013). Nevertheless, the isoelectric point of goat milk caseins is 4.2, which is different to the cow one (4.7) (Rojas-Castro et al., 2007). Then, at 4.7 pH the goat milk caseins are starting the aggregation and the lesser caseins aggregated the worse gel network formation and therefore the more syneresis resulting. Finally, the fermentation at 37 ºC could influence a better whey retention according to (Bensmira et al., 2012).

In summary, ultrafiltration demonstrated to be a better way of goat milk concentration than the addition of powdered milk, as it improved the whey retention in the final product. Additionally, the addition of *L. plantarum* C4 could be interesting because it

did not change the texture properties of the final product and it could contribute to obtain a healthier final product due to its probiotic effects.

4.3. Characteristics of the standardised fermented goat milk

4.3.1. Dry extract measurement

This parameter depends mainly on the fat, protein and carbohydrates contents of the milk and for this reason it can vary widely depending on the milk used. The dry extract of UFM was previously selected (chapter 1) because it demonstrated to be adequate to reach good texture parameters, increasing in that way other nutritional compounds on the obtained fermented milk. Reported values for fermented milks ranged from 8.10-9.88% (Merin, 2000; Quintana López, 2011) when skimmed milk was used, to 17.8 % when was employed whole milk previously concentrated (Martín-Diana et al., 2003).

4.3.2. Spontaneous syneresis

The mean value for syneresis of PFM was very low (0.20 ± 0.25 %), which means a good coagula formation with an adequate whey retention, due mainly to a good protein concentration and manufacturing process (Jacek Domagała, 2009). It has been also demonstrated above the importance of the acidity in this process, overall if the ultrafiltration was not carried out (paragraph 4.2).

4.3.3. pH, acidity and D/L- lactic acid levels

In comparison to reported values for fermented goat milks, despite the pH of the PFM was in the range (from 3.83 to 4.32 g lactic acid/100g), the acidity was slightly higher (from 0.876 to 1.08 g lactic acid/100g), and in the upper limit reported for fermented cow milks (from 0.5 to 1.1 g lactic acid/100g; Dave et al., 1997; Erkaya et al., 2011; González-Martinez et al., 2002; Güler-Akin et al., 2009; Quintana, 2011). However, it is in the range reported for fermented milks when concentration was carried out to the milk, where a bit higher acidity was found (from 1.07 to 1.15 g lactic acid/100g; Marafon et al., 2011). These researchers showed that titratable acidity content in yoghurts was influenced by the type of protein used to fortify the total solid content of the milk base, and then, the increased protein concentration in PFM could be the reason of its high value.

According to Beal et al. (1999), the lactic acidification by the starter strains is influenced by the quality of the milk, the strains used and the incubation temperature. Furthermore, he described that the post-acidification process contributes also to the acidity and it is mainly affected by the strains used, the storage temperature and time. The acidity in our samples was measured the fifth day of storage and, despite *L. plantarum* C4 demonstrated to be lactose +, its effect on the fermented milk acidity had not been previously studied (Bujalance, 2006).

In relation with the lactic acid isomers, the D-lactic is produced by *L. bulgaricus* and it was attributable to the post-acidification, whereas the L-lactic is produced by *S. thermophilus* and it is usually more concentrated in non stored fermented milks (Beal et al., 1999; Feller et al., 1989; Homons, 1999; Kneifel, 1993). Therefore, the proportion of both isomers depends on the fermentation intensity by the fermenting bacterias and the storage time and conditions. The percentage of L/D-lactic acid in PFM was 57/43, which mean a similar fermenting activity of both St bacteria. These values were very similar to five fermented cow milks reported by Kneifel et al. (1993) fermented with commercial strains, which ranged from 55/45 to 60/40. Among the other samples analysed by those authors high variavility was observed (36-100% L-lactic acid and 0-64 % D-Lactic acid), but generally higher L-lactic concentration was found, as it was observed in analysed samples. Other authors reported different amount of these isomers, from 0.13 to 0.6 g L-Lactic acid/100g and from 0.5 to 0.93 g D-lactic acid/100g.

4.3.4. Lactose levels

The lactose content is one of the main responsible in the curd formation and, independently of the protein concentration, when lactose concentration was lesser than 2% in milk it led to a soft curd that could not be considered as a yoghurt-like product (Álvarez et al., 1998). In this sense, the lactose concentration in UFM used for the fermentation was 4.92 g/100g, percentage high enough to get a good curd according to those findings. The lactose of UFM was reduced in PFM to 2.44g/100g, which reinforces the fermentation by the strains, making this by-product more digestible than the milk. This percentage in PFM was in the range reported for fermented goat milks by

other authors, which goes from 1.19 to 4.59 g/100g (Kneifel et al., 1993; Martín-Diana et al., 2003; Merin, 2000; Quintana, 2011).

In spite of the high acidity found in PFM, no much galactose (one of the end products of milk fermentation) was measured, whereas other authors reported values up to 1.64 g/100g (Quintana, 2011). It could be owing to *L. plantarum* C4 metabolized this carbohydrate because it is galactose + (Bujalance, 2006). However, in fermented cow milks and kefir, the lactose and galactose percentage reported by Quintana (2011) was similar to the one found by us (lactose: 2.82 and 2.97%; galactose: 0.68 and 0.37%; respectively). This support the hypothesis that these differences could be due to the different fermenting strains used (Kneifel et al., 1993).

4.3.5. Protein concentration

The protein concentration of PFM was much higher than the reported for fermented goat milks (from 2.97 to 3.99 %; Merin, 2000; Y. Park, 2000; Quintana, 2011) as consequence of the concentration carried out by ultrafiltration. However, compared to fermented milks made with milk concentrated by different methods, the differences were lower. The values for this milks ranged from 3.54 to 5.90 % (when concentrated x1.5) to 5.9 % (when concentrated x2) (Magenis et al., 2006; Rinaldoni et al., 2009). On the other hand, the addition of skim milk powder performed by others led to lesser protein concentration than those found by us in the present study, even adding 2 or 4% the protein concentration ranged from 2.8 to 4.85 g/100g respectively (Herrero et al., 2006; Ünal et al., 2013).

Due to the high protein concentration of PFM, the known better characteristics of goat milk proteins than cow milk ones, together with ultrafiltration process, which did not alter the proteins by heating, we could consider PFM a better source of proteins than other reported fermented milks.

4.3.6. Fat concentration

In Spain the skimmed milk consumption is rising in decrease of the whole fat milk (MAGRAMA, 2012). The fat concentration was lower than the detection limit of the method, considered the developed fermented goat milk as skimmed, which will allow us to commercialize it section of light, 0%, healthy products, etc.

4.3.7. Mineral (Ca, P, Mg, Zn, Cu and Fe) levels

Milk and dairy products are excellent sources of certain minerals. Recently, the mineral concentration of goat milk was reported higher than these of cow milk for Ca, Mg, P, Cu, K, Fe, Mn and I (Haenlein, 2001; Park et al., 2007; Raynal-Ljutovac et al., 2008; Rutherfurd et al., 2006; Sanz Ceballos et al., 2009). Although, the ultrafiltration increased the mineral concentration of skimmed milk, as discussed in chapter 1, the concentration of analysed minerals in the experimental fermented goat milks was in the range of values reported by others (Table 2.9). Those reported values are mainly from commercial fermented goat milks and probably, the high mineral concentration reported could be a result of the increase in total solids with the addition of dairy ingredients, usually carried out in the fermented milk manufacturing and not reported (Slačanac et al., 2010). Particularly, the reported concentration of Ca in fermented milks concentrated in proteins ranged from 118 mg/100g, when concentrated with a 2% of whey protein concentrate, to 197 mg/100g when concentrated x2 by ultrafiltration (Martín-Diana et al., 2003; Rinaldoni et al., 2009). The P concentration found by us is a bit higher than that reported. This could be owing to the increase in casein concentration with the ultrafiltration, because Ca, P and Mg are associated to milk as calcium colloid phosphate, which is responsible, together with hydrophobic interactions, of the linkage of the sub-micelles for the micelles formation (Gösta, 2003; Moreno et al., 2013). Nevertheless, the concentration of Mg is in the range reported by others. The Ca/P relationship is in the ratio reported for dairy products (Table 2.11), which improves its absorption due to if it is higher than 1.5 the Ca is excreted and if it is lower than 1 it is not well absorbed (Baró Rodríguez et al., 2010). Due to the 90-95% of Zn is linked to caseins, its mean concentration was higher than most a of reported values for commercial fermented milks (De la Fuente et al., 2003). Finally, the Cu and Fe, which are bonded to the fat globule, decreased their concentration with the skimming process and did not increased with the ultrafiltration (De la Fuente et al., 2003).

Table 2.9. Reported mineral concentration of different goat fermented milks.

Mineral	n	PFM	Reported	Reference
Ca (mg/100g)	10	154.1 ± 18.54	111.0-197.0	(Güler et al., 2009; Martín-Diana et al., 2003; Navarro-Alarcón et al., 2011; Park, 2000; Quintana-López, 2011)
P (mg/100g)	10	138.2 ± 9.448	78-127.7	(Bergillos-Meca et al., 2013; Güler et al., 2009; Park, 2000; Quintana-López, 2011)
Ca/P		1.12	1-1.5	(Baró-Rodríguez et al., 2010)
Mg (mg/100g)	10	16.564 ± 3.352	8.79-39.90	(Güler et al., 2009; Martín-Diana et al., 2003; Park, 2000; Quintana-López, 2011)
Zn (mg/100g)	10	0.640 ± 0.047	0.370-0.514	(Güler et al., 2009; Park, 2000; Quintana-López, 2011)
Cu (µg/100g)	10	33.99 ± 3.348	21-80	(Güler et al., 2009; Park, 2000; Quintana-López, 2011)
Fe (µg/100g)	10	67.27 ± 14.84	48-102	(Güler et al., 2009; Park, 2000)

PFM: Probiotic fermented goat milk manufactured with skimmed milk concentrated by ultrafiltration and fermented with *L. bulgaricus, S. thermophilus* plus *Lactobacillus plantarum* C4 by the standardised procedure.

4.4. Sensorial analysis

The colour of the fermented milk is the first attribute perceived by the consumer. The acidification and coagulation processes in set-style yoghurt have been demonstrated a shift toward to yellow colour of the milk (Kneifel et al., 1992). In addition, it is known that the cow milk is richer than cow one in vitamim A, which gives to the milk a slight yellow hue and could be the reason why cow yoghurt was perceived as the one with the least pure white. However, both fermented goat milks showed some differences. Agata et al. (2012) stated that as much higher is the incubation temperature, it is concomitantly higher is this colour. In that sense we could hypothesize that GFM was fermented a higher temperature than the PFM. In addition, the colour correlation with syneresis and smoothness, which both are improved at low incubation temperature, can support that hypothesis.

A curd formed by big grains with high syneresis is a negative characteristic that could lead to the consumer to reject the fermented milk (Domagała, 2009). The importance of pH on the viscosity and syneresis was previously observed, and small correlation of

these parameters was found in sensorial analysis. In that sense, when the water is better retained in the protein network there will be fewer tendency to syneresis (Domagala et al., 2012). However, other parameters such as total solids content, concentration of Ca^{2+} and fat, pH, fermentation temperature and preheat treatment of the milks are also important (Bensmira et al., 2012; Jacek Domagała, 2012)

Since the vapor phase odor is first perceived when opening a yoghurt pot, its quality would influence the consumer's preference (Ott et al., 1997). It is widely reported that volatile compounds generated during fermentation process and responsible of the final aroma depend, in addition to the milk used, on the strain and conditions used for the fermentation as well as the storage time and conditions (Domagala, 2008; Hruskar et al., 1995; Imhof et al., 1994, 1995). For this reason is not easy to establish the reason of the different aromas found in the fermented milks due to the unknown manufacturing conditions for the commercial ones. The yoghurt aroma is generally ascribed to acetaldehyde produced by St, mainly by *L. bulgaricus,* from threonine. It has been even described the concentration at which yoghurt aroma is optimum and despite other volatile organic aroma compounds have been identified, their contribution to yoghurt quality has not been clearly demonstrated (Beal et al., 1999; Hruskar et al., 1995). As goat milk protein contains more threonine residues than cow milk protein it must be expected more acetaldehyde levels in yoghurts produced from this milk (Marshall et al., 1986). However, due to the acetaldehyde formation in yoghurt production occurred within the first 6 h, the fermentation conditions would influence the concentration of this metabolite (Imhof et al., 1994). Nevertheless, aroma parameters, and more specifically, acetaldehyde aroma was less perceived in PFM than in the other two fermented milks. This lack of flavour was also reported in fermented goat milks concentrated by ultrafiltration by Kavas et al., (2003). It has been also reported that many starter organisms metabolise acetaldehyde to ethanol, decreasing during storage the acetaldehyde concentration, but *S. thermophilus* and *L. bulgaricus* do not have alcohol dehydrogenase (Hruskar et al., 1995). However, not many studies have been carried out regarding the alcohol dehydrogenase activity of *L. plantarum* and none about the *L. plantarum* C4. It was described in *L. plantarum* an enzyme that has activity toward benzyl alcohol, which also showed activity against other volatile compounds

involved in determining food aroma (Landete et al., 2008). Therefore *L. plantarum* C4 could influence be the responsible of the lesser aroma found in PFM. According to Hruskar et al. (1995), dyacetil concentration increases during storage and it is especially important if acetaldehyde is low because it can enhance the yoghurt flavor, but for the analysed fermented milks it was not a descriptor, probably because they were recently made.

On the other hand, the goat flavour is considered a negative quality parameter and some researchers recommended remove it to be preferred in comparison to fermented cow by-products (Domagala, 2008; Shekarchian, 2013). In both fermented goat milk analysed goat aroma was not highly perceived, however, goat taste was perceived in by more than 60% of the panellists. Due to the fact that this flavour is caused by the short chain fatty acids, this taste was probably soften by the skimming process. In addition, fermentation decreases this flavour and owing to this taste increases during storage, this kind of fermented milks must be consumed as soon as possible to avoid the increasing of the goat flavour (Domagala et al., 2012).

The taste fineness, which was weakly correlated with aroma fineness, was the factor which most influences the overall acceptability. The taste fineness was mainly influenced by the sweetness in a positive way and acidity in a negative way, relation supported by others (Lucey, 2002). Some authors reported that when milk concentration by ultrafiltration was used to manufacture fermented milks, lower acidity is perceived in yoghurts due to the higher buffering capacity of the concentrates by their concentration of proteins, however, no significant differences were found among samples (Domagala et al., 2012). Finally, despite the differences in the profile, no significant differences were observed in overall acceptability and then the products would be accepted in a similar way.

5. Conclusions

A new probiotic skimmed fermented goat milk (PFM) has been developed using concomitantly the classical starter cultures *L. bulgaricus* and *S. thermophilus* plus the probiotic strain *L. plantarum* C4. This PFM was manufactured with skimmed goat milk concentrated by ultrafiltration, which gave the best syneresis and viscosity parameters. Additionally, the bacterial strains used were viable at 10^7cfu/mL even after *in vitro* gastrointestinal digestion. When compared with two commercial fermented milks PFM showed in sensorial analysis the best visual parameters and viscosity. Finally, its low lactose (2.44%) and fat concentration (< 0.1 %), high protein proportion (5.83%) and good mineral content (Ca [154 ± 19 mg/100g], P [138 ± 9 mg/100g], Mg [16.56 ± 3.35 mg/100g], Zn [0.640 ± 0.047 mg/100g], Cu [34.0 ± 3.3 µg/100g] and Fe [67.3 ± 14.8 µg/100g), together with the overall acceptability described by the panellist, could lead us to consider this PFM such a good dairy product to be commercialised.

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CHAPTER 3

Biological activities of different fractions from two novel fermented goat milks

Biological activities of different fractions from two novel fermented goat milks.

Abstract

In addition to the already known benefits of the fermented milks on the organism, nowadays is increasing the interest, and hence the research, on their benefits beyond their nutritional value. The total antioxidant capacity (TAC) determined by different methods (ORAC, ABTS, DPPH and FRAP) as well as the angiotensin-I-converting-enzyme inhibitory (ACEi) and the antibacterial activities against *Escherichia coli* and *Micrococcus luteus* of different fractions of two novel fermented skimmed goat milks, were assayed. The first milk concentrated by ultrafiltration was (a) fermented with the classical starter bacteria (St) *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus,* obtaining the sample UY (Ultrafiltered yoghurt); (b) another one with St plus the probiotic strain *Lactobacillus plantarum* C4, obtaining the PFM (Probiotic fermented milk). From these fermented milks were separated: the WHEY fraction (fermented milk fraction after centrifugation); Permeate (P) and Retentate (R) after WHEY cation exchange and the fractions after P ultrafiltration through 3 kDa cut-off membrane < 3 kDa (P<3) and > 3 kDa (P>3). In general, no different activities were observed in UY and PFM. The peptides present in the P<3 fraction showed the highest TAC in ORAC assay and ACEi activities. However, the TAC of cationic peptides and proteins (at pH 4.5) against $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals, in comparison with other fractions was the highest. Those peptides and proteins in PFM also showed low antibacterial activity against *E. coli,* which could be due to some peptides released by *L. plantarum* C4. Despite the small bioactive peptides could be responsible of most of ACEi and antioxidant activities, the WHEY had also an important bioactivity, which reinforces the benefits of the fermented milk intake, in this case from goat milk, in the prevention of cardiovascular diseases associated to oxidative stress and hypertension.

1. Introduction

The yoghurt is a widely consumed dairy product owing to the fact that it fulfils many current dietary needs and its consumption has been reported to be able to exert a number of health benefits (Farvin et al., 2010). Furthermore, the yoghurt is a great source to supply several bacterial strains as long as it exists an appropriate compatibility among all of them (Erdmann et al., 2008). For this reason many fermented milks contain several probiotic strains, which increase the already known benefits of these dairy products. Milk fermentation by classical starter bacteria (St) (*Lactobacillus delbrueckii* subsp. *Bulgaricus* and *Streptococcus thermophilus*) changes the milk properties and increases its digestibility by a decrease in the lactose concentration and pH. This process could also release biological active peptides from their inactive forms present in the corresponding sequence of the precursor protein. The specific sequence and length of released peptides depends on two main factors: (a) the precursor protein, which is different in sequence depending on the animal species and even on the breed (Li et al., 2013); (b) the starter bacteria, because the proteolytic system is inherent to each bacteria strain. The healthy benefits of these bioactive peptides may be attributed to their demonstrated antibacterial, antioxidant, antihypertensive, antithrombotic, immunomodulatory and opioid activities among others (Korhonen, 2009). Many of the bioactive peptides have demonstrated to have multi-functional properties. Nevertheless, its specific activity depends on the amino acid composition as well as sequence. In this sense, it is well known that anionic peptides do not affect gram-negative bacteria, with negatively charged outer membrane, which likely repel anionic peptides (Demers-Mathieu et al., 2013). Despite this, a positively charged peptide does not ensure antibacterial activity and the action mechanism of milk-derived antimicrobial peptides remains uncertain (Benkerroum, 2010). Anyway, several biopeptides with antibacterial activity useful for a further application in industry have been discovered (Benkerroum, 2010).

Among the different functions of bioactive peptides, antioxidant properties are very important because high levels of reactive oxygen species and free radicals in the organism are associated to several diseases like cancer, diabetes, cardiovascular diseases, arthritis, allergies as well as to the aging (Chang et al., 2013; Unal et al.,

2012). In addition, their presence in food causes quality deterioration and shelf life reduction by lipid oxidation (Li et al., 2013). It is known that the defense systems of organisms are at many times not enough to prevent oxidative damage. Some researchers have stated that antioxidant peptides present in the food system play a vital role in the maintenance of antioxidant defense systems in the organism by preventing the formation of free radicals or by scavenging free radicals and reactive oxygen species, and others even recommended their supplementation (Chang et al., 2013; Gupta et al., 2009). An increasing number of food protein hydrolysates and antioxidant peptides have been found to exhibit antioxidant activity, especially in bovine milk casein (Hernández-Ledesma et al., 2005; Li et al., 2013). Furthermore, peptides can act synergistically with non-peptide antioxidants enhancing their protective effect (Kitts et al., 2003).

In vitro measurement of antioxidant activity is key in the evaluation of the antioxidant potential of bioactive peptide-enriched preparations. Due to the complex nature of antioxidants, there is no a single technique to measure the total antioxidant capacity (TAC) of a food system. Therefore, a variety of analytical techniques are employed with this aim, which can roughly be classified into two types namely the assays based on hydrogen atom transfer (HAT) reactions and those based on electron transfer (ET) (Huang et al., 2005). Then, to study the antioxidant activity of any sample it is necessary to use at least one assay of each type, and having in mind that each assay has different mechanism of action, reason for what it evaluates the TAC in a different way, the use of even more than one method of each type can give a more complete information (León-Ruiz et al., 2013).

The most widely studied activity of milk bioactive peptides is their ability to inhibit the ACE (Angiotensin-I-converting enzyme) and most of the biologically active peptides generated from milk proteins have demonstrated an ACE-inhibitory activity (ACEi) (Martínez-Maqueda et al., 2012). It is mainly due to hypertension is a chronic disease which has to be controlled overall because it is a risk factor for cardiovascular disease and stroke, and ACE plays a crucial role in the blood pressure regulation. Even though, its inhibition leads to a decrease in the level of angiotensin II and a corresponding increase in the level of bradykinin, yielding an overall reduction in the blood pressure

(Donkor et al., 2007). Although the inhibitory capacity of milk derived peptides is lower than that for chemically designed drugs, their production from natural sources could represent a healthier and more natural alternative for this chronic treatment, without the side-effects associated to antihypertensive drugs (Donkor et al., 2007; Fitzgerald et al., 2004). It is known that most of publications on ACEi and antihypertensive peptides are about peptides from cow milk (Korhonen, 2009). However, in recent years goat milk proteins have become an important alternative source of ACEi bioactive peptides (Espejo-Carpio et al., 2013; Haque et al., 2007; Park et al., 2007).

Only few studies focused on the bioactivity of fermented goat milk peptidic fractions. In addition, the use of a milk concentrated in proteins produced by a local breed of goat, and the employment of the probiotic strain *L. plantarum* C4 for the fermentation, could gave interesting results not studied before.

Therefore, the aim of this research was to study the TAC, ACEi and antibacterial activities of some fractions obtained from two novel fermented skimmed goat milks.

2. Materials and methods

2.1. Samples

Five samples of probiotic fermented milk manufactured with skimmed goat milk concentrated by ultrafiltration and fermented with St plus the probiotic strain *Lactobacillus plantarum* C4 (PFM) and 5 samples of goat milk yoghurt made with skimmed goat milk concentrated by ultrafiltration and fermented with St (UY) were manufactured as described in chapter 2 (paragraph 3.1.2.3) for the standardised procedure. Each batch was made in different week and each sample was analysed by triplicate.

2.2. Sample fractionation

It was carried out in three steps as schematized in figure 3.1.

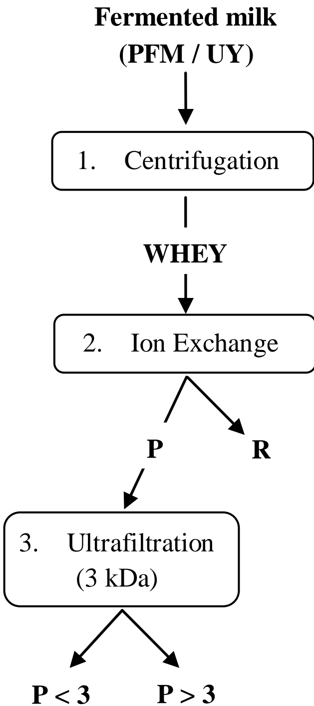


Figure 3.1. Samples fractionation diagram.

UY: Yoghurt manufactured with skimmed goat milk concentrated by ultrafiltration and fermented with *L. bulgaricus* and *S. thermophilus* (St); PFM: Probiotic fermented goat milk manufactured with skimmed milk concentrated by ultrafiltration and fermented with St plus *L. plantarum* C4; WHEY: Fermented milk supernatant after centrifugation; P: Ion exchange (IEX) permeate; R: IEX retentate; P<3: P fraction with less than 3kDa; P>3: P fraction with more than 3kDa.

2.2.1. First step: WHEY obtaining

All the samples were centrifuged at 3000*g* and 4 ºC for 30 minutes (Sigma 2-16PK, Sartorius, Goettingen, Germany). Then the supernatant was separated, freeze-dried and stored at refrigeration temperature and dry atmosphere until the analysis. Before the following fractionation, freeze-dried samples were dissolved in water up to the initial volume and filtered through 0.22 µm size pore filters Millex® - GS (Merck Millipore Ltd., Cork, Ireland) under laminar flow and stored in sterile containers.

The WHEY was fractionated in two steps:

2.2.1. Second step: cation exchange

Sartobind filter MA-15 Units (Sartorius, Goettingen, Germany), which are a strong acidic cation exchanger, were used. The procedure was carried out according the

operating instructions following four steps: (a) equilibration with 10 mL of 10mM potassium phosphate buffer at pH 4.5; (b) loading with 5 mL of sample; (c) washing with 10 mL of equilibration buffer; (d) and elution with 5 mL of elution buffer (equilibration buffer + 1 M NaCl at pH 4.5). Then, the cation exchange units were cleaned with 0.2 N NaOH for 30 minutes and equilibrated with 10 mL of equilibration buffer. All steps were conducted at 3 drops/second.

With this method were obtained two fractions for each sample: Permeate (P) composed by anionic or zwitterion peptides and proteins at pH 4.5 that permeates when loading the sample) and Retentate (R) composed by cationic peptides and proteins at pH 4.5 retained in the resin and extracted in the elution step. We will refer to them as peptides because we assume that the bioactivity is because of them.

2.2.2. Third step: Ultrafiltration

The ion exchange permeates were fractionated using 3 kDa cut-off ultrafiltration units (Vivaspin20, Sartorius, Goettingen, Germany), which yield two different products: (1) the P<3 or fraction with compounds sized less than 3 kDa anionic or zwitterions peptides and (2) the P>3 or the fraction with more than 3 kDa anionic or zwitterions peptides and proteins. As in the previous fractions we will refer to them as peptides.

2.3. Total soluble protein content

The total protein content of the samples was determined based on the bicinchonic acid (BCA) assay according to the previously optimized method by Welderufael et al. (2010). For this aim, 2 mL of the BCA working reagent (copper sulphate solution: BCA solution at a ratio of 1:50; Sigma-Aldrich, Steinheim, Germany) and 100 µL of sample were mixed. The resulting mixture was incubated for 30 minutes at 37 ºC and the absorbance was measured at 562 nm within 10 minutes using an Ultrospec 1100 pro UV/Visible spectrophotometer (Amersham Biosciences, Little Chalfont, UK). Serial dilutions of bovine serum albumin (Sigma-Aldrich, Steinheim, Germany) were used as standard and bidistilled water as blank.

2.4. Total antioxidant capacity (ORAC, ABTS, DPPH and FRAP assays)

2.4.1. The ORAC assay

The TAC using the oxygen radical antioxidant capacity assay (ORAC) was determined according to the method described by Huang et al., (2002) slightly modified. For this aim, 20 µL of sample, water or trolox, 60 µL of freshly prepared and warmed at 37 ºC 2,2-azobis(methylpropionamidine)dihidro (AAPH, Sigma-Aldrich, Steinheim, Germany) reagent and 120 µL of fluorescein 70nM were mixed in this order in each well of a transparent 96-well polystyrene microplate (Biogen Científica, Spain). The AAPH reagent contained 12mM AAPH in 75mM sodium phosphate buffer at pH 7.4. Then, the microplate was automatically shaken before the first reading, and the fluorescence at 485 nm and emission at 528 nm were recorded every 3-4 minutes for 120 minutes until the relative fluorescence intensity was less than 5% of the initial reading value. The linear trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Sigma-Aldrich, Steinheim, Germany) calibration curve was made from 4 to 40 µM/L. Results were expressed as µmol equivalents of trolox per mL of sample (µmol TEAC / mL).

2.4.2. The ABTS assay

The antioxidant capacity was estimated in terms of radical scavenging activity following the procedure described by others (Re et al., 1999). Briefly, the ABTS$^{\bullet+}$ was produced by reacting 7 mM 2,2-azino-bis-(-3ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, Sigma-Aldrich, Steinheim, Germany) stock solution with 2.45 mM potassium persulphate and allowing the mixture to stand in darkness at room temperature for 12-16 h before use. The ABTS$^{\bullet+}$ solution was diluted with an ethanol:water (50:50) mixture in order to obtain an absorbance of 0.70 ± 0.02 at 730 nm. Then, 20 µL of sample, water or trolox standard and 280 µL of diluted ABTS$^{\bullet+\cdot+}$ solution were placed on a transparent 96-well polystyrene microplate (Biogen Científica, Spain). Absorbance readings were taken every 60 s for 20 minutes on a FLUOStar Omega microplate reader (BMG Labtech, Germany) with temperature control (37 ºC). The calibration was performed as described previously, with a trolox

stock solution used to perform the calibration curves from 0.1 to 0.01 mg/L. Results were expressed as µmol equivalents of trolox per mL of sample (µmol TEAC / mL).

2.4.3. The DPPH assay

The antiradical activity of different samples was estimated according to the procedure reported by Brand-Williams (1995), which was adapted to a microplate reader. A 20 µL sample, water or trolox volume was mixed with 280-µL of freshly prepared methanolic solution of 2,2-diphenyl-1-1-picrylhydrazyl 95% (DPPH, Sigma-Aldrich, Steinheim, Germany) at 37ºC. Readings of maximum absorbance at 520 nm were taken every 60 seconds during 60 minutes using FLUOStar Omega microplate reader (BMG Labtech, Germany). The temperature was maintained at 37ºC during the reaction. Trolox stock solutions were used to perform the calibration curves as described above. For this method, it has been referred that the higher initial purple colour disappearance, the greater the antiradical activity. Results were expressed as µmol trolox equivalents per mL of sample (µmol TEAC/ mL).

2.4.4. The FRAP assay

For the FRAP determination the ferric reducing ability of each sample solution was estimated according to the procedure described by Benzie et al. (1996) and adapted to a microplate reader. First, 280 µL of FRAP reagent were prepared freshly and warmed at 37ºC. Afterwards, they were mixed at each well of a transparent 96-well polystyrene microplate (Biogen Científica, Spain) with 20µL of sample, water or trolox. The FRAP reagent contained 2.5 mL of a 10 mM 2,4,6-tris(2piridil)s-triazina (TPTZ, Sigma-Aldrich, Steinheim, Germany) solution in 40 mM HCl, plus 2.5 mL of 20 mM $FeCl_3 \cdot H_20$, and 25 mL of 0.3 M acetate buffer at pH 3.6. Readings of maximum absorbance (at 595 nm) were taken every 60 s using a FLUOStar Omega microplate reader (BMG Labtech, Germany). The temperature was maintained at 37ºC and the reaction was monitored for 30 minutes. Trolox stock solutions were used from 0.2 to 0.010 mg/L concentrations and obtained results were expressed as µmol equivalents of trolox per mL of sample (µmol TEAC / mL).

2.5. Measurement of the ACEi% activity

The ACE-inhibitory activity of the samples and fractions was measured following the HPLC-based method described by Gonzalez-Gonzalez et al. (2011) with some modifications. The enzymatic assay was based on the hydrolysis of the substrate N-Hyppuryl-His-Leu (HHL) into hippuric acid (HA) by ACE. For this purpose, 90µL of 5 mM HHL in a 0.1 M sodium phosphate buffer (pH 8.2) with 0.3 M NaCl were incubated with 30µL of ACE (60 mU/mL; Sigma-Aldrich Inc., St Lous, MO, USA) and 10 µL of sample, captopril as positive control (Sigma-Aldrich, Steinheim, Germany) or buffer as blank at 37 ºC for 60 minutes. Therefore, after stop the reaction with HCl, the HA was determined by RP-UHPLC, using a Thermo Scientific Accela UHPLC system (Santa Clara, USA) with thermostated compartment sample injector at 10 ºC and a C18 analytical column (Extrasyl-ODS2, 250 x 4.0 mm, 5 mm, Tecknokroma, Barcelona, Spain) thermostated at 37 ºC. The injection volume was 10µL and the photodiode array detector was set at 228nm. The flow rate was 1 mL/min with an isocratic solution of acetonitrile 12.5% and trifluoroacetic acid 0.1% in milli-Q water over 8 minutes. The percentage of ACEi (ACEi%) was calculated based on the hippuric acid released in the samples relative to that released by the control sample: ACEi%= $[(HA_{control} - HA_{sample}) / HA_{control}] \times 100$. The ACEi activity was expressed also as the inhibitory efficiency ratio: IER = ACEi% / protein concentration (Gonzalez-Gonzalez et al., 2013). The IC_{50} values of the captopril were determined by making serial dilutions of the captopril and plotting the inverse of their ACEi % versus the inverse of their total protein concentration. The IC_{50} was determined from the resulting linear equation and expressed as µM.

2.6. Evaluation of the antibacterial activity

This activity was studied using two bacterial strains: one Gram-negative, *Escherichia coli* K-12 (*E. coli*) and another Gram-positive, *Micrococcus luteus* (*M. luteus*). Before the assay all samples were filtered through 0.22 µm size pore filters (Millex® - GS, Merck Millipore Ltd., Cork, Ireland) under laminar flow and stored in sterile containers. Every measurement was done in triplicate and sterile Phosphate Buffered Saline (PBS, Sigma-Aldrich, Steinheim, Germany) was assayed as blank.

2.6.1. The well diffusion assay

The antibacterial activity of the WHEY, P and R fractions of PFM and UY was assayed by the well diffusion assay, based on the method described by León-Ruiz et al. (2013). Briefly, a tube of sterilized agar culture media (Nutrient Agar [NA] for *E. coli* and Tryptone Soy Agar [TSA] for *M. luteus;* Oxoid, Thermo Scientific, Basingstoke, UK) at ~ 50 ºC was seeded with 100 µL of bacteria culture onto broth culture media (Nutrient Broth [NB] for *E. coli* and Tryptone Soy Broth [TSB] for *M. luteus*; Oxoid, Thermo Scientific, Basingstoke, UK) and poured onto the plates. The final density of bacterial cultures in NB and TSB was 6-8x10^8cfu/mL (controlled by plate count). When the agar was solidified (~ 30 minutes), 3 separate 6 mm diameter wells were punched into the agar with a sterile cylinder. Then, 50 µL of sample was placed to each well and the plates were incubated for 24 h at 37 ^{o}C in the case of *E. coli* and at 30 ºC for 48-72 h in the case of *M. luteus*. Finally, inhibition zones were measured from the edge of the wells.

2.6.2. The spot assay

This assay of antibiosis was carried out according to the method described by (Mohankumar et al., 2011) with some modifications. The agar was inoculated with the bacteria prepared as described above. Instead of doing wells, three 20 µL drops of each sample were put on the agar and the plates were incubated as also described above. Inhibition zones were measured from the edge of the drop.

2.6.3. The co-culture assay

In this assay 4.5 mL of broth culture (NB for *E. coli* and TSB for *M. luteus*), 0.5 mL of the sample and 50 µL of the bacteria suspension (growth in NB or TSB at ~ 6-8x10^8cfu/mL), were cultured all together. This mixture was incubated under stirring at 37 ºC for *E. coli* and 30 ºC for *M. luteus*. Aliquots at t=0, 2, 4, 8 and 24 h were taken, plated out and incubated 24h at 37ºC in NA for *E.coli* and 48-72h at 30 ºC in TSA for *M. luteus*. Finally, the colonies were counted and the mean for each plate was calculated and expressed as cfu/mL.

2.7. Statistical analysis

Homogeneity of variance was first assessed using the Levene test at a significance level of 5% ($p < 0.05$). Statistical significance of data was then tested using the t-student test. The normal distribution of the samples was assayed with the Shapiro-Wilk test at a significance level of 5% ($p < 0.05$). Finally, evaluation of the relationship between different assays was carried out by computing the relevant correlation coefficient at the $p < 0.05$ confidence level by Pearson linear correlation (if normal distribution of the samples) or Spearman linear correlation (if no normal distribution of the samples). Analyses were performed using SPSS 15.0 program (Windows version; SPSS Inc., Chicago, IL).

3. Results

When the different parameters were measured in the fractions, obtained values referred to all soluble compounds present in the fermented milks. However, its further fractionation allows us to relate the activity of the compounds present in these fractions according to their physicochemical characteristics according to which we separated them.

3.1. Total protein analysis

A significantly higher protein concentration was observed for UY in WHEY and P fractions (Table 3.1). Inoculation of samples was carried out after concentration by ultrafiltration, so, the same concentrated milk was half inoculated with St and the other half with St and *L. Plantarum* C4. In this sense, the higher protein concentration observed in UY cannot be due to a higher concentration during ultrafiltration, but to other reasons as any difference during the fermentation process by LAB.

All analysed fractions showed significantly different protein concentration among them. It was also observed a bit protein lost during the treatment. In the IEX method it could be due to the washing step, where we measured a loss of proteins during the standardisation method (around 10-20% of the inoculated protein), and in the ultrafiltration process because some proteins could remain attached to the ultrafiltration membrane.

Table 3.1. Total protein content in the different fractions of fermented milks (mg/mL) (mean ± SD)

	n	WHEY	P	R	P(<3)	P(>3)
UY	5	6.782±0.773*	5.658±0.548*	0.436±0.096	2.238±0.145	1.315±0.377
PFM	5	5.698±0.661*	4.305±0.843*	0.355±0.055	2.083±0.127	0.975±0.142
Mean value	10	6.163±0.868 a*b**	4.846±0.990 a*c**	0.388±0.076 b**c**	2.145±0.143 b**c**	1.186±0.225 b**c**

UY: Yoghurt manufactured with skimmed milk concentrated by ultrafiltration (UFM) and fermented with the classical starter bacteria (St) *L. bulgaricus* and *S. thermophilus*; PFM: Probiotic fermented goat milk manufactured with UFM and fermented with St and *L. plantarum* C4; WHEY: Fermented milk supernatant after centrifugation; P: IEX (Ion exchange) permeate; R: IEX retentate; P<3: P fraction with less than 3 kDa; P>3: P fraction with more than 3 kDa. Differences between UY and PFM are signaled in the column; * p<0.05. Differences among fractions are signaled with letters in the same row; the same letter indicates significant differences ($^{letter}p$<0.05), ^{letter}* p<0.01, ^{letter}** p<0.001).

3.2. Total antioxidant capacity

The highest TAC of the fermented milk fractions was measured by ORAC, reaching more than 2.927 ± 0.043 μmol TEAC/mL in the UY yoghurt (Figure 3.2a). However, for the other assays used, milk fractions did not reach the 0.4 μmol TEAC/mL (Figures 3.2b, 3.2c and 3.2d). The P<3 fraction demonstrated the highest activity in ORAC and DPPH assays, whereas in the FRAP and ABTS assays it showed less activity than WHEY and P, fractions with similar TAC in all the assays used.

Therefore, fractionation by IEX did not result in increased activity as WHEY and P samples had similar TAC according to all methods. However, fractionation by size did result in differences in activity: whereas the P>3 fraction showed different behaviour (more or less TAC than P) depending on the method used, TAC of P<3 fraction was always higher than P (Figure 3.2 and table 3.2).

Regarding the differences among the analysed fermented milks, in ORAC, ABTS and FRAP assays almost all analysed fractions showed significantly higher antioxidant activity for UY than for PFM, (Table 3.2, Figures 3.2a, 3.2b and 3.2d). The only fraction that did not had significant differences between PFM and UY values was the P<3. Nevertheless, against DPPH radical, the PFM demonstrated significantly higher TAC than UY in the WHEY fraction.

For all the assayed antioxidant methods, with the exception of the DPPH, significant correlation (p<0.001) was observed between TAC and protein concentration (r^2: ORAC=0.596, ABTS=0.821 and FRAP=0.902). According to those results, the low

TAC observed in R fraction could be caused by its low protein concentration. This correlation of TAC with the protein concentration was in accordance to our previous hypothesis that attributed this activity to the peptides present in those fractions. In order to foundfind which one of the fractions had the most active peptides, the results of antioxidant activity were also expressed as µmol TEAC/mg of protein (Figure 3.3).

In general, no differences were observed between the TAC of UY and PFM. It is noteworthy that the most active fractions when expressed in mL were not the same as when expressed by mg of protein (Table 3.3). The most active peptides measured with the ORAC assay were in the P<3 fraction, reaching more than 1µmol TEAC/mg protein (Figure 3.3a and table 3.3). However, the peptides with highest TAC against $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals were present in R (~ 0.4 µmol TEAC/mg protein; Figures 3.3b and 3.3c, respectively and table 3.3), whereas in FRAP less differences were observed among fractions.

Finally, the TAC (TEAC/mL) of the fractions obtained by different methods was significantly ($p<0.001$) correlated among them ($r^2>0.700$ for ABTS-FRAP, r^2 0.500-0.600 for the others), with the exception of DPPH, which was not correlated with the other methods. However, when the TAC was expressed as mg of protein was only were significantly correlated ($p<0.001$) DPPH-ABTS ($r^2=0.878$) and ORAC-FRAP ($r^2=0.651$). This could be due to the different methods measured different activities, which reinforces the importance of using different methods to measure this activity.

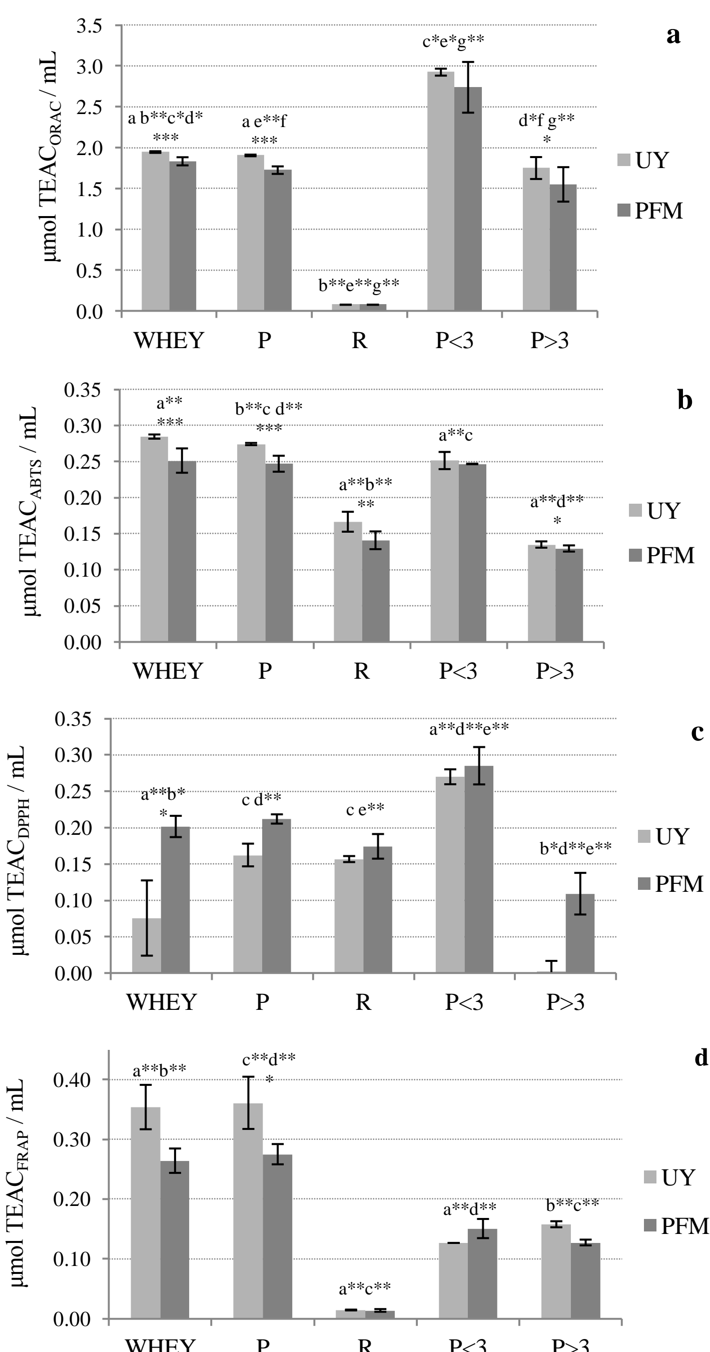


Figure 3.2. TEAC (Trolox equivalent antioxidant capacity) for mL of fermented milk fractions. **(a)** TEAC$_{ORAC}$; **(b)** TEAC$_{ABTS}$; **(c)** TEAC$_{DPPH}$ and **(d)** TEAC$_{FRAP}$

UY: Yoghurt manufactured with skimmed goat milk concentrated by ultrafiltration (UFM) and fermented with the classical starter bacteria (St) *L. bulgaricus* and *S. thermophilus*; PFM: Probiotic fermented goat milk manufactured with UFM and fermented with St and *L. plantarum* C4); WHEY: Fermented milk supernatant after centrifugation; P: IEX (Ion exchange) permeate; R: IEX retentate; P<3: P fraction with less than 3kDa; P>3: P fraction with more than 3kDa. Significantly differences between UY and PFM are signalled on the top of the bars as follows: *p<0.05; **p<0.01; ***p<0.001; among fractions, same letters indicate significant differences (^{letter} p<0.05; ^{letter}* p<0.01; ^{letter}** p<0.001).

Table 3.2. Antioxidant activity (μmol TEAC/mL) of the fermented milk fractions.

Method	Sample	n	WHEY	P	R	P<3	P>3
FRAP	UY	5	0.354 ± 0.037 ^{a**b**}	*0.361 ± 0.044 ^{a**b**}	0.014 ± 0.001 ^{a**c**}	0.126 ± 0.004 ^{a**d**}	0.157 ± 0.005 ^{b**c**}
	PFM	5	0.264 ± 0.020 ^{a**b**}	*0.275 ± 0.017 ^{c**d**}	0.013 ± 0.002 ^{a**c**}	0.150 ± 0.016 ^{a**d**}	0.127 ± 0.005 ^{b**c**}
ABTS	UY	5	***0.284 ± 0.003 ^{a**}	***0.274 ± 0.002 $^{b**c\ d**}$	**0.166 ± 0.014 ^{a**b**}	0.251 ± 0.012 ^{a**c}	*0.135 ± 0.004 ^{a**d**}
	PFM	5	***0.251 ± 0.017 ^{a**}	***0.247 ± 0.011 $^{b**c\ d**}$	**0.141 ± 0.012 ^{a**b**}	0.247 ± 0.000 ^{a**c}	*0.129 ± 0.004 ^{a**d**}
ORAC	UY	5	***1.949 ± 0.010 $^{a\ b**c*d*a}$	***1.908 ± 0.010 $^{a\ e**f}$	0.081 ± 0.001 ^{b**e**g**}	2.927 ± 0.043 ^{c*e*g**}	*1.754 ± 0.134 $^{d*f\ g**}$
	PFM	5	***1.836 ± 0.050 $^{a\ b**c*d*}$	***1.728 ± 0.0046 $^{a\ e**f}$	0.081 ± 0.001 ^{b**e**g**}	2.741 ± 0.311 ^{c*e*g**}	*1.553 ± 0.211 $^{d*f\ g**}$
DPPH	UY	5	*0.075 ± 0.052 ^{a**b}	0.162 ± 0.016 $^{c\ d**}$	0.157 ± 0.004 $^{c\ e**}$	0.270 ± 0.010 ^{a**d**e**}	-0.010 ± 0.014 ^{d**e**}
	PFM	5	*0.201 ± 0.015 ^{a**b}	0.212 ± 0.006 $^{c\ d**}$	0.174 ± 0.017 $^{c\ e**}$	0.285 ± 0.026 ^{a**d**e**}	0.109 ± 0.029 ^{b*d**e**}

Table 3.3. Antioxidant activity (μmol TEAC/mg protein) of the fermented milk fractions.

Method	Sample	n	WHEY	P	R	P<3	P>3
FRAP	UY	5	0.055 ± 0.001 $^{a\ b**c*}$	0.064 ± 0.002 $^{a\ d**}$	0.009 ± 0.000 ^{b**d**e**f*}	0.061 ± 0.003 ^{c*e**}	0.067 ± 0.004 ^{f*}
	PFM	5	0.047 ± 0.005 $^{a\ b**c*}$	0.059 ± 0.009 $^{a\ d**}$	0.037 ± 0.001 ^{b**d**e**f*}	0.073 ± 0.006 ^{c*e**}	0.065 ± 0.003 ^{f*}
ABTS	UY	5	0.042 ± 0.004 ^{a**b*}	*0.049 ± 0.005 ^{a**}	0.388 ± 0.050 ^{a**c**}	0.112 ± 0.002 ^{a**c**}	0.052 ± 0.010 ^{b*c**}
	PFM	5	0.045 ± 0.008 ^{a**b*}	*0.059 ± 0.012 ^{a**}	0.395 ± 0.035 ^{a**c**}	0.122 ± 0.000 ^{a**c**}	0.061 ± 0.020 ^{b*c**}
ORAC	UY	5	0.290 ± 0.033 ^{a*b**}	*0.339 ± 0.031 $^{a\ c**d*}$	0.191 ± 0.044 ^{b**c**}	1.310 ± 0.102 ^{b**c**}	0.668 ± 0.084 ^{b**c**}
	PFM	5	0.326 ± 0.044 ^{a*b**}	*0.409 ± 0.063 ^{a*c**}	0.230 ± 0.034 ^{b**c**}	1.347 ± 0.264 ^{b**c**}	0.854 ± 0.009 ^{b**c**}
DPPH	UY	5	**0.018 ± 0.002 $^{a\ b**}$	0.029 ± 0.011 $^{a\ c**d*}$	0.383 ± 0.101 ^{b**c**e**}	0.120 ± 0.003 ^{b**d*f}	0.004 ± 0.006 ^{e**f}
	PFM	5	**0.035 ± 0.004 $^{a\ b**}$	0.050 ± 0.010 $^{a\ c**d*}$	0.498 ± 0.058 ^{b**c**e**}	0.106 ± 0.043 ^{b**d*f}	0.084 ± 0.007 ^{e**f}

TEAC:Trolox equivalent antioxidant capacity; UY: Yoghurt manufactured with skimmed goat milk concentrated by ultrafiltration (UFM) and fermented with the classical starter bacteria (St) *L. bulgaricus* and *S. thermophilus*; PFM: Probiotic fermented goat milk manufactured with UFM and fermented with St and *L. plantarum* C4; WHEY: Fermented milk supernatant after centrifugation; P: IEX (Ion exchange) permeate; R: IEX retentate; P<3: P fraction with less than 3kDa; P>3: P fraction with more than 3kDa. Significantly differences: between UY and PFM are signalled as follows: *p<0.05; **p<0.01; ***p<0.001 in the same column at the left of the value; among fractions, same letters indicate significant differences (^{letter}p<0.05; ^{letter}* p<0.01; ^{letter}** p<0.001) in the same row.

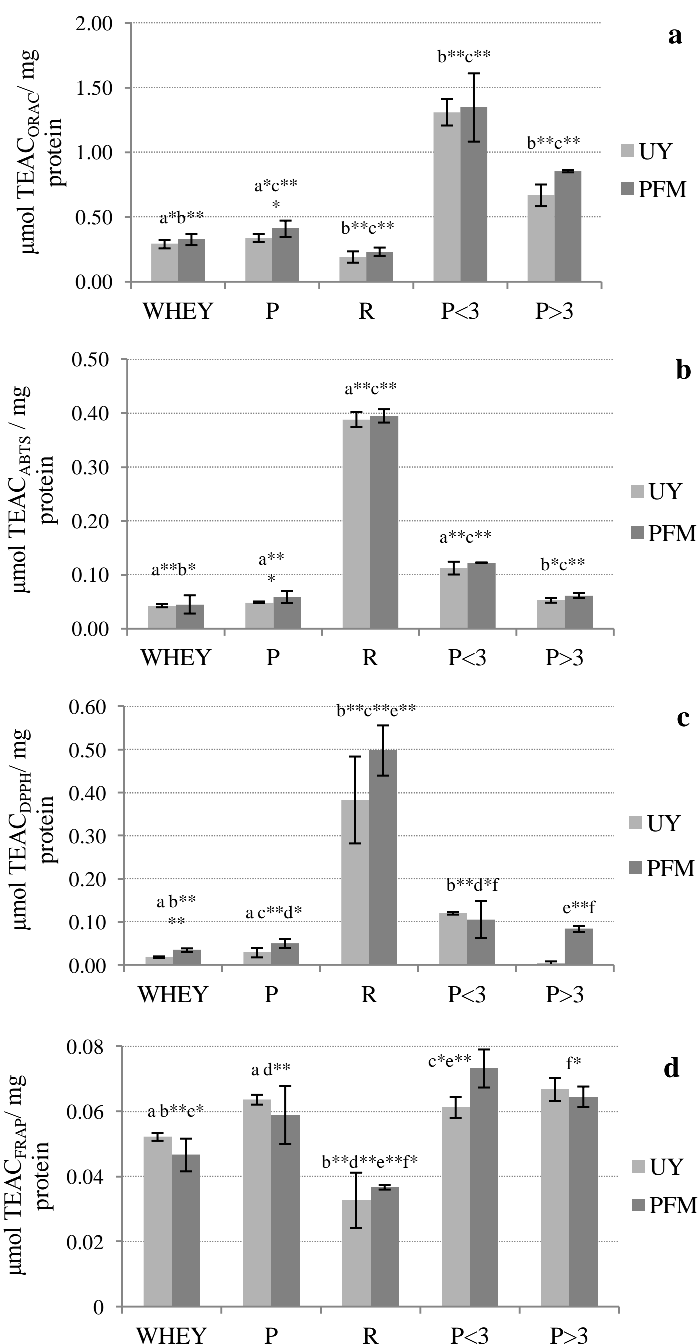


Figure 3.3. TEAC (Trolox equivalent antioxidant capacity) for mg of protein in the fermented milk fractions. **(a)** $TEAC_{ORAC}$; **(b)** $TEAC_{ABTS}$; **(c)** $TEAC_{DPPH}$ and **(d)** $TEAC_{FRAP}$

UY: Yoghurt manufactured with skimmedgoat milk concentrated by ultrafiltration (UFM) and fermented with the classical starter bacteria (St) *L. bulgaricus* and *S. thermophilus*; PFM: Probiotic fermented goat milk manufactured with UFM and fermented St and *L. plantarum* C4; WHEY: Fermented milk supernatant after centrifugation; P: IEX (Ion exchange) permeate; R: IEX retentate; P<3: P fraction with less than 3 kDa; P>3: P fraction with more than 3 kDa. Significantly differences (on the top of the bars) between UY and PFM are signalled as follows: *$p<0.05$; **$p<0.01$; ***$p<0.001$; among fractions, same letters indicate significant differences ($^{letter}p<0.05$; $^{letter}*\ p<0.01$; $^{letter}**\ p<0.001$).

3.3. ACEi% activity

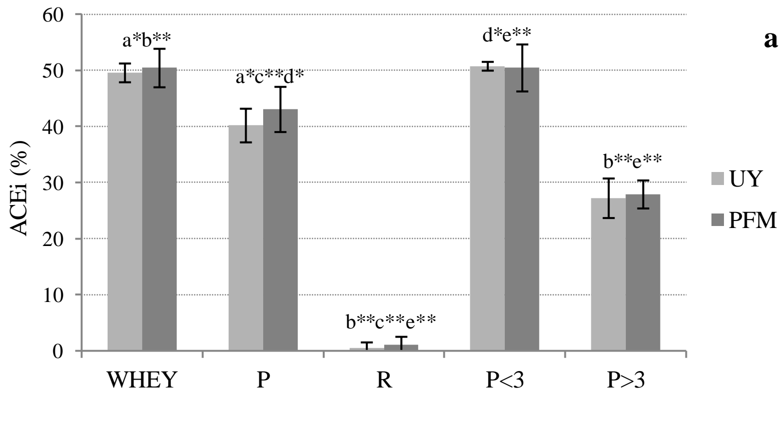
Firstly, the measured IC_{50} of captopril was 0.023 µM, in the range considered as correct by the manufacturer (0.021 ± 0.013 µM). This result confirms the reliability of the reagent used.

In figure 3.4a the ACEi activities of the different fractions expressed as percentage of inhibition were collected. The fractions with significantly highest activity were WHEY and P<3, whereas R did not show any activity. Between UY and PFM no significant differences were found for any of analysed fractions.

As happened for TAC, ACEi activity was significantly ($p<0.001$) correlated with protein concentration ($r^2= 0.640$), and when the results were expressed as IER, the fractions with the highest activity were not the same as when expressed as ACEi% (3.4b).

As expected, the smaller peptides the higher ACEi, founding significantly highest ACEi activity of peptides in the P<3 fraction, followed by the peptides in P>3 fraction (Figure 3.4b). In that sense, the fractionation by size leads to an increase on the activity.

To finish, we also calculated the mU of enzyme inhibited by the WHEY of the fermented milks, and taking into account that each pot had 200 g of fermented milk, the ACEi activity of the WHEY in this weight of fermented milk was calculated. We obtained that the mean value of inhibition for PFM and UY corresponding to the WHEY fraction was 9,200 mU of ACE, while the one corresponding to P<3 fraction (the most active fraction) was considerably higher (26,161 mU of ACE).



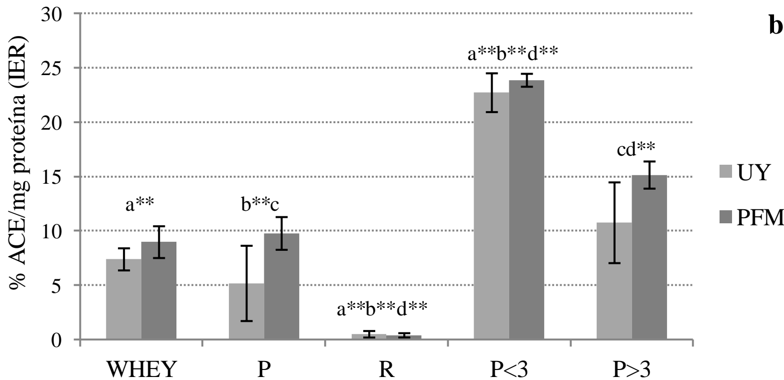


Figure 3.4. Angiogensin-I-converting-enzyme inhibitory activity (ACEi) of UY and PFM expressed as **(a)** Percentage of ACE inhibition. **(b)** Inhibitory efficiency ratio (IER).

UY: Yoghurt manufactured with skimmed goat milk concentrated by ultrafiltration (UFM) fermented with the classical starter bacteria (St) *L. bulgaricus* and *S. thermophilus*; PFM: Probiotic fermented goat milk manufactured with UFM fermented with St and *L. plantarum* C4; WHEY: Fermented milk/yoghurt supernatant after centrifugation; P: IEX (Ion exchange) permeate; R: IEX retentate; P<3: P fraction with less than 3kDa; P>3: P fraction with more than 3kDa. Significantly differences (on the top of the bars) between UY and PFM are signalled as follows: **p*<0.05; ***p*<0.01; ****p*<0.001; among fractions, same letters indicate significant differences ($^{letter}p$<0.05; ^{letter}* *p*<0.01; ^{letter}** *p*<0.001).

3.4. Antibacterial activity

After the well diffusion assay, no antibacterial activity of the supernatants against *E. coli* was observed. By contrast, around WHEY and P fraction, *E. coli* grew even better than in the control assay (Figure 3.5).

Nevertheless, in the spot assay for both WHEY and P fractions *E.coli* did not grow where the drop was placed probably due to the low pH of these samples, which contained (WHEY = 4.26; P = 4.59). However, R fraction, with higher pH (6.97) due to the cationic peptides were located, did not show any activity (Figure 3.6)

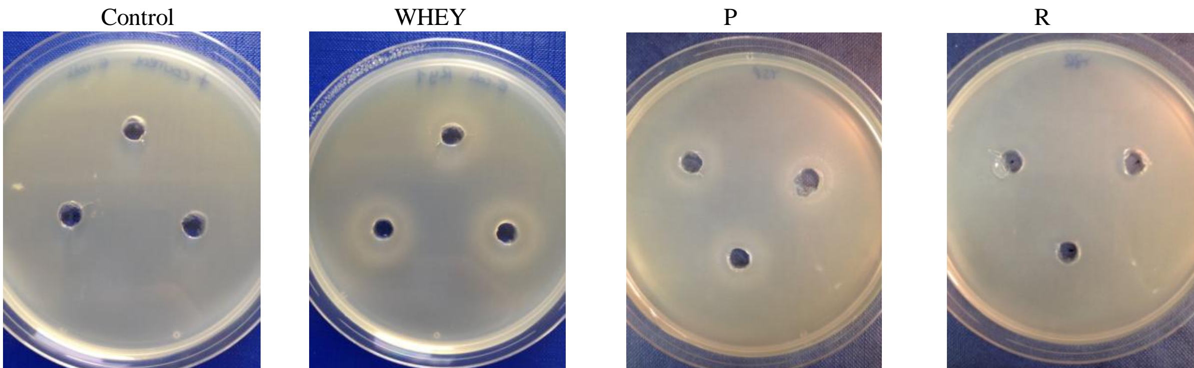


Figure 3.5. Pictures from well diffusion against *E. coli.*

Control: Sterile phosphate buffered saline; WHEY: Fermented milk supernatant after centrifugation; P: IEX (Ion exchange) permeate; R: IEX retentate.

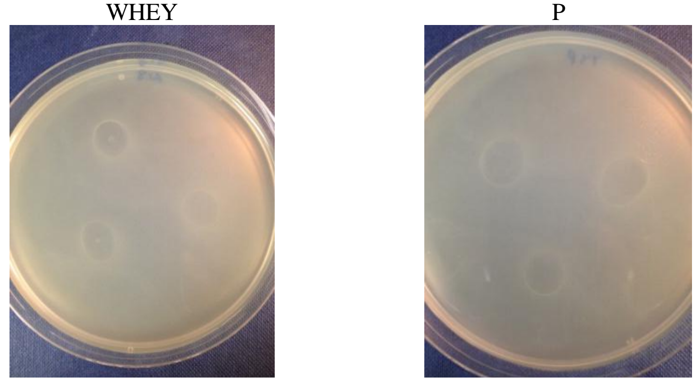


Figure 3.6. Pictures from spot test against *E. coli.*

WHEY: Fermented milk supernatant after centrifugation; P: IEX (Ion exchange) permeate

Figure 3.7. Pictures from well diffusion assay against *M. luteus*

Control: sterile phosphate buffered saline; WHEY: Fermented milk supernatant after centrifugation; P: IEX (Ion exchange) permeate; R: IEX retentate.

In relation to *M. luteus,* we did not show any inhibition neither in the well diffusion assay nor in the spot test. Contrarily, even higher growth was found around the well of the WHEY fraction compared to the other fractions where no effect was shown (Figure 3.7).

Additionally, the co-culture assay was carried out to evaluate more precisely the possible inhibition of *E. coli* by the studied fractions. As result, in both UY and PFM, any of the fractions showed high antibacterial activity and the pathogen grew almost as much as in the control (Figure 3.8). However, after 24 h significant differences in *E. coli* viable bacteria among control and WHEY and P of both fermented milks, and R in PFM were found. This inhibition could be due to the acid pH of these samples, because the WHEY and P fractions had almost the same value for both samples (Table 3.4). However, due the different pH of R fraction, closer to the pH of the control, its action could be be to the cationic peptides isolated in this fraction. This different pH found among fractions could confirm that separation correctly happened according to the charge.

Due to the low inhibition observed, the further separation of the fractions, especially R, would be interesting to clarify the peptides responsible of this activity and if they were specifically released by *L. plantarum* C4.

Table 3.4. Final pH of the co-culture supernatants at 24h.

Sample	n	WHEY	P	R	Control
UY (TSB)	5	5.04 ± 0.07	5.06 ± 0.01	7.46 ± 0.07	7.30 ± 0.18
PFM (NB)	5	4.91 ± 0.07	4.83 ± 0.01	6.64 ± 0.01	6.85 ± 0.12

The pH was measured in the supernatant of the culture media mixed with the fractions after the assay. TSB: Tryptone soy broth culture media; NB: Nutrition broth culture media; WHEY: Fermented milk supernatant after centrifugation; P: IEX (Ion exchange) permeate; R: IEX retentate; Control: Sterile PBS.

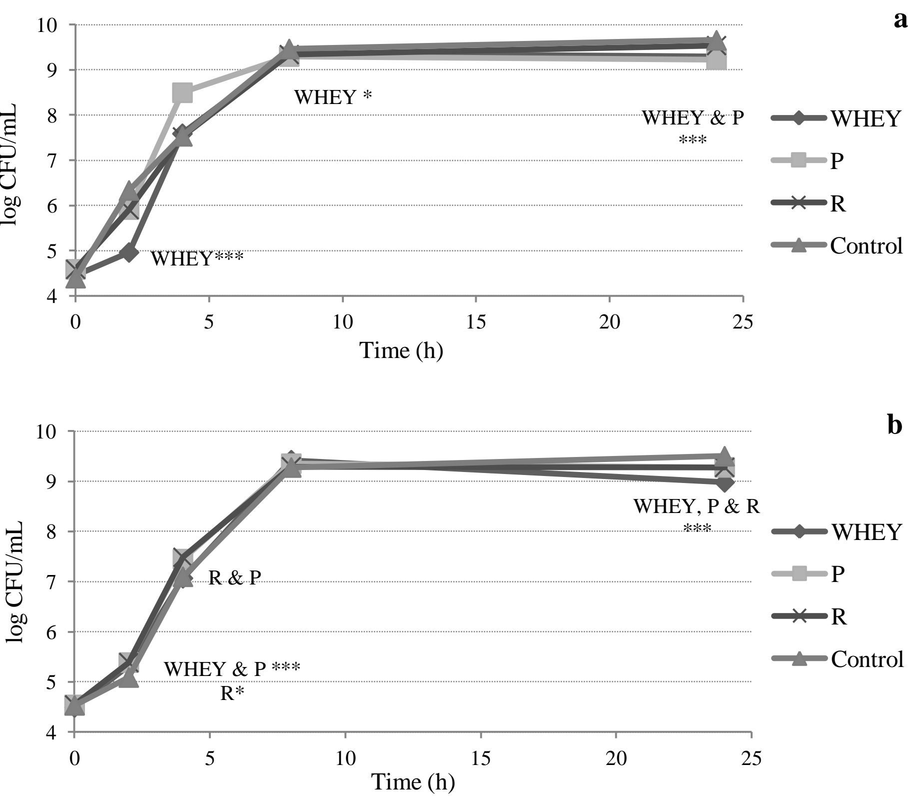


Figure 3.8. Viable *E.coli* after co-culture with the different fractions from **(a)** UY and **(b)** PFM

UY: Yoghurt manufactured with skimmed milk concentrated by ultrafiltration (UFM) fermented with the classical starter bacteria (St) *L. bulgaricus* and *S. thermophilus*; PFM: Probiotic fermented goat milk manufactured with UFM fermented with St and *L. plantarum* C4; WHEY: Fermented milk supernatant after centrifugation; P: IEX (Ion exchange) permeate; R: IEX retentate; Control: Sterile PBS. Significantly differences at specific time between any fraction and the Control are signalled as follows: **p*<0.05; ***p*<0.01; ****p*<0.001, together with the significantly different fraction name.

4. Discussion

In the following discussion, due to most of the research was done in cow milk, is important have in mind the different proportion in proteins and amino-acid sequences of the 4 main proteins between cow and goat milk. These differences (α_{s2}-casein [CN]: 11.7% difference; β-CN: 9.4% difference; κ-CN: 16.3% difference; and β-lactoglobulin [LG]: 8.3% difference) could influence the cleavage of different peptides with different activities (Geerlings et al., 2006).

4.1. Total proteins

The BCA assay has been described as a reliable method to measure the concentration of peptides with more than three amino-acids (Wiechelman et al., 1988). In that sense, one

of the reasons of the different protein concentration of PFM and UY in WHEY and P fractions could be the cleavage, by the different strains during the fermentation, of small peptides or amino acids no detectable by this methods (Donkor et al., 2007). On the other hand, the lost of proteins during the washing step during the IEX procedure has been previously reported (Welderufael et al., 2010).

4.2. Total antioxidant capacity

As it was described above, TAC was different depending on the method used to measure it and on the way to express the obtained results. In other studies, the TAC of LAB has been reported. However, because all samples were previously sterilized, it should not influence the measured TAC (Lin et al., 1999). In addition, in WHEY fraction, water-soluble antioxidant compounds could be also present, but they reported very low TAC (Zulueta et al., 2009).

It has been demonstrated that the TAC of dairy products is mainly due to the peptides activity. Some authors agreed on that the main contribution to TAC comes from casein fractions in milk and suggested that this effect is due to the self oxidation of amino-acid residues of the caseins as well as its derived peptides. Additionally, they reported that this activity cannot be replaced by free amino acids because it is the primary structure of casein itself which plays a determining role (Farvin et al., 2010). Among the caseins that releases antioxidant peptides, β-CN could be preferably degraded by lactic acid bacteria because it is more unstructured and accessible to cleavage, and therefore hydrolyzed to a greater extent (Chang et al., 2013). On the other hand, β-LG and lactoferrin had been reported as key components for their high scavenging activity, releasing also peptides with this activity (Hernández-Ledesma et al., 2007; Lindmark-Månsson et al., 2000).

The different TAC results obtained by the different methods, attributed above to their mechanism of action, could be specifically due to differences in the solubility and reactivity of radicals as well as their different diffusivity in the reaction medium (Aloğlu et al., 2011).

According to Huang et al. (2005), the used antioxidant assays are classified as follows. ABTS, DPPH and FRAP are ET-based assays that measure the antioxidant's reducing capacity. Particularly, ABTS and DPPH assess the antioxidant activity by reaction with

free radicals ($ABTS^{\bullet+}$ or $DPPH^{\bullet}$ respectively) and FRAP by Fe^{3+} reduction (Niki, 2011). Even though this measured capacity is not directly related to its radical scavenging capacity, it is an important parameter for antioxidants. However, the ORAC is an HAT-based assay that quantifies the hydrogen atom donation capacity and applies a competitive reaction scheme, being more relevant to evaluate the TAC of radical chain-breaking. Additionally, the ORAC assay is considered useful for food samples because of its greater specificity and capability of responding to a greater number of antioxidant compounds (Zulueta et al., 2009).

Regarding the ET-based assays, DPPH results were not well correlated with the other assays, probably owing to the organic media used along the reaction, different to the used for the other assays. However, despite it was described a notable limitation when it was used to interpret the role of hydrophilic antioxidants, the TAC measured by this method was as representative as the measured by the other methods (Tang et al., 2010).

On the other hand, the FRAP was the one with slightly higher TAC values for the fractions, but the lowest TAC when expressed as mg of proteins. Due to the high correlation between FRAP and proteins we could state that the peptides present in the fractions demonstrated less Fe^{3+} reducing activity in comparison to other measured antioxidant activities. Despite some authors have reported that the FRAP assay is suitable to measure the TAC of low molecular weight peptides, this method demonstrated low reactivity with whey proteins (Chen et al., 2003). In addition, notwithstanding the capability of lactose to reduce Fe^{3+}-TPTZ, this assay was carried out at acid pH and the reducing activity at this pH may be suppressed in part by the protonation on antioxidant compounds (Huang et al., 2005).

Lastly, the ABTS assay has been described as a suitable and sensitive procedure to determine the TAC in milk and milk fractions (Chen et al., 2003). It could be due to this radical is soluble in aqueous and organic media, and the method is carried out at neutral pH, where there are no interferences due to protonation (acid pH) or proton dissociation (basic pH) (Arnao, 2000).

The TAC of peptides has been described as remarkably dependent on factors like molecular weight, amino acid composition and sequence (Zhang et al., 2012). Many authors reported that most of milk protein-derived peptides with antioxidant activity have less than 20 amino-acid residues (Chang et al., 2013; Donkor et al., 2007;

Hernández-Ledesma et al., 2005; Farvin et al., 2010; Unal & Akalın, 2012). According to this, the P<3 fraction, which should contain these peptides, was the one with the highest TAC measured by ORAC. Nevertheless, Virtanen et al. (2007) reported the contrary, supporting higher scavenging activity against the $ABTS^{\bullet+}$ radical of peptides with more than 4kDa. However, the peptides with significantly highest TAC against $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals were found in the R fraction, which consisted of cationic peptides at pH 4.5 no separated by size. These findings agree with the results reported by Ren et al., (2008), who stated that basic peptides had greater capacity to scavenge hydroxyl radical than acidic or neutral ones.

Few studies have indicated that the radical scavenging activity is strain-specific and higher proteolysis is not always associated with higher TAC (Aloğlu et al., 2011; Uluko et al., 2014; Virtanen et al., 2007). However, between PFM and UY significant differences were not observed in P<3 fraction (µmol TEAC / mL) and almost none in any fraction when results were expressed as mg of protein. In that sense we can hypothesize that the putative probiotic strain *L. plantarum* C4 by itself or its interaction with St did not produce enough concentration of any antioxidant peptide to be appreciated when measured the TAC of the fractions.

On the other hand, in spite of it was demonstrated in humans the antioxidant activity of a fermented goat milk due to the use of an antioxidative strain, no data have been found about TAC for the WHEY fraction of fermented goat milks and only few data for cow ones (Balakrishnan et al., 2014; Kullisaar et al., 2003). In addition, due to it does not exist a well standardised way to express the results, TAC is measured in different units such as percentage, TEAC, gallic acid equivalents, ascorbic acid equivalents, etc. becoming more difficult the comparison among the results obtained and the reported values.

Regarding the WHEY of commercial fermented milk, Hernández-Ledesma et al. (2005) reported moderate TAC. It is known that goat milk has more *β*-CN than cow milk, and in particular, the analysed fermented milks were concentrated in caseins, being therefore expected more *β*-CN derived peptides in these fermented goat milks than in cow fermented milk. Notwithstanding, results were in the range reported by others for WHEY fraction of fermented milks measured with ABTS, with values ranging from 0.2774 to 2.0356 µmol TEAC/mL (Aloğlu et al., 2011; Moslehishad et al., 2013).

However, as expected, it showed a generally higher TAC in comparison with reported values for the WHEY fraction of the milk (0.489 in UHT and 1.078 µmol TEAC/mL in pasteurized milk; Zulueta et al., 2009). This finding is related with the fact that the proteolytic activity of the fermenting strains are able to release the antioxidative peptides from milk proteins (Moslehishad et al., 2013).

On the other hand, PFM and UY were made only in 6 hours whereas some authors reported that TAC increases with the fermentation time up to 24-48 h (Chang et al., 2013; Gonzalez-Gonzalez et al., 2011; Unal et al., 2012; Virtanen et al., 2007). It should be taken into account that some studies showed low TAC of WHEY fraction, but after fractionation by HPLC were obtained fractions with higher TAC (Aloğlu et al., 2011). Consequently, future research should be focused in the fractioning process by HPLC and peptide identification in these fractions in order to study the possible presence of any antioxidant peptide responsible of the TAC of the WHEY fractions.

Finally, Saura-Calixto (2006) reported a total antioxidant intake in a typical Spanish diet of 3,549 µmol TEAC (ABTS) and 6,014 µmol TEAC (FRAP). Taking into account the WHEY obtained from a unit of fermented milk sample (200 g), the percentage for which this WHEY participate in this total antioxidant intake is 0.75% for the ABTS and 0.50% for the FRAP (Saura-Calixto & Goñi, 2006). However, the total antioxidant activity of the fermented milk should be higher if we consider the precipited fraction, with precipited caseins and bacteria for which an antioxidant activity has also been reported (Farvin et al., 2010).

4.3. ACEi activity

Despite the strain used in fermentation is one of the main influencing factors in the ACEi peptides synthesis (Chobert et al., 2005), no differences between PFM and UY were observed. *L. bulgaricus* demonstrated to be one of the most proteolytic microorganism as well as a great producer of ACEi peptides (Muguerza et al., 2006; Papadimitriou et al., 2007). This statement suggests, as for TAC, that the probiotic strain *L. plantarum* C4 did not produce ACEi peptides or it did, but not in enough concentration to show their effects beyond those of the produced by St. It was previously reported high ACEi activity of supernatants obtained from milk fermented

with 4 strains of *L. bulgaricus* (more than 50%) and none ACEi activity of supernatants fermented with 3 strains of *L. plantarum* (Chen et al., 2012). Nevertheless, Gonzalez-Gonzalez et al. (2011) found a strain of *L. plantarum* able to produce a supernatant with high ACEi activity at 24 h of fermentation, despite it started the pH fall and hydrolysis after 24 h. However, not always an increase in the fermentation time leads to more active products (Donkor et al., 2007; Welderufael et al., 2012).

ACEi% reported values for fermented milk WHEY are very variable depending on the strain used. For milks fermented with *L. bulgaricus* and *S. thermophilus,* whereas the most of reported values are around the 50%, they range from 25% to 70% of ACEi% activity (Chobert et al., 2005; Donkor et al., 2007; Papadimitriou et al., 2007). Other researchers tested 13 strains at 3 different final pH and found that the maximum inhibitory activity was 51% for milk fermented until pH 4.3 with *Lactococcus lactis* 3906. Contrarily, the milk fermented with *S. thermophilus* did not reach the 18% of ACEi activity (Nielsen et al., 2009). Others demonstrated a negative correlation between pH and ACEi activity of milk fermented with two strains of *L. helveticus* and two species of the *Lactococcus* genus reporting a range from 8 % to 50% ACEi activity (Otte et al., 2011). However, higher values of ACEi activity were found in milk fermented with other strains like Kumis bacteria, ranging from 10.11 to 74.27 % and up to 100% when fermented with St plus *L. acidophilus* L10, *L. casei* L26 and *B. lactis* B94 (Chaves-López et al., 2011; Donkor et al., 2007).

On the other hand, the ACEi activity has been demonstrated to be related with the ionic calcium (Ca^{2+}), which depending on its concentration may activate or inhibit the ACE (Gonzalez-Gonzalez et al., 2011). In chapter 1 we demonstrated that UFM was concentrated in caseins and we hypothesized that the ultrafiltration process changed the calcium distribution, probably increasing the Ca^{2+}. Additionally, the most potent antihypertensive and ACE-inhibitory peptides are generated from caseinates and casein fractions (Contreras et al., 2009). These could be two of the reasons by which the developed fermented goat milks are in the upper range of ACEi % activity reported. In addition, the relatively high temperature used for the pasteurization could increase ACEi activity of the final fermented milk as Chobert et al. (2005). Finally, it should be taken into account that, despite for *L. plantarum* did not influence the ACEi activity within the fermentation time, one of its strains was reported to be the best γ-amino

butyric acid (GABA) synthesizer. This is a non-protein amino acid with hypotensive effect, different from that of ACEi peptides, in rats and humans when added to fermented milks (Nejati et al., 2013). Then, it would be of interest a further study of GABA production by the probiotic *L. plantarum* C4 because maybe it could act on hypertension by this way.

Much research has been done about the influence on ACEi potential of small peptides (up to six amino acids) sequence, physicochemical characteristics of hidrophobicity and amino acid charge at the C-terminal position. (Contreras et al., 2009; Gobbetti et al., 2000; Haque et al., 2007; López-Fandiño et al., 2006; Ortiz-Chao et al., 2009; Tsai et al., 2008). However, when peptides have more than 6 amino acids, binding mechanisms to ACE still has not been clarified and more factors as steric effects must be taken into account (Jing et al., 2014; Papadimitriou et al., 2007; Pripp et al., 2004)

P fractions (P<3 and P>3) were mainly composed by anionic and neutral peptides at pH 4.5 and were probably all charged negatively at the working pH of the ACE assay (pH 8.3), were the most active (Figure 3.4b). In particular, the highest activity of peptides in the P<3 fraction is supported by many authors who reported that ACEi peptides usually contain between 2–12 amino acids, although higher active peptides have been also identified (López-Fandiño et al., 2006). In this sense, Contreras et al. (2009) described a 4 times higher ACEi activity of the fraction with less than 3 kDa in comparison with the WHEY one, and until 40 times higher than more than 3 kDa fraction such as others also reported (Gómez-Ruiz et al., 2006; Quirós et al., 2005; Quirós et al., 2007). On the other hand, cationic peptides separated at pH 4.5 were no longer positively charged all of them at pH 8.3. In that sense it was difficult to predict the charge of the R fraction at working pH and if it influenced their low activity or if it was caused by their low protein concentration or by other unknown factors. From these results we could conclude that peptides absorbed by the membrane (cationic at pH 4.5) were less active than the neutral and negatively charged. However, further investigation about the peptide composition of the fractions is required.

To finish, the captopril tablet for the treatment of low arterial hypertension has a content of 12.5 mg of captopril (Sypniewski et al., 1996), which inhibits 9,733,555mU ACE. As it was described above, WHEY and P<3 fractions in 200g of fermented milk inhibited 9,200 and 26,161 mU ACE activity, which is 1,058 and 372 times lower than

that reported for the captopril tablet, respectively. Nevertheless, having in mind that the assayed fermented goat milk is a foodstuff it could be considered an important activity.

4.4. Antibacterial activity

In our experiment a charge-based assay was considered to measure the antibacterial activity of the fractions, in view of the current consensus that ionic charge is crucial for the attachment of peptides to the bacterial membrane (Demers-Mathieu et al., 2013). For instance, the gram-negative bacteria were inhibited by positively charged peptides and gram-positive bacteria by negatively charged peptides. Nevertheless, other unknown physicochemical and structural properties are important factors for the antibacterial activity (Baranyi et al., 2003). In fact, the mechanism of action of milk-derived antimicrobial peptides remains uncertain, due to the diversity of sequences, sizes, structures and physicochemical characteristics such as net charge, charges on the peptide, isoelectric point, widths of the helix and amphipathicity (Benkerroum, 2010; Demers-Mathieu et al., 2013)

In the well diffusion assay, any effect was observed on *E. coli* and *M. luteus*. However, in the drop assay an inhibition was observed on the WHEY and P drop against *E. coli*, which could be due to the low pH of these samples. This is in accordance with some authors who reported an effect of negatively charged peptides on gram-negative bacteria, probably due to the factors described above. (Benkerroum, 2010; Pellegrini et al., 2001). Those fractions, as described above (paragraph 3.4), had very similar and low pH, which could cause the inhibition. However, when the co-culture assay was carried out, R fraction of PFM also showed a small and significant inhibition at 24 h. The effect of this fraction, only composed by peptides, could be due to some released peptides as caprine lactoferricin by *L. plantarum*. Caprine lactoferricin is a peptide with known antibacterial activity against *E. coli* with reported inhibitory effect by fermented milk (López Expósito et al., 2006).

Finally we could hypothesize that the low pH of the samples is the principal cause of the small antibacterial activity observed against *E. coli,* and maybe some cationic peptides could have antibacterial activity. However, further research is required to isolate and test smaller fractions.

5. Conclusions

The present research focused on the study of the biological activity of fermented milk fractions of two novel fermented skimmed goat milks. A remarkable TAC and high ACEi activity were shown by both fermented goat milks, and the WHEY was in general one of the most active fractions in all the assays. Regarding the peptides activity, the smallest showed the highest ACEi% activity and the highest TAC measured by the ORAC assay. However, positively charged peptides at pH 4.5 had the highest TAC against $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals, in comparison with the other fractions. Despite the WHEY and the anionic fractions of both fermented milks having small antibacterial activity, the cationic fraction at pH 4.5 of PFM had also antibacterial activity, which could be due to some peptides released by *L. plantarum* C4 during the fermentation. Finally, the activities attributed to the WHEY fraction can lead us to think about the benefits of the fermented milk intake. However, further research must be done to investigate the activity of more restrictive fractions as well as the identification of individual peptides responsible of these effects.

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CHAPTER 4

In vitro gastrointestinal digestion of two novel fermented goat milks released bioactive peptides

In vitro gastrointestinal digestion of two novel fermented goat milks released bioactive peptides

Abstract

In the present research the released peptides from goat milk, two novel fermented goat milks, and the fermented milk products after *in vitro* gastrointestinal digestion (GID) were identified. Fermented milks were manufactured with skimmed goat milk concentrated by ultrafiltration and fermented (a) with the classical starter bacteria (St) *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* and (b) with St plus the probiotic strain *Lactobacillus plantarum* C4. After *in vitro* GID: (S) Soluble, (D) Dialyzable and (ND) Non dialyzable fractions were obtained. In overall, 121 peptides were identified, the majority were released from β-Casein (CN) (53%) followed by α_{s1}-CN (19%) and α_{s2}-CN (17%). Only 11 were released from κ-CN and 3 from β-Lactoglobulin. Despite several peptides were identified in milk and fermented milks, most of the peptides were released after *in vitro* GID. Only few different peptides were found exclusively in PFM, but in spite of this, *L. plantarum* could be the responsible for some peptides solubilization. Some of the identified peptides had been previously reported as bioactive peptides or share any structure homology with these peptides. The identified peptides with reported bioactivity in D fraction (bioaccesible peptides) were the following: α_{s1}-CN f(157-164) DAYPSGAW and f(180-193) SDIPNPIGSENSGK; α_{s2}-CN f(90-96) VQKFPQY and β-CN f(191-205) YQEPVLGPVRGPFPI, f(191-207) YQEPVLGPVRGPFPILV, f(197-207) GPVRGPFPILV and f(197-206) GPVRGPFPIL. The three last peptides correspond to bioactive fragments described in cow milk. The peptide f(106-119) HKEMPFPKYPVEPF, released from β-CN was also identified in S fraction (soluble in the gut) These peptides in S and D fractions might be absorbed and probably exert their activity in the organism. Finally, because only few sequences were equal in UY and PFM before and after digestion, and due to the fact that peptides are absorbed mainly in the gut, we consider essential the knowledge of peptides breakage after *in vitro* GID.

1. Introduction

Biologically active peptides are food-derived peptides that exert (beyond their nutritional value) a physiological, hormone-like effect in humans (Erdmann et al., 2008). The activity of these peptides is based on their inherent amino acid composition and sequence and there are known peptides with demonstrated antihypertensive, antioxidant, antibacterial, anticancer, immunomodulatory, mineral-binding and opioid activities (Donkor et al., 2007; Korhonen, 2009; Minervini et al., 2003; Muguerza et al., 2006). These peptides, which are inactive within the sequence of the precursor protein, can be released by enzymatic proteolysis during *in vitro* gastrointestinal digestion (GID) or food processing. (Hernández-Ledesma et al., 2004). They usually contain 2–20 amino-acid residues per molecule, but in some cases they may consist of more than 20 amino acids.

They are found in milk, egg, meat and fish of various kinds as well as in many plants. But milk proteins are currently the main source of several biofunctional peptides and the daily intake of milk and milk products has proved to be physiologically important to both neonates and adults (Erdmann et al., 2008). In particular, fermented milk products are an excellent source of bioactive peptides (Donkor et al., 2007). Milk fermentation involves a number of metabolic pathways, in which metabolites contribute to confer chemical, biochemical and nutritional attributes to fermented milk, including the production of bioactive peptides (Chaves-López et al., 2014).

Proteinases of lactic acid bacteria may hydrolyze more than 40% of the peptide bonds of α_{s1}-Casein (CN) and β-CN, producing oligopeptides of 4 to 40 amino acid residues. (Minervini et al., 2003). More specifically, the proteolytic system of lactic-acid bacteria (LAB) comprises: Cell-wall bound proteinase that initiates the degradation of extracellular milk protein into oligopeptides, peptide transporters that take up the peptides into the cell, and various intracellular peptidases that degrade the peptides into shorter peptides and amino acids (Liu et al., 2010).

The proteolytic activity is influenced by the type of dairy product, the technology adopted and, specially, the lactic acid bacteria (LAB) strain (Gobbetti et al., 2002). In this regard, the proteolytic activity of the probiotic strain *Lactobacillus plantarum* C4,

isolated by our research group (Bujalance et al., 2007) and with demonstrated probiotic activity and other benefits on health, has never been tested (Bergillos-Meca et al., 2014; Bujalance et al., 2007).

However, other reports have claimed that, in some cases, milk fermentation is not enough to release active sequences from milk proteins but can produce several oligopeptides which will generate the bioactive form after subsequent GID (Hernández-Ledesma et al., 2004). Following digestion, bioactive peptides can either be absorbed through the intestine to enter the blood circulation intact and exert systemic effects, or produce local effects in the gastrointestinal tract (Erdmann et al., 2008). In addition, and despite the reports about its high digestibility and nutritional properties there is also a lack of information on the peptides released after the simulated GID of goat milk (Minervini et al., 2009). In any case, difficulties in peptide identification limit the knowledge on bioactive peptide formation and release from the precursor proteins. Milk protein hydrolysates are known for their complexity and can contain up to hundreds of different peptide sequences. For this reason, identification of bioactive peptides in fermented dairy products is a labor-intensive and difficult task (Hernández-Ledesma et al., 2004).

The main objective of this research was to identify the peptides produced by fermenting microorganisms (Classical starter bacteria [St] *Lactobacillus delbrueckii* subsp. *bulgaricus* plus *Streptococcus thermophilus*) or by the probiotic strain *Lactobacillus plantarum* C4) during the manufacturing of the developed probiotic skimmed fermented goat milks and after their *in vitro* GID. A special focusing was fixed on the identification of biologically active sequences or precursors of these as well as to identifiy bioactive sequences generated through *in vitro* GID.

2. Materials and methods

2.1. Samples

Three samples of skimmed goat milk concentrated by ultrafiltration (UFM) were manufactured as described in chapter 1 (paragraph 2.1), from raw goat milk samples collected in different weeks. The ultrafiltrate, discarded in other cases, was used to

measure the water-soluble peptides in goat milk. With the UFM, two different fermented goat milks were manufactured in our laboratory as described in chapter 2 (paragraph 3.1.2.3): (1) UY (Ultrafiltered yoghurt), inoculated with St and (2) PFM (Probiotic fermented milk) inoculated with St plus the probiotic strain *L. plantarum* C4.

2.2. Isolation of peptide fractions

The peptides in the ultrafiltrate (n=3) and fermented goat milk samples (n=3) were isolated based on the method developed by Farvin et al., (2010) with some modifications. Fermented goat milk samples were centrifuged at 4,500 rpm for 30 min at 4ºC. The precipitate was discarded and the supernatant, as well as the ultrafiltrate of the milk, was adjusted to pH 2.0 by addition of HCl. The acidified supernatant was filtered through a 30 kDa cut off ultrafiltration membrane. The retentate fraction was discarded and 100 ml of the filtrate (< 30 kDa) was applied to Dowex 50 WX2 cation exchange column (2.6 x 10 cm, H^+-form, 200-400 mesh, Serva, Heidelberg, Germany). After washing with 60 ml of milli-Q water, peptides were eluted with 200 ml of 2M aqueous ammonia. Ammonia was firstly evaporated *in vacuo* and then samples obtained from milk ultrafiltrate enluates (Milk [E]) and fermented milk whey eluates (WHEY [E]: Which includes both UY and PFM eluates; UY [E] and PFM [E]) were freeze dried. This procedure was carried out by duplicate.

2.3. *In vitro* gastrointestinal digestion of the fermented goat milk samples

UY and PFM samples were subjected to *in vitro* GID by using the following procedures. All digestions were performed in duplicate and digestion blanks were prepared with bidistilled water instead of the sample.

2.3.1. Solubility assay

This assay was done as described by Perales et al., (2007) with some modifications (Bergillos-Meca et al., 2013). Twenty grams of fermented milk were weighted and after addition of 60 ml of milliQ water, homogenization was carried out under sonication. A first step of gastric digestion was carried out at pH 2.0, with freshly prepared pepsin solution, at 30ºC during 2 h in a shaken water-bath. After that, the flask was cooled in an ice-bath for 10 minutes. Then, in the intestinal stage, an amount of freshly prepared pancreatin/bile solution was added to the gastric digest, previously adjusted to pH 6.5,

and the incubation was runned at 37ºC in the shaken water-bath for 2 h. Digested samples were maintained 10 minutes at room temperature and after that they were immersed in a water-bath at 100ºC for 5 minutes to inactivate the enzymes. Subsequently, they were cooled in an ice-bath for 10 minutes and the pH was adjusted to 7.2. Finally, the digests were centrifuged at 3,500*g* for 1h at 4ºC and the supernatants, the soluble fraction (S), were freeze dried and kept until the analysis.

2.3.2. Dialysis assay

This procedure was carried out according to Bergillos-Meca et al. (2013) to identify the bioaccesible peptides. It comprised a gastric step, common to that of the solubility, followed by an intestinal step where dialysis was included (dialysis bag: molecular weight 12-14 kDa; Visking 45mm x 27mm, Medicell International, London, UK). Dialysis tubing, containing bidistilled water and $NaHCO_3$ equivalent to titratable acidity measured previously, were placed in the flasks and incubated in the shaken bath at 37ºC for 30 min, after which pancreatin-bile extract mixture was added to the flask and incubated for 2 h. Dialyzable (D) and non dialyzable (ND) fractions were weighted and freeze dried and stored until the assay.

2.4. Total protein analysis: Bicinconic acid assay

This method was carried out with the Thermo Scientific™ Pierce™ BCA™ Protein Assay kit according to the instructions, in a 96 well plate using a FLUOStar Omega microplate reader (BMG Labtech, Germany). Serial dilutions with bovine serum albumin (provided with the kit) were used as standard. Results were expressed as mg/mL.

2.5. Analysis by on-line reverse-phase ultra high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS)

The milk and fermented milk samples after ion exchange and UY and PFM digested samples were analysed.

Before injection, all samples were redissolved in water-FA (0.1%) at 2 mg/mL protein concentration and centrifuged at 10,000*g* to precipitate all impurities. If turbidity was

shown, also a filtration step throught 0.45µm µm size pore filters (Millex® - GS , Merck Millipore Ltd., Cork, Ireland) was carried out.

Chormatographic analysis of the samples was performed with an Acquility UPLC® system (Waters Technologies, Cerdanyola del Vallès, Spain) with an ACQUITY UPLC BEH 130 column, a C18 column 100 mm of length, 2.1mm of internal diameter, 1.7 µm of particle size and 130Å of pore diameter (Waters Technologies, Cerdanyola del Vallès, Spain). The UPLC system was connected online to a quadrupole-time of flight MS/MS detector, equipped with an electrospray ionization source (Bruker Daltonik, Bremen, Germany). Solvent A was water with 0.1% formic acid and solvent B was acetonitrile with 0.1% formic acid and the flow used was 0.2 mL/min. The peptide fractions were eluted with an isocratic gradient after 1 min of pure solvent A, up to 35%B within 28 min, then in 2 min 70% of solvent B was reached and maintained during 2.5 minutes. The injection volume was 15 µL and the absorbance was monitored at 214 nm. The nebulizer pressure was set at 2 bar, the temperature of the source at 180ºC and the capillary voltage at 4.5 kV Spectra were recorded over the mass/charge (m/z) range 50-1500 and 3 spectra were averaged in the MS and in the MS(n) analyses. The signal threshold to perform auto MS(n) analyses was 5,000 counts and three precursor ions were isolated within a range of 100-1500 m/z and fragmented with a voltage ramp depending of the isolation mass of the precursor ion, from 20 to 70eV. Data were processed with Data Analysis™ (version 4.0, Bruker Daltonik, Bremen, Germany). The m/z spectral data were processed with Biotools (Version 3.2, Bruker Daltonik, Bremen, Germany) where the deconvoluted mass spectra were matched against the main goat milk proteins (α_{s1}-CN, α_{s2}-CN, β-CN, κ-CN, α-Lactoalbumin [LA] and β-LG)

3. Results and discussion

3.1. Protein concentration of the samples

Despite the high concentration of proteins of the UFM (6.07g/100g) and fermented milks (UY: 5.85 g/100g and PFM: 5.83 g/100g), their eluates after ion exchange separation showed much less concentration (Milk [E] and WHEY [E]: Table 4.1). This could be due to the fact that these eluates were only composed by peptides. On the other

hand, the protein concentration of digested samples was higher than the eluate ones. The reasons could be the following: the no purification of peptides by IEX resine and the presence of digestive proteases

Table 4.1. Protein concentration of the samples for in 100g of milk or fermented milk.

	n	Milk (E) (g/100g ± SD)	WHEY (E) (g/100g ± SD)	D (g/100g ± SD)	ND (g/100g ± SD)	S (g/100g ± SD)
UY	3		0.013 ± 0.003	0.95 ± 0.04	2.36 ± 0.20	1.91 ± 0.28
		0.005 ± 0.001	a** b**	a** b**	a**	b**
PFM	3	a** b**	0.009 ± 0.002	0.88 ± 0.05	2.23 ± 0.11	1.86 ± 0.29
			a** b**	a** b**	a**	b**

UY: Ultrafiltered goat yoghurt manufactured with milk concentrated by ultrafiltration (UFM) and fermented with (St) *L. bulgaricus* and *S. thermophilus*. PFM: Probiotic fermented goat milk manufactured with UFM and fermented with St plus *L. plantarum* C4. Milk (E): Milk whey eluate after cation exchange at pH 2; WHEY (E): Fermented milks whey eluate after cation exchange at pH 2; D: Dialyzable fraction after *in vitro* gastrointestinal digestion (GID); ND: Non dialyzable fraction after *in vitro* GID; S: Soluble fraction after *in vitro* GID. Statistically differences processed samples are signalled with the same letter in row. $p<0.001$ (letter**).

The D fraction had significantly lower protein concentration than S and ND fractions (Table 4.1). Peptides in this fraction were bioacessible, and then, they could be able to cross the intestinal barrier by different mechanisms. Around 15% of total proteins in fermented milks were found in this fraction and then, they could be absorbed as peptides in the gut (Figure 4.1). Peptides in the soluble fraction were approximately the double of dialyzed peptides. One half of S peptides were dialyzed, but the other half, despite being not dialyzed, they could also be absorbed in the gut because they are soluble. Non soluble peptides were not identified, but they were in part in ND fraction. Those peptides could exert some action but only in the intestinal tube. It is to notice that with the sum of ND and D fractions we did not reach 100%. It could be due to the hard conditions applied for protein digestion where peptides smaller than 3 amino acid residues were released and are not detected by the BCA assay.

Finally, no significant differences were observed between UY and PFM samples.

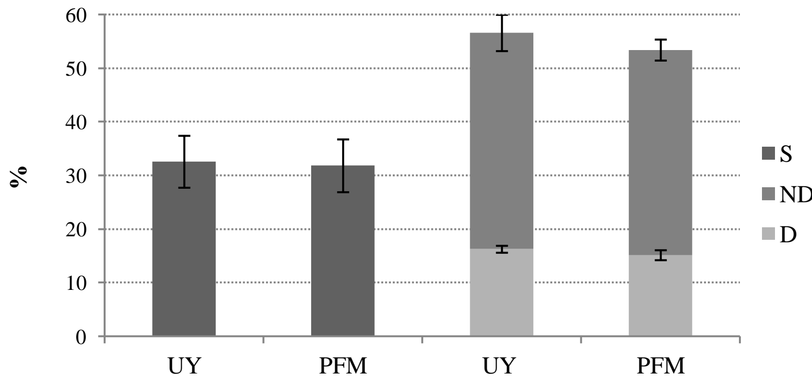


Figure 4.1. Percentage of peptides in the different fractions after *in vitro* gastrointestinal digestion (GID) in comparison to their total proteins before GID.

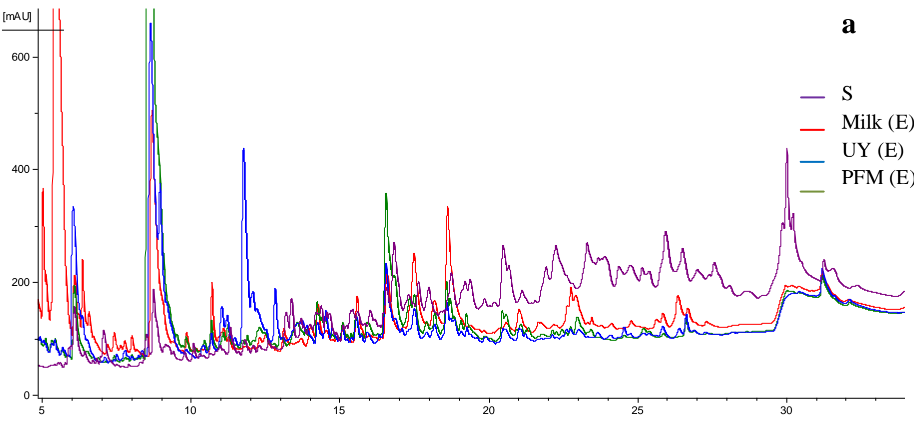
UY: Yoghurt manufactured with skimmed goat milk concentrated by ultrafiltration (UFM) and fermented with the classical starter bacteria (St) *L. bulgaricus* and *S. thermophillus*. PFM: Probiotic fermented goat milk manufactured with UFM fermented with St plus *L. plantarum* C4. D: Dialyzable fraction after *in vitro* GID; ND: Not dialyzable fraction after *in vitro* GID; S: Soluble fraction after *in vitro* GID.

3.2. Peptides sequencing by UPLC-MS/MS

3.2.1. UPLC-UV chromatographic profile

All the analytical samples corresponding to milk, fermented milk and digestion products were analysed by RP-UPLC coupled to on-line Q-TOF mass spectrometer. Figure 4.2.a shows the chromatographic UV profile of milk, fermented milk and digestion product samples. It can be appreciated that the eluates of milk and fermented milks presented more intense peaks than the digestion product fraction S, but after 20 minutes of running several signals were appreciated in S which weren't in the others. Despite this, the intensity of MS spectrum was higher in the digested samples (Figure 4.2b).

Figure 4.3a shows the UV profile of digested PFM fractions. It is observed a high number of peaks, despite low intensity. However, they MS spectrum showed a good intensity (4.3b). This could indicate the presence of high variety of peptides due to the different proteolysis processes, firstly with the fermentation and afterwards in the *in vitro* digestion with peptidases. Finally, high differences were observed between UY and PFM samples neither in UV profile nor in MS spectrum (Figure 4.4). In that sense, the probiotic strain *L. plantarum* C4 did not have enough proteolytic activity to produce different peptides in enough concentration to be observed in UV chromatogram.



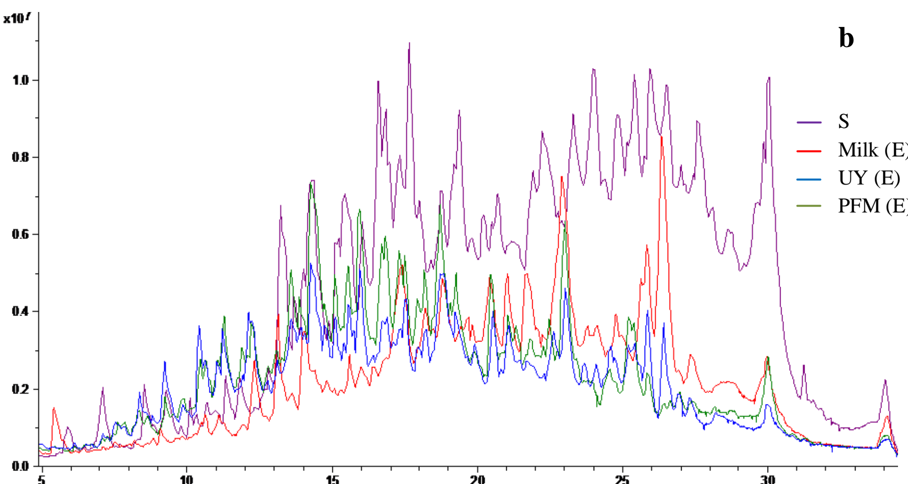
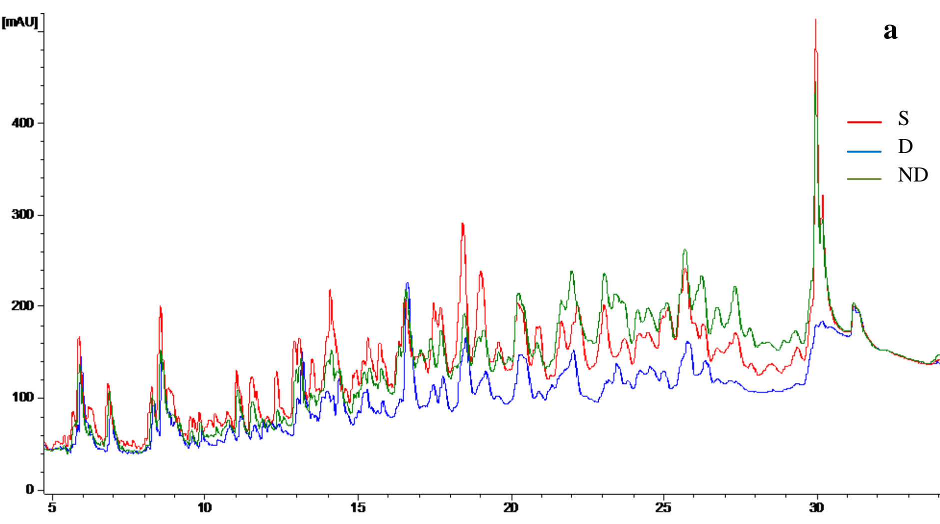


Figure 4.2. (a) UV at 214 nm and (b) MS profiles of different samples.

UF: Goat milk soluble compounds after cation exchange; UY: Cation exchange eluate of yoghurt manufactured with skimmed goat milk concentrated by ultrafiltration (UFM) and fermented with the classical starter bacteria (St) *L. bulgaricus* and *S. thermophilus*; PFM: Cation exchange eluate of a probiotic fermented goat milk manufactured with UFM fermented with St plus *L. plantarum* C4; S: Soluble fraction of PFM after *in vitro* gastrointestinal digestion.



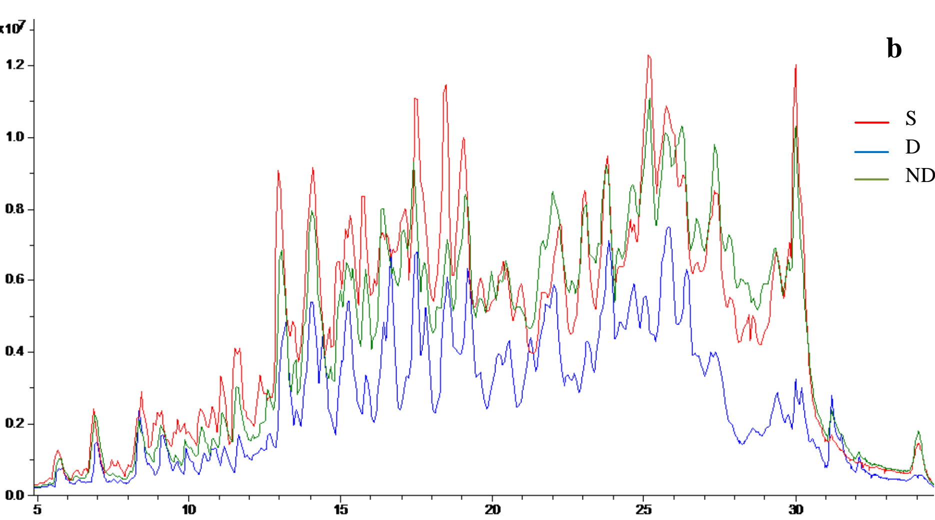
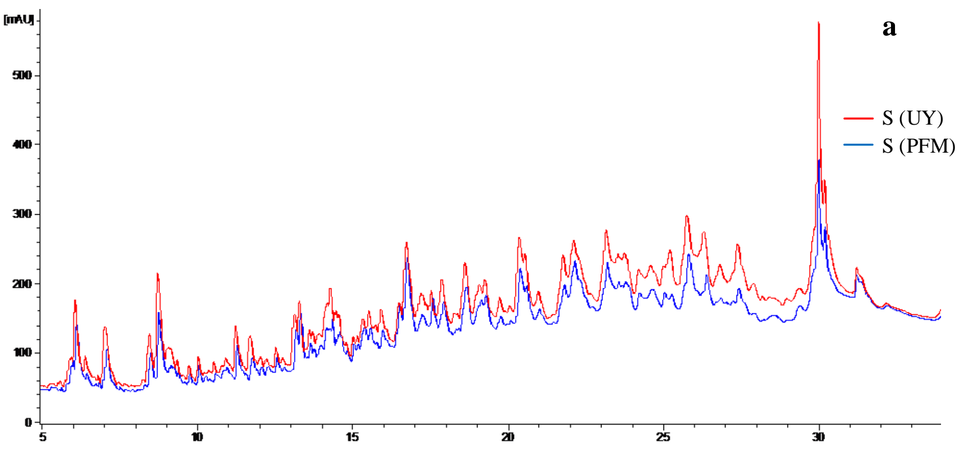


Figure 4.3. (a) UV at 214 nm and (b) MS profiles of the different fractions after *in vitro* gastrointestinal digestion of PFM.

PFM: Probiotic fermented goat milk manufactured with skimmed milk concentrated by ultrafiltration and fermented with *L. bulgaricus* and *S. thermophilus* and *L. plantarum* C4. S: Soluble fraction after *in vitro* gastrointestinal digestion (GID); D: Dialyzable fraction after *in vitro* GID; ND: Not dialyzable fraction after *in vitro* GID.



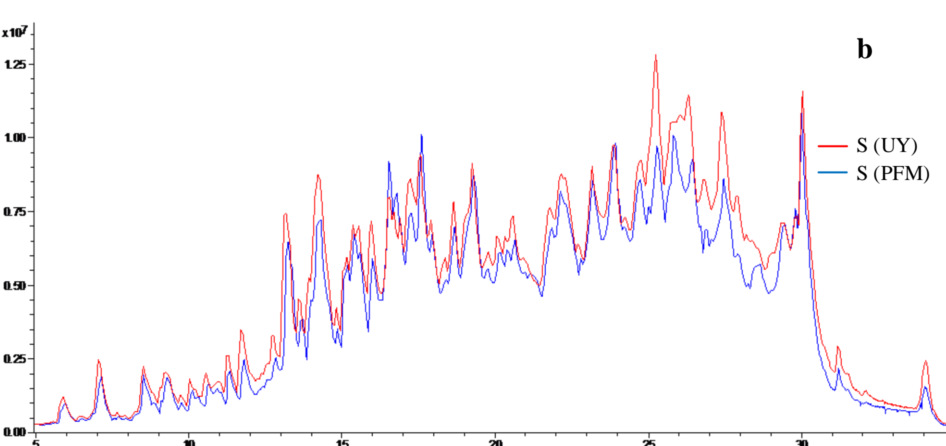


Figure 4.4. (a) UV at 214 nm and (b) MS profiles of S fraction from UY and PFM.

S: Soluble fraction after *in vitro* gastrointestinal digestion; UY: Yoghurt manufactured with skimmed goat milk concentrated by ultrafiltration (UFM) and fermented with *L. bulgaricus* and *S. thermophilus* (St); PFM: Probiotic fermented goat milk manufactured with UFM fermented with St plus *L. plantarum* C4.

3.2.2. Peptides identification and sequencing

The search for the masses and partial sequences was carried out using a database of caprine milk proteins, corresponding to the main genetic variants. For the α_{s1}-CN genetic variant, a RP-UPLC analysis was carried out in the isolated casein, corroboring that the present in our milk was the main genetic variant (Data not shown) (Feligini et al., 2005). 121 different peptides were identified, of which 64 correspondent to β-CN fragments and 23 to α_{s1}-CN fragments.

Figures 4.5 and 4.6 show the peptides identified in the different samples derived from α_{s1}-CN and β-CN respectively and those reported with structure overlapping with the identified by us. Because the reported are referred to peptides found in different dairy samples from different animal species, differences in the sequence were found. There were also identified 20 fragments correspondent to α_{s2}-CN, 11 to κ-CN 3 to β-LG (Table 4.2).

Despite its UV chromatographic profile, the sample where the least peptides were identified was the Milk (E), because it was not subjected to hydrolysis and the presence of them was due to its natural presence on the milk due to the skimming and ultrafiltration are soft processes that should not lead to proteolysis. In WHEY (E) only few more peptides were identified. The samples where more peptides were identified were the digested fractions, in spite of their UV chromatographic profile, as it was suspected according to its MS profile.

Bacteria, particularly LAB, are well known peptide releasers, due to their production during their growth. (Liu et al., 2010). Depending on the bacteria used, the fermentation process as well as the starting proteins (which depends on the milk used, the milk pre-treatment, if it has been concentrated and the concentration method, etc.), the released peptides will be different.

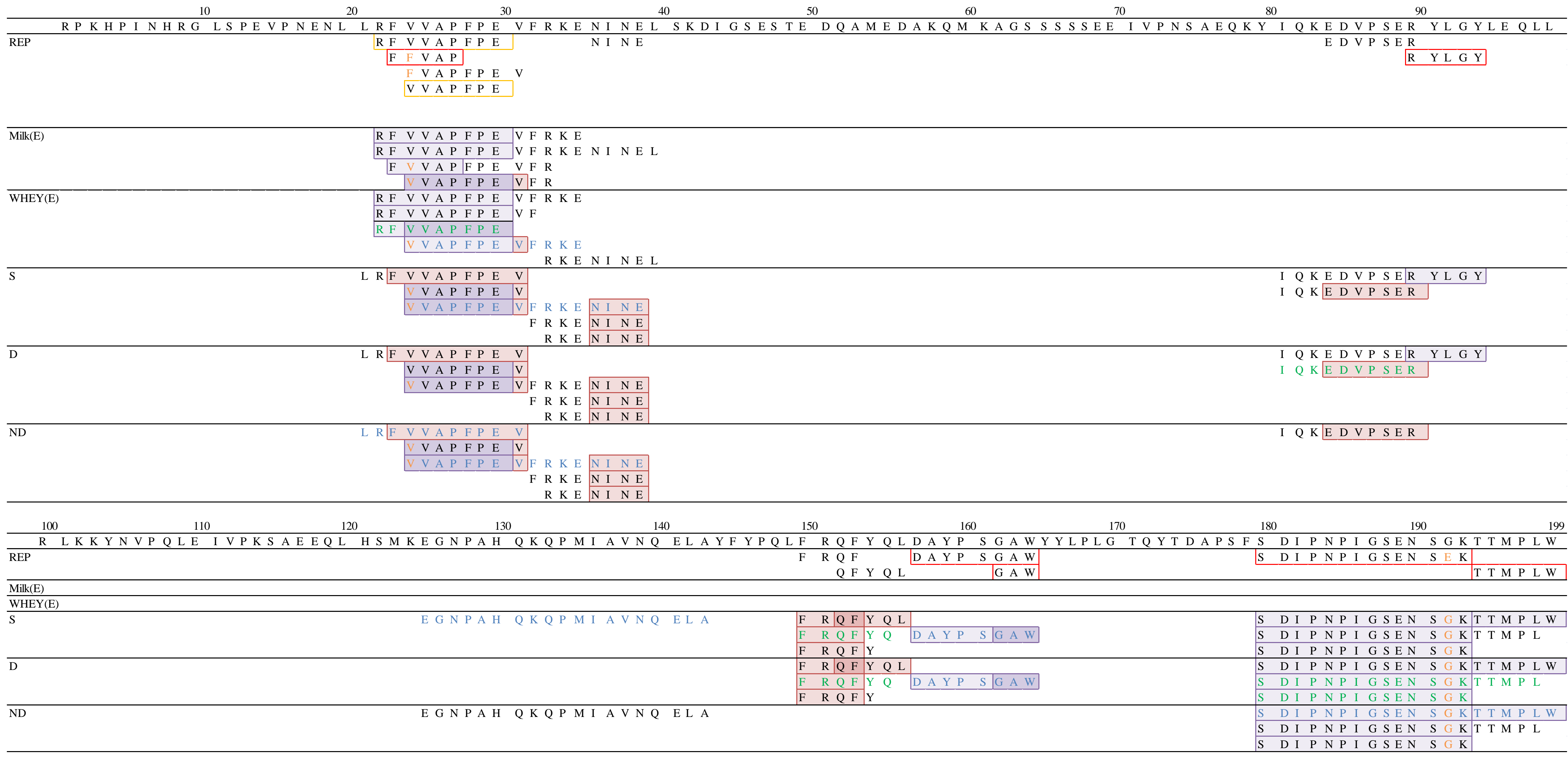


Figure 4.5. α_{s1}-CN protein sequence and derived peptides.

REP: Reported by other authors: those with shares any structure homology with identified peptides, including either the N or the C-terminal part of the peptide; Milk (E): Milk whey eluate after cation exchange at pH 2; WHEY: Fermented milks eluate after cation exchange at pH 2; S: Soluble fraction after *in vitro* gastrointestinal digestion (GID) of fermented milks; D: dialyzable fraction after *in vitro* GID of fermented milks; ND: Non dialyzable fraction after *in vitro* GID of fermented milks.

Colours: Peptides in both UY (Goat yoghurt manufactured with milk concentrated by ultrafiltration (UFM) and fermented with *L. bulgaricus* and *S. thermophilus*(St)) and PFM (Probiotic fermented goat milk manufactured with UFM and fermented with St and *L. plantarum* C4) are in black; peptides exclusively in UY in green and peptides oexclusively in PFM in blue. Reported sequences encircled with red have demonstrated biological activity and those encircled with yellow were identified in an active mixture. Peptides similar to reported sequences were in pink when the reported peptide did not show any biological activity; in lilac when it did. When two or more reported sequences were in the same peptide region, the colour of this coincident region was increased one tone with each peptide coincidence. Then, the more coincidences, the more intense colour. Serine (S) amino acids in red are phosphorilated, amino acids in orange are those different between reported sequences and identified peptides due to the different milk source.

References: (Benkerroum, 2010; Fitzgerald et al., 2006; Gómez-Ruiz et al., 2006; Gútiez et al., 2013; Haque et al., 2007; Hernández-Ledesma et al., 2004; Hernández-Ledesma et al., 2007; Kopf-Bolanz et al., 2014; Losito et al., 2006; Martínez-Maqueda et al., 2012; Phelan et al., 2009; Picariello et al., 2010; Ricci et al., 2010; Silva et al., 2005)

a

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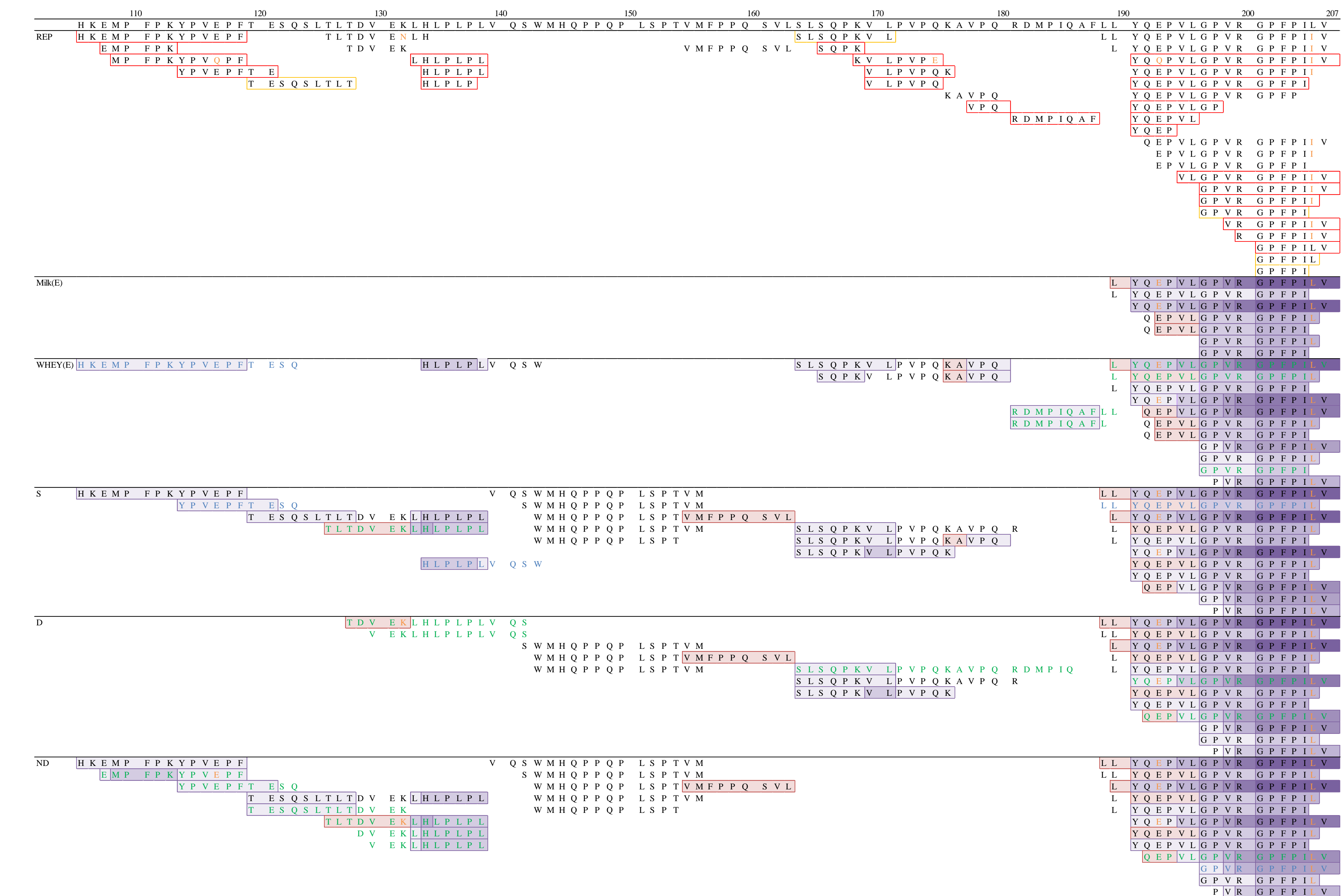
                10                  20                  30                  40                  50                  60                  70                  80                  90                  100
      R E Q E E L N V V G E T V E S L S S S E E S I T H I N K K I E K F Q S E E Q Q Q T E D E L Q D K I H P F A Q A A S L V Y P F T G P I P N S L P Q N I L P L T Q T P V V V P P F L Q P E I M G V P K V K E T M V P K
REP   R E L E E L N V V G E I V E    K I E K F Q S E E    L V Y P F T G P I P N    N I L P L    G V P K V K E
      R E Q E E L    I E K F Q S E E Q Q Q T E D E L Q D K    L V Y P F P G P I    P L T Q T P    G V P K V K
      R E Q E E    I E K F Q S E E Q Q Q T E D E L Q    L V Y P F P G P    T Q T P V V V P P F L Q P E    G V P K
      L N V V G E T V E    F Q S E E Q Q Q T E D E L Q D K I H P F    L V Y P    T P V V V P P F L Q P E V M    V P K V K
      L N V V    F Q S E E Q Q Q T E D E L Q D K I H P    T G P I P N S L P Q    T P V V V P P F L Q P E V
      N V V G E T V E    S E E Q Q Q T E D E L Q D K    S L P Q    P P F L Q P E V
      T E D E L Q D K I H P F
      D E L Q D K I H P F
      E L Q D K I H P F
      D K I H P F A Q
      D K I H P F
      D K I H P
      I H P F
      H P F A Q
Milk(E)    F Q S E E Q Q Q T E D E L Q D K
WHEY(E)    R E Q E E L    S L S S S E E S I T H I N K
           R E Q E E L N V V G E T V E    S S S E E S I T H I N K
S     R E Q E E L    K I E K F Q S E E Q Q Q T E D E L Q D K I H P F    L V Y P F T G P I P N S L P Q N I L P L T Q T
      R E Q E E L N V V    K I E K F Q S E E Q Q Q T E D E L Q D K I H P F A Q    P L T Q T P V V V P P F L Q P E I M G V P K V K E
      I E K F Q S E E Q Q Q T E D E L Q D K I H P    P L T Q T P V V V P P F L Q P E I M G V P K
      F Q S E E Q Q Q T E D E L Q D K I H P F    P L T Q T P V V V P P F L Q P E I M
      F Q S E E Q Q Q T E D E L Q D K I H P    T Q T P V V V P P F L Q P E I M G V P K V K E
      T E D E L Q D K I H P F    P V V V P P F L Q P E I M G V P K V K E
      P V V V P P F L Q P E I M G V P K V K
      P V V V P P F L Q P E I M G V P K
      P V V V P P F L Q P E I M
D     R E Q E E L    L V Y P F T G P I P N S L P Q N I L P L T Q T
      P L T Q T P V V V P P F L Q P E I M G V P K V K E
      P L T Q T P V V V P P F L Q P E I M G V P K
      P L T Q T P V V V P P F L Q P E I M
      P L T Q T P V V V P P F L Q P E I
      P V V V P P F L Q P E I M G V P K V K E
      P V V V P P F L Q P E I M G V P K V K
      P V V V P P F L Q P E I M G V P K
      P V V V P P F L Q P E I M
ND    R E Q E E L N V V    K I E K F Q S E E Q Q Q T E D E L Q D K I H P F    P F T G P I P N S L P Q N I L P L
      R E Q E E L    I E K F Q S E E Q Q Q T E D E L Q D K I H P    P F T G P I P N S L P Q    P L T Q T P V V V P P F L Q P E I M G V P K V K E
      I E K F Q S E E Q Q Q T E D E L Q D K    P L T Q T P V V V P P F L Q P E I M G V P K
      F Q S E E Q Q Q T E D E L Q D K I H P F    P L T Q T P V V V P P F L Q P E I M
      F Q S E E Q Q Q T E D E L Q D K I H P    P L T Q T P V V V P P F L Q P E I
      T E D E L Q D K I H P F    T Q T P V V V P P F L Q P E I M G V P K V K E
      P V V V P P F L Q P E I M G V P K V K E
      P V V V P P F L Q P E I M G V P K V K
      P V V V P P F L Q P E I M G V P K
      P V V V P P F L Q P E I M
```

Figure 4.6. *β*-CN protein sequence and derived peptides **(a)** (from the residue 0 to 105) and **(b)** (from the residue 106 to 207).

REP: Reported by other authors, those that shared any structure homology with the identified peptides, including either the N or the C-terminal part of the peptide; Milk (E): Milk whey eluate after cation exchange at pH 2; WHEY: Fermented milks eluate after cation exchange at pH 2; S: Soluble fraction after *in vitro* gastrointestinal digestion (GID) of fermented milks; D: dialyzable fraction after *in vitro* GID of fermented milks; ND: Non dialyzable fraction after *in vitro* GID of fermented milks.

Colours: Peptides in both UY (Goat yoghurt manufactured with milk concentrated by ultrafiltration (UFM) and fermented with *L. bulgaricus* and *S. thermophilus*(St)) and PFM (Probiotic fermented goat milk manufactured with UFM and fermented with St and *L. plantarum* C4) are in black; peptides only in UY in green and peptides only in PFM in blue. Reported sequences encircled with red have demonstrated biological activity and those encircled with yellow were identified in an active mixture. Peptides similar to reported sequences in pink when the reported peptide did not show any biological activity; in lilac when it did. When two or more reported sequences were coincident in the same peptide region, the colour of this coincident region was increased one tone with each peptide coincidence. Then, the more coincidences, the more intense colour. Serine (S) amino acids in red are phosphorilated, amino acids in orange are those different between reported sequences and identified peptides due to the different milk source.

References: (Cruz-Huerta et al., 2015; El-Salam et al., 2013; Geerlings et al., 2006; Gobbetti et al., 2000; Gómez-Ruiz et al., 2006; Gútiez et al., 2013; Hernández-Ledesma et al., 2004; Losito et al., 2006; F Minervini et al., 2003; Otte et al., 2011; Papadimitriou et al., 2007; Phelan et al., 2009; Picariello et al., 2010; Quirós et al., 2005; Ricci et al., 2010; Robert et al., 2004; Farvin et al., 2010; Sánchez-Rivera et al., 2014; Schieber et al., 2000).



(Chang et al., 2013; Donkor et al., 2007; El-Salam et al., 2013; Fitzgerald et al., 2004; Fitzgerald et al., 2006; Geerlings et al., 2006; Gobbetti et al., 2002; Gómez-Ruiz et al., 2006; Gútiez et al., 2013; Haque et al., 2007; Hernández-Ledesma et al., 2004; Hernández-Ledesma et al., 2005; Hernández-Ledesma et al., 2007; Losito et al., 2006; Minervini et al., 2003; Otte et al., 2011; Papadimitriou et al., 2007; Phelan et al., 2009; Picariello et al., 2010; Plaisancié et al., 2013; Quirós et al., 2007; Ricci et al., 2010; Robert et al., 2004; Farvin et al., 2010; Sánchez-Rivera et al., 2014; Schieber et al., 2000; Silva et al., 2005)

Table 4.2. Peptides identified in all analysed samples and released from α_{s2}-CN, κ-CN and β-LG

Milk (E)	WHEY (E)	D	ND	S	Sequence	Protein (Range)	Meas. Mr	Calc. Mr	z
	✓				VVRNANEEEY	α_{s2}-CN (44-53)	1,221,585	1,221,563	2+
		✓✓			YQKFPQY	α_{s2}-CN (90-96)	972,385	972,471	2+
			✓✓	✓✓ [red]	LQYPYQGPIVLNPWDQV	α_{s2}-CN (97-113)	2,028,985	2,029,031	2+
		✓✓	✓✓	✓✓	LQYPYQGPIVLNPWDQVK	α_{s2}-CN (97-114)	2,157,185	2,157,126	2+
		✓✓	✓✓ [red]	✓✓	LQYPYQGPIVLNPWDQVKR	α_{s2}-CN (97-115)	2,313,278	2,313,227	2+
				✓✓ [green] ✓ [red]	PYQGPIVLNPWDQVK	α_{s2}-CN (100-114)	1,752,985	1,752,920	2+
		✓✓			PYQGPIVLNPWDQVKR	α_{s2}-CN (100-115)	1,909,178	1,909,021	3+
✓✓	✓✓				QGPIVLNPWDQVK	α_{s2}-CN (102-114)	1,492,785	1,492,804	2+
✓	✓✓				QGPIVLNPWDQVKR	α_{s2}-CN (102-115)	1,649,078	1,648,905	3+
✓	✓✓				QGPIVLNPWDQVKRN	α_{s2}-CN (102-116)	1,763,078	1,762,948	3+
			✓✓ [red]	✓✓ [red]	NAGPFTPTVNR	α_{s2}-CN (116-126)	1,172,585	1,172,594	2+
	✓✓				NAGPFTPTVNRE	α_{s2}-CN (116-127)	1,301,585	1,301,636	2+
✓	✓✓				AGPFTPTVNRE	α_{s2}-CN (117-127)	1,187,585	1,187,593	2+
	✓✓ [green]				AGPFTPTVNREQL	α_{s2}-CN (117-129)	1,428,785	1,428,736	2+
	✓✓ [red]				FTPTVNREQL	α_{s2}-CN (120-129)	1,203,585	1,203,625	2+
	✓ [red]				KTKLTEEEKNRLN	α_{s2}-CN (151-163)	1,601,978	1,601,874	3+
		✓ [green]	✓✓	✓✓	TKLTEEEKNRLN	α_{s2}-CN (152-163)	1,473,878	1,473,779	3+
		✓✓	✓✓ [red]	✓✓	TKLTEEEKNRLNF	α_{s2}-CN (152-164)	1,620,878	1,620,847	2+
		✓	✓✓	✓✓	LTEEEKNRLN	α_{s2}-CN (154-163)	1,244,585	1,244,636	2+
✓✓		✓✓			TNAIPYVRYL	α_{s2}-CN (199-208)	1,208,585	1,208,655	2+
		✓✓	✓✓	✓✓	VLSRYPSYGLN	κ-CN (31-41)	1,267,585	1,267,656	2+
		✓✓	✓✓	✓✓	SRYPSYGLN	κ-CN (33-41)	1,055,585	1,055,504	2+
		✓✓	✓✓ [red]	✓✓	SRYPSYGLNY	κ-CN (33-42)	1,218,585	1,218,567	2+
	✓				SRYPSYGLNYYQQRPV	κ-CN (33-48)	1,989,878	1,989,970	3+
		✓✓ [green]			SRYPSYGLNYYQQRPVAL	κ-CN (33-50)	2,174,078	2,174,091	3+
		✓ [red]	✓ [green] ✓✓ [red]	✓✓ [green]	YYQQRPV	κ-CN (42-48)	952,493	952,477	1+
		✓✓	✓✓	✓✓	YYQQRPVAL	κ-CN (42-50)	1,136,585	1,136,598	2+
		✓✓	✓✓	✓✓	INNQFLPYPY	κ-CN (51-60)	1,267,693	1,267,624	1+
		✓✓	✓✓	✓✓	VRSPAQTLQ	κ-CN (67-75)	998,585	998,551	2+
		✓✓			VRSPAQTLQW	κ-CN (67-76)	1,184,585	1,184,630	2+
	✓✓				AVRSPAQTLQ	κ-CN (66-75)	1,069,585	1,069,588	2+
		✓✓ [green] ✓ [red]	✓	✓	ISLLDAQSAPLRV	β-LG (29-41)	1,381,785	1,381,793	2+
		✓✓	✓✓ [red]	✓✓	LDAQSAPLRV	β-LG (32-41)	1,068,585	1,068,593	2+
		✓✓	✓✓	✓✓	YVEELKPTPEGNL	β-LG (42-54)	1,487,785	1,487,751	2+

According to the number of ticks: ✓: Low signal intensity; ✓✓: High signal intensity.
According to the colour: ✓: In UY and PFM; ✓ [green]: In UY; ✓ [red] In PFM
UF: Milk soluble compounds after cation exchange; WSE: Water-soluble extract of fermented milks after cation exchange; D: Dialyzable fraction after *in vitro* gastrointestinal digestion (GID); ND: No dialyzable fraction after *in vitro* GID; S: Soluble fraction after *in vitro* GID; CN: Casein; Meas.: Measured; Mr: Relative molecular mass; Calc.: Calculated; z: Charge; UY: Goat yoghurt manufactured with milk concentrated by ultrafiltration (UFM) and fermented with *L. bulgaricus* and *S. thermophilus* (St); PFM: Probiotic fermented goat milk manufactured with UFM and fermented with St and *L. plantarum* C4

The classical starter bacteria have been widely studied due to their use in the yoghurt elaboration. *L. bulgaricus* demonstrated one of the highest proteolytic activities among LAB (Papadimitriou et al., 2007). However, the majority of the peptides were identified in both UY and PFM samples, reinforcing the hypothesis that *L. plantarum* C4 did not

have high proteolytic acitivity in comparison to the classical starter bacteria, at least in the fermented milks fermentation time. Some peptides, such as the released between the 29 and 54 amino acid residues, were identified in both fermented milks ND fraction but only in the soluble fraction of the PFM. Then, we could hypothesize that the probiotic strain metabolism could produce compounds that favour this solubility in any way.

The bioactive peptides mechanism of action has been widely studied but it is not well known yet. In general, bioactive peptides have less than 20 amino acid residues, and ACEi peptides usually contain between 2-12 (López-Fandiño et al., 2006). For instance, despite their activity has been linked to the C-terminal region composition and sometimes the N-terminal region influences the ACEi activity of peptides with less than 6 amino acid residues, the cause of higher molecular weight peptides activity is still unknown. (Fitzgerald et al., 2004; Haque et al., 2007). ACEi activity of the small peptides is related to the presence in C-terminal position of basic, aromatic or ranched chain amino acids as W, Y, F, L, as well as P. (Contreras et al., 2009; Gobbetti et al., 2000; Haque et al., 2007; López-Fandiño et al., 2006; Quirós et al., 2005; Tsai et al., 2008). The presence of basic amino acids such as K or R, at the C-terminal or penultimate chain position also influences the ACEi activity of the peptide (Gómez-Ruiz et al., 2006; Hernández-Ledesma et al., 2002; Ortiz-Chao et al., 2009).

Regarding the antioxidant activity, the presence of the hydrophobic amino acid residues V or L at the N-terminus and P, H, or Y in the amino acid sequence are related with antioxidant peptides, and the presence and position of W, Y and M are thought to be responsible for the antioxidant activity (Aloğlu at al., 2011; Farvin et al., 2010). In addition, casein derived peptides with E and D have been reported as able to inhibit lipid peroxidation and acid and basic amino acids played an important role in metal chelation (Farvin et al., 2010). An example of an antioxidant peptide that fulfills those characteristics is VKEAMAPK (*β*-CN (f98–105)) produced by the cell envelope protease PrtS of *S. thermophilus* (Miclo et al., 2012).

Some of the identified peptides present those characteristics and could therefore show this activity. The prediction of the peptides activity based on their sequence is a speculative work and examples have been reported. For instance, Hernández-Ledesma

et al. (2004) reported that the peptide SQSKVLPVPQ (β-CN f181-190) may contain potential antioxidant activity, given that this peptide contains the fragment VLPVPQ (β-CN f185-190) (Chang et al., 2013), despite the first peptide did not have the V residue at N-terminus.

Antibacterial activity, in turn, has been related with the peptide charge but the mechanism of action of those peptides remain uncertain due to the influence of other unknown physicochemical and structural properties (Baranyi et al., 2003; Demers-Mathieu et al., 2013).

Almost all peptides identified had less than 20 amino acid residues and could therefore be potentially active. In bovine milk, the α_{s1}-CN structure is composed of four parts: (1) hydrophilic region (f1–12), (2) hydrophobic region (f13–40), (3) hydrophilic region (f41-99), (4) hydrophobic region (f100–199) (Kumosinski et al., 1993). Due to this sequence is similar to the goat milk one, we could assume that the behavior of the protein against hydrolysis will be similar. In the present study almost all peptides were released from the hydrophobic regions, being the most hydrolyzed region the corresponding to the N-terminal hydrophobic region (f22-38). Miclo et al. (2012) reported that the *S. thermophilus* cleavages gave rise to more peptides within the first 40 amino acid residues. In that sense, peptides identified on this region could be released by this strain. In addition, these authors defined the f41-91 region as very resistant to hydrolysis, and we only found two peptides on it.

Two reported bioactive peptides were found in our samples: SDIPNPIGSENSGK, with demonstrated antibacterial activity against *Listeria inocua* (Benkerroum, 2010) and DAYPSGAW, with ACEi activity (IC_{50} = 98μM) (Fitzgerald et al., 2006). The first was present in all digested fractions and the second in the S and D fraction of PFM. In that sense, both would be released after GID and be bioaccesible to be absorbed.

The α_{s1}-CN region of bovine milk f(23-34), identified as antihypertensive in humans, and corresponding to the peptide FFVAPFEVFGK, was released also in our samples (Fitzgerald et al., 2004). In this regard, some peptides in Milk (E) and WHEY (E), contain the equivalent region. However, due to the different sequences from cow milk, as well as the lack of some amino acid residues, the activity will be probably different.

In addition, other identified peptides as VVAPFPEV and RFVVAPFPEV showed high sequence coincidence with other peptides which were isolated from antibacterial active fractions (VVAPFPE and RFVVAPFPE) (Losito et al., 2006). To finish with α_{s1}-CN, the peptide FRQFY released in S and D fractions had not been reported but being 5 amino acid residues long and with a Tyr in C-terminus, it could probably show ACEi activity according to the characteristics of ACEi peptides described previously.

In accordance to results reported by others, the highest number of peptides was derived from β-CN, because it is the major protein in goat milk and its higher susceptibility to cleavage in comparison to α_{s1}-CN and α_{s2}-CN. It is more unstructured and more accessible to enzymes, being therefore hydrolyzed more easily (Chang et al., 2013). The hydrolysis of this protein by *S. thermophilus* and *L. bulgaricus* has been described as due to their cell envelope proteases, Prt.S and PrtB respectively (Chang et al., 2012; Courtin et al., 2002).

The highest breakage in β-CN was between residues 76-100 and 189-207, being this C-terminal region where more peptides with reported active sequences (in cow milk) were found. Miclo et al. (2012) reported this region as more accessible to the cell envelope protease of *S. thermophilus.* Eight reported peptides were found in this region, three of which (PVRGPFPILV, PVRGPFPIL and GPVRGPFPI) with reported activity of the equivalent sequences in cow milk: GPVRGPFPIIV (immunomodulatory, antihypertensive and in antibacterial fraction [Hernández-Ledesma et al., 2005; Losito et al., 2006; Otte et al., 2011; Silva et al., 2005]), GPVRGPFPII and GPVRGPFPI (both present in an antibacterial fraction [Losito et al., 2006]). Despite this, and due to the different C-terminal sequence, the activity of identified peptides would probably be different to the reported by others. Those differences between goat and cow protein sequences were previously reported as the probable reason of the different ACE-inhibitory activity shown for example between GPFPILV (IC_{50}=424 μM) derived from caprine β-CN and LLYQQPVLGPVRGPFPIIV (IC_{50}=22 μM) released from bovine β-CN by hydrolysis with *Lactobacillus helveticus* CP790 proteinase (Quirós et al., 2005).

Other interesting region is that released between the 29 and 54 amino acid residues. The C-terminal sequence of peptides identified in this region is the same as reported peptides with ACEi activity: DELQDKIHPF, ELQDKIHPF, DKIHP, DKIHPF, IHPF

and HPFAQ (Gútiez et al., 2013; Hernández-Ledesma et al., 2004; Otte et al., 2011; Quirós et al., 2005; Ricci et al., 2010).

Finally other peptides were identified in other β-CN regions with reported activity (Figure 4.6b), such as HKEMPFPKYPVEPF (which was identified in two fractions with opioid activity [Plaisancié et al., 2013]) or with reported active sequences, in the region f64-180 and f114-139, with demonstrated antihypertensive, ACEi, antioxidant, opioid, and antibacterial activity. The smaller peptide from this protein was REQEEL. Despite its analogue peptide in bovine milk (RELEEL) did not demonstrate ACEi activity (IC_{50}>1000μM) (Maeno et al., 1996), it would be interesting study its activity because the different amino acid is positively charged.

In α_{s2}-CN, the majority of the peptides belonged to the 97-115 region of the protein and in k-CN to the fragment 33-48. Most of these released peptides had not been identified by others total or partially. However, the reported ACEi peptide YQKFPQY and some active sequences were found (Table 4.3). κ-CN sequence used to the identification was the variety A, with 171 amino acid residues. No peptide was generated from the glycomacropeptide region (f106-169) at the C-terminus, which could be due to the glycan chains protection against hydrolysis (Chang et al., 2013).

Finally, the low amount of peptides identified derived from whey proteins (3 from β-LG and none from α-LA) could be due to the low susceptibility of these proteins to the proteolytic action of LAB and to its relative resistance to GID for their high compact formation (Picariello et al., 2010; Quirós et al., 2005). In addition, those proteins were described as more resistant to GID proteolysis than caseins, whose susceptibility to proteolysis increases with the pre-treatment as heating or fermentation (Kopf-Bolanz et al., 2014).

Table 4.3. Sequences identified in α_{s2}-CN, κ-CN and β-LG, from digested or undigested fermented milks, which share structure homology with previously described peptides.

Sequence	Reported sequence (protein fragment)	Activity	Reference
YQKFPQY	**YQKFPQY** (α_{s2}-CN f90-96)	ACEi (IC_{50} = 95.46 μg/mL)[a]	(El-Salam et al., 2013)
TNAI**PYVRYL**	**PYVRYL** (α_{s2}-CN f202-208)	ACEi (IC_{50}=2.4 μM) Antioxidant Antibacterial	(Quirós et al., 2005) (Chang et al., 2013) (Benkerroum, 2010)
TNAIPY**VRYL**	**VRYL** (α_{s2}-CN f204-208)	ACEi (IC_{50}=24.1 μM)	(Fitzgerald et al., 2006)
SRYPSYGLN	**SRYPS** (κ-CN f33-37)	nr[b]	(Hernández-Ledesma et al., 2004)
SRYPSYGLNY	**SRYPS** (κ-CN f33-37) **YPSYGLNY** (κ-CN f38-42)	nr Opioid antagonist	(Hernández-Ledesma et al., 2004) (Silva et al., 2005)
SRYPSYGLNYYQQRPV	**SRYPS** (κ-CN f33-37)	nr	(Hernández-Ledesma et al., 2004)
IN**NQFLPYPY**	**NQFLPYPY** (κ-CN f53-60)	nr	(Papadimitriou et al., 2007)
LDAQSAPLRV	**LDAQSAPLR** (β-LG f32-40)	ACEi (IC_{50} nr)	(Kopf-Bolanz et al., 2014)
YVEELKPTPEGNL	**YVEEL** (β-LG f42-46)	Antioxidant	(Kopf-Bolanz et al., 2014)

[a]IC_{50}: peptide concentration needed to inhibit 50% the original ACE activity.
[b]nr: No reported information.

4. Conclusions

Several peptides were identified in the ion exchange eluates of Milk and fermented milks WHEY, as well as in the fermented milks *in vitro* products of digestion (S, D and ND). Only few different peptides were observed between UY and PFM, which could indicate the small proteolytic activity of *L. plantarum* C4. However, the passage of some peptides to the D fraction was favored in this product. The samples where less peptides were identified were the least treated, whereas the digested ones showed the highest number of peptides. In addition, almost all the sequences identified after GID were not present before it. The majority of identified peptides had less than 20 amino acid residues, which is the typical maximum size of bioactive peptides. Among the released sequences the following reported active peptides were identified: (1) Bioaccesible in the gut (in D fraction): DAYPSGAW (α_{s1}-CN: f157-164), SDIPNPIGSENSGK (α_{s1}-CN: f179-193), YQEPVLGPVRGPFPILV (β-CN: f191-207), YQEPVLGPVRGPFPI (β-CN: f191-205), GPVRGPFPILV (β-CN: f191-207), GPVRGPFPIL (β-CN: f191-205) and VQKFPQY (α_{s2}-CN: f90-96); (2) Soluble in the

gut (In S fraction): HKEMPFPKYPVEPF (β-CN f106-119). However, other peptides with active sequences and with probable activity were also identified. Most of the identified bioactive peptides or sequences were in S and D fractions, being able to be absorbed and probably to exert their activity on the organism. This, together with the different peptidic composition before and after GID remarks the importance of studying the released peptides after digestion, as they will be the candidates to be absorbed or to exert their activity at the intestinal wall. However, further investigation is required to establish the breakage mechanisms and the *in vivo* activity of those released peptides.

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CONCLUSIONS / CONCLUSIONES

Conclusions

1. The ultrafiltration process increases the content of multiple physicochemical and nutritional parameters of the analyzed goat milks. Specifically, the high mineral concentration measured in skimmed goat milk concentrated by ultrafiltration, particularly for Ca, P, Mg and Zn, as well as its low fat content and high casein concentration, made this milk more appropriate for goat yogurt manufacturing than raw and skimmed milks.
2. The novel probiotic fermented goat milk, manufactured with the skimmed goat milk concentrated by ultrafiltration, showed optimum syneresis and viscosity. Moreover, as result of the sensorial analysis carried out by the trained panellists, it showed the best visual parameters and viscosity as well as an acceptable overall acceptability, similar to the acceptance of analyzed commercial fermented milks.
3. The three strains used in the fermentation remained viable up to the fifth week of storage and even after *in vitro* gastrointestinal digestion.
4. Nutritional and physicochemical parameters of the novel fermented milk are similar to those reported by others in fermented milks, with the exception of Zn levels, which were higher than those reported.
5. The antioxidant and angiotenisin-I converting enzyme inhibition (ACEi) were the more remarkable activities exerted by the developed probiotic and no probiotic ferემented milks. The anionic and neutral peptides with molecular weight lower than 3 kDa were specially actives in ORAC assay and inhibiting the ACE enzyme. However, cationic peptides showed the highest activity against $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals.
6. After 24 h of co-cultive of the different fermented milk fractions, certain activity was found against *Escherichia coli*, highlighting that the fraction with cationic peptides showed this activity only in the probiotic fermented milk.
7. The number of released peptides increased when the milk was fermented, as well as after *in vitro* digestion, identifying the least number of peptides in milk and the most in the digested samples.

8. The size of identified peptides in probiotic and no probiotic fermented goat milks, their digested fractions and in the milk, as well as their homologous sequence to peptides with recognized biological activity, led us to believe that they were the main responsible of the antioxidant and ACEi activities. In addition, their presence in soluble and dialyzed fractions after *in vitro* digestion showed their capacity to be absorbed and exert their activity in the organism.

General conclusion

The new probiotic fermented goat milk developed had adequate physicochemical, nutritional and organoleptic characteristics. It showed remarkable antioxidant and high angiotensin-I-converting enzyme inhibitory activities, which could be due to the peptides released during the fermentation, some of which show homologous sequences to peptides with already known biological activity. In addition, the release of high variety of potentially bioactive peptides with capacity to be absorbed after *in vitro* gastrointestinal digestion could imply their activity *in vivo*. Therefore, we could consider the developed fermented milk as a healthy alternative to the widely commercialized cow yoghurt.

Conclusiones

1. El proceso de ultrafiltración aumentó el contenido de gran cantidad de parámetros fisicoquímicos y nutricionales de las leches de cabra analizadas. En concreto, el alto contenido mineral medido en la leche de cabra desnatada concentrada por ultrafiltración, particularmente de Ca, P, Mg y Zn, así como su bajo contenido en grasa y su alta concentración en caseína la hace más apropiada para la fabricación de yogur que las leches entera y desnatada.
2. La nueva leche fermentada probiótica de cabra, elaborada a partir de la leche desnatada de cabra concentrada por ultrafiltración, presentó una viscosidad y sinéresis óptimas. Además, como resultado del análisis sensorial llevado a cabo por panelistas entrenados presentó mejores parámetros visuales y viscosidad, así como una aceptable aceptación general, semejante a la de las leches fermentadas comerciales analizadas.
3. En la leche fermentada desarrollada, las tres cepas usadas se mantuvieron viables hasta la quinta semana de almacenamiento e incluso tras someterse a un proceso de digestión *in vitro*.
4. Los valores nutricionales y físico-químicos de la nueva leche fermentada son similares a los determinados por otros autores en leches fermentadas, resaltando el Zn, cuyos niveles fueron netamente superiores.
5. Las actividades biológicas más destacables ejercidas por las leches fermentadas probiótica y no probiótica desarrolladas, fueron la antioxidante y la inhibidora de la enzima convertidora de la angiotensina-I (ACEi). Los péptidos aniónicos y neutros de peso molecular menor a 3 kDa fueron específicamente activos en el ensayo de actividad antioxidante ORAC ye inhibiendo la ACE. Sin embargo, los péptidos catiónicos mostraron la mayor actividad antioxidante frente a los radicales $ABTS^{\bullet+}$ y $DPPH^{\bullet}$.
6. Hemos observado una cierta actividad antimicrobiana frente a *Escherichia coli* tras 24 h de co-cultivo de distintas fracciones de las leches fermentadas, destacando la que contiene péptidos catiónicos la cual manifiestó esta actividad únicamente en el yogur probiótico.

7. El número de péptidos liberados se incrementó cuando al leche de cabra se sometió al proceso de fermentación, así como tras la digestión *in vitro,* identificándose el número más bajo en la leche y el más alto en los digeridos.
8. El tamaño de los péptidos identificados en las leches fermentadas desarrolladas, probiótica y no probiótica, en sus digeridos correspondientes y en la leche, así como su secuencia homóloga a la de algunos péptidos de reconocido valor biológico, nos induce a pensar que son los principales responsables de las actividades antioxidante y ACEi. Además, su presencia en las fracciones soluble y dializada tras el proceso de digestión *in vitro* manifiesta su capacidad para ser absorbidas y así ejercer su actividad en el organismo.

Conclusión general

El nuevo fermentado probiótico de leche de cabra desarrollado presentó unas características físico-químicas, nutricionales y organolépticas adecuadas. Éste mostró destacable actividad antioxidante y una alta actividad inhibidora de la enzima convertidora de la angiotensina-I, que podrían ser debidas a los péptidos liberados durante la fermentación, algunos de los cuales presentan secuencias aminoacídicas comunes a péptidos con actividades biológicas conocidas. Además, la liberación de una gran variedad de péptidos posiblemente bioactivos y con capacidad de ser absorbidos tras la digestión gastrointestinal *in vitro*, podría implicar la actividad de los mismos *in vivo.* Por tanto, podríamos considerar la leche fermentada desarrollada como una alternativa saludable al yogur de vaca ampliamente comercializado.

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Anexe – Answer sheet

Sensorial evaluation of fermented goats' milk

Name:

Date:

Sample:

Descriptive scale of perception:

No perceptible	Starts the perception	Weak	Moderate	Strong	Very strong
0	1	2	3	4	5

VISUAL

1. Please, rate from 1 to 5 the following parameters

	Grey/yellow white	Pure white
- Colour		

	0	5
- Syneresis		

	0	5
- Smoothness		

2. Please, tick if any of those defects is perceived.
   - Absence of curd homogenity ☐
   - Floury ☐
   - Lumps ☐
   - Bubbles ☐
   - Others ☐

AROMA

3. Please, rate from 1 to 5 the overall aroma perception

- Aroma fineness 0 |—|—|—|—|—| 5
- Aroma intensity 0 |—|—|—|—|—| 5
- Aroma persistence 0 |—|—|—|—|—| 5

4. Please, tick from none to three times in function of the intensity perceived for the following attributes

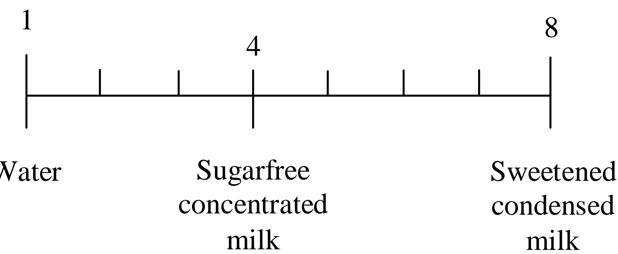
- Fermentation ☐☐☐
- Acetaldehyde ☐☐☐
- Dyacetil ☐☐☐
- Goat ☐☐☐
- Flower ☐☐☐
- Fruit ☐☐☐
- Others ☐☐☐

5. Please, tick from none to three times in function of the intensity perceived for the following defects

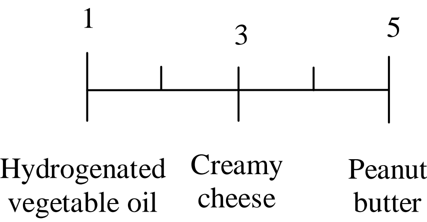
- Cooked milk ☐☐☐
- Others ☐☐☐

TEXTURE

6. Viscosity



7. Stickiness



TASTE

8. Please, rate from 1 to 5 the taste perception

- Taste fineness 0 ——— 5
- Taste intensity 0 ——— 5

9. Please, rate from 1 to 5 the following parameters

- Sweetness 0 ——— 5
- Acidity 0 ——— 5
- Bitterness 0 ——— 5

10. Please, tick from none to three times in function of the intensity perceived for the following attributes or defects

- Goat ☐☐☐
- Fruit ☐☐☐
- Astringent ☐☐☐
- Spicy ☐☐☐
- Metallic ☐☐☐
- Salty ☐☐☐
- Insipid ☐☐☐
- Dirty ☐☐☐
- Rancid ☐☐☐
- Yeast like ☐☐☐
- Cooked milk ☐☐☐
- Humidity ☐☐☐

11. Please, rate from 1 to 5 the overall acceptability of the fermented milk

0 ——— 5