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**Integrated green strategies for the management,
recovery and recycling of expired milk**

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La cosa più importante è non smettere mai di domandare. La curiosità ha il suo motivo di esistere. Non si può fare altro che restare stupiti quando si contemplano i misteri dell'eternità. È sufficiente se si cerca di comprendere soltanto un poco di questo mistero tutti i giorni. Non perdere mai una sacra curiosità.

Albert Heinstein

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ABSTRACT

The management of food waste and the adoption of biopolymers to replace plastics are crucial challenges for transitioning to a circular economy. This thesis addresses the knowledge gap regarding the reuse of expired pasteurized milk for producing biopolymeric films suitable for food packaging, combining technological innovation and environmental sustainability. Specifically, the research activities focused on the following objectives: optimizing experimental conditions for producing and functionalizing sodium caseinate (NaCas) from expired milk through chemical and physical treatments; developing a NaCas-based packaging prototype and assessing its performance in keeping low-moisture cheese snacks; conducting a life cycle assessment (LCA) of the production process from NaCas powder to the packaging prototype.

In Chapter 1, the study explored the production of NaCas powders from expired milk, managing lipid content, and selecting plasticizers and casting methods to optimize film properties. Various experimental conditions were tested to ensure the quality and functionality of the NaCas films, resulting in a defined experimental protocol that can be used for film production. Chapter 2 focused on preparing film-forming solutions and producing films, investigating the role and characteristics of different amounts of fat in the powder, as well as different sonication techniques for degassing the film-forming solution and dispersing fat. The different films have been characterized for their mechanical, thermal, optical and barrier properties. The detailed analysis included testing different formulations and processing conditions, which demonstrated that converting expired milk can yield films with properties comparable to some synthetic materials. Chapter 3 provided for the application of treatments such as LED exposure with photosensitizers and alkaline conditions to promote protein cross-linking, to enhance films properties. These treatments were designed to enhance the structural properties of the films, leading to improved performance in practical applications. Chapter 4 described the development of a NaCas packaging prototype for low-moisture cheese snacks. This activity involved collaborative efforts with Lactalis Italia to optimize the weldability of the NaCas film and the mechanical strength of the welds to obtain a flow-pack. Effectiveness in preserving product quality was evaluated by comparing the experimental prototype with the commercial packaging. Chapter 5 presented an LCA to evaluate the environmental impacts of the production process, from NaCas powder manufacturing to the final packaging prototype.

This comprehensive assessment identified several opportunities to improve sustainability through process modifications, suggesting solutions to reduce environmental impact through scale-up of some production steps and the incorporation of eco-friendly additives.

The results demonstrate the potential of expired milk as a valuable resource for developing sustainable biopolymeric films. By exploiting expired pasteurized milk, this research provides an effective solution for reducing food waste and raw material consumption, contributing to a more circular economy and alignment with SDGs.

Future improvements include investigation of additives to reduce water vapor susceptibility of the film as well as mechanical properties, optimizing techniques for industrial scale-up, and implementing strategies to further reduce the environmental impact of the films. Additionally, exploring alternative production methods and new applications for the NaCas films can lead to broader adoption in the food packaging industry.

INTRODUCTION AND LITERATURE REVIEW

General Introduction

The global population is projected to reach 9.7 billion by 2050 (UN, 2019), presenting an unprecedented demand on agri-food systems to sustainably nourish the world (FAO, 2018). Meeting this demand while remaining within the planet's ecological boundaries is imperative (Rockström et al., 2020). Recognizing these global challenges, the United Nations launched the 2030 Agenda for Sustainable Development in 2015, which comprises 17 Sustainable Development Goals (SDGs) to address critical issues such as poverty, inequality, climate change, environmental degradation, and responsible consumption. This framework for sustainable development emphasizes the interconnected nature of economic, social, and environmental dimensions, advocating for a holistic approach to tackle the complex issues underlying global challenges. With less than a decade remaining to achieve these targets, transforming agrifood systems is crucial to fulfilling the SDGs.

In alignment with the 2030 Agenda, the European Union has adopted the SDGs as a central framework to drive sustainability across its member states. The EU's Green Deal—aimed at achieving climate neutrality by 2050—emphasizes reducing food waste and advancing circular economy principles. Among the 17 SDGs, Goal 12, “Responsible Consumption and Production,” is pivotal for promoting sustainable production and consumption patterns, reducing waste generation, and increasing resource efficiency across sectors.

The dairy sector, integral to global food security, has a significant role within this context. While it contributes to cover dietary needs worldwide, the dairy industry is rather unique in its processing system, including generation of large amounts of product losses and waste. Recent estimates indicate that approximately 20% of dairy products are wasted worldwide at various stages across the supply chain, from production to retail and consumption (FAO, 2012). Due to the direct economic impact, optimizing process efficiency represents the main topic the dairy industry has focused efforts to minimize food waste. In addition, some ways to reduce wastes by utilizing by-products are already in use, such as reutilization of whey at the cheese plant. While some dairy products such as milk powder and UHT milk contribute very little to food waste, one significant source of waste is pasteurized milk. Due to the mild heating conditions of conventional HTST pasteurization, pasteurized milk is susceptible to spoilage by microorganisms that survive the treatment (Lott et al., 2023). Furthermore, some degradative phenomena occur during shelf storage of pasteurized milk that impair its overall quality attributes (Limbo et al., 2021). It is normally accepted that food manufacturers can reprocess (rework) their products before the expiration date. Reworking unsaleable products into saleable commodities is a way to recovery costs and minimize waste (Rush et al., 2022). However, this approach is hardly feasible in the case of pasteurized milk on the market

because of its very short shelf life. Excess unsold product is unlikely reworked before expiration and, therefore, becomes a waste no longer suitable for human consumption. Current destinations are mainly direct usage in animal feeding and soil fertilization. A more widespread transition from a linear economy—where resources are consumed and discarded—to a circular economy, which emphasizes resource regeneration, reuse, and recycling, would be advisable also in the pasteurized milk chain (UN, 2021).

Waste and by-product management, however, is not the sole sustainability issue the dairy sector faces. Plastic pollution has also emerged as a critical environmental crisis, driven by the massive use of single-use plastics, especially in food packaging. Known as the “Plastic Era,” this period has seen an exponential increase in plastic production, contributing to pervasive environmental pollution. The United Nations Environment Programme (UNEP) estimates that over eight million metric tonnes of plastic enter the oceans annually, posing severe risks to marine biodiversity and human health (UNEP, 2021). Mounting environmental concerns and the push to reduce single-use packaging waste have intensified interest in alternative materials like biopolymers. Biopolymers, derived from renewable biological sources such as corn starch, proteins, and polysaccharides, offer a promising alternative to conventional plastics (Muneer et al., 2021). Unlike oil-based polymers, biopolymers are often biodegradable and compostable, which significantly reduces waste management burdens and environmental impact.

Research on biopolymers for food packaging is rapidly advancing, with growing attention to innovation and adoption within this field. Transitioning to sustainable packaging alternatives could help mitigate plastic pollution and support sectors like dairy in implementing eco-friendly, responsible practices, thereby lowering their environmental footprint.

In this Ph.D. thesis project, these environmental concerns are addressed, along with the need to improve resource efficiency in the dairy industry—specifically by reusing expired pasteurized milk from retail shelves as a raw material to produce a biopolymer film suitable for packaging applications. In pursuit of this goal, the following literature review examines the currently available research on biomaterials derived from milk proteins, with a particular focus on films and coatings. It explores existing production techniques, methods for enhancing film properties, and the different functionalities that milk protein-based films can offer in sustainable food packaging implementation.

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Literature review - Strategies for exploiting milk protein properties in making films and coatings for food packaging: A review

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Abstract

Biopolymers of different natures (carbohydrates, proteins, etc.) recovered from by-products of industrial processes are increasingly being studied to obtain biomaterials as alternatives to conventional plastics, thus contributing to the implementation of a circular economy. The food industry generates huge amounts of by-products and waste, including unsold food products that reach the end of their shelf life and are no longer usable in the food chain. Milk proteins can be easily separated from dairy waste and adapted into effective bio-based polymeric materials. Firstly, this review describes the relevant properties of milk proteins and the approaches to modifying them for subsequent use. Then, we provide an overview of recent studies on the development of films and coatings based on milk proteins and, where available, their applications in food packaging. Comparisons among published studies were made based on the formulation as well as production conditions and technologies. The role of different additives and modifiers tested for the performances of films and coatings, such as water vapor permeability, tensile strength, and elongation at break, were reviewed. This review also outlines the limitations of milk-protein-based materials, such as moisture sensitivity and brittleness. Overall, milk proteins hold great potential as a sustainable alternative to petroleum-based polymers. However, their use in food packaging materials at an industrial level remains problematic.

1. Introduction

Dairy industries operate worldwide and produce many different food products, such as drinking milk, cheese, butter, fermented milk, and milk powders, where milk solids are recovered with different efficiency. Around 4–11 million tons of dairy waste is disposed of every year, and because of its high organic content, it represents a real hazard for the environment (Ahmad et al., 2019). In addition, milk and dairy products include fresh products with an extremely short (1–2 weeks) shelf life. These products create further waste if they surpass their expiry date without being consumed. A practical example is pasteurized milk bottled in clear PET (polyethylene terephthalate) bottles and displayed in shops and supermarkets. Limbo et al. (2020) emphasize that, depending on refrigeration conditions, the shelf life of this type of milk can be somewhat extended; however, this extension could cause decay of its nutritional and sensory properties due to light exposure. Expired milk quickly coagulates, sours, and spoils; therefore, it is often disposed of without recycling and taking advantage of its high-quality nutrients. Expired milk can be alternatively used for animal feed or soil fertilization.

The biotechnological valorization of whey has attracted great interest from researchers, while the management of expired milk or, more generally, dairy waste has received scarce attention in the literature until very recently. The use of dairy waste as a substrate in microbial fermentation reactors for producing value-added compounds (Sar et al., 2022), for the extraction of selected components by green processes (Kamal et al., 2023), and as a conductive material for batteries (Zhao et al., 2023) are among the recently proposed strategies. However, transferring these novel approaches to technological processes that can be used in industrial production remains problematic. Dairy waste differs in its composition and the characteristics of individual components; therefore, its reuse implies that targeted technologies are developed. Often pre-treatments are necessary steps in order to stabilize the product, remove contaminants, or simply concentrate targeted components, increasing the efficiency of the subsequent process. Components isolated from dairy waste or expired dairy products are normally sanitized and turned into powders to be used as new raw materials. The purity grade, in terms of residual microbiological and chemical contaminants, of these raw materials will define their suitability for further uses. As for any raw material or ingredients, safety and quality characteristics shall comply with the legal requirements provided for specific usages, either in food or non-food areas. Thus, the commercial valorization of the obtained derivatives will depend on both their origin and final destination. In principle, these ideal achievements help to mitigate environmental impacts while creating new employment opportunities within the framework of a sustainable industrialization.

The packaging sector also has an impact on the environment. Food products are generally packed after processing with the aim of both preventing microbial spoilage and preserving their

sensory and quality properties during shelf life. The European Union policy on the sustainability of the whole food chain (European Commission, 2020) and the “zero waste” goal of the Food and Agriculture Organization of the United Nations (FAO) (FAO, 2019) have encouraged food packaging manufacturers to find alternative, green packaging solutions; therefore, researchers are studying materials that can replace conventional plastic. Above all, the production of biodegradable packaging materials based on natural biopolymers is being prioritized, with these biopolymers being obtained from renewable sources or by-products of different origins (Hassan et al 2018; Karaca et al., 2019). Biopolymers derived from by-products or waste are already available and sometimes underutilized resources, and their use reduces disposal costs (Korycki, A. et al., 2022). Biopolymers such as proteins, polysaccharides and lipids, with the aid of some additives, appear to be promising raw materials (Awasthi et al., 2022; Mastropetros et al., 2022). Biopolymers can be obtained from a variety of animal or plant sources; some can be chemically synthesized from bio-based monomers (e.g., polylactic acid) or synthesized by microorganisms (e.g., cellulose, polyhydroxyalkanoates, xanthan) (Thulasisingh et al., 2021). An overview of the main biopolymer classes based on their origin and chemical nature is shown in Figure 1.

Among polysaccharides, the most common biopolymers include starch, cellulose, alginic and hyaluronic acid, dextran, chitosan, and chitin (Verma et al., 2016). Due to their widespread availability, low processing costs, and wide range of properties and compositions, polysaccharide polymers are commonly used for various biological applications, such as food packaging, tissue engineering, and bioplastic formation (Di Donato et al., 2020; Verma, et al., 2016). With respect to proteins, the most used biopolymers are collagen, gelatin, soy protein, and milk proteins due to their biodegradable and biocompatible properties. This class of biopolymers also has excellent mechanical strength, heat resistance, and water resistance, making it suitable for storage films, packaging films, and heat-processed products (Gautam et al., 2022). Aliphatic polyesters are another class of polymeric macromolecules, with polylactic acid and polyhydroxy butyrate being the most significant materials.

Currently, multiple advantages, including high accessibility and biodegradability, widespread natural sources, mechanical properties, and the controlled release of additives and bioactive compounds, have sparked broader investigations on protein biopolymers and related applications compared with other biopolymers (Calva-Estrada, et al., 2019). This review focuses on the main features of milk-protein-based films and coatings and discusses the available strategies used to improve their technological properties. The native properties of milk proteins are preliminarily presented in order to provide the reader with the correct background knowledge. Furthermore, the main strategies to modify these properties to improve milk protein performances are discussed. Finally, different uses and applications of

milk-protein- based films and coatings are considered, depending on their formulations and additives.

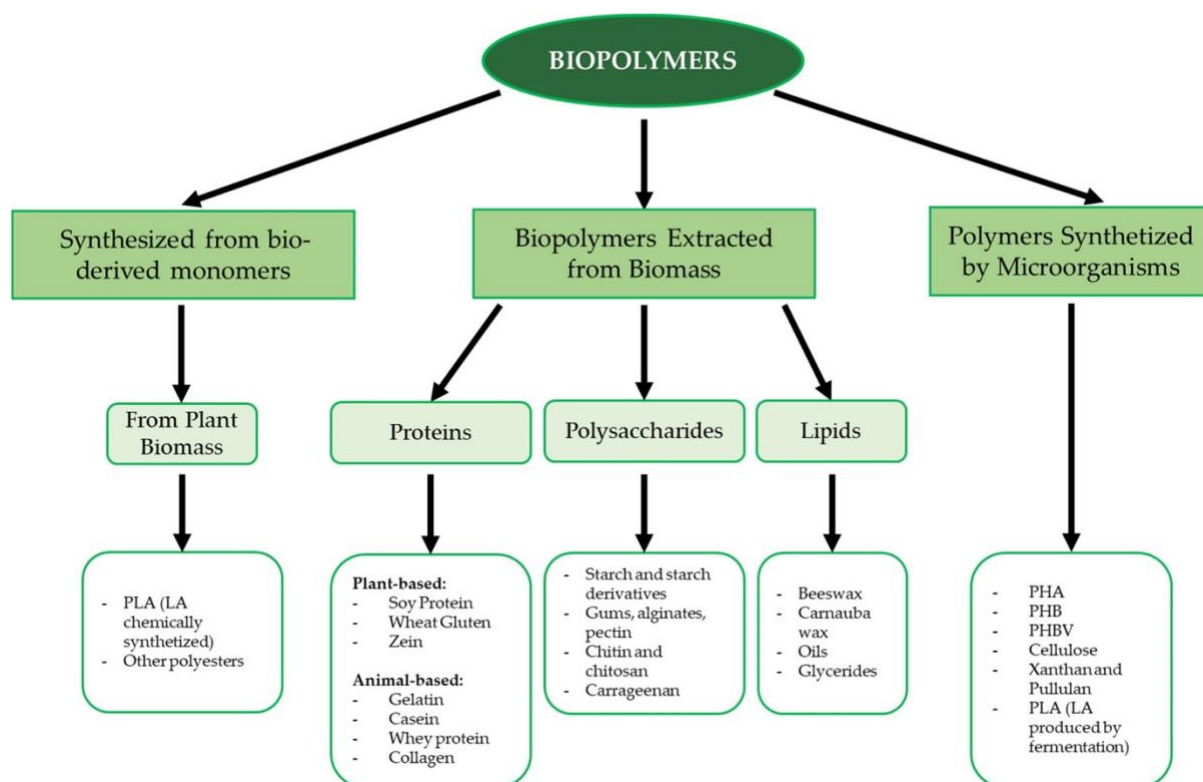


Fig. 1. Schematic description of the main biopolymer classes. Abbreviations: PLA (polylactic acid), PHA (polyhydroxy alkanate), PHB (polyhydroxy butyrate), PHBV (polyhydroxy butyrate-valerate).

2. Milk Proteins: Native Structures and Properties

Milk protein fractions include numerous proteins. Caseins are the most abundant (around 75–78%), being a class of phosphorylated proteins; the remaining 22–25% is represented by whey proteins, which are broadly defined as “soluble proteins”, and minor components such as proteose peptones. The relevant physico-chemical characteristics of milk proteins are described below and summarized in Table 1.

Table 1. Relevant properties of milk proteins.

	α_{s1} -Casein	α_{s2} -Casein	β -Casein	κ -Casein	α -La	β -Lg
Molecular mass (Da)	23,614	25,230	23,983	19,023	14,174	18,362
Concentration g L ⁻¹	10	2.6	9.3	3.3	1.2	3.2
Pro	17	10	35	20	2	8
Cys	0	2	0	2	8	5
Glu	24	25	18	13	8	16
Asp	8	11	5	1	9	11
SerP	8	11	5	1	0	0
Glucidic residues	0	0	0	0–5	0	0
Hydrophobicity (kJ/res)	4.9	4.7	5.6	5.1	4.7	5.1
Isoelectric pH	4.16–4.49	4.68–5.13	4.50–5.29	5.43–6.12	4.66–4.90	4.64–4.98
Net charge/residue	–0.10	–0.07	–0.06	–0.02	–0.02	–0.04

Abbreviations: Pro (proline), Cys (cysteine), Glu (glutamic acid), Asp (aspartic acid), SerP (phosphoserine).

2.1. Caseins: Characteristics and Properties

Casein is defined as the protein fraction present in milk that precipitates at a pH of 4.6 at 20 °C (Swaigood, 2013). Casein includes four principal components, α s1, α s2, β and κ , representing nearly 40, 10, 35, and 15% of the whole fraction, respectively, and with a molecular weight ranging from 20 to 25 kDa (Huppertz, 2013). The primary structure of casein is characterized by a high content of proline, which interrupts the α -helices and β -sheet secondary structures, giving a disordered structure to the molecule. The tertiary structure is also loose and unexpressed. Consequently, caseins have a flexible structure with an open shape that can adapt to the surrounding environment. These features make them susceptible to proteolysis but resistant to heat treatments. The quaternary structure is called a micelle, with a protein matrix containing dispersed calcium phosphate clusters (De Kruif et al., 2014). The most recent studies on the structure of casein micelles depict them as highly hydrated sponge-like colloidal particles. Huppertz et al. (2017) reported that water binds to 1 g casein in different ways: around 0.5 g of water directly binds to the protein, 1 g is associated with the glycosylated end of κ -casein, and 1.8 g occupies the pores of the micelles for a total hydration of ~3.3 g of water for 1 g of casein. Such a strong hydration occurs because casein micelles have a net negative charge in milk at a pH of 6.6–6.7. When milk is acidified to a pH of approximately 4.6, the micelles become more protonated, the calcium phosphate progressively solubilizes, and the casein molecules bind in small aggregates of insoluble acid casein.

Individual casein fractions display rather characteristic features, mostly depending on their amino acid composition and conformational flexibility. Caseins easily interact with multiple target molecules and thus are biologically fundamental in binding to surfaces and organizing in macroscopic networks (McMahon & Oommen 2013). The α s1-casein has a molecular weight of 23.6 kDa and a disordered “random coil” structure due to its high content (8.4%) of proline (Swaigood, 2013). Differently, despite its similar molecular weight (25.2–25.4 kDa), the α s2-casein molecule can be divided into five distinct regions: two regions (residues 1–41 and 42–80) represent the typical casein phosphorylated regions with low hydrophobicity and high charge; a third region (residues 81–125) form a slightly positively charged region with high hydrophobicity; a fourth region (residues 126–170) with a negative charge and low phosphate content; finally, the last region (residues 171–207) has a high positive charge and high hydrophobicity (Huppertz, 2013). β -casein has a molecular weight of 24.0 kDa and a random tangle shape with many β -turn structures. β -casein is the most hydrophobic among caseins, with a negatively charged hydrophilic N-terminal region formed by 40 amino acids residues. Its hydrophobicity increases in the C-end region. These properties of β -casein, unlike other unstructured disordered proteins, determine its ability to self-assemble into micelles under physiological conditions and even in acidic conditions. The hydrophobic part of the β -casein molecule is inside the micelle, while the hydrophilic part bearing the phosphorylation centers

(mainly Ser-P residues) is located outside (McMahon & Oommen, 2013). The κ -casein is the smallest of caseins (molecular weight of 19.0 kDa); it is a glycoprotein that contains about 5% of carbohydrates and two cysteine residues. In fact, the C-terminal part, named glycomacropeptide, is hydrophilic due to the presence of a glucidic moiety. The κ -casein is found on the external part of the micelle in a heterogeneous way, described as a brush layer (Swaisgood, 2013). It was also demonstrated that κ -casein does not have a stable three-dimensional structure, and this allows for changes in the conformation at different pH and temperature (Mirdha & Chakraborty, 2019).

2.2. Whey Proteins

The proteins found in whey after either isoelectric precipitation or enzyme coagulation of caseins represent around 17% of protein substances in milk. Whey proteins include β -lactoglobulin (β Lg), α -lactalbumin (α -La), bovine serum albumin (BSA), immunoglobulins (Igs), lactoferrin (Lf), lactoperoxidase (LP), and other indigenous enzymes (Wynn & Sheehy, 2013). The proteose peptones (PP) are also considered part of the whey protein fraction (Innocente et al., 2011).

The β -Lg is the most abundant (51%) whey protein, with a molecular weight ranging from 18.20 to 18.36 kDa (Sawyer, 2013; Guyomarc'h et al., 2014). This protein exists as a dimer of two identical subunits, where each monomer entails one sulfhydryl group and two disulfide bonds. β -Lg is largely insoluble in distilled water, and salt dramatically increases its solubility. In this regard, the unusual solubility is the result of a strong salt binding due to the unique distribution of surface charges, and thus dipoles, at a neutral pH (Sawyer, 2013). Thermal denaturation of β -Lg occurs at 70–75 °C, whereas aggregates form at 78–82 °C. The precipitation of β -Lg occurs before the precipitation of α -La due to lower heat resistance. The α -La, representing 22% of the whey protein fraction, is a small globular protein formed by a single polypeptide chain with a molecular mass of around 14 kDa. In the polypeptide chain, there are eight cysteine residues that form four disulfide bonds and thus no free cysteine sulfhydryl groups (Guyomarc'h et al., 2014). The α -La is a calcium-regulating protein and, more in general, can act as a metal carrier. The presence of a Ca^{2+} ion inside the molecule stabilizes its conformation; hence, removing calcium increases sensitivity to heat denaturation. The BSA represents 7–8% of the whey protein fraction and has a molecular mass of around 66 kDa (Wynn & Sheehy, 2013). An important property of BSA is its ability to bind in a reversible way to multiple ligands; therefore, this protein can be used as a carrier for fatty acids and other lipids (Madureira et al., 2007). The Igs include three main classes: IgG, IgA and IgM, with IgG including IgG1 and IgG2. They are the largest whey proteins with a molecular mass of up to around 180 kDa, and thus, they are highly susceptible to destabilization and precipitation.

3. Approaches and Methodologies for Modifying the Properties of Milk Proteins

Due to the presence of many side chains and charged groups in the constitutive amino acids, proteins are amphiphilic molecules with a high reactivity. In this respect, milk proteins have unique properties that make them highly suitable for non-food applications, such as the production of biomaterials in the form of films and coatings. To further improve these properties, milk proteins can be intentionally modified by different approaches, with heat treatments and crosslinking being the most common.

3.1. Thermal Treatments

Thermal treatments are normally adopted by the dairy industry as part of the manufacturing processes that convert milk into safe food products. However, depending on the operating conditions, thermal treatments can induce changes to milk proteins in food mostly associated with the appearance of undesirable modifications in sensory and nutritional properties. These modifications, generally referred to as heat damage, have been the topic of extensive research for many years and further motivated the development of non-thermal processes that could avoid these drawbacks (Nunes & Tavares, 2019; D'Incecco et al., 2021).

Differently, less attention has been paid to heat-induced changes that can be of interest for proteins destined for non-food applications. Heat can lead to protein unfolding, with the exposure of hydrophobic groups, aggregation, and flocculation. In general, the energy associated with thermal treatments is enough to destroy noncovalent interactions in the native structure, such as hydrogen bonds and hydrophobic interactions, thus modifying protein conformation and causing the exposure of hydrophobic amino acids residues and/or free sulfhydryl groups. β -Lg is most affected by thermal treatments among milk proteins. At a relatively low temperature (67–78 °C), this structural change is determined by unfolding, whereas at higher temperatures (>78 °C), the aggregation process becomes significant (Considine et al., 2007). This process occurs in three steps: initiation, propagation, and termination. During the first step, the β -Lg dimers reversibly divide into monomers; subsequently, active monomers accumulate via an irreversible exposition of free sulfhydryl groups previously hidden inside the molecule. During propagation, aggregates form due to the formation of newly arranged disulfide bonds. Finally, in the termination step, active intermediates react to create larger aggregates without exposed/reactive SH groups (El-Salam et al., 2009).

Unlike β -Lg, α -La undergoes very limited aggregation due to the absence of free thiols in this protein. Both β -Lg and α -La in the unfolded status preferentially bind to the surface of casein micelles by reacting with κ -casein (Guyomarc'h et al., 2014). When severe heat treatments are

applied, other inter and intramolecular interactions occur, leading to the formation of insoluble protein aggregates, also entrapping fat globules (Anema, 2020; Pellegrino et al., 2022). Aside from heating conditions, the protein concentration and pH of the medium also regulate the degree of whey protein binding to casein instead of self-aggregation, and this dramatically impacts milk protein functionality and behavior (Cattaneo et al., 2012).

Although casein micelles themselves are very stable under heat treatment, exposure to high temperatures causes a change in the mineral equilibrium and, thus, can induce the partial disintegration of micelles (Beliciu et al., 2012). Moreover, when milk proteins undergo thermal treatments in the presence of reducing sugars, such as lactose in milk, the α -amino groups of lysine residues can react with the reducing end of the sugar through the Maillard reaction (El-Salam & El-Shibiny, 2018). Cardoso et al. (2018) observed that susceptibility to the glycation of milk proteins individually mixed with different sugars in the dry state follows the order: α -La > β -Lg > β -casein. The extent of protein glycation influences many of their physico-chemical and functional properties, such as solubility, heat stability, gelation, and foaming properties. Structural changes arising from glycation generally determine a more flexible protein structure that enables molecules to move faster in an aqueous solution, improving their solubility as well as emulsion formation properties and foaming capacity. This last property is ensured by faster adsorption at the air/water interface. However, changes are very dependent on reaction conditions and pathways followed by the Maillard reaction. Consequently, controlled glycation could represent a useful tool for producing modified milk proteins with tailored properties (Lund & Ray, 2017).

3.2. Protein–Protein Crosslinking

Inducing covalent crosslinks at either intra- or intermolecular levels is another approach to modifying protein structure and functionality. Chemical, physical, and enzymatical treatments can be applied to induce protein crosslinking since proteins have several reactive groups (e.g., side chains of glutamine, lysine, tyrosine, cysteine) (Friedman, 1999; McKerchar et al., 2019). Disulfide bonds are naturally occurring crosslinks in whey proteins, which may rearrange when subject to thermal treatments, enzymatic catalysis, or oxidative conditions, with a significant impact on protein functionality (Anema, 2020). The creation of inter- and intra-molecular disulfide bonds leads to the formation of a stronger, more rigid protein network with enhanced resistance to proteolysis (Buchert et al., 2010). The combination of extreme pH/high temperatures/low moisture conditions dramatically promotes the formation of protein crosslinks starting from a dehydroalanine residue intermediate, which derives from cysteinyl or phosphoserine residues and further reacts with the ϵ -amino group of lysyl residues. Among these crosslinks, lysinoalanine is the most widely studied in milk proteins (Al-Saadi & Deeth, 2008; Cattaneo, et al., 2012). The competition existing between the Maillard reaction and

dehydro-alanine-mediated crosslinking towards lysine residues has been highlighted (Cattaneo et al., 2012; Nielsen et al., 2022). Oxidation phenomena are definitely less investigated in milk proteins. Nevertheless, the riboflavin-mediated photo-oxidation of milk proteins is rather well documented as a mechanism leading to the formation of crosslinks, such as di-tyrosine and di-tryptophan (Dalsgaard et al., 2007; Fuentes-Iemus et al., 2018; Hellwig, 2019). Huang et al. (2022) studied the oxidative modification of α -La and reported that a moderate oxidation improved the emulsifying and foaming properties of the protein, while an extensive oxidation induced an increasing aggregation and impaired emulsion and foam stability. In the field of milk proteins, a particular importance is given to enzymatic crosslinking. This approach can be used to improve protein networking in order to reach specific features in terms of the stability, viscosity, and thickening of the protein matrix. Enzymatic crosslinking was studied for both casein and whey proteins (Chen, et al., 2018; Schorsch, et al., 2000; Raak & Corredig, 2022). In particular, the enzymatic crosslinking of casein with microbial transglutaminase (mTGase) has received much attention in recent years as a potential tool for improving the texture and properties of dairy products [48,51–53] (Chen, et al., 2018; Gharibzahedi, & Chronakis, 2018; Kouravand et al., 2020; Li et al., 2021). It has been demonstrated, for example, that sodium caseinate crosslinked by mTGase, when added to skim milk, increases the stiffness of the resulting acid gels (Raak & Corredig, 2022). This enzyme is able to catalyze the reaction between the γ -carboxy amide of glutamine and the ϵ -amino group of lysine residues, resulting in stable protein polymerization (Romeih & Walker, 2017). TGase crosslinking improved the water solubility, water vapor permeability, tensile strength (TS), wettability, and thermal stability of casein, whereas excessive crosslinking led to contrasting results (Liu et al., 2021).

4. Films and Coatings from Milk Proteins

Based on the aforementioned context, the remarkable characteristics of milk proteins can be consistently exploited in the production of materials as alternatives to plastic. The overall scheme, from the recovery of milk proteins to the production of films and coatings, is represented in Figure 2. In particular, the recovery of residual milk proteins from by-products or waste from the dairy industry chain can contribute to a more sustainable circular system. Food packaging materials are based on two different objects, films and coatings, which, apart from thickness, do not substantially differ in composition (Díaz-Montes & Castro-Munoz, 2021; Kandasamy et al., 2021). Nevertheless, they have different uses.

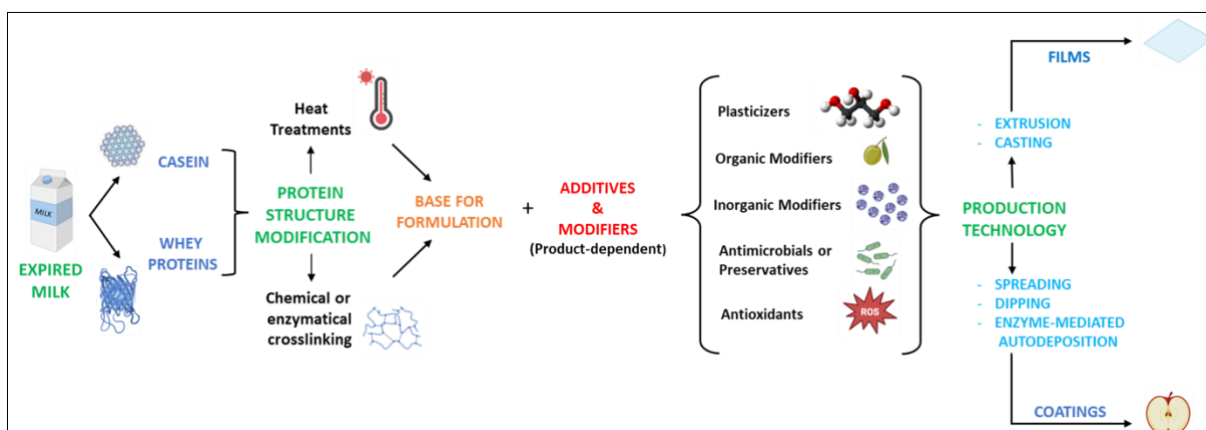


Fig. 2. Overall approach to producing films and coatings from modified milk proteins.

Films can be used to make pouches, wraps, capsules, or sleeves. Alternatively, coatings are directly applied to the surface of a material. Depending on its type, the coating might be removed from the coated material itself, but it is usually designed not to be discarded separately. Therefore, the coating is normally considered part of the final material. Edible films and coatings, in particular, are designed to be ingested together with the food. In this case, coatings are best applied on the surface of the food product by various approaches, as discussed later.

Casein itself possesses the ability to form films or coatings from aqueous solutions without further processing. This behavior is caused by its random-coil nature and the large number of electrostatic, hydrophobic, and intermolecular hydrogen bonds. Its additional useful properties include high thermal stability and biodegradability and the capability of forming micelles and binding a variety of ions and molecules. Acid-precipitated casein is easily turned into soluble caseinates through neutralization with an alkali (e.g., sodium, potassium, or calcium hydroxide). Nevertheless, single casein molecules in a water solution have a strong tendency to spontaneously aggregate depending on the solution concentration, the temperature, and the presence of Ca^{2+} ions (Cheng et al., 2022). Due to these features and behaviors, which can be easily tempered towards the required performances, casein in the form of caseinates is the most investigated among milk proteins as a material for producing films and coatings.

Whey proteins can be efficiently recovered from cheese whey, thus exploiting the potential of the most abundant waste of the dairy industry (Fernandes et al., 2020; Ramos et al., 2012; Schmid et al., 2019). Globular proteins have the ability to unfold and form new polymeric structures via crosslinking under suitable conditions. These characteristics make whey proteins excellent raw materials for producing films as opposed to other film-forming biopolymers. Studies that focus on the development of films or coatings where caseinates or whey proteins represented a minor constituent are not considered in this review.

4.1. Films from Casein and Whey Proteins: Formulation and Production Methodologies

The scientific literature describes different formulations that can be used for milk protein film production, with different complexities depending on the adopted conditions and required properties. The type of protein used in building the film matrix is even more important. Although prepared under the same conditions, films obtained from either caseinate, or whey proteins may have significantly different features, behaviors, and performances (Fematt-Flores et al., 2022). Sabil et al. (2021) studied the characteristics of films simply obtained from water solutions of sodium caseinate and found 9.5% (w/w) to be the caseinate concentration that maximizes yield, TS, and thickness. A similar concentration was shown to be optimal by Picchio et al. (2018). However, due to the brittleness of the protein films, the addition of a plasticizer is usually recommended, as discussed below.

Essentially, protein films can be produced using two different methodologies: solvent casting or extrusion. Solvent casting is mostly used at a laboratory scale since it is easily implemented and does not require expensive equipment. Generally, the solvent casting process uses a pouring solution prepared by dispersing caseinate or whey protein isolates (5–10% w/w) in water or a blend of various solvents. A plasticizer is usually added, the pH value is adjusted to 8–9, and then the solution is heated up to 90–95 °C for 30 min for protein denaturation. The film is obtained by casting the solution on a smooth surface with an average casting volume of 0.1 mL/cm² to obtain a thickness of about 100 µm. After casting, the film is normally dried at 20–30 °C and 40–50% RH in order to achieve the best structure before peeling off (Alizadeh-Sani et al., 2020; Brzoska et al., 2018; Schmid et al., 2019; Wu et al., 2020).

The extrusion process is normally used for large-scale industrial production due to the continuous operating conditions. Extrusion consists of a dry process that uses a co-rotating twin screw extruder. For caseinate film production, the powder is introduced into the first zone of the extruder using a gravimetric feeder. In the second zone, the plasticizer (usually glycerol) is introduced through a valve. Temperature progressively increases up to 70–75 °C from hopper to die, which has approximately a 5 cm width and 1 mm thickness. The film thickness is about 500 µm, depending on the formulation. Residence time into the twin-screw extruder, with a flow powder rate of 2 kg/h and screw speed of 170 rpm, may vary between 2 and 6 min (Chevalier et al., 2018).

Casein and Whey Protein Pre-Treatments

As discussed before, protein crosslinking could be an interesting approach to improving film properties. However, it seems to be scarcely explored in the literature, specifically on the topic of milk proteins. Pereda et al. (2009) evaluated the most relevant characteristics of casted films prepared from caseinate previously crosslinked with glutaraldehyde (GTA). The TS increased

in the samples prepared with more than 5% GTA compared to the untreated caseinate. This enhancement in TS is due to the crosslinking reaction between GTA and caseinate, where the free amino groups of basic amino acids, such as lysine, in the polypeptide chains of casein reacted with the aldehyde groups of GTA. This reaction resulted in the formation of Schiff bases, which created a more interconnected and rigid protein network. The increased crosslinking reinforced the structural integrity of the caseinate matrix, leading to improved mechanical properties, including higher tensile strength. These results suggest the formation of a network with high chain-segment mobility, promoting the diffusion of water molecules in agreement with the increased water vapor permeability (WVP). Nevertheless, improved behavior was also observed in films from caseinate crosslinked by thermal treatment (Pereda et al., 2009). The enzymatic crosslinking of caseinate was obtained using TGase, laccase or tyrosinase, and the derived casted films were characterized comparatively (Juvonen et al., 2011). The tyrosinase-crosslinked film was proven to perform best in terms of solubility resistance, contact angle, and structural homogeneity. Picchio et al. (2018) used tannic acid (TA) for crosslinking casein. As highlighted by FTIR analysis, the incorporation of TA produced an appreciable shifting in the amide I and amide II bands, with a relative increase in the former signal, which was attributed to a strong chemical bonding between the phenolic compound and the protein. Increasing the crosslinker concentration improved the TS but compromised the elongation at break (EAB). Adding 8% of TA produced a decrease in the moisture content of the film from 24% to 11%. Meanwhile, using 4% of TA led to a decrease in the WVP. A recent study (Jahromi et al., 2020) investigated the effects of treating sodium caseinate powder by dielectric barrier discharge (DBD) cold plasma at 7 kV and 10 kHz frequency on its structural, thermal and film-forming characteristics. The treatment resulted in an increased β -structure and reduced the random coil of the protein, with a positive impact on its interfacial activity in solution. The microstructure of the derived film, obtained using a casting method, was more uniform, and the mechanical strength improved compared to untreated caseinate film. Similar effects were observed by directly treating the casein film (Wu et al., 2020).

Commercial whey proteins that can be used as raw materials, mainly whey protein concentrates (WPC) and whey protein isolates (WPI) with a protein content of 60–80% or >95%, respectively, are normally derived from cheese whey and require a preliminary heat treatment for extensive denaturation. Notably, Abdalrazeq et al. (2019) showed that films produced at pH 12 without heat treatment were more resistant, flexible and opaque compared to films produced at pH 7 with a heat treatment at 80 °C for 25 min. Similarly, casted films were obtained from a WPC previously submitted to different functionalizing pre-treatments: heat treatment, enzymatic crosslinking by TGase, and ultrasound (Cruz-Diaz et al., 2019). Ultrasound-treated films showed increased TS and puncture strength, which could be caused by increased hydrophobic interactions of the unfolded protein molecules. The TGase-treated

films had a lower water content, likely due to the reduced capacity of binding water for crosslinked proteins. Overall, heat-treated films had the best mechanical properties (TS, EAB, puncture strength). Díaz et al. (2016) tested the effects of ultraviolet radiation ($0.12\text{--}12\text{ J cm}^{-2}$ at 254 nm) applied to a film-forming WPC solution (8% protein) compared to those induced by heat treatment (80 °C for 20 min). UV radiation at the highest doses induced whey protein denaturation and aggregation comparable to heat treatment, with α -La being more extensively affected than β -Lg. The derived film had a higher TS, elastic modulus, puncture strength and lower solubility compared to control films. Aside from a rearrangement of disulfide bonds, these effects were also attributed to the concomitant oxidation of UV-absorbing amino acids, mainly tyrosine and tryptophan, which may form intermolecular covalent crosslinks.

4.2. Additives and Modifiers

In order to improve the properties of casein or whey protein films, compared to plastic films, some additives are normally required in the formulation to obtain the appropriate features, depending on the food packaging requirements. Furthermore, films can perform as an active packaging material by releasing selected substances beneficial to the packed food items (Khan et al., 2021; Mohamed et al., 2020). Additives can be of natural origin (e.g., essential oils, plant extracts, phenols, enzymes) or inorganic (e.g., minerals, clays) and may have more than a single role or promote side effects. Depending on the added substance, the protein matrix may change in structure and thus in the properties of the derived film (Chevalier et al., 2018).

4.2.1. Plasticizers

Plasticizers work by disrupting protein–protein interactions and, consequently, increasing the intermolecular free volume and the mobility of polymer chains, allowing good flexibility of the film. However, the sorption of water molecules into the film matrix increases the permeability coefficient and lowers the glass transition temperature (Brzoska et al., 2018). Glycerol is the most widely used plasticizer for both casein and whey protein film production, especially for edible films (Hernandez-Izquierdo et al., 2008). Chevalier et al. (2018) added different concentrations (13.2–24.2%) of glycerol in the formulation of a casein film and found a positive effect on its hydrophilicity (i.e., water contact angle values lower than 90°). In a whey-protein-based film, a glycerol concentration of 30% based on a protein gave the highest TS, while lower concentrations had no plasticizing effect (Galus & Lenart, 2019). Sorbitol is another studied plasticizer. The study of Brzoska et al. (2018) highlighted that caseinate films containing sorbitol were stronger and less flexible compared to films containing glycerol. This was attributed to a couple of reasons: (i) polyol plasticizers interact with polar amino acid residues in biopolymers, resulting in a decreased intermolecular protein cross-linking; (ii) the

high hygroscopicity of polyols facilitates the sorption of water molecules. Nevertheless, moisture barrier properties were much better in glycerol-plasticized films.

4.2.2. Organic Modifiers

Plant extracts have a wide range of biological, antioxidant and antimicrobial activities and are generally recognized as safe (GRAS) products; thus, their use is also suitable for the fortification of edible films. The addition of essential oils primarily aims to modify the water barrier properties of the film by increasing the content of hydrophobic groups (Krochta et al., 1990). Pereda et al. (2015) developed a sodium caseinate film supplemented with a linseed-oil-based resin (LOR). The WVP decreased, compared with the control, and was the lowest when the LOR addition was 10–15%. Alongside this effect, the addition of LOR caused a decrease in TS, whereas the EAB was not significantly affected. Alizadeh-Sani et al. (2020) produced a casein film reinforced with rosemary essential oil and zinc oxide (ZnO) nanoparticles using glycerol as a plasticizer. Along with the expected antimicrobial effect of both additives, tested against some bacterial strains, the film demonstrated good water barrier and mechanical properties (flexibility and strength) and moisture resistance. Another type of active composite film, produced through solvent casting, was proposed by Ranjbaryan et al. (2019) with an application in perishable food. The authors used sodium caseinate, two different amounts (2.5 and 5% w/w based on sodium caseinate) of cellulose nanofibers, and a cinnamon essential oil nanoemulsion. While the supplementation of cellulose nanofibers increased the crystallinity and reduced the porosity of the film, as confirmed by scanning electron microscopy (SEM), the further addition of essential oil induced a reduction in the WVP and moisture adsorption. The addition of cellulose in the form of nanocrystals (CNCs) has also been proposed to improve the mechanical properties of whey protein films (Sukyai et al., 2018). The CNCs were incorporated into the water solution of whey proteins at a concentration of up to 8% (w/w based on whey protein) before casting and caused an increased TS and Young's modulus of the film, while the WVP decreased. Including rapeseed oil in the formulation of a whey protein film decreased its water hydrophilicity, thus moisture content and solubility in water; on the other side, the film was more permeable to oxygen and carbon dioxide (Galus & Kadzinska, 2018). As expected, the WVP in the presence of rapeseed oil was significantly higher when the temperature was 5 °C instead of 25 °C (Galus & Kadzinska, 2018).

Plant extracts are usually rich in phenolic compounds, many of which are attracting increased interest as natural free-radical scavengers (antioxidants). Brzoska et al. (2018) investigated the effects of quercetin, an antioxidant compound, added to casein film formulations. Aside from other tested variables, a 100% radical scavenging activity was observed in undiluted extracts using the DDPH method. Furthermore, light transmittance measurements revealed

that the incorporation of quercetin provided UV-light barrier effects at wavelengths between 300 and 400 nm. Interestingly, Fernandes et al. (2020) proposed using a whey-protein-based edible film as a carrier of prebiotics, namely galacto-oligosaccharides (GOS) and xylo-oligosaccharides (XOS). In their study, these incorporated components reduced the melting point and WVP due to the overall increased hydrophobicity. Mechanical tests detected a lower TS and higher EAB. Based on previous evidence of the improved functionality of soy proteins resulting from the addition of γ -amino butyric acid (GABA), He et al. (2021) tested the effects of introducing different concentrations of this amino acid in the recipe of a whey-protein-based film. The presence of GABA increased both the EAB and WVP of the film but reduced TS. The authors reported that GABA concentrations higher than 1% (w/v) impaired the hydration and thermal properties of the film.

4.2.3. Inorganic Modifiers

This category includes a miscellaneous group of inorganic substances that have been added in casein or whey protein film formulation with the aim of improving selected properties. The advent of nanotechnologies has increased the use of inorganic nanoparticles in film preparation to enhance or modify their techno-functional characteristics. Nanoparticles of TiO₂ (Alizadeh-Sani et al., 2020a) or ZnO (Alizadeh-Sani et al., 2020b) were incorporated in caseinate films at concentrations of 1–2% based on casein. Both TiO₂ and ZnO nanoparticles increased the thermal stability of the film while decreasing its transparency and, acting against the plasticizer, also decreasing mechanical properties (strength, flexibility, stiffness). The WVP of the casein film significantly decreased due to the hydrophobic nature of the nanoparticles. In addition, the incorporation of water-impermeable particles into the casein matrix generated a tortuous pathway for vapor particles to move through (Alizadeh-Sani et al., 2020a). Interestingly, caseinate films containing TiO₂ and ZnO nanoparticles exhibited remarkable antimicrobial activity. The addition of halloysite in Na caseinate film production was investigated by Kajthunyakarn et al. (2019). Halloysite (HS) is a natural clay used as an additive and adsorbent in drug systems, acting as a drug carrier and allowing the sustained-release effect of drugs on dissolution. Films with different caseinate–HS ratios were prepared using a casting method and physico-chemical and mechanical properties, as well as drug permeability, were investigated. The results show that Na caseinate may interact with HS through hydrogen bonds between amine and amide groups of SC and hydroxyl groups of HS. The caseinate–HS film had a transparency like that of the unfortified film, while thermal stability was improved. The incorporation of HS caused a decrease in the puncture resistance and EAB of the films. Furthermore, the lowered water absorption resulted in delayed drug penetration through the film. A previous study by the same authors evaluated magnesium aluminum

silicate (MAS), a layered montmorillonite clay, as an additive for modifying the properties of sodium caseinate films, achieving similar results (Kajthunyakarn et al., 2018).

Schmid et al. (2017) investigated the effects of reactive additives, such as sodium sulfite (SS), sodium dodecyl sulfate (SDS) and urea in whey protein film and observed different complex interactions, often counteracting each other. The presence of SS did not affect the oxygen permeability and WVTR, even though the importance of hydrophobic interactions and hydrogen bonds increased. Urea caused an increase in WVTR and oxygen permeability but without intermolecular changes. SDS was the most effective additive for permeability reduction at concentrations as low as 1%.

4.2.4. Antimicrobial Agents and Preservatives

The antimicrobial activity of agents incorporated into foods may be reduced by compounds within the food matrix. Contrastingly, the incorporation of antimicrobial compounds into the packaging in contact with food may result in a selective and steady migration of these compounds from the packaging material to the food surface, where they subsequently disperse (Kandasamy et al., 2021).

Alongside the array of aspects related to the actual effectiveness of the antimicrobial agent included in the material, other factors must be considered regarding its possible interference with the physical or mechanical properties of the film. The effects of plant extracts and essential oils on active casein or whey-protein film formulation have already been discussed. Colak et al. (2015) successfully produced a caseinate film containing hen egg lysozyme (E1105), a food additive that exhibits antibacterial activity against most Gram-positive and some Gram-negative bacteria. Under the optimized conditions for the extrusion process (twin-screw extrusion at 65 °C and a glycerol content of 25 or 20%), the relevant properties of the film were not affected by the presence of lysozyme, and the enzyme retained about 26% activity. Lysozyme was also incorporated in a whey protein film matrix, either as a free molecule or complexed with polyacrylic acid (Ozer et al., 2016). The complexed form proved to be released more slowly than the free form (up to 500 h instead of 24), creating a long-term antimicrobial effect against *Listeria innocua*. Chevalier et al. (2018) investigated the antimicrobial activity against *Escherichia coli* of a film obtained from rennet casein mixed with a potassium sorbate and different natural waxes (beeswax, Candelilla, and Carnauba wax). A twin-screw extrusion process was adopted. The sorbate proved to inhibit *E. coli* even at low concentration (2%) and over 20 days of storage at 15 °C, confirming that the casein matrix is a good preservative carrier. Furthermore, the addition of 10% of potassium sorbate revealed a plasticizing action on the film, but only beeswax decreased the WVP by 20%. Yoplac et al. (2022) developed a sodium caseinate film, plasticized by sorbitol (Sb), with the incorporation of citral microparticles

(CMs) as an antimicrobial agent. The authors used a response surface methodology to optimize the proportion of components in the formulation and other parameters, such as opacity and elastic modulus. Using this methodology, based on FT-IR and MID (mid-infrared) spectroscopy measurements, the optimal conditions were NaCas:Sb = 1:0.91 and NaCas:CMs = 1:0.95. However, SEM revealed discontinuities and micro-holes on the surface of the film, likely due to the incorporation of citral microparticles and the insufficient elimination of air bubbles during mixing, highlighting the crucial role of film settling conditions aside from the optimization of the formulation. Differently, Agudelo-Cuartas et al. (2020) obtained a homogeneous structure in a whey-protein-based-film where both natamycin and an oil-in-water nanoemulsion of α -tocopherol were added. Aside from an effective inhibitory activity against various microbial species, inclusion induced an increase in the EAB, opacity, UV–vis light barrier, and WVP of the film. Vanden Braber et al. (2020) developed a whey protein film added with low quantities of a water-soluble derivative of chitosan (WCh) and neutralized with sodium tripolyphosphate, which showed excellent antifungal activity against *Aspergillus niger* (100% inhibition).

The incorporation of viable lactic acid bacteria strains into the film for the in situ production of bacteriocins is a novel approach. In the study by Mozaffarzogh et al. (2020) probiotic strains of various *Lactobacillus spp.* and *Bifidobacterium spp.*, all showing an inhibitory effect towards biogenic amine formation, were individually incorporated in sodium caseinate films containing carboxymethyl cellulose (CMC) and intended to extend the shelf life of fresh trout fillets. Two separated solutions of NaCas–glycerol and CMC–glycerol were mixed in 1:1 ratio; then, each strain was incorporated at a final concentration of 9 log CFU/mL, and the mixture was cast in a Petri dish. Although still preliminary, the results were encouraging since the film provoked a 2-week delay in microbial growth in fish fillets, compared with the control, due to the migration of the probiotic strains from the film to the fish (Mozaffarzogh et al., 2020). The bacteriocin-producer *Lactobacillus curvatus* 54M16 was incorporated in a whey-protein-based film and tested against *Listeria innocua* C6 (La Stora et al., 2020). The presence of bacterial cells improved the elasticity and the percentage of EAB of the film while having no effect on WVP. Sogut et al. (2022) observed that probiotic lactic acid bacteria were more stable in films obtained from blends of whey proteins and carrageenan than in films obtained from whey proteins only.

An overview of the above-described studies is reported in Table 2. Although most of these studies focused on innovative approaches, very few included testing possible applications in food packaging. To the best of the authors' knowledge, no studies thus far have explored an industrial application of milk protein films in the food packaging sector.

Table 2. Overview of the recent studies aimed at developing milk-protein-based films.

Type of Material	Aims	Formulation	Production Conditions	Main Results	Reference
Edible Casein Sheets	Develop films from different casein types. Evaluation of glycerol (Gly) concentration on the extrusion process	Rennet casein/acid casein/sodium caseinate (NaCas); Gly 13.2%/24.2% <i>w/w</i> on protein.	Extrusion + conditioning at 23 °C/50% relative humidity (RH) for 48 h	Gly concentration, RH, and type of casein are relevant parameters. The higher the Gly concentration, the higher the susceptibility to water on the film.	[68]
Edible Casein Film	Study of properties of films obtained using different casein concentrations	Casein 7.5/8.5/9.5% <i>w/w</i>; Gly (amount not indicated).	Casting + drying (oven) at 50 °C for 5 h + conditioning at 27 °C for 2 days	Higher casein concentration (9.5%) showed higher yield, increased thickness, and tensile strength (TS).	[63]
Edible Casein Film	Use of dielectric barrier discharge (DBD) cold plasma technology for improving casein film properties	Casein; Gly; 0.5 M NaOH; Rate of 5:1:30 by weight.	Casting on polyacrylic plates + drying at 35 °C/50% RH for 48 h Films loaded onto quartz reaction device, treated with DBD-50 Plasma Reactor at different conditions of voltage and time	DBD cold plasma improved mechanical and barrier performances: increased TS, elongation at break (EAB); decreased water vapor permeability (WVP).	[67]
Casein Film	Develop a NaCas-based film added with linseed oil resin (LOR)	NaCas 2.5% <i>w/w</i>; LOR 0.05/0.1/0.15/0.2% <i>w/w</i> on protein.	Casting on Teflon Petri dish + drying at 35 °C for 10 h + conditioning at 23 °C-50% RH for 3 days	LOR decreased film WVP; minimum was reached with 10–15% addition. Decrease in tensile modulus (TM) and TS; EAB remained stable.	[81]
Casein Film	Casein crosslinking with tannic acid (TA) to obtain a film with better physico-chemical properties for food packaging	Casein 10% <i>w/w</i>; Carbonate buffer; Gly 50% <i>w/w</i> on protein; TA 4/8/10/15/20% <i>w/w</i> on protein.	Casting on silicon molds + drying at room temperature (RT) for 7 days	TA was a good crosslinker for casein, as confirmed by FTIR. Protein network was modified by concentration of phenolic acid. Increased TS and decreased EAB; TA improved water resistance of the film.	[71]
Active Casein Film	Develop a NaCas-based film including microencapsulated citral as antimicrobial	- NaCas 5% <i>w/w</i>; Sorbitol (Sor) - NaCas:Sor = 1:0.5 to 1:1.5 <i>w/w</i>; Citral microparticles (CM) - NaCas:CM = 1:0.5 to 1:1.5 <i>w/w</i>.	Casting + drying at 25 °C-55% RH for 24 h	Best film resistance at NaCas:Sor = 1:0.91 and NaCas:CM = 1:0.95. Mechanical properties were acceptable, but SEM revealed surface discontinuity.	[93]

Table 2. Cont.

Type of Material	Aims	Formulation	Production Conditions	Main Results	Reference
Active Casein-CMC film	Develop a carboxymethyl cellulose (CMC)-NaCas-based film containing 5 probiotic bacterial strains and application on trout filets.	• NaCas/Gly (5 g + 3.75 mL in 100 mL water); • CMC/Gly (1 g + 0.75 mL in 100 mL water); • Solutions mixed 1:1; • Added species: <i>L. acidophilus</i> / <i>L. reuteri</i> / <i>L. casei</i> / <i>L. rhamnosus</i> / <i>B. bifidum</i>.	Casting on Petri dish + drying at ambient conditions for 48 h	During 14-day refrigerated storage, delay in both spoilage bacteria growth and formation of biogenic amines compared to the control. Films containing <i>L. acidophilus</i> were most effective.	[96]
Active Casein Film	Develop casein active films reinforced with ZnO nanoparticles and rosemary essential oil (REO) and properties investigation	• NaCas 8% w/w; • Gly 4.5% w/w; • ZnO NPs; • REO 1–2% v/w on protein.	Casting on Petri dish + drying in oven at 30 °C-50% RH for 24 h + conditioning at 25 °C-50% RH	ZnO + REO increased barrier, mechanical properties, and humidity resistance. WVP reduction, strength and flexibility increase. Good antimicrobial activity against tested bacteria.	[82]
Active Casein Film	Develop a NaCas film added with cinnamon essential oil nanoemulsion (CEO-NE) as antimicrobial and cellulose nanofibers (CNF) as mechanical reinforcement	• NaCas 4% w/v; • CEO-NE; • CNF; • Gly 37.5% w/w on protein.	Casting on polystyrene Petri dish + drying at 25 °C-50% RH for 48 h	Presence of CNF increased film crystallinity, SEM revealed decreased surface roughness. Decrease in both WVP and adsorption of humidity. Improved mechanical properties and controlled release CEO-NE due to CNF; CEO-NE showed a little antimicrobial activity.	[83]
Active Casein Film	Study of effects derived from type of plasticizer and lipid concentration included in NaCas-based films. Investigation of quercetin (QC) antioxidant effect	• NaCas 10% w/w; • Gly/Sor 66% w/w protein; • Oleic acid 35% w/w protein; • Beeswax (BW) 0/6.6/10/15/20% on DM; • QC 1% w/w total dispersion.	Casting on Petri dish + drying at 23 °C-40% RH for 3 days	Water vapor transmission rate (WVTR) was significantly lower in Sor-plasticized films. Adding 10% BW strongly decreased WVTR in the presence of Gly. Sor improved TS and Young's modulus (YM) but worsened EAB. QC extracts showed an effective radical scavenging activity.	[65]

Table 2. Cont.

Type of Material	Aims	Formulation	Production Conditions	Main Results	Reference
Edible WP film	Investigating and testing properties of WP edible films	• WPI at 7/8/9/10% w/w; • Gly at 30/40/50/60% w/w on protein.	Casting on petri dishes + drying at 25°-50% RH × 24 h + conditioning at 25°-53% RH × 48 h before testing	Increasing protein concentration leads to lower opacity and moisture adsorption of films and increased TS and EAB. Films with higher glycerol concentration showed weakened mechanical resistance and higher moisture adsorption rates.	[79]
WP Edible Film	Improve the physical characteristics of whey protein isolate (WPI)-based film using γ -aminobutyric acid (GABA)	• WPI 6% w/v; • GABA 0.0/0.1/0.5/1.0% w/v; • Gly 1:25 w/w on protein; • pH = 8.	Pouring on perspex sheet + drying overnight	Films made with GABA had an increased EAB and WVP and decreased TS and light transmittance. Lowest concentrations of GABA enhanced hydration and thermal properties.	[86]
Active WP Edible Film	Develop antifungal films from WPI added with low quantities of a water-soluble derivative of chitosan (WSCCh)	• WPI 5% w/v; • Na tripolyphosphate 2 mM; • Gly 1.8% w/v; • WSCCh 1.5/3% w/w on protein.	Casting on polystyrene Petri dish + drying at 60 °C for 3 h in oven + conditioning at 20 °C-58% RH for 48 h	WSCCh acts as a crosslinking agent through H bonds, causing a decrease in EAB and solubility. WPI/WSCCh films had excellent fungistatic activity and barrier effects. <i>Aspergillus niger</i> was 100% inhibited, while <i>Penicillium roqueforti</i> was more resistant.	[95]
WP Edible film	Develop an edible film using WPC previously treated with heat, high-power ultrasound (US) and/or crosslinked with microbial TGase	• WPC 8% w/w + Gly 50% on protein + different treatments: - no treatment - crosslinking - heat treated (HT) - HT + crosslinking - US for 15 or 60 min - US + crosslinking	Casting on Plexiglass Petri dish + drying in oven at 35 °C for 18 h + conditioning at 50% RH for 96 h before peeling + 20 °C-50% RH for 48 h	US treatment slightly decreased WVP and increased puncture strength and TS of the films. TGase crosslinking increased puncture deformation values and affected film color. Heat-treated films had the best mechanical properties and thus were tested as separation material for cheese slices.	[74]

Table 2. Contd.

Type of Material	Aims	Formulation	Production Conditions	Main Results	Reference
Active WP film	Develop a film based on WPC added with natamycin (Nat) and/or α -tocopherol (α TOC) nanoemulsion	- WPC 8% w/w + Gly + control αTOC 2% - Nat 300 ppm αTOC 2% + Nat 300 ppm	Solvent casting on polystyrene Petri dishes + drying in oven at 30 °C for 12 h + conditioning at 25 °C-58% RH for 7 days	Addition of α TOC and Nat significantly decreased TS and EM and increased EAB. Opacity, UV light barrier, and WVP of composite film also increased. Antioxidant activity and antimicrobial effect against <i>C. albicans</i> , <i>P. chrysogenum</i> , and <i>S. cerevisiae</i> were evidenced.	[94]
Active WP film	Develop a WPI film carrying a controlled release system for lysozyme (LYZ) based on pH-responsive polyacrylic acid (PAA)/LYZ complex incorporated within the matrix	- WPI 5% w/w + Gly 50% + free LYZ - PAA/LYZ complex - free LYZ:PAA/LYZ complex = 1:1 - Total LYZ amount was constant	Casting on polypropylene substrate + drying overnight at 25 °C-40% RH	PAA molecular weight affected the surface charge and hydrophilicity of the films. Incorporating PAA/LYZ complex into film extended its release time up to 500 h due to a low diffusivity. A 5.7 log reduction in bacterial population within 72 h was observed. Free LYZ was hardly effective against <i>Listeria innocua</i> .	[91]
WP film reinforced with CNCs	Develop a WPI film reinforced through addition of cellulose nanocrystals (CNC) extracted from sugar bagasse	- WPI 5% w/w; - Gly 50% dry matter; - CNC from 0% to 8% w/w.	Casting on Teflon mold + drying in incubator at 50 °C for 15 h + conditioning 25 °C- 50% RH for 48 h	Lightness and transparency of the films decreased with increasing WPI content. CNC increased film hydrophilicity (lower water contact angle values), increased TS and YM, and reduced the WVP. Oxygen permeability did not change.	[84]
WP film added with Sodium Sulfite, SDS and Urea	Evaluate effects of reactive additives sodium sulfite (SS), sodium dodecyl sulfate (SDS), and urea on properties of WP films	- WPI 10% w/w; - Gly 66.7% w/w on protein; - Additives (SS, SDS and urea) 1 to 20%.	Casting on Petri dish + conditioning at 23 °C-50% RH for 9 days at least	SS led to increased number of hydrophobic interactions and H bonds and slightly decreased number of disulfide bonds. O ₂ permeability decreased from 68 to 46 cm ³ with 1% SDS addition. WVTR decreased with 20% SDS addition.	[89]

Table 2. Cont.

Type of Material	Aims	Formulation	Production Conditions	Main Results	Reference
WP edible films modified with UV radiation	Evaluate effects of UV radiation on WPC treating either the film-forming solution or the film	• WPC 8% w/w; • Gly 50% w/w on protein; • UV radiation doses: 0.12 J cm^{-2}, 4.0 J cm^{-2}, 12.0 J cm^{-2}.	UV exposure in a stainless-steel exposure chamber of a microprocessor-controlled UV radiation system	UV radiation of solutions increased free-SH groups and induced formation of aggregates. Derived films showed significantly higher TS, puncture strength and puncture deformation. Solubility was lower than for finished films exposed to UV. When UV was applied to solutions, films were more tinted.	[75]
WP edible film added with rapeseed oil	Develop WPI films added with rapeseed oil (RSO)	• WPI 8% w/w; • Gly 50% w/w on protein; • RSO 0/1.0/ 2.0/3.0% (w/w).	Casting on Petri dish + drying at 25 °C-50% RH for 24 h + conditioning at 25 °C-50% RH for 48 h before testing	Presence of RSO decreased film WVP and water hydrophilicity, increased permeability to O ₂ and CO ₂ . WVP and diffusion coefficient values were higher for films stored at 5° C than at 25° C.	[85]
WP film produced under alkaline conditions	Develop WP-derived materials obtained under alkaline conditions and with no heat-treatment	• WPI 1% w/v; • Gly 30/40/50% w/w on protein; • Poliglicolic acid; • NaOH 0.1 N to pH 7/12.	Casting on Petri dish + drying at 25 °C-45% RH for 48 h	Casting films containing either 40 or 50% of Gly at pH 12 led to the production of more resistant and flexible materials than at pH 7. Opacity was also higher for films obtained at a pH of 12.	[73]
Active WP film	Develop a WPC active coating, incorporated with seaweed (<i>Fucus vesiculosus</i>) extract (SWE) as antioxidant	• WPC 8% w/w; • Gly 8% w/w; • SWE 1% w/w.	Casting on aluminum foil surface + drying at room temperature (RT) for 3–4 days. Chicken breasts were vacuum packaged with the films.	Presence of SWE improved thickness, TS, and EM of the film. The active film also inhibited lipid oxidation at the surface of chicken breast for up to 25 days of storage.	[99]

Table 2. Cont.

Type of Material	Aims	Formulation	Production Conditions	Main Results	Reference
Active edible WP film	Study viability and antimicrobial activity of bacteriocin-producing lactic acid bacteria (LAB) incorporated in a WP film in presence or absence of nutrients	- WP 13% <i>w/v</i> + Inulin 4% + Gelatin 6% + Gly 6% + - Control, no addition <i>L. curvatus</i> 4% <i>v/v</i> in m-MRS broth <i>L. curvatus</i> 4% in distilled water	Casting into Petri dishes + drying at 30 °C-50% RH for 24 h	The presence of LAB reduced viscosity of film-forming solutions and improved elasticity and EAB of film. WP-based films ensured high viability of LAB strains during 28-day storage at 4 °C. Adding MRS broth slightly affected the viability but was needed to achieve a good antimicrobial activity against <i>L. innocua</i> .	[97]
WP film added with XOS and GOS	Develop a WP film added with xylooligosaccharide (XOS) and galactooligosaccharide (GOS).	- WP: amount depends on prebiotics concentration; - Gly 30% <i>w/w</i>; - XOS, GOS 0–10–20–30% <i>w/w</i>; Total film solid conc.: 10%.	Casting onto Petri plates + drying at 27 °C for 24 h + conditioning (vacuum) at 75% RH for 48 h before testing	XOS and GOS addition resulted in films with similar structure (cross-sectional SEM), with lower TS and higher EAB than control films. Prebiotics reduced WVP, despite the higher hydrophobicity evidenced by contact angle reduction.	[61]
WP film added with probiotics	Develop a composite film made with WP and carrageenan (CA) as a carrier of probiotic strains	- WPI at 5% <i>w/w</i>; - Gly 50% <i>w/w</i> on protein; - Addition of strains; Same trial with: - CA 1% <i>w/w</i>; - Gly 35% <i>w/w</i> on CA powder and with blended solutions.	Casting onto Teflon-coated plates + drying at RT + conditioning at 25 or 4 °C-53% RH, before testing	Significant decrease in cell counts observed for all strains in both WPI and CA films during storage at 25 °C, whereas counts were stable in blended films. Multi-strain cultures presented the same behavior. Incorporation of probiotic bacteria influenced WVP and color values of films, decreasing TS and EAB.	[98]

4.3. Coatings from Casein and Whey Protein: Formulation and Production Methodologies

The production of coatings, starting from biodegradable raw materials such as proteins, is a still-developing sector, and the vast majority of coatings are produced using a synthetic material coupled with a biopolymer. As stated above, this review does not consider mixed materials.

Both caseinates and whey proteins can be used to produce coatings, with the latter being more frequently preferred in the case of edible coatings due to its absence of taste, superior nutritional value and lower price (Schmid et al., 2019). Both protein types shall be used in combination with other compounds that improve mechanical behavior and minimize sensitivity to the moisture of the protein matrix (Jalilzadeh et al., 2020; Valentino et al., 2020). Protein-based coatings have the disadvantages of brittleness and poor water resistance. In this regard, Picchio et al. (2017) proposed to modify casein by conjugating methacrylic groups to side amino groups in the protein chain, thus increasing its hydrophobicity. The chemically modified casein polymerized more efficiently, and the obtained material had enhanced technological properties, in particular, water resistance.

Compared to film preparation via a casting technique, the coating technology offers important advantages. Coatings are normally layered on a plastic or cellulosic support, and thus their formulation can be simpler. Recently, edible coatings were developed that can be directly applied to the surface of perishable foods. Depending on their formulation, the coating creates a barrier able to prevent moisture loss, mechanical damages, oxidation, or microbial spoilage (Nian et al., 2022). This implies, however, that direct contact between the coating and food surface without air gaps is obtained. The film-forming dispersions for direct coatings are also widely used in the pharmaceutical industry to protect capsules and tablets.

4.3.1. Coating Application Methods

In coating applications, different approaches can be used, depending on the functionality. Spraying or spreading the formulated solution uniformly on a surface with an applicator is a relatively common method. A suitable method for edible coatings is to dip a food product into a solution for a short time, usually a few minutes. After dipping, a dripping step is required for the removal of excess liquid, and then a final drying step allows the coating to cake. This final step is usually carried out at room temperature so that the coating performance is not affected. Furthermore, dipping technology is particularly suitable for food products that may be affected by high temperatures. Enzyme-mediated autodeposition has been proposed as an alternative to these conventional techniques for casein coating production. This approach exploits the ability of casein micelles to lose solubility after enzymatic cleavage via chymosin (Ruediger et

al., 2016; Strube et al., 2015). The enzyme (chymosin) is immobilized on a support, which is further coated with destabilized casein particles. Due to the enzyme immobilization via spacer molecules, it is possible to carefully control the process. The results of this study have revealed that the use of immobilized single-chymosin molecules can form a continuous casein monolayer.

4.3.2. Additives and Modifiers

Additives added in the formulation of coatings vary depending on the type of product (inert supports or food items) that is coated. Dávalos-Saucedo et al. (2018) developed a whey-protein-based coating intended for application on eggshells, thus increasing the shelf life of eggs. Essentially, pectin (20%) was crosslinked to the protein via TGase in the film-forming solution. The obtained coating reduced both the weight loss of eggs and post-wash bacterial penetration. The same approach was successfully used for coating roasted peanuts to prevent oxidation (Rossi-Márquez et al., 2021). Decreases in peroxide value and water content were observed in the coated product compared with the uncoated one. Valentino et al. (2020) developed coatings with good antioxidant properties by adding rosemary oil or gallic acid to a sodium caseinate solution (4% w/w). Minimally processed fennels were coated via the dipping process, and the dry layer was 0.7–6 µm thick. Other additives, such as tea polyphenols, lemongrass essential oil (LGEO) and lemon essential oil (LEO), were used as antioxidants in whey protein coatings by Ming et al. [108] and Galus et al. [109], respectively. Tea polyphenols increased both the zeta-potential and surface hydrophobicity of the solution. Pieces of fresh-cut apple coated with this solution demonstrated a reduction in browning, compared with the uncoated control, with the increased addition of polyphenols ranging from 0.1% to 0.5% (w/v). The same antioxidant activity was achieved by adding LEO and LGEO in a whey protein coating applied to fresh-cut pears. In the study performed by Mileriene et al. (2021), a liquid whey protein concentrate (12.34% dry matter), was prepared from fresh sweet whey by ultrafiltration and the addition of sunflower oil (4% w/w) and cinnamon extract (6% w/w), and with glycerol as the plasticizer. It was then homogenized and immediately applied on the surface of fresh acid-curd cheeses (350 g each) via dipping. The results highlight a strong antimicrobial effect of the coating during 1 month of cheese storage, with decreasing counts of yeasts and molds and no significant changes in composition and sensory properties.

Natamycin (E235) is commonly used for the surface treatment of cheeses and raw sausages against the growth of yeasts and molds. This compound was added (0.07% w/w) to an acid casein (7.5% w/w) solution that was tested for coating Kashar cheese (Yangilar et al., 2015). The antimicrobial activity was effective throughout a 90-day ripening period. Similarly, natamycin was added in a whey-protein-based coating, also containing lysozyme-xantan gum

conjugate, which was layered on an ultrafiltrated white cheese (Jalilzadeh et al., 2020). This coating avoided the growth of *Penicillium chrysogenum* but also reduced the growth of pathogenic bacteria such as *Escherichia coli* O157: H7 and *Staphylococcus aureus*, which were intentionally inoculated on the cheese surface. Furthermore, the authors observed a reduced moisture loss of coated cheese compared to the uncoated control during a 60-day ripening, with no effect on taste.

The antimicrobial activity of ZnO was exploited with the assembly of casein-based composite coatings, including hollow ZnO nanospheres (Liu et al., 2017) or ZnO nanoparticles (Pereira et al., 2018; Wang et al., 2017). Inclusion usually requires both chemical and mechanical action (prolonged stirring or ultrasonication) and the addition of a casein emulsifier/stabilizing agent, such as caprolactam. In general, the inclusion of inorganic nanoparticles, such as ZnO, TiO₂, and SiO₂, in casein-based coating materials also improves hydrophobicity, mechanical properties, covering ability, and self-cleaning properties under the action of UV light (Wang et al., 2017, Xu et al., 2016).

As previously described for casein or whey-protein-based films, the use of probiotics in a formulation of coatings that are capable of producing antimicrobial agents in situ seems to be a promising approach. Pereira et al. (2018) separately incorporated two different commercial probiotic strains (from *Bifidobacterium animalis* or *Lactobacillus casei*, respectively) in the whey protein solution, which was then used for coating ham slices. Both coated and uncoated (control) ham slices were modified and atmosphere-packed. The main outcome of the storage trials was a decrease in water loss in coated ham compared to the control, with no color changes. The inhibition of common spoilage bacteria was achieved using viable probiotic cell numbers remaining at ca. 10⁸ CFU/g during 45 days of storage at 4 °C. Such high levels of the tested probiotic species can also be directly beneficial for human gut flora. Wang et al. (2019) developed a whey protein coating (0.5% w/w) where carvacrol was added to preserve fresh-cut cheddar cheese in synergy with whey protein nanofibrils (WPNFs). WPNFs, prepared via a 10 h filtrate incubation at 80 °C under prolonged magnetic stirring, improved technological and functional properties, such as foaming and emulsifying properties, self-supporting gelling ability, and viscosity. The obtained coating had a smoother and continuous film surface with a higher antimicrobial and antioxidant activity with respect to traditional WP coating.

In summary, different from what was observed for the films, several recent studies proposed applications of milk-protein-based coatings in food packaging. An overview of the aforementioned studies, focusing on the latest advances in this field, is shown in Table 3.

Table 3. Overview of the recent applications of milk-protein-based coatings in food packaging.

Type of Material	Aims	Formulation	Production Technology	Application	Main Results	Reference
Antioxidant WP coating	Develop a WP coating incorporated with tea polyphenols (TP) for preserving fresh-cut apples	- WP 5–10% <i>w/v</i>; - Glycerol (Gly) 50% <i>w/w</i>; - TP powder 0.1–0.5% <i>w/v</i>.	Dipping for 5 min + draining for 10 min + storing at 20 °C for 24 h	Fresh-cut apples	Antioxidant activity increased with increasing TP concentration. Coated slices showed lower browning during 24 h storage.	[108]
WP edible antioxidant coating	Develop WP edible coatings with incorporated lemon (LEO) and lemongrass (LGEO) essential oils for preserving fresh-cut pears	- WPI acq. Solution 8% water + WPI; - Gly 50% <i>w/w</i> dry basis; - LEO 1% <i>w/w</i>; - LGEO 0.5% <i>w/w</i>.	Dipping for 2 min + draining on a filter paper + packing under modified atmosphere + storing at 4 °C-80 % relative humidity (RH) for 28 days	Fresh-cut pears	Presence of LEO and LGEO reduced O ₂ and CO ₂ permeability of the film. Coating caused reduction in color changes and loss in hardness of pear slices.	[109]
Active casein coating	Develop an active casein coating by adding antioxidant substances. Investigate effects of NaCas concentration on coating properties	- NaCas 4/8/10/12/14% <i>w/v</i>; - Gly 0.4/0.8/1.0/1.2/1.4% <i>w/v</i>; - Gallic Acid 0.005% <i>w/v</i>; - Rosemary Essential Oil 1.5% <i>v/v</i>.	Dipping for 2 min + draining on metallic grids for 10 min	Fresh fennels	Thickness of dry coating was 0.6–7.2 µm, depending on NaCas concentration, with good antioxidant properties. Water vapor permeability (WVP) was highest for films with 4 and 14% NaCas.	[101]
Composite WP-pectin coatings	Develop WP-pectin complex coating enzymatically reticulated by TGase and applied to eggshells to increase egg shelf life and to roasted peanuts to prevent oxidation	- WPI 4.8% <i>w/v</i>; - Sor 2.4% <i>w/v</i>; - Pectin 4.8% <i>w/v</i>; - Final solution: WPI/PEC-4:1; - TGase 8 U/g of WPI.	Eggs: Dipping for 1 min in the solution + drying for 10 min at room temperature (RT) Peanuts: Dipping for 10 s + drying at RT for 10 min + packing in sealed low-density polyethylene bags	Eggshells, Roasted peanuts	Eggs: Coating maintained a higher yolk index and albumen CO₂ content, reduced weight loss and increased albumen and yolk pH. Compared to uncoated eggs, eggshell strength was higher and post-wash bacterial penetration was lower. Peanuts: Coating reduced the peroxide value. TGase reticulation decreased WVP and thus seed water absorption.	[106,107]

Table 3. Cont.

Type of Material	Aims	Formulation	Production Technology	Application	Main Results	Reference
Antimicrobial WP coating	Develop an edible active coating for improving shelf life of fresh-curd cheese	• LWPC (liquid WP conc.) 5.0% w/w; • Gly 5% w/w on protein basis (pb); • Guar Gum 0.7% w/w pb; • Tween 0.2% w/w pb; • Sunflower oil 4% w/w pb; • Cinnamon CO₂ extract 6% w/w pb.	Dipping into coating solution for 3 s + drying on perforated metal trays at 12 °C for 30 min + vacuum packing	Fresh-curd cheese	After 31-day ripening, coating had no effects on cheese moisture, color, texture, flavor. Coating had strong antimicrobial effect, and thus efficiently extended cheese shelf life compared to uncoated control cheese.	[110]
Antimicrobial casein coating	Investigate the efficacy of a casein/natamycin (NTM) coating for the control of mold growth on cheese surface	• Acid casein 7.5% w/w; • Gly 2.5% w/w; • NTM 0.07% w/w.	Dipping cheese into the solution twice for 60 s + leaving to drip for 2 h + draining at 22 °C for 1 h	Kashar cheese	The casein/NTM coating suppressed mold growth during 90-day ripening without adverse effects on cheese quality but a slight decrease in ripening.	[111]
WP nanofibril-based antimicrobial and antioxidant coating	Develop a coating using WPI nanofibrils (WPNFs), incorporating carvacrol (CA) for preserving fresh-cut cheddar cheese	• WPNFs 5% w/w; • Gly 5% w/w; • CA 0.5% w/w.	Dipping cheese pieces into the solution for 60 s + draining at RT for 30 min + storage at 4 °C for 10 days	Fresh-cut cheddar cheese	WPNFs-CA coating had smooth and continuous surface, promoting lower weight losses and better textural properties in cheese. Antimicrobial activity was higher than in traditional films due to CA.	[116]
Active antimicrobial WP edible coating	Develop WP coating incorporated with probiotics and investigate its antimicrobial activity on sliced ham preservation	• WPI 10% w/vl • Gly 5% w/vl • Strain centrifuged pellet 5% w/w (<i>B. animalis</i> Bb-12® or <i>L. casei</i>-01).	Slice immersion for 2 min in the solution + draining for 30 s + storage at 4 °C for 45 days	Sliced ham	Coating decreased water loss of ham without changes in color. Probiotics incorporated in coating inhibited growth of <i>Staphylococcus</i> spp., <i>Pseudomonas</i> spp., <i>Enterobacteriaceae</i> and yeasts / molds during storage.	[115]

Table 3. *Cont.*

Type of Material	Aims	Formulation	Production Technology	Application	Main Results	Reference
Active edible WP film/coating	Develop a coating containing a nanoemulsion of cocoa-liquor (nCL) for improving physical and functional properties and prolonging the shelf life of muffins	• WPC 8% w/w • Gly 5/6/7% w/w • nCL 0/1/2% w/w (different microfluidization conditions).	Dipping muffins into the solution for 5 s + drying at 40 °C for 15 min	Muffins	nCL modified the secondary structure of the WP (FTIR), decreasing mechanical properties, solubility and WVP of the film. Moisture loss during storage at 20–50% RH was lower for coated muffins compared to the uncoated control.	[117]

4.4. Relevant Properties of Milk-Protein-Based Films and Coatings and Future Developments

As widely discussed previously, films and coatings from milk proteins can protect food from changes in moisture, the loss of volatiles, oxidation, and microbial spoilage. For food packaging applications, the gas and vapor barriers are certainly the most sought-after properties for the preservation of sensitive foods: common food packaging polymers with high O_2 barrier properties have gas transmission rates in the range of $0.1\text{--}0.5\text{ cm}^3\text{ m}^{-2}\text{ d}^{-1}\text{ bar}^{-1}$ at $23\text{ }^\circ\text{C}$ and $0\%\text{RH}$ and vapor permeability (WVP) in the range of $1\text{--}5 \times 10^{10}\text{ g H}_2\text{O Pa}^{-1}\text{ s}^{-1}\text{ m}^{-1}$ at $23\text{ }^\circ\text{C}$. Many biopolymers have interesting oxygen barriers but are more sensitive to water vapor. It is, therefore, necessary to combine the properties of different materials using multilayer and coating structures (Awasthi et al., 2022). In this latest research, the deposition of very thin films and coatings of biopolymers on the surface of plastics and paper can reduce permeability to gas and moisture (Fotie et al., 2020; Fotie et al., 2023), delay the migration of contaminants (Guazzotti et al., 2014; Guazzotti et al., 2015) and improve the surface properties of the materials on which they are deposited (Li et al., 2013).

Whey protein layers with an optimized plasticizer content present good oxygen and moisture barrier properties (Schmid et al., 2012). The crosslinked protein network provides an oxygen barrier in the same range of ethylene vinyl alcohol (EVOH) with average ethylene contents ($<2\text{ cm}^3\text{ m}^{-2}\text{ d}^{-1}\text{ bar}^{-1}$ at $23\text{ }^\circ\text{C}$ and $0\%\text{RH}$), thus confirming its potential application in multilayer structures. In this way, biopolymers can contribute to the replacement of more expensive oil-based barrier resins, such as polyvinylidene chloride (PVDC), ethylene vinyl alcohol (EVOH) and aromatic polyamide (MXD6), commonly used in multilayer films. The same authors highlighted that the deposition of whey proteins on a PET film guarantees high transparency (i.e., light transmission $>95\%$), temperature stability and interesting mechanical performance, as well as good adhesion to the substrate (Schmid et al., 2012). In a more recent study, a whey protein solution was layered on a PET film that was previously treated to improve the interfacial compatibility of the two layers. The final material, also including layers of nylon and linear low-density polyethylene, showed a reduced oxygen transmission rate compared with the same multilayer material lacking the protein layer (Joo et al., 2018). A comparable effect was obtained by spreading the whey protein solution on a poly-lactic acid (PLA) film (Weizman et al., 2017). Performance measurements showed a 90% improvement in the oxygen barrier and 27% improvement in the water vapor barrier. This latter effect was achieved by adding either pectin (0.2%, w/w) or a commercial clay (Cloisite-30B) (0.5%, w/w) to the protein solution. Table 4 shows the water vapor permeability and mechanical properties (expressed as TS, and EAB) of casein and whey protein films. We can infer from these data that formulations of milk-protein-based films, with an appropriate amount of plasticizer, also offer a good barrier to water vapor diffusion through the thickness, without significantly compromising

the mechanical performances of the material. A new milestone in the development of whey protein usage has been achieved, demonstrating the biodegradability of the whey coatings using enzymatic detergents (Schmid et al., 2012). In this context, the application of whey proteins and, more generally, milk-protein-based coatings in multilayer structures opens the door to a new generation of recyclable materials. A whey-protein-based layer could facilitate the separation of conventional petroleum-based plastics.

Table 4. Selected parameters of milk-protein-based films and coatings.

Type of Material	Formulation	Thickness μm	WVP ($10^{10} \text{ g H}_2\text{O Pa}^{-1} \text{ s}^{-1} \text{ m}^{-1}$) (ASTM Method E96-95)	TS MPa (ASTM D1708-93)	EAB % (ASTM D1708-93)	Reference
Casein film	• NaCas 2.5% <i>w/v</i> ;	110	5.4	57.0	4.0	[81]
	• NaCas 2.5% <i>w/v</i> + linseed oil resin/protein weight ratios: 0.05/0.1/0.15/0.2;	110	2.2–5.0	23.4–41.2	2.7–4.7	
	• NaCas 2.5% <i>w/v</i> + Gly;	110	/	13.6	11.6	
	• NaCas 2.5% <i>w/v</i> + tung oil.	110	/	25.8	3.0	
		μm	($10^{10} \text{ g H}_2\text{O Pa}^{-1} \text{ s}^{-1} \text{ m}^{-1}$) (ASTM E96M-10)	MPa (23°C, 50%RH)	% (23°C, 50%RH)	
Casein film	• Casein 10% <i>w/w</i> ;	800	11.0	2.6	458	[71]
	• Gly 50% <i>w/w</i> on protein;					
	• Casein 10% <i>w/w</i> ;					
	• Gly 50% <i>w/w</i> on protein;					
	• Tannic acid 4/8/10/15/20% <i>w/w</i> on protein.					
Active WP film	• WPI 5% <i>w/v</i> ;	149	2.1	5.4	19	[95]
	• Sodium TPP 2 mM;					
	• Gly 1.8% <i>w/v</i> ;					
	• WPI 5% <i>w/v</i> ;					
	• Sodium TPP 2 mM;					
	• Gly 1.8% <i>w/v</i> ;	139–141	1.9–2.3	3.9–5.0	7–13	
	• WSch 1.5/3% <i>w/w</i> on protein.					

Table 4. *Cont.*

Type of Material	Formulation	Thickness	WVP	TS MPa (ASTM D882)	EAB % (ASTM D882)	Reference
WP film reinforced with CNCs	• WPI 5% <i>w/w</i> ;	/	/	2.30	46.07	[84]
	• Gly 50% dry matter;					
	• WPI 5% <i>w/w</i> ;					
	• Gly 50% dry matter;					
	• CNC (from 0% to 8% <i>w/w</i>).					
Antimicrobial casein-based flexible coating	• Casein 10.9 g + TEA 2.6 g	/	/	0.49	55.23	[113]
	• + 86 mL water;					
	• Caprolactam sol. 25% added drop-wise;					
	• ZnO NPs;					
Casein-based TiO ₂ nanocomposite coating	• ZnO NPs 0.1/0.5/1.0/2.0%.	/	/	0.97–1.65	52.51–64.77	[114]
	• Casein 15.7 g + TEA 3.9 g					
	• + 80 mL water;					
	• Caprolactam sol. 25% added drop-wise;					
	• Selected additives;					
	• Nano-TiO ₂ 0.0/0.1/0.2/0.3/0.4 g.	/	/	0.55–1.25	102–120	

WVP—water vapor permeability; TS—tensile strength; EAB—elongation at break; / = not determined.

5. Conclusions

The purpose of this review was to present the current status of research on food packaging materials based on milk proteins. This use of milk proteins is certainly of great interest to the food and the packaging industry because they can be used as “edible” materials, but in a broader and more modern sense, they can also improve and refine the technical and functional performances of conventional packaging materials. In fact, the current demand for food packaging is shifting towards the development of sustainable materials from renewable sources with high barrier properties and good processability. These materials can be used in packaging lines and to protect the quality of the most sensitive foods during their shelf life. Although great progress has been made on this topic, most findings remain at the laboratory stage. Milk proteins appear to be suitable for use in existing techniques as the main component in material formulations to produce both films and coatings. However, other aspects still hinder the upscaling of the proposed experimental materials to industrial application. The preparation of the film-forming matrices currently requires the usage of various additives, whose safety and suitability for food contact should be fully assessed. Issues such as long-term stability or the migration behavior of those substances into food have only been partly addressed thus far.

Nevertheless, this review evaluates recent advances in food preservation and food packaging with novel materials obtained from milk proteins, exploring the advantages and disadvantages of this approach. Directly comparing characteristics and technological performances of materials developed in different studies is very difficult since they are often evaluated under different conditions or reported using different units. However, gas barrier, antioxidant, antibacterial, mechanical, and water resistance properties, as well as the efficacy in the preservation of food items, were fully explored in every single study. The modification of milk protein structure using various approaches, such as crosslinking, either chemical or enzymatic, and physical treatments based on unconventional technologies, appears as a promising strategy for empowering their functional properties.

Overall, the suitability of milk proteins as biomaterials for composite film production seems to be consolidated. The commercial application of milk-protein-based materials in food packaging still requires studies that evaluate crucial aspects such as their sustainability and behavior and the long-term stability of additives included in packaged food items.

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AIMS AND OBJECTIVES

Today, critical issues such as planetary health, resource management, global warming, and waste generation demand urgent attention. Reuse and recycling of waste have become priorities, with research increasingly focused on optimizing resources and reducing raw material consumption. Such efforts aim to decrease land use and greenhouse gas (GHG) emissions.

This Ph.D. project, co-funded by the Programma Operativo Nazionale (PON) and Lactalis Italia S.p.A. through Azione IV.4, emphasizes advancing sustainable practices in the dairy sector. Specifically, it seeks to valorize the casein fraction of pasteurized milk withdrawn from retail shelves when nearing expiration, by developing an innovative film suitable for packaging applications. The thesis project is divided into five chapters, each addressing specific objectives:

- **Chapter 1:** Outlines background experiments carried out to define overall conditions for film production, including evaluation of various approaches for NaCas preparation from milk, selection of a plasticizing agent, optimization of formulation to achieve appropriate workability, and assessment of different deposition techniques to create the film.
- **Chapter 2:** Focuses on investigating the optimal conditions for producing neat NaCas films with properties suitable for application as packaging materials while respecting the eco-friendly and sustainability targets. By refining composition and production parameters, neat NaCas films achieve properties that enable their development into novel materials with functional, active, or intelligent packaging capabilities.
- **Chapter 3:** Explores green techniques to improve material performances and to enhance film properties. Focus was in promoting protein cross-linking within the casein matrix to strengthen the intermolecular bonds between protein chains. This would result in improved mechanical strength, flexibility, and/or barrier properties of the NaCas film. To foster these interactions, natural cross-linking induced by non-toxic treatments are tested.
- **Chapter 4:** Focuses on the design and development of a prototype flow-pack tailored for low-moisture cheese snacks. The effectiveness of the prototype is assessed throughout the product's storage up to 20 days by evaluating the water activity as well as the moisture content, oxidation levels through the monitoring of volatile oxidation compounds, enzyme activities through the changes of free amino acid profile. the change over time of the modified atmosphere within the prototype. This chapter provides insights into packaging performance, system and packaging-product interaction, and presents a real application for food packaging.

- **Chapter 5:** This chapter focuses on evaluating the environmental impact of NaCas film production through a comprehensive Life Cycle Assessment (LCA). The study aimed to quantify and compare the environmental footprints of the production process at both laboratory and pilot plant scale, highlighting differences in resource efficiency, energy use, and waste generation. By identifying key hotspots and areas for improvement, the assessment provided insights into the sustainability of scaling up NaCas film production. The ultimate goal was to support the development of environmentally responsible manufacturing processes that align with the principles of green chemistry and sustainable material design.

EXPERIMENTAL SECTION

Chapter 1 - Development of experimental conditions for the production of NaCas-based film

Abstract

With the aim of developing a NaCas-based film, baseline investigations were needed to address key challenges, including optimizing NaCas extraction from expired milk, selecting appropriate plasticizer formulations, and identifying suitable techniques for film production. Since a different amount of milk fat is retained in NaCas powders depending on the preparation protocol, the impact on film microstructure and performances is addressed. A second skimming step significantly improved film uniformity by reducing fat coalescence. Formulation tests identified a 10% NaCas solution with 30% glycerol as optimal for achieving a balance between mechanical properties and processability, while confocal microscopy confirmed that glycerol did not interfere with fat microstructural analysis.

For the film-forming process, materials such as silanized glass, food-grade silicone, and Teflon were evaluated for plate in mould. Teflon emerged as the most suitable material, producing smooth, defect-free films without compound migration. Alternative techniques, including automated solution spreading and extrusion, demonstrated potential for industrial scalability but require further refinement to address challenges such as mixing and dosing of ingredients and bubble formation.

1. Introduction

In the light of the previously reported considerations, I decided to explore the potential of expired milk as a source of casein for producing neat NaCas films for food packaging. Since no previous research has adopted such approach, the setup of the experimental conditions—from purifying the caseins to finalizing the film formulation—required careful testing and balancing of various factors, not always leading in successful results. The optimization steps, that are illustrated in this chapter, were fundamental to establish the experimental basis for developing a functional and reproducible film prototype.

The main challenges lie in the setup of the conditions for extracting the casein fraction from expired pasteurized milk; management of the amount of residual fat in the NaCas powder; set up of the film-forming solution production to reduce the presence of irregularities in the film; identifying the best support material for depositing the solution to obtain an easily peelable film; and obtain the experimental protocols for the development of NaCas films.

2. Suitability of expired pasteurized milk for sodium-caseinate powder production

The aim of the present project was to work pasteurized milk soon after the expiration date, assuming that it is recalled from the market at the end of its commercial profitability. In Italy, pasteurized milk has a shelf-life of 7 days to ensure the best quality product to the consumer. It is known that pasteurized milk undergoes some unavoidable changes during storage in the refrigerated display counters (Limbo et al., 2020). The most frequent event that causes spoiling of pasteurized milk is the acidification promoted by microbial growth, with the rate of this last being strongly dependent on milk storage temperature. Given the destination designed for pasteurized milk within this project, i.e. the isoelectric precipitation of casein, a limited acidification should not be regarded as problematic. Nevertheless, the pH value of the batches of pasteurized milk used in this study 1-3 days after expiration was always higher than 6.4. Among other modifications affecting pasteurized milk stability, a limited proteolysis occurs due to plasmin activity, since this native enzyme is not inactivated by the thermal process. Plasmin primarily cleaves β -casein releasing the soluble proteose peptones (Egito et al., 2003; Ismail & Nielsen, 2010). In a previous study, accumulation of proteose peptones was monitored in commercial samples of pasteurized milk stored under proper conditions (5 ± 1 °C) up to 13 days, i.e. 6 days beyond the expiration date, and the content reached 900 mg/L (Limbo et al., 2020). Since proteose peptones remain in the whey fraction, the recorded amount would represent around 4% of loss in recovered casein. Similarly, Wang et al. (2019) report that the content of intact casein in HTST pasteurized milk decreased by 3-4% after 15 days of storage at 6 °C.

The content of proteose peptones has been evaluated in all the batches of pasteurized milk used in this study 1-3 days after expiration and values always lower than 900 mg/L were recorded, confirming negligible yield losses in casein recovery.

Semi-skimmed pasteurized milk was chosen for this study since it represents the most widespread type on the Italian market, along with ultra-high temperature treated milk (UHT) (Merlino et al., 2021). Also, we wanted to avoid obtaining a high-fat powder with possible undesirable properties. Specific attention was also paid to reduce residual lactose content by washing the acid casein with water exhaustively. This aimed at preventing the Maillard reaction during the subsequent heat treatment that would make casein to turn brown.

For the production of NaCas powders, a method based on (i) isoelectric precipitation of casein, (ii) its solubilization with NaOH and (iii) final lyophilization, was chosen due to ease of both implementation and customization in terms of processed milk amount (Gerna et al., 2025). For the lyophilization, the NaCas solution was placed in a steel tray, frozen and then freeze-dried for 24-36 hours. The resulting dried pellet (Figure 1, panel A) was immediately ground to prevent hydration (Figure 1, panel B) and stored in moisture-resistant plastic bottles in a dry environment. P_LAB_PS was obtained. The final protocol is described in Chapter 2.



Fig. 1. Appearance of NaCas powder just after freeze-drying (panel A) and after grinding (panel B).

3. Managing the residual fat content in the NaCas powders

Since the casein was recovered from a homogenized pasteurized milk, a certain quantity of fat was expected to be retained by casein itself due to the casein-fat globule interactions (Cano-

Ruiz & Richter, 1997). Theoretically, without removing any fat, a powder with a fat content around 38% would be obtained. Indeed, some fat was separated and discarded upon cold centrifugation after casein precipitation. Nevertheless, the fat content of the obtained powder was still high, around 32%. This fat content proved to be quite repeatable when different batches of NaCas were prepared, indicating rather stable interactions between casein and MFGM that could guarantee a consistent behavior of casein and fat in the film under development. Therefore, it was decided to produce additional NaCas powders with a lower fat content, in order to investigate the role of milk fat in the derived NaCas films. With this aim, an additional centrifugation step of acid casein solution was carried out at 40 °C and the obtained oily fat layer was removed using a pipette. The casein pellet was then processed as for the other powder. A powder having a further reduced fat content was prepared from pasteurized skimmed milk (0.1% fat).

To observe how fat is present in the powder with the highest content (32%), a sample film was produced, and the microstructure was analyzed with Confocal Laser Scanning Microscopy (CLSM).

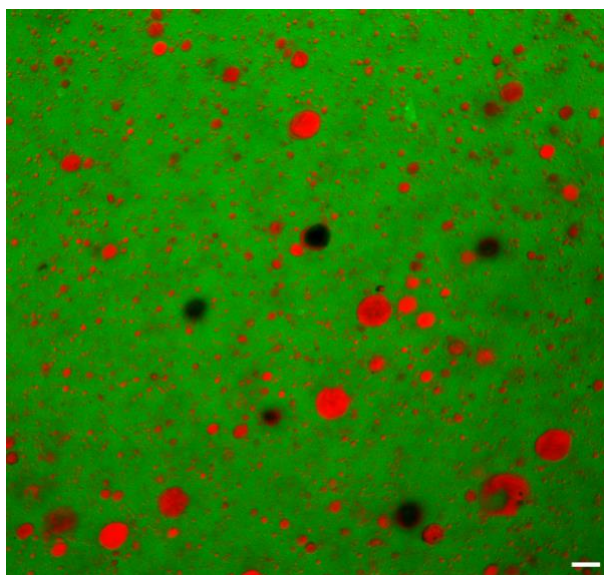


Fig. 2. Morphology of fat structures (in red) and protein network (in green) in the sample film produced with the powder with 32% fat and observed by CLSM. Bar length is 10 μm .

Looking at the film microstructure in Figure 2, it can be seen that fat (in red) was spatially and dimensionally unevenly distributed within the protein matrix (in green), with a large number of small globules together with some large structures (up to 10 μm in diameter) due to coalescence phenomena likely originating during the film drying process which occurs through

Brownian motions (Long et al., 2012; Huppertz et. al., 2020). Some large air bubbles were also present (in black).

To better understand the effect of reducing the fat content in the powders, the image analysis (ImageJ software) was performed on many CLSM images of sample films obtained from the powder prepared from partly skimmed milk (PS) and after further reduction of fat (RF). Figure 3 shows that the diameter of fat structures formed in the film changes as the fat content of the powder changes. In the film produced with P_LAB_PS powder, the frequency of fat structures with diameters greater than 30 μm is very low (close to few units), yet these large structures account for the majority of the fat in the film, as indicated by % volume. In contrast, in the film produced with F_LAB_RF powder, most to the fat content is contributed by structures with diameter length up to 10 μm . These differences further highlighted the interest of investigating the behavior and performances of films obtained from NaCas powders where significantly different amounts of milk fat were retained.

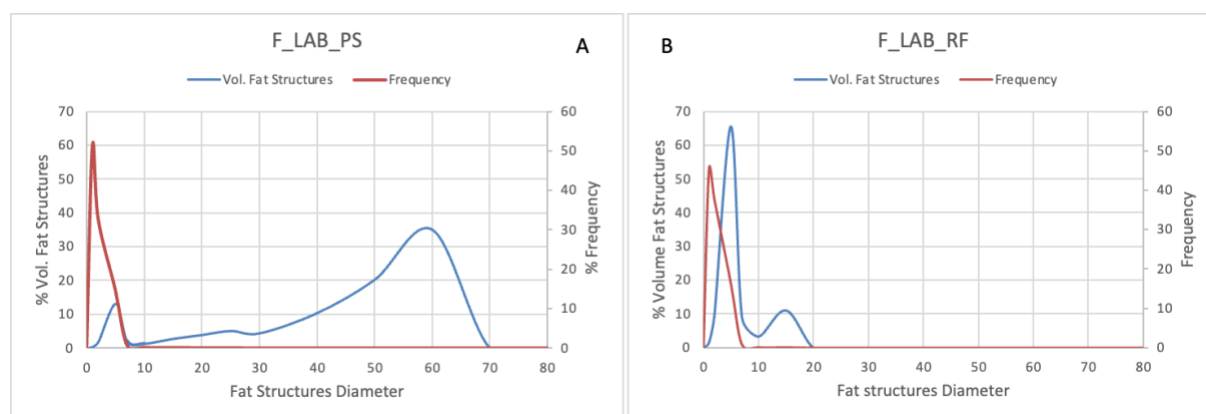


Fig. 3. Data obtained by image analysis of CLSM images of (panel A) a film produced with P_LAB_PS powder and (panel B) a film produced with P_LAB_RF powder. The % volume distribution of fat structures (blue line) and frequency (red line) in relation to diameter can be observed.

4. Development of the film-forming solution and selection of a plasticizer

A variety of formulations are reported in the literature for the production of NaCas films, including those of Khan et al. (2021), Sabil et al. (2021), Kozempel & Tomasula, (2004), Bonnaillie et al. (2014) and Juvonen et al. (2011), all based on industrial powders of NaCas. For the first time, to the best of my knowledge, the idea here was to deal with NaCas powders containing different amounts of milk fat and prepared with a scalable laboratory procedure. Based on the above-mentioned literature, water solutions with protein concentrations from 5 up to 15 % (w/v) were tested. As a fast screening test, the solutions were cast in Petri dishes

(0.1 mL/ cm²) to visually assess their spreadability and then were left to dry for 48 hours. The solutions containing 5% and 7.5% NaCas had a very low viscosity and resulted in film thickness of 20-40 µm and 60-85 µm, respectively. These thicknesses were too low for having a self-supporting film. In contrast, the solution containing 15% NaCas had excessively high viscosity, which prevented proper spreadability. Additionally, it incorporated numerous air bubbles during solubilization, which were difficult to remove by sonication or vacuum treatment. The resulting film had a thickness between 190 and 220 µm, making it too thick for practical application as a packaging material. The solution with 10% NaCas concentration showed an optimal viscosity for spreading in the Petri dish and formed films with thickness in the range 90-130 µm after drying.

Meanwhile, glycerol was tested as plasticizer. The literature review identified glycerol as the most frequently used plasticizer, due to its suitability for flexible film production. The concentrations used were in the range from 30 % to 35 %, in some papers with respect to total solids, while in others with respect to protein concentration. A concern was about whether glycerol could interfere with the detection of fat when analyzed by CLSM, particularly in the analysis of lipid structures within the film. In sample preparation for CLSM, lipids undergo staining with Nile Red, an acetone soluble dye that is excited at 485 nm and emits between 530 and 590 nm. According to current knowledge, the glycerol molecule at high concentrations (>50%) may interfere in the quenching of Nile Red fluorescence, as extensively discussed by Pick & Rachutin-Zalogin (2012). It was thus necessary to assess whether Nile Red binds to the propyl backbone of glycerol and thus could also emit fluorescence when irradiated by the microscope laser, creating artifacts during the detection of the fat structures. In this case, a commercial NaCas with a very low fat content (1%) was used in order to minimize fat interference. Two different solutions were prepared with a protein concentration of 10% w/v and glycerol was added to one of them in the amount of 30% of the protein weight. Both solutions were stained with Nile Red and Fast Green according to D’Incecco et al. (2020) and analyzed by CLSM.

Figure 4 shows microstructural images of the solutions, where the protein network is shown in green and the lipid phase in red. Both images looked the same, regardless the addition of glycerol, with a comparable number of small residual lipid structures (white arrows) remaining after the skimming process. This observation suggests that the glycerol in the solution did not interact with the stain. Notably, in addition to the small lipid structures, light green aggregates, indicated by red arrows, are also visible. These aggregates likely correspond to NaCas particles that did not fully solubilize during the solution preparation.

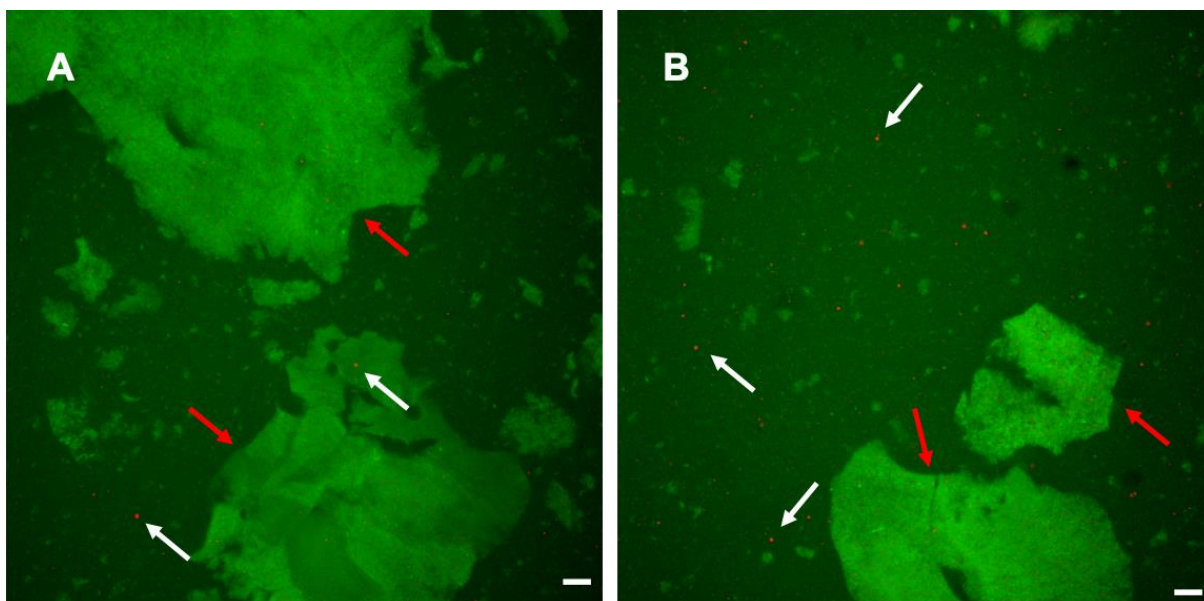


Fig. 4. CLSM images of films produced from 10% NaCas solution either without (Panel A) or with (Panel B) addition of 33% glycerol. White arrows indicate the very small fat structures present in the powder (1% fat). Red arrows indicate particles of NaCas powder that did not solubilize in the film forming solutions. Bar length is 10 μm .

5. Different solution-casting materials evaluation and development of film production process

Different materials were investigated for best casting the NaCas solutions. The film size chosen for these tests was a 10x10 cm square, the edges of which were delimited by silicon strips. Materials suitable for solution-casting must have a smooth surface, free of roughness, allowing to obtain films free of irregularities and also allowing easy peeling after drying. Three different materials were tested: (i) silanized glass, (ii) food-grade silicone and (iii) Teflon. In order to obtain a homogeneous thickness of the film, plates of these materials were mounted on a rectangular wooden support with adjustable steel leveling feet that would allow for the perfect flatness of the surface.

For the film production, the solutions were cast onto the molds using 0.1 mL/cm² as described by Juvonen et al. (2011). The molds were left to dry at room temperature (21-23 °C) and ambient relative humidity (RH) for 48 h. After the drying period, the films were peeled off from the molds, individually sandwiched between two sheets of aluminum foil, and stored in a polystyrene box at 4 °C until analyses.

The film-forming solution was first cast onto a silanized glass plate and left to dry at room temperature for two days (Figure 5A). Upon peeling, the resulting film appeared glossy and defect-free, with a smooth, pinhole-free surface on the glass-contact side. However, the

film adhered strongly to the glass surface, resulting in breakage when force was applied for removal. Consequently, silanized glass was deemed unsuitable as a casting material.

Films produced by casting on food-grade silicone plate and following the same drying method were easily peeled off after two days, yielding smooth surfaces with a slight mat finish on the side in contact with the silicone and free from irregularities. However, as documented by Liu et al. (2021), repeated use of silicone mats or extreme temperature conditions can lead to the release of non-intentionally added substances (NIAS).

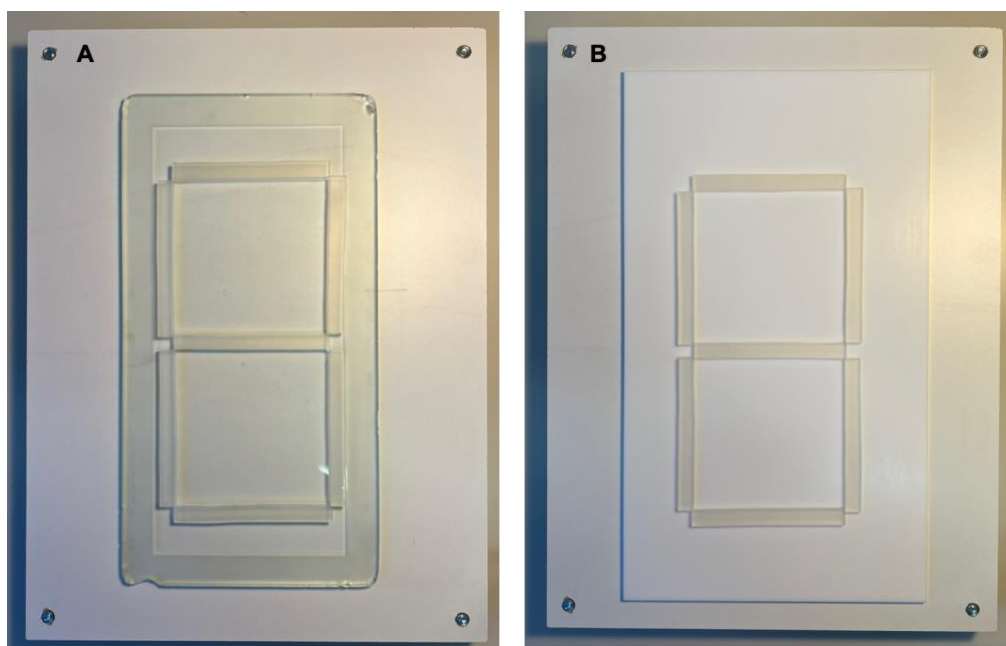


Fig. 5. Wooden support with the four adjustable steel leveling feet on which the silanized glass plate (panel A) and Teflon plate (panel B) are placed, respectively, for solution-casting.

This risk is heightened when silicone lacks an adequate final curing process at high temperatures (200 °C for 6-8 hours) to eliminate unreacted side-products. Migration primarily involves compounds such as volatile methylsiloxanes, which are lipophilic and environmentally persistent. FTIR analyses of both the film and the silicone mat were conducted to determine whether any migration of these substances into the film occurred during the contact period. The conditions described by Parichanon et al. (2021) were followed using a PerkinElmer FTIR SpectrumTM 100 series spectrometer with the single-reflection sampling plate of a 1.8-mm round germanium surface. Spectra were recorded within the range of 650 to 4000 cm^{-1} at a 4 cm^{-1} resolution.

The FTIR spectra (Figure 6A) evidenced that both the NaCas-based film and the food-grade silicone exhibit the characteristic peaks of methylsiloxane compounds, identified by multiple Si-O-Si bonds (Mawhinney et al., 1997). Those peaks are not inherently present in the NaCas-based film, suggesting that these substances likely migrated from the silicone mat into the film

during the drying process. Due to the migration of some substances that are recognized as ambient persistent, it was decided to abandon this material as a support for solution casting.

By casting the NaCas solution on the Teflon plate, good quality films were obtained, with a visually very smooth surface and perfect peelability when compared with previous material used. For the Teflon plate, it was also verified by FTIR analysis that no migration of compounds from the material to the film had occurred. From the spectra in Figure 6B, the two characteristic bands of Teflon, consisting of the symmetrical and asymmetrical stretching of CF_2 bonds, are not found in the films. Furthermore, the spectrum of the NaCas film in contact with the Teflon plate matches the spectrum of the film that has not been in contact with the material. For all these reasons, Teflon was adopted as a suitable material for solution casting in the subsequent tests.

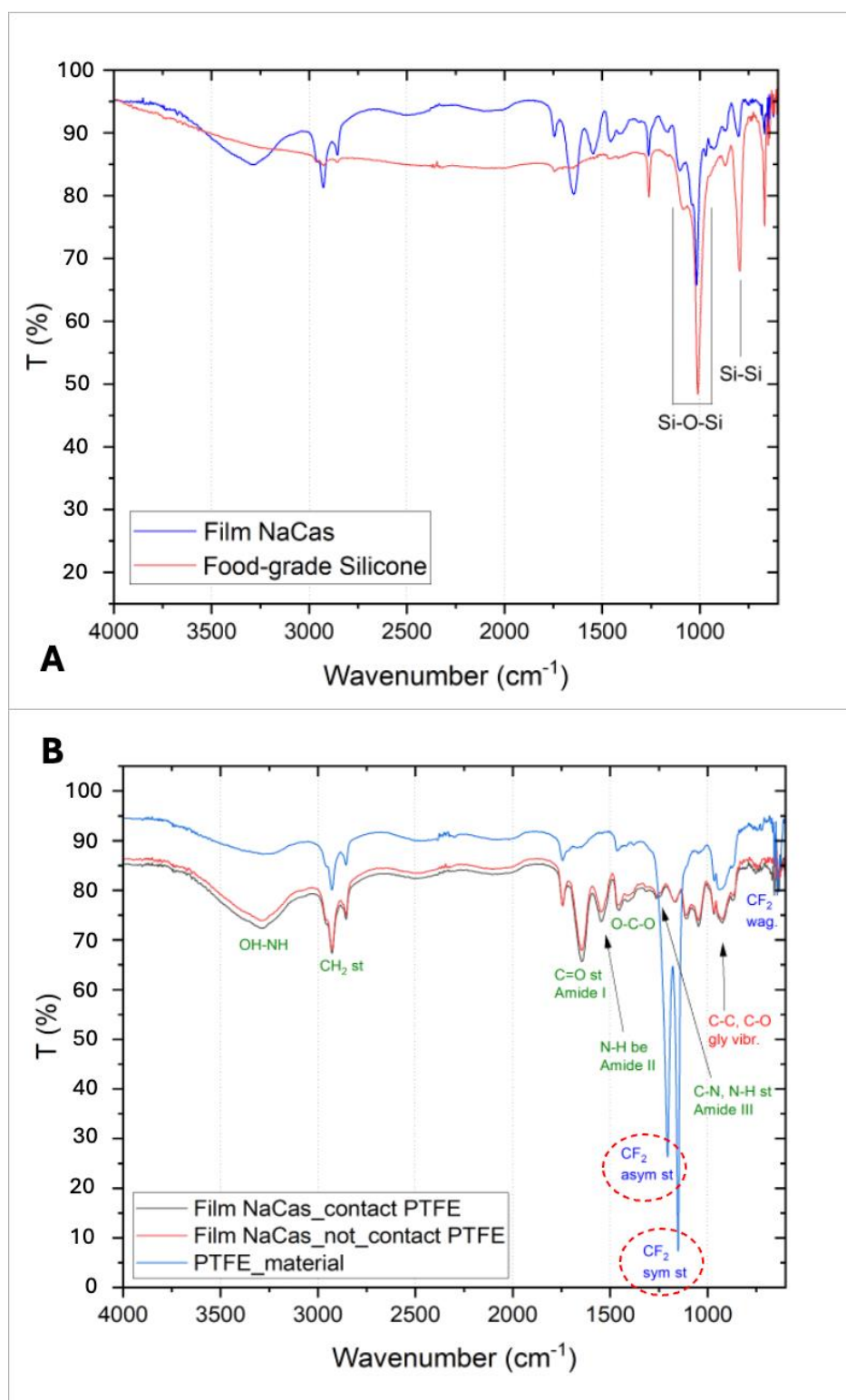


Fig. 6. FTIR spectra obtained to evaluate migration phenomena. Panel A: spectra of the silicon mat used for casting (red line) and the obtained NaCas-based film (blue line). The bands related to Si-O-Si and Si-Si bonds are indicated in the figure by the black lines. Panel B: spectra of the Teflon plate (PTFE: polytetrafluoroethylene) used for casting (blue line), the obtained NaCas-based film (black line) and of the NaCas-based film not in contact with the Teflon plate (red line). The bands related to CF₂ bonds asymmetric stretching and symmetric stretching are indicated by the red dashed circles.

6. Alternative methods to solution casting for the production of NaCas-based films

As discussed in the literature review, various techniques are available for producing NaCas-based films, each with unique properties and distinct scalability for industrial applications.

To speed up the casting step, an automated flat-sheet casting machine was tested that allows spreading the NaCas solution by the Porometer Memcast™ unit (Porometer, APTCO Technologies NV, Nazareth, Belgium), as shown in Figure 7.

For each sample, 32 mL of NaCas solution (made with P_LAB_PS powder) was spread over a 39 × 16 cm surface to a liquid thickness of 500 μm, with a spreading speed of 0.2 cm s⁻¹. A self-adhesive plastic film (d-c-fix®, Konrad Hornschuch AG, Weissach, Germany) was applied to the steel casting plate to facilitate the peeling of the film after drying. The casting plate was kept at 30°C, and the film was left to dry for 24 hours before peeling.

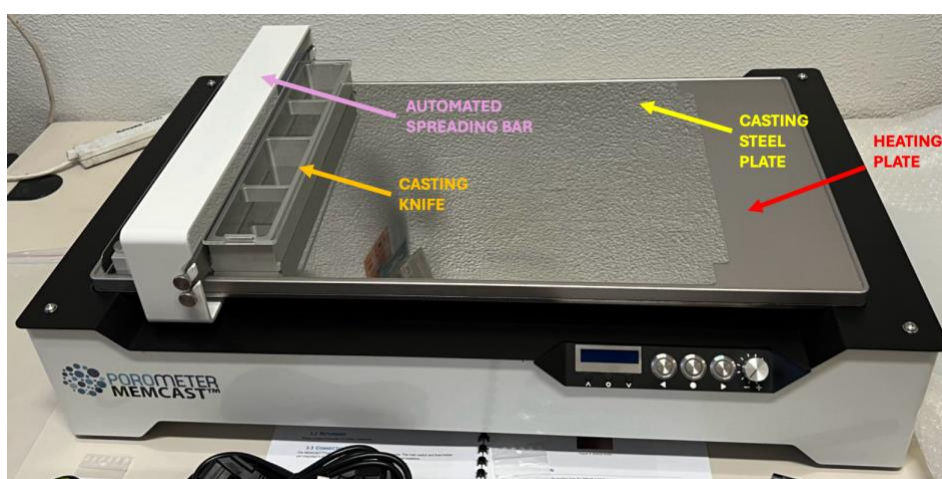


Fig. 7. Porometer Memcast™ automated flat-sheet casting module.

The scanning electron microscopy (SEM) was adopted to examine the film surface for irregularities. For SEM preparation, approximately 10 × 5 mm portions of each film were mounted on 12 mm diameter circular specimen holder stubs (Agar Scientific, Essex, UK) using double-sided carbon tape (Carbon Tabs, 9 mm, Agar Scientific, Essex, UK) and coated with platinum via a sputter coater (ACE600, Leica Microsystem, Wetzlar, DE). Surface imaging was performed with a Field-Emission SEM Sigma (Zeiss, Oberkochen, DE) at an accelerating voltage of 5 kV and a working distance of 5 mm.

In additions, oxygen transmission rate (OTR) of the films was measured at 23 °C and 50% RH using a PermeO2 instrument (ExtraSolution, PermTech, Pieve Fosciana, Lucca, IT) to compare the barrier properties of films obtained through Memcast™ and solution casting on

Teflon plate as above described. The film was mounted on a "surface reducer" composed of two aluminum sheets, allowing a permeation surface of 25 cm². The samples were individually clamped within the diffusion cell and conditioned for 4 h before the measurement started. The oxygen volumetric flow rate per unit area of the film as a function of time (OTR, cm³ m⁻² 24h⁻¹) was continuously monitored until a steady state was reached.

The dry film obtained with Memcast™ had a smooth, visually defect-free surface with a uniform thickness of about 140 ± 5 μm. The applied adhesive plastic made the film peeling operation easy. The microstructure of the film surface analysed by SEM resulted in the image shown in Figure 8.

Looking at the film surface in contact with the Memcast™ plate, it was possible to observe the presence of numerous irregularities and bubbles, with diameters ranging from 1 to 10 μm. In addition, at each bubble a small pinhole is present; these small holes did not affect the entire thickness of the film but greatly increased the permeability of the film to oxygen.

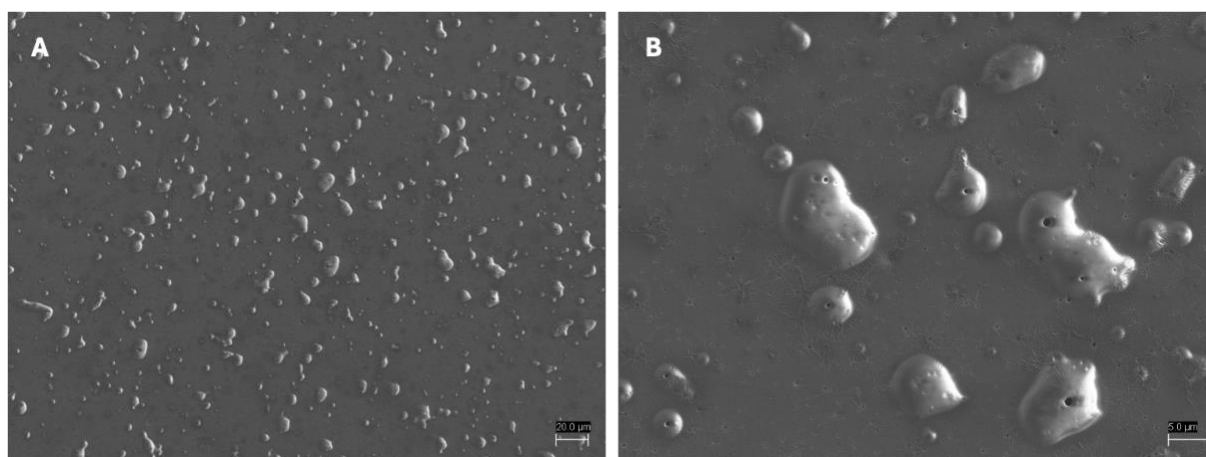


Fig. 8. SEM micrographs of the film obtained through spreading using Memcast™. The micrographs show the surface of the film in contact with the Memcast™ plate. The magnification in panel A is 1000x and the bar length is 20 μm, while in panel B the magnification is 5000x and the bar length is 5 μm.

Thus, the OTR analysis resulted in an oxygen flux value (in cm³ m⁻² 24h⁻¹) that is beyond the limit detectable by the instrument. The film F_LAB_PS_B produced by solution casting has an OTR of 5.9 ± 0.5. The formation of these bubbles and associated pinholes can be related to the heating of the Memcast™ plate to 30 °C, which creates faster evaporation of the free water present in the film, leading to the formation of irregularities through cavitation phenomena.

Solution casting is a simple laboratory-scale method that is widely applied in research studies and was also adopted in this PhD project (Belyamani et al., 2014). However, alternative methods are also worth investigation.

Because of the interest and novelty of our casein powder preparation, it was decided to conduct some side experiments to explore a twin-screw extrusion process to produce NaCas pellets, followed by a blown extrusion to form the films.

The extrusion process involves producing NaCas pellets using a twin-screw extruder, which are then fed into a blown-film extruder to produce films with uniform thickness (Colak et al., 2015). This section focuses solely on the preliminary production of NaCas-based pellets, investigating different formulations and their behavior under various operational conditions. Experimental NaCas extrudates were produced using a bench-top twin-screw extruder with conveying screws (HAAKE™ MiniCTW Micro-Conical Twin Screw Compounder - Thermo Fisher Scientific, Cleveland, OH, USA). The interpenetrate screws had a length of 100 mm, a diameter of 15 mm and the slit die used had a diameter of 2 and 1 mm. The NaCas powder was introduced in the feeder together with water, glycerol and olive oil using a force feeder. The amount of water, plasticizer and olive oil were used to control both the viscosity of the melt and the engine torque of the extruder, which was attempted to be kept constant during extrusion, except for the starting phase of the test, when the ingredients have yet to mix. As hydrophilic plasticizers, water and glycerol naturally reduce the overall viscosity when added to caseinate powder. Preliminary trials were dedicated to optimize the relative amounts of NaCas, water and olive oil in the operating chamber that was always kept at 80 °C.

The subsequent tests performed included the following conditions:

- Trial 1: 5 g NaCas P_COM_S; 30% (w/w) ultrapure water; 15% (w/w) analytical-grade bi-distilled glycerol; 4% (w/w) olive oil. Extrusion parameters: operating chamber temperature at 80°C, rotation screw speed 100 rpm, with a 4-minute residence time before the extrusion (nozzle diameter: 2 mm).
- Trial 2: 5 g NaCas P_LAB_RF; 25% (w/w) ultrapure water; 15% (w/w) analytical-grade bi-distilled glycerol. Extrusion parameters: operating chamber temperature at 80°C, rotation screw speed 100 rpm, with a 4-minute residence time before the extrusion (nozzle diameter: 2 mm).
- Trial 3: 6 g NaCas P_LAB_RF; 15% (w/w) ultrapure water; 15% (w/w) analytical-grade bi-distilled glycerol. Extrusion parameters: operating chamber temperature at 80°C, rotation screw speed 100 rpm, with a 5-minute residence time before the extrusion (nozzle diameter: 1 mm).

In trial 1, the formulation produced a workable paste. While the extrudate was excessively wet and showed slight expansion upon exiting the nozzle, it exhibited a yellowish color (Figure 9A), suggesting that the high water and olive oil content may have reduced the Maillard reaction. Trial 2 involved the removal of the oil fraction, and slight reduction of the water content. The screw speed was again gradually increased to 100 rpm. The resulting extrudate was firmer,

less sticky, and brownish in color, with no significant expansion observed upon exiting the nozzle, as shown in Figure 9B.

In trial 3, the water content was further reduced to 15%, while maintaining a constant glycerol level. The screw speed was gradually increased from 10 to 100 rpm, resulting in a final torque of 1.7-2.2 N, well within the instrument's maximum limit of 10 N. The extrudate, extruded through a 1 mm nozzle, displayed considerable expansion upon release (Figure 9C), with a dark brown color and a firmer consistency. This outcome likely reflects a more pronounced Maillard reaction due to the lower water availability (Cardoso et al., 2011).

This series of trials highlights the critical balance between ingredient composition, moisture content, and operational parameters in optimizing the extrusion process for NaCas pellet production.

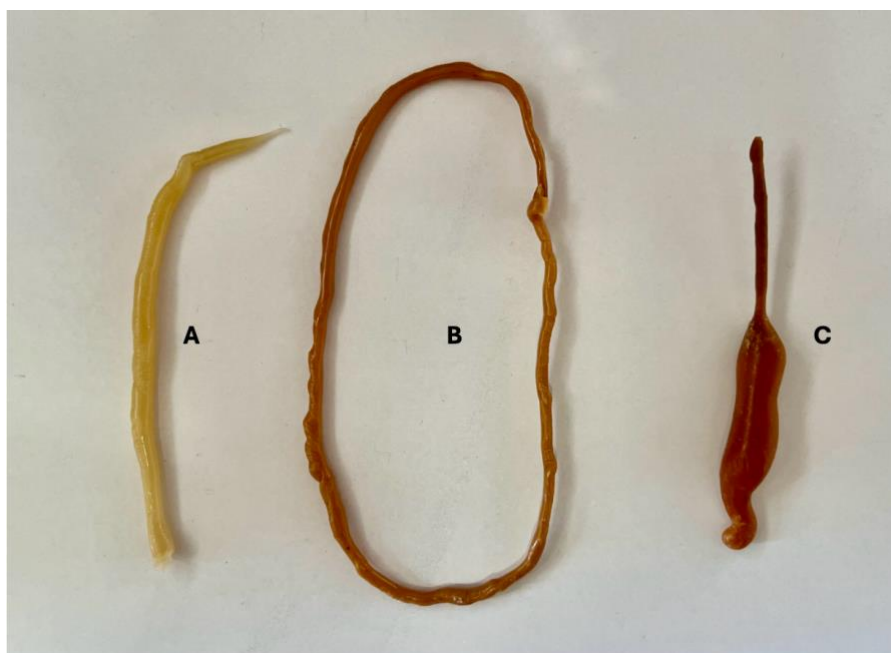


Fig. 9. NaCas-based extrudates obtained from the following experimental conditions: A) 5 g NaCas (P_COM_S), 30% water, 15% glycerol, 4% olive oil, residence time: 4 min; B) 5 g NaCas (P_LAB_RF), 25% water, 15% glycerol, residence time: 4 min; C) 6 g NaCas (P_LAB_RF), 15% water, 15% glycerol, residence time: 5 min. The temperature of the chamber was 80 °C and the screw speed 100 rpm in all trials.

This series of trials provided a preliminary assessment of formulations for the production of NaCas-based extrudates. However, these formulations differ significantly from those required for NaCas-based films. As such, further in-depth investigations are necessary to optimize the concentrations of powder, water, and glycerol, along with exploring the potential inclusion of a hydrophobic component. Additionally, the incorporation of an amphiphilic agent should be considered to stabilize the two phases, ensuring a more uniform and functional material suitable for film production.

Conclusions

Based on literature knowledge and previously acquired experience, baseline experimental work was crucial for setting the main conditions for the development of the NaCas-based films. Key advancements included the successful extraction of NaCas obtaining powders with different contents of residual fat, with high fat levels leading to undesirable aggregation and coalescence phenomena. An additional skimming treatment reduced the fat content, producing films with improved uniformity and size of fat structures. A 10% NaCas solution with 30% glycerol was identified as an optimal formulation, balancing mechanical properties with ease of processing. Importantly, glycerol was confirmed not to interfere with microstructural analyses.

For the casting process, testing of plates made of different materials highlighted that food-grade silicone offered ease of film removal and smooth surfaces, though concerns regarding potential migration of compounds emphasized the need for an alternative material. The Teflon plate made it possible to obtain a film with a smooth and regular surface, not allowing any compounds to migrate into the film, and for this reason it was chosen as a suitable material for the continue of the experiments, covered in the next chapter.

Alternative methods, such as automated spreading and extrusion, demonstrated potential scalability for industrial applications but require further optimization to address issues such as ingredient mixing, moisture control, and material viscosity.

These findings establish a robust framework for producing NaCas-based films, addressing both methodological challenges and practical considerations for future applications. The set-up of the experimental conditions obtained in this chapter provide the foundation on which the research project is based, particularly the investigation of the role of different concentrations of fat in the powders for film production and the role that different techniques for bubbles removal from the film-forming solution play in the properties of the films, topics discussed in Chapter 2.

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Chapter 2 - Upcycling Casein from Expired Pasteurized Milk into films – Development and characterization of films made from sodium caseinates

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Abstract

The present study focuses on the upcycling of expired pasteurized milk into sodium caseinate (NaCas) that was used to develop sustainable packaging films. Three NaCas types were prepared from pasteurized milk with a different fat content and a commercial NaCas was used as a control. The film forming solutions were added with glycerol as a plasticizer and were treated with two different sonication modes, bath or probe, before casting and drying in ambient conditions. Results showed that NaCas with varying milk fat content led to notable differences in properties of the derived films. Films with higher fat content (25%) were thicker and with a more irregular and heterogeneous microstructure, as observed by SEM and CLSM. Sonication, particularly probe sonication, reduced the size of milk fat structures in the derived films. Fat content also impacted on mechanical properties, such as tensile strength and elongation at break, as well as on oxygen permeability which showed higher values in films with high fat content. A significant improvement in the oxygen barrier was achieved when bath sonication was used. Thermal analysis revealed distinct transitions related to both fat content and processing, affecting melting and crystallization in the different films. Films with high fat content were hazier than low-fat films. By properly combining milk fat content and sonication conditions, NaCas obtained from expired pasteurized milk can become a raw material for upcycling this high-impact waste into neat films for green packaging applications.

Keywords

Expired pasteurized milk, sodium-caseinate films, mechanical properties, thermal properties, packaging material, ultrasonic treatment.

1. Introduction

The increasing demand for milk and its derivatives in multiple countries has driven a significant growth in the global dairy industry, leading to a parallel increase in waste generated by the entire supply chain. Among dairy products, fluid milk has been estimated to constitute around 67% of total dairy food loss by weight in the United States, and such substantial food loss can also have a significant impact on the profitability of retailers (Endara et al., 2023). Pasteurized milk represents a premium type of drinking milk since it receives the minimum heat treatment to guarantee its microbiological safety without compromising the integrity of nutrients. The duration of pasteurized milk's shelf-life depends on factors such as the quality of the initial raw milk, the processing conditions, and the storage conditions (Ziyaina et al., 2018). Consumers detect the decline in milk quality through off-flavors, which can stem from chemical, physicochemical, or microbiological alterations in the product (Zygoura et al., 2004). Among these defects, light-induced off-flavors are likely the most prevalent in pasteurized milk (D'Incecco et al., 2024; Limbo et al., 2020). Expired pasteurized milk is no longer suitable for human consumption and becomes a waste even though it contains high-value components. At present, expired pasteurized milk is mostly destined to animal feeding, like other dairy wastes, or utilized as a substrate of anaerobic digestion processes for producing biofuels and bio-products (Ahmad et al., 2019; Donzella et al., 2022; Okamoto et al., 2019). Nevertheless, besides other milk components, milk proteins are recognized as the fraction most suitable for non-food technological applications (De Gregorio et al., 2024; Indrie et al., 2023; Zhu et al., 2023). Due to the growing interest in the development of sustainable packaging materials, biopolymers from various animal and plant sources are extensively studied to replace synthetic polymers. However, processes for extracting these biopolymers are mostly complex and expensive, thus limiting their commercial interest. In contrast, casein is readily recovered from fluid milk with a yield of about 27 g/L; it is safe, biocompatible, and biodegradable. In this context, producing films from casein obtained from dairy wastes like expired milk represents an option attracting considerable interest (Costa et al., 2021; Gerna et al., 2023).

Casein is the major protein fraction of milk. Native casein consists of four different proteins (α s1, α s2, β , κ -casein) associated into large complexes stabilized by calcium phosphate nanoclusters and called micelles. Due to the high content of hydrophobic amino acids (35-40%) and the low degree of secondary structure, casein molecules form hydrophobic and hydrogen bonds establishing robust interactions (contacts) between polymeric chains. The

presence of polar amino acid residues unevenly distributed contributes to the remarkable thermal stability of casein (Markoska et al., 2020).

Casein is typically separated from milk by isoelectric precipitation at pH 4.6. With the increasing acidity, casein micelles swell due to the dissociation of calcium ions and the individual casein molecules associate and sediment. The sedimented casein is then treated with Na or Ca hydroxide to obtain water soluble caseinates, and dried. Solutions of Na caseinate (NaCas) have proven to have good emulsifying and foaming properties but also efficacy in forming coatings. In fact, casein has been successfully used as coating substance for paper and paperboard (Khwalidia, 2010; Kim et al., 2023) or as edible coating for foods to provide moisture, oxygen or aroma barrier (Miele et al., 2022; Khan, et al., 2021). Casein-based coatings can also represent a support that releases bioactive compounds (Picchio, et al., 2018).

Different from coatings, stand-alone NaCas films are rather brittle and susceptible to cracking during the drying process due to the strong intermolecular cohesion of individual protein chains. The use of a plasticizer has proven to be essential to reduce these drawbacks and to improve the mechanical properties of the film. Commonly used plasticizers are low-molecular weight compounds, such as glycerol or sorbitol, that form hydrogen bonds with the polymeric matrix thus reducing the tensile strength of the film and increasing its flexibility. Furthermore, compared to commonly used synthetic polymers, casein-based films have rather low water vapor barrier and thermal properties. To overcome these limitations, composite films have been produced incorporating into the film-forming solution various reinforcing components, such as chitosan (Wai et al., 2022), gelatin-coupled cellulose (Yao et al., 2019), microcrystalline cellulose (Deng et al., 2023), and TiO₂ nanoparticles (Hoyos Merlano et al., 2023). However, the use of composite films is limited by the high costs of production since this often relies on generating nanostructures of the compound to be incorporated (Garavand et al., 2022). Other studies have shown that properties of casein-based films can be improved by protein crosslinking using different agents. Genipin (Qiu et al., 2020), tannic acid (Picchio et al., 2018), and transglutaminase (Zhang et al., 2014) are the most widely used crosslinking compounds because of their low toxicity compared to aldehydes such as formaldehyde or glutaraldehyde. Enzymatically crosslinked NaCas was successfully used to form stand-alone films (Juvonen et al., 2011). Nevertheless, it is well known that selected amino acids may crosslink upon heating and that the involved reactions are promoted by alkaline pH conditions and lack of lactose (Anema, 2021; Nielsen et al., 2022). Manufacturing conditions of NaCas at both laboratory and industrial level proved to induce formation of lysinoalanine (LAL), a dehydroalanine-mediated crosslink involving phosphoserine and lysine residues (Cattaneo et al., 2012). Similarly, adjacent tyrosine residues may spontaneously crosslink in milk exposed to light (D’Incecco et al., 2024). Generating these crosslinks in NaCas as a strategy for

improving selected properties of the derived films is still unexplored. The addition of lipids to NaCas has been investigated with the aim of compensating for the high hydrophilicity of the derived films. Indeed, the barrier properties of lipid-containing materials are directly influenced by factors such as the degree of unsaturation, the length of the aliphatic chains, and the nature of chemical functionalities of lipid components themselves (Devi et al., 2024). Free fatty acids were either emulsified in the caseinate solution (Fabra et al., 2011) or covalently grafted onto the casein chains before preparing the film (Tichané et al., 2023). However, to the best of our knowledge, no studies have dealt with the effects of milk fat globules, primarily composed of triacylglycerols (TAGs) in their native form in milk, on the properties of NaCas films.

Ultrasonication has been proposed as a high-efficiency green technology for improving physicochemical properties of biopolymer-based films (Mi et al., 2023). Compared with other physical methods that also have advantages, ultrasonication of film-forming solutions looks to be effective in promoting a more uniform and denser microstructure of the derived films. This effect is mainly permitted by bubble collapse during cavitation causing a better mixing of fat, protein, and water phases. Despite this, effects of ultrasonication treatment of NaCas solutions on the derived films have been scarcely investigated (Dutta & Sit, 2023).

In this work, a set of eight NaCas-based films were obtained under different conditions and characterized in terms of structural, thermal, and mechanical properties. NaCas powder samples were prepared at laboratory scale from expired pasteurized milk with a different fat content, while a commercial NaCas sample was used as reference material. Water solutions of NaCas were prepared with the sole addition of glycerol as plasticizer and submitted to ultrasound treatment under different conditions. Neat films were obtained by the casting method. The aim of the study was to investigate the conditions that can allow production of neat non-edible NaCas films with properties supporting their use as packaging material. This knowledge will contribute to the development of clean processes and green approaches in the packaging sector. Depending on composition and production conditions, neat NaCas films could assume the right features for further developing novel materials with functional, active or intelligent properties.

2. Materials and Methods

2.1 Experimental design

The outline of the experimental design and the sample codes are shown in Fig. 1. The present study has been developed at three levels, i.e. the powders (P) of NaCas, the derived film-forming solutions (S), and the final films (F). Three independent experiments were carried out on different occasions. In each experiment, all the film types were produced in order to perform the whole set of analyses. Additional samples were freshly prepared when necessary.

All powders were prepared at laboratory scale (LAB) and a commercial sample (COM_S) was used as control. Two powders were obtained starting from partially skimmed milk as it is (PS) or after further fat removal (reduced fat, RF); in addition, a skimmed (S) powder was obtained starting from skimmed milk, as described below. For each powder, the respective film-forming solution underwent a sonication treatment using either an ultrasonic bath (B) or an immersion probe (P).

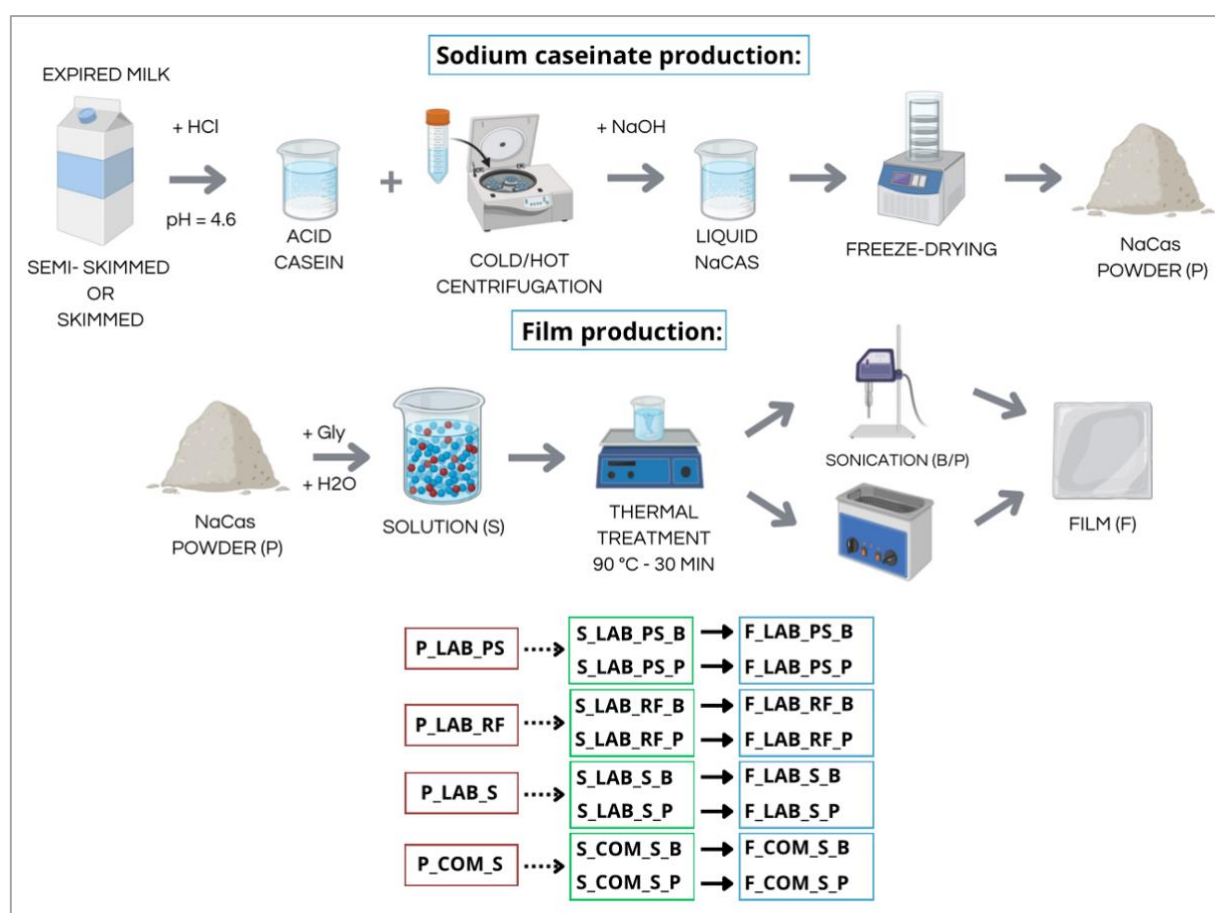


Fig. 1. Experimental design showing the production of sodium caseinate powders (P), film-forming solutions (S) with two different sonication treatments (B/P) and the production of the final films (F). The respective sample codes used throughout the text are specified at the bottom (Figure created with BioRender).

2.2 Preparation and characterization of Na caseinate powders

Partially skimmed (fat 1.5%, w/v) pasteurized milk was collected from a local supermarket 1-2 days before expiration. All 1-L bottles had a pH of about 6.6 at opening. Subsequently, milk pH was adjusted to 4.6 with 2N HCl to induce casein precipitation. Acid whey was removed by centrifugation at 4000 g for 15 min at 4°C using a Sorvall® RC-5B centrifuge (Thermo Fisher Scientific, Milan, IT). The fat layer atop the vial was removed with a spatula and the supernatant was discarded. The casein pellet was suspended in ultrapure water (MilliQ, Waters), agitated

with a magnetic stirrer for 15 min and centrifuged as mentioned before. A second washing was carried out. The pellet was then dispersed in a minimal volume of water (150 mL water/100 g pellet) and solubilized by dropwise addition of 2N NaOH under stirring until pH stabilized to 7. The solution was subsequently poured into a stainless-steel tray and freeze-dried (Lio10P, 5Pascal, Milan, IT) for approximately 24 h. The freeze-dried NaCas was ground, transferred into a plastic bottle with a tight stopper and stored in a cool, dry place until analyses. This process yielded P_LAB_PS powder.

Reduced fat milk for preparing P_LAB_RF powder was obtained by centrifugation of the partially skimmed milk at 40 °C (8000 g for 20 min) and removal of the oily supernatant with a Pasteur pipette. Differently, the P_LAB_S powder was directly prepared from commercial pasteurized skimmed milk (fat 0.3% w/v). In both cases, the same conditions for casein separation, washing and freeze-drying described for the P_LAB_PS were followed.

A commercial NaCas powder (P_COM_S) was purchased from “Società Ingredienti e Additivi – S.I.A. S.r.l.” (Desio, MB, IT) and used as a reference powder.

All powders were analyzed for protein (ISO, 2014), fat (ISO, 2022), moisture (ISO, 2006), and ash (ISO, 2008) content following the respective ISO Standard methods. Analyses were performed in triplicate.

2.3 Preparation and characterization of film-forming solutions

Solutions of NaCas (10% protein in water, w/v) were prepared following the procedure used by Juvonen et al. (2011) with some modifications. The powder was dissolved in ultrapure water under stirring for 10 min. The solution was then heated to 90 °C and stirred for 30 min using a magneto-thermic stirrer (C-MAG HS 4, IKA, Staufen, DE). Afterward, the solution was stirred for an additional 10 min at room temperature to allow it to cool down. Analytical grade bi-distilled glycerol (VWR Chemicals, Milan, IT) was added at a concentration of 33.3% (w/w), and the solution was stirred for an additional 10 min to ensure homogeneous dispersion of glycerol. The resulting solutions (30 mL) underwent a 10-min continuous sonication in an ultrasonic bath Transonic T310 (GEASS, Turin, IT) operating at a frequency of 37 kHz. The temperature of water in the bath was controlled and kept below 25 °C. Samples S_LAB_PS_B, S_LAB_RF_B, S_LAB_S_B, and S_COM_S_B were obtained. Alternatively, the solutions (30 mL) were sonicated using an ultrasonic cell disruptor (Soniprep 150 Plus, MSE, UK) with a probe of 9.5 mm operating at a frequency of 23 kHz. The probe was directly immersed into the solution, about 1 cm deep below the liquid surface, and the amplitude of the sonication was kept constant at 10.6 μ m. During sonication, the beaker containing the sample was kept in an ice bath to prevent overheating of the solution. Three 30-s sonication cycles, with a 2-min rest

between cycles, were applied to obtain samples S_LAB_PS_P, S_LAB_RF_P, S_LAB_S_P, and S_COM_S_P. The final pH values for the different solutions were in the range 6.95-7.09.

2.3.1 Rheological analyses

All the NaCas solutions were characterized in terms of flow behavior, determining the flow and viscosity curves by a Physica MCR 102 rheometer (Anton Paar GmbH, Graz, AT), supported by the software RheoCompass (v. 1.21, Anton Paar GmbH) and equipped with a cone-plate geometry with 50 mm diameter (CP50). The analyses were performed in triplicate at 25 °C, with a shear rate range of 0.1-500 s⁻¹. The power law model was used to fit experimental data and calculate the consistency coefficient (K) and flow behavior index (n) (Steffe, 1996).

2.4 Production and characterization of films

The solutions were cast onto Teflon molds measuring 10 x 10 cm, using 0.1 mL/cm² as described by Juvonen et al. (2011). The molds were left to dry at room temperature (21-23 °C) and ambient relative humidity (RH) for 48 h. After the drying period, the films were peeled off from the molds, individually sandwiched between two sheets of aluminum foil, and stored in a polystyrene box at 4 °C until analyses. The moisture content of the films was determined on 3 x 3 cm portions kept in a laboratory oven at 102 °C until constant weight was reached. Three replicates were carried out for each film. The approximate composition of each film was calculated based on the composition of the parent mother solution and the actual moisture content. This allowed us to have an estimate of the content of milk fat and glycerol separately. Film thickness was the average of values measured in 9 random positions using a Mitutoyo QuantuMike Digimatic micrometer (Takatsu-ku, Kawasaki, Kanagawa, JP).

2.4.1 Microstructural characterization

Microstructural investigation of films was performed by confocal laser scanning microscopy (CLSM) as described by D'Incecco et al. (2020) with some modifications. Three representative portions of each film (around 3 × 3 mm) were sampled using a razor blade and fixed in 1 mL of fixative solution (glutaraldehyde 3%, paraformaldehyde 2% in 20 mM phosphate-buffered saline (PBS), w/v; Agar Scientific, Stansted, UK) for 2 h. Samples were washed three times in the same buffer for 10 min each and stained with Nile Red (Sigma Aldrich, St Louis, MO, USA) to visualize triglycerides of milk fat. The Nile Red stock solution, 1 mg/mL in 80% dimethyl sulfoxide (Sigma Aldrich), was tenfold diluted in MilliQ water prior staining of sample in a watch glass for 5 min. The stained sample was mounted on a microscope slide and covered by a coverslip using the Eukitt mounting medium (Sigma

Aldrich). An upright confocal laser scanning microscope A1+ (Nikon, Minato, JP) equipped with a 60x oil immersion objective was used to visualize samples. The excitation and emission wavelengths were set at 488 nm and 520–590 nm respectively (Bettera et al., 2023). Ten images of each film were taken at different positions in the sample. Three representative images were analyzed using the open-source software ImageJ (National Institute of Health, Bethesda, MD, USA) to calculate the number of particles, surface area, Feret diameter and circularity. The surface of the upper side of the films was investigated by scanning electron microscopy (SEM). A portion of around 10 × 5 mm of each film was mounted on circular specimen holder stub of 12 mm diameter (Agar Scientific, Essex, UK) with double carbon tape (Carbon Tabs 9 mm, Agar Scientific, Essex, UK) and platinum coated with a sputter coater ACE600 (Leica Microsystem, Wetzlar, DE). Observations were carried out using a Field-Emission SEM Sigma (Zeiss, Oberkochen, DE) operating at 5 kV with 5 mm working distance.

2.4.2 Thermal and thermo-mechanical properties

For thermal characterization, a differential scanning calorimetry (DSC) analysis was performed following the procedure of Plumier et al. (2024) and Pankaj et al. (2014) with some modifications. A NEXTA DSC600 (Hitachi High-Tech, Tokyo, JP) equipped with an electric cooling system was used, by applying heating/cooling/heating cycles at a rate of 10 °C/min in the range of temperature from -20 to 220 °C. Film aliquots of about 5 mg were inserted into a crimped aluminum pan (an empty pan was used as a reference). Melting temperature (T_m) and glass transition temperature (T_g) were determined where possible.

Dynamic mechanical thermal analysis (DMTA) was performed with a NEXTA DMA200 (Hitachi High-Tech, Tokyo, JP) equipped with a liquid nitrogen cooling system. Film samples (5 x 10 mm) were analyzed in tension mode from -150 °C to 220 °C. A sinusoidal strain (amplitude: 10 μ m, frequency: 1 Hz) was applied on the specimens and the resulting storage (E') and loss (E'') moduli, as well as the loss tangent ($\tan\delta$), were plotted as a function of temperature. Prior to analyses, the samples were conditioned at 40 °C and a RH of 50% for 48 h.

2.4.3 Mechanical and barrier properties

The mechanical properties of the films, including tensile strength (TS), elongation at break (EAB), and Young's modulus (YM), were evaluated at room temperature following the protocol ASTM D882-18 and Lin et al. (2020) with modifications. A TA.HDplus Texture Analyser (Stable Micro Systems, Godalming, UK) was used, equipped with suitable grips for tensile tests. Prior to testing, the films were conditioned at 50% RH and 20 °C. Rectangular strips of film (20 x 100 mm) were clamped with an initial grip separation of 60 mm and a crosshead speed of 20

mm min⁻¹ was used until film break. The values of EAB (%), TS (MPa), and YM (MPa) were calculated from the force-strain curves obtained, as the average of at least eight determinations.

Oxygen transmission rate (OTR) of the films was measured at 23 °C and 50% RH using a PermeO2 instrument (ExtraSolution, PermTech, Pieve Fosciana, Lucca, IT). The film was mounted on a "surface reducer" composed of two aluminum sheets, allowing a permeation surface of 25 cm². The samples were individually clamped within the diffusion cell and conditioned for 4 h before the measurement started. The oxygen volumetric flow rate per unit area of the film as a function of time (OTR, cm³ m⁻² 24h⁻¹) was continuously monitored until a steady state was reached.

Water vapor transmission rate (WVTR) of the films was determined at 23 °C and 65% RH using a TotalPerm (ExtraSolution). The film was prepared and conditioned as for OTR measurement. The water vapor flow rate per unit area of the film as a function of time (WVTR, g m⁻² 24h⁻¹) was continuously monitored until a steady state was reached.

To compare the OTR and WVTR values of different films, which possess different thicknesses from each other, the derived values of oxygen permeance (KPO₂) and water vapor permeance (KPWV) were calculated, respectively. The KP is derived from the following equation (Eq. 1) and considers a unit thickness and a unit partial pressure difference (1bar):

$$KP = \frac{l \cdot Q}{A \cdot t (p_1 - p_2)} \quad (1)$$

Where KP = permeance of the gas, l = thickness of the film, Q = gas flow, A = area, t = time, $(p_1 - p_2)$ = partial pressure difference.

2.4.4 Optical properties

Color measurement was performed using a portable tristimulus colorimeter Chroma Meter CR-400 (Konica Minolta, Nieuwegein, NL), with standard illuminant C, and a white standard plate was used for instrument calibration and as the background of the films. The three CIE color parameters (L^* : lightness, a^* : green-red, b^* : blue-yellow) were measured with nine readings per film. The measured coordinates were used to calculate total color difference (DE) with respect to the control caseinate films (F_COM_S_B and F_COM_S) using the Eq. (2) shown below:

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

Different color indexes were calculated as described by Milovanovic et al. (2020); D’Incecco et al. (2020). Whiteness (WI) and yellowness (YI) indexes were calculated using equations Eq. (3) and Eq. (4), respectively:

$$WI = 100 - [(100 - L^*) + a^{*2} + b^{*2}]^{1/2} \quad (3)$$

$$YI = 142.86 b^*/L^* \quad (4)$$

The haze and UV-visible transmission spectra of the films were measured as described by Taglialatela Scafati et al. (2011), using a Lambda 650 Perkin Elmer spectrophotometer (Milan, IT) equipped with a 150 mm integrating sphere, according to ASTM D1003-21.

2.5 Statistical treatment of data

Statistical analysis of data was performed using the SPSS Win 12.0 software Version 27 (IBM Corp., Chicago, IL, USA). The results are presented as mean $\pm$ standard deviation, except for thickness and image analysis outputs where results are expressed as median $\pm$ standard error. To determine the significance of differences between samples and treatments, one-way analysis of variance (ANOVA), multi-factor ANOVA (factors: COMPOSITION and TREATMENT) as well as Tukey’s test ($p < 0.05$) were performed. Instead, for non-homoscedastic variables (i.e., mechanical properties), Dunnett post-hoc test was used considering F_COM_S as the reference. Finally, for non-homogeneously distributed data with non-homoscedastic variances (i.e., thickness and image analysis data), the nonparametric Kruskal-Wallis test was used, with comparisons of homogeneous subsets to determine the difference between groups identified according to the 'COMPOSITION' factor. Principal component analysis (PCA) was conducted after the Bartlett’s test of sphericity was verified ($p < 0.05$) and using components with eigenvalue >1 . Only variables with loading >0.65 were selected: Fat, Moisture, Feret diameter, TS, YM, EAB, KPWW, KPO₂, WI, YI and Haze. Before PCA, data were normalized using the “standardize” function.

3. Results and Discussion

3.1 Powders characterization

The gross composition of the powders is shown in Table 1.

Table 2. Gross composition (g/100g) of experimental and commercial NaCas powders.

Sample	Protein	Fat	Moisture	Ash
P_LAB_PS	55.82 ± 0.99 ^a	32.13 ± 0.07 ^a	6.29 ± 0.00 ^a	3.21 ± 0.00 ^a
P_LAB_RF	67.07 ± 0.80 ^b	22.71 ± 0.01 ^b	3.83 ± 0.07 ^b	4.17 ± 0.10 ^b
P_LAB_S	91.76 ± 0.56 ^c	1.01 ± 0.01 ^c	1.75 ± 0.07 ^c	4.51 ± 0.06 ^c
P_COM_S	87.83 ± 0.20 ^d	1.04 ± 0.01 ^c	7.32 ± 0.03 ^a	3.53 ± 0.03 ^d

Results are expressed as mean ± standard deviation (3 replicates). Data in the same column followed by different superscript letters are significantly different ($p < 0.05$) according to Tukey test.

As expected, large compositional differences were observed among samples. In fact, the process for casein separation was intentionally carried out starting from batches of pasteurized milk having a different fat content, as described in section 2.2. This resulted in powders with considerably different contents of fat (from 1 to 32 g/100g) and protein (from 56 to 92 g/100g). Commercial pasteurized milk undergoes a homogenization process that, besides reducing the size of the fat globules, induces their interaction with casein (Cano-Ruiz & Richter, 1997). Therefore, it is reasonable to assume that the fat present in the powders is mainly represented by small fat globules bearing casein micelles bound on the surface and that, consequently, co-precipitate with casein upon milk acidification. The removal of fat from partially skimmed milk by means of centrifugation at 40 °C to produce P_LAB_RF was effective, as the milk TAGs are liquid at this temperature and allow to reduce the interaction with casein, obtaining a higher yield. As expected, the P_LAB_S powder obtained from skimmed milk showed the highest protein content (91.76 ± 0.56 g/100g) and a very low-fat content (1.01 ± 0.01 g/100g). Overall, the composition of this powder was close to that of the commercial NaCas (P_COM_S) used as a control, except for the significantly lower moisture content. The difference in moisture content of experimental powders is probably due to the lyophilization process at pilot-scale.

3.2 Film-forming solution characterization

The NaCas solutions were prepared with a constant concentration of 10% protein and 33% glycerol. The viscosity curves are shown in Fig. S1 while Table 2 reports the derived values of consistency coefficient (K) and flow behavior index (n) calculated by the power law equation.

Table 2. Values of consistency coefficient (K) and flow behavior index (n) calculated for experimental and commercial NaCas solutions.

Sample	K (Pa s ⁿ)	n
S_LAB_PS_B	0.054 ± 0.002	0.990 ± 0.007
S_LAB_PS_P	0.176 ± 0.006	0.980 ± 0.004
S_LAB_RF_B	0.124 ± 0.002	0.980 ± 0.005
S_LAB_RF_P	0.144 ± 0.006	0.978 ± 0.003
S_LAB_S_B	0.038 ± 0.003	1.004 ± 0.015
S_LAB_S_P	0.041 ± 0.002	0.991 ± 0.008
S_COM_S_B	0.036 ± 0.002	0.992 ± 0.001
S_COM_S_P	0.044 ± 0.003	0.989 ± 0.015
COMPOSITION	< .001	.012
TREATMENT	< .001	.073
COMP X TREAT	< .001	.632

All samples showed a nearly Newtonian behavior (n values near to 1) and viscosity increased with the increase in solid matter, confirming observations of Valentino et al. (2020), although it has been shown by Pitkowski et al. (2008) that shear thinning behavior is found in sodium caseinate suspensions at high concentrations. In general, K and viscosity values were higher when solutions were treated by the sonication probe; indeed, the factor “treatment” resulted significant ($p < 0.001$). This effect was most evident in partially skimmed sample (S_LAB_PS), suggesting that the difference in K values could be due to a different fat dispersion determined by bath or probe sonication, possibly leading to a higher number of fat globules with probe sonication. The literature extensively confirms that sonication decreases fat globule size and increases their number. These effects intensify with increasing treatment power, temperature and duration (Gregersen et al., 2019, Sun et al., 2024). Consistently, we did not observe creaming of fat in the solutions after the sonication treatments. The effect of sonication mode was almost negligible in skimmed samples (S_LAB_S and S_COM_S) which also displayed the lowest values of viscosity and K, confirmed by the significance of the factor “composition”. According with the above, the Tukey test revealed that all solutions were significantly different from each other for the 'composition' factor, except for the S_LAB_S and S_COM_S solutions. The effects of sonication mode on fat dispersion are specifically addressed in film characterization section. It is worth mentioning here that ultrasound treatments also affect structure and conformation of proteins, besides improving disruption of protein aggregates in reconstituted powders (Chandrapala & Leong, 2015). Han et al. (2020) reported that ultrasound treatment reduced secondary structure of casein and increased exposure of

hydrophobic amino acid residues, supporting a different behavior of casein at fat interface in sonicated solutions. The cause of protein unfolding is conducted to cavitation events that result in high temperature and pressure reached locally. Further studies to assess the potential role of casein modifications induced by ultrasonication are needed.

3.3 Film composition and thickness

The percent composition of the films was calculated based on the composition of the corresponding film-forming solution and the actual moisture content retained into the film after drying (Table 3). All films proved to have a similar moisture content except for the two films produced from the commercial NaCas, whose moisture content was unexpectedly higher. Schou et al. (2005) reported the moisture content of NaCas films to be strongly dependent on the glycerol to protein ratio. However, this ratio was kept constant to 0.33 in all our films. As planned, the films primarily differed for the content of milk fat, that ranged from 0.7 to 25%. Different from what occurs for glycerol, TGs are confined within globular structures that are unevenly distributed within the matrix of single films. To appreciate the specific role of milk fat, its content was obtained by calculation.

The thickness of NaCas films varied across the different formulations. F_LAB_PS and F_LAB_RF samples, both produced from experimental powders with a relevant fat content, showed comparably high thickness values; instead, thickness values were lower for F_COM_S and F_LAB_S films, where the fat content is very low. Therefore, differences in thickness can be attributed to the initial powder composition, specifically to the presence of milk fat and possible aggregates created by sonication. Differently, the type of sonication did not bring to significant differences in the thickness of the films ($p > 0.05$). F_COM_S films had a thickness comparable to the NaCas films obtained by Lin et al. (2020), regardless of the use of genepin as a crosslinker. However, as thickness of protein films strongly depends on preparation conditions, comparing our results with literature data is difficult. Statistical analysis (nonparametric Kruskal-Wallis test) confirmed significant differences in film thickness for the 'composition' factor ($p < 0.001$), namely for the fat content, whereas the 'treatment' factor (P/B) was not significant ($p = 0.215$).

Table 3. Gross composition (g/100g) and thickness (μm) of the experimental films

Sample	Protein	Fat	Glycerol	Ash	Moisture	Thickness
F_LAB_PS_B	43.9	25.3	14.6	4.9	11.3	123 $\pm$ 2
F_LAB_PS_P	43.6	25.1	14.5	4.9	11.8	138 $\pm$ 4
F_LAB_RF_B	52.1	17.6	17.3	3.0	10.0	142 $\pm$ 9
F_LAB_RF_P	51.1	17.3	17.0	2.9	11.7	134 $\pm$ 6

F_LAB_S_B	64.7	0.7	21.5	1.2	11.8	87 ± 1
F_LAB_S_P	65.0	0.7	21.6	1.2	11.4	93 ± 2
F_COM_S_B	58.4	0.7	20.1	4.9	15.9	76 ± 1
F_COM_S_P	58.1	0.7	20.0	4.8	16.4	90 ± 1
COMPOSITION						< .001
TREATMENT						.215

The composition of each film was calculated based on the composition of the parent solution and the actual moisture content obtained experimentally. Thickness values are expressed as median ± standard error. Results of Kruskal Wallis non-parametric statistical analysis (bottom) concerning the factors 'composition' and 'treatment' were considered as significant when $p < 0.05$.

3.3.1 Film microstructure

CLSM was applied to specifically investigate the organization of the lipid phase within films prepared using different powder formulations and two different pre-treatments applied to the film-forming solutions. CLSM was employed to investigate the film below its surface while SEM was specifically used for surface analysis. Representative images of films are shown in Fig. 2 where the lipid phase is displayed in red. Indeed, a homogeneous microstructure was observed under the surface of each film type. Besides the expected larger presence of fat in F_LAB_PS and F_LAB_RF compared to F_LAB_S and F_COM_S, a different size distribution and organization of the fat structures is outlined. Image analysis confirmed that the size of fat structures decreased while decreasing the fat content and it was also affected by the treatment applied (Table 4). Indeed, probe sonication caused a significant increase in the number of fat structures in PS and RF films; additionally, the median of Feret diameter of the lipid structures decreased by about 25 and 26% in F_LAB_PS_P and F_LAB_RF_P film respectively, determining a more uniform size distribution of the structures, as indicated by the lower standard error compared to the corresponding bath-treated samples. Also, the median area decreased by around 68% for both PS and RF films. This effect could be attributed to the different effectiveness of the sonication treatments applied to the solutions. The experimental conditions of the two sonication instruments were set to operate as far as possible in comparable ways. Nevertheless, ultrasonic baths typically generate a weak ultrasonication of about 20-40 W/L, with a highly non-uniform distribution (Asadi et al., 2019). In sharp contrast, ultrasonic probe devices can deliver up to 20,000 W/L into the fluid, showcasing an effectiveness up to 1,000 times higher than that of ultrasonic baths.

The effects of the sonication treatments were less pronounced in LAB_S and COM_S films, likely due to their lowest fat content. The median circularity of the fat structures was equal to 1.0 ± 0.001 for all samples (data not shown).

Coalescence of fat occurred above all in films with high fat content, especially when subjected to bath sonication, as evidenced by the presence of structures larger than 1-2 μm , which is roughly the largest size of fat globules found in homogenized pasteurized milk from which this fat originates. The fat structures with Feret diameter larger than 5 μm were 1.48%, 0.08%, 1.04%, 0.02%, 0.10%, 0% in F_LAB_PS_B, F_LAB_PS_P, F_LAB_RF_B, F_LAB_RF_P, F_LAB_S_B, F_LAB_S_P, respectively, while only smaller structures were detected in the two films prepared from commercial NaCas. The coalescence of fat globules caused the area and Feret's diameter of fat structures to be not normally distributed with few events with large diameters. Similar phenomena of coalescence were observed among fat globules during natural creaming of raw milk at temperatures higher than 22°C (D'Incecco et al., 2018). While it is well established that the cavitation phenomena induced by ultrasound treatment of film-forming solutions overall affect physicochemical properties of the derived biopolymer-based films (Mi et al., 2023), specific effects induced on fat behavior involving hydrophobic domain distribution are less elucidated and even less in films. Literature observations on ultrasound treatment of milk clearly showed a reduction of fat globule size, depending on the power level, which promoted an increased association of casein with the milk fat globule membrane to compensate for the increased surface area, as it occurs for conventional homogenization, without loss of constitutive membrane proteins (Gregersen et al., 2019). The fat structures in our samples can be assumed to behave much more like fat globules, regardless the size. However, other modifications can occur during film drying that need to be investigated.

Table 4. Image analysis of fat structures observed by CLSM in experimental films

Sample	Fat Structures (number)	Area (μm^2)	Feret Diameter (μm)
F_LAB_PS_B	3443 $\pm$ 68	1.3 $\pm$ 5.9	1.1 $\pm$ 1.2
F_LAB_PS_P	15157 $\pm$ 637	0.4 $\pm$ 0.9	0.9 $\pm$ 0.6
F_LAB_RF_B	2301 $\pm$ 106	1.2 $\pm$ 3.0	1.2 $\pm$ 1.0
F_LAB_RF_P	19489 $\pm$ 350	0.4 $\pm$ 0.6	0.9 $\pm$ 0.6
F_LAB_S_B	596 $\pm$ 81	0.3 $\pm$ 0.9	0.7 $\pm$ 0.4
F_LAB_S_P	891 $\pm$ 63	0.3 $\pm$ 0.3	0.7 $\pm$ 0.4
F_COM_S_B	704 $\pm$ 80	0.3 $\pm$ 0.7	0.6 $\pm$ 0.4
F_COM_S_P	503 $\pm$ 223	0.2 $\pm$ 0.2	0.5 $\pm$ 0.3
COMPOSITION	< .001	< .001	< .001
TREATMENT	.041	< .001	< .001

Data are expressed as median $\pm$ standard error of 3 images analyzed per sample. Results of Kruskal-Wallis statistical analysis (bottom) concerning the factors 'composition' and 'treatment' were considered significant when $p < 0.05$.

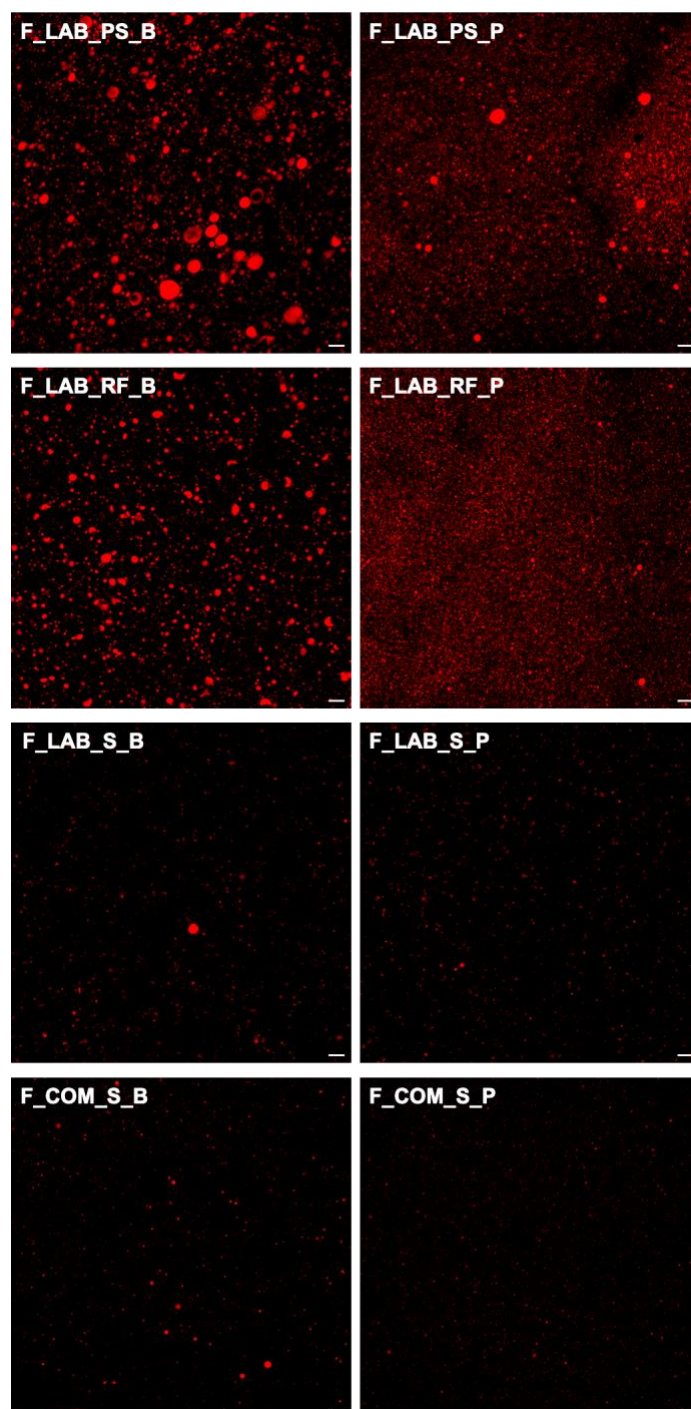


Fig. 2. Morphology of fat structures in experimental films observed by CLSM. Fat is stained by Nile Red and is visualized in red. Bar length is 10 μm .

The surface microstructure of the films investigated using SEM revealed significant differences primarily depending on film composition and, to a lesser extent, on the type of sonication treatment applied (Fig. 3). Films with the highest fat content showed an uneven surface

characterized by roughness and presence of many openings like pores, often circular. The number and size of pores decreased in films with lower fat content and, regardless of the fat content, when the probe treatment was applied. Indeed, the average size of pores ranged from $12.01 \pm 6.9 \mu\text{m}$ in F_LAB_PS_B to $0.28 \pm 0.2 \mu\text{m}$ in F_COM_S_B and from $5.90 \pm 4.8 \mu\text{m}$ to $0.31 \pm 0.1 \mu\text{m}$ in the probe-treated samples (Table S1). Even though F_LAB_S and F_COM_S films had similar fat and glycerol contents, the former showed large pores that were nearly absent in the latter. This trend was attenuated in probe-treated films. The presence of more pronounced irregularities on the surface of the F_LAB_PS_B and F_LAB_RF_B films could be due to the faster rising of largest fat structures during film drying (Fabra et al., 2012; Aloui et al., 2019). Interestingly, Fabra et al. (2011) used NaCas solutions added with oleic and stearic acids as lipid phase and observed the formation of a laminate-like structure within the derived films, with fewer irregularities and a more continuous protein network when a more intense homogenization was used to prepare the emulsion.

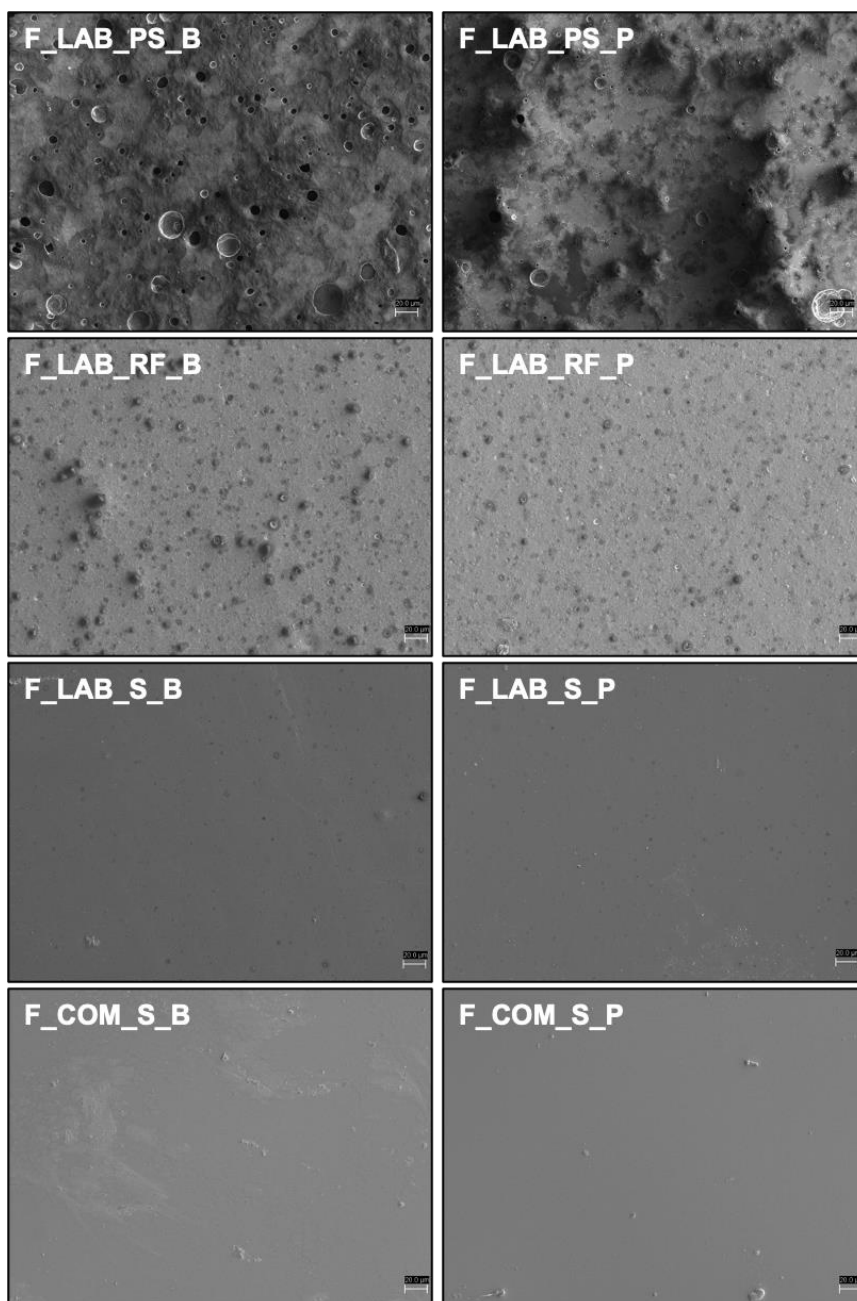


Fig. 3. SEM micrographs of experimental films (bar length is 20 µm).

3.3.2 Thermal and thermo-mechanical behavior

DSC analysis was performed to investigate the thermal behavior of the different films. Since the films were produced at lab scale, the first heating cycle was also considered, where the thermal history of the different films is seen (Fig. 4A). It was expected that the composition of the starting NaCas determines differences in the thermal behavior of the material, but it is also important to emphasize how different treatments may change it in films with similar composition.

In the first heating cycle, multiple endothermic transitions were observed for each film, likely attributable to the complexity of the matrix, with the presence of species having different molecular masses. Probe sonication treatment appeared to induce a decrease of melting temperatures in the samples containing more fat (PS and RF), probably by promoting a better dispersion and a low size of fat structures within the protein network as was observed by CSLM (Fig. 2). The glass transition temperature (T_g) represents a structure-dependent attribute. For proteins, T_g increases with the intensity of both intra- and intermolecular interactions, but these were largely shielded by the plasticizer in the samples under study. In general, T_g is difficult to detect for biopolymers such as casein since, unlike crystalline polymers, amorphous materials do not have a defined T_g (Colak et al., 2015a; Kristo et al., 2007). These reasons can explain why no T_g was detected in the first heating cycle (Fig. 4A) at the studied temperature range. In the second heating cycle (Fig. 4B), due to the suppression of the effect of thermal history of the material, it was clearly visible the progressive melting of milk TAGs occurring between 10 and 40 °C, the intensity of the endothermic transition being dependent on the amount of fat in the films. These DSC profiles largely overlap those reported by Ren et al. (2019) for homogenized pasteurized milk stored at 4 °C. For samples containing high amounts of TAGs, the T_g was difficult to appreciate, likely because the melting of milk fat covers a wide temperature range, overlapping the glass transition of the amorphous matrix of the biopolymer (Fabra et al., 2010). The presence of lipids can therefore be assumed to result in a lower order in the polymers. Slight T_g was detected only for F_LAB_S films by calculating the second derivative: 73.5 and 70.2 °C for F_LAB_S_B and F_LAB_S_P, respectively. Similar T_g s were reported by Colak et al. (2015b), with a T_g of 78 °C for NaCas films, and by Zhao et al. (2018) with a T_g of 73.4 °C for NaCas powder. Fig. 4C shows the first cooling cycle, where crystallization phenomena occurring from 200 and –20 °C could be evaluated. In films with the highest fat content, two crystallization peaks were detected: at 16°C and 7°C for F_LAB_PS_B and at 15°C and 6°C for F_LAB_PS_P. This shift in the crystallization temperatures, although of small entity, points to the different types of sonication adopted in our experiments, with the probe sonication resulting stronger. Chandrapala & Leong (2015) report that ultrasonication of milk fat affects crystallization by decreasing the induction time and generating smaller crystals. The effects strongly depend on the processing conditions, thus probe sonication, having a higher intensity, had a higher impact on fat crystallization. However, it has to be underlined that ultrasound treatment can also cause changes in the membrane of the fat globules, thus modifying the interactions within fat globules and with casein micelles, making the occurring phenomena very complicated (Chandrapala & Leong, 2015). Only one crystallization peak at 8°C was observed for the two films with reduced fat content (F_LAB_RF_P and F_LAB_RF_B) and none for the films from skimmed NaCas. Due to the complex composition of TAGs in natural milk fat and the associated variability, its crystallization upon cooling begins at about

22 °C and is complete at 12 °C (Walstra et al., 1995). However, a temperature as low as 3 °C must be reached for homogenized milk.

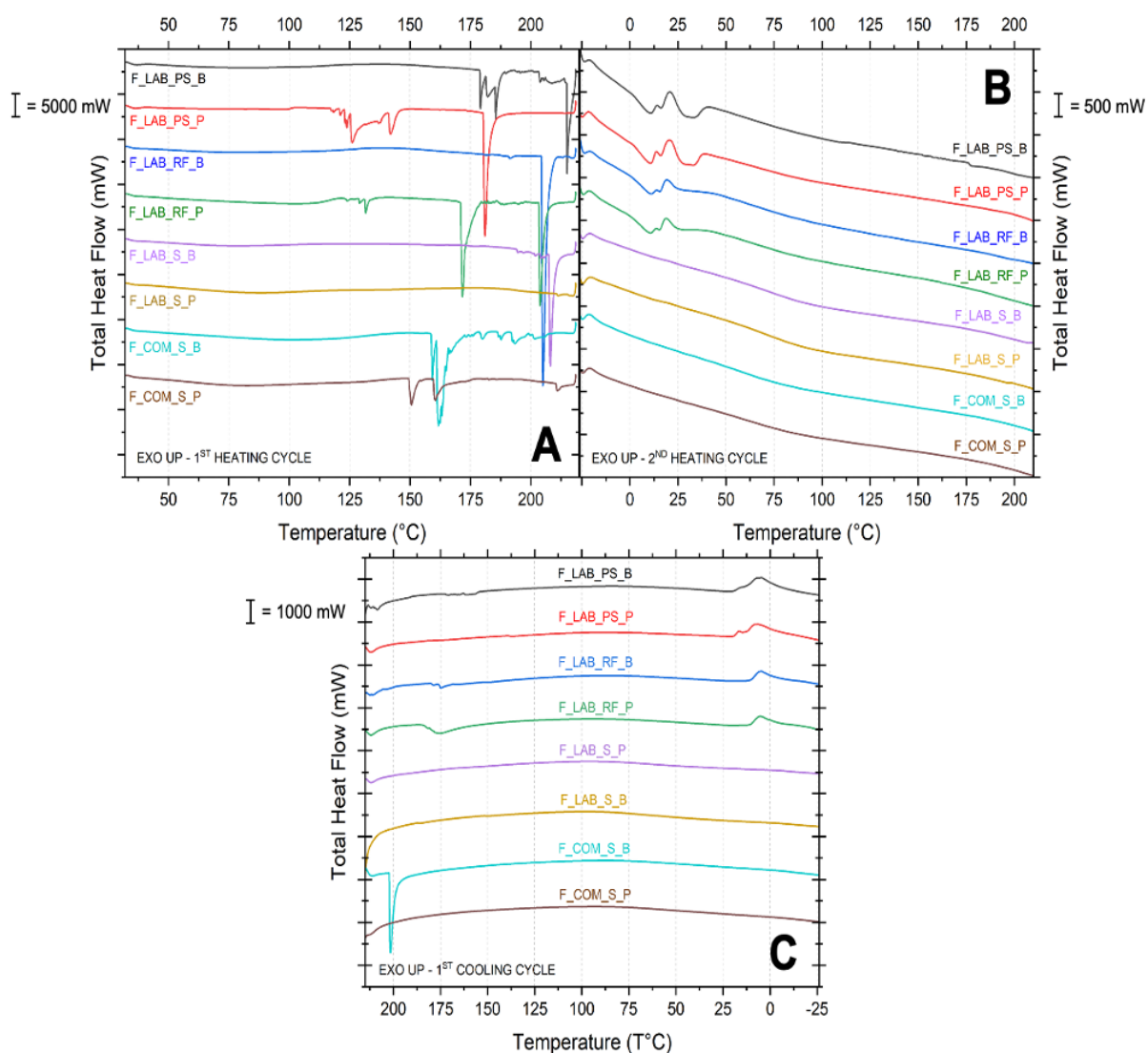


Fig. 4. DSC thermograms of experimental films prepared with different NaCas powders and treatments. Panels refer to: (A) first heating cycle; (B) second heating cycle; (C) first cooling cycle.

The DMTA was adopted to evaluate the thermo-mechanical behavior of selected experimental films. Measurements were obtained after a 48-h film conditioning step at 40 °C and 50% RH. In Fig. 5 the curves refer to storage modulus (E'), loss modulus (E'') and loss factor ($\tan \delta$) as a function of the temperature, in panel A, B and C, respectively. The E' represents the energy stored by the material. All the samples showed two main relaxation processes. The first relaxation occurred as the temperature increased from -50 °C to 50 °C and was associated to the glycerol relaxation (Quijada-Garrido et al., 2007). The ratio between the loss modulus and the storage modulus (representing the energy dissipated by the material) is expressed by the

loss factor ($\tan \delta$), which represents the T_g both at the relative and absolute maximum points. While the actual T_g of pure glycerol was observed to transpire at a lower temperature, it is anticipated to shift towards higher temperatures when incorporated as a constituent in polymer systems, due to a decreased mobility (Pereda et al., 2011). This was evident in the plot of panel C, where the T_g shifted to $-26\text{ }^{\circ}\text{C}$ for the F_COM_S_P film, to $-16\text{ }^{\circ}\text{C}$ for the F_LAB_S_B and F_LAB_S_P films, and to $-14\text{ }^{\circ}\text{C}$ for films with a high fat content. At temperatures close to $100\text{ }^{\circ}\text{C}$, the modulus increased because of the evaporation of the water (plasticizing agent) that was initially present in the conditioned films. Additionally, films displayed various secondary transition temperatures, corresponding to distinct alterations in protein-protein interactions or partial denaturation of tertiary or secondary protein structures. Protein-based thermoplastics exhibit characteristics close to those of semi-crystalline polymers, presenting multiple structural relaxations and transition temperatures such as the T_g and melting point (T_m). It is noteworthy that the occurrence of T_g and T_m does not invariably indicate complete unfolding of proteins into a fully amorphous structure, as α -helices and β -sheets can persist (Bier et al., 2014; Bonnaillie & Tomasula, 2015). The second relaxation process observed at temperatures higher than $100\text{ }^{\circ}\text{C}$ (Fig. 5C) could be associated to the T_g of the NaCas and it was referred as the main relaxation by Kristo et al. (2008).

F_LAB_S_B film had the highest initial storage modulus, as confirmed by the highest tensile strength measured in mechanical properties (Table 5), suggesting higher stiffness at low temperatures. F_LAB_S_B and F_LAB_S_P films therefore have a higher stiffness and rigidity than films with a fat content and the commercial film. Films with a higher fat content, on the other hand, have a lower storage modulus but possess more plasticity, as confirmed by the higher EAB at room temperature, except for the F_LAB_PS film where the excessive presence of fat caused a weakened protein network. The probe sonication treatment made it possible to obtain more rigid films with a more ordered structure compared with those obtained by sonication in an ultrasonic bath. In fact, F_LAB_RF_B showed a more pronounced decrease in storage modulus with increasing temperature than the other samples, it also has a higher and narrower peak, indicating a more defined T_g and greater energy dissipation at that temperature. This more rigid structure in film F_LAB_RF_P has been confirmed by the increased compactness observed in CLSM images where the fat structures are smaller in diameter and with an area that has a smaller standard deviation. The higher rigidity of this film could be related also to the different fat crystallization induced by sonication and to the interactions of fat globules with casein, as already commented for the DSC results.

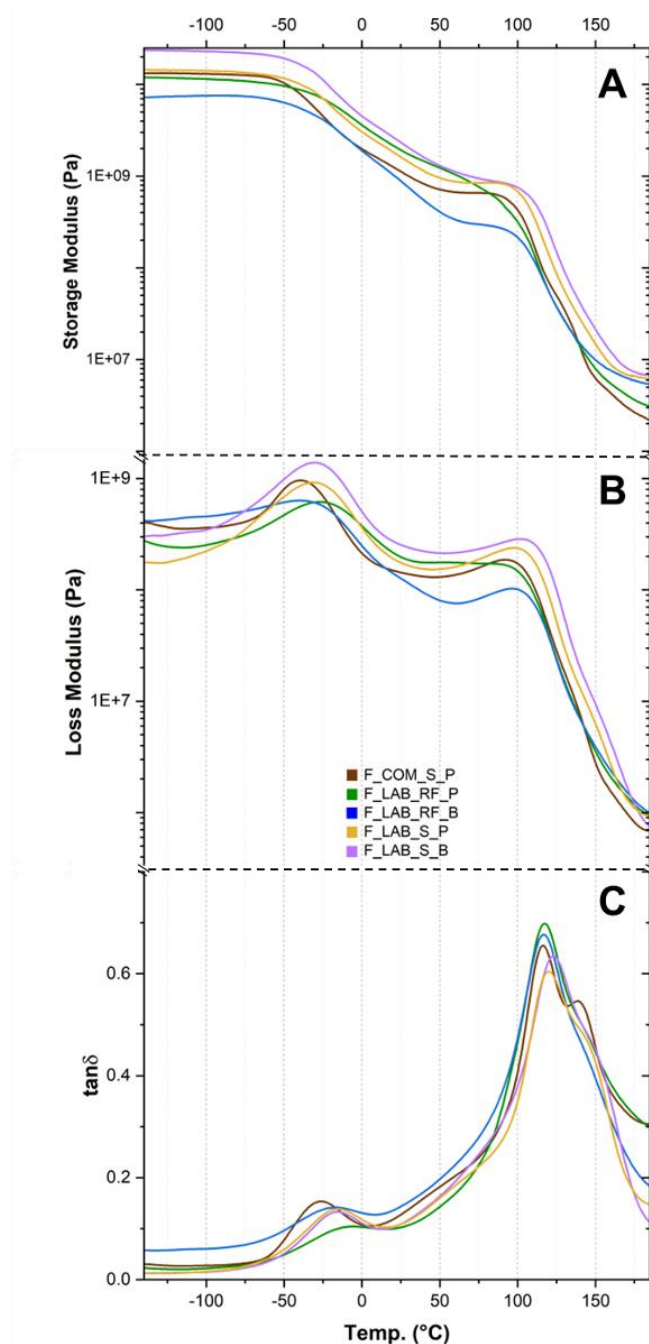


Fig. 5. DMTA plots showing the storage modulus (A), the loss modulus (B) and the loss factor ($\tan\delta$) (C) of selected experimental films.

3.3.3 Mechanical and gas-barrier properties

Mechanical properties are crucial for film performance in packaging applications, as adequate mechanical strength and elasticity guarantee the film's integrity and withstand breakages upon stresses. Interactions between film components as well as strength of the inter- and intra-molecular network bonds influence the mechanical and gas-barrier characteristics of films (Bonnaillie et al., 2014). Thus, the related parameters that could be most informative of the

produced films were evaluated and the results are shown in Table 5. The values of OTR and WVPR were expressed as KPO_2 and $KPWV$ respectively.

Table 5. Mechanical (TS= tensile strength; EAB= elongation at break; YM= Young's modulus) and gas-barrier properties (KPO_2 = oxygen permeability constant; $KPWV$ = water vapor permeability constant) of experimental films.

Sample	TS (MPa)	EAB (%)	YM (MPa)	KPO_2 ($cm^3 \mu m m^{-2} d^{-1} bar^{-1}$)	$KPWV$ ($g \mu m m^{-2} d^{-1}$)
F_LAB_PS_B	4.3 ± 0.4	73 ± 9	72 ± 4	627 ± 74	321692 ± 14192
F_LAB_PS_P	3.4 ± 0.3	57 ± 9	65 ± 3	814 ± 69	388978 ± 24264
F_LAB_RF_B	5.8 ± 0.8	124 ± 15	120 ± 15	525 ± 99	435363 ± 9363
F_LAB_RF_P	5.4 ± 0.4	126 ± 19	67 ± 10	724 ± 94	391692 ± 11044
F_LAB_S_B	12.0 ± 0.8	112 ± 11	167 ± 16	435 ± 104	293984 ± 13863
F_LAB_S_P	7.9 ± 0.5	116 ± 8	80 ± 11	614 ± 130	309148 ± 13797
F_COM_S_B	7.2 ± 1.1	108 ± 16	154 ± 22	342 ± 61	243451 ± 5846
F_COM_S_P	6.8 ± 0.5	123 ± 9	78 ± 5	450 ± 54	283352 ± 8901
COMPOSITION	< .001	< .001	< .001	< .001	< .001
TREATMENT	< .001	.690	< .001	< .001	.003
COMP x TREAT	< .001	.008	< .001	.781	< .001

Data are expressed as mean ± standard deviation. Results of multifactorial statistical analysis (bottom) concerning the factors 'composition', 'treatment' and their interaction were considered significant when $p < 0.05$.

TS values were rather close for all samples, except for F_LAB_S_B and F_LAB_S_P films, which showed the highest values. The data indicates a stiffness increase in these films, as confirmed by the high YM values. This suggests that the presence of milk fat in PS and RF films affected their mechanical properties far behind that of glycerol, likely because the large fat structures observed by the CLSM weakened the protein network and increased elasticity of the material. Consistently, films obtained by sonication with the probe had systematically lower TS values than the corresponding bath-sonicated films, due to the reduced size of fat structures obtained with the former treatment. Unexpectedly, films obtained from commercial NaCas showed TS values lower than F_LAB_S_B and F_LAB_S_P films despite the comparable content of milk fat. It can be hypothesized that the industrial manufacturing process of the commercial caseinate as well as the slightly higher moisture content of the derived films could play a role in determining a lower TS compared to the corresponding lab-prepared samples, given the substantial absence of a role of milk fat in both cases. Both RF films displayed the highest EAB values, despite their intermediate fat content. Generally, when oil or fat in the form of emulsified droplets is incorporated into a biopolymer-based film, it

becomes more flexible (higher EAB values) and less stiff (decrease in TS) (Bertan et al., 2005; Fabra et al., 2010). An excessive presence of fat in the film, on the other hand, may lead to a decrease in EAB due to the excessive weakening of the protein network. Based on the CLSM observations, it can be confirmed that milk fat was more homogeneously dispersed in RF samples, notably in those obtained with probe sonication, compared with the PS samples. Jahromi et al. (2020) and Pereda et al. (2015) found EAB values in NaCas films slightly lower than in the present work, likely due to the lack of a plasticizer in their formulation. YM was lower in the films with a higher fat content. According to some authors (Ferreira et al., 2014; Aloui et al., 2019), this behavior may be attributed to the formation of structural discontinuities caused by a high lipid content, which can diminish the cohesive forces within the polymer network, consequently reducing the mechanical resistance and stiffness of the film. The probe sonication of the solutions significantly reduced the YM of the derived films, almost halving the values compared to those of the corresponding bath-sonicated films. This effect was not observed for the F_LAB_PS films.

The two-factors ANOVA showed that both 'composition' and 'treatment' as well as their interaction had a significant effect on TS and YM, while EAB was only influenced by the factor 'composition'. The results of the post-hoc test highlighted how the control group (F_COM_S) was statistically different from all the experimental groups (F_LAB_PS; F_LAB_RF; F_LAB_S) regarding TS, whereas for both EAB and YM, the control group was not significantly different from the F_LAB_S films (not shown).

The oxygen and water vapor permeance (K_{PO_2} and K_{PWV}) presented in Table 5 were determined for NaCas films in this study, considering the composition (fat content of NaCas used) and the applied treatment (P/B).

All films demonstrated good oxygen barrier characteristics, emphasizing the significance of fat content in the films ($p < 0.001$) and its impact on increasing oxygen permeability. This result aligns with the findings of Janjarasskul et al. (2014), which showed that in whey protein isolate-based films incorporating candelilla wax, oxygen permeability increased with higher wax concentrations. This effect is attributed to the compatibility between non-polar oxygen and non-polar wax. Furthermore, considering the microstructure of the films, it is assumed that the removal of fat globules from the matrix reduces void spaces at the interface between the protein and the lipid phase. This hypothesis is also supported by Chick & Hernandez, (2002), who observed similar behavior in casein films when supplemented with different types of waxes.

Interestingly, the values were significantly different ($p < 0.05$) also depending on the sonication mode. It can be argued that the more homogeneous and fine distribution of the fat structures within the protein network achieved by probe sonication may allow more oxygen to pass

through (higher KP values) due to the higher affinity of the gas with the lipid phase. Nonetheless, the KPO_2 values of the NaCas films obtained in the present work were comparable to those of polyvinylidene chloride (PVDC) films, but an order of magnitude higher than ethylene vinyl alcohol (EVOH) films (Piergiovanni & Limbo, 2010). Furthermore, NaCas-based films had a lower oxygen permeability than films produced from polysaccharides (Khwaldia et al., 2004). This can be attributed to their increased polarity and more linear (non-ring) structure, which results in higher cohesive energy density and lower free volume.

Regarding the water vapor permeance (KPWV), both the film composition and the sonication treatment had a significant influence on it. The sonication treatment, which reduces the diameter of the fat structures and increases their distribution and frequency within the continuous phase, was expected to reduce the WVTR in films with a relevant fat content, but this occurred for the F_LAB_RF samples only, while an opposite effect was observed in the other samples. Although water vapor permeability was expected to decrease in samples with a high fat content, it appears to be higher in sample F_LAB_RF. The presence of fat may lead to greater disruptions within the film's matrix, resulting in a higher number of voids at the interface between the protein and the fat. In fact, moisture transfer occurs through the protein network that forms the continuous phase. Differently, Dutta & Sit (2023) observed that WVP was almost halved in neat casein films when the native casein solution was bath sonicated at 35 kHz for up to 30 min before glycerol addition. In general, the WV permeability values measured for the films are higher than those measured for casein films containing wax (Galus et al., 2020).

3.3.4 Optical properties

Additional differences among the films were examined by assessing color parameters (L^* , a^* and b^*) and the derived color indices, i.e. ΔE , yellowness index (YI) and the whiteness index (WI). In addition, the haze of the films was measured. The data are shown in Table 6.

Table 6. Optical properties and haze values of experimental NaCas films

Film sample	a^*	b^*	L^*	ΔE	WI	YI	Haze (%)
F_LAB_PS_B	-1.46 ± 0.20	10.45 ± 0.87	92.27 ± 0.08	4.66 ± 0.89	89.08 ± 0.86	16.19 ± 1.35	93.6 ± 0.0
F_LAB_PS_P	-1.40 ± 0.10	10.11 ± 0.41	92.29 ± 0.10	4.31 ± 0.41	89.43 ± 0.40	15.64 ± 0.64	82.4 ± 2.3
F_LAB_RF_B	-1.11 ± 0.04	15.91 ± 0.52	90.45 ± 0.15	10.15 ± 0.54	83.76 ± 0.52	25.13 ± 0.87	92.4 ± 0.7
F_LAB_RF_P	-1.22 ± 0.05	14.77 ± 0.45	90.76 ± 0.15	8.99 ± 0.45	84.87 ± 0.44	23.25 ± 0.72	70.6 ± 0.8
F_LAB_S_B	-0.35 ± 0.03	5.09 ± 0.13	92.29 ± 0.22	0.89 ± 0.16	94.19 ± 0.13	7.87 ± 0.22	20.3 ± 0.6
F_LAB_S_P	-0.33 ± 0.02	5.11 ± 0.08	92.15 ± 0.09	0.85 ± 0.08	94.16 ± 0.07	7.93 ± 0.13	20.2 ± 1.8
F_COM_S_B	-0.27 ± 0.02	5.95 ± 0.16	92.20 ± 0.13	REF.	93.42 ± 0.15	9.22 ± 0.25	16.3 ± 0.4

F_COM_S_P	-0.25 ± 0.02	5.99 ± 0.16	92.07 ± 0.13	REF.	93.38 ± 0.15	9.29 ± 0.26	15.4 ± 0.8
COMPOSITION	< .001	< .001	< .001		< .001	< .001	< .001
TREATMENT	.964	.082	.597		< .001	< .001	< .001
COMP X TREAT	.015	< .001	< .001		< .001	< .001	< .001

Results of multifactorial statistical analysis (bottom) concerning the factors 'composition', 'treatment' and their interaction were considered significant when $p < 0.05$. REF= reference used to calculate ΔE compared to other films.

Compared to skimmed (S) films, film samples containing fat (PS and RF) returned lower values on the green-red scale (a^*) and higher values on the blue-yellow scale (b^*), indicating a greater presence of the green and yellow components, respectively. Indeed, significant differences were determined among films depending on 'composition' and not on the sonication treatment. Differences among L^* values were less remarkable although statistically significant with respect to fat content. The greatest difference in terms of total color ($\Delta E^* > 3$) was observed between the reference films (F_COM_S_B and F_COM_S_P) and the F_LAB_RF films, followed by the F_LAB_PS films. Meanwhile, no visual differences ($\Delta E^* < 1$) were observable for films free of milk fat (F_LAB_S and F_COM_S), regardless of the sonication mode. Whiteness (WI) and yellowness (YI) indices were highly and inversely influenced by both fat content and sonication mode. Haze was determined since it characterizes the cloudy or turbid appearance of an otherwise transparent specimen and represents the percentage of light that is deflected more than 2.5° from the incident light direction (Taglialatela Scafati et al., 2011). Haze is caused by light scattering that occurs due to the difference between the refractive indices of two phases. This is observed in films composed of blends, where the minor component (milk fat in this case) forms isolated domains dispersed within the matrix (casein). In this context, the observed morphology of the films, the amount of fat, and the sonication treatment are likely to influence the haze of the films. In fact, it can be observed that, as the fat content decreased, thereby altering its dispersion within the protein matrix, the haze value tended to decrease (Table 6). The sonication mode applied to the film-forming solution significantly influenced the haze value by altering the size of fat globules. Specifically, probe sonication led to a reduction in the size of fat structures, as shown by CLSM analysis (Fig. 2), which in turn resulted in a decrease in light scattering and a corresponding reduction in haze (Table 6). Conversely, films treated using bath sonication exhibited higher haze values, which may be attributed to their greater surface roughness, as observed by SEM (Fig. 3). This finding aligns with a previous study, which reported that films with higher surface roughness and crater-like morphology exhibited increased haze (Cheng et al., 2020). It is worth mentioning, in this regard, that films with high haze values have light barrier properties that could be requested in some packaging applications. Aloui et al. (2019) reported that NaCas films had the highest light barrier properties when added with 30-40% of lipid fraction.



Fig. 6. Transparency of experimental films of the present study. The films were individually placed on a white paper to cover the logo of University of Milan (diameter of 3 cm) and photographed.

3.4 Principal component analysis

Considering that a large multiparametric dataset was collected in this study, the principal component analysis (PCA) was performed to assess which variables were primarily differentiating the NaCas films (Fig. 7). The first two principal components explained 77% of the total variability of the dataset, considering 11 out of 29 variables. Loadings for the selected variables that contribute to the positioning of the films in the vector space are shown in the score plot (Fig. 7A).

Samples F_LAB_S and F_COM_S distributed in the negative part of PC1, while the samples with a higher fat content were mainly distributed in the positive part of PC1, except for F_LAB_PS samples sonicated with probe. The distribution of the samples along the positive part of PC1 was greatly affected by KPWW, YI, Feret diameter of fat structures and haze, while the positioning along the negative part of PC1 was driven by the WI, as well as the moisture content of the films.

Mechanical properties of films mostly drove their positioning along the positive part of PC2 while KPO₂, KPWW and fat content placed samples along the negative part of PC2. Samples treated with bath positioned at PC1 values higher than the corresponding probe-sonicated samples: this behavior was evident for films containing higher amount of fat. The same positioning effect was evident for samples containing small amounts of fat, but which were positioned at higher PC2 values than the corresponding probe-sonicated samples. Besides the Feret diameter of fat structures, this positioning was essentially caused by higher values

of KPO₂ in probe-sonicated films than in bath-sonicated ones, as well as by a higher haze in F_LAB_PS_B and F_LAB_RF_B compared to F_LAB_PS_P and F_LAB_RF_P.

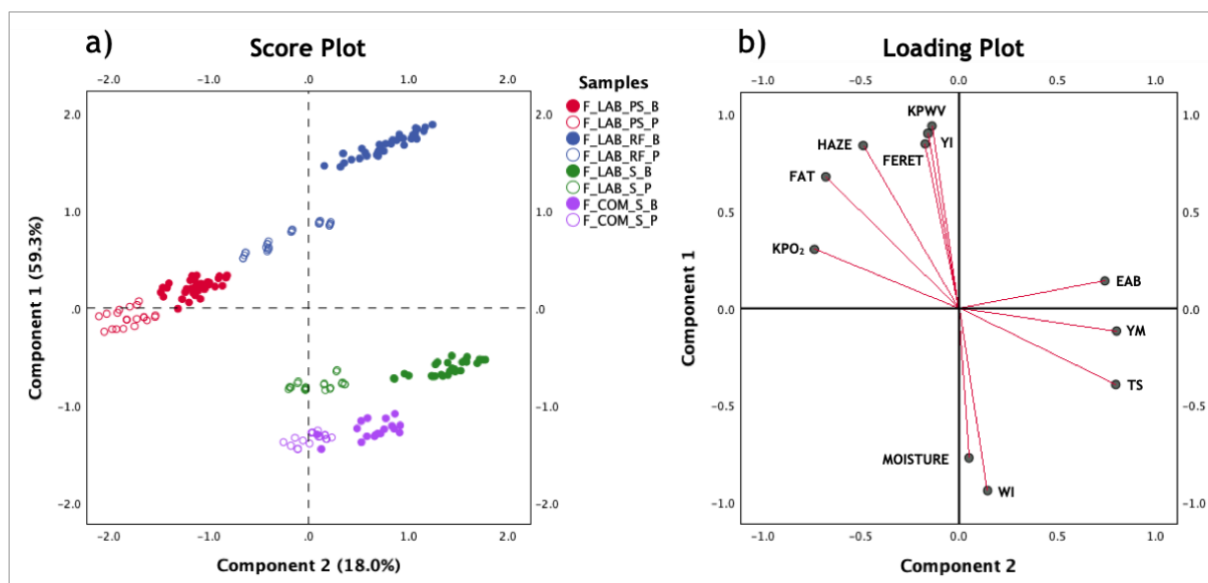


Fig. 7. Principal component analysis of NaCas films. Panel A shows the score plot where different colors denote films with different fat content. Solid circles denote bath sonicated samples and empty circles denote probe sonication. Panel B shows loadings of the selected variables of films: Fat= fat content, Moisture= moisture content, TS= tensile strength, YM= Young's modulus, EAB= elongation at break, KPWW= Water vapour permeability, KPO₂= Oxygen permeability, WI= whiteness index, YI= yellowness index, Haze= haze value, Feret= Feret diameter of the fat structures.

4. Conclusions

In this study, the upcycling of expired pasteurized milk was explored by developing and characterizing a novel packaging solution based on the production of not-edible Na-Cas films containing part of the endogenous milk fat. The fat content in the starting milk directly influenced the fat content remaining in the NaCas and thus in the derived film. Furthermore, treatment of the film forming solutions by two different ultrasonication methods led to significant microstructural differences in the films, as evidenced by CLSM and SEM. Probe sonication was more effective than bath sonication in reducing the size of fat structures and surface discontinuities in the films but negatively influenced their oxygen barrier properties. Our results demonstrated that both the ultrasound treatment and the content of milk fat affect selected properties of the final films that may be further improved depending on the film destination. Although properties of casein-based films have been widely studied, the unique role of endogenous milk fat in NaCas films was highlighted for the first time. Keeping milk fat into the film carries some advantages over the usual caseinate fortification with lipids from other sources. In particular, the proposed approach leads to an increased recovery of solids from

the expired milk, with a corresponding increase in the process yield, as a higher amount of film is obtained from a given amount of milk, and a lower production cost because no casein purification is necessary nor addition of ingredients but glycerol. A potential application could involve combining the film with a water-resistant material to create a multilayer composite with both water resistance and a high oxygen barrier. Alternatively, the strong water affinity of NaCas films could be leveraged to develop water-soluble packaging for controlled detergent release. Further research should investigate how to modulate the proposed conditions to obtain film performances targeted towards specific packaging applications.

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Chapter 3 - Functionalization of NaCas-based films: an exploratory investigation of promoting casein cross-linking by means of the exposure of NaCas solutions to LED light and to alkaline pH

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Abstract

This chapter explores the functionalization of sodium caseinate (NaCas) films using environmentally friendly approaches to enhance their mechanical, chemical, and structural properties for sustainable packaging applications. NaCas, known for its film-forming capabilities, requires functionalization to address limitations such as hydrophilicity and moderate mechanical properties of the obtained films. Two green methodologies were employed: (1) riboflavin-mediated photo-oxidation under LED light to induce di-tyrosine (di-Tyr) crosslinks and (2) thermal treatment under alkaline conditions to promote lysinoalanine (LAL) crosslinks.

Riboflavin-mediated photo-oxidation generated singlet oxygen, facilitating oxidative modifications in tyrosine residues to form di-Tyr crosslinks. The inclusion of riboflavin significantly increased di-Tyr formation, confirmed through HPLC and immunogold TEM. Alkaline thermal treatment induced LAL formation via β -elimination reactions, with LC-MS adapted for quantifying variations in LAL content based on treatment intensity.

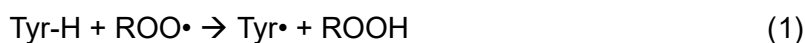
Characterization using FTIR spectroscopy revealed structural changes, such as shifts in amide and carboxylate groups, confirming successful crosslinking. Mechanical testing demonstrated that di-Tyr crosslinks enhanced film ductility, while LAL crosslinks increased tensile strength and stiffness. Limitations included constrained di-Tyr formation due to the availability of adjacent tyrosine residues and sensitivity of LAL formation to processing conditions.

These results establish a sustainable pathway for developing high-performance NaCas films with tailored properties for active and intelligent packaging applications. Future research should refine process parameters, investigate synergistic green crosslinking methods, and evaluate the long-term performance of functionalized films in real-world scenarios.

1. Introduction

Sodium caseinate (NaCas), a water-soluble derivative of casein, is produced by dissolving acid-precipitated casein with sodium hydroxide, followed by lyophilization. As previously noted, Casein exhibits strong film-forming properties due to its random coil structure and molecular interactions, which result from hydrogen bonds, electrostatic forces, and hydrophobic interactions (Rezvani *et al.*, 2013). Due to the high proportion of polar groups, films obtained from NaCas are good barriers for non-polar substances such as oxygen, carbon dioxide and volatile organic compounds. On the other hand, NaCas films are not a good barrier to water vapor because of relatively high hydrophilicity, which could therefore change film functionality. One of the possible strategies for improving the water vapour barrier properties, as well as mechanical properties, may be through the promotion of cross-linking of the NaCas network, using different approaches (Belyamani *et al.*, 2014; Lin, Wang & Weng, 2020). Creating crosslinks among the protein chains results in a strengthened three-dimensional network with modified techno-functional properties, including a decreased uptake of moisture. Affordability and costs of this approach are surely advantageous if compared to novel approaches like the addition of nanoparticles as fillers (Kumar & Singh, 2008). The cross-linking process involves the formation of chemical bonds among different protein chains. Based on the type of created bond, there are basically three main types of crosslinks: covalent crosslinking, ionic bonding, and physical crosslinking involving hydrogen bonds and Van der Waals interactions. Another classification divides crosslinking agents into three primary types: chemical, physical, and enzymatic (Garavand *et al.*, 2017). Crosslinking reactions of proteins have been extensively investigated because of the various implications in many sectors, i.e. technological, functional, nutritional, toxicological. Indeed, most of the interest is toward covalent crosslinkers as they are normally irreversible and, when form at intermolecular level, induce formation of polymers and stable aggregates (Cardoso *et al.*, 2019; Raak *et al.*, 2018; Friedman, 1999; Pellegrino *et al.*, 1999).

Among the numerous approaches that can be used for crosslinking caseins, attention of the present project will focus on two green mechanisms as they do not require any reagent addition. The first one involves riboflavin-mediated photo-oxidation reactions, where riboflavin (RF) adsorbs light at wavelengths of 380 and 450 nm in presence of oxygen to form reactive singlet oxygen ($^1\text{O}_2$). This radical acts as a photo initiator to induce oxidation of selected amino acids, such as tyrosine or tryptophan. Radicals of Tyrosine residues create crosslinks by forming di-tyrosine (di-Tyr) bonds (Lee *et al.*, 2023). In particular, the peroxy radicals ($\text{ROO}\cdot$) participate in the pathway of protein- $\text{ROO}\cdot$ reaction (1).



Under low oxygen conditions or a high flux of free radicals, these pathways compete with the self-reaction of tyrosine, leading to the formation of inter-protein crosslinks and dimers (Fuentes-Lemus *et al.*, 2018).



Formation of di-Tyr through this mechanism has been observed also in individual casein fractions (Fuentes-Lemus *et al.*, 2018) and in casein recovered from pasteurized milk packaged in clear PET bottles (D’Incecco *et al.*, 2024).

The second approach involves the β -elimination reaction under alkaline conditions leading to the formation of the crosslink lysinoalanine (LAL). LAL is an unnatural amino acid that forms in proteins undergoing heat treatments at alkaline conditions. Its formation occurs in a two-step mechanism: β -elimination reaction at cysteine or serine residues to form dehydroalanine, followed by reaction with lysine (Friedman, 1999). Factors influencing LAL formation include pH, temperature, and exposure time (Koning & Rooijen, 1982). Since LAL formation involves lysine residues, its formation is strongly depressed in presence of reducing sugars due to the competition of Maillard reaction (Nielsen *et al.*, 2022). Consistently, highest levels of LAL were found in low-lactose dairy products such as casein and caseinate powders (Pellegrino *et al.*, 1996; Cattaneo *et al.*, 2012; Calabrese *et al.*, 2009).

The regulatory landscape surrounding food packaging materials is evolving, with agencies like the European Food Safety Authority (EFSA) and the U.S. Food and Drug Administration (FDA) emphasizing the need for comprehensive safety assessments of new materials, including those that may release compounds into food. As biopolymers and nanocomposites become more prevalent, understanding the interactions between these materials and food components will be crucial for ensuring consumer safety. The safety of food packaging materials is an area of growing concern, especially because the food industry increasingly adopts biopolymers and nanocomposites that may come in contact with the food. The migration of substances from packaging materials into food is a critical safety concern. Various studies have highlighted that some contaminants, including plasticizers and other chemical residues, can migrate from food packaging into food products, potentially leading to health risks (Panou, 2024). This is particularly relevant for biopolymer-based packaging, where the interaction between the packaging material and food can influence the migration rates of compounds. Both di-Tyr and LAL have been recognized as potentially toxic when ingested as free molecules. The former is involved in the development of neurological diseases like Alzheimer’s (Maina *et al.*, 2023) while the latter is reported to be nephrotoxic (Friedman, 1999). However, these crosslinks form between two amino acid residues of the casein backbone and thus are stably bound and not subject to migration phenomena.

The two above mentioned mechanisms have been used to produce functionalized NaCas films and represent the focus of this chapter. In order to evaluate the respective potential effects of the functionalization treatments, this study was conducted starting from freshly produced commercial pasteurized milk.

2. Materials and Methods

2.0 Experimental Design

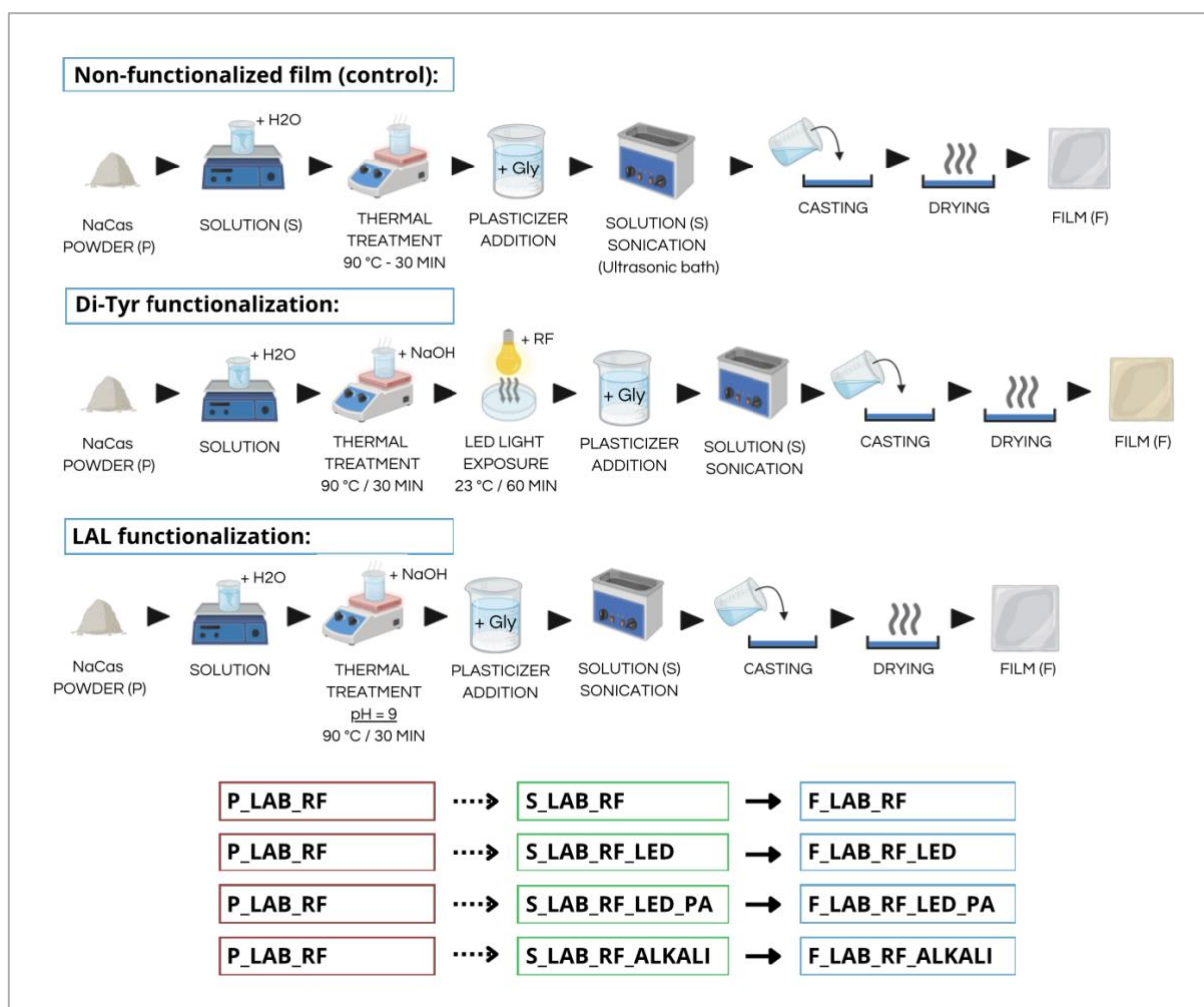


Fig 1. Experimental design showing the processes of NaCas functionalization and film production. The first stage shows the production of the film from untreated NaCas solution (control), the second stage shows the production of the functionalized film by exposing the solution to LED light, and the third stage shows the process of film production after functionalization by heat treatment carried out at alkaline pH.

2.1 Preparation of the film-forming solutions for the casein functionalization study

A large batch of P_LAB_RF powder was prepared from freshly produced pasteurized milk using the procedure described in Chapter 2, Section 2.3. The powder was then used to prepare

the solutions to be functionalized by the two different approaches as well as a non-functionalized solution that was used as control. Lastly, the solutions were used to produce the corresponding films. The composition of the powder is reported in Table 1.

Table 1. Gross composition (g/100g) of P_LAB_RF NaCas powder, used to produce functionalized solutions.

Sample	Protein	Fat	Moisture	Ash
P_LAB_RF	69.34 ± 0.70	23.42 ± 0.03	3.65 ± 0.10	3.46 ± 0.09

Preparation of S_LAB_RF solution (untreated control) and reference samples

The film-forming solution S_LAB_RF was prepared as described in Chapter 2 and used as control. Briefly, the powder was dissolved in ultrapure water under stirring for 10 min. The solution was then heated to 90 °C and stirred for 30 min using a magneto-thermic stirrer (C-MAG HS 4, IKA, Staufen, DE). Afterward, the solution was stirred for an additional 10 min at room temperature to allow it to cool down. Analytical grade bi-distilled glycerol (VWR Chemicals, Milan, IT) was added at a concentration of 33.3% (w/w), and the solution was stirred for an additional 10 min to ensure homogeneous dispersion of glycerol. The resulting solution (30 mL) underwent a 10-min continuous sonication in an ultrasonic bath Transonic T310 (GEASS, Turin, IT) operating at a frequency of 37 kHz to remove air bubbles and prevent film discontinuities.

In addition, two reference samples were prepared with the same procedure and from two different raw materials: (i) S_LAB_RF_MILK_LIGHT was obtained from a commercial sample of pasteurized milk that was exposed to fluorescent light (tubular fluorescent lamps TL-D super 58 W/840, 5.240 lm, Philips, Italy) in a vertical display cabinet for 12 days; (ii) S_COM_S was obtained from a commercial sample of NaCas purchased from “Società Ingredienti e Additivi – S.I.A. S.r.l.” (Desio, MB, IT).

Preparation of solutions for the LED light exposure trial

A batch of S_LAB_RF solution (10% protein in water, w/v) was prepared as described for the control and divided into two aliquots, both destined to LED illumination: one batch as it is (S_LAB_RF_LED), the other one after the addition of riboflavin as a photo-activator (PA) (S_LAB_RF_LED_PA). The PA solution (riboflavin from Sigma-Aldrich, St. Louis, MO, USA) was prepared in ultrapure water by stirring and subsequent 30-s sonication to achieve complete solubilization, then was added to the NaCas solution to reach a final concentration of 35 µmol/L (Fuentes-Lemus et al., 2018). The two solutions (30 mL) were poured into Petri dishes with an area of 113 cm² and subjected to LED illumination for 60 min. During light

exposure, solutions were mixed every 15 min to ensure homogeneous exposure. The Petri dishes were positioned under the lamp (Multispectrum 900000920, Atena Lux, Gruaro, Venezia, IT) at a distance of 38 cm, as shown in Figure 2-A. The spectral irradiance is shown in Figure 2-B.

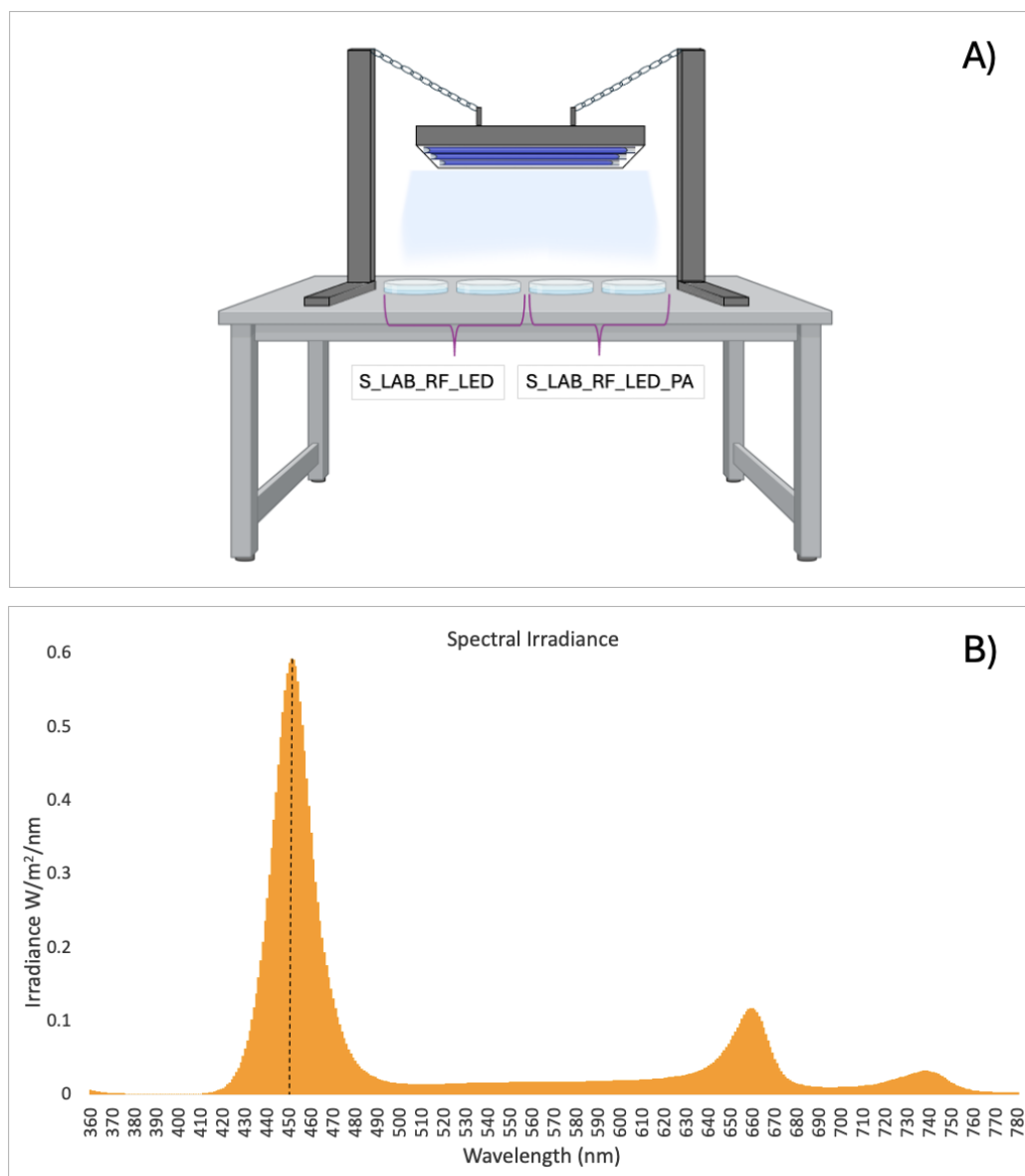


Fig. 2. E for LED-light exposure of NaCas solutions (A), and spectral irradiance of the LED-light lamp used in the experiment (B).

Preparation of solution for the alkali treatment trial

The S_LAB_RF_ALKALI solution of NaCas (10% protein in water, w/v) was prepared as described for the control. The initial water solution was adjusted to pH 9 by dropwise addition of 1N NaOH and kept under stirring until pH stabilized. The solution was then heated to 90 °C

and stirred for 30 min and further processed as previously described for the control. The S_LAB_RF was the control solution for this trial.

All of the obtained solutions were lyophilized before further analyses.

2.2 Identification and quantification of the crosslinks formed

2.2.1 Di-Tyr analysis and quantification by HPLC

The determination of di-Tyr was carried out as described by D’Incecco *et al.* (2024). The NaCas powders were submitted to acid hydrolysis. A 20-mg sample of protein was added with 8 mL of 6 N HCl in a glass vial and hydrolyzed under vacuum at 110 °C for 23 hours. After filtration, 2 mL of the hydrolysate was evaporated at 30 °C using a Rotavapor (Büchi Italia, Cornaredo, MI, Italy) and carefully resuspended in 250 µL of 0.1% (v/v) trifluoroacetic acid in milliQ water. A 10-µL aliquot of the sample was analyzed by HPLC using an Alliance 2695 system (Waters, Milford, MA, USA) with a Hitachi L2480 fluorescence detector (VWR, Milan, Italy) set at 285 nm for excitation and 400 nm for emission. The chromatographic separation was performed on a Platinum EPS C18 column (4.6 mm i.d. × 150 mm, 100 Å pore size, 3 µm particle size; Grace Alltech, Italy), maintained at 30 °C and eluted with a gradient of solvent A (0.1% (v/v) trifluoroacetic acid in milliQ water) and solvent B (0.1% (v/v) trifluoroacetic acid in acetonitrile) at a flow rate of 0.8 mL/min. The gradient program was as follows: 2% B for 1 minute, increasing from 2% to 5% B over 14 minutes, from 5% to 98% B over 10 minutes, and held at 98% B for 5 minutes (washing step). The column was re-equilibrated at 2% B for 5 minutes before the next injection. Chromatographic data were processed using Empower2 software (Waters). Di-Tyr content was quantified using an external standard method and expressed as µg/g of protein. The exact protein content of the hydrolysate was determined in 2 mL using the Kjeldahl method (ISO 8968-1/IDF 20-1). The method validation in terms of limits of detection (LOS) and quantification (LOQ) was carried out in the publication of D’Incecco *et al.* (2024).

2.2.2 Transmission electron microscopy of milk with immune gold labelling of di-Tyrosine

Transmission electron microscopy (TEM) with immune gold labelling was used to localize the di-Tyr crosslinks in casein micelles. A semi-skimmed milk exposed to light for 14 days was used in this trial. Native casein micelles were recovered from milk by ultracentrifugation at 80,000g for 60 minutes at 4 °C using a SW 28 rotor in a L5-75 analytical ultracentrifuge (Beckman Coulter Inc., CA, USA). The resulting pellet was collected and embedded in resin following the protocol outlined by D’Incecco (2022). Briefly, the moist pellet was mixed at a 1:1 ratio (w/w) with low-temperature gelling agarose (3% in water, melted at 35–40 °C; VWR, Milan, Italy). This mixture was layered on a microscope slide, allowed to solidify, and then sectioned into 1-mm³ cubes. Four cubes from each sample were fixed in 1 mL of a

glutaraldehyde (1%) and paraformaldehyde (4%) solution prepared in sodium cacodylate buffer (w/v; Agar Scientific, Stansted, UK). The cubes were washed in the same buffer for 30 minutes and subsequently post-fixed in a 1% osmium tetroxide solution containing 1% potassium ferrocyanide in the dark (EMS, Hatfield, PA, USA). Another 30-min washing was performed with the same buffer. The cubes were then dehydrated in graded ethanol solutions and embedded in London Resin White (EMS, Hatfield, PA, USA) within molds, preventing air bubbles entrapment, and cured at 60 °C for 24 hours. Ultrathin sections, approximately 70 nm thick, were obtained from the resin blocks using a diamond knife on a PowerTome XL ultramicrotome (RMC Boeckler, USA). These sections were mounted on 200-mesh nickel grids coated with a supporting film. Immunogold labeling was performed by floating the grids with the sections facing downward on drops of specific solutions, as described by D’Incecco (2022). The sections were initially incubated in 50 mM glycine in 20 mM PBS at room temperature for 20 minutes, followed by rinsing with PBS drops. These were then treated with 50 mM PBS containing 1% BSA and 0.01% Tween 20 (Aurion, Wageningen, NL). Overnight incubation at 4 °C was carried out with a primary monoclonal antibody against di-Tyr (Biorbyt, Cambridge, UK), diluted 1:100 or 0.1:100 in PBS. After thorough washing in PBS, the sections were incubated for 2 h at room temperature with a secondary anti-mouse serum conjugated to 15-nm colloidal gold, raised in goat (Biorbyt, Cambridge, UK). After additional PBS washes, the sections were post-fixed with 0.2% glutaraldehyde for 5 minutes. The grids were then floated on distilled water and dried on filter paper. Before imaging, the sections were stained with 2% aqueous uranyl acetate and 2% aqueous lead citrate. Observations were conducted using a 120 kV Talos L120C TEM (Thermo Fisher Scientific, Waltham, USA).

2.2.3 LAL analysis and quantification by LC-MS

To determine the LAL content, acid hydrolysis pre-treatment of the NaCas powders is required. About 20-mg sample of each lyophilized powder P_LAB_RF_ALKALI, P_COM_S and P_LAB_RF_LED_PA were added with 8 mL of 6 N HCl in a glass vial and hydrolyzed under vacuum at 110 °C for 24 hours. After filtration, 2 mL of the hydrolysate was evaporated at 40 °C using a Rotavapor (Büchi Italia, Milan, IT) and carefully resuspended in 1 mL of ultra-pure water by a sonication treatment for 15 min. The LC-MS analysis was carried out at UNITECH OMICS platform (University of Milan, Milan, IT). Samples were added of 1 mL water and centrifuged at 10’000 g for 5 min, then diluted in a ratio of 1:25 with ultra-pure water (analytical grade for LC-MS). The method used for the analysis was that of Akilloğlu *et al.* (2022) with modifications. Samples have been analyzed using Agilent 1290 II LC (Agilent, Santa Clara, CA, USA) connected to an Agilent 6495 LC/TQ (Agilent, Santa Clara, CA, USA). Chromatographic separation was achieved on a Kinetex® Hilic (Phenomenex, Torrance, CA,

USA), 100 mm (Length) x 2.1 mm (ID) x 1.7 μ m (Particle Size) using mobile phase A (0.1% formic acid in water with ammonium formate 1M) and mobile phase B (90% acetonitrile with 0.1% formic acid and ammonium formate 1M). Flow rate: 400 μ L/min. Total run-time: 11 min (Table 2). The sample injection volume was 5 μ L. A calibration curve has obtained by analyzing four different concentration of LAL solutions (0.50, 0.75, 1.0, and 1.5 μ g/mL), prepared from a standard solution of LAL 100 μ g/mL (lysinoalanine 2HCl, F-1195.0050, Bachem).

Table 2. Elution program.

N.	Time	Flow (μ L/min)	%A	%B
1	0.00	Run		
2	0.00	400	20	80
3	2.00	400	20	80
4	3.50	400	75	25
5	4.50	400	95	5
7	6.00	400	95	5
8	6.10	400	20	80
9	11.00	400	20	80
10	11.00	Stop Run		

Mass spectrometry spectra were collected in positive ion mode and operated in MRM (Multiple Reaction Monitoring); details are compiled in Table 3.

Table 3. Details of the experimental conditions for MS.

Compound name	Parent Ion (Da)	Product Ion (Da)	Collision Energy (CE)
LAL (H ⁺)	234.2	84.1	28

2.3 Production and characterization of films obtained from functionalized NaCas

The solutions were cast onto Teflon molds measuring 10 x 10 cm, using 0.1 mL/cm² as described in Chapter 2. The molds were left to dry at room temperature (21-23 °C) and ambient relative humidity (RH) for 48 h. After the drying period, the films were peeled off from the molds, individually sandwiched between two sheets of aluminum foil, and stored in a polystyrene box at 4 °C until analyses. Film thickness was obtained as the average of values measured in 9 random positions using a Mitutoyo QuantuMike Digimatic micrometer (Takatsu-ku, Kawasaki, Kanagawa, Japan).

2.3.1 Mechanical properties

The mechanical properties of the films, including tensile strength (TS) and elongation at break (EAB), were evaluated at room temperature following the protocol ASTM D882-18 (ASTM, 2018) and Lin, Wang & Weng, (2020) with modifications. A TA.HDplusC Texture Analyser (Stable Micro Systems, Godalming, UK) was used, equipped with suitable grips for tensile tests. Prior to testing, the films were conditioned at 50% RH and 20 °C. Rectangular strips of film (20 x 100 mm) were clamped with an initial grip separation of 60 mm and a crosshead speed of 20 mm min⁻¹ was used until film break. The values of EAB (%) and TS (MPa) were calculated from the force-strain curves obtained, as the average of eight determinations at least. To facilitate a comprehensive comparison, the mechanical properties of the films used as unfunctionalized control (F_LAB_RF) and the reference samples (F_LAB_RF_MILK_LIGHT and F_COM_S) were analyzed and used as reference points for evaluating the functionalized films.

2.3.2 Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy was performed as in the article by Mena-Prado et al. (2024) in the range of 450 cm⁻¹ to 4000 cm⁻¹ and an accumulation of 40 scans using a Perkin Elmer Spectrum Two instrument (Perkin Elmer instruments, Shelton CT, USA) equipped with an attenuated total reflection (ATR) module on the different functionalized NaCas films.

3. Results and Discussion

3.1 Di-Tyr formation in NaCas film-forming solutions exposed to LED light

Di-Tyr detection was carried out using the method developed by D'Incecco et al., (2024). The chromatograms of the different samples analyzed are shown in Figure 3. Di-Tyr elutes as a main peak with a shoulder, likely given by a minor stereoisomer, in a clean area of the chromatogram. No presence of di-Tyr was detected in the control solution S_LAB_RF (not functionalized), nor in the solution that underwent alkaline thermal treatment (S_LAB_RF_ALKALI). As a reference, the pattern obtained from a sample of pasteurized milk exposed to fluorescent light for 12 days in a display cabinet was included in the Figure (D'Incecco et al., 2024). In that sample, di-Tyr was detected with an amount of 183 µg/g of NaCas powder, suggesting the role of activator of the riboflavin naturally present in milk.

Riboflavin is a water-soluble vitamin naturally present in milk at a concentration of 1.8-2 mg/L (4.8-5.3 µmol/L) (Poulsen et al., 2015; Fracassetti et al., 2018). According to Fuentes-Lemus et al. (2018), riboflavin in milk acts as a photo-activator, facilitating oxidative modifications in

the tyrosine residues of casein molecules through the formation of cross-links, which can be either intra- or inter-molecular.

With the aim of inducing extensive crosslinking in our NaCas, the concentration of riboflavin was increased up to 35 $\mu\text{mol/L}$, i.e. about 7 times higher than in natural milk. No detectable levels were found in caseinate. The chromatograms of the two samples exposed to LED light for 60 min both evidenced the formation of di-Tyr although in very different amounts. Only 3 $\mu\text{g/g}$ of di-Tyr formed in the solution directly exposed to LED light (S_LAB_RF_LED), whereas 351 $\mu\text{g/g}$ formed when riboflavin was added as photo-activator before exposure (S_LAB_RF_LED_PA). Dalsgaard et al. (2011) demonstrated that, in a protein solution containing a photosensitizer (riboflavin, 13 $\mu\text{mol/L}$) and exposed to light, the amount of di-Tyr sharply increased within the first 10 min and was more or less three times higher in β -casein than in globular proteins like β -lactoglobulin or BSA, due to the different structural characteristics of the protein molecules.

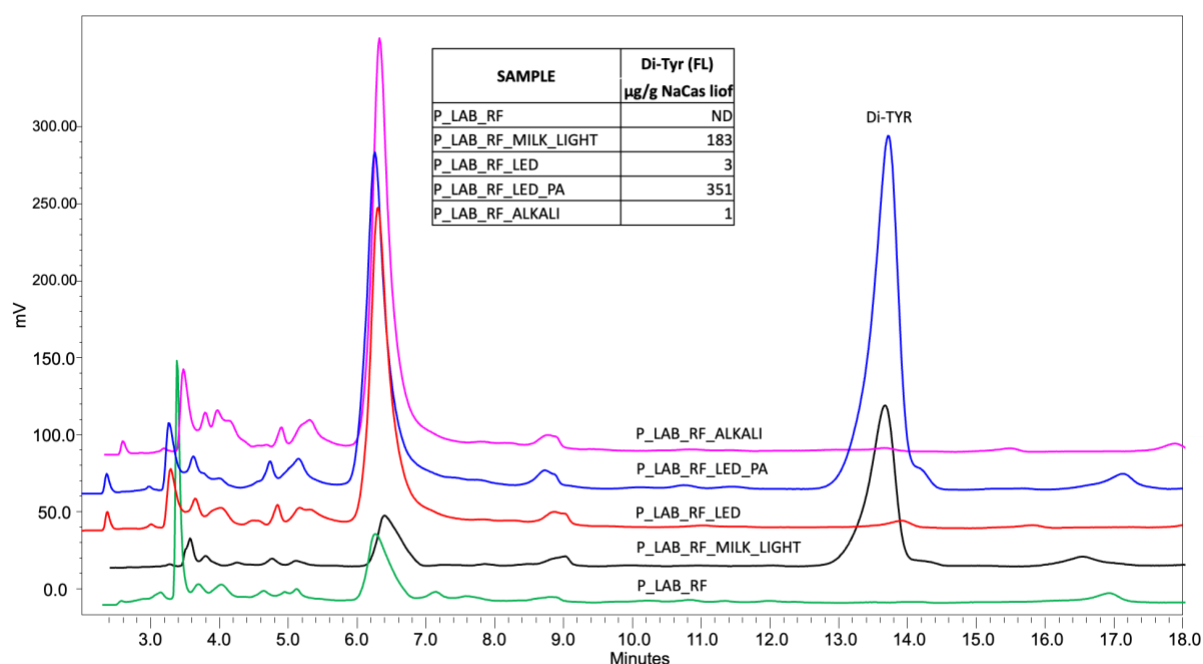


Fig. 3. HPLC chromatograms and quantification of di-Tyr in: control solution (green); NaCas from milk exposed to fluorescent light 6 days in cabinet (black); solution exposed to LED light without PA (red) and in presence of PA (blue); solution that underwent to alkaline thermal treatment (violet).

The high formation of di-Tyr (183 $\mu\text{g/g}$ NaCas) in caseinate obtained from milk exposed to light in a display cabinet can be attributed to the presence of riboflavin during milk exposure. In contrast, riboflavin was removed during the washing steps in NaCas production, possibly explaining the significantly lower di-Tyr formation (3 $\mu\text{g/g}$ NaCas) when the derived solution was exposed to LED light. Overall, these data indicated that the addition of a compound acting

as PA, namely riboflavin, strongly increased the amount of di-Tyr with a potential interest to our aim of modifying properties of NaCas films. However, the interest is mostly towards di-Tyr crosslink occurring at intermolecular level and the available information was not clarifying this aspect yet.

3.2 Localization of Di-Tyr in casein micelles

The positioning of di-Tyr in native casein micelles in milk exposed to light was studied using the transmission electron microscopy (TEM) after immuno-gold labelling of the molecule. The study employed an unconjugated monoclonal antibody along with a secondary antibody conjugated to 15-nm colloidal gold. Ultrathin sections of pellets from ultracentrifuged milk revealed well-preserved casein micelles in partially skimmed milk exposed to light. As shown in Figure 4, anti-di-Tyr labeling indicated gold particles distributed along the edges of casein micelles, with none observed inside. This distribution may be attributed to κ -casein, which is a primary target for photo-oxidation reactions due to its surface localization on the casein micelle and its higher tyrosine content compared to β -casein, which is mostly located in the inner part of the micelle. This might explain the presence of gold nanoparticles at the micelle edges. Furthermore, immunogold labeling indicates that the bond formation between two tyrosine residues occurs within a single casein micelle, supporting the hypothesis that this bond is intra-molecular in nature.

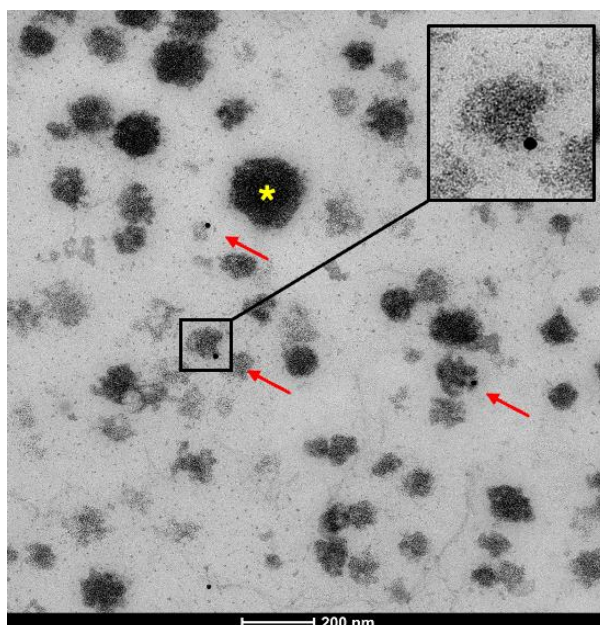


Fig. 4. Transmission electron micrograph of immunolocalization of di-Tyr in casein micelles pelleted by ultracentrifugation of partially skimmed pasteurized milk exposed to light for 14 days. Gold-labelled di-Tyr (black dots signaled by red arrows) is localized at the periphery of the casein micelle (yellow “*”) as in the magnified frame.

3.3 Lysinoalanine (LAL) formation in NaCas film-forming solutions heated at alkaline conditions

The quantification of LAL crosslinks in NaCas films that underwent alkaline thermal treatment is crucial due to their potential impact on the mechanical properties of the films. LAL arises from the nucleophilic addition of the ϵ -amino group of Lys to the dehydroalanine residues, which are produced via the β -elimination reaction from Cys or P-Ser residues (Pellegrino et al., 1996). This crosslink may contribute to the mechanical stability and durability of NaCas-based films but can also reduce their workability and plasticity.

Liquid Chromatography-Mass Spectrometry (LC-MS) represents a powerful and precise analytical approach for detecting LAL crosslinks, enabling both qualitative identification and quantitative analysis. In this study, the application of LC-MS to accurately analyze LAL in NaCas films was crucial, because of the advantages of a robust and interference-free detection of this technique.

Results from the LAL determination are shown in Table 4.

μg LAL/mg protein

Table 4. Amounts of LAL determined in different NaCas powders. The LAL content refers to the protein content of the powder.

SAMPLE	LAL (mg/g protein)
P_LAB_RF (control)	n.d.
P_LAB_RF_MILK_LIGHT	n.d.
P_COM_S	2.85 ± 0.35
P_LAB_RF_LED_PA	1.43 ± 0.03
P_LAB_RF_ALCALI	3.40 ± 0.21

The control sample (P_LAB_RF) and the P_LAB_RF_MILK_LIGHTs was free of LAL, as expected, while a level of 2.85 mg/g protein was found in a commercial sample of NaCas (P_COM_S) that was analysed as a positive control. High amounts of LAL in NaCas have also been previously reported, i.e. up to 35 mg/100 g of proteins (Cattaneo et al., 2012), and up to 50 mg/100 g of proteins (Resmini et al., 2003). The authors demonstrated that the formation of LAL and furosine (FUR) are correlated in opposite ways to the presence of lactose. The presence of lactose promotes the Maillard reaction that therefore prevails over β -elimination reactions, leading to the production of FUR. In contrast, the numerous washes adopted in industrial manufacturing of commercial NaCas remove lactose almost totally and allow lysine residues to be mainly involved in LAL formation instead of FUR. This mechanism also occurred for experimental NaCas produced under alkaline condition and after undergoing heat treatment. The adopted conditions lead to formation of 3.40 μg /g protein of LAL in the powder

P_LAB_RF_ALCALI, confirming the effectiveness of the treatments applied. A lower amount of LAL was found in the powder exposed to LED light, even if the pH was neutral. This can be explained because LAL formation occurs upon severe heat treatment even at neutral pH (Anema, 2021).

3.4 Films characterization

The thicknesses of the films were determined and are shown in Table 5.

Table 5. Thicknesses of the different functionalized films.

SAMPLE	THICKNESS (μm)
F_LAB_RF (control)	145 ± 30^a
F_LAB_RF_MILK_LIGHT	139 ± 51^a
F_LAB_RF_LED	145 ± 14^a
F_LAB_RF_LED_PA	152 ± 15^a
F_LAB_RF_ALCALI	142 ± 4^a

Thickness values are expressed as mean $\pm$ standard deviation. Results of one-way ANOVA were considered as significant when $p < 0.05$.

The thickness of functionalized NaCas films did not vary across the different treatments applied ($p > 0.05$). As all the powders used in the production of the films had a fat content of 23%, this parameter did not influence the thickness of the derived films.

3.4.1 Mechanical properties of the films

The determination of mechanical properties is essential to assess the tensile strength (TS) of a material under applied stress. This analysis also provides insights into key attributes such as strength, ductility, and flexibility. Packaging films, in particular, must possess adequate mechanical properties to endure the stresses encountered during transportation, storage, handling, and usage in real applications (Hager, Vallons, & Arendt, 2012). Ensuring sufficient mechanical strength is crucial for maintaining the structural integrity of these films (Sun, Wang, Kadouh, & Zhou, 2014).

In this context, the mechanical parameters evaluated included TS and elongation at break (EAB). TS refers to the maximum stress a film can withstand before failure. EAB, expressed as the percentage increase in the film's original length, reflects its ductility and indicates the extent of deformation the film can undergo before breaking. For polyolefin-based packaging films, elongation values can reach several hundred percent, whereas rigid plastics typically exhibit much lower values, often below 5%. EAB provides an important measure of a material's ability to bend and deform without fracturing, making it a critical parameter for applications

involving energy absorption through plastic deformation. Higher elongation values are associated with greater material toughness (Lin et al., 2020).

The purpose of these analyses was to understand whether functionalization of the films through the different treatments has led to changes in the mechanical properties of the films. Depending on the intended use of the packaging, a stiffer or more deformable film may contribute to better packaging performance. Thus, TS and EAB could be most informative and were evaluated on the functionalized films. The measured values are shown in Figure 5 while Table 6 is shown to recall all the levels of the tested crosslinks present in samples.

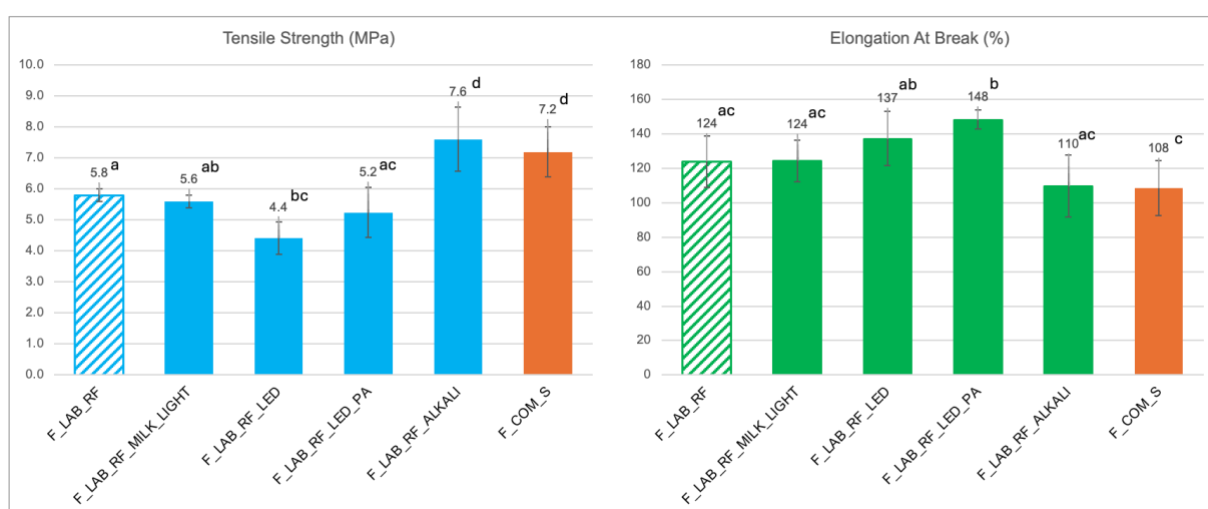


Fig. 3. Mechanical properties (Tensile strength-TS and Elongation at Break-EAB) of the functionalized films. F_LAB_RF film (control, unfunctionalized) is indicated by striped bars while the F_COM_S film (reference) is indicated by the orange solid bar. Data in the same graph followed by different superscript letters are significantly different ($p < 0.05$) according to one-way ANOVA and Tukey test.

Table 6. Levels of di-Tyr and LAL previously determined in the experimental powders used for producing the films tested in Figure 5.

Sample	LAL (mg/g protein)	Di-Tyr ($\mu\text{g/g}$ NaCas)
P_LAB_RF (control)	ND	ND
P_LAB_RF_MILK_LIGHT	ND	183
P_LAB_RF_LED	-	3
P_LAB_RF_LED_PA	1.43 ± 0.03	351
P_LAB_RF_ALKALI	3.40 ± 0.21	1
P_COM_S	2.85 ± 0.35	ND

Values describing mechanical properties were rather similar among unfunctionalized films. The F_LAB_RF_MILK_LIGHT film showed no significant differences from the control film (F_LAB_RF) for both TS and EAB, despite the relevant amount of di-Tyr ($183 \mu\text{g/mg}$ NaCas)

in the former that formed upon exposure of the milk to light in the display cabinet. In addition, the sample P_LAB_RF_LED_PA, which contains nearly twice the amount of di-Tyr (351 $\mu\text{g}/\text{mg}$ NaCas), exhibited comparable TS and EAB values respect to films without di-Tyr. Overall, the crosslinking di-Tyr, irrespective of its concentration, did not exert a significant impact on the mechanical properties of the films. The observed inefficacy could be attributed to the low quantities formed or to the predominantly intramolecular nature, rather than intermolecular, of this bond. No information is currently available in the literature regarding the localization of di-Tyr in caseinate to explain such behavior. However, immunolocalization studies conducted in milk have demonstrated that di-Tyr forms at the intramolecular level without inducing protein aggregation (D’Incecco et al., 2024). In contrast, the F_LAB_RF_ALKALI film, which contained the highest concentration of LAL, showed the highest TS among the experimental samples, comparable to that of the F_COM_S sample. As expected, the sample with the highest TS also exhibited the lowest EAB, highlighting the well-established inverse relationship between these two mechanical properties. These results suggest that a denser protein network forms in films subjected to thermal treatment under alkaline conditions, which enhances film stiffness. In fact, it has been reported that the formation of LAL crosslinks can occur intermolecularly, contributing not only to protein aggregation but also to changes in protein functionality (Nielsen et al., 2020). Notably, both the F_LAB_RF_ALKALI and F_COM_S films exhibited the highest LAL content (Table 6), which likely explains their similar mechanical characteristics.

3.4.2 FTIR analysis of functionalized NaCas films

FTIR spectroscopy was performed to investigate possible crosslinking in the NaCas films, after the functionalization with light exposure and alkaline treatment. Firstly, a careful review of the recent literature was made to identify the bands and their respective functional groups, as well as the bonds formed within the spectrum. The spectrum and the fingerprint of the different NaCas films is shown in Figure 6.

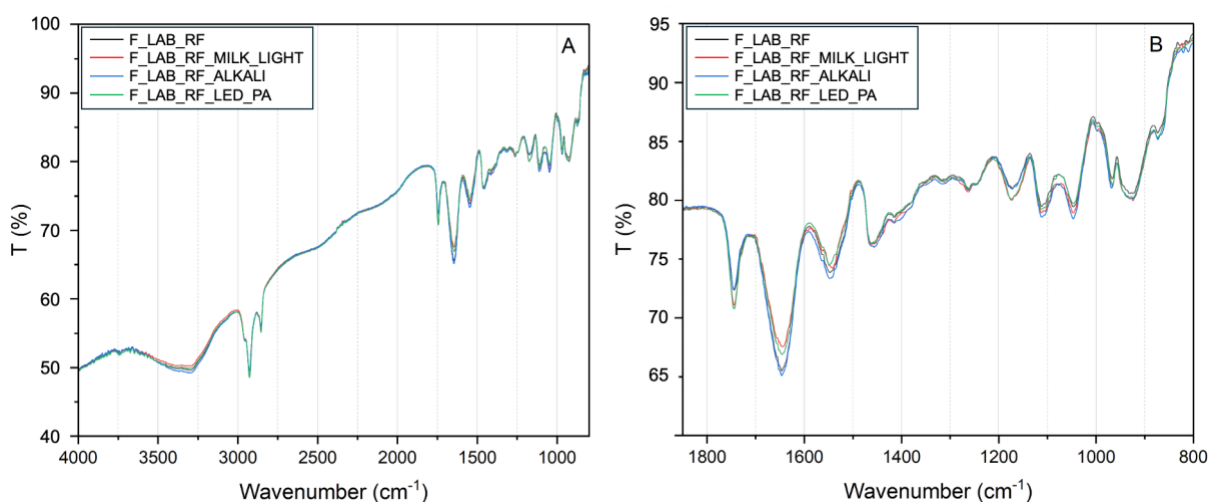


Fig. 4. FTIR spectra of the NaCas films; A) full spectrum of the films, B) fingerprint of the molecule, with wavenumbers from 800 to 1850 cm^{-1} . F_LAB_RF: control film (non-functionalized); F_LAB_RF_MILK_LIGHT (reference sample from milk exposed to fluorescent light); F_LAB_RF_ALKALI: film obtained from NaCas solution heat-treated at alkaline condition; F_LAB_RF_LED_PA: film obtained from NaCas solution exposed to LED light in presence of riboflavin.

Casein typically shows absorption bands at 3400 – 3300 cm^{-1} attributed to O-H and N-H stretching vibrations, while the peak at 2960 cm^{-1} is related to the stretching vibrations of $-\text{CH}_2$ groups (Picchio et al., 2018; Diak et al., 2007). In this wavelength range, no particular shifts attributable to higher O-H or $-\text{CH}_2$ bonds were observed, but only a higher intensity of the band at 3400 cm^{-1} for the F_LAB_RF_ALKALI film, related to the amount of water in the film.

A focus of the FTIR spectra, on protein structure, can be seen in Figure 7.

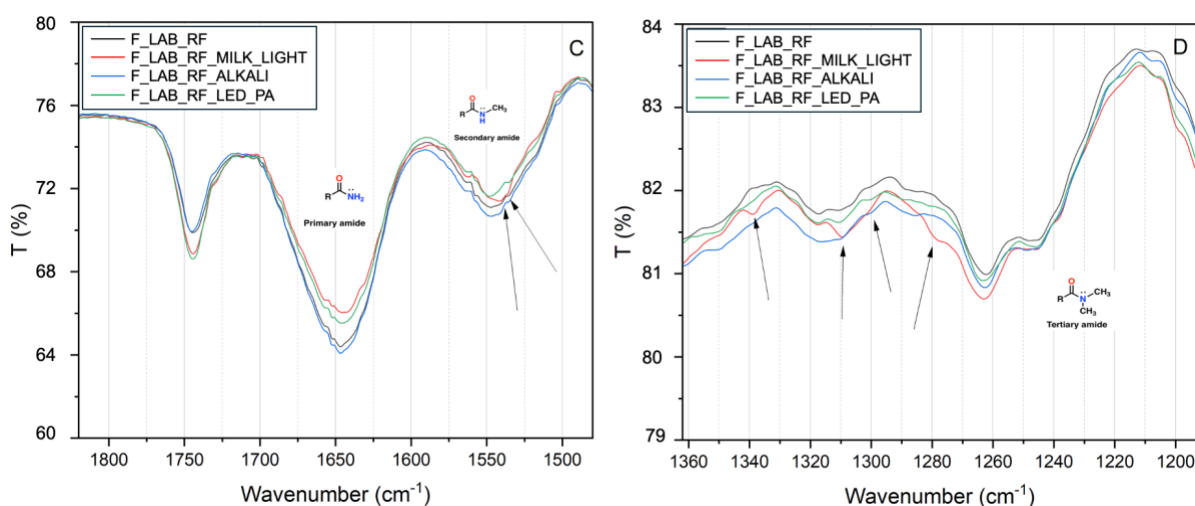


Fig. 5. FTIR spectra with a focus on the protein structure in the range of wavenumbers from 1450 to 1850 cm^{-1} (C) and from 1150 to 1360 cm^{-1} (D).

Peaks at 1640 cm^{-1} are attributed to amide I groups (C=O stretching), while peaks between 1550 and 1510 cm^{-1} are attributed to amide II (N-C=O symmetric stretching and N-H bending) (Arrieta et al., 2013; Picchio et al., 2018; Indrie et al., 2023). Finally, the bands around 1400 cm^{-1} could be attributed to the carboxylate group (O-C-O) (Diak et al., 2007) while the bands from 1240 to 1260 cm^{-1} could be assigned to amide III (C-N and N-H stretching). In this range of wavenumbers, shifts can be observed in the F_LAB_RF_LED_PA film for secondary and tertiary amides, thus assuming a higher presence and higher number of amides II and amides III, while a shift is observed for the F_LAB_RF_ALKALI film in the O-C-O bonds. In addition, films that underwent exposure to light showed a higher intensity of the bands at 1750 cm^{-1} , attributable to an increase in protein secondary structure and also attributable to C=O stretching linked to the photo-oxidations of proteins. While from 1650 to 1640 cm^{-1} , a higher intensity is seen in the F_LAB_RF_ALKALI films and the nonfunctionalized film, attributable to a greater presence of random structures in the protein conformation.

Finally, a focus on the wavelengths from 825 to 1125 cm^{-1} and a particular band of assonance around 1135 cm^{-1} was done, to investigate C-O bond stretching in C-OH bonds (Pellissari et al., 2009) and can be seen in Figure 8.

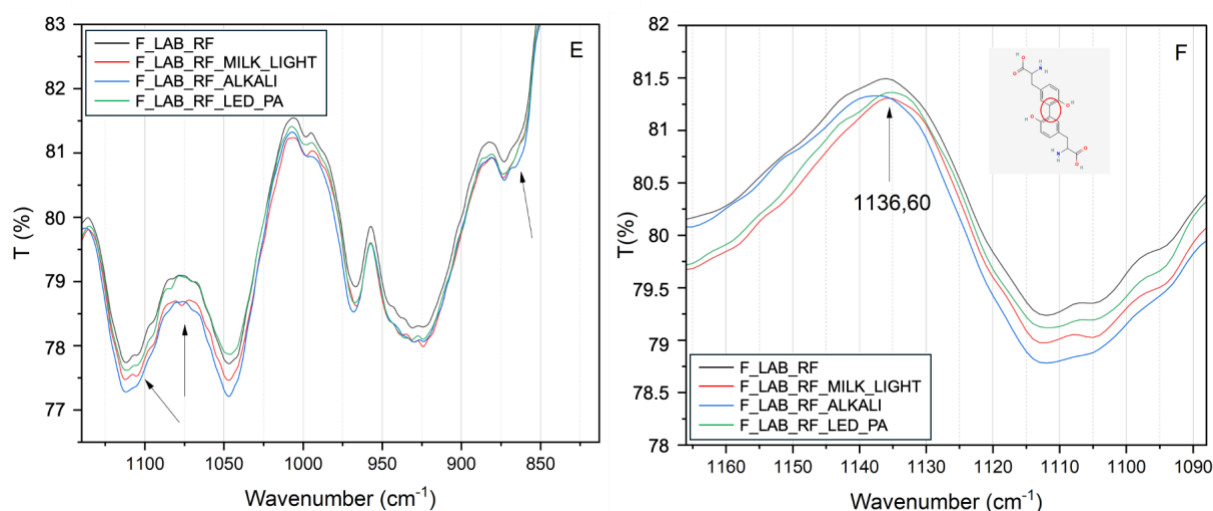


Fig. 6. FTIR spectra with a focus on the lower wavenumbers, in the range from 820 to 1125 cm^{-1} (E) and on the band located at 1136 cm^{-1} (F).

The band attributable to C-O bending in NaCas films is found at 1120 cm^{-1} , while the other bands from 925 to 970 cm^{-1} are attributable to monocationic Na^+ interactions (Fernandez et al., 2003). In addition, typical bands associated with the presence of glycerol in films, between 800 and 1150 cm^{-1} , are present in this region (Chevalier et al., 2018); these bands represent the strong binding that occurs between sodium caseinate and the hydroxyl groups of glycerol.

Finally, at 1136.60 cm^{-1} the band attributing to the formation of di-Tyr in the films was identified, as we can observe there is a shift for the films exposed to light, with a more pronounced shift for the F_LAB_RF_LED_PA film thus confirming a higher production of Di-Tyr, as also found by chromatographic analysis.

4. Conclusions

This study demonstrates the effectiveness of green functionalization techniques in improving the properties of NaCas films, offering a sustainable solution for packaging applications. Two distinct methodologies, riboflavin-mediated photo-oxidation and alkaline thermal treatment, were applied to enhance the mechanical and structural characteristics of NaCas films. Each approach introduced unique crosslinking mechanisms that partly affected the films' functional performance.

Riboflavin-mediated photo-oxidation leveraged the photosensitizing properties of riboflavin under LED light to generate singlet oxygen radicals, which facilitated oxidative modifications of tyrosine residues, forming di-Tyr crosslinks. The addition of RF significantly increased di-Tyr production compared to control samples, as confirmed by HPLC. TEM revealed that di-Tyr crosslinks in milk were localized primarily at the edges of casein micelles, where κ -casein, susceptible to oxidation, is more accessible. This functionalization enhanced the films' elasticity and plasticity, as evidenced by increased EAB. However, the extent of di-Tyr formation was constrained by the spatial arrangement of tyrosine residues within the casein molecules, limiting the overall crosslinking potential.

Alkaline thermal treatment promoted LAL formation through β -elimination reactions, producing a strengthened protein network. LC-MS quantification confirmed substantial LAL content in treated samples, correlating with increased TS and reduced EAB. The improved rigidity and durability of the films were comparable to those of commercial NaCas products, highlighting the efficacy of this functionalization method.

Characterization of the functionalized films using FTIR spectroscopy revealed significant molecular changes, including shifts in amide and carboxylate group bands, indicative of successful crosslinking. Mechanical testing further demonstrated the complementary effects of the two treatments: di-Tyr crosslinks enhanced film elasticity and deformability, while LAL crosslinks improved tensile strength and stiffness.

Despite these promising results, certain limitations were noted. Di-Tyr formation was limited by the availability of tyrosine residues, while the effectiveness of LAL crosslinking required precise control of alkaline processing conditions. These challenges highlight the need for further optimization of the functionalization processes. The crosslinking mechanisms investigated in

this chapter were developed for nonedible NaCas-based films, as the possible effects of formed crosslinks upon the digestive process were not investigated.

Future research should focus on refining the parameters for both functionalization methods to maximize crosslink formation and enhance material properties. Additional studies could explore the synergistic effects of combining these techniques with other green crosslinking agents, potentially broadening the functionality of NaCas films. Moreover, investigating the long-term stability and barrier properties of functionalized films under real-world conditions is essential to validate their industrial scalability. Finally, scaling up the production processes and conducting feasibility analyses will be critical for integrating these materials into commercial packaging systems.

In conclusion, this study establishes a strong foundation for the development of sustainable NaCas-based films with customizable properties. By addressing the limitations and advancing the functionalization techniques, NaCas films can emerge as a viable alternative to synthetic packaging materials.

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Chapter 4 - Development and test of a NaCas-based flow-pack prototype for packing low-moisture cheese snacks in modified atmosphere - In partnership with Lactalis Italia S.p.A.

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Abstract

This chapter explores the development and testing of NaCas-based modified-atmosphere flow-packs for packaging low-moisture cheese snacks. Optimal weldability conditions for the NaCas films were identified, with the F_LAB_S_B film demonstrating superior mechanical properties and thermal stability. A storage test was performed after producing the NaCas-based flow-packs and packaging the cheese-based snacks, also using secondary packaging with reduced WVTR to prevent moisture transfer. The test showed that NaCas-based flow packs exhibited greater moisture transfer than control polymer packs causing increased moisture in cheese snacks, particularly in the first ten days. Despite the higher moisture uptake, NaCas-based flow-packs maintained the quality parameters of cheese snacks along the storage test. The study highlights the dual role of NaCas packaging in sorbing and releasing volatile compounds related to lipid oxidation and rancidity, raising considerations on the migration of compounds from packaging to food and vice versa, namely scalping phenomena. Future research should focus on enhancing barrier properties, investigating migration phenomena and microbiological aspects to ensure the feasibility of NaCas-based packaging for industrial applications.

1. Introduction

The global demand for sustainable alternatives to synthetic polymers has led to the development of biopolymers as promising materials for food packaging. Biopolymers, derived from renewable sources such as proteins, starch, and cellulose, offer the potential for biodegradability and reduced environmental impact (Yadav *et al.*, 2018).

Among the uses of these biopolymers, some of them can be used for food contact; more specifically, for food packaging (Bhaskar *et al.*, 2023). Weldability is a crucial property for the successful application of biopolymers in food packaging, as it determines the strength and integrity of the sealed joints. Parameters such as temperature, pressure, and time significantly influence the quality of the welds (Sikorska *et al.*, 2020). Techniques like heat sealing, ultrasonic welding (Talaniuk *et al.*, 2023), and radiofrequency (RF) welding are commonly employed to achieve effective seals in biopolymer films. Despite the potential of biopolymers, welding these materials poses several challenges. The thermal sensitivity of many biopolymers often results in inconsistent weld quality, while their moisture absorption properties and tendency to crystallize complicate the control of the welding process. Additionally, the variability in material composition makes it difficult to standardize welding parameters, leading to issues such as reduced weld strength and integrity (Taherimehr *et al.*, 2021). Several strategies have been explored to enhance the weldability of biopolymers, including material modifications such as the incorporation of plasticizers to reduce brittleness and improve flexibility. Process optimization, focusing on fine-tuning the temperature, pressure, and time during welding, has shown potential in improving weld quality. Additionally, emerging techniques like laser welding offer more precise control, reducing the likelihood of thermal degradation in biopolymers.

Sodium-caseinate (NaCas) offers excellent film-forming properties and biodegradability, making it a promising candidate for food packaging applications (Audic *et al.*, 2007). However, its weldability presents several challenges due to its sensitivity to moisture and temperature, which can lead to difficulties in achieving consistent and robust seals.

This research aims to address these challenges by first identifying the key parameters affecting weld quality of NaCas films, through the investigation of the best conditions of temperature, time and pressure, based on the film characteristics obtained in Chapter 2. Subsequently, a flow-pack system was developed for packaging cheese-based snacks with reduced water activity and moisture content, followed by an in-depth investigation of the package-product interaction, during a preliminary storage test. In this storage test, physico-chemical and biochemical changes were monitored within a shorter timeframe than the shelf-life indicated by the manufacturer and the storage temperature was set at 23 °C. Temperature is the most used factor to accelerate degradation processes in food and biological products (Haouet *et al.*, 2019). The study involved monitoring the water activity and moisture levels of the snacks over storage time. Additionally, a qualitative and quantitative analysis of lipid oxidation compounds and free amino acids were performed at three intervals during the storage test. Changes in the active atmosphere, initially introduced during the food packaging phase, were also tracked to assess their impact on product stability.

2. Materials and Methods

2.1 Experimental design

The experimental design is outlined in Figure 1. This study was conducted on two distinct levels. The first level focused on optimizing the sealing conditions—specifically, time, temperature, and pressure—for the various films developed in Chapter 2. These films differed in fat content and were processed using two distinct sonication technologies. The second level involved choosing one of the films developed in Chapter 2, taking into consideration both the different thermal, mechanical and barrier properties of the films and the output obtained from the weldability optimization conditions, to produce flow-packs designed to package cheese snacks with reduced moisture content. These flow-packs were stored under controlled humidity and temperature conditions to assess the stability of the modified atmosphere introduced during packaging, the lipid oxidation volatile compounds and changes in moisture content in the cheese snacks over the storage period. A control flow-pack, composed of a plastic polymeric material with a high-water vapor and oxygen barrier, was included for comparison.

Given the well-documented susceptibility of NaCas-based films to water vapor, a secondary protective packaging was implemented to ensure that external ambient water vapor does not permeate into the experimental flow-packs, thereby preserving their integrity. This secondary flow-pack, made of plastic polymeric material with a high-water vapor barrier but low oxygen barrier, was used to create a multi-pack containing three individual cheese snack flow-packs. This setup ensured the isolation and protection of the experimental packaging system during the storage period. A single cheese snack flow-pack was also stored as additional control.

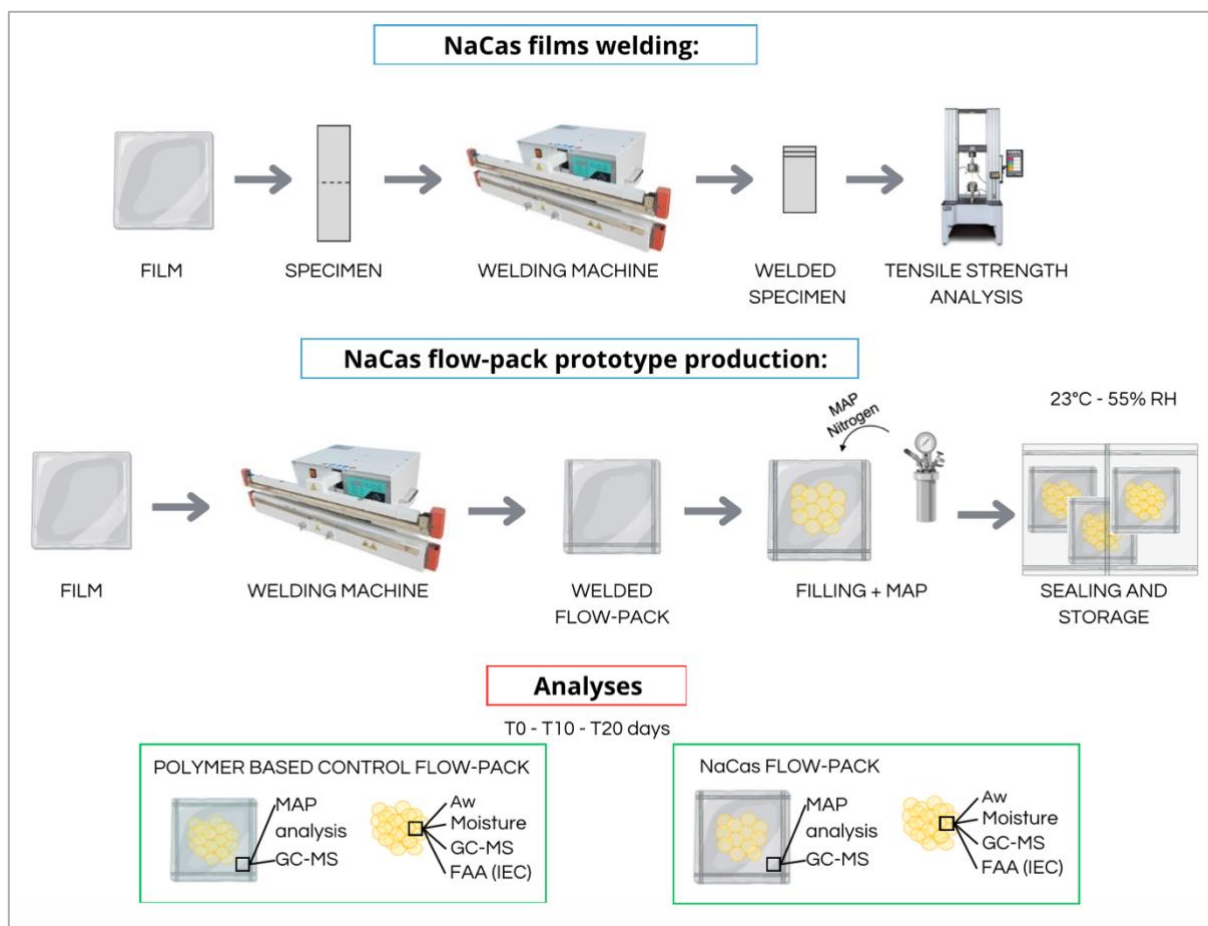


Fig. 1. Experimental design showing the production and testing of NaCas film welds using a thermo-sealer for thin plastic films. The production process of the flow-packs (both polymer-based control and NaCas-based) and the packaging of the cheese snacks are depicted at the bottom; finally, the analyses performed on the different flow-packs for their characterization are shown (The figure was created with BioRender website).

2.2 Set up of the best weldability conditions in NaCas-based films

To achieve welds with suitable mechanical properties that ensure both effective food preservation and packaging integrity, an experimental design was developed to determine the optimal conditions for welding. The investigation considered different film formulations, the same ones developed in Chapter 2, and considered various combinations of welding time, pressure, and temperature to identify the most effective parameters.

2.2.1 Film production and specimen preparation

All the films produced and characterized in Chapter 2 were considered for identifying the one that would allow to obtain welds with a high tensile strength, suitable to produce a flow-pack for packaging uses. The gross composition of the films is reported here for clarity and shown in Table 1.

Table 3. Gross composition (g/100g) of experimental and commercial NaCas films, as shown in Chapter 2, section 3.3.

Sample	Protein	Fat	Glycerol	Ash	Moisture
F_LAB_PS_B	43.9	25.3	14.6	4.9	11.3
F_LAB_PS_P	43.6	25.1	14.5	4.9	11.8
F_LAB_RF_B	52.1	17.6	17.3	3.0	10.0
F_LAB_RF_P	51.1	17.3	17.0	2.9	11.7
F_LAB_S_B	64.7	0.7	21.5	1.2	11.8
F_LAB_S_P	65.0	0.7	21.6	1.2	11.4
F_COM_S_B	58.4	0.7	20.1	4.9	15.9
F_COM_S_P	58.1	0.7	20.0	4.8	16.4

Once dried, the films were carefully peeled off from the molds and specimens were prepared following ISO 527 guidelines, cutting the films into strips of 15 × 100 mm.

2.2.2 Welding and tensile testing at breakage of specimens

Specimens were conditioned for about 2 hours at 23 °C - 55% RH before being tested in an environmental test chamber CTC 256 (Mettler GmbH, Büchenbach, DE). After conditioning, the samples were welded using a thermo-sealing machine for plastic films (Digital Accutemp, Gandus Saldatrici, Cornaredo, IT) using different conditions listed in Table 2.

Table 2. Temperature, pressure, and time conditions investigated for NaCas-based film sealing.

Temperature (°C)	Pressure (bar)	Time (s)
90	3.5	0.2
110	4.25	0.5
130	5	0.8

The heat-sealing bar used consists of an upper and a lower sealing bar, both of which can be thermostatically controlled; both were covered with a Teflon-coated scotch to prevent the film from sticking to the steel of the bars. All combinations of temperature, pressure and time were investigated, following the experimental scheme shown in Table 3. For each trial presented in the table, 5 different sealed specimens were produced with the same conditions to ensure repeatability and reliability of the result obtained.

Table 3. Experimental conditions used for the welding of NaCas-based film specimens. The table shows the standard order, the order in which the tests were performed (Run order) and the different combinations of temperature, pressure and time for each welding test. Some combinations of the different parameters are repeated, to increase robustness of the experimental design.

Std order	Run order	Temperature (°C)	Pressure (bar)	Time (s)
17	1	110	4.25	0.5
16	2	110	4.25	0.5
5	3	90	4.25	0.2
6	4	130	4.25	0.2
11	5	110	3.5	0.8
4	6	130	5	0.5
1	7	90	3.5	0.5
8	8	130	4.25	0.8
3	9	90	5	0.5
9	10	110	3.5	0.2
7	11	90	4.25	0.8
2	12	130	3.5	0.5
15	13	110	4.25	0.5
12	14	110	5	0.8
13	15	110	4.25	0.5
10	16	110	5	0.2
14	17	110	4.25	0.5

After welding, the specimens were conditioned for 2 hours at 23°C - 55% RH before testing. Specimens thickness was determined using a Mitutoyo QuantuMike Digimatic micrometer (Takatsu-ku, Kawasaki, Kanagawa, JP). The force at weld failure (TF) was determined using a floor electronic dynamometer (AG5E, Acquati, Arese, IT) following the parameters provided in the regulation ISO 527. The crosshead space was 60 mm, and the two edges of the specimen were secured to the instrument by two clamps. Mean and standard deviation of each set of specimens (5 specimens for each type of condition) were calculated to understand the overall best welding conditions.

2.3 Development of NaCas-based flow-packs and packaging for reduced moisture cheese snacks

2.3.1 Film production and flow-pack preparation

As mentioned later in this chapter (Section 3.1), the film chosen for the NaCas-based flow-pack production was F_LAB_S_B. Once dried, the films were carefully peeled off from the molds, edges of the film that were uneven in thickness were removed, and jigs were prepared to then weld the flow-pack. The NaCas jigs were composed of two identical films with the size

of 90 x 90 mm. Control flow-packs of the same dimensions were prepared using a poly-coupled plastic polymer film consisting of oriented polyamide (OPA)/solventless adhesive/polyethylene (PE). The control packaging had $OTR < 1.1 \text{ cm}^3 \text{ m}^{-2} \text{ 24h}^{-1}$ and $WVTR < 4.2 \text{ g m}^{-2} \text{ 24h}^{-1}$ (technical datasheet information). Due to this high barrier characteristic, it was adopted as a reference flow-pack in terms of performance for food preservation, to be compared with the performance of the experimental NaCas flow-pack. Figure 2 shows the jigs to produce flow-packs.

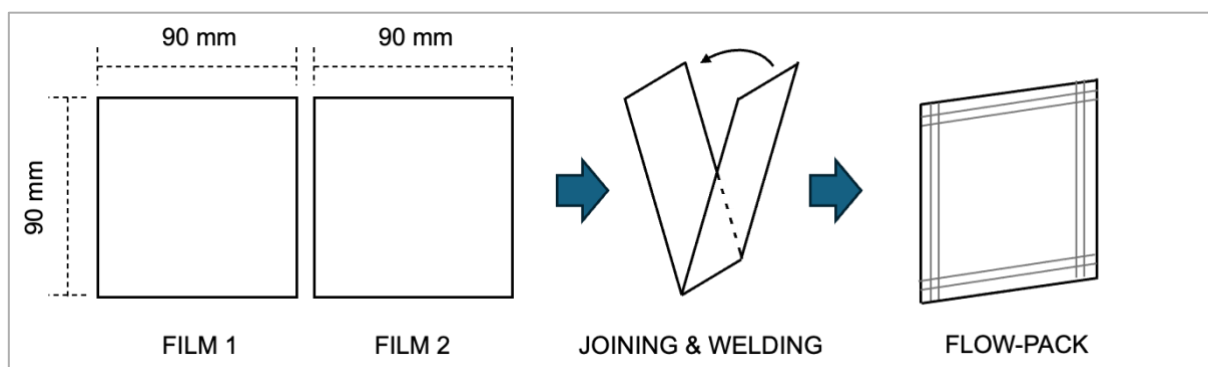


Fig. 2. Jigs for flow-pack production, consisting of two films of the same size (90 x 90 mm) joined and heat-sealed. The production process is the same for both NaCas experimental and control flow-packs.

To produce the flow-pack, the two 90 x 90 mm NaCas films are overlapped and heat-sealed with a thermo-sealing machine (Digital Accutemp, Gandus Saldatrici, Cornaredo, IT) on three sides, leaving one side open for the introduction of cheese snacks and modified atmosphere. For NaCas-based flow-pack weldings, the bars of the thermo-sealing machine were set at 135 °C with a sealing time of 1 s, while for the control flow-packs the temperature was set at 145 °C with a sealing time of 0.5 s. For the packaging and storage test, commercially available cheese snacks from a single lot were provided by Lactalis Italia. Four g of cheese snacks were introduced in each flow-pack, a nitrogen flow at about 25 L/min was flushed for 10 s within the pack, and then the flow-pack was immediately sealed through the thermo-sealing machine.

A multi-pack produced from a poly-coupled plastic polymer (polypropylene(PP)/solventless adhesive/white PP) with a $WVTR$ of $7.2 \text{ g m}^{-2} \text{ 24}^{-1}$ and an OTR of $2200 \text{ cm}^3 \text{ m}^{-2} \text{ 24}^{-1}$ (technical datasheet information) was produced and welded on three sides, using the same thermo-welding machine and using a bar temperature of 145 °C for 0.5 s. Three NaCas flow-packs containing the cheese snacks were inserted for each multi-pack, three multi-packs were produced for each type of material to monitor storage times: T_0 , at the start of the test; T_{10} , at 10 days of storage; and T_{20} , at 20 days of storage, as depicted in Figure 3.

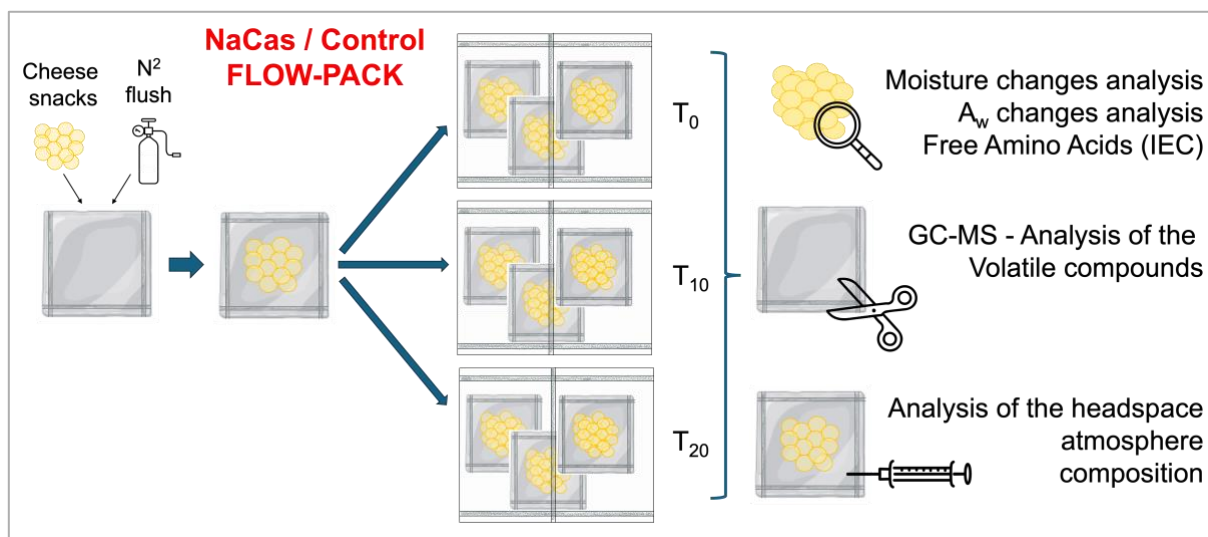


Fig. 3. Experimental design for production and storage test of NaCas-based flow-pack inserted in a multi-pack and a control polymer-based flow-pack, both containing low-moisture cheese snacks.

The storage test was conducted using room temperature conditions; the multi-packs were stored in an environmental test chamber CTC 256 (Mettler GmbH, Büchenbach, DE) at 23°C - 55 % RH.

2.3.2 Analyses of cheese snacks and flow-packs during the storage test

For the storage test, commercial cheese snacks (100 g) from the same lot were provided by the company. The gross composition, shown in Table 4, was obtained from the product label, while other parameters, i.e. moisture, water activity, volatile compounds and free amino acids, were directly determined to characterize the food before and during the storage period.

Table 4. Gross composition (g/100g) of cheese snacks.

Gross composition (g/100g)	
Fat	49.4
Protein	39.0
Salt	2.7
Carbohydrates	< 0.1

The packed cheese snacks were stored for 0, 10 and 20 days at 23 °C and 55 % RH to obtain three different samples for each packaging type.

The moisture content was determined on 3 g of grinded cheese snack. The sample was kept in a laboratory oven at 102 °C until constant weight was reached. Three replicates were carried out.

The water activity (a_w) was evaluated using an Aqua Lab model Series 3TE instrument (Decagon Devices, Pullman, WA, USA) on 3 g of grinded cheese snacks. Three replicates were carried out.

Volatile compounds (VOCs) including hexanal, nonanal, 2-heptanone, 2-nonanone, 2,3-butanedione, 3-hydroxy,2-butanone, hydroxy,2-propanone, butanoic acid, 2-methyl butanoic acid and hexanoic acid were considered as key odorants related to rancidity and lipid oxidation in cheese (Renoldi et al., 2024; Dalsgaard et al., 2010). The volatile compounds of the cheese snacks were evaluated following the method of D’Incecco et al. (2023) with some modifications. The method for the volatile compounds determination foreseen firstly a headspace solid phase micro extraction (SPME) followed by gas chromatography-mass spectrometry (GC-MS). A gas chromatograph Trace 1300 coupled with a TSQ 8000 Evo Mass Spectrometer (Thermo Scientific, Milan, IT), equipped with an automatic autosampler (Gerstel MPS) was used for the analyses. About 2 g of grinded cheese snacks was weighed into 20 mL glass vial, 20 μ L of internal standard was added (2-methyl pentanol (50 μ g/mL; Sigma-Aldrich, Milan, Italy)). Samples were equilibrated at 45 °C for 15 min and then exposed to the Carboxen-polydimethylsiloxane-divinylbenzene fiber (CAR/PDMS/DVB 50/30 μ m $\times$ 1 cm, 23 Ga) (Supelco, Bellefonte, PA, USA) for 10 min at 45 °C. The injector was set at 250 °C and the injection mode was splitless for 0.75 min with a desorption time of 15 min. The gas-chromatographic separation was carried out with a DB-WAX UI MS column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m) (Agilent, Santa Clara, CA, USA) using helium as carrier gas at a flow rate of 1 mL/min. The oven temperature was initially set at 40 °C and held for 5 min, then ramped at 5 °C/min up to 220 °C and held for 15 min. The temperatures of the transfer line and the source were 240 °C and 200 °C, respectively. The mass spectrometer operated in electron ionization mode at 70 eV using full scan mode, registering the m/z from 40 Da up to 350 Da. The ions used for identification were chosen according to the NIST MS Search 2.0 library and validated by external standard comparisons of ion fragmentation patterns. The abundance of each volatile compound was determined through the ratio of the target compound areas and the internal standard area (A/A_{is}). Analyses were carried out in duplicate. For the determination of some volatile compounds of particular interest (hexanal, 2-hydroxypropanone, internal standard), the Single Ion Monitoring (SIM) method was used. Results are expressed as the sum of the amounts of selected compounds responsible for cheese rancidity and oxidation.

Free amino acids (FAA) were also monitored during the storage test following the method of Pellegrino et al. (2024) with some modifications. FAA was monitored in the cheese snacks at the beginning of the storage test (T0) and after 20 days of storage (T20) in flow-pack packaged within the polypropylene-based maxi-pack. An additional check was carried out on cheese snacks packed in NaCas-based flow-packs which, however, were not placed inside the maxi-pack and were directly kept in the climate chamber. A 2.5 g aliquot of cheese snack

was added to 40 mL of Na citrate buffer in a beaker, maintained under magnetic stirring for 15 min and then homogenized with an Ultra-Turrax (5 min - 11,000 rpm). The extract was filtered using a paper filter and 10 mL of the filtrate was transferred into a 25 mL volumetric flask, with an addition of 10 mL of 7.5% (w/v) 5-sulphosalicylic acid (pH 1.7) that was added dropwise under continuous agitation, diluted to the volume mark with Na citrate buffer and then filtered. Finally, 10 mL of filtrate was transferred in a 100 mL flask, with norleucine added as internal standard, made up to the volume mark with 0.2 N lithium citrate with a pH of 2.2, and filtered through a 0.2 μ m regenerated cellulose filter (Merck, Milan, IT) prior to the injection. The free amino acid (FAA) content was determined using ion exchange chromatography (IEC) according to Hogenboom et al. (2017). A Biochrom 30+ amino acid analyzer (Errecci, Milan, Italy) was used. The injection volume of the samples was 100 μ L. The determination was carried out by the external calibration method and the data were expressed on a cheese snacks protein basis and as a relative percentage. Cheese snacks protein content was determined according to the label of the product. Data are expressed as mean $\pm$ standard deviation.

For each flow-pack sample the headspace atmosphere has been analyzed and the volatile compounds were detected. Headspace atmosphere (residual oxygen) was analyzed using a precision headspace gas analyzer CheckMate 3 Dansensor (Ametek Mocon, Brooklyn Park, MN, USA).

The volatile compounds of the film were evaluated following the method of D’Incecco et al. (2023) with some modifications. The method for the volatile compounds determination foreseen firstly a headspace solid phase micro extraction (SPME) followed by gas chromatography-mass spectrometry (GC-MS). A gas chromatograph Trace 1300 coupled with a TSQ 8000 Evo Mass Spectrometer (Thermo Scientific, Milan, IT), equipped with an automatic autosampler (Gerstel MPS) was used for the analyses. About 50 cm² of each film coming from a flow-pack (NaCas and control) was put into 20 mL glass vial. Samples were equilibrated at 70 °C for 10 min and then exposed to the Carboxen-polydimethylsiloxane-divinylbenzene fiber (CAR/PDMS/DVB 50/30 μ m $\times$ 1 cm, 23 Ga) (Supelco, Bellefonte, PA, USA) for 20 min at 70 °C. The injector was set at 250 °C and the injection mode was splitless for 0.75 min with a desorption time of 10 min. The gas-chromatographic separation was carried out with a DB-WAX Ultra Inert MS column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m) (Agilent, Santa Clara, CA, USA) using helium as carrier gas at a flow rate of 1 mL/min. The oven temperature was initially set at 40 °C and held for 5 min, then ramped at 5 °C/min up to 220 °C and held for 15 min. The temperatures of the transfer line and the source were 240 °C and 200 °C, respectively. The mass spectrometer operated in electron ionization mode at 70 eV using full scan mode, registering the m/z from 40 Da up to 350 Da. The ions used for identification were chosen

according to the NIST MS Search 2.0 library and validated by external standard comparisons of ion fragmentation patterns. Analyses were carried out in duplicate. Results are expressed as the sum of the amounts of selected compounds responsible for cheese rancidity and oxidation.

2.4 Statistical analysis

For each type of film used for flow-pack production, results are expressed as mean $\pm$ standard deviation. Statistical differences between mean values ($p < 0.05$) were determined, after checking for homogeneity of variances with Levene's test, using a one-way ANOVA followed by Tukey' post-hoc test.

For the sum of volatile compounds present at the different times of the storage test, the results were expressed as mean $\pm$ standard deviation. In order to determine whether significant differences existed between the experimental and control flow-packs ($p < 0.05$) between the means, the *t-test* was used.

Statistical analysis of data was performed using the SPSS Win 12.0 software Version 27 (IBM Corp., Chicago, IL, USA).

3. Results and Discussion

3.1 Optimization of weldability conditions in NaCas-based films

The evaluation of weldability conditions for NaCas-based films focused on identifying the optimal combination of temperature, pressure, and welding time to produce weldings with the mechanical integrity required for food packaging applications. These parameters were systematically varied according to the experimental design (Table 3) to ensure reproducibility and reliability in determining the most effective conditions for heat-sealing. For each type of film, the best weldability conditions were identified, and then a film type was chosen considering its mechanical, thermal, and barrier properties, as well as the best value of TF, that would enable the production of food packaging, as discussed in the next section.

The results for the weldability tests for each film type, reporting the temperature, pressure and time conditions that resulted in the highest TF (N) values for each film, are shown in Table 5.

All films exhibited similar tensile force (TF) in their welds, with the exception of the F_COM_S_B film, which demonstrated a significantly higher TF compared to the others. The temperature range used for welding was determined based on results obtained from thermal analyses of the films, combined with preliminary tests conducted on the specimens. For casein-based biopolymer films, the welding temperature range appears to be lower than that typically required for other biopolymers, such as polylactic acid (PLA) (Brosda et al., 2018).

Table 5. Shows the results of weldability tests for each type of NaCas film, with the parameters that resulted in higher TF of welds for each respective film.

NaCas Film	Welding conditions			Max Welding TF (N)
	Temperature (°C)	Pressure (bar)	Time (s)	
F_LAB_PS_B	110	3.5	0.8	1.4 ± 0.5 ^a
F_LAB_PS_P	90	4.25	0.2	3.4 ± 0.6 ^b
F_LAB_RF_B	110	3.5	0.2	1.3 ± 0.1 ^a
F_LAB_RF_P	110	3.5	0.2	1.3 ± 0.1 ^a
F_LAB_S_B	130	5	0.5	3.6 ± 1.2 ^b
F_LAB_S_P	110	4.25	0.5	2.8 ± 0.9 ^b
F_COM_S_B	110	5	0.8	6.2 ± 1.2 ^c
F_COM_S_P	130	5	0.5	3.6 ± 1.0 ^b

Results for Max Welding TF are expressed as mean ± standard deviation (5 replicates). Data in the same column followed by different superscript letters are significantly different ($p < 0.05$) according to One-way ANOVA and Tukey post-hoc test.

As outlined in Chapter 2, the mechanical properties of the films revealed that those with higher fat content (F_LAB_PS) exhibited reduced TS and EAB, likely due to the excessive weakening of the protein network caused by fat structures. This characteristic also impacted the TF values of the film welds, particularly for the sonicated film processed using an ultrasonic bath. Interestingly, the film sonicated with an ultrasonic probe produced higher TF welds. This improvement can be attributed to the better dispersion and smaller, more homogeneously distributed fat structures within the film, which enhanced the cohesion of the protein network, even when a welding temperature 20 °C lower than that of the ultrasonic bath-processed film was applied.

Films F_LAB_RF showed weak welds, with TF values similar to those of the F_LAB_PS_B film. The low TF observed in these films may be linked to their lower elastic and viscous modulus values, as identified through dynamic mechanical analysis (DMA). This behavior was particularly evident in the F_LAB_RF_B film. Conversely, films made from skimmed experimental NaCas exhibited higher TF values in their welds, with the F_LAB_S_B film achieving the highest TF among all experimental films. As confirmed by thermal properties obtained via DMA, this film displayed higher elastic and viscous moduli, contributing to more consistent welds at elevated welding temperatures. In contrast, the viscous modulus of the F_LAB_S_P film was shifted to lower temperatures by approximately 10 °C, which may explain its relatively lower TF values.

Films produced using commercial NaCas exhibited two distinct TF values depending on the sonication method. The F_COM_S_B film achieved the highest TF welds across all films, while the TF values of the F_COM_S_P film welds were statistically comparable to those of the F_LAB_S films.

Based on the results of the weldability tests, a single film type was selected to produce prototype flow-packs for cheese snack packaging. The choice of the F_LAB_S_B film was driven by its favorable properties, as detailed in Chapter 2. Thermal analysis via differential scanning calorimetry (DSC) revealed that this film exhibited melting temperatures above 170 °C, a predominantly amorphous structure, and a glass transition temperature of 73.5 °C, determined using second-derivative calculations. Furthermore, the mechanical properties of the F_LAB_S_B film reinforced its selection, as it demonstrated the highest TS and Young's modulus (YM) among the tested formulations, despite its lower EAB.

In addition, the low haze value of the F_LAB_S_B film was a critical factor, as it enhanced transparency, allowing clear visibility of the contents—a feature highly desirable to consumers (Laraichi et al., 2020). Considering the susceptibility of cheese snacks to lipid oxidation, the film's ability to limit oxygen permeability was also a key factor. The F_LAB_S_B film exhibited the lowest KPO₂ among the tested formulations. Moreover, fluctuations in relative humidity could impact not only the chemical and physical characteristics of the biopolymer but also the permeability to organic vapors, as emphasized by Piergiovanni & Limbo (2010). To address this, the F_LAB_S_B film was also selected for its low KP WV among the experimental films, excluding those produced with commercial NaCas.

3.2 Storage test of cheese snacks packaged in NaCas-pack

The development of the flow-pack and cheese snack storage tests enabled a detailed investigation of the performance of biopolymer-based packaging, such as NaCas, in comparison to a conventional polymer film commonly used in food packaging. These tests also investigated the changes occurring within the food matrix—specifically cheese-based snacks—and allowed for a direct comparison of their properties when packaged in NaCas films versus traditional plastic films.

3.2.1 Moisture and water activity of cheese snacks during the storage test

Moisture content and water activity are critical parameters for preserving the freshness and quality of low-moisture foods (Khoshgozaran et al., 2012; Renoldi et al., 2024). The cheese snacks exhibited an initial moisture content of $2.82 \pm 0.01\%$. Such a low moisture content, together with the highly porous surface, make the snacks extremely prone to moisture

absorption. Thus, the chosen model food was useful to detect even very small changes in moisture content and water activity.

Figure 4 shows the changes in moisture content and water activity of cheese snacks over the storage period. During storage, an increase in moisture content was observed for both samples packaged in NaCas-based and control packaging, with increments noted at the intermediate time point (10 days) and the final time point (20 days). However, the increase in moisture was more pronounced in samples packaged with NaCas-based films, leading to a final difference of 24%. Specifically, the cheese snacks packaged in NaCas flow-pack exhibited a greater rate of moisture gain during the first 10 days of storage, while the rate slowed down between 10 and 20 days. In contrast, the control samples exhibited a linear increase in moisture throughout the entire 20-day storage period. The increase in moisture observed in both types of packaging is mainly attributed to residual moisture present within the flow-pack at the time of packaging. In addition, in the experimental flow-pack, the greater increase in moisture is due to the high permeability of the NaCas film to water vapor (Gerna et al., 2025), with the absorption of environmental moisture being mitigated by the secondary packaging that have high water-vapor barrier properties.

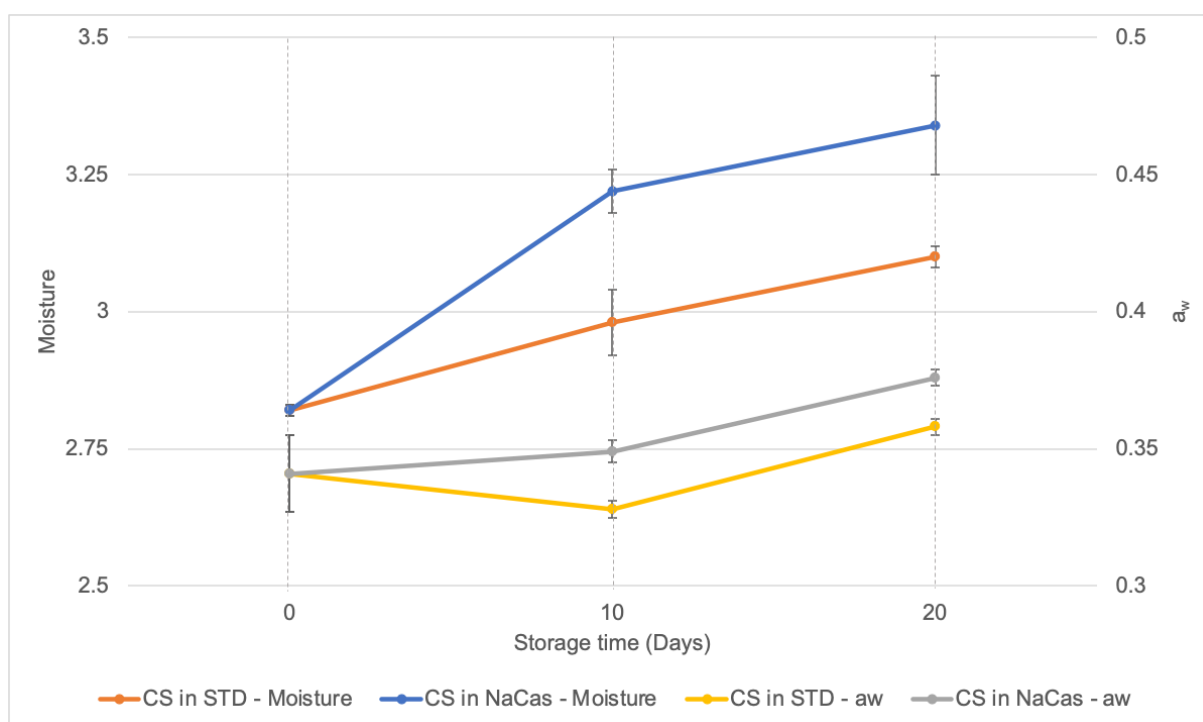


Fig. 4. Trends of moisture (g/100g) and water activity (a_w) in cheese snacks (CS) packaged in control (STD) and in NaCas flow-packs in the multi-pack.

The initial value of water activity of cheese snacks was 0.341 ± 0.014 . During storage, water activity also increased overall, for both cheese snacks packaged in NaCas flow-packs and those packaged in the control flow-pack. In the control flow-packs, an initial slight decrease

in water activity was observed. This trend was not observed for the NaCas flow-pack, where water activity increased gradually, to a value of 0.349 ± 0.004 at day 10 of storage, to a maximum of 0.376 ± 0.003 at the end of the storage test. From 10 to 20 days, increases of water activity paralleled in the two packages.

3.2.2 Headspace atmosphere during the storage test

The oxygen content in the headspace of packaged cheese snacks was measured immediately after packaging and nitrogen flushing, for both the control flow-pack and the experimental NaCas flow-pack, to determine the oxygen percentage at time 0. The same analysis was repeated at two subsequent storage times: 10 days and 20 days post-packaging (Table 6).

As a control, the oxygen percentage within the multi-packs was also monitored at time 0, day 10, and day 20 of the storage test to ensure its consistency. The oxygen content remained stable throughout the storage period in both the experimental and control multi-packs, with an average oxygen concentration of $21.2 \pm 0.1\%$.

The oxygen concentration within the NaCas flow-packs showed no significant variation during the storage period, confirming the effectiveness of the high oxygen barrier properties of NaCas-based films in preventing oxygen ingress. In the control flow-packs, a slight initial decrease in residual oxygen percentage was observed, followed by a final increase of 0.9% relative to the initial concentration. However, this variation was not statistically significant, indicating minimal oxygen exchange in both packaging systems.

Table 7. Residual oxygen present inside control and experimental flow-packs of NaCas during storage time. Acronyms: FP (Flow-pack).

Flow-Pack residual O ₂ (%)		
Storage Time (days)	NaCas FP	Standard FP
0	2.0 ± 0.5	2.7 ± 1.7
10	2.1 ± 0.6	2.4 ± 0.9
20	2.0 ± 0.5	3.6 ± 1.1

A One-way ANOVA was performed on the flow-packs residual oxygen for the three different storage times, after checking for normality of data (Shapiro-Wilk Test) and homogeneity of variances (Levene's Test), and no significant difference was observed between storage times ($p > 0.05$).

3.2.3 Volatile compounds related to rancidity and lipid oxidation in cheese snacks and flow-packs

Selected volatile compounds associated with lipid oxidation and rancidity were analysed in cheese snacks packaged in NaCas-based flow-packs and further inserted in the sealed multi-pack, to assess the efficacy of the experimental packaging in preserving product quality

compared to the commercial flow-pack in use for packaging the low-moisture cheese snacks. Lipid oxidation is a well-documented cause of off-flavor formation, leading to a reduction in product quality and potential consumer rejection, as previously reported by various authors (Roberts et al., 1994; Bulat & Topcu, 2020).

Figure 5 illustrates the trends of the total concentration of volatile compounds (hexanal, nonanal, 2-heptanone, 2-nonanone, 2,3-butanedione, 3-hydroxy-2-butanone, hydroxy-2-propanone, butanoic acid, 2-methyl butanoic acid, and hexanoic acid) that can be associated with lipid oxidation and rancidity in cheese snacks stored in both NaCas-based flow-packs and control flow-packs. Additionally, these compounds were monitored in the material of flow-packs themselves to assess potential migration or scalping phenomena (Arvanitoyannis & Kotsanopoulos, 2014; Gupta et al., 2024).

At the initial storage time, cheese snacks exhibited a high concentration of volatile compounds, indicating lipid oxidation already in progress, likely caused by heat treatments applied during snack production starting from ripened cheese. High levels of 2,3-butanedione, 3-hydroxy-2-butanone, and butanoic acid were observed (Table S1), aligning with findings from Munir et al. (2022). Notably, specific volatile compounds, such as nonanal and 2,3-butanedione, were detected in greater amounts within the NaCas flow-packs compared to the control flow-packs. After 10 days of storage, a general decrease in volatile compounds was observed, except for a significant increase in 2-heptanone in both packaging types. This methyl ketone is strongly associated with the development of off-flavors in cheese products (Xi et al., 2022). Despite the overall decline in cheese snacks, a significant increase in volatile compounds, particularly ketones and acids, was detected in the material of the flow-packs themselves. This increase was more pronounced in the NaCas-based material compared to the commercial control (Table S2). The presence of volatile compounds in the two materials may be attributed to the phenomenon of 'scalping' (Gupta et al., 2024). Scalping, or sorption, occurs when packaging materials adsorb or absorb flavor compounds, lipids, or moisture from the food product (Vimala Bharathi et al., 2018). While this phenomenon can reduce the sensory quality of food, particularly products sensitive to odors and flavors, the greater susceptibility of NaCas-based packaging to scalping highlights a critical area for further improvement in biopolymer-based materials.

During the first 10 days of storage, the decrease of volatile compounds in cheese snacks was significantly more pronounced compared to the slower reduction observed between days 10 and 20. This trend coincided with an increased sorption of volatile compounds by the packaging material, particularly in the NaCas-based packaging. Notably, within the total volatile compounds detected, the NaCas flow-pack itself contributed to the formation of specific compounds.

In cheese products, microbial metabolic pathways can also contribute to the production of rancidity-related volatile compounds, as highlighted by McSweeney & Sousa (2000). Despite the low moisture and water activity in the snacks, even after the considered storage time, the high value of Water Vapor Transmission Rate (WVTR) of the NaCas-based flow-pack may have facilitated enzyme activity during storage.

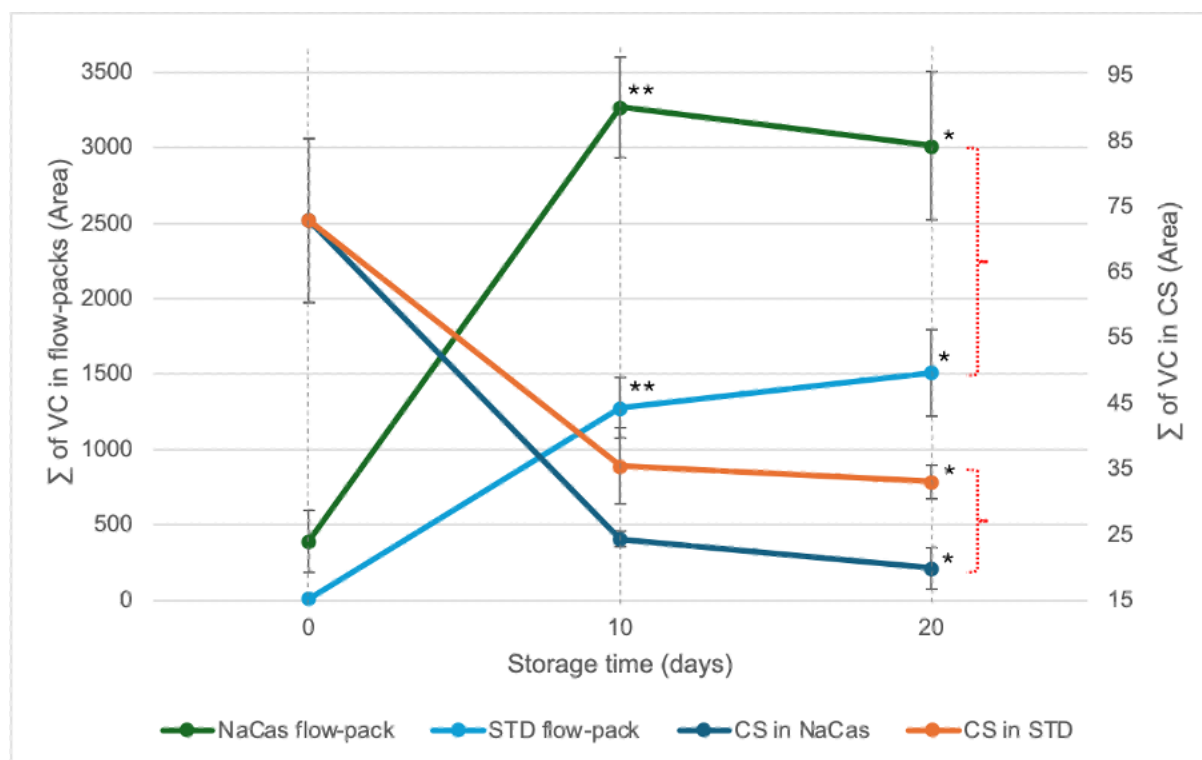


Fig. 5. Evolution of rancid-related and oxidation-related volatile compounds (VC) (peak area) in cheese snacks (CS) and flow-packs. Results are expressed as the sum of corrected peak area counts of selected compounds and shown as mean $\pm$ standard deviation. * and ** indicate statistically significant difference at $p < 0.05$ and $p < 0.01$ between the two sums of volatile compounds, respectively. The sums of volatile compounds in cheese snacks and the sums of volatile compounds in flow-pack materials were statistically analyzed separately.

At the end of the 20-day storage period, higher amounts of volatile compounds were detected in cheese snacks stored in the standard polymer flow-pack. Furthermore, additional volatile compounds, such as nonanal and hexanoic acid, were generated within the NaCas-based packaging itself, suggesting a dual effect of sorption and compound formation.

Future studies should focus on investigating the impact of these packaging-derived volatile compounds on food quality and safety. Additionally, detailed monitoring of potential migration phenomena from the packaging material to the food matrix is essential to determine the suitability of NaCas-based flow-packs for real-world food packaging applications.

3.2.4 Free amino acids in cheese snacks

The free amino acids (FAA) represent rather stable end products of casein proteolysis occurring in cheese during ripening (Ardo et al., 2017). It is known that FAA mostly accumulate in cheese and the total content can increase up to 25-28% of cheese protein in very long ripened cheeses (D’Incecco et al., 2020). The relative pattern of FAA has been adopted as a fingerprint of selected cheese types, particularly PDO cheeses (Lima et al., 2019; Pellegrino et al., 2021; Pellegrino et al., 2024). In addition, some amino acids may undergo degradation during cheese ripening due to residual activity of specific enzymes (Maifreni et al., 2013; D’Incecco 2016).

Although the type of cheese and the processing conditions used for making the snacks considered in this study were not known, it was hypothesized that some changes could occur in the FAA profile of the cheese snacks during the 20-day storage. The obtained results are shown in Table 7 for selected amino acids while the whole set of data is given in Table S3. During the 20-day storage period, the total content of FAA of the snacks increased from 6.43 to 7.41 mg/g protein when stored in the commercial package and to 7.08 mg when stored in the NaCas flow-pack contained in the multi-pack, indicating that proteolytic activity progressed, although slowly, in the cheese snacks. Apparently, the FAA content did not increase in the snacks stored in single NaCas flow-pack (no multi-pack), but it should be considered that the observed value is the result of simultaneous formation and degradation of single amino acids. The content of stable amino acids, such as Ile, Leu, increased in all samples, with slightly lower levels reached in the snacks stored in single NaCas flow-packs. Since the contents of Met, Val, His, Lys, Phe significantly increased in the other two samples, a decrease can be assumed for these FAA in the snacks stored in single NaCas flow-packs although their net content was unchanged compared with the corresponding contents in the snack at T0. These amino acids easily undergo enzymatic degradation phenomena such as decarboxylation and deamidation that bring to formation of amines, organic acids among other organic compounds (Maifreni et al., 2013; Natrella et al., 2024). These phenomena are strongly accelerated by the observed increase moisture content. Differently, the content of free Tyr did not change in all the samples stored for 20 days compared to the not-stored control (t0). This amino acid is highly sensitive to oxidation; therefore, its degradation could have occurred in all the samples to a comparable extent, possibly laying on the low content of oxygen that remained or entered the package during storage. Overall, these data point to a decisive role of the additional package adopted for producing the multi-pack in protecting the cheese snack from degradative effects, mostly through prevention of moisture absorption.

Table 8. Content of selected free amino acids (mg/g protein) determined in cheese snacks (CS) packaged in different modes and stored for 20 days (T20) compared with the respective initial content (T0).

Sample name	Thr	Val	Met	Ile	Leu	Tyr
CS - T0	0.16 ± 0.00	0.37 ± 0.00	0.12 ± 0.00	0.25 ± 0.00	0.84 ± 0.01	0.19 ± 0.02
CS in NaCas - T20	0.16 ± 0.00	0.4 ± 0.01**	0.13 ± 0.00*	0.28 ± 0.00***	0.9 ± 0.02**	0.18 ± 0.00
CS in NaCas - T20 (no maxi-pack)	0.15 ± 0.00**	0.36 ± 0.01	0.12 ± 0.00	0.26 ± 0.00**	0.82 ± 0.01*	0.16 ± 0.00
CS in STD - T20	0.17 ± 0.00***	0.42 ± 0.01**	0.14 ± 0.00***	0.29 ± 0.01***	0.95 ± 0.02***	0.2 ± 0.00

	Phe	Lys	His	Arg	Total
CS - T0	0.42 ± 0.01	0.63 ± 0.00	0.14 ± 0.00	0.1 ± 0.00	6.43 ± 0.01
CS in NaCas - T20	0.45 ± 0.01*	0.69 ± 0.01**	0.18 ± 0.00***	0.09 ± 0.00**	7.08 ± 0.13**
CS in NaCas - T20 (no maxi-pack)	0.42 ± 0.01	0.62 ± 0.01	0.15 ± 0.00	0.08 ± 0.00***	6.44 ± 0.07
CS in STD - T20	0.48 ± 0.00***	0.74 ± 0.01***	0.19 ± 0.00***	0.07 ± 0.01**	7.41 ± 0.08***

Results are expressed as mean ± standard deviation of three analytical replicates. Abbreviations: in NaCas: packaged in experimental NaCas flow-pack including multi-pack; In NaCas (no maxi-pack): packaged in single experimental NaCas flow-pack; in STD: packaged in commercial flow-pack. *, **, and *** indicate statistically significant difference at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, between the cheese snacks sample at t20 and the t0 for each amino acid.

Conclusions

This chapter examined the development and testing of NaCas-based modified atmosphere flow-packs for low-moisture cheese snacks, achieving significant insights into their performance and potential applications. The optimization of weldability conditions highlighted the importance of parameters such as temperature, pressure, and time in achieving high tensile strength, with the F_LAB_S_B film emerging as the most suitable candidate due to its superior mechanical properties, thermal stability, and transparency.

The storage tests revealed that NaCas-based flow-packs exhibited higher moisture uptake compared to control polymer packs. Specifically, cheese snacks packaged in NaCas flow-packs showed a greater rate of moisture increase during the first ten days, followed by a slower increase from days 10 to 20. In contrast, the control packs exhibited a more linear increase in moisture over the entire storage period. Despite this higher moisture uptake, NaCas-based flow-packs maintained advantageous mechanical and aesthetic properties, making them appealing for consumer packaging.

Water activity analysis indicated that both types of packaging allowed some degree of increase in free water in the cheese snacks, but NaCas-based flow-packs demonstrated a greater increase in a_w value. Despite this, the NaCas flow-pack proved to be an effective packaging when placed inside a maxi-pack that limits moisture transmission rate.

The analysis of volatile compounds related to lipid oxidation and rancidity underscored the dual role of NaCas-based flow-pack in both sorbing and generating certain volatile compounds. This phenomenon, coupled with the high WVTR of NaCas films, suggests potential limitations related to increased enzyme activity and low barrier performance, which may affect the shelf life and sensory quality of packaged products. Despite these challenges, provided humidity conditions are controlled by a secondary packaging, the NaCas flow-pack was able to maintain the quality parameters of the cheese snacks.

Future research should focus on improving the barrier properties of NaCas films, studying migration phenomena to ensure product safety and integrity, and microbiological studies to investigate possible bacterial growth. The possibility of introducing anti-microbial agents into the packaging formulation could be an interesting application. Scalability of production processes and evaluation of consumer acceptance will be key factors to enhance the interest in the production of NaCas-based packaging.

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Chapter 5 - From expired pasteurized milk to packaging films: a screening LCA study from NaCas powder production to a prototype for food packaging

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Abstract

This study presents a Life Cycle Assessment (LCA) of a sodium caseinate (NaCas)-based flow-packaging, focusing on the environmental impacts of all the stages of the process, in particular NaCas powder, films, and flow-packs for food packaging applications. The analysis identified the NaCas powder production phase as the most impactful, largely due to energy-intensive processes such as freeze-drying and the environmental footprint of milk as a raw material. A further additional analysis considered the whole NaCas flow-pack production process without including the environmental impact of milk, as it was treated as a zero-impact waste. The film and flow-pack production phases exhibited lower impacts, with the flow-pack phase accounting for 16% of the total environmental burden, mainly influencing “Human Toxicity,” “Water Use,” and “Resource Use, Fossil.” Strategies to reduce environmental impacts were proposed, including the use of non-thermal welding techniques like ultrasonic sealing, the elimination of freeze-drying through in-line processes, and optimization of energy use. Future work will explore the scalability and industrial feasibility of these innovations, aiming to establish NaCas-based flow-packs as a sustainable alternative to conventional food packaging materials.

1. Introduction

Sodium caseinate, a water-soluble protein derived from casein, has gained attention in the field of food packaging due to its film-forming properties and potential applications in biodegradable materials. In recent times, the Life Cycle Assessment (LCA) methodology has been extensively utilized in the packaging sector to pinpoint environmental hotspots and

evaluate more eco-friendly solutions through comparative analysis (Molina-Besch et al., 2018; Vendries et al., 2020; Wohner et al., 2019). The life cycle assessment (LCA) of sodium caseinate films is crucial for understanding their environmental impact compared to conventional plastic films and also evaluate the reduction in food waste. Considering the intricate connection between the environmental impact of food packaging and its role in minimizing food waste through its technical performance, it is crucial to acknowledge that these dynamics are largely influenced by the type of food the packaging contains (Casson et al., 2022).

LCA is a systematic approach for evaluating the environmental impacts of a product from the extraction of raw materials to the disposal of waste. An LCA involves gathering data on various inputs and outputs, including emissions, waste, and resource usage, which is compiled into a life cycle inventory (LCI). This data is then analyzed using impact assessment methods to determine environmental consequences such as climate change, global warming, eutrophication, acidification, and both human and ecosystem toxicity. The results can be categorized by impact or aggregated into broader damage categories, such as 'human health,' 'ecosystem quality,' and 'resource depletion' as defined in the Ecoindicator-99 methodology, often following a normalization process. Normalization typically involves scaling impact scores to the average annual contribution of an individual, allowing for a combined view of the various categories, which then become unitless. Additionally, due to differing significance among environmental impacts, weights may be applied to these normalized scores.

The LCA for a packaging product that is made of natural polymer, like NaCas films, can be performed by following the International Organization for Standardization (ISO) framework guidelines (Ponnusamy & Mani, 2022). Although LCA studies follow standard guidelines, there remains a degree of flexibility in interpretation and application by practitioners. Consequently, comparing results across different studies can be challenging and should be approached cautiously due to various methodological discrepancies—such as differences in functional units, allocation methods, and the types of inventory data used (Yates & Barlow, 2013).

The environmental implications of using sodium caseinate films are significant. The biodegradability of sodium caseinate, coupled with its low-cost production and availability, positions it as a sustainable alternative to traditional petroleum-based plastics (Valentino *et al.*, 2020). Studies have highlighted that sodium caseinate films can be produced with minimal environmental impact, especially when compared to conventional plastic films, which contribute significantly to pollution and waste (Ríos-de-Benito *et al.*, 2021). In summary, sodium caseinate films present a viable option for sustainable food packaging solutions. NaCas films mechanical and barrier properties, coupled with antimicrobial functionalities, make them suitable for various applications in the food packaging. The ongoing research into optimizing these films for enhanced performance and reduced environmental impact

underscores their potential as a green alternative to conventional packaging materials. The novelty of this study seeks to analyze through environmental impact assessment the benefit and reduction of using new resources in producing sodium caseinate from expired milk.

2. Materials and Methods

The basic idea of LCA is that all environmental burdens associated with the production of NaCas powder and NaCas film should be evaluated, starting from the raw material contribution to the finished product. This method is developed according to the international standard ISO 14040-14044.

2.1 Goal and scope definition

The objective of this screening study is to employ LCA to evaluate the environmental impact associated with the production of a NaCas-based film intended for cheese snack packaging. The assessment included the entire production chain, including the synthesis of NaCas powder, the fabrication of the NaCas film, and the development of the prototype packaging on a laboratory scale. Additionally, this study aims to identify the production steps with the highest environmental impact to facilitate the proposal of a scaled-up production process with a reduced environmental footprint. In addition, this study aims to identify the production steps with the highest environmental impact and an identification of the causes, with the goal of identifying them and proposing process improvement solutions.

As a preliminary investigation, this screening LCA provides an initial evaluation, with the understanding that each phase will require a more detailed analysis in future studies. A comprehensive scale-up assessment would enable significant reductions in environmental impacts, particularly those associated with the energy-intensive use of laboratory-scale instruments and equipment, which tend to have disproportionately high environmental burdens.

2.1.1 Declared unit

In accordance with the ISO 14040 standard, the declared unit serves as a reference point to which all associated inputs and outputs are linked. In this study, the declared unit was identified by the production of a single flow-pack of NaCas measuring 9 x 9 cm, the pack being composed of two NaCas films of the same size, welded through a heat-sealing machine, as discussed in detail in Chapter 3. Impacts from the nitrogen MAP and the packaged product (cheese snacks) were not considered for the present study.

2.1.2 Definition of the System and System boundaries

The system analyzed in this study included all activities associated with the production of NaCas powder, starting from the raw material—pasteurized skimmed milk—through the casein extraction process, neutralization to achieve water-soluble NaCas, and culminating in the production of the NaCas-based film and packaging prototype. Within these processes, all input flows of raw materials and energy, as well as output flows of materials destined for disposal, were systematically accounted for. The transformation from NaCas powder to the final prototype was carried out at the University of Milan using laboratory-scale equipment, which served as the basis for environmental calculations.

System boundaries determine which unit processes are included in the LCA study; the reference flows considering NaCas powder production, NaCas film production, and packaging prototype production are shown in Figure 1.

2.2 Life Cycle Inventory (LCI)

Inventory analysis included the collection of data and application of calculation methods to quantify the key inputs and outputs of a product system, as mentioned by the ISO 14044. All inputs and outputs gathered during the study should be analyzed in alignment with the goals and scope of the LCA. Additionally, the data within the LCI serve as the foundation for conducting the life cycle impact assessment.

For this study, the information required for the inventory phase was derived from the processes and operations detailed in the previous chapters. In particular, the data collection referring to the production of NaCas P_LAB_S powder was carried out following the development of the production process, detailed in Section 2.2 of Chapter 2. Solution and film data collection, specifically on the S_LAB_S_B solution and F_LAB_S_B film, was sourced during the production process, detailed in Sections 2.3 and 3.4 of Chapter 2. Regarding prototype production, the data collection was carried out after the production process detailed in Section 2.3 of Chapter 4.

All materials used in this study were thoroughly characterized, including analytical instruments, laboratory equipment, and reagents. The inventory phase accounted for the quantities of raw materials consumed, the energy usage of the instruments and equipment employed, and the waste generated throughout the production process. This comprehensive approach ensures that all relevant inputs and outputs are accurately represented in the inventory phase.

For the present study, the inventory of each step was performed separately and reported, starting from the production of the powder and ending with the production of the packaging prototype.

2.2.1 NaCas powder production LCI

For the production of NaCas P_LAB_S powder, 1 L of expired skimmed milk was recovered. For isoelectric precipitation of caseins, a magnetic stirrer and pHmeter were used for a time of 45 min, hydrochloric acid (HCl 2N) was added to reach the isoelectric point of caseins under continuous stirring. At the end, a centrifugation process for 20 min was carried out that yielded about 750 mL of acid whey and 265 mL of acid casein. To the acid casein, 500 mL of ultrapure distilled water was added and mixed under stirring with a magnetic stirrer for 20 min. The solution was then again centrifuged for 20 min, resulting in about 500 mL of washing water to be disposed of and the acid casein. This washing and centrifugation step was repeated twice. To the washed acid casein, 300 mL of ultrapure distilled water was added, homogenized with a homogenizer for 5 min and then brought to pH 7 using a pHmeter and about 17 mL of sodium hydroxide (NaOH 2N) was added under stirring for 45 min. The wet NaCas was then lyophilized

through a freeze-dryer for 36 h obtaining about 45 g of NaCas pellet, finally grounded with a blender for 10 min to obtain a powder. Inputs and outputs of NaCas powder production and related processes are shown in Table 1.

Table 1. Shows the production process of NaCas powder, indicating the raw material input (in blue), inputs (in green), processes (in white), outputs (in yellow) and final product (in pink). For each step the quantity, instrumentation, time, temperature, rated output and actual output has been reported.

NaCas Powder Production						
	Amount	Equipment	Time	Temperature	Power	Energy consumption
Pasteurized milk	1000 mL	-	-	-	-	-
HCl 2N	~15 mL	-	-	-	-	-
Isoelectric precipitation of casein	-	pHmeter	45 min	-	3.3 W + 10 W	10 Wh
	-	Centrifuge	20 min	4°C	4620 W	1540 Wh
Acid whey	~750 mL	-	-	-	-	-
Ultrapure water	~500 mL	-	-	-	-	-
Isoelectric casein washing	-	Stirrer	20 min	-	10 W	3.5 Wh
	-	Centrifuge	20 min	4°C	4620 W	1540 Wh
Washing water (H ₂ O + whey)	~500 mL	-	-	-	-	-
Ultrapure water	~500 mL	-	-	-	-	-
Isoelectric casein washing	-	Stirrer	20 min	-	10 W	3.5 Wh
	-	Centrifuge	20 min	4°C	4620 W	1540 Wh
Washing water (H ₂ O + whey)	~500 mL	-	-	-	-	-
Ultrapure water	~300 mL	-	-	-	-	-
NaOH 2 N	~17 mL	-	-	-	-	-
Homogenization	-	Homogenizer	5 min	-	600 W	50 Wh
pH 7 equilibration	-	pHmeter	45 min	-	3.3 W + 10 W	10 Wh
NaCas Lyophilization	-	Freeze-dryer	~36 h	-	1500 W	54000 Wh (4L sol.)
	-	Grinder	10 min	-	1000 W	170 Wh
NaCas powder	~45 g/L	-	-	-	-	-

2.2.2 NaCas film production LCI

The production process for the NaCas film involved the use of 14.6 g of P_LAB_S powder combined with 130 mL of ultrapure distilled water, measured using a precision balance with an estimated operation time of 5 minutes. The ingredients were mixed together using a magnetic stirrer for 10 minutes. Heat treatment of the solution was then performed using a magnetic heating stirrer. The heating plate required approximately 15 minutes to reach a temperature of 140°C, sufficient to maintain the temperature at 90°C at the core of the solution. This heat treatment was conducted for 30 minutes, with the plate temperature held constant. During this process, an estimated 20 mL of water evaporated.

Subsequently, the solution was cooled to a temperature of 35–40 °C in 10 minutes using the same magnetic stirrer. At this point, 4.5 g of analytical grade bidistilled glycerol was added, and the mixture was stirred for an additional 10 minutes. To remove entrapped air bubbles, the solution was subjected to ultrasonic bath sonication for 10 minutes.

The final steps involved manual casting of the solution onto molds and allowing the films to dry at room temperature for 48 hours, a phase that required no energy consumption from

equipment. The process yielded two films, each with dimensions of 20 × 35 cm. Inputs and outputs of NaCas film production and related processes are shown in Table 2.

Table 2. Shows the production process of NaCas film, indicating the raw material input (in blue), inputs (in green), processes (in white), outputs (in yellow) and final product (in pink). For each step the quantity, instrumentation, time, temperature, rated output and actual output has been reported.

NaCas Film Production						
	Amount	Equipment	Time	Temperature	Power	Energy consumption
NaCas powder	14.6 g	-	-	-	-	-
Water	~130 mL	-	-	-	-	-
Weighing of powder and stirring	-	Scale	5 min	-	10 W	1 Wh
	-	Stirrer	10 min	20°C	10 W	2 Wh
Thermal treatment of the solution	-	Heat - Stirrer	15 min	20 - 90°C	270 W	68 Wh
	-	Heat - Stirrer	30 min	90°C	270 W	135 Wh
Water evaporation	~20 mL	-	-	-	-	-
Glycerol	4.5 g	-	-	-	-	-
Cooling and mixing	-	Stirrer	10 min	-	10 W	2 Wh
	-	Stirrer	10 min	-	10 W	2 Wh
Solution degassing	-	Ultrasonic bath	10 min	-	100 W	17 Wh
Casting	-	-	-	-	-	-
Drying	-	-	172800	-	-	-
Water evaporation	~95 mL	-	-	-	-	-
NaCas film	2x 20 x 35 cm	-	-	-	-	-

2.2.3 NaCas flow-pack prototype production LCI

A prototype NaCas flow-pack was produced, starting from a NaCas film with the size of 20 x 35 cm. Jigs were prepared by cutting out the film and preparing two 9 x 9 cm films. From the same NaCas sheet, six 9 x 9 cm films can be obtained. From a single NaCas sheet, approximately 214 cm² of wasted scraps are obtained, out of 700 cm² of sheet surface.

Once the jigs are prepared, the two films are conditioned in a climate chamber for 2 hours at 23 °C and 55% RH; up to 20 films can be placed in the climate chamber at the same time. Once the films are conditioned, they are joined and sealed on three-sided with a heat sealer, 4 g of cheese snacks per flow-pack is added and nitrogen is flushed at 25 L/min for 10 sec, after which the fourth side of the packaging is immediately sealed, the total sealing time was estimated to be 5 min. The cheese snacks and the nitrogen flush were not considered in this study. The final product is a 9 x 9 cm flow-pack. The process yielded two films, each with dimensions of 20 × 35 cm. Inputs and outputs of NaCas flow-pack prototype production and related processes are shown in Table 3.

Table 3. Shows the production process of NaCas flow-pack prototype, indicating the raw material input (in blue), inputs (in green), processes (in white), outputs (in yellow) and final

product (in pink). For each step the quantity, instrumentation, time, temperature, rated output and actual output has been reported.

NaCas Flow-Pack prototype Production						
	Amount	Equipment	Time	Temperature	Power	Energy consumption
NaCas film	2 (9 x 9 cm)	-	-	-	-	-
Jigs preparation	-	Cutter	-	-	-	-
Film scraps	~20 mL	-	-	-	-	-
Film conditioning	(20 film)	Climatic chamber	120 min	23 °C - 55 % RH	1000 W	2000 Wh
Film welding	-	Heat-sealing machine	5 min	-	2500 W	208 Wh
Flow-pack prot.	1 (9 x 9 cm)	-	-	-	-	-

2.2.4 Life cycle impact assessment (LCIA)

The impact assessment phase of LCA is designed to evaluate the significance of potential environmental impacts based on the results obtained from the life cycle inventory analysis. This phase typically involves linking inventory data to specific environmental impact categories and interpreting those impacts. The scope, level of detail, and selected impact categories, along with the methodologies employed, are determined by the defined goals and scope of the study (Zambelli et al., 2022). In order to analyze the environmental impact in this study, the SimaPro V 9.1.1.1. software (PRè Sustainability, Amersfoort, NE) was used. Environmental Footprint 3.1 V1.01 / EF 3.1 was used as a method to calculate the environmental impact related to the entire process. This method considers 16 impact categories that are reported in Table 4.

Table 4. Impact categories, acronyms and unit of the EF 3.1 method used.

Impact category	Acronyms	Unit
Acidification	AC	mol H ⁺ eq
Climate change	CC	kg CO ₂ eq
Ecotoxicity, freshwater	ETF	CTUe
Particulate matter	PM	disease inc.
Eutrophication, marine	EM	kg N eq
Eutrophication, freshwater	EFW	kg P eq
Eutrophication, terrestrial	ET	mol N eq
Human toxicity, cancer	HTC	CTUh
Human toxicity, non-cancer	HTNC	CTUh
Ionizing radiation	IR	kBq U-235 eq
Land use	LU	Pt
Ozone depletion	OD	kg CFC11 eq
Photochemical ozone formation	POF	kg NMVOC eq
Resource use, fossils	RUF	MJ
Resource use, minerals and metals	RUMM	kg Sb eq
Water use	WU	m ³ depriv.

3. Results and Discussion

According to the purpose and goal and scope of this study, the environmental impacts of the entire production chain of the NaCas flow-packaging prototype were analyzed. This made it possible to highlight the hotspots that impact the most and to analyze their causes. Two different scenarios in the entire production chain were analyzed, both starting from cradle to gate (flow-pack disposal was not considered). In the first scenario, the environmental impacts from the entire milk production process are considered by significantly increasing the contribution of this raw material; in the second scenario, on the other hand, the environmental contribution of milk was not considered, in order to better demonstrate how using a waste for flow-pack production contributes and promoting a circular economy allows a saving on environmental impacts.

For each individual phase then (i.e. powder, film and flow-pack production), a focus on environmental impacts was made to better understand which operation or materials, involved the highest impact.

3.1 Scenario 1: *LCIA* of the flow-pack production process considering the impact of milk

Table 5 represents the environmental impact divided into percentages, highlighting the main hotspots deriving from the inputs and outputs of the production process from NaCas powder to flow-packaging prototype, considering the environmental impact from milk production. To better understand the results of this method, the final environmental impact was divided into raw materials, ingredients, and the stages that constitute the process.

Among all five components of the process, the one that seems to have the highest environmental impact is the powder production process where we find an average impact of 65% among the impact categories, this result can be explained mainly by the large consume of electricity used during the freeze-drying process, but it will be investigated in the following sections when a focus will be placed only on the NaCas powder production process.

It is important to consider how a large part of the impact is due to the milk component, particularly “Land use” and “Terrestrial eutrophication,” which cover 53% and 30% of their impact categories, respectively.

The ingredients used, such as glycerol, ultrapure distilled water, and reagents, along with the film production process, have only a marginal role in the overall environmental impact of the internal process due to the small quantities required.

Table 5 shows only an overview of the impact and does not allow to go into the details of individual stages to investigate which operation impacts the most, so in accordance with the goal of the study, the three stages consisting of powder, film, and flow-pack production should be treated as individual stages for the identification of their respective main hotspots.

Table 5. Environmental impact percentages responsibilities of the entire production process, considering the impacts related to milk. Impact categories acronyms are listed in Table 4.

Impact category	Unit	Total	Milk	Ingredients	Powder production	Film production	Flow pack production
AC	mol H+ eq	2.47 10 ⁻³	16%	1%	66%	1%	16%
CC	kg CO2 eq	6.40 10 ⁻¹	7%	0%	73%	2%	18%
ETF	CTUe	1.63	24%	2%	57%	2%	14%
PM	disease inc.	1.34 10 ⁻⁸	22%	1%	60%	1%	15%
EM	kg N eq	4.88 10 ⁻⁴	25%	3%	56%	2%	14%
EFW	kg P eq	1.04 10 ⁻⁴	5%	3%	72%	2%	18%
ET	mol N eq	5.41 10 ⁻³	30%	1%	54%	1%	13%
HTC	CTUh	1.31 10 ⁻⁹	2%	1%	77%	2%	19%
HTNC	CTUh	3.44 10 ⁻⁹	24%	2%	58%	2%	14%
IR	kBq U-235 eq	7.87 10 ⁻²	8%	0%	72%	2%	18%
LU	Pt	3.73	53%	2%	36%	1%	9%
OD	kg CFC11 eq	1.59 10 ⁻⁸	17%	1%	64%	1%	16%
POF	kg NMVOC eq	1.74 10 ⁻³	5%	0%	75%	2%	18%
RUF	MJ	10.1	4%	0%	75%	2%	19%
RUMM	kg Sb eq	1.26 10 ⁻⁶	12%	1%	69%	1%	17%
WU	m3 depriv.	4.34 10 ⁻¹	3%	1%	77%	0%	19%

Analyzing the environmental impact of the entire production process without the environmental contribution of milk (Table 6), it can be seen how the percentages related to the film and flow-pack production process, and ingredients, increase only slightly, underscoring how the impact is preponderant in the production of NaCas powder.

Table 9. Environmental impact percentages responsibilities of the entire production process, without considering the impacts related to milk. Impact categories acronyms are listed in Table 4.

Impact category	Unit	Total	Ingredients	Powder production	Film production	Flow pack production
AC	mol H+ eq	2.07 10 ⁻³	1%	78%	2%	19%
CC	kg CO2 eq	5.97 10 ⁻¹	0%	79%	2%	19%
ETF	CTUe	1.23	3%	76%	3%	18%
PM	disease inc.	1.05 10 ⁻⁸	1%	78%	2%	19%
EM	kg N eq	3.64 10 ⁻⁴	4%	75%	2%	18%
EFW	kg P eq	9.93 10 ⁻⁵	4%	76%	2%	19%
ET	mol N eq	3.78 10 ⁻³	2%	77%	2%	19%
HTC	CTUh	1.29 10 ⁻⁹	1%	78%	2%	19%
HTNC	CTUh	2.59 10 ⁻⁹	3%	77%	2%	19%
IR	kBq U-235 eq	7.22 10 ⁻²	0%	79%	2%	19%
LU	Pt	1.76	4%	76%	2%	19%
OD	kg CFC11 eq	1.31 10 ⁻⁸	2%	78%	2%	19%

POF	kg NMVOC eq	1.65 10 ⁻³	0%	79%	2%	19%
RUF	MJ	9.66	0%	79%	2%	19%
RUMM	kg Sb eq	1.11 10 ⁻⁶	1%	78%	2%	19%
WU	m3 depriv.	4.19 10 ⁻¹	1%	80%	0%	20%

3.2 NaCas Powder Manufacturing Process LCIA

To determine which unit process or ingredient contributed most significantly within each individual phase, a detailed analysis was conducted for each phase. Figure 2 illustrates the impact assessment associated with the production phase of NaCas powder, including the environmental impact of milk as a raw material.

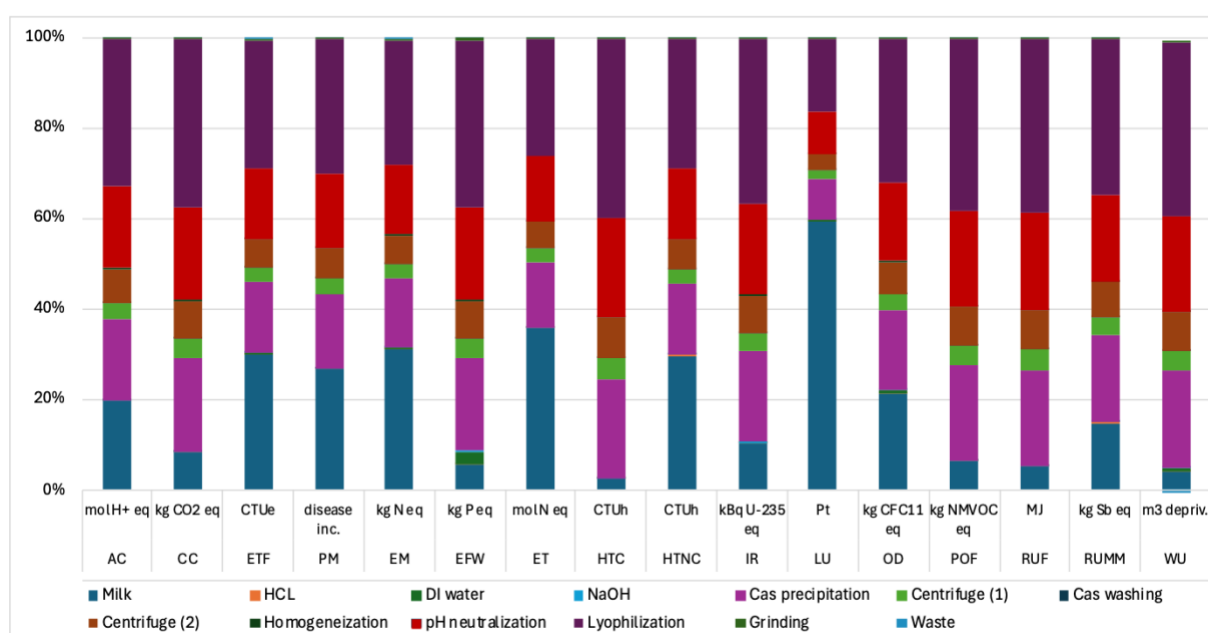


Fig. 2. Hotspots for the NaCas powder production process based on a factor subdivision. Impact categories acronyms are listed in Table 4.

The data reveal a heterogeneous distribution of impacts across the various impact categories during this production phase. For certain categories, such as “Terrestrial eutrophication” and “Land use,” milk emerges as a primary contributor, accounting for 59% and 36% of the impacts, respectively. However, milk also remains a significant factor in all other categories, even if its contribution is lower.

The largest contributions across impact categories are predominantly associated with energy-intensive processes, particularly freeze-drying, which is identified as the most impactful step. Other notable contributors include pH neutralization and casein precipitation, which have significant impacts due to their prolonged processing times. Conversely, the contributions of formulation ingredients such as NaOH, DI water, and HCl are minimal across all impact categories, likely due to the relatively small quantities used in the process.

In the context of an industrial scale-up, energy-intensive processes would be conducted on a larger scale, which is expected to lower the environmental impact per gram of NaCas. Additionally, industrial plants could implement continuous in-line operations, potentially eliminating the freeze-drying step by directly producing the film-forming solution from wet NaCas. While this modification would reduce energy consumption, it would likely alter the thermal properties of the resulting films, necessitating careful re-evaluation and optimization to ensure product performance remains consistent.

3.3 NaCas Film Manufacturing Process LCIA

After assessing the environmental impacts of the NaCas powder production phase, a similar analysis was conducted for the NaCas film production process. This phase is comparatively more streamlined, involving fewer operations and using a reduced number of ingredients and reagents.

Going into the details of the NaCas film production process, Figure 3 shows how the environmental impact is distributed across the various production steps. This analysis provides a clearer understanding of which specific step contributes most significantly to the overall environmental burden, offering valuable insights for potential process optimization.

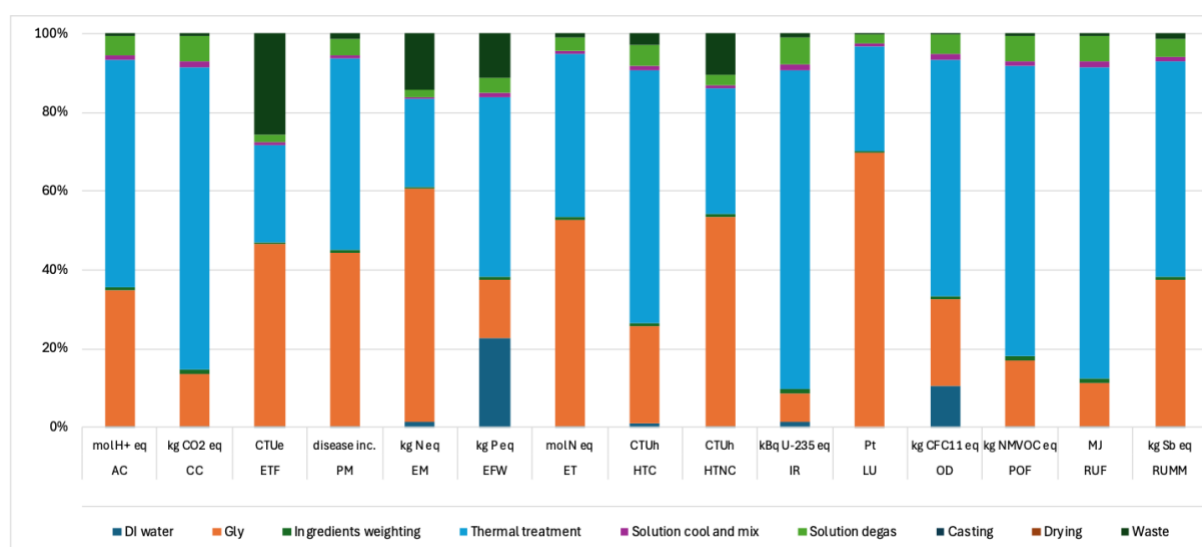


Fig. 3. Hotspots for the NaCas film production process based on a factor subdivision. Impact categories acronyms are listed in Table 4.

Looking at the different impact categories, it is evident that the heat treatment to which the NaCas solution is subjected, and the addition of glycerol are the most impactful process and ingredient, respectively. Heat treatment has an average impact of 53% while glycerol impacts 34% on average. In the impact categories of “Fresh water eutrophication” and “Ozone depletion,” distilled water also has a significant environmental impact, 23% and 11%,

respectively. Finally, water evaporation during the thermal process and during the film drying process also causes a significant impact, only on the “Ecotoxicity, fresh water” impact category by 26%.

3.4 NaCas Flow-pack prototype Manufacturing Process *LCIA*

Finally, after assessing the environmental impact of the NaCas film production phase, a final analysis was conducted for the NaCas flow-packaging production process. As with the previous phase, the impact percentages for the entire NaCas flow-pack production process are depicted in Table 6, providing an understanding of how much the flow-pack production impacts the whole process if compared with the other two process steps.

The flow-pack production phase accounts for an average of 16% of the total environmental impact across all process steps. The most affected impact categories are “Human Toxicity,” “Water Use,” and “Resource Use, Fossil.” This phase involved two energy-intensive operations: the conditioning of films and the welding of flow-packs, both of which contribute significantly to the overall environmental impact of this phase. The influence of these operations on the environmental impact of this stage is further illustrated in Figure 4.

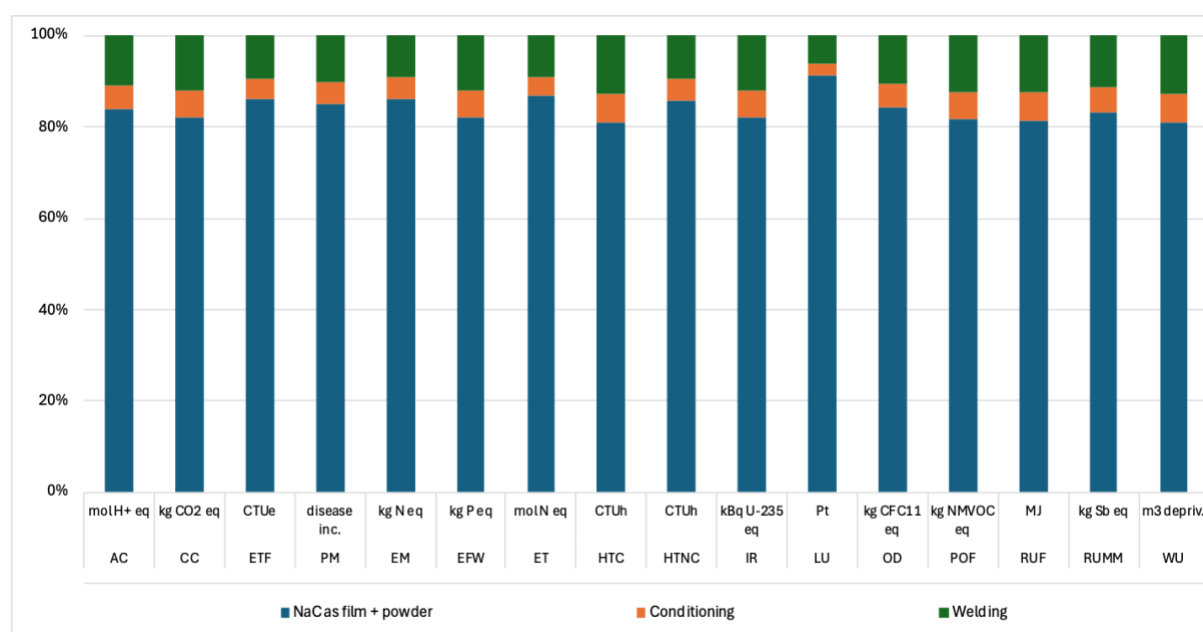


Fig. 4. Hotspots for the NaCas flow-pack production process based on a factor subdivision and compared with the others (powder and film) phases. Impact categories acronyms are listed in Table 4.

Figure 4 compares the environmental impact of the NaCas flow-pack production phase to the overall process. The analysis reveals that the ‘conditioning’ operation has a lower overall

impact than the 'welding' operation. However, it is important to note that, in terms of electrical power consumption, the 'conditioning' process is more energy-consuming. The reduced impact observed in the graph is due to the ability to condition multiple flow-packs simultaneously in a single operation, which reduces the energy consumption per individual flow-pack of this step. This efficiency highlights the potential for optimizing resource use within this stage of the process.

To reduce the environmental impact of the flow-pack welding phase, innovative and non-thermal welding techniques, such as ultrasonic sealing, present a promising alternative. (Talaniuk et al., 2023) Ultrasonic sealing uses high-frequency vibrations to generate localized heat, significantly reducing energy consumption compared to traditional thermal methods. This approach not only lowers emissions related to energy use but also enhances process efficiency by eliminating preheating and cooling phases. A detailed LCA of these approaches is recommended to evaluate their full potential compared to conventional methods.

4. Conclusions

This chapter presented a screening LCA study to evaluate the environmental impacts associated with the production of NaCas powder, NaCas films, and NaCas-based flow-packs. The findings highlighted significant environmental hotspots across the production chain, with the NaCas powder production phase contributing the highest impact due to energy-intensive processes such as freeze-drying and milk-derived raw material inputs. In contrast, the NaCas film and flow-pack production phases exhibited comparatively lower impacts, with the flow-pack phase accounting for an average of 16% of the total environmental impact.

Among the specific impact categories, the powder production stage heavily influenced "Land Use" and "Terrestrial Eutrophication," while the flow-pack stage contributed substantially to "Human Toxicity," "Water Use," and "Resource Use, Fossil." Energy consumption was identified as a key driver across all phases, particularly in operations such as powder lyophilizing, film conditioning, welding, and heat treatments.

To address these challenges, innovative strategies were proposed to enhance sustainability, including the adoption of non-thermal welding technology such as ultrasonic sealing, which could significantly reduce energy demand and emissions. Furthermore, continuous in-line production processes and the elimination of freeze-drying could improve resource efficiency while maintaining product performance.

Future research should focus on scaling up these alternative processes and conducting detailed LCAs to assess their feasibility and environmental benefits at an industrial scale. By integrating innovative solutions and optimizing energy use, NaCas-based flow-packs have the

potential to become a sustainable alternative for food packaging, advancing circular economy principles in the food sector.

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GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

This Ph.D. thesis has focused on addressing the critical issue of the depreciation of expired pasteurized milk, a valuable resource that, once withdrawn from the market, currently ends up being mainly destined to animal feeding or biogas production, despite the high biological value of the components still contained in it. By providing a more valuable alternative to its current destinations, this research aims to reduce waste and create an upcycled application for this undervalued resource. By leveraging expired pasteurized milk as a resource for producing NaCas-based biopolymer films for packaging, this research proposes innovative solutions aligned with the principles of a circular economy and SDGs.

The research began by exploring the feasibility of converting expired pasteurized milk into sodium caseinate (NaCas) powders, which serve as the foundation for biopolymeric film production. A key challenge was the variability in the raw material, particularly the fat content, which was successfully managed to ensure consistent powder quality. This step established a robust protocol for powder production, forming a reliable base for the subsequent development of film-forming solutions.

Building on these fundamentals, through optimization of formulations and processing conditions, the films achieved mechanical and barrier properties comparable to those of certain synthetic plastics. However, their susceptibility to humidity emerged as a critical limitation, highlighting the need for further enhancements in moisture resistance.

To address these limitations and improve the films' characteristics, easy to apply functionalization techniques were explored. Light-induced cross-linking and alkaline treatments proved to be effective in enhancing the structural integrity and mechanical properties of the films. These modifications improved their mechanical performances, demonstrating the potential to overcome some of the constraints identified in earlier stages of the research.

A significant milestone was achieved in the collaborative development of a flow-pack prototype for low-moisture cheese snacks in partnership with Lactalis Italia. This phase of the research combined theoretical advancements with practical application, optimizing film weldability while ensuring packaging compliance with cheese snack storage. Storage tests have demonstrated the ability of NaCas films to effectively preserve cheese snacks from moisture adsorption while avoiding excessive increase in water activity, providing a viable alternative to conventional packaging, despite some volatile compounds inherent in lipid oxidation being found to be higher than with standard packaging. Nonetheless, challenges such as welding stability under diverse storage conditions and scalability of production processes were identified as areas for refinement.

The environmental sustainability of the entire process was critically evaluated through a life cycle assessment (LCA), which encompassed every stage from powder production to prototype development. The LCA findings identified key contributors to energy consumption identifying the powder production process as the most energy-intensive on a laboratory scale. Within the production of the powder, the freeze-drying process and the thermal treatment resulted to be the most impactful, proposing alternative solutions for reducing energy consumption and environmental impacts.

This body of work not only demonstrates the feasibility of upcycling expired milk into functional materials but also underscores the importance of integrating environmental considerations into every stage of material development. Limitations identified throughout the study—such as the sensitivity of films to moisture, the reliance on energy-intensive processes, and scalability constraints—provide a clear roadmap for future research.

To address these challenges, future efforts should focus on integrating hydrophobic additives to enhance moisture resistance and exploring alternative production techniques, such as blown extrusion or thermo-formability, to improve scalability and reduce energy demands.

By transforming expired pasteurized milk into sustainable biopolymeric films, this research contributes to global efforts to reduce food waste and tried to find a valuable alternative to plastic. It provides a model for integrating waste management with innovative material development, aligning with the United Nations Sustainable Development Goals and FAO 2030 agenda, fostering a more circular and sustainable economy. This work represents a meaningful step toward rethinking how industrial by-products can be harnessed to address pressing environmental challenges, paving the way for a more responsible and sustainable packaging industry.

APPENDICES - SCIENTIFIC PRODUCTION

Supplementary Materials

Chapter 2 – Supplementary

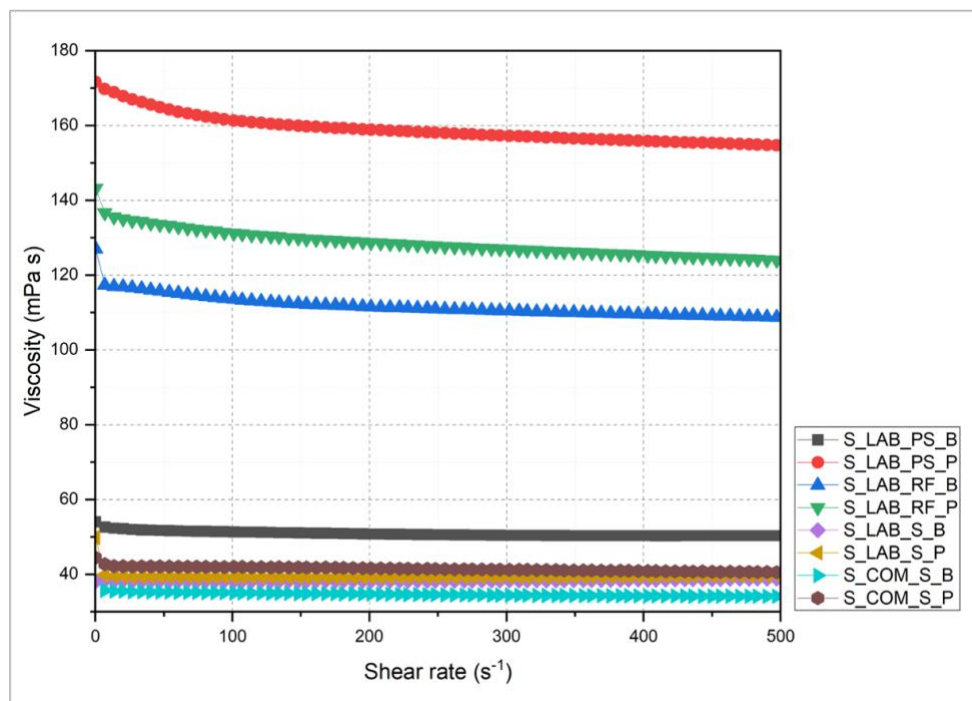


Fig. S1. Viscosity curves of experimental and commercial NaCas solutions.

Table S1. Size of pores on the films' surface.

Sample	Pores (μm)	Pores (μm)*
	Mean ± st.dev.	Median ± st.error
F_LAB_PS_B	12.01 ± 6.92	9.25 ± 1.55
F_LAB_PS_P	5.90 ± 4.85	3.52 ± 1.08
F_LAB_RF_B	3.11 ± 1.56	2.96 ± 0.35
F_LAB_RF_P	2.55 ± 0.81	2.59 ± 0.18
F_LAB_S_B	1.30 ± 0.53	1.30 ± 0.17
F_LAB_S_P	0.70 ± 0.27	0.74 ± 0.09
F_COM_S_B	0.28 ± 0.20	0.21 ± 0.08
F_COM_S_P	0.31 ± 0.11	0.28 ± 0.04
COMPOSITION		< .001
TRATMENT		0.034

Data are expressed as median ± standard error. Results of Kruskal-Wallis statistical analysis (bottom) concerning the factors 'composition' and 'treatment' were considered significant when p-value < 0.05.

Chapter 4 – Supplementary

Table S1. Volatile compounds identified in cheese snacks (CS), packaged in NaCas flow-pack and standard flow-pack (STD) at different storage times. Results are expressed as peak area counts corrected in peak area of the internal standard and shown as mean $\pm$ st. deviation.

Chemical class	Volatile compound	CS in NaCas			CS in STD		
		Storage Time (days)			Storage Time (days)		
		T0	T10	T20	T0	T10	T20
<i>Aldehydes</i>	Hexanal	0.01 $\pm$ 0.00	0.2 $\pm$ 0.00	0.19 $\pm$ 0.01	0.01 $\pm$ 0.00	0.2 $\pm$ 0.00	0.19 $\pm$ 0.03
	Nonanal	ND	0.04 $\pm$ 0.00	0.04 $\pm$ 0.00	ND	0.02 $\pm$ 0.00	0.02 $\pm$ 0.00
<i>Ketons</i>	2-heptanone	0.38 $\pm$ 0.17	5.94 $\pm$ 0.23	5.55 $\pm$ 0.44	0.38 $\pm$ 0.17	7.26 $\pm$ 1.31	6.33 $\pm$ 0.21
	2-nonanone	0.04 $\pm$ 0.01	0.89 $\pm$ 0.04	0.8 $\pm$ 0.07	0.04 $\pm$ 0.01	0.96 $\pm$ 0.21	0.83 $\pm$ 0.10
	2,3-butanedione	4.51 $\pm$ 2.39	1.8 $\pm$ 0.04	1.62 $\pm$ 0.02	4.51 $\pm$ 2.39	2.5 $\pm$ 0.41	2.34 $\pm$ 0.04
	3-hydroxy-2-butanone	56.41 $\pm$ 6.93	9.3 $\pm$ 0.66	7.08 $\pm$ 1.57	56.41 $\pm$ 6.93	14.07 $\pm$ 2.56	13.29 $\pm$ 0.78
	hydroxy-2-propanone	0.01 $\pm$ 0.00	0.04 $\pm$ 0.01	0.03 $\pm$ 0.00	0.01 $\pm$ 0.00	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01
<i>Acids</i>	Butanoic acid	5.96 $\pm$ 1.58	3.79 $\pm$ 0.16	2.9 $\pm$ 0.66	5.96 $\pm$ 0.58	6.53 $\pm$ 0.77	6.55 $\pm$ 1.02
	2-methyl Butanoic acid	4.57 $\pm$ 1.23	1.31 $\pm$ 0.04	0.86 $\pm$ 0.20	4.57 $\pm$ 1.23	2.72 $\pm$ 1.23	2.32 $\pm$ 0.21
	Hexanoic acid	0.97 $\pm$ 0.25	0.98 $\pm$ 0.03	0.76 $\pm$ 0.02	0.97 $\pm$ 0.25	1.14 $\pm$ 0.27	1.13 $\pm$ 0.12

Table S2. Volatile compounds identified in NaCas flow-pack and standard flow-pack (STD), at different storage times. Results are expressed as peak area counts corrected in peak area of the internal standard and shown as mean $\pm$ st. deviation.

Chemical class	Volatile compound	NaCas Flow-Pack			STD Flow-pack		
		Storage Time (days)			Storage Time (days)		
		T0	T10	T20	T0	T10	T20
<i>Aldehydes</i>	Hexanal	27.94 $\pm$ 14.99	50.56 $\pm$ 0.36	33.88 $\pm$ 3.48	0.37 $\pm$ 0.06	5.39 $\pm$ 0.24	5.66 $\pm$ 1.44
	Nonanal	230.62 $\pm$ 134.80	367.34 $\pm$ 38.39	231.18 $\pm$ 6.59	8.82 $\pm$ 2.01	53.84 $\pm$ 7.92	47.82 $\pm$ 8.64
<i>Ketons</i>	2-heptanone	ND	233.76 $\pm$ 31.19	233.08 $\pm$ 5.3	ND	90.08 $\pm$ 22.42	131.86 $\pm$ 21.42
	2-nonanone	2.07 $\pm$ 0.76	47.1 $\pm$ 14.46	47.23 $\pm$ 2.74	2.63 $\pm$ 0.52	119.25 $\pm$ 1.15	162.21 $\pm$ 41.94
	2,3-butanedione	78.89 $\pm$ 37.98	227.46 $\pm$ 7.99	252.81 $\pm$ 47.38	0.17 $\pm$ 0.01	5.58 $\pm$ 0.44	8.06 $\pm$ 1.32
	3-hydroxy-2-butanone	28.57 $\pm$ 9.26	631.73 $\pm$ 0.54	723.56 $\pm$ 149.05	ND	26.6 $\pm$ 0.72	34.73 $\pm$ 1.48
	hydroxy-2-propanone	7.79 $\pm$ 4.33	16.79 $\pm$ 1.08	12.00 $\pm$ 1.46	0.08 $\pm$ 0.01	0.90 $\pm$ 0.15	1.62 $\pm$ 0.46
<i>Acids</i>	Butanoic acid	14.14 $\pm$ 0.27	527.93 $\pm$ 91.09	434.68 $\pm$ 28.11	0.55 $\pm$ 0.07	90.82 $\pm$ 8.36	91.91 $\pm$ 8.58
	2-met Butanoic acid	ND	307.76 $\pm$ 36.47	257.89 $\pm$ 39.14	ND	42.6 $\pm$ 3.12	43.55 $\pm$ 5.18
	Hexanoic acid	ND	856.87 $\pm$ 116.36	787.85 $\pm$ 208.88	ND	840.06 $\pm$ 156.97	980.05 $\pm$ 198.57

Table S3. Free amino acid content (mg/g protein) detected in cheese snacks at different stages of the storage test.

Sample name	Asp	Thr	Ser	Asn	Glu	Gln
CS - T0	0.03 ± 0	0.16 ± 0	0.14 ± 0.01	0.33 ± 0.01	0.95 ± 0.01	0.26 ± 0
CS in NaCas - T20	0.03 ± 0	0.16 ± 0	0.15 ± 0	0.35 ± 0.01*	1.04 ± 0.02**	0.39 ± 0.03**
CS in NaCas - T20 (no maxi-pack)	0.03 ± 0	0.15 ± 0**	0.13 ± 0	0.33 ± 0	0.94 ± 0.01	0.33 ± 0.02**
CS in STD - T20	0.04 ± 0**	0.17 ± 0***	0.15 ± 0	0.35 ± 0*	1.08 ± 0.02**	0.4 ± 0.02***
	Gly	Ala	Cit	Val	Met	Ile
CS - T0	0.12 ± 0	0.29 ± 0.01	0.07 ± 0.01	0.37 ± 0	0.12 ± 0	0.25 ± 0
CS in NaCas - T20	0.14 ± 0**	0.27 ± 0.01*	0.14 ± 0**	0.4 ± 0.01**	0.13 ± 0*	0.28 ± 0***
CS in NaCas - T20 (no maxi-pack)	0.13 ± 0	0.26 ± 0**	0.13 ± 0.01**	0.36 ± 0.01	0.12 ± 0	0.26 ± 0**
CS in STD - T20	0.15 ± 0***	0.3 ± 0*	0.14 ± 0**	0.42 ± 0.01**	0.14 ± 0***	0.29 ± 0.01***
	Leu	Tyr	Phe	Gaba	Orn	Lys
CS - T0	0.84 ± 0.01	0.19 ± 0.02	0.42 ± 0.01	0 ± 0	0.31 ± 0	0.63 ± 0
CS in NaCas - T20	0.9 ± 0.02**	0.18 ± 0	0.45 ± 0.01*	0.01 ± 0	0.29 ± 0***	0.69 ± 0.01**
CS in NaCas - T20 (no maxi-pack)	0.82 ± 0.01*	0.16 ± 0	0.42 ± 0.01	0.01 ± 0	0.25 ± 0***	0.62 ± 0.01
CS in STD - T20	0.95 ± 0.02***	0.2 ± 0	0.48 ± 0***	0.01 ± 0	0.3 ± 0	0.74 ± 0.01***
	His	Arg	Pro	Total		
CS - T0	0.14 ± 0	0.1 ± 0	0.72 ± 0.03	6.43 ± 0.01		
CS in NaCas - T20	0.18 ± 0***	0.09 ± 0**	0.82 ± 0.02**	7.08 ± 0.13**		
CS in NaCas - T20 (no maxi-pack)	0.15 ± 0	0.08 ± 0***	0.77 ± 0.03	6.44 ± 0.07		
CS in STD - T20	0.19 ± 0***	0.07 ± 0.01**	0.85 ± 0.02**	7.41 ± 0.08***		

Results are expressed as mean ± standard deviation. Acronyms: CS (cheese snack); “in NaCas (packaged in experimental NaCas flow-pack); STD (packaged in standard flow-pack); T0, T20 (day 0 and 20 of the storage test). “*” indicate statistically significant difference (p<0.05); “**” indicate statistically significant difference (p<0.01); “***” indicate statistically significant difference (p<0.001) between the cheese snacks samples within the same amino acid.

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Publications

Pellegrino, L., Hogenboom, J., Rosi, V., Sindaco, M., **Gerna, S.**, & D'Incecco, P. 2022. Focus on the Protein Fraction of Sport Nutrition Supplements. *Molecules*, 27, 3487.

Gerna, S., D'Incecco, P., Limbo, S., Sindaco, M., & Pellegrino, L. 2023. Strategies for Exploiting Milk Protein Properties in Making Films and Coatings for Food Packaging: A Review. *Foods*, 12(6), 1271.

Gerna, S., D'Incecco, P., Limbo, S., Alamprese, C., & Pellegrino, L. 2025. Upcycling casein from expired pasteurized milk into films – Development and characterization of films made from sodium caseinate. *Applied Food Research*. UNDER PEER REVIEW – Waiting for reviewers' acceptance

Non-peer reviewed dissemination

Gandolfi, I., **Gerna, S.**, D'Incecco, P. & Pellegrino, L. 2024. Dairy packaging processing: thinking about green strategies for recycling. IDF dairy sustainability outlook, International

Dairy Federation. Website: <https://shop.fil-idf.org/products/issue-8-idf-dairy-sustainability-outlook-dairy-processing>

Oral communications to national and international conferences

Gerna, S. Integrated green strategies for the management, recovery and recycling of waste in a dairy factory. Lake Como of Advanced Studies, Como, July 16-19th, 2024.

Gerna, S. Integrated green strategies for waste management in dairy factories: Upcycling casein from expired pasteurized milk into films. 28° Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology, Catania, Italy, September 18th -20th, 2024.

Gerna, S., D’Incecco, P., Limbo, S., Alamprese, C., & Pellegrino, L. Development of sodium-caseinate packaging films: upcycling the casein from expired pasteurized milk in a circular economy approach. 10th International Conference on Food Chemistry & Technology, Valencia, November 25th – 27th, 2024.

Poster communications to national and international conferences

Gerna, S., D’Incecco, P., Pellegrino, L. Integrated Green Strategies for the Management, Recovery, and Recycling of Waste in a Dairy Factory. 26° Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology, Asti, Italy, September 19th - 21th, 2022.

Gerna, S. Strategie green per il miglioramento delle proprietà di film di caseina ottenuti da latte fresco pastorizzato scaduto. 7° congresso lattiero caseario AITeL (Associazione Italiana Tecnici del Latte): LATTE E DERIVATI: Una filiera che si confronta con la sostenibilità, Cremona, September 7th, 2023.

Gerna, S., D’Incecco, P., Pellegrino, L. Integrated Green Strategies for the Management, Recovery, and Recycling of Waste in a Dairy Factory. 27° Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology, Portici, Italy, September 13th - 15th, 2024.

Gerna, S., D’Incecco, P., Limbo, S., Alamprese, C., Pellegrino, L. Green strategies to improve chemical, mechanical and gas-barrier properties of casein films obtained from expired

pasteurized milk. 9th International Conference on Food Chemistry & Technology, Paris, November 27th – 29th, 2024.

Awards

Award for originality and scientific validity of the research provided by the scientific committee of 7th National Dairy Conference AITeL, Cremona, September 7th, 2023.

Award for The Best Oral Presentation provided by the scientific committee of the 10th International Conference on Food Chemistry & Technology, Valencia, November 25th – 27th, 2024.

Other PhD activities

Co-supervisor of a bachelor thesis (Faculty of Agricultural and Food Science, University of Milan) titled “Sustainable strategies for casein film production from expired milk” supervised by Dr. Paolo D’Incecco.

Teaching activities

Lecture on “Integrated green strategies for the management, recovery and recycling of waste in a dairy factory, a focus on microstructure analysis of NaCas-based films: CLSM and SEM microscopy.” in the course held by Dr. Paolo D’Incecco: Advanced techniques of microscopy, microanalysis and image analysis applied to foods.

Overview of completed training activities

PhD Course	Scientific Field	Organizer and location	Year
Sustainability concepts in food technologies – Methodological approaches and case studies	AGR/15	Prof. Lavelli UNIMI, Milan, Italy	2022
E-sensing technologies & chemometrics	AGR/15	Prof. Alamprese UNIMI, Milan, Italy	2022
Fundamental of statistics (for veterinary and animal science)	AGR/17	Prof. Crepaldi UNIMI, Milan, Italy	2022

Advanced methods in the characterization of macromolecules in food systems	AGR/15	Prof. Barbiroli UNIMI, Milan, Italy	2023
Transferable skills	Hours	Year	
Academic writing	4	2022	
Language coaching: Interpersonal/presentation skills	4	2022	
IP and patent	2	2022	
Open Science	4	2022	
Data management	3	2022	
Communication and new media	5	2022	
CV and interview	3	2022	
Research evaluation	4	2022	
Introduction and valorization of research	2	2022	
Research Integrity – part I	6	2023	
Research Integrity – part II	2	2023	
Introduction to Open Science and research integrity	1.5	2023	
Academic writing II	4	2023	
Open access and open licenses	1.5	2023	
Public with the rights retention strategy: Is it for me?	1.5	2023	
Language coaching: Interpersonal and interview skills	4	2023	
Self-branding: competencies of PhDs	3	2023	
Workshop for the production of scientific content for new media	3	2023	
Grantsmanship I e II	4	2023	
The art of grantsmanship	4	2024	
Searching work with a PhD	4	2024	
Teaching and learning I and II	9	2024	
Public speaking	4	2024	
Preparing a dissemination plan for a research project	2	2024	
Advanced lecture on using IP to do innovation	2	2024	
The Integrated Sustainability Assessment for Global Health	5	2024	
Exploitation by means of business creation: spin-off and entrepreneurship from an academic point of view	4	2024	

Preprint and Open Peer Review	1.5	2024
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