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PhD Thesis

VALORISATION OF WHEY AND SECOND CHEESE WHEY: ELABORATION OF HIGH ADDED VALUE PRODUCTS

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VALORISATION OF WHEY AND SECOND CHEESE WHEY: ELABORATION OF HIGH ADDED VALUE PRODUCTS

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DECLARACIÓN CONFLICTO DE INTERESE

Eu, Arona Figueroa Pires, declaro que esta tese de doutoramento non presenta conflicto de interese.

En Lugo, Novembro 2025

Fdo: Arona Pires

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LIST OF PUBLICATIONS

The results reproduced in Articles 1, 2, 3, 4 and 5 of this thesis have been published in international journals. Find below the correspondence between the PhD chapters and the published articles.

Article 1

Title: Application of Ultrafiltration to Produce Sheep's and Goat's Whey-Based Synbiotic Kefir Products

Authors: Arona Pires [1,2], Gözdenur Tan [3], David Gomes [1], Susana Pereira-Dias [1,4], Olga Díaz [2], Angel Cobos [2] and Carlos Pereira [1,4,*]

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Article 2

Title: Whey Cheeses Containing Probiotic and Bioprotective Cultures Produced with Ultrafiltrated Cow's Whey

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Authors: Arona Pires [1,2], Agata Bozek [3], Hubert Pietruszka [3], Katarzyna Szkolnicka [3], David Gomes [1], Olga Díaz [2], Angel Cobos [2] and Carlos Pereira [1,4,*]

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Article 3

Title: Evaluation of the Characteristics of Sheep's and Goat's Ice Cream, Produced with UF Concentrated Second Cheese Whey and Different Starter Cultures

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Article 4

Title: Sheep's Second Cheese Whey Edible Coatings with Oregano and Clary Sage Essential Oils Used as Sustainable Packaging Material in Cheese

Authors: Arona Pires [1,2], Hubert Pietruszka [3], Agata Bozek [3], Katarzyna Szkolnicka [3], David Gomes [1], Olga Díaz [2], Angel Cobos [2] and Carlos Pereira [1,4,*]

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Article 5

Title: Edible Films based on Ovine Second Cheese Whey with Oregano Essential Oil

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"The important thing is not to stop questioning; curiosity has its own reason for existing"

Albert Einstein

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ABBREVIATIONS AND SYMBOLS

AA- Amino Acid

ACE- Angiotensin I-Converting Enzyme

α-La- α-Lactalbumin

ANOVA- Analysis of Variance

BCAAs- Branched-Chain Amino Acids

β-Lg- β-Lactoglobulin

BHA- Butylhydroxyanisole

BHT- Butylhydroxytoluene

BOD- Biochemical Oxygen Demand

C- Fresh Cheese Control

C: Control Cheese Without Coating

ºC- Degree Celsius

CFU/mL- Colony Forming Unit per Liter

CMP- Caseinomacropeptide

COD- Chemical Oxygen Demand

CW- Cheese Whey

DF -Diafiltration

DNA- Deoxyribonucleic acid

DPPH- 2,2-diphenyl-1-picrylhydrazyl

EAB- Elongation at Break

ED- Electrodialysis

EK- Fresh Cheese with Commercial Kefir Culture

EKABT5- Fresh Cheese with Commercial Kefir Culture and Probiotic Culture

EOs- Essential Oils

EU- European Union

FAO- Food and Agriculture Organization

FTIR- Fourier transform infrared spectroscopy

g- Grams

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γ-Radiation - Gamma radiation

g/L or g L-1 - Grams per Liter

G-Kefir- Sheep's Ice Cream with Kefir Culture

G-Probiotic- Sheep's Ice Cream with Mixed Probiotic Culture

G-Yoghurt- Sheep's Ice Cream with Yoghurt Culture

h- Hour

HBA- Hydrogen Bond Acceptor

HBD- Hydrogen Bond Donor

HDPE- High-Density Polyethylene

Igs- Immunoglobulins

INRA- Institute National de la Recherche Agronomique

IUPAC- International Union of Pure and Applied Chemistry

JCR-Journal citation report

kDa- kiloDalton

kPa- kiloPascal

kg- kilogram

LA5- Fresh Cheese with Probiotic *Lactobacillus acidophilus*

LA5+FQ- Fresh Cheese with *Lactobacillus acidophilus* + *Lactobacillus paracasei* + *Lactobacillus rhamnosus*

LC-HRMS- Liquid Chromatography High-Resolution Mass Spectrometry

L- Liter

LDPE- Low-Density Polyethylene

LF- Lactoferrin

LWC- Liquid Whey Concentrate

LSCWC- Liquid Second Cheese Whey Concentrate

LP- Lactoperoxidase

Ly- Lysozyme

m- Meter

m^2- Square Meter

MF- Microfiltration

MIC- Minimum Inhibitory Concentration

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µm- Microns or Micrometer

mm- Millimeter

MPa- Mega Pascal

Mt- Millions of tons

MW- Molecular Weight

N- Control Cheese with Natamycin

NADES- Natural Deep Eutectic Solvent

NF- Nanofiltration

Pa- Pascal

pI- Isoelectric Point

pH- pH (Hidrogen Potential)

PDO- Protected Designation of Origin

PGI- Protected Geographical Indication

PLA- Polylactic Acid

PP- Polypropylene

PVC- Polyvinyl Chloride

%- Percentage

% w/v- Percent Weight/ Volume

PESH- Polyethersulfone

PET- Polyethylene Terephthalate

PHAs- Polyhydroxyalkanoates

PS-Polystyrene

PS-Polysulfone

RC- Regenerated Cellulose

RO- Reverse Osmosis

RNA- Ribonucleic acid

s- Seconds

SA- Serum Albumin

SCFAs- Short-Chain Fatty Acids

SCW- Second Cheese Whey

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SCWF- Films with Ovine Second Cheese Whey

SCWP- Second Cheese Whey Powder

SCWPC- Second Cheese Whey Powder Concentrate

SEM- Scaning Electron Microscopy

S-Yoghurt- Sheep Ice Cream with Yoghurt Culture

S-Kefir- Sheep Ice Cream with Kefir Culture

S-Probiotic- Sheep Ice Cream with Mixed Probiotic Culture

SPI- Soy Protein Isolate

TGA- Thermogravimetric Analysis

TBHQ- Tert-butylhydroquinone

TK- Fresh Cheese with Traditional Kefir Culture

TKABT5- Fresh Cheese with Traditional Kefir Culture and Probiotic Culture

TS- Tensile Strength

UF- Ultrafiltration

UV- Ultra Violet

YM-Young's Modulus

WC: Cheese with SCW:WPI Coating

WCO- Cheese with SCW:WPI Coating Containing Oregano Essential Oil

WCS- Cheese with SCW:WPI Coating Containing Clary Sage Essential Oil

WP- Whey Powder

WPC- Whey Protein Concentrate

WPI- Whey Protein Isolate

WPH- Whey Protein Hydrolysate

WS- Films of WPI-SCW

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WS 1:2O2: Films of WPI-SCW Mixture in a Protein Proportion 1:2 with 2% EO

WVP- Water Vapour Permeability

VOCs- Volatile Organic Compounds

XRD-X-Ray Diffraction

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Resumo en Galego

VALORIZACIÓN DE SORO E SEGUNDO SORO DE QUEIXOS: ELABORACIÓN DE PRODUTOS DE ALTO VALOR ENGADIDO

O soro e o segundo soro de queixos o queixería son subprodutos das industrias lácteas procedentes da elaboración de queixo e requeixo, respectivamente. Ambos dous subprodutos poden causar un impacto ambiental alto; con todo, debido ás súas propiedades nutricionais e tecnofuncionais, pódense utilizar para a produción de produtos deshidratados. Estas investigacións non son suficientes porque as pequenas e medianas empresas lácteas teñen a limitación dos altos custos que representan as tecnoloxías necesarias, sobre todo para os elaboradores tradicionais de queixos de ovella e cabra. O desenvolvemento de procesos e sistemas máis eficientes e económicos para reducir a contaminación ambiental e a creación de novos alimentos de alto valor engadido poderían aumentar o valor económico destes subprodutos.

O obxectivo desta tese foi crear novos alimentos ou produtos de valor engadido a partir de soros e segundos soros de queixería bovinos, ovinos e caprinos para permitir que as industrias lácteas pequenas e medianas poidan introducilos nas súas empresas.

Proponse a aplicación da tecnoloxía de filtración con membranas para obter concentrados de proteínas líquidos, evitando a etapa de secado, podendo usarse directamente para a fabricación de alimentos innovadores. Desenvolvéronse tres tipos de alimentos: quefires simbióticos baseados en concentrados por ultrafiltración de soros de queixería de ovella e cabra, fermentados con grans de quefir ou cultivos iniciadores comerciais de quefir, con ou sen a adición dun cultivo probiótico comercial e con inulina; requeixos contendo probióticos con ou sen cultivos protectores usando concentrados por ultrafiltración de soro bovino; e en terceiro lugar, xeados de ovella e cabra elaborados con concentrados por ultrafiltración de segundos soros de queixería contendo inulina como prebiótico e fermentados con cultivos iniciadores comerciais de iogur, quefir e probióticos. Determináronse as propiedades físico-químicas, microbiológicas e sensoriais das mostras.

Finalmente, secáronse os concentrados líquidos, investigando a súa aplicación na industria alimentaria como material de envasado (elaboración de películas e cubertas comestibles). Aplicáronse cubertas comestibles elaboradas con segundo soro de queixería de ovella coa adición de aceites esenciais de ourego e xarxa en queixos para a substitución das cubertas comerciais.

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Summary in English

VALORISATION OF WHEY AND SECOND CHEESE WHEY: ELABORATION OF HIGH ADDED VALUE PRODUCTS

Whey and second cheese whey are by-products of dairy industries resulting from cheese and whey cheese manufacture, respectively. Both by-products can cause a high environmental impact; however, due to their nutritional and techno-functional properties, they can be used to develop dehydrated, high value, products. However, this option does not solve the problem for their disposal/valorisation because the small and medium size dairy companies are not able to support the high cost of the necessary technologies, particularly in traditional manufacturers of sheep and goat cheeses. The development of more efficient and economic processes and systems to reduce environmental pollution and the creation of new foods of high added value could help solving the problem by increasing the economic value of these by-products.

The aim of this thesis was to create new foods or value-added products by using bovine, ovine and caprine whey and second cheese whey and enabling the small and medium dairy industries to include them in their product's portfolio.

Membrane filtration technology is proposed to obtain liquid whey protein concentrates, which can be used directly or further processed for the manufacture of innovative foods. Three different foods were developed: synbiotic kefir products based on sheep and goat ultrafiltrated concentrated cheese whey, fermented by kefir grains or commercial kefir starter, with or without the addition of a commercial probiotic culture and with inulin; whey cheeses containing probiotics with or without protective cultures using ultrafiltrated concentrated bovine cheese whey; and, sheep´s and goat´s ice creams produced with ultrafiltrated concentrated second cheese whey, with inulin added as a prebiotic and fermented with yoghurt, kefir and commercial probiotic cultures. The physicochemical, microbiological, and sensory properties of the obtained products were determined.

Finally, the liquid concentrates were dried and their application in the food industry as packaging material (elaboration of edible coatings and films) was investigated. Besides, sheep´s second cheese whey edible coatings with oregano and clary sage essential oils were applied in cheeses to substitute commercial coatings.

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Resumen en Castellano

VALORIZACIÓN DEL SUERO Y DEL SEGUNDO SUERO DE QUESOS: ELABORACIÓN DE PRODUCTOS DE ALTO VALOR AÑADIDO

El suero y el segundo suero de quesos o quesería son subproductos de las industrias lácteas procedentes de la elaboración de queso y requesón, respectivamente. Ambos subproductos pueden causar un impacto ambiental alto; sin embargo, debido a sus propiedades nutricionales y tecnofuncionales, se pueden utilizar para la producción de productos deshidratados. Estas investigaciones no son suficientes porque las pequeñas y medianas empresas lácteas tienen la limitación de los altos costes que representan las tecnologías necesarias, sobre todo para los elaboradores tradicionales de quesos de oveja y cabra. El desarrollo de procesos y sistemas más eficientes y económicos para reducir la contaminación ambiental y la creación de nuevos alimentos de alto valor añadido podrían aumentar el valor económico de estos subproductos.

El objetivo de esta tesis fue crear nuevos alimentos o productos de valor añadido a partir de sueros y segundos sueros de quesería bovinos, ovinos y caprinos para permitir que las industrias lácteas pequeñas y medianas puedan introducirlos en sus empresas.

Se propone la aplicación de la tecnología de filtración con membranas para obtener concentrados de proteínas líquidos, evitando la etapa de secado, pudiendo usarse directamente para la fabricación de alimentos innovadores. Se desarrollaron tres tipos de alimentos: kéfires simbióticos basados en concentrados de ultrafiltración de sueros de quesería de oveja y cabra, fermentados con gránulos de kéfir o cultivos iniciadores comerciales de kéfir, con o sin la adición de un cultivo probiótico comercial y con inulina; requesones conteniendo probióticos con o sin cultivos protectores usando concentrados por ultrafiltración de suero bovino; y en tercer lugar, helados de oveja y cabra elaborados con concentrados por ultrafiltración de segundos sueros de quesería conteniendo inulina como prebiótico y fermentados con cultivos iniciadores comerciales de yogur, kéfir y probióticos. Se determinaron las propiedades físico-químicas, microbiológicas y sensoriales de las muestras.

Finalmente, se secaron los concentrados líquidos, investigando su aplicación en la industria alimentaria como material de envasado (elaboración de películas y cubiertas comestibles). Se aplicaron cubiertas comestibles elaboradas con segundo suero de quesería de oveja con la adición de aceites esenciales de orégano y salvia en quesos para la sustitución de las cubiertas comerciales.

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RESUMO EXTENSO EN GALEGO

INTRODUCIÓN

O soro ou lactosoro de queixo o de queixería é un subproduto obtido durante a elaboración de queixos. Obtense ao separar a callada e representa o 80-90% do volume do leite utilizado, contendo máis do 50% dos nutrientes do devandita leite. O de soro bovino adoita ter a seguinte composición: lactosa (4,5-5%), proteínas (0,6-0,8%), graxas (0,4-0,5%) e minerais (0,4-1,0%) (Yadav et al. 2016). Se se refuga, constitúe un importante problema ambiental xa que presenta unha alta demanda bioquímica de osíxeno (DBO de ao redor de 40-50 g/L) debido ao seu alto contido de lactosa. As cantidades de lactosa, proteína e graxa son mesmo maiores nos soros de orixe caprina e sobre todo nos ovinos (Pires et al. 2021b). As empresas lácteas teñen altos custos se o tratan como efluente industrial, pero o interesante é aproveitalo xa que é un subproduto que ten excelentes propiedades nutricionais e tecnofuncionais.

Unha solución para a súa valorización é a elaboración de requeixo, un produto tradicional elaborado mediante tratamento térmico a temperaturas de ao redor de 90ºC durante 20-30 minutos do soro de queixería que pode ser previamente acidificado; con ese quecemento precipítanse as proteínas do soro formando unha matriz que atrapa a graxa e outros sólidos presentes. Con todo, ten unha serie de desvantaxes tales como un baixo rendemento que fai que o gasto enerxético necesario para obtelo sexa alto, unha curta vida útil e o feito de xerar outro subproduto, o segundo soro de queixería, cun alto contido de lactosa (Barukčić et al. 2019; Osorio-González et al. 2022; Stamatelatou et al. 2011). Este tamén mostra unha alta demanda bioquímica de osíxeno (DBO maior de 30 g/L), obténdose aproximadamente 18 L por cada kg de requeixo fabricado e, como o soro de queixería, tamén pode aproveitarse polas propiedades nutricionais e tecnofuncionais dos seus constituíntes. Lactosa (4,5-5,0%), minerais (0,5-1,5%), proteínas (0,15-0,3%) e graxa (0,15-0,3%) son os principais compoñentes sólidos do segundo soro bovino. Os de orixe ovina adoitan presentar uns maiores valores de proteína e graxa, contendo 4,2-5,5% de lactosa, 0,5-1,8% de proteína, 0,3-2,5% de graxa e 0,5-1,0% de minerais (Carvalho et al. 2013; Maragkoudakis et al. 2016; Pereira et al. 2002; Pires et al. 2022; Pires et al. 2021b; Secchi et al. 2012; Sommella et al. 2016).

Para o aproveitamento destes dous subprodutos (soro e segundo soro de queixería), pero principalmente no soro de queixería, utilízanse procesos de concentración (ósmose inversa ou evaporación) e de separación por membranas (ultrafiltración, microfiltración e nanofiltración), cromatografía de intercambio iónico e adición de enzimas seguidos de técnicas de secado que permitirán recuperar os materiais retidos e permeados, para ser utilizados en novos produtos. Así, pódense obter lactosoro en po, concentrados e illados de proteínas, hidrolizados de proteínas e lactosa; ao redor do 50% do soro de queixería xerado no mundo aprovéitase desta maneira (Mollea et al. 2013; Pires et al. 2021b). O aproveitamento do segundo soro de queixería é moito menor sendo necesario máis estudos para a súa valorización.

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A maior limitación para que as pequenas e medianas empresas lácteas poidan utilizar estes dous subprodutos son os altos custos que representan estas tecnoloxías, sobre todo para os elaboradores tradicionais de queixos de ovella e cabra. Con todo, o resultado das análises de custo-beneficio dos procesos integrados de ultrafiltración/nanofiltración sinalan que son economicamente viables en produción a pequena escala (Macedo et al. 2021b). Mediante procesos de separación selectiva de membranas de baixo custo, que poderían aplicarse a pequenas e medianas empresas lácteas, pódense obter concentrados proteicos líquidos, que á súa vez se poden aplicar directamente na elaboración de alimentos, aforrándose custos. Ademais, a creación de novos produtos con estes concentrados podería conducir a aumentar o valor destes subprodutos e xustificar así o investimento nestas tecnoloxías. O desenvolvemento de produtos diferentes é unha oportunidade de valorizar áreas periféricas rurais mediante a transformación de subprodutos en alimentos de alto valor engadido (Marnotes et al. 2021; Tulla 2019). Desta maneira, tamén contribúen a un concepto de economía circular e sustentabilidade.

A industria desenvolveu un gran número de alimentos mellorados nutricionalmente que teñen un gran éxito no mercado. Por tanto, os produtos lácteos tradicionais cambiaron e as industrias lácteas deben evolucionar cara a unha nova xeración de produtos lácteos con características diferenciais en relación coas súas propiedades nutricionais e de saúde (Jeličić et al. 2008; Ryan and Walsh 2016). O desenvolvemento de novos produtos lácteos que conteñan prebióticos e probióticos está a aumentar debido para os efectos beneficiosos que teñen para a saúde humana. Os alimentos probióticos conteñen microorganismos que teñen propiedades terapéuticas pola súa actividade antimicrobiana, hipocolesterolémica, de mantemento dun bo equilibrio da microbiota intestinal e pola súa actividade anticanceríxena (Madureira et al. 2005a). A utilización de soros de ovella e cabra para elaborar estes novos produtos, por exemplo quefires ou xeados de leites fermentados, axudaría a mellorar a competitividade dos produtores de queixos de ovella e cabra. Ademais, o crecente interese nos produtos de leite de cabra e os seus derivados, debido ás súas características nutricionais e nutracéuticas (por exemplo, a menor alergenicidad das súas proteínas e o seu maior contido de oligosacáridos) fai da utilización do soro de queixería de cabra unha oportunidade para transformar este subproduto en produtos lácteos de alto valor engadido (Macedo et al. 2021b). Igualmente pódese dicir para o aproveitamento do segundo soro de queixería de orixe caprina. Ademais de probióticos, tamén é interesante a adición de cultivos bioprotectores a alimentos; esta incorporación pode ser importante naqueles con baixa vida útil, como é o requeixo, o que axudaría a incrementar a súa vida media. Dentro destes novos produtos, está a alcanzar gran interese a elaboración películas e cubertas comestibles a partir de proteínas do lactosoro (Kandasamy et al. 2021; Rosseto et al. 2023), habéndoselle a miúdo engadido como axentes antimicrobianos, aceites esenciais de plantas tales como comiño (Nemati et al. 2023), cravo (Fahrullah et al. 2021), limón, bergamota (Çakmak et al. 2020), ourego, romeu, allo (Seydim and Sarikus 2006; Seydim et al. 2020), estragón (Socaciu et al. 2020) e canela (Bahram et al. 2014). Estes estudos sería interesante realizalos con películas e cubertas elaboradas con segundos soros de queixería e aplicalas en recubrimentos de queixos para alongar a súa vida útil.

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OBXECTIVOS

O obxectivo xeral foi crear novos alimentos ou produtos de valor engadido a partir de soros e segundos soros de queixería bovinos, ovinos e caprinos para permitir que as industrias lácteas pequenas e medianas utilicen eficientemente estes subprodutos e así melloren a súa rendibilidade. En cada artículo, o obxectivo particular foi o desenvolvemento de diferentes novos alimentos ou produtos.

No artículo 1 foi a elaboración de novos quefires simbióticos baseados en concentrados proteicos líquidos obtidos mediante ultrafiltración de soros de queixería de ovella e cabra fermentados con gránulos de quefir ou cultivos iniciadores comerciais de quefir, con ou sen a adición dun cultivo probiótico comercial, e suplementados con inulina. Observouse a evolución dos fluxos de ultrafiltración durante a concentración e estudáronse as características dos novos produtos obtidos mediante análises fisicoquímicos, microbiolóxicos e sensoriais.

No artículo 2 foi a elaboración de requeixos contendo probióticos con ou sen cultivos protectores usando concentrados por ultrafiltración de soro bovino. Determináronse as características fisicoquímicas, microbiolóxicas e sensoriais dos alimentos elaborados.

No artículo 3 foi a elaboración de novos xeados de ovella e cabra con concentrados por ultrafiltración de segundos soros de queixería contendo inulina como prebiótico e fermentados con cultivos iniciadores comerciais de iogur, quefir e probióticos. Avaliáronse as propiedades fisicoquímicas, microbiolóxicas e sensoriais dos alimentos obtidos.

No artículo 4, investigouse a eficacia de cubertas comestibles elaboradas con segundo soro de queixería de ovella na conservación de queixos. Para iso, recubríronse queixos de leite de vaca con cubertas comestibles elaboradas con deshidratados obtidos con segundo soro de queixería de ovella coa adición de aceites esenciais de ourego e xarxa e comparáronse con queixos sen cuberta ou recubertos con natamicina. Determináronse as características fisicoquímicas e microbiolóxicas dos queixos.

No artículo 5 foi a elaboración de películas comestibles elaboradas con deshidratados obtidos con segundo soro de queixería de ovella con ou sen a adición de aceite esencial de ourego. Determináronse diversas características das películas como permeabilidade ao vapor de auga, grosor, contido de extracto seco, solubilidade en auga, densidade, cor e opacidade, propiedades mecánicas, actividade antioxidante, estructura da película por FTIR, estabilidade térmica por TGA, cristalinidade por XRD y microestructura por SEM.

ARTÍCULO 1. APLICACIÓN DA ULTRAFILTRACIÓN PARA PRODUCIR QUEFIRES SIMBIÓTICOS BASEADOS EN SOROS DE QUEIXERÍA DE OVELLA E CABRA

O quefir é un leite fermentado pola acción dunha microbiota moi complexa (bacterias acedo lácticas, acéticas e lévedos) que actúan en simbioses. Tradicionalmente para a súa fermentación utilízanse os gránulos de quefir, onde se atopan estes microorganismos nunha matriz de proteínas e polisacáridos. Presenta moitos compostos bioactivos como

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exopolisacáridos (principalmente kefirano), péptidos bioactivos e ácidos orgánicos, (sobre todo acedo láctico), aos que se lles atribúe moitos efectos beneficiosos para a saúde como actividade antimicrobiana, anticancerígena e estimuladora do sistema inmune. Actualmente tamén se elabora quefir con cultivos iniciadores comerciais, pero sinálase que o seu potencial funcional é diferente ao obtido con gránulos de quefir pola distinta composición microbiana de ambos os tipos (Vieira et al. 2021). A elaboración de quefir simbiótico con gránulos ou cultivos comerciais de quefir coa adición de prebióticos como inulina e microorganismos probióticos a partir de soro concentrado de ovella e cabra é un produto novo e que pode ter importantes propiedades funcionais.

Elaboráronse catro tipos diferentes de quefires simbióticos de ovella e cabra baseados en concentrados proteicos líquidos obtidos mediante ultrafiltración de soros de queixería ovinos e caprinos: os dous primeiros con cultivos de quefir comercial (con ou sen cultivo probiótico) e os dous últimos con gránulos de quefir tradicional (con ou sen cultivo probiótico). Todos os quefires tiñan a adición de inulina. O cultivo probiótico contiña *Bifidobacterium bifidus*, *Lactobacillus acidophilus* e *Streptococus thermophilus*.

Estudouse a evolución do fluxo e a concentración volumétrica durante a concentración, observando que coa ultrafiltración puidéronse alcanzar altos niveis de proteína (16,4% para ovino e 7,8% para caprino) nos concentrados proteicos líquidos obtidos. Os quefires de ovella tiñan unha textura sólida, mentres que os quefires de cabra eran líquidos. Todas as mostras mostraron recontos de bacterias acedo lácticas superiores a log. 7 ufc/mL, o que indica a boa adaptación dos microorganismos ás matrices alimentarias. En canto á avaliación sensorial, atopouse que os quefires de ovella foron os mellor clasificados, pero en xeral a aceptación destes novos produtos non foi moi positiva. Aínda que hai que traballar máis en mellorar a aceptabilidade sensorial destes quefires, a súa elaboración pode ser unha gran oportunidade para que pequenas e medianas empresas lácteas con equipos de ultrafiltración valoricen soros de queixería de ovella e cabra.

ARTÍCULO 2. REQUEIXOS CONTENDO CULTIVOS PROBIÓTICOS E BIOPROTECTORES PRODUCIDOS CON SORO DE QUEIXERÍA ULTRAFILTRADO DE VACA

A elaboración de requeixo a partir de soros de queixería é un proceso que ten baixos rendementos, é necesario un alto consumo de enerxía para obtelo e ten unha vida útil curta. A aplicación da ultrafiltración ao soro antes da súa elaboración e a adición de cultivos bioprotectores poden reducir estas desvantaxes. Ademais, a adición de probióticos pode aumentar o valor engadido deste produto.

Elaboráronse tres tipos de requeixos bovinos con concentrados líquidos obtidos por ultrafiltración de soros de queixería: un control sen adición algunha, outro coa adición de *Lactobacillus acidophilus* e, finalmente, un terceiro coa adición de *Lactobacillus acidophilus* máis un cultivo bioprotector que contén *Lacticaseibacillus paracasei* e *Lacticaseibacillus rhamnosus*. O requeixo bovino almacenouse en refrixeración durante 28 días.

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Os produtos coa adición de cultivos mostraron valores de pH máis baixos e acidez titulable máis alta en comparación co control. Observáronse pequenas diferenzas entre produtos en canto aos parámetros de cor durante o tempo de almacenamento. As mostras con cultivos engadidos foron máis firmes e tiveron un reconto de bacterias acedo lácticas da orde de log. 8-9 ufc/g. Os requeixos elaborados co cultivo bioprotector presentaron menores niveis de lévedos e mofos; por tanto, observouse o impacto beneficioso do cultivo bioprotector sobre a vida útil do alimento, retardando a súa deterioración. En canto á avaliación sensorial, os queixos con cultivos bioprotectores obtiveron as puntuacións máis altas en todos os parámetros avaliados. Pódese concluír que o uso de ultrafiltración no soro bovino asociado á adición de cultivos probióticos e bioprotectores pode ser de gran utilidade para reducir o consumo enerxético do proceso de fabricación, alongar a vida útil do requeixo e mellorar as súas propiedades sensoriais, ademais de conseguir que estes produtos teñan boas propiedades para a saúde humana.

ARTÍCULO 3. AVALIACIÓN DAS CARACTERÍSTICAS DE XEADOS DE OVELLA E CABRA, PRODUCIDOS CON CONCENTRADOS DE ULTRAFILTRACIÓN DE SEGUNDOS SOROS DE QUEIXERÍA E DIFERENTES CULTIVOS INICIADORES

Os produtos lácteos conxelados poden ter propiedades tanto de leites fermentados como de xeados e ser portadores de bacterias lácticas, prebióticos e probióticos. A produción de iogures conxelados con prebióticos e probióticos crea novas oportunidades para o desenvolvemento de produtos lácteos con propiedades beneficiosas para a saúde. A utilización de segundos soros de ovella e cabra para a produción de xeados con estas propiedades funcionais é de gran interese.

Os xeados elaboráronse con concentrados proteicos líquidos obtidos por ultrafiltración a partir de segundos soros de queixería procedentes de requeixos de ovella e cabra. A concentración proteica dos concentrados foi do 9,58% para ovino e 2,96% para caprino. Obtivéronse tres tipos de produtos, xa que os concentrados (todos coa adición de inulina) fermentáronse con cultivos comerciais de iogur, quefir ou probióticos antes da súa conxelación. Os probióticos eran *Bifidobacterium bifidus, Lactobacillus acidophilus e Streptococus thermophilus*. As propiedades dos xeados avaliáronse durante 120 días de almacenamento conxelado. Os xeados de ovella tiveron unha maior dureza e viscosidade complexa que os de cabra, relacionadas co seu maior contido proteico. Tamén presentaron maior "overrun" (incremento no volume de xeado tras conxelación pola incorporación de aire) e menor porcentaxe de derretemiento. É destacable que o reconto de bacterias lácticas indicou que as características probióticas dos xeados mantivéronse por encima do mes de almacenamento en conxelación. Todos os xeados presentaron unha boa aceptación polo panel de consumidores, pero os xeados elaborados co segundo soro de queixería de ovella presentaron mellor valoración en canto a textura, sabor e aroma que os xeados elaborados co segundo soro de queixería de cabra. A adición de prebióticos e probióticos aumentou o valor nutricional dos xeados ademais de incrementar a funcionalidade destes produtos; con iso, ponse en valor uns subprodutos da industria

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láctea, os segundos soros de queixería de ovella e cabra, que ata o de agora pouco reutilízanse, axudando a reducir o seu impacto ambiental.

ARTÍCULO 4. CUBERTAS COMESTIBLES DE SEGUNDO SORO DE QUEIXERÍA OVINO CON ACEITES ESENCIAIS DE OUREGO E XARXA USADAS COMO MATERIAL DE ENVASE SOSTIBLE EN QUEIXO

A utilización de segundos soros de queixería na produción de películas e cubertas comestibles e a súa utilización como material de envasado foi moi escasa, só un artigo (Alfano et al. 2022) fai referencia ao seu emprego. Tamén a aplicación de cubertas baseadas en proteínas de lactosoro e compostos antimicrobianos ou extractos de plantas en queixos é limitada (Mileriene et al. 2021; Ramos et al. 2012a; Ramos et al. 2012c).

Con tecnoloxía de membranas (ultrafiltración e diafiltración) obtivéronse concentrados proteicos líquidos de segundo soro de queixería de ovella, que se concentraron mediante ósmose inversa e logo secáronse mediante liofilización. Os deshidratados obtidos tiveron un alto contido de proteínas (54,7%) e utilizáronse para crear recubrimentos de queixos.

As cubertas elaboráronse mesturando estes liofilizados procedentes de segundo soro de queixería de ovella (SCW) con illado de proteína de lactosoro (WPI) e empregando glicerol como plastificante, tendo a solución final de SCW:WPI/plastificante 8% de proteína (p/v) na proporción 2:1 SCW:WPI e nunha relación de proteína/plastificante 1:1. A estes recubrimentos agregáronselles aceites esenciais de ourego (*Origanum compactum*) e de xarxa (*Salvia sclarea*), para mellorar as súas propiedades antimicrobianas. Aplicáronse a queixos elaborados con leite de vaca. Comparáronse cinco tipos de queixos: un sen recubrimento, outro recuberto cunha solución de natamicina, un terceiro con recubrimento de SCW:WPI e os dous últimos cubertos con SCW:WPI e aceite esencial de ourego ou de xarxa

A eficiencia dos recubrimentos antimicrobianos avaliouse mediante o seguimento das propiedades fisicoquímicas e microbiolóxicas dos queixos durante os 28 días de maduración. As cubertas elaboradas con segundo soro de queixería de ovella evitaron a evaporación excesiva dos queixos, conservando mellor a humidade, e tiveron menores valores de dureza, mastigabilidade e gomosidade ao final da maduración. As diferenzas de cor entre produtos con e sen cuberta non foron evidentes. A nivel microbiano, non houbo grandes diferenzas entre os produtos estudados. Os resultados obtidos permiten concluír que as cubertas a base de proteínas do segundo soro de queixería de ovella son unha alternativa prometedora aos recubrimentos comerciais, e tamén son outra forma para que as pequenas e medianas empresas lácteas dean valor ao segundo soro de queixo a un prezo razoable.

ARTÍCULO 5. PELÍCULAS COMESTIBLES DE SEGUNDO SORO DE QUEIXERÍA OVINO CON ACEITE ESENCIAL DE OUREGO

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Unha posible aplicación do segundo soro de queixería de ovella é a elaboración de películas comestibles, o cal non foi estudado ata a data deste traballo. É interesante comparar as súas propiedades coas elaboradas con illados de proteínas de lactosoro e as da combinación de ambos os deshidratados. A adición de aceite esencial de ourego é

debido aos seus coñecidos efectos antimicrobianos e antioxidantes. Os filmes elaboráronse, como no caso das cubertas do capítulo anterior, con liofilizados procedentes de segundo soro de queixería de ovella (SCW) e illados de proteína de lactosoro (WPI), sendo glicerol o plastificante utilizado. A concentración final de proteína na solución do filme foi do 8% (p/v). Elaboráronse once películas diferentes, as cinco primeiras sen aceite esencial, dous só con WPI ou SCW, e as tres restantes coa proporción 2:1, 1:1 e 1:2 de SCW:WPI. As seis restantes fixéronse coas tres combinacións de SCW:WPI (2:1, 1:1 e 1:2) ás que se lles agregou aceite esencial de ourego (*Origanum compactum*) en dúas concentracións.

Observouse que a diferente proporción de SCW/WPI influía nalgunhas características (cor e propiedades mecánicas) dos filmes e a adición de aceite esencial de ourego incrementou a actividade antioxidante das películas ademais de modificar outras características como a permeabilidade ao vapor de auga e a forza tensil dos filmes. Tanto a incorporación de SCW como a de aceites esenciais influíron na estrutura secundaria das proteínas, a estabilidade térmica, microstructura e cristalinidad dos filmes.

CONCLUSIÓNS

O traballo desenvolvido nesta tese demostrou que a valorización de soros e segundos soros de queixería de diversas orixes (bovino, caprino e ovino) mediante un proceso de baixa complexidade e baixo custo, presenta un enorme potencial para a súa aplicación en pequenas e medianas empresas lácteas. As tecnoloxías utilizadas permitiron crear novos alimentos de valor engadido, beneficiosos para a saúde e de fácil implementación como son os xeados e quefires simbióticos e os requeixos con probióticos e cultivos bioprotectores. Os produtos son de sinxela preparación e con custos de procesamento relativamente baixos.

Esta estratexia permite solucionar os problemas ambientais asociados á produción de soro e segundo soro de queixería en unidades industriais, así como fortalecer as súas estratexias de mercado. Doutra banda, permite demostrar a diversificación do uso de proteínas do segundo soro de queixería en aplicacións non convencionais, concretamente na produción de películas e cubertas comestibles. Estes recubrimentos poden ser produtos alternativos aos recubrimentos comerciais que existen actualmente.

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INTRODUCTION

1. The importance of the cheeses in Spain and Portugal

Milk production is considered the most important agricultural activity in the European Union, having supplied approximately 161 million tons of raw milk, representing 12% of the total value of agricultural production in 2021 (Jongeneel et al. 2023). Cow's milk is the most used milk in cheese making, with almost 8 million tons of cheese being produced in the EU in 2022. Goat, sheep and buffalo dairy products are produced in smaller quantities, but their production is increasing all around the world (Difonzo et al. 2023). The consumption of goat/sheep milk has been increasing, particularly because cow milk proteins are a common cause of allergies in infants (Rodrigues et al. 2024).

In 2024 cheese exports ascended to a total of 2.94 million metric tons, the main exporters being Australia, Belarus, the European Union, New Zealand, United Kingdom and the United States (https://apps.fas.usda.gov/ accessed in 19/02/2025). In Spain, more than 200 types of cheeses exist which form part of the historical legacy. Spain consumed a total of 4 233 million tons of milk and dairy products in 2023, which represents a slight variation of +0,16% compared to the previous year (https://www.tridge.com/news/the-consumption-of-dairy-products-in-spain-stagnat accessed in 19/02/2025). In Portugal, a total of 1 969 million tons of total milk were consumed in 2022, which represents a slight decrease compared to the previous year (2 036 million tons of total milk were consumed) (https://www.clal.it/en/?section=produzioni_cheese, accessed in 19/02/2025).

The European Commission created Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI) quality labels (Council Regulation No. 2081/92, 1992) to protect some cheeses with cultural impact. By the year 2022, around 198 PDO and around 55 PGI quality labels have been awarded in the EU. Italy is the country with the highest number of registered PDO cheeses (53 cheeses), followed by France (47), Spain (26) and Greece (22). In Portugal, 12 PDO and 1 PGI cheeses are registered, all of which are produced with raw milk (Dias 2022). These cheeses with PDO designation are Queijo do Pico, Queijo de Cabra Transmontano and Queijo de Cabra Transmontano Velho, Queijo Rabaçal, Queijo Terrincho, Queijo de Azeitão, Queijo Serra da Estrela, Queijo de Nisa, S. Jorge Cheese, Serpa Cheese, Évora Cheese and Beira Baixa Cheese (Cardinali et al. 2022). Several types of Spanish cheese are internationally recognized, such as Manchego (La Mancha) and Idiazábal (Basque Country) (Diana et al. 2014).

In the year 2023, in Portugal the total production of cheese was 86 078 tons (Table 1), of which around 63 558 tons were cow's cheese, 11 290 tons were sheep's cheese, and 3 908 tons were goat's cheese.

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Table 1: Milk and cheese consumption in Portugal in 2023

Year	Milk type	L (millions of tons)	Cheese type	tons
2023	Cow's milk	1 927 561	Cow's cheese	63 558
	Ovine's milk	1 884 478	Ovine's cheese	11 290
	Caprine's milk	24 526	Caprine's cheese	3 908
	Total milk	18 557	Mix cheese	7 321
			Total cheese	86 078

Adapted (INE) accessed in 19/02/2025

Cheese is generally characterized as a fresh or ripened product that is obtained by coagulating of milk or cream or a mixture of them. Cheeses have easy digestion and are rich in vitamins, minerals , amino acids and proteins (Diana et al. 2014). In fresh cheese, there is no curing period, semi-cured cheese has a ripening time of more than 1 month, and, in the case of cured cheeses the ripening period is, at least, 3 months, which makes these cheeses drier with a stronger, and more persistent flavour (Diana et al. 2014).

2. Whey. Importance and composition

Cheese whey (CW) is the liquid fraction that remains after milk coagulation to make cheese. Dairy companies produce millions of tons of by-products annually, with CW, being the main by-product. Global CW production already exceeds 200 million tonnes per year, and Europe contributed approximately 52.2 million tonnes in 2023 (Malos et al. 2025).

The dairy industry is one of the most polluting industries, as it generates around 0.2 to 10 L of effluents per L of processed milk (Garcia et al. 2022). Worldwide, more than 40% of CW is discarded, leading to the loss of valuable nutrients and potentially polluting the environment (Tița et al. 2024). Large amounts of cheese whey are generated worldwide as a by-product of cheese production. The cheese industry produces approximately 145 Mt of whey annually, 54% of which is reused in new food products, 60 Mt are used as animal feed, fertilizer or waste and 6 Mt are destined for non-food uses (Uribarrena et al. 2024).

Cheese whey contains more than half of the solids present in the original whole milk, including whey proteins, lactose, water-soluble vitamins and minerals (Garcia et al. 2022).

Approximately 8 to 10 L of milk are necessary to produce 1 kg of cheese, and if the resulting whey is untreated, its disposal causes a very significant problem for the environment due to its high Biochemical Oxygen Demand (BOD) and high Chemical Oxygen Demand (COD) (Castelli and Du Vale 2013; Jelen ; Sommella et al. 2016). Some years ago, CW, was treated as sewage by small and medium-scale food industry (Sommella et al. 2016) and when CW is discharged into water courses such as rivers, it reduces the dissolved oxygen, hampers biodegradability, and poses a major risk to aquatic

life, as well as to the environment and human health (Yadav et al. 2015). If it is discarded directly into the soil, it causes excessive oxygen consumption, impermeabilization, eutrophication and toxicity in the area where it is discarded (Uribarrena et al. 2024). However, CW has been recognized as a rich source of bioactive and functional compounds such as lactose, minerals and especially proteins, with excellent properties for human health. The use of membrane technologies made possible to valorise its components, particularly proteins and peptides, and create new products with excellent nutritional and functional properties for human consumption (Castelli and Du Vale 2013; Sommella et al. 2016).

CW contains about 65 g of total solids/L. It represents 85-95% of the milk volume, retains about 55% of the milk nutrients and approximately 20% of the total protein content (Delgado-Macuil et al. 2025; Ryan and Walsh 2016). These characteristics make CW a valuable resource for various food products and supplements, especially due to its valuable protein content.

The origin of milk (bovine, ovine or caprine), influences the nutritional and functional composition of CW, as well as, the types of cheese manufactured (depending if is rennet or acid coagulation) and, generally, the factors that affect milk composition such as, breed, seasonal cycles, feed and lactation phase also have impact on CW composition. This by-product is divided in two different types: sweet and acid CW, being the processing technique used to remove casein from the milk that influences the type of whey (Delgado-Macuil et al. 2025). The BOD values for sweet and acid whey are 40-102 g/L and 42-62 g/L respectively, and for the COD are 27-60 g/L and 35-51g/L, respectively (Buchanan et al. 2023).

Sweet bovine whey has a pH around 6-7, containing approximately 6-10 g L^{-1} of protein, 5-6 g L^{-1} of fat, 46-52 g L^{-1} of lactose and 2.5-4.7 g L^{-1} of minerals, and it results from the production of most types of cheese or some casein products (Argenta and Scheer 2020; Guo and Wang 2019; Ryan and Walsh 2016; Siso 1996). In the first step, rennet is added to milk as the initial stage in the production of cheese and sweet whey. The rennet works by curdling the casein present in the milk leading to the formation of curd. Then, the curd is strained from the remaining liquid (CW). The rennet induced coagulation of casein occurs at pH 6.0-6.5 (Argenta and Scheer 2020).

Acid bovine whey has a pH of approximately 4-6, 6-8 g L^{-1} protein, 5-6 g L^{-1} fat, 44-46 g L^{-1} lactose and 4.3-7.2 g L^{-1} minerals (Argenta and Scheer 2020; Guo and Wang 2019; Siso 1996). This whey results from the activity of lactic acid bacteria or by the addition of lactic acid or mineral acids (hydrochloric or sulphuric acid) to coagulate the casein for the manufacture of most types of industrial caseins, or results from the production of acid curdled cheeses, such as cottage cheese. Lactose content is generally higher in sweet whey compared to acid whey, but in acid whey, the mineral content normally exceeds the sweet whey mineral content (Pintado et al. 2001; Siso 1996). The values of total solids (lactose, proteins and fat) are even higher in ovine and caprine whey when compared to that of bovine whey (Table 2).

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Lactose, representing 70-75 % of the total solids, is the main component responsible for the high values for BOD and COD (Carvalho et al. 2013; Kotoulas et al. 2019; Ryan and Walsh 2016). CW proteins are a relatively heterogeneous group, that includes β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins and low molecular peptides (Gilmour et al. 2024).

Table 2: Average composition of bovine, ovine and caprine whey (% w/v).

	Bovine CW	Ovine CW	Caprine CW
Total solids (%w/v)	6.0-7.0	7.6-10.5	7.07-10.8
Lactose (%w/v)	4.2-5.0	4.3-6.1	5.02- 6.7
Proteins (%w/v)	0.7-0.9	1.6-1.8	1.2-0.63
Fat (%w/v)	0.1-0.8	1.2-2.5	0.84
Minerals (%w/v)	0.5-0.6	1.0-1.8	0.57
pH	5.6-6.3	5.3-5.9	6.34

Adapted from (Fancello et al. 2024; Henriques et al. 2011; Macedo et al. 2015; Pintado et al. 1999; Sanmartín et al. 2012)

Recently, particular attention has been given to the benefits of CW regarding to the nutritional, biological and techno-functional value of its proteins. CW proteins are recognized for their high quality, attributed to their digestibility and essential amino acid (AA) content (Gilmour et al. 2024). More recently, great interest has emerged in relation to minor components, such as whey protein derived bioactive peptides with antihypertensive, antioxidant and antimicrobial activities, and non-digestible oligosaccharides, which are claimed to behave as dietary fibers and prebiotics, important in human health (Macedo et al. 2015; Ostertag et al. 2021).

The described processes for whey solids concentration include evaporation, ultrafiltration (UF), nanofiltration (NF) or reverse osmosis (RO), before dehydration by spray or freeze drying. However, these processes cannot be applied by small and medium companies as they are expensive and require large installations (Cassano et al. 2019).

3. Whey cheese

In many countries, such as Portugal, Spain, Italy and Turkey, a significant amount of CW is used to produce whey cheeses by thermal aggregation of whey proteins, but other lactic products with nutritional and medical potential are also made (Pires et al. 2021a; Prazeres et al. 2012; Ryan and Walsh 2016). CW of bovine, sheep, goat, or buffalo origin is normally used to produce these products. Whey cheeses are called *Ricotta* in Italy, *Anthotyros* in Greece, *Urda* in Serbia and Romania, *Lor* in Turkey, *Anari* in Cyprus, *Skuta* in Croatia and Serbia, *Gjetost* in Norway, *Mesost* in Sweden, *Mysuostur* in Iceland, *Myseost* in Denmark, *Requeijão* in Portugal and *Requesón* in Spain and Mexico (Bintsis and Papademas 2023).

Whey cheese (WC) is manufactured through thermal processing of whey, which allows the recovery of most of its original dry matter. CW can be previously acidified, as is the

case with *Ricotta* cheese, followed by heating at temperatures around 85-90 °C during 20-30 minutes, to allow coagulation and subsequent precipitation of the proteins present in the CW originating the separation of the cheese whey mass (Madureira et al. 2005a; Maragkoudakis et al. 2016; Sommella et al. 2016). The yield of whey cheese is variable because it depends on the origin of the whey (bovine, ovine or caprine) and depends on the process used, but unless the whey is previously concentrated, this yield is lower than 4-6% and generates large quantities of a by-product known as second cheese whey (SCW) (Pires et al. 2021b; Pontonio et al. 2021).

The composition, as well as the high volumes produced, associated to the environmental impact of CW and SCW, are drivers for the great interest in their valorisation. It should be emphasized that ovine SCW contains a protein and mineral content higher than that presented by bovine whey (Table 2).

4. Second cheese whey. Importance and composition

Second cheese whey (SCW) or exhausted whey or deproteinized whey, also known as *Sorelho* in Portugal and *Scotta* in Italy, appears as a white to yellow/green opalescent liquid who contains suspended solids (Fancello et al. 2024). This is the by-product resulting from whey cheese production (e.g. *Requeijão*), referred to in the previous section (Maragkoudakis et al. 2016) (Figure 1). SCW also has a high organic content (BOD and COD). In most cases, small and medium companies do not have capacity to valorise the SCW components for human consumption and this product is used as animal feed, or sometimes discarded. Thus, the high volumes of CW and SCW produced remain a problem for cheese producers as they pose valuation/environmental challenges (Macedo et al. 2011). According to environmental and health regulations, regulation (EC) no. 1069/2009 and regulation (EC) no. 1774/2002, the handling and disposal of SCW is subject to regulations to prevent environmental pollution and disease transmission (Fancello et al. 2024).

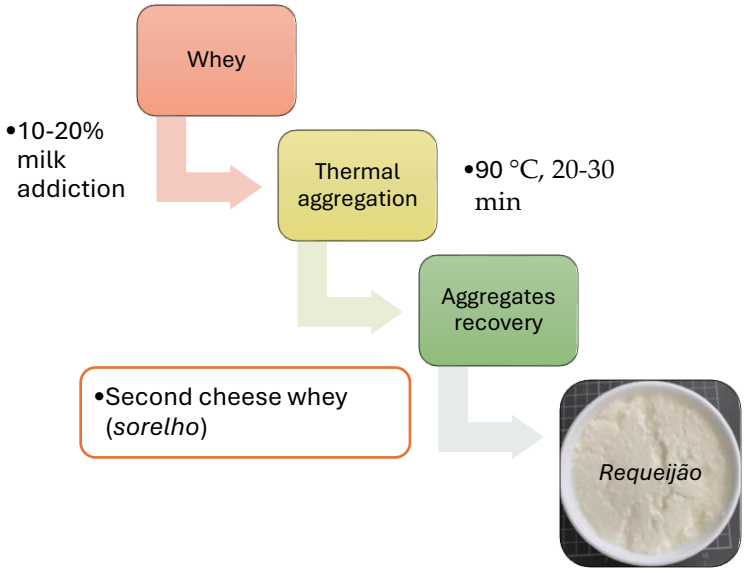


Figure 1: Scheme of whey cheese (*Requeijão*) manufacturing and second cheese whey (*Sorelho*) production (Pires et al. 2021b).

SCW is widely produced in Southern Europe and in the Mediterranean area, particularly in Italy, Spain, Portugal, Greece, Turkey and Romania, being also widely produced in Brazil (Fancello et al. 2024), where it can be used for animal feed but, a large proportion is still treated as waste. In Italy about 15% of the whey obtained annually is used to produce *Ricotta* cheese generating about 1 million tons of SCW (Maragkoudakis et al. 2016).

In the past, easy and economical solutions were used by dairy industries to discard SCW such as pulverization of the product on fields or elimination in rivers, lakes and ocean (Tirloni et al. 2020). Currently, most of SCW is used as supplement feed for livestock. Therefore, for environmental prevention and non-transmission of diseases, the direct discharge of *sorelho* into the environment is prohibited and when transported it must comply with transport regulations and be treated as waste or organic material (Fancello et al. 2024).

This by-product is a natural source of proteins, mostly in the denatured state, soluble peptides, oligosaccharides, lactose, non-protein nitrogen, hydro-soluble vitamins, a variety of minerals (potassium and magnesium) and free amino acids (Minhalma et al. 2007; Monti et al. 2018). Generally, its high salt concentration is due to the addition of NaCl during the production process of whey cheese, but it can be a good substrate in biotechnological processes to produce commercial high-value compounds (Pereira et al. 2015a; Sansonetti et al. 2009; Secchi et al. 2012). Some of the applications of SCW are as a fermentation medium for culture media, for biofuels, bioplastics (such as polyhydroxyalkanoates) as well as for biomass production (Naziri et al. 2023). Some studies also use SCW for food products with good protein characteristics and health benefits (Piskorz et al. 2019).

SCW contains around 50% of the original dry matter of the correspondent CW. Lactose and minerals largely contribute to its dry matter, but residual fat and non-thermally precipitated nitrogen components are still present (Pereira et al. 2002). The composition of SCW varies considerably, depending on the origin of the whey (bovine, ovine or caprine) and the process used to make the cheese (Table 3). SCW resulting from bovine whey contains proteins (0.15-0.22%), mineral salts (1.0-1.13%), and lactose (4.8-5.0%), and has a biological oxygen demand (BOD) of 30-50 g L^{-1} and a chemical oxygen demand (COD) of 60-80 g L^{-1}. SCW obtained from ovine whey is characterized by a higher protein content (Secchi et al. 2012; Sommella et al. 2016) and contains around 6.71% total solids, 0.49% of proteins, 0.53% fat and 2.08% of minerals (Pereira et al. 2002).

Because salt is added to milk during the manufacturing of cheese, the mineral content of SCW is relatively high (Carvalho et al. 2013; Macedo et al. 2015; Minhalma et al. 2007). This by-product has acidic characteristics with pH values around 3-6 (Carvalho et al. 2013). High concentrations of nutrients (ammonium nitrogen (0.06-0.270 g L^{-1})) and total phosphorus (0.006-0.5 g L^{-1}) are also present in SCW (Pires et al. 2021a).

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Table 3: Average composition of bovine and ovine second cheese whey (% w/v).

	Bovine SCW	Ovine SCW
Total solids (%w/v)	6.08	6.71-8.3
Lactose (%w/v)	4.8-5.0	4.5-5.7
Proteins (%w/v)	0.15-0.22	0.49-1.2
Fat (%w/v)	0.1-0.3	0.2-0.53
Minerals (%w/v)	1.0-1.13	1.7-2.08
pH	6.12	5.5-6.3

Adapted from (Fancello et al. 2024; Henriques et al. 2011; Macedo et al. 2015; Pintado et al. 1999; Sanmartín et al. 2012)

SCW contains proteins such as β-lactoglobulin (β-Lg), α-lactalbumin (α-La), immunoglobulins (Igs), serum albumin (SA). These proteins play an important role in human health as well as in animal health (Fancello et al. 2024). SCW contains higher amounts of saturated and ω-6 fatty acids and lower amounts of monounsaturated, ω-3 fatty acids and conjugated linoleic acid, in relation to *Ricotta* cheese whey (*Scotta*) probably due to the very low content of fat found in *Scotta* (Bergamaschi and Bittante 2017). The concentration of volatile organic compounds (VOCs) in SCW it is higher than in CW (+136%) and this large difference in values results from physicochemical and microbial changes occurring during the processing of CW. The main VOC in terms of relative abundance present in SCW is 3-methyl-butan-1-ol (Fancello et al. 2024).

5. Whey and second cheese whey proteins

Whey proteins are essential for human nutrition and have a number of physiological properties, including the ability to bind metals, support immunological and digestive processes, and provide a significant supply of essential amino acids (Mollea et al. 2013). Whey proteins are rich in branched-chain amino acids (BCAAs), which are important for muscle health and as metabolic regulators in protein and glucose homeostasis and lipid metabolism (weight control). They also contain sulphur-containing amino acids, which regulate metabolism, protein folding and function, and are precursors of the powerful intracellular antioxidant glutathione. The essential amino acids in whey represent also a better source compared to egg, meat and soya proteins (Dullius et al. 2018; Smithers 2015). There has been an increase in interest in whey as a potential source of bioactive compounds that can reduce the risk of disease (Pihlanto 2011). Interest in the major and minor proteins and peptides present in CW and SCW is growing as the techniques used to study these components become more sophisticated (Chen et al. 2019a; Smithers 2015). Whey proteins have a compact globular structure, with a relativity uniform distribution of non-polar, polar and charged groups. These proteins include β-lactoglobulin (β-Lg), α-lactalbumin (α-La), immunoglobulins (Igs), serum albumin (SA), lactoferrin (LF), lactoperoxidase (LP) and enzymes (approximately 60), in addition to other protein components, such glycomacropeptide or caseinomacropeptide (CMP), which is released from casein in the first step of enzymatic curdling (Dinika et al. 2020;

Madureira et al. 2010; Onwulata and Huth 2009; Pihlanto 2011; Tavares 2011). These proteins have an amino acid profile different from caseins (a greater amount of sulfur-containing amino groups), are easily denatured by heat and are dephosphorylated (Tavares 2011).

Whey proteins can be utilized in a variety of ways, including as components of modified meal compositions or as straightforward protein supplements (Rojas et al. 2020). β-lactoglobulin (β-Lg) is the major protein in whey, representing around 50% of the total bovine whey proteins (Dinika et al. 2020; Ostertag et al. 2021). It has a globular structure with 162 amino acid residues, has a molecular weight (MW) of approximately 18.4 kDa and a pI value of 5.1 (Fee et al. 2013; Tavares 2011). It is a valuable source of essential amino acids, specially branched-chain amino acids (isoleucine, leucine and valine), and is used in sports and dietary nutrition due to its excellent technofunctional and nutritional properties (Toro-Sierra et al. 2013). β-Lg is used in emulsification, foaming, gelling, as well as in the manufacture of protein hydrolysates for ingredient formulation (Chen et al. 2019a), and improves the absorption of retinol. However, it is recognized as an allergen (Buchanan et al. 2023) due to its absence in human milk.

α-lactalbumin (α-La) is the second major protein in whey, with a globular structure and representing around 20-25% of bovine whey proteins (around 3.5% in total bovine milk) (Dinika et al. 2020; Malos et al. 2025). It is a calcium metalloprotein composed by 123 amino acids, being a strong Ca^{2+} binding protein. Its molecular weight is about 14.2 kDa and it has a pI value of 4.2 (Fee et al. 2013). α-La is produced by the mammary glands' epithelial cells and is involved in the regulation of lactose synthesis (Gallo et al. 2024). This protein is more resistant to heat denaturation than β-Lg at neutral pH. Its denaturation is reversible below 75 ºC (Tavares 2011). Its nutritive properties are very important, being the α-La purified fractions utilized in infant foods to reduce their allergenic potential as well as to adjust its protein content close to that of human milk (Toro-Sierra et al. 2013). α-La is also very important for its antihypertensive and antioxidant activities, anti-obesity potential and the anti-tumour activity observed in the complex between human α-La and oleic acid called HAMLET (human alpha-lactalbumin made lethal to tumour cells) (Argenta and Scheer 2020; Hernández-Ledesma et al. 2011). Likewise, the complex between bovine α-La and oleic acid (BAMLET) also killed tumour cells (Argenta and Scheer 2020; Hernández-Ledesma et al. 2011).

Serum albumin (SA) represents around 5-6% of bovine whey proteins, has 582 amino acids residues, a MW of 69 kDa and a pI value of around 4.7-4.9, with high ligand-binding capacity. It acts as a transporter for fatty acids, hormones, metal ions, and drugs (Malos et al. 2025). Biological functions of SA include fatty-acid binding, an antimutagenic function. SA has good gelling properties and is widely used in food and therapeutic applications, cancer prevention and as an antioxidant (Fee et al. 2013; Madureira et al. 2010). It is also a good source of essential amino acids (Buchanan et al. 2023).

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Immunoglobulins (Igs), which make up about 5–8% of whey proteins (Malos et al. 2025), are γ-globulins found in the blood and body fluids of all lactating species. All Igs have a basic structural unit consisting of four polypeptide chains, two heavy chains with a

molecular mass of around 50-70 kDa and two light chains with a molecular mass of around 25 kDa, held together by disulfide bonds. These proteins are widely used by the immune system to identify and neutralise bacteria, viruses and other antigens, provide various types of immunity to the body and have anti-cholesterol, antimicrobial and antiviral properties. Human immunoglobulins are divided into different classes: IgA, IgD, IgE, IgG and IgM, but bovine milk and colostrum contain mainly IgG, IgM and IgA (Fee et al. 2013; Besediuk et al. 2024; Madureira et al. 2010).

Lactoferrin (LF) is a single-chain polypeptide characterised as an iron-binding glycoprotein, also known as lactotransferrin. It is present at high levels in various biological fluids, especially in milk and colostrum (Gallo et al. 2024). Human colostrum contains the highest amount of LF (5 g/L) (Lin et al. 2021). LF has a molecular weight of 80 kDa, is composed of 691 amino acid residues and has a pI of 8.6. Lactoferrin is one of the minor proteins found in bovine whey (around 1% of whey proteins). It is important for iron transport and absorption in the young intestine, for defence against pathogens and also has antioxidant, anti-inflammatory and antimicrobial activities (Argenta and Scheer 2020; Tavares 2011).

Lactoperoxidase (LP), which represents 0.25‑0.5% of whey proteins (Malos et al. 2025), has a single polypeptide chain with a molecular weight of around 78 kDa, with 612 amino acid residues and a pI value of 9.6. This protein is less important than lactoferrin in terms of nutraceutical activity, but it is very important for milk preservation (Fee et al. 2013) and has antimicrobial, bacteriostatic, bactericidal and antifungal activities (Argenta and Scheer 2020; Tavares 2011).

Caseinomacropeptide (CMP) is a hydrophilic glycopeptide derived from the action of chymosin on κ-casein during the milk-clotting process in cheese manufacturing (Janer et al. 2004). It is composed by the 64 C-terminal amino acids of κ-casein and is rich in branched-chain amino acids (BCAA). CMP is most commonly found in dairy products, being released into whey by enzymatic digestion during cheese-making processes; however, it is also found in its free form in cow's milk and whey, although in lower quantities (Gallo et al. 2024). In sweet cheese whey, CMP makes up 15-20% of the total protein (Pillai et al. 2024). It is adequate for special diets, due to its high content of BCAA (valine and isoleucine) and low levels of methionine. Besides, it has not phenylalanine and due to this, it is adequate in phenylketonuria patients´diets. CMP interacts with toxins, viruses and bacteria due to its carbohydrate fraction, preventing various pathogens from binding to cells. Studies have also shown that CMP protects against acid erosion of teeth. CMP also has immunomodulatory and prebiotic activities (Hernández-Ledesma et al. 2011).

Lysozyme (Ly) is a 14.4 kDa antibacterial enzyme found in plants and animals. In animals, it is present in various biological fluids such as saliva, tears and milk. Human milk, especially colostrum, has high levels of lysozyme (0.2-0.9 g/L), whereas bovine colostrum has significantly lower levels. Like lactoferrin, the antibacterial activity of lysozyme plays a role in reducing the complexity of the intestinal microbiota, increasing resistance to colonisation by some bacterial species, including pathogens, while

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promoting the growth of beneficial bacteria and improving recovery from various gastrointestinal pathological conditions (Gallo et al. 2024).

In Table 4 the protein composition of bovine milk and bovine, ovine and caprine whey proteins and their functional properties are presented.

Table 4: Protein composition of bovine milk and bovine, ovine and caprine whey proteins and their functional properties (% of total proteins).

Whey Proteins (% of total proteins)	Bovine Milk	Bovine whey	Ovine whey	Caprine whey	Functional Properties
ß-Lactoglobulin	7-12	53.3-66.0	73.1	46.6	Pro-vitamin A transfer
α-Lactalbumin	2.5	15.0-20.0	17.9	18.3	Synthesis of lactose
Serum Albumin (SA)	0.7-1.3	6.0-7.0	2.7	5	Transfer fatty acids
Immunoglobulins	1.9-3.3	11.0-13.3	-	-	Immunity
Lactoferrin	-	0.7-3.3	1.6	2	Bacteriostatic agent
Lactoperoxidase	-	0.5-1.0	-	-	Antibacterial agent
Caseinomacropeptide	-	15-20*	-	-	Regulation of cell growth and differentiation
Enzymes	-	0.5	-	-	-
Lysozyme	-	0.0002	.	-	-

*In sweet cheese whey

Adapted from (De Wit 1998; Díaz et al. 2004; Hernández-Ledesma et al. 2011; Janer et al. 2004; Onwulata 2008; Ostertag et al. 2021)

The amount and state of whey proteins in foods depend on several factors: the origin of the whey (type of cheese and type of milk), the production process and process conditions used in cheese production, and the conditions used in the manufacture of whey powder, whey protein concentrates (WPCs) or whey protein isolates (WPIs), namely pH and heat treatment, as well as the characteristics of the food matrix in which they are incorporated, together with the operational conditions under which they are manufactured (Pelegrine and Gasparetto 2005).

The technological properties of whey have proven distinguished health benefits when hydrolysed, due to the resulting bioactive peptides (Yadav et al. 2015). Over several years, scientists have focused their studies and investigation on bioactivities of compounds associated with whey protein-derived peptides (Hernández-Ledesma et al. 2011; Tavares et al. 2012).

Bioactive peptides are known as specific protein fragments (during food processing and storage the bioactive peptides are released from their inactive state in the parent protein structure) who have a positive effect on body functions and promote good health, a positive effect on physiological and metabolic functions, as well as, antimicrobial, antihypertensive, antioxidative, anticytotoxic, immunomodulatory, opioid, and mineral-carrying activities. Additionally, the process of fractionating proteins into peptides enhances the functional quality of whey proteins, including their solubility, emulsifying ability, and texture, so increasing their value in food (Abadía-García et al. 2013; Argenta and Scheer 2020; Madureira et al. 2010; Park and Nam 2015). Bioactive peptides derived from proteins, promote health and affect the major physiological systems (cardiovascular, immunological, digestive, and nervous) (Quirós et al. 2009).

Natural digestion of whey proteins in the gastrointestinal tract is not enough to achieve a positive health effect. Thus, the bioactive peptides must result from industrial processing of hydrolysed whey and second cheese whey (Dullius et al. 2018; Siso 1996). These processes will be discussed in the next section. One of the main problems with the industrial use of CW and SCW is that their protein concentration is relatively low, which implies the use of concentration processes to guarantee high hydrolysis productivity (Tavares and Malcata 2013). The food industry benefits with the development of membrane separation techniques because it has taken advantage of their relatively easy scale-up, as well as to the fact of being inexpensive when compared with preparative chromatographic techniques. Another benefit is that membrane separation technics do not require drastic heat treatments (Tavares and Malcata 2013; Tolkach and Kulozik 2005).

To concentrate and separate proteins from other components present in whey, several methods, such as ultrafiltration (UF), diafiltration (DF), nanofiltration (NF), ion exchange chromatography, electrophoresis, crystallization and precipitation have been used. These techniques have been applied not only to separate the major compounds (β-lactoglobulin and α-lactalbumin), but also to separate minor compounds, such as lactoperoxidase, lactoferrin and immunoglobulins, as well as other compounds such as the biologically active peptides obtained by enzymatic or chemical modification of proteins (Pihlanto 2011). Several types of products can be obtained from CW: whey powder (WP), whey protein concentrates (WPC), whey protein isolates (WPI), whey protein hydrolysates (WPH), delactosed whey, demineralized whey, whey permeate and lactose powder (Jeewanthi et al. 2015; Kotoulas et al. 2019). For SCW it is also possible to obtain second cheese whey powder (SCWP) and second cheese whey protein concentrates (SCWPC) (Pires et al. 2024a).

6. Products based on whey proteins: Whey powders, whey protein concentrates (WPC), whey protein isolates (WPI) and whey protein hydrolysates (WPH). Main properties

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Whey proteins are highly valued and can be obtained in many different forms, such as whey powder (8-12% protein and over 70% lactose), WPC, a dry product containing between 30-89 % protein and WPI containing more than 90% protein and 3% lactose.

WPH contain around 80% proteins that have been hydrolysed by specific enzymes, thus being more easily metabolized and having nutraceutical properties (Argenta and Scheer 2020; Kotoulas et al. 2019; Onwulata and Huth 2009). WPC, WPI and WPH are the best ways to use and valorise whey proteins. The different variations in the composition and functional properties result from several factors, such as the source and composition of the milk (bovine, caprine or ovine), heat treatment, fractionation and isolation techniques, the conditions in which cheeses are produced (type of cheese, rennet type, process modifications) and storage and sanitation conditions (Schmidt et al. 1984). However, the main cause for the variations in proteins content and state are the factors related to the obtention of cheese whey (sweet or acid) rather than the process of production of the WPC and WPI (De la Fuente et al. 2002).

Processing conditions, such as thermal treatments, pH and type and quantity of salts also play a significant role in the techno-functional properties of WPC, WPI and WPH products. Although whey products are highly valued, globally, only about 50% of the produced whey is valorised in such forms. WPC and WPI have a wide range of food applications due to their high protein content and can function as water-binding, gelling, emulsifying and foaming agents. Benefits of WPC and WPI in food applications include their high protein and valuable amino acid content, low calorie, low fat, low sodium content, lack of toxic compounds and antinutritional factors, good emulsification capacity, compatibility with other ingredients as well as easy of availability and the perception that it as a “natural” product (Onwulata and Huth 2009).

Processing treatments used in the manufacture of WPC and WPI may sometimes cause some protein denaturation, which tends to affect their functionality (Pihlanto 2011; Sanmartín et al. 2018). In the production of WPC and WPI, the most used techniques for the pre-concentration of whey are ultrafiltration (UF), diafiltration (DF), nanofiltration (NF) reverse osmosis (RO), electrodialysis (ED) and microfiltration (MF) (Madenci and Bilgiçli 2014). To produce WPC or WPI the UF and/or diafiltration (DF) processes are employed and afterwards, the concentrated whey is pasteurized, evaporated and dehydrated (Figure 2).

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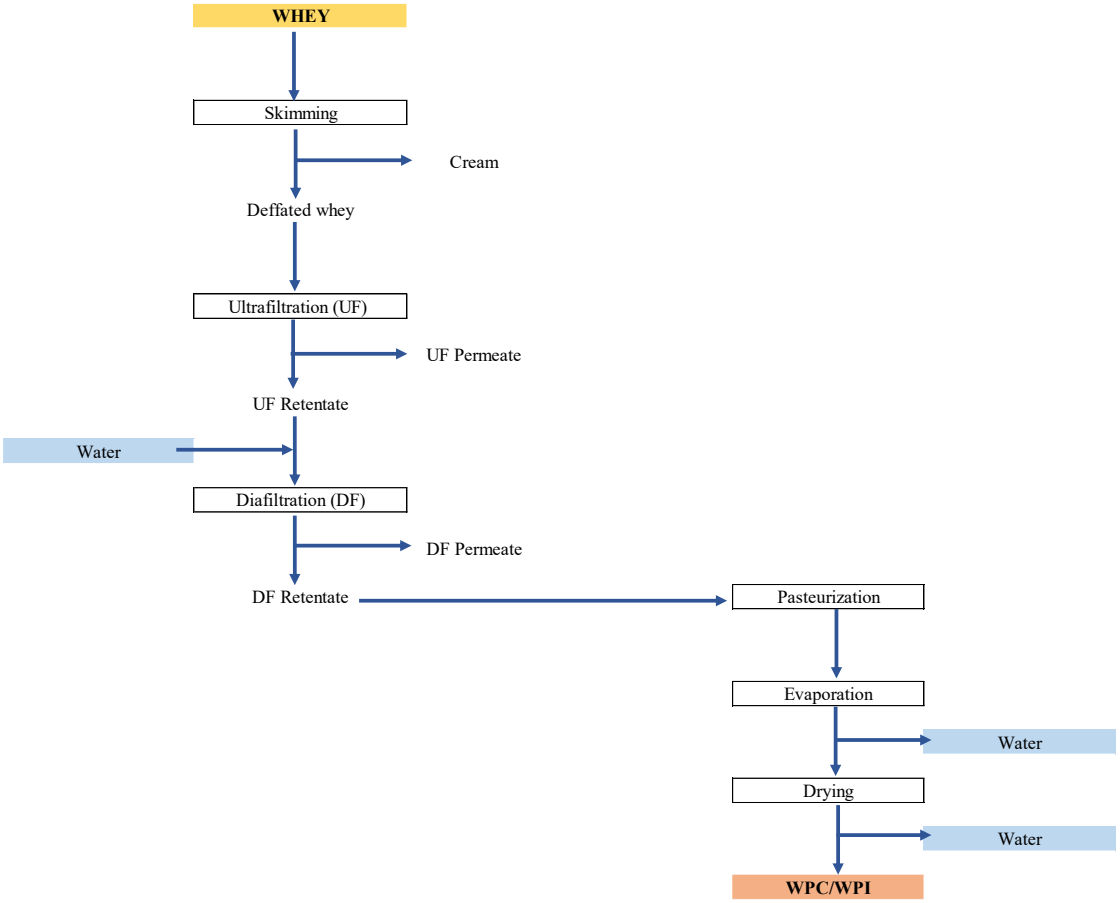


Figure 2: Representative scheme about the process for obtaining whey protein concentrates (WPC) and whey protein isolate (WPI) (Pires et al. 2021b).

The manufacturing process for WPC includes a last drying step by freeze or spray drying. Adsorption methods like ion exchange chromatography, which offers an additional level of membrane selectivity, can also be utilized for WPI manufacturing. The resultant product has more techno-functional properties than WPC and has a higher economic value (Foegeding et al. 2002).

In the case of whey protein hydrolysates production, the digestive enzymes trypsin, pepsin, and chymotrypsin, plant enzymes mainly papain, bromelain and cardoon enzymes, as well as bacterial proteases, mainly those originating from *Bacillus licheniformis* and *Bacillus subtilis,* or a mixture of some of these enzymes, are used in specific conditions of temperature an pH that favour the hydrolysis process (Jeewanthi et al. 2015; Tavares and Malcata 2013). WPH are soluble over a wide pH range, have good viscosity in solution due to water binding, promote cohesion, adhesion, elasticity and improve fat emulsification and whipping. WPH can form flexible edible films, as is the case of WPC or WPI. WPH are good emulsifiers and when used in addition with polysaccharides the emulsification ability improves. This also improves their colour and texture. Regarding flavour, it depends on the hydrolysis conditions. Their techno-functional and biological properties depend on the type of enzyme used in terms of specificity and selectivity, the hydrolysis conditions (enzyme-to-substrate ratio,

incubation temperature, pH, time) and the source of the original protein (native or denatured) (Jeewanthi et al. 2015).

Enzymatic hydrolysis of proteins allows the selection of the protein substrate and enzyme specificity to improve the yield of bioactive peptides (Pihlanto 2011; Siso 1996). The WPH contain peptides which increase the digestibility, bioactivity, and nutritional properties of foods to which they are added. WPH can be used in many different applications, such as gelling agents, emulsifiers or foaming agents, depending on the nature of the peptides produced and the degree of protein hydrolysis (Mota et al. 2006).

Favourable properties for human health, such as positive effects on the cardiovascular system, good opioid activities, anti-thrombotic effects and antioxidant effects, are examples of what some of these peptides can induce (Park and Nam 2015; Tavares and Malcata 2013). Several studies have confirmed the applicability of health-promoting peptides to functional foodstuffs. The hypotensive effect of anti-hypertensive peptides depends on their capacity to reach their target organs intact after oral administration (Quirós et al. 2009).

These peptides can also be assimilated by microorganisms, such as *Kluyveromyces marxianus*, and have also been used in the preparation of phosphopeptide complexes, which are interesting for the intestinal absorption of minerals (Siso 1996).

Some of the bioactive peptides reported in the literature are the α- and β-lactophorin (derived from α-lactalbumin and β-lactoglobulin), which have opioid agonist ACE inhibition, non-opioid stimulatory effect on ileum and ileum contraction, lactoferroxin (derived from lactoferrin), which act as opioid antagonist and serophorin (derived from serum albumin) which have opioid activity and casoplatelins (derived from glycomacropeptide), which have antithrombotic activity (Gerdes et al. 2001; Tavares 2011).

The most studied and commercially available peptides are inhibitors of angiotensin I-converting enzyme (ACE) and anti-microbial agents. ACE inhibitory peptides, derived by proteolytic digestion of α-lactalbumin and serum albumin, are effective in lowering blood tension and play a role in enhancing cardiovascular health, being highly beneficial in a wide range of bioactivities (Pihlanto 2011; Quirós et al. 2009; Smithers 2015). Fermentation with highly proteolytic strains of lactic acid bacteria is a successful strategy to produce ACE-inhibitory and antihypertensive peptides (Quirós et al. 2009). Other important peptide, lactoferricin, generated by the action of pepsin on lactoferrin under acidic conditions as in the human digestive system, has antimicrobial, antifungal, antiviral, antitumor and anti-inflammatory activities (Smithers 2015). Dullius *et al.* reviewed the benefits of bioactive whey peptides available for food processing and compared their production processes and development in laboratory conditions as opposed to industrial production (Dullius et al. 2018).

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Hydrolysis of whey proteins has also been used to modify their techno-functional properties, such as solubility, viscosity, emulsifying and foaming properties, as well as to improve the nutritional properties of foods (Brandelli et al. 2015). In view of the nutraceutical and techno-functional potential of SCW from buffalo's milk processing, Sommella *et al.* studied the SCW profile, and isolated a peptide fraction (226 peptides belonging to α, β, and k caseins), with molecular weights lower than 3 kDa that revealed a high complex profile. The peptidomic analysis highlighted a wide presence of valuable potential bioactive peptides, with possible health benefits (Sommella et al. 2016).

Another study conducted by Monari *et al.* reported the enzymatic valorisation of the protein fraction of SCW by means of UF with membrane cut-offs from 0.5 to 4 kDa. The obtained protein-enriched fractions were used for the optimization of enzyme-based digests aimed at producing potentially bioactive peptides (Monari et al. 2019).

7. Valorisation of cheese whey and second cheese whey technologies by membrane separation process

7.1. Membrane separation process

Due to the high pollutant capacity of whey, but chiefly due to its high nutritional and functional value in combination with its versatility to create new products, the dairy industry developed various technologies to take advantage of the solid constituents of such by-product (Ryan and Walsh 2016). However, the valorisation of whey is a challenge for small/medium size industries since they do not have the appropriate equipment and technology. Therefore, a large part of the traditional cheese processing industries does not have effective CW or SCW treatment systems that allow for their valorisation (Minhalma et al. 2007). The development of membrane techniques and their application in the production of dairy products has been essential since the process is relativity easy and not expensive when compared to other technologies. Besides, when applied in the treatment of whey, these techniques do not reduce the quality and properties of the products obtained (Argenta and Scheer 2020; Tavares and Malcata 2013; Tolkach and Kulozik 2005).

In the traditional process of whey demineralization, whey is concentrated by evaporation or reverse osmosis (RO) followed by demineralization of the concentrated whey using ion exchange columns. These processes are widely used at an industrial scale but imply high investment and operation costs (Cassano et al. 2019). For the separation and purification of whey proteins, centrifugation and membrane technologies are the most used (Mollea et al. 2013; Yada 2017). For the obtention of whey protein concentrates, it is necessary to selectively concentrate the solid components and then further concentrate the product by reverse osmosis or evaporation, before dehydration. Membrane filtration has been important in concentrating desirable whey components and removing those that are undesirable, but equally important has been the evolution of drying technologies (Smithers 2015).

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Different membrane filtration techniques, such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), reverse osmosis (RO), and electrodialysis are used to obtain WPC, WPI or WPH. Besides these, for WPI production, adsorption techniques such as ion exchange are also used (Onwulata and Huth 2009; Yada 2017).

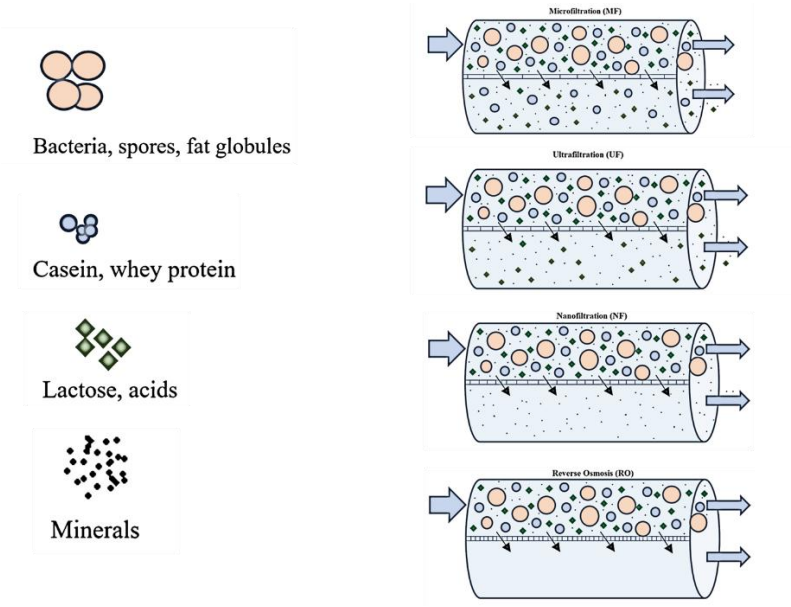


Figure 3: Process separation illustration of different membrane types (Pires et al. 2021b).

Variations in whey manufacture led to differences in functionality. These variability may depend on the whey source, whey handling conditions, pretreatment, ultrafiltration and microfiltration membrane performance, as well as on spray dryer conditions and characteristics (Yada 2017).

With the increasing evolution and utilization of membrane technologies in the dairy industry and their ease of operation, it is a possible, and can be an economic option, their use for the treatment of CW and SCW in view of their use as ingredients for food products, such as salad dressings or fermented drinks.

UF membranes have low rejections (high permeability) for lactose, and salts (NaCl, KCl) and have high rejections of the nitrogen compounds and fat. Besides, the processing of UF permeates by nanofiltration has two major advantages. Firstly, the production of a clean effluent and the reduction of wastewaters due to the possible reuse of some water in the process (*e.g.* as water used in cleaning processes). Secondly, the production of lactose concentrates with potential application in pharmaceuticals, sugar, cellulose fibers and food industry. The NF process has the advantage of simultaneously concentrating and demineralizing the whey, leading to a reduction of the total costs (equipment, energy) and the reduction of the wastewater disposal.

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Membrane phenomena were discovered and investigated in the middle of the eighteenth century, primarily to investigate barrier qualities and related phenomena. The first

commercial membranes for practical applications were manufactured by Sartorius in Germany after World War I, the know-how necessary to prepare these membranes originating from the early work of Zsigmondy (Mulder 2012). In the dairy industries, the 1960s marked a significant turning point for the commercial use of membrane filtration techniques (Minhalma et al. 2007).

From a research technique, membrane processing has become a significant industrial method for processing milk and whey. Nowadays membrane processes are used in a wide range of applications, and their number is still growing. Meanwhile, major advances in concentrated and dry milk products, cheese, and milk fat-based spreads have occurred (Mulder 2012; Pintado et al. 2001; Smithers 2015).

In the food industry, a group of French researchers from INRA (Institute National de la Recherche Agronomic), started using membrane technologies to concentrate the milk used in cheesemaking. Membrane processing allowed for uniform composition of the cheese milk and starter cultures activity has become more predictable (Henning et al. 2006).

After these first steps, the growth of these new technologies has been exponential and had a strong impact on the food industry and the agribusiness sectors. Several food products depend on membrane separation processes for their production. Examples of such products and applications are fruit and vegetable juices, wines and some grape products, the clarification of beer and the valorisation of fish proteins (Daufin et al. 2001). However, the dairy industry can be considered as the main sector of the food industry benefiting from these technologies.

Membrane separation processes are extensively utilized to obtain proteins and lactose concentrates from CW and SCW (de Oliveira Morais 2017; Minhalma et al. 2007; Prazeres et al. 2012). Membrane processes consist of passing a liquid through a semi-permeable membrane. In this process two fractions are obtained: the retentate or concentrate, which is the portion containing molecules that cannot pass through the membrane, and the permeate or filtrate which is the fraction that crosses the membrane. Usually the different types of membrane are described with reference to their pore size or their cut-off point with respect to molecular mass (Smithers 2015).

Five different types of membranes can be used in tangential filtration processes, sometimes in combination: reverse osmosis (RO), electrodialysis (ED), nanofiltration (NF), ultrafiltration (UF), and microfiltration (MF) (Onwulata and Huth 2009). Membrane processes present some advantages when compared to other, more traditional, separation processes, namely, the reduction of wastewater production, with the possibility of reuse and production of a clean effluent, low energy consumption, especially when compared with evaporation and distillation processes, ease of combination with other separation processes, and use of moderate process conditions, which is a very important issue in the food industry. Furthermore, additives and solvents are not required and the

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separation of components from a mixture is highly selective (Argenta and Scheer 2020; Minhalma et al. 2007).

Regarding whey valorisation, membrane processes are followed by spray drying to obtain a dry (less than 5% moisture) product, and the combination of these processes is utilized to produce whey powders with different protein contents. Table 5 presents a comparison of filtration techniques, comparing pore sizes and the type of components retained in each process when applied to milk (Onwulata and Huth 2009).

Table 5: Differences in comparison of membrane separation of milk components.

Type	Pore size (nm)	Retained compounds	MW of compound (kDa)
MF	200-4.000	Bacteria, fat globules and casein micelles	100-500
UF	20-200	Whey proteins, fat globules	1-100
NF	< 2	Lactose, divalent salts	0.1-1
RO	< 2	Monovalent salts	< 0.1
Electrodialysis	-	Removal of salt and deacidification of solutions containing neutral components	-
Pervapouration	-	Used for volatile organic pollutants	-

Adapted from (Chen et al. 2018; Onwulata and Huth 2009).

The separation process of whey components by ultrafiltration (UF) began in 1971 (Onwulata and Huth 2009). UF has been the most widely used process to separate the protein fraction (concentrate the native or pre-denatured whey proteins for the production of whey protein powders with different protein contents) from the lactose fraction, the former being retained by the UF membranes, while the second constitutes the solid fraction of the permeate (Atra et al. 2005; Yee et al. 2007).

The obtained permeate of UF contains about 90% of the total whey solids. This permeate is an excellent source of lactose (that can be applied in the sweets industry or in pharmaceutical and fermentation procedures), as well as it contains minerals, non-protein nitrogen compounds such as urea, free amino acids, creatine and creatinine, which are present in the initial whey and pass through the membrane (Atra et al. 2005; Neville 2006).

Generally, to concentrate whey proteins, the molecular weight cut-off used is 10 kDa and usually is performed at temperatures below 50-55 ºC, with a transmembrane pressure around 300 kPa. Whey retentate consists of protein, fat, and insoluble salts, lactose and soluble minerals that did not cross the membrane, and the solid component of the

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permeate is mostly lactose (Onwulata and Huth 2009). The concentration of lactose and soluble salts in the retentate remains similar to that of the feed.

The recovery of the components presents in the UF permeate has been carried out by several processes, namely RO, which, more recently, has been replaced by nano filtration (NF). This process has been used by the dairy industry mainly to concentrate and demineralize acid whey and sweet whey. NF has an important function in the dairy industry for valorisation of whey because it allows for protein concentration and partial demineralization of the product. Compared to RO, it provides an energy gain of approximately 45%, as it uses lower transmembrane pressures. When used as an alternative to evapouration (or reverse osmosis) and electrodialysis to concentrate and demineralize the whey, energy and environmental costs can be significantly reduced (Lipnizki 2010).

Thus, the sequence of UF/NF operations allows for the separation and recovery of the various whey fractions, predominantly the protein fraction (retentate) and the fraction consisting mainly of lactose and salts (permeate).

Many different membranes for the UF process were studied, but we indicate two works applied to SCW. Macedo *et al.* compared the performance of three ultrafiltration membranes used for SCW processing. In this study the authors compared the RC70PP (cellulose regenerated acetate), ETNA10PP (composite fluoro polymer) and UFX10pHt (polysulphone permanently hydrophilic) membranes with similar MW cut-off (10 kDa). The results showed better performance for the RC70PP membrane because it had the highest permeate fluxes, the lowest irreversible fouling, good selectivity (compared with ETNA10PP membrane) and the water recovery fluxes was around 100%. The higher hydrophilicity of membrane RC70PP justified these results (Macedo et al. 2015).

Monti *et al.* studied the chemical composition of bovine *Ricotta* cheese exhausted whey (*Scotta*) of different origins and evaluated combined membrane technologies to separate and recover single constituents. The authors used a membrane fractionation system composed by two UF and one NF process for testing different combinations of membranes and operational parameters to separate residual proteins, peptides and recover lactose. The results showed the difficulty for the evaluation of the membrane differences because protein denaturation phenomena occurring during the production of *Ricotta* negatively influenced the attempt to apply membrane technologies to separate and recover residual chemical constituents. The principal problem was the fouling phenomena and protein-membrane interactions that inhibited a complete separation between whey proteins and peptides. However, the authors refer an important result in the application of one UF process, followed by NF process, that allowed for the separation of one pure fraction, including whey proteins and peptides, and of another with 80% of the original lactose concentrated. The membranes used in this study were based on Polyethersulfone (PESH), Polysulfone (PS), or Regenerated Cellulose (RC) for the UF process while for the NF Process one with Composite Thin Film was used (Monti et al. 2018).

7.2. Fouling in membranes processes

The major problems that can occur in membrane processes are related to fouling or with the concentration polarization phenomena, which are responsible for the decline of the permeation fluxes, and for changing the selectivity of the processes (Chen et al. 2019a; Macedo et al. 2015). These phenomena must be minimized due to their effect on reducing permeate flux and membrane selectivity, along with the costs of implementing cleaning cycles required to restore productivity (Chen et al. 2019a). Fouling of UF and MF membranes by proteins is a major challenge in the bioprocessing and dairy industries (Tanudjaja et al. 2022). Many studies confirm that whey proteins (β-lactoglobulin and α-lactalbumin) and minerals, especially, calcium and phosphate, are the main contributors for the fouling of ultrafiltration membranes (Jeantet et al. 2000; Macedo et al. 2015; Wen-Qiong et al. 2019). Other components long-term affecting the performance of UF membranes are residues from processing, such as curd, residual lipids, CMP, enzymes and microorganisms (Macedo et al. 2015). These components probably adsorb onto the membrane surface, promote gelation in the polarized layer or induce pore blocking.

Fouling depends on many physical and chemical factors such as protein and calcium concentration, temperature, pH, ionic strength and specific interactions (Ding et al. 2019).This occurrence not only reduces the flow, but also makes cleaning operations more difficult and expensive (Gerdes et al. 2001; Macedo et al. 2015).

During the filtration process, the solution is transported to the membrane surface and, due to its semipermeable nature, a portion of the solvent, with or without solutes, passes through the membrane. This causes a higher concentration of solutes on the membrane surface than in the solution, so some of these compounds return to the core of the flux. The development of a concentration gradient of the components retained near the membrane is denominated concentration polarization. In particular, porous membranes, such as MF and UF membranes, are highly susceptible to this type of fouling (Chen et al. 2019a). However, this problem is reversible, if the membrane is cleaned with water and appropriate cleaning solutions, the flow can be recovered if the process has not advanced (Mulder 2012). Membrane cleaning is very important in order to recover as much flux as possible, thus maintaining the filtration rate and also extending the useful life of the membrane (Tanudjaja et al. 2022).

A study showed that ultrasound is an effective method for cleaning membranes. This technique specifically works well for ultrafiltration membranes that have become contaminated with concentrated whey protein solutions. The findings highlight the potential of ultrasound as a valuable cleaning approach in maintaining membrane performance (Luján-Facundo et al. 2017).

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7.3. Applications, valorisation, and opportunities for whey and second whey cheese products

Whey and whey protein concentrates have many applications in the food industry due to the excellent nutritional and techno-functional properties of the whey proteins. They have a high content of essential amino acids, are soluble in a wide pH range, have emulsifying, foaming and gelling properties, in addition, their relatively low cost. Thus, they are used in the production of new dairy products, meats, salad dressings, bakery and confectionery products, drinks and foods for athletes, among others.

Through advanced membrane technologies, it is possible to separate and obtain a specific type of dairy proteins, with a concentration that can be used more efficiently in the food industry (Jana 2022). These protein foods provide functionality, solubility, viscosity, gelling, emulsification and foaming to the foods in which they are inserted, and are also used as specialized ingredients (Jana 2022). Since whey proteins have antioxidant, anti-inflammatory, blood pressure-lowering, anti-obesity and appetite-suppressing effects, which allow the creation of new products with excellent techno-functional properties (Yiğit et al. 2023).

The application of WPI and WPC (in concentrated liquid form or as powders) in dairy products is a way of improving these products' nutritional level, as well as their sensorial properties. Many studies were carried out to create new dairy products with better characteristics by using such products (Gursel et al. 2016; Henriques et al. 2013b; Pires et al. 2021a). Some examples of dairy products that have used liquid or powdered WPI or WPC to improve their characteristics are yoghurts, fresh cheeses, dairy drinks, butters, salad dressings and cheese (Borges et al. 2020; Gursel et al. 2016; Henriques et al. 2013b; Karam et al. 2013; Marnotes et al. 2021). WPI and WPC are also added to processed or creamy cheeses and coffee creams, to improve their texture, making them creamier, improving the colour and appearance and increasing the nutritional value by increasing the protein and calcium contents (Borges et al. 2020; Jana 2022).

The use of probiotics in protein-rich whey drinks has demonstrated potential benefits for brain/psychological and cardiovascular activities (Forkwa et al. 2022). These drinks promote digestive health, improving gut flora balance and nutrient digestibility (Soumati et al. 2023).

Recently new dairy products with the addition of second cheese whey have also been created (*e.g.* dairy beverages, salad dressings, ice cream) (Borges et al. 2020; Piskorz et al. 2019).

A very important food for children is breast milk (approximately 40% casein to 60% whey protein), so creating a suitable milk for children is an important challenge, since bovine milk contains 80% casein and 20% whey proteins. The use of WPC and WPI proved to be adequate for the formulation of infant milk, as they allow the addition of the necessary amount of proteins to be able to approximate the values contained in breast milk (Mc Entee et al. 2023).

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Whey proteins contribute to the reduction of problems associated with childhood allergies. In infant formulas, various hydrolysed versions of β-lactoglobulin are often used to reduce the likely allergic reactions in children (Tariq et al. 2013).

WPI, WPC and WPH are also used for the manufacture of dietetic foods and special foods for athletes (*e.g.* drinks), as they have high digestibility (Soumati et al. 2023) and are an excellent source of essential amino acids, improving immunity, antioxidation, relieving fatigue, increasing muscle exercise capacity and strengthening bone quality (Liu 2020). These drinks are carefully formulated to provide a blend of high-quality whey protein, essential carbohydrates and vital electrolytes to meet athletes' nutritional needs (Soumati et al. 2023).

Traditionally, bread is very important food for populations. So, the addition of whey powder and calcium carbonate to bread, allows protein and calcium to be added to the bread (reaching 78% of nutritional recommendations for adults), with good sensory acceptance and purchase intention. This product is especially valuable for people, especially children, who do not have access to the main sources of protein and calcium provided by dairy products (Batista et al. 2018).

Due to the high lactose content, CW and SCW can carry out alcoholic fermentation and thus can be used as a source to obtain bioethanol. Its biotechnological reuse is beneficial as it allows us to transform waste into renewable energy sources (Zoppellari and Bardi 2013).

Cheese whey can be used to develop sustainable packaging materials and thus help reduce the environmental impact of the food industry. The production of polylactic acid (PLA) and polyhydroxyalkanoates (PHAs) through the anaerobic digestion of whey, as well as the use of WPI, and WPC to create edible films allows the creation of new sustainable packaging.

PLA and PHAs are bio-based polymers that can replace petroleum-based plastics (Soumati et al. 2023). Whey-derived PLA is used in films for packaging, food coatings, and textile applications (Soumati et al. 2023). In addition to PHAs and PLA, WPI and WPC are favourable in the development of edible films. These edible films present an innovative and ecological alternative to conventional packaging materials.

Edible films made from WPI and WPC are biodegradable, allowing for the reduction of environmental impact and waste generation. Furthermore, through the incorporation of bioactive compounds and antimicrobial agents, as is the case of lactoferrin, their functionality is increased and extends the shelf-life of packaged products. Lactoferrin in edible films has antimicrobial properties, inhibiting the growth of spoilage microorganisms, contributing to better food preservation, ensuring its quality for longer (Soumati et al. 2023).

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Edible films have also been shown to be effective when functional ingredients such as probiotics and prebiotics are used. The use and incorporation of probiotics in films maintains the molecular structure and reduces the moisture content and solubility of the films (Odila Pereira et al. 2019).

Another important aspect in edible films and coatings is the use of nanomaterials in combination with WPI and WPC that improve the properties of these edible films. The incorporation of immunoglobulins as nanomaterials improves the performance of films and coatings (Soumati et al. 2023).

8. Probiotics, prebiotics and symbiotics

Nowadays, human beings are increasingly concerned about their health and therefore take healthy eating habits into consideration (Kumar et al. 2022b). Since the 1970s, there has been increasing concern about the use of additives and preservatives in food that may not be beneficial to human health. Thus, consumers identify additives with the letter "E" as “harmful chemicals in food products,” and have increasingly sought foods free of these additives (Fernandes et al. 2025). Researchers are also increasingly exploring the development of food products with symbiotic (probiotic plus prebiotic activities) benefits. Symbiotic foods have gained popularity due to their functional, nutritional, physiological and therapeutic characteristics (Kumar et al. 2022b).

Probiotics are live microbial food supplements, with benefits for human health when consumed in adequate quantities. Recently, new definitions have been added to the probiotic terminology such as parabiotics and postbiotics. Some of the main strains of probiotics are lactic acid bacteria (LAB) such as *Lactobacillus, Bifidobacterium, Streptococcus*; non-lactic acid-producing bacterial genera, such as *Bacillus, Propionibacterium*; non-pathogenic yeasts such as *Saccharomyces*; and non-spore-forming, non-flagellated coccobacilli (Zepeda-Hernández et al. 2021).

On the other hand, prebiotics are substrates that are selectively used by microorganisms, providing a health benefit. Different ingredients based on dietary fiber and soluble dietary fibber are being studied as potential prebiotics. Compounds such as inulin, resistant starch, pectin, pectin-like compounds and β-glucans are recognized as prebiotic compounds. There are non-carbohydrate compounds that have been proposed as prebiotics: minerals, polyphenols and polyunsaturated fatty acids are some examples. Additionally, milk contains many different oligosaccharides, which can also function as prebiotics. In fact, breast milk-like oligosaccharides from cow's milk are currently being studied to develop new therapeutic food products (Zepeda-Hernández et al. 2021).

Symbiotics are currently referred to as “a mixture comprising live microorganisms and substrate(s) used selectively by host microorganisms that confer a health benefit to the host.” A complementary symbiotic must consist of a probiotic plus a prebiotic. In contrast, a synergistic symbiotic contains a substrate that is designed to be used selectively by co-administered microorganisms. Fermentation of prebiotics by probiotics and microbiota

increases the levels of short-chain fatty acids (SCFAs) such as acetate, propionate and butyrate. For intestinal epithelial cells, butyrate is an important source of energy. SCFAs have potential anti-inflammatory and antioxidant activity; and affect cell proliferation, cell differentiation, mucus secretion and have barrier function (Zepeda-Hernández et al. 2021).

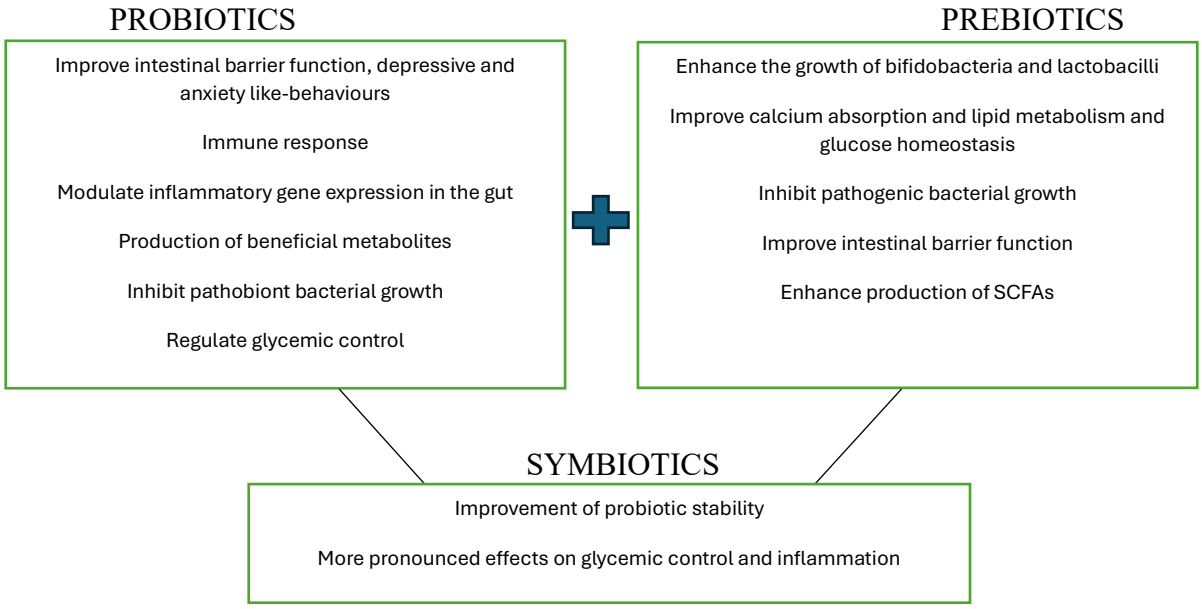


Figure 4: Probiotics, prebiotics and symbiotics principal characteristics for health.

Fermented dairy products, rich in live beneficial bacteria, are traditional and essential probiotic sources in the human diet. During fermentation, bacteria convert lactose into lactic acid, giving a distinct flavour and extending shelf-life (Utebaeva et al. 2024). The dairy industry is the largest sector where probiotics are used, such as sour/fermented milk, yoghurt, cheese, butter/cream, ice cream, and infant formulas. These probiotics are used as a starter culture alone or in combination with traditional starters, or incorporated into dairy products after fermentation, where their presence confers many functional characteristics to the product (*e.g.* improved aroma, flavour and textural characteristics), as well as confer many health-promoting properties, such as improving intestinal health (Gao et al. 2021).

Two probiotic strains widely used in dairy production are *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. *Lactobacillus acidophilus* is known for its ability to adhere to the intestinal mucosa, colonizing the intestine and exerting health-promoting effects such as reducing cholesterol levels, strengthening the immune system and preventing infections. On the other hand, *Bifidobacterium bifidum* is crucial for maintaining intestinal health, producing acetic and lactic acids that reduce intestinal pH and inhibit pathogenic bacteria. This strain also improves intestinal barrier function and modulates the immune system (Utebaeva et al. 2024).

Incorporating these probiotics into dairy products not only improves their nutritional profile but also provides health benefits that go beyond basic nutrition. For example, studies have shown that dairy products fermented with *Lactobacillus acidophilus* and

Bifidobacterium bifidum can alleviate symptoms of lactose intolerance, reduce the risk of certain gastrointestinal illnesses, and even improve mental health (Utebaeva et al. 2024).

Despite their benefits, one of the main challenges in incorporating *Lactobacillus acidophilus* and *Bifidobacterium bifidum* into fermented dairy products is ensuring the viability of these probiotics during production and storage. Factors such as pH, composition and storage conditions significantly impact the survival of probiotics. Probiotic bacteria, especially bifidobacteria, are sensitive to oxygen, which inhibits their growth in fermented dairy products. Strategies such as adjusting the redox potential and adding antioxidants are employed to increase viability (Utebaeva et al. 2024).

The objective of the study carried out by Plessas *et al.* was the evaluation of the probiotic *Lactobacillus paracasei* SP5, previously isolated from dairy products, as a starter culture for the production of salty white cheese "trahanas", to provide starter culture protection and prebiotic effect. Thus, the use of freeze-dried starter culture, free or immobilized, improved the aromatic profile of the cheeses, leading to a salty white cheese with overall quality and improved sensory characteristics. The results indicated a potential industrial use of freeze-dried *L. paracasei* SP5 as a starter culture for the production of good quality functional white savoury cheeses (Plessas et al. 2021).

9. Biopolymer based edible films and coatings

9.1. Introduction, properties and types

The accumulation and slow degradation of fossil-based plastics in the environment has caused serious ecological problems (Tavares et al. 2021). The global plastic packaging market is predicted to grow from USD 1.18 Trillion in 2025 to USD 1.43 Trillion in 2030 (https://www.mordorintelligence.com/industry-reports/global-packaging-market, assessed in 3 March 2025). According to the Flexible Packaging Association, flexible packaging is the most used in food, and contributes for more than 60% of the total packaging market (Flexpack). The packaging of food products has a great impact on the environment, as it consumes a lot of raw materials. However, due to the durability of plastic, design flexibility, lightness and low price, plastic became advantageous over other materials. After processing, food products are generally packaged to prevent microbial spoilage and to preserve their organoleptic properties and their quality during their shelf-life and transportation (Calva-Estrada et al. 2019; Gerna et al. 2023; Tavares et al. 2021). Plastic films produced based on petroleum, such as polyethylene terephthalate (PET), high-density polyethylene (HDPE), low-density polyethylene (LDPE), polypropylene (PP), polyvinyl chloride (PVC) and polystyrene (PS), are widely used for packaging since they are easy to produce (Zhang et al. 2023), widely available, have good mechanical strength (tensile strength (TS), elongation at break (EB) and Young's modulus) and help to preserve the good functional properties of food (permeability to water vapour, oxygen, carbon dioxide and aromatic compounds) (Abdullah et al. 2022).

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The tensile strength (TS), Young's modulus (YM), elongation at break (EAB) are some of mechanical properties that are necessary for the edible protein films to have good application potential (Shah et al. 2023). Tensile strength represents the maximum stress

the film can support before breaking. Elongation at break is the film's ability to resist changes in shape without breaking. Young's modulus demonstrates the stiffness of the material and the flexibility of the film (Shah et al. 2023). Water vapour permeability (WVP) is also an important property of food packaging because it affects the shelf-life of products (Bhaskar et al. 2023). However, traditional packaging materials present problems at the recycling level, fossil fuel reserves are finite. Besides, plastics can impose a series of risks and health problems when they enter in the food chain as microplastics (*e.g.* cancer) (Hammam 2019) and can be associated to the migration of harmful additives (cadmium or mercury) (Campanale et al. 2020; Pavli et al. 2018). These reasons, are the driver to continue developing sustainable fully biodegradable packaging materials, including edible films and coatings, providing a long shelf-life for bioactive substances in foods and promoting safe packaging integration (Pavli et al. 2018). In this way, packaging materials obtained from biopolymers (proteins, polysaccharides and lipids) represent a good alternative to the petroleum-based materials in order to mitigate the environmental problems, since they are obtained from renewable sources, or industrial by-products that are biodegradable (Gerna et al. 2023; Kandasamy et al. 2021).

Films based on biopolymers or biodegradable polymers have been extensively studied in recent years (Dirpan et al. 2023). Biopolymer materials are derived from renewable sources or by-products. They are increasingly used in the food packaging industry because of their relative abundance, film-forming capacity, biodegradability and nutritional value (Calva-Estrada et al. 2019; Gerna et al. 2023; Ramos et al. 2013). The biopolymers can be obtained from different animal and vegetable sources, extracted from biomass, synthesized from bio-monomers, and produced by microorganisms (Ranganathan et al. 2020; Shaikh et al. 2021). Due to their economic value, the most common biopolymers are polysaccharides (cellulose, starch, chitosan, pectins), and proteins (soy, gelatin, zein, sunflower) (Castro-Rosas et al. 2016; Gerna et al. 2023). Therefore, the development of edible films and coatings from biopolymers is one of the promising strategies in the recent years for the replacement of synthetic polymers.

Recently, edible films and coatings emerged as an alternative to conventional plastics and synthetic films, with the main advantage being that they can be consumed with food products, have the potential to stabilize food structures (extend shelf-life, maintain structural integrity), are biodegradable, renewable, non-toxic and minimize environmental problems (Pérez et al. 2016; Soukoulis et al. 2017; Tavares et al. 2021). Biopolymers derived from renewable agricultural resources or industrial by-products are used in the formulation of edible films and coatings (Tavares et al. 2021).

Edible films and coatings are obtained from polysaccharides, proteins, lipids, or the combination of these components. Recently protein-based materials seem to be the most appealing because they also provide good nutritional value (Galus and Kadzińska 2016). These materials are non-toxic, anti-allergic and biodegradable, generating water, CO_2 and biomass under the action of physical factors and decomposing microorganisms (Gheorghita Puscaselu et al. 2021). In addition, edible films and coatings also have the ability to carry active compounds such as antioxidants and antimicrobials within the film

matrix, which allows to improve their safety and the shelf-life of edible products (Hoque et al. 2021). Powders from natural products, essential oils, dyes, and flavourings, can be incorporated into the film-forming solutions. These substances can be consumed together with the packaging material and can improve the functional characteristics of the final product (Gheorghita Puscaselu et al. 2021).

Films based on whey protein isolate (WPI) and whey protein concentrate (WPC) show great interest due to their high transparency, good flexibility, excellent nutritional characteristics, absence of odour and taste, and they have a good water vapour barrier (He et al. 2022). In addition, protein-based edible films can be applied inside heterogeneous foods at the interfaces between different component layers (Wittaya 2012). However, they have some problems related to texture, namely poor tensile strength, which can arise when WPI is used alone in film systems. Therefore, an important parameter to be discussed is the improvement of the physical properties of WPI films (He et al. 2022).

Films derived from biopolymers also present some disadvantages since their production is limited by high costs, when compared to synthetic plastics derived from fossil petroleum, and also present disadvantages such as higher permeability to water vapour and oxygen, fragility, low thermal resistance, low mechanical properties, vulnerability to degradation and low processability when compared to conventional plastics (Abe et al. 2021; Pop et al. 2019).

For many years, foods have been preserved and given an extended shelf-life by the application of edible films and coatings. (Gaspar and Braga 2023). Fruit was protected with wax in China in the 12th century, which marked the beginning of the usage of coatings for food preservation. Japan was the first country to adopt edible films created from soy for fruit preservation and shining. However, there was little interest in these films because of the limited variety of materials used. Edible packaging did not attract as much attention as techniques like refrigeration, sterilization or radiation, smoking, regulated and/or modified atmosphere, and so forth. Food preservation techniques have developed and grown over time, making it possible to prepare, store, and eat food throughout the year (Pop et al. 2019).

A film or coating is formed from a combination of biopolymers and different additives placed in an aqueous medium and must have a thickness of less than 0.3 mm (Díaz-Montes and Castro-Muñoz 2021). The preparation and application processes are the primary distinctions between a coating and a film. The solution to prepare films and coatings is similar, but coatings are applied to the surface of the food products by dipping, spraying or brushing, whereas films are applied by wrapping the food. However, in both edible films and coatings, rigid matrices are formed with similar characteristics.

Films and coatings protect against ultraviolet light (UV), transport solutes between food and the atmosphere (salts, additives and pigments), control the exchange of water vapour and gases (oxygen, carbon dioxide, nitrogen and ethylene), form a good protective barrier against mechanical damage, increase the shelf-life of the product, may contain bioactive components (antioxidants) and healthy microorganisms (probiotics) that are beneficial to

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health and have antimicrobial and antifungal effects (Díaz-Montes and Castro-Muñoz 2021; Peltzer et al. 2017; Pop et al. 2019).

Edible and biodegradable films reduce the rate of oxygen absorption, the migration of lipids, gases and flavours and prevent the development of undesirable micro-organisms during the storage of food products (Tavares et al. 2021).

The use of edible films plays an important role in encapsulating aromas, polyphenols, vitamins, microorganisms, etc. These films are formed by a preformed thin matrix of a solution or by the dispersion of long chain polymers. It is necessary to remove the solvent from the solution or dispersion, to form the film matrix, thus reducing the distance between the polymers and favouring their interaction. This interaction causes an intertwining of the polymeric chains that increase the viscosity through the formation of a polymeric network (Peltzer et al. 2017). Encapsulation protects against oxidation, isomerization, degradation during storage or processing, extending shelf-life and protecting components from nutritional loss. Furthermore, it can be used to control the release of functional or bioactive components when ingested since the encapsulated component remains intact in the stomach and when it reaches a specific physiological pH, it starts to be released in the intestine. Controlling the release of an additive can extend the shelf-life of foods. The use of encapsulation is important due to several factors such as masking off-flavours, colours or odours, increasing the viability of bacteria, since encapsulation facilitates cell handling and allows dosage control (Peltzer et al. 2017).

Films can be produced from materials with film-forming capacity and the components used are classified into three categories: hydrocolloids, lipids and composites (Pereira et al. 2016). Even if they are not consumed with food, they can be more quickly and easily degraded when compared to plastic materials (Pop et al. 2019).

Films based on hydrocolloids (polysaccharides and proteins) and lipids are the most used materials, but films based on polysaccharides, due to the presence of a large number of hydroxyl groups and hydrogen bonds, favour the formation of the film and are thus easy to obtain. Different properties can be observed between films and coatings made from negatively charged gums (alginate, pectin or carboxymethylcellulose) (Pop et al. 2019).

The proteins used for the films and coatings are usually of animal origin (gelatin, casein, whey protein, collagen, or ovalbumin). However, plant-based proteins are important for a vegetarian diet (corn, soy, wheat, cottonseed, peanuts, and rice). For the formation of films and coatings, the protein is initially denatured by heat or pH adjustment, followed by a conglomeration of peptide chains through new intermolecular interactions. This type of film is particularly suitable for meat products, as it has an affinity for the hydrophilic surfaces present in meat (Pop et al. 2019).

Lipids (waxes, paraffins, glycerides) do not form cohesive films, unlike hydrocolloids, and are therefore used especially for coatings, or mixed with polysaccharides to obtain an optimized barrier to water vapour (Díaz-Montes and Castro-Muñoz 2021; Pop et al. 2019).

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Depending on the biopolymers used to form edible films, and depending on environmental conditions such as humidity, these films may contain water of hydration that acts as a plasticizer by fitting into the polymeric chains, spacing them, decreasing the transition temperature glass, and improving flexibility. So, water of hydration affects the mechanical and barrier properties of the film. Thus, edible films are classified as hydrophilic, hydrophobic, or films containing both hydrophilic and hydrophobic components (Peltzer et al. 2017).

Hydrophilic films are films made of polysaccharides and proteins that strongly interact with water. Hydrophobic films are formed from natural lipids, fats and waxes. Since lipids have low polarity and therefore have a weak interaction with water, these films have good barrier properties to water vapour, whereas the barriers to oxygen and carbon dioxide are weak. Composite, hydrophilic and hydrophobic, films combine the advantages and lessen the disadvantages of each component. Hydrophilic components are a good oxygen barrier, and hydrophobic components are a good water vapour barrier. These films can be formed by a single layer or by multilayers. Firstly, the layer of the film based on polysaccharides or proteins is formed and then the lipid layer is applied. The properties of these films have been studied showing good barrier properties, but separation and fracture of the lipid layer has been observed as a disadvantage (Peltzer et al. 2017).

The properties of films and coatings are very important as they control the transmission of water vapour and other gases, allowing for the safety and prolonging the shelf-life of food. To obtain films with good properties, they must have good flexibility, optical transparency, thermal stability, mechanical resistance, biodegradability and high gas barrier properties (Cazón et al. 2017). For consumers, transparency and sensory properties are also very important (Yaashikaa et al. 2023).

The barrier properties, such as water vapour permeability (WVP) of a polymeric film protect food and extend its shelf-life (Cazón et al. 2017). The measurement of WVP in the films allows quantifying the amount of water that permeates per unit area and time (kg/m.s.Pa). WVP of films and coatings is an important parameter to be evaluated because it prevents dehydration and also prevents the absorption of moisture from the environment into the food (Cazón et al. 2017). Relative humidity, temperature and water resistance are also extremely important, and are affected by film and coating thickness (Matloob et al. 2023). Mechanical properties of films, including tensile strength (TS), percentage of elongation at break (EAB) and Young's modulus (YM), depend on the composition of the film and the nature of its components. These properties allow the film to resist external stresses, maintaining its integrity and thus allowing food to be protected (Cazón et al. 2017). However, mechanical properties are influenced by the type of material and manufacturing method of films and coatings (Díaz-Montes and Castro-Muñoz 2021).

Edible films and coatings are classified in three ways:

1) hydrocolloids, based on proteins (example: whey protein, casein, gelatin, wheat gluten, soy protein, etc.) or polysaccharides (alginates, cellulose and its derivatives, starch, chitosan, etc.).

2) lipids (waxes, acylglycerols and fatty acids);

3) composites (blends containing two or more components to produce films or coatings with improved physicochemical properties) (Tavares et al. 2021).

Table 6 describes the principal biopolymers used and their functionalities for edible films and coatings.

Table 6: Main biopolymers used and functionality in the manufacture of edible films and coatings

Materials	Example of materials	Properties	Functionality
Polysaccharides	Alginate, Pectin, Cellulose, Starch, Chitosan, Agar	Thickeners Gellants Emulsifiers Stabilizers Coating	Polysaccharides form the structure of a solid polymer matrix.
Proteins	Whey protein, Gelatin, Casein, Collagen, Ovalbumin	Gellants Thickeners Stabilizers Foaming Firmness Coating	Transport of antimicrobials and antioxidants. They control the transport of gases (oxygen).
Lipids	Waxes Paraffin Glycerides	Coating	Avoid drying or dehydration and give flexibility. Prevent moisture migration and water vapour transmission.

Adapted from (Díaz-Montes and Castro-Muñoz 2021; Pop et al. 2019; Priya et al. 2023).

Hydrocolloids, proteins, polysaccharides and lipids can be used in the preparation of edible films and coatings, and can be of vegetable or animal origin (Alipour et al. 2023). The group of hydrocolloids or gums is composed of polysaccharides and proteins (Pavli et al. 2018). Hydrocolloids obtained from animal or vegetable sources are widely used in food systems as gelling agents, thickeners and stabilizers (Alipour et al. 2023). Hydrocolloids are obtained by the hydrophilic dispersion of colloids and are composed of long-chain polymers. They tend to swell when into contact with water and can partially or completely disperse, forming stable gels that show changes in their physicochemical properties, viscosity, thickness, and stability (Liyanapathiranage et al. 2023). Some examples of hydrocolloids are alginate, agar, xanthan, carrageenan, guar, pectin and locust bean gum (Zibaei et al. 2021). The hydroxyl functional groups of polysaccharides allow them to be widely used in the preparation of hydrocolloid films due to their water solubility. In the case of proteins, they are also used to form hydrocolloid films, in some cases due to the structural hydrophilicity and intrinsic polydispersity of protein colloids (Liyanapathiranage et al. 2023). Most polysaccharides used for edible films and coatings are cellulose derivatives, dextrans, inulin, alginate, carrageenan, starch derivatives, pectin derivatives, chitosan, seaweed extracts and galactomannans (Pavli et al. 2018).

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Composite films are heterogeneous in nature and are obtained by combining biopolymers that mix hydrophobic and hydrophilic materials to achieve desired properties. Depending on the number of biopolymers combined, they are categorized as binary or ternary. Combinations of synthetic and/or natural composites and polymers are also used, giving rise to binary films and coatings such as protein-protein, carbohydrate-carbohydrate, carbohydrate-protein and lipid-based binary films (Liyanapathiranage et al. 2023).

9.2. Protein based films and coatings

Proteins are fibrous or globular in their natural form. Fibrous proteins are generally insoluble in water, are fully extended and closely associated in parallel structures, usually through hydrogen bonding, to form fibres. They are the main structural material of animal tissues (example collagen). Globular proteins are soluble in water or aqueous solutions like acids, bases or salts. The globular proteins fold into intricate spherical structures held together by a combination of hydrogen, ionic, hydrophobic and covalent (disulfide) bonds. The chemical and physical properties depend on the relative amounts of the constituent amino acid residues and their arrangement along the protein polymer chain. They have many functions in living systems (Wittaya 2012).

Proteins are found in a variety of plant and animal sources (Mihalca et al. 2021). Proteins are excellent raw materials for the production of films. The excellent barrier and mechanical properties of protein films make them a material with enormous potential for use in food packaging (Chen et al. 2019b). Animal proteins used in films can be whey proteins, casein, gelatin, collagen, fish myofibrillar protein, egg white protein and keratin. Vegetable proteins can be wheat gluten, corn zein, mung bean, soy protein, peanut protein, and cottonseed protein. Protein-based films have a high affinity for polar substances (water) but have a low affinity for non-polar substances (oxygen, aromas and oils). The addition of some other materials can reduce their sensitivity to humidity. However, permeability and humidity depend on the concentration and type of plasticizer used to form the film (Sothornvit and Krochta 2005). When compared to polysaccharides, proteins have lower water vapour permeability (Pop et al. 2019). As an example, a soy protein-based film has 260, 500, 540, and 670 times less permeability to oxygen than low-density methyl cellulose, polyethylene, starch, and pectin-based films (Yashwant et al. 2023). The tensile strength of protein films is best when compared with polysaccharide films (*e.g.* the tensile strength of gelatin protein films is 68 MPa, and the tensile strength of chitosan polysaccharides films is 41 MPa) (Khin et al. 2024).

To produce protein-based films, the proteins are dissolved or dispersed in solvents (water or ethanol) which are subsequently evaporated to obtain the packaging. The formation process of these films is favoured by heat or acid conditions. Milk proteins are classified as caseins and whey proteins, which can form malleable, transparent and tasteless films. Caseins form stable films at different pH values, different temperatures and different salt levels. Antioxidants, food colouring or antibacterial compounds can be added to improve the properties of the films (Mihalca et al. 2021).

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Protein films based on WPI or WPC demonstrated better water vapour barrier properties (WVP) than competing protein-based films (corn, zein and isolated soy protein), and according to Ramos et al., they can be comparable to synthetic polymer films available on the market (Table 7). However, their mechanical characteristics suffer some limitations (TS, EAB and YM values are lower when compared with plant proteins based films) (Ramos et al. 2012b). WPI films are stronger and more flexible when compared with WPC films. Protein films show better gas barrier properties, have specific mechanical characteristics and a greater intermolecular binding capacity when compared to films based on polysaccharides and lipids (Murrieta-Martínez et al. 2018; Ramos et al. 2012b).

Table 7: Mechanical properties of different protein sources.

Protein Source	TS (MPa)	EAB (%)	YM (MPa)	WVP (g mm/m² h kPa)	Thickness (µm)
Soy*	1,59	105,3	87,4	-	82
Zein	4	118	-	4	-
Milk Casein	2-77	2-130	-	1,6-11	-
WPC	0,7-0,9	18	-	6.2-12.8	132
WPI	0,9-1,49	33	24,71	4,57	119,86
Grass pea*	8,59	68,3	483,0	-	110

TS (MPa) tensile strength; EAB (%) elongation break; YM (MPa) young's modulus; WVP (g mm/m² h kPa) water vapour permeability; WPC whey protein concentrate; WPI whey protein isolate *(at pH7).
Adapted from (Abdalrazeq et al. 2019; Chen et al. 2019b; Etxabide et al. 2023).

9.2.1. Improvement of protein film's properties

Various methods are being studied to improve protein films, to make them stronger and less permeable. Modification of proteins (denaturation), use of other macromolecules (polysaccharides and lipids), use of bioactive compounds (microbial agents, probiotics, antioxidants), and plasticizers, are some examples (Linares-Castañeda et al. 2023).

Cross-linking is an effective way to improve waterproofing, cohesion, rigidity, mechanical strength and water barrier (Wittaya 2012). The cross-linking by the formation of stronger intermolecular covalent bonds in protein networks closer to the molecule's packaging and the reduction of polymer mobility are methods that are being used in the formation of protein films. Protein cross-linking can be done through heating, acid/base treatments, addition of aldehydes, phenolic compounds, $CaCl_2$, enzymes, irradiation and ultrasonic/microwave-assisted treatment (Calva-Estrada et al. 2019).

As explained earlier, the functional properties of films and coatings can be improved by chemical, physical or enzymatic cross-linking. A long chain structure with low permeability and greater tensile strength should result through increased protein interaction with chemical treatments. Enzymatic crosslinking techniques use enzymes such as lipoxygenase, polyphenol oxidase, transglutaminase and peroxidase to crosslink proteins. In this case, a specific enzyme known as transglutaminase, which is capable of catalysing covalent cross-linking processes between proteins, forms high molecular weight biopolymers (Purewal et al. 2023). The use of γ-radiation or UV radiation modifies the conformation of proteins (Díaz et al. 2016; Lacroix et al. 2002) oxidizes amino acids, breaks covalent bonds, and produces free radicals within the protein, being able to transform proteins with greater molecular weight through electrostatic and hydrophobic

interactions, the creation of disulfide bonds and interprotein cross-linking reactions (Purewal et al. 2023).

Combining lipids and proteins to create a continuous, cohesive network improves the performance of films and coatings. The lipids disperse within the hydrocolloid matrix, forming emulsified films, or they can form a layer on top of the hydrocolloid matrix, leading to the development of bilayer films. Emulsified films received great attention, through the "microvoid model", according to which, the transfer of gases and vapours occurs through microvoids that emerge during the drying of the emulsion, forming a hydrocolloid matrix. Another way is the "microvia model", in which mass transfer occurs through the highly polymeric matrix itself. Thus, the addition of lipids to films and protein coatings gives flexibility to the film and can interrupt interactions between polymer chains (Purewal et al. 2023).

However, these methods do not change the sensitivity to humidity that protein films have, since it is not possible to replace all reactive proteins. Another alternative method for improving the barrier and mechanical properties of protein films is developed by combining the properties of several different polymers (composite films) (Calva-Estrada et al. 2019). Protein-Protein Composite Films (Tsai and Weng 2019), Protein-Polysaccharide Composite Films (Fitriani et al. 2021), Protein-Lipid Composite Films (Erdem and Kaya 2022), Protein-Polymer Composite Films (Li et al. 2021), and Nanocomposite Films (Jafarzadeh et al. 2023) are the composite films that are being studied.

Blends of proteins for the development of biodegradable films allows for an improvement in their physical and/or mechanical properties when compared to isolated protein films (Calva-Estrada et al. 2019). The microstructural and physical properties of WPI, and gelatin composite films were investigated by Jiang *et al*. The authors observed the formation of a compact film in which a shrinkage of gelatin molecules was observed in the whey protein isolated/gelatin ratio 50:50. However, this aggregation process made the microstructure of the film discontinuous and led to lower perforation resistance of the film. The deformation and the water vapour permeability of the composite films increased with the gelatin content, but in terms of the moisture content and the solubility, significant variations were not observed (Jiang et al. 2010).

The improvement in the mechanical properties of protein-polysaccharide films is attributed to the strong intermolecular interactions (hydrogen bonding, dipole-dipole bond formation and charge effects) between hydroxyl groups of the polymer chains. Furthermore, cross-linking and heat treatment allow bonds between protein and polysaccharide chains (via Maillard reactions) and thus improve the mechanical properties of the polymer network (Calva-Estrada et al. 2019). Chakravartula and coworkers studied edible films composed with different proportions of pectin, alginate and WPC formulated with a simplex centroid mixture design. The matrices showed good film formation capacity, except for whey protein at a certain concentration, with thickness, elastic and optical properties correlated to the initial viscosity of the solution. Whey protein reduced the viscosity of the starting solutions compared to alginate or pectin

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solutions and decreased mechanical strength as well as water affinity. Pectin influenced the yellowing index, while alginate and whey protein affected the opacity of the film. Whey protein favoured opacity, gas barrier properties and originated dense structures, resulting from polysaccharide-protein aggregates. All films showed good thermal stability (Chakravartula et al. 2019).

The moisture barrier properties of protein-based films can be improved by adding lipids to their formulation and thus improve their mechanical properties. The addition of hydrophobic compounds to protein-based film formulations such as waxes, fats, oils and fatty acids is important to improve their properties. Protein-lipid composite films consist of a continuous phase (matrix protein) and a dispersed phase (lipid) typically produced by emulsification, or thin layer by layer lamination. In the case of emulsification, lack of homogeneity with the film matrix due to differences in lipid droplet size distribution is a problem. In the case of thin layer by layer lamination they face problems in terms of cracking and/or delamination (Calva-Estrada et al. 2019).

In the case of films and coatings produced with denatured whey protein, hydrogen and disulfide bonds play a major role in the formation of films and coatings, while using whey protein in its native form only hydrogen bonds contribute to the formation of films and coatings (Kandasamy et al. 2021). Denatured whey protein films are insoluble in water, since their solubility decreases with temperature. They have higher tensile values, and the WVP remains identical when compared to native whey protein films. The latter are soluble in water and have lower traction values. Its opacity is also higher than the opacity of denatured whey protein films. Thus, increasing time and temperature in the film-forming solutions greatly improves firmness, robustness and extensibility. Therefore, the covalent cross-linking produced by thermal denaturation in whey proteins is responsible for the insolubility, increased mechanical resistance and O_2 barrier properties of the film, without changing the WVP of the film (Kandasamy et al. 2021).

An alternative way to improve protein-based films is the use of nanocomposites. Polymeric nanocomposites exhibit improved packaging properties due to their nanosized dispersion (Rhim and Ng 2007). Due to the nanometric size of its particles, application in biopolymers leads to improvements in mechanical and barrier properties. About the mechanical properties of nanocomposites, there are large increases in the material's stiffness [Young's modulus (YM)] and they can delay the passage of gases and water vapour, thus improving barrier properties. Another advantage is that nanocomposites do not affect the transparency of the films, since their dimensions are smaller than micro and macro composites (Schmid et al. 2017). An important advantage is that biologically active ingredients can be added to impart the desired functional properties to packaging materials resulting from nanocomposites, thus offering enormous potential for application in the active food packaging industry (Rhim and Ng 2007).

Studies have also been carried out to optimise protein-based films using conventional and unconventional techniques. Thus, the use of thermal and non-thermal treatments to improve the properties of biodegradable edible protein films has been developed. The application of non-thermal and thermal treatments has been successful since it has been

possible to form protein films with good mechanical and functional properties (Yashwant et al. 2023). Yashwant and co-workers provided an extensive review for study the impact of non-thermal and thermal techniques on protein-based films properties and their principal applications (Yashwant et al. 2023).

9.2.2. Whey protein films and coatings

The use of whey protein to make films has received a lot of attention, since they are edible and biodegradable, have excellent mechanical properties, and are transparent, flexible, colourless and odourless. Besides, they also represent a benefit to the environment because made possible the recovery the by-products from cheese manufacturing and create a new product with good nutritional and functional properties (Ramos et al. 2013).

Whey protein films are generally obtained from whey protein isolates (WPI) and whey protein concentrates (WPC) (Ramos et al. 2013). WPI have proteins with hydrophobic and thiol groups placed in the globular structure. Heat-induced denaturation of such proteins alters the 3D network, opening their structure, exposing sulfhydryl and hydrophobic groups that interact with other molecules and form strong covalent disulfide intermolecular bonds (Tonyali et al. 2018). Commercial WPC and WPI contain about 35-80% and 90% protein respectively, and their main components are β-lactoglobulin and α-lactalbumin, which are easily dissolved in water. WPI and WPC differ in their protein levels as well as in the levels of other constituents such as lipids, minerals and, particularly, lactose. These differences strongly influence the intermolecular bonds of the films produced from WPC and WPI and, consequently, affect their barrier, mechanical and thermal properties (Ramos et al. 2013; Tsai and Weng 2019).

The edible whey protein film is formed by a highly interactive dry polymeric network and has a three-dimensional gel-like structure. Depending on the techniques for forming these materials, the final films can result in a spatially rearranged gel array that includes all additional film-forming agents. These films are obtained through the casting method, which consists of pouring the solution onto a flat surface to produce a dry gel, and thus applying this film as an envelope to food products. In the case of coatings, food products are immersed in the film-forming solution (approximately between 30 s and 60 s) to ensure complete surface exposure with good adhesion and perfect integrity. Both films and coatings based on whey are flexible, colourless and transparent. Whey films contain some distinguishing features such as their amphiphilic nature, denaturing and electrostatic charges, and other factors such as charge density and hydrophilic-hydrophobic balance, which can modify their conformation as these factors define the physical and mechanic properties of films and coatings (Kandasamy et al. 2021).

Films made from whey protein have also received a lot of attention as they are edible and biodegradable. They have good physical properties at low relative humidities, are impermeable to oxygen, and have a good aroma, but have high water vapour permeability due to their hydrophilic nature. This property can be significantly improved by combining these films with hydrophobic materials such as essential oils (EOs) or plant extracts, which have been shown to reduce WVP by increasing the content of hydrophobic groups

(Alipour et al. 2023; Gerna et al. 2023). Another advantage of these films is that they are easily dissolved in water and can be prepared using water as film-forming solvent (Tsai and Weng 2019). The films obtained from whey protein are characterized by transparency, flexibility, lack of odour and taste, which favour their acceptance by consumers (Tavares et al. 2021).

9.2.3. Methods to obtain whey protein films and coatings

Protein-based films have an initial amino acid sequence that determines the interactions between the protein chains themselves, as well as with other components of the film. For the formation of a protein film, the structure of the chain and its conformation depend on the rearrangement of amino acids (Schmid and Müller 2019). For whey proteins in particular, the thiol groups contained in the cysteine residues can form disulfide bonds. β-Lg (50% of total whey proteins) is the principal protein responsible for aggregation and gelling behaviour of whey proteins. β-Lg has five cysteine residues, of which four of them form two S-S bonds and the fifth cystein is hidden in center of native β-Lg structure and it is reactive when this protein is denatured being decisive for film and coating formation (Kandasamy et al. 2021).

α-La is the second largest fraction of whey proteins (20% of total whey proteins) and contains eight cysteine residues forming four S-S bonds. Calcium ions help to maintain the tertiary structure and provide stabilization against heat denaturation (Kandasamy et al. 2021); so, it has a higher thermal stability as compared to β-Lg. Another important whey protein, serum albumin (SA) (8% of total whey proteins) contains 17 disulfide bonds and one free thiol (Kandasamy et al. 2021). Whey protein's ability to change chain conformations and interact with each other to form modified three-dimensional networks are excellent properties for use in films and coatings (Schmid and Müller 2019). The process for formation of the whey protein films is the same used for other protein film's formation. The first step in preparing a film is the unfolding of the protein's native state, relieving low-energy intermolecular bonds. Protein unfolding and dissociation can be caused by various treatments, such as temperature change, pH change, shear forces, or addition of organic solvents or salts (Schmid and Müller 2019).

For preparation of whey films and coatings it is necessary to dissolve WPC or WPI (5–12%) in distilled water and adjust the pH of the solution (normally to 7 or 8). This step is named "solvent process", where proteins are dispersed and solubilized in a solvent, such as water or ethanol (Kandasamy et al. 2021; Zink et al. 2016). Plasticizers are placed in the film or coating forming solution (Kandasamy et al. 2021; Zink et al. 2016). Then it is necessary to heat the solution (80-90 °C, 10-30 min) for protein denaturation. The process for thermal denaturation ("thermoplastic process") involves several phenomena at pH 6.0. First, at a temperature greater than 40 ºC, β-Lg dimers dissociate into monomers. Denaturation occurs at 65 °C and unfolds the β-Lg molecule exposing hydrophobic and thiol groups, which bond to other smaller β-Lg-containing aggregates or to other thiol-containing proteins. These smaller aggregates then interact to produce high molecular weight irreversible aggregates. The temperature for the irreversible denaturation of β-Lg is 69 ºC and for α-La is 80 ºC. β-Lg acts in the polymerization of WP and defines the

degree of denaturation in WP. The aggregation rate is also affected by the free sulfhydryl groups that exist on β-Lg.

In these films other ingredients can be added before or after heating. Ingredients that can be heated (prebiotics, starches, blends) are included early in the process, while heat-sensitive ingredients (antioxidants, antimicrobial compounds, probiotics) are included after heating. Whey films that are produced without heat treatment break very easily during the drying process due to weak intermolecular interactions that occur (Kandasamy et al. 2021).

9.2.4. Advantages and disadvantages of protein edible films and coatings

For be benefit for human health, protein films and coatings have other advantages such as being made from natural materials, safe to eat, are environmentally friendly, and may include colorants, antioxidants, and anti-browning agents (Hassan et al. 2018). Edible protein films and coatings help to preserve the antioxidants, phenolics, and additives of the food for a long time (Liyanapathiranage et al. 2023).

Materials for edible films and coatings should be chosen according to specific food applications, food products and the main mechanisms of food quality deterioration involved (Han 2005).

Despite the studies carried out and the progress in the development of protein films and coatings as packaging materials, their physical properties are still inferior to those of synthetic packaging, as they have a weaker structure and lower elongation capacity. Regarding barrier properties, these protein films have a sensitive permeability (sometimes highest permeability) to water vapour due to their hydrophilic nature when compared with some polymeric synthetic films. Besides, since the cohesive energy density of proteins is strong, protein based in biopolymers are more fragile (Calva-Estrada et al. 2019; Chen et al. 2019b). Another disadvantage is the inability to make large films (>25 cm), difficulties in maintaining a uniform thickness in the film, and a long drying time (2-3 days) (Kumar et al. 2022a).

To improve its structure and functionality, several methods are under investigation. Methods such as plasticisation, cross-linking through the formation of stronger intermolecular covalent bonds, the development of composite films by blending with other polymers, the application of nanotechnology through the incorporation of different nanoparticles are the main focus of research (Calva-Estrada et al. 2019; Oymaci and Altinkaya 2016).

9.3. Plasticizers in edible films and coatings

Plasticizer was defined by the International Union of Pure and Applied Chemistry (IUPAC) as “a substance or material incorporated in a material (usually a plastic or elastomer) to increase its flexibility, workability, or distensibility” (Chen et al. 2019b).

Plasticizers are generally required for the formation of edible films and coatings, especially when polysaccharides or proteins are used as base materials. Plasticizers and

emulsifiers are added to film-forming solutions to improve their mechanical properties or to increase stability when lipids and hydrocolloids are combined, to prevent film breakage and to facilitate handling and storage, as they provide flexibility and elasticity by reducing intermolecular forces and increase polymer chain mobility (Díaz et al. 2019).

9.3.1. Effect of plasticizer type on film and coating properties

The type of base material, plasticizers, bioactive compounds, and other components employed are critical for optimal film-forming (Dinika et al. 2020). Plasticizers for films can be defined as small, low molecular weight, non-volatile compounds added to polymers or proteins to reduce brittleness, provide flexibility, and increase film strength. As a specific definition for coatings, plasticizers reduce peeling and cracking, improving the flexibility and strength of the coating. In general, plasticizers reduce forces along polymer chains, thereby increasing free volume and chain movements (Sothornvit and Krochta 2005). Plasticizers are defined in two different ways: (1) plasticizers that are capable of forming hydrogen bonds and thus interact with polymers, interrupting the polymer-polymer bond and maintaining the distances between polymer chains and; (2) plasticizers that interact with water, retaining more water molecules, and thus leading to a higher moisture content and a larger hydrodynamic radius (Ramos et al. 2012b).

The most common plasticizers used are polyols (glycerol, sorbitol, and polyethylene glycol 400), mono-, di-, or oligosaccharides (glucose, fructose-glucose syrups, sucrose, honey), and lipids and their derivatives (phospholipids, fatty acids, surfactants). In some cases, water molecules can also act as plasticizers in starch films, causing a phenomenon known as retrogradation, which is a rearrangement of amorphous starch chains in the presence of moisture during storage. Plasticizers have low molecular weight and are incorporated in film-forming materials as they lower the glass transition temperature (Castro-Rosas et al. 2016; Pavli et al. 2018; Sothornvit and Krochta 2005). An important parameter of hydrophilic plasticizers is the fact that they adsorb water, which affects the water barrier properties also leading to the destabilization of proteins. Thus, water plasticizes the hydrophilic phases, affecting the physicochemical properties and leading to possible changes in the properties of the material (Huntrakul and Harnkarnsujarit 2020).

Protein-based films require incorporation of plasticizers to reduce their brittleness, to allow easier removal from casings when formed, and to support and impart plastic properties. Plasticizing molecules lead to decreases in intermolecular forces along the polymeric chains, thus improving the flexibility, extensibility, tenacity and tear strength of the film, but have the disadvantage of decreasing its mechanical strength and barrier properties (Ramos et al. 2013). Glycerol produces the best effects on whey protein films, leading to more stable, flexible and less brittle films under various relative humidities (Ramos et al. 2013). Most plasticizers have hydroxyl groups that form hydrogen bonds with biopolymers and increase the free volume and flexibility of the film matrix. Plasticizers have different numbers of hydroxyl groups and are in different physical states (solid or liquid), which causes them to change the degree of rigidity and softening of the film (Sothornvit and Krochta 2005).

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The barrier properties of whey protein films are superior to those of polysaccharide films. Nevertheless, these protein films have limitations regarding their mechanical characteristics since the molecular weight of the WPI has a strong influence on the fragility of the film. Thus, the use of some additional structuring agents, such as plasticizers to avoid fragility, emulsifiers to stabilize the base emulsion, lipids to increase WVP and polysaccharides to improve barrier properties is widely adopted (Kandasamy et al. 2021).

Whey protein edible films and coatings plasticizer content varies between 10% to 60% (w/w). These levels depend on the properties of the film and on the type of plasticizer used. Generally, the proportions of plasticizers in concentrated whey protein films are lower, since non-protein components such as fat and lactose can interfere with film formulations (Kandasamy et al. 2021). The plasticizer glycerol used at very high levels in whey protein films improves moisture content and solubility, and water vapour barrier but in turn reduces mechanical strength, Young's modulus and glass transition temperature (Kandasamy et al. 2021). The evaluation of the effect of two different plasticizers (glycerol and sorbitol) on the mechanical and barrier properties of whey protein films was carried out by Anker *et al*. The moisture content of WPI/glycerol films decreased until the 45th day and then, remained constant until the 120th day. This affected the mechanical properties (increased stress at break, decreased strain at break and increased glass transition temperature). Barrier properties were not affected (Anker et al. 2001). Shaw and coworkers evaluated the effects of many different plasticizers, like glycerol, xylitol and sorbitol, on the physical properties of whey protein isolate films. These authors found that increasing the glycerol or sorbitol content led to increases in moisture content, water vapour permeability and elongation, but led to decreases in tensile strength, elasticity and glass transition temperatures of the films. Increasing the xylitol content had no effect on permeability, moisture content or glass transition temperature, but decreased the elongation, tensile strength and elasticity of the films. They also concluded that the moisture content of the films correlated well with the glass transition temperatures and that the differences in the physical properties of the films depending on the type and concentration of the plasticizer can be attributed to differences in the hygroscopic and crystalline properties of the plasticizers (Shaw et al. 2002).

The study by Huntrakul *et al*., demonstrates the effects of plasticizers glycerol, xylitol, sorbitol, polyethylene glycol 400 (PEG 400) and oleic acid on water sorption, phase transitions and other physical, mechanical, barrier and stability properties of WPC and of mixed WPC-carboxymethilcellulose films. The effects of water sorption at 50 and 75% relative humidity on film stability were also studied. The results obtained demonstrated that the use of plasticizer to modify the properties and stability of the films based on mixtures of proteins and polysaccharides has a positive impact on bioplastic film properties (Huntrakul and Harnkarnsujarit 2020). Perez *et al.* developed and characterised edible films made from WPC and plasticised with different levels of glycerol and/or trehalose to evaluate new edible film formulations. In addition, changes in the mechanical properties of the film during storage at room temperature and under freezing conditions were verified. The authors concluded that both films had good transparency, as the

addition of trehalose to the film formulation proved effective in preventing the Maillard reaction, which darkens WPC-based films and can also generate potentially toxic by-products that can cause a loss of nutritional value from the whey constituents of the edible film. However, they also found that the use of trehalose did not significantly improve the physicochemical properties of the films during long-term storage at different temperatures (Pérez et al. 2016).

The evaluation of the impact of fish gelatin hydrolysates with varying degrees of hydrolysis (23, 61, and 95%) employed as plasticizer at varied levels (30-60%) on the fish myofibrillar protein film was another significant study conducted by Nuanmano and colleagues. Fish myofibrillar protein films added with gelatin hydrolysate exhibited a decrease in stiffness and tensile strength with increasing level of gelatin hydrolysate and showed significant improvement in WVP properties when compared to films containing glycerol in the same amounts. However, they found these films showed increased yellowing and decreased transparency (Nuanmano et al. 2015).

As alternatives to ionic liquids, deep eutectic solvents have started to gain prominence in green chemistry, since they offer several advantages, such as biodegradability, low toxicity, and low cost. By combining different mixtures, which associate by complexation with a hydrogen bond acceptor (HBA) (halide salts of quaternary ammonium or phosphonium cations) and a hydrogen bond donor (HBD), such as citric acid, urea, glycerol, they form a liquid solution with varying properties and present a depression in their melting temperature when compared to the freezing points of their individual components. Natural deep eutectic solvents are recently being studied as plasticizers for the formation of food films and coatings. They have excellent antifreeze properties, low cost, non-volatility, non-toxicity, high biodegradability and have antimicrobial properties that offer biosafety (Almeida et al. 2018; Mercadal et al. 2024). Natural deep eutectic solvents are composed of constituents such as sugars, alcohols, choline derivatives, amino acids and organic acids (such as acetic acid). When combined with pectin, starch, cellulose, chitosan and agar, they produce flexible, transparent films with improved tensile strength (Boiteux et al. 2023).

The use of natural deep eutectic solvents was tested by Mercadal and coworkers. They described the use of this type of solvents in soy protein isolate (SPI) for manufacturing functional packaging materials. A neoteric eutectic solvent based on choline chloride/tannic acid and a mixture of choline chlorie/citric acid to be integrated into the protein matrix in high proportions were used. The characterization was carried out by evaluating morphological, physicochemical and mechanical properties, as well as their antioxidant and antimicrobial activities. The authors concluded that these films had excellent resistance to water swelling, showed good tensile strength and a greater plasticizing effect when compared to traditional protein films with glycerol as a plasticizer. Furthermore, the reduction in water vapour permeability highlights the dense interconnection between protein chains. In terms of opacity, they observed high opacity and very effective antimicrobial activity (Mercadal et al. 2024).

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9.3.2. Advantages and disadvantages of plasticizers in films and coatings

Films and coatings can be fragile due to intermolecular forces (hydrogen bonds, electrostatic forces, hydrophobic bonds, and disulfide bonds). Therefore, the use of plasticizers is necessary to interrupt intermolecular forces, providing greater flexibility and toughness. But plasticizers increase the permeability of the film to moisture, oxygen, aroma, and oils, which are disadvantages. Thus, there are advantages and disadvantages associated to the use of plasticizers in edible films and coatings (Sothornvit and Krochta 2005). They function as plasticizers and as antiplasticizers. Plasticization involves the reduction of intermolecular forces between polymer chains, thus improving the flexibility of the film and coating. Antiplasticization can occur at lower concentrations and temperatures of the plasticizer, reduces the free volume of the polymer, interferes with the interaction between the polymer and the plasticizer gives rigidity to the film due to the presence of rigid plasticizer molecules adjacent to the polar groups of the polymer. Furthermore, when a low concentration of a plasticizer addition occurs, there may be an increase in the crystallinity of the polymer since there is a reduction in the energy barrier for a change of state of the polymer, and the formation of ordered structures within amorphous regions increases. Antiplasticization decreases the WVP of the edible films and coatings, and increases the elastic modulus and brittleness, the opposite behaviour of plasticization (Sothornvit and Krochta 2005). Therefore, plasticizers may have the disadvantage of increasing the permeability of the film. Other disadvantages are that plasticizers are subject to aging, extraction, migration, volatility, and exudation. These phenomena limit their effectiveness and leave levels of plasticizer that can be harmful to the properties of the film (Sothornvit and Krochta 2005). A recent alternative to improve the properties of plasticizers in films (until now, only applied in polysaccharides films) is the use of NADES because, as previously mentioned, they have antifreeze properties, low cost, are non-volatile, non-toxic, have high biodegradability and antimicrobial properties that offer biosafety (Mercadal et al. 2024).

9.4. Functional agents in edible films and coatings

Edible films with functional agents such as antimicrobials, antioxidants and enzymes have been studied and used to improve the shelf-life and quality of food products. The addition of different additives (organic acids, essential oils, plant extracts, antibacterial compounds and probiotic microorganisms) in the films and coatings has a great interest for food preservation, since oxidation and microbial deterioration are the main problems that affect food quality and safety (Benbettaïeb et al. 2019; Pop et al. 2019; Tavares et al. 2021). Films and coatings can be used not only as protectors, but also as carriers of bioactive substances (antimicrobial compounds, probiotics, anti-browning compounds, Ω -3 fatty acids and other nutraceuticals). Active food packaging with bioactive molecules acts as a traditional protection system and promotes consumers health (Figure 5) (Pop et al. 2019).

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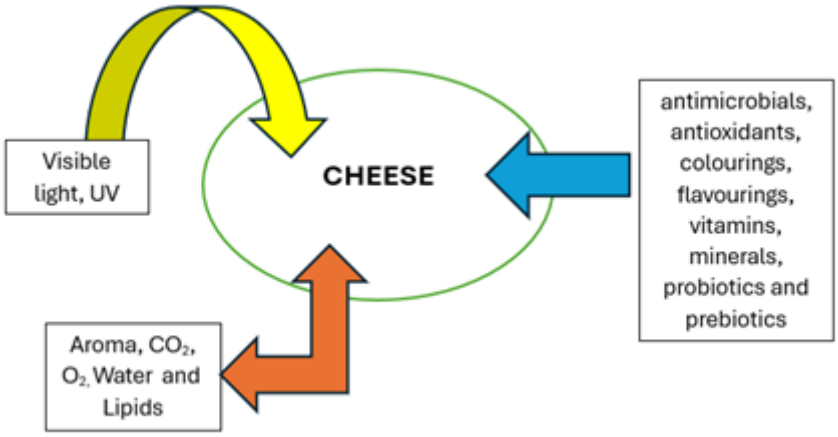


Figure 5: Example of the functional properties of films and coatings on cheese.

9.4.1. Prebiotics and probiotics

In recent years, the consumers are demanding healthier and natural foods, minimal processing and reduction or absence of additives. Foods that contain probiotics and prebiotics confer health benefits when consumed in adequate amounts (Sogut et al. 2022). The recommended dose for good benefits for human consumes of probiotic viable cells is that of 10^{8-9} per day (Pavli et al. 2018; Pop et al. 2019).

Foods that naturally contain probiotics (for example, *Lactobacillus acidophillus*, *Bifidobacterium* sp. and other probiotic *Lactobacillus* species) or prebiotics can be used as dietary supplements, as they are good for combating lactose intolerance (Pavli et al. 2018) increase resistance to invasion by pathogenic bacteria in the intestine (Pereira et al. 2016) stimulate the immune system and also help against cancer (Le Leu et al. 2010; Pavli et al. 2018).

Thus, the use of probiotics and prebiotics in foods that naturally contain them or by their incorporation has been increasing since they allow to produce healthy and safe products. However, despite the benefits of these products, ensuring the viability of microorganisms during the processing and storage and, after consumption, through its transit in the digestive tract has been a challenge for the industry. The number of probiotic microorganisms may decrease depending on their production and storage (oxygen, heat treatments applied, mechanical processes and osmotic stress mechanisms are factors that may reduce their viability).

The joint use of probiotics and prebiotics for food packaging is a very important innovation for the industry. Edible films and coatings incorporating probiotics and prebiotics represent a novel strategy that should be widely studied. Recently, edible films or coatings with probiotics and prebiotics have been the object of several studies (Pavli et al. 2018; Pereira et al. 2016; Sogut et al. 2022). However, the application of probiotics in edible films is not an easy practice and represents a difficult task during food processing and storage. The techniques used for these processes must overcome osmotic and thermal stress and/or acid-induced stress that can occur during food preparation and storage (Peltzer et al. 2017). The first study of films and coatings incorporating probiotics was

carried out by Tapia *et al*. This study describes the use of alginate-gellan coatings and films for coating fresh apples and papayas through the incorporation of *Bifidobacterium lactis* Bb-12®. Viable probiotic cell numbers greater than 10^6 CFU/g were maintained for 10 days of storage. Since then, many studies are being done on the incorporation of probiotics in edible films and coatings with different approaches (Tapia et al. 2007).

9.4.2. Essential oils

The addition of antioxidants to retard lipid oxidation in foods, in order to improve the quality and extend the shelf-life of foods (*e.g.* processed meat products) is necessary. Synthetic antioxidants such as butylhydroxyanisole (BHA), butylhydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) are widely used industrially. However, the use of these compounds has been questioned as raising safety issues. So, the use of spices and herbs as natural antioxidants could be an important advance for the food industry to protect food. Its effectiveness depends on several factors, such as dose and mode of application (Akcan et al. 2017).

Essential oils (EOs) are natural antimicrobial compounds, since in nature they protect plants from bacteria, fungi, and viruses, being used in food products to extend their shelf-life. Generally, they are obtained from plants or spices, being excellent sources of biologically active compounds, like terpenoids and phenolic acids (Ochoa-Velasco et al. 2021). Essential oils are extracted from different parts of the plant (roots, bark, leaves, seeds, and fruits) through steam distillation methods, mechanical processes, dry distillation for essential oils (EO) and solvent extraction for plant extracts. Essential oils and plant extracts have numerous safety advantages when compared to chemical preservatives and are approved by the Food and Drug Administration (Sharafi et al. 2023).

The antimicrobial activity of EOs is attributed to their phenolic content, mainly flavonoids and their derivatives. The mechanisms of antimicrobial activity of phenolic compounds are related to the destruction of the cell wall and cytoplasmic membranes, preventing the synthesis of DNA, RNA, proteins and polysaccharides in bacteria and fungi (Sharafi et al. 2023).

Oregano (*Origanum vulgare L.*) essential oil has been extensively studied in the last five years due to its inhibitory action against microorganisms that cause problems in cheese conservation. For example, the presence of *Aspergillus flavus* can be controlled by applying 0.02% (v/v) oregano EO to milk before making cheese, thus preventing its presence for at least 15 days. With the same concentration, the presence of *Escherichia coli* was eliminated after 3 days (Fernandes et al. 2025). Examples of the use of essential oils such as cinnamon, bergamot and lemon incorporated into edible films, as these promote antimicrobial efficacy and improve the film's barrier properties, are reported (Çakmak et al. 2020). Akcan and coworkers evaluated the effectiveness of natural antioxidant extracts of laurel (*Laurus nobilis* L.) and salvia (*Salvia officinalis*) on edible films made of whey protein isolate applied to cooked meatballs, over frozen storage at 18 °C for 60 days. This study showed that the application of an antioxidant active pack with

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laurel and/or salvia to meatballs was effective in delaying oxidative changes in these foods (Akcan et al. 2017).

9.4.3. Antibacterial and antifungal compounds

Many compounds such as organic acids, enzymes, fungicides and natural compounds (spices and essential oils) have been studied as antimicrobial agents for food packaging (Pérez et al. 2011). Antimicrobial compounds can influence the sensorial, mechanical, and optical characteristics and properties of edible films and coatings (Dinika et al. 2020). Antimicrobial packaging materials are used as food preservatives because they improve the barrier properties of the packaging material and help increase the shelf-life of a food product by impeding microbial development and food degradation (Dinika et al. 2020). Furthermore, antimicrobial films and coatings have been proved as capable of extending the lag phase of microorganisms, reducing the rate of microbial growth, prolonging the shelf-life, and promoting safety of food (Dinika et al. 2020).

Braber *et al*., developed antifungal whey protein isolate (WPI) films with low amounts of a water-soluble derivative of chitosan. These films acted as a cross-linking agent through hydrogen bonds with whey proteins and thus reduced the solubility and elongation of the films. The mechanical resistance and barrier to water vapour were maintained. These films showed excellent antifungal and barrier effects against the growth of fungi that spoil food (Braber et al. 2021).

Another important antimicrobial agent, with antioxidant and anticancer activities is carvacrol (Pérez et al. 2011). Carvacrol is a compound found in essential oils like oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), peppermint (*Lepidium flavum*), wild bergamot (*Citrus aurantium bergamia*) and other plants. The antimicrobial activity of this compound is higher than most other volatile compounds present in essential oils, and this is due to the presence of the free hydroxyl group, its hydrophobicity and phenol fraction (Sharifi-Rad et al. 2018). Ochoa-Velasco *et al*., studied both *in vitro* and *in vivo*, the efficacy of thymol and carvacrol compounds in edible starch films and coatings against *Colletotrichum gloeosporioides*. The authors determined the minimum inhibitory concentration (MIC) of carvacrol and thymol at different pH values, and the films and coatings were applied to papaya and mango. Both carvacrol and thymol were capable of reducing the incidence of symptoms of anthracnose, while preserving the quality of the fruit by reducing the senescence rate (Ochoa-Velasco et al. 2021).

Potassium sorbate is the salt of sorbic acid which restricts the activity of fungi, yeasts and aerophilic bacteria. Potassium sorbate ionizes forming sorbic acid when dissolved in water. At pH of 6.5 it is very effective, and as the pH decreases its effectiveness increases. It is more effective when in non-dissociated form, due to its great ability to penetrate the cytoplasmic membrane of bacteria (Pérez et al. 2011). The incorporation of potassium sorbate into edible WPC films with two different pH values was tested by these authors. It was verified that the addition of potassium sorbate in the WPC films inhibited the growth of non-O157 STEC isolated strains (Pérez et al. 2011).

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According to Yu *et al.* chitosan films were prepared with the addition of different deep eutectic solutions: choline chloride:glycerol (1:2), choline chloride:acetylsalicylic acid (1:1), choline chloride:vitamin C (2:1), choline chloride:malonic acid (1:1), choline chloride:citric acid (1:1) and choline chloride:lactic acid (1:2), in proportions of 20% and 60%. The effect of these solvents on the films was evaluated by their microstructure and their mechanical and optical properties, water resistance, as well as regarding antioxidant and antimicrobial properties. They concluded that the chitosan film with deep eutectic solutions based on choline and acetylsalicylic acid presented the best mechanical, optical, antioxidant and antimicrobial properties, being a good prospect for application in packaging with food active ingredients (Yu et al. 2023).

9.5. Applications of WPI and WPC films and coatings

WPI and WPC are included in various foods such as dairy products, infant formulas, sports nutrition foods, meats, baked goods, sweets, snacks, beverages and other food products to develop the functionality and improve the benefits of foods (Sharma and Chauhan 2018). WPI and WPC are also very important in the formation of edible films and coatings. An edible whey film is like a dry, highly interacting polymer network with a three-dimensional gel-like structure (Kandasamy et al. 2021). Films and coatings made from whey proteins have the main advantages of being colourless, odourless, flexible and transparent, with excellent mechanical properties and barrier properties when compared to some films and coatings based on polysaccharides and other polymers. protein base. They have high water vapour permeability, low tensile strength and excellent oxygen permeability when compared to other protein-based films (Kandasamy et al. 2021). These films have demonstrated excellent nutritional value and the potential to deliver active ingredients (antimicrobials, antioxidants, probiotics), meaning that the properties of films that have very good properties for industrial applications do not change much (Kandasamy et al. 2021).

9.5.1. Applications in cheeses

Cheese is a product that can be prepared with different types of milk (bovine, goat and sheep). It is a very diverse product in terms of texture, aromas, flavours and appears in different forms, being part of the human diet due to its nutritional composition, with a high amount of proteins, calcium, minerals and vitamins. Cheese is a dairy food that can be consumed directly (Costa et al. 2018).

The global consumption of cheese has increased significantly over the years and therefore the cheese industry aims to increase the shelf-life of this product without affecting its quality and safety. One of the main losses during marketing occurs during storage, as it is common for cheese to be contaminated by microorganisms such as bacteria, moulds and yeasts, meaning that these cheeses can no longer be consumed (Costa et al. 2018). Some of the materials used for cheese packaging, such as polyethylene, polyamide, polypropylene, are not biodegradable and are not edible, therefore leading to environmental problems, and also leading to problems with legislation which is very strict

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with regard to the migration of compounds existing in cheese packaging (Costa et al. 2018).

The cheese industry is one of the sectors where edible films and coatings based on WPI or WPC have a good application potential, as some studies have shown (Costa et al. 2018). Therefore, the use of films and coatings based on WPI and WPC is an added value for the preservation of cheeses, as well as being protein-rich and carrying active ingredients, they are an added value regarding for human nutrition.

Table 8 presents some examples of the application of films or coatings made with of WPI or WPC in cheeses.

Table 8: Examples of the application of films or coatings made with whey proteins on cheeses.

Whey Protein-Based Film/Coating Composition	Function	Food Products	References
WP with antimicrobial properties	Improved functionality	Ripened cheese	(Henriques et al. 2013a)
WPC with *Lactobacillus buchneri*	Reduction or eliminate *Penicillium nordicum*	Cheese	(Guimarães et al. 2020)
WPC	Improve oxygen permeability	Cheddar cheese	(Wagh et al. 2014)
WPI with essential oils	Reduction of microbial contamination during storage	Kasar cheese	(Seydim et al. 2020)
WPI with natamycin/chitooligosaccharides/lactic acid	Reduce moisture loss Inhibit pathogens	Cheese	(Ramos et al. 2012a)
WPC with cumin essential oil	Improve the shelf-life	Cheese	(Nemati et al. 2023)
WP with chitosan	Reduced microbial growth, delay acidity development, extend shelf-life	Ricotta cheese	(Di Pierro et al. 2011)
WPC	Improved separation and shelf-life	Slices cheese	(Cruz-Diaz et al. 2019)
WPC with cinnamon	efficiently extend shelf-life	Acid Curd Cheese	(Mileriene et al. 2021)
WPC with rosemary and sage extracts	Improve spoilage or pathogenic bacteria appeared	Cheese	(Kontogianni et al. 2022)
WPC with green tea (*Camellia sinensis* L.) extract	Preserve and improved shelf-life	Fresh cheese	(Robalo et al. 2022)
Furcellaran/WPI with yerba mate and white tea extracts	Improve shelf-life	Fresh soft rennet-curd cheese	(Pluta-Kubica et al. 2020)
Furcellaran-WPI with green tea or pu-erh extracts	Improve shelf-life	Acid-curd cheese	(Pluta-Kubica et al. 2021)
WPI with incorporation of A511 bacteriophage	Control of Listeria monocytogenes	Cheese	(García-Anaya et al. 2023)
WPC	Improve shelf-life	Mustafakemalpasa sweet cheese	(Guldas et al. 2010)

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9.5.2. Aplications in other foods

The good properties of WPI and WPC films and coatings, make these films and coatings excellent for continuing research into materials for sustainable food packaging and with very good functional and nutritional properties (Chen et al. 2019b).

Each different food needs specific storage requirements, so packaging materials must meet specific storage conditions for each specific food (Chen et al. 2019b).

Protein-based films and coatings materials are a strong candidate to meet the necessary requirements for food packaging through modification. Furthermore, the nutritional and protein biodegradation capabilities of these films and coatings make these films and coatings a good option for food packaging (Chen et al. 2019b). When compounds with good functional properties are added (*e.g.* essential oils), the effectiveness of these films and coatings tends to increase. Thus, through the studies carried out on films and coatings made with WPC and WPI, we can verify that they improve the quality of the products throughout the storage time, in other cases they preserve the moisture of the food and also help to maintain the colour of the food for more time. In Table 9 some examples of the application of films or coatings made with of WPI or WPC in other foods are presented.

Table 9: Examples of the application of films or coatings made with of WPI or WPC in other foods.

Whey Protein-Based Film/Coating Composition	Function	Food Products	References
WPI with Cinnamon, cumin, thyme essential oil	Antimicrobial action	Fresh meat	(Badr et al. 2014)
WPI with sodium lactate, ε-polylysine	Antimicrobial action	Fresh meat cuts	(Zinoviadou et al. 2010)
WPI or WPC or hydroxypropyl methylcellulose, and beeswax or carnauba wax	Colour protection	Apples	(Perez-Gago et al. 2005)
WP with sodium alginate	Improved shelf-life	Kilka fish	(Seyfzadeh et al. 2013)
WPC with olive oil	Improve shelf-life and sensory properties	Peanuts	(Javanmard 2008a)
WPI	Improved shelf-life	Pistachios	(Javanmard 2008b)
WPC with glycerol or sorbitol	Quality preservation	Salmon fish	(Rodriguez-Turienzo et al. 2011)
WPC and rice oil	Improved shelf-life	Kiwi fruit	(Hassani et al. 2012)
WPI with sodium carboxymethyl-cellulose	Improved shelf-life	Asparagus	(Tzoumaki et al. 2009)
WPI with casein	Improved quality	Cookies and Muffins	(Onwulata 2008)
WPC with *Origanum virens* essential oil	Controlling microbial spoilage and prevents lipid oxidation and colour fading	Traditional Portuguese sausages (paínhos and alheiras)	(Catarino et al. 2017)

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Table 9: Examples of the application of films or coatings made with of WPI or WPC in other foods (continued).

Whey Protein-Based Film/Coating Composition	Function	Food Products	References
WPI with tarragon essential oil	Quality and shelf-life	Brook trout fish	(Socaciu et al. 2021)
WPI with essential oils ginger and chamomile	Improve shelf-life	Rainbow trout fillets	(Oğuzhan Yıldız and Yangılar 2017)
WPI and WPI with flaxseed oil blended with beeswax	Quality and improved shelf-life	Plums	(Reinoso et al. 2008)
WPC and WPI	Eggshell breaking strength of fresh eggs	Eggs	(Caner and Yüceer 2015)
WP with aqueous clove (*Syzygium aromaticum* L.) extract	Prevent bacterial growth and oxidative spoilage	Trout fillet	(Bolandi et al. 2025)
WPC	Extended the quality of fresh grapes	Grapes	(Sajimon et al. 2023)

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HYPOTHESIS AND OBJECTIVES

1. Hypothesis

Food industries produce a varied and significant amount of waste, which constitutes a serious problem for the environment. Until recently, these residues were not valorised and were generally used for animal feed, transformed into biomass fuels or discarded into the environment. Their specific composition (high organic content) requires adequate treatments, required by legislation. However, for small and medium-sized companies it can involve a very high cost, that they sometimes cannot support. One of the possibilities for minimizing these costs involves the recovery of valuable compounds present in those by-products with potential for valorisation. With the environmental problems that have been increasing and with the scarcity of some foods, especially in developing countries, where nutritional deficiency is a huge problem, the reuse of by-products from the food industry can be an advantage. Until recently, only some of these industrial residues could be valued because they still contain compounds that can be used again to prepare new products beneficial to human health.

In recent years, new technologies have been proposed to be able to use these agro-industrial residues more efficiently, not only in terms of their reuse for agriculture or the food industry, but also to produce unusual and innovative products for other sectors. One of the most interesting areas for the reuse of industrial waste, due to its functional and nutritional compounds, as well as its bioactive compounds, are by-products from the dairy industry. These compounds can help in the development of unexplored products, resulting from the interconnection between the food, pharmaceutical and cosmetic industries and may create new business areas, which can include functional foods, nutraceuticals and nutricosmetics.

Whey (CW) and second cheese whey (SCW) are by-products from the manufacture of cheese and whey cheese, respectively. These are produced in high volumes, and have a very high biochemical charge, which can damage the environment. However, CW and SCW also have high nutritional and functional values, as they retain a large amount of the total solids present in milk (especially lactose and soluble proteins). The direct use of CW is one of the oldest and most common practices for managing this product. It can be applied by farmers as biofertilizer or for livestock feed or used to produce whey cheese. However, through the production of whey cheese, SCW is obtained, which is also applied by farmers as biofertilizer or given as animal feed, or otherwise discarded. These practices for reusing CW and SCW are insufficient since the quantity of these by-products generated is quite high, and especially in the case of SCW, it does not currently have any major uses of interest. Therefore, the use of membrane technologies to recover some components of CW and SCW, such as proteins and lactose, is a promising alternative to enhance the potential functional and nutritional properties of these by-products. Through membrane technologies new products such as whey protein concentrates (WPC) and

whey protein isolates (WPI) can be obtained. However, these alternatives involve a final drying step, which limits their application in medium/small companies. Therefore, the direct use of concentrated CW or SCW in the liquid form is the most promising option for small/mediun sacale dairy companies.

Packaging is essential to protect food from environmental interactions, maintain its quality and preserve the shelf-life of these foods. The most widely used packaging materials are petroleum-derived polymers, which represent enormous environmental problems, as their degradation is difficult and very slow. Thus, in recent years, the use of biopolymers that are biodegradable and some of them may even be edible has been investigated as new materials for manufacturing packaging. Whey proteins, coming from by-products such as CW and SCW are some biomaterials that allow us to create edible films and coatings with functional and nutritional properties beneficial to human health. Films based on CW and SCW can show good mechanical and barrier properties.

This work proposes new direct uses of CW and SCW liquid concentrates to create new products with functional and nutritional properties beneficial to human health. Particular importance will be given to the SCW, since this by-product is currently seldom valued, but still contains nutritional compounds (*e.g.* proteins) with high nutritional value for human health.

2. Objectives

The main objective of the research activities developed during this doctoral training period were focused on finding alternatives for the valorisation of cheese whey and second cheese whey, obtaining liquid and/or dehydrated whey (CW) and second cheese whey (SCW) protein concentrates through the use of ultrafiltration/diafiltration (UF/DF), which allow for the valorisation of these by-products in medium and small dairy companies. In the case of dryed products, subsequent dehydration processes were used to obtain dried whey protein concentrates (WPC). Two lines of research were developed for the application of these by-products; the direct conventional application of liquid WPC from different origins (bovine, caprine and ovine), in the formulation of new food products, and the nonconventional use of dehydrated WPC in the development of edible films and coatings. Since the intention was to develop all processing steps, all of them were executed and investigated, and several objectives were identified:

1-Applications of membrane technologies, specifically UF/DF to produce liquid CW and SCW protein concentrates. Evaluation of the efficiency of membrane processes: evaluation of ultrafiltration fluxes and volumetric concentration factor during CW and SCW concentration.

2-Development of innovative dairy products (fermented probiotic/prebiotic products such as kefir, whey cheese and ice cream) using liquid CW and SCW protein concentrates as the main ingredient.

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3-Evaluation of physicochemical characteristics (total solids, minerals, proteins, fats, colour, pH, viscosity, texture, titratable acidity), microbiological (lactic acid bacteria, total

bacteria, moulds and yeasts) and sensory characteristics (appearance, flavour, texture) of these products.

4- Production of dehydrated products from sheep SCW protein concentrates and their evaluation for potential applications in edible films and coatings, with optimal functional and nutritional properties, since these have never been developed. Application of essential oils (*e.g.* oregano and sage essential oils) in films and coatings to improve their functional and microbiological properties.

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MATERIAL AND METHODS

1. General material, equipments and reagents

1.1. General material and equipments

To carry out this work, the following material and equipment was used:

analytical balances (METTLER TOLEDO, ACCULAB ALC-310.3, KERN EWJ); pH (HI 9025 Hanna Instruments pH meter and CRISON pH METER GLP 21); homogenizer (APV Rannie™ Blue Top); incubation chamber (Jenogand, Y—1000); oven (Schutzart DIN 40050-IP20 Memmert™); freeze-dried (Lyph-Lock freeze dryer); electric muffle furnace (Nabertherm™, LE 4/11/R6 and HD-23 Hobersal™); digester (Digestion System 6 1007 Digester Tecator™, Foss Analytical); colourimeter (Minolta Chroma Meter, model CR-200B); centrifuge (SuperVario-N Funke Gerber™); rotary evaporator RII (Buchi™); rheometer (Rheostress 6000 rheometer equipped with a plate-to-plate TMP35 bottom plate and a P35 TiL upper plate); texture analyser (Stable Micro Systems™ texture analyzer, TA.XT Express Enhanced); Rotronic (Hygrolab equipped with an HC2-AW cell); Ultra-Turrax (T18 basic, IKA™, Staufen, Germany); heating magnetic stirrer (Arex digital PRO, Velp ™ Scientifica, VELP Scientifica Srl., Usmate, Italy, connected to a temperature controller (VTF Digital Thermoregulator, VELP Scientifica Srl., Usmate, Italy); milk analyser (Lactoscan, ultrasonic); polypropylene boxes (Ondipack™, Makro), plates (Biokar Diagnostics), climate chamber (Ensayos Terlab S.L., Barcelona, Spain), electronic digital micrometer (Selecta, Barcelona, Spain), spectrophotometer X-Rite (mod. SP60; Grand Rapids, MI, USA), spectrophotometer (mod. 6850 UV/Vis; Jenway, Bibby Scientific, Stone, UK) and texturometer (mod. EZ Test and the Trapezium Data Processing System software Shimadzu Corporation, Tokyo, Japan); Jasco FT/IR-4600 equipped with the ATR Pro One accessory with an internal reflectance element of ZnSe (wave number range of 15000-550 cm^{-1}) (Jasco Corporation, Tokyo, Japan); thermogravimetric analyzer with differential scanning calorimetric capability (Mettler Toledo, mod. TGA/DSC1; Schwerzenbach, Switzerland); attenuated A JEOL-JSM 6360LV scanning electron microscope (Jeol, Ltd., Tokyo, Japan); X-ray diffractometer (Bruker D8 Advance with a LYNXEYE XE-T detector, Bruker, Germany)

1.2. Reagents

All chemical products used in this work were of analytical quality, supplied by Merck, Panreac, Riedel de Haen and Sigma.

- Whey protein isolate (WPI) (NatWPI90, 90% w/w protein, 5.5% lactose, 1.0% fat, 2.5% ashes) supplied by InLeit Ingredients S.L.U. (Curtis, Spain)
- Glycerol (Panreac, Barcelona, Spain)
- Oregano and clary sage (Plena Natura, Amadora Portugal)
- Potassium carbonate

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- Methanolic DPPH (2,2-diphenyl-1-picrylhydrazyl; Sigma-Aldrich, St. Louis, MO, USA)
- Methanol
- 0.1 M NaOH
- Petroleum ether
- Sulfuric acid
- MRS, M17, CRB Biokar Diagnostics (Allonee, France).
- $CaCl_2$ (Tecnilac, Viseu, Portugal),
- *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, and *Streptococcus thermophilus* (Mesófilo Plus Starter, Enzilab, Leça do Balio, Portugal),
- Granulated lysozyme (0.025% w/v) (Biostar, Rua das Picarotas, Portugal)
- Animal rennet (>92 g/100 g chymosin, supplied by Tecnilac, Viseu, Portugal)
- Natamycin (Nataseen™-L, Siveele, supplied by Enzilab, Leça do Balio, Portugal)
- *Lactobacillus acidophilus* (LA5™ Chr. Hansen, Hoersholm, Denmark) *Lacticaseibacillus paracasei* and *Lacticaseibacillus rhamnosus* (FreshQ4™, Chr. Hansen, Hoersholm, Denmark) (LA5FQ4).
- Inulin (Fibruline™, Cosucra, supplied by Induxtra de Suministros, Moita, Portugal)
- Commercial kefir culture: Exact™ Kefir 1 (CHR Hansen, Hoersholm, Denmark)
- Yoghurt culture: Yoflex™(YF-L903, CHR Hansen, Hoersholm, Denmark)
- Kefir culture: ExactTM Kefir 1 (CHR Hansen, Hoersholm, Denmark)
- A mixture of probiotic cultures: (*Bifidobacterium* BB-12, *Lactobacillus acidophilus* LA-5) and Streptococcus thermophilus, at a concentration of 0.005% (w/v). ABT-3TM (CHR Hansen, Denmark).

2. Sample preparation

The whey and second whey cheese samples ovine and caprine, respectively, came from Portuguese dairy industries. The bovine whey resulted from the production of cow's milk cheese at the dairy pilot plant of the Polytechnic Institute of Coimbra's School of Agriculture (Coimbra, Portugal). The ultrafiltration of the samples was similar in all cases. A total of 500 L of sheep's and goat SCW and 200 L of bovine whey were ultrafiltrated in a pilot plant to obtain liquid whey concentrates. All experiments were performed in triplicate.

To carry out the ultrafiltration (UF) processes for whey and second cheese whey, a pilot plant produced by Proquiga Biotech SA equipment (A Coruña, Spain) was used, equipped with an organic UF membrane (3838 PVDF/polysulfone) with an effective filtration area of 7 m^2 and 10 kDa cut-off, provided by FipoBiotech, Pontevedra, Spain (Figure 1). The process was carried out at 40–45 °C at a transmembrane pressure of 3-3.5 bar aiming at a volumetric concentration factor (VCF = Volume feed/Volume retentate) of 5 for bovine whey and 20 for sheep and goat whey and for sheep and goat second cheese whey

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obtaining 25 L of of ovine and caprine liquid whey and second whey concentrates and 40 L of bovine liquid whey concentrate.



Figure 6: Equipment for membrane separation process used.

The 25 L of ovine and caprine liquid whey concentrates (LWC´s) were pasteurized (65 °C, 30min) and then homogenized at 15 MPa using a Rannie™ model Bluetop homogenizer (Copenhagen, Denmark). The final product was frozen at -25 ± 2 °C until they were used to produce goat and sheep kefir as well as cheese whey formulations.

The 25 L of ovine and caprine liquid second cheese whey concentrate (LSCWC´s) were pasteurized (65 °C, 30min) and then homogenized at 10 MPa using a Rannie™ model Bluetop homogenizer (Copenhagen, Denmark). The final product was frozen at -25 ± 2 °C until it was used to produce goat and sheep ice cream formulations.

The 40 L of bovine liquid whey concentrate (LWC) were used to produce the whey cheese directly.

In order to produce sheep second cheese whey powders, 500 L of sheep's SCW were also ultrafiltrated in the same pilot plant, in the same way and with the same VCF (20) as it was indicated before, thereby obtaining 25 L of concentrate. Afterwards, to the concentrate, 225 L of process water were added, and the diluted concentrate was submitted to diafiltration (VCF=10). For reverse osmosis (RO) was used a ORM (Belas, Portugal) equipment equipped with 2.5 S seawater pressure vessel and a 1 m^2 Filmtec membrane SW302540 (Dow Chemical Company, Midland, TX, USA). The diafiltrated concentrate was pasteurized (75 °C, 30 min) and then was concentrated by reverse osmosis (RO) in a pilot plant developed by ORM equipment (Belas, Portugal) equipped with 2.5 S seawater pressure vessel and a 1 m^2 Filmtec membrane SW302540 (Dow Chemical Company, Midland, TX, USA). The RO concentrate was freeze-dried in a Lyph-Lock freeze dryer (Labconco Corporation, Kansas City, MI, USA) in order to obtain the ovine SCW powder.

3. Manufacture of products

To create the various new food products and to make the coatings with SCW:WPI and essential oils, several different techniques were used. In the following sub-sections, all formulations used, and their respective equipment will be described.

3.1. Sheep´s and goats´s kefir products

Sheep or goat liquid whey concentrates, LWCs (approximately 12 L) were thawed under refrigeration at 0 °C for 24 h. Subsequently, the samples were heated to 80 ± 2 °C and 2.5% (w/v) inulin (Fibruline™, Cosucra, supplied by Induxtra de Suministros, Moita, Portugal) was added, being the mixture homogenized at 15 MPa. The mixtures were then cooled to 38 °C, divided into four portions (3 L each), and inoculated with one of the following cultures:

1. Commercial kefir culture: Exact™ Kefir 1 (CHR Hansen, Hoersholm, Denmark) mesophilic and thermophilic culture (*Debaryomyces hansenii*, *Lactococcus lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* biovar *diacetylactis*, *L. lactis* subsp. *lactis*, *Leuconostoc* and *Streptococcus thermophilus*) at a concentration of 0.01% (w/v) (EK)

2. A mixture of a commercial kefir culture (Exact™ Kefir 1, CHR Hansen, Hoersholm, Denmark) and a probiotic culture containing *Bifidobacterium bifidus*-BB12, *Lactobacillus acidophilus*-LA5 and *S. thermophilus* (ABT-5™, CHR Hansen, Denmark) at 0.01% each one (EKABT5)

3. A traditional kefir culture at a concentration of 2.5% v/v (TK)

4. A mixture of the traditional kefir culture at 2.5% v/v and probiotic culture (ABT-5™) at 0.01% (TKABT5).

The inoculated goat or sheep LWCs were placed in an incubation chamber (Jenogand™ Y 1000, Copenhagen, Denmark) at 37 °C and the pH and titratable acidity were monitored until the products reached a target pH of 4.5. The fermentation process was stopped by rapid cooling to 20 °C in less than 30 min. Afterward, the fermented products were placed in the refrigeration chamber at 4 ± 2 °C for 12 h, approximately. After this process, the goat and sheep kefir products were stored under refrigeration in a chamber at 0 ± 2 °C for 30 days. Products were evaluated at the 1st, 10th, 20th, and 30th days of storage.

3.2. Bovine whey cheeses

A total of 2% (v/v) full-fat milk (3.2% protein; 3.6% fat), 1% (w/v) salt, 2% (w/v) starch (Pregeflo™ CH 20, Roquette Freres, Lestrem, France) and 0.04% (v/v) $CaCl_2$ (51% w/v, supplied by Tecnilac, Viseu, Portugal) were added to the bovine liquid whey concentrate (LWC). The mixture was heated to 90–95 ºC for 20 min to allow coagulation and subsequent precipitation of whey proteins. The precipitated fraction was separated from the remaining liquid and divided into three similar portions which were submitted to different treatments: control CW without any addition (C); CW with the addition of 10% (w/w) full-fat milk fermented with the probiotic *Lactobacillus acidophilus* (LA5™ Chr. Hansen, Hoersholm, Denmark) (LA5); and CW with the addition of 10% (w/w) full-fat

milk fermented with the probiotic culture (LA5) plus a protective culture containing *Lacticaseibacillus paracasei* and *Lacticaseibacillus rhamnosus* (FreshQ4™, Chr. Hansen, Hoersholm, Denmark) (LA5FQ4). Both cultures were previously inoculated in the milk and incubated for 15 h at 30 ºC according to the manufacturer's instructions.



Figure 7: Example of cow's milk whey cheese produced.

The probiotic cultures were selected based on their commercial availability and proven activity. According to the manufacturer, the bioprotective culture has a combination of traditional lactic acid bacteria that inhibits unwanted yeasts and moulds in fermented dairy products, and its effect is obtained through active participation in the natural fermentation process. The whey cheeses were packaged in closed polypropylene boxes (Ondipack™, Makro, Carnaxide, Portugal) and stored under refrigerated conditions at 4 ± 1 ºC for 28 days. Consecutive analyses were performed on the 1st, 7th, 14th, 21st and 28th days of storage.

3.3. Sheep´s and goat´s ice creams

The sheep's or goat's liquid second whey concentrates (LSCWC's) were thawed under refrigeration for 24 h. Subsequently, the samples were heated at 65 °C and homogenized at 10 MPa using APV Rannie™ model Blue Top (Copenhagen, Denmark). Afterwards, they were heated to 85 °C and recirculated through the homogenizer valve. The concentrates were then cooled to 44 °C and divided into 6 L portions, to which the remaining ingredients were added, in the following proportions (% w/v): sucrose (12%); inulin (5%), citric acid (0.4%) and xanthan gum (0.1%). The mix was allowed to mature for 12 h at 4 ± 2 °C. Finally, each mixture was inoculated with one of the following cultures:

1. Yoghurt culture: Yoflex™ (YF-L903, CHR Hansen, Hoersholm, Denmark) thermophilic yoghurt starter culture (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) at a concentration of 0.005% (w/v)

2. Kefir culture: ExactTM Kefir 1 (CHR Hansen, Hoersholm, Denmark) mesophilic and thermophilic culture (*Debaryomyces hansenii*, *Lactococcus lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis biovar diacetylactis*, *L. lactis* subsp. *lactis*, *Leuconostoc* and *Streptococcus thermophilus*) at a concentration of 0.005% (w/v)

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3. A mixture of probiotic cultures: (Bifidobacterium BB-12, *Lactobacillus acidophilus* LA-5) and *Streptococcus thermophilus*, at a concentration of 0.005% (w/v). ABT-3TM (CHR Hansen, Denmark).

The inoculated sheep or goat mixtures based on LSCWCs were placed in an incubation chamber (Jenogand, model Y-1000, Copenhagen, Denmark) at 43 °C, and the pH was monitored until it reached a value of 4.6. Fermentation was stopped by rapid cooling to 20 °C in less than 30 min. Afterwards, the fermented mixtures were placed in the refrigeration chamber at 4 ± 2 °C for 12 h. For each formulation, 6 L batches of ice cream were produced and placed in a horizontal frozen yoghurt freezer for 40 min. Immediately after freezing, the temperature of ice creams was -6 ± 1 °C. After this process, the ice creams were packaged in 500 mL polypropylene boxes and stored in a freezer at -21 ± 1 ºC for 120 days. All experiments were performed in triplicate.



Figure 8: Example of the preparation of sheep's liquid second whey concentrate ice cream.

3.4. Cheeses containing sheep´s second cheese whey coatings

3.4.1. Coatings with sheep´s second cheese whey powder

Whey protein isolate (WPI) (NatWPI90, 90% w/w protein, 5.5% lactose, 1.0% fat, 2.5% ashes) supplied by InLeit Ingredients S.L.U. (Curtis, Spain) and sheep's SCW powder (54.7% w/w protein, 14.3% fat, 7.9% ashes) were weighed in order to originate a final concentration of protein in the cheese coating solution of 8% (w/v) and a protein proportion of 2:1 SCW:WPI. The WPI was dissolved in distilled water by slow stirring for 30 min at 20 °C. Then, glycerol (Panreac, Barcelona, Spain) was added in a 1:1 protein/plasticizer ratio. The pH was adjusted to 7.0 with 0.1 M NaOH. Afterwards, the WPI/plasticizer solution was heated to 90 °C for 15 min with stirring. After cooling to 30 ºC, the WPI/glycerol solution was separated into three equal portions to prepare the coatings containing sheep's SCW as follows: coating solution without essential oils; coating solution with oregano essential oil; coating solution with clary sage essential oil. The essential oils (oregano and clary sage, Plena Natura, Amadora Portugal) (1% of the protein weight in solution) and the SCW powder were added simultaneously to the previously prepared WPI/plasticizer solution, and the mixture was homogenized using an Ultra-Turrax (mod. T18 basic, IKA™, Staufen, Germany). The final pH was adjusted to

7 with 0.1 M NaOH, and the solution was stored in refrigeration until used. Summarizing, the final SCW:WPI/plasticizer solution had 8% (w/v) protein (in the proportion 2:1 SCW:WPI) and a ratio of 1:1 protein/plasticizer.

3.4.2. Cheeses

Cheese manufacture was carried out according to Borges and coworkers (Borges et al. 2020) with some modifications. Cylindrical cheeses of approximately 250 g were produced at Polytechnic Institute of Coimbra's School of Agriculture (Coimbra, Portugal) dairy pilot plant and used as cheese food model for sheep's SCW-based coating application. A local producer supplied the bovine milk (3.2% protein, 3.5% fat, 4.2% lactose). Milk pasteurization occurred at 75 ± 1.0 ºC for 30 s in a plate and frame heat exchanger (Albinox, S. Pedro do Sul, Portugal). After stabilizing the temperature at 29.5 ± 0.5 °C, 0.04% of a 51% w/v $CaCl_2$ solution (Tecnilac, Viseu, Portugal), 0.01% w/v starter culture containing *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, and *Streptococcus thermophilus* (Mesófilo Plus Starter, Enzilab, Leça do Balio, Portugal), and granulated lysozyme (0.025% w/v) (Biostar, Portugal) were added. Finally, 0.0025% w/v animal rennet (>92 g/100 g chymosin, supplied by Tecnilac, Viseu, Portugal), previously diluted in water, was added. The milk coagulated in approximately 45 min, and once coagulation was complete, the curd was cut into small pieces (2 cm^3) to promote the drainage of whey. After half of the whey was drained, an equal amount salt water (1% w/v salt, at 30 °C) was added to the curd, and the mixture was thoroughly stirred before final draining of the whey for 15 min. The curds were placed in plastic molds before being pressed and stored in a refrigerated chamber at approximated 8–9 °C for 24 h. Next day, the cheeses were immersed in a brine solution (18–20 °Baumé) for 1 h 30 min. On the following day, the different coatings were applied by immersion of the cheeses on the coating solution:

a) control cheese without coating;

b) control cheese with natamycin;

c) cheese with SCW:WPI coating;

d) cheese with SCW:WPI coating containing oregano essential oil;

e) cheese with SCW:WPI coating containing clary sage essential oil.

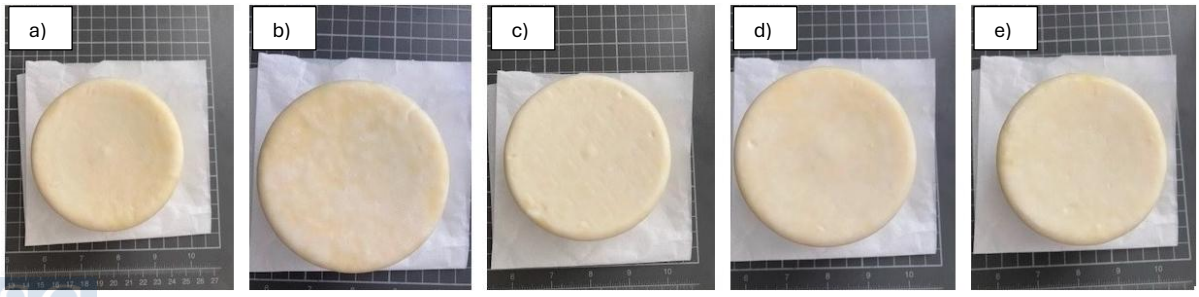


Figure 9: Example of cheese samples, a)-Control; b)-Cheese with natamycin coating; c)-Cheese with SCW coating; d)-Cheese with SCW coating with oregano essential oil; e)-Cheese with SCW coating with clary sage essential oil.

The solution of natamycin (Nataseen™-L, Siveele, supplied by Enzilab, Leça do Balio, Portugal) containing 3.2 g/L in distilled water was previously prepared, and the cheeses were submerged in it for one minute. The cheeses were maintained at *ca.* 10 °C, and the operation was repeated on the following day. Afterwards, the cheeses were transferred to the ripening room, where they were maintained for 28 days at 10 ± 2 °C, 80–90% R.H.

3.5. Films with sheep´s second cheese whey powder

Whey protein isolate (WPI) (NatWPI90, 90% w/w protein, 5.5% lactose, 1.0% fat, 2.5% ashes) supplied by InLeit Ingredients S.L.U. (Curtis, Spain) and sheep's second cheese whey powders (SCW) (54.7% w/w protein, 14.3% fat, 7.9% ashes) were used to prepare films. Firstly, different films were obtained with five different concentrations of WPI and SCW. WPI and SCW were weighed to obtain a final concentration of protein in the film solution of 8% (w/v). Five films with WPI and SCW were prepared.

1. WPI: films only with whey protein isolate;

2. WS 2:1, films of WPI-SCW mixture in a protein proportion of 2:1;

3. WS 1:1, films of WPI-SCW mixture in a protein proportion of 1:1;

4. WS 1:2, films of WPI-SCW mixture in a protein proportion 1:2;

5. SCW: films only with sheep´s second cheese.

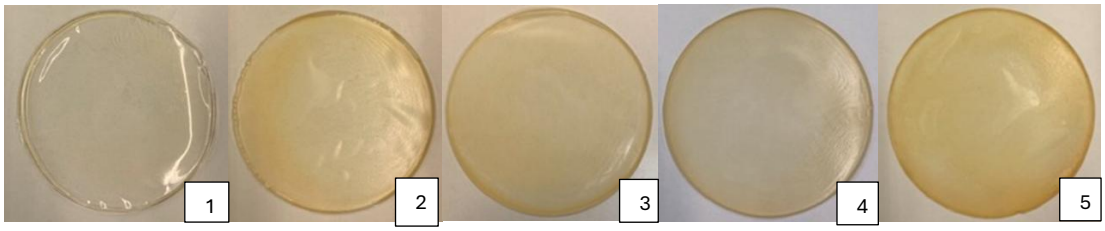


Figure 10: Films samples with WPI and SCW: 1)-WPI; 2)-WPI-SCW 2:1; 3- WPI-SCW 1:1, 4)- WPI-SCW 1:2; 5)-SCW.

The films were prepared in the following way: WPI were dissolved in distilled water by slow stirring in a magnetic stirrer for 30 min at 20 °C. Then, glycerol (Panreac, Barcelona, Spain) was added in a 1:1 protein/plasticizer ratio. The pH was adjusted to 7.0 with 0.1 M NaOH. Afterwards, the WPI/plasticizer solution was heated to 90 °C for 15 min with stirring in a digital magnetic stirrer (VELP Scientifica Srl., Usmate, Italy) connected to a temperature controller (VTF Digital Thermoregulator, VELP Scientifica Srl., Usmate, Italy). In the films with SCW, after cooling to 30 ºC, the SCW powder was added simultaneously to the previously prepared WPI/plasticizer solution, and the mixture was homogenized using an Ultra-Turrax (mod. T18 basic, IKA™, Staufen, Germany) at 15 500 rpm for 3 min. The final pH was adjusted to 7 with 0.1 M NaOH. After degassing under vacuum for 15 min, film forming solutions (0.22 g cm^{-2}) were poured into Plexiglas Petri dishes of 14 cm diameter and dried at 50 °C for 20 h in an air force cabinet. After, the dried films were kept at 25 °C and 45% relative humidity for 48 h prior to peeling they off in a climate chamber (Ensayos Terlab S.L., Barcelona, Spain) and were stored in

desiccators at room temperature and 45% relative humidity for further testing. The experiments were performed in triplicate.

Secondly, six different films were prepared with the addition of oregano (*Origanum compactum*) essential oil (Plena Natura, Amadora, Portugal). The films were prepared in the following way: the preparation of WPI/plasticizer solution after heat treatment and cooling was separated into six equal portions to prepare the films containing the three proportions of WPI-SCW (2:1, 1:1 and 1:2) and two different proportions of oregano essential oil (1 and 2% of the powder weight in solution). The essential oil and the SCW powder were added simultaneously to the previously prepared WPI/plasticizer solution, and the mixtures were homogenized using an Ultra-Turrax homogenizer. The final pH was adjusted to 7 with 0.1 M NaOH, and the films were formed as indicated before.

Six different films with oregano essential oil were obtained.

1.WS 2:1O1: films of WPI-SCW mixture in proportion 2:1 with 1% oregano essential oil;

2.WS 1:1O1: films of WPI-SCW mixture in proportion 1:1 with 1% oregano essential oil;

3.WS 1:2O1: films of WPI-SCW mixture in proportion 1:2 with 1% oregano essential oil;

4.WS 2:1O2: films of WPI-SCW mixture in proportion 2:1 with 2% oregano essential oil;

5.WS 1:1O2: films of WPI-SCW mixture in proportion 1:1 with 2% oregano essential oil;

6.WS 1:2O2: films of WPI-SCW mixture in proportion 1:2 with 2% oregano essential oil.

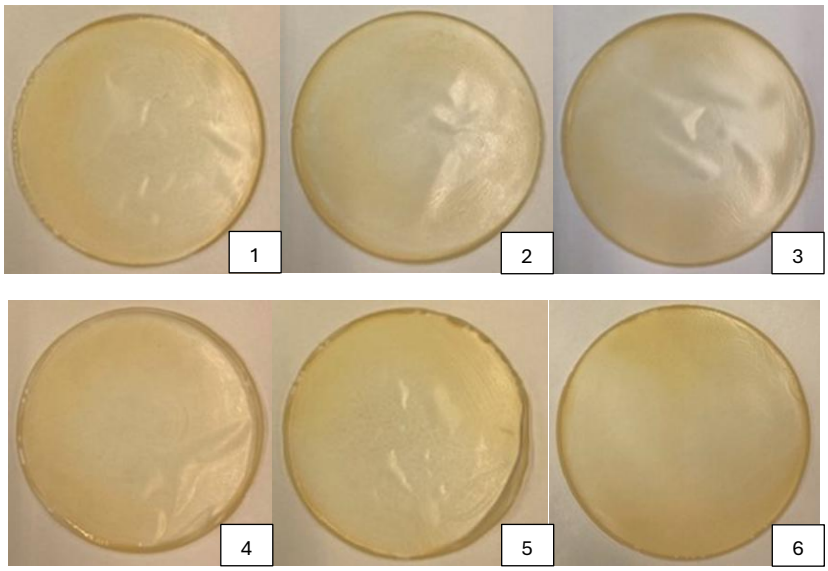


Figure 11: Films samples with oregano essential oil, 1)-WPI-SCW 2:1 with 1% essential oil 2)-WPI-SCW 1:1 with 1% essential oil; 3- WPI-SCW 1:2 with 1% essential oil, 4)- WPI-SCW 2:1 with 2% essential oil ; 5)-WPI-SCW 1:1 with 2% essential oil; WPI-SCW 1:2 with 2% essential oil.

4. Physico-chemical analysis of the foods

4.1. pH and titratable acidity

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The pH was measured directly in the samples with a HI 9025 Hanna Instruments pH meter (Leighton Buzzard, UK). The pH meter was previously calibrated with 7.01 (HI5007) and 4.01 (HI5004) Hanna buffer solutions. The titratable acidity, expressed in g of lactic acid/L, was determined by means of titration using a 0.1 N NaOH solution according to the different techniques described and depending on the products. For most products it was used the NP 701:1982 technique (NP 701:1982) for yoghurts. The exception were cheese products, to which was used the AOAC 920.124 technique for cheeses (AOAC 920.124). For each sample, three determinations were made both for pH and for titratable acidity.

4.2. Compositional analysis

The total solids content in general, was determined by drying the samples (105 °C) until constant weight in a Schutzart DIN 40050-IP20 Memmert™ oven (Schwabach, Germany) (AOAC 1997, NP 703: 1982, NP-1598:1983). The ash content was determined by incineration of dry samples in an Nabertherm™, model LE 4/11/R6 electric muffle furnace (Bremen, Germany) at 550 °C (AOAC 935.42:1997).

The fat content was determined by the Gerber method (SuperVario-N Funke Gerber™ centrifuge, Berlin, Germany) with differences regarding different samples. For sheep kefir whey cheeses, bovine whey cheeses with probiotic and bioprotective cultures and cheese with SCW coatings, it was measured using 3.0 g of cheese, previously homogenized and placed in the Van Gulik butyrometer where 15 mL of sulfuric acid (95% w/w) were added. Complete dissolution of the cheese sample in the butyrometer was carried out in a water bath at approximately 65 ºC under stirring. After dissolution, 1 mL of isoamyl alcohol was added and immediately stirred. Sulfuric acid was then added until it reached 35% on the butyrometer scale. The butyrometer was then closed and shaken again. The butyrometer was centrifuged for 10 min at 1200 rpm at 65 °C. The fat content (% w/w) was read immediately after centrifugation using the butyrometer scale (NP 2105: 1983). For goat kefir whey cheeses, the Gerber method for yoghurts was applied. The following were placed in succession in the Gerber butyrometer: 10 mL of concentrated sulphuric acid (65% w/w), 11 mL of the sample and 1 mL of isoamyl alcohol. The butyrometer was closed with the rubber stopper and shaken until complete homogenised. The butyrometer was centrifuged in the Gerber centrifuge for 10 min at 1 200 rpm at 65 °C. The fat content (%w/w) was read immediately after centrifugation using the butyrometer scale (NP 1923: 1987).

The fat content for the SCW ice-cream was determined according Mojonnier Acid/Base Hydrolysis method (AOAC 952.06:1997).

The total nitrogen content (N) was determined by the Kjeldahl method in the Digestion System 6 1007 Digester Tecator™ (Foss Analytical, Häganäs, Sweden). 0.5 g of sample was weighed and placed in a digestion tube. Two copper catalyst tablets (Kjeldahl tablets with 0.3% $CuSO_4.5H_2O$, PanReac) and 24.0 ml of concentrated sulphuric acid were then added. The tubes were placed in the digestion unit, keeping them at a temperature of 400 ºC until complete clarification. After digestion, 100.0 ml of a 33% (w/v) NaOH solution

were added to the sample (Distillation Unit VELP©SCIENTIFICA). Distilled ammonia was collected in 25.0 ml of 4% boric acid solution. Then, it was titrated with 0.1 N HCl solution in the presence of bromocresol green indicator (AOAC. 1997). The percentage of total nitrogen was determined according to the following equation:

N (%) = (14.007 x (V-Vo) x N x 100) / (mg of sample)

where:

N = Normality of HCl

V = Volume of HCl used in the titration (ml)

Vo = Volume of HCl used in the blank titration (ml)

The total protein content was calculated by multiplying the total nitrogen result obtained by 6.38. All analyses were performed in triplicate.

4.3. Water activity

Cheese water activity (a_w) was measured in a Rotronic Hygrolab (Bassersdorf, Switzerland) laboratory device for a_w measurement equipped with an HC2-AW cell at 20 ºC. Water activity was determined by cutting a circle of cheese (2.25 cm radius and 0.5 cm height) and placing it in cell until a constant value of equilibrium relative humidity (E.R.H.) was attained.

4.4. Colour parameters

The colour parameters for all samples were determined with a colorimeter (model CR-200B Chroma Meter, Minolta™, Tokyo, Japan) using illuminant C and 10º standard observer; the aperture diameter was 1 cm. It was previously calibrated with a white standard (CR-A47: Y = 94.7; x 0.313; y 0.3204). The CIEL*a*b* system was used to measure the colour coordinates. Three measurements were taken for each sample. Total colour difference (ΔEab*) was calculated as follows:

$$\Delta Eab^* = [(L^* - L^*0)^2 + (a^* - a^*0)^2 + (b^* - b^*0)^2]^{1/2}$$

where L*0, a*0, and b*0 and L*, a*, and b* were the values measured for the samples under comparison.

4.5. Texture parameters

A Stable Micro Systems texture analyser, model TA.XT Express Enhanced (Godalming, UK) was used for all analyses. Depending on the products to be analysed different probes and different conditions were used.

To perform the texture analysis of sheep and goat kefir samples, a TPA test was carried out with a penetration distance of 15 mm at 1 mm/s using a cylindrical acrylic probe with a diameter of 25.4 mm and a height of 38.1mm.

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For the analysis of bovine whey cheese with probiotic and bioprotective cultures an acrylic cylindrical probe (25.4 mm diameter, 38.1 mm height) with a penetration distance of 15.0 mm at 2 mm/s was used.

For ice creams made with SCW a 6 mm cylindrical probe was used, and the penetration distance was 25.0 mm at 1 mm/s.

For cheese samples with coatings, the penetration distance was 20.0 mm at 2 mm/s, and a cylindrical probe with a diameter of 6 mm was used.

The Specific Expression PC software (version 1.1.9.0) was used to calculate the results of all analyses performed.

4.6. Overrun

Overrun is the increase in the volume of the ice cream after freezing due to the incorporation of air. The method was followed measuring the weight of the mixture and that of ice cream with the same volume (Skryplonek et al. 2017). The determinations were done in triplicate.

Overrun [%] = (weight of mixture-weight of ice cream/weight of ice cream) *100

4.7. Meltdown rate

The meltdown rate was determined by placing 30 g of ice cream on a stainless-steel screen with a 1 x 1 mm opening, located on top of a beaker. After 45 min at 20 ± 1 °C, the weight of the melted sample collected in the beaker was measured (n=3). The meltdown rate was expressed as the percentage of the melted ice cream weight divided by the initial ice cream weight (Skryplonek et al. 2017). The determinations were done in triplicate.

Meltdown [%] = (weight of melted ice cream/initial weight of ice cream) *100

4.8. Rheological parameters

Rheological tests were performed at 5 ºC with the aid of a Rheostress 6000 rheometer equipped with a plate-to-plate TMP35 bottom plate and a P35 TiL upper plate. Data acquisition was carried out using the HAAKE RheoWin Job Manager software (version 4.82.0002, ThermoHaake™, ThermoFisher Scientific, Waltham, MA, USA). The measuring system utilized a cone and plate geometry, specifically C60/Ti-0.052 mm (35 mm diameter and 1° angle). Stress sweep tests were conducted at 1 Hz to determine the linear viscoelastic rheological behaviour of all samples. Values for the elastic and viscous moduli (G′ and G″) and the complex viscosity (η*) were obtained at frequencies ranging from 0.05 to 1.0 Hz. The samples' complex viscosities were fitted to the power law model according to following equation, and the consistency index k (Pa.s^{n-1}) and the power law index (n) were determined. This model is typically used for shear-thinning fluids such as weak gels and low-viscosity dispersions.

$$\eta^* = k\,(\dot{r})^{\,n-1}$$

k = flow consistency index

$\dot{r}$ = shear strain

n = flow behaviour index (Pa.s^n)

5. Microbiological analyses

The microbial counts of lactic acid bacteria (LAB) of the genera *Lactobacillus* sp. and *Lactococci* sp. were determined after production and during the storage and depending on the type of sample analysed. *Lactobacilli* sp. and *Lactococci* sp. were enumerated on plates at 37 °C for 48 h on M17 agar (in aerobiosis) and on MRS agar (in anaerobiosis) (Biokar Diagnostics, France), respectively, according to ISO 7889, IDF 117 (2003) (ISO 7889 IDF 117). Yeasts were enumerated in plates at 25 °C according to ISO 6611 IDF 94 (2204) (IDF 94: 2004). Total mesophilic aerobic counts were enumerated at 30 °C for 72 h on plate count agar (PCA) according to Gonzalez-Fandos *et al.* (Gonzalez-Fandos et al. 2000). Analyses were carried out in triplicate along with two controls for each medium and results are expressed as log CFU/g of product.

6. Sensory analyses

Consumer preference tests were carried out with an untrained panel of at least thirty people. The hedonic test was used to determine the degree of acceptability of the products, evaluating the parameters: aroma, texture, flavour and general appreciation, using a scale from 1 to 9 (1= I don't like it at all to 9 = I like it a lot) (Stone et al. 2020). Panel members were also asked to rank the samples according to their preference, from 1-most preferred to 4-least preferred, in accordance with ISO 8587 (1988) (ISO 8587:1988).

7. Film analysis

7.1. Thickness

Film thickness was determined using a 0-25 mm electronic digital micrometer with 0.001 mm resolution (Selecta, Barcelona, Spain), at 20 points of three films selected randomly.

7.2. Water vapour permeability

Water vapour permeability (WVP) was measured according to the method described by Díaz et al. (Díaz et al. 2019), based on the ASTM E-96-93 method (ASTM. E 96-93—Standard test method for water vapor transmission of materials. In Annual Book of ASTM Standard; American Society for Testing and Materials: Philadelphia 1993), with some modifications. Film samples (0.032 ± 0.001 m exposed area diameter) were attached to glass cups containing dried silica gel and were placed in desiccators with a saturated solution of potassium carbonate and maintained in a chamber at 25 °C. Weight gain of the cups was measured twice a day for 5 days. Measurements were performed in triplicate. WVP was calculated using the following formula:

$$WVP\ (ng\ s^{-1}\ m^{-1}\ Pa^{-1}) = (w \times L)/(A \times \Delta t \times \Delta P)$$

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where w is the weight gain of the cell (ng) during time of permeation Δt (s); L is the film thickness (m); A is the permeation area (m^2), and ΔP is the partial water vapour pressure difference (Pa) across the two sides of the film.

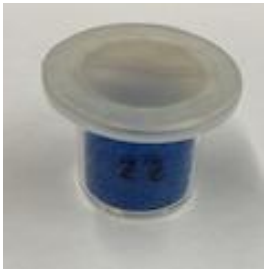


Figure 12: Example of Water vapour permeability (WVP) of film sample.

7.3. Dry matter content, solubility and density

Dry matter content and solubility were determined according to the method described by Yildiz *et al.* (Yildiz et al. 2022). Film samples (2 cm x 2 cm) were weighed (W1) and dried at 105 °C until constant weight (W2). The dried samples were then immersed in 25 mL of water at 25 °C for 24h and afterwards they were dried again at 105 °C for 24h and the final weight was determined (W3). Dry matter and solubility were calculated as:

$$\text{Dry matter (\%)} = 100 - \left[\frac{W_1 - W_2}{W_1} \times 100\right]$$

$$\text{Solubility (\%)} = \frac{W_2 - W_3}{W_2} \times 100$$

Density of films was determined in accordance with the method of Sun et al. (Sun et al. 2017). Density was determined by dividing the weight of the film by its volume. The volume of the film was calculated by multiplying its area by its thickness. All measurements were done in triplicate.

7.4. Colour and opacity

Colour parameters and opacity of films were determined in the CIE L*a*b* colour space according to the methods reported by Díaz and coworkers (Díaz et al. 2016), using a spectrophotometer X-Rite (mod. SP60; Grand Rapids, MI, USA). The colour of three different films in three random positions was measured.

The difference in colour (ΔE*) of films regarding the control film colour values was calculated as:

$$\Delta E^* = \sqrt{(L^* - L_0)^2 + (a^* - a_0)^2 + (b^* - b_0)^2}$$

where L^*, a^* and b^* are the color values for the film added of nanoclay and L_0, a_0 and b_0 are the color values for the control film. Whiteness (WI) index of films was calculated according to Saberi et al. (Saberi et al. 2016), using the equations:

$$WI = 100 - \sqrt{(100 - L)^2 + a^2 + b^2}$$

where L, a and b are the color parameter values of the sample.

Opacity values were calculated according to Márquez-Reyes *et al*. (Márquez-Reyes et al. 2022) as:

$$\text{Opacity (\%)} = \frac{Y_B}{Y_W} \times 100$$

where Y_B is the opacity of the film against a black background and Y_W is the opacity of the film against a white background.

7.5. Antioxidant activity

DPPH radical scavenging capacity of the films was determined using the method described by Vargas *et al.* (Vargas et al. 2017). Film samples (1 cm^2) were submerged in tubes containing 4.0 mL of a 0.06 mM methanolic DPPH (2,2-diphenyl-1-picrylhydrazyl; Sigma-Aldrich, St. Louis, MO, USA) solution. The tubes were maintained in darkness at 25 °C for 2 h 30 min. The film samples were separated, and the absorbance of the solution was measured at 517 nm in a spectrophotometer (mod. 6850 UV/Vis; Jenway, Bibby Scientific, Stone, UK). Methanol was used as blank. A calibration curve was prepared with DPPH (Kocakulak et al. 2019), using different concentrations of DPPH in methanol (0.006-0.06 mM). The determinations were done in duplicate. Antioxidant activity was expressed as milligrams of DPPH degraded per gram of dry film weight and was calculated as follows:

Antioxidant activity (mg DPPH/g dry film) = C x dilution rate/dry film weight in g

where C is the value obtained from calibration curve as milligrams of DPPH per mL of the difference between the absorbances of the DPPH reagent and the sample.

7.6. Mechanical properties

Tensile strength, elongation at break, elastic modulus, puncture strength and puncture deformation were tested according to the ASTM D882 method (ASTM. D882—Standard test method for tensile properties of thin plastic sheeting. In Annual Book of ASTM Standard; American Society for Testing and Materials: Philadelphia 2000), as described by Díaz *et al.* (Díaz et al. 2016). After cutting, film samples were stored in a desiccator at 20 °C and 45% RH for 48 h before analysis. A texturometer mod. EZ Test and the Trapezium Data Processing System software (Shimadzu Corporation, Tokyo, Japan) were used for mechanical properties determination.

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Figure 13: Example of mechanical properties determination in film sample.

7.7. Fourier transform infrared spectroscopy (FTIR)

The attenuated total reflectance-Fourier transform infrared spectra of films were obtained with a Jasco FT/IR-4600 equipped with the ATR Pro One accessory with an internal reflectance element of ZnSe (wave number range of 15000-550 cm^{-1}) (Jasco Corporation, Tokyo, Japan). Determinations were done in triplicate. The spectra were measured between 400-4000 cm^{-1} by co-adding 16 scans at 1 cm^{-1} resolution. Data treatments were performed with PeakFit software version 4.12 (SYSTAT Software, Richmond, CA, USA), Second derivative spectra were calculated on data (previously base-line corrected and smoothed with a Savitsky-Golay function to remove possible noise) for the identification and intensity determination of individual band positions.

7.8. Thermogravimetric analysis (TGA)

Thermogravimetric analysis of the films was performed according to the method described by Díaz *et al.* (Díaz et al. 2019), using a thermogravimetric analyzer with differential scanning calorimetric capability (Mettler Toledo, mod. TGA/DSC1; Schwerzenbach, Switzerland). The first derivative of the TGA curve (DTG) was calculated by means of PeakFit software (version 4.12; SYSTAT Software, Richmond, CA, USA).

7.9 Microstructure analysis by scanning electron microscopy (SEM)

The attenuated A JEOL-JSM 6360LV scanning electron microscope (Jeol, Ltd., Tokyo, Japan) operated at 15 kV was used to observe the microstructure of the films. The sample preparation was in accordance with the description of Díaz *et al.* (Díaz et al. 2016).

7.10. X-ray diffraction (XRD)

The crystallinity of films was studied by using an X-ray diffractometer (Bruker D8 Advance with a LYNXEYE XE-T detector, Bruker, Germany) with a CuKα1 radiation

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beam (λ = 1.5406 Å) in the angular range of 3 to 50 (2θ) at a voltage of 40 kV and current of 40 mA.

8. Statistical Analysis

In general, the statistical analyses described were carried out for all products. Before statistical analysis, normal distribution was tested using the Kolmogorov-Smirnov test. One-way and two-way ANOVA and Tukey's post hoc test were used to test and compare the statistical significance of differences between means, respectively. The results of the sensory classification test were evaluated using the chi-square test. For all average evaluations, a significance level of $p < 0.05$ was used (IBM SPSS Statistics version 19 for Windows; 2010; SPSS Inc., Chicago, IL, USA, and IBM SPSS Statistics software for Windows version 27 (2021; IBM Corp, Armonk, NY, USA). Graphs were produced using the Statistica Software, version 8 (Stasoft Europe, Hamburg, Germany).

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RESULTS. PUBLISHED ARTICLES

Manuscript I-Application of Ultrafiltration to Produce Sheep's and Goat's Whey-Based Synbiotic Kefir Products

The results from this article have been published as:

Arona Pires [1,2], Gözdenur Tan [3], David Gomes [1], Susana Pereira-Dias [1,4], Olga Díaz [2], Angel Cobos [2] and Carlos Pereira [1,4,*] (2023), Application of Ultrafiltration to Produce Sheep's and Goat's Whey-Based Synbiotic Kefir Products, *Membranes, 13, 473.*

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Abstract:

Membrane filtration technologies are the best available tools to manage dairy byproducts such as cheese whey, allowing for the selective concentration of its specific components, namely proteins. Their acceptable costs and ease of operation make them suitable for application by small/medium-scale dairy plants. The aim of this work is the development of new synbiotic kefir products based on sheep and goat liquid whey concentrates (LWC) obtained by ultrafiltration. Four formulations for each LWC based on a commercial kefir starter or traditional kefir, without or with the addition of a probiotic culture, were produced. The physicochemical, microbiological, and sensory properties of the samples were determined. Membrane process parameters indicated that ultrafiltration can be applied for obtaining LWCs in small/medium scale dairy plants with high protein concentration (16.4% for sheep and 7.8% for goats). Sheep kefirs showed a solid-like texture while goat kefirs were liquid. All samples presented counts of lactic acid bacteria higher than log 7 CFU/mL, indicating the good adaptation of microorganisms to the matrixes. Further work must be undertaken in order to improve the acceptability of the products. It could be concluded that small/medium-scale dairy plants can use ultrafiltration equipment to valorize sheep's and goat's cheese whey-producing synbiotic kefirs.

Keywords: caprine; cheese whey; kefir; ovine; prebiotics; probiotics; ultrafiltration

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1. Introduction:

The food industry produces high volumes of byproducts, with considerable environmental impacts. However, in most cases, the nutritional content of such byproducts makes them an important subject for valorization (Banaszewska et al. 2014). Cheese whey (CW), buttermilk (BM), and second cheese whey (SCW) are the byproducts resulting from the production of cheese, butter, and whey cheeses, respectively. Both CW and BM have excellent nutritional and techno-functional properties(Barukčić et al. 2019; Borges et al. 2020; Henriques et al. 2017; Henriques et al. 2019). Due to its high organic load, and with an estimated worldwide production of 190 million tons/year, CW represents an opportunity for bioenergy and biochemical production (Asunis et al. 2020). This byproduct represents 80–90% of the milk volume and contains more than 50% of milk nutrients. It has the following composition (% w/v) in the case of bovine CW: 4.5–5% lactose, 0.6–0.8% protein, 0.4–0.5% lipids, and 0.4–1% mineral salts (Yadav et al. 2016). Lactose is responsible for the high Biochemical Oxygen Demand (BOD) of whey (ca. 40–50 g/L). The values of total solids (lactose, proteins, and fat) are even higher in ovine and caprine whey when compared to bovine whey (Pires et al. 2021a). The potential environmental impact of CW represents a problem for the small/medium-scale dairy companies that do not possess the capacity to produce whey powder (WP), whey protein concentrates (WPC), or other whey derivatives, such as whey protein isolates (WPI) and hydrolysates (WPH). This problem is particularly relevant for producers of traditional cheeses based on sheep and/or goat milk. In the case of these producers, sheep and/or goat CW is often used to produce whey cheeses (WC). However, this option has some disadvantages, particularly the high energy input required to produce WC, its short shelf-life, and the fact that the byproduct of such process, deproteinized whey, also known as SCW, presents the same environmental constraints of CW, which result from its high lactose content (Pires et al. 2022). Pires et al. (Pires et al. 2021a) made an extensive review of the potential alternatives to valorize CW or SCW. Very promising developments have been made to convert these byproducts into value added commodities such as biofuels, bioplastics, bacterial cellulose, food colors and flavors, bioactive peptides, and single-cell proteins, as reported by several authors (Barukčić et al. 2019; Osorio-González et al. 2022; Sebastián-Nicolás et al. 2020). The dairy industry manages whey mainly by membrane separation or fermentation (Zotta et al. 2020). Due to the low protein content of cheese whey, membrane processes allow industries to concentrate and fractionate its components, operating at ambient or mild temperatures that respect their functional properties at a cost-effective production. The main membrane process applied in whey protein products manufacture is ultrafiltration (Pavoni et al. 2020). The use of membrane separation technologies, including UF, in the dairy industry and whey valorization has been extensively reviewed (Aslam et al. 2021; Barba 2021; Paladii et al. 2021; Reig et al. 2021; Wen-Qiong et al. 2019). The most valuable commercial products obtained in the dairy industry are those based on the highest protein concentration, usually in the form of dehydrated powders which guarantee a long shelf-life. Most commercial dry products are based on bovine whey proteins; they include concentrates (WPC), with protein contents between 35% and 80% manufactured by ultrafiltration, and isolates (WPI), with higher

protein concentrations (more than 90%) that can be obtained by ultrafiltration/diafiltration or ion chromatography (Macedo et al. 2021b).

The recent growing interest in caprine products, attributed to their specific nutritional and nutraceutical characteristics, (i.e., lower allergenicity of their proteins and higher content of oligosaccharides), makes the recovery of goat CW an opportunity to transform this byproduct into value-added novel dairy products (Macedo et al. 2021a). The results of cost–benefit and sensitivity analyses show that an integrated process of ultrafiltration/nanofiltration (UF/NF) is economically viable in small/medium cheese producers (Macedo et al. 2021b). Sheep dairy products, other than cheese, are also being developed and are looked at as an opportunity to valorize peripheral rural areas through the transformation of dairy products into competitive value-added commodities (Marnotes et al. 2021; Silva et al. 2023b; Tulla 2019).

Concomitantly, increasing awareness of health has increased demand for healthy food which is confirmed by raising market sales of functional foods. Functional foods are defined as foods that, in addition to their nutritional value, contain ingredients that act specifically on body functions associated with the control or reduction of the risk of developing some diseases. One such food is kefir. This product is a natural acidic-alcoholic fermented milk drink traditionally produced by milk fermentation using kefir grains. Kefir grains are composed of a complex population of bacteria and yeasts embedded in a polysaccharide-protein matrix. Lactic acid bacteria, acetic acid bacteria, and yeasts are the dominant microorganisms. Kefir can provide probiotic benefits, such as intestinal microecological balance regulation, and antibacterial and anti-inflammatory activity (Lv et al. 2021).

A systematic review by Vieira et al. (Vieira et al. 2021) reports that the bioactive compounds more commonly found in kefir were exopolysaccharides, including kefiran, bioactive peptides, and organic acids, especially lactic acid. It has been indicated that the kefir peptides have therapeutic potential for the treatment of Alzheimer's disease (Malta et al. 2022). The existence of a relationship between the improvement in skin parameters and the changes in the intestinal microbial balance after kefir consumption has also been reported (Alves et al. 2022). The bioconversion of whey by kefir lactic acid bacteria (LAB) may be effective in reducing obesity and obesity-related diseases (Youn et al. 2022). Other authors (Chang et al. 2022; de Vasconcelos et al. 2022; Lai et al. 2022) report that milk kefir enhances bone microarchitecture and metabolism, has osteoprotective effects, and can be used as a nutritional supplement to accelerate fracture healing. The antimicrobial activity of kefir microorganisms derives from the microorganisms' capacity to adhere to the intestinal epithelium, preventing the adhesion of pathogens, among other properties. Bacteria and yeasts isolated from kefir have been shown to have in vivo and in vitro antimicrobial activity against enteropathogenic bacteria and spoilage fungi (González-Orozco et al. 2022). Some authors developed synbiotic drinks from milk fermented by kefir grains and supplemented by inulin, quinoa flour, or with the addition of specific probiotic bacteria (Kef and Arslan 2021; Lv et al. 2021; Sebayang et al. 2022). However, most available milk-based kefir products on the market are produced without

kefir grains; they use commercial kefir cultures (Agarbati et al. 2021). This study will also aim to develop new synbiotic kefir products based on sheep and goat UF concentrated CW, fermented by kefir grains or commercial kefir starter, with and without the addition of a commercial probiotic culture, and supplemented with inulin.

2. Materials and Methods

2.1. Production of Liquid Whey Concentrates

The sheep and goat CW were supplied by external dairy companies and processed at the dairy pilot plant of Escola Superior Agrária de Coimbra (Coimbra, Portugal). Each type of whey (500 L) was subjected to ultrafiltration (UF) in a pilot plant supplied by Proquiga Biotech SA (A Coruña, Spain), equipped with an organic UF membrane (3838 PVDF/polysulfone) with an effective area of filtration of 7 m2 and 10 kDa cut-off, supplied by FipoBiotech, Pontevedra, Spain. The process was carried out at 40–45 °C, at a transmembrane pressure of 3.0–3.5 bar, aiming at a volumetric concentration factor (VCF = Vol. Feed/Vol. Retentate) of 20. The UF concentration step allowed for the obtention of 25 L of goat and 25 L of sheep liquid whey concentrate (LWC). The goat and sheep LWCs obtained were pasteurized (65 °C, 30 min) and then homogenized at 15 MPa using a Rannie™ model Bluetop homogenizer (Copenhagen, Denmark). LWCs were frozen at -25 ± 2 °Cuntil they were used to produce goat and sheep kefir formulations.

2.2. Manufacture of Goat and Sheep Kefir Products

Sheep or goat LWCs (approximately 12 L) were thawed under refrigeration at 0 °C for 24 h. Subsequently, the samples were heated to 80 ± 2 °C and 2.5% (w/v) inulin (Fibruline™, Cosucra, supplied by Induxtra de Suministros, Moita, Portugal) was added, being the mixture homogenized at 15 MPa. The mixtures were then cooled to 38 °C, divided into four portions (3 L each), and inoculated with one of the following cultures:

1. Commercial kefir culture: Exact™ Kefir 1 (CHR Hansen, Hoersholm, Denmark) mesophilic and thermophilic culture (Debaryomyces hansenii, Lactococcus lactis subsp. cremoris, L. lactis subsp. lactis biovar diacetylactis, L. lactis subsp. lactis, Leuconostoc and Streptococcus thermophilus) at a concentration of 0.01% (w/v) (EK);

2. A mixture of a commercial kefir culture (Exact™ Kefir 1, CHR Hansen, Hoersholm, Denmark) and a probiotic culture containing Bifidobacterium bifidus-BB12, Lactobacillus acidophilus-LA5 and S. thermophilus (ABT-5™, CHR Hansen, Denmark) at 0.01% each one (EKABT5);

3. A traditional kefir culture at a concentration of 2.5% v/v (TK);

4. A mixture of the traditional kefir culture at 2.5% v/v and probiotic culture (ABT-5™)

at 0.01% (TKABT5). The inoculated goat or sheep LWCs were placed in an incubation chamber (Jenogand™ Y 1000, Copenhagen, Denmark) at 37 °C and the pH and titratable acidity were monitored until the products reached a target pH of 4.5. The fermentation process was stopped by rapid cooling to 20 °C in less than 30 min. Afterward, the fermented products were placed in the refrigeration chamber at 4 ± 2 °C for 12 h,

approximately. After this process, the goat and sheep kefir products were stored under refrigeration in a chamber at 0 ± 2 °C for 30 days. Products were evaluated at the 1st, 10th, 20th, and 30th days of storage.

2.3. Physicochemical Analysis

The total solids content of kefir products was determined by drying the samples in a Schutzart DIN 40050-IP20 Memmert™ oven (Schwabach, Germany), according to NP 703: 1982 for yogurt. The ash content was determined by incineration of dry samples in an Nabertherm™, model LE 4/11/R6 electric muffle furnace (Bremen, Germany). The fat content was determined by the Gerber method (SuperVario-N Funke Gerber™ centrifuge, Berlin, Germany) according to NP 2105:1983 for sheep kefir, and NP 1923:1987 for goat kefir. The total N content was determined by the Kjeldahl method in the Digestion System 6 1007 Digester Tecator™ (Foss Analytical, Häganäs, Sweden) following the AOAC (1997) standard. To calculate the percentage of protein the conversion factor of 6.38 was used. All analyzes were performed in triplicate.

2.3.1. pH and Titratable Acidity

The pH of products was directly determined with a HI 9025 HANNA Instruments pH meter (Leighton Buzzard, UK), to monitor the evolution of the pH over fermentation, immediately after production and on the 10th, 20th, and 30th days of storage. The titratable acidity, expressed in % lactic acid, was determined by titration using a 0.1 N NaOH solution according to NP 701: 1982 for yogurts (NP 701: 1982;).

2.3.2. Color Parameters

The color of kefir samples was determined with a Minolta™ Chroma Meter, model CR-200B colorimeter (Tokyo, Japan) calibrated with a white standard (CR-A47: Y = 94.7; x 0.313; y 0.3204). The following conditions were used: illuminant C, 1 cm diameter aperture, 10° standard observer. The color coordinates were measured in the CIEL*a*b* system. Color difference (ΔEab*) was calculated as:

$$\Delta Eab^* = [(L^* - L^{*0})^2 + (a^* - a^{*0})^2 + (b^* - b^{*0})^2]^{1/2} \quad (1)$$

where L^{*0}, a^{*0}, and b^{*0} and L^*, a^*, and b^* were the values measured for the samples under comparison. A matrix of ΔEab^* values between products was constructed. Three measurements were taken for each sample.

2.3.3. Rheological Properties

The rheological properties of the kefir products were evaluated in a rheometer (Rheostress 1, ThermoHaake ™, Thermo Fisher Scientific, Waltham, MA, USA) in oscillatory mode. The measurement system consisted of a cone and plate geometry, C60/Ti-0.052 mm (35 mm diameter and 1° angle). Stress sweep tests were performed at 1 Hz to investigate the rheological linear viscoelastic behavior of the samples. The elastic modulus (G′), the viscous modulus (G″), and the complex viscosity (η*) of the products were evaluated in the range of 0.3 to 6.5 rad/s at 3 Pa.

2.3.4. Texture Parameters

A Stable Micro Systems™ texture analyzer, model TA.XT Express Enhanced was used to perform the texture analysis of the sheep kefir samples and the results were calculated using the Specific Expression PC software, version 1,1,12 (Godalming, Surrey, UK). A TPA-type test was run with a penetration distance of 15 mm at 1 mm/s using an acrylic cylindrical probe with a diameter of 25.4 mm and a height of 38.1 mm.

2.4. Microbiological Analysis

The microbial counts of lactic acid bacteria (LAB) of the genera Lactobacillus spp. And Lactococcus spp. were performed after production and over storage. Lactococci and lactobacilli were enumerated on plates at 37 °C for 48 h on M17 agar (in aerobiosis) and on MRS agar (in anaerobiosis) (Biokar Diagnostics, Beauvais, France), respectively, according to ISO 7889, IDF 117 (2003) (IDF 117: 2003). Yeasts were enumerated in plates at 25 °C according to ISO 6611 IDF 94 (2204) (IDF 94: 2004). Analyses were carried out in triplicate along with two controls for each medium and results are expressed as log CFU/g of product.

2.5. Sensory Analysis

Consumer preference tests were conducted with an untrained panel within 7 days of storage. A hedonic test was performed for the kefir samples in order to evaluate the aroma, texture, flavor, and global appreciation on a scale from 1 to 9 (1 = I don't like it at all to 9 = I like it very much) using a non-trained panel with 30 members (Stone et al. 2020). The members of the panel were also asked to rank the samples according to their preference, from 1-most preferred to 4-less preferred, according to ISO 8587 (1988) (ISO 8587: 1988).

2.6. Statistical Analysis

Prior to statistical analysis, normal distribution was evaluated using the Kolmogorov–Smirnov test. The differences among formulations produced with the same LWC were analyzed by one-way ANOVA and the means were compared by Tukey's post hoc test. The same statistical treatment was performed to study the effect of storage time on the hardness of sheep kefir samples. The differences between each formulation produced with different LWC (sheep or goat) were compared by t-test for independent samples. For all mean evaluations, a significance level of $p < 0.05$ was used (IBM SPSS Statistics for Windows (version 27); 2021; IBM Corp, New York, NY, USA).

3. Results

3.1. Performance of UF Process

Figure 1 presents the evolution of the UF fluxes over time. The results are the average of two concentration trials. It is clear that higher filtration flux was obtained during the concentration of goat CW. In both cases, average fluxes are of the order of 30 and 20 $Lm^{-2}h^{-1}$, respectively for goat and sheep CW, which indicates the feasibility of the operation of ultrafiltration units in small-scale cheese plants, processing volumes of ca.

500– 1000 L milk per day. The figure also presents the calculated evolution of the volumetric concentration factor during the concentration process. This information indicates that it is possible to concentrate whey to obtain high levels of protein and/or fat with the objective to obtain the desired levels of these nutritional components, namely protein, in the LWCs.

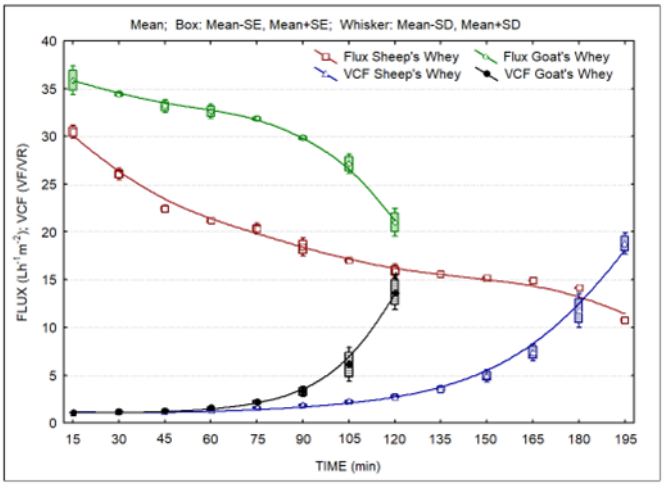


Figure 1. Evolution of ultrafiltration fluxes and volumetric concentration factor during concentration of sheep and goat whey. Fit by distance weighted least squares.

3.2. Physicochemical Characteristics of LWCs and Kefir Samples

The physicochemical characteristics of the original whey used in the trials and of the LWCs used in the production of kefir samples are displayed in Table 1. It is evident the higher protein content of the sheep LWC, while in the case of fat, the goat LWC presented higher values. It has to be referred that, despite its higher fat content, goat cheese whey had higher UF fluxes than sheep whey. Hence, it can be concluded that the protein level of the feed had a major impact on UF performance.

Table 1. Composition of sheep and goat whey and of liquid whey concentrates (LWCs).

PRODUCTS	Dry matter (%)	Protein (%)	Fat (%)	Ashes (%)
Sheep's Whey	8.63 ± 0.68	1.32 ± 0.09	1.18 ± 0.43	2.15 ± 0.27
Sheep's LWC	34.74 ± 0.68	16.35 ± 0.36	14.12 ± 0.50	1.98 ± 0.67
Goat's Whey	6.61 ± 0.09	0.73 ± 0.01	1.76 ± 0.47	1.03 ± 0.24
Goat's LWC	32.23 ± 0.10	7.84 ± 0.45	19.32 ± 0.12	1.55 ± 0.07

The physicochemical characteristics of the different kefir formulations produced are displayed in Figure 2. As expected, the products originating with sheep LWC present higher levels of protein, which are nearly double the ones of the products obtained from goat whey. Conversely, goat whey products present higher levels of fat, which results from the higher fat level in the original goat CW.

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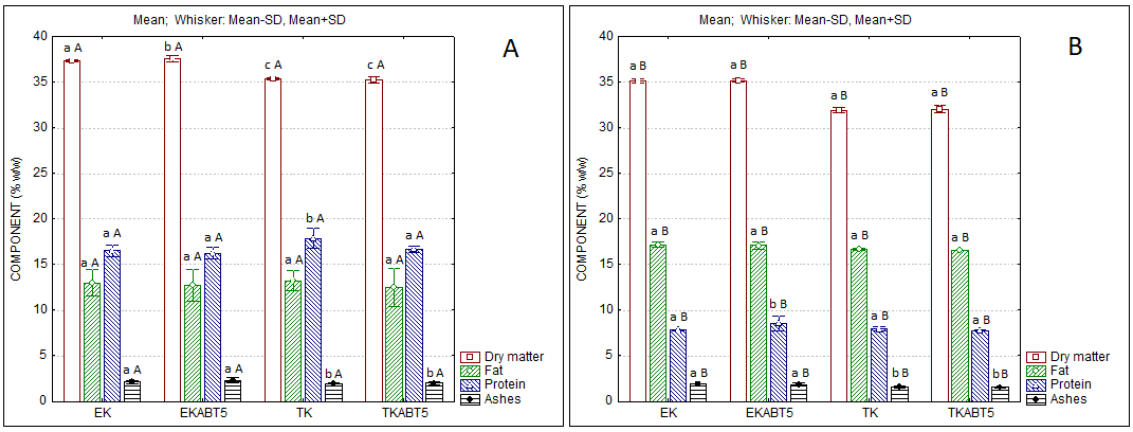


Figure 2. Composition of kefir samples produced with sheep (A) and goat (B) liquid whey concentrates. (Mean; Standard Deviation-SD). Different lowercase letters a, b, and c indicate significant differences ($p < 0.05$) among formulations produced with the same LWC. Different capital letters A and B indicate differences ($p < 0.05$) between each formulation produced with different LWC (sheep or goat). EK = Commercial kefir; EKABT5 = Commercial kefir + probiotic culture; TK = Traditional kefir; TKABT5 = Traditional kefir + probiotic culture.

Figure 3 presents the evolution of the pH and titratable acidity of kefir products over 30 days of refrigerated storage. It is clear the lower pH and the higher acidity of goat kefir products. The sheep kefir products produced with commercial kefir starter without and with the addition of the probiotic culture (EK and EKABT5) presented the highest pH and the lowest acidity values, while the traditional kefir without or with the addition of the probiotic culture (TK and TKABT5) presented pH values below 4.5 and acidity values of the order of 1.3% lactic acid, immediately after production. The acidity increased to values higher than 1.5 on days 10 and 20 and presented a sharp increase from the 20th to the 30th day of storage. In the case of goat kefir samples, only TKABT5 surpassed values of 1.5% lactic acid. The use of the probiotic culture had impacts on these parameters, decreasing the pH and increasing the titratable acidity, both in sheep and in goat kefir products.

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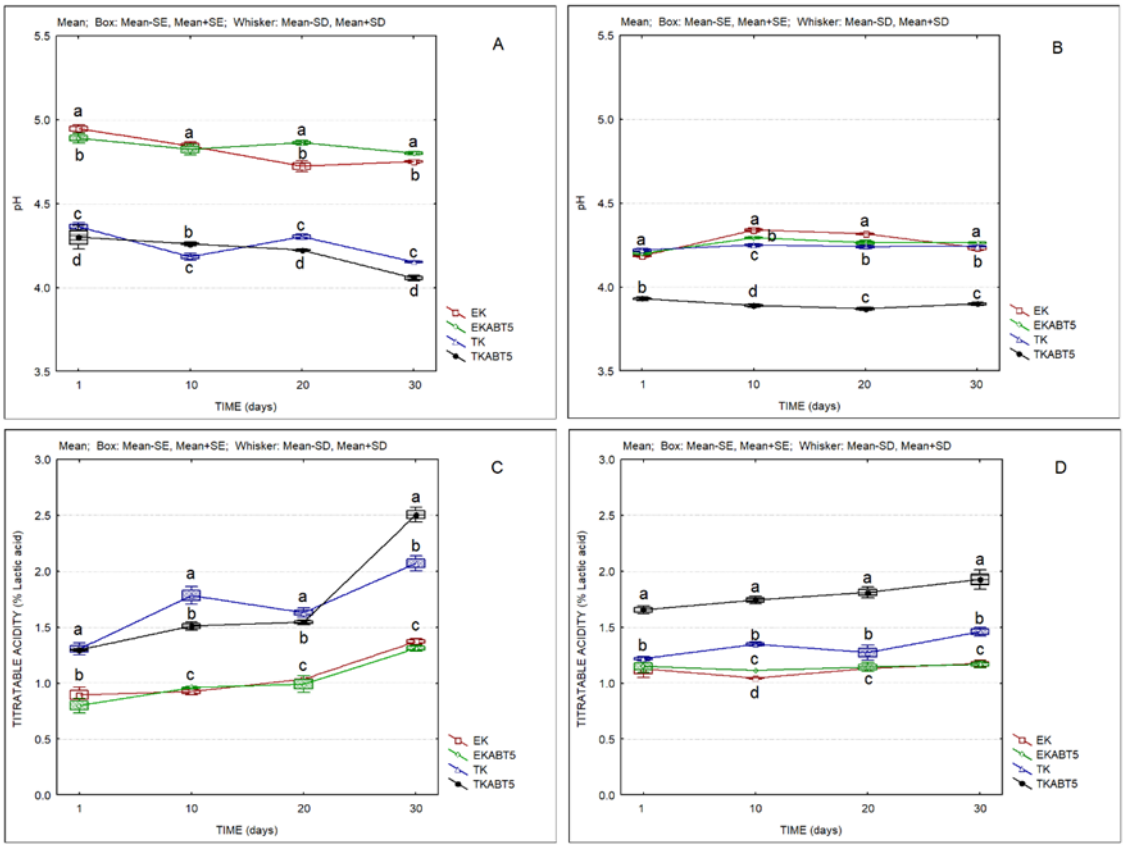


Figure 3. pH and titratable acidity of kefir samples produced with sheep and goat liquid whey concentrates. (A) pH of sheep kefir samples; (B) pH of goat kefir samples; (C) Titratable acidity of sheep kefir samples; (D) Titratable acidity of goat kefir samples. (Average values; Standard Error-SD; Standard Deviation-SD). EK = Commercial kefir; EKABT5 = Commercial kefir + probiotic culture; TK = Traditional kefir; TKABT5 = Traditional kefir + probiotic culture. Different lowercase letters a, b, and c indicate significant differences ($p < 0.05$) among formulations produced with the same LWC.

Figure 4 shows the elastic (G′) and viscous (G″) moduli as well as the complex viscosity of sheep and goat kefir products. Sheep kefir products presented a solid-like texture while goat kefir products were liquid. Regarding sheep kefir products, the highest values of G′ were obtained for TK and TKABT5 (Figure 4A). EK and EKABT5 presented G′ values ca. one log cycle lower as compared to the ones produced with the traditional kefir. These observations are confirmed by the evaluation of the complex viscosity (Figure 4B). In all the goat kefir products, the viscous modulus (G″) was higher than the elastic modulus (G′) (Figure 4C), indicating the liquid nature of the products, which is also confirmed by their complex viscosity (Figure 4D).

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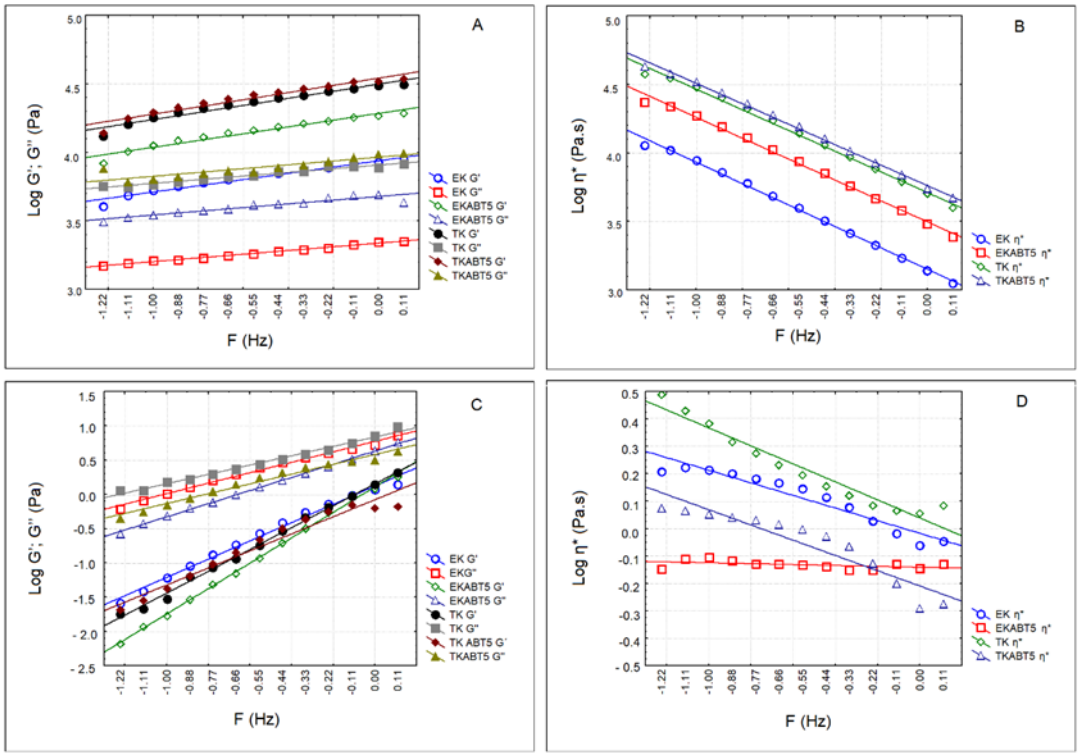
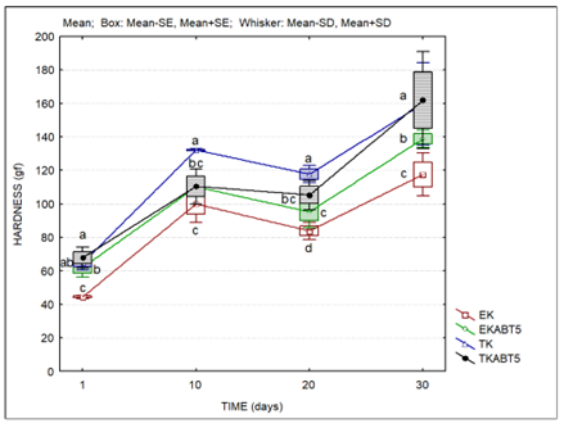


Figure 4. Elastic (G′) and viscous moduli (G″) and complex viscosity (η*) of kefir samples produced with sheep and goat liquid whey concentrate. (A) G′ and G″ of sheep kefir samples; (B) η* of sheep kefir samples; (C) G′ and G″ of goat kefir samples; (D) η* of goat kefir samples. EK = Commercial kefir; EKABT5=Commercial kefir + probiotic culture; TK = Traditional kefir; TKABT5 = Traditional kefir + probiotic culture.

The texture evaluation performed on sheep kefir products is displayed in Figure 5. It is clear the tendency for the increase in the hardness of sheep kefir products between the first and the 30th days of storage. Most probably, this result could be due to the increased links in the protein matrix of the products and/or to the separation of water from the protein matrix (syneresis), which increased the hardness of the protein/lipid matrix.



Figure–5. Hardness of kefir samples produced with sheep liquid whey concentrates. (Average values; Standard Error-SD; Standard Deviation-SD). Different lowercase letters a, b, c, and d indicate significant differences ($p < 0.05$) among the days of storage. EK = Commercial kefir; EKABT5 = Commercial kefir + probiotic culture; TK = Traditional kefir; TKABT5 = Traditional kefir + probiotic culture.

Figure 6 shows the evolution of the color parameters of all kefir samples over storage. It can be observed that both sheep and goat kefir products show a slight increase in the lightness parameter L* over time (Figure 6A,B). Goat kefir products presented higher values for parameter a* (red-green axis) as compared to sheep kefir products (Figure 6 B,C). However, the main difference between sheep and goat kefir products was observed in parameter b* (blue-yellow axis), with goat products presenting values of the order of 4, increasing to 6 on the 30th day of storage, while the lowest value registered for sheep samples was of the order of 6. This observation reflects the more intense yellow color for these samples. Tables S1 and S2 of Supplementary Materials indicate the values of ΔEab* between different products and for the same product over storage. In most cases these values are higher than 1, indicating that color differences can be detected by a common observer. Fewer differences were observed between kefir products based on goat LWC (ΔEab* values <1).

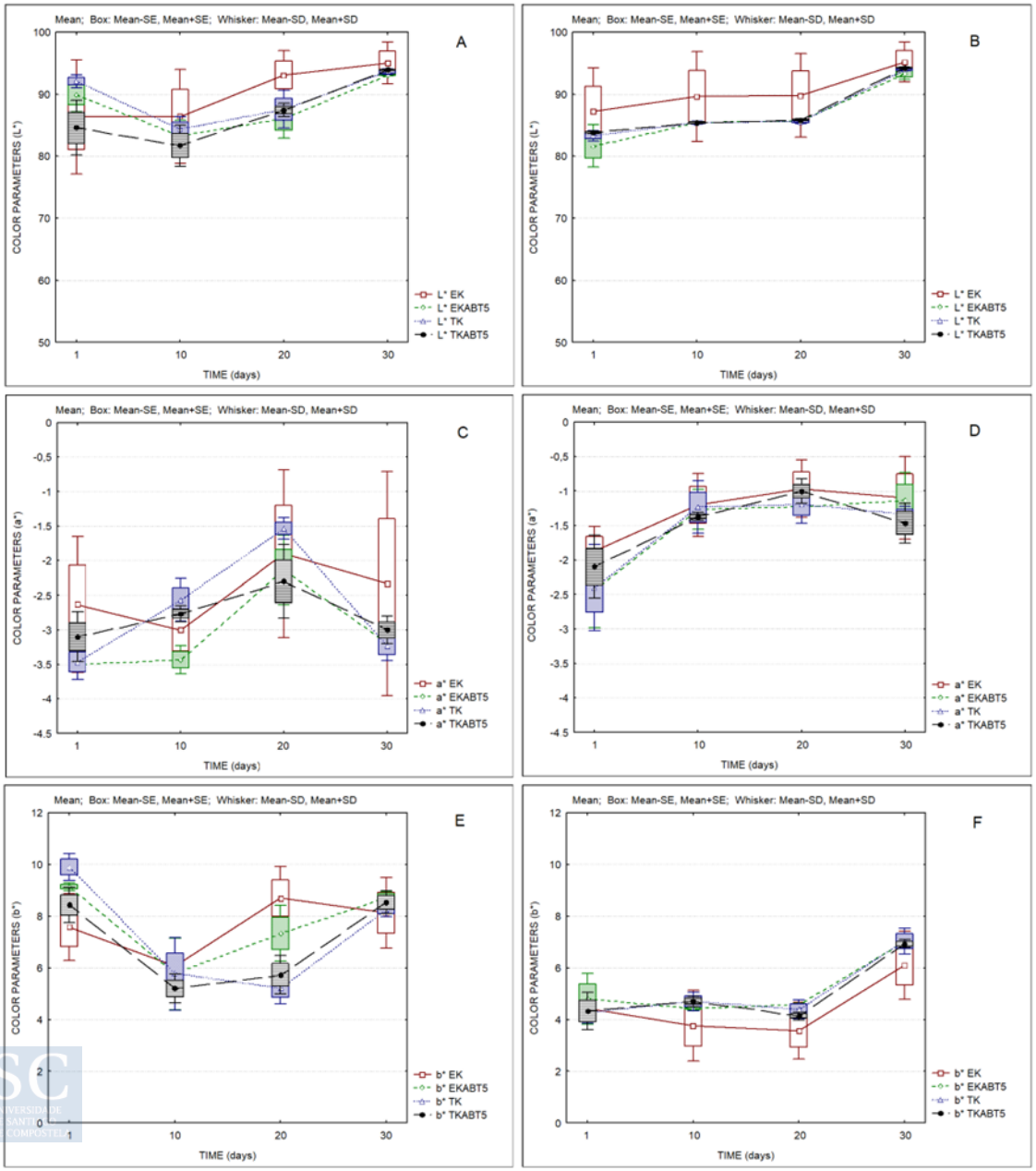


Figure 6. Color parameters of kefir samples produced with sheep and goat liquid whey concentrate. (A) L* of sheep kefir samples; (B) L* of goat kefir samples; (C) a* of sheep kefir samples; (D) a* of goat kefir samples; (E) b* of sheep kefir samples; (F) b* of goat kefir samples. (Average values; Standard Error-SD; Standard Deviation-SD). EK = Commercial kefir; EKABT5 = Commercial kefir + probiotic culture; TK = Traditional kefir; TKABT5 = Traditional kefir + probiotic culture.

3.3. Microbiological Characteristics of Kefir Products

Regarding the evolution of the microbial composition, Figure 7 compares the counts of lactobacilli, lactococci, and yeasts of the different products during storage. In all cases, the products presented counts of lactobacilli, lactococci, and yeasts of the order of, or higher than, log 7 CFU/g, indicating the good adaptation of LAB and yeasts to the LWC, and the probiotic potential of the products over the storage period.

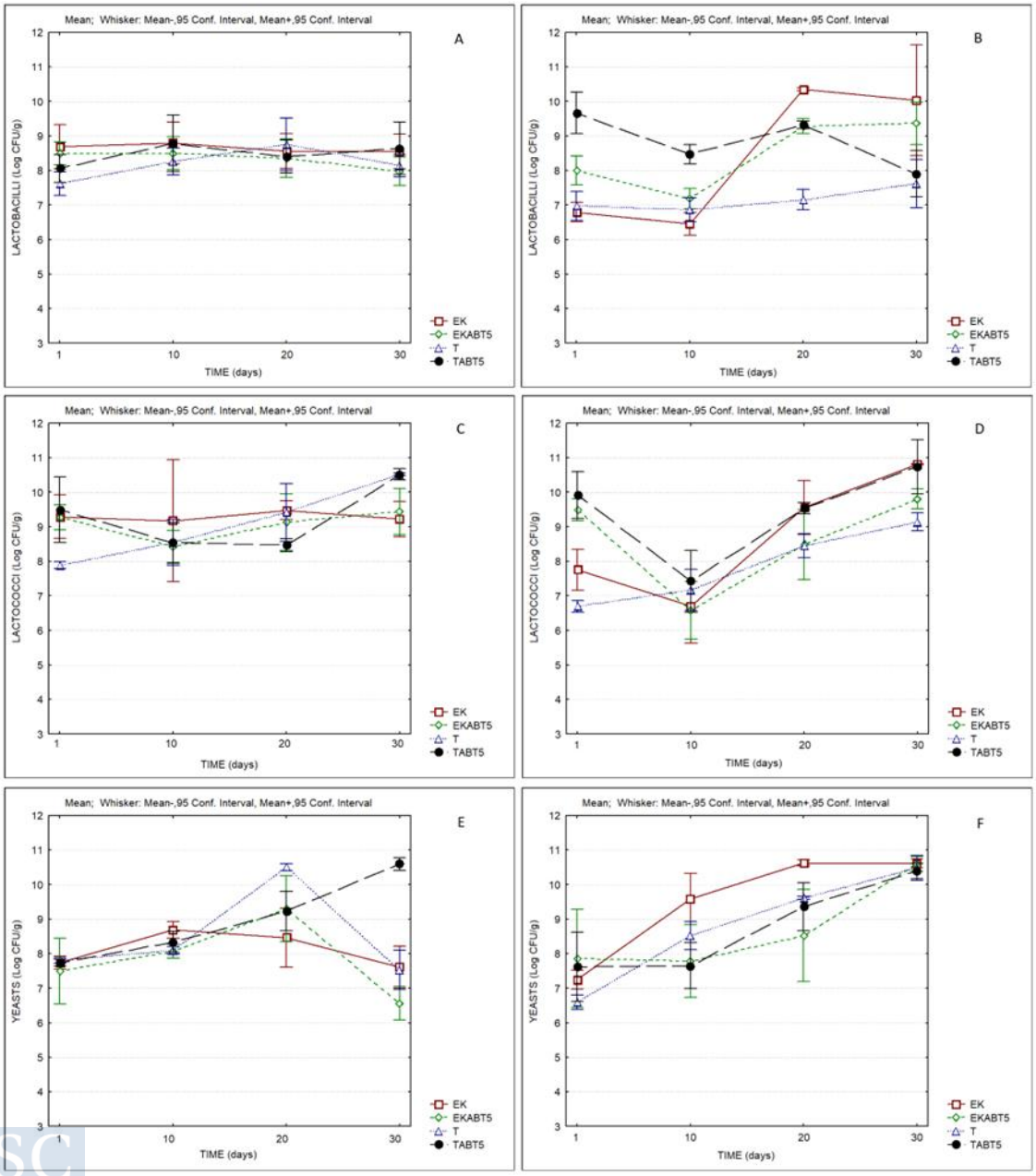


Figure 7. Microbial characteristics of kefir samples produced with sheep and goat liquid whey concentrate. (A) Lactobacilli counts of sheep kefir samples; (B) Lactobacilli counts of goat kefir samples; (C) Lactococci

counts of sheep kefir samples; (D) Lactococci counts of goat kefir samples; (E) Yeasts counts of sheep kefir samples; (F) Yeasts counts of goat kefir samples (Average values and 0.95 confidence interval). EK = Commercial kefir; EKABT5 = Commercial kefir + probiotic culture; TK = Traditional kefir; TKABT5 = Traditional kefir + probiotic culture.

Concerning the sensory evaluation of the different products, it is clear that further work must be undertaken in order to improve the acceptability of the products. Considering the results presented in Table 2, it can be concluded that sheep kefir products were below an acceptable score of 6, regarding the aroma and taste of samples S-TK and STKABT5. Concerning goat products, both G-EK and G-EKABT5 presented unacceptable scores for aroma, taste, and texture. The ranking test allowed the separation of samples into different groups. Regarding sheep products, samples S-EK and S-EKABT5 were ranked 1st and 2nd respectively, being S-TK and S-TKABT5 ranked 3rd and 4th. The opposite pattern was observed with goat kefir samples which were ranked in the following order: 1st G-TKABT5; 2nd G-TK; 3rd G-EKABT5; 4th G-EK.

Table 2. Sensory scores of kefir samples produced with sheep and goat LWCs.

PRODUCTS	S-AROMA	S-COLOR	S-TASTE	S-TEXTURE
S-EK	5.9 ± 1.5^{aA}	7.0 ± 1.6^{aA}	6.3 ± 2.0^{aA}	7.1 ± 1.7^{aA}
S-EKABT5	6.0 ± 1.8^{aA}	7.0 ± 1.5^{aA}	6.3 ± 2.0^{aA}	7.2 ± 1.4^{aA}
S-TK	4.5 ± 1.5^{bA}	6.8 ± 1.6^{aA}	4.1 ± 1.9^{bA}	5.6 ± 2.0^{bA}
S-TKABT5	4.8 ± 1.9^{bA}	6.8 ± 1.7^{aA}	3.8 ± 1.8^{bA}	5.6 ± 1.9^{bA}
G-EK	4.9 ± 2.0^{abB}	7.5 ± 1.6^{aA}	3.7 ± 2.3^{aB}	5.2 ± 1.7^{aB}
G-EKABT5	4.7 ± 2.0^{aB}	7.6 ± 1.7^{aA}	3.1 ± 1.8^{aB}	5.6 ± 2.1^{abB}
G-TK	5.8 ± 1.7^{abB}	7.9 ± 1.3^{aB}	5.5 ± 2.2^{bB}	6.5 ± 1.6^{bB}
G-TKABT5	6.0 ± 2.0^{bB}	8.0 ± 1.1^{aB}	5.8 ± 2.1^{bB}	6.4 ± 2.0^{abA}

Different superscript letters a and b indicate significant differences between formulations produced with the same LWC. Different superscript capital letters A and B indicate differences between each formulation produced with different LWCs.

4. Discussion

Considering the performance of the UF process, the direct concentration of sheep or goat CW can lead to the production of LWCs with interesting nutritional properties, which can subsequently be used in the production of fermented synbiotic dairy products with excellent nutritional characteristics. As expected, at higher volumetric concentration factors, the increased solids concentration of retentates started to affect UF filtrate fluxes negatively. This was particularly evident in the case of sheep whey due to its higher protein content. One must also point out the fact that the total solids concentration factors of the retentates do not reflect the VCF 20 aimed. This is due to the dilution of the volume of product with approximately 15 L of water retained in dead volumes at the beginning of the operation and, the need to push the final volume of retentate with the introduction of water in the system. Both these situations originate a certain amount of dilution of the feed and of the retentate, with higher impacts when working in batch mode and when low feed volumes are used. This was particularly evident in the case of goat whey. The characteristics of the UF processing unit used can fit the needs of small-scale dairy industries that, currently, do not possess the capacity to valorize whey. Macedo and

coworkers (Macedo et al. 2021a) had already reported that an integrated process of ultrafiltration (UF)/nanofiltration (NF) is economically viable in small/medium-sized cheese dairies processing of ca. 3500 L milk/day and they observed that UF membranes of 10 kDa (the same pore size that we used) have higher permeate fluxes than membranes of 1 kDa. The efficiency of the process performed in the present work allows us to consider that the process is feasible even in smaller scale units (i.e., processing ca. 500 L milk/day). Higher protein contents in UF liquid concentrates from sheep cheese whey could be obtained by using diafiltration and adding calcium chloride as has been reported by Pavoni et al. (Pavoni et al. 2020); however, these operations increase the complexity of the valorization process.

It is also important to remark that as a result of the UF process with a VCF of 20 a high volume of whey permeate is obtained (ca. 95 % of the original feed volume). This UF permeate contains most of the lactose of the original whey which is responsible for its high BOD. Although UF concentration allows for the obtention of a valuable product (i.e., LWC), this operation does not solve completely the environmental problem associated with cheese whey. UF whey permeates must be treated by physicochemical treatment (e.g., nanofiltration) or fermentation in order to overcome environmental problems. The application of NF to UF whey permeates allows for the obtention of lactose concentrates and reduces the environmental impact, as has been reported in goat whey treatment (Macedo et al. 2021a).

Regarding the physicochemical characteristics of the kefir samples obtained, one can refer that they reflect the composition of the LWCs obtained. The higher values of protein reached in sheep LWC in relation to goat LWC influenced the rheological properties (G′, G″, η*) of the kefir. The lower levels of protein in goat kefir was the cause for their liquid nature. Marnotes et al. (Marnotes et al. 2021) also observed that sheep yogurts before freezing were solid, and goat yogurts were viscous liquids due to differences in protein concentrations of the LWCs used as raw materials.

Some differences in kefir from sheep and goats have also been reported. Guangsen et al. (Guangsen et al. 2021) compared the kefir produced with ovine and caprine milk and observed significantly higher values of pH in the parameter b* of ovine kefir. The high counts of lactobacilli, lactococci, and yeasts (>log 7 CFU/g) indicate the good adaptation of microorganisms to the ovine´s and goat´s LWCs. Commercial and traditional kefir did not show particular differences regarding their microbial counts. Several studies compared the microbiological characteristics of kefir produced by commercial cultures or grains (Biçer et al. 2021; Guangsen et al. 2021; Guclu et al. 2021; Wang et al. 2022). Guangsen et al. (Guangsen et al. 2021) report that the counts of lactic acid bacteria were higher in the sheep kefir than in goat kefir. These authors indicated that the sheep kefir was found better than goat kefir due to the microbiological characteristics, volatile compounds, and sensory profile.

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Biçer et al. (Biçer et al. 2021) evaluated the bacterial microbiota of five commercial and one traditional kefir and observed that the microbial diversity in traditional kefir is higher than in commercial kefir, being Lactobacillaceae and Streptococaccceae, the most

important families in traditional kefir. Guclu et al. (Guclu et al. 2021) studied industrial kefirs (plain kefirs (without fruits) and fruit kefirs) and kefir fermented using kefir grains. They observed counts of total aerobic mesophilic bacteria in the order of log 5.5–8.0 CFU/mL in plain kefir and of log 6.2–8.5 CFU/mL in fruit kefir; higher counts were found in kefir fermented with grains. They did not find any differences in the counts of lactic acid bacteria in plain and fruit kefir measured at two different times of storage. Wang et al. [49] observed that the microbiological and sensory characteristics of the kefir fermented by a (Wang et al. 2022) compound strain starter and by kefir grains were similar.

Kef & Arslan (Kef and Arslan 2021) observed in cow's milk kefir and goat milk kefir a decrease of streptococci and lactobacilli counts during the storage of the products, slowly until the 7th day and quickly until the 14th day of storage. These results are different than those found in our work since a decrease in the microbiological counts during the 30 days of storage was not observed.

In relation to sensory analysis, it has been reported that the ovine kefir is more appreciated than the caprine kefir (Guangsen et al. 2021). The sensory scores of our ovine and goat kefirs showed significant differences, but some sensorial aspects of both kefirs can be improved. It must be referred that the composition of the LWCs can be tailored according to the needs, particularly concerning the levels of solids, protein, and fat desired for the fermented products. It has also to be referred that, other ingredients such as fruit syrups or jams can be used in the formulations according to consumer's preferences. Therefore, there is a large margin to improve the sensory acceptability of the products. The improvement of the acceptability of the products will largely increase the feasibility of this approach, envisaging the valorization of CW through the production of fermented dairy products with potentially positive health effects. Furthermore, these products can have a shelf-life of ca. 30 days, which is larger than the one of conventional whey cheeses traditionally produced by small-scale dairy companies.

5. Conclusions

Considering the feasibility of the use of UF concentration of CW in small/medium scale cheese plants processing small ruminant's milk, the present work proves that this operation can allow for the valorization of such byproducts, reducing therefore the environmental impact of the operation and allowing for the development of functional foods with proven health benefits. However, this does not solve the environmental problem associated with cheese whey due to the high amount of permeate obtained, which must be further processed. Despite the need to improve the sensory acceptability of the kefir products developed, it can be considered that this approach represents an excellent opportunity for such companies, which in most cases face the production of CW as a problem and do not take advantage of the potential opportunities to valorize it.

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Manuscript II-Whey Cheeses Containing Probiotic and Bioprotective Cultures Produced with Ultrafiltrated Cow's Whey

The results from this chapter have already been published as:

Arona Pires [1,2], Agata Bożek [3], Hubert Pietruszka [3], Katarzyna Szkolnicka [3], David Gomes [1], Olga Díaz [2], Angel Cobos [2] Carlos and Pereira [1,4,*, (2024), Whey Cheeses Containing Probiotic and Bioprotective Cultures Produced with Ultrafiltrated Cow's Whey, *Foods* 13(8), 1214.

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Conceptualization, C.P., A.P., O.D. and A.C.; methodology, C.P., A.P., O.D. and A.C.; validation, C.P., K.S., O.D. and A.C.; investigation, A.P., H.P., A.B. and D.G.; resources, C.P.; data curation, C.P., O.D., A.C. and K.S.; writing—original draft preparation, A.P., C.P., O.D. and A.C.; writing—review and editing, C.P, O.D., A.C. and K.S.; supervision, C.P.; project administration, C.P.; funding acquisition, C.P. All authors have read and agreed to the published version of the manuscript

Abstract:

Bovine whey cheese (WC) is a product from southern European countries that presents some challenges: its production process involves high energy inputs; the yield is low; and WC has a short shelf life. The application of ultrafiltration (UF) to bovine whey before manufacture of WC and the employment of protective cultures can reduce these disadvantages. The objective of this research was the production of whey cheeses using ultrafiltrated bovine cheese whey with added probiotics or probiotics plus protective cultures. Three types of WC were produced: control CW without any addition (C); CW with the addition of the probiotic Lactobacillus acidophilus (LA5); and CW with the addition of Lactobacillus acidophilus plus a protective culture containing Lacticaseibacillus paracasei and Lacticaseibacillus rhamnosus (LA5FQ4). The WCs were stored under refrigerated conditions for 28 days. The products with added cultures presented lower pH values and higher titratable acidities when compared to the control. Sample LA5 presented the lowest pH and the highest titratable acidity, while LA5FQ4 presented intermediate values. Slight differences were observed between products regarding color parameters, chiefly resulting from storage time. The samples with added cultures were firmer when compared to the control, with LA5 cheeses showing the highest values at the end of the storage. Lactic acid bacteria (LAB) counts were on the order of log 8–9 CFU/g for the products with added cultures. Lower levels of yeasts and molds were detected on the sample with the protective culture (LA5FQ4), so that by the end of storage they presented counts one log cycle

lower than C and LA5. Hence, the beneficial impact of the protective culture on the shelf life of the product is evident. Regarding sensory evaluation, LA5FQ4 cheeses obtained the highest scores for all parameters evaluated. It can be concluded that the use of UF associated with the addition of protective cultures can be very useful to reduce the energy consumption of the manufacturing process, to prolong the shelf life of WC and to improve its sensory properties.

Keywords: whey cheese; ultrafiltration; probiotics; protective cultures.

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Introduction:

Cheese whey (CW) results from cheese production, and large amounts are generated worldwide. CW contains over 50% of the total solids found in the original whole milk; these include lactose, whey proteins, minerals and B vitamins (Pintado et al. 1996; Pintado and Malcata 2000). About half of the CW generated worldwide is valorized through the production of whey powder, whey protein concentrates, whey protein isolates, whey protein hydrolysates or dried lactose (Mollea et al. 2013; Pires et al. 2021b). In some countries, part of the whey is used to produce whey cheeses, known as Requeijão (Portugal), Requesón (Spain) or Ricotta (Italy) (Pintado et al. 2001). Whey cheese is a traditional product manufactured by heat treatment of whey, which allows for the recovery of most of the protein and fat fractions of CW (Garcia et al. 2022). Whey, previously acidified or not, is heated at 90–95 ◦C to precipitate whey proteins. The protein aggregates form a matrix that traps the fat and other solids that are present in the whey. The liquid remaining after production of whey cheeses is called "deproteinized" whey (Pereira et al. 2007). Whey cheeses are mostly produced with whey resulting from the production of sheep's or goat's milk cheeses. However, cow's whey can also be used to produce such cheeses, although the yield is lower. Despite the interesting nutritional value of whey cheeses, only a small fraction of the CW produced is valorized as whey cheese. Only about 15% of the CW obtained annually in Italy is used in the production of Ricotta cheese (Maragkoudakis et al. 2016). The low valorization of whey through the production of whey cheese (WC) results from the fact that WC production is an energy-intensive process, which is associated with the short shelf life of the product and represents a challenge for producers.

To overcome the difficulties related to the low yield and high energy consumption associated with WC production, selective concentration of CW by ultrafiltration (UF) can represent a major aid for producers. Tangential filtration technologies such as UF or nanofiltration (NF) can be considered the best available tools to manage dairy byproducts. These technologies allow the selective concentration of specific components of whey, namely proteins. The proteins and fat of the CW are concentrated in the retentate, while water, lactose and salts permeate through the membrane; the main solid component of the permeate is lactose. The concentration of lactose and salts of both the retentate and permeate remain similar to that of the original CW (Pires et al. 2021b). So, this selective concentration process offers advantages over evaporation for the production of fermented dairy products, since high levels of lactose can originate products with high acidity. CW is concentrated by UF prior to whey cheese manufacturing, drastically reducing the volume of product to be submitted to the thermal treatment applied to aggregate whey proteins, thus reducing the energy required to produce whey cheeses (Henriques et al. 2017; Henriques et al. 2019; Marnotes et al. 2021; Monti et al. 2018; Pires et al. 2022; Pires et al. 2023; Pires et al. 2021a). Some authors also propose the use of transglutaminase to increase the yield in whey cheese production (Ceren Akal 2023).

Several attempts have also been made to extend the shelf life of whey cheeses, most of them based on the use of modified atmosphere packaging (Arvanitoyannis et al. 2011;

İrkin 2011; Papaioannou et al. 2007; Temiz et al. 2009) and, more recently, using lactic acid bacteria, including probiotics, as bioprotective cultures (Carrero-Puentes et al. 2022; Hilmi et al. 2019; Irkin and Yalçın 2017; Kaminarides et al. 2018; Spanu et al. 2018). Regarding the incorporation of probiotic strains in Requeijão, considerable research has been performed by Madureira and coworkers (Madureira et al. 2005a; Madureira et al. 2011a; Madureira et al. 2011b; Madureira et al. 2005b; Madureira et al. 2011c; Madureira et al. 2011d; Madureira et al. 2013; Madureira et al. 2008). However, color, textural and sensory properties of the resulting probiotic whey cheeses can be different than those of control WC, which can lead to lower acceptance by consumers. In a previous work, we evaluated the characteristics of cow's WC containing probiotics that was produced with whey concentrated by UF (Pires et al. 2021a).

To the best of our knowledge, this is the only work in which such a strategy was applied. In that research, we used kefir and bifidobacterium cultures with the aim of increasing the shelf life of WC. However, it was observed that the counts of yeasts and molds quickly increased after seven days of refrigerated storage. So, it was concluded that other techniques, such as modified atmosphere packaging (MAP), should be applied to increase the shelf life of whey cheeses. In the present work, we tested an innovative approach, namely the use of probiotics together with protective cultures. This solution may also represent an interesting possibility to increase the shelf life of WC at lower costs compared to MAP.

Hence, the objective of this work was to produce whey cheeses with added probiotics or probiotics plus protective cultures, using UF concentrated bovine cheese whey. The products were compared with control whey cheeses without added cultures.

2. Materials and Methods

2.1. Production of Liquid Whey Concentrates

Sweet cheese whey resulted from the production of cow's milk cheese at the dairy pilot plant of the Polytechnic Institute of Coimbra's School of Agriculture (Coimbra, Portugal). CW (200 L) was submitted for ultrafiltration (UF) in a pilot plant (Proquiga Biotech SA, A Coruña, Spain) using a 3838 polyvinylidenefluoride/polysulfone UF membrane (effective filtration area of 7 m^2; 10 kDa cut-off) supplied by FipoBiotech (Vigo, Spain). The membrane cut-off was selected because it retains almost all of the fat and protein of cheese whey while allowing for high filtration fluxes. The process was conducted at 40–45 ◦C with a transmembrane pressure of 3.0–3.5 bar, aiming to achieve a volumetric concentration factor (VCF = vol. feed/vol. retentate) of 5. After the UF concentration step, 40 L of liquid whey concentrate (LWC) was obtained.

2.2. Manufacture of Whey Cheeses

A total of 2% (v/v) full-fat milk (3.2% protein; 3.6% fat), 1% (w/v) salt, 2% (w/v) starch (Pregeflo™ CH 20, Roquette Freres, Lestrem, France) and 0.04% (v/v) CaCl2 (51% w/v, supplied by Tecnilac, Viseu, Portugal) was added to the LWC. The mixture was heated

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to 90–95 ◦C for 20 min to allow coagulation and subsequent precipitation of whey proteins. The precipitated fraction was separated from the remaining liquid and divided into three similar portions which were submitted to different treatments: control CW without any addition (C); CW with the addition of 10% (w/w) full-fat milk fermented with the probiotic Lactobacillus acidophilus (LA5™ Chr. Hansen, Hoersholm, Denmark) (LA5); and CW with the addition of 10% (w/w) full-fat milk fermented with the probiotic culture (LA5) plus a protective culture containing Lacticaseibacillus paracasei and Lacticaseibacillus rhamnosus (FreshQ4™, Chr. Hansen, Hoersholm, Denmark) (LA5FQ4). Both cultures were previously inoculated in the milk and incubated for 15 h at 30 ◦C according to the manufacturer's instructions.

The probiotic cultures were selected based on their commercial availability and proven activity. According to the manufacturer, the bioprotective culture has a combination of traditional lactic acid bacteria that inhibits unwanted yeasts and molds in fermented dairy products, and its effect is obtained through active participation in the natural fermentation process. The whey cheeses were packaged in closed polypropylene boxes (Ondipack™, Makro, Carnaxide, Portugal) and stored under refrigerated conditions at 4 ± 1 ◦C for 28 days. Consecutive analyses were performed on the 1st, 7th, 14th, 21st and 28th days of storage.

2.3. Physicochemical Analysis

Dry matter of samples was determined by drying according to NP 703:1982 for yoghurt (NP 703:1982), in an oven (Schutzart DIN 40050-IP20 Memmert™, Schwabach, Germany). Ashes were determined by incineration of dry samples in an HD-23 Hobersal™ electric muffle furnace. The fat content was determined using the Gerber method (SuperVario-N Funke Gerber™centrifuge, Berlin, Germany) in accordance with NP 2105:1983 (NP 2105:1983), Total nitrogen content was quantified by the Kjeldahl method (Digestion System 6 1007 Digester Tecator ™, Foss Analytical, Häganäs, Sweden) following the AOAC (1997) standard (AOAC 1997). The percentage of protein was calculated using a conversion factor of 6.38. The analyses were conducted in triplicate.

2.3.1. pH and Titratable Acidity

The pH values were directly determined with the aid of an HI 9025 HANNA Instruments pH meter immediately after production and on the 1st, 7th, 14th, 21st and 28th days of storage. Acidity was determined according to NP 701:1982 for yoghurts (NP 701:1982), by titration with a 0.1 N NaOH solution and was expressed as the percentage of lactic acid.

2.3.2. Color Parameters

The color of whey cheese samples was determined with a colorimeter (Minolta™ Chroma Meter, model CR-200B, Tokyo, Japan) calibrated with a white standard (CRA47: Y = 94.7; x 0.313; y 0.3204) using illuminant C and a 1 cm diameter aperture and a 10◦

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standard observer. Color coordinates were measured in triplicate in the CIEL*a*b* system. Color difference (ΔEab^*) was calculated as the following:

$$\Delta Eab^* = [(L^* - L^{*0})^2 + (a^* - a^{*0})^2 + (b^* - b^{*0})^2]^{1/2} \quad (1)$$

where L^{*0}, a^{*0} and b^{*0} and L^*, a^* and b^* were the values measured for the samples under comparison.

2.3.3. Rheological Parameters

Rheological tests were performed at 5 ◦C with the aid of a Rheostress 6000 rheometer equipped with a plate-to-plate TMP35 bottom plate and a P35 TiL upper plate. Data acquisition was carried out using the HAAKE RheoWin Job Manager software (version 4.82.0002, ThermoHaake™, ThermoFisher Scientific, Waltham, MA, USA). The measuring system utilized a cone and plate geometry, specifically C60/Ti-0.052 mm (35 mm diameter and 1◦ angle). Stress sweep tests were conducted at 1 Hz to determine the linear viscoelastic rheological behaviors of all samples. The measurements were made on the 1st, 14th and 28th days of storage. Values for the elastic and viscous moduli (G′ and G″) and the complex viscosity (η*) were obtained at frequencies ranging from 0.05 to 1.0 Hz. The samples' complex viscosities were fitted to the power law model according to Equation (2), and the consistency index k ($Pa.s^{n-1}$) and the power law index (n) were determined. This model is typically used for shear-thinning fluids such as weak gels and low-viscosity dispersions.

$$\eta^* = k\,(\dot{r})^{\,n-1} \quad (2)$$

k = flow consistency index

$\dot{r}$= shear strain

n = flow behavior index ($Pa.s^n$)

2.3.4. Texture Parameters

The textural properties of WC samples were evaluated using a Stable Micro Systems ™ texture analyzer (mod. TA.XT Express Enhanced, Godalming, UK) using an acrylic cylindrical probe (25.4 mm diameter, 38.1 mm height) with a penetration distance of 15 mm at 2 mm/s. The results were calculated using the Specific Expression PC software (version 1.1.9.0).

2.4. Microbiological Analysis

The microbial counts of lactic acid bacteria (LAB) were evaluated at the 1st, 7th, 14th, 21st and 28th days of storage. Microbial enumeration was performed on plates incubated at 37 ◦C for 48 h on M17 agar for presumptive lactococci (in aerobic conditions) and on MRS agar (under anaerobic conditions) for presumptive lactobacilli (including lacticaseibacilli) according to ISO 7889, IDF 117 (2003) (ISO 7889, IDF 117). Yeasts and molds were counted in accordance with ISO 6611, IDF 94 (2204) (ISO 6611, IDF 94) on plates incubated at 25 ◦C. Culture media were supplied by Biokar Diagnostics (Allonee,

France). The results are expressed as log CFU/g of product, and the analyses were performed in triplicate with two controls for each medium.

2.5. Sensory Analysis

Analysis of the sensory characteristics was conducted in isolated booths with the same lighting and temperature conditions. In the acceptance test, the samples, identified by three-digit random numbers, were rated for aroma, taste, texture and overall impression using a 9-point hedonic scale, where 1 = dislike extremely and 9 = like extremely. The evaluation was carried out using a non-trained panel consisting of 30 members (Stone et al. 2020). The panel members were also asked to rank the samples in accordance with their preference, from most preferred (1) to least preferred (3), according to ISO 8587 (1988) (ISO 8587:1988). The members of the panel were informed of the objectives of the test and signed the informed consent form in use at the Polytechnic of Coimbra, giving their consent for the treatment and publication of results.

2.6. Statistical Analysis

The influence of storage time, the kind of whey cheese and their interaction on all the determined parameters were analyzed by a two-way ANOVA. The differences among whey cheeses and among different days of storage were assessed by one-way ANOVA and the means were compared using the Tukey's post hoc test. For all mean evaluations, a significance level of $p < 0.05$ was used (IBM SPSS Statistics for Windows, version 27, 2021; IBM Corp, Armonk, NY, USA). Graphs were generated using the Statistica Software, version 8 (Stasoft Europe, Hamburg, Germany).

3. Results

Table 1 represents the protein, fat and ashes contents of the different WC samples. Non-significant differences were observed regarding the protein content of whey cheeses, but the products with added microbial cultures presented higher fat and lower mineral contents. These differences probably resulted from the added milk in which the cultures were pre-incubated (which was added at a level of 10% w/w of the whey cheeses).

Table 1. Gross chemical composition of whey cheeses. C-Control; LA5-Whey cheese with probiotic LA5 culture; LA5FQ4-Whey cheese with probiotic LA5 culture plus FQ4 protective culture.

	Protein in dry matter (%w/w)	Fat in dry matter (%w/w)	Ashes in dry matter (%w/w)
C	29.75 ± 2.07^{a}	21.07 ± 0.41^{a}	7.72 ± 0.79^{a}
LA5	33.08 ± 4.59^{a}	24.41 ± 1.36^{b}	6.18 ± 0.47^{b}
LA5FQ4	33.94 ± 0.06^{a}	24.24 ± 0.24^{b}	6.71 ± 0.38^{b}

a,b: Same superscript letter (a) in a column indicates no significant differences.

The changes in the dry matter and in the water activity of the products are presented in Figure 1. Significant differences were observed regarding the evolution of the dry matter of samples, either between products or for the same product at different days of storage.

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The samples LA5 and LA5FQ4 presented slightly higher solids contents when compared to the control, except at the 14th day of storage. However, the two-factor ANOVA did not indicate differences resulting from the interaction of factors. Differences were also observed regarding the water activity of whey cheese samples, chiefly during the first 7 days of refrigerated storage (Table S1).

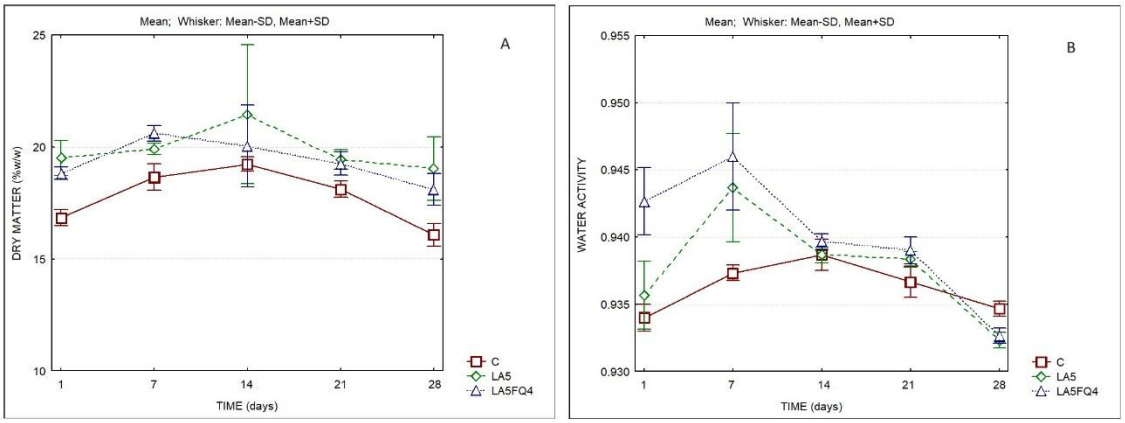


Figure 1. Dry matter and water activity of whey cheeses. C—control; LA5—whey cheese with probiotic LA5 culture; LA5FQ4—whey cheese with probiotic LA5 culture plus FQ4 protective culture. (A) Dry matter; (B)Water activity.

Significant differences were also observed regarding the pH values and titratable acidities (Figure 2 and Tables 2 and S1). As expected, the products with added cultures presented lower pH values and higher titratable acidities when compared to the control. The sample LA5 presented the lowest pH and the highest titratable acidity. LA5FQ4 presented intermediate values (pH 5.47 and TA 0.33% lactic acid at the end of storage). These values probably played an important role regarding the better acceptance of this sample by consumers, as will be discussed later.

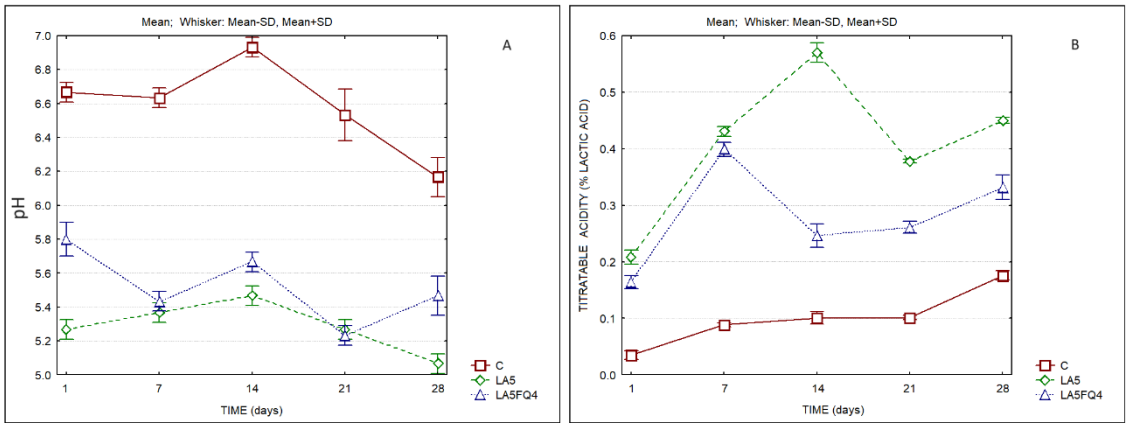


Figure 2. pH and titratable acidity of whey cheeses. C—control; LA5—whey cheese with probiotic LA5 culture; LA5FQ4—whey cheese with probiotic LA5 culture plus FQ4 protective culture. (A) pH; (B) Titratable acidity.

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Table 2. Two-way ANOVA of physicochemical parameters of whey cheese samples.

Two-way ANOVA

		F	*p value*
Dry matter	Time	7.70	0.000
	Product	15.08	0.000
	Interaction	0.67	0.715
a_w	Time	27.85	0.000
	Product	15.31	0.000
	Interaction	5.83	0.000
pH	Time	45.81	0.000
	Product	1118.10	0.000
	Interaction	11.34	0.000
Titratable acidity	Time	368.49	0.000
	Product	2508.79	0.000
	Interaction	109.05	0.000

The evolution of the color parameters of the whey cheeses is shown in Figure 3. The lightness (L*) ranged between 80 and 90 over the storage duration. The lowest L* values were observed for sample LA5FQ4 (Table S2). The a* values decreased between the 1st and the 14th day, then tended to increase slightly. Conversely, the b* values increased from the 1st to the 14th day, then decreased.

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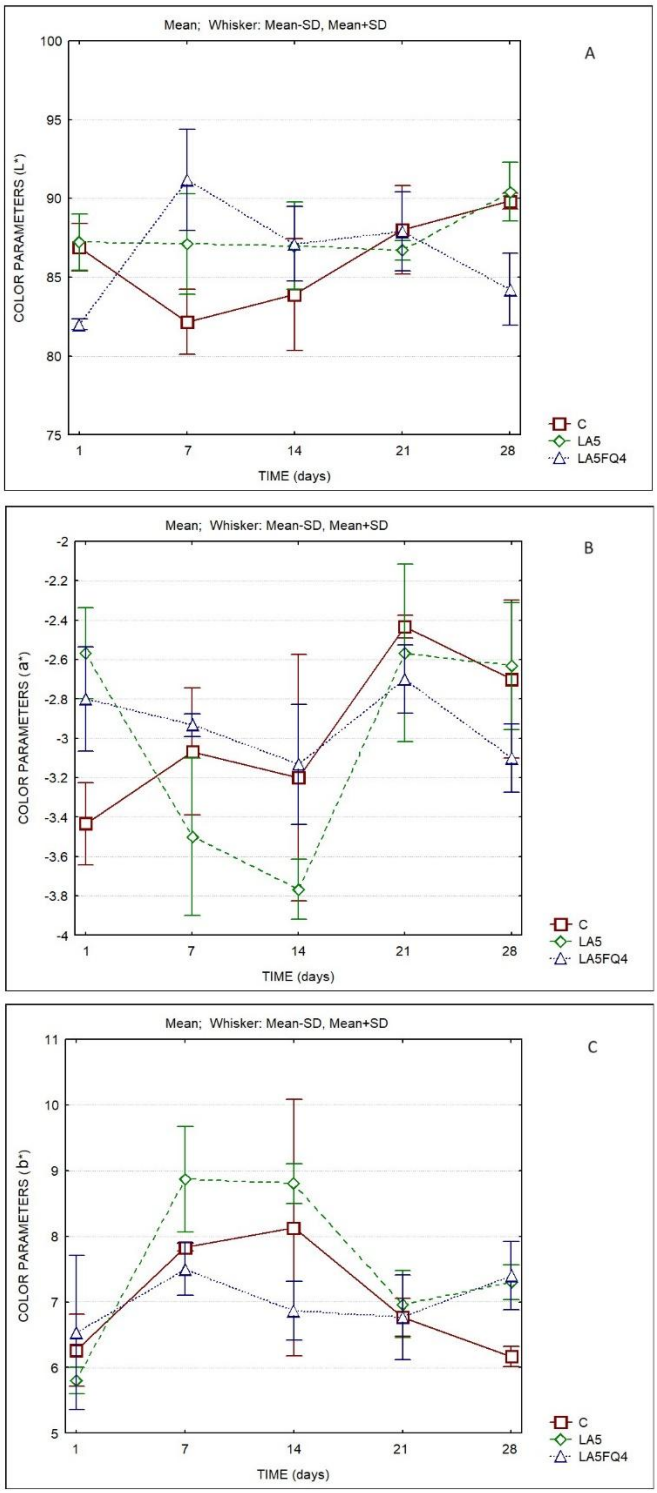


Figure 3. Color parameters (CIEL*a*b*) of whey cheeses. C—control; LA5—whey cheese with probiotic LA5 culture; LA5FQ4—whey cheese with probiotic LA5 culture plus FQ4 protective culture. (A) L*; (B) a*; (C) b*.

A better indicator of the differences in color are the values of ΔEab* (Table 3). It can be observed that no color differences were detected between the control and LA5 at the 1st, 21st and 28th days (values < 1), while the sample LA5FQ4 was like the control at the 21st day of storage, being different at all other days at which they were compared.

Significant differences were observed for the same sample at different days of storage, with this trend being more evident between the 7th and the 1st day, chiefly for the control (C) and for LA5FQ4. The sample LA5 presented lower differences between the 1st and the 7th day of storage and between the 14th and the 7th day, as well as between the 21st and the 14th day. In Table 4, it can be observed that the L* parameter was not different between products (p = 0.176) nor between storage times (p = 0.101). Additionally, no differences were observed between products regarding parameters a* (p = 0.815) and b* (p = 0.087).

Table 3. ΔEab* values between products and over time for each product.

ΔEab* values between products					
Storage time (days)	1	7	14	21	28
Products					
LA5 *vs* C	0.54	15.24	5.19	0.87	0.82
LA5FQ4 *vs* C	12.24	47.75	6.03	0.04	16.52
ΔEab* values for the same product at different storage days					
		C	LA5	LA5FQ4	
7d *vs* 1d		23.96	5.15	42.49	
14d *vs* 7d		6.27	5.25	13.29	
21d *vs* 14d		1.23	0.82	17.44	
28d *vs* 21d		4.58	6.25	2.91	

Table 4. Two-way ANOVA of color parameters of whey cheese samples.

CIEL*a*b* color parameters		Two-way ANOVA	
		F	*p value*
L *	Time	2.14	0.101
	Product	1.84	0.176
	Interaction	5.81	0.000
a *	Time	8.85	0.000
	Product	0.21	0.815
	Interaction	3.71	0.004
b*	Time	10.82	0.000
	Product	2.66	0.087
	Interaction	2.38	0.041

Figure 4 presents the rheological parameters of whey cheese samples at the 1st, 14th and 28th days of storage. The elastic modulus (G′) was always higher than the viscous modulus (G″). Hence, the loss tangent, or tan δ (ratio of G″/G′), was lower than 1 (C—0.187 ± 0.017; LA5—0.230 ± 0.024; LA5FQ4—0.211 ± 0.013). These values show that the samples had mainly elastic behavior. Whey cheese samples exhibited a flow behavior that was dependent on shear as the complex dynamic viscosity (η*) decreased linearly with the increase in frequency on a double logarithmic scale (Figure 4B, D, F). So, whey cheese samples showed a non-Newtonian shear-thinning or pseudoplastic behavior. Additionally, the power law parameters indicated the pseudoplastic, or shear-thinning, nature of the products (δ<1). Pseudoplastic fluids are those whose behavior is independent of time and that have a lower apparent viscosity at higher shear rates. In general, these fluids are solutions of large, polymeric molecules in a solvent with smaller molecules. An

explanation for such behavior is that the large molecular chains tumble randomly, affecting large volumes of fluid at low shear, but gradually align in the direction of increasing shear and produce less resistance, and consequently lower dynamic viscosity values. Table 5 presents the regression equations for the log/log plots of dynamic viscosity/frequency. Sample LA5 presented significantly higher values for G′, G″ and η* over the storage period. This is evident in Table 6, which displays the calculated power law parameters.

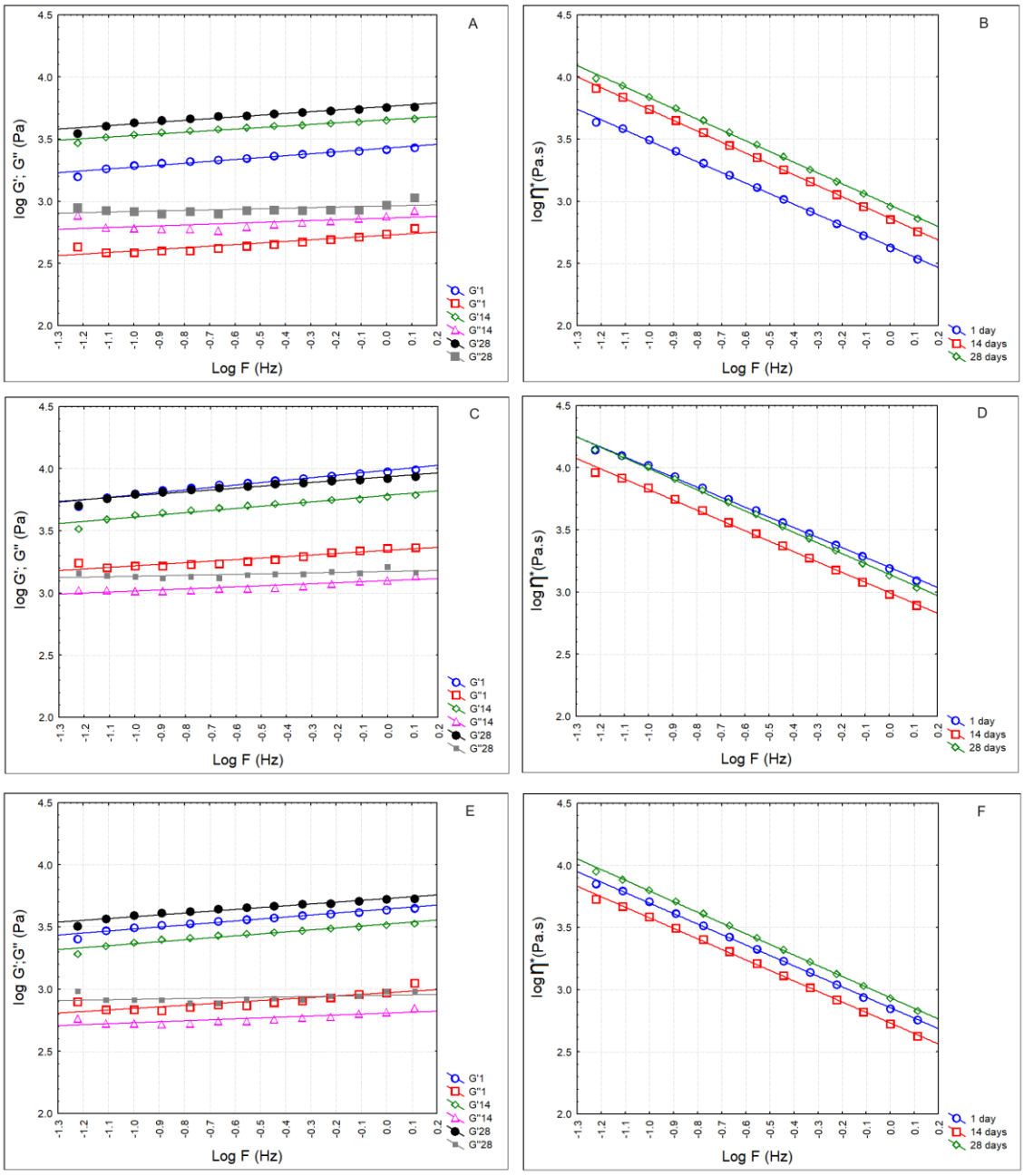


Figure 4. Rheological parameters of whey cheeses over storage. (A) G′—elastic modulus; G″— viscous modulus; (B) η*—dynamic viscosity of control cheese. (C) G'—elastic modulus; G″—viscous modulus; (D) η*—dynamic viscosity of LA5 (whey cheese with probiotic LA5 culture); (E) G′—elastic modulus; G″—viscous modulus; (F) η*—dynamic viscosity of LA5FQ4 (whey cheese with probiotic LA5 culture plus FQ4 protective culture).

Table 5. Regression equations for the log/log plots of dynamic viscosity/frequency ($r^2 > 0.99$).

Products/Storage Time	C	LA5	LA5FQ4
1 day	y = 2.637 – 0.849*x	y = 3.200 – 0.808*x	y = 2.855 – 0.842*x
14 days	y = 2.863 – 0.876*x	y = 2.996 – 0.830*x	y = 2.733 – 0.846*x
28 days	y = 2.969 – 0.864*x	y = 3.143 – 0.852*x	y = 2.936 – 0.859*x

C-Control; LA5-Whey cheese with probiotic LA5 culture; LA5FQ4-Whey cheese with probiotic LA5 culture plus FQ4 protective culture.

Table 6. Power Law parameters of whey cheese samples.

Products	k (kPa.s)	n	r^2
C	4.46 ± 1.45	0.972 ± 0.016	0.869
LA5	11.72 ± 4.45	0.977 ± 0.089	0.875
LA5FQ4	5.06 ± 0.92	0.989 ± 0.009	0.871

C-Control; LA5-Whey cheese with probiotic LA5 culture; LA5FQ4-Whey cheese with probiotic LA5 culture plus FQ4 protective culture.

The firmness of the samples was also determined using a texturometer (Figure 5). The determined values agreed with the rheological parameters obtained with the rheometer. The samples with added cultures were firmer when compared to the control throughout the storage period. These differences were more marked at the beginning and at the end of the storage period. Sample LA5 presented the highest values by the end of storage.

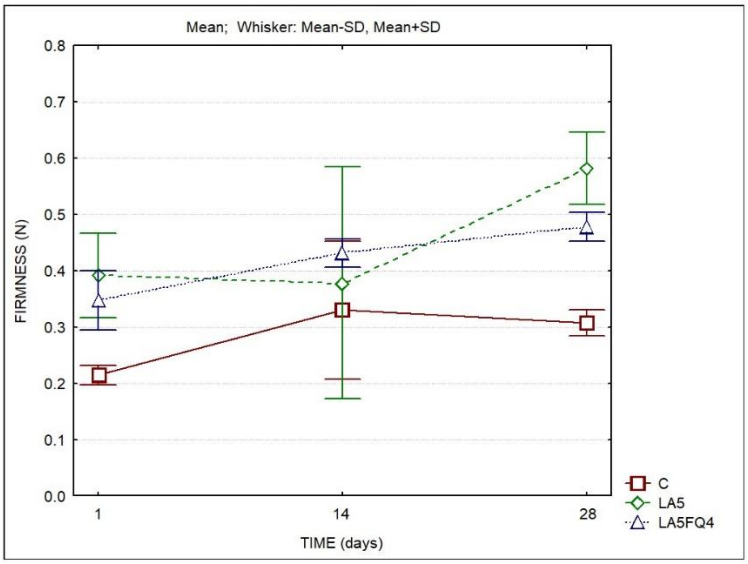


Figure 5. Firmness of whey cheeses over storage. C—control; LA5—whey cheese with probiotic LA5 culture; LA5FQ4—whey cheese with probiotic LA5 culture plus FQ4 protective culture.

The microbiological characteristics of the whey cheeses are displayed in Figure 6 (and in Table S3). Lactococci and lactobacilli (including lacticaseibacilli) counts were on the order of log 8–9 CFU/g for the products with added cultures. The control sample presented levels of lactococci and lactobacilli of ca. 6.5 and 5.5 log CFU/g, respectively, at the beginning of storage, but their counts increased steadily towards the end of

refrigerated storage (to ca. log 8 CFU/g). It is worth mentioning the lower levels of yeasts and molds detected on the sample with the protective culture (LA5FQ4), which by the end of storage presented counts one log cycle lower than C and LA5. Hence, the beneficial impact of the protective culture (FQ4™) on the shelf-life of the product is evident. The high levels of presumptive lactococci that were observed probably resulted from the low selectivity of the M17 culture media, which allows for the growth of other lactic acid bacteria, or even non-LAB, as reported non-LAB, as reported by other authors (Freitas et al. 1996; Potes and Marinho 2007). Table 7 presents the two-way ANOVA test and clearly indicates the significant differences in the microbiota of whey cheeses among products and among different.

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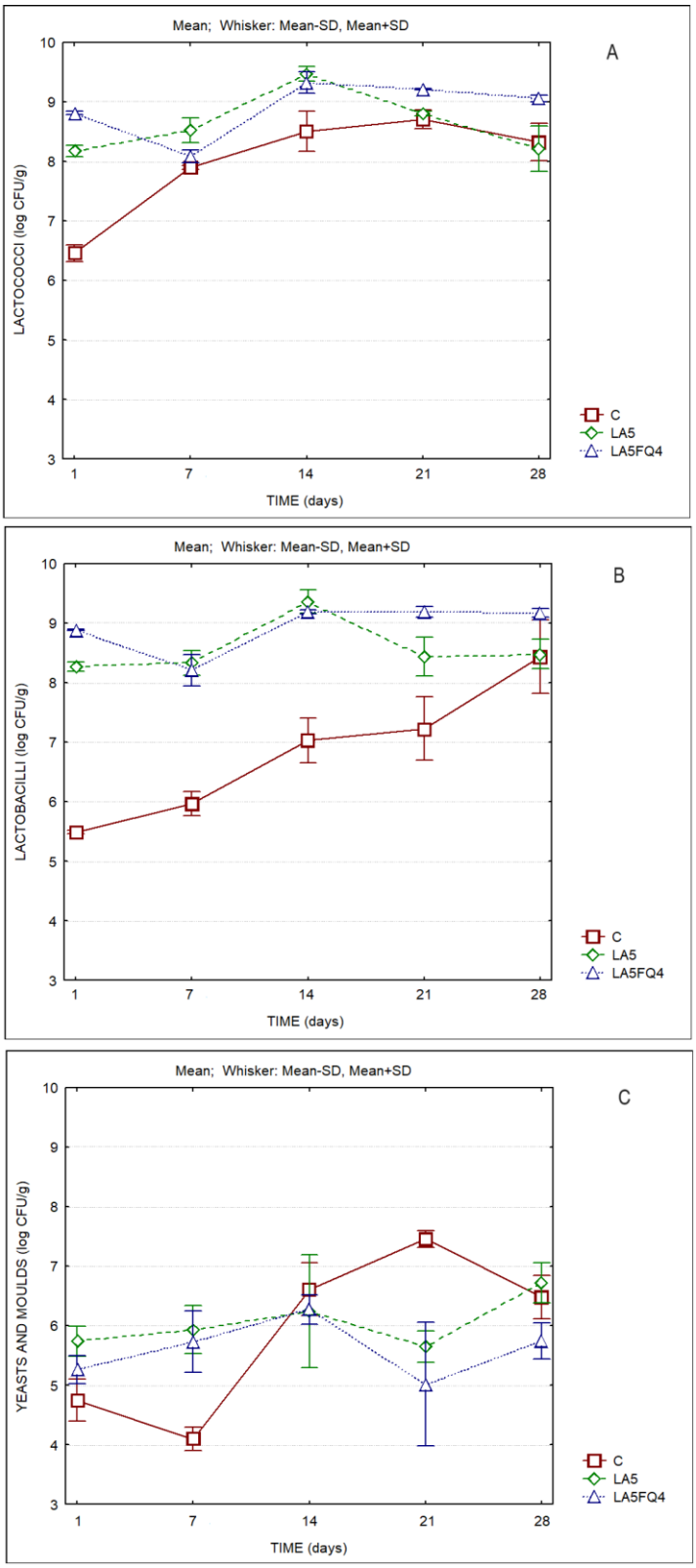


Figure 6. Microbial parameters of whey cheeses over the storage period. (A) Lactococci; (B) lactobacilli; (C) yeasts and molds. C—control; LA5—whey cheese with probiotic LA5 culture; LA5FQ4— whey cheese with probiotic LA5 culture plus FQ4 protective culture.

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Table 7. Two-way ANOVA of microbiological parameters of whey cheese samples.

Microbial groups		Two-way ANOVA	
		F	*p value*
Lactococci	Time	68.77	0.000
	Product	48.47	0.000
	Interaction	18.54	0.000
Lactobacilli	Time	170.83	0.000
	Product	23.10	0.000
	Interaction	10.77	0.000
Yeasts and Moulds	Time	4.58	0.014
	Product	14.13	0.000
	Interaction	8.31	0.000

Figure 7 presents the sensory evaluation of the whey cheeses. Significant differences were found regarding the taste (p = 0.0031) and the global evaluation (p = 0.0032). No differences were observed for the aroma (p = 0.1963) or for the texture (p = 0.4455) of the products. Whey cheese containing probiotics (LA5) obtained lower scores for the taste and for the global evaluation, while the sample LA5FQ4 obtained the highest scores for all evaluated parameters. The ranking test also differentiated this sample in comparison with the control (C) and LA5, which were ranked second and third, respectively. The higher acidity of sample LA5 was the reason for its lower acceptance.

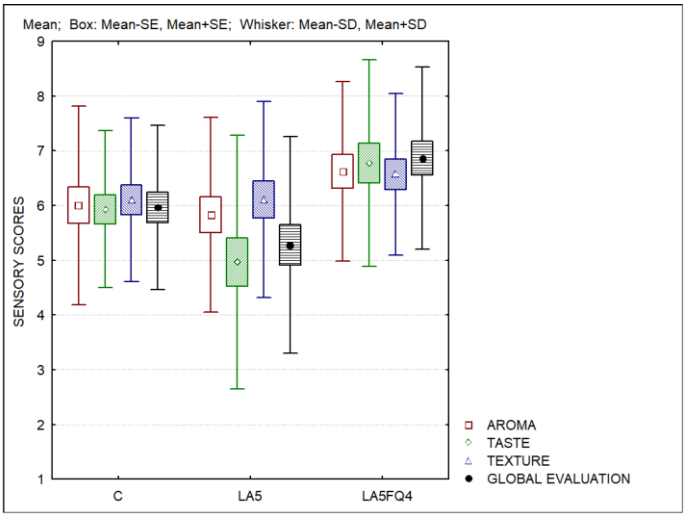


Figure 7. Sensory evaluation of whey cheeses. C—control; LA5—whey cheese with probiotic LA5 culture; LA5FQ4—whey cheese with probiotic LA5 culture plus FQ4 protective culture.

4. Discussion

Whey cheeses showed lower values for dry matter, protein and fat than those observed in a previous works (Pires et al. 2021a), in which whey cheeses with added fermented cream as the vehicle for probiotics presented a higher level of total solids (22–25% w/w), protein (33–36%, dry basis) and fat (30–33% dry basis). These differences probably resulted from the type of product used to pre-incubate the cultures (cream). Garcia and coworkers prepared goat's probiotic whey cheeses using a similar approach and reported higher

values matter (25–27%), but lower average values for fat (25.91 ± 4.05%, dry basis) and protein (43.18 ± 1.96%, dry basis) contents (Garcia et al. 2022).

The color values were different than those reported in whey cheeses with kefir or probiotics (Pires et al. 2021a), where the values of L* were higher (between 94 and 96), a* values were lower (between −3.0 and −3.5) and b* values were higher (between 8 and 9). We have not found studies about the effect of inclusion of probiotic bacteria in the color of WC.

Regarding the rheological properties, the whey cheeses with added cultures were firmer when compared to the control products. On the contrary, Pires et al. (Pires et al. 2021a) observed that the addition of probiotics and kefir caused decreases in the elastic modulus (G′) of the whey cheeses. They indicated that the cultures used (Bifidobacterium spp. and a commercial kefir culture) could weaken the protein structure. In the present study, the cultures of *Lactobacillus acidophilus, Lacticaseibacillus paracasei* and *Lacticaseibacillus rhamnosus* did not affect the protein structure. On the contrary, the whey cheeses with *Lactobacillus acidophilus* showed higher firmness. Madureira et al. (Madureira et al. 2011b; Madureira et al. 2011c) studied the rheological characteristic of whey cheeses manufactured with probiotic bacteria *Lacticaseibacillus casei (Madureira et al. 2011b)* or *Lacticaseibacillus casei* and *Bifidobacterium animalis* (Madureira et al. 2011c). They reported that in all the whey cheeses, the elastic modulus (G′) was higher than the viscous modulus (G″), typical of solid viscoelastic foods. They also observed, as in our study, that the probiotic whey cheeses were firmer, more elastic and more viscous than non-inoculated whey cheeses. They indicated that this was due to the lower pH of these cheeses, indicating that there is a positive correlation between acidity and hardness of cheeses and that higher acidity values lead to firmer matrices. In the cheeses of our study, the whey cheeses with added cultures had higher titratable acidities and lower pH values than control whey cheeses and, consequently, presented higher firmness; LA5 whey cheeses showed the highest firmness values by the end of refrigerated storage and had the lowest pH.

Madureira et al. (Madureira et al. 2011b; Madureira et al. 2011c) also indicated that higher acidity values negatively affected the sensory acceptance of probiotic whey cheeses. In the present study, despite LA5FQ4 whey cheeses having higher acidities than the control whey cheeses, they obtained higher sensorial evaluations. It was also reported that whey cheeses inoculated with cultures of *Lactobacillus* spp. have better sensory scores than whey cheeses with *Bifidobacterium* spp. due to the production of acetic acid by the latter, which has negative consequences in sensorial evaluation (Madureira et al. 2011c).

Results for viable cell counts of goat's probiotic whey cheeses (containing *Lacticaseibacillus rhamnosus* and *Bifidobacterium animalis*) presented an average of log 8.24 CFU/g on the final day of storage (Garcia et al. 2022), whereas in the present study microbial counts of lactic acid bacteria (LAB) were on the order of log 9 CFU/g for LA5FQ4 and log 8 CFU/g for LA5. Although the levels of probiotic bacteria that have beneficial consequences for health are normally strain- and host-dependent, it has been indicated that concentrations of at least log 7 CFU/g are necessary for a positive effect

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(Guarner et al. 2012; Tripathi and Giri 2014). Hence, it can be concluded that even at the end of storage, probiotic counts of LA5 and of LA5FQ4 were at levels that allow for that claim.

It must be noted that by the end of storage, the control sample presented values higher than log 8 CFU/g for bacteria counted on M17 and MRS agar, but these values resulted from adventitious contamination and subsequent growth during storage. However, during the first two weeks of storage the counts were much lower than the ones observed in the samples with added cultures, and therefore the acidity values were lower. Another possibility for the lower acidity of the control sample can be related to a lower capacity for production of lactic acid by the bacteria present in this sample. Whey cheeses with kefir culture or *Bifidobacterium* spp. showed lower counts of lactic acid bacteria (log 7 UFC/g) after 21 days of storage (Pires et al. 2021a). These results may indicate that the cultures used in the present study have a better adaptation to the whey cheese matrix. Additionally, Pires et al. (Pires et al. 2021a) did not observe reduction in the counts of yeasts and molds in the whey cheeses with *Bifidobacterium* spp. and commercial kefir culture in relation to control whey cheeses, while in the present study the protective culture reduced yeast and mold counts by one log cycle. The protective culture with *Lacticaseibacillus paracasei* and *Lacticaseibacillus rhamnosus* (together with *Lactobacillus acidophilus*) showed a better effect on the shelf-life of the product than the culture with only the probiotic Lactobacillus acidophilus.

The research of Madureira and coworkers indicated that whey cheese protected the probiotic strains during transit throughout the simulated gastrointestinal system, therefore comprising a promising carrier of those bacteria. Probiotic bacteria *(Lacticaseibacillus casei* and *Bifidobacterium animalis*) added to whey cheese also showed positive effects concerning control of spoilage and pathogenic microorganisms, thus promoting the extension of the shelf life of the product (Madureira et al. 2005a; Madureira et al. 2011a; Madureira et al. 2011d). Hence, the approach of adding probiotic or probiotic plus protective cultures to whey cheeses can increase the shelf life of whey cheeses while providing beneficial health effects to consumers.

5. Conclusions

The concentration of cow's whey by ultrafiltration proved to be a very useful tool to reduce the volume of whey to be submitted to the thermal treatment applied to precipitate the whey proteins. Using a volumetric concentration factor of 5, one can estimate that the energy requirements for the process can be reduced by over 70%. The probiotic whey cheeses (with *Lactobacillus acidophilus* and with a protective culture containing *Lacticaseibacillus paracasei* and *Lacticaseibacillus rhamnosus*) manufactured using UF-concentrated bovine cheese whey showed the lowest levels of yeasts and molds at the end of the refrigerated storage. However, the probiotic whey cheeses showed some differences (lower pH values, higher titratable acidities and firmer textures) when compared to control whey cheeses. The product with a probiotic plus a bioprotective culture obtained the best global sensory evaluation. These results indicate that the addition

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of protective cultures with *Lacticaseibacillus* strains can be very useful to prolong the shelf life of whey cheeses while also improving the sensory evaluation of whey cheeses and providing the health benefits associated with the consumption of probiotics.

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Manuscript III-Evaluation of the Characteristics of Sheep's and Goat's Ice Cream, Produced with UF Concentrated Second Cheese Whey and Different Starter Cultures

The results from this chapter have already been published as:

Arona Pires [1,2], David Gomes [1], João Noronha [1], Olga Díaz [2], Angel Cobos [2] and Carlos Dias Pereira [1,3,*], (2022), Evaluation of the Characteristics of Sheep's and Goat's Ice Cream, Produced with UF Concentrated Second Cheese Whey and Different Starter Cultures, *Foods* 11, 4091.

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Author Contributions

Conceptualization, C.D.P.; Methodology, D.G., J.N., O.D., A.C. and C.D.P.; Investigation, A.P., D.G. and C.D.P.; Resources, C.D.P.; Data curation, O.D. and A.C.; Writing – original draft, A.P. and C.D.P.; Writing – review & editing, A.P., O.D., A.C. and C.D.P.; Supervision, O.D., A.C. and C.D.P.; Funding acquisition, C.D.P. All authors have read and agreed to the published version of the manuscript.

Abstract:

Second cheese whey (SCW) is the by-product resulting from the manufacture of whey cheeses. In the present work, sheep (S) and goat (G) SCW concentrated by ultrafiltration (UF) were used in the production of ice creams. Concentrated liquid SCW samples with inulin added as a prebiotic were fermented with yoghurt, kefir and probiotic commercial cultures before being frozen in a horizontal frozen yoghurt freezer. The physicochemical, microbiological and sensory properties of the products were evaluated over 120 days of frozen storage. The products presented significant differences regarding these properties, specifically the higher total solids and protein contents of sheep's ice creams, which were higher compared to their goat ice cream counterparts. Sheep's ice creams also presented higher hardness and complex viscosity, which increased with storage. These ice creams also presented higher overrun and lower meltdown rates. The color parameters of the ice creams showed significant differences between formulations resulting from storage time. In all cases, Lactobacilli sp. cell counts were higher than log 6 CFU/g at the first week of storage. In the case of sheep's ice creams these values were maintained or increased until the 30th day, but decreased until the 60th day. Lactococci sp. counts surpassed log 7 CFU/g in all products, and these values were maintained until the end of storage, except in the case of G-Yoghurt and G-Kefir. Concerning the products containing probiotics, the sum of *Lactococci* sp. and *Lactobacilli* sp. counts was of the order log 8–9 CFU/g until the 60th day of storage, indicating that the probiotic characteristics of ice creams were maintained for at least 2 months. All products were well accepted by the consumer panel. Sheep's SCW ice creams were better rated regarding aroma, taste and texture. However, only the ranking test was able to differentiate preferences among formulations.

Keywords: ovine; caprine; second cheese whey; ice cream; Kefir; yoghurt; probiotics

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1. Introduction

Cheese whey (CW) is the by-product resulting from the manufacturing of cheese. In some countries, CW is used to produce whey cheeses, such as *Ricotta* (Italy), *Requesón* (Spain) or *Requeijão* (Portugal). Particularly in the case of *Requesón* or *Requeijão*, the whey results from sheep's or goat's cheeses. However, not all the whey resulting from the manufacture of small ruminant's cheeses is transformed into whey cheeses, with a large proportion being used as animal feed or directly discarded (Pintado et al. 2001; Pires et al. 2021a). Even in the case of cow's cheese whey, half of the global production is not valorized, despite its valuable nutritional composition (Pires et al. 2021a). Second cheese whey (SCW), also called deproteinized whey, is the byproduct resulting from the production of whey cheeses. In previous works, we evaluated the characteristics and functional properties of sheep's and goat's whey and SCW. In addition, solutions for the valorization of these by-products were also proposed (Díaz et al. 2004; Pereira et al. 2007; Sanmartín et al. 2012, 2013, 2018). In some cases, the solutions envisaged their direct utilization after a concentration step performed by ultrafiltration (UF) (Borges et al. 2020; Henriques et al. 2013b; Pereira et al. 2015b). Particularly in the case of SCW, only a few papers describe its characteristics and propose solutions for its treatment or valorization (Macedo et al. 2021b; Macedo et al. 2015).

Other authors evaluated this material as a growth media to produce microalgae (Russo et al. 2021; Tatoulis et al. 2015; Tsolcha et al. 2016). However, small scale dairy plants rarely apply such methodologies to valorize SCW. A possible solution can be the utilization of liquid SCW concentrated by ultrafiltration (UF) or nanofiltration (NF) as the main ingredient to produce fermented drinks or other dairy products, as is the case of ice cream. We recently evaluated the performance of sheep's and goat's whey in the production of frozen yoghurts (Marnotes et al. 2021).

Fermented dairy products are commonly produced in different parts of the world. Furthermore, the use of specific microbial cultures such as probiotic bacteria offers opportunities to develop innovative foods with extended shelf lives. Frozen dairy products can have properties of both yoghurt and ice cream, and can be the carriers of lactic acid bacteria, prebiotics and probiotics. The production of frozen yoghurts or ice creams with low fat or lactose levels and/or with added prebiotics and probiotics created new opportunities for the development of dairy foods with health-promoting properties. An increasing number of papers describe the formulations and characteristics of such products (Al-Shawi and Ali 2020; Camelo-Silva et al. 2021; Campidelli et al. 2021; Guerra et al. 2018; Junyusen et al. 2017; Pimentel et al. 2022; Sabet-Sarvestani et al. 2021; Shahein et al. 2022; Silantjeva et al. 2022; Skryplonek et al. 2017; Skryplonek et al. 2019; Tekin et al. 2017; Utpott et al. 2020). The production of ovine's and caprine's yoghurts and frozen yoghurts has also followed this trend. Several research works refer to the use of ewe's (Balthazar et al. 2017a; Balthazar et al. 2017b; Kowalczyk et al. 2021a; Kowalczyk et al. 2021b; Moschopoulou et al. 2021; Sert and Mercan 2021) and goat's (Acu et al. 2020; Açu et al. 2017; Alves et al. 2009; Bezerra et al. 2015; Carvalho et al.

2022; Chacón-Villalobos et al. 2016; Da Silva et al. 2015; McGhee et al. 2015; Paula et al. 2020; Ranadheera et al. 2013; Şanlidere Aloğlu et al. 2018; Türkmen et al. 2018; Verruck et al. 2020) milks in the production of ice creams with functional properties. In addition, several authors reported the potential medicinal properties of small ruminant's milks and dairy products (Balthazar et al. 2021; Nadelman et al. 2017; Nayik et al. 2022).

Hence, the objective of this research was to manufacture ice creams using goat and sheep liquid SCW concentrates (LSCWCs) produced by UF, and to study their physicochemical, microbiological and sensory properties in order to evaluate the feasibility of the use of this by-product by the dairy industry. No reports were found in the literature regarding the manufacture of ice creams with goat and sheep liquid SCW concentrates.

2. Materials and Methods

2.1. Production of Liquid Whey Concentrates

Sheep and goat second cheese whey (SCW), supplied by external dairy companies, was transported to the pilot plant of the Escola Superior Agrária de Coimbra (Coimbra, Portugal), where it was processed. A total of 500 L of each type of SCW (ewe's or goat's) were subjected to ultrafiltration (UF) in a Proquiga Biotech SA pilot plant (A Coruña, Spain) equipped with a UF organic membrane (3838 PVDF/polysulfone) with an effective filtration area of 7 m2 and a 10 kDa cutoff, supplied by FipoBiotech, Spain. The process was carried out at 40–45 °C at a transmembrane pressure of 3 bar aiming at a volumetric concentration factor (VCF = Vol. feed/Vol. retentate) of 20, obtaining 25 L of concentrate. The concentrate was pasteurized (65 °C, 30 min) and then homogenized at 10 MPa using an APV Rannie™ homogenizer model Blue Top (Copenhagen, Denmark). Pasteurized and homogenized LSCWC were frozen at −25 °C until the moment they were used to produce ice creams.

2.2. Manufacture of Ice Creams

The sheep's or goat's LSCWC's were thawed under refrigeration for 24 h. Subsequently, the samples were heated at 65 °C and homogenized at 10 MPa using APV Rannie™ model Blue Top (Copenhagen, Denmark). Afterwards, they were heated to 85 °C and passed through the homogenizer valve again. The concentrates were then cooled to 44 °C and divided into 6 L portions, to which the remaining ingredients were added in the following proportions (% w/v): sucrose (12%); inulin (5%), citric acid (0.4%) and xanthan gum (0.1%). The mix was allowed to mature for 12 h at 4 ± 2 °C. Finally, each mixture was inoculated with one of the following cultures:

1. Yoghurt culture: Yoflex™ (YF-L903, CHR Hansen, Hoersholm, Denmark) thermophilic yoghurt starter culture (Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus) at a concentration of 0.005% (w/v);

2. Kefir culture: ExactTM Kefir 1 (CHR Hansen, Hoersholm, Denmark) mesophilic and thermophilic culture (Debaryomyces hansenii, Lactococcus lactis subsp. cremoris, L. lactis subsp. lactis biovar diacetylactis, L. lactis subsp. lactis, Leuconostoc and Streptococcus thermophilus) at a concentration of 0.005% (w/v);

3. A mixture of probiotic cultures: (Bifidobacterium BB-12, Lactobacillus acidophilus LA-5) and Streptococcus thermophilus, at a concentration of 0.005% (w/v). ABT-3TM (CHR Hansen, Denmark).

The inoculated sheep or goat mixtures based on LSCWCs were placed in an incubation chamber (Jenogand, model Y—1000, Copenhagen, Denmark) at 43 °C, and the pH was monitored until it reached a value of 4.6. Fermentation was stopped by rapid cooling to 20 °C in less than 30 min. Afterwards, the fermented mixtures were placed in the refrigeration chamber at 4 ± 2 °C for 12 h. For each formulation, 6 L batches of ice cream were produced and placed in a horizontal frozen yoghurt freezer for 40 min. Immediately after freezing, the temperature of ice creams was −6 ± 1 °C. After this process, the ice creams were packaged in 500 mL polypropylene boxes and stored in a freezer at −21 ± 1 °C for 120 days. All experiments were performed in triplicate.

2.3. Physico-Chemical Analysis

2.3.1. Compositional Analysis

Dry matter was determined by drying the samples in a Schutzart DIN 40050-IP20 Memmert™ oven (Schwabach, Germany) according to the AOAC (1997) procedure for frozen yoghurt (AOAC 1997). The ash content was determined by the incineration of dry samples in a Nabertherm™, model LE 4/11/R6 electric muffle furnace (Bremen, Germany) at 550 °C for 4 h, according to the AOAC method 935.42 (AOAC 1997). The fat content was determined according to the AOAC method 952.06 (AOAC 1997). The total N content was determined by the Kjeldahl method in the Digestion System 6 1007 Digester Tecator™ (Foss Analytical, Häganäs, Sweden) following the AOAC standard, and the conversion factor of 6.38 was used to calculate the percentage of protein (AOAC 1997). All analyses were performed in triplicate.

2.3.2. pH and Titratable Acidity

The pH was determined with a HI 9025 Hanna Instruments pH meter (Leighton Buzzard, UK) in order to monitor its evolution over fermentation, immediately after the production of the fermented products and on the 1st, 30th, 60th and 120th days of storage of ice creams. The pH meter was previously calibrated with 7.01 (HI5007) and 4.01 (HI5004) Hanna buffer solutions. The titratable acidity, expressed in g of lactic acid/L, was determined by means of titration using a 0.1 N NaOH solution according to the technique described in NP 701:1982 for yoghurts and AOAC (1997) for frozen yoghurts (NP 701:1982, AOAC 1997). For each sample, three determinations were made both for pH and for titratable acidity.

2.3.3. Color Analysis

The color of the ice creams was determined with a Minolta Chroma Meter, model CR-200B colorimeter (Tokyo, Japan) calibrated with a white standard (CR-A47: Y = 94.7; x 0.313; y 0.3204). The following conditions were used: illuminant C, 1 cm diameter aperture, 10° standard observer. The color coordinates were measured in the CIEL*a*b* system. Color difference (ΔEab*) was calculated as:

$$\Delta Eab^* = [(L^* - L^{*0})^2 + (a^* - a^{*0})^2 + (b^* - b^{*0})^2]^{1/2} \quad (1)$$

where L^{*0}, a^{*0}, and b^{*0} and L^*, a^*, and b^* were the values measured for the samples under comparison. A matrix of ΔEab^* values between products was constructed. Five measurements were taken for each sample.

2.3.4. Rheological Analysis

The rheological properties of the different samples were determined in a Rheostress 1 rheometer (ThermoHaake™, ThermoFisher Scientific, Waltham, MA, USA) in oscillatory mode. The measurement system consisted of a cone and plate geometry, C60/Ti-0.052 mm (35 mm diameter and 1° angle). Stress sweep tests were conducted at 1 Hz to deter-mine the linear viscoelastic range of the yoghurts. The complex viscosity (η^*) of the products was evaluated in the range of 0.3 to 6.5 rad/s at 3 Pa. Three measurements were taken for each sample.

2.3.5. Texture Analysis

A Stable Micro Systems texture analyzer, model TA.XT Express Enhanced (Godalming, UK), was used to evaluate the hardness of the frozen samples one day after production, and the results were calculated using Specific Expression PC software.

2.3.6. Overrun

Overrun is the increase in the volume of the ice cream after freezing due to the incorporation of air. The method described by Skryplonek et al. (Skryplonek et al. 2017) was followed, measuring the weight of the mixture and that of ice cream with the same volume. The determinations were done in triplicate.

Overrun [%] = (weight of mixture-weight of ice cream/weight of ice cream) * 100

2.3.7. Meltdown Rate

The meltdown rate was determined by the method described in Skryplonek et al. (Skryplonek et al. 2017) by placing 30 g of ice cream on a stainless-steel screen with a 1* 1 mm opening, located on top of a beaker. After 45 min at 20 ± 1 °C, the weight of the sample collected in the beaker was measured (n = 3). The meltdown rate was expressed as the percentage of the melted ice cream weight divided by the initial ice cream weight. The determinations were done in triplicate.

Meltdown [%] = (weight of melted ice cream/initial weight of ice cream) * 100

2.4. Microbiological Analysis

The microbial counts of lactic acid bacteria (LAB) of the genera *Lactobacillus* sp. And *Lactococci* sp. were determined after production and during the storage of frozen samples (1, 30, 60 and 120 days). *Lactobacilli* sp. and *Lactococci* sp. were enumerated on plates at 37 °C for 48 h on M17 agar (in aerobiosis) and on MRS agar (in anaerobiosis) (Biokar

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Diagnostics, France), respectively, according to ISO 7889, IDF 117 (2003) (ISO 7889, IDF 117:2003). In this process, 1 mL of dilutions of 10^{-5}, 10^{-6} and 10^{-7} were inoculated in triplicate along with two controls for each medium.

2.5. Sensory Analysis

Consumer preference tests were conducted with an untrained panel at the 7th, 30th, 60th and 120th days of storage. The hedonic test was used to determine the degree of acceptability of the products (Stone et al. 2020). A category-type scale with an odd number (five) categories (1 = "I don't like it at all" to 5 = "I like it very much") was used. A neutral midpoint (neither like nor dislike) was included.

The six formulations were presented to panelists, who were asked to evaluate the characteristics of aroma, taste, color and texture using a hedonic scale of 1 to 5. Thirty-one consumers participated in the panel. The members of the panel were also asked to rank the samples according to their preference, from 1 (most preferred) to 6 (less preferred) (ISO 8587:1988).

2.6. Statistical Analysis

Prior to statistical analysis, normal distribution was tested using the Kolmogorov–Smirnov test. The one way ANOVA and the Tukey's post hoc test were used to test and compare, the statistical significance of differences among means, respectively. Results from the sensory ranking test were evaluated by the chi square test. For all mean evaluations, a significance level of $p < 0.05$ was used (IBM SPSS Statistics version 19 for Windows; 2010; SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

Table 1 presents the composition of goat and sheep LSCWCs. The values of dry matter, protein, fat and ashes in sheep LSCWC were higher than those observed in goat LSCWC. The pH values for sheep and goat liquid CSW concentrates were 6.51 and 6.36, respectively. The titratable acidity from goat LSCWC (1.02 ± 0.007%) was lower than that observed in sheep LSCWC (1.95 ± 0.07%).

Table 1. Composition of goat and sheep liquid second cheese whey concentrates (LSWC).

LSCWC	Dry matter (%)	Protein (%)	Fat (%)	Ashes (%)
Goat	12.38±0.01	2.96±0.003	1.60±0.001	1.57±0.004
Sheep	26.84±0.75	9.58±0.27	2.10±0.001	1.65±0.01

The compositions of the ice creams are shown in Figure 1. Significant differences in dry matter were found among all samples. The protein content was higher in sheep's SCW ice creams when compared to goat's SCW ice creams. Sheep's ice creams presented slightly lower fat contents and higher ash contents when compared to goat's ice creams.

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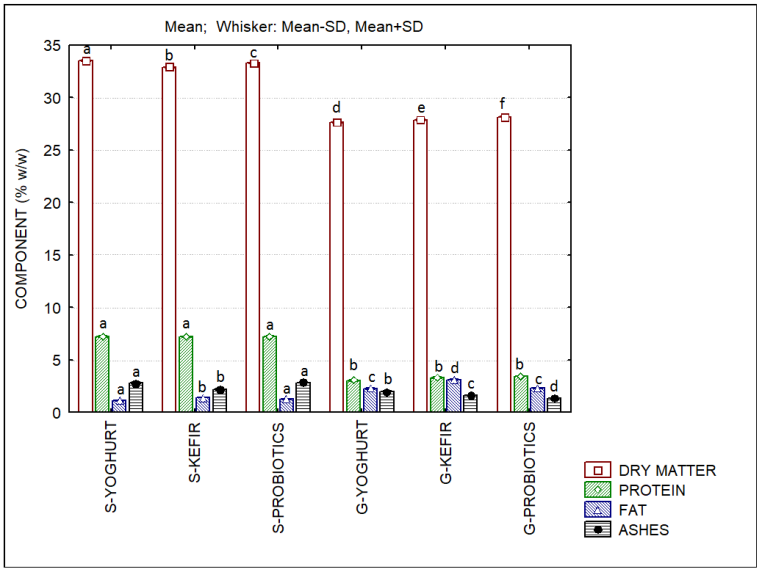


Figure 1. Chemical composition of sheep's and goat's second cheese whey ice creams produced with different starter cultures. Different letters of the same component indicate significant differences ($p < 0.05$) among ice creams.

Figure 2 shows the pH and titratable acidity of the different ice creams. The decrease in pH over storage time in all samples is clear. In the beginning of storage, goat's SCW ice creams presented lower pH values when compared to their sheep's counterparts, and this pattern was maintained until the end of storage, except for goat's ice cream fermented with probiotics, whose pH is similar to all of the sheep's ice creams at the first and 120th days of storage. However, the titratable acidity values do not reflect the evolution of pH, since sheep's ice creams present higher values when compared to goat's ice creams, and the opposite pattern was expected. The explanation for this finding can be attributed to the buffering effect exerted by whey proteins, as reported by other authors (Onwulata et al. 2006).

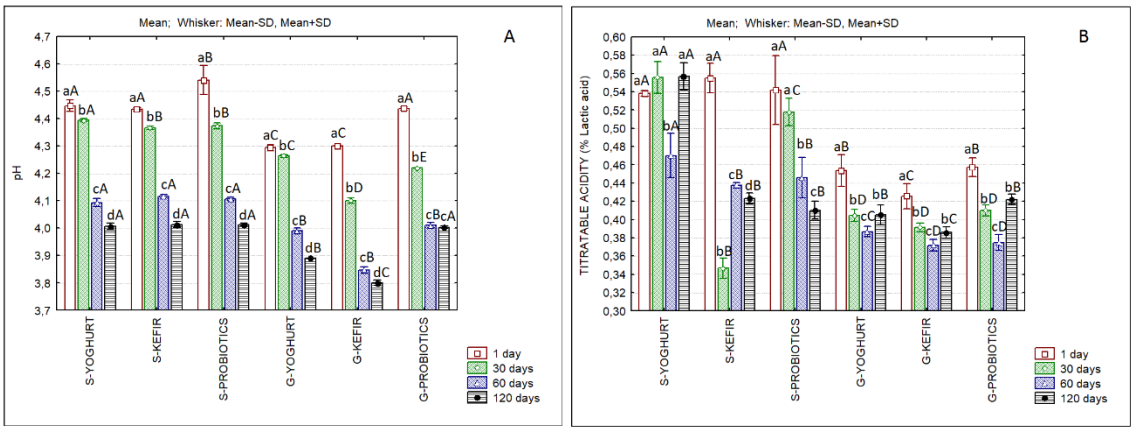


Figure 2. pH (**A**) and titratable acidity (**B**) of sheep's and goat's second cheese whey ice creams produced with different starter cultures over 120 days of frozen storage. Different lowercase letters in each type of ice cream indicate significant differences ($p < 0.05$) among storage days and different capital letters in each storage day indicate significant differences ($p < 0.05$) among ice creams.

The hardness results of sheep's and goat's SCW ice creams produced with different starter cultures are presented in Figure 3. The texture results indicate higher values of hardness

in the sheep's ice creams with a tendency to increase over storage, exceptions being the cases of S-Yoghurt and S-Kefir at the 30th day of storage. At the end of storage, the hardness of sheep's ice creams is clearly higher when compared to goat's ice creams. The significantly higher amount of protein in sheep's formulations, which more than doubled that of goat's formulations, can explain these results. Small strain rheological tests (Figure 4) confirm the patterns observed with the texture analyzer. Sheep's ice creams containing probiotics always presented the highest values for the complex viscosity (η*). S-Yoghurt, S-Kefir and S-Probiotics present higher values than their goat's SCW ice cream counterparts. However, goat's ice cream containing yoghurt (G-Yoghurt) presented η* values similar to that of its sheep's counterpart at the 60th day of storage. At this moment, goat's ice cream containing probiotics (G-Probiotics) presented higher values when compared with S-Yoghurt and S-Kefir.

Figure 5 presents the evolution of L^* (lightness), a^* (red-green axis) and b^* (blueyellow axis) of sheep's and goat's SCW ice creams produced with different starter cultures over storage. Significant differences were observed between products and over storage time. Tables 2 and 3 describe the color differences (ΔEab^*) of sheep's and goat's SCW ice creams produced with different starter cultures over storage and among products at the same storage day. With a few exceptions, ΔEab^* values are higher than 1, indicating that a normal observer could detect color differences between ice creams (https://techkonusa.com/a-simple-review-of-cie-%CE%B4e-color-difference-equations/. (accessed on 10 November, 2022)). It has to be pointed out that the exceptions were observed with respect to the same product at different storage times (e.g., the case of S-Probiotics between the 60th and 120th days of storage).

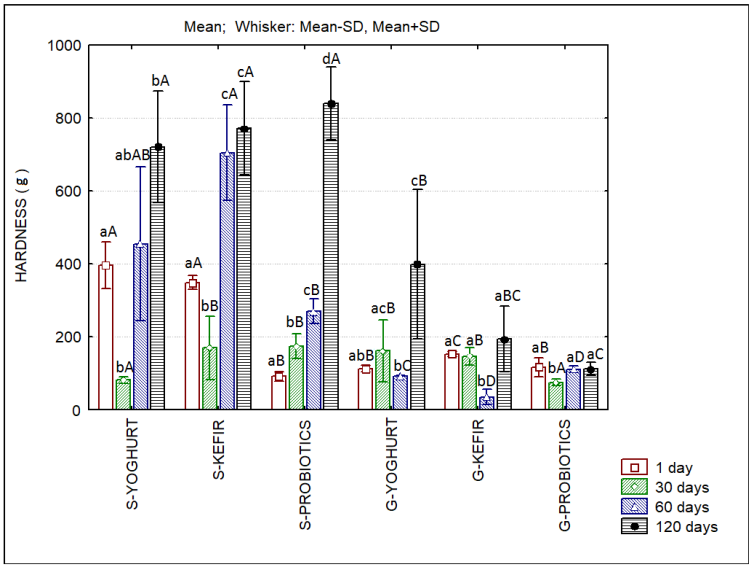


Figure 3. The hardness of sheep's and goat's second cheese whey ice creams produced with different starter cultures. Different lowercase letters in each type of ice cream indicate significant differences ($p < 0.05$) among storage days and different capital letters in each storage day indicate significant differences ($p < 0.05$) among ice creams.

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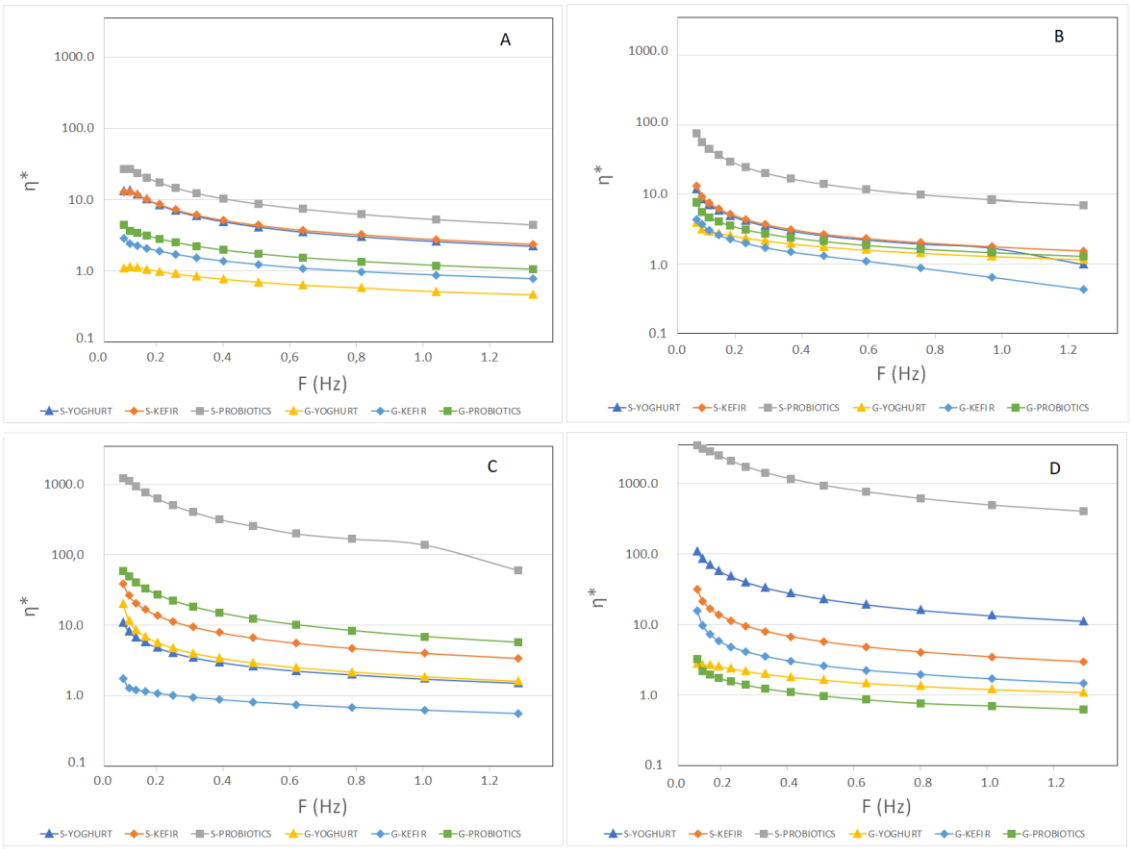


Figure 4. Complex viscosity (η*) of sheep's and goat's second cheese whey ice creams produced with different starter cultures over storage. 1 day (**A**); 30 days (**B**); 60 days (**C**); 120 days (**D**).

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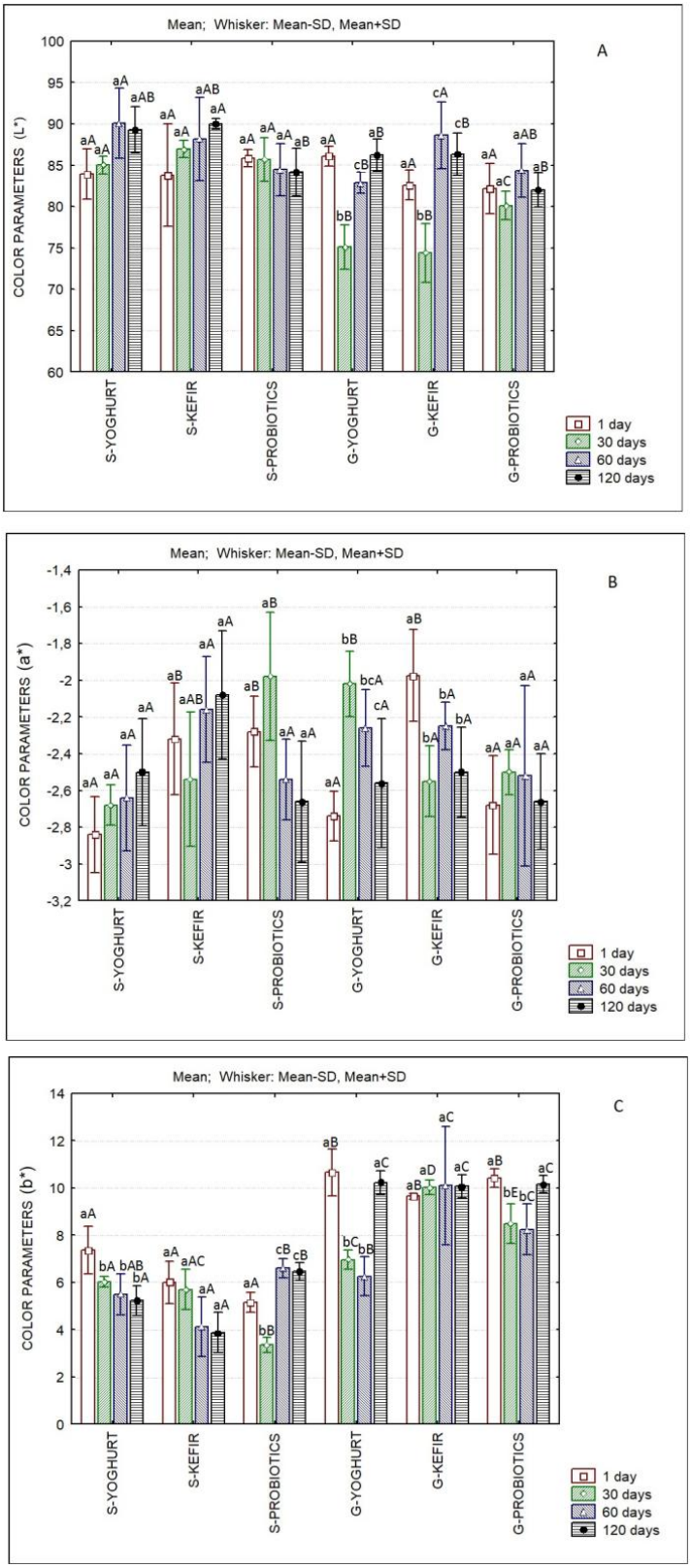


Figure 5. Evaluation of color parameters of sheep's and goat's second cheese whey ice creams produced with different starter cultures over storage. L* (A); a* (B); b* (C). Different lowercase letters in each type of ice cream indicate significant differences ($p < 0.05$) among storage days and different capital letters in each storage day indicate significant differences ($p < 0.05$) among ice creams.

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Table 2. Color differences (ΔEab*) of sheep's and goat's second cheese whey ice creams produced with different starter cultures over storage.

Products	1d. vs 30d.	1d. vs 60d.	1d. vs 120d.	30d. vs 60d.	30d. vs 120d.	60d. vs 120d.
S-Yoghurt	3.6	35.4	24.1	22.2	13.3	1.9
S-Kefir	24.8	45.9	35.3	11.6	8.1	12.3
S-Probiotics	4.8	5.6	5.6	13.5	10.7	0.8
G-Yoghurt	72.7	17.9	1.0	34.1	72.8	18.0
G-Kefir	37.2	32.5	11.2	119.3	80.4	16.8
G-Probiotics	8.2	17.3	0.5	17.8	13.8	13.8

Table 3. Color differences (ΔEab*) of sheep's and goat's second cheese whey ice creams produced with different starter cultures. Comparison between products at the same storage time.

Product's comparison	1 day	30 days	60 days	120 days
S-Kefir *vs* S-Yoghurt	9.8	3.6	13.7	4.2
S-Probiotics *vs* S-Yoghurt	9.9	5.5	24.8	17.1
S-Probiotics *vs* S-Kefir	22.2	7.3	14.4	23.7
G-Yoghurt *vs* S-Yoghurt	14.3	54.2	33.7	23.2
G -Kefir *vs* S-Yoghurt	4.1	65.8	35.9	20.9
G-Probiotics *vs* S-Yoghurt	7.7	18.0	32.6	46.5
G-Kefir *vs* S-Kefir	19.7	96.9	28.1	27.7
G-Probiotics *vs* S-Kefir	22.8	30.2	23.5	53.2
G-Probiotics *vs* S-Probiotics	26.0	31.7	8.9	11.3
G-Yoghurt *vs* G-Kefir	9.6	13.7	34.5	5.4
G-Yoghurt *vs* G-Probiotics	29.8	30.0	5.7	22.5
G-Kefir *vs* G-Probiotics	2.3	18.4	21.9	15.1

The overrun values (Table 4) are higher in sheep's ice creams when compared to goat's ice creams; however, in all cases, overrun values can be considered low when compared to data referred by other authors (Marnotes et al. 2021). Regarding the meltdown rate, S-Yoghurt and S-Probiotics presented lower values, with the latter presenting the higher resistance to melt.

Table 4. Overrun and meltdown rate of sheep's and goat's second cheese whey ice creams produced with different starter cultures. Different letters in each column indicate significant differences ($p < 0.05$) between ice creams.

Products	Overrun (%)	Meltdown Rate (%)
S-Yoghurt	18.34 ± 3.12^{a}	43.39 ± 2.16^{a}
S-Kefir	20.93 ± 5.30^{ab}	61.10 ± 6.95^{b}
S-Probiotics	30.92 ± 4.64^{b}	21.45 ± 2.22^{c}
G-Yoghurt	13.53 ± 1.06^{c}	73.94 ± 1.28^{b}
G-Kefir	13.87 ± 9.79^{c}	71.08 ± 2.26^{b}
G-Probiotics	12.51 ± 7.07^{c}	63.37 ± 6.32^{b}

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Few works refer to the use of dairy by-products in the manufacture of ice cream. De Meneses et al. (de Meneses et al. 2021) evaluated ice cream production with ricotta or cheese whey and buttermilk and the impact of such ingredients on quality parameters.

The results indicated a significant reduction in fat content in ice creams with different by-products (40% on aver-age). The instrumental texture and color of the ice creams with ricotta and cheese whey and buttermilk showed a slight change, showing greater hardness and color intensity, which were not observed by consumers and did not affect the sensory tests. All ice cream samples had good overall liking values. Moschopoulou et al. (Moschopoulou et al. 2021) tested the employment of ovine and caprine whey protein concentrates (WPC) in the production of three ovine ice creams with 5% of lipids. The first ice cream had bovine skimmed milk powder (SMP) (35% protein), the second one contained ovine-caprine WPC with 65% protein (WPC65), and the third ice cream had ovine/caprine WPC with 80% protein (WPC80). The authors observed that the ice cream with SMP melted faster and showed higher overrun than ice creams with WPCs. Regarding hardness, the ice cream with SMP presented lower values when compared with the other samples. In color parameters, ice cream with SMP was significantly brighter, while ice creams with WPCs were more yellow. For the sensory properties, the flavor scores of ice cream with 65% protein WPC were similar to those of ice cream with SMP. Our results showed higher values for meltdown in goat ice creams. Regarding overrun, sheep ice creams presented higher values, most probably due to their higher protein content, which allowed for more air incorporation in the matrix. In hardness, sheep ice creams presented higher values and this can be attributable, once again, to their higher protein content. Both sheep and goat ice cream hardness tended to increase over storage. McGhee et al. (McGhee et al. 2015) tested three goat milk ice creams manufactured using milk with three levels of lipids (3.64%, 2% and 0.71%) and studied their textural and sensory characteristics. The results showed very important increases in firmness and consistency in all three types of goat ice creams after 1 day of frozen storage. The increases of these properties were also important after 56 days of frozen storage. There was a slight decrease in overall acceptability of the three goat ice creams as the storage period increased, but they were acceptable after 8 weeks of frozen storage. Regarding hardness, we also observed an increase in hardness of all sheep ice creams and in goat´s ice cream containing yoghurt, but mainly after 120 days of storage.

Pimentel et al. (Pimentel et al. 2022) reviewed the technological and sensory aspects of probiotic ice creams and reported that these products showed satisfactory sensory properties as well as probiotic survival (>6 log CFU/g during storage and simulated gastrointestinal conditions). In our products, Lactobacilli sp. cell counts were higher than log 6 CFU/g at the first week of storage (Figure 6A). In the case of sheep's ice creams, these values were maintained or increased until the 30th day, but decreased until the 60th day of frozen storage. However, S-Kefir presented counts of ca. log 8 CFU/g at the end of storage. Lactococci sp. counts surpassed log 7 CFU/g in all products, and these values were maintained until the end of storage, except in the case of G-Yoghurt and G-Kefir (Figure 6B). It must be stressed that, in the case of ice creams containing probiotic cultures (SProbiotics and G-Probiotics), the sum of Lactococci sp. and Lactobacilli sp. counts was of the order of log 6–7 CFU/g until the 30th day of storage, indicating that the probiotic characteristics were maintained, at least for 1 month.

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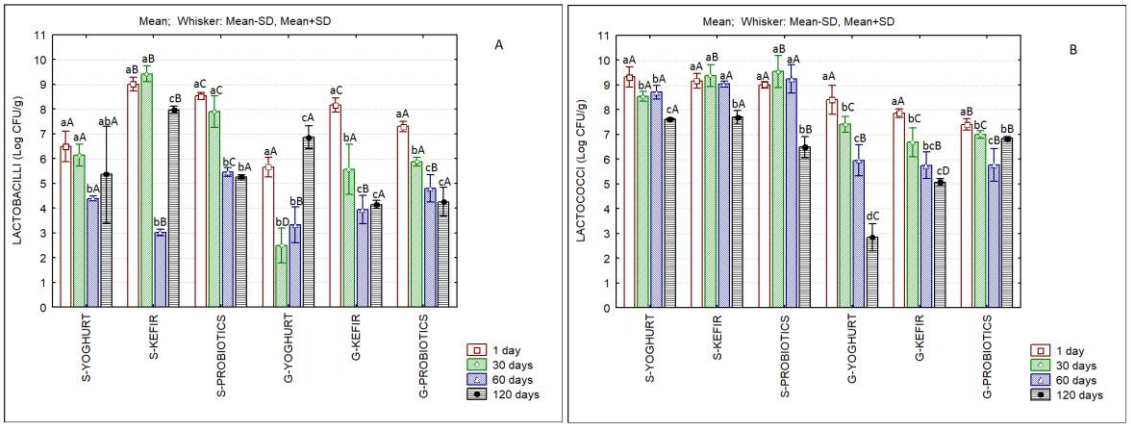


Figure 6. Evaluation of microbial counts of sheep's and goat's second cheese whey ice creams produced with different starter cultures over storage. *Lactobacilli* sp. (**A**); *Lactococci* sp. (**B**). Different lowercase letters in each type of ice cream indicate significant differences ($p < 0.05$) among storage days and different capital letters in each storage day indicate significant differences ($p < 0.05$) among ice creams.

Other manuscripts reported high probiotic counts in ice creams. Senaka Ranadheera et al. (Ranadheera et al. 2013) observed that the manufacturing process of probiotic ice cream originates a reduction in the viable cell numbers, but the products showed values of log 7–8 CFU/g over one year storage at −20 °C. Variations in body, texture and taste of the product were apparent after 12 weeks of storage. Camelo-Silva et al. (Camelo-Silva et al. 2021) reported that concentrated milk protected Bifidobacterium BB-12 cells over storage and during in vitro gastrointestinal assays. For the ice cream manufactured with concentrated milk, a high bifidobacteria protective effect in the descending colon was observed, with probiotic viable cell counts and recovery rate values equal to 9.88 log CFU/g. Silva et al. (Da Silva et al. 2015) observed that the addition of the probiotic bacteria Bifidobacterium animalis subsp. Lactis BLC1 in the manufacture of goat's milk ice cream decreased the pH, but it had no effect on physicochemical properties, overrun or the melting behavior of this product. After 120 days of frozen storage, a high survival rate was detected. In addition, fine sensory scores were reported and adequate probiotic viability was maintained throughout the 120 days of frozen storage in this ice cream. Açu, et al. (Açu et al. 2017) produced ice cream with goat's milk, milk powder, prebiotics and *Lactobacillus paracasei* subsp. *paracasei* and *Bifidobacterium longum* + *Bifidobacterium bifidum* combined culture (as probiotic cultures). Açu, et al. (Acu et al. 2020) also observed that these synbiotic ice creams maintained their probiotic properties during storage and had good sensory scores. de Paula et al. (Paula et al. 2020) manufactured goat milk ice creams with Lactobacillus rhamnosus or Lactobacillus paracasei as probiotic cultures, and these microorganisms had high viability levels during storage. The added probiotics maintained viability levels above 8 Log CFU/g during storage. Inulin added ice creams that showed lower overrun, while the hardness and meltdown rate increased in comparison with milk cream formulations. These products were well accepted by consumers. Shahein et al. (Shahein et al. 2022) evaluated the microbiological and physicochemical characteristics of frozen yoghurt made with probiotic strains in combination with Jerusalem artichoke tubers powder (JATP) used as a fat and sugar replacer. Samples with JATP contained viable counts of Bifidobacterium bifidum BGN4

and Lactobacillus casei Lc-01 of 7 log CFU/g during 90 days of storage, as compared to the control sample. The addition of JATP also increased the acidity and enhanced the overrun melting resistance and viscosity of frozen yoghurts. The addition of JATP up to 10% also increased sensory attributes. Al-Shawi et al. (Al-Shawi and Ali 2020) evaluated probi- 347 otic (Lactobacillus acidophilus), and synbiotic (L. acidophilus and inulin) ice creams and concluded that L. acidophilus counts were higher in synbiotic ice cream. Synbiotic ice cream received the highest overall acceptance scores. Sabet-Sarvestani et al. (Sabet-Sarvestani et al. 2021) produced synbiotic ice cream using Lactobacillus casei/Lactobacillus plantarum and fructooligosaccharides, and they observed a beneficial role of these prebiotics for the growth of probiotics. Kowalczyk et al. (Kowalczyk et al. 2021a) observed that the survival of Bifidobacterium animalis ssp. lactis BB-12 in sheep's milk ice cream at the 21st day of storage exceeded log 10 CFU/g. The overrun of the sheep's milk ice cream was within the range of 78.50% to 80.41%. These values are clearly higher than the ones obtained by us. However, the differences could result from the fact that overrun values have a high dependence on the type of freezer used. Kowalczyk et al. (Kowalczyk et al. 2021b) also observed that the freezing process reduced the population of probiotic bacteria cells in sheep´s milk ice cream with inulin from 0.8, 1.0 and 1.1 log CFU/g in products with L. *acidophilus* LA-5, *Lacticaseibacillus paracasei* L-26, and *Lacticaseibacillus casei* 431, respectively. With regard to sensory quality, all products produced in the present work were well accepted by the consumer's panel up to the 120th day of storage (Figure 7). Only the ranking test was able to differentiate preferences between products. Sheep's ice creams were preferred in comparison to goat's ice creams, specifically regarding taste. The ranking test indicated that at the 7th day of storage, the order of preference was as follows: S-Kefir, S-Probiotics, S-Yoghurt, G-Probiotics, G-Yoghurt and G-Kefir. However, no differences were observed between S-Yoghurt, G-Probiotics and G-Yoghurt. At the 30th day of storage, the order of preference was: S-Probiotics, S-Kefir, S-Yoghurt, G-Yoghurt G-Kefir and G-Probiotics. Again, no differences were observed between S-Yoghurt, G-Yoghurt and G-Kefir. At the 60th day of storage, the order of preference was: S-Kefir, S-Yoghurt, S-Probiotics, G-Kefir, G-Probiotics and G-Yoghurt. At this time, sheep's and goat's ice creams were clearly separated with regard to consumer's preference. At the end of storage, the order of preference was S-Kefir, S-Probiotics, G-Yoghurt, S-Yoghurt, G-Kefir, G-Probiotics.

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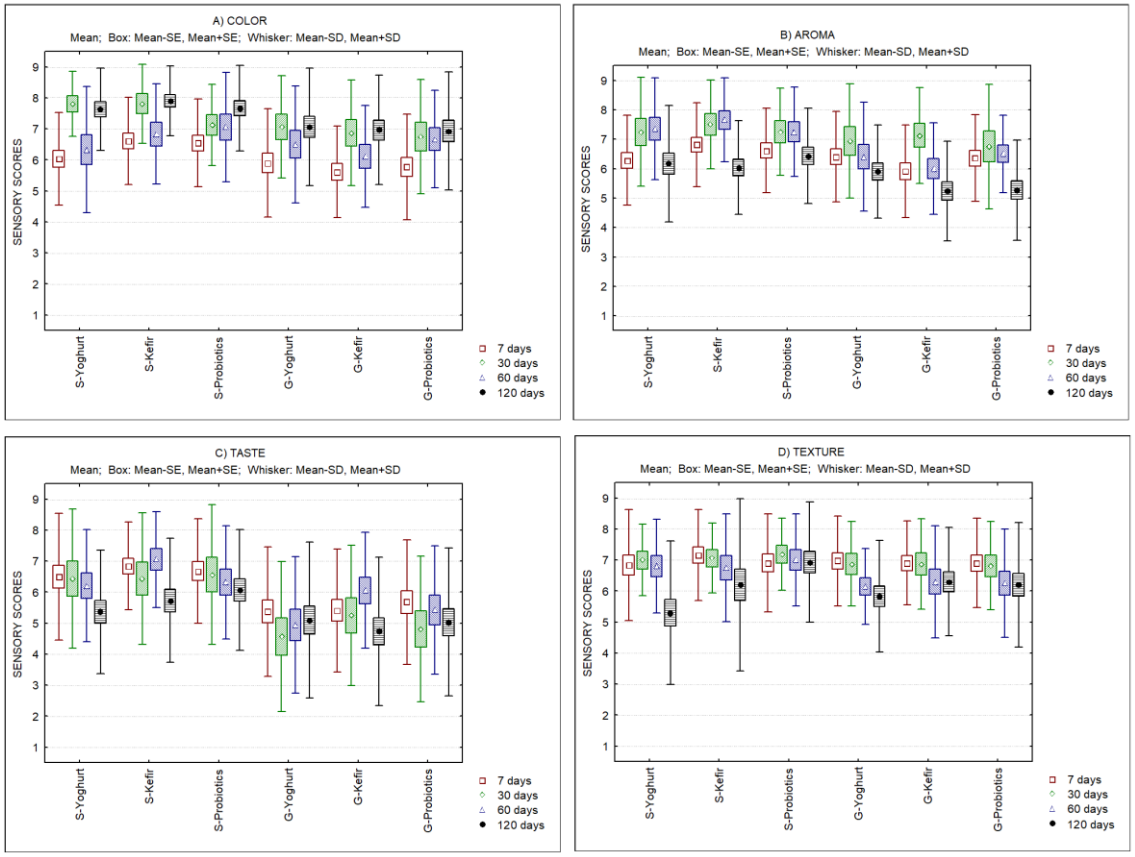


Figure 7. Sensory scores of sheep's and goat's second cheese whey ice creams produced with different starter cultures over 120 days of storage. Color (**A**); Aroma (**B**); Taste (**C**); Texture (**D**).

4. Conclusions

The production of sheep's or goat's ice creams can represent an interesting opportunity for the valorization of SCW since it is infrequently reused. Whey and second cheese whey usually represent a problem to small and medium scale dairy processing units. These plants do not have adequate equipment to process SCW (i.e., concentration and drying equipment) and usually these by-products are offered for livestock feeding or are discarded into the public sewage system. The use of ultrafiltration, or even nanofiltration, to concentrate SCW originates liquid second cheese whey concentrates (LSCWCs) which can be directly used in the development of novel dairy products. This solution allows for the reduction in negative environmental effects resulting from the direct discharge of SCW. Furthermore, the addition of prebiotics and the production of fermented products containing probiotic bacteria increases the nutritional value of LSCWC's and their market potential. The results of the present work have practical applications in small and medium scale dairy plants by reducing their environmental impact and improving their profitability via value-added products, which is crucial for sustainable rural development.

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Manuscript IV - Sheep's Second Cheese Whey Edible Coatings with Oregano and Clary Sage Essential Oils Used as Sustainable Packaging Material in Cheese

The results from this chapter have already been published as:

Arona Pires [1,2], Hubert Pietruszka [3], Agata Bozek [3], Katarzyna Szkolnicka [3], David Gomes [1], Olga Díaz [2], Angel Cobos [2] and Carlos Pereira [1, 4, *], (2024), Sheep's Second Cheese Whey Edible Coatings with Oregano andClary Sage Essential Oils Used as Sustainable Packaging Material in Cheese, *Foods* 13, 674.

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Conceptualization, C.P., A.P., O.D. and A.C.; methodology, C.P., A.P., O.D. and A.C.; validation, C.P., K.S., O.D. and A.C.; investigation, A.P., H.P., A.B. and D.G.; resources, C.P.; data curation, C.P., O.D., A.C. and K.S.; writing—original draft preparation, A.P., C.P., O.D. and A.C.; writing—review and editing, C.P, O.D., A.C. and K.S.; supervision, C.P.; project administration, C.P.; funding acquisition, C.P. All authors have read and agreed to the published version of the manuscript

Abstract:

Sheep's second cheese whey (SCW), the by-product resulting from whey cheese production, was used as a component of cheese coatings containing oregano (Origanum compactum) and clary sage (Salvia sclarea) essential oils (EOs). SCW powder was obtained by the ultrafiltration/diafiltration of SCW followed by reverse osmosis and freeze drying. The coatings were produced with a mixture of SCW and whey protein isolate (WPI) using glycerol as plasticizer. Model cheeses were produced with cow´s milk and those containing SCW:WPI coatings; those with and without EOs were compared to controls without coating and with a commercial coating containing natamycin. At the end of ripening (28 days), the cheeses containing EOs presented higher water activity (ca. 0.930) and moisture content, as well as lower titratable acidity. Concerning color parameters, significant differences were also observed between products and as a result of ripening time. However, the use of SCW:WPI coatings did not significantly influence the color parameters at the end of ripening. Regarding texture parameters, the cheeses containing SCW:WPI coatings presented significantly lower values for hardness, chewiness, and gumminess. Significant differences were also observed for all microbial groups evaluated either between products and as a result of ripening time. In all cases, lactobacilli and lactococci counts surpassed log 7–8 CFU/g, while the counts of yeasts and molds increased steadily from ca. log 3 to log 6 CFU/g. The lowest counts of yeasts and molds were observed in the samples containing natamycin, but nonsignificant differences between products were observed. In conclusion, SCW:WPI cheese coatings can successfully substitute commercial coatings with the advantage of being edible packaging materials manufactured with by-products.

Keywords: edible coatings; cheese; second cheese whey; essential oils; oregano; salvia

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1. Introduction

Whey cheeses (e.g., Requeijão, which is produced in Portugal) are obtained by heating cheese whey (CW) (90–95 ◦C for ca. 20 min) to precipitate whey proteins. The protein aggregates originate a matrix that entraps fat and other solids present in whey (Madureira et al. 2005a; Maragkoudakis et al. 2016). Second cheese whey (SCW) is the liquid remaining after whey cheese production and represents more than 90% of the original CW volume. Lactose (4.5–5.0%), salts (0.5–1.5%), proteins (0.15–0.3%), and fat (0.1–0.3%) represent the major solid components of bovine SCW (Pires et al. 2021b; Secchi et al. 2012). SCW derived from sheep whey is typically characterized by a higher protein content, thus containing 7.5–10.5% dry matter, 4.2–5.5% lactose, 0.5–1.8% protein, 0.3–2.5% fat, and 0.5–1.0% minerals (Carvalho et al. 2013; Pereira et al. 2002; Secchi et al. 2012). The high volumes of SCW produced (about 18 L per kg of whey cheese) represent a problem for cheese producers, as this by-product poses environmental challenges due to its very high chemical and biochemical oxygen demand (COD and BOD5 values higher than 30 g/L) (Carvalho et al. 2013; Maragkoudakis et al. 2016; Pires et al. 2021b). Most SCW is used as supplement feed for livestock, either directly or as an additive (Mariotti et al. 2020), but high amounts are still discarded without treatment. However, this by-product has important nutrients such as denatured proteins, nonprotein nitrogen such as soluble peptides and free amino acids, oligosaccharides, lactose, and hydrosoluble vitamins and minerals (Minhalma et al. 2007; Monti et al. 2018; Pereira et al. 2002; Pires et al. 2021b).

SCW can serve as a substrate in biotechnological processes for producing high-value commercial compounds such as lactic acid, bioethanol, lactobionic acid or polyhydroxyalkanoates (Bosco et al. 2021; Carvalho et al. 2013; De Giorgi et al. 2018; Macedo et al. 2015; Sansonetti et al. 2009; Tesfaw 2023; Zoppellari and Bardi 2013). The obtention of bioactive peptides from SCW was also proposed (Monari et al. 2019; Sommella et al. 2016). Some manuscripts are related to the use of SCW as a biotechnological substrate for probiotic or starter cultures (Chessa et al. 2020; Coronas et al. 2023; Naziri et al. 2023). Others refer to its potential to produce biofuel products (Russo et al. 2021; Tsolcha et al. 2016; Vasilakis et al. 2022) .The use of SCW to produce fermented drinks or as an ingredient in food formulations, was also evaluated (Borges et al. 2020; Díaz et al. 2004; Garcia et al. 2022; Lopes et al. 2019; Pires et al. 2022; Pires et al. 2021b). However, further research work on the valorization of SCW is still required, and the difficulties related with the technology transfer from researchers to dairy producers need to be overcome.

The utilization of dairy by-products on the production of edible films and coatings was generally based on the use of cheese whey (CW) (Cruz-Diaz et al. 2019; Galus and Lenart 2019; Pérez et al. 2016). Revisions on this subject have also been published (Kandasamy et al. 2021; Nemati et al. 2023). CW films or coatings are often added with antimicrobial agents, namely essential oils obtained from plants such as cumin (Nemati et al. 2023), clove (Fahrullah et al. 2021), lemon, bergamot (Çakmak et al. 2020), oregano, rosemary, garlic (Seydim and Sarikus 2006; Seydim et al. 2020), tarragon (Socaciu et al. 2020), and

cinnamon (Bahram et al. 2014). Other plant-based ingredients were also tested (Barba et al. 2015; Kontogianni et al. 2023; Pluta-Kubica et al. 2020; Robalo et al. 2022). Chitosan (Yangılar 2015), lactic and propionic acids, chitooligosaccharides (Ramos et al. 2012d), and enterocins (Silva et al. 2023a) were also evaluated in whey-based films.

Several authors used previously fermented CW to produce edible films as carriers of lactic acid bacteria (Cosentino et al. 2018; Dopazo et al. 2022; Guadalupe et al. 2023; Guimarães et al. 2020; Hernández-Carranza et al. 2023; Pereira et al. 2016; Sáez-Orviz et al. 2022)or probiotics (Gagliarini et al. 2019). Finally, other research works refer to the proteins and peptides with biological activity present in CW films (Dinika et al. 2020). However, the evaluation of SCW in the production of edible films and coatings was almost ignored, and its potential as a sustainable packaging material was neglected. To the best of our knowledge, only one work reported (Alfano et al. 2022) the use of SCW for such applications. The application of coatings based on whey proteins for cheeses is also limited. Whey protein isolates (Ramos et al. 2012a; Ramos et al. 2012c) and whey protein concentrates (Mileriene et al. 2021; Vasiliauskaite et al. 2023; Vasiliauskaite et al. 2022) have been used to elaborate coatings to wrap cheeses by incorporating antimicrobial compounds (Ramos et al. 2012a; Ramos et al. 2012c), natural plant extracts (cinnamon) (Mileriene et al. 2021), or protective lactic acid bacteria (Vasiliauskaite et al. 2023; Vasiliauskaite et al. 2022) to extend the shelf life of the product.

The aim of the present work was to test the efficacy of sheep's SCW-based edible coatings on bovine milk cheeses. The cheeses containing SCW edible coatings, without the addition of antimicrobial agents (WC), with added oregano (Origanum vulgare) essential oil (WCO), or with clary sage (Salvia sclarea) essential oil (WCS), were compared with cheeses without coating (C) or with a commercial coating containing natamycin (N).

2. Materials and Methods

2.1. Production of Sheep Second Cheese Whey Powders

The sheep's SCW was supplied by an external dairy company. A total of 500 L of sheep's SCW were ultrafiltrated in a pilot plant (Proquiga Biotech SA, A Coruña, Spain) equipped with an organic ultrafiltration membrane (3838 PVDF/polysulfone) (effective filtration area of 7 m^2 and 10 kDa cutoff) (FipoBiotech, Vigo, Spain). The process was performed at a temperature ranging between 40 and 45 ◦C, at a transmembrane pressure of 3–4 bar, with a volumetric concentration factor (VCF) of 20 (VCF = Vol. feed/Vol. retentate), thereby obtaining 25 L of concentrate. To the concentrate, 225 L of process water were added, and the diluted concentrate was submitted to diafiltration (VCF = 10). The diafiltrated concentrate was pasteurized (75 ◦C, 30 s) and was further concentrated by reverse osmosis (RO) in a pilot plant developed by ORM (Belas, Portugal) equipped with 2.5 S seawater pressure vessel and a 1 m^2 Filmtec membrane SW302540 (Dow Chemical Company, Midland, TX, USA). The RO concentrate was freeze-dried in a Lyph-Lock freeze dryer (Labconco Corporation, Kansas City, MI, USA) in order to obtain the SCW powder.

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2.2. Manufacture of the Coatings with Sheep Second Cheese Whey Powder

Whey protein isolate (WPI) (NatWPI90, 90% w/w protein, 5.5% lactose, 1.0% fat, 2.5% ashes) supplied by InLeit Ingredients S.L.U. (Curtis, Spain) and sheep's SCW powder (54.7% w/w protein, 14.3% fat, 7.9% ashes) were weighed in order to originate a final concentration of protein in the cheese coating solution of 8% (w/v) and a protein proportion of 2:1 SCW:WPI.

The WPI was dissolved in distilled water by slow stirring for 30 min at 20 ◦C. Then, glycerol (Panreac, Barcelona, Spain) was added in a 1:1 protein/plasticizer ratio. The pH was adjusted to 7.0 with 0.1 M NaOH. Afterwards, the WPI:plasticizer solution was heated to 90 ◦C for 15 min with stirring. After cooling to 30 ◦C, the WPI:glycerol solution was separated into three equal portions to prepare the coatings containing sheep's SCW as follows: coating solution without essential oils; coating solution with oregano essential oil; coating solution with clary sage essential oil.

The essential oils (oregano and clary sage; Plena Natura, Amadora Portugal) (1% of the protein weight in solution) and the SCW powder were added simultaneously to the previously prepared WPI/plasticizer solution, and the mixture was homogenized using an Ultra-Turrax (mod. T18 basic, IKA™, Staufen, Germany). The final pH was adjusted to 7 with 0.1 M NaOH, and the solution was stored in refrigeration until used.

Summarizing, the final SCW:WPI/plasticizer solution had 8% (w/v) protein (in the proportion 2:1 SCW:WPI) and a ratio of 1:1 protein/plasticizer.

2.3. Manufacture of Cheeses

Cheese manufacture was carried out according to Borges et al. (Borges et al. 2020) with some modifications. Cylindrical cheeses of approximately 250 g were produced at Escola Superior Agrária (ESAC, Coimbra, Portugal) dairy pilot plant and used as cheese food model for sheep's SCW-based coating application. A local producer supplied the bovine milk (3.2% protein, 3.5% fat, 4.2% lactose). Milk pasteurization occurred at 75 ◦C (±0.1 ◦C) for 30 s in a plate and frame heat exchanger (Albinox, S. Pedro do Sul, Portugal). After stabilizing the temperature at 29.5 ± 0.5 ◦C, 0.04% of a 51% w/v CaCl2 solution (Tecnilac, Viseu, Portugal), 0.01% w/v starter culture containing *Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. Cremoris,* and *Streptococcus thermophilus* (Mesófilo Plus Starter, Enzilab, Leça do Balio, Portugal), and granulated lysozyme (0.025% w/v) (Biostar, Rua das Picarotas, Portugal) were added. Finally, 0.0025% w/v animal rennet (>92 g/100 g chymosin, supplied by Tecnilac, Viseu, Portugal), previously diluted in water, was added. The milk coagulated in approximately 45 min, and once coagulation was complete, the curd was cut into small pieces (2 cm^3) to promote the drainage of whey. After half of the whey was drained, an equal amount salt water (1% w/v salt, 30 ◦C) was added to the curd, and the mixture was thoroughly stirred before final draining of the whey for 15 min. The curds were placed in plastic molds before being pressed and stored in a refrigerated chamber at approximated 8–9 ◦C for 24 h. Next day, the cheeses were immersed in a brine solution (18–20 ◦Baumé) for 1 h 30 min. On the

following day, the different coatings were applied by immersion of the cheeses on the coating solution:

1. C: control cheese without coating;

2. N: control cheese with natamycin;

3. WC: cheese with SCW:WPI coating;

4. WCO: cheese with SCW:WPI coating containing oregano essential oil;

5. WCS: cheese with SCW:WPI coating containing clary sage essential oil.

The solution of natamycin (Nataseen™-L, Siveele, supplied by Enzilab, Leça do Balio, Portugal) containing 3.2 g/L in distilled water was previously prepared, and the cheeses were submerged in it for one minute.

The cheeses were maintained at ca. 10 ◦C, and the operation was repeated on the following day. Afterwards, the cheeses were transferred to the ripening room, where they were maintained for 28 days at 10 ± 2 ◦C, 80–90% R.H.

2.4. Physicochemical Analysis

Cheese sample total solids contents were measured by drying in a Schutzart DIN 40050-IP20 Memmert™oven according to NP 1598:1983 for cheese (NP 1598:1983). The cheese samples' ash content was determined through incineration after drying in a HD-23 Hobersal™ electric muffle furnace. The Gerber method was used for fat content determination in a SuperVario-N Funke Gerber™centrifuge according to NP 2105:1983 (NP 2105:1983). The total nitrogen content was quantified in a Digestion System 6 1007 Digester Tecator™ using the Kjeldahl method (AOAC 1997). A conversion factor of 6.38 was applied to calculate the protein content of cheeses. All determinations were performed in triplicate.

2.4.1. pH and Titratable Acidity

The pH of cheeses was directly determined using a pH meter (HI 9025 HANNA Instruments) in order to monitor its changes between the 1st and the 28th days of storage. The evolution of the titratable acidity (TA) (% lactic acid) was measured by titration according to AOAC 920.124 for cheese (AOAC 2002).

2.4.2. Water Activity

Cheese water activity (a_w) was measured in a Rotronic Hygrolab (Bassersdorf, Switzerland) laboratory device for aw measurement equipped with an HC2-AW cell at 20 ◦C. Water activity was determined by cutting a circle of cheese (2.25 cm radius and 0.5 cm height) and placing it in cell until a constant value of equilibrium relative humidity (E.R.H.) was attained.

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2.4.3. Color Parameters

The color parameters of rind and paste of cheese samples were determined (according to Borges et al. (Borges et al. 2020) with some modifications) with a colorimeter (model CR-200B Chroma

Meter, Minolta™, Tokyo, Japan) using illuminant C and 10° standard observer; the aperture diameter was 1 cm. It was previously calibrated with a white standard (CR-A47: Y = 94.7; x 0.313; y 0.3204). The CIEL*a*b* system was used to measure the color coordinates. Three measurements were taken for each sample. Total color difference (ΔEab^*) was calculated as follows:

$$\Delta Eab^* = [(L^* - L^{*0})^2 + (a^* - a^{*0})^2 + (b^* - b^{*0})^2]^{1/2} \quad (1)$$

where L^{*0}, a^{*0}, and b^{*0} and L^*, a^*, and b^* were the values measured for the samples under comparison (C vs. N vs. WC vs. WCO vs. WCS; 1st day vs. 7th, 14th day vs. 7th day, 21th day vs. 14th day, and 28th day vs. 21st day).

2.4.4. Texture Parameters

Texture analysis (TPA test) of cheese samples was carried out (according to Borges et al. (Borges et al. 2020) with some modifications) using a texture analyzer (model TA.XT Express Enhanced, Stable Micro Systems™, Godalming, UK). The penetration distance was 20 mm at 2 mm/s, and a cylindrical probe of 6 mm diameter was used. The Specific Expression PC software (version 1.1.9.0) was utilized for results calculation.

2.5. Microbiological Analysis

Lactic acid bacteria (LAB) of the genera Lactobacillus spp. and Lactococcus spp. Were counted on M17 agar (in aerobiosis) and on MRS agar (in anaerobiosis) plates (Biokar Diagnostics, Allonne, France) at 37 ◦C for 48 h, respectively, according to ISO 7889 IDF 117 (2003) (ISO 7889 IDF 117 2003). Yeasts and molds were enumerated according to ISO 6611 IDF 94 (2004) (ISO 6611 IDF 94 2004) in plates at 25 ◦C. Total mesophilic aerobic counts were enumerated at 30 ◦C for 72 h on plate count agar (PCA) according to Gonzalez-Fandos et al. (Gonzalez-Fandos et al. 2000).

Microbial analyses were carried out on the 1st, 7th, 14th, 21st, and 28th days of ripening in triplicate, together with two controls for each medium. Results are presented as the log CFU/g of cheese product.

2.6. Statistical Analysis

A two-way ANOVA was used to analyze the effects of days of ripening and type of cheese and their interaction on all the parameters determined. The differences among cheese samples and the effect of days of ripening were assessed using one-way ANOVA, and the means were compared using Tukey's post hoc test. A significance level of $p < 0.05$ was used for all mean evaluations. Statistical analyses were carried out using the IBM SPSS Statistics software for Windows version 27 (2021; IBM Corp, Armonk, NY, USA). Graphs were produced using the Statistica Software, version 8 (Stasoft Europe, Hamburg, Germany).

3. Results

Table 1 presents the compositional parameters of the cheese samples that, according to the Portuguese standard, are the basis for the classification of cheeses. Nonsignificant differences were observed regarding these parameters. All the cheeses produced were full-fat (≥45 <60% fat in dry matter) and semihard cheeses (54–63% moisture in the defatted cheese).

Table 1. Gross chemical composition of cheeses.

Cheese Sample	Protein in dry matter (%w/w)	Fat in dry matter (%w/w)	Moisture in defatted cheese (%w/w)
C	31.05 ± 1.08[a]	46.02 ± 1.43[a]	61.27 ± 1.40[a]
N	28.92 ± 1.30[a]	48.00 ± 2.16[a]	62.86 ± 0.91[a]
WC	30.08 ± 1.17[a]	48.07 ± 1.79[a]	62.28 ± 1.26[a]
WCO	31.89 ± 3.06[a]	49.26 ± 0.89[a]	60.73 ± 0.78[a]
WCS	29.61 ± 0.85[a]	49.07 ± 2.06[a]	62.79 ±0.57[a]

C-Control; N-cheese with natamycin; WC-cheese with SCW coating; WCO- cheese with SCW coating with oregano essential oil; WCS- cheese with SCW coating with clary sage essential oil. Same superscript letter (a) in a column indicates no significant differences.

The evolution of the dry matter and of the water activity of the different cheeses over ripening can be observed in Figure 1. Significant differences regarding dry matter and the water activity were observed among samples and during ripening, as can be observed in Table 2. The variation in dry matter was most evident between the 1st and the 7th day. The dry matter of the WC cheese showed a very significant increase between the 14th and the 21st day and presented the highest content of solids by the end of storage (Table S1 of the Supplementary Materials). The values of dry matter at the end of ripening were lower in cheeses containing the coating with essential oils. This fact was reflected in the significantly higher values of water activity of these samples (Table S1 of the Supplementary Materials).

Significant differences were also observed for the pH and the titratable acidity (TA) of the different cheeses (Figure 2). Control cheeses showed significantly higher values of TA than the other cheeses at the first day of ripening; these cheeses also presented higher values of TA at the other days of ripening except in the 21st day. It was observed that, despite its lower pH, the cheese with oregano essential oil presented the lowest titratable acidity by the end of the ripening time. Lower values of TA were also observed for WC and WCS samples compared to C and N cheeses at the end of ripening.

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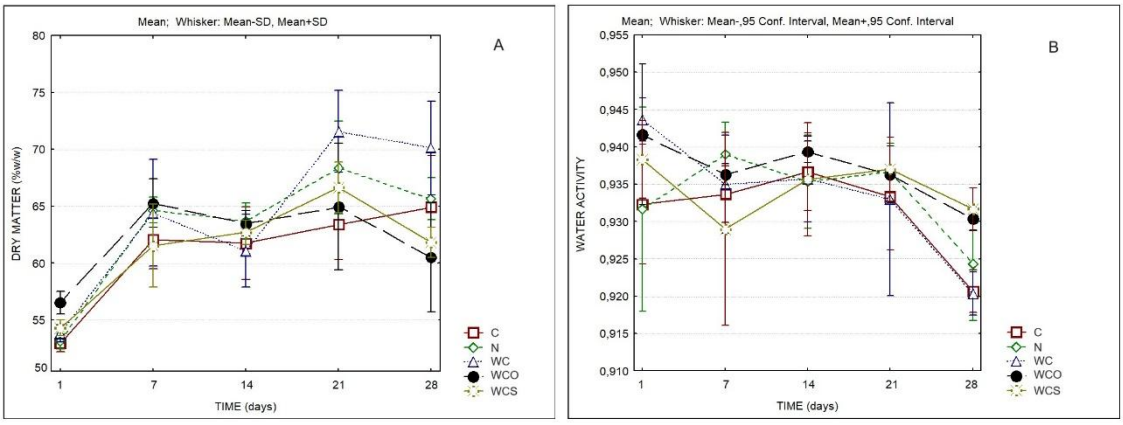


Figure. 1. Dry matter (**A**) and water activity (a_w) (**B**) of cheese samples. C-Control; N-Cheese with natamycin coating; WC-Cheese with SCW coating; WCO-Cheese with SCW coating with oregano essential oil; WCS-Cheese with SCW coating with clary sage essential oil.

Table 2. Two-way ANOVA of physicochemical characteristics of cheese samples.

Physicochemical analysis		two-way ANOVA	
		F	*p value*
Dry matter	Time	41.42	0.000
	Product	2.92	0.030
	Interaction	1.89	0.045
a_w	Time	48.83	0.000
	Product	7.90	0.000
	Interaction	5.98	0.000
pH	Time	2.91	0.031
	Product	5.68	0.000
	Interaction	2.82	0.003
Titratable acidity	Time	16.68	0.000
	Product	17.35	0.000
	Interaction	2.67	0.004

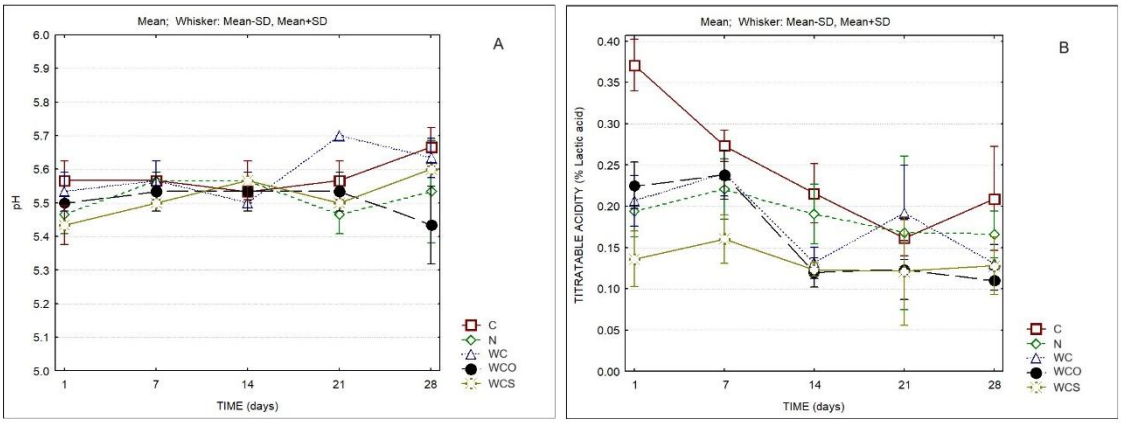


Figure 2. pH (**A**) and titratable acidity (**B**) of cheese samples. C—Control; N—Cheese with natamycin coating; WC—Cheese with SCW coating; WCO—Cheese with SCW coating with oregano essential oil; WCS—Cheese with SCW coating with clary sage essential oil.

Regarding the color parameters, the reduction in lightness of the rind over storage (Figure 3A) and the increase in parameter b* (increasing yellowness) from the first to the 7th day (Figure 3E) is clear. Parameter b* of the rind increased from values around 15 to values of ca. 20 between the 1st and the 7th day of ripening and were maintained, or slightly

reduced, at the end of ripening. Parameter b* of the paste (Figure 3F) had the same pattern as was observed in the rind, but, in this case, the b* values increased to just 16–19.

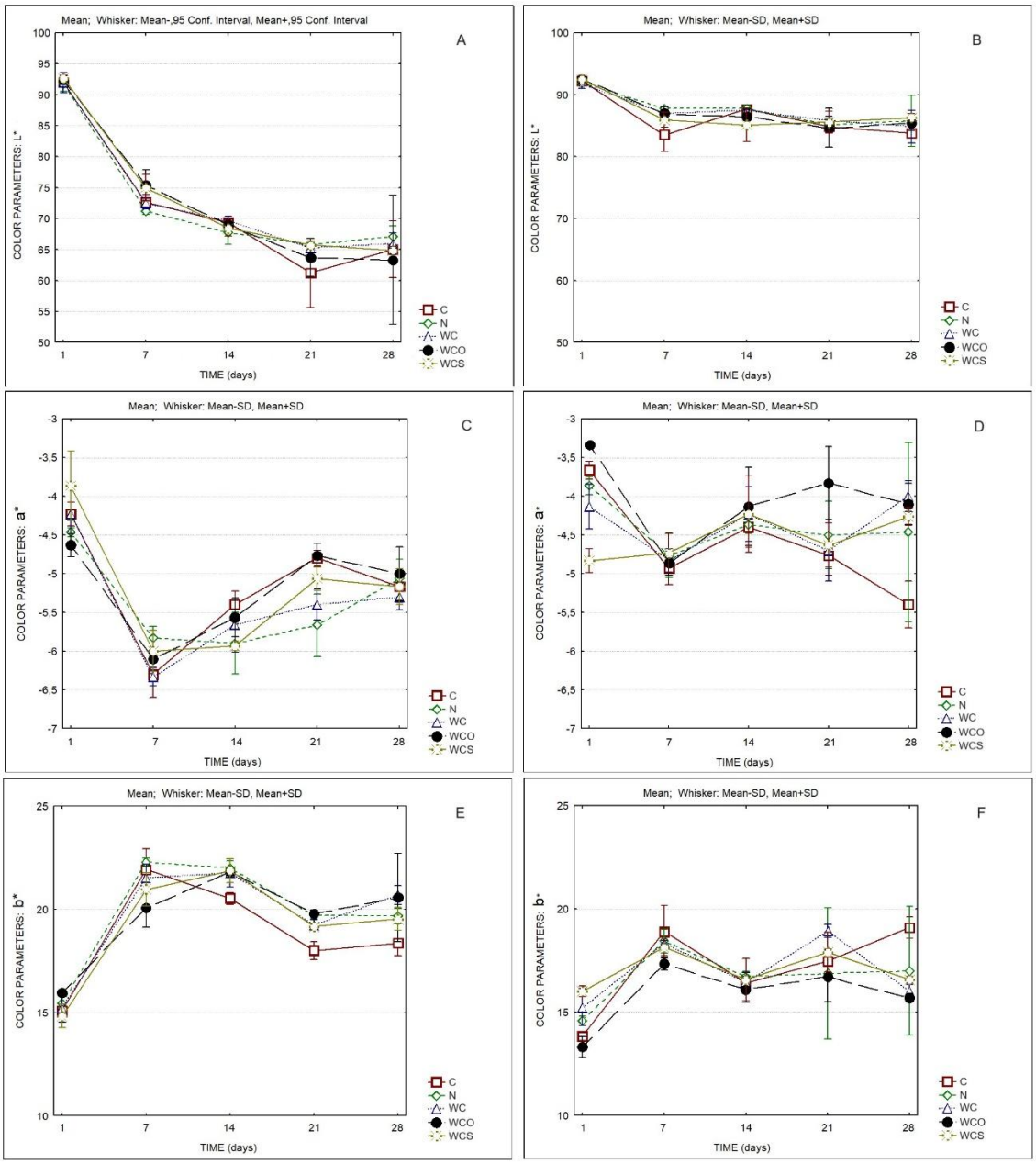


Figure 3. Color parameters of cheese samples. (**A**) L* rind; (**B**) L* paste; (**C**) a* rind; (**D**) a* paste; (**E**) b* rind; (**F**) b* paste. C-Control; N-Cheese with natamycin coating; WC-Cheese with SCW coating; WCO-Cheese with SCW coating with oregano essential oil; WCS-Cheese with SCW coating with clary sage essential oil.

The lightness of the paste of all samples (Figure 3B) also decreased, but the decrease was less pronounced when compared to the color of the rind. Parameter a* (green-red axis) of the rind and of the paste of cheeses (Figure 3C,D) decreased from the first to the 7th day, thus showing an increase toward the end of ripening, which was less pronounced on the paste. The exception was the paste of the control cheese, in which a* decreased with storage time.

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Table 3 presents the ΔEab* values between cheese samples (compared to the control—C) and for the same sample at different days of storage (compared to the 1st day of storage). It is evident that the differences between the cheese samples at the same day of storage are less marked than the differences observed for the same sample at different days of storage. At the first day of storage, the ΔEab* values between the samples and the control were lower than one, thereby indicating that a common observer cannot detect color differences. Exceptions were WC vs. C and WCS vs. C. The differences between samples tended to increase over ripening and reached the highest values observed at the 21st day. On the 28th day, the color differences were not so marked as at the 21st day. On the contrary, considering the evolution of the color of each cheese sample over time, very high ΔEab* values were observed between the 1st and the 7th day. Lower values were observed between the 14th and the 7th day, thereby increasing between the 21st and the 14th day. Between the 28th and the 21st day, the color differences were less marked, both in the rind and in the paste.

Table 3. ΔEab* values of cheese samples over storage.

ΔEab* values between products at different ripening days					
Ripening time (days)	1	7	14	21	28
Cheese samples				RIND	
N vs. C	0.4 ± 0.7	1.6 ± 0.8	2.7 ± 1.1	17.9 ± 10.3	4.2 ± 4.9
WC vs. C	0.7 ± 0.5	0.3 ± 0.4	1.2 ± 0.9	19.2 ± 13.0	5.4 ± 5.3
WCO vs. C	0.6 ± 0.2	7.1 ± 6.5	1.0 ± 0.5	8.5 ± 10.2	15.0 ± 12.2
WCS vs. C	0.7 ± 0.7	3.5 ± 2.0	1.7 ± 1.4	12.7 ± 11.3	2.4 ± 2.4
				PASTE	
N vs. C	0.3 ± 0.1	12.2 ± 11.7	0.4 ± 0.4	3.9 ± 4.7	14.6 ± 9.3
WC vs. C	1.7 ± 1.1	8.0 ± 8.5	0.2 ± 0.2	2.2 ± 2.9	9.3 ± 3.6
WCO vs. C	0.4 ± 0.4	11.4 ± 16.6	1.8 ± 2.7	6.5 ± 4.7	8.9 ± 0.6
WCS vs. C	3.2 ± 0.6	4.5 ± 4.4	5.6 ± 7.9	1.0 ± 1.5	7.4 ± 1.4
ΔEab* values for the same product at different ripening days					
Cheese Sample	C	N	WC	WCO	WCS
Ripening time (days)				RIND	
7 vs. 1	213.5 ± 15.6	234.5 ± 9.2	210.0 ± 21.1	155.3 ± 23.4	179.4 ± 26.1
14 vs. 7	7.5 ± 0.8	6.3 ± 3.0	4.9 ± 1.5	22.4 ± 5.7	21.6 ± 4.8
21 vs. 14	39.7 ± 19.1	16.2 ± 20.1	37.1 ± 24.9	16.7 ± 4.7	8.2 ± 2.5
28 vs. 21	7.7 ± 2.5	7.2 ± 9.5	18.9 ± 15.2	9.7 ± 7.4	0.6 ± 0.2
				PASTE	
7 vs. 1	55.4 ± 32.0	16.9 ± 3.4	17.2 ± 4.5	26.0 ± 11.4	24.3 ± 5.3
14 vs. 7	14.3 ± 13.7	1.8 ± 0.9	2.7 ± 0.8	2.4 ± 1.3	3.0 ± 2.4
21 vs. 14	5.2 ± 2.6	7.3 ± 3.5	4.8 ± 1.6	3.7 ± 2.6	2.5 ± 1.9
28 vs. 21	2.8 ± 1.2	9.3 ± 2.3	8.6 ± 2.2	5.6 ± 5.1	2.2 ± 0.2

Taking into attention the values of the two-way ANOVA test (Table 4), significant differences among products and days of ripening regarding the color parameter of the rind and of the paste were detected. However, as it can be seen in Table S2 of the Supplementary Materials, no significant differences were observed among different cheeses in all the color parameters (L*, a*, and b* of the rind and of the paste) at the end of the ripening.

Figure 4 presents the external aspect of the representative cheeses over the storage period. It can be observed that all cheeses showed low external contamination with molds over

storage. However, at the 21st and 28th days, some cheeses containing oregano EO presented some visible contamination by molds.

Table 4. Two-way ANOVA of color parameters of cheese samples.

Color Parameters		two-way ANOVA	
Rind		*F*	*p value*
L*	Time	1478.07	0.000
	Product	2.71	0.040
	Interaction	4.74	0.000
a*	Time	114.13	0.000
	Product	2.56	0.050
	Interaction	3.49	0.000
b*	Time	100.44	0.000
	Product	3.07	0.025
	Interaction	3.63	0.000
Paste			
L*	Time	51.68	0.000
	Product	1.35	0.264
	Interaction	1.31	0.227
a*	Time	11.06	0.000
	Product	5.44	0.001
	Interaction	3.09	0.001
b*	Time	25.26	0.000
	Product	3.73	0.010
	Interaction	1.80	0.059

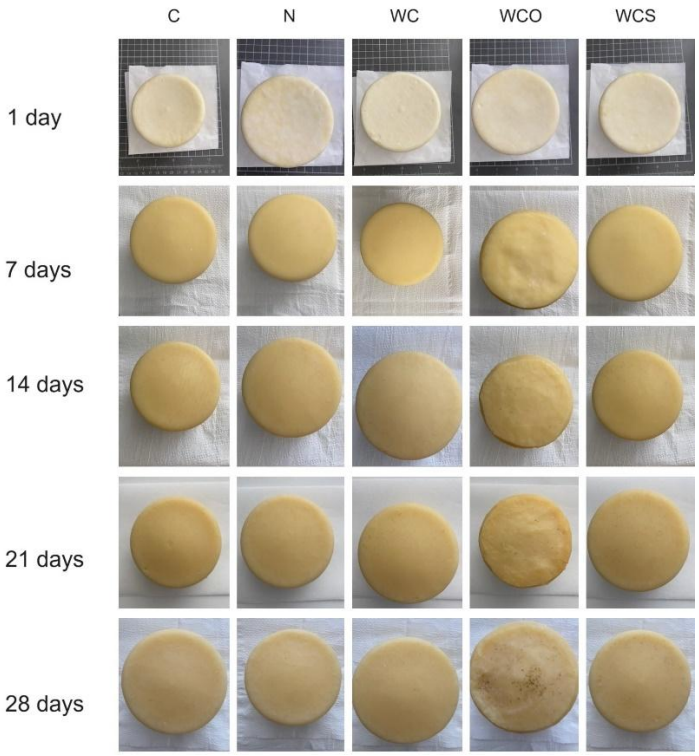


Figure 4. External aspect of representative cheese samples over the ripening period. C—Control; N—Cheese with natamycin coating; WC—Cheese with SCW coating; WCO—Cheese with SCW coating with oregano essential oil; WCS—Cheese with SCW coating with clary sage essential oil.

Figure 5 displays the texture parameters of cheese samples. Significant differences were observed for all parameters as a result of ripening time (Table 5). The cheeses with higher ripening times showed significant higher values for hardness, chewiness and gumminess, and significant lowest values for adhesiveness and resilience (Table S3 of supplementary materials). The cheeses containing SCW coatings presented lower values for hardness, chewiness, gumminess, and cohesiveness (Fig.5A, C, D, E) when compared to C and N.

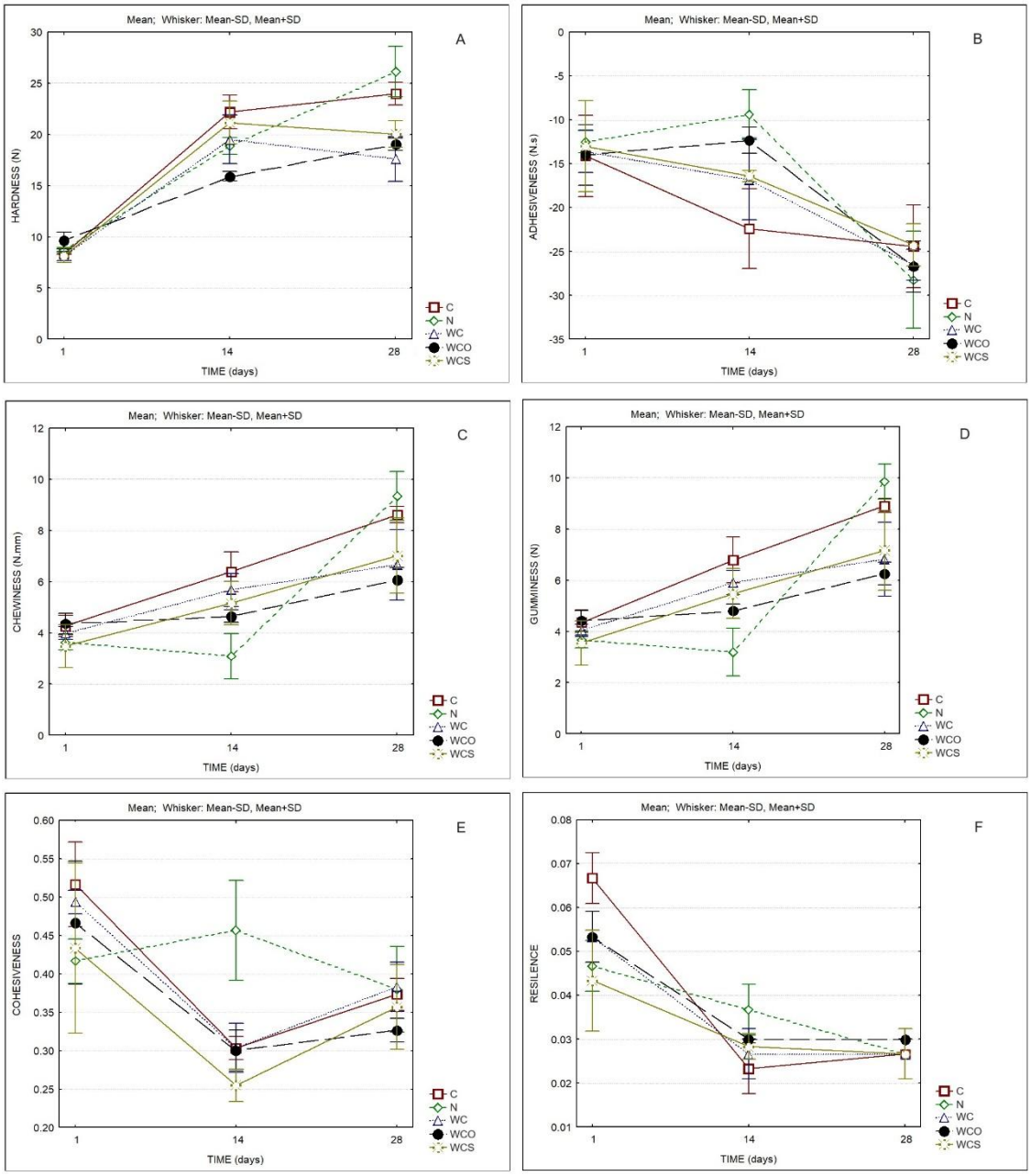


Figure 5. Texture parameters of cheese samples. (**A**) Hardness; (**B**) Adhesiveness; (**C**) Chewiness; (**D**) Gumminess; (**E**) Cohesiveness; (**F**) Resilience. C—Control; N—Cheese with natamycin coating; WC—Cheese with SCW coating; WCO—Cheese with SCW coating with oregano essential oil; WCS—Cheese with SCW coating with clary sage essential oil.

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Table 5. Two-way ANOVA of texture parameters of cheese samples.

Texture Parameters		two-way ANOVA	
		F	*p value*
Hardness	Time	267.79	0.000
	Product	9.94	0.000
	Interaction	25.80	0.000
Adhesiveness	Time	47.96	0.000
	Product	1.28	0.300
	Interaction	2.45	0.038
Chewiness	Time	84.76	0.000
	Product	4.40	0.007
	Interaction	6.72	0.000
Guminess	Time	90.62	0.000
	Product	4.98	0.004
	Interaction	7.72	0.000
Cohesiveness	Time	30.28	0.000
	Product	2.54	0.061
	Interaction	3.40	0.007
Resilience	Time	88.87	0.000
	Product	1.46	0.239
	Interaction	3.99	0.003

The microbiological evaluation of the different cheese samples is displayed in Figure 6. Significant differences were observed for all microbial groups over storage (Table 6). In all cases, the counts of lactobacilli and lactococci surpassed Log 7–8 CFU/g, while the counts of yeasts and molds increased steadily from ca. Log 3 CFU/g to Log 6 CFU/g. The type of cheese coating significantly influenced the counts of lactobacilli, yeasts, and molds (Table 6). The lowest counts of yeasts and molds were observed in the samples with natamycin. However, nonsignificant differences were observed between products at the end of storage (Table S4).

Table 6. Two-way ANOVA of microbiological characteristics of cheese samples.

Microbiological analysis		two-way ANOVA	
		F	*p value*
Lactococci	Time	35.66	0.000
	Product	9.58	0.000
	Interaction	12.63	0.000
Lactobacilli	Time	22.64	0.000
	Product	1.17	0.343
	Interaction	8.65	0.000
Mesophilic Aerobic Bacteria	Time	9.37	0.000
	Product	2.27	0.070
	Interaction	3.48	0.000
Yeasts and Moulds	Time	52.60	0.000
	Product	3.13	0.019
	Interaction	1.29	0.225

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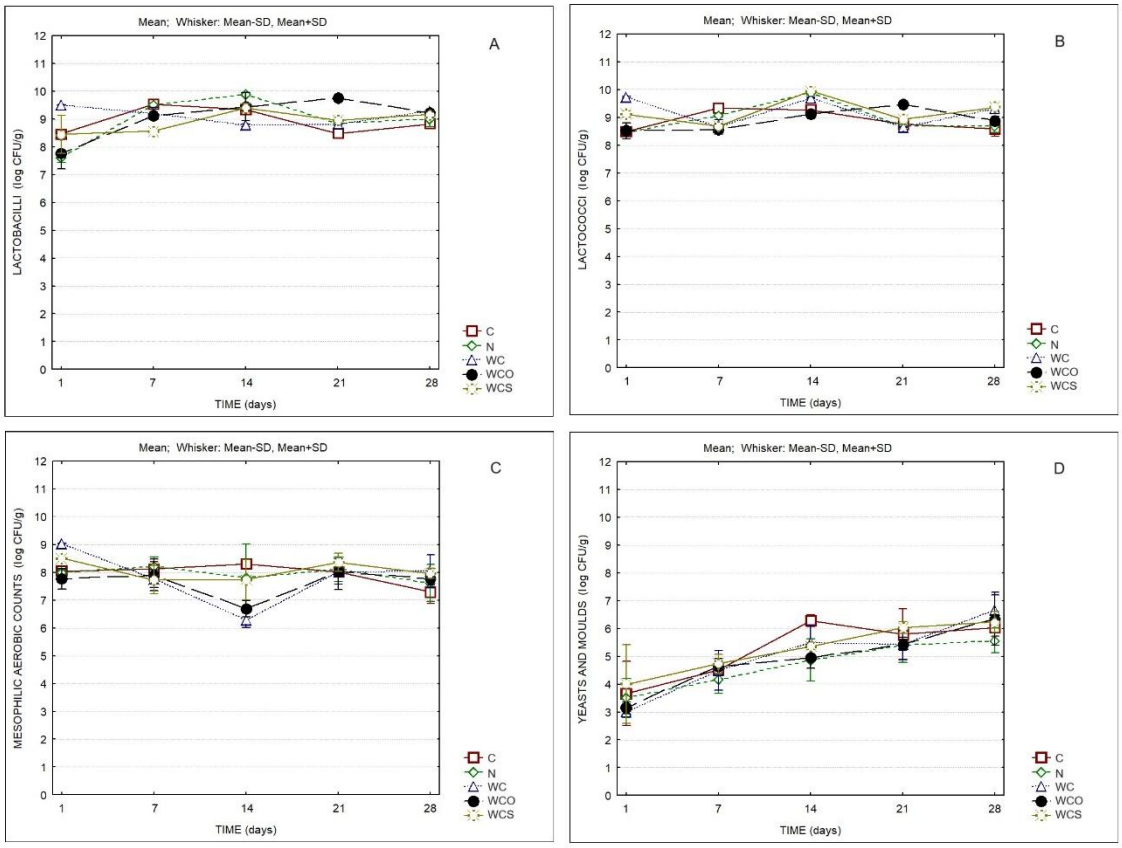


Figure 6. Microbiological parameters of cheese samples. (**A**) Lactobacilli; (**B**) Lactococci; (**C**) Mesophilic aerobic counts; (**D**) Yeasts and molds. C—Control; N—Cheese with natamycin coating; WC—Cheese with SCW coating; WCO—Cheese with SCW coating with oregano essential oil; WCS—Cheese with SCW coating with clary sage essential oil.

4. Discussion

Dairy products, namely cheeses, are commonly contaminated with molds, which produce visible or non-visible defects that lead to significant economic losses. Methods to reduce contamination include good manufacturing and hygiene practices, as well as air filtration and the manual cleaning of cheeses during ripening. Control methods such as inactivation treatments, temperature control, or modified atmosphere packaging are also used. However, fungal spoilage remains a problem for cheese producers despite technological advances in existing preservation methods. New preservation technologies have been introduced in recent years, including bioprotective cultures or edible coatings containing antimicrobial agents (Garnier et al. 2017; Junior and Tribst 2022; Meloni et al. 2023; Ribeiro et al. 2021; Sanna et al. 2023; Vasiliauskaite et al. 2023; Vasiliauskaite et al. 2022).

We only found one work referring the use of SCW as a material to produce edible coatings. In the work performed by Alfano and coworkers (Alfano et al. 2022), SCW was microfiltered, and the MF permeate was concentrated by UF using 100 kDa membranes. The UF permeate obtained was also concentrated using 10 kDa membranes. The 10 kDa permeate was further concentrated on nanofiltration membranes. With this approach, different retentate fractions were obtained and used to make films or to serve as a primary substrate for probiotic cultures. These authors report that only extensively diafiltrated

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SCW powder, with more than 50% protein on a dry weight basis, proved to form films in the presence of glycerol.

In the present work, SCW was immediately concentrated by UF (10 kDa), and the UF concentrate was diafiltrated to reduce lactose and to increase the ratio of nitrogen components on the SCW powder. Hence, a powder with almost 55% (w/w) crude protein was obtained. Our preliminary essays aiming at evaluating the potential of sheep's SCW diafiltrated powder as a potential material to produce edible films and coatings indicated that the SCW powder showed poor film forming properties. The best film forming properties were obtained by mixing SCW powder with WPI in the proportions 2:1 or 1:1. The amount of fat in our SCW and the strong thermal treatment used for the production of whey cheeses, as is the case of Requeijão, might have affected the film forming properties of the sheep's SCW powder. Therefore, the decision regarding the proportions of both types of whey proteins was based on the maximization of the utilization of SCW powder (i.e., 2:1 SCW:WPI).

Regarding the physicochemical characteristics of the cheese samples, it is worth noting the beneficial effect that oregano and clary sage EOs added to SCW:WPI coatings with respect to the retention of moisture. Water loss reduction in cheeses with coatings containing oregano essential oil has also been reported by Artiga-Artigas et al. (Artiga-Artigas et al. 2017). This is a consequence of the vapor permeability reduction due to the decrease in films' hydrophilic portion due to the incorporation of essential oil, as reported by Lee and coworkers (Lee et al. 2015). The addition of a hydrophobic agent to hydrophilic polymer matrices decreases their affinity to the water and enhances their ability to act as a barrier to water vapor permeation (Christaki et al. 2021). This fact had a particular influence on the texture properties of samples WCO and WCS, with the reduction of hardness, chewiness, and gumminess, which can be considered positive regarding the desired sensory characteristics of the cheese type tested. Cheeses with whey protein coatings (with or without essential oils) exhibited lower hardness values than control batches at 28 days of storage. Decreases in this parameter have also been observed in cheeses coated with hydrolyzed WPC solutions with added marjoram essential oil (Tarhan and Şen 2022).

Despite the significant differences regarding the microbiological counts of the different cheese samples obtained, it can be considered that the antimicrobial agents used in the coatings (natamycin, oregano essential oil, and clary sage essential oil) did not affect the bacteria of the starter culture used. In all cases, the lactobacilli and lactococci counts surpassed log 8.0 CFU/g. Exceptions were the lactobacilli counts of theNand WCS samples at the day after production (log 7.6 and 7.8 CFU/g respectively). EOs may negatively affect the growth of lactic acid bacteria of cheese starter cultures, but they are relatively resistant to their antimicrobial compounds. It has also been reported that they are not affected (Christaki et al. 2021). In the present study, lactobacilli counts were not significantly affected by EO addition to SCW, while lactococci counts increased in SCW-coated cheeses.

In all cases, the counts of yeasts and molds were lower than log 4.5 CFU/g until the 7th day, thereby increasing steadily to ca. log 6 CFU/g at the end of ripening. The lowest value (5.6 log CFU/g) at the end of ripening was observed for the sample containing natamycin. Hence, it cannot be concluded that either the commercial coating or the coatings based on SCW with EOs were more efficient in reducing yeast and mold counts when compared to the negative control (C).

The antimicrobial activity of the coatings with essential oils depends on the EO concentration and also on the active compounds; in oregano EO is mainly carvacrol, and in sage EO are camphor, α-thujone, and 1,8-cineole (Khedher et al. 2017; Tarhan and Şen 2022). However, the efficacy of essential oils depends on various factors, including the microorganism strains, the EO composition and concentration, the environmental conditions, and the chemical composition of the food to which they are added. If the EO is incorporated into a film or coating, the composition of the latter may also modify its effects. In dairy products, proteins could interact with phenolic compounds and limit the EO activity against microorganisms (Christaki et al. 2021). It has been reported in sodium alginate-based coatings (Artiga-Artigas et al. 2017) that concentrations of oregano EO lower than 2% w/w seem not to be enough to produce the inhibition of pathogenic and spoilage microorganisms (e.g., molds and yeast) in cheese.

Several authors report on the positive effect of Eos as antimicrobial agents in whey films as such or applied as coatings in cheese. Fahrullah and coworkers (Fahrullah et al. 2021) report that the addition of clove essential oil at different levels inhibited the growth of E. coli, Salmonella sp., S. aureus, and molds in whey-based films. However, high incorporation levels were used (5–15%), and this fact may influence the sensory properties of the food products in which the films are applied.

Çakmak and coworkers (Çakmak et al. 2020) tested lemon and bergamot essential oils as antimicrobials in whey-based films. Although bergamot oil had a strong antimicrobial activity against E. *coli* and S. *aureus*, lemon oil was a poorer antimicrobial against S. aureus. Both Eos also showed efficacy against *Aspergillus niger*. These authors also refer that water vapour transmission rates and water vapor permeability of edible films are important factors to be considered, and it is expected that both the plasticizer and the EO composition affect those parameters. Their results demonstrated that 3.5% EO in the film composition originated a better barrier property against water vapor. This was also observed in our work, even with lower levels of EOs addition.

Seydim and coworkers (Seydim et al. 2020) tested WPI films containing 2% (w/v) oregano and garlic EOs, nisin, and natamycin. E. *coli* O157:H7 inoculated in Kasar cheese containing oregano EO showed significant decreases on the first day and at the end of storage in comparison to cheeses without WPI film. Similar results were obtained with nisin. No significant *Penicillium* spp. reduction was observed on the cheese samples with antimicrobial WPI film on the first day. The cheese samples with natamycin presented the highest microbial reduction. At day 15, the cheese samples with oregano EO showed a 1.20 log reduction of *Penicillium* spp. compared to the control groups without WPI film. Similarly, the cheese with natamycin showed a 1.78 log reduction in comparison to the control. The WPI films incorporated with oregano EO exhibited higher antimicrobial activity against E. *coli* O157:H7, while the higher bactericidal effect on L. monocytogenes was observed in WPI films containing nisin. Cheese samples containing garlic EO showed higher microbial content when compared with cheeses containing oregano EO. Cheeses containing natamycin presented lower levels of *Penicillium* spp. In the present study, natamycin also showed the best results regarding the inhibition of molds.

Seydim and Sarikus (Seydim and Sarikus 2006) refer that the direct addition of EOs to foods decreases bacterial populations but may change the sensory characteristics of the products. Oregano and garlic Eos exhibited greater inhibitory zones on S. *aureus*, S. *enteritidis*, L. *monocytogenes*, E. *coli*, and L. *plantarum* than rosemary EO-incorporated films. Ture et al. (Ture et al. 2011) investigated the antifungal effect of films containing

natamycin on Kasar cheeses inoculated with *Aspergillus niger* and P. roqueforti during 30 days of storage. The authors found that protein-based films were more effective than carbohydrate-based films. An oregano EO antifungal effect was also observed on chitosan-based coatings on cheeses (Cano Embuena et al. 2017).

Bahram and coworkers (Bahram et al. 2014) report that films containing cinnamon (CEO) (0.8 and 1.5% v/v) exhibited remarkable antibacterial activity against both Gram-positive and Gram negative strains, as well as a good inhibitory effect on the fungi tested. However, no inhibition zone was observed in the film disk at a concentration of 0.8% CEO. With 1.5%, a clear inhibition zone was observed.

Ramos and coworkers observed that the addition of natamycin to edible films manufactured with whey protein isolate and glycerol had an important effect against yeasts but had no activity against bacteria (Ramos et al. 2012d). Fajardo and coworkers observed that the addition of natamycin to coatings with chitosan caused a reduction in molds and yeasts in cheese samples after 27 days of storage (Fajardo et al. 2010). Yangilar (Yangılar 2015) reported that the application of films only with chitosan and whey proteins to cheeses reduced the mold counts compared to the cheeses without these films.

As a final remark, taking in consideration that, in Portugal, most of the SCW produced results from ovine whey cheeses, which are curdled with vegetable rennet (*Cynara cardunculus*), and since this curdling agent has an intense proteolytic activity that can generate peptides with biological impact, namely antihypertensive and antimicrobial activities, the use of ovine SCW-based edible coatings resulting from such cheeses can ultimately reveal such biological functions. In addition, the previous fermentation of SCW by lactic acid or probiotic bacteria can further enhance the potential for the recovery and valorization of such a by-product. Therefore, it is important to develop research activities aiming at highlighting the potential of SCW as a potential ingredient of functional food products.

5. Conclusions

Second cheese whey, the by-product of whey cheese production, due to its composition, which includes valuable proteins and other nutrients, is an object of food technologists' interest. The study demonstrated that SCW resulting from ovine whey cheese production may be used to prepare edible cheese coatings, which additionally were fortified with oregano and clary sage essential oils. The coatings were used to protect the surface of a bovine milk rennet curdled cheese model. The cheese with SCW coatings without and with EOs were characterized by lower titratable acidity in comparison to the control samples. The results also indicate that WCS and WCO coatings prevent the cheeses from excessive evaporation, which results in higher moisture. Moreover, the samples containing SCW coatings had lower values of hardness, chewiness, and gumminess at the end of ripening. The differences in colour between samples with and without coatings were not evident. Coatings based on SCW did not affect the viability of the starter bacteria cultures. However, tested films were not more efficient in reducing yeast and mould counts comparing to the control cheese without coating and to cheese with a commercial coating containing natamycin. To conclude, SCW:WPI coatings without and with oregano and clary sage EO may successfully substitute commercial coatings.

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Manuscript V - Edible Films based on Ovine Second Cheese Whey with Oregano Essential Oil

The results from this chapter have already been published as:

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Conceptualization, A.P., A.C., C.P. and O.D.; methodology, A.P. and O.D.; validation, A.P., A.C. and O.D.; formal analysis, A.C. and O.D.; investigation, A.P. and O.D.; resources, A.C., C.P. and O.D.; writing—original draft preparation, A.P., A.C. and O.D.; writing—review and editing, A.P., A.C., C.P. and O.D.; visualization, A.C. and O.D.; supervision, A.C., C.P. and O.D.; project administration, A.C. and O.D.; funding acquisition, A.C. and O.D. All authors have read and agreed to the published version of the manuscript.

Abstract:

The aim of this study was to produce edible films using ovine second cheese whey (SCW) powder, alone or combined with whey protein isolate (WPI). SCW is a byproduct obtained in the manufacture of ovine whey cheeses. In this instance, it was dehydrated after increasing the protein concentration by ultrafiltration/diafiltration. Furthermore, the effects of the addition of oregano (Origanum compactum) essential oil (EO) in two proportions to the films produced with a mixture of SCW powder and WPI were studied. The water vapour permeability, solubility, color, opacity, antioxidant activity, and the mechanical properties of the films were determined. In addition, we determined the films' structure, by FTIR; thermal stability, by TGA; and microstructure and crystallinity, by XRD. SCW combined with WPI can be used to prepare edible films, but their properties were found to be affected depending on the proportion of each product. The substitution of WPI by SCW caused decreases in water solubility (from 81.44 to 66.49% D.M.), modified the color and decreased tensile strength (from 1.57 to 0.17 MPa), and decreased the elongation at break (from 52.17 to 3.57%), the puncture strength (from 2.40 to 0.20 MPa) and the deformation (from 18.92 to 0.93%) of the films. EO addition to the SCW–WPI films increased the antioxidant activity of the films (from 0.97 to 2.19 mg DPPH/g). It also modified other characteristics of the films such as the water solubility and the tensile strength. Both SCW and EO incorporations influenced the secondary structure of proteins and the thermal stability, microstructure and crystallinity of the films.

Keywords: edible packaging; sheep cheese whey; essential oils; antioxidant; mechanical properties, FTIR, TGA, microstructure, XRD

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1. Introduction:

The preservation of food products, extending their shelf life, and the sustainability of companies are some of the most important challenges that industries face. Food that is not stored safely and hygienically can cause serious negative health effects. The most used packaging is plastic, followed by glass, metal and paper (Nilsson et al. 2024). Due to its durability, design flexibility, lightness and low price, plastic packaging has become advantageous over other raw materials for packaging food products(Pires et al. 2024b).

Plastic films derived from petroleum are widely utilized due to their ease of production, cost-effectiveness, and satisfactory mechanical properties. These films aid in preserving the quality of food products by restricting the permeation of water vapor, oxygen, carbon dioxide, and aromatic compounds. However, conventional packaging materials are associated with challenges related to recycling, inability to degrade after disposal, detrimental environmental and wildlife impacts, and utilization of finite fossil fuel reserves. Additionally, the presence of microplastics and the potential migration of harmful additives from plastic packaging into food pose significant health risks and concerns (Pires et al. 2024b). Regulation 2025/40 of the European Union restricts the utilization of certain single-use plastics and minimizes the weight and volume of packaging, the waste produced and the inclusion of some additives in the materials (The European Parliament and the Council of the European Union. Regulation (EU) 2025/40 of 19 December 2024 on Packaging and Packaging Waste 2025).

The food sector is one of the biggest contributors to global greenhouse gas emissions (approximately 26%) and, consequently, food waste contributes to the negative impact of climate change, since much of the food produced is not consumed. Therefore, to minimize these negative impacts, new packaging must be able to guarantee food quality and safety, as well as increase the shelf life of food and be edible or easily recyclable (Nilsson et al. 2024). These reasons are why we continue to develop sustainable and fully biodegradable packaging materials, including edible films and coatings, providing a long shelf life for food and thus promoting the safe integration of packaging (Nilsson et al. 2024). Thus, packaging materials obtained from biopolymers such as proteins, polysaccharides and lipids are a good alternative to petroleum-derived materials (synthetic materials) since they are obtained from renewable sources or biodegradable industrial by-products and do not adversely affect the environment (Gaspar and Braga 2023; Pires et al. 2024b).

Protein-based films are a sustainable and efficient alternative for food packaging (Chen et al. 2019b). These films are produced from proteins extracted from various sources, such as cheese whey, soy, wheat and corn (Kandasamy et al. 2021; Soliman and Furuta 2009; Xu and Li 2023). They have advantages in terms of biodegradability, are edible, and have good barrier properties that help preserve food against moisture, oxygen and light (Pires et al. 2024b).

Whey protein-based edible films have become an interesting innovation in the food industry as a food packaging material, due to their sustainability and nutritional

characteristics. The whey is a by-product of cheese production, and the protein is concentrated mainly by membrane processes that obtain whey protein concentrate (WPC) and whey protein isolate (WPI). These products contain valuable essential amino acids and are useful to produce edible films and coatings(Cruz-Diaz et al. 2019; Etxabide et al. 2023; Galus and Kadzińska 2016; Pires et al. 2024b; Pires et al. 2021b). These edible films can reduce food waste and act as barriers against oxygen and aromatic compounds with good mechanical properties (Pires et al. 2021b). It has been also indicated that WPI films in substitution of fossil polymers could be useful to reduce environmental contamination due to the reduction of electricity consumption (Etxabide et al. 2023). These studies have been done with protein films obtained from bovine whey. We have not found works related to the application of ovine whey in edible films.

The second cheese whey (SCW) is a by-product generated in the whey cheese production. Whey cheese is obtained by heating whey at 85–90 °C for 20–30 min to facilitate the denaturation of whey proteins, which results in their aggregation; SCW is the liquid remaining after protein aggregates recovery (Pires et al. 2021b). Although a high proportion of this product is discarded without treatment, it contains proteins, mainly in a denatured state, that are beneficial to human health, can be used in the manufacture of foods such as fermented drinks or dressings, and can also be useful in the production of edible films and coatings with excellent nutritional and functional properties (Pires et al. 2021b). However, the use of SCW in the production of edible coatings and films has been mostly ignored, and its potential as a sustainable packaging material has remained unstudied.

To date, only one study has been found referring to the use of SCW as a material for the production of edible films (Alfano et al. 2022). Alfano et al. investigated the use of buffalo second cheese whey (SCW) to obtain films. They treated buffalo SCW with microfiltration and ultrafiltration and obtained two retentates of 100 kDa and 10 kDa. They obtained removable and reusable film with a thickness of 100 μm only with the retentate of 10 KDa using glycerol as plasticizer. The characteristics of the films were not described in this study (Alfano et al. 2022). There are no studies of films obtained with ovine SCW.

In one article of our research group, Pires et al. (Pires et al. 2024a) used sheep SCW in a mixture with WPI with 8% (w/v) protein (in the proportion 2:1 SCW/WPI) and with glycerol as plasticizer in order to prepare cheese coatings with or without oregano or clary sage essential oils. To obtain SCW powder, ultrafiltration (10 kDa cutoff), diafiltration, reverse osmosis and lyophilization processes were used. Cheeses with SCW/WPI coatings had higher water activity, higher moisture content (due to prevent high evaporation), lower titratable acidity and lower chewiness, hardness and gumminess at the end of ripening than cheeses without coating or with commercial coating with natamycin. Additionally, it was concluded that coatings made with SCW and WPI can successfully replace commercial coatings(Pires et al. 2024a).

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The addition of essential oils, such as oregano essential oil, to films manufactured with WPC or WPI has been investigated due to their antimicrobial and antioxidant effects

(Oliveira et al. 2017; Sajimon et al. 2023; Seydim and Sarikus 2006; Seydim et al. 2020). However, this incorporation has some effects in some properties as water permeability and flexibility of the films (Çakmak et al. 2020; Oliveira et al. 2017; Sajimon et al. 2023).

The development of edible films with a mixture of SCW and WPI and the addition of essential oils could also be interesting for food packaging. However, their properties are different to those of the coatings and must be studied before application in the food industry because the proportion of SCW, WPI and essential oils can influence their efficiency.

One objective of this work was to develop edible films based on ovine SCW powders, alone or combined with different proportions of bovine WPI, and to compare the water vapor permeability, solubility, color, opacity, antioxidant activity, mechanical properties, structure (by FTIR), thermal stability (by TGA), microstructure and crystallinity (by XRD) of the films. The second aim was to study these properties in films obtained with ovine SCW and WPI incorporating oregano (Origanum compactum) essential oil (EO) in two different percentages.

2. Material and Methods

2.1. Materials

Bovine whey protein isolate (NatWPI90, 90% w/w protein, 1.0% fat, 2.5% ashes) was supplied by InLeit Ingredients S.L.U. (Curtis, Spain). Ovine second cheese whey powder was obtained after ultrafiltration/diafiltration, pasteurization, reverse osmosis and freeze drying of SCW, as indicated by Pires et al. (Pires et al. 2024a). The composition of the powder was: 54.7% w/w protein, 14.3% fat, 7.9% ashes.

2.2. Films preparation

Firstly, five different films were obtained with five different concentrations of WPI and SCW powders. WPI and SCW were weighed to obtain a final protein concentration of 8% (w/v) in the film-forming solution. The films were prepared in the following manner: The WPI was dissolved in distilled water using a magnetic stirrer at 20 °C for 30 min and glycerol (Panreac, Barcelona, Spain) was incorporated at a protein-to-plasticizer ratio of 1:1. The pH was then adjusted to 7.0 using 0.1 M NaOH. Thereafter, the WPI–plasticizer solution was heated to 90 °C for 15 min with constant stirring. This process was conducted in an electronic magnetic stirred hot plate (VELP Scientifica, Usmate, Italy) connected to a digital temperature controller (VTF Digital Thermoregulator, VELP Scientifica, Usmate, Italy) to ensure precise temperature regulation. Then, the solutions were cooled to room temperature and degassed under vacuum for 15 min. The films composed exclusively of SCW were prepared in a similar manner except that the heating step was omitted. In the films containing WPI + SCW (in three proportions of WPI:SCW, 2:1, 1:1, and 1:2), the SCW powder was incorporated into the WPI–plasticizer solution after the cooling step. The mixture was then homogenized using an Ultra-Turrax (model T18 basic; IKA™, Staufen,

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Germany) for 3 min operating at 15,500 rpm. Finally, the pH of the solution was readjusted to a value of 7 using 0.1 M NaOH. Subsequently, degassing was conducted under vacuum for 15 min. Thereafter, all film-forming solutions (0.22 g cm^{-2}) were dispensed into Plexiglas Petri dishes (14 cm diameter) and were subjected to drying at 50 °C for 20 h within an air force cabinet. Before being peeled off, the dried films were transferred to a climate chamber (Ensayos Terlab S.L., Barcelona, Spain) and maintained at 45% relative humidity and 25 °C for 48 h. The peeled films were finally stored in a desiccator at 45% relative humidity and room temperature for additional testing. The experiments were conducted in triplicate.

Secondly, six different films were prepared with the addition of oregano (Origanum compactum) essential oil (Plena Natura, Amadora, Portugal). The films were prepared in the following way: the preparations of WPI:plasticizer solution after heat treatment and cooling were separated into six equal portions to prepare the films containing the three proportions of WPI-SCW (2:1, 1:1 and 1:2) and two different proportions of oregano essential oil (EO) (1 and 2% of the powder weight in solution). The EO and the SCW powder were added simultaneously to the previously prepared WPI/plasticizer solution and the mixtures were homogenized using an Ultra-Turrax homogenizer. The final pH was adjusted to 7 with 0.1 M NaOH, and the films were formed as indicated before. The experiments were performed in triplicate.

Summarizing, eleven different films were obtained.

1. WPI: films only with whey protein isolate.
2. WS 2:1: films of WPI-SCW mixture in a protein proportion of 2:1.
3. WS 1:1: films of WPI-SCW mixture in a protein proportion of 1:1.
4. WS 1:2: films of WPI-SCW mixture in a protein proportion 1:2.
5. SCW: films only with ovine second cheese whey.
6. WS 2:1O1: films of WPI-SCW mixture in protein proportion 2:1 with 1% EO.
7. WS 1:1O1: films of WPI-SCW mixture in protein proportion 1:1 with 1% EO.
8. WS 1:2O1: films of WPI-SCW mixture in protein proportion 1:2 with 1% EO.
9. WS 2:1O2: films of WPI-SCW mixture in protein proportion 2:1 with 2% EO.
10. WS 1:1O2: films of WPI-SCW mixture in protein proportion 1:1 with 2% EO.
11. WS 1:2O2: films of WPI-SCW mixture in protein proportion 1:2 with 2% EO.

2.3. Film thickness

Film thickness was measured as per the method described by Cobos and Díaz (Cobos and Díaz 2023) using a 0-25 mm electronic digital micrometer with 0.001 mm accuracy (Selecta, Barcelona, Spain) at 20 random points of three films.

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2.4. Water vapor permeability

Water vapor permeability (WVP) was determined according to the ASTM E-96-93 method (ASTM. E 96-93—Standard test method for water vapor transmission of materials. In Annual Book of ASTM Standard; American Society for Testing and Materials: Philadelphia 1993) with some modifications (Cobos and Díaz 2023). Measurements were conducted in triplicate. WVP was calculated using Equation (1):

$$WVP\ (ng\ s^{-1}\ m^{-1}\ Pa^{-1}) = (w \times L)/(A \times \Delta t \times \Delta P) \quad (1)$$

where w is the weight increase of the cell (ng) for time of permeation Δt (s); L is the thickness of the film (m); A is the permeation area (m^2), and ΔP is the difference in water vapor pressure (Pa) through the film.

2.5. Dry matter content, water solubility and density

The determinations of dry matter content and water solubility were conducted in accordance with the methods outlined by Yildiz et al. (Yildiz et al. 2022). Film samples measuring 2 × 2 cm^2 were initially weighed (*W1*). These were dried at 105 °C until a constant weight was achieved (*W2*). The dried samples were then dipped in 25 mL water at 25 °C for 24 h and subsequently dried again at 105 °C for 24 h. Finally, the samples were weighed (*W3*) once again. The dry matter and water solubility were calculated using Equations (2) and (3), respectively.

$$\text{Dry matter}\ (\%) = 100 - \left[\frac{W_1 - W_2}{W_1} \times 100\right] \quad (2)$$

$$\text{Solubility}\ (\%) = \frac{W_2 - W_3}{W_2} \times 100 \quad (3)$$

In accordance with the method outlined by Sun et al. (Sun et al. 2017) the density of the films was ascertained by dividing the weight of the films by their volume. This volume was determined by multiplying the area of the film by its thickness. All measurements were carried out in triplicate.

2.6. Color and opacity

Color and opacity of films were evaluated in the CIE L*a*b* color space according to Díaz et al (Díaz et al. 2016), The determinations were undertaken at three random points of three films in a spectrophotometer X-Rite (mod. SP60; Grand Rapids, MI, USA).
The difference in color (*ΔE**) of films regarding the WPI film's color values was calculated as follows:

$$\Delta E^* = \sqrt{(L^* - L_0)^2 + (a^* - a_0)^2 + (b^* - b_0)^2} \quad (4)$$

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where L^*, a^* and b^* are the color values for the film added of nano clay and L_0, a_0 and b_0 are the color values for the control film.
Whiteness (*WI*) index of films was calculated according to Saberi et al. (Xu and Li 2023) using the equations:

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

where L, a and b are the color parameter values of the sample.
The calculation of opacity values was conducted in accordance with Márquez-Reyes et al. (Márquez-Reyes et al. 2022) as:

$$\text{Opacity (\%)} = \frac{Y_B}{Y_W} \times 100 \quad (6)$$

where Y_B is the opacity of the film against a black background and Y_W is the opacity of the film against a white background.

2.7. Mechanical properties

The mechanical properties of films were determined using a texturometer model EZ Test and the Trapezium2 (version 1.03SP) data processing system software (Shimadzu Corporation, Tokyo, Japan), according to the method reported by Diaz et al. (Díaz et al. 2016) based on the ASTM D882 method (ASTM. D882—Standard test method for tensile properties of thin plastic sheeting. In Annual Book of ASTM Standard; American Society for Testing and Materials: Philadelphia 2000). The samples of the films were cut and stored in a desiccator maintained at 20 °C with relative humidity of 45% for 48 h prior to testing.

2.8. Antioxidant activity

In order to ascertain DPPH radical scavenging capacity, the procedure outlined by Vargas et al. (Vargas et al. 2017) was followed. Film specimens (1 cm^2) were placed into tubes containing 4.0 mL of a 0.06 mM methanolic DPPH (2,2-diphenyl-1-picrylhydrazyl; Sigma-Aldrich, St. Louis, MO, USA) solution. The tubes were then maintained in conditions of darkness at a temperature of 25 °C for a period of two hours and thirty minutes. Following this, the film samples were separated, and the absorbances of the solutions were measured at 517 nm using a spectrophotometer (model 6850 UV/Vis; Jenway, Bibby Scientific, Stone, UK). Methanol was utilized as a blank. Calibration curve was prepared with DPPH (Kocakulak et al. 2019), using concentrations of DPPH ranging from 0.006 mM to 0.06 mM in methanol. The determinations were carried out in duplicate. The antioxidant activity was expressed in terms of milligrams of DPPH degraded per gram of film weight and was calculated using Equation (7).

Antioxidant activity (mg DPPH/g dry film) = *C* x dilution rate / dry film weight in g (7)

where C is the value obtained from the calibration curve as mg of DPPH per mL of the difference between the absorbance of the DPPH reagent and the sample.

2.9. Fourier transform infrared spectroscopy (FTIR)

The attenuated total reflection (ATR)–Fourier transform infrared (FTIR) spectra of the films were obtained by means of a Jasco FT/IR-4600 instrument equipped with the ATR Pro One accessory (manufactured by Jasco Corporation, Tokyo, Japan). The experimental determinations were conducted in triplicate. The spectra were measured between 400 and 4000 cm^{-1} by performing 16 scans at 1 cm^{-1} resolution. PeakFit software version 4.12 (SYSTAT Software, Richmond, CA, USA) was used to process the resulting data. For the identification and intensity determination of individual band positions, the second derivative spectra were calculated on data previously baseline corrected and smoothed with a Savitsky–Golay function to remove possible noise.

2.10. Thermogravimetric analysis (TGA)

Thermogravimetric analysis of the films was carried out in accordance with the method outlined by Díaz et al. (Díaz et al. 2019). Film samples (4.6–6.0 mg) were heated from 30 to 600 °C in an aluminum pan at a rate of 10 °C/min under a nitrogen atmosphere (30 mL/min). The measurements were carried out in duplicate. This analysis was performed using a thermogravimetric analyzer equipped with differential scanning calorimetry functionality (model TGA/DSC1; Mettler Toledo, Urdorf, Switzerland). The calculation of the first derivative of the TGA curve (DTG) was conducted by using PeakFit software (version 4.12; SYSTAT Software, Richmond, CA, USA).

2.11. Microstructure analysis by scanning electron microscopy (SEM)

An attenuated A JEOL-JSM 6360LV scanning electron microscope (Jeol, Ltd., Tokyo, Japan) operating at 15 kV was used to observe the microstructure of the films. The sample preparation was in accordance with the description of Díaz et al. (Díaz et al. 2016).

2.12. X-ray diffraction (XRD)

The crystallinity of films was studied using an X-ray diffractometer (Bruker D8 Advance with a LYNXEYE XE-T detector, Bruker, Karlsruhe, Germany) with a CuKα1 radiation beam (λ = 1.5406 Å) in the angular range of 3 to 50 (2θ) at a voltage of 40 kV and current of 40 mA.

2.13. Statistical analysis

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The evaluation of data was conducted utilizing IBM SPSS Statistics for Windows version 28.0.1.0, developed by IBM Corporation (NY, USA). Before statistical analysis, the data

were checked for outliers, and normal distribution was tested using the Kolmogorov–Smirnov test. One-way analysis of variance (ANOVA) and Tukey's post hoc test were utilized for the purpose of testing and comparing, respectively, the statistical significance of differences among means. The statistical significance of differences in means was established with a significance level of $p < 0.05$. A two-way ANOVA was used to analyze the effects of the substitution of WPI by SCW and of the addition of oregano essential oil and their interaction on all of the determined parameters (only in samples with the WPI–SCW mixture: 2, 3, 4, 6, 7, 8, 9, 10, 11).

3. Results and Discussion

3.1. Visual appearance

Figure 1 shows the visual appearance of the films. No important differences were observed among the films. Sajimon et al. (Sajimon et al. 2023) reported that films with high levels of oregano essential oil had a reddish tint. However, this was only observed in samples with 4% of essential oil. The addition of 2% of EO did not cause an unpleasant appearance of the films.

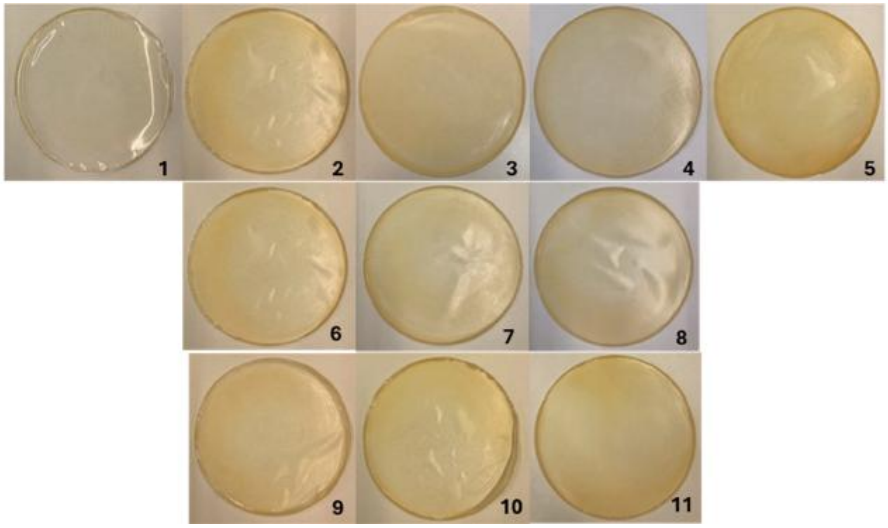


Figure 1. Visual appearance of the films described in material and methods section. 1) WPI; 2) WS 2:1; 3) WSC1:1; 4) WS1:2; 5) SCW; 6) WS2:1O1; 7) WS1:1O1; 8) WS1:2O1; 9) WS2:1O2; 10) WS1:1O2; 11) WS1:2O2.

3.2. Water vapor permeability, thickness, dry matter content, solubility and density

Water vapor permeability (WVP), thickness, dry matter content, solubility and density values of films are shown in Table 1. Two-way ANOVA of these characteristics in films of WPI–SCW mixture are shown in Table 2. No significant effects of the substitution of WPI by SCW and the addition of EO were found in the water vapour permeability. However, the films produced with the addition of 2% of EO showed lower values of WVP than the corresponding films with the incorporation of 1% of EO and these samples showed lower values than films without EO. It has been reported that the addition of essential oils reduces the vapor permeability of the films due to that, because of the way

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in which the incorporation of a hydrophobic agent, such as an essential oil, decreases the hydrophilic portion of the films and their affinity to the water and enhances their ability to act as a barrier to water vapor permeation(Çakmak et al. 2020; Christaki et al. 2021; Lee et al. 2015). A different result was observed by Oliveira et al. (Oliveira et al. 2017) one which indicated that the addition of oregano essential oil increased the WVP due to the oil not binding to the proteins, leaving empty spaces through which water can permeate. Thus, the effects of the addition of oregano EO in the water permeability of the films are not clear.

Table 1. Water vapor permeability (WVP), thickness, dry matter (D.M.), solubility and density (mean ± SD) of films described in material and methods section.

Film	WVP ($ng\ s^{-1}\ m^{-1}\ Pa^{-1}$)	Thickness µm	Dry matter (g/100 g)	Solubility (% D.M.)	Density (g/cm^3)
WPI	0.156 ± 0.04	150.67 ± 58.66	57.77 ± 0.9 a	81.44 ± 0.94 d	1.43 ± 0.15 a
WS 2:1	0.135 ± 0.05	166.33 ± 40.27	63.34 ± 1.7 ab	79.04± 3.22 cd	1.73 ± 0.27 ab
WS 1:1	0.089 ± 0.01	131.83 ± 33.45	65.59 ± 2.04 b	78.86 ± 2.85 cd	2.07 ± 0.06 ab
WS 1:2	0.104 ± 0.03	172.33 ± 40.55	66.69 ± 2.24 b	77.68 ± 2.44 bcd	1.49 ± 0.23 a
SCW	0.141±0.03	160.17 ± 16.37	66.79±1.20 b	66.49±3.64 a	1.98 ± 0.16 ab
WS 2:1O1	0.137±0.05	124.17 ± 11.51	67.93±0.55 b	71.54±0.99 ab	3.27 ± 0.06 b
WS 1:1O1	0.141±0.009	173.83 ± 5.30	66.34±0.53 b	74.59±0.27 abcd	1.76 ± 0.22 ab
WS 1:2O1	0.147±0.03	184.00 ± 38.94	63.86±1.00 b	74.05±1.95 abcd	1.69 ± 0.58 ab
WS 2:1O2	0.120±0.03	139.67 ± 25.15	63.33±1.68 ab	80.16±4.43 cd	2.27 ± 0.73 ab
WS 1:1O2	0.102±0.003	123.00 ± 17.32	66.56±2.97 b	74.12±6.64 abcd	2.53 ± 1.11 ab
WS 1:2O2	0.117±0.007	142.33 ± 6.25	64.83±4.25 b	68.48±2.98 ab	2.11 ± 0.96 ab

Means in the same column with different letters (a-d) are significantly different ($p < 0.05$).

Table 2. Two-way ANOVA of water vapor permeability (WVP), thickness, dry matter, solubility and density of WS films.

		Two-way ANOVA	
		F	***p value***
WVP	Proportion WPI/SCW	0.98	0.393
	Addition of OE	3.40	0.056
	Interaction	0.69	0.609
Thickness	WPI/SCW	2.05	0.158
	OE	2.21	0.139
	Interaction	2.07	0.127
Dry matter	WPI/SCW	0.88	0.432
	OE	0.65	0.534
	Interaction	2.60	0.071
Solubility	WPI/SCW	2.57	0.104
	OE	6.01	0.010
	Interaction	3.65	0.024
Density	WPI/SCW	2.78	0.089
	OE	2.18	0.141
	Interaction	2.54	0.076

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No significant effects of the substitution of WPI by SCW and the addition of EO were found in thickness of the films. Other researchers also indicated that the addition of essential oils did not modify the thickness of the films (Pedro et al. 2023). However, Sajimon et al (Sajimon et al. 2023) have observed that the incorporation of oregano essential oil causes an increase in film thickness due to the way in which that addition causes disruption to the structure. This different result could be due to the different homogenizers used. No remarkable effects of the substitution of WPI by SCW or the addition of EO were found in density of the films. Despite the compositional differences between SCW and WPI (higher lipid and ash contents in SCW), the effective homogenization of the film-forming solutions likely maintained structural uniformity, preventing significant changes in the mass-to-volume ratio. In relation to the dry matter content, it is remarkable that the films made only with WPI showed lower values of dry matter than the films with a mixture WPI–SCW or with only SCW. The dry matter content of the films was not influenced by the incorporation of OE, though other researchers have indicated that essential oil addition can increase the dry matter content of the films (Mahdavi et al. 2018; Sajimon et al. 2023).

Significant effects of the addition of EO and significant interactions between this incorporation and the substitution of WPI by SCW in the water solubility of the films were observed. In the films without EO, the addition of SCW caused a decrease in the water solubility of the films, with the films with only SCW showing significantly lower values than the other films. This result could be due to the way in which SCW has higher levels of fat than WPI and the way in which the hydrophobic properties of lipids can decrease the water solubility of the films. The water solubility of proteins is also related to the protein structure and the bonds among proteins (Oliveira et al. 2017). The denatured state of the SCW proteins probably increased the amount of intermolecular disulfide bonds, which made the SCW films more resistant to solubilization in water. The substitution of WPI by SCW also significantly decreased the solubility of the films in the films with 2% of EO but this was not observed in films with 1% of EO. This different effect of the substitution of WPI by SCW in films with different concentration of EO should be investigated in future experiments. The films with the addition of oregano EO showed lower solubility than the films without EO, except in the film WS 2:1O2. The decrease of water solubility by the addition of oregano essential oil has also been observed in other works, and attributed to the hydrophobic properties of the lipids (Oliveira et al. 2017; Sajimon et al. 2023).

3.3. Color

The CIEL*a*b* color values, total color difference (ΔE), opacity and whiteness index are shown in Table 3. Two-way ANOVA of the color of the films with the WPI–SCW mixture are shown in Table 4. The substitution of WPI by SCW significantly influenced the color parameters. The addition of SCW to the films decreased the value of L* but only in samples without essential oil. The substitution of WPI by SCW increased the values of a* and b* in the films with or without EO. Total color difference (ΔE) presented significant changes with the addition of SCW, with values of ΔE that are higher than 2.

This indicates that inexperienced observers can detect differences in the films with different proportions of WPI and SCW (Mokrzycki and Tatol 2011). The color parameters of the films (*L*, a*, b**) and their opacity were not influenced by the addition of EO. However, Sajimon et al. (Sajimon et al. 2023) observed that the incorporation of oregano essential oil decreased the lightness, whereas the influence in *a** and *b** are contradictory because it was found to depend on the amount of EO. The decrease of lightness was attributed to the fact that the oregano essential oil was included in small globules in the structure of the films. The different homogenizers in the preparation of the mixtures and the different brands used could influence both the distribution of EO and the color of the films.

Table 3. CIE *L*a*b** color parameters, total color difference (*ΔE*), opacity and whiteness index (mean ± SD) of films described in material and methods section.

Film	L*	a*	b*	ΔE	Opacity	Whiteness
WPI	92.96 ± 0.49 c	-3.04 ± 0.09 ab	3.15 ± 2.04 a	0.00± 0.00 a	14.91± 0.25 a	91.59 ± 1.19 d
WS 2:1	90.08± 1.35 b	-3.11 ± 0.10 a	7.04 ± 1.59 ab	4.92 ± 2.18 ab	18.55 ±0.22 b	87.43 ± 1.92 cd
WS 1:1	88.16± 1.25 ab	-2.89± 0.13 abc	9.86± 2.16 bcd	8.33 ± 4.13 bc	22.00 ± 1.18 de	84.30 ± 2.26 bc
WS 1:2	88.51 ± 0.97 ab	-2.46 ± 0.32 bc	13.00 ±2.27 cd	10.83 ± 2.93 bcd	26.82 ± 0.59 g	82.46 ± 2.22 b
SCW	86.16 ± 1.32 a	-1.10± 0.52 d	18.11 ±2.21 e	16.58 ± 2.22 d	23.81 ± 1.79 ef	77.17 ± 2.48 a
WS 2:1O1	88.60 ±1.00 ab	-3.06 ± 0.09 ab	8.86 ±1.45 bc	7.21 ± 1.21 bc	19.55 ±0.66 bcd	85.17 ± 0.40 bc
WS 1:1O1	88.52±0.62 ab	-2.92±0.17 ab	11.60±1.33 bcd	9.54 ± 2.37 bc	21.28±0.65 cde	83.41 ± 1.24 bc
WS 1:2O1	88.09±0.60 ab	-2.25±0.21 c	14.67±1.68 de	12.53 ±1.65 cd	27.61±1.16 g	80.96 ± 1.59 ab
WS 2:1O2	88.82±0.74 b	-3.03±0.07 ab	9.63±0.71 bc	7.74 ± 0.90 bc	18.83±0.73 bc	84.94 ± 1.00 bc
WS 1:1O2	88.91±0.27 b	-2.85±0.15 abc	11.52±1.07 bcd	9.33 ± 2.99 bc	21.86±0.59 de	83.75 ± 0.84 bc
WS 1:2O2	88.44±0.12 ab	-2.58±0.16 abc	13.15±1.54 cde	11.01 ± 1.70 bcd	24.83±0.82 fg	82.28 ± 1.16 b

Means in the same column with different letters (a-g) are significantly different ($p < 0.05$).

Table 4. Two-way ANOVA of color parameters of WS films.

		Two-way ANOVA	
Color Parameters		***F***	***p value***
L*	WPI/SCW	2.24	0.135
	OE	0.82	0.455
	Interaction	1.27	0.317
a*	WPI/SCW	34.18	**0.000**
	OE	0.55	0.586
	Interaction	1.30	0.307
b*	WPI/SCW	22.78	**0.000**
	OE	3.08	0.071
	Interaction	0.56	0.698
ΔE	WPI/SCW	8.961	**0.002**
	OE	1.267	0.306
	Interaction	0.273	0.892
Opacity	WPI/SCW	207.33	**0.000**
	OE	3.54	0.050
	Interaction	4.24	**0.014**
Whiteness	WPI-SCW	15.03	**0.000**
	OE	2.43	0.117
	Interaction	0.56	0.693

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3.4. Mechanical properties

The mechanical properties of the films and the two-way ANOVA of these properties of the films with the WPI–SCW mixture are shown in Tables 5 and 6, respectively. All of these properties (tensile strength, elongation at break, elastic modulus, puncture strength and deformation and elastic modulus) were significantly influenced by the substitution of WPI by SCW. The films with SCW only and with the highest proportion of SCW showed the lowest values of tensile and puncture strengths, elongation at break and puncture deformation. These effects in the mechanical properties of the films could limit the use of the highest levels of SCW in the food industry. This could be because SCW has high levels of denatured proteins, soluble peptides, lactose and non-protein nitrogen(Pires et al. 2021b).

The addition of EO also significantly influenced the mechanical properties of the films. It was observed that films with 1% of EO showed lower values of tensile strength and elongation at break than films without EO; however, the films with 2% EO showed higher values of tensile strength than the corresponding films with 1% EO.Sajimon et al. (Sajimon et al. 2023) observed the different evolution of these parameters, depending on the amount of oregano essential oil. They detected increases in tensile strength and elongation percentage with the addition of up to 2% of EO in WPI films, but that the incorporation of 3% decreased both values (Sajimon et al. 2023). It has been observed that the addition of other essential oils increases the tensile strength and the elongation of the films(Ghoshal 2022; Mahdavi et al. 2018; Pedro et al. 2023; Ramos et al. 2013) and has been explained by the way these compounds can become intercalated between the whey proteins, acting as plasticizers (Pedro et al. 2023). The incorporation of EO decreased significantly the values of puncture deformation of the films.

Table 5. Mechanical properties (mean ± SD) of films described in material and methods section.

Film	Tensile Strength at Maximum (MPa)	Elongation at Break (%)	Puncture Strength (MPa)	Puncture Deformation (%)	Elastic modulus (MPa)
WPI	1.57 ± 0.73 ef	52.17 ± 17.32 ef	2.40 ± 0.66 e	18.92± 5.74 h	7.41 ± 2.08 a
WS 2:1	1.17 ± 0.18 de	57.26 ± 14.77 ef	1.74 ± 0.14 d	15.71 ± 3.89 gh	15.00 ± 8.45 bc
WS 1:1	1.19 ± 0.22 de	45.20 ± 9.83 de	1.87 ± 0.36 de	9.57 ± 2.40 def	11.24 ± 2.60 abc
WS 1:2	0.46± 0.14 ab	19.56 ± 7.79 abc	0.51± 0.16 abc	3.55 ± 1.33 abc	16.75 ± 4.22 c
SCW	0.17 ± 0.05 a	3.57 ± 1.18 a	0.20 ± 0.03 a	0.93 ± 0.28 a	9.25 ± 4.13 ab
WS 2:1O1	1.02± 0.25 cd	30.32 ± 9.27 bcd	2.07 ± 0.59 de	12.34 ± 2.42 fg	12.29 ± 3.04 abc
WS 1:1O1	0.61 ± 0.12 abc	34.32 ± 10.99 cd	0.94 ± 0.11 bc	7.62 ± 1.11 cde	8.07 ± 2.99 a
WS 1:2O1	0.41±0.08 a	15.01 ± 4.65 ab	0.47±0.11 ab	2.56±0.95 ab	7.09 ± 1.54 a
WS 2:1O2	1.90±0.36 f	68.22 ± 14.30 f	1.77±0.48 d	11.14±3.47 ef	14.66 ± 3.27 bc
WS 1:1O2	0.95±0.16 bcd	27.59 ± 8.70 bc	1.05±0.17 c	5.52±0.94 bcd	9.91 ± 2.08 ab
WS 1:2O2	0.55±0.09 abc	17.98 ± 3.34 abc	0.66±0.13 abc	3.57±1.00 abc	7.54 ±2.37 a

Means in the same column with different letters (a-h) are significantly different ($p < 0.05$).

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Table 6. Two-way ANOVA of mechanical properties of WS films.

		Two-way ANOVA	
Mechanical properties		***F***	***p value***
TS	WPI/SCW	104.22	**0.000**
	OE	28.09	**0.000**
	Interaction	14.07	**0.000**
EB	WPI/SCW	70.25	**0.000**
	OE	13.53	**0.000**
	Interaction	11.73	**0.000**
PS	WPI/SCW	112.63	**0.000**
	OE	3.88	**0.026**
	Interaction	11.03	**0.000**
PD	WPI/SCW	117.44	**0.000**
	OE	10.66	**0.000**
	Interaction	2.76	**0.035**
EM	WPI/SCW	8.12	**0.000**
	OE	11.12	**0.000**
	Interaction	3.47	**0.013**

TS—tensile strength, EB—elongation at break, PS—puncture strength, PD—puncture deformation, EM—elastic modulus.

3.5. Antioxidant activity

Table 7 shows the DPPH radical scavenging capacity of films. The two-way ANOVA of the DPPH radical scavenging capacity of the films with a WPI–SCW mixture are shown in Table 8. No significant effects of the substitution of WPI by SCW in the antioxidant activity of the films were observed. The addition of EO significantly increased the antioxidant activity of the films, as has been reported (Sajimon et al. 2023), being a very significant factor in the increase of the values (from 0.97–1.19 in samples without EO to 2.00–2.19 in films with 2% of EO). The samples with 2% of EO showed higher values than those with 1% of EO. The films with a WPI–SCW mixture with the highest concentration of EO could be very useful in foods with a high content of polyunsaturated fatty acids to reduce oxidation of the lipids.

Table 7. DPPH radical scavenging capacity (mean ± SD) of films described in material and methods section.

Film	Antioxidant activity (mg DPPH/g film)
WPI	1.06 ± 0.27 a
WS 2:1	1.19 ± 0.31 a
WS 1:1	1.04± 0.16 a
WS 1:2	0.97 ± 0.16 a
SCW	1.09± 0.13 a
WS 2:1O1	1.52± 0.18 abc
WS 1:1O1	1.35± 0.08 a
WS 1:2O1	1.48±0.15 ab
WS 2:1O2	2.08±0.21 cd
WS 1:1O2	2.00±0.30 bcd
WS 1:2O2	2.19±0.40 d

Means with different letters (a-d) are significantly different ($p < 0.05$).

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Table 8. Two-way ANOVA of DPPH radical scavenging capacity of WS films.

		Two-way ANOVA	
Antioxidant activity		***F***	***p value***
DPPH	WPI/SCW	1.02	0.376
	EO	57.79	**0.000**
	Interaction	0.60	0.667

3.6. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of films can be observed in Figures 2 and 3. Some bands corresponding to Amide I region (1600–1700 cm^{-1}) showed changes in the wave number (WN) in SCW added films compared with WPI films. This region is governed by the stretching vibration of the C=O and C–N groups and provides information about the secondary structure of proteins (Ramos et al. 2013). Bands observed at 1610-1634 cm^{-1} and at 1675-1683 cm^{-1} correspond to β-sheet structures; bands at 1641-1646 cm^{-1} are attributed to random coil or unordered structure. 1652 cm^{-1} band has been assigned to α-helix, and 1660-1668 cm^{-1} and 1692 cm^{-1} bands to β-turn (Stuart 2004). The mean values of each secondary structure were 56.9% β-sheet, 15.8% random coil, 10.6% α-helix and 16.7% β-turn. β-sheet structure proportion was higher than values reported by other authors (Pereira et al. 2010; Ramos et al. 2013). High levels of β-sheet structures are often observed in aggregate proteins, particularly those that have undergone extensive thermal denaturation (Ramos et al. 2013) as in the case for samples to which SCW was added.

In bands corresponding to the β-sheet structure of proteins, several differences were found: the band at 1625 cm^{-1} in the WPI films shifted to a lower WN (1623 cm^{-1}) in WPI–SCW films. Furthermore, a new band at 1628 cm^{-1} was observed in the latter. Both bands have been assigned to the intramolecular β-sheet structure (Stuart 2004).

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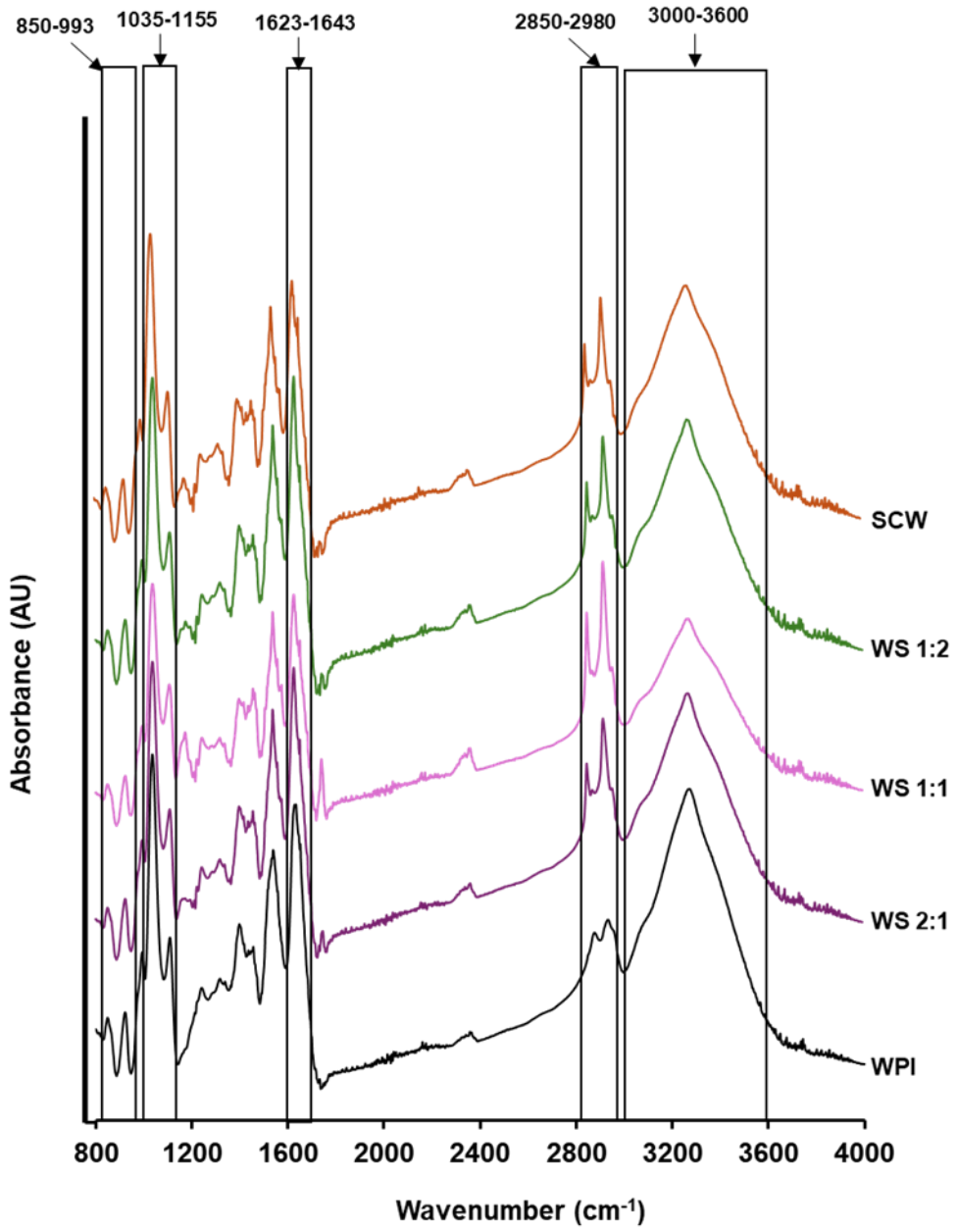


Figure 2. FTIR spectra of films with WPI and/or SCW described in material and methods section.

The band at 1643 cm^{-1} in WPI films also showed a shift to a lower WN in SCW-added films, 1640 cm−1, in which another new band was found at 1646 cm^{-1}. The appearance of a new band corresponding to random coils has been observed after emulsion formation in caseins dispersions (Daniloski et al. 2022).

The spectral region from 2850 to 2980 cm−1 is assigned to the stretching vibrations of the C–H bonds from methyl groups (Dinache et al. 2020; Ramos et al. 2013). Two bands showed changes in their WN depending on the sample. The band at 2843 cm−1 in the WPI films shifted to a higher WN (2850–2856 cm^{-1}) in WPI–SCW films, with or without EO. By contrast, the band at 2932 cm^{-1} in the WPI films shifted to a lower WN (2922–2925 cm^{-1}) in films with SCW. These vibration frequencies have been assigned to the symmetrical and asymmetrical C–H stretching of -CH_2 from aliphatic chains, probably

related to lipids (Stuart 2004; Dinache et al. 2020). The intensity of the bands in this spectral region increased in SCW- and EO-added samples compared with WPI films, probably due to the high lipid content of the SCW. However, the increment of the number of -CH_2 vibrations could also be due to the decrease in the interactions between lipids and proteins. Changes in the 2922 cm−1 band have been related to emulsion stability (Dinache et al. 2020).

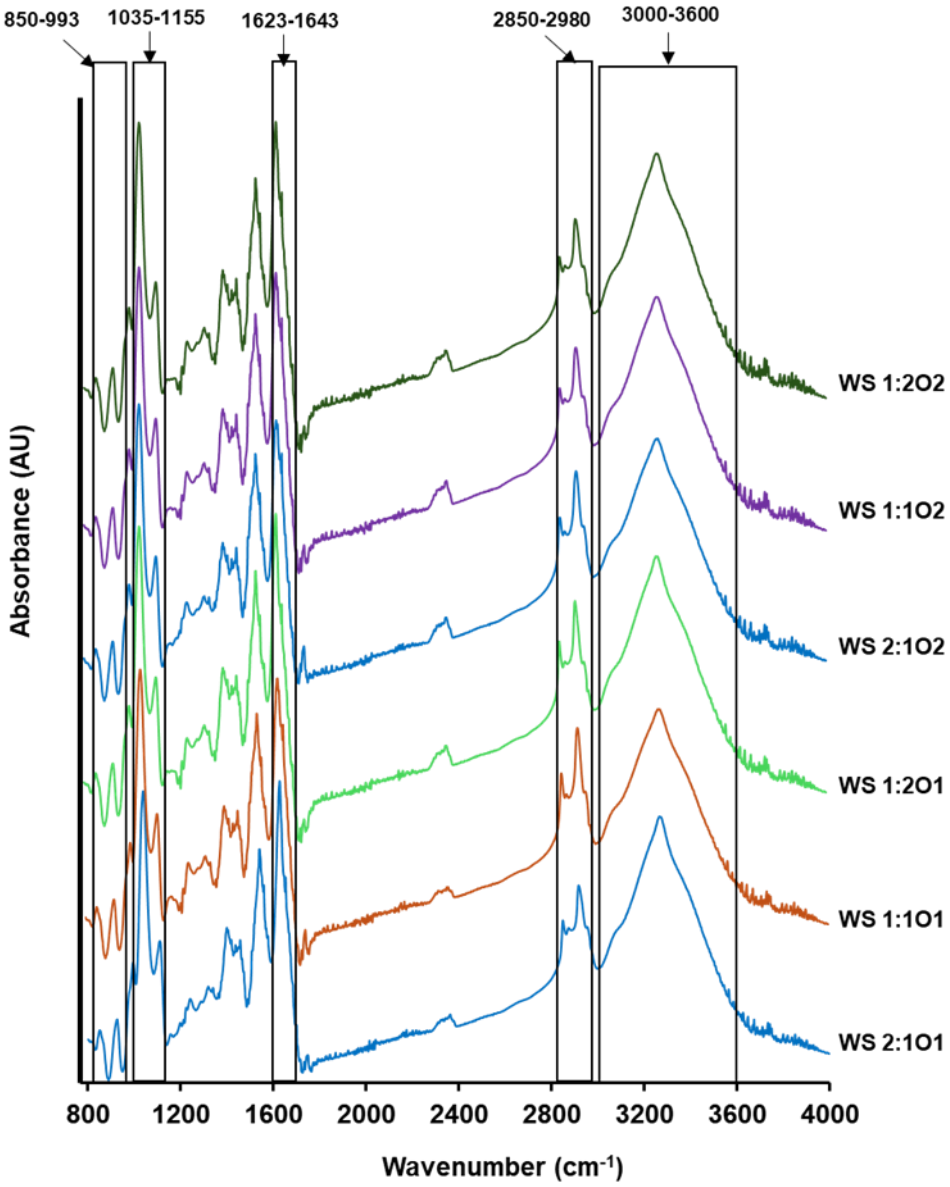


Figure 3. FTIR spectra of films with oregano essential oil described in material and methods section.

The absorption peaks located in the spectral range between 3000 cm^{-1} and 3600 cm^{-1} are related to free and bound O—H and N—H groups (Ramos et al. 2013). There were no differences in band wave numbers, although the intensity of the peaks in this region was lower in WPI–SCW films (lesser O–H vibrations) than in the WPI samples. This result may be derived from a higher degree of interactions by hydrogen bonding in the protein network, which involve a reduction in the availability of free -OH groups (Ramos et al. 2013).

Several absorption bands of glycerol were found in the spectral range between 800 and 1150 cm^{-1}. Bands at 850, 921 and 993 cm^{-1} are assigned to the vibrations of the backbone C—C bond, and band found at 1035 cm^{-1} corresponds to C—O in C1 and C3 (Matsakidou et al. 2019; Ramos et al. 2013). The intensity of these bands decreased in films with SCW, which can be attributed to the decrease in the interactions among proteins and the plasticizer. Other bands were observed in this region. The band at 1075 cm^{-1} increased its intensity in films with SCW and is related to the C–O stretching band in C–OH (Arrieta et al. 2013), although peaks at approximately 1080 cm^{-1} are commonly attributed to OH stretch of carbohydrates such as lactose (Andrade et al. 2019). The band at 975 cm^{-1} also increased intensity, which could be due to the interactions of protein with Na^{+} in the SCW-added films. Both bands, together with the band at 1155 cm^{-1} that was only observed in the SCW-added samples, have also been related to interactions between protein and phosphate groups(Arrieta et al. 2013). SCW showed higher ash content than WPI, so more interactions among proteins and minerals may be possible.
The addition of EO did not influence the FTIR spectra of films; the intensity and wave numbers of bands, and new peaks due to EO were not observed. This lack of modifications could be due to the low proportion of oregano essential oil added to film-forming solutions, which did not produce chemical changes; similar observations have been reported by other authors (Wang et al. 2024).

3.7. Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) and the first derivative of TGA (DTG) plots of the thermal degradation pattern of protein films are shown in Figure 4. DTG curves of percent weight loss versus temperature are obtained from raw thermogravimetric data. Table 9 shows the weight loss and residual weight of the films. Four weight loss stages were observed, as has also been reported by other authors [(Wang et al. 2023). The first period was 32-120°C and corresponded to the evaporation of free water and hydrogen-bonded water to the film matrix; weight loss was around 9.4%.

The second period occurred in the range of 120–235 °C and involved the thermal decomposition of glycerol, the degradation of low molecular weight compounds (including essential oils) and the vaporization of structure-bound water within the film (Wang et al. 2024). The lowest weight losses were observed in WS films compared with WPI and SCW samples, and they were lower at the highest SCW proportion; interactions among WPI and SCW compounds (denatured proteins, lipids) may improve the thermal stability of WS films. The weight loss at the third stage (235–370 °C) was primarily caused by polymer degradation. The highest weight loss was found in WS 1:2 films. The fourth period was in the range of 370–600 °C; polymer carbonization was responsible for the weight loss above 370 °C.

Total weight loss was in the range of 86.3–88.6%, and the residual weight between 11.3% and 13.7%. The films with the highest values of residual weight were the mixture of WPI–

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SCW in a 2:1 proportion (WS 2:1) and the presence of EO did not modify this parameter. EO addition did not influence the weight loss of films.

Table 10 shows the temperature peaks observed at each stage. Three peaks were observed at around 63 °C, 193 °C and 313 °C in all of the films. Nevertheless, two peaks (at 257 °C and 313 °C) were only found at the third stage in the WS films, which involved the presence of two chain structures with different thermal stabilities. The peak at around 313 °C appeared in all samples and may correspond to the degradation of parts of the protein matrix in which molecules established better molecular interactions. The peak at 257 °C in the polymer degradation stage could indicate a lower thermal stability of films when SCW was added to WPI, which may be due to the degradation of the denatured proteins from the second cheese whey protein concentrate that interacted with whey proteins from WPI. This peak was found at lower temperatures, 246–252 °C, in WS films with EO. The interaction of EO with SCW and WPI proteins may cause this decrease. However, weight losses were similar in all samples, as mentioned above. Further studies are needed for the determination of the positive or negative influence of SCW and EO addition on the thermal stability of WPI in view of the industrial application of these films in food packaging.

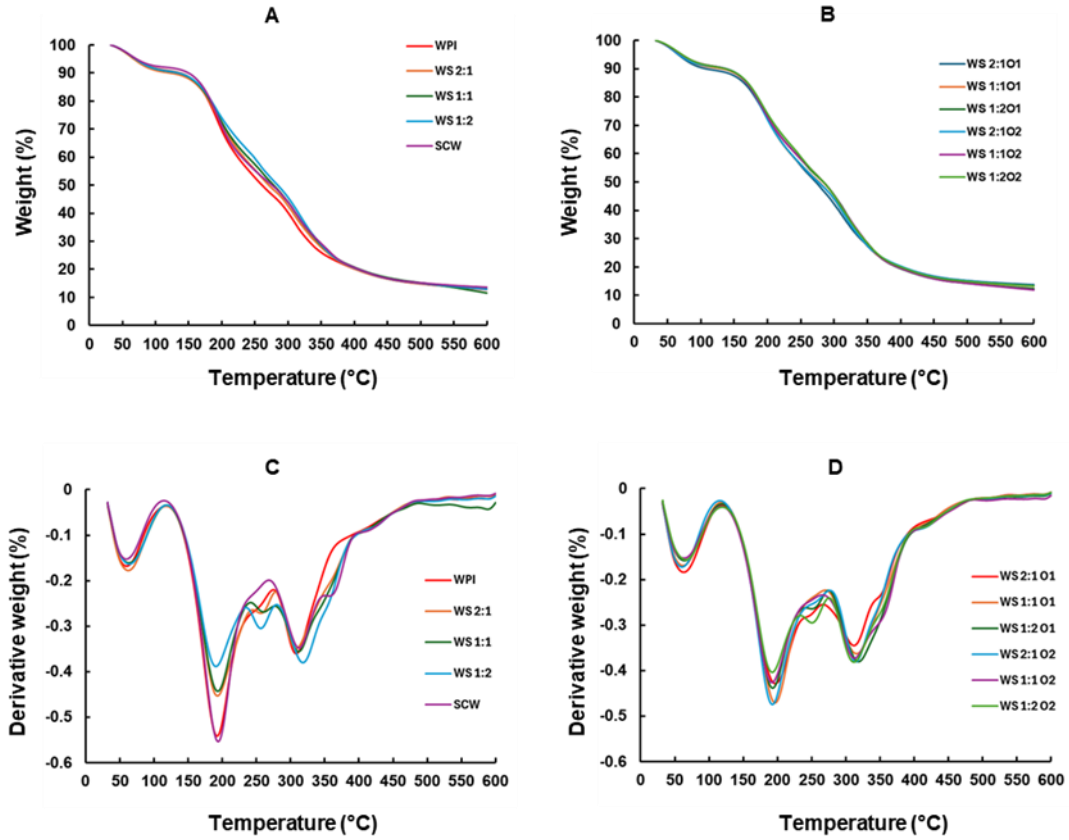


Figure 4. Thermogravimetric analysis (TGA) (A, B) and first derivative of TGA (DTG) (C, D) curves of films described in material and methods section.

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Table 9. Thermal degradation of films described in material and methods section: weight loss and residual weight (%).

Film	Stage I 32-120°C	Stage II 120-235°C	Stage III 235-370°C	Stage IV 370-600°C	Total weight loss	Residual weight
WPI	9.21	34.76	33.38	10.55	87.28	12.72
WS 2:1	9.94	31.24	35.41	10.58	86.70	13.30
WS 1:1	9.39	28.64	36.57	13.05	88.64	11.36
WS 1:2	9.42	26.70	39.22	11.39	87.19	12.81
SCW	8.09	32.55	34.09	11.02	86.44	13.56
WS 2:1O1	10.49	28.70	36.49	9.76	86.34	13.66
WS 1:1O1	9.57	30.79	36.57	10.10	86.79	13.21
WS 1:2O1	9.21	28.01	38.65	10.91	87.68	12.32
WS 2:1O2	9.22	30.31	36.35	10.30	86.65	13.35
WS 1:1O2	9.13	28.64	38.44	11.55	88.20	11.80
WS 1:2O2	9.20	27.32	39.38	10.40	86.72	13.28

Table 10. Thermal degradation of films described in material and methods section: Temperature peaks (°C).

Film	Stage I 32-120°C	Stage II 120-235°C	Stage IIIa 235-370°C	Stage IIIb 235-370°C
WPI	62.7	193.0	nd	308.1
WS 2:1	62.3	192.8	257.3	307.8
WS 1:1	63.5	193.2	257.4	311.3
WS 1:2	63.8	190.6	257.3	318.3
SCW	61.1	194.0	nd	312.5
WS 2:1O1	63.6	195.9	246.2	311.9
WS 1:1O1	62.2	197.0	247.2	315.8
WS 1:2O1	65.4	193.1	249.9	316.8
WS 2:1O2	61.2	192.6	251.3	314.4
WS 1:1O2	64.4	193.4	251.7	314.4
WS 1:2O2	66.1	192.3	250.3	312.5

Nd=not detected.

3.8. Microstructure

Scanning electron microscopy was used to analyze the film microstructure. Figure 5 shows the surface images of experimental films. The WPI film surface was smooth, uniform and dense, while SCW films displayed a rough surface with aggregates of different sizes, probably due to the presence of denatured proteins and lipids. The WS films showed pinholes and cavities probably caused by lipid droplets from the SCW powder used in film-forming solutions; these droplets increased their size, and their distribution was more irregular proportionally to the amount of SCW in the mixture. When EO was added, more parts of the images appeared in a darker color compared with other sites of the film images; similar observations have been reported in WPI films containing free thyme extract (Aziz and Almasi 2018).

Figure 6 presents the cross-sectional images of the films. The WPI films exhibited a rough and compact image due to the protein aggregates that formed the film matrix. The SCW

films showed a coarser appearance with cavities, due to the presence of highly denatured proteins. The WS 2:1 samples had a compact structure with small, sinuous creases. The increase of SCW content made the cross-sectional morphology of films rougher with larger aggregates, particularly in the WS 1:2 films. This phenomenon may be associated with the diminution of tensile strength and elongation values in films with higher proportions of SCW. In the case of the WS 1:2 films, the large aggregates were likely formed by denatured proteins from SCW, thereby impairing the mechanical properties of these films. The addition of EO modified the microstructure of the films. Larger aggregates and an increase in void sizes were observed in the WS 2:1 O2 and in the WS 1:1 and WS 2:1 films at both EO concentrations. The increase in roughness in protein films added with essential oils has been attributed to the attraction among the matrix components and EO, which at high concentrations can lead to the formation of agglomerates (Wang et al. 2024). However, the nature of these interactions is likely to vary depending on the structural characteristics of the proteins involved. In the WS 2:1 O2 films, the presence of trabeculae that intersect voids was observed. These structures are likely the result of the interaction between WPI proteins and EO molecules, potentially contributing to enhanced mechanical properties, such as tensile strength and elongation, in comparison with the WS 2:1 and WS 2:1 O1 films. As previously documented by other researchers, an enhancement of these properties was observed when increasing concentrations of essential oil were added (Wang et al. 2024). The aforementioned effect was not observed in films with lower WPI content, likely due to the EO concentration exceeding the limit over which WPI proteins can interact with EO components. It is possible that denatured SCW proteins exhibited a diminished capacity for binding EO compounds due to changes in their structure.

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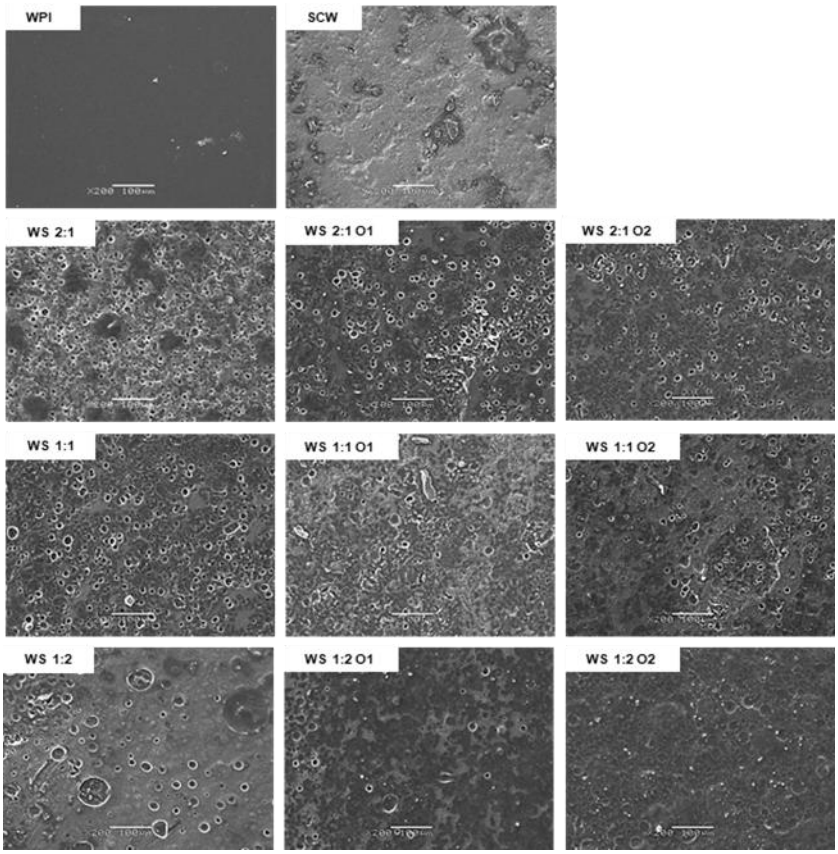


Figure 5. Surface image of films described in material and methods section.

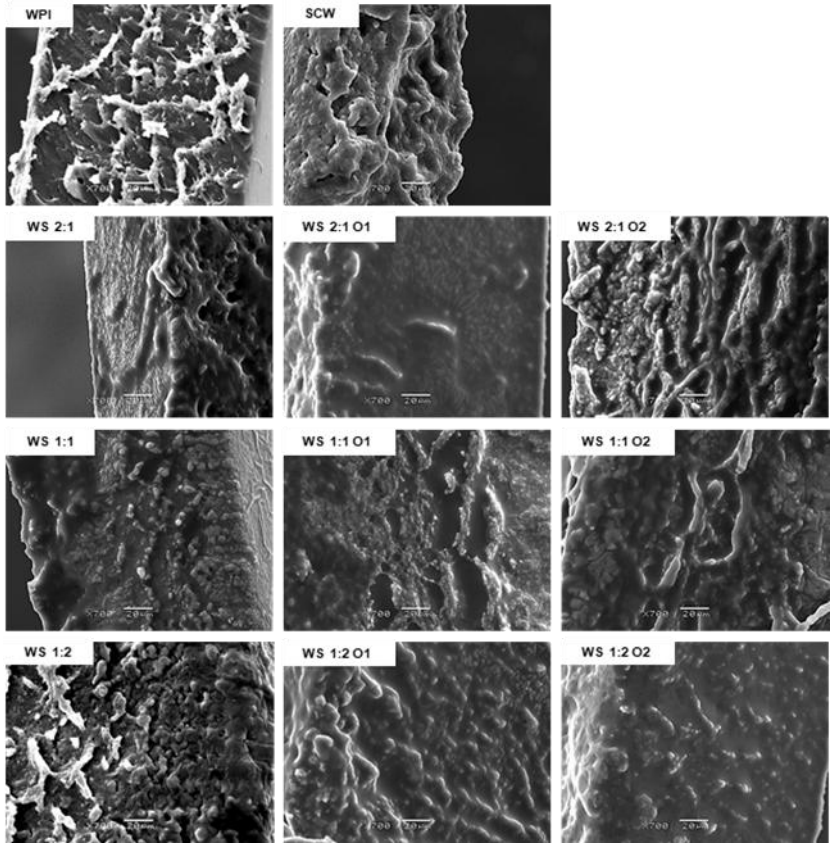


Figure 6. Cross-sectional images of films described in material and methods section.

3.9. X-ray diffraction (XRD)

As illustrated in Figure 7A, the diffractograms of the films WPI, SCW, and the mixtures of both (WS) reveal the occurrence of structural changes in two angular zones, depending on the sample. These changes may involve inter- or intra-molecular contacts of the polymeric chains or aggregates that become crystalline, as evidenced by the observation of peaks rather than bands. The low-angle zone, which corresponds to peaks and bands at 2θ in the range of 3° to 15°, shows the structural changes in the films. The diffractograms of the WPI films exhibit a poorly structured system, while the addition of SCW at varying proportions (all WS films) results in the formation of crystalline structures with peaks at 3.8, 5.8, and 9.8°. In the high angle disturbance zone (15° to 30°), the WS 1:2 and SCW films exhibit variations indicative of a higher degree of structuring, suggesting enhanced homogeneity in the inter- or intra-molecular contacts. Denaturing agents, such as heating, produce conformational changes in whey proteins that can induce the formation of novel peaks in diffractograms, as has been reported by other authors (Gomide et al. 2021). The addition of SCW powder to film-forming solutions involves the incorporation of denatured whey proteins that may affect the crystalline structure of films.
The diffractograms of the WS films to which oregano essential oil was added are shown in Figure 7B. In the low-angle zone, peaks showing a crystalline component are also observed in all of the samples, as has been mentioned above. In the WS 1:2 films with added EO, the relative intensities of peaks 3.8, 5.8 and 9.8° were found to be higher than in the WS 1:2 sample without any addition. Furthermore, in the high-angle zone, two small peaks (indicated by arrows in Figure 7B) appear in the WS 1:2 O1 and O2 films. It may be possible that the essential oil interacted with the denatured whey proteins, present in greater proportion in these samples and increased the crystallinity of the films. Amjadi et al. (Amjadi et al. 2021) have reported that the incorporation of orange peel essential oil improves the crystallinity of WPI-based films.

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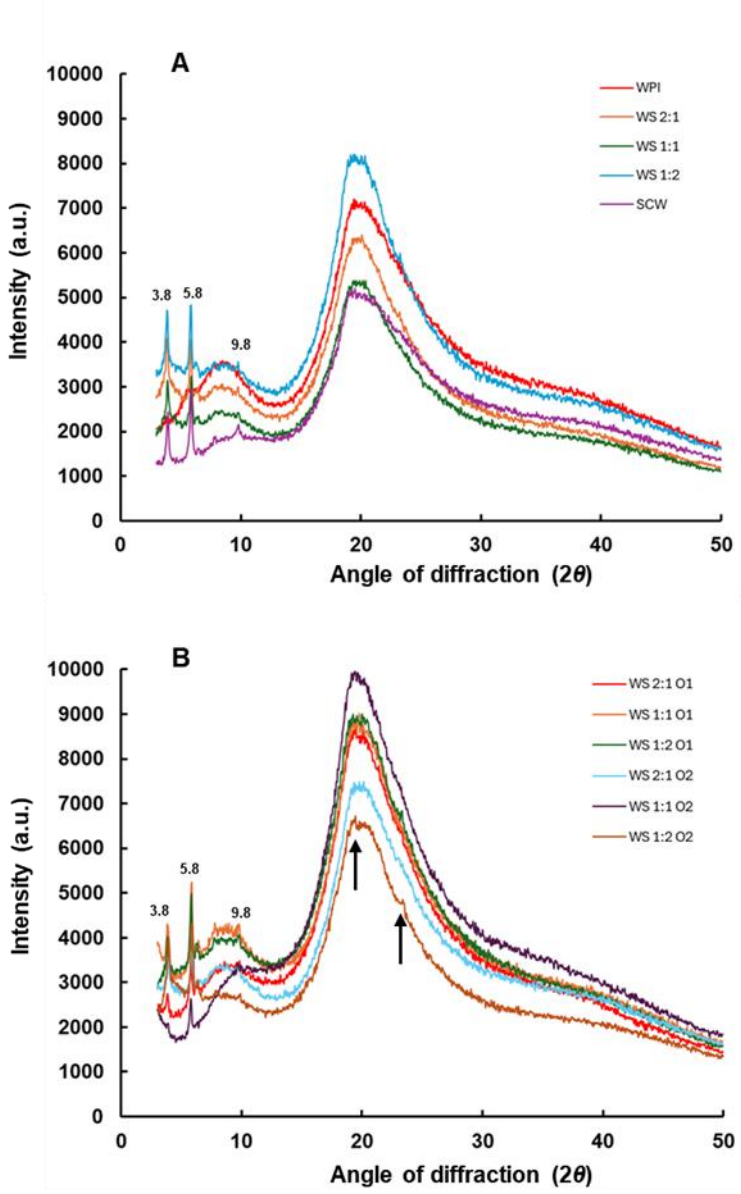


Figure 7. XRD patterns for films with WPI and/or SCW (A) and films with oregano essential oil (B) described in material and methods section.

4. Conclusions

SCW powder, a by-product resulting from ovine whey cheese production, when combined with WPI, may be used to prepare edible films, but they showed important differences in their properties depending on the proportion of each product. The films with only SCW or a high proportion of SCW had the lowest values in tensile strength, elongation at break, puncture strength and puncture deformation. These effects in the mechanical properties of the films could limit the application of the highest levels of SCW in foods. The addition of oregano essential oil to the SCW–WPI films is useful to increase the antioxidant activity of the films, though it must also be considered that it can modify the water solubility and the mechanical properties of these films. The mixture of WPI and

SCW at a 2:1 proportion, with the addition of 2% oregano essential oil, yielded films that exhibited the highest values of tensile strength and elasticity, and good antioxidant capacity.

Consequently, these films can be regarded as the most efficient films for food packaging among those examined in this study. However, in view of their practical application, it is necessary to investigate other important parameters that affect their properties, such as the pH of the film-forming solutions, the incorporation of alternative plasticizers or the drying temperature, to improve their characteristics.

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GENERAL DISCUSSION

In the dairy industry, several wastes and by-products are produced, which in many cases are not reused and thus can represent an environmental problem when discarded inappropriately. Due to their high content of organic matter, the responsible management of them is of particular importance in the context of the circular economy.

As described in the introduction of this thesis, studying and proving effective innovative solutions for the valorisation of whey and, particularly, second cheese whey is currently limited. Thus, in the present work, their valorisation included the creation of new food products through the separation of their protein content, and the creation of edible films and coatings that allow the extension of the foods shelf-life and at the same time, add health benefits to these products.

Although there are in the literature several studies carried out to valorise whey, the second cheese whey is still not valued at all, and this by-product also has very relevant nutritional properties. Therefore, for these reasons, in the present work, new ways of valorisation of the whey and especially the second cheese whey were studied. The solutions involved the use of selective separation techniques by means of low-cost membranes, which can be applied in small and medium-sized dairy companies.

In this section an integrative discussion is presented, which gives coherence and unity to the work carried out.

1. Application of Ultrafiltration to Produce Cow's, Sheep's and Goat's Liquid Whey Concentrates

As it has been indicated in the introduction of this thesis, the use of selective separation processes with low-cost membranes (UF, NF and MF) can be applied in small and medium-sized dairy companies. The use of these methods allowed to obtain liquid whey protein concentrates, which were directly applied in the manufacture of new dairy products with high nutritional and functional properties.

Membrane filtration technologies have become the most promising available tools to selectively concentrate high value components present in CW and SCW (*i.e.* proteins). The acceptable costs and ease of operation make them suitable for application in small and medium-sized dairy plants.

In this work, new symbiotic products, based on liquid whey concentrates (LWC) from cow, sheep and goat were obtained by ultrafiltration and characterized.

The evolution of the UF fluxes of sheep and goat whey over time are presented in Figure 1 of article 1. The results are the average of two concentration trials. Higher filtration fluxes were obtained during the concentration of goat's whey. However, at a nutritional level, the product obtained presents less protein, less dry matter and less ash. In both cases, average fluxes are of the order of 30 and 20 $Lm^{-2}h^{-1}$ for goat and sheep CW,

respectively, which indicates the feasibility of the operation of ultrafiltration units in small-scale cheese plants, processing volumes of *ca.* 500 – 10 000 L milk per day. That figure also presents the calculated evolution of the volumetric concentration factor during the concentration process. It was observed that it is possible to concentrate whey to obtain high levels of protein (up to 16 %) and fat (up to 14 %), in the LWCs.

Then the composition of the cow's, sheep's and goat's samples obtained by membrane separation processes was evaluated (Table 10). The values for cow cheese whey and cow LWC are taken from the literature (Henriques et al. 2011).

Table 10. Composition of cow's, sheep's and goat's liquid whey, liquid whey concentrates and liquid second cheese whey concentrates.

PRODUCTS	Dry matter (%)	Protein (%)	Fat (%)	Ashes (%)
Cow's Whey	6.92 ± 0.04*	0.89 ± 0.00*	0.78 ± 0.00*	0.50 ± 0.02*
Cow's LWC	19.53 ± 0.20*	6.12 ± 0.24*	7.82 ± 0.00*	0.61 ± 0.00*
Sheep's Whey	8.63 ± 0.68	1.32 ± 0.09	1.18 ± 0.43	2.15 ± 0.27
Sheep's LWC	34.74 ± 0.68	16.35 ± 0.36	14.12 ± 0.50	1.98 ± 0.67
Goat's Whey	6.61 ± 0.09	0.73 ± 0.01	1.76 ± 0.47	1.03 ± 0.24
Goat's LWC	32.23 ± 0.10	7.84 ± 0.45	19.32 ± 0.12	1.55 ± 0.07
Sheep's LSCWC	20.84 ± 0.75	9.58 ± 0.27	2.10 ± 0.001	1.65 ± 0.01
Goat's LSCWC	12.38 ± 0.01	2.96 ± 0.003	1.60 ± 0.001	1.57 ± 0.004

LWC- liquid whey concentrate; LSCW- liquid second cheese whey concentrate.
*Values from (Henriques et al. 2011)

Based on the data, some important aspects can be observed, such as the nutritional value of LWCs. Among them, sheep LWC stands out due to its higher amount of protein and dry matter content, which makes it highly nutritious.

Membrane separation processes significantly increased the nutrient concentrations in the LWC products, compared to their respective whey and second cheese whey initial products. Membrane filtration processes offer several advantages in the dairy industry. They allow the concentration of proteins and other nutrients, improving the nutritional value of the samples, which can be reused to obtain new dairy products. These processes also are effective in removing microorganisms and other contaminants, resulting in safer products. Since these technologies operate at lower temperatures, they are more energy-efficient processes and minimize product losses during processing. The use UF process allowed to conclude that this process can be applied even in dairy factories that processes *ca.* 500 L of milk/day.

Therefore, we can conclude from this work that ultrafiltration processes are not only feasible but also advantageous for small dairy plants. This enhances the use of functional ingredients and improves production efficiency, allowing dairy factories to become more competitive in the market by offering high-quality value-added products. Despite some initial investment, the economic gain resulting from the reuse of these by-products can far outweigh this value. Also, it contributes to a circular economy concept and reduces the environmental damage.

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2. Application of Concentrates Obtained by Membrane Technologies in Innovative foods

Articles 1, 2 and 3 describe the use of liquid concentrates obtained from whey and second cheese whey with the addition of probiotics, prebiotics and starter cultures to produce new foods.

By using liquid concentrates from sheep and goat whey, fermented drinks with kefir culture were developed. A fresh cheese (whey cheese) was obtained using liquid whey concentrates from cow's whey with the addition of bioprotective cultures. Finally, liquid concentrates from sheep and goat second cheese whey we were used to create a frozen dairy product.

In the first article, four different types of symbiotic sheep and goat kefirs were produced based on liquid concentrates obtained by ultrafiltration of sheep and goat cheese whey: the first two with commercial kefir cultures (with or without probiotic culture) and the last two with traditional kefir grains (with or without probiotic culture). All kefirs had the addition of inulin. The probiotic culture contained *Bifidobacterium bifidus*, *Lactobacillus acidophilus*, and *Streptococus thermophilus*.

Kefir is a milk product that results from the action of a very complex microbiota (lactic acid bacteria, acetic acid bacteria, and yeasts) acting in symbiosis. Kefir can be produced with cow's, goat's, sheep's or buffalo milks. It has an acidic flavour and creamy texture. The name Kefir is derived from the word "kef", which means "pleasant taste" in Turkish (Farag et al. 2020). The composition of kefir depends on the milk origin, temperature and time-related fermentation conditions. The fermentation process enriches the content of vitamins B_1, B_{12}, K, folic acid, calcium and amino acids, increasing the health benefits of Kefir. Kefir generally contains 89–90% of moisture, 0.2% of lipid, 3.0% of protein, 6.0% of sugar, 0.7% of mineral content and 1.0% of lactic acid and alcohol, respectively. Kefir contains around 1.98 g/L of CO_2 and 0.48% of alcohol (Gao and Li 2016). The smell and taste are due to the volatile and non-volatile compounds generated during fermentation through lipolysis, glycolysis and proteolysis. Its physical-chemical properties include an acidic pH of approximately 4.6 and an alcohol percentage of 0.5%–2%. Kefir has many health benefits, such as anti-stress, immunomodulatory, cholesterol-lowering, anti-allergic, anti-asthmatic, antimicrobial, anti-cancer and chemoprevention properties against colon cancer, in addition to its beneficial gastrointestinal effects (Farag et al. 2020). Traditionally, kefir grains are used for the fermentation process. The microorganisms are found in a matrix of proteins and polysaccharides. It contains many bioactive compounds such as exopolysaccharides (mainly kefiran), bioactive peptides, and organic acids (especially lactic acid), which are attributed to have many beneficial health effects such as antimicrobial, anticancer, and immune-stimulating activity. Kefir is also currently produced with commercial starter cultures, but it is noted that its functional potential is different from that obtained with kefir grains due to the distinct microbial composition of both types (Vieira et al. 2021). The production of symbiotic kefir with commercial kefir grains or traditional cultures with the addition of prebiotics such as

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inulin and probiotic microorganisms using concentrated sheep's and goat's whey, originates novel products that may have important functional properties.

Regarding the physicochemical characteristics, goat Kefir products had lower pH and higher acidity. Sheep Kefir products produced with commercial yeast with and without the addition of probiotic culture (EK and EKABT5) presented the highest pH values and the lowest acidity values. Thus, the use of probiotic culture had an impact on the pH, as well as on the titratable acidity, both in Kefir-based products of ovine and caprine origin. These values of pH in Kefir fermented products decrease when compared with pH of ovine and caprine milk, and in terms of titrable acidity, the values of Kefir fermented products are higher when compared with ovine and caprine milk. Therefore, the use of kefir grains decreases the pH and increases the titratable acidity of fermented products, both of sheep and goat origin (La Torre et al. 2024).

In terms of viscosity, it was found that sheep-based products are more viscous than goat-based products, and in terms of texture, sheep kefir products are harder.

Regarding the evolution of the microbial composition, the counts of presumptive lactobacilli and presumptive lactococci and yeasts of the different products during the storage time were higher than log 7 CFU/g, indicating the good adaptation of LAB and yeasts to LWC and the probiotic potential of the products over the storage time.

Regarding sensory evaluation (Figure 14), products based on sheep's whey were preferred comparing to products based on goat's whey, since goat products always have a characteristic goat's milk flavour. Farag *et al.* state that sensory characteristics are influenced by the type of milk used, but these authors found that Kefirs produced from cow, goat or sheep milk had similar microbiological properties (Farag et al. 2020). Gul *et al.* compared the rheological, textural, colour and sensory properties of Kefir samples produced with buffalo or cow's milks, using two different sources of microbial fermentation, namely Kefir grains and starter cultures. Buffalo milk Kefir demonstrated to have a higher polysaccharide content than cow milk Kefir, and the authors explain the use of buffalo milk for Kefir production had positive effects on the water retention capacity and firmness value of the resulting Kefir samples. The buffalo's milk Kefir samples showed higher viscosity, higher consistency index, storage modulus and loss values when compared to the samples made with cow's milk. In terms of sensory evaluation and colour properties of Kefir samples improved when buffalo milk was used (Gul et al. 2018).

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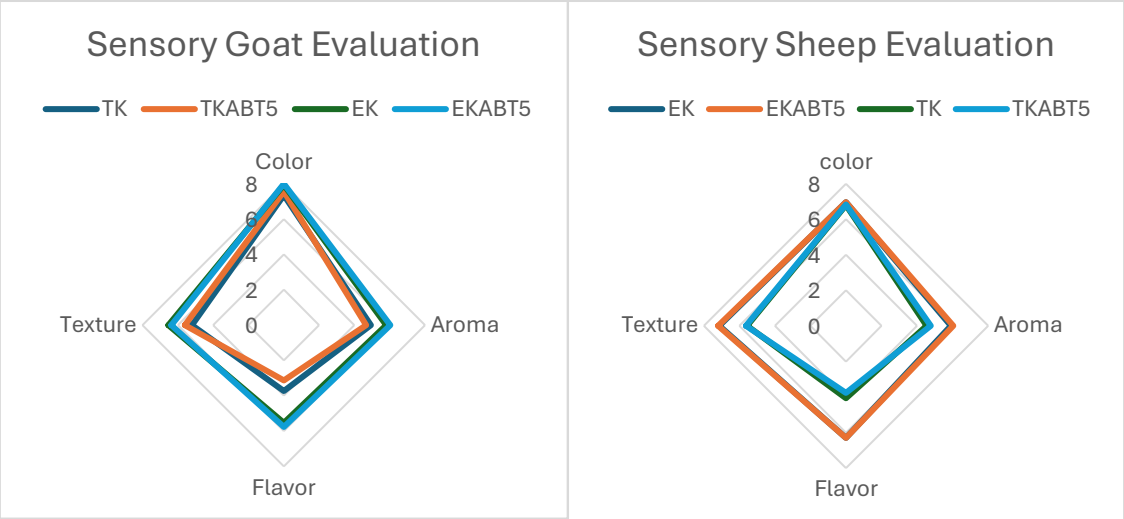


Figure 14: Sensory evaluation of goat and sheep products (data obtained from article 1). TK = Traditional kefir; TKABT5 = Traditional kefir + probiotic culture, EK = Commercial kefir; EKABT5 = Commercial kefir + probiotic culture.

Kurnick et al. 2024 produced fermented and neutral (non-fermented) drinks from cheese whey (CW) and second cheese whey (SCW) from goat and sheep. All products proved to be microbiologically safe, and regarding their sensory evaluation, the use of ingredients/additives that masked the unpleasant taste of the CW resulted in more favourable evaluations. However, unfermented samples were preferred due to added coffee and sweet/vanilla flavours. Overall results indicated that the treatment of fermented and neutral beverages overcame any differences observed in relation to the animal source or the type of whey (CW or SCW) and that, for the fermented samples, the animal source was more important than the type of whey (Kurnick et al. 2024).

Kefir-based products have been shown to be beneficial to human health, and although some aspects need to be improved at the sensory level, this is a promising way for small and medium-sized companies to reuse their by-products that would otherwise be discarded. These new products offer many nutritional benefits and thus also help combat food shortages and poor nutrition worldwide.

In the second article, whey cheeses containing probiotic and bioprotective cultures were produced with ultrafiltered cow's whey. The production of whey cheese from whey is a process that has low yields, requires a high energy consumption of energy for its production and has a short shelf-life. The application of ultrafiltration to the whey before its transformation in whey cheese and the addition of bioprotective cultures can reduce these disadvantages. Furthermore, the addition of probiotics can increase the functional value of this product.

Three types of bovine fresh cheese were prepared with liquid whey concentrates obtained by ultrafiltration of cheese whey: a control without any addition, another with the addition of *Lactobacillus acidophilus* and, finally, a third with the addition of *Lactobacillus acidophilus* plus a bioprotective culture containing *Lacticaseibacillus paracasei* and

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Lacticaseibacillus rhamnosus. The fresh cheese was stored under refrigeration for 28 days.

The results showed that the whey cheeses with the added cultures showed lower pH values and higher titratable acidity compared to the control. Small differences between products were observed regarding color parameters during storage. The samples with added cultures were firmer and had a lactic acid bacteria count in the range of 8–9 log cfu/g. The fresh cheeses made with the bioprotective culture had lower levels of yeast and molds. Therefore, the beneficial impact of the bioprotective culture on the shelf-life of the food was observed, delaying its deterioration. Regarding the sensory evaluation, cheeses with bioprotective cultures (LA5+FQ) obtained the highest scores in all the parameters evaluated (Figure 15).

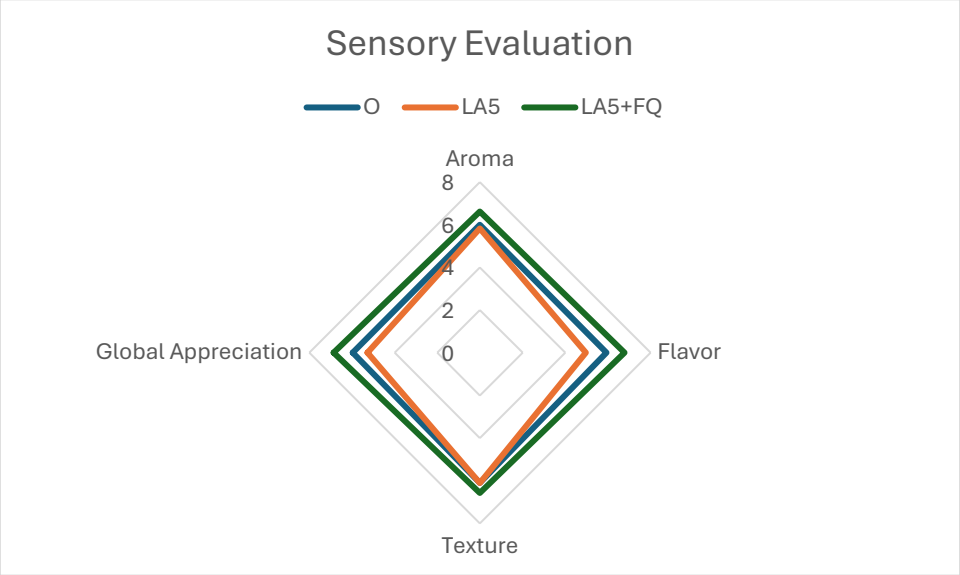


Figure 15: Sensory evaluation of fresh cheese with and without bioprotective cultures (data obtained from article 2). O—Original control; LA5—whey cheese with probiotic LA5 culture; LA5FQ4—whey cheese with probiotic LA5 culture plus FQ4 protective culture.

Madureira et al. 2005b, studied the viability of probiotic strains (*Lactobacillus acidophilus*, *Lactobacillus paracasei*, *Bifidobacterium animalis* and *Lactobacillus brevis*) after incorporation into whey cheese for 28 days of storage. These whey cheeses were made in the traditional way for preparing *Requeijão*, with some incorporating sugar and others salt. The results showed that all considered strains were able to maintain their initial viable numbers. The *L. paracasei* LCS-1 strain, and *L. acidophilus* strain exhibited the highest viability in plain whey cheese at the end of the storage period. Thus, these authors concluded that bacterial species and the nature of the matrix had the most important effect on viable counts, while storage time was the least important (Madureira et al. 2005b). Comparing our results with those obtained by Madureira et al. 2005b, we found that the protective cultures used in bovine whey cheese positively influenced these products, resulting in better viability. These products made with protective cultures show several advantages.

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It can be concluded that the use of ultrafiltration of bovine whey combined with the addition of probiotic and bioprotective cultures can be very useful in reducing the energy consumption of the manufacturing process, extending the shelf-life of the whey cheese and improving its sensory properties, in addition to ensuring that these products have beneficial properties for human health.

Frozen dairy products can have properties of both fermented and frozen milks and be carriers of lactic bacteria, prebiotics and probiotics. The production of yoghurts with prebiotics and probiotics creates new opportunities for the development of dairy products with beneficial properties for health. The use of second cheese whey from sheeps and goats to produce ice creams with these functional properties is of great interest (article 3).

The ice creams were made with liquid second cheese whey concentrates obtained by ultrafiltration from ovine and caprine second cheese whey. Figure 16 shows the ultrafiltration fluxes observed in the production process of sheep's and goat's second cheese whey liquid concentrate. Better fluxes were observed in the concentration of sheep's SCW despite the higher concentration of solid products observed in this product.

The protein concentration of the concentrates was 9.58% for sheep and 2.96% for goat. Three types of products were obtained, since the concentrates (all with the addition of inulin) were fermented with commercial cultures of yoghurt, kefir or probiotics before freezing. The probiotics used were *Bifidobacterium bifidus, Lactobacillus acidophilus* and *Streptococus thermophilus.* The properties of the ice creams were evaluated during 120 days of frozen storage. Sheep's SCW ice creams had greater hardness and complex viscosity than goat ice creams, related to their higher protein content. They also presented higher "overrun" (increase in ice cream volume after freezing due to the incorporation of air) and lower melting percentage. It is noteworthy that the lactic acid bacteria count indicated that the probiotic characteristics of the ice creams were maintained over the first month of frozen storage, then those values decreased in some samples. All ice creams were well accepted by the consumer panel, but ice creams made with ovine SCW were rated higher in terms of texture, flavour and aroma than ice creams made with SCW from goat's whey cheese. The addition of prebiotics and probiotics increased the nutritional value of the ice creams in addition to increasing the functionality of these products; with this, some by-products of the dairy industry, namely SCW from sheep's and goat's cheeses, which until now have not been used in human nutrition, were valorised, helping to reduce their environmental impact.

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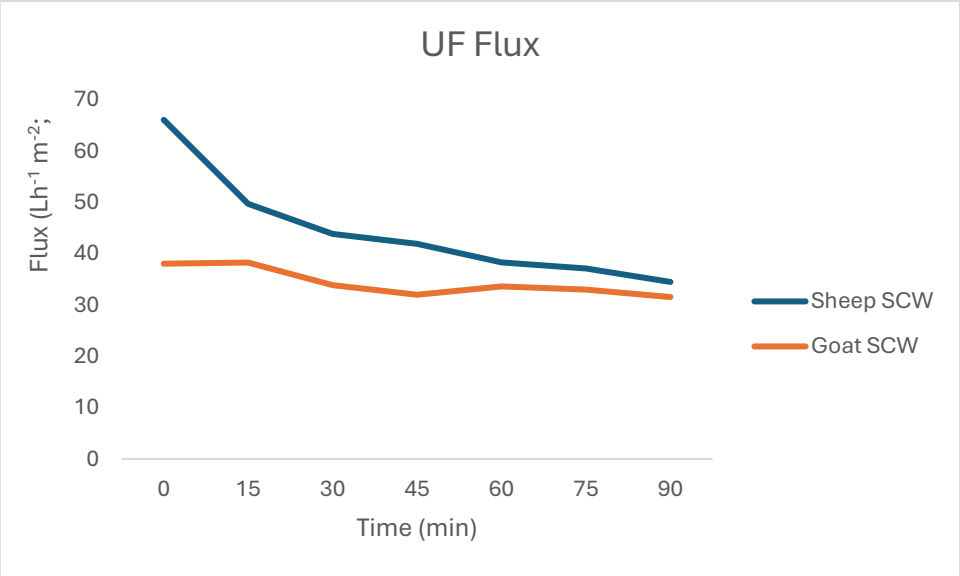


Figure 16: UF flux process for obtain the sheep and goat second cheese whey liquid concentrate.

In an article of our research group, Marnotes et al. (2021) used goat's and sheep's liquid whey concentrates (LWCs) produced by ultrafiltration (UF) for the manufacturing of frozen yoghurts. Firstly, natural yoghurts using only goat's and sheep's LWCs as raw material were developed. Next, after production, these yoghurts were used to produce frozen yoghurts with different concentrations of added inulin. The physicochemical characteristics of sheep's and goat's yoghurts were significantly different regarding dry matter, protein, fat and minerals. Sheep's yoghurts were solid, while goat's yoghurts behaved as a viscous liquid. Frozen yoghurts with different levels of inulin addition, also presented significant differences concerning physicochemical and microbiological characteristics. Overrun was similar for all formulations except for that produced with ewe's LWC containing 5.0% inulin, which presented a significantly higher value. Higher meltdown rates in goat's frozen yoghurts were observed. The survival rates of lactic acid bacteria were lower than the data reported for similar products. Concerning sensory acceptance, both products showed encouraging results (Marnotes et al. 2021).

Comparing the results obtained with the work carried out by Marnotes et al. (2021), we see that both sheep ice creams made with SCW, and frozen sheep yoghurts made with cheese whey have a higher solids content, higher protein and mineral content when compared to the same products made with SCW and CW from goats. Regarding texture, in both cases, products made from goat SCW and CW had a more liquid and viscous texture, while products made from sheep SCW and CW had a more solid texture. The melting rates in both studies were shown to be higher in products obtained from goat SCW and CW.

At a microbiological level, in both studies, it was found that there was a decrease in lactic bacteria counts over the storage time. But although LAB counts decreased over time, ice creams made with SCW maintained presumptive *Lactococci* sp. and *Lactobacilli* sp. counts of log 8–9 CFU/g for up to 60 days, indicating good probiotic stability.

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Regarding the sensory evaluation, Marnotes et al. (2021), obtained positive acceptance for both yoghurts, but without a clear differentiation regarding the flavour and texture profile between the goat and sheep products. In our trial (article 3), sheep ice creams were better evaluated in terms of aroma, flavour and texture, with consumers preferring them compared to goat ice creams (Figure 17).

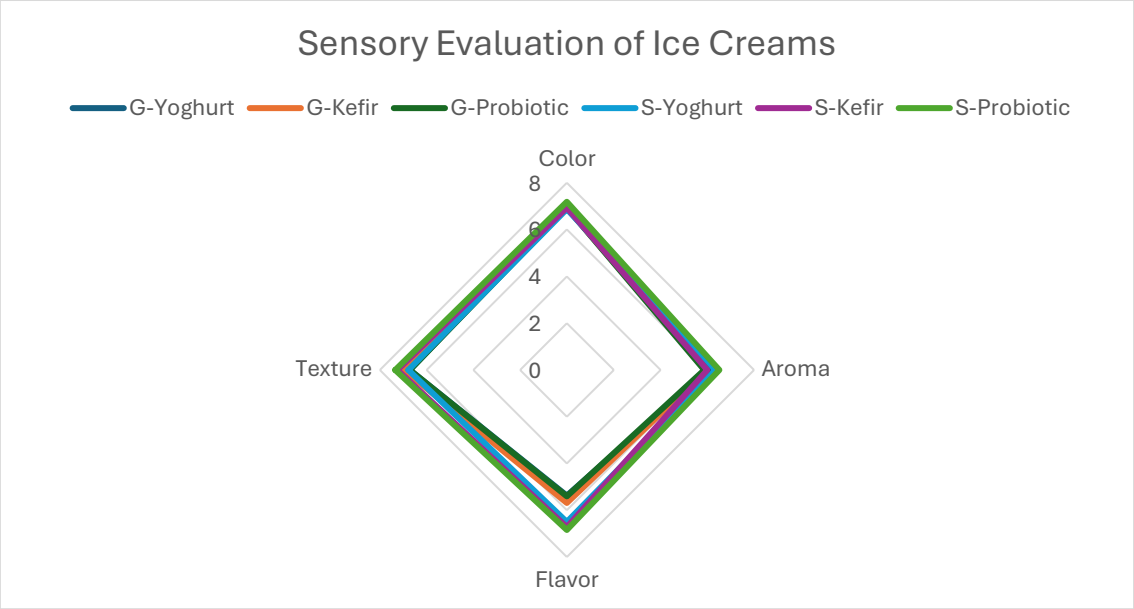


Figure 17: Sensory evaluation of ice creams (data obtained from article 3). G-Yoghurt- goat ice cream with Yoghurt culture, G-Kefir- goat ice cream with Kefir culture, G-Probiotic- goat ice cream with probiotic culture, S-Yoghurt- sheep ice cream with Yoghurt culture, S-Kefir- sheep ice cream with Kefir culture, S-Probiotic- sheep ice cream with probiotic culture.

Therefore, we can conclude that these two studies proved that there are interesting alternatives to produce new products obtained with SCW and CW, and that the products obtained with sheep SCW and CW were suitable in terms of physical-chemical characteristics. However, the survival of probiotics deserves more attention in future studies.

The use of SCW from sheep and goats in the production of ice cream, especially with probiotic cultures, showed good results in terms of bacterial viability and sensory properties.

In general, sheep ice creams had better sensory acceptance, maintaining good probiotic characteristics during storage. Therefore, we can conclude that both studies indicate that the use of whey concentrates and second cheese whey from sheep and goat milk offers good potential to diversify the supply of dairy products, with nutritional and functional benefits associated with probiotic cultures. However, these products also present challenges regarding microbiological stability during storage.

Mykhalevych et al. (2024) used liquid whey concentrates in the composition of ice cream, combining with other whey protein powders. This study evaluated the main rheological, thermophysical, colour, microstructure, ice crystal analysis and ice cream quality indicators and probiotic character during storage. Thus, whey protein isolate has been

shown to have a significant effect on the rheological properties of ice cream mixes based on hydrolysed whey concentrates, *i.e.,* an increase in viscosity indicators, contributes to the formation of a structure with a greater water retention capacity, ensuring a uniform distribution of air bubbles in the ice cream and contributes to obtaining a product with a high whipping rate.

According to microbiological analysis, the developed ice cream types could be classified as probiotic based on the amount of *Lactobacillus acidophilus* in all samples presenting values not less than 6.2 log CFU/g (Mykhalevych et al. 2024). When we compare this study with ours results, we can state that the use of liquid protein concentrates to make ice cream can improve the general properties of ice cream, as well as helping with sustainability and innovation.

3. Application of powder concentrates obtained by membrane and freeze-drying technologies to develop edible coatings for cheeses

Article 4 describes the use of protein concentrate powders from the second sheep's cheese whey, with the addition of essential oils to produce coatings on food products.

The use of SCW in the production of edible films and coatings and its use as packaging material has been very limited; only one article (Alfano et al. 2022) refers the use of SCW to obtain films. The application of coatings based on whey proteins with antimicrobial compounds or plant extracts in cheeses is also limited (Mileriene et al. 2021; Ramos et al. 2012a; Ramos et al. 2012b).

Liquid protein concentrates of sheep's SCW were obtained using membrane technology (ultrafiltration and diafiltration) The UF/DF retentates were further concentrated by reverse osmosis and then dried by freeze-drying. The obtained dehydrated products had a high protein content (54.7%) and were used to create the cheese coatings (Table 11).

The protein content present in whey powders and in the SCWpowders, is one of the most important requirements to obtain edible films and coatings with optimal characteristics. Comparing the protein content in the SCW concentrate powders of goat and sheep milk the latter has a greater amount (up to 6% more). Due to this reason, coatings were produced with the sheep's SCW powders.

Table 11. Composition of sheep's and goat's second cheese whey powders.

PRODUCTS	Dry matter (%)	Protein (%)	Fat (%)	Ashes (%)	pH
Sheep	96.58±0.08	54.74±1.84	14.81±0.11	8.05±0.86	6.52±0.00
Goat	96.60±0.04	48.82±0.13	3.65±0.42	11.06±0.20	6.36±0.00

This study (article 4) explored the potential of using sheep's SCW, a by-product of whey cheese production, as a major component in the cheese coatings production. The coatings were created by combining sheep's SCW with whey protein isolate (WPI) and glycerol as a plasticizer. The focus of the study was to examine the effects of these coatings,

especially when oregano (*Origanum compactum*) and clary sage (*Salvia sclarea*) essential oils (EOs) were used.

The coatings were prepared by mixing these freeze-dried products from SCW with WPI and using glycerol as a plasticizer, with the final solution of SCW:WPI/plasticizer having 8% protein (w/v) in the ratio 2:1 SCW:WPI and in a protein/plasticizer ratio of 1:1. Essential oils of oregano (*Origanum compactum*) and clary sage (*Salvia sclarea*) were added to these coatings, to improve their antimicrobial properties. The use of EOs in cheeses presents great advantages since they have excellent antimicrobial and antioxidant properties, improve flavour and aroma, present functional benefits for human health, are a natural alternative to the use of synthetic preservatives and help in the sustainability and innovation of new food products.

The coatings were applied to cheeses made with cow's milk. Five types of cheeses with different coatings were compared: one cheese was prepared without coating, another coated with a natamycin solution, a third one with a SCW:WPI coating and the last two coated with SCW:WPI and either oregano or clary sage essential oil. The coatings made with SCW and WPI were compared to a commercial coating containing natamycin, which is typically used to prevent mold growth. The efficiency of the antimicrobial coatings was evaluated by monitoring the physicochemical and microbiological properties of the cheeses during 28 days of ripening.

Coatings made with SCW prevented excessive moisture evaporation of the cheeses, retained moisture better, and cheeses presented lower hardness, chewiness and gumminess values at the end of ripening. The cheeses with the SCW:WPI coatings containing EOs had higher water activity (around 0.930) and moisture content compared to the control samples. These cheeses also showed lower titratable acidity. These results suggest that coatings can influence the texture, making the cheese softer and less chewy, as well as influence the acidity of the cheese, making them more pleasant to taste.

The colour differences between the products with and without coating were not evident. There were slight differences in the colour of the cheeses due to the coatings and ripening time, but the SCW:WPI coatings did not have a significant impact on the colour at the end of the ripening period.

At the microbial level, no major differences were observed among the studied products. Lactobacilli and lactococci counts remained high (log 7-8 CFU/g) in all the products and yeasts and molds counts increased steadily during the ripening period. In cheeses with natamycin coatings they were slightly lower. Overall, there were no significant differences in microbial counts between cheeses with SCW:WPI coatings and cheeses containing commercial coatings, which demonstrates the effectiveness of the developed coatings in preserving the shelf-life of the cheeses.

The results obtained in this study allow to conclude that coatings based on protein from SCW are a promising alternative to commercial coatings and are also another way for small and medium-sized dairy companies to reuse SCW, enhancing the value of this by-product.

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Mileriene et al. 2021, studied an antimicrobial coating made from liquid whey protein concentrate and fortified with chinese cinnamon bark extract (*Cinnamomum cassia*). This study focuses on improving the shelf-life of fresh cheese, using cinnamon for its antimicrobial properties (Mileriene et al. 2021). Thus, these authors intended to extend the shelf-life of fresh, ripened cheese using a bioactive coating, in addition to evaluating the impact of different types of packaging (no packaging and vacuum packaging). The aim was to improve shelf-life and microbiological safety. The study demonstrated that the coating had no effect on pH, lactic acid, protein and fat content and there was also no effect of the coating on the taste of the curd cheese. Thus, the edible coating based on liquid whey protein concentrate with the incorporation of cinnamon extract efficiently extended the shelf-life of perishable fresh curd cheese, increased its functional value and contributed to a more sustainable production process.

In summary, both studies propose innovative alternatives to improve the shelf-life of cheeses, but with different focuses and methodologies. The first study emphasizes a more sustainable and sensorially compatible solution, while the second offers a more effective solution for microbiological control of fresh cheeses, particularly under more controlled storage conditions. For these reasons, the valorisation of sheep SCW is a reliable option, decreasing the environmental impact and using a circular economy concept.

4. Application of powder concentrates obtained by membrane and freeze-drying technologies in edible films

One potential application of sheep's SCW is the production of edible films, which has not been studied to date. In article 5, the properties of the produced edible films were compared with those made with whey protein isolates and with the combination of both dehydrated ingredients. Besides, the obtained films were also made incorporating different percentages of oregano (*Origanum compactum*) essential oil. The addition of oregano essential oil is justified due to its known antimicrobial and antioxidant effects. The films were made, as in the case of the coatings in the article 4, with freeze-dried sheep's second cheese whey (SCW) and whey protein isolates (WPI), with glycerol as the plasticizer. The final protein concentration in the film solution was 8% (w/v). Eleven different films were produced: the first five without essential oil, two with only WPI or SCW, and the remaining three with SCW:WPI ratios of 2:1, 1:1, and 1:2. The remaining six were made with the three SCW:WPI combinations (2:1, 1:1, and 1:2) to which oregano essential oil (*Origanum compactum*) was added at two concentrations.

The water vapour permeability, thickness, dry matter content, water solubility, density, color and opacity, mechanical properties, antioxidant activity, FTIR, TGA, and microstructure by scanning electron microscope (SEM) of the films were evaluated.

The combination of SCW with WPI caused a decrease in the water solubility. The films prepared with SCW showed a solubility of 66.49 ± 3.64% in D.M, while films prepared only with WPI alone showed a solubility of 81.44 ± 0.94% in D.M. Regarding the visual aspect of the developed films, significant colour changes were observed. Films prepared with SCW showed a more yellow colour, decrease in tensile strength (for films prepared

with SCW it was 0.17 ± 0.05 MPa, and for films prepared with WPI it was 1.57 ± 0.73 MPa), elongation at break (for films prepared with SCW it was 3.57 ± 1.18% and for films prepared with WPI it was 52.17 ± 17.32%), puncture resistance (for films prepared with SCW it was 0.20 ± 0.03 MPa and for the films prepared with WPI was 2.40 ± 0.6 6MPa) and puncture deformation (for the films prepared with SCW was 0.93 ± 0.28% and for the films prepared with WPI was 18.92 ± 5.74%). In general, the differences in these values are ten times greater for films prepared with WPI when compared to films prepared with SCW. Thus, the mechanical properties of the films (tensile strength, elongation at break, modulus of elasticity, resistance to puncture and deformation, and modulus of elasticity) were significantly influenced by the replacement of WPI by SCW. The higher the proportion of SCW present in the films, the lower the values of tensile and puncture strength, elongation at break and puncture deformation. These results may have been due to the fact that SCW presents high levels of denatured proteins, soluble peptides, lactose and non-protein nitrogen. This conclusion can also be reinforced from the results presented by SEM, since the surface of the WPI film was smooth, uniform and dense, while the films prepared only with SCW presented a rough surface with aggregates of different sizes.

The addition of EO promoted changes in the microstructure of the films. Larger aggregates and an increase in void sizes were observed in the the blend of both materials, WS 2:1 O2 and in the WS 1:1 and WS 2:1 films at both EO concentrations. The increase in roughness can be attributed to the chemical interactions among the matrix components and EO, which at high concentrations can lead to the formation of agglomerates.

It was also found that depending on the structural characteristics of the proteins present in the films, these interactions are affected. In the WS 2:1 O2 films, the presence of trabeculae that intercept voids was observed. Therefore, these structures are the result of the interaction between the WPI proteins and the molecules of the essential oil, leading to an improvement on the mechanical properties, such as tensile strength and elongation, compared to the WS 2:1 and WS 2:1 O1 films. The above effect was not observed in films with lower WPI content, probably due to the EO concentration exceeding the threshold above which WPI proteins can interact with EO components. It is possible that denatured SCW proteins have a decreased ability to bind EO compounds due to changes in their structure.

Zhang et al. (2024) studied the incorporation of milk-derived antimicrobial peptides (αs_2-casein151–181 and αs_2-casein182–207) into whey protein-based edible films, focusing on their antimicrobial properties and potential applications in cheese packaging. The peptides αs_2-casein151–181 and αs_2-casein182–207 showed antimicrobial activity similar to or even greater than nisin, particularly against *Escherichia coli*. Nisin, although effective, showed weaker activity against Gram-negative bacteria, particularly *E. coli*, in contrast to milk-derived peptides. Whey protein films containing αs_2-casein 151–181 effectively inhibited the growth of yeast, mould, and psychotropic bacteria (which can spoil dairy products) in soft cheeses stored at refrigeration temperatures (*ca.* 4 ˚C). The films also shown effectiveness by controlling the growth of a mixed culture of *E. coli* and

B. subtilis on the cheese surface, preventing contamination during storage for more than 28 days (Zhang et al. 2024).

Zhang et al. (2024) demonstrated in the same study that the mechanical properties were influenced by the incorporation of antimicrobial peptides into whey protein films. It resulted in an increased film opacity and improved water vapour barrier properties, which are important to preserve food quality. However, the films became more brittle, as demonstrated by the reduced tensile strength and elongation at break values. Regarding the stability, the film containing αs_2-casein 151–181 has proved to be the most promising, particularly in saline or acidic solutions, as evidenced by scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). This stability makes it suitable for use in food packaging, where contact with different food environments is common. The study demonstrated that antimicrobial peptides incorporated into films were more effective than direct application of the peptides in solution. This suggests that the controlled release of antimicrobial agents from the films provides a sustained protective effect. Films with αs_2-casein 151–181 also acted as a barrier, preventing post-processing contamination of the cheese during storage (Zhang et al. 2024).

In our study, the addition of EO to the films also influenced their mechanical properties. The films prepared with 1% EO showed lower tensile strength and elongation at break values than those prepared without EO. However, the films prepared with 2% of EO showed higher tensile strength values than the corresponding films with 1% oregano essential oil.

The addition of the EO in the SCW-WPI films has been shown to have positive effect, since it can modify the water solubility (for the SCW-WPI films with Oregano EO) and mechanical properties of these films (only in the SWC-WPI films with 2% EO), and increases the antioxidant activity of the films (the addition of EO to films made with SCW–WPI caused the antioxidant activity to increase, going from 0.97 to 2.19 mg DPPH per gram).

At the structural level, the addition of SCW affects the structure of proteins in the films. Changes were observed in FTIR spectra. Particularly, the bands of the Amide I region (1600-1700 cm^{-1}), which reflect the secondary structure of the proteins. The main types of structures found were β-sheets (more than 56%), in addition to α-helices, β-strands and random chains. With the addition of SCW, shifts occurred, and new bands emerged, indicating changes in protein conformation, especially an increase in β-sheet structures, which are common in heat-denatured proteins (Ramos et al. 2013).

In the region of 2850-2980 cm^{-1}, related to the C—H groups of lipids, there were also changes in the bands, suggesting higher lipid content or changes in the interactions between lipids and proteins, possibly influenced by the high lipid content in SCW. Furthermore, in the range of 3000-3600 cm^{-1}, related to O—H and N—H groups, there was a reduction in the intensity of the peaks, indicating greater interaction through hydrogen bonds in the protein network, which reduces the availability of free hydroxyl groups. In the 800-1150 cm^{-1} region, signals of glycerol and interactions with minerals,

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such as sodium and phosphorus, present in SCW were detected, which increased the interaction between proteins and minerals due to the higher ash content in SCW.

The addition of EO in the films did not influence the FTIR spectra of films probably due to the low amount of added EO as it has been indicated by other authors (Wang et al. 2024).

Bahram et. al. 2014 studied WPI films with two different percentages of cinnamon essential oil added (0.8% and 1.5%). These authors found that a difference in the results analyzed by FTIR only occurred when using cinnamon essential oil at 1.5%. According to these authors, the signal in the control film and in the film incorporated with 0.8% was observed at 3.245 cm^{-1}, but the signal in the film incorporated with 1.5% EO was at 3.232 cm^{-1}. The same was observed for the signals at 1.627 and 1.025 cm^{-1}, which became less describable and flatter when 1.5% EO was added to the film. In general, the authors describe that these differences in absorption bands and their intensity can be attributed to the coverage of the protein's functional groups, such as –NH and –OH, with the functional groups of cinnamon essential oil and some special interactions that made them less invisible in the spectra. The other signals appear the same for the control film as well as for the films with 0.8 and 1.5% EO (Bahram et al. 2014).

In order to study the crystalline structure of the film samples, X-Ray Diffraction (XRD) analysis was carried out. Figure 7a in article 5, presented the diffractograms of WPI, SCW, and the blend of both (WS) films. Figure 7a reveals the occurrence of structural changes in two angular zones, indicating that the WPI, SCW and WS films can have a semi-crystalline structure. The fractionograms of WPI films exhibit a poorly structured system, but the addition of SCW in varying proportions (WS) results in the formation of more crystalline structures. These changes are likely due to the involvement of inter- or intramolecular contacts of polymer chains or aggregates, which cause the structure to become more crystalline. When proteins are denatured to prepare WPI, SCW, and WS films through heating, conformational changes occur in the whey proteins, which can induce the formation of new peaks in the diffractograms when new rearrangements occur to form the films. When we added essential oils to the WS films, we observed changes in signal intensity. These differences in intensity were also observed with the amount of SCW added to the WPI films (Figure 7b in article 5). These differences in intensity were also observed with the amount of SCW added to the WPI films. For the WS 1:2 films with added EO, the relative intensities of the 3.8, 5.8, and 9.8º peaks were higher when compared to the WS 1:2 films without EO, and this may be because the essential oil interacted with the denatured whey proteins when present in a higher proportion and then increased the crystallinity of the films.

Amjadi et. al. 2021 studied the crystal structure of WPI films without and with the addition of essential oil from an orange peel nanoemulsion using X-Ray Diffraction (XRD). The authors observed that the diffraction spectral profiles of the film samples with EO showed no new peaks or peak movement when compared to the WPI-based film. However, they also observed an increase in the intensity of these signals, which the authors explained by the addition of orange peel nanoemulsions that improves the

crystallinity of the WPI-based films. Furthermore, the authors stated that the results obtained demonstrated the compatibility between the incorporated biopolymer and the EO (Amjadi et al. 2021).

Regarding the thermal stability of the developed films analyzed by thermogravimetric analysis (TGA) and their derivatives (DTG), some differences were observed. We evaluated the thermal degradation behavior of WPI, SCW, and WS films, as well as those with the addition of essential oil (EO). The results obtained showed that the films' weight loss occurs in four distinct phases during heating, reflecting the processes of evaporation, decomposition, and carbonization.

The first phase involves the evaporation of free water and hydrogen-bonded water, resulting in a 9.4% mass loss. The second phase involves the decomposition of glycerol, the vaporization of structural water, and the degradation of low-molecular-weight compounds, such as essential oils. The third phase involves polymer degradation, and the final phase involves carbonization.

Films containing only WS showed lower mass loss in the first and second phases, especially as the amount of SCW increased. Thus, the interactions between proteins and denatured lipids in SCW increase thermal stability. In the third phase, the greatest loss observed was in WS films at a 1:2 ratio. Overall, the total mass loss in the films ranged from 86.3% to 88.6%, leaving residues between 11.3% and 13.7%. Films with a higher WPI and SCW ratio (2:1 ratio) had higher residual mass, indicating greater thermal resistance. The addition of EO did not significantly alter the mass loss or degradation peaks. The temperature peaks identified in the DTG technique occurred around 63 °C, 193°C, and 313°C in all films, corresponding to different degradation processes. Furthermore, in the WS films, additional peaks were detected at 246–252 °C and 257 °C, indicating chain structures with different levels of thermal stability. The addition of EO nas quantiddades de 1 e 2% may influence thermal stability dos filmes in a subtle way.

The antioxidant activity of the WPI, SCW and WS films were analised by DPPH method (2,2-diphenyl-1-picrylhydrazyl). For the WPI films with the various amounts of SCW, the antioxidant activity did not show great differences between them.

However, the addition of EO in two diferents percentages (1% and 2%) significantly increased the antioxidant activity of the films (from 0.97–1.19 mg DPPH/g Film in the samples without EO to 2.00–2.19 mg DPPH/g Film in the films with 2% EO). As expected, samples with 2% EO showed higher values than those with 1% EO. The antioxidant activity of bioactive films incorporating EOs is affected by the EO concentration.

Khan *et. al.,* conducted an extensive review explaining that depending on the concentration of EO used to make films of various types, the antioxidant and antimicrobial properties are affected. The greater the amount of EO used in the film, the better its antioxidant activity and antibacterial properties (Khan et al. 2023). Seydim *et al.* found that WPI films containing garlic or oregano EOs applied to sliced Kasar cheese restricted microbial growth, thus ensuring a longer shelf-life (Seydim et al. 2020).

In conclusion the incorporation of SCW and EO influenced the secondary structure of proteins, the thermal stability, microstructure and crystallinity of the films.

Further research is still needed to determine whether the combination of SCW and EO can be beneficial or detrimental to the thermal stability of films, especially considering their application in food packaging. However, despite improving these properties, the use of EO in edible films also affects sensory properties.

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CONCLUSIONS

1.Regarding the use of liquid whey concentrates and liquid second whey concentrates in the manufacture of innovative diary products

1. The application of ultrafiltration (UF) to sheep and goat cheese whey allowed to obtain liquid whey concentrates (LWC) with high protein concentration (16.4% for sheep and 7.8% for goats). Differences in the texture were observed between symbiotic kefirs manufactured with these ovine or caprine LWC, showing kefirs obtained with ovine LWC a solid-like texture while kefirs based on caprine LWC were liquid. All samples presented counts of lactic acid bacteria higher than log 7 CFU/mL, indicating the good adaptation of microorganisms to the matrixes. Further work must be undertaken to improve the sensory acceptability of the ovine and caprine whey-based symbiotic kefirs.

2. The use of UF to bovine whey before manufacture of whey cheese (WC) could be very useful to reduce the energy consumption of the manufacturing process. The incorporation of probiotic cultures (*Lactobacillus acidophilus*) reduced pH values, increased the titratable acidity and increased the firmness of the UF concentrated bovine WC. The addition of a protective culture (*Lacticaseibacillus paracasei* and *Lacticaseibacillus rhamnosus*) to these probiotic whey cheeses reduced the levels of yeasts and molds at the end of the refrigerated storage. Besides, the WC with a probiotic plus a bioprotective culture obtained the best global sensory evaluation. It can be concluded that the addition of protective cultures with *Lacticaseibacillus* strains can be very useful to prolong the shelf life of whey cheeses while also improving the sensory evaluation of whey cheeses and providing the health benefits associated with the consumption of probiotics.

3. The application of UF to concentrate ovine and caprine second cheese (SCW) whey originated liquid second cheese whey concentrates (LSCWC) which can be directly used in the production of ice creams with inulin and different starter cultures (yoghurt, kefir and probiotic commercial cultures). These sheep and goat ice creams presented significant differences, specifically the highest total solids and protein contents of sheep ice creams. The ovine ice creams also presented higher hardness and complex viscosity, higher overrun and lower meltdown rates. The probiotic characteristics of sheep and goat ice creams were maintained for at least 2 months. All products were well accepted by the consumer panel, but sheep ice creams were better rated regarding aroma, taste and texture. The addition of prebiotics and the production of fermented products containing probiotic bacteria increases the nutritional value of LSCWC and their market potential.

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4. To conclude, the application of ultrafiltration to concentrate bovine, ovine and caprine CW and SCW originates LWCs and LSCWC which can be directly used in the development of novel dairy products such as synbiotic kefir, probiotic whey cheeses with protective cultures and synbiotic ice creams. The results of the present work have practical applications in small and medium scale dairy plants by reducing their environmental impact from the direct discharge of CW and SCW and improving their profitability via value-added products, which is crucial for sustainable rural development.

2.Regarding the use of ovine second cheese whey powder to obtain edible films and coatings

1. Ovine second cheese whey (SCW) powder, obtained by the ultrafiltration/diafiltration of SCW followed by reverse osmosis and freeze drying, was used as a component of cheese coatings containing whey protein isolate (WPI) and oregano (*Origanum compactum*) and clary sage (*Salvia sclarea*) essential oils (EO). These SCW-WPI coatings prevent the cheeses from excessive evaporation, and these cheeses have lower values of hardness, chewiness, and gumminess at the end of ripening in comparison to cheeses with a commercial coating containing natamycin. Coatings based on SCW does not affect the viability of the starter bacteria cultures. In conclusion, SCW-WPI cheese coatings can successfully substitute commercial coatings with the advantage of being edible packaging materials manufactured with by-products.

2. Edible films using ovine SCW powder, alone or combined with WPI were developed. The films with only SCW or a high proportion of SCW showed the worst mechanical properties that can limit their application in foods. The addition of oregano EO increased the antioxidant activity of the films but also modified other properties such as the water solubility and the tensile strength. The mixture of WPI and SCW at a 2:1 proportion, with the addition of 2% oregano essential oil, yielded films that exhibited the highest values of tensile strength and elasticity, and good antioxidant capacity. Consequently, these films can be regarded as the most efficient films for food packaging among those examined in this study. Both SCW and EO incorporations influenced the secondary structure of proteins and the thermal stability, microstructure and crystallinity of the films.

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FUTURE PERSPECTIVES

1.Future perspectives

Based on the studies presented in this doctoral thesis, the future perspectives for the use of cheese whey (CW) and second cheese whey (SCW) are quite promising. Here are some directions that can be explored, as some problems have emerged in the creation of new food products as well as in the creation of edible coatings and films.

Evaluation of Production Parameters: To make commercial application viable, it is essential to study practical aspects, such as production scale, costs, drying time and compatibility with existing industrial processes. This will facilitate the transition from the laboratory to large-scale production.

Sustainability and Valorisation of By-products: Using CW and SCW, a by-product that is often discarded, contributes to the sustainability of the dairy production chain. Future research may explore the use of other agro-industrial by-products or residues in the formulation of new products with improved nutritional and functional properties as well as new edible coatings and films also with improved functional, mechanical, and barrier properties in order to promote the circular economy and sustainability.

Masking the Flavour of Products: One of the main difficulties presented in the new food products produced with SCW is the characteristic flavour, which may not be attractive to consumers. Future research focusing on incorporating ingredients that mask or balance this flavour, such as natural flavourings, spices, or ingredients that complement the sensory profile of the final product is a promising strategy.

Optimization of Film Properties: Different factors, such as the pH of the film forming solution, alternative plasticizer types, and drying temperatures, can improve the mechanical, barrier, and thermal stability properties of films. This will make the coatings more resistant and suitable for application a real scenario.

Enhanced Antimicrobial Properties: Although coatings and films with oregano and sage essential oils have shown potential, their effectiveness against fungi and yeasts can still be improved. Future research may explore combinations of antimicrobial ingredients (different essential oils or fruits) or incorporation techniques that increase the controlled release of these compounds, extending product protection.

Applications in Various Products: In addition to cheese, these films can be adapted to protect other sensitive foods, such as fruits, vegetables, meats or bakery products. Studies can evaluate the effectiveness of these coatings in different contexts, considering factors such as humidity, temperature and storage time.

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Annex I.

Suplementary tables:

ARTICLE 1

Supplementary Tables 1 and 2 in article 1 are available in the original article as Table S1 and Table S2.

Table S1.-Color difference values (ΔEab*) between different sheep's kefir products, and for the same product over storage.

Table S2.-Color difference values (ΔEab*) between different goat's kefir products, and for the same product over storage.

ARTICLE 2Supplementary Tables 1, 2 and 3 in article 2 are available in the original article as Table S1, S2 and S3.

Table S1. Physichochemical parameters of whey cheese samples. Average values and standard deviation (±).

Table S2. CIEL*a*b* parameters of whey cheese samples. Average values and standard deviation (±).

Table S3. Microbiological counts of whey cheese samples. Average values and standard deviation (±).

ARTICLE 4Supplementary Tables 1, 2, 3, and 4 in article 4 are available in the original article as Table S1, S2, S3 and Table S4.

Table S1. Physicochemical parameters of cheese samples. Average values and standard deviation (±).

Table S2. CIEL*a*b* parameters of cheese samples. Average values and standard deviation (±).

Table S3. Texture parameters of cheese samples. Average values and standard deviation (±).

Table S4. Microbiological counts of cheese samples (log CFU/g). Average values and standard deviation (±).

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ARTICLE 1 **Table S1.-**Color difference values (ΔEab*) between different sheep's kefir products, and for the same product over storage.

	EK 1d	EKABT5 1d	TK 1d	TKABT5 1d	EK 10d	EKABT5 10d	TK 10d	TKABT5 10d	EK 20d	EKABT5 20d	TK 20d	TKABT5 20d	EK 30d	EKABT5 30d	TK 30d
EKABT5 1d	40.4														
TK 1d	18.3	4.6													
TKABT5 1d	0.8	31.3	12.7												
EK 10d	12.9	91.0	58.1	17.2											
EKABT5 10d	2.3	33.7	15.6	1.3	14.0										
TK 10d	6.5	43.8	24.7	5.4	10.5	1.7									
TKABT5 10d	8.2	74.0	45.1	10.9	1.0	7.9	5.2								
EK 20d	25.0	2.2	1.2	17.6	66.9	20.0	28.3	52.4							
EKABT5 20d	5.3	33.2	16.8	2.9	16.5	1.4	1.6	9.6	19.1						
TK 20d	24.9	13.5	10.8	17.1	50.9	14.3	15.4	37.9	7.7	10.2					
TKABT5 20d	12.5	15.7	7.5	7.4	34.8	5.5	7.6	24.3	7.6	4.1	2.2				
EK 30d	51.4	0.8	8.9	40.8	105.6	42.8	52.9	86.9	5.0	41.2	16.1	20.8			
EKABT5 30d	50.3	0.9	8.9	39.7	102.6	41.0	50.3	84.2	4.8	39.2	14.1	19.0	0.1		
TK 30d	59.1	2.3	12.9	47.4	114.2	48.5	57.9	94.6	7.8	46.2	17.3	23.7	0.5	0.4	
TKABT5 30d	60.2	2.2	12.9	48.5	117.2	50.4	60.6	97.4	7.9	48.1	19.2	25.5	0.4	0.5	0.1

Table S2.-Color difference values (ΔEab*) between different goat's kefir products, and for the same product over storage.

	EK 1d	EKABT5 1d	TK 1d	TKABT5 1d	EK 10d	EKABT5 10d	TK 10d	TKABT5 10d	EK 20d	EKABT5 20d	TK 20d	TKABT5 20d	EK 30d	EKABT5 30d	TK 30d
EKABT5 1d	2.6														
TK 1d	1.7	1.8													
TKABT5 1d	0.2	4.0	2.3												
EK 10d	2.3	8.8	4.2	1.5											
EKABT5 10d	2.5	9.2	5.0	1.7	0.1										
TK 10d	2.4	8.6	3.9	1.6	0.0	0.2									
TKABT5 10d	2.3	8.5	3.8	1.5	0.0	0.2	0.0								
EK 20d	3.7	11.4	6.0	2.5	0.2	0.2	0.2	0.2							
EKABT5 20d	2.2	8.5	4.2	1.4	0.0	0.1	0.0	0.0	0.2						
TK2 0d	3.4	11.1	6.0	2.4	0.2	0.1	0.3	0.3	0.0	0.2					
TKABT5 20d	3.7	11.6	6.4	2.6	0.2	0.2	0.3	0.3	0.0	0.2	0.0				
EK 30d	49.2	66.4	47.0	45.2	30.8	31.9	30.3	30.6	27.6	31.8	29.0	28.2			
EKABT5 30d	60.3	78.6	57.1	55.9	39.9	41.3	39.3	39.6	36.3	41.0	37.9	37.0	0.6		
TK 30d	59.9	78.9	57.5	55.3	39.3	40.6	38.8	39.1	35.6	40.4	37.1	36.2	0.6	0.1	
TKABT5 30d	57.6	76.3	55.4	53.1	37.4	38.7	36.9	37.3	33.8	38.5	35.4	34.4	0.4	0.2	0.0

ARTICLE 2

Table S1. Physicochemical parameters of whey cheese samples. Average values and standard deviation (±).

DRY MATTER			
Storage time (days)	**C**	**LA5**	**LA5FQ4**
1	16.83 ± 0.36 a,A	19.50 ±0.77 a,B	18.82 ± 0.28 a,b,B
7	18.66 ± 0.59 b,A	19.90 ± 0.25 a,B	20.61 ± 0.34 b,B
14	19.23 ± 0.33 b,A	21.46 ± 3.10 a,A	20.04 ± 1.82 a,b,A
21	18.11 ± 0.36 b,A	19.44 ± 0.43 a,B	19.25 ± 0.52 a,b,B
28	16.07 ± 0.51 a,A	19.04 ± 1.42 a,B	18.10 ± 0.71 a,A,B
aw			
Storage time (days)	**C**	**LA5**	**LA5FQ4**
1	0.934 ± 0.00 a,A	0.936± 0.00 a,b,A	0.943 ± 0.00 b,c,B
7	0.937 ± 0.00 c,A	0.944 ± 0.00 c,A,B	0.946 ± 0.00 c,B
14	0.938 ± 0.00 c,A	0.939 ± 0.00 b,c,A	0.940 ± 0.00 b,A
21	0.937 ± 0.00 b,c,A	0.938 ± 0.00 b,c,A	0.939 ± 0.00 b,A
28	0.935 ± 0.00 a,b,B	0.932 ± 0.00 a,A	0.933 ± 0.00 a,A
pH			
Storage time (days)	**C**	**LA5**	**LA5FQ4**
1	6.67 ± 0.06 b,C	5.27 ± 0.06 a,b,A	5.80 ± 0.10 d,B
7	6.63 ± 0.06 b,B	5.37 ± 0.06 b,c,A	5.43 ± 0.06 a,b,A
14	6.93 ± 0.06 c,C	5.47 ± 0.06 c,A	5.67 ± 0.06 c,d,B
21	6.53 ± 0.15 b,B	5.27 ± 0.06 b,A	5.23 ± 0.06 a,b,A
28	6.17 ± 0.12 a,C	5.07 ± 0.06 a,A	5.47 ± 0.12 b,c,B
TITRATABLE ACIDITY			
Storage time (days)	**C**	**LA5**	**LA5FQ4**
1	0.035 ± 0.01 a,A	0.208 ± 0.01 a,C	0.164 ± 0.01 a,B
7	0.089 ± 0.00 b,A	0.431 ± 0.01 c,C	0.399 ± 0.01 d,B
14	0.100 ± 0.01 b,A	0.570 ± 0.02 d,C	0.246 ± 0.02 b,B
21	0.101 ± 0.00 b,A	0.378 ± 0.00 b,C	0.261 ± 0.01 b,B
28	0.176 ± 0.01 c,A	0.451 ± 0.01 c,C	0.332 ± 0.02 c,B

Different superscript letters (a,b,c,d) indicate differences for the same product in different ripening days. Superscript capital letters (A,B,C) indicate differences between products at the same ripening day.

Table S2. CIEL*a*b* parameters of whey cheese samples. Average values and standard deviation (±).

L*			
Storage time (days)	**C**	**LA5**	**LA5FQ4**
1	86.90 ± 1.49 $_{a,b,B}$	87.23 ± 1.79 $_{a,B}$	82.00 ± 0.35 $_{a,A}$
7	82.17 ± 2.07 $_{a,A}$	87.10 ± 3.20 $_{a,A,B}$	91.17 ± 3.20 $_{b,B}$
14	83.90 ± 3.56 $_{a,b,A}$	87.00 ± 2.76 $_{a,A}$	87.13 ± 2.37 $_{a,b,A}$
21	88.00 ± 2.82 $_{a,b,A}$	86.70 ± 0.61 $_{a,A}$	87.90 ± 2.51 $_{a,b,A}$
28	89.83 ± 0.51 $_{b,B}$	90.43 ± 1.85 $_{a,B}$	84.23 ± 2.29 $_{a,A}$
a*			
Storage time (days)	**C**	**LA5**	**LA5FQ4**
1	-3.43 ± 0.21 $_{a,B}$	-2.57 ± 0.23 $_{a,A}$	-2.8 ± 0.26 $_{a,A}$
7	-3.07 ± 0.32 a,A	-3.50 ± 0.40 b,c,A	-2.93 ± 0.06 a,A
14	-3.20 ± 0.62 a,A	-3.77 ± 0.15 c,A	-3.13 ± 0.31 a,A
21	-2.43 ± 0.06 a,A	-2.57 ± 0.45 a,A	-2.70 ± 0.17 a,A
28	-2.70 ± 0.40 a,A	-2.63 ± 0.32 a,b,A	-3.10 ± 0.17 a,A

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b*			
Storage time (days)	**C**	**LA5**	**LA5FQ4**
1	6.27 ± 0.55 ^{a,A}	5.80 ± 0.20 ^{a,A}	6.53 ± 1.17 ^{a,A}
7	7.83 ± 0.06 ^{a,A,B}	8.87 ± 0.80 ^{c,B}	7.50 ± 0.40 ^{a,A}
14	8.13 ± 1.95 ^{a,A}	8.80 ± 0.30 ^{c,A}	6.87 ± 0.45 ^{a,A}
21	6.77 ± 0.29 ^{a,A}	6.97 ± 0.51 ^{a,b,A}	6.77 ± 0.64 ^{a,A}
28	6.17 ± 0.15 ^{a,A}	7.30 ± 0.26 ^{b,B}	7.40 ± 0.52 ^{a,B}

Different superscript letters (a,b,c) indicate differences for the same product in different ripening days. Superscript capital letters (A,B) indicate differences between products at the same ripening day.

Table S3. Microbiological counts of whey cheese samples. Average values and standard deviation (±).

LACTOCOCCUS			
Storage time (days)	**C**	**LA5**	**LA5FQ4**
1	6.46 ± 0.14 ^{a,A}	8.18 ± 0.10 ^{a,B}	8.81 ± 0.03 ^{b,C}
7	7.90 ± 0.03 ^{b,A}	8.53 ± 0.21 ^{a,B}	8.08 ± 0.11 ^{a,A}
14	8.51 ± 0.34 ^{c,A}	9.47 ± 0.12 ^{b,B}	9.33 ± 0.18 ^{c,B}
21	8.71 ± 0.16 ^{c,A}	8.80 ± 0.03 ^{a,A}	9.21 ± 0.01 ^{c,B}
28	8.32 ± 0.31$^{b, c,A}$	8.21 ± 0.39 ^{a,A}	9.06 ± 0.06 ^{b,c,B}
LACTOBACILLUS			
Storage time (days)	**C**	**LA5**	**LA5FQ4**
1	5.49 ± 0.02 ^{a,A}	8.27 ± 0.08 ^{a,B}	8.89 ± 0.01 ^{b,C}
7	5.96 ± 0.20 ^{a,A}	8.33 ± 0.21 ^{a,B}	8.21 ± 0.26 ^{a,B}
14	7.03 ± 0.38 ^{b,A}	9.36 ± 0.20 ^{b,B}	9.19 ± 0.04 ^{b,B}
21	7.23 ± 0.53 ^{b,A}	8.44 ± 0.33 ^{a,B}	9.19 ± 0.09 ^{b,C}
28	8.43 ± 0.62 ^{d,A}	8.48 ± 0.25 ^{a,A}	9.18 ± 0.07 ^{b,B}
YEASTS AND MOULDS			
Storage time (days)	**C**	**LA5**	**LA5FQ4**
1	4.75 ± 0.35 ^{a,A}	5.74 ± 0.25 ^{a,B}	5.27 ± 0.24 ^{a,b,A,B}
7	4.10 ± 0.20 a,A	5.93 ± 0.40 ^{a,B}	5.73 ± 0.51 ^{a,b,B}
14	6.61 ± 0.44 ^{b,c,A}	6.24 ± 0.95 ^{a,A}	6.27 ± 0.25 ^{b,A}
21	7.46 ± 0.14 ^{c,B}	5.65 ± 0.27 ^{a,A}	5.02 ± 1.04 ^{a,A}
28	6.48 ± 0.37 ^{b,B}	6.72 ± 0.34 ^{a,B}	5.74 ± 0.30 ^{a,b,A}

Different superscript letters (a,b,c,d) indicate differences for the same product in different ripening days. Superscript capital letters (A,B,C) indicate differences between products at the same ripening day.

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Table S1. Physicochemical parameters of cheese samples. Average values and standard deviation (±).

Storage time (days)	C	N	WC	WCO	WCS
			DRY MATTER (%w/w)		
1	52.9 ± 0.7 ^{a,A}	53.1 ± 0.6 ^{a,A}	53.8 ± 0.8 ^{a,A}	56.5 ± 1.0 ^{a,B}	54.3 ± 0.7 ^{a,A}
7	62.1 ± 2.5 ^{b,A}	64.7 ± 1.1 ^{b,A}	64.4 ± 4.7 ^{b,c,A}	65.3 ± 2.1 ^{a,A}	61.5 ± 3.7 ^{b,A}
14	61.8 ±3.2 ^{b,A}	63.7 ±1.6 ^{b,A}	61.1 ±3.2 ^{a,b,A}	63.5 ±1.1 ^{a,A}	62.7 ±1.1 ^{b,A}
21	63.4 ± 3.1 ^{b,A}	68.4 ± 4.1 ^{b,A}	71.6 ± 3.6 ^{c,A}	65.0 ± 5.6 ^{a,A}	66.6 ± 2.2 ^{b,A}
28	64.9 ± 4.5 ^{b,A}	65.6 ± 1.9 ^{b,A}	70.1 ± 4.1 ^{b,c,A}	60.5 ± 4.8 ^{a,A}	61.8 ± 1.3 ^{b,A}
			WATER ACTIVITY		
1	0.933 ± 0.0 ^{b,A,B}	0.932 ± 0.0 ^{ab,A}	0.944 ± 0.0 ^{c,C}	0.942 ± 0.0 ^{c,B,C}	0.938 ± 0.0 ^{b,A,B,C}
7	0.934 ± 0.0 ^{b,A,B}	0.939 ± 0.0 ^{b,B}	0.935 ± 0.0 ^{b,A,B}	0.936 ± 0.0 ^{b,A,B}	0.929 ± 0.3 ^{a,A}
14	0.937 ± 0.0 ^{b,A}	0.935 ± 0.0 ^{b,A}	0.936 ± 0.0 ^{b,A}	0.939 ± 0.0 ^{b,c,A}	0.936 ± 0.0 ^{ab,A}
21	0.933 ± 0.0 ^{b,A}	0.937 ± 0.0 ^{b,A}	0.933 ± 0.0 ^{b,A}	0.936 ± 0.0 ^{b,A}	0.937 ± 0.0 ^{ab,A}
28	0.921 ± 0.0 ^{a,A}	0.924 ± 0.0 ^{a,A}	0.920 ± 0.0 ^{a,A}	0.930 ± 0.0 ^{a,B}	0.932 ± 0.0 ^{ab,B}
			pH		
1	5.6 ± 0.1 ^{a,A}	5.5 ± 0.1 ^{a,A}	5.5 ± 0.1 ^{a,b,A}	5.5 ± 0.0 ^{a,A}	5.4 ± 0.1 ^{a,A}
7	5.6 ± 0.1 ^{a,A}	5.6 ± 0.1 ^{a,A}	5.6 ± 0.1 ^{a,b,A}	5.5 ± 0.1 ^{a,A}	5.5 ± 0.0 ^{a,b,A}
14	5.5 ± 0.1 ^{a,A}	5.6 ± 0.1 ^{a,A}	5.5 ± 0.0 ^{a,A}	5.5 ± 0.1 ^{a,A}	5.6 ± 0.1 ^{b,c,A}
21	5.6 ± 0.1 ^{a,A}	5.5 ± 0.1 ^{a,A}	5.7 ± 0.0 ^{c,B}	5.5 ± 0.1 ^{a,A}	5.5 ± 0.0 ^{a,b,A}
28	5.7 ± 0.1 ^{a,A}	5.5 ± 0.2 ^{a,A}	5.6 ± 0.1 ^{b,c,A}	5.4 ± 0.1 ^{a,A}	5.6 ± 0.0 ^{c,A}
			TITRATABLE ACIDITY (% lactic acid)		
1	0.37 ± 0.0 ^{c,C}	0.19 ± 0.0 ^{a,A,B}	0.21 ± 0.0 ^{a,b,A,B}	0.23 ± 0.0 ^{b,B}	0.14 ± 0.0 ^{a,A}
7	0.27 ± 0.0 ^{b,c,B}	0.22 ± 0.0 ^{a,A,B}	0.24± 0.0 ^{b,B}	0.24 ± 0.0 ^{b,B}	0.16 ± 0.0 ^{a,A}
14	0.22 ± 0.0 ^{a,b,C}	0.19 ± 0.0 ^{a,B,C}	0.13 ± 0.0 ^{a,A,B}	0.12 ± 0.0 ^{a,A}	0.12 ± 0.0 ^{a,A,B}
21	0.16 ± 0.0 ^{a,A}	0.17 ± 0.1 ^{a,A}	0.19 ± 0.1 ^{a,b,A}	0.12 ± 0.0 ^{a,A}	0.12 ± 0.1 ^{a,A}
28	0.21 ± 0.1 ^{a,b,B}	0.17 ± 0.0 ^{a,A,B}	0.13 ± 0.0 ^{a,A,B}	0.11± 0.0 ^{a,A}	0.13 ± 0.0 ^{a,A,B}

C-Control; N-cheese with natamycin; WC-cheese with SCW coating; WCO-cheese with SCW coating with oregano essential oil; WCS- cheese with SCW coating with clary sage essential oil. Different superscript letters (a,b,c) indicate significant differences for the same product in different ripening days. Superscript capital letters (A,B,C) indicate significant differences between products at the same ripening day.

Table S2. CIEL*a*b* parameters of cheese samples. Average values and standard deviation (±).

Storage time (days)	C	N	WC	WCO	WCS
			L*RIND		
1	92.0 ± 0.2 ^{d,A}	91.7 ± 0.5 ^{d,A}	91.9 ± 0.7 ^{d,A}	92.4 ± 0.3 ^{d,A}	92.7 ± 0.2 ^{d,A}
7	72.6 ± 0.5 ^{c,A}	71.2 ± 0.2 ^{c,A}	72.6 ± 0.4 ^{c,A}	75.4 ± 1.0 ^{c,B}	75.0 ± 0.9 ^{c,B}
14	69.3 ± 0.1 ^{c,B}	67.8 ± 0.8 ^{b,A}	69.6 ± 0.4 ^{b,B}	69.0 ± 0.4 ^{b,A,B}	68.5 ± 0.5 ^{b,A,B}
21	61.2 ± 2.3 ^{a,A}	65.8 ± 0.2 ^{a,B}	65.3 ± 0.2 ^{a,B}	63.7 ± 1.2 ^{a,b,A,B}	65.7 ± 0.3 ^{a,b,B}
28	65.0 ± 1.8 ^{b,A}	67.1 ± 0.7 ^{a,b,A}	66.0 ± 0.7 ^{a,A}	63.4 ± 4.2 ^{a,A}	64.9 ± 0.2 ^{a,A}
			a*RIND		
1	-4.2 ± 0.2 ^{a,A,B}	-4.5 ± 0.1 ^{a,A,B}	-4.2 ± 0.4 ^{a,A,B}	-4.6 ± 0.2 ^{a,B}	-3.9 ± 0.5 ^{a,A}
7	-6.3 ± 0.3 ^{d,A}	-5.8 ± 0.2 ^{b,c,A}	-6.3 ± 0.1 ^{c,A}	-6.1 ± 1.0 ^{c,A}	-6.0 ± 0.3 ^{c,A}
14	-5.4 ± 0.2 ^{c,A}	-5.9 ± 0.4 ^{c,A}	-5.7 ± 0.4 ^{b,c,A}	-5.6 ± 0.3 ^{b,c,A}	-5.9 ± 0.1 ^{c,A}
21	-4.8 ± 0.1 ^{b,A,B}	-5.7 ± 0.4 ^{b,c,C}	-5.4 ± 0.2 ^{b,B,C}	-4.8 ± 0.2 ^{a,A}	-5.1 ± 0.2 ^{b,A,B,C}
28	-5.2 ± 0.2 ^{b,c,A}	-5.1 ± 0.3 ^{a,b,A}	-5.3 ± 0.2 ^{b,A}	-5.0 ± 0.3 ^{a,b,A}	-5.2 ± 0.2 ^{b,A}
			b*RIND		
1	15.1 ± 0.3 ^{a,A}	15.4 ± 0.5 ^{a,A}	15.2 ± 0.7 ^{a,A}	16.0 ± 0.1 ^{a,A}	14.8 ± 0.6 ^{a,A}
7	21.9 ± 1.0 ^{c,A,B}	22.3 ± 0.2 ^{c,B}	21.5 ± 0.7 ^{b,A,B}	20.1 ± 0.9 ^{b,A}	21.0 ± 0.8 ^{c,d,A,B}
14	20.5 ± 0.3 ^{c,A}	22.0 ± 0.4 ^{c,B}	21.8 ± 0.7 ^{b,B}	21.8 ± 0.2 ^{b,B}	21.9 ± 0.6 ^{d,B}
21	18.0 ± 0.4 ^{b,A,B}	19.7 ± 0.2 ^{b,A,B}	15.2 ± 3.7 ^{a,A}	19.8 ± 0.2 ^{b,B}	19.2 ± 0.2 ^{b,A,B}
28	18.4 ± 0.6 ^{b,A}	19.7 ± 0.4 ^{b,A}	20.7 ± 0.5 ^{b,A}	20.6 ± 2.1 ^{b,A}	19.5 ± 0.6 ^{b,c,A}
			L*PASTE		
1	92.3 ± 0.1 ^{c,A}	92.0 ± 0.2 ^{b,A}	91.7 ± 0.7 ^{b,A}	92.6 ± 0.4 ^{b,A}	92.6 ± 0.5 ^{b,A}
7	83.5 ± 2.7 ^{a,A}	87.8 ± 0.2 ^{a,b,B}	87.0 ± 1.0 ^{a,A,B}	87.0 ± 1.2 ^{a,A,B}	86.0 ± 1.2 ^{a,A,B}

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14	87.7 ± 0.4 ^{b,A}	87.7 ± 0.6 ^{a,b,A}	87.5 ± 0.2 ^{a,A}	86.4 ± 1.1 ^{a,A}	85.1 ± 2.7 ^{a,A}
21	84.9 ± 1.1 ^{a,b,A}	85.2 ± 0.9 ^{a,A}	85.9 ± 0.7 ^{a,A}	84.7 ± 3.2 ^{a,A}	85.6 ± 1.7 ^{a,A}
28	83.8 ± 0.4 ^{a,A}	85.8 ± 4.1 ^{a,A}	84.9 ± 2.7 ^{a,A}	85.4 ± 1.2 ^{a,A}	86.3 ± 0.6 ^{a,A}
			a*PASTE		
1	-3.7 ± 0.1 ^{a,A,B}	-3.9 ± 0.1 ^{a,B,C}	-4.1 ± 0.3 ^{a,b,C}	-3.3 ± 0.1 ^{a,A}	-4.8 ± 0.2 ^{a,D}
7	-4.9 ± 0.2 ^{b,c,A}	-4.8 ± 0.3 ^{a,A}	-4.8 ± 0.2 ^{b,A}	-4.9 ± 0.2 ^{b,A}	-4.7 ± 0.3 ^{a,A}
14	-4.4 ± 0.3 ^{b,A}	-4.4 ± 0.1 ^{a,A}	-4.2 ± 0.4 ^{a,b,A}	-4.1 ± 0.5 ^{a,b,A}	-4.2 ± 0.5 ^{a,A}
21	-4.8 ± 0.3 ^{b,c,A}	-4.5 ± 0.4 ^{a,A}	-4.7 ± 0.4 ^{a,b,A}	-3.8 ± 0.5 ^{a,A}	-4.6 ± 0.3 ^{a,A}
28	-5.4 ± 0.3 ^{c,A}	-4.5 ± 1.2 ^{a,A}	-4.0 ± 0.2 ^{a,A}	-4.1 ± 0.3 ^{a,b,A}	-4.3 ± 0.1 ^{a,A}
			b*PASTE		
1	13.8 ± 0.1 ^{a,A}	14.6 ± 0.2 ^{a,A,B}	15.2 ± 0.9 ^{a,B,C}	13.3 ± 0.5 ^{a,A}	16.0 ± 0.3 ^{a,C}
7	18.9 ± 1.3 ^{c,A}	18.4 ± 0.6 ^{a,A}	18.3 ± 0.1 ^{b,A}	17.3 ± 0.3 ^{b,A}	18.1 ± 0.4 ^{b,A}
14	16.4 ± 0.3 ^{b,A}	16.7 ± 0.3 ^{a,A}	16.4 ± 0.5 ^{a,A}	16.1 ± 0.6 ^{b,A}	16.6 ± 1.0 ^{a,b,A}
21	17.5 ± 0.6 ^{b,c,A}	16.9 ± 3.2 ^{a,A}	18.9 ± 0.3 ^{b,A}	16.7 ± 1.2 ^{b,A}	17.9 ± 1.0 ^{b,A}
28	19.1 ± 0.5 ^{c,A}	17.0 ± 3.1 ^{a,A}	16.0 ± 0.3 ^{a,A}	15.7 ± 0.2 ^{b,A}	16.6 ± 0.2 ^{a,b,A}

C-Control; N-cheese with natamycin; WC-cheese with SCW coating; WCO-cheese with SCW coating with oregano essential oil; WCS- cheese with SCW coating with clary sage essential oil. Different superscript letters (a,b,c,d) indicate significant differences for the same product in different ripening days. Superscript capital letters (A,B,C) indicate significant differences between products at the same ripening day.

Table S3. Texture parameters of cheese samples. Average values and standard deviation (±).

			HARDNESS (N)		
Storage time (days)	**C**	**N**	**WC**	**WCO**	**WCS**
1	8.4 ± 0.1 ^{a,A,B}	8.7 ± 0.3 ^{a,A,B}	8.1 ± 0.5 ^{a,A}	9.6 ± 0.8 ^{a,B}	8.2 ± 0.7 ^{a,A}
14	22.2 ± 1.6 ^{b,A}	18.9 ± 0.82 ^{b,B}	19.5 ± 2.4 ^{b,A,B}	15.9 ± 0.5 ^{b,C}	21.2 ± 2.1 ^{b,A,B}
28	24.0 ± 1.1 ^{b,B,C}	26.1 ± 2.5^{c,C}	17.6 ± 2.2 ^{b,A}	19.1 ± 0.6 ^{c,A}	20.0 ± 1.3 ^{b,A,B}
			ADHESIVENESS (N.s)		
1	-14.1 ± 4.6 ^{a,A}	-12.5 ± 1.3 ^{a,A}	-13.6 ± 2.4 ^{a,A}	-14.0 ± 3.5 ^{a,A}	-13.0 ± 5.2 ^{a,A}
14	-22.4 ± 4.5 ^{a,B}	-9.3 ± 2.8 ^{a,A}	-16.8 ± 4.6 ^{a,A,B}	-12.3 ± 1.5 ^{a,A}	-16.4 ± 0.7 ^{a,A,B}
28	-24.4 ± 4.7 ^{a,A}	-28.2 ± 5.5 ^{b,A}	-26.5 ± 1.8 ^{b,A}	-26.7 ± 2.9 ^{b,A}	-24.3 ± 2.4 ^{a,A}
			CHEWINESS (N.mm)		
1	4.27 ± 0.41 ^{a,A}	3.64 ± 0.30 ^{a,A}	3.99 ± 0.26 ^{a,A}	4.36 ± 0.41 ^{a,A}	3.48 ± 0.84 ^{a,A}
14	6.39 ± 0.78 ^{b,B}	3.09 ± 0.89 ^{a,A}	5.68 ± 0.64 ^{a,b,B}	4.65 ± 0.24 ^{a,A,B}	5.16 ± 0.84 ^{a,b,B}
28	8.62 ± 0.32 ^{c,A,B}	9.35 ± 0.96 ^{b,B}	6.67 ± 1.38 ^{b,A,B}	6.06 ± 0.50 ^{b,A}	7.02 ± 1.47 ^{b,A,B}
			GUMMINESS (N)		
1	4.3 ± 0.5 ^{a,A}	3.7 ± 0.3 ^{a,A}	4.0 ± 0.2 ^{a,A}	4.4 ± 0.4 ^{a,A}	3.5 ± 0.9 ^{a,A}
14	6.8 ± 0.9 ^{b,B}	3.2 ± 0.9 ^{a,A}	5.9 ± 0.5 ^{a,b,B}	4.8 ± 0.3 ^{a,A,B}	5.5 ± 1.0 ^{a,b,B}
28	8.9 ± 0.3 ^{c,A,B}	9.9 ± 0.7 ^{b,B}	6.8 ± 1.4 ^{b,A}	6.3 ± 0.4 ^{b,A}	7.2 ± 1.6 ^{b,A,B}
			COHESIVENESS		
1	0.517 ± 0.1 ^{b,A}	0.417 ± 0.0 ^{a,A}	0.493 ± 0.0 ^{c,A}	0.467 ± 0.1 ^{b,A}	0.433± 0.1 ^{a,A}
14	0.303 ± 0.0 ^{a,A}	0.457 ± 0.1 ^{a,B}	0.303 ± 0.0 ^{a,A}	0.300 ± 0.0 ^{a,A}	0.255 ± 0.0 ^{a,A}
28	0.373 ± 0.0 ^{a,A}	0.380 ± 0.1 ^{a,A}	0.383 ± 0.0 ^{b,A}	0.327 ± 0.0 ^{a,A}	0.357 ± 0.1 ^{a,A}
			RESILIENCE		
1	0.067 ± 0.0 ^{b,B}	0.047 ± 0.0 ^{a,A}	0.053± 0.0 ^{b,A,B}	0.053 ± 0.0 ^{b,A,B}	0.043 ± 0.0 ^{a,A}
14	0.023 ± 0.0 ^{a,A}	0.037 ± 0.0 ^{a,b,B}	0.027 ± 0.0 ^{a,A,B}	0.030 ± 0.0 ^{a,A,B}	0.028 ± 0.0 ^{a,A,B}
28	0.027 ± 0.0 ^{a,A}	0.027 ± 0.0 ^{b,A}	0.027± 0.0 ^{a,A}	0.030 ± 0.0 ^{a,A}	0.027 ± 0.0 ^{a,A}

C-Control; N-cheese with natamycin; WC-cheese with SCW coating; WCO-cheese with SCW coating with oregano essential oil; WCS- cheese with SCW coating with clary sage essential oil. Different superscript letters (a,b,c) indicate significant differences for the same product in different ripening days. Superscript capital letters (A,B,C) indicate significant differences between products at the same ripening day.

Table S4. Microbiological counts of cheese samples (log CFU/g). Average values and standard deviation (±).

			LACTOCOCCI		
Storage time (days)	**C**	**N**	**WC**	**WCO**	**WCS**

1	8.5 ± 0.0 ^{a,A}	8.5 ± 0.0 ^{a,A}	9.7 ± 0.0 ^{b,B}	8.5 ± 0.3 ^{a,A}	9.1 ± 0.1 ^{b,c,A,B}
7	9.3 ± 0.1 ^{b,B}	9.1 ± 0.0 ^{b,A,B}	8.7 ± 0.3 ^{a,A}	8.6 ± 0.2 ^{a,b,A}	8.7 ± 0.1 ^{a,A}
14	9.3 ± 0.1 ^{b,A}	9.9 ± 0.1 ^{c,B,C}	9.7 ± 0.0 ^{b,B}	9.1 ± 0.0 ^{b,c,A}	10.0 ± 0.0 ^{d,C}
21	8.8 ± 0.1 ^{a,b,A,B}	8.7 ± 0.0 ^{a,b,A}	8.6 ± 0.0 ^{a,A}	9.5 ± 0.0 ^{c,C}	8.9 ± 0.0 ^{a,b,B}
28	8.6 ± 0.3 ^{a,A}	8.7 ± 0.2 ^{a,b,A,B}	9.3 ± 0.1 ^{b,B,C}	8.9 ± 0.1 ^{a,b,c,A,B,C}	9.4 ± 0.0 ^{c,C}
LACTOBACILLI					
1	8.5 ± 0.0 ^{a,A,B}	7.6 ± 0.2 ^{a,A}	9.5 ± 0.0 ^{b,B}	7.8 ± 0.6 ^{a,A}	8.5 ± 0.7 ^{a,A,B}
7	9.5 ± 0.0 ^{b,B}	9.5 ± 0.0 ^{c,d,B}	9.2 ± 0.2 ^{a,b,B}	9.1 ± 0.1 ^{a,b,B}	8.6 ± 0.0 ^{a,A}
14	9.3 ± 0.0 ^{b,A,B}	9.9 ± 0.0 ^{d,B}	8.8 ± 0.1 ^{a,A}	9.5 ± 0.5 ^{b,A,B}	9.4 ± 0.2 ^{a,A,B}
21	8.5 ± 0.2 ^{a,A}	8.9 ± 0.0 ^{b,A,B}	8.8 ± 0.1 ^{a,A,B}	9.7 ± 0.1 ^{b,C}	9.0 ± 0.1 ^{a,B}
28	8.8 ± 0.0 ^{a,A}	9.0 ± 0.3 ^{b,c,A}	9.3 ± 0.0 ^{b,A}	9.2 ± 0.0 ^{b,A}	9.2 ± 0.0 ^{a,A}
TOTAL MESOPHILIC AEROBIC COUNTS					
1	8.0 ± 0.0 ^{b,A,B}	8.0 ± 0.1 ^{a,A,B}	9.0 ± 0.0 ^{c,C}	7.8 ± 0.4 ^{b,A}	8.5 ± 0.0 ^{a,B,C}
7	8.1 ± 0.3 ^{b,A}	8.2 ± 0.4 ^{a,A}	7.8 ± 0.3 ^{b,A}	7.9 ± 0.6 ^{b,A}	7.7 ± 0.5 ^{a,A}
14	8.3 ± 0.2 ^{b,C}	7.8 ± 1.2 ^{a,B,C}	6.3 ± 0.3 ^{a,A}	6.7 ± 0.3 ^{a,A,B}	7.7 ± 0.7 ^{a,A,B,C}
21	8.0 ± 0.2 ^{b,A}	8.1 ± 0.4 ^{a,A}	8.0 ± 0.5 ^{b,A}	8.0 ± 0.7 ^{b,A}	8.4 ± 0.3 ^{a,A}
28	7.3 ± 0.4 ^{a,A}	7.6 ± 0.7 ^{a,A}	8.0 ± 0.6 ^{b,A}	7.8 ± 0.2 ^{b,A}	8.0 ± 0.2 ^{a,A}
YEASTS AND MOULDS					
1	3.7 ± 1.2 ^{a,A}	3.5 ± 0.7 ^{a,A}	3.0 ± 0.1 ^{a,A}	3.2 ± 0.2 ^{a,A}	4.0 ± 1.4 ^{a,A}
7	4.5 ± 0.3 ^{a,b,A}	4.2 ± 0.5 ^{a,b,A}	4.5 ± 0.7 ^{b,A}	4.6 ± 0.3 ^{b,A}	4.7 ± 0.3 ^{a,b,A}
14	6.3 ± 0.2 ^{c,B}	4.9 ± 0.8 ^{b,c,A}	5.5 ± 0.6 ^{b,A,B}	5.0 ± 0.4 ^{b,A}	5.4 ± 0.1 ^{b,c,A,B}
21	5.8 ± 0.9 ^{b,c,A}	5.4 ± 0.6 ^{b,c,A}	5.4 ± 0.6 ^{b,A}	5.4 ± 0.2 ^{b,c,A}	6.0 ± 0.2 ^{b,c,A}
28	6.0 ± 0.3 ^{c,A}	5.6 ± 0.4 ^{c,A}	6.7 ± 0.5 ^{c,A}	6.3 ± 0.9 ^{c,A}	6.2 ± 0.4 ^{c,A}

C-Control; N-cheese with natamycin; WC-cheese with SCW coating; WCO-cheese with SCW coating with oregano essential oil; WCS- cheese with SCW coating with clary sage essential oil. Different superscript letters (a,b,c) indicate significant differences for the same product in different ripening days. Superscript capital letters (A,B,C) indicate significant differences between products at the same ripening day.

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Whey and second cheese whey are by-products of dairy industries resulting from cheese and whey cheese manufacture. Both by-products can cause a high environmental impact; however, due to their nutritional and techno-functional properties, they can be used to develop dehydrated, high value products. The development of more efficient and economic processes and systems to reduce environmental pollution and the creation of new foods of high added value could help solving the problem. The aim of this thesis was to create new foods or value-added products by using bovine, ovine and caprine whey and second cheese whey and enabling the small and medium dairy industries to include them in their product's portfolio.