Universitat de Girona

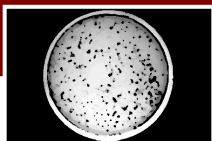# ADVANCES IN ANIMAL BLOOD PROCESSING: DEVELOPMENT OF A BIOPRESERVATION SYSTEM AND INSIGHTS ON THE FUNCTIONAL PROPERTIES OF PLASMA

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Advances in animal blood processing: development of a biopreservation system and insights on the functional properties of plasma

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Advances in animal blood processing: development of a biopreservation system and insights on the functional properties of plasma

by

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Advances in animal blood processing: development of a biopreservation system and insights on the functional properties of plasma

Memòria presentada per Eduard Dàvila Ribot, inscrit en el programa de doctorat de Ciències Experimentals i de la Salut, itinerari de Biotecnologia, per optar al grau de Doctor europeu per la Universitat de Girona.

Eduard Dàvila Ribot

Novembre, 2006

Dolors Parés Oliva, Professora titular de l'Àrea de Tecnologia dels Aliments del Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària i investigadora de l'Institut de Tecnologia Agroalimentària de la Universitat de Girona,

CERTIFICA:

Que el Llicenciat en Ciència i Tecnologia dels Aliments Eduard Dàvila Ribot ha portat a terme sota la seva direcció a l'Àrea de Tecnologia dels Aliments de la Universitat de Girona el treball titulat *Advances in animal blood processing: development of a biopreservation system and insights on the functional properties of plasma*, que presenta en aquesta memòria per optar al grau de Doctor europeu.

I per a què consti als efectes oportuns, signa la present a Girona, el 24 de novembre de 2006.

Vist i plau

Dra. Dolors Parés Oliva

Dolors Parés Oliva, Professora titular de l'Àrea de Tecnologia dels Aliments del Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària i investigadora de l'Institut de Tecnologia Agroalimentària de la Universitat de Girona,

Eduard Dàvila Ribot, que ha inscrit la tesi doctoral titulada *Advances in animal blood processing: development of a biopreservation system and insights on the functional properties of plasma* amb el número de registre 695 (11/11/2004),

DECLAREN

Que aquesta tesi forma part dels projectes de recerca AGL2001-0888 i AGL2004-06077/ALI, i que està sotmesa a la propietat intel·lectual compartida amb els investigadors de l'àrea de Tecnologia dels Aliments de la Universitat de Girona que participen en ambdós projectes d'acord amb el que s'estipula en els Reials Decrets RD 1326/2003 i RD 1/1996.

I per a què consti als efectes oportuns, signen la present a Girona, el 24 de novembre de 2006.

Dra. Dolors Parés Oliva   Eduard Dàvila Ribot

Voldria agrair...

... a la Dolors, l'ajuda i l'orientació que durant quatre anys m'ha ofert, sempre immediata, i la possibilitat d'haver estat gaudint d'una feina que m'ha omplert durant aquest temps.

... a totes les tecnogirls: la Nuri, la Mònica, la Sònia, l'Elena, la Paula, la Carme, l'Anna Maria, la Maria, les Annes, l'Ariadna i la Núria, la valuosa ajuda que sempre m'han ofert, i perquè han format part d'un gran equip de treball que ha fet de la feina també una diversió.

... a tots els membres de l'INTEA, tots els mitjans on he realitzat experiments i els cops de mà que m'han donat quan he necessitat quelcom.

... al Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària, als Serveis de Recerca i a la Universitat de Girona, les instal·lacions i els mitjans on he realitzat aquest treball.

... a l'Agència de Gestions i Ajuts Universitaris i de Recerca, de la Generalitat de Catalunya, les beques concedides durant aquest període.

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... a l'Ester, la Cristina i la Núria, els seus treballs, que han estat de gran ajuda per mi, i a la Lucero, la seva col·lecció de bacteris làctics.

... al menjador en general, els mecanismes de selecció natural que s'aprenen.

... als meus amics, el seu sofert interès i perquè quan ha calgut han aportat aquella guspira.

... als meus pares, germana i a la resta de tota la meva família, tot, perquè us trobeu més enllà del suport i l'afecte, i sou un impuls incessant.

... a qui ens ha deixat durant aquests quatre anys, la il·lusió que elles tenien.

... a la Rosa, tot, perquè ni la inspiració ni la transpiració tenen sentit sense tu.

A tu, Rosa

SUMMARY

Blood is a by-product obtained in large amounts in industrial slaughterhouses with a high potential of valorisation. Currently, most of the blood collecting systems are not subjected to strict hygienic measures hence it becomes a product with low microbiological quality. As a result, the use of blood for consumption purposes is not a stimulating prospect from an economic point of view, because the intrinsic worth allowing the development of high value-added products is normally lost.

In order to improve the interest in the valorisation of blood, it would be necessary to develop an easy, low-cost system that guaranteed its quality during collection and storage. Also, it would be necessary to enhance the obtaining of blood-derived products through the use of their functional properties, to develop functional food ingredients.

Chapter I of the present dissertation is included within a research project that suggests lactic acid bacteria (LAB) as a blood biopreservative culture: a simple, inexpensive system to keep the stability of blood both in terms of microbiological and physicochemical quality, during its storage.

Firstly, the identification of a collection of LAB strains by means of genotypic methods was carried out. These strains had been previously isolated from blood collecting tanks, and their antagonistic capacity against contaminant indicators had been studied. The identification results established that the diversity of LAB found at slaughterhouses is gathered by the following species: *Lactococcus garvieae*, *Lactobacillus reuteri*, *Lactobacillus murinus*, *Enterococcus raffinosus*, *Enterococcus dispar*, *Enterococcus hirae*, *Lactobacillus mucosae* and *Streptococcus bovis*. The former four accounted for the 95% of the total isolated colonies, and all of them were equally found in all the screened slaughterhouses.

Secondly, the preservation ability in blood stored at 15 ºC of 12 strains that showed inhibitory qualities in plate assays was investigated. Five out of 12 strains inhibited the growth of indicator microorganisms. A positive effect on the slowdown of spoilage processes –mainly by preventing from hemolysis and loss of functionality– caused by the inoculation of LAB was observed. The two strains that showed the best results were selected for further preservation experiments at 5 ºC. It was decided to supplement blood with 2% inulin, a selective energy source to favour LAB's activity. Preservation experiments of inulin-enriched blood proved that the addition of the selected strains entailed positive effects against the growth of undesirable bacteria (coliforms, *Pseudomonas* spp., hemolytic and proteolytic bacteria). Finally, it was concluded that the inoculation of the strain PS99 (*E. raffinosus*) may be adequate to maintain the quality of blood stored at refrigeration temperatures, especially useful to prevent microbial spoilage in cases of cold-chain breakdown.

Chapter II is framed within a research project that investigates ways to improve the use of blood as a food ingredient, which, in the case of plasma, is focused on the functionality of its main protein constitu-

ents. The knowledge of the contribution of each constituent can be used to improve the functional properties of plasma-based ingredients.

The protein fractions investigated were: whole plasma, serum, albumin and globulins. The functional properties studied at varying pH conditions (from 4.5 to 7.5) were: heat-induced gelation, solubility, foaming and emulsifying properties. Besides the determination of the functionality of each sample, biochemical investigations were performed with differential scanning Calorimetry, FT-Raman spectroscopy and electrophoresis. Protein-protein interactions were also studied: the role of fibrinogen was elucidated by comparing plasma and serum samples, and theoretical mixtures of albumin and globulins –obtained from the results of both fractions on its own– as compared to serum samples, allowed the determination of interactions between the major constituents. On the other hand, a method to characterise the microstructure of protein gels was developed, which will be useful to investigate the structural conformation of each protein fraction.

The results of gelation studies showed that each fraction displayed distinct thermal properties, which contributed in a particular way to the development of the protein aggregates network when plasma was subjected to heat-induced gelation. Covalent bonds, weak unions and changes in the secondary structure components were confirmed to participate in the gelation process of all the studied fractions. The obtained gels were different in terms of their texture profile and water-holding capacity. Furthermore, a specific interaction between albumin and fibrinogen during gelation was determined, and the development of strong gels at every pH was attributed to the globulins fraction, which were responsible for the strength of gels when mixed with albumin, because of the weakness of the latter.

In relation to solubility measurements, all protein fractions showed high values, above 85%. Surface properties, conversely, presented remarkable differences between fractions, in some cases enhanced by pH conditions. The foaming capacity of all samples was high, with the exception of globulins at pH 6.0 and 7.5, and the stability of the foams was higher in albumin and plasma samples at pH 6.0. The emulsifying capacities were similar for all fractions, but the stability of emulsions was extremely different; both globulins and albumin at pH 7.5 showed a several-fold larger stability than serum and plasma, and that of the same fractions at acidic conditions.

Hence, the functional properties of plasma could be engineered to specific food requirements by reformulating its natural composition and enhanced by controlling pH.

The results presented in this thesis dissertation may help the valorisation of porcine blood from industrial slaughterhouses, thanks to the acquired knowledge about the improvement of blood preservation and the development of plasma-based food ingredients with interesting functional properties.

RESUM

La sang és un subproducte amb un alt potencial de valorització que s'obté en quantitats importants en els escorxadors industrials. Actualment, la majoria de sistemes de recollida de la sang no segueixen unes mesures d'higiene estrictes, pel que esdevé un producte de baixa qualitat microbiològica. Conseqüentment, l'aprofitament de la sang és una sortida poc estimulant des del punt de vista econòmic, ja que acostuma a perdre les qualitats que permetrien l'obtenció de productes d'alt valor afegit.

Per millorar l'interès per a la seva valorització, caldria implementar un sistema senzill i poc costós que n'asseguré la qualitat durant la recollida i l'emmagatzematge. Així mateix, també caldria potenciar l'obtenció de productes derivats de la sang mitjançant l'aprofitament de les seves propietats funcionals pel que fa a la fabricació d'ingredients alimentaris.

El capítol I del present treball s'inclou dins d'un projecte que proposa la inoculació de bacteris de l'àcid làctic (LAB) com un cultiu bioconservador de la sang, un sistema senzill i de baix cost que cerca l'estabilitat de la sang, tant microbiològica com fisicoquímica, durant el període del seu emmagatzematge.

En primer lloc, es va realitzar la identificació d'una col·lecció de soques de bacteris làctics mitjançant mètodes genotípics. Aquestes soques havien estat aïllades anteriorment en tancs de recollida de sang, i s'havia estudiat la seva capacitat antagonista davant alguns microorganismes indicadors. La seva identificació va permetre conèixer que la diversitat dels LAB presents en les instal·lacions estava recollida en les següents espècies: *Lactococcus garvieae*, *Lactobacillus reuteri*, *Lactobacillus murinus*, *Enterococcus raffinosus*, *Enterococcus dispar*, *Enterococcus hirae*, *Lactobacillus mucosae* i *Streptococcus bovis*. Les quatre primeres espècies ja contenien el 95% de totes les colònies aïllades, i aquestes es trobaven igualment distribuïdes en totes les instal·lacions mostrejades.

En segon lloc, es va avaluar la capacitat bioconservadora en sang mantinguda a 15 ºC de 12 soques seleccionades segons la inhibició que havien demostrat en anteriors assajos de conservació en placa. Els resultats van mostrar que 5 de les 12 soques inhibien el creixement dels microorganismes indicadors utilitzats, i van evidenciar els efectes positius de la presència de LAB vers les alteracions fisicoquímiques provocades per la microbiota contaminant autòctona, especialment pel que fa al grau d'hemòlisi i l'alteració de les propietats funcionals del plasma. Les dues soques que van mostrar els millors resultats van ser seleccionades per a assajos de bioconservació a 5 ºC. Es va decidir enriquir la sang amb inulina, una font d'energia selectiva per a l'activitat dels LAB. Els assajos de conservació de sang enriquida amb inulina al 2% van demostrar que l'addició de les soques tenia efectes positius enfront al creixement de coliforms, *Pseudomonas* spp., bacteris hemolítics i proteolítics, i es va concloure que la inoculació de la soca PS99 (*E. raffinosus*) podia ser adequada per al manteniment de la qualitat de la sang en condicions de refrigeració, especialment útil pel que fa a la prevenció del deteriorament en casos de trencament de la cadena del fred.

El capítol II s'emmarca dins d'un projecte que cerca la millora de l'aprofitament integral de la sang que, en el cas de la fracció plasmàtica, es centra en l'estudi de la funcionalitat dels seus principals constituents. Conèixer la contribució dels components majoritaris ha de permetre la millora de la funcionalitat dels ingredients alimentaris derivats.

Les fraccions que es van investigar van ser: el plasma sencer, el sèrum, l'albúmina i les globulines. Les propietats funcionals que es van estudiar, a diferents condicions de pH (de 4,5 a 7,5), van ser: la gelificació, la solubilitat, la capacitat escumant i la capacitat emulgent. A més d'establir els resultats de cadascuna de les mostres pel que fa a la propietat d'interès, es van realitzar estudis de tipus bioquímic mitjançant anàlisi calorimètrica diferencial, espectroscòpia FT-Raman i electroforesi. Per altra banda, es van determinar les interaccions proteïna-proteïna entre els constituents: la comparació entre el plasma i el sèrum va desvetllar la contribució del fibrinogen, i la comparació entre el sèrum i les fraccions aïllades d'albúmina i globulines, les interaccions entre ambdues fraccions. Es va posar a punt un mètode per caracteritzar la microestructura dels gels, que permetrà l'estudi de la conformació estructural de les principals fraccions.

Els resultats de les propietats de gelificació van demostrar que cadascun dels components posseïa diferents propietats tèrmiques, que contribuïen de forma específica en l'agregació proteica del plasma quan aquest es sotmetia a un procés de gelificació induït per calor. Es va determinar que la formació d'enllaços covalents, el desenvolupament d'unions dèbils i els canvis en els components de l'estructura secundària intervenien en la formació de gels de cadascuna de les fraccions. Aquests, van mostrar propietats macroscòpiques particulars pel que fa al seu perfil de textura i a la capacitat de retenció d'aigua. Es va descobrir que el fibrinogen interaccionava de forma selectiva amb l'albúmina durant la gelificació, que les globulines formaven gels compactes en totes les condicions de pH assajades i que la interacció de les globulines amb l'albúmina conferia una duresa notablement superior a l'esperada en gels de mescles d'ambdues fraccions. Pel que fa a la solubilitat de les fraccions, totes van mostrar valors elevats, superiors al 85%. Les propietats superficials, per altra banda, van evidenciar diferències notables entre les mostres, modificades en alguns casos per les condicions de pH. La capacitat escumant de les fraccions va ser elevada, amb l'excepció de les globulines a pH 6,0 i 7,5, i la estabilitat de les escumes va ser superior en les mostres d'albúmina i plasma a pH 6,0. La capacitat emulgent de totes les fraccions va ser similar, però la estabilitat de les emulsions va demostrar grans diferències; a pH 7,5, tant les globulines com l'albúmina presentaven una estabilitat molt superior a la del sèrum i el plasma, o a la de les mateixes fraccions a pH àcid.

Amb tot això, es pot concloure que les propietats funcionals del plasma poden ser reconduïdes cap a requeriments alimentaris específics mitjançant la reformulació de la seva composició natural, potenciant-la amb el control de les condicions de pH en què es troba.

Els resultats presentats en aquesta tesi poden ajudar a la valorització de la sang porcina d'escorxadors industrials, mitjançant els coneixements adquirits pel que fa a la millora del seu sistema de recollida i del desenvolupament d'ingredients alimentaris amb interessants propietats funcionals.

RESUMEN

La sangre es un subproducto que se obtiene en cantidades importantes en los mataderos industriales con un alto potencial de valorización. Actualmente, la mayoría de sistemas de recolección de la sangre no utilizan unas medidas de higiene estrictas, por lo que ésta se convierte en un producto de baja calidad microbiológica. Consecuentemente, su aprovechamiento resulta poco estimulante desde el punto de vista económico, puesto que las cualidades que permiten la obtención de productos de alto valor añadido suelen perderse.

Con el fin de mejorar el interés por su valorización, convendría implementar un sistema sencillo y poco costoso que asegurase la calidad de la sangre durante su recolección y almacenaje. Asimismo, convendría potenciar la obtención de productos derivados de la sangre mediante el aprovechamiento de sus propiedades funcionales en lo que se refiere a la producción de ingredientes alimenticios.

El Capítulo I se incluye dentro de un proyecto que propone la utilización de bacterias del ácido láctico como cultivo bioconservador de la sangre, un sistema sencillo y de bajo coste para lograr la estabilidad de la sangre, tanto microbiológica como fisicoquímica, durante su almacenaje.

En primer lugar, se llevó a cabo la identificación de una colección de cepas de bacterias lácticas mediante métodos genotípicos. Estas cepas habían sido aisladas anteriormente en tanques de recolección de sangre, y se había estudiado su capacidad antagonista frente a ciertos microorganismos indicadores. Su identificación estableció que la diversidad de cepas LAB presentes en las instalaciones correspondía únicamente a la siguientes especies: *Lactococcus garvieae*, *Lactobacillus reuteri*, *Lactobacillus murinus*, *Enterococcus raffinosus*, *Enterococcus dispar*, *Enterococcus hirae*, *Lactobacillus mucosae* y *Streptococcus bovis*. Las cuatro primeras agrupaban el 95% de todas las colonias aisladas y se hallaban igualmente distribuidas en todas las instalaciones muestreadas.

En segundo lugar, se determinó la capacidad de conservación en sangre mantenida a 15 ºC de 12 cepas seleccionadas según la inhibición que habían demostrado en anteriores ensayos de conservación en placa. Los resultados mostraron que 5 de las 12 cepas utilizadas inhibían el crecimiento de los microorganismos indicadores, y evidenciaron los efectos positivos de la presencia de LAB frente a alteraciones fisicoquímicas provocadas por la microbiota contaminante, especialmente en la alteración de las propiedades funcionales del plasma. Las dos cepas que mostraron los mejores resultados fueron seleccionadas para ensayos de bioconservación a 5 ºC. Se decidió enriquecer la sangre con inulina, una fuente de energía selectiva para la actividad de las LAB. Los ensayos de bioconservación de sangre enriquecida con inulina al 2% demostraron que la adición de las cepas tenía efectos positivos frente al crecimiento de coniformes, *Pseudomonas* spp., bacterias hemolíticas y proteolíticas, y se concluyó que la inoculación de la cepa PS99 (*E. raffinosus*) podía ser adecuada para el mantenimiento de la calidad de la sangre en condiciones de refrigeración, especialmente en cuanto a la prevención de su deterioro en casos de rotura de cadena del frío.

El Capítulo II se enmarca dentro de un proyecto que persigue la mejora del aprovechamiento integral de la sangre que, el caso de la fracción plasmática, se centra en el estudio de la funcionalidad de sus principales constituyentes. Conocer la contribución de los componentes mayoritarios puede permitir la mejora de la funcionalidad de los ingredientes alimenticios que de ellos se derivan.

Las fracciones que se investigaron fueron: el plasma, el suero, la albúmina y las globulinas. Las propiedades funcionales que se estudiaron, bajo distintas condiciones de pH (de 4,5 a 7,5), fueron: la gelificación, la solubilidad, y las capacidades espumantes y emulgentes. Además de establecer los resultados de todas las muestras en lo que se refiere a su funcionalidad, se realizaron estudios bioquímicos mediante análisis calorimétrico diferencial, espectroscopia FT-Raman y electroforesis. También se investigaron las interacciones proteína-proteína entre los diferentes constituyentes: la comparación entre el plasma y el suero desveló la contribución del fibrinógeno, y la comparación entre el suero y la albúmina y las globulinas aisladas, las interacciones entre ambas. Por otro lado, se desarrolló un método para caracterizar la microestructura de los geles que permitirá el estudio de la conformación estructural de todas las fracciones.

Los resultados de las propiedades de gelificación demostraron que todos los componentes poseían distintas propiedades térmicas, que contribuían de forma específica en la agregación proteica sufrida por el plasma cuando se sometía a un proceso de gelificación inducido por calor. Se determinó que la formación de enlaces covalentes, el desarrollo de uniones débiles y los cambios en los componente de la estructura secundaria intervenían en la formación de los geles de todas fracciones. Éstos, a su vez, mostraron propiedades macroscópicas particulares en cuanto a su perfil de textura y a la capacidad de retención de agua. Se descubrió que el fibrinógeno interaccionaba de forma selectiva con la albúmina durante la gelificación, que las globulinas formaban geles compactes en todas las condiciones de pH estudiadas y que la interacción de las globulinas con la albúmina confería una dureza en los geles notablemente superior a la esperada en mezclas de ambas fracciones. En cuanto a la solubilidad de las fracciones, todas mostraron valores elevados, superiores al 85%. Las propiedades superficiales, por otro lado, evidenciaron diferencias notables entre las muestras, modificadas en ciertos casos por el pH. La capacidad espumante de las fracciones fue elevada, con la excepción de las globulinas a pH 6,0 y 7,5, y la estabilidad de la espumas fue mayor en las muestras de albúmina y plasma a pH 6,0. La capacidad emulgente de todas las fracciones fue similar, pero la estabilidad de las emulsiones demostró grandes diferencias. A pH 7.5, las globulinas y la albúmina presentaron una estabilidad muy superior a la del plasma y el suero, y a la de las mismas fracciones a pH ácido.

Con todo esto, se concluyó que las propiedades funcionales del plasma podían ser reconducidas hacia requerimientos alimentarios específicos, mediante la reformulación de su composición natural, potenciando dichos efectos a través del control del pH.

Los resultados presentados en esta tesis pueden ayudar a la valorización de la sangre porcina de mataderos industriales, mediante los conocimientos adquiridos relativos a la mejora de su sistema de recolección y al desarrollo de ingredientes alimenticios con propiedades funcionales interesantes.

The present thesis dissertation is the outcome of a 4-year period of research (2003-2006). It has been divided into four main parts (see scheme below). Firstly, a **general introduction** about the attributes and the use of blood precedes the following two chapters.

In Chapter I, the experiments concerning the **preservation of blood** are detailed; these experiments were carried out to accomplish the objectives of the project AGL2001-0888, which included the genotypic identification of a collection of lactic acid bacteria (LAB) strains and the preservation of blood by means of LAB inoculation.

In Chapter II, some investigations about the **functionality of plasma** and its protein fractions are presented; these belong to a research line within the project AGL2004-06077/ALI, which focuses on the improvement of the functionality of blood and its fractions, namely plasma and red blood cells (RBC).

Both Chapter I and II are organised as follows: i) a brief introduction to the specific subject therein discussed, ii) the objectives of the chapter, iii) a section that describes all the materials and methods used, iv) the results of the experiments as published or submitted articles, v) a general discussion section that discusses the obtained results as a whole and includes some relevant results not presented in the publications, vi) the conclusions, and vii) the cited references.

Finally, a section of **concluding remarks** terminates the dissertation with some general comments and a list of future prospects.
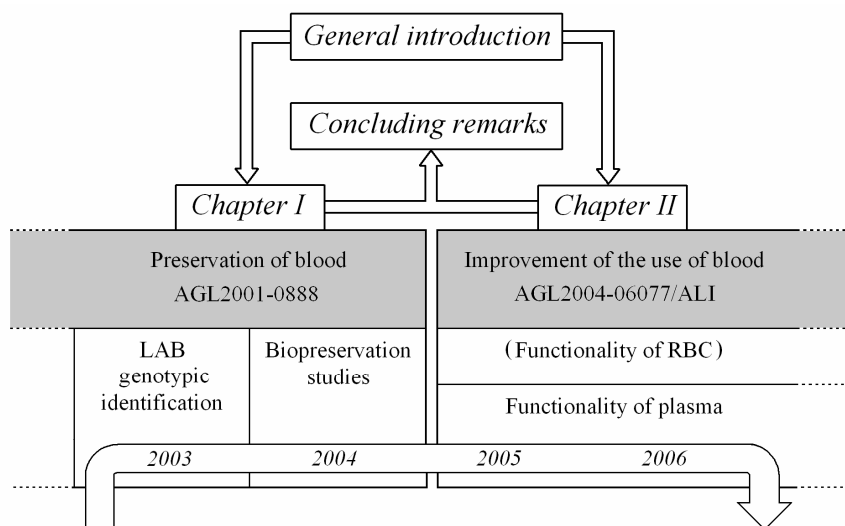
Organisation of this dissertation, where the results of a 4-year period investigating the preservation of blood and the functionality of plasma are presented.

INDEX OF TABLES

INDEX OF FIGURES

INDEX OF SCHEMES

ABBREVIATIONS USED

ANOVA	Analysis of variance
A_ε	Lacunarity
BAB	Blood agar base
BSA	Bovine serum albumin
D_f	Fractal dimension
dNTP	Deoxyribonuclease triphosphate
DSC	Differential scanning calorimetry
EAI	Emulsifying activity index
EDTA	Ethylene diamine tetra-acetic acid
ESI	Emulsifying activity index
FC	Foaming capacity
FS	Foaming stability
G'	Elastic modulus
G''	Viscous modulus
GLM	General linear model
GRAS	Generally recognized as safe
I_k	Interaction index
LAB	Lactic acid bacteria
MRS	de Man, Rogosa and Sharpe (agar/broth)
PCA	Principal component analysis
PCR	Polymerase chain reaction
PIA	*Pseudomonas* isolation agar
R_0	Autocorrelation function cut-off
RBC	Red blood cells
rDNA	Ribosomal deoxyribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
TAE	TRIS Acetate EDTA
TPA	Texture profile analysis
TRIS	Tris(hydroxymethyl)aminomethane
VRBL	Violet red bile lactose
WHC	Water-holding capacity
δ	Phase shift

GENERAL INTRODUCTION

GENERAL INTRODUCTION

REFERENCES

GENERAL INTRODUCTION

BLOOD AT THE INDUSTRY

Blood is a common by-product of the meat industry, which is obtained in large volumes especially in industrial slaughterhouses. Although there are no data about the exact quantity of collected blood, a rough amount can be estimated. In Spain, near 40 millions pigs were slaughtered in 2005 (FAOSTAT, 2005); if we consider that nearly 3 L of blood are obtained from each pig (Ockerman and Hansen, 1988), it can be deduced that the amount collected annually exceeds 115 000 tons. Moreover, taking into account a typical protein content around 18% (Putnam, 1975), this volume of blood is equivalent to the production of 21 000 tons of protein.

In spite of the significance of this sum, an important part of the collected blood is not considered but a waste. The reason lies in the lack of suitable facilities in the slaughterhouses to properly collect and handle blood. Hence, a high percentage of the mentioned 21 000 tonnes of protein becomes a waste –thus causing damaging environmental consequences, instead of making use of a product with high valorisation potential. Blood generates a high-contaminating residual flow, since its biological oxygen demand (BOD) can reach 250 000 mg L^{-1}, and the chemical oxygen demand (QOD), 375 000 mg L^{-1} (Tritt and Schuchardt, 1992; Mitjà, 1994). According to the Spanish current regulations (Real Decreto 849/1986 and 606/2003), the QOD of the residual water drained to the sewer system for livestock slaughtering facilities cannot exceed 160 mg L^{-1}. Therefore, the fulfilment of this restriction requires expensive decontamination treatments and large amounts of water.

On the other hand, the collection of blood in most of the slaughterhouses where it is considered a by-product is carried out together with other by-products, being all the organic materials treated unspecifically in large digesters. This process avoids part of the costs of elimination because it excludes the decontamination treatments, but the obtained end-product does not have a remarkable added value and the possibility of valorisation remains unexploited.

Initially, blood is sterile for alive and healthy animals; thus, all the contaminating microbiota which is incorporated before it reaches the collecting vessel is due to the bleeding practice and the draining system. Although there are several collecting systems that can be employed, they are basically classified into two major categories. On the one hand, open-draining systems, where animals are slaughtered and blood drains downwards thanks to gravity, until it reaches the collecting vessel. No specific precautions are taken to guarantee the absence of strange elements and low bacterial counts. Blood comes into contact with the carcass and is contaminated with surface organisms. Washing water, even vomit and faeces, can fall into the vessel. The quality of this blood classifies it as veterinary blood (Rodríguez, 1994). On the other hand, closed-draining systems allow hygienic blood collection, a high-quality product with low microbiological counts whose further uses include human consumption. In this case, blood is collected with a hollow knife through vacuum piping directly from the cut in the throat of the slaughtered animals to the refrigerated

vessel, and any contact with the skin of the animal is impeded. The drawbacks of this system are the high investment it implies and the slowdown of the slaughtering chain speed. For these reasons, only few abattoirs in Spain have it in their facilities.

One alternative to the use of a hollow knife is the implementation of a well-designed slaughtering system, which permits the hygienic collection of blood, combining an open-drainage with physical barriers to undesirable contamination.

LEGAL FRAMEWORK

During the last few years, the legal framework for the use of blood has been determined by animal health issues. Diseases like encephalopathies, examples of which are the well-known BSE or *scrapie*, have caused important changes in the current legislation, thus leading the regulation of blood derivatives towards a complex situation. The utilisation of blood is nowadays legislated by two guidelines: the Directive 77/99/CEE and the Regulation 1774/2002/CEE. For non-ruminant animals, blood is classified into three categories depending on the inspections to which it has been subjected (see Scheme 1).
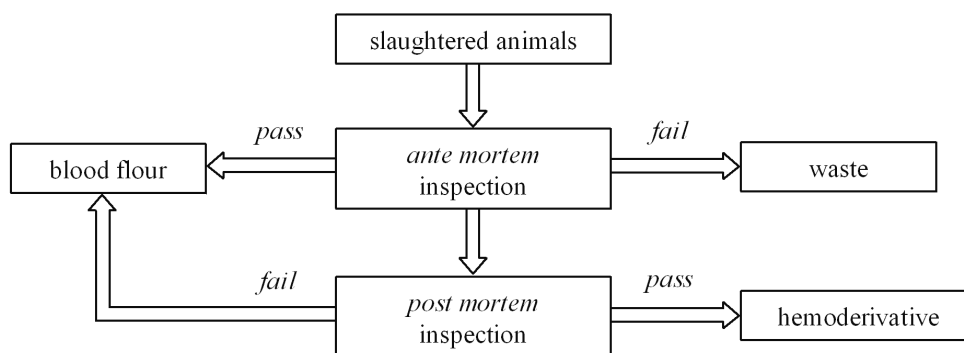
Scheme 1. Utilisation of blood from non-ruminant animals allowed by the current legislation.

First, blood is considered hazardous material if it comes from animals that do not pass the *ante mortem* inspection. It is treated as a waste that must be eliminated because no further uses are allowed. Second, blood can be treated to obtain *blood flour* if it comes from animals that pass the *ante mortem* inspection but do not pass or are not subjected to the *post mortem* inspection. This blood is coagulated and dried, and it is transformed into a protein powder that can be used for animal feed, as a fertiliser or for the elaboration of cellular cement. However, blood flour is subjected to several restrictions: although it can be used for animal

feed, cattle cannot be fed, nor can it be used as a fertiliser in grazing pastures. Moreover, blood flour should always be sterilised using a thermal treatment of 20 min at 133 ºC and 3 bars before any use. This measure was postponed until 2006, but no modifications have been proposed to date. Third, blood from animals that pass both *ante* and *post mortem* inspections can be processed to produce *hemoderivatives*, products which are able to be used for animal feed as well as for human, medical or nutritional consumption. The denomination of *hemoderivative* instead of *blood flour* excludes these products from the imposed restrictions concerning thermal treatments, because safety is guaranteed by other means.

DESCRIPTION OF BLOOD

Physicochemical description

Blood is composed of two fractions, namely red blood cells (RBC) and plasma. Red cells are the solid fraction, which correspond to 30~40% of blood weight, and they are dispersed into the liquid fraction, the plasma, which represents up to 60%. The most important cellular elements are red corpuscles (erythrocytes), white corpuscles (leucocytes) and platelets. Within this fraction, total protein content ranges from 28 to 38%, and haemoglobin is the major protein constituent (Wismer-Pedersen, 1988). Plasma contains 6~8% proteins, mainly albumin, globulins and fibrinogen (Ockerman and Hansen, 1988). Table 1 shows the composition of blood with more detail.

Table 1. Constituents of blood and its fractions –plasma and red blood cells (RBC)– by percentage of weight (Ockerman et al., 1988).

Constituent	Blood	Plasma (60%)	RBC (40%)
Water	80	90.8	60.8
Salts	0.9	0.8	1.1
Fat	0.2	0.1	0.4
Protein	17	7.9	35.1
Albumin	2.8	4.2	-
Globulins	2.2	3.3	-
Fibrinogen	0.3	0.4	-
Haemoglobin	10	-	30
Other	1.1	0.4	2.6

Blood has an important nutritive value. On the one hand, it can be considered as a good food enricher thanks to the high protein content, since it not only means an increase in loin yield but because it also solves amino acid deficiencies. Blood proteins even show an efficacy index larger than casein (Young et al., 1973). On the other hand, blood has high iron content binded to haemoglobin, which is the best bioavailable form (Reizenstein, 1980). Table 2 contains the composition in essential amino acids of blood and plasma, and an equilibrated food formulation proposed by the FAO. It can be seen that the amino acidic profile is not as good as it should to be considered well-equilibrated, due to the deficit in isoleucine and methionine.

Table 2. Essential amino acid contents (g/100 g protein) of blood and plasma, and proposed requirements according to FAO.

Amino acid	Blood[a]	Plasma[a]	FAO proposal[b]
Ile	0.4-0.9	1.0-3.4	1.8
Leu	12.4-13.6	9.2-10.1	2.5
Lys	9.2-9.7	6.5-9.2	2.2
Met (+Cys)	1.3-1.8	0.6-1.3	2.4
Phe (+Tyr)	7.0-8.0	5.1-5.7	2.5
Thre	4.7-5.2	2.6-7.1	1.3
Trp	1.4	0.6-1.0	0.7
Val	8.0-9.1	6.8-7.4	1.8

[a] According to Ockerman et al. (1988).
[b] According to FAO/WHO (1973).

Microbiological description

As explained above, collecting systems determine the microbiological quality of blood. While closed-draining systems -i.e. the use of a hollow knife- allow obtaining a product with low bacterial counts, open-draining systems expose blood to many sources of contamination. In the latter case, blood gets in touch with the carcass, the stomach content, etc.

Undesirable bacteria entail the presence of potential pathogens, as well as spoilage microorganisms. Furthermore, the fact that blood is indeed such a nutritive medium sets an environment without any hurdle to prevent the development of contaminating microbiota (Carretero and Parés, 2000). Hence, there is no natural resistance to count on to limit bacterial growth. Besides the initial microbial counts, storage time and temperature turn out to be two key features to keep this product with an acceptable microbiological

quality (Parés, 1998). Figure 1 shows the counts of several microorganisms groups which are typically found in blood from open-draining systems, that is, collected without special hygienic measures.

Blood may carry pathogenic bacteria, mostly coming from the intestinal content. Among them, it can be found *Salmonella*, *Escherichia coli* enteropathogenic, *Shigella* and *Yersinia enterocolitica*. The presence of pathogenic strains of *Staphylococcus aureus* is also important, because it entails the risk to thrive if blood is not stored at refrigeration temperatures. In this case, thermoresistant toxins could be produced, prevailing even in derivative products.

The absence of hygienic cautions during collection is also important in relation to spoilage bacteria, since initial counts can easily reach 10^6 cfu mL^{-1} (Ockerman et al., 1988; Parés, 1998). High counts determine the shelf life of blood, because the development of spoilage microorganisms provokes severe damages to blood functionality.

Since blood is usually stored at refrigeration temperatures, a selective pressure towards the development of psychrotrophic bacteria is exerted. Taking into account that *Pseudomonas* is the dominating group within psychrotrophs (Erikson and Von Bockelmann, 1975), bacterial growth is led to the development of microorganisms with important damaging abilities, especially concerning proteins.

Another contaminating group is *Enterobacteriaceae* (Lynn Knipe, 1988). Besides the potential hazard for the consumer's health, these species can alter severely the functionality of blood by means of proteolytic activities, gas formation or organic acids production.
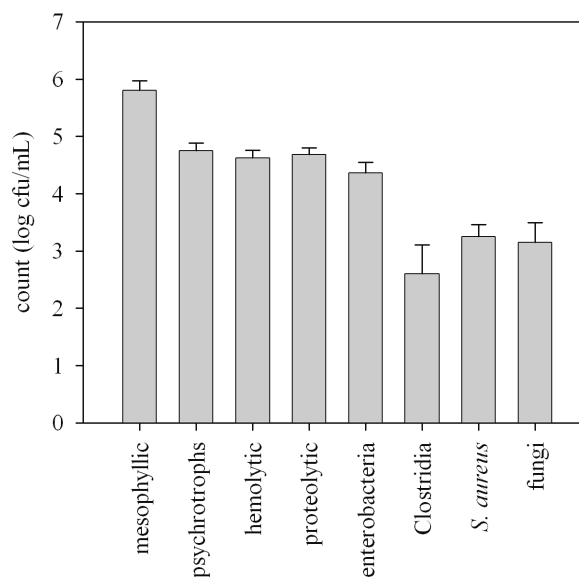
Figure 1. Typical microbial contamination of blood collected in open
systems (Parés, 1998).

As it will be further explained in Chapter I, the microbiological quality of blood is a key point to monitor and control, either to guarantee the safety for the consumer health or to prevent the spoilage of blood derivatives. Microbiological quality has to be taken into account especially because the storage of blood at refrigeration temperatures does not guarantee *per se* such control, since it merely redirects microbial growth.

APPLICATIONS OF BLOOD

Blood has several potential applications, some of which are displayed in Table 3. For the food industry, incorporation of blood –or some of its fractions– into foodstuff is derived from its nutritional value and good functional properties, including its potential role as a colouring agent. It is interesting to remark that the addition of blood in some of these foods, like meat products, would not actually involve the use of any strange substance, since blood is naturally found in this kind of products.

Table 3. Utilisation of blood in different sectors (Divakaran, 1980).

Sector	Utilisation
Food industry	Emulsifying, stabiliser, gelling, thickening, foaming, clarifying, colouring, enricher or fortifying agent
Animal feed	Supplement, vitamin stabiliser, dairy substitute or enricher
Fertilisers	Seed coating, pH stabiliser, mineral enricher
Laboratory	Culture media, active coal, catalase, peptones, globulins, albumin
Medical	Agglutination tests, immunoglobulins, fractionation techniques, clotting factors, fibrinogen, fibrin, serotonin, plasminogen
Pharmaceutics	Cosmetics
Industry	Adhesive, insecticide coadjutant, cellular concrete, fire extinguisher, resin, leather, fabric ceramics and plastic additive

Blood proteins can be useful ingredients in the formulation of food products (Tybor et al., 1975; De Vuono et al., 1979; Howell and Lawrie, 1984). Plasma proteins, for example, form heat-induced gels (Howell et al., 1984; Parés, et al., 1998) that can be used in meat or bakery products. They are good emulsifying agents (Nakamura et al., 1984; Parés and Ledward, 2001), and they possess a foaming capacity simi-

lar to egg white (De Vuono et al., 1979). Summarising, it is well worth to study the applications of blood thanks to the interesting functional properties of its proteins, with great potential uses for the food industry. A more detailed analysis of these properties will be treated in Chapter II.

CURRENT STATUS OF BLOOD VALORISATION

The high susceptibility to microbial spoilage often turns blood and its fractions into a non-suitable ingredient for the food industry. In addition, the manufacturing processes used to obtain blood-derived protein powders, which typically include a concentration step (ultrafiltration or evaporation at low pressure) and a dehydration step (spray-drying), do not reduce the bacterial counts (Parés, 1998; Dailloux et al., 2002; Toldrà, 2002). The lack of microbial lethal treatments neither prevents the growth of spoilage microorganisms nor the pathogenic during storage, and their presence can jeopardize consumers' health and the nutritional and functional properties of protein powder.

The difficulties to collect blood in adequate conditions, due to technical as well as economical aspects, lead to an infra-utilisation of the collected blood. Furthermore, the presence of a demanding market and high added-value blood derivative products is very feeble, and this fact does not encourage the conversion of blood from a source of expenses to a source of benefits in most cases. This situation creates a loop within which the food industry has a low interest for blood due to its low quality, and it has a low quality because the non-existence of economical interests which may improve it. If an alternative was developed – this being cost-effective and easily put into practice– this closed-cycle could therefore be guided towards a new situation, where the collection of blood would allow the obtaining of high added-value products. Taking advantage of blood's potential would give an incentive to both the food industry and the slaughterhouses, and it would transform this by-product into a co-product, with real functional and nutritional applications.

A diagram representing the associations between industrial slaughterhouses and the food industry, in relation to the utilisation of blood, is shown in Scheme 2. As mentioned above, there exists a feedback between both industries through the motivation to develop high added-value products that require a sanitary collecting system. The two main subjects developed in the present dissertation –blood preservation by lactic acid bacteria (LAB) and functionality studies– may have a positive effect on the key steps of this diagram.
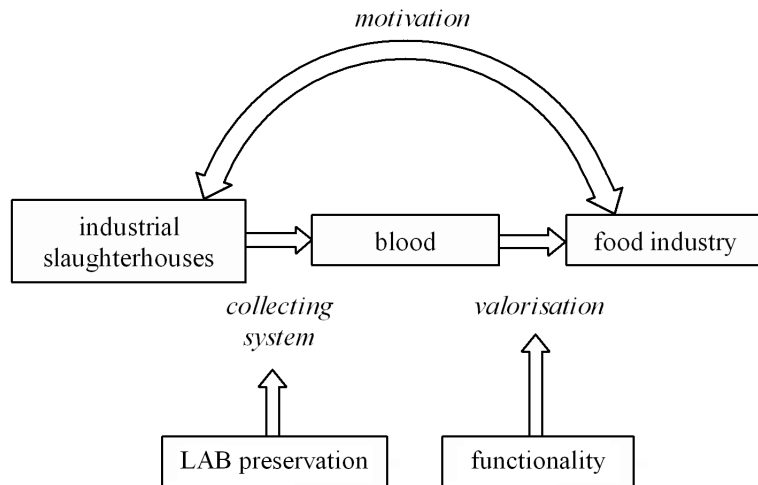
Scheme 2. Associations between industrial slaughterhouses and the food industry through the utilisation of blood. Blood preservation by lactic acid bacteria (LAB) and functionality studies may have an effect on the key steps of this diagram.

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Chapter I

BIOPRESERVATION OF BLOOD

INTRODUCTION

OBJECTIVES

MATERIAL AND METHODS

RESULTS AND DISCUSSION

- ▪ **Article 1. Identification and antagonistic activity of lactic acid bacteria occurring in porcine blood from industrial slaughterhouses—a preliminary study**

- ▪ **Article 2. Preservation of porcine blood quality by means of lactic acid bacteria**

- ▪ **General Discussion**

CONCLUSIONS

REFERENCES

INTRODUCTION

The work presented in this chapter has been framed within the project entitled "Development of a preservation system for porcine blood from industrial slaughterhouses. Application of biopreservation and high hydrostatic pressure as combined methods", with the financial support of the Spanish Government from the Institutional Program CICYT (2001-2004), referenced as AGL2001-0888. The general aim of this project was the development of a biopreservation system to improve, by means of lactic acid bacteria (LAB) inoculation, the microbiological quality of blood from slaughterhouses, extending its shelf life and providing a simple and economical preservation alternative. The development of such a technology may be applied to hygienically collected blood, and the improvement of the overall quality through reduction of microbial hazards, concerning both the spoilage of blood and the consumer's health, would give as outcome a product with high-added value. The objectives pursued were: i) the isolation and identification of lactic acid bacteria from blood collecting tanks of industrial slaughterhouses, ii) the investigation of the antagonistic activity of the isolated strains, iii) the improvement and formulation of the preservative cultures for industrial scale-up, iv) the evaluation of biopreservation as a method to improve the use of blood, and iv) the evaluation of biopreservation and high-hydrostatic pressures as combined treatments to improve the use of blood.

BIOPRESERVATION

Blood is a by-product with high possibilities of valorisation when being high-quality, which is limited by the microbial contamination. The undesirable presence of microorganisms can be controlled by means of an appropriate collecting system that, in most cases, is not implemented in slaughtering lines. Moreover, blood to be processed requires rapid handling, because refrigeration itself does not guarantee the stability for a long time. Due to these drawbacks, biopreservation appears as an alternative for the improvement of blood's quality towards its use as an ingredient for foodstuff.

Biopreservation can be defined as an extension of the shelf-life of foods with an increase of their safety thanks to the utilisation of specific microorganisms or their metabolites (Aymerich and Hugas, 1998). Taking advantage of a microbiota, especially that autochthonous, pursues the aim of inhibiting or reducing the growth of spoiling or pathogenic bacteria.

The utilisation of biopreservative cultures is associated in many cases to the group of microorganisms belonging to lactic acid bacteria (LAB) (Ross et al., 2002). As it will be later explained, they are used in many fermentation processes, and they also show promising results concerning food safety. In any case, the selection of biopreservative strains needs to fulfil certain requisites before being successfully used (Holzapfel et al., 1995). First of all, the biopreservative culture must not be a hazard for consumers' health,

and it must not be pathogenic, synthesise toxins, biogenic amines or some other metabolites that might have a negative effect on human beings. Furthermore, its presence must have positive effects on the product.

The use of LAB covers a broad range of food. Their inhibitory abilities have been studied against several spoiling and pathogenic bacteria: *Escherichia coli*, *Pseudomonas* spp., *Salmonella*, *Clostridium*, *Listeria, Staphylococcus*, fungi and yeasts (Adams and Halls, 1988; De Vuyst and Vandamme, 1994; Batish et al., 1997). Its abilities have been as well tested in a variety of food products, examples of which are: refrigerated meats (Raccach and Baker, 1978; Ahn and Stiles, 1990; Golf et al., 1996; Buncic et al., 1998), fermented meats (Garriga et al., 1993; Monfort et al., 1996; Aymerich and Hugas, 1998; Garriga et al., 1998; Fragoso and Fernández, 2000), fermented vegetables (Fleming et al., 1975), canned vegetables (Edwards et al., 1999; Skinner et al., 1999), fish (Schöder et al., 1979; Kang and Lee, 1999), dairy (Hurst, 1972; Speck, 1981; Batish et al., 1997; Buyong et al., 1998; Bachrouri et al., 2002).

In all the above mentioned studies, positive –or positive-promising– effects were described when LAB were present, with no significant disadvantages. As a matter of fact, the addition of LAB into foodstuff does not normally alter its nutritional value and the induced changes in taste and flavour are usually well-appreciated (Lindgren and Dobrogosz, 1990).

In the case of blood, the inoculation of LAB just after the collection may provide a safer and better quality product for both the processing industry and the end consumer. On the one hand, it is known that spoilage microorganisms and foodborne pathogens easily grow in such a nutritive medium (Carretero and Parés, 2000). On the other, the preservation of blood's microbiological quality during bleeding and collection entails the implementation of well-designed facilities which, in most cases, are non-existent. Hence, LAB inoculation could reduce microbial hazards without the need of changing the slaughterhouses' facilities or increasing the hygienic collection requirements.

There exist some experiences concerning the inoculation of blood with LAB. Earlier works investigated the biopreservation of blood intended as animal feed, evaluating at the same time the probiotic effect of the presence of LAB (Marland, 1984). It has been described that the simultaneous addition of anticoagulant and LAB to blood during the bleeding of animals extends the shelf-life of blood to several weeks, even though if good hygienic practices have not been not followed during the collection (Morgan, 1984; Pinel, 1985).

In these works, blood was enriched with carbohydrates and stored at room temperature to favour the growth of LAB (Morgan, 1984); these conditions caused a decrease in the pH from 7.2 to 4.7, consequently showing a positive effect regarding biopreservation although being against the interest of maintaining the functionality of blood for other purposes than animal feed. It is reasonable to think that the addition of carbohydrates could be helpful in the case that antagonistic effects were not achieved, because LAB need the presence of fermentable sugars and blood has a poor concentration of this energy source [0.07% (Duarte et

al., 1999)]. Furthermore, the addition of sugars could prevent the oxidation of iron, hence an improvement of hemoderivatives stability may be achieved (Toldrà, 2002).

Studies from our group have reported that the addition of glucose enhances the antagonistic activity of LAB (Parés et al., 2004). However, glucose can be widely used by microorganisms and the addition of such a nutritive compound could play a negative effect on blood's preservation by promoting the development of undesirable bacteria. Therefore, carbohydrates specifically selected to favour LAB's growth should be used, always considering the risk of loss of functionality due to an excessive lowering of pH. In this case, fructooligosaccharides could be used as selective energy source since they have been proved to be able to favor LAB's growth, without increasing the population of contaminant bacteria (Wang and Gibson, 1993; Gibson et al., 1995; Kaplan and Hutkins, 2000).

LACTIC ACID BACTERIA

Lactic acid bacteria have been involved for thousands of years in food fermentations, one of the most ancient preservation techniques. First signs of LAB utilisation date back in 6000 BC, describing the fermentation of milk, meat and vegetables (Fox, 1993). Obviously, the application of LAB to foodstuff was not carried out being aware of the preservation technique that was being used, but it proved indeed to be effective since these bacteria started their preservation mechanisms. Nowadays, the challenge has become the understanding of these mechanisms in order to take advantage of them in the industrial production of foods.

LAB are considered GRAS (*Generally Recognized As Safe*) microorganisms because they have historically proved to be safe for the health of consumers (Schilinger et al., 1996; Hugas, 1998). Then again, it is true that some LAB have been isolated from infected or immunodepressed persons, but it is not worth to consider them but opportunistic pathogens if we take into account the conditions through which these widespread microorganisms infect people, (Facklam and Elliott, 1995).

LAB are gram-positive rods and cocci, non-spore forming, aerotolerant anaerobes that require fermentable carbohydrates and mainly produce lactic acid during the fermentation of glucose (Vandamme et al., 1996). However, this definition includes more bacteria than the LAB strains historically accepted, and the list is subsequently narrowed by pointing out the positive role that LAB play in the fermentation process of food products. In spite of the frequent changes in LAB's taxonomic classification, the widely accepted genera include: *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Vandamme et al., 1996; Stiles and Holzapfel, 1997). Some authors also include the genus *Bifidobacterium* because of its probiotic role, although it belongs to a different phylogenetic group (Vandamme et al., 1996).

Typically, LAB are mesophyllic bacteria, but they are also able to grow at temperatures ranging from

5 to 45 ºC, and they tolerate both acid and alkaline environments (Wood and Holzapfel, 1996). LAB are weakly proteolytic and lipolytic, and they require the presence of free amino acids, nitrogenated bases, and vitamins (Jay, 1996). Their metabolism is based on fermentative processes, homo- or heterofermentative, oxidising carbohydrates in all cases (Axelsson, 1998). They produce a great variety of products, mainly organic acids, alcohol and carbon dioxide (Ray and Daeschel, 1992), although they often generate aromatic molecules, vitamins and bioactive peptides (Ross et al., 2002).

The antimicrobial mechanisms of LAB that allow the biopreservation of food are due to the combined action of several metabolites produced during the fermentative reactions (Caplice and Fitzgerald, 1999). The production of organic acids (lactic, acetic and propionic) has an antagonistic effect on the microbiota thanks to the inhibition of the active transport processes, reactions and the modification of their membrane potential (De Vuyst et al., 1994) this effect is caused by both the dissociated and undissociated forms of acids. Among them, acetic acid has the most severe antimicrobial effect, and it acts over fungi, yeasts and bacteria (Blom and Mortvedt, 1991). Moreover, all organic acids cause a decrease of the pH, thus contributing to biopreservation. LAB's lack of catalase causes as well an accumulation of hydrogen peroxide (H_2O_2), what can inhibit the presence of some microorganisms due to the oxidation of the lipids and proteins of their membrane (Lindgren et al., 1990). However, the presence of enzymes such as peroxidase entails the degradation of this molecule, and antimicrobial action of H_2O_2 does therefore not always take place (Caplice and Fitzgerald, 1999). The carbon dioxide produced during heterolactic fermentations creates an anaerobic microenvironment which is toxic for some aerobic bacteria. It contributes to decrease the pH as the rest of organic acids (De Vuyst et al., 1994).

A few LAB strains are able to produce protein compounds with a noteworthy antimicrobial effect, which are known as bacteriocins (Ross et al., 2002). Some of them are highly specific, like lactococcins, and some other have a wide antimicrobial spectrum, like nisin. These growth inhibitors usually act over the membrane of targeted microorganisms, causing a depolarisation or stopping their synthesis (Abee et al., 1995). There is a growing interest in the use of bacteriocins for the improvement of food safety, but nisin is the only one being industrially produced so far (Cleveland et al., 2001).

Identification of lactic acid bacteria

LAB have been traditionally identified and classified by means of phenotypic characteristics (Pot et al., 1994). Most of the techniques used are, however, extremely time-consuming and sometimes, they are not specific enough to reach the species level (Gancheva et al., 1999). For these reasons, during the past few years, genotypic identification techniques have been incorporated and, thanks to their specificity and rapidness, have become an essential tool in bacterial identification (Tynkkynen et al., 1999).

Genotypic identification is usually carried out through the following steps: first, a portion of one

gene –or the whole gene itself–, is amplified with the polymerase chain reaction (PCR), using two primers with universal or specifically-designed features (Lane, 1991). Then, the amplified fragment is sequenced, and finally compared to previously identified sequences. In some cases, this comparison matches with a previously identified microorganism. In some others, it is similar but not identical; the taxonomic classification depth is then subjected to the percentage of likelihood.

Genetic characterisation of LAB normally targets universal genes, present in all microorganisms. These genes belong to high-conservative regions within the bacterial genome, containing just a few variable sub-regions where the tasks of identification are placed (Holzapfel et al., 2001). An example of such a region is the 16S rDNA gene, which is popular in taxonomic studies of both closely and far related microorganisms (Baker et al., 2003). The rDNA regions are structural genes that encode ribosomal subunits, and the 16S rDNA gene contains the code of the bacterial ribosomal subunit known as 16S rRNA, with its 9 variable regions, namely V1-V9 (Tung et al., 2002).
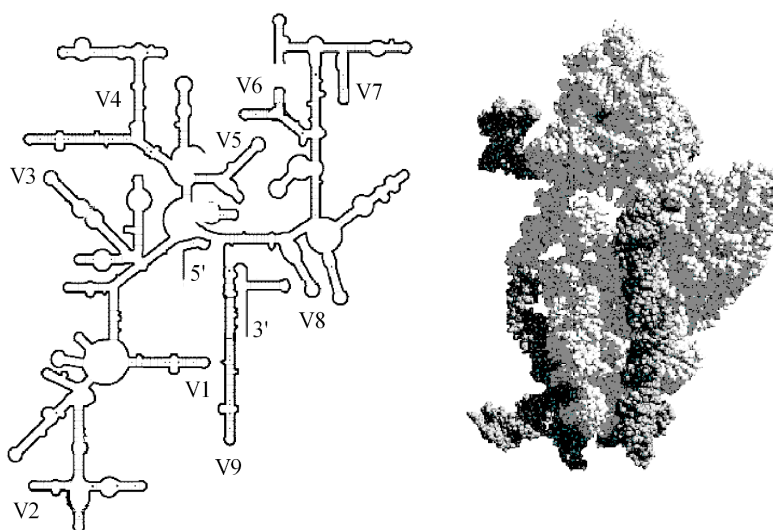
Figure 2. Structure of the bacterial 16S rRNA. Left, secondary structure displaying the 9 variable regions V1-V9. Right, tertiary structure with some of the variable regions in dark grey (Tung et al., 2002).

The sequence of the 16S rDNA has been widely used for LAB classification. In the literature, identification studies of LAB isolated from different origins can be found, examples of which are: meat products (Zhang and Holley, 1999; Yost and Nattress, 2000, 2002; Papamanoli et al., 2003), dairy products (Blaiotta et al., 2002; Randazzo et al., 2002; Duthoit et al., 2003; Fortina et al., 2003), fish and seafood (Gonzalez et al., 2000; Ringo et al., 2000; Kobayashi et al., 2003), vegetables (Kelly et al., 1998; Kim et al., 2003; Ran-

dazzo et al., 2004) and human sources (Walter et al., 2001; Heilig et al., 2002; Nielsen et al., 2003). It has been verified that the first regions V1-V3 of the 16S rDNA gene do contain enough information to characterise at the species level (Klijn et al., 1991; Tannock et al., 1999).

When the genetic information of a strain of interest has been sequenced, the identification is accomplished throughout alignment algorithms which perform multiple pairwise alignments using a database of previously sequenced bacteria. Sequences are compared, and a score based on distances or similarities is calculated. Positive identification is achieved when a high score –depending of the sequence length– is reached. Although the results of these comparisons can be presented in different ways, one of the most widely used is the construction of a phylogenetic tree.

A phylogenetic tree presents the evolutionary history of a set of species, hence the similarity of pairs of bacterial strains. It is usually a rooted, leaf-labelled tree, where internal nodes represent ancestral relationships and the leaves represent evolved species comprising larger similarities (Page, 1998). Several methods can be used to build a tree (UPGMA, neighbour-joining, minimum evolution, maximum parsimony and maximum likelihood) although the one that best fits a large set of sequences is the neighbour-joining algorithm (Saitou and Nei, 1987). The principle of this method is to find pairs of operational taxonomic units, or neighbours, which minimize the total branch length at each stage of clustering, using evolutionary distance data.

OBJECTIVES

The experiments presented in this chapter are the continuation of the study of Zamora (2003), in which a collection of lactic acid bacteria (LAB) was formed with strains isolated from the blood collecting tanks of several industrial slaughterhouses. The antagonistic abilities of those strains were as well tested in plate against contaminant indicators (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas fluorescens* and *Bacillus* spp.), to seek out bacterial inhibitors among the common lactic microbiota found in blood. The results proved that several strains showed encouraging results.

The present work continues the task of finding bacterial inhibitors to be eventually used as a biopreservative culture to improve the microbiological quality of blood. In addition, the characterization of the LAB strains collection should provide relevant information about the common lactic flora found at the slaughterhouse facilities. The following objectives were pursued:

i. The identification of the strains from the LAB collection by means of genotypic methods, in order to know which species of lactic acid bacteria inhabit the blood collecting tanks of industrial abattoirs.

ii. The evaluation of LAB inoculation as a blood preservation system, in relation to the control of the growth of contaminant microorganisms and the maintenance of blood's functional properties. This aim was further subdivided into

ii. I. A first step to screen the antagonistic and preservation abilities of the LAB strains that had shown inhibitory skills in plate in order to select the strains showing best aptitudes. The experiments were carried out in blood stored at 15 ºC for 72 h.

ii. II. A second step to evaluate the antagonistic and preservation skills of the LAB strains selected in the previous screening tests in blood stored at 5 ºC for 144 h, imitating real storage conditions.

MATERIAL AND METHODS

EXPERIMENTAL DESIGN

The ability of LAB to preserve blood in terms of microbiological safety and functional properties was evaluated at different conditions with several strains. Scheme 3 illustrates the experimental design followed in Chapter I.
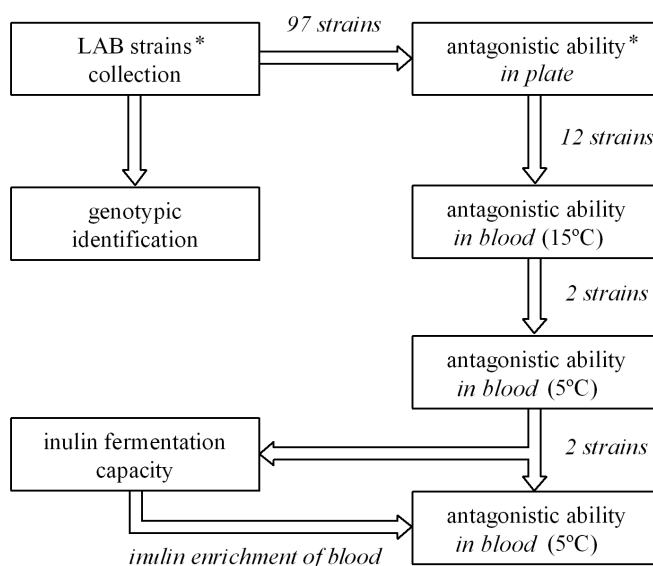
Scheme 3. Experimental design followed during blood biopreservation studies. (*) Done in a previous work (Zamora, 2003).

Ninety-seven LAB strains previously isolated from industrial slaughterhouses (Zamora, 2003) were identified in the present study by means of genotypic techniques. Afterwards, the antagonistic activity of the 12 strains that had shown best results in plate (Zamora, 2003) was evaluated in blood, by inoculating and maintaining it at 15 ºC for a period of 72 h. These experiments monitored the growth of contaminant microbiota and some indicators of blood functionality, and allowed the rapid screening of the biopreservative abilities at low temperatures. Eventually, two strains from the starting 12 were selected for further studies on blood stored at 5 ºC for 144 h. These experiments led to the search of a selective energy source to improve the inhibitory activity of LAB against contaminant indicators, and the inulin fermentation capacity of both strains was investigated. Then, assays on the biopreservation of inulin-enriched blood were performed with both strains.

LACTIC ACID BACTERIA STRAINS

All the LAB strains of the present work belonged to a collection of strains previously isolated from blood collecting tanks of several industrial slaughterhouses (Zamora, 2003). These strains were preserved at -80 ºC in MRS broth (Oxoid CM0359) and glycerol (20%). Frozen LAB strains were recovered by spreading plate culturing in MRS agar (Oxoid CM0361) incubated at 30 ºC for 72 h under anaerobic conditions (anaerogen, Oxoid).

LACTIC ACID BACTERIA IDENTIFICATION

The identification of 97 LAB isolates was carried out by genotypic methods. However, additional biochemical tests were necessary to complete the classification. All the steps followed during this process are presented in Scheme 4 and further explained in the sections below.
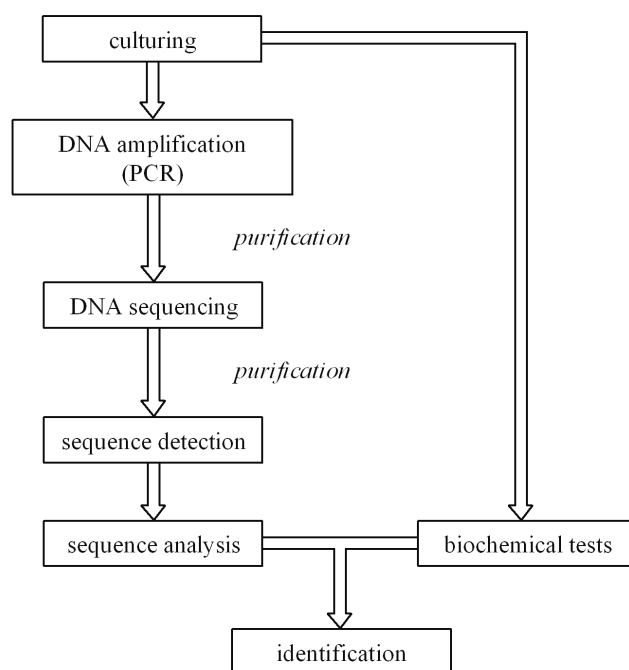
Scheme 4. Identification process of lactic acid bacteria strains.

16S rDNA gene sequencing

LAB strains were plated in MRS agar. One colony of each plate was picked and resuspended in 100 μL of distilled water. This suspension of cells was directly used as DNA template for the polymerase chain reaction (PCR), after checking out that DNA extraction was not necessary to carry out the amplification reac-

tion. Amplification reactions were performed in polypropylene tubes of 200 µL, each one of them containing a final volume of 50 µL with the reagents displayed in Table 4. The primers used in this study were selected for their universality in targeting 16S rDNA gene (Lane, 1991) and the corresponding sequences are detailed in Table 5.

Table 4. PCR amplification reaction formulation.

Reagent	Final concentration	Volume (µL)
Water		34.20
PCR buffer[a]	1x	5.00
MgCl2[a]	3 mM	3.00
dNTPs[a]	200 µM	1.00
Primer 8f[b]	150 nM	0.75
Primer 1492r[b]	150 nM	0.75
Taq polymerase[a]	1 U	0.30
DNA template		5.00
Total		50.00

[a] Invitrogen Life Technologies, California, USA.

[b] Roche diagnostics, Mannheim, Germany.

Table 5. Details of the primers used for DNA amplification.

Primer	Orientation	Sequence (5'-3')
8f	forward	AGTTTGATCCTGGCTCAG
1492r	reverse	ACGGTTACCTTGTTACGACTT

The PCR tubes were kept within ice until the amplification reaction. Reactions were carried out in a GenAmp PCR system 9700 thermocycler (PE Applied Biosytems, California, USA), using the programmed conditions detailed in Table 6. Amplification products were kept at 4 ºC until its utilisation and checked out by running electrophoreses of samples in 1.5% agarose gels (Roche diagnostics, Mannheim, Germany) in TAE (1x)[1] buffer. Each lane of the gel contained 2 µL of the PCR products, 2 µL of loading buffer[2] and 6

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[1] TAE(50x). TRIS, 484 g L^{-1}; glacial acetic acid, 57.1 mL L^{-1}; EDTA, 0.5 M; pH 8.0

[2] Bromophenol blue, 0.25% p/v; xylene cyanol, 0.25% p/v; glycerol, 30% p/v

µL of water. As well, a DNA molecular weight marker (1 Kb plus ladder; Invitrogen Life Technologies) was included in order to assess whether the size of the amplified fragments was around 1500 bp, as expected with the used primers. Electrophoreses were run at 100 V for 30 min. When finished, gels were stained for 20 min with ethidium bromide (1 µg mL^{-1}) in TAE (1x) and visualised under UV light. A typical example of amplification products is shown in Figure 3.

Table 6. Conditions used in the PCR amplification reaction.

Nº cycles	T (ºC)	Duration
1	95	2 min
35	95	1 min
	52	1 min
	72	1 min 27 s
1	72	5 min
1	4	-

1650 bp
1000 bp
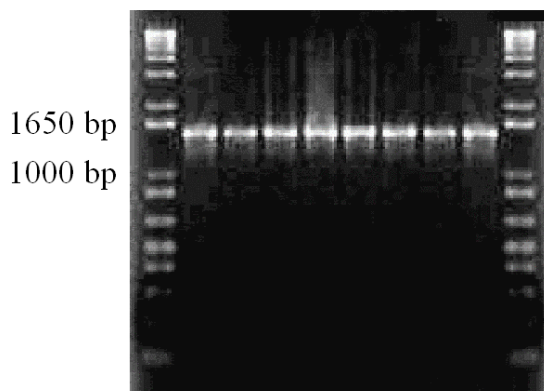
Figure 3. Electrophoresis of eight amplification products (centred lanes) and DNA molecular weight markers (side lanes). Typical bands of roughly 1500 bp can be observed.

In order to eliminate the presence of reagents that could interfere or inhibit the sequencing reactions, PCR amplification products were purified with the QIAEX II kit (Qiagen GmbH, Hilden, Germany). DNA fragments larger than 100 bp were separated from the residual primers, mineral salts, dNTPs, etc. The PCR products were diluted in QX I buffer (1:4) and 3 µL of QIAEX II silica-gel were added. These dispersions were vortexed every two minutes, during 10 minutes, and left to precipitate at room temperature. Disper-

sions were then centrifuged at 13 000 rpm for 30 s in an Eppendorf 5415D centrifuge (Eppendorf, Hamburg, Germany) and the supernatants were discarded. The precipitates were washed twice with 500 µL PE[3] buffer, and left to dry at room temperature.

Dried pellets were resuspended in 20 µL of TE[4] buffer and centrifuged at 13 000 rpm for 30 s to recover the supernatant. This step was carried out twice, until 40 µL of purified amplification products were obtained. Electrophoreses of purified products were run, as described above, in order to verify that amplified DNA was not lost during the purification step.

The sequencing reaction was performed in polypropylene tubes of 200 µL with the BigDye Terminator v3.1 kit (Applied Biosystems) and the reagents listed in Table 7. Reactions were carried out in a GenAmp PCR system 9700 thermocycler, using the program conditions detailed in Table 8.

Table 7. Sequencing reaction formulation.

Reagent	Volume (µL)
Water	4
BigDye buffer[a]	1
Primer 8f (10 µM)	1
BigDye terminator[a]	3
DNA template	1
Total	10

[a] Applied Biosystems

Sequenced products were then precipitated. After vortexing each sample, 1 µL of EDTA 125 mM, 1 µL of sodium acetate 3 M, and 25 µL of ethanol 100% were added. The tubes were inverted 3-4 times to mix the solution and were incubated at room temperature for 15 min. The solutions were transferred into 1.5 mL tubes and then centrifuged at 13 000 rpm for 30 min. The remaining supernatant was removed using a fine glass pipette attached to a vacuum suction. After that, 35 µL of ethanol 100% were added, and pellets were vortexed centrifuged again at 13 000 rpm for 15 min. The supernatant was removed again and the DNA pellets were dried at 30 ºC for 15 min. Dried samples were wrapped with aluminium foil and stored at 4 ºC.

Pellets of DNA were resuspended in 30 µL Template Suppression Reagent (Applied Biosystems, MA, USA) and left rehydrating at room temperature for 20 min. Suspensions were vortexed and denatured

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[3] Sodium phosphate, 20 mM; EDTA, 1 mM; pH 7.2

[4] TRIS/HCl, 10 mM; EDTA, 1 mM; pH 8.0

in a water bath at 94 ºC for 3 min. Afterwards, they were immediately cooled within ice. The 30 µL were transferred into ABI 310 microtubes (PE Applied Biosytems) and the sequenced DNA fragments were separated in a capillary sequencer with fluorescence ABI PRISM TM 310 Genetic Analyzer (PE Applied Biosytems). The resulting corresponded to partial 16S rDNA sequences.

Table 8. Conditions used in the sequencing reaction.

Nº cycles	T (ºC)	Duration
1	94	3 min
25	96	10 s
	50	5 s
	60	4 min
1	4	-

Sequences analysis

The obtained partial 16S rDNA sequences (averaged 400 bp) were edited with the free software CHRO-MAS v1.45 (http://www.technelysium.com.au/chromas.html) and submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) to identify the LAB isolates by comparison of their nucleotide chains with other 16S rDNA sequences included in the database (Madden et al., 1996). However, in most cases, a clear identification with a single species was not possible. Instead, the generated list contained more than one species that could be identified with the submitted LAB strains, what suggested that further identification analysis was required.

From the output similarity list, the proposed species with higher scores were selected. The sequences of the 16S rDNA genes of all the selected species, as well as other LAB species from pig origin already isolated (Russell, 1979; Robinson et al., 1981; Pryde et al., 1999; Simpson et al., 2000; Hill et al., 2002; Leser et al., 2002), were downloaded from the Aligned 16S rDNA Collection of the CASCADE-P project (DeSantis et al., 2003). The 96 sequences of the present work, together with the compiled 16S rDNA genes of the other LAB species, were aligned using the CLUSTALW tool (http://www.ebi.ac.uk/ clustalw). The resulting alignment was edited to obtain fragments of the same length for all the included species in order to calculate a dendrogram.

The dendrogram was constructed using the free software MEGA v2.1 (Kumar et al., 2001), with the tool DNAsp (Rozas and Rozas, 1995) to convert the alignment from CLUSTALW format to MEGA's. The grouping method was the neighbour-joining (Saitou and Nei, 1987), the distances matrix was calculated using the Jukes-Cantor model (Jukes and Cantor, 1969; Rzhetsky and Nei, 1995) and a total of 1000 itera-

tions were performed for bootstrapping (Efron et al., 1996). In order to better appreciate the differences between distances, an external species (*Mycobacterium* spp.) was included to root the tree. Matching of species was carried out by clustering, based on similarity analysis. When one cluster clearly gathered a single species with a LAB isolate, the LAB strain was positively identified. In the cases where more than one species was clustered with the LAB isolates, showing more than 98% similarity, biochemical tests were performed to resolve the identification.

After positive identification of the LAB isolates, the 97 sequences were uploaded to the EMBL database (http://www.ebi.ac.uk/embl) and they were assigned to the accession numbers from AJ877963 to AJ878059.

Biochemical tests

Carbohydrate fermentation tests were performed in tubes containing filter sterilised phenol red broth[5]. The medium also contained 1% of the carbohydrate to be fermented, which, in the case of enterococci, was L-arabinose (Manero and Blanch, 1999), and, in the case of streptococci, mannitol (Devriese et al., 1998). Tubes were inoculated with the LAB strain and incubated at 37 ºC for 24 h. Results were considered positive if the broth turned yellow after incubation.

BIOPRESERVATION EXPERIMENTS

Blood collection and handling

All the blood samples used in the present work were obtained from two industrial abattoirs. In order to collect blood under high hygienic conditions, all samples were taken directly from the draining blood during the bleeding of slaughtered animals. Samples were collected in 5 L sterile recipients containing trisodium citrate to prevent coagulation (1% w/v) and maintained at chilled conditions until processing.

Blood samples were split into aliquots of 2~3 L. One was treated as a control and the rest were inoculated with the strains tested in each replicate. Control and inoculated samples were kept either at 15 ºC for 72 h or at 5 ºC for 144 h, maintained in gentle, continuous agitation with a six-blade stirrer (65 mm Ø) in 5 L laboratory fermentors (Biostat B5, B. Braun Biotech Int., Melsungen, Germany).

The inoculation of blood with the LAB strains was carried out as follows; pure cultures of LAB were grown by spreading plate culturing in MRS agar (Oxoid CM361) incubated at 30 ºC for 72 h under anaerobic conditions (anaerogen, Oxoid). One colony was picked from the plate and was inoculated in MRS broth

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[5] Proteose peptone, 10 g L^{-1}; beef extract, 1 g L^{-1}; sodium chloride, 5 g L^{-1}; phenol red, 18 mg L^{-1}

at 15 ºC for 72 h until stationary phase. Cultures were centrifuged at 10 000 *g* at 10 ºC for 10 min and the supernatant was discarded. Precipitated cells were resuspended in small amounts of blood, and then added to the corresponding sample in order to obtain an inoculum size around 10^7 cfu mL^{-1}.

Microbiological analysis

The evolution of microbial growth was measured by counts every 24 h. Samples of 10 mL were removed from control and inoculated blood and serially diluted in sterile tryptone water[6]. Either 1 or 0.1 mL of these dilutions were plated in Petri plates and suitably incubated for microbiological counts (see table 9). Micro-bial counts were determined according to Equation 1.

Table 9. Microbiological counts in blood.

Group	Medium	Technique	Volume	Incubation
LAB	MRS Agar (Oxoid CM361)	Pour plate	1 mL	48 h 30 ºC
Total aerobic	BAB (Oxoid CM55)	Pour plate	1 mL	48 h 30 ºC
Coliforms	VRBL (Oxoid CM107)	Pour plate	1 mL	24 h 37 ºC
Pseudomonas spp.	PIA (Difco, 292710)	Spread plate	0.1 mL	48 h 25 ºC
Proteolytic	Frazier agar (Adsa Micro 1-272)	Pour plate	0.1 mL	48 h 30 ºC
Hemolytic	BAB +7% sheep blood (Oxoid SR51C)	Pour plate	1 mL	48 h 25 ºC

Equation 1

$$\text{cfu mL}^{-1} = \frac{c}{(n_i + 0.1 n_j) \cdot d \cdot V}$$

Where *c* is the sum of all colonies counted in plates containing between 30 and 300 colonies (15 and 150 for *Pseudomonas* spp.), *n* is the number of plates counted in the *i* and *j*-th dilution, *d* is the factor of the smallest dilution and *V* is the inoculated volume.

Functional properties of blood

The maintenance of the functional properties in stored blood was evaluated during storage. The level of hemolysis and changes in the gelation properties of plasma proteins were chosen as indicators of degradation processes that could be taking place within control or inoculated blood samples.

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[6] Tryptone, 10 g L^{-1}; NaCl, 5 g L^{-1}

Measure of the hemolysis level

Hemolysis of blood is detected by the presence of hemoglobin in the plasma fraction due to the red blood cells breakdown and it can be used as a parameter related to blood quality (Parés, 1998). The concentration of haemoglobin in plasma can be monitored by absorbance measures, since the spectrum of this protein shows a pronounced peak at 414 nm. Samples of blood were taken from both control and inoculated to measure hemolysis every 24 h. Blood was centrifuged at 2250 *g* at 10 ºC for 15 min and plasma fraction was separated. The increase of the hemolysis level was monitored by measuring the absorbance at 414 nm of an appropriate dilution of plasma (1:5 or 1:10) in a Shimatzu UV-160A spectrophotometer (Shimatzu Co., Kyoto, Japan).

Texture profile analysis (TPA)

From the plasma samples obtained as described above, gels were obtained as follows. Plasma aliquots adjusted to pH 7.0 using either HCl or NaOH 1 N were poured into plastic membranes of 25 mm diameter (Wienie-pak 2350/84, Teepak LLC, Lommel, Belgium). The membranes were properly sealed and heated in a water bath at 80 ºC for 45 min. After that, samples were immediately cooled to 20 ºC and stored at 4 ºC for 24 h to age before texture measurements.

Gel samples were cut into cylindrical portions of 24 mm diameter and 12 mm height, and texture profile analysis (TPA) was determined by uniaxial two-cycle compression test with a TA-XT2 texturometer (Stable Micro Systems Ltd., Surrey, UK) using a cylindrical aluminium plunger of 50 mm diameter at a compression rate of 1 mm s^{-1} and to a final deformation of 30%. Stress was measured as a function of the applied strain, which corresponded to the ratio of deformation to the initial height of the gels.

As described by Bourne (2002), hardness was determined as the maximum force applied to the gels during the first cycle of compression. Springiness was calculated from the quotient of the time needed to reach the maximum compression between the second and the first cycle. Cohesiveness was calculated as the proportion of the area, which corresponds to the compression work, between the second and the first cycle. The way these measurements were performed is further detailed in the materials and methods section concerning the gelation properties of plasma (see page 92).

Water-holding capacity (WHC)

The water-holding capacity (WHC) of gels was determined with a technique combining filtration and centrifugation. Gel samples were cut into cylindrical portions of 8 mm diameter and 14 mm height and were placed into hollow PVC containers with a polyester net (100 μm Ø; Henry Simon, Cheshire, UK) fixed at the bottom. The plastic containers were suspended inside centrifuge tubes and they were centrifuged at

4000 *g* at 15 ºC for 10 min. The water released during the centrifugation step was collected and the results were reported as the percentage of weight of released water in relation to the sample weight.

Inulin-enrichment of blood

LAB strains were tested for carbohydrate fermentation by using API 50CH strips and the API CHL médium (Biomérieux, Marcy-l'Etoile, France). Tests were performed according to the instructions of the manufacturer and the results, which were observed after incubation at 30 °C for 48 h, were positive for the fermentation of inulin for the two selected LAB strains (TA43 and PS99).

The inulin fermentation capacities of TA43 and PS99 and four previously isolated indicator microorganisms (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus* spp. and *Pseudomonas fluorescens*) were evaluated using a Bioscreen C microplate reader (Labsystems Corporation, Helsinki, Findland). Two hundred microliters of a microorganism suspension in the appropriate culture media [MRS broth for LAB and Nutritive Broth (Oxoid, CM1) for all the indicators] containing 0%, 0.5%, 1% or 2% inulin (Fibrulose F97, Cosucra, Warcoing, Belgium) were used to obtain the growth curves at 30 °C. The optical density (OD) at 600 nm was measured every 15 min for 48 h with pre-shaking at medium intensity for 10 s prior to OD measurements. Five replicates of the experiment were performed to draw the mean growth curve. The growth rate, calculated as the slope of the straight line corresponding to the exponential growth phase transformed into natural logarithm, and the area under the growth curve, which was used as an indicator of the growth capacity of bacteria, were calculated.

Once the optimum concentration of inulin for LAB's growth was chosen, the enrichment of blood with this fructooligosaccharide was performed. An aliquot of blood was separated before the inoculation with the LAB strain, and it was used to disperse inulin. After solubilisation, this aliquot was added to the corresponding sample of blood, to give a 2% inulin final concentration.

DATA PROCESSING

Descriptive and statistical data analyses were calculated using SPSS v13.0 for windows (SPSS Inc, Chicago, IL). The normal data distribution was verified by the Kolmogorov-Smirnov procedure, and the homogeneity of variances was confirmed by the Levene test. One-way ANOVA and Tukey's post hoc tests were calculated with a significance level of $p < 0.05$. A general linear model (GLM) was as well used to investigate the effect of LAB inoculation on the evolution of microbial contamination of chilled blood.

RESULTS AND DISCUSSION

The results obtained in the biopreservation experiments are presented in this chapter as the following articles:

- **Identification and antagonistic activity of lactic acid bacteria occurring in porcine blood from industrial slaughterhouses—a preliminary study**

  Eduard Dàvila, Lucero M. Zamora, Maria Pla, Carmen Carretero, Dolors Parés

  International Journal of Food Microbiology, 2006, 107(2), 207 – 211

- **Preservation of porcine blood quality by means of lactic acid bacteria**

  Eduard Dàvila, Elena Saguer, Mònica Toldrà, Carmen Carretero, Dolors Parés

  Meat Science, 2006, 73(2), 386 – 393

After these articles, a general discussion follows, which includes comments about the two papers and some features that have been not included there.

GENERAL DISCUSSION

Identification and distribution of lactic acid bacteria strains

The successful identification of the 97 strains indicated that blood collecting tanks in slaughterhouses do contain lactic acid bacteria. Identification results showed very low variability, since only four species (*Lactobacillus reuteri*, *Lactococcus garvieae*, *Lactobacillus murinus* and *Enterococcus raffinosus*) represented more than 95% of the total isolates. These overpowering species probably have the greatest ability to survive and grow in blood at refrigeration temperatures. Furthermore, as it can be observed in the distribution of species in the slaughterhouses (Fig. 4), all the four predominant strains were found in all the collecting tanks; hence, they probably are the dominating contaminant LAB species in those facilities.
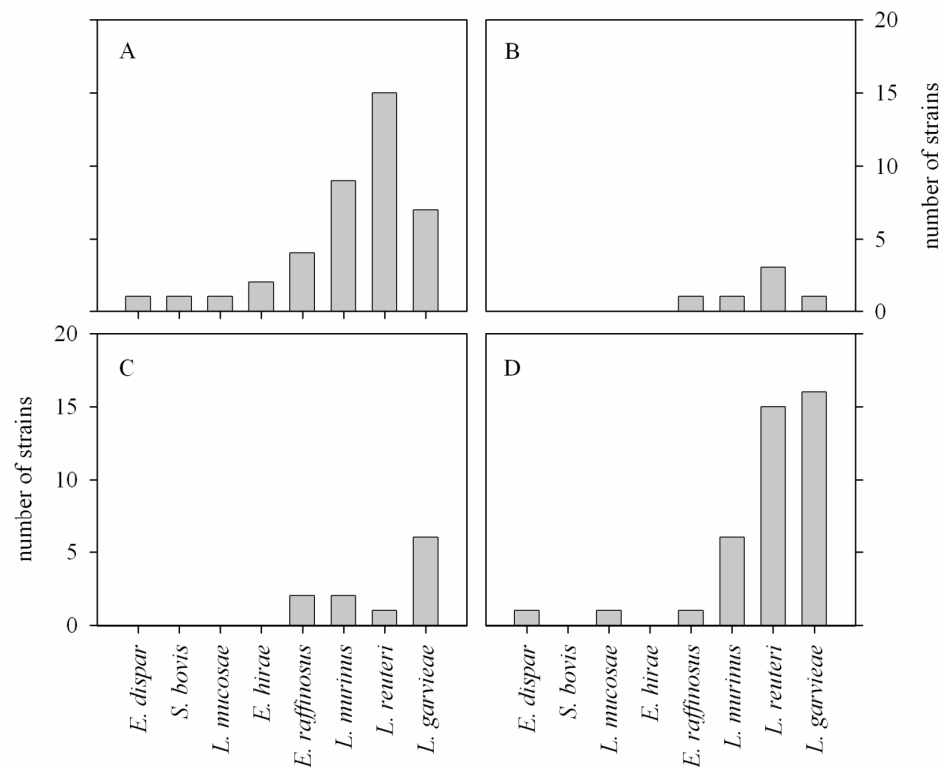
Figure 4. Distribution of isolated LAB strains from blood collecting tanks of four industrial slaughterhouses (A, B, C and D).

Most of the identified strains coincided with other LAB species that had been previously isolated from pig gut (Russell, 1979; Robinson et al., 1981; Pryde et al., 1999; Hill et al., 2002; Leser et al., 2002), being *E. raffinosus* and *L. garvieae* the two only exceptions. Notwithstanding, they have also been reported to be present in animals: the currently considered "soil-bacterium" *E. raffinosus* in the oral tract of cats (Devriese et al., 1992) and faeces (Pinto et al., 1999), and *L. garvieae* in various mammals (Teixeira et al.,

1996; Dewhirst et al., 1999). In conclusion, it is reasonable to think that LAB species found in blood samples come from contamination with bacteria mainly from the skin and the gastrointestinal tract of slaughtered pigs. These species also proved to be able to grow in blood and some of them showed antagonistic effects against several indicator microorganisms, namely *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas fluorescens* and *Bacillus* spp.

Inhibition tests in plate

Inhibition experiments in plate (Zamora, 2003) allowed the selection of 12 strains on the basis of their antagonistic skills to carry out further studies in blood. These strains are detailed in Table 10, where it can be observed that the selected strains coincided with the dominating species mentioned above. The four predominant LAB strains showed as a whole different inhibition ranges, but the widest spectrum belonged to *L. murinus*, followed by *L. reuteri*, *L. garvieae* and *E. raffinosus*.

L. murinus was isolated for the first time in the oral cavity of mice (Dewhirst et al., 1999) but it has also been identified in the gastrointestinal tract of pigs (Leser et al., 2002) and other mammals (Greetham et al., 2002). Although there are no studies about its antagonistic properties against other bacteria, it has shown the largest inhibitory spectrum as compared to all tested species. Its inhibitory properties placed it as a good candidate for biopreservation, since three strains (TA36, TA73 and TA76) inhibited the growth of all tested indicators (Zamora, 2003).

L. reuteri, the most frequently identified species, has been previously isolated from pig intestine and faeces and it is considered a good probiotic in fermented foods, pharmaceutical products and for animal nutrition (Simpson et al., 2000). It is a bacteriocin-producer of interest, since it synthesizes a broad-spectrum antimicrobial substance termed reuterin (Caplice et al., 1999). It showed wide and good spectra in inhibition tests; *S. aureus* and *Bacillus* spp. were the most sensitive indicators to its antagonistic activity. Three strains (TA43, TA62 and TA78) were able to inhibit all indicators and the first one of these showed halo sizes larger than 4 mm in antagonistic tests in plate against all indicators, except for *Bacillus* spp. (Zamora, 2003).

L. garvieae was the second one in isolating frequency. Most of the *L. garvieae* strains showed good inhibitory skills, especially for *S. aureus*, and many of them were able form inhibition halos for the rest of the indicators. Only one strain (TA20) had the ability to inhibit all indicators (Zamora, 2003). Although *L. garvieae* is considered to be a fish pathogen (Eldar et al., 1999), it can be found in foodstuff (Coppola et al., 2001) and some strains of *L. garvieae* even produce a bacteriocin named garviecin L1-5, which could be effective in preventing the growth of other bacteria (Villani et al., 2001).

E. raffinosus stood out for its inhibition abilities against gram-positive indicators: 100% of the tested strains showed inhibition halos against *S. aureus* and 90% against *Bacillus* spp. Nevertheless, it did not

inhibit *E. coli* at all, and only 12% of the tested strains could reduce the growth of *P. fluorescens* (Zamora, 2003).

These results suggested that biopreservation with LAB isolated from blood of industrial slaughterhouses could be a way to reduce the growth of undesirable bacteria, since several strains were capable of inhibiting contaminant indicators.

Table 10. List of the 12 selected LAB strains in antagonistic tests in plate.

Strain[a]	Identification
PS7	*E. raffinosus*
PS14	*L. garvieae*
PS22	*L. garvieae*
PS23	*L. garvieae*
PS48	*L. garvieae*
PS95	*L. garvieae*
PS99	*E. raffinosus*
TA20	*L. garvieae*
TA43	*L. reuteri*
TA73	*L. murinus*
TA75	*L. murinus*
TA76	*L. murinus*

[a] Codification of the strain according to the collection of Zamora (2003).

Inhibition tests in blood

Typically, blood collecting tanks in slaughterhouses maintain the collected blood at 4-6 ºC. Since all the reactions are slowed down at these low temperatures, microbial growth time is prolonged. For this reason, a first round of inhibition experiments was performed at 15 ºC in order to obtain preliminary screening results in a short-term period as selective criteria for the use of LAB species.

Blood preservation experiments by means of LAB inoculation focused on two issues. Firstly, the inhibitory abilities of the LAB strains against natural contaminants of blood, by monitoring the growth of two widespread undesirable groups of microorganisms –coliforms and *Pseudomonas* spp. Secondly, the effect

of the presence of LAB –at an inoculum size around 10^7 cfu mL^{-1}– on the functionality of blood, by monitoring the degree of hemolysis of the red blood cells and the gelation properties of plasma proteins. The selected strains in this preliminary step would eventually be used for further experiments in blood stored at 5 ºC.

Depending on the temperature conditions, blood can be a highly-unstable product during storage (Ockerman et al., 1988). This fact was confirmed during preservation experiments at 15 ºC; if no preservation mechanisms were applied to hygienically collected blood, counts of contaminant microbiota increased 5 logs within the first 72 h of storage.

When LAB strains were inoculated in blood maintained at 15 ºC, it was found that 5 out of the initial 12 showed antagonistic effects after 72 h of storage. These strains were: PS22, PS48, PS99, TA20 and TA43. However, the best results did not exceed 2 logs of growth inhibition, what resulted insufficient as preservation technique but it suggested that better results may be achieved by modifying the storage conditions. Besides, the lack of fermentable carbohydrates utilisable as energy source caused a decrease in the counts of inoculates, thus suggesting that neither of these conditions were favourable for the activity of LAB.

The decrease of the pH due to the presence of LAB was not important. A typical evolution of the pH for the strain PS99 and a control blood sample is shown in Figure 5. As it can be observed, the pH of the inoculated sample tended to decrease only 0.2-0.4 units from the initial value. Control samples conversely suffered an increase of the pH probably to protein degradation processes.
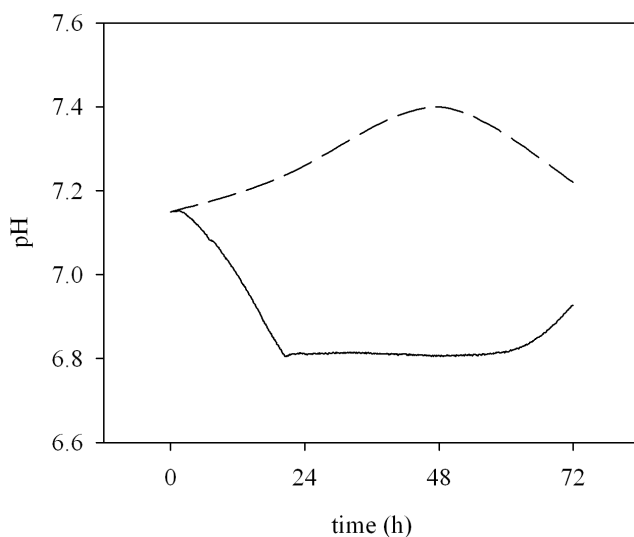
Figure 5. Example of the evolution of the pH in inoculated blood (PS99, solid line) and control blood (dashed line).

Then again the addition of LAB did not represent a risk for the maintenance of functional properties of blood. Even though the population of LAB often exceeded 10^7 cfu mL^{-1}, no undesirable changes were related to inoculation but to the natural contaminant microorganisms. In addition, an association between the counts of total contaminant microbiota and the loss of functionality, in terms of the hemolysis level, was obtained. At 15 ºC, from 48 h of storage onwards, the risk of complete loss of the functionality was about to happen since the capacity to separate blood into red blood cells and plasma was sometimes completely lost. The fact that this threat was common in control samples but it was not in the inoculated ones, evidenced that LAB may have some preservation capacity in relation to degradation processes, besides no negative effects. LAB strains played a positive role in the preservation of blood because its functionality tended to be better in inoculated samples, even when no microbial antagonism was detected.

Finally, two strains, PS99 and TA43, were selected for further studies at 5 ºC. In these experiments, the inoculation of each strain separately, as well as the co-inoculation of both of them, was assayed in blood and inulin-enriched blood.

When preservation experiments were performed in blood at 5 ºC, the effect of refrigeration temperatures slowed the growth of the contaminant microbiota because, on average, only an increment of 2-3 logs was observed after 144 h-storage. Unfortunately, low temperatures also affected the activity of inoculated strains and their antagonistic effects were weakened. Only a slight decrease in the counts of coliforms, *Pseudomonas* spp., hemolytic and proteolytic bacteria due to the presence of either PS99 or TA43 was observed. In the best case (PS99 against proteolytic bacteria) a reduction of 1 log was achieved after 96 h of storage.

API 50CH (Biomérieux, Marcy-L'Étoile, France) strips of fermentable carbohydrates were used to investigate potential energy sources for PS99 and TA43 strains in order to find a way to selectively favour their development and activity –therefore their inhibitory skills, via blood supplementation. Since the two strains proved to be able to ferment inulin, this fructooligosaccharide was selected as a supplement because it has been described that it can favour LAB's growth with no further privileges on undesirable microbiota (Wang et al., 1993; Gibson et al., 1995; Kaplan et al., 2000). The growth of PS99, TA43 and several contaminant indicators was then studied at different concentrations of inulin (0, 0.5, 1 and 2%) in a Bioscreen C microplate reader (Labsystems Corporation. Helsinki, Findland). An example of the growth curves of the strain PS99 is shown in Fig 6, where it can be observed that the maximum growth was achieved at a concentration of 2%. Similar results were obtained for the strain TA43, and there were no significant differences in the growth of contaminant indicators. Finally, a concentration of 2% was selected for blood enrichment.
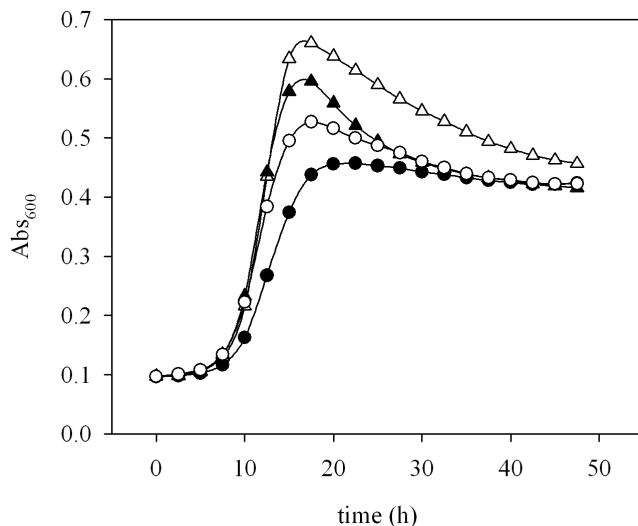
Figure 6. Example of the evolution of the Abs (600 nm) reflecting the growth of the strain PS99 using inulin at 0 (●), 0.5 (○), 1 (▲) and 2% (△) as energy source.
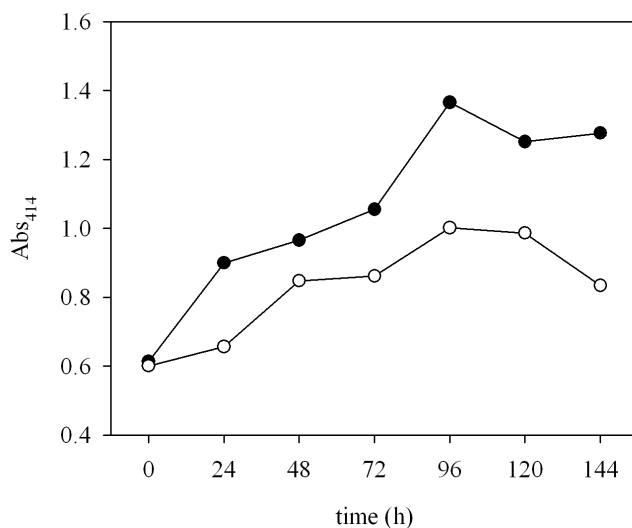
Figure 7. Average evolution of the hemolysis level in plasma as determined by absorbance measurements at 414 nm in control blood (●) and inulin-enriched blood inoculated with strain PS99 (○), stored at 5 ºC (n=4).

The inoculation of LAB proved to successfully reduce the presence of contaminant indicators during the storage period at 5 ºC of inulin-enriched blood, since the presence of this fructooligosaccharide enhanced the antagonistic activity of the inoculated strains. The maximum concentration of inulin in blood

(2%) –before LAB started consuming it– proved not to affect the gelation properties, i.e. hardness of plasma gels from inulin-enriched blood was 2.78 ± 0.62 N, whereas hardness of gels from non-enriched blood was 3.11 ± 0.42 N. Furthermore, no differences were found in the gelation properties of plasma along the storage period when blood was inoculated. The level of hemolysis in plasma, which reflected the degradation of red blood cells, was maintained and kept below the one of control samples when LAB strains were inoculated, as Figure 7 displays. Functional properties were therefore not affected neither by the presence of inoculated LAB strains nor by the presence of inulin.

On the other hand, the temperature of 5 ºC on its own seemed to be effective to guarantee the microbiological safety of blood within the first 48 h of storage, since the microbial counts did not raise enough to imperil the potential valorisation of this product. Taking into account the self-stability of blood within the first hours of storage at refrigeration temperatures, and the fact that LAB prevented spoilage processes, not only at 5 ºC but also when blood was stored at 15 ºC, it could be stated that inoculation may be specially useful to prevent the development of undesirable bacteria and the worsening, or even loss, of functional properties in the case of cold-chain breakdowns. The strain PS99, identified as *E. raffinosus*, was the best inhibitor in the biopreservation of blood, and its inoculation could be used as a feasible method of taking advantage of this abundant by-product.

The mechanism used by PS99 to inhibit the growth of undesirable bacteria is not known. Therefore, it would be valuable information to find it out. For example, it would be interesting to carry out studies about the production of bacteriocins, since it is known that several *Enterococcus* species are able to synthesize such compounds (Aymerich et al., 1996).

Use of PS99 (*E. raffinosus*) for the biopreservation of blood

The strain PS99 belongs to the group of enterococci. This genera of bacteria typically colonises raw foods of animal origin by intestinal or environmental contamination and can survive during fermentative processes (Giraffa, 2002). Like many other lactic acid bacteria, enterococci are found in fermented foods contributing to the development of desirable aromatic compounds, and they are as well used as probiotic or biopreservative microorganisms (Aymerich et al., 2000; Hugas et al., 2003, Martin et al., 2004). In addition, some species of enterococci are able to synthesize enterocin, which is able to control the growth of some foodborne pathogens (Aymerich et al., 2000; Sarantinopoulos et al., 2002; Ananou et al., 2005a, 2005b).

However, enterococci have been considered as spoilage microorganisms in a variety of food products (Houben, 1982; Gordon and Ahmad, 1991). Although they belong to the group of LAB, their consideration as GRAS is debated (Giraffa et al., 1997). Furthermore, during the last years, some cases of nosocomial infections caused by enterococci have been reported, being the resistance of the involved strains to antibiot-

ics, specially to vancomycin, the most specific cause of concern (Edwards, 2000). Most strains of meat enterococci are fortunately susceptible to antibiotics (Teuber et al., 1999; Bodnaruk et al., 2001). Particularly, strains isolated from pork carcasses have shown no vancomycin resistance (Knudtson and Hartman, 1993). Moreover, in spite of the reservations the use of Enterococci may entail, there exist some strains which are widely used as starters or probiotic organisms, like *Enterococcus faecium* K77D, and approved for dairy products by the UK Advisory Committee on Novel Foods and Processes (ACNFP, 1996). The use of *E. raffinosus* for the preservation of blood should therefore not be a cause of concern initially, although some considerations to ensure the safety of its inoculation should be taken into account.

In any case, the presence of *E. raffinosus* at the levels required for biopreservation is a parameter of concern. For example, a legal framework concerning blood derivatives might be implemented in a future, and regulate total counts as a microbiological criteria limited to a maximum permitted. In this case, blood may not pass the sanitary controls due to the inoculated bacteria. Thus, it would be interesting to investigate some ways for the removal of the inoculum. Pasteurisation treatments by means of thermal processes should obviously not be applied to blood, because loss of protein functionality would occur, if not the gelation of the product. As an alternative, non-thermal methods that have appeared as emerging technologies could provide the answer: high pressure processing, controlled instantaneous decompression, pulsed electric fields, high intensity pulsed light, X-rays or electron beams. Other methods based on oscillating magnetic fields, such as ohmic heating, dielectric heating or microwaves, imply some heating.

Among these treatments, the one that seems to best fit blood requirements may be high hydrostatic pressure (HHP). The use of such a technology during the manufacture of blood products would provide, additionally to LAB biopreservation, benefits in the microbial quality through the concept of hurdle technology (Leistner and Gorris, 1995; Leistner, 1996). Previous works on the application of HHP to blood have shown encouraging results in relation to microbial quality improvement and maintenance –even enhancement– of functional properties (Parés et al. 2000; Parés et al., 2001; Toldrà et al., 2002).

In this study, some experiments concerning the effect of HHP in combination with biopreservation by means of LAB inoculation were performed. An HHP treatment of 450 MPa for 15 min at 20 ºC was applied to plasma samples, because these conditions were effective in reducing the contaminant microbiota (Parés et al., 2001). For red blood cells, 400 MPa for 15 min at 20 ºC were applied, because more severe treatments entailed the formation of a thick paste or even the solidification of this fraction (Toldrà et al., 2002). A discontinuous isostatic press (ACB, Nantes, France) was used, and control and inoculated inulin-enriched blood samples were pressurised after 72 h of blood storage. Microbial counts were carried out to determine the presence of contaminant indicators and the inoculated LAB (Table 11).

After HHP treatments, the counts of coliforms, hemolytic and proteolytic bacteria in plasma and red blood cells (RBC) fractions were always lower than the detection limit of the plating technique, that is, either 1 or 10 cfu mL^{-1} (see Table 9). No colonies of *Pseudomonas* spp. were observed in two replicates,

whereas growth was detected in the other two. Parés et al. (2001) reported 2 to 3 log reduction in total aerobic counts in plasma at the same processing conditions, what agrees with the present findings.

Table 11. Counts (log cfu mL^{-1}) of microorganisms in control and pressurised fraction samples from blood stored at 5 °C for 72 h (mean ± SD, n=4). The applied HHP treatment was 450 MPa for plasma and 400 MPa for red blood cells, both for 15 min at 20°C.

Microorganism	Plasma		Red blood cells		Control blood[c]
	control[a]	HHP[b]	control[a]	HHP[b]	
Coliforms	1.49 ± 0.70	<0	2.09 ± 0.92	<0	2.15 ± 0.33
Hemolytic	2.01 ± 0.36	<0	2.17 ± 0.50	<0	2.41 ± 1.45
Proteolytic	1.90 ± 0.72	<1	2.08 ± 0.74	<1	3.55 ± 0.06
Pseudomonas spp.	2.77 ± 0.33	1.56[d] ± 0.79	2.73 ± 0.60	1.75[d] ± 1.05	3.47 ± 0.78
LAB	7.13 ± 0.80	0.74 ± 0.81	7.06 ± 0.67	4.51 ± 0.58	

[a] Non-pressurised samples from inulin-enriched blood inoculated with strain PS99.

[b] Pressurised samples from inulin-enriched blood inoculated with strain PS99.

[c] Non-pressurised samples from non-inoculated control blood.

[d] Average counts from the plates containing colonies (n=2).

Inoculated *E. raffinosus* showed a different survival response depending on the studied fraction. *E. raffinosus* proved to be extremely sensitive to the pressurization treatment in plasma samples, since a 7 log reduction was nearly achieved. Conversely, a 2.5 log reduction was found in RBC. These differences may be explained by the milder treatment applied (400 MPa vs. 450 MPa), and by the larger content of proteins in RBC (more than 35%) which may provide resistance to pressure-induced damage and greatly increase the ability of bacteria to survive HHP treatments.

Different behaviours have been described for LAB species in relation to endurance to HHP treatments. For example, Hugas et al. (2002) reported an inactivation of total LAB cells in meat products comprised between 1.58 and 4.57 log cfu mL^{-1} after a treatment of 600 MPa for 6 min at 31 °C; a reduction comprised between 3 and 4 log units have been described for lactobacilli species after pressurisation treatments from 300 to 500 MPa (Hugas et al., 2002; Wuytack et al., 2002; Mallidis et al., 2003). In the case of enterococci, Moerman (2005) reported a reduction of 1.5 log units for *Enterococcus faecalis* in a cooked pork product after a treatment of 400 MPa at 20 °C during 30 min; Hugas et al. (2002) described a 4 log reduction for *Enterococcus faecium* after a treatment of 500 MPa at 40 °C during 10 min in a spiked meat model, and a maintenance of total *Enterobacteriaceae* below 10 cfu g^{-1} for long-storage meat products pressurised at 600 MPa at 31 °C during 6 min.

According to the results referenced in the literature, the inactivation levels achieved for *E. raffinosus* are satisfactory, and it could be concluded that HHP treatments applied to blood fractions may be useful in the development of blood products. Furthermore, they contributed to the improvement of the sanitary quality of blood fractions through the concept of hurdle technology. Besides, these treatments proved to have no relevant effects on the functionality of blood proteins, because solubility, gelation, thermal, emulsifying and foaming properties remained similar to control samples (results not shown).

Industrial application of blood biopreservation

The next step, beyond the experiments performed in blood at a laboratory scale, is the application of biopreservation at an industrial scale. The participation of the meat industry to put into operation such a system at the facilities of an industrial slaughterhouse is a key action to confirm its suitability, and it would make it possible to value this process as the method to improve the microbiological quality of the collected blood.

Yet prior to the implementation of the biopreservation system, it has to be developed a method to obtain pure cultures of PS99 –the LAB strain proposed for inoculation– at an industrial scale, since large quantities of this strain will be needed to inoculate blood with. Although this can be done by growing PS99 strains in bioreactors in appropriate culture media, the uncertainty that needs to be worked out is the way to obtain these cultures under a suitable form to be used for the industry, i.e. in a dehydrated state, with guarantees about the endurance of PS99 after going through these conditions and maintain its antagonistic capacity. This issue has been investigated in the work of Zamora et al. (2006), where the survival rates of several LAB strains after freezing, freeze-drying or spray-drying processes were measured. It was shown that, for PS99, freeze-dried cultures stored at 5 ºC for 60 days entailed a 0.1 log reduction of viable cells, whereas spray-dried cultures, stored for the same period of time, suffered a 0.5 log reduction. Therefore, the strain PS99 may be produced at large quantities, then be dehydrated by means of a low-cost technique such as spray-drying, and finally obtain a dry culture to inoculate blood successfully.

At this moment, both the biopreservation system and the obtaining of LAB cultures at an industrial scale investigated so far are awaiting for the support of the meat industry to be tested in a running slaughtering line.

CONCLUSIONS

- The identification of lactic acid bacteria (LAB) strains confirmed the presence of this group of microorganisms in blood collecting tanks of industrial slaughterhouses. The identified species were *Lactococcus garvieae*, *Lactobacillus reuteri*, *Lactobacillus murinus*, *Enterococcus raffinosus*, *Enterococcus dispar*, *Enterococcus hirae*, *Lactobacillus mucosae* and *Streptococcus bovis*.

- A low diversity of LAB strains contaminated blood, because the former four of the above mentioned species gathered the 95% of the total isolates. These strains are widespread LAB with the greatest ability to survive in such an environment, since they were found in all industrial facilities and their isolation from animal sources has been previously reported.

- The inoculation of selected LAB strains protected blood from spoilage, because a positive association was found between inoculated strains and functionality maintenance. However, if blood was not supplemented with a selective energy source, the antagonistic activity of LAB against undesirable contaminant bacteria was not enough to prevent their growth, neither at 15 ºC nor at 5 ºC.

- Inulin proved to be a good selective carbon source to be used as a supplement in blood, since inhibition of undesirable bacteria was enhanced when it was present at 2% and blood was inoculated with strains PS99 or TA43.

- The strain PS99, identified as *Enterococcus raffinosus*, showed the best preservative abilities in blood stored at 5 ºC. Its inoculation reduced the presence of contaminant indicators (coliforms, *Pseudomonas* spp., hemolytic and proteolytic bacteria) especially within the first hours of storage. Additionally, no changes in the functionality of blood plasma were observed after 144 h-storage of inoculated blood.

- The refrigeration temperature (5 ºC) on its own was enough to slow down the microbial growth in hygienically-collected blood within the first 48 h of storage. Hence, the inoculation of PS99 could be seen as an effective way to prevent damages in this by-product especially in the case of cold-chain breakdown during storage or handling of blood.

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Chapter II

FUNCTIONAL PROPERTIES OF PLASMA

INTRODUCTION

OBJECTIVES

MATERIAL AND METHODS

RESULTS AND DISCUSSION

- **Article 3. Heat-induced gelation of porcine blood plasma proteins as affected by pH**

- **Article 4. Fourier Transform Raman spectroscopy study of heat-induced gelation of plasma proteins as influenced by pH**

- **Article 5. Studies on plasma protein interactions in heat-induced gels by differential scanning calorimetry and FT-Raman spectroscopy**

- **Article 6. Structure of heat-induced plasma protein gels studied by fractal and lacunarity analysis**

- **Article 7. Characterization of plasma protein gels by means of image analysis**

- **Article 8. Surface functional properties of blood plasma protein fractions**

- **General Discussion**

CONCLUSIONS

REFERENCES

INTRODUCTION

The present dissertation has also been framed within the project entitled "Integral use of blood from industrial slaughterhouses", with the financial support of the Spanish Government from the Institutional Program MICYT (2004-2007), referenced as AGL2004-06077/ALI. The general aim of this project was the investigation of a system to attain the full-use of porcine blood from slaughterhouses, to develop high value-added blood derivatives. This would be achieved through the investigation of the functionality of blood for the food industry and the complete exploitation of blood to minimize process wastes. In this case, the proposed objectives were: i) the obtaining of high quality food ingredients from blood fractions (plasma powder and hydrolysates of red blood cells), from a functional and nutritive standpoint, and ii) the nutritive use of the residue from the hydrolysation process of red blood cells.

The improvement of functional properties of plasma proteins has been the object of previous studies carried out in our research group. This enhancement has been tackled from different approaches. One the one hand, it is known that high-pressure processing (HPP) causes a variety of changes that may be of benefit in relation to functional properties. Parés and Ledward (2001) studied the emulsifying and gelling properties of plasma as influenced by HPP, and it was found that high-pressure treatments up to 300 MPa did not affect the functionality of proteins, although the effects of treatments at pressures above 400 MPa caused some pH-dependent changes; Parés et al. (2000) also studied the effect of (HPP) on plasma, and they showed that even though structural protein modifications had occurred as a consequence of pressurization at 450 MPa, the functional properties of heat-induced gels were not significantly affected. On the other hand, enzymatic modifications may lead to an enhancement of the functionality thanks to crosslink reactions between plasma proteins. Saguer et al. (2004, 2007) showed that an enzymatic treatment with microbial transglutaminase enhanced the textural properties and WHC of plasma gels at pH 5.5, thus improving their gelling properties at acidic conditions –which are reduced due to protein denaturation. Currently, studies on the effect of combined treatments –HHP plus enzymatic– are being performed.

Since plasma contains a rich variety of proteins –each one with particular functional properties– the enrichment or removal of some protein constituents may entail an improvement of the functionality. Plasma properties depend to a large extent on the occuring protein-protein interactions, as it will be further explained; hence, taking advantage of these interactions may be a different approach to undertake investigations about functionality improvement.

The Chapter II of this dissertation compiles the work carried out to accomplish part of the objectives of the above mentioned project, concerning the functionality studies of the plasma fraction. Properties such as gelation, emulsifying and foaming capacities were studied to improve the understanding of their mechanisms and the role that each protein constituent plays in these properties. This was investigated to make progress in the development of a reformulated plasma-based food ingredient, with enhanced functionality.

BLOOD PLASMA

Plasma, the main fraction of blood (~60%), is a naturally occurring protein mixture like whey, soy or egg. Blood plasma is a common by-product of the meat industry, with a high potential for utilization due to its abundance and its useful functional properties, especially for the manufacturing of foodstuff. The complex mix of plasma proteins is listed in Table 12, where the huge variety of existing molecules can be observed. Nevertheless, they can be summarised into no more than three major groups according to a relevant content for functionality studies. This classification includes: albumin, globulins and fibrinogen, a molecular representation of which is depicted in Figure 8.

Albumin, a globular heart-shaped protein with a molecular weight of 66-69 kDa and an isoelectric point (pI) around 4.8, is the most abundant of plasma proteins, representing up to 60% of the protein content (Putnam, 1975; Cheftel et al., 1985). The three-dimensional configuration of serum albumin is composed of three homologous domains (I, II, III), predominantly helical and extensively cross-linked by several disulfide bridges (Carter and Ho, 1994). It is characterized by a low content of tryptophan and methionine, and a high content of cystine and charged amino acids such as aspartic, glutamic, lysine and arginine. Its primary structure is responsible for the high solubility of this protein, since it confers a high total charge, about 185 ionized groups per molecule at pH 7.0 and 90 at pH 2. Albumin is composed of circa 580 amino acids in a single chain and its sequence contains 17 disulfide bridges and one free cysteine in position 34 (Carter et al., 1994).

Globulins, which are further subdivided into α, β and γ fractions, comprise a heterogeneous group of globular proteins that include a variety of enzymes, carrier and antigenic proteins, and account for 40% of the plasma protein content. The most abundant subgroup within globulins corresponds to the immunoglobulins. The range of molecular weights of globulins spreads from few to hundreds kDa and they have a variety of pI, mostly comprised between 5 and 7. In the case of immunoglobulins, the typical tertiary structure is a Y-shaped assembly of two pairs of light and heavy chains stabilised by disulfide bridges, at the edge of which is located the antigen binding site. The most abundant protein within this group is immunoglobulin G (IgG), followed by α_1-antitrypsin and transferrin (Putnam, 1975; Cheftel et al., 1985).

Fibrinogen, a single protein that represents 3% of the total content, is a multidomain fibrous protein of 340 kDa and pI near 5.5 involved in blood coagulation, since its main function is the formation of the three-dimensional network of fibrin fibres during blood clotting (Putnam, 1975; Cheftel et al., 1985). It is made of three pairs of non-identical polypeptide chains, Aα, Bβ and γ, (AαB$\beta\gamma)_2$, forming two identical subunits. Both the subunits and the chains are linked together by 29 disulfide bonds to form a symmetrical trinodular structure (Privalov and Medved, 1982; Weisel et al., 1985).

Table 12. Detailed protein composition of blood plasma (Sigma-Aldrich, 2006).

Protein	Molecular Weight (Da)	Amount (mg/100 mL)
Albumin	66 500	3000-5000
Prealbumin, thyroxine-binding	54 980	10-40
Retinol-binding protein	21 000	3-6
α-Globulins		
α$_1$-Acid glycoprotein	40 000	55-140
α$_1$-Antitrypsin	54 000	200-400
9.5 S α$_1$-Glycoprotein	~250 000	3-8
GC Globulin	52 000	20-55
Ceruloplasmin	132 000	15-60
3.8 S Histidine-rich α$_2$-glycoprotein	58 500	5-15
α$_2$-Macroglobulin	725 000	150-420
α$_1$B-Glycoprotein	68 000	15-30
α$_1$T-Glycoprotein	85 000	5-12
α$_1$-Antichymotrypsin	68 000	30-60
α$_1$-Microglobulin	26 000	4-9
Zn-α$_2$-Glycoprotein	41 000	2-15
α$_2$HS-Glycoprotein	49 000	40-85
3.1 S Leucine-rich α$_2$-glycoprotein	49,600	2-3
8 S α$_3$-Glycoprotein	220 000	3-5
Serum cholinesterase	348 000	0.5-1.5
Thyroxine-binding globulin	54 000	1-2
Inter-α-trypsin inhibitor	~160 000	20-70
Transcortin	55 700	~7
Haptoglobulin Type 1-1	86 000	100-220
Haptoglobulin Type 2-1	~200 000	160-300
Haptoglobulin Type 2-2	~400 000	120-260
β-Globulins		
Hemopexin	60 000	50-115
Transferrin	79 500	200-320
β$_2$-Glycoprotein I	~48 000	15-30
β$_2$-Glycoprotein II	63 000	12-30

Protein	Molecular Weight (Da)	Amount (mg/100 mL)
β_2-Glycoprotein III	35 000	5-15
C-Reactive Protein	105 000	<1
Fibronectin	440 000	25-40
Immunoglobulins		
Immunoglobulin G	150 000	800-1800
Immunoglobulin A	$(160\ 000)_n$	90-450
Immunoglobulin M	950 000	60-250
Immunoglobulin D	175 000	<15
Immunoglobulin E	190 000	<0.06
Low-molecular weight proteins		
Lysozyme	14 000	0.5-1.5
Basic protein B_1	11 000	–
Basic protein B_2	8 800	<1
0.6 S γ_2-Globulin	5 100	<1
2 S γ_2-Globulin	14 000	0.1
Post γ-globulin	13 260	–
Complement components		
C_1q Component	400 000	10-25
C_1r Component	166 000	–
C_1s Component	83 000	1-2
C_2 Component	102 000	2-3
C_3 Component	185 000	55-120
C_4 Component	200 000	20-50
C_5 Component	185 000	4-15
C_6 Component	105 000	7
C_7 Component	92 500	6
C_8 Component	163 000	8
C_9 Component	71 000	23
C_1 Esterase inhibitor	104 000	15-35
Factor B	90 000	–
Factor D	24 000	–

Protein	Molecular Weight (Da)	Amount (mg/100 mL)
Factor H	155 000	–
C_4 Binding Protein	540 000	–
Properdin	220 000	2-3
Coagulation proteins		
Antithrombin III	58 000	(20-40)
Prothrombin	72 000	(5-10)
Antihemophilic factor (Factor VIII)	$(100\ 000)_n$	(1-2)
Plasminogen	92 000	(6-25)
Fibrin-stabilizing factor (Factor XIII)	320 000	(1-4)
Fibrinogen	340 000	200-450
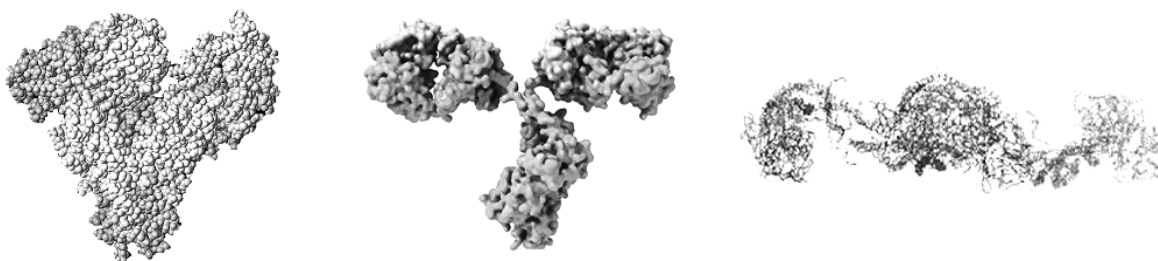
Figure 8. Molecular representation of major plasma proteins. From left to right: albumin, immunoglobulin G and fibrinogen [Source: University of Arizona (2006)].

FUNCTIONAL PROPERTIES

The concept functional property has been defined as "any property besides nutritional attributes that influences an ingredient's usefulness in food" (Boye et al., 1997). Some of the functional properties determine the sensory attributes of foods but they can also modify the physical behaviour of foods and their ingredients during manufacturing or storage (Cheftel et al., 1985).

Proteins are a group of molecules with interesting functional property, widely used in the food industry because their incorporation into food products permits the development of desired attributes. The func-

tionality of a protein is related to their physical, chemical and conformational attributes, which are influenced by its chemical environment and thermo-mechanical history (Harwalkar and Ma, 1996; Damodaran, 1997). These properties can be classified into the following categories (Yada et al., 1994).

- Hydration properties, which depend on water-protein interactions, such as hydration, solubility, dispersability, swelling, water-holding capacity.

- Rheological properties, which depend on protein-protein interactions, such as precipitation, gelation and texturization.

- Surface properties, such as emulsification and foaming capacity.

- Sensory properties, such as taste, flavour, colour and texture.

- Other: adhesion, cohesion, film formation, etc.

However, all these categories have to be seen as interrelated rather than totally independent. For example, gelation entails protein-protein as well as water-protein interactions, and the protein matrix developed after protein aggregation can have good water-holding capacity due to water entrapment.

To date, some research has been conducted to study the functionality of plasma proteins (Tybor et al., 1975; De Vuono et al., 1979; Howell and Lawrie, 1984a, 1984b; King et al., 1989; O'Riordan et al., 1989; Raëker and Johnson, 1995; Oshodi and Ojokan, 1997; Parés et al., 1998a; Parés et al., 1998b; Duarte et al., 1999; Parés et al., 2000; Parés et al., 2001; Silva and Silvestre, 2003; Ramos-Clamont et al., 2003). Their ability to gel under thermal treatments and to form good emulsions and foams are the best properties they display, therefore they can be considered as good functional ingredients for the food industry. For example, plasma can be incorporated in meat products to take advantage of its gelling and thickening properties, and the bakery industry can use it as egg replacer, not only for economic and functional reasons (Howell, 1995), but also for consumer's health purposes.

Solubility

Besides inherent characteristics like amino acids composition, the solubility of proteins depends mostly on environmental parameters, such as pH, ionic strength, solvent and temperature (Cheftel et al., 1985). It is a key parameter influencing proteins' functionality because, overall, undissolved protein makes little or no contribution to the functional performance, specially in those properties depending on the surface activity (Halling, 1981). In some cases, however, the performance of proteins with high solubility is not as good as when being slightly denatured. For example, Parés et al. (2001) reported an increase in the emulsifying

capacity of plasma proteins when pressurised at 400 MPa at pH ≥ 6.5, due to a partial unfolding of proteins that enhanced the surface hydrophobicity, although their solubility may be lowered.

Solubility is useful for monitoring the optimal conditions of transformation processes, starting with the extraction and purification of proteins. The development of good ingredients for the food industry is subjected to their ability to dissolve in the food matrix, thus a high solubility is often needed. It must be noted that solubility is highly sensitive to heating; consequently, thermal treatments typically entail the insolubilisation of proteins.

Foaming properties

Foams are dispersed systems that contain at least two distinct phases: a liquid continuous phase which surrounds the disperse phase, bubbles of the incorporated gas. Foams are characterised by a large air-water interface area, wherein proteins tend to accumulate and constitute an interfacial layer, thereby determining the surface properties of the whole system (Halling, 1981; Dickinson and McClements, 1996).

Foams can be created by bubbling, whipping and shaking. In all these methods, the volume fraction incorporated is variable and the formation process includes some stages similar to breakdown processes in emulsions, because gas emulsions break down by bubble rise and drainage. In addition, the mechanical stress of the foaming process itself can promote the coalescence of the air bubbles (Halling, 1981).

The distribution of proteins along the thin lamellae between air bubbles and the liquid phase determines the maximum level of gas incorporation as well as the stability of the foam. This is mainly determined by three factors: the interfacial tension, the viscosity of the continuous phase and the structural properties of the adsorbed layer, such as the surface flexibility (Zayas, 1997). The surface activity of proteins is responsible for the former and for the rate at which the adsorption process takes place. The viscosity of the liquid phase is influenced by the protein therein present, therefore all factors affecting the solubility of proteins may enhance the stability of foams, thus holding up leakage phenomena through steric repulsions (Dickinson, 1986). In addition, environmental factors, like ionic strength or pH conditions, influence enormously the foaming properties of proteins because the degree of unfolding, as well as the electric charge of molecules, determine the exposure of their residues and their steric and electrostatic repulsive effects. Therefore, protein-protein interactions may promote a better foaming capacity or, in contrast, quite the opposite. The best conditions need to be established in order to enhance the formation of foams.

Emulsifying properties

Like foams, emulsions are dispersed systems containing two immiscible liquids: disperse droplets contained within a continuous phase. Most emulsions found in food are O/W or W/O (oil-in-water or water-in-

oil, respectively), and the size of disperse droplets usually ranges from 0.1 to 50 μm (Cheftel et al., 1985). Typically, an aqueous solution or suspension is vigorously stirred or subjected to turbulent mixing while oil or melted fat is run in steadily (Halling, 1981).

The emulsification process causes the creation of an interfacial surface between both liquids, which increases geometrically as the diameter of the droplets decreases. Emulsions, like foams, are not stable, and they suffer breakdown processes which include: creaming, drainage, flocculation and coalescence. The mechanisms that favour the stabilisation of emulsions, or even increase the emulsifying capacity, are the same as with foams, that is, the ones depending on the surface activity of the adsorbed molecules and the properties of the continuous phase, such as viscosity (Dickinson, 1986). In general, soluble proteins with high surface hydrophobicity show good emulsifying properties (Dickinson, 1986; Dickinson and Mc Clements, 1996) and, since a notable number of food products are emulsions, proteins are considered essential emulsion stabilisers thanks to their ability to texturize via film formation (Kinsella et al., 1994).

Gelation properties

One of the most interesting properties of plasma proteins is their ability to form gels when they are thermally treated –and when a concentration threshold is achieved. Plasma proteins reorganize into self-assembled aggregates that develop a three-dimensional network, forming consistent gels that entrap water within the protein matrix (Hermansson, 1982; Howell et al., 1984a; Oshodi and Ojokan, 1997; Parés et al., 1998a). In Figure 9, the intertwined steps occurring during heat-induced gelation are depicted.

The functionality of the gels is determined by both the spatial distribution of the protein aggregates and the contribution of covalent and non-covalent bonds to the developed network. The intrinsic properties of the proteins as well as newly-created bonds, which depend on the gelation conditions, modify protein-protein and water-protein unions. Such modifications involve changes in the secondary structure –like β-sheet formation–, in the hydrophobicity –like exposure of amino acid residues– and in covalent bonds –like disulphide bonds exchange reactions (Lin and Koenig, 1976; Nonaka et al., 1993; Alizadeh-Pasdar et al., 2002; Ikeda, 2003; Ngarize et al., 2004). The resulting size, shape and spatial arrangement of the protein aggregates may vary widely. Consequently, gels will display different macroscopic properties, like textural behaviour and water-holding capacity.

Electrostatic interactions, hydrogen bonds, disulfide bonds, dipole-dipole interactions and hydrophobic interactions are the forces involved in folding and stabilizing the native protein structure. However, during heat-induced gelation proteins unfold partially (Hermansson, 1978). When amino acid residues are exposed, intermolecular interactions between these sites occur. The same forces contribute then to the development of new structures that, in most cases, impede the renaturation of the unfolded molecules upon cooling, and the process becomes therefore irreversible.

native   unfolded

network   aggregation
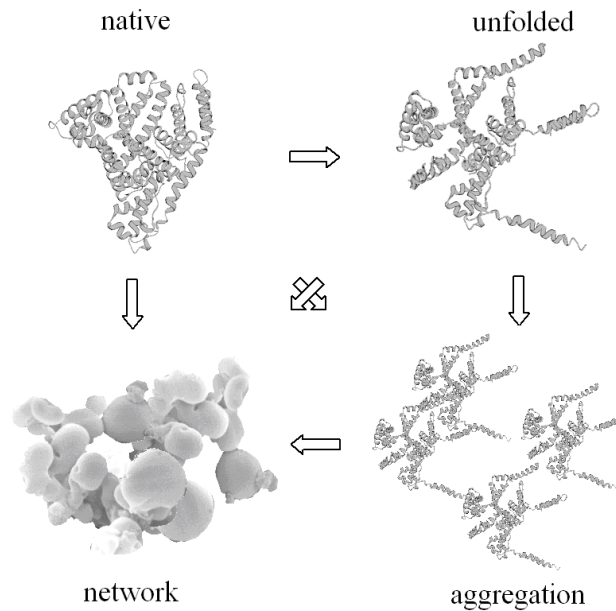
Figure 9. Transformation of proteins from the native form into the protein network during heat-induced gelation, with intertwined unfolding and aggregation processes.

PROTEIN-PROTEIN INTERACTIONS

Protein-protein interactions are common phenomena during food processing that can modify the functionality of single proteins. These processes lead to synergism, phase separation and/or precipitation (Howell et al., 1984a; Howell, 1992, 1995; Comfort & Howell, 2002) and they can be seized to improve the functionality of single proteins.

As described above, plasma is a complex blend of proteins. This mixture contains globular and fibrous proteins, sized few to hundreds kDa, highly or poorly cross-linked, with high or low hydrophobicity. This variety suggests that, when a functional property is evaluated, not individual protein phenomena but a global sum of them all are observed. Therefore, the presence of a single constituent may cause either an enhancement or a worsening, but it would not be detected since it is the overall result what is being measured. It would be interesting to isolate fractions and check their own functionality and their contribution to the global functionality of plasma via protein-protein interactions. With this knowledge, the natural-occurring plasma protein profile could be modified to develop ingredients with enhanced functionality, steered to specific food requirements.

OBJECTIVES

The objectives that follow were pursued, and the experimental determinations were carried out as a function of pH, using three conditions (pH 4.5, 6.0 and 7.5) to account for the typical variety of pH found in food products:

     i.   The study of the gelation properties of plasma and its major protein constituents, using different approaches to account for textural, thermal and molecular properties.

     ii.   The study of the hydration and surface properties of plasma and its major protein constituents, in relation to their solubility and their ability to form and stabilise foams and emulsions.

     iii.   The elucidation of the role of each fraction in the overall functionality of plasma, in order to acquire knowledge about the formulation of plasma-derived ingredients for specific food requirements.

In spite of the main objectives mentioned above, the gelation properties of plasma proteins should be also studied in terms of gels microstructural properties to fully understand and describe their mechanisms, as well as the way other variables, such as pH, affect them and, consequently, their macroscopic properties. An approach based on image analysis has been developed in order to achieve this goal, thus proceeding to study the microstructural properties of the protein network and the porosity of heat-induced gels. This methodology has only been applied to describe plasma gels, but the results herein presented show that image analysis is a powerful tool in this field, and it may be successfully applied to the rest of the protein fractions to give valuable information about their gelation properties.

MATERIAL AND METHODS

EXPERIMENTAL DESIGN

All the experiments carried out in Chapter II, concerning the functional properties of blood plasma proteins, are summarised in Scheme 5. The functionality of plasma and its protein fractions were studied by means of several analytical methods, which are listed in italics. Although the results of most of them are interrelated, they are shown under the main subject of study.

The main functional properties of plasma, those derived from its gelation and surface features, were studied in terms of the texture profile analysis (TPA), rheology and water-holding capacity (WHC), and solubility, foaming, and emulsifying capacities, respectively. As well, differential scanning calorimetry (DSC), FT-Raman spectroscopy, electrophoresis, and image analysis by means of scanning electron microscopy (SEM) studies were performed to better understand their mechanisms and the interactions taking place between protein constituents.
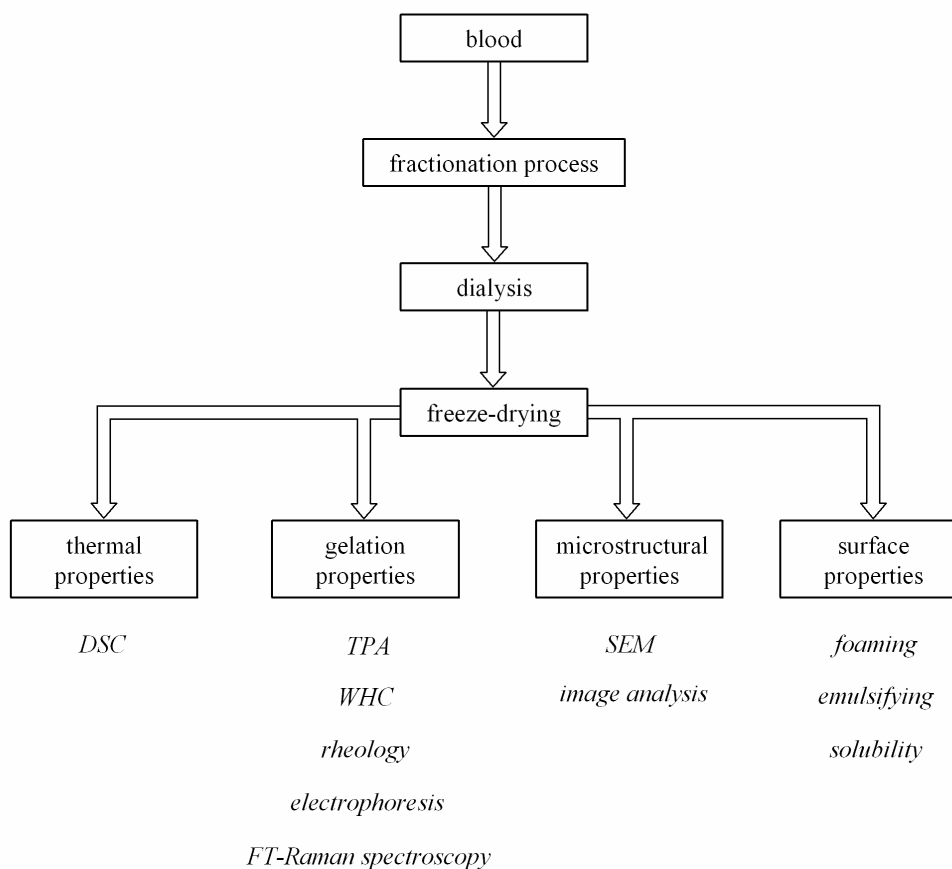
Scheme 5. Experimental design followed in the functionality studies of plasma and its fractions.

BLOOD PLASMA PROTEINS

All the blood samples were collected at the bleeding line of an industrial abattoir as blood drained out of the wound of the slaughtered animals. Samples were collected in 5 L sterile recipients containing trisodium citrate to prevent coagulation (1% w/v in blood), and they were maintained in refrigeration until plasma was separated at the laboratory by centrifuging blood at 2530 g at 4 ℃ for 15 min (Sorvall RC 5C Plus, DuPont Co., Newtown, CT) and decanting.

Fractionation process

Plasma protein fractions were separated by the salting out method, using the fractionation protocol described by Spadaro et al. (2003) with sight modifications. The saturation percentages of ammonium sulphate to precipitate the protein fractions were chosen according to the results of preliminary experiments. In these, a series of plasma samples at different saturation levels were centrifuged. Afterwards, SDS-PAGE electrophoreses of the supernatants and the precipitates were run (with the conditions detailed later on); a picture of the gels is shown in Figure 10, where the differences along the lanes can be observed. The molecular weights of fibrinogen, globulins and albumin from porcine blood reported by Howell and Lawrie (1983) (Table 13) were used to identify the protein bands and select the saturation thresholds, which were set at 20% for fibrinogen, 60% for globulins and 75% for albumin.
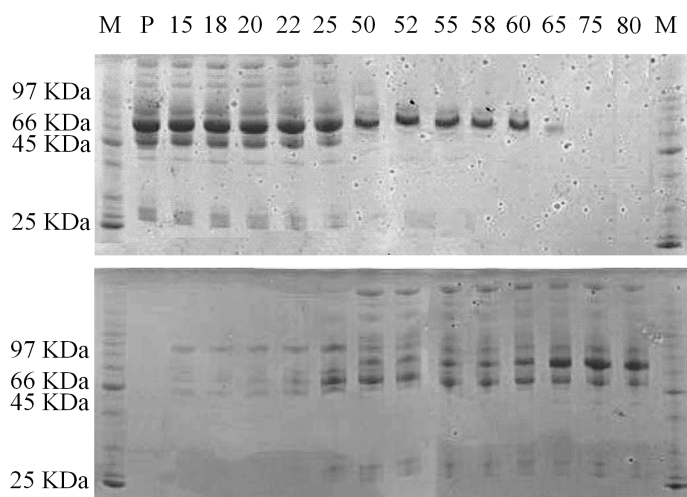
Figure 10. SDS-PAGE gels of the supernatants (up) and the precipitates (down) of plasma solutions saturated with ammonium sulphate. Lanes correspond to: molecular weight marker (M), fresh plasma (P) and the saturation percentage (15-80%).

Table 13. Main plasma proteins and their molecular weights as described by SDS-PAGE (adapted from Howell et al., 1983).

Protein	Molecular weight (kDa)
α-globulins	150
	130
	125
	115
	110
	105
β-globulins	92
β-,γ-globulins	78
β-globulins, α-globulins	76.5
Albumin	65-69
fibrinogen, α-,β-globulins	60
γ-,α-globulins	56
β-globulins	52
	48
Fibrinogen	40
γ-globulins	33
β-globulins	27
	15
	12
	11

All the precipitation steps were carried out in an ice bath, using a 100% saturated solution of ammonium sulphate in 10 mM Tris-HCl pH 7.4 at 4 ºC, which was added drop by drop to protein solutions–initially fresh plasma– to increase the saturation of salt and progressively precipitate each protein fraction. Scheme 6 shows the steps followed during the fractionation process.

Fibrinogen, precipitated at 20% saturation, was removed by centrifugation (10 000 g at 4 ºC for 15 minutes). The recovered fibrinogen precipitates were washed once with a 20% saturated solution of ammonium sulphate and then centrifuged again; fibrinogen pellets were dissolved in 10 mM Tris-HCl pH 7.4.

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                          ┌─────────────┐
                          │    blood    │
                          └─────────────┘
                                 │
        ┌─────────────┐          │          ┌──────────────────┐
        │   plasma    │ ◄────────┼────────► │  red blood cells  │
        └─────────────┘          │          └──────────────────┘
               │          centrifugation
               │
               │   20% saturation
               │      NH₄SO₄        ┌─────────────┐
               ├──────────────────► │  fibrinogen │
               │                    └─────────────┘
               ▼
        ┌─────────────┐
        │    serum    │
        └─────────────┘
               │
               │   60% saturation
               │      NH₄SO₄        ┌─────────────┐
               ├──────────────────► │  globulins  │
               │                    └─────────────┘
   75% saturation
      NH₄SO₄
               ▼
        ┌─────────────┐
        │   albumin   │
        └─────────────┘
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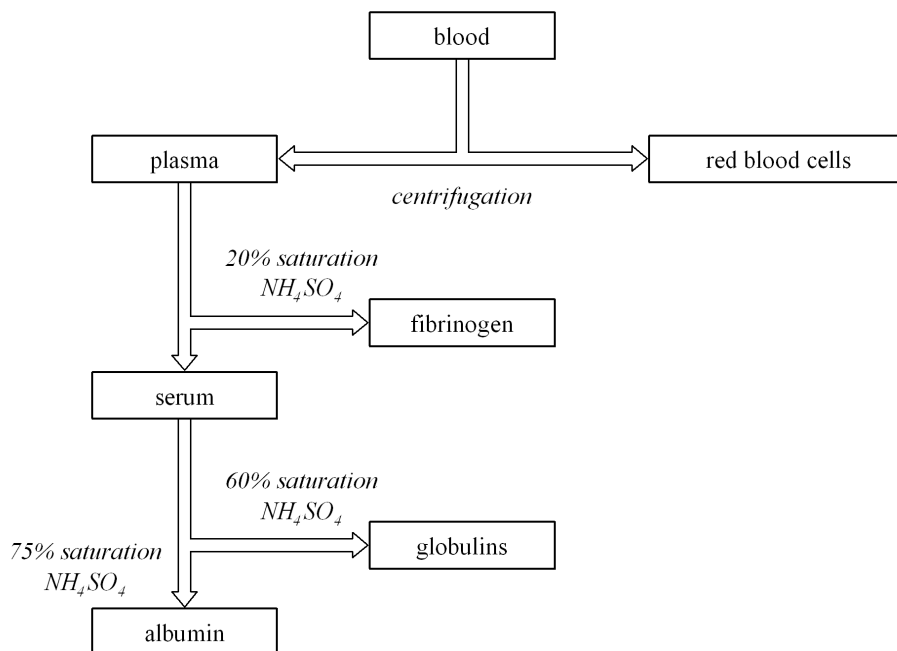
Scheme 6. Fractionation process of blood into red blood cells and plasma. The latter was further separated into fibrinogen, globulins and albumin.

After precipitating fibrinogen, the remaining supernatant –that is, serum– contained globulins and albumin. Ammonium sulphate was then added up to 60% saturation to precipitate globulins, which were recovered by centrifugation, washed once with a 60% saturated solution of ammonium sulphate and centrifuged again; globulins precipitates were dissolved in 10 mM Tris-HCl pH 7.4.

The remaining supernatant after globulins were removed contained albumin, which was also precipitated by increasing the ammonium sulphate saturation up to 75%. Albumin was recovered by centrifugation, washed once with a 75% saturated solution of ammonium sulphate and then re-centrifuged. Albumin precipitates were also dissolved in 10 mM Tris-HCl pH 7.4.

The purity of protein isolates was confirmed by SDS-PAGE electrophoresis. Figure 11 shows an example of SDS-PAGE gel in reducing conditions, where it can be observed the three polypeptide chains of fibrinogen, the diversity of globulins and the major single band of albumin.

All the resuspended protein fractions, together with serum and plasma aliquots, were exhaustively dialysed against distilled deionised water at 5 ºC with a membrane of 12-14 kDa pore diameter (Medicell International Ltd, London, UK) in order to reduce the content of ammonium sulphate. This step also diminished the presence of salts naturally present in plasma, as well as the content of trisodium citrate, added as anticoagulant. Dialysed solutions were frozen at -80 ºC for 24 h and freeze-dried in a Virtis

Unitop SQ freeze dryer (The Virtis Co., Gardiner, NY) at -15 ºC and 15 ºC for the primary (sublimation) and the secondary (desorption) drying stages, respectively. The proximate composition of protein powders for moisture (ISO R-1442), total protein (ISO R-937), and ash content (ISO R-936) was determined, and it was used to calculate the amount of powder required to prepare the protein solutions at the desired concentration. Dried proteins were vacuum-packed and stored under refrigeration temperatures until use.
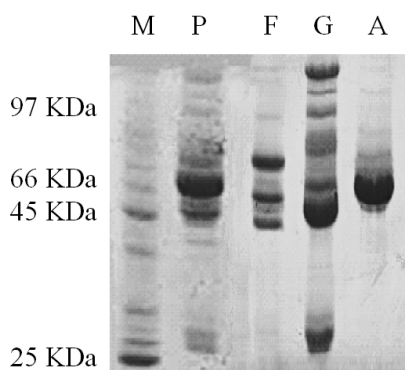
Figure 11. SDS-PAGE of plasma and the isolated protein fractions. Lanes correspond to: molecular weight marker (M), fresh plasma (P), fibrinogen (F), globulins (G) and albumin (A).

PROTEIN SOLUTIONS

Protein solutions were prepared in distilled deionised water at different protein and salt concentrations depending on the experiment to perform. Table 14 summarizes all the conditions. The pH of the solutions was adjusted with HCl or NaOH and they were maintained in mild stirring for 1-2 h to reach ionic equilibrium prior to experimentation.

Table 14. Characteristics of protein solutions used in each experimental procedure.

Experiment	Buffer	(mM)	pH	Protein conc. (%wt)
DSC	trisodium citrate	20	4.5	5%
	monosodium phosphate	50	6.0, 7.5	5%
	monosodium phosphate	50	7.0	6%
Electrophoreses	trisodium citrate	20	4.5	5%
	monosodium phosphate	50	6.0, 7.5	5%
Rheology	trisodium citrate	20	4.5	5%
	monosodium phosphate	50	6.0, 7.5	5%
Texture, WHC	sodium chloride	50	4.5, 6.0, 7.5	8%
Microstructure analysis	sodium chloride	50	4.5-7.5 (Δ0.5)	6-11% (Δ1%)
FT-Raman spectroscopy	sodium chloride	50	4.5, 6.0, 7.5	15%
	monosodium phosphate	50	7.0	15%
Solubility	deionised water	-	4.5, 6.0, 7.5	1%
Foaming, emulsifying	deionised water	-	4.5, 6.0, 7.5	0.5%

THERMAL PROPERTIES

Differential scanning calorimetry (DSC)

Heat-induced conformational changes (denaturation and aggregation phenomena) of protein solutions were monitored by DSC either with a DSC7 (Perkin Elmer, Norwalk, USA) or a DSC822 (Mettler Toledo, Switzerland), using stainless-steel or aluminium pans, respectively, with the corresponding buffer in the reference cell. Samples were first maintained at 25 or 35 ºC for 1 min to equilibrate, and then subjected to a heating ramp to reach 100 ºC at a scan rate of 3 ºC min^{-1}. If a second scan was performed to check the irreversibility of the reactions, a cooling step at 50 ºC min^{-1} to 25 ºC, an isothermal step at 25 ºC for 1 min and a second scan from 25 to 100 ºC was applied after the first scan.

DSC thermograms were edited with Origin v7.0 (OriginLab Corporation, Northampton, MA) and the following parameters were calculated at least in triplicate: T_p, defined as the peak temperature at maximum heat flow or shoulders within the range of interest, and ΔH, as the enthalpy change involved in the overall

heat-induced reactions within the protein molecules, determined from the area between the curve and a straight line drawn from the beginning to the end of the transition (Relkin, 1994).

Protein-protein interactions in mixtures were investigated by comparison of experimental and theoretical data, in terms of differences between both. Theoretical DSC curves were constructed from the sum of the thermograms of single fractions, multiplied by the proportion of each fraction present in the protein mixture being analysed.

Deconvolution of thermograms

Thermograms were deconvoluted to separate the contribution of each fraction within the protein mixtures. The DSC curves of single fractions gave an insight into the temperature ranges within which they were located and, with the aid of the second derivative, these features were confirmed. The number of inflection points in the second derivatives of DSC curves was used to set the number and placement of peaks to be fitted during the deconvolution process. Thermograms were smoothed with the Savitsky-Golay 5-point algorithm and deconvoluted using a nonlinear least-squares curve-fitting subroutine with Gaussian type functions with 50 iterations to converge. These calculations were performed using Origin v7.0 with the Peakfitting module (OriginLab Corporation, Northampton, MA).The enthalpy of deconvoluted peaks was calculated as the relative area below fitted curves in relation to the total area of the studied thermogram, expressed as J g^{-1}. The average coefficient of variation for the fitted peaks determinations did not exceed 10% for enthalpy and 0.9% for T_p.

GELATION PROPERTIES

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoretic solutions contained 50 μL of protein solution in 950 μL of TRIS/EDTA buffer with sodium dodecyl sulphate (SDS) for non-reducing conditions. For reducing conditions, 2% β-mercapto-ethanol was added. Molecular weights were determined using molecular mass standards (LMW marker: 14.4-97 kDa, Amersham Biosciences, Uppsala, Sweden). All experiments were performed in a Phastsystem apparatus (Pharmacia Biotech, Orsay, France) using the running conditions described in Table 15. The SDS-PAGE electrophoreses were carried out in 8-25% acrylamide gradients gels (Amersham Biosciences), with SDS buffer strips (Amersham Biosciences) placed at the electrodes. Gels were stained with Coomassie blue (PhastSystem, 1990). Firstly, gels were submerged in the staining solution[7] for 8 min at 50 ºC. After that,

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[7] Methanol, 30%; Acetic acid, 10%; Distilled water, 60%; Coomassie blue, 0.1%

three consecutive washes were carried out for 5, 10 and 8 min at 50 °C with the destaining solution[8]. Finally, SDS-PAGE gels were poured with the preservation solution[9] for 5 min at 50 °C.

Table 15. Running conditions of SDS-PAGE electrophoreses.

Step	Conditions	T (°C)	Duration (Vh)
Preelectrophoresis	250 V - 10 mA - 3 W	15	1
Aplication	250 V - 1 mA - 3 W	15	1
Separation	250 V - 10 mA - 3 W	15	60

SDS-PAGE bands analysis

Protein bands in electrophoretic gels were analysed using Image Scanner and Image Master Software (Pharmacia Biotech). Bands corresponding to individual proteins were identified according to the typical plasma protein profile shown in Figure 11. Since protein bands intensity faded as total protein content diminished, aggregation phenomena could be monitored through the decrease in the bands intensity in relation to a fresh sample which acted as reference. Protein aggregates were identified in non-reducing SDS-PAGE and type and percentage of aggregated proteins were both determined in reducing SDS-PAGE.

Texture profile analysis (TPA)

According to the protocol detailed in page 29, protein solutions were poured into cylindrical plastic membranes and submerged in a water bath at 80 °C for 45 min to provoke the gelation of samples. Gels were cut into cylindrical portions and texture profile analysis (TPA) was determined by a two-cycle uniaxial compression test with a TA-XT2 texturometer (Stable Micro Systems Ltd., Surrey, UK). As displayed in Figure 12, hardness, measured in N, was determined at the maximum force applied to the gels during the first cycle of compression (S_{max}). Springiness, adimensional value that refers to the capacity of recovering the initial shape after a compression, was calculated as the quotient of the times needed to reach the maximum compression from the start of each cycle (t_2/t_1). Cohesiveness, adimensional, corresponds to the compression work, was calculated as the proportion of the area between the second and the first cycle (A_2/A_1).

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[8] Methanol, 30%; Acetic acid, 10%; Distilled water, 60%

[9] Glycerol, 10%; Acetic acid, 10%; Distilled water, 80%
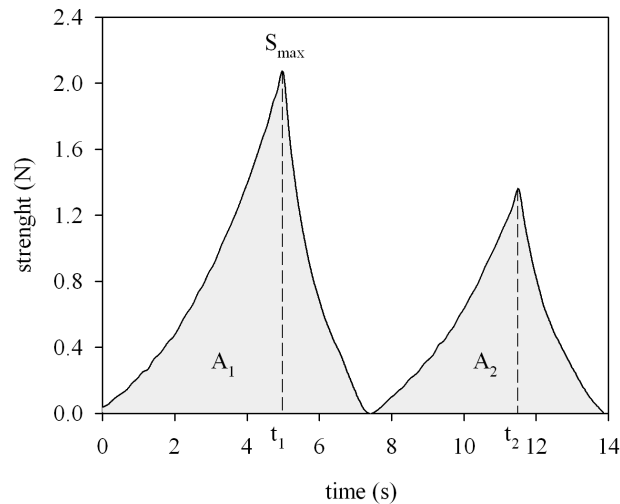
Figure 12. Typical two-cycle compression plot of a plasma protein gel.

Water-holding capacity (WHC)

The water-holding capacity of gels was determined with a technique combining filtration and centrifugation, further described in page 30. Gel samples were cut into cylindrical portions and were placed into hollow containers with a polyester net fixed at the bottom, depicted in Figure 13. These plastic containers were suspended inside centrifuge tubes and they were centrifuged. The water released during the centrifugation step was collected and the results were reported as the percentage of weight of released water in relation to the sample weight.
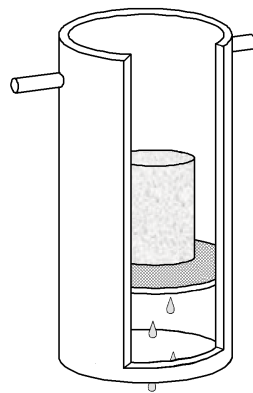
Figure 13. Illustration of the gadget used in water-holding capacity measurements.

Rheological measurements

Dynamic viscoelastic measurements to study the gelation of plasma protein solutions were carried out on a Rheometric Fluids Spectrometer RFSII (Rheometrics Scientific, Piscataway, NJ) fitted with coaxial cylinders (R1/R2=0.97) and thermostatically controlled. Temperature sweeps at the maximum speed permitted for the instrument (~2.5 ºC min^{-1}) were recorded from room temperature to 85 ºC. Samples were covered with mineral oil to prevent evaporation during heating. The oscillatory strain was set at 5%, to keep the measures within the domain of linearity, and the oscillation frequency was 1 rad s^{-1}. Evolution of elastic (G') and viscous (G'') moduli and phase shift (δ) were recorded.

FT-Raman spectroscopy

FT-Raman spectroscopy was used to monitor structural characteristics of fresh protein solutions and gels at a molecular level. Spectra were recorded on a Perkin Elmer 2000 FT-Raman spectrometer (Beaconsfield, Buckinghamshire, UK), with a spectral resolution set at 4 cm^{-1} and laser power at 1437 mW. The frequency calibration of the instrument was undertaken using the sulphur line at 217 cm^{-1}. All the data presented were based on 128 co-added spectra. The original spectra in the 100-3500 cm^{-1} region were baseline corrected and normalised using the phenylalanine peak near 1005 cm^{-1} (Howell et al., 2001) with Origin v7.0 software. The tentative assignment of the major bands in the spectra to vibrational motions of various side chains or peptide backbone was based on comparison to FT-Raman spectra reported in the literature (Nonaka et al., 1993; Li-Chan, 1996; Howell et al., 2001; Ngarize et al., 2004a). Table 16 presents the corresponding tentative assignments.

A detailed analysis of the amide I region (1600-1700 cm^{-1}) was performed with the Peakfitting module to calculate the secondary structure components of each sample. Spectral data within this region was smoothed with the Savitsky-Golay 5-point algorithm and deconvoluted using a nonlinear least-squares curve-fitting subroutine with Gaussian-type functions, using the assignment of secondary structure components reported in the literature (Susi et al., 1967; Susi and Byler, 1988; Dong et al., 1990; Dong and Caughey, 1994; Vanstokkum et al., 1995; Dong et al., 2000; Ngarize et al., 2004b). The percentage of each component (α-helix, β-sheet, β-turns and random coil) was determined as the corresponding fitting peak area in relation to the area of all the fitted range. Peak intensities and secondary structure components were expressed as the average of the replicate spectra, with a typical coefficient of variation of 10%.

Interactions in protein mixtures were investigated by comparison of experimental and theoretical data. Theoretical FT-Raman spectra for protein mixtures were constructed from the sum of single fractions, multiplied by the proportion of each fraction present in the mixture being analysed. Protein-protein interactions were described according to the differences between calculated and measured data.

Table 16. Tentative assignments of major bands in the FT-Raman spectra of plasma proteins.

Band assignment	wavenumber (cm^{-1})
S-S stretching	510
Tryptophan	760
Tyrosine doublet	850/830
Amide III' (α-helix)	940
Amide III' (random coil)	960
Phenylalanine	1034
CN or CH stretching	1060
CN stretching	1130
Amide III (β-sheet)	1240
Amide III (α-helix)	1320
CH deformation, Tryptophan	1345
COO$^-$ Aspartic, Glutamic acids	1425
CH$_2$ or CH$_3$ bend	1450
Amide I	1600-1700
CH$_3$ symmetrical and R$_3$–CH stretching (aliphatic amino acids)	2880
CH stretching (aromatic, aliphatic, charged amino acids)	2930
=CH stretching (aromatic amino acids)	3060

MICROSTRUCTURAL PROPERTIES

Scanning electron microscopy (SEM)

Plasma gels obtained as described above were cut into slices of 3x1x1 mm and were fixed for 3.5 h at 4 ºC in a buffer containing 2.5% glutaraldehyde, 2% paraglutaraldehyde, 0.1 M cacodylate at pH 7.4. Fixed samples were rinsed twice in 0.1 M cacodylate buffer for 10 min at room temperature and were maintained in the same buffer overnight at 4 ºC prior to the dehydration step. Samples were dehydrated in a graded series of ethanol (50%, 75%, 90% and 3x100%) for 20 min/step before proceeding to critical point drying with an Emitech K850 machine (EM Integrated Technology, Kent, UK). Dried samples were coated with gold in an Emitech K550 sputter coater. Protein gels were examined with a Zeiss DSM960A scanning electron microscope (Carl Zeiss, Electric Optics Division, Oberkochen, Germany) at an accelerating voltage of 15 kV.

Gels were focused on different magnification levels, from 500 to 30 000x. An intermediate zoom at 10 000x was selected for the description of the microstructure, because it exposed the aggregates of the protein network in detail. Figure 14 shows a zoomed sequence of a gel, keeping the focused area. It can be observed that the 10 000x magnification rendered the best results.

Treatment of SEM images

The SEM micrographs were analyzed with the public domain software ImageJ v1.34n (Rasband, 2005) and the FracLac v2.0a plugin for ImageJ (Karperien, 2004). Micrographs are 256 grey level pictures of 1020x970 pixels, which are defined by three dimensions: two spatial coordinates, representing its position on the plane, and its brightness value (0-255). If the grey level is considered a third spatial coordinate, images can be presented as 3D objects, being high values of Z the white-tending pixels and low values the dark-tending ones. This representation is displayed Figure 15A.
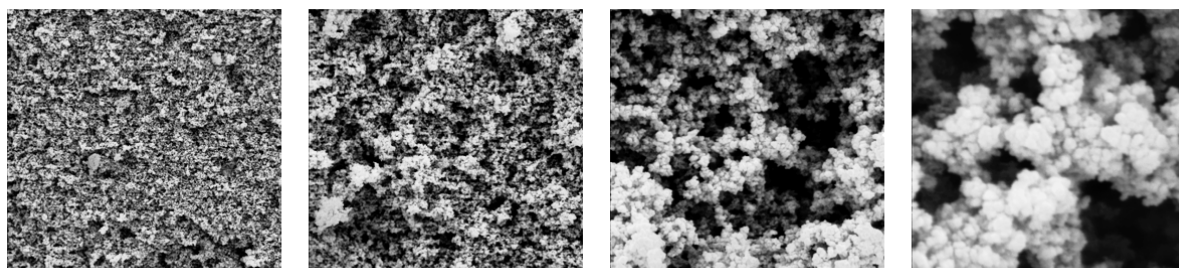
Figure 14. From left to right, scanning electron microscopy micrographs of 7% plasma protein gels at a magnification level of 500x, 2000x, 10 000x and 30 000x.

Micrographs have to be transformed into binary images prior to microstructural measurements such as fractal dimension (D_f) and lacunarity (A_ε). The process of binarization transforms the image into black/white through forcing pixels to fall into these two categories after thresholding. The election of the binarization threshold can be viewed as setting a plane that cuts the Z-axis (Figure 15B). All the points above the plane will be considered protein aggregates (Figure 15C), consequently they will be transformed into white pixels (Figure 15D). On the contrary, points below the plane will be considered empty spaces, and such cavities will be transformed into black pixels.
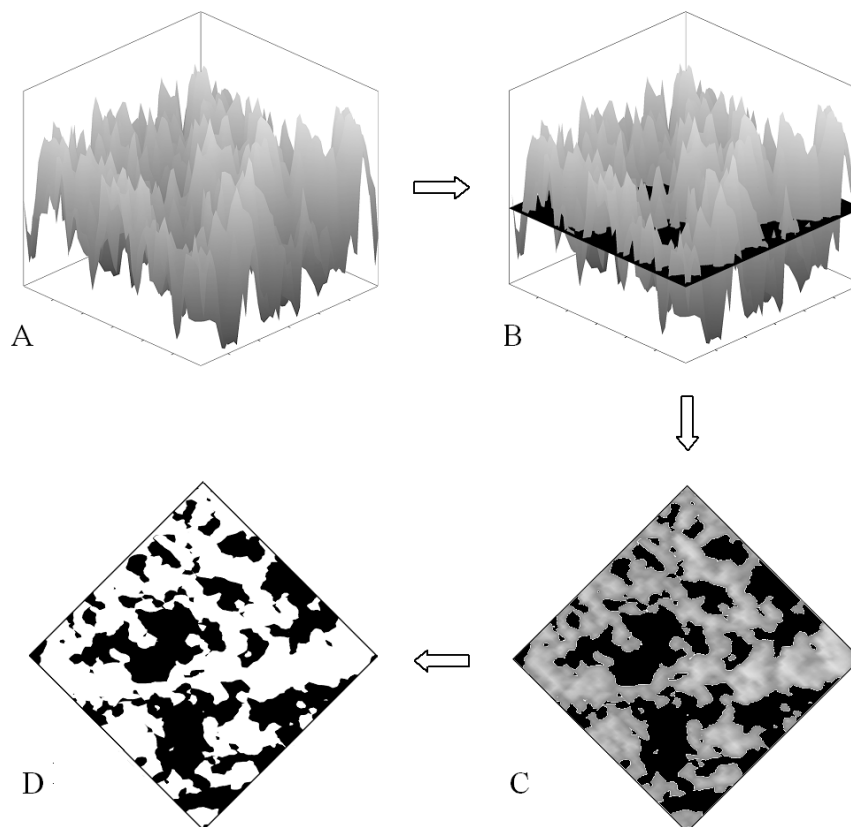
Figure 15. Binarization process of SEM micrographs of plasma protein gels.

Two different thresholds were used depending on the measure. The grey level corresponding to the median of the grey level histogram was chosen for D_f calculation. The grey level at which thresholding the image resulted in the largest number of cavities was used for A_ε determination. In the latter case, it must be noted that the number of cavities of a binarized image increases with the grey level used for thresholding, up to a value where the size of the cavities is large enough to coalesce with other neighbour cavities, thus resulting in a drop of the number of total cavities from that threshold on.

Determination of the fractal dimension (D_f)

The fractal dimension of plasma protein gels was calculated by means of two approaches. Firstly, a rheological method was applied to verify the nature of protein aggregates as a mass-fractal. Afterwards, image analysis was used to determine D_f values.

Rheological fractal model

The scaling model of Wu and Morbidelli (2001), which relates microstructural parameters of colloidal gels to their macroscopic elastic properties, was used. The elasticity of gels (E) and their limit of linearity (γ_0) are proportional to a power of particle concentration (φ), according to Equations 2 and 3. As described by Hagiwara et al. (1998), values of E were determined as the slope from the linear part of the stress-strain curve at strain values below 5%. Values of γ_0 were considered as the strain at which the ordinate of the stress curve showed a 5% deviation from the elasticity line extrapolation, following Equation 4.

The exponents assigned to the particle concentration (A and B) were calculated with Equations 5-7, where the parameter x represents the backbone fractal dimension or tortuosity of the network (typically comprised between 1 and 1.3 for colloidal gels), and the parameter α accounts for the three possible gelation regimes: strong-link ($\alpha = 0$), weak-link ($\alpha = 1$) and transition gels ($0 < \alpha < 1$).

Equation 2	$E \propto \varphi^A$
Equation 3	$\gamma_0 \propto \varphi^B$
Equation 4	$(\sigma - E)/ E = 0.05$
Equation 5	$A = \beta/(3 - D_f)$
Equation 6	$B = (2 - \beta)/(3 - D_f)$
Equation 7	$\beta = 1 + (2 + x)(1 - \alpha)$

Image analysis

D_f values of the protein aggregates were calculated using the box counting method (Kaye, 1989). The basics of the box counting method are as follows: firstly, there is an iteration process of placing a grid of decreasing size over an image; then, the number of boxes in the grid containing pixels –assigned to protein aggregates– is counted for each grid size. Then, the D_f is based on the calculation of the scaling rule found in the counts, given by Equation 8, where N_ε is the number of boxes in the grid at a certain scale containing pixels and ε is the corresponding scale. Since the determination of D_f by image analysis is based on a two-

dimensional space, that is, the picture itself, it is necessary to add an extra dimension to the calculated D value to actually represent the three-dimensional feature of the protein gel, as shown in Equation 9.

Equation 8 $\qquad\qquad\qquad\qquad\qquad\qquad D = -\log N_\varepsilon/\log \varepsilon$

Equation 9 $\qquad\qquad\qquad\qquad\qquad\qquad D_f = D + 1$

Determination of lacunarity (A_ε)

Lacunarity (A_ε) was calculated as the variation in the number of pixels at each grid placed in the image during a standard box counting, according to Equation 10. Where A_ε is the lacunarity for the grid of size ε. A_ε depends on the square of the pixels coefficient of variation, being σ the standard deviation of the pixels count among all boxes of size ε, and μ the average number of pixels for the same grid size.

Equation 10 $\qquad\qquad\qquad\qquad\qquad\qquad A_\varepsilon = (\sigma/\mu)^2$

Spatial variability

Spatial variability of SEM images was modelled by an omni-directional semivariogram included in the package S+SpatialStats for S-Plus 6.0 (Insightful Corp., Seattle, WA). The spatial autocorrelation pattern was adjusted with the function detailed in Equation 11, where half the variance (γ) at different distances corresponds to the average squared difference between the grey level values of any two pixels i, j that are h distance apart, being N(h) the number of pairs of such sites. In order to calculate the range of the variogram (R_0), that is, the cut-off distance of the autocorrelation plot, a graph of γ vs. log (h) was constructed. A regression line was fitted in the linear part of the graph, and R_0 was determined as the distance at which the γ vs. log (h) curve showed a 5% deviation from the calculated regression line.

Equation 11 $\qquad\qquad\qquad\qquad 2\gamma(\mathrm{h}) = \dfrac{1}{N(\mathrm{h})} \sum_{N(\mathrm{h})} \left[z(s_i) - z(s_j)\right]^2$

SURFACE PROPERTIES

Protein solubility

The solubility of plasma protein fractions was determined with the method described by Morr et al. (1985). Aliquots of 50 mL of protein solutions were centrifuged at 20 000 g for 30 min at 15–20°C, and protein solubility was calculated as the percentage of soluble protein in the supernatant relative to the total protein content in the sample. Nitrogen determinations were carried out with the Kjeldahl procedure (AOAC, 1995).

Foaming properties

Samples (200 mL) of protein solutions were whipped in a Braun Multimix M700 mixer (Braun Española S.A., Barcelona, Spain) with two whisks (Ø = 5 cm) at 1000 rev min^{-1} for 10 min. Samples were placed on a rotational plate during whipping in order to form a homogeneous foam. Afterwards, the foaming capacity (FC) was determined as the volume (mL) of foam after 2 min at rest. The foaming stability (FS) was determined using the gravimetric method that follows; a measured quantity of foam was placed in a dry stainless steel sieve to let the drainage of the released liquid, and the remaining foam was weighted every 10 min. Results were expressed as weight percentage of initial foam.

Emulsifying properties

Samples (150 mL) of protein solutions were mixed with 50 mL of commercial corn oil. The mixtures were homogenised using a hand-operated laboratory piston-type homogeniser (MFC Microfluidizer™ Series 5000, Microfluidics Corporation, Newton, MA, USA) with recirculation, at a flow rate of 40 L h^{-1} and a homogenisation pressure of 12 MPa, at 25 ºC. Emulsions were collected after 1.5 min of processing.

Aliquots of fresh emulsions and after 10 min at rest were diluted 2500-fold with 0.1% SDS. The absorbance of the diluted emulsions was determined at a wavelength of 500 nm in a Cecil CE 1021 UV-visible spectrophotometer (Cecil Instruments, Cambridge, UK). For each solution, results were reported as Emulsifying Activity Index (EAI) according to Equation 12 and as Emulsion Stability Index (ESI) according to Equation 13 (Pearce and Kinsella, 1978):

Equation 12 $$EAI = 2T/(\Phi \times C)$$

Equation 13 $$ESI = (T/\Delta T) \times \Delta t$$

Where T is Abs_{500}, Φ is the volume fraction of the dispersed phase (0.25), C is the weight of protein

per unit of volume of aqueous phase before the emulsion is formed, and ΔT is the change in Abs_{500} during Δt (between t=0 and t=10 min).

Determination of protein-protein interactions

Interactions between proteins were investigated with the Interaction Index proposed by Howell and Lawrie (1984a). According to Equation 14, I_k is the interaction index for the property k, M_{exp} is the experimental measured value and M_{teor} is a theoretical calculated value, derived from the sum of the contributions of single fractions measured in isolation, in proportion to the concentration of each in the mixture.

Equation 14
$$I_k = 100x\frac{(M_{exp} - M_{teor})}{M_{teor}}$$

DATA PROCESSING

Descriptive and statistical data analyses were calculated using SPSS v13.0 for windows (SPSS Inc, Chicago, IL). The normal data distribution was verified by the Kolmogorov-Smirnov procedure, and the homogeneity of variances was confirmed by the Levene test. One-way ANOVA and pairwise post hoc tests (Tukey and Fisher's Least Square Difference) were calculated with a significance level of $p < 0.05$. As well, a general linear model (GLM) with repeated measures was used for the analysis of measurements along time or space (like foam stability and lacunarity curves). Nonparametric bivariate correlations were determined for pairs of measured parameters by means of the Spearman's rho. Principal component analysis (PCA) was performed to reduce the dimensionality of large sets of data. The extraction method used was the correlations matrix and an orthogonal rotation with the Varimax method was included. Eigenvalues and variability gathered by principal components (PC) were calculated and sample scores in the first four PC were selected.

RESULTS AND DISCUSSION

The results obtained in the functional experiments are presented in this chapter as the following articles:

- **Heat-induced gelation of porcine blood plasma proteins as affected by pH**
  Eduard Dàvila, Dolors Parés, Gérard Cuvelier and Perla Relkin
  Meat Science, in press

- **Fourier Transform Raman spectroscopy study of heat-induced gelation of plasma proteins as influenced by pH**
  Eduard Dàvila, Dolors Parés and Nazlin K. Howell
  Journal of Agricultural and Food Chemistry, 2006, 54(20), 7890-7897

- **Studies on plasma protein interactions in heat-induced gels by differential scanning calorimetry and FT-Raman spectroscopy**
  Eduard Dàvila, Dolors Parés and Nazlin K. Howell
  Food Hydrocolloids, in press

- **Structure of heat-induced plasma protein gels studied by fractal and lacunarity analysis**
  Eduard Dàvila and Dolors Parés
  Food Hydrocolloids, 2007, 21(2), 147-153

- **Characterization of plasma protein gels by means of image analysis**
  Eduard Dàvila, Mònica Toldrà, Elena Saguer, Carmen Carretero and Dolors Parés
  Lebensmittel Wissenschaft und Technologie, in press

- **Surface functional properties of blood plasma protein fractions**
  Eduard Dàvila, Elena Saguer, Mònica Toldrà, Carmen Carretero and Dolors Parés
  European Food Research and Technology, in press

After these articles, a general discussion follows, which includes comments about the six papers and some features that have been not included there.

GENERAL DISCUSSION

Functional properties measurements

The functionality of plasma proteins was studied in terms of the most important attributes they possess, that is to say: gelation, surface and hydration properties. These determinations were carried out as a function of pH, choosing three conditions that could reflect the range of pH typically found in foods, from highly-acidified to neutral products. A summary of the previously exposed functionality results is presented in Table 17, where the differences among fractions and pH conditions can be observed along columns and rows, respectively.

Solubility measurements (S) showed that albumin remained 100% soluble in all conditions. Globulins were slightly less soluble, with a minimum at pH 6.0, which coincided with the average of their range of isoelectric points. Plasma and serum displayed similar features, both having a lower solubility than the former two fractions.

The emulsion activity indexes (EAI) of all fractions were quite alike, mostly comprised between 500 and 550 $m^2\ g^{-1}$. The highest value was registered for albumin at pH 6.0, whereas plasma at pH 7.5 showed the lowest one. The emulsion stability index (ESI) displayed disparate results depending on the fraction and the pH; albumin increased near 20-fold its lowest value as pH moved from 4.5 to 7.5, and the stability of globulins remained similar at pH 4.5 and 6.0 but boosted at pH 7.5.

The largest foaming capacity (FC) was observed in albumin samples. Serum and plasma performed likewise, although their FC was kept slightly below albumin. Globulins had a good FC at pH 4.5, which was reduced in half at pH 6.0 and 7.5. The foaming stability (FS) of all fractions was similar. In general, foams were more unstable at pH 7.5; both albumin and plasma samples at acidic conditions showed the most stable foams.

The hardness of protein gels also revealed a disparate behaviour among fractions and pH conditions. Albumin gels were the softest ones, even at pH 6.0 it was not possible to carry out textural measurements because gels were too crumbly. Whereas serum and plasma gels were tougher as pH increased –this effect was much more evident for plasma samples–, globulins performed alike for all pH conditions. Both the springiness and cohesiveness were quite similar for all the samples. Conversely, this was not the case for the water-holding capacity (WHC); albumin gels were the ones that released the least amount of water, followed by plasma gels. In general, the best results were achieved at pH 7.5.

Plasma proteins have as a whole a good functionality profile, which, in some cases, is subjected to the fraction studied and to the pH conditions.

Table 17. Averaged results of the functional properties of plasma proteins as a function of pH (see Table 18 for further details).

Property	pH	Sample			
		Albumin	Globulins	Serum	Plasma
Solubility (%)	4.5	99.89	91.70	86.84	86.83
	6.0	100.00	83.06	84.63	85.70
	7.5	100.00	96.41	91.09	92.36
EAI ($m^2 g^{-1}$)	4.5	519.56	532.08	552.03	521.55
	6.0	633.02	554.51	571.60	535.56
	7.5	506.81	560.03	511.80	441.58
ESI (min)	4.5	39.92	97.93	88.29	60.02
	6.0	240.92	96.25	206.83	87.42
	7.5	717.61	611.00	145.42	135.54
FC (cm^3)	4.5	604.76	560.25	515.74	589.05
	6.0	602.14	298.45	589.05	583.81
	7.5	604.76	243.47	568.10	555.01
FS (%min)[a]	4.5	3263	3086	2997	3416
	6.0	3353	2638	2625	3397
	7.5	2887	2721	2477	2554
Hardness (N)	4.5	0.291	1.090	0.799	0.487
	6.0	-	1.074	0.966	0.757
	7.5	0.452	1.123	1.110	1.267
Springiness	4.5	0.807	0.926	0.900	0.785
	6.0	-	0.932	0.905	0.896
	7.5	0.885	0.925	0.935	0.934
Cohesiveness	4.5	0.480	0.528	0.497	0.488
	6.0	-	0.528	0.528	0.526
	7.5	0.482	0.525	0.533	0.545
WHC (%)	4.5	54.77	57.29	60.18	61.98
	6.0	-	62.95	58.79	57.32
	7.5	36.28	62.40	55.14	41.64

[e] Calculated by integration and expressed as the total area below foam stability curves (larger areas mean higher stabilities).

Interactions between protein fractions

As previously described, plasma is a natural-occurring mixture that contains a rich variety of proteins, with different chemical and structural properties. The diversity of amino acid composition, molecular sizes, electrical charges, etc., means that each protein fraction does not have to replicate whole plasma properties; they may instead display their own functionality profile, which can be widely different. In addition, cooperative effects between fractions when they are mixed are likely to promote protein-protein interactions, leading to synergistic or worsening phenomena. The study of such interactions constitutes a research line towards the development of isolates-based products which, on the basis of fractions' functionality, would broaden and improve food applications of plasma proteins. This kind of research has already been conducted on whey proteins, and interactions between albumin, α-lactalbumin, β-lactoglobulin and lysozyme have been described and proposed for the improvement of some properties, such as gelation (Poole et al., 1984; Matsudomi et al., 1991; Howell, 1992; Hines et al., 1993; Matsudomi et al., 1993, 1994; Howell, 1995; Gezimati et al., 1996).

In the present Chapter, the cooperative effects between fractions have been elucidated through the determination of interaction indexes. Experimental results were compared with the theoretical assumption that no interactivity was taking place during the functionality measurements, and interaction values for mixtures were derived. Table 18 summarizes the interaction indexes obtained in the experiments. The results therein presented can be interpreted as the percentage of the variance that should be assigned to protein-protein interactions. Therefore, positive values reveal synergistic effects, whereas the negative ones involve a reduction in the functionality (except for WHC, which is expressed as percentage of released water and positive values mean larger amounts of liberated water, that is a lower ability to retain water).

The co-occurrence of albumin and globulins entailed a slight reduction in solubility although it has been described that the presence of albumin prevents the spontaneous aggregation of immunoglobulins at room temperature (Soltis and Hasz, 1982) –what was evident because serum solutions were clear and globulins were turbid. Large interactive effects were observed in the stability of emulsions, because serum showed better results at acidic conditions (pH 4.5 and 6.0) and it did not show the good properties of isolated fractions at pH 7.5. The opposite effect was observed in the foaming capacity of samples, and a general decrease was observed in the stability of foams when both fractions were present. The most important interactive effects were observed in the textural properties of gels, especially at pH 6.0, where serum performed alike globulins even though albumin alone formed a pasty mass instead of firm gels.

The presence of fibrinogen did not represent a significant reduction in the solubility of plasma. This protein is highly insoluble and, even though the formation of fine strands could be observed during the solubilisation of plasma due to the self-aggregation of fibrinogen, these aggregates did not constitute a relevant loss of soluble protein because fibrinogen is found at a low concentration (~3%). The elimination of fibrinogen caused an enhancement in the emulsifying properties, especially concerning the stability of

emulsions. In relation to foaming properties, the presence of fibrinogen entailed an increase in the foaming capacity at pH 4.5 and more stable foams at both pH 4.5 and 6.0. This protein also affected the gelation properties of plasma, making it more sensitive to pH conditions: whereas it favoured the formation of the strongest gels at pH 7.5, softer gels were obtained at acidic conditions as compared to serum, as it can be observed in Table 17. In addition, the WHC of plasma at pH 7.5 was enhanced in relation to serum. There-fore, the presence of fibrinogen entails positive effects on the gelation properties at pH 7.5, and negative effects at acidic conditions.

Table 18. Summarized interaction indexes of albumin, globulins and fibrinogen for func-tional properties as a function of pH. Interactions between albumin and globulins were cal-culated from serum samples, whereas the interactivity of fibrinogen was deduced from plasma measurements [S (solubility), FC (foaming capacity), FS (foaming stability), EAI (emulsion activity index), ESI (emulsion stability index), WHC (water-holding capacity)].

| Property | Interaction index $(I_k)^a$ | | | | | |
| | albumin-globulins[b] | | | fibrinogen-serum[c] | | |
	pH 4.5	pH 6.0[d]	pH 7.5	pH 4.5	pH 6.0	pH 7.5
S (%)	−9.73	−8.39	−7.41	+0.01	+1.26	+1.39
EAI ($m^2 \, g^{-1}$)	+5.11	−4.37	−3.57	−5.52	−6.31	−13.72
ESI (min)	+33.72	+17.64	−78.28	−32.02	−57.73	−6.79
FC (cm^3)	−11.80	+26.55	+28.48	+14.21	−0.89	−2.30
FS (%min)	−5.85	−13.40	−11.92	+13.98	+29.41	+3.11
Hardness (N)	+22.80	+99.88	+47.20	−39.05	−21.64	+14.14
Springiness	+4.58	+115.78	+3.54	−12.78	−0.99	−0.11
Cohesiveness	−0.87	+122.22	+6.31	−1.81	−0.35	+2.36
WHC (%)[e]	+8.87	−29.44	+14.79	+2.99	−2.50	−24.48

[a] Calculated as $I_k = 100 \times (M_{exp} - M_{teor})/M_{teor}$, where M_{exp} is the experimental measured value and M_{teor} is the value derived from theoretical assumptions.

[b] Interaction index of albumin and globulins, comparing mixture (serum) vs. isolated fractions, assuming a natural proportion in serum of 55% albumin and 45% globulins.

[c] Interaction index of fibrinogen with the rest of the serum proteins, comparing the presence of this protein (plasma) vs. its absence (serum).

[d] Experimental values of albumin were considered 0 for texture attributes and 100% for WHC.

[e] Expressed as percentage of released water, thus a positive value accounts for a lower ability to retain water.

Biochemical aspects

The experiments carried out to study the thermal properties and the behaviour of plasma proteins during heat-induced gelation revealed interesting features of each constituent. It was observed in DSC experiments that globulins were the fraction that showed thermal transitions at the highest temperatures, preceded by albumin and the first transition of fibrinogen. However, when electrophoreses of heated plasma samples were run, it could be observed that the first proteins undergoing aggregation were globulins, while albumin remained in the soluble phase to a larger extent. This feature has also been observed for immunoglobulins and BSA in milk (Calvo et al., 1995; Law and Leaver, 2000), and it may suggest that globulins undergo unfolding and aggregation reactions at a faster rate than albumin, although these thermal transitions start at higher temperatures. In contrast, it may take longer for albumin to develop larger aggregates, because this single protein may form soluble aggregates before developing a protein network. As observed in rheology measurements, the early increase in the storage modulus of albumin samples may reflect the raise in viscosity due to the formation of soluble aggregates as evidenced in electrophoreses. For these reason, it would be interesting to carry out rheological studies of globulins alone during heating. They would probably reflect an increase of G' at slightly higher temperatures than albumin, yet absolute values of G' would achieve larger values due to the formation of the protein network more rapidly. In spite of the ability of albumin to prevent the aggregation of globulins in solution previously mentioned this protein may promote the aggregation between globulins during heating, thus accounting for the faster aggregation observed in electrophoreses. As described by Hines et al. (1993), mixing BSA and β-lactoglobulin increased the aggregation rate of the latter during heating, whereas the rates of BSA remained similar. This effect could also be occurring in plasma and serum, and the fact that the denaturing enthalpies of both samples were larger than those of albumin and globulins alone may reflect a change in the energy of the aggregation processes due to this effect.

When plasma and serum samples were run in DSC experiments, at a protein concentration where denaturation and aggregation processes of the major constituents are both superimposed (Relkin, 1996), a relevant effect of the presence of fibrinogen was observed. From the comparison of theoretically constructed DSC curves –from those of single fractions– a specific interaction between fibrinogen and albumin was deduced. Most likely, the unfolded molecules of fibrinogen initiate cross-linking reactions with the exposing sites of albumin molecules from 60 ºC onwards. This behaviour has also been described for mixtures of α-lactalbumin and BSA (Havea et al., 2000), and α-lactalbumin and β-lactoglobulin (Boye and Alli, 2000). It has been therefore concluded that when thermal transitions of different proteins are overlapped and reactive sites –such as free thiol groups– are available, interactions between constituents may happen. In this case, although FT-Raman spectra showed that changes in the disulphide region were responsible for such interaction, it remains unclear which is the specific reaction that binds fibrinogen with albumin. The former has 29 disulphide bonds with no free groups to form covalent unions, and the latter has 17 disulfide bridges and one free thiol; even so, changes in the environment of S-S groups are somehow involved.

The physical properties of the final gels varied considerably, and this was a consequence of the described interactions between proteins. Albumin gels were the weakest, probably because this single fraction requires either higher temperatures or longer holding times to form stronger gels. As pointed out elsewhere (Howell et al., 1984a; Foegeding et al., 1986; Matsudomi et al., 1991), some proteins, including plasma proteins, develop stronger gels when the thermal treatment increases within a temperature range. Conversely, globulins formed strong gels at the assayed conditions, probably because of the ability mentioned before to aggregate more rapidly. The results of TPA of serum and plasma gels also showed the interactions between globulins and albumin, and fibrinogen.

The study of these interactions at a molecular level pointed out that both covalent bonds and hydrophobic interactions play a key role in the development of the protein network. However, the specific reactions and the relative contribution of each type of union to the macroscopic properties are not completely clear. For example, an increase of the disulphide bonds in FT-Raman spectra has been reported in mixtures of β-lactoglobulin and lysozyme and a decrease in mixtures of α-lactalbumin and β-lactoglobulin (Howell et al., 1996; Li-Chan, 1996); it is obvious that disulphide bonds proposed changes in both mixtures but, like the results presented for plasma proteins, further chemical analyses would be necessary to specify the number of oxidation or exchange reactions that take place, for example by using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). In order to elucidate the relative contribution of intermolecular reactions it would also be interesting to perform some research concerning the variation of gels hardness with the presence of SDS, urea, dithiothreitol (DTT), N-ethylmaleimide (NEM) and mercaptoethanol (ME) (Matsudomi et al., 1994; Relkin, 1998). Then again, changes in the secondary structure, which also play a key role in the development of gels (Boye et al., 1996), were well established by FT-Raman spectroscopy, and differences between fractions were found.

By considering all the above mentioned aspects, a model for the gelation of plasma proteins can be proposed. This is presented in Figure 16, where all the steps involved in the heat-induced gelation are depicted.

Firstly, all the protein constituents are found in solution, in their native forms. Section 16A illustrates this situation, with all the fractions maintained roughly at the proportion they are found within plasma. When samples are heated and the temperature reaches 55 ºC, fibrinogen molecules start unfolding (16B). As temperature increases, albumin molecules begin to unfold (16C) and interact with the already unfolded fibrinogen (16D); since albumin outweighs fibrinogen by a factor of 50:3, collisions between albumin and fibrinogen are more likely to occur than fibrinogen-fibrinogen impacts, therefore interspecific reactions take place instead of intraspecific. When the temperature increases some globulins start unfolding (16E) but
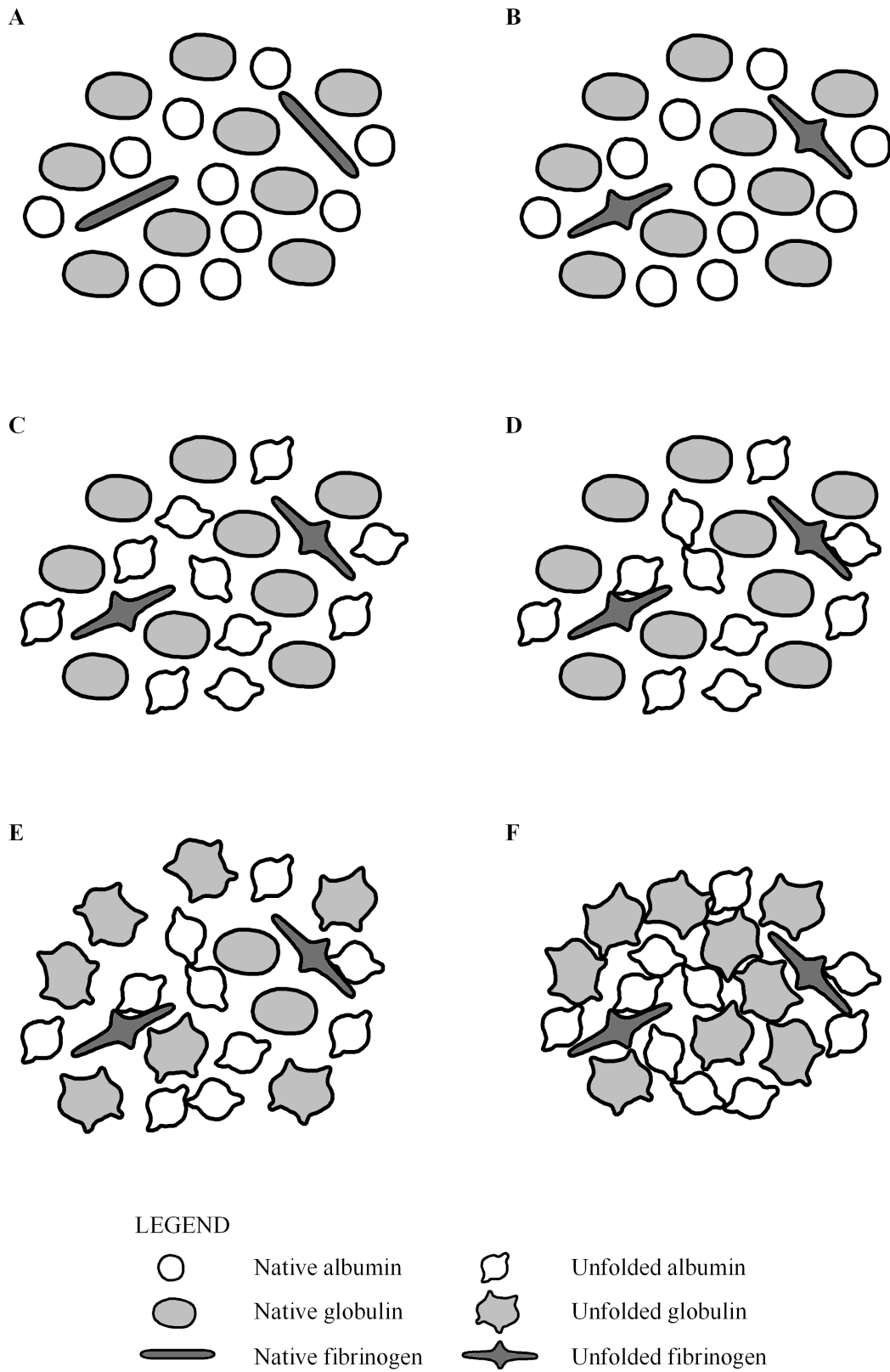
Figure 16. Proposed model for the gelation of plasma proteins. Sections A to F represent the consecutive steps occurring during heating.

as described above, the ability of globulins to form larger aggregates, before albumin begins to form large protein clusters, leads to the development of the protein matrix that forms the final gel network (16F). Afterwards, while gels are held at high temperatures, the creation of new protein-protein unions is allowed, which will contribute to the macroscopic properties of the gels. Of course, the nature of these interactions is subject to the pH conditions because, as previously described, these features vary considerably with pH.

The above presented model is only a speculation, conjectured from the experimental data obtained in the present dissertation. It would be interesting to carry out microstructural studies under the electron microscope in combination with immunochemistry, using antibodies for each protein constituent to corroborate such hypothesis. This way, the microstructure of gels may reveal the manner proteins aggregates pile up, and confirm or not the suggested distribution. This proposal is based on an experiment performed by Comfort et al. (2002), who studied the gelation of mixtures of soya and whey protein isolates and observed phase separation phenomena that explained the macroscopic differences they were measuring in protein gels.

The study of the microstructure of the gels provides relevant information about the gelation properties of proteins. Combined with macroscopic properties and molecular investigations, a deep understanding of the gelation as a whole can be achieved. Hence, the microstructure of gels can be explored by means of image analysis. However, the objectiveness in the discussion of the results that usually comes out from widely used methods, such as textural measurements or spectroscopy, is rarely attained in image analysis.

Most of the works that study the microstructural features of protein gels use visual descriptors to ascertain characteristics or differences between samples, for example, in terms of coarseness or the shape of the aggregates (Langton et al., 1996; Boye et al., 1997; Handa et al., 1998; Kanno et al., 1998; Relkin et al., 1998; Boye et al., 2000; Parés et al., 2000). Some others rely on the concept of the fractal dimension, considered hitherto the only method based on image analysis to describe features quantitatively (Peleg, 1993). In the present dissertation, a detailed investigation of the microstructural properties of plasma gels has been performed. After the confirmation that plasma proteins gels could be considered as mass-fractals, the study of the microstructure was carried out by means of several techniques, namely: fractal dimensionality, spatial variability, and cavities size, counts and heterogeneity (lacunarity). The combination of techniques has permitted a successful and comprehensive description of the plasma protein network, in addition to the changes it undergoes at different pHs. With the development of such a procedure, the gelation properties of single fractions, as well as the gelation of plasma induced by other methods than heating or at different conditions, can be the subjects of further investigations leading to objective and quantitative conclusions.

A few aspects concerning the surface properties can also be pointed out. The protein-air or protein-oil interactivity can be explained by factors including molecular elasticity and hydrophobicity. These, together with the ability to adsorb at the interface, determine properties such as foaming or emulsifying capacity. In the presented study, albumin showed the best results overall, thus confirming that this protein has

interesting attributes in relation to the development and maintenance of two-phase systems. Globulins, on the contrary, showed worse results than those expected from their theoretical hydrophobicity and variety of molecules, most likely due to their tendency to form aggregates rather than to adsorb at the interfaces. On the other hand, fibrinogen contributed positively in the foaming ability of plasma thanks to its high surface-activity (Lee et al., 1974), especially in the stabilisation of foams. Fibrinogen overcomes its low bulk proportion and it is capable of gradually adsorbing at the interface, since albumin and globulins are several-fold less effective competitors. Unfortunately, this property was never observed when the dispersed phase was oil.

The stability of foams and emulsions depend to a large extent on what happens within the continuous phase. The affinity of molecules may lead to the formation of aggregates, and the shift in the concentration equilibrium of monomers consequently promotes the migration of the protein molecules adsorbed at the interface into the continuous phase, thus destabilising the system. The pH conditions play a key role in this behaviour, for they facilitate proteins to mount up when molecules collide if the electrostatic repulsion is reduced. For example, albumin emulsions at pH 4.5 were quite unstable due to the pH conditions, because they favoured protein aggregation, and due to the mechanical work exerted by the homogenisation valve, which caused partial unfolding and accelerated molecular collisions. The outcome of both factors was an increase in the viscosity of the continuous phase that evidenced the formation of albumin aggregates, which left few molecules available to stabilise the emulsion.

Conversely, these phenomena were slightly different in the formation of foams. Probably, the whipping process provided more energy to the system, increasing the unfolding of molecules to a larger extent. These, in turn, may display better adsorption aptitudes other than aggregation reactions.

Development of functional ingredients

The purpose of knowing the functionality of plasma and its protein constituents is the possibility to develop reformulated plasma-based products steered to meet specific food requirements. Currently, one can take advantage of the functional properties of whole plasma and use it, for example, as a thickening agent in non-acid meat products –because it is well-known that plasma has excellent gelling properties. Yet, what if a high-acid meat product needed a gelling agent to develop a particular consistency? Indeed, it should contain larger amounts of plasma in order to compensate the loss of strength caused by the pH reduction; otherwise, changing the ingredient might be recommended.

The role each major constituent plays in the functionality of plasma has been investigated in the present dissertation. With this knowledge, a solution to the above exposed problem could be easily provided. In that case, making use of a serum-based product may satisfactorily meet the exemplified requirement. If a higher viscosity is needed at acidic conditions, the use of globulins alone could solve it. Using this exam-

ple, a wide range of new ingredients could be developed from plasma proteins. On the one hand, ingredients derived from the partial or total elimination of a major constituent, most likely fibrinogen, could be produced. On the other hand, the isolated constituent itself may be commercialized, and combining both, plasma protein engineered blends could be prepared.

The results reported in Table 17 should be followed in order to formulate the functional ingredient, taking into account the property required for the food product of interest. Another example to illustrate this could be the following: there is an industrial demand for a non-acid salad dressing not containing egg products for allergenic reasons; then, a protein ingredient with good emulsifying properties is needed, and plasma proteins are suitable candidates thanks to an excellent functionality/cost ratio. Since albumin and globulin showed good emulsifying activities and have formed stable emulsions, they are known to fit these requirements considering the results obtained in emulsification experiments. Thus, both isolated albumin and globulins could be recommended as the functional ingredient to be used.

All the experiments carried out may certainly be considered just as a screening step. Questions about whether ionic strength or the presence of other macromolecules-such as polysaccharides or fats- affects the functionality results should be asked. Therefore, model food systems should be the scenario to test the functionality of plasma proteins henceforth, to demonstrate in real conditions the worth of this by-product for the food industry.

CONCLUSIONS

- Whole plasma, serum, globulins and albumin samples exhibited distinct thermal and gelation properties that led to the formation of heat-induced gels (80ºC - 45 min) with remarkable differences in textural attributes. The weakest gels were those from albumin which, together with whole plasma, showed a high pH-dependence. Stronger gels in all pH conditions, quite similar to those of serum, were obtained for the globulins fraction.

- A specific interaction between albumin and fibrinogen during heat-induced gelation has been detected in calorimetric measurements. The formation of such complexes entails synergistic effects at pH 7.5 for both textural attributes and water-holding capacity, but has negative effects at acidic conditions.

- FT-Raman spectroscopy confirmed the contribution of both covalent and hydrophobic bonds in the gelation of plasma proteins. Changes in secondary structure components were also detected, resulting in a loss of helix and formation of beta structures. All protein fractions displayed similar characteristics with slight quantitative differences in the intensities of some bands.

- The combined use of several image-based techniques has been successfully used in investigating the microstructure of plasma gels, which act as a mass-fractal. The fractal dimension, spatial variability, and cavities size, counts and lacunarity described quantitatively several features of the protein network, and they may be applied in the study of the microstructure of single protein fractions.

- Most of the protein fractions at varying pH conditions showed good solubility, emulsifying and foaming properties. Albumin was the more soluble fraction (100%), yet the rest of the fractions were at least 85% soluble. All the fractions had similar emulsifying activity, but both albumin and globulins at pH 7.5 formed emulsions several-fold more stable than the rest. All the fractions also displayed similar foaming capacities, except for globulins at pH 7.5. Albumin and plasma developed the most stable foams.

- Protein-protein interactions play a key role in the development of the functional properties, because important interaction indexes have been determined at varying pH conditions. Albumin and globulins interactions affected significantly the stability of emulsions, the foaming capacity and, especially, the textural parameters of gels. The presence of fibrinogen also affected both the stability of emulsions and foams, and some of the textural parameters.

- The formulation of plasma-based functional ingredients, by considering their functionality when mixed at different proportions at different pH conditions, should have a major influence on the use of the formulated product. Taking advantage of this knowledge should allow the production of ingredients intended to meet specific food requirements.

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CONCLUDING REMARKS

CONCLUDING REMARKS

The starting point of the present dissertation was a research project conducted to develop a preservation system for porcine blood from industrial slaughterhouses. The continuation was a second project, the main objective of which was the improvement of the integral use of blood. The motivation that laid behind both research studies was the currently existing problem with (porcine) blood, which involves two aspects: the quality requirements during its collection and the lack of incentives to produce high value-added derivatives.

In the late 70s and 80s, several studies were published to report the interesting attributes of blood fractions in terms of their nutritional values and their functional properties. All these studies were performed on plasma and red blood cells samples with good sanitary conditions, obtained by buying them to a reagents company or in the course of a careful collection. Using such non-deteriorated and microbial risk-free products, all the statements therein concluded –which stated that blood proteins should be used as food ingredients– could be put into practice without any further consideration. Unfortunately, as many of the studies also pointed out, the reality is that most of the blood that is collected at slaughterhouses lacks quality standards, and therefore it may have a negative impact on its functionality or risk consumers' health.

Currently, there exist blood-derived products for human consumption in the market. Safety measures are obviously being applied to some of the collected blood. However, these products account only for a slight amount of the total blood generated in the meat industry. Hence, the problem with the collection and valorisation still remains unsolved for the most part of blood.

During the carrying out of this thesis, several abattoir facilities have been visited. It has been confirmed that, in most cases, blood is collected at the slaughter line by a drainpipe and then it is transported to large collecting tanks. Many drawbacks have been noticed: strange materials contaminate blood because of the non-selective method used, the pumps and elbows of the piping system increase the hemolysis of blood, the size of the tanks favours the sedimentation of solids, and the microbial counts are usually too elevated. A general improve of these facilities should be undertaken in order to collect blood in better conditions. For example, plasma can be separated from blood immediately after the slaughter of animals by using a continuous centrifuge. This measure was observed in one of the abattoirs, and plasma could then be sold separately because this step allowed a better handling of this fraction.

Engineering solutions would help this improvement. Yet, one important aspect that ought to be guaranteed is the absence of potential risks for the consumer and spoilage microorganisms. With respect to this, a simple and low-cost solution may be the inoculation of lactic acid bacteria into blood. The effects of LAB inoculation concurrently with the enrichment with a selective energy source –that is, inulin– have been studied in Chapter I. Although refrigeration temperatures were enough to guarantee the maintenance of

blood quality, as long as it fulfilled good sanitary conditions, the initial quality itself could not be improved. Hence, in those cases were the initial contamination entailed a potential hazard, the presence of LAB was an effective way to control or reduce the growth of undesirable bacteria. Inoculation could be used as a method to prevent, at the very least, spoilage processes in the case of cold-chain breakdown. In addition, the use of complementary techniques, such as high hydrostatic pressure, proved to be helpful thanks to synergistic effects via the hurdle concept and the elimination of the inoculated strain.

Conversely, some work concerning the functionality of plasma has been done in Chapter II. A fundamental knowledge about the development of plasma-based ingredients has been provided by studying the contribution of the major protein constituents in the behaviour of plasma at varying pH conditions, with regard to gelation and surface properties. New functional ingredients may therefore be formulated by taking advantage of the presented insights. Specific food requirements, i.e. the need of a thickening agent at acidic conditions, can be achieved by using plasma proteins but modifying its protein profile or using single fractions.

The results herein presented may help to deal with the problem of treating blood as a residue instead of a by-product, or even, a co-product. The research performed in Chapter I and II, respectively, may have a positive influence in both the above-mentioned facets, namely collection and valorisation, and lend a hand to the use of blood by the food industry.

Future prospects

As it has been previously expressed, new tests may be carried out to make a step forward the end at which this dissertation has come, and to answer some of the raised questions. All these future prospects may be summarised in the following points:

i. The application of the biopreservation of blood at an industrial scale. The presented results have demonstrated that inoculation of lactic acid bacteria improved the microbiological quality of blood and prevented spoilage processes at a laboratory level. In previous studies, the applicability of spray-drying or freeze-drying to preserve lactic acid bacteria strains and obtain industrial cultures was demonstrated. Thus, the participation of the meat industry to put into practice such a biopreservation system *in situ*, that is in the facilities of an industrial slaughterhouse, is the next step to do some progress in the valorisation of blood.

ii. The inhibition mechanisms of the strain PS99. In inhibition tests, it has only been observed the results against undesirable bacteria, but the means through which it inhibits the growth of micro-

organisms are not known. It may be able to synthesize a bacteriocin or another compound with antimicrobial effects, and it would be valuable information to determine it.

iii. The use of model food systems to investigate the functionality of plasma proteins in real conditions. As it has been shown, plasma proteins displayed good functional properties, which confirmed their suitability to develop food ingredients. Like in the case of egg or whey proteins, there are plenty of products to benefit of the use of plasma proteins: meat products, bakery products, etc. Nonetheless, the next step to confirm this fact and develop specific plasma-based formulations should be the functionality tests in model food systems, where other macromolecules, such as fats or polysaccharides, are present and may alter the performance of plasma proteins.

iv. The obtaining of plasma-based ingredients at an industrial scale. Two main questions have to be solved in order to obtain food ingredients from plasma proteins:

iv. I. First, one important aspect concerning their use is the undesirable taste they may give to food products. Fortunately, undesirable taste imparting components of plasma can be removed by ultrafiltration, since aromatic compounds are not retained in this process. Studies about the ultrafiltration of plasma could then be carried out to determine at which conditions aromatic compounds are removed and whether this process affects the functionality of the resulting product.

iv. II. Second, a procedure to isolate plasma protein fractions at an industrial scale should be developed. According to what has already been mentioned, some functional properties show better results depending on the fraction being present, hence these ones should be obtained separately in order to formulate specific food ingredients. Like the process of milk cracking, a combination of solvents, pH conditions or salt concentrations should be investigated to selectively precipitate the protein fractions and develop an effective low-cost industrial process of plasma fractionation.

v. Biochemical analyses. Further experiments are required to elucidate the contribution of intermolecular reactions involved in the functional properties of plasma proteins. For example, disulfide bonds play a key role in the development of heat-induced gels, but their relative contribution in relation to hydrophobic interactions is not known. New tests using chemical reagents, and the

comparison of the functionality results in the presence of reducing agents, unfolding agents, etc., may give insights on the relative importance of these reactions.

vi. Microstructural analyses. The analysis at a microstructural level may provide valuable information about the property under study. It would be interesting to take into account:

vi. I. First, the application of the developed image analysis techniques to study heat-induced gels of each protein fraction. Microscopic attributes may reveal the formation of different structures, such as fine-strands instead of large globular aggregates, which could explain the differences in the macroscopic properties of the gels.

vi. II. Second, the study of microstructural properties under the electron microscope in combination with immunochemistry, using antibodies for each protein constituent, may reveal the way through which proteins aggregate and aggregates pile up. This would elucidate whether interspecific or intraspecific protein reactions occur during the gelation process, and help to corroborate the proposed gelation model.

vi. III. The use of image analysis has been only applied to investigate the microstructural properties of heat-induced gels. Moreover, this technique could be also useful to study the properties of foams and emulsions. For example, measuring the size distribution of air bubbles or oil droplets can be related to the capacity to form foams and emulsions, respectively, and monitoring breakdown processes may help to understand the stabilisation mechanisms of biphasic systems by plasma proteins.