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Combining plant and dairy proteins in food colloid design

Emma B. A. Hinderink[1,2,a], Adeline Boire[3], Denis Renard[3], Alain Riaublanc[3], Leonard M. C. Sagis[4], Karin Schroën[2], Saïd Bouhallab[5], Marie-Hélène Famelart[5], Valérie Gagnaire[5], Fanny Guyomarc'h[5] and Claire C. Berton-Carabin[2,3]

Abstract

The use of plant proteins to design colloidal food systems is a hot topic in the current context of the protein transition. However, replacing animal-derived proteins (in particular, dairy proteins) that have been traditionally used for this purpose by plant proteins is a challenge from various perspectives, and in particular, because of drastically different solubility and functionality. A possible route to mitigate these issues is to combine plant and dairy proteins, providing that their interactions can be understood from the molecular to the macroscopic scale. This review addresses the major advances that have occurred in the field of such blend-based systems, all the way from their behaviour in aqueous dispersions to their potential applications in gels, foams and emulsions.

Addresses

[1] TiFN, P.O. Box 557, 6700 AN, Wageningen, the Netherlands
[2] Laboratory of Food Process Engineering, Wageningen University, Bornse Weilanden 9, 6708 WG, Wageningen, the Netherlands
[3] INRAE, UR 1268, Biopolymères, Interactions, Assemblages, F-44316, Nantes, France
[4] Laboratory of Physics and Physical Chemistry of Foods, Wageningen University, Bornse Weilanden 9, 6708 WG, Wageningen, the Netherlands
[5] INRAE, Institut Agro, STLO, F-35042, Rennes, France

Corresponding author: Berton-Carabin, Claire C (claire.berton-carabin@inrae.fr)
[a] Current address: Delft University of Technology, Faculty of Applied Sciences, Department of Chemical Engineering, van der Maasweg 9, 2629 HZ, Delft, the Netherlands.

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Keywords

Legume proteins, Animal proteins, Multiphase systems, Food structuring, Protein solubility, Non-protein components, Fluid interfaces, Protein aggregates, Hierarchical structures.

Abbreviations

NAP, Napin; SPI, soy protein isolate; β-lg, β-lactoglobulin; WPI, whey protein isolate; PPI, pea protein isolate; LF, lactoferrin; LLPS, liquid–liquid phase separation; LSPS, liquid–solid phase separation; LYS, lysozyme; α-lac, α-lactalbumin.

Introduction

Proteins are an inherent part of many commonly consumed foods. They not only hold a nutritional role as sources of essential amino acids, but also a number of techno–functional roles. For example, they are vital in achieving desired macroscopic properties of the food product, such as its appearance or texture. This is particularly important when considering food matrices at the colloidal scale, where proteins are crucial in forming or stabilising relevant structures such as aggregates, particles, droplets, bubbles, or combinations thereof. These properties depend themselves on intrinsic characteristics of the proteins, and primarily on their amino acid composition and sequence, which control their molecular structure, native assembly state, charge and solubility [1]. In addition, the structure, assembly state and functionality of proteins may be largely modified by the processes applied to yield protein ingredients (e.g. concentrates and isolates from laboratory scale to commercial ones) from raw materials, and to formulate the end-food products.

The mechanisms underlying the structure–function relationship in protein-based food colloids are thus inherently complex, with various relevant scales (from molecular to macroscopic properties) and a number of intertwined parameters potentially involved. A large part of the research in this area has been performed on animal-derived proteins, in particular, dairy and egg proteins. The latter have therefore been well-characterised in that respect, although some of the related mechanisms still remain the subject of intense investigation. Nevertheless, a new challenge in this research field stems from the ongoing so-called protein transition, which aims at reducing the consumption of animal-derived proteins while promoting that of alternative food proteins, of which plant proteins are the

major ones. Such a partial replacement not only poses a number of questions regarding nutritional aspects (e.g. amino acid composition, digestibility), but also has important consequences when it comes to the aforementioned functional properties for structuring food colloids.

Edible plant proteins may originate from various biological sources, including legumes (e.g. pea, faba bean), oilseeds (e.g. rapeseed, sunflower, hemp seed) or cereals (e.g. wheat, sorghum) [2]. According to the classification proposed by Osborne at the beginning of the 20th century, they are usually classified in four categories depending on their solubility in various aqueous-based media and susceptibility to heat-induced denaturation: (a) albumins are soluble in water and tend to coagulate upon heating; (b) globulins are insoluble in water, yet soluble in dilute salt solutions (e.g. 0.1M NaCl); (c) prolamins are insoluble in water, yet soluble in aqueous media containing 50–70% ethanol/water mixture or diluted acidic media, and they are heat-resistant; and (d) glutelins are also insoluble in water, yet soluble in dilute alkaline solutions (e.g. 0.1M NaOH).

In plant seeds, storage proteins may self-organise as dense, glassy micron-sized structures named protein bodies [3]. Such a supramolecular organisation has no equivalent in e.g. dairy proteins, which are, in essence, adapted to water-rich media. The latter subdivide into (a) whey proteins, which are globular and heat-sensitive (the main ones being β-lactoglobulin (β-lg), lactoferrin (LF) and α-lactalbumin (α-lac)); and (b) caseins, which have a disordered tertiary structure, and are assembled as micelles in milk. Contrary to plant protein bodies, casein micelles are highly hydrated colloidal structures (3–4 gram water per gram casein). In acidic conditions, they destabilise and get dissociated, which is the basis for preparing sodium caseinate, a common food ingredient. Besides, enzymatic and/or isoelectric destabilisation are commonly used to form dairy gels such as yoghurt or cheese.

The low solubility of most plant protein ingredients is a major barrier in achieving full replacement of dairy proteins for the stabilisation of colloidal food systems. Different strategies may be considered to unlock such applications: (a) reconsider the production processes of plant protein ingredients in order to avoid extensive protein aggregation owing to solvents and heat, and the removal of fractions with high technological potential, such as albumins [4]; (b) make use of this low solubility to achieve food structuring via alternative routes, such as the formation of Pickering emulsions stabilised by insoluble protein particles [5]; and/or (c) use plant proteins in mixtures with dairy proteins, which can be a promising integrated approach to address the sustainability incentive for promoting the use of plant proteins, while mitigating potential drawbacks in terms of nutritional and functional properties [6,7].

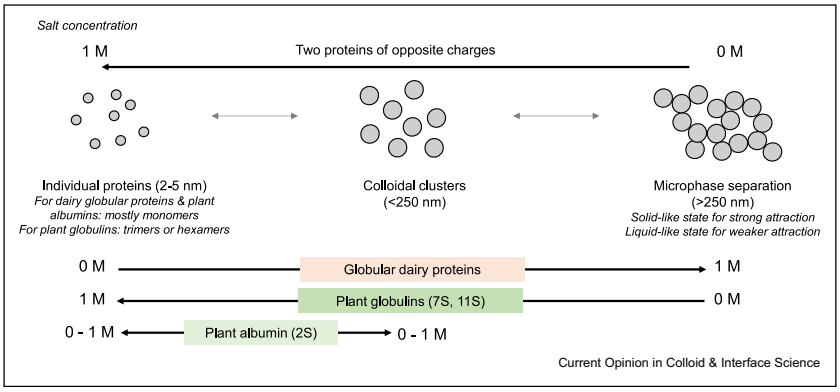
The present article focuses on recent advances in designing food colloidal systems (aggregates, gels, foams, emulsions) based on mixtures of dairy and plant proteins. We first revisit the behaviour of such mixtures in aqueous dispersions, which is an important basis to understand the mechanisms occurring in systems that are further structured by network formation (gels) or incorporation of a non-aqueous phase (foams, emulsions – i.e. interface-dominated systems). For some of these structures and systems, experimental data with plant-dairy protein mixtures are still scarce, which is why we also propose some possible outcomes based on findings obtained with other protein mixtures. Finally, we discuss some scientific questions and technological challenges that will need to be further addressed in the realm of food colloids made of plant-dairy proteins mixtures, including the potential huge effect of non-protein components in the plant protein ingredients.

Aqueous dispersions

Dairy and plant proteins are diverse in terms of molecular structure, supramolecular assembly, and solubility in food-relevant conditions. Some of them are folded into well-defined globular 3D structures. Those include whey proteins, such as β-lg or LF, but also plant albumins, such as napin (NAP), and plant globulins such as pea legumin. The behaviour of such globular proteins in solution is usually well-described by a colloidal model with a repulsive potential, including electrostatic and steric repulsion, and an attractive potential, including van der Waals interactions [1]. The interaction potential is modulated by pH, ionic strength, solvent composition, and temperature. It should be noted that the applicability of such a colloidal model to plant albumins/globulins has never been demonstrated, even though NAP has been compared to a Janus particle upon adsorption at an oil-water interface [8]. Most of the sequences of plant-based globulins and albumins contain low-complexity regions comprising polar and/or charged amino acids. They are expected to form disordered loops at the surface of their globular structure [1]. This may limit the applicability of an incompressible colloidal model to predict their behaviour. Besides, a major difference between whey globular proteins/plant albumins and plant globulins is the ionic strength dependence of their assembly (Figure 1). While the former are highly soluble at low-ionic strength, the latter form highly aggregated structures as shown in Figure 1 (top). A range of dairy/plant proteins lack a well-ordered 3D structure; this is the case for partially or fully disordered proteins such as caseins and wheat prolamins [9].

Several scenarios may be encountered when mixing two proteins in their native-like state and/or under mild physico–chemical conditions, depending on their charge and intrinsic conformation (Table 1). One of the

Figure 1



Effect of ionic strength on heteroprotein assembly in the case of oppositely charged proteins (top) and on the assembly state of dairy globular proteins, plant globulins, and plant albumins (bottom).

most investigated scenarios in the past five years involves associative assembly of oppositely charged proteins. Among dairy–plant proteins, acid proteins such as β-lg, α-lac, caseins, and pea globulins, may form electrostatic complexes with basic proteins such as LF and rapeseed NAP, under specific ionic strength, pH, and stoichiometry conditions. Accordingly, three different structures may be formed: (a) a homogeneous dispersion of intermolecular soluble nanocomplexes; associative phase separation with either (b) liquid–liquid phase separation (LLPS, complex coacervation) or (c) liquid–solid phase separation (LSPS, precipitation), leading to phases highly enriched with proteins in both cases. The driving forces towards coacervates or precipitates in the enriched phase depend on the charge densities and the strength of the attractive forces between the two mixed biopolymers. Coexistence of coacervates and precipitates has been reported in some cases, including protein-based systems [10]. In general, when the electrostatic attraction between biopolymers is very strong, the association occurs in a random way and precipitates are therefore favoured. The strong attraction between macromolecules leads to a 'frozen' state, with low-molecular flexibility and low ability for structural rearrangements. Consequently, precipitates are described as a dense and less hydrated phase exhibiting a solid-like viscoelastic behaviour. In contrast, a coacervate is a highly hydrated phase, depending on the mixed systems. Weaker electrostatic interactions are involved during the formation of coacervates, which exhibit liquid-like viscoelastic behaviour. It is generally assumed that neutral soluble complexes are initially formed and subsequently associate to yield neutral micron-sized (spherical) particles, followed by coalescence and liquid–liquid phase separation [11]. The driving force for the formation of precipitates and coacervates arises from desolvation, driven by or at least coupled to the release of counterions. The greater the potential fields of both proteins, the greater the overlap between these potentials (owing to charge proximity when the two proteins approach), the greater the degree of desolvation, promoting precipitation at the expense of coacervation.

Studies of such systems primarily focused on mixtures of proteins, mainly of dairy or egg origin, selected for their high purity, availability and high solubility [11]. From combined protein systems, evidences were drawn confirming that complex coacervation in heteroprotein systems is a generic process that can occur in all oppositely charged mixtures, provided that the experimental conditions were relevant, considering the physico–chemical and structural properties of the involved proteins. In case of plant-dairy protein mixtures of opposite charges, the first scenario of soluble complexes was shown for a mixture of anionic β-lg with cationic rapeseed NAP for pH ranging from 6.5 to 7.5 [12]. Using isothermal titration calorimetry, β-lg and NAP were found to interact with each other leading to nanometer-sized clusters, as evidenced by dynamic light scattering, but no microscopic phase separation was observed. Conversely, LLPS or LSPS was observed between β-lg and another cationic protein of similar size and charge, lysozyme (LYS), depending on pH conditions. Molecular simulations suggested that the charge anisotropy of NAP would lead to the formation of smaller NAP-β-lg clusters as compared to LYS-β-lg ones. These results are in contrast with previous data obtained on mixtures of animal proteins, where protein supercharging and charge patchiness were found to promote phase separation and to determine whether the condensed phase was liquid- or solid-like [13]. A patchiness parameter was defined, representing the charge correlation between neighbouring sites on the protein surface, which correlates well with the likelihood of phase separation [14]. The role of other parameters such as molecular

Table 1

Examples of plant–animal protein mixtures and related assemblies in aqueous media. For older and more complete references, please refer to the recent review by Wu et al. (2020) [7].

Animal protein	Plant protein	Type of assembly	Highlights	References
Dairy proteins				
Lactoferrin	Pea proteins isolate	Associative phase separation – LLPS	Small angle X-ray scattering experiments revealed the internal structure of densely assembled coacervates, with a roughly spherical size distribution with a max around 80nm at pH 5.4	[19]
Lactoferrin	Soy proteins	Associative phase separation – LLPS	The SPI/LF interaction improved the heat-stability of heat-sensitive lobe in lactoferrin	[21]
β-casein	Napin	Associative phase separation – LLPS	The aggregation/assembly between the two proteins is controlled by pH, ionic strength and protein mass ratio in an independent manner. Addition of salt or chelating agents of divalent cations suppressed the aggregation process	[23]
Casein micelles	Pea proteins	Co-solubility	The denaturation temperature of five pea proteins increased in the presence of casein, indication a chaperone-like activity of casein micelles	[24]
Whey proteins	Pea proteins	Co-solubility Co-precipitation	Heat-treatment and pH drive co-solubility versus co-precipitation of mixed protein micro-particles	[25]
Whey proteins	Rapeseed proteins		RP modified the microstructural and/or denaturation properties of WP, the effect being dependent on the protein mixing ratio	[20]
β-lactoglobulin	Napin	Stable colloidal clusters	Charge anisotropy drives protein–protein assembly and subsequent potential phase separation	[12]
Egg proteins				
Lysozyme	Soy proteins	Associative phase separation – LSPS	The formed complexes exhibited solid–liquid phase separation under pH, stoichiometry, sodium chloride, and assembling time conditions tested	[22]
Lysozyme	Pea proteins	Associative phase separation – LSPS	Strong enthalpy–driven interactions under low ionic strength with two-successive exothermic phases	Bouhallab et al. (unpublished results)
Ovalbumin	Pea proteins	Co-solubility	Weak interaction, no detectable assembly	Bouhallab et al. (unpublished results)
Egg proteins	Soy proteins	Co-solubility Co-precipitation	Heat-treatment and pH driven co-solubility or co-precipitation of mixed protein micro-particles	[26]

flexibility of NAP [15] may be involved in this contrasted outcome; this molecular flexibility parameter deserves to be investigated in detail since several plant proteins display disordered loops [1]. Beyond the specific case of dairy–plant proteins, the mechanism that prevents the growth of the protein–protein nanocomplexes is still under investigation. Similarly, the liquid–liquid phase separation process may lead to kinetically arrested metastable multidroplet state instead of a macroscopic phase separation. The mechanisms behind these kinetically arrested states are not clear yet. It was attributed to residual electrostatic repulsion owing to the absence of charge compensation [16] and/or to the exhaustion of available valencies within smaller clusters [17]. Besides the fundamental interest of such a topic, the functional properties of such stable clusters have not yet been investigated. They may provide a new route to stabilise interfaces as was found for protein–polysaccharide complexes [18].

Besides soluble complexes as described for a β-lg-NAP mixture, heteroprotein complex coacervation was evidenced for other dairy–plant protein mixtures. The fine tuning of protein–protein stoichiometry and of the aqueous phase composition allowing for appropriate formation of heteroprotein coacervates was specifically studied by mixing bovine LF with pea or soy proteins [19–22], and rapeseed NAP and bovine β-casein [23]. It was found that LF and the soluble fraction of pea proteins isolated through an industrial process form complex coacervates in a narrow range of pH comprised between 5.0 and 5.8. The presence of dense assembled coacervates was highlighted using small angle X-ray scattering, which revealed a roughly bimodal size distribution with maximum sizes of 80 nm at pH 5.4 [19]. For NAP-β-casein mixture, the reversible LLPS was enhanced by increased temperature while remaining reversible. Heteroprotein complex coacervation was also evidenced for a mixture of LF and soy proteins [21], whereas only LSPS was observed for lysozyme and soy proteins [22]. Most studies have investigated the associative properties of plant protein isolates containing several types of proteins, mostly 7S and 11S globulins. Therefore, the mechanisms associated with the observed phase separation could not be unambiguously ascribed to a given type of protein. In near future, the use of pure individual fractions of each protein could provide a deeper understanding of the mechanisms involved as suggested by Ainis et al. [12]. This strategy may be a tedious task given the high polymorphism of plant proteins. Besides, electrostatic attraction between oppositely charged proteins is usually observed at low-ionic strength, (Figure 1, bottom). While plant albumins are in a monomeric state over a wide range of pH and ionic strength, plant globulins may be highly aggregated below 100 mM ionic strength. This implies that plant globulins may be present as clusters in conditions, where LLPS and/or LSLP with dairy proteins were observed. The effect of the aggregation state of plant globulins on such a mechanism has not been investigated.

In theory, another scenario called segregative phase separation could occur when mixing two proteins in conditions involving electrostatic repulsion (i.e. both proteins having alike strong net charges). It may be observed in case of thermodynamic incompatibility between the two proteins, similarly to protein–polysaccharide mixtures. Incompatibility of protein mixtures depends strongly on the conformational state of the proteins and is enhanced by protein denaturation [27]. Limited literature on mixtures of whey protein isolate (WPI) and plant globulins reported that segregative phase separation does not happen and that co-aggregation occurs only to some extent [28]. However, segregative phase separation can occur when large aggregates of a single type of protein are already present before mixing [29]. One may therefore wonder if segregative phase separation could occur between oligomeric (frequently found in the plant kingdom) and monomeric dairy proteins.

Besides associative or segregative phase separation that may occur upon mixing dairy and plant proteins, dairy proteins may help stabilising plant proteins against precipitation. Some proteins, such as caseins, possess chaperone-like functions, owing to their flexible and unfolded random coil conformation, thereby altering the heat-induced aggregation of many proteins such as whey proteins, ovalbumin and ovotransferrin [30]. This particular function is being explored in plant–dairy protein mixed systems since plant proteins are highly susceptible to self-aggregation [31]. Enhanced water solubility of hydrophobic proteins by association with chaperone proteins is a challenging strategy adopted to change the unfolding-folding pathway of binary proteins in order to assemble novel protein composites [30]. Molten globules (i.e. proteins with a native-like secondary structure but disordered tertiary structure) obtained in high-alkaline environment can be exploited to promote hydrophobic effects between heteroproteins, forming novel water-soluble protein hydrocolloids at neutral pH [32,33]. Relying on such an approach, several proteins such as rice proteins (mainly rice glutelin) (Wang, Chen et al., 2019), wheat prolamins [33] and scallop myofibrillar proteins [34], generally insoluble in water, were prepared into water-dispersible composites. One major finding was that the co-folded secondary structures had a weakened structural folding compared to each individual constituent. Another alternative to stabilise plant proteins alone (or plant–dairy protein mixtures) can be to use inorganic particle-based stabilisation (e.g. proteins adsorb onto titanium dioxide nanoparticles) [35]. Functional assemblies, such as dispersible and/or heat-resistant microparticles, may also be formed by combining heat-treatment and pH to

co-precipitate whey and pea proteins, or egg white and soy proteins [25,26].

To sum up, many researchers are currently searching for alternatives to create plant proteins-based supramolecular assemblies with both tuneable structures and high dispersibility. Behind the classically proposed chemical and enzymatic treatments, optimized mixing of plant and animal proteins (e.g. dairy proteins) could be a promising alternative way to improve solubility and functionality of food proteins in a context of sustainability.

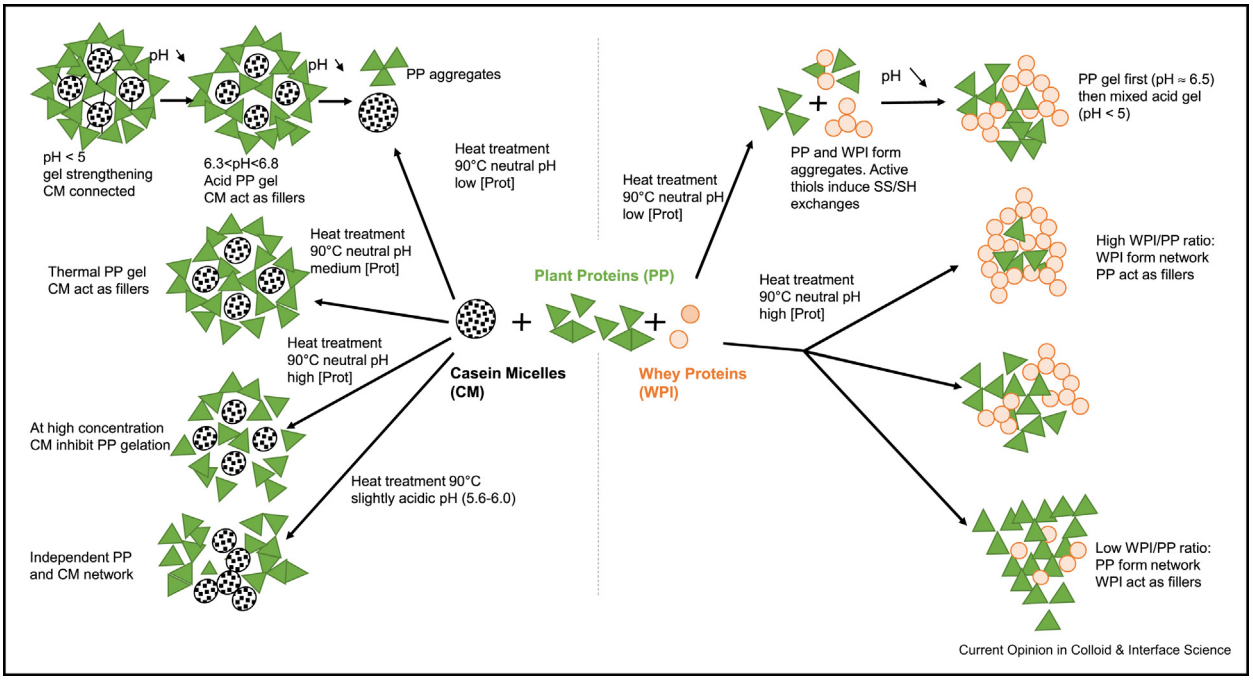
Gelation

A substantial number of studies have investigated the gelation of plant–dairy protein mixtures, and mainly used plant protein isolates or the non-precipitated fraction after centrifugation. Recent reviews have extensively reported on studies that mixed plant and milk proteins to form gels [6,7,36,37]. In most cases, the plant fraction was soy or pea proteins, although wheat, canola, sunflower or hemp proteins have also caught attention. Heat-induced and cold-set gels are both based on the heat-induced denaturation of globular proteins, which form primary aggregates through thiol/disulfide exchanges and/or hydrophobic effects. In general, these aggregates further extend, upon further heating or become insoluble upon acidification or salt addition. This general mechanism applies as well to gels made of plant–dairy protein mixtures, even though the final microstructure and texture of such mixed gels depend on the type of ingredients that are involved (Figure 2).

Heat-induced and cold-set gels

When only casein micelles are mixed with plant globular proteins to form gels, the resulting networks are often weaker and less homogeneous i.e. with larger pores, than for their single-ingredient counterparts, especially when the casein micelles alone are in a sufficient concentration and at an adequate pH (i.e. pH < 6.0) to sustain the formation of a gel [38]. When mixing micellar casein and soy protein isolate (1/1, w/w) in water at pH 7.0, the resulting heat-induced gels exhibited lower yield stress and lower elastic and viscous moduli than those of a gel made of only soy proteins. By systematically varying the respective concentrations of micellar casein and plant protein isolates (pea or soy), Schmitt et al. (2019) unravelled an antagonistic effect of the mixture on both the elastic moduli and the connectivity of the heat-induced gel [39]. Similarly, the heating of pea legumin or pea protein isolate with casein micelles yielded gels with low storage moduli (G'<10Pa) upon acidification, due to very large and insoluble aggregates, although heated mixtures of vicilin and casein micelles led to

Figure 2



Schematic diagram of gelling behaviours of plant proteins (mainly soy and pea proteins, green triangles) in mixed system with whey proteins (orange spheres) or casein micelles (black–white spheres) during heat treatments and/or acidification, according to the studies by Mession et al. and Alves et al. [24,36].

stiffer gels [24]. The antagonistic effect of mixing legume proteins and caseins was accounted for by the withdrawal of calcium from casein micelles by the legume proteins (or by residual phytates that are naturally present in legume seeds), and by the chaperone and/or hindrance effects that caseins exert on the aggregation of heat-denatured globular proteins [6,38]. In these studies, electrophoresis analyses showed that although casein micelles could interfere with the heat-induced denaturation and aggregation of the globular plant proteins, no heat-induced interactions could be identified that involved both plant proteins and κ-casein, probably because of the lack of reactive free thiols in native legume globulins. A lack of interactions between caseins and hemp proteins upon heating was reported by Chuang et al. (2019) with heat-induced (90 °C, 15min, I = 0.5M) mixed aggregates of: (a) κ-casein and hemp proteins, by thiol/disulfide exchanges, owing to the presence of a free thiol group on the latter; (b) hemp proteins and αS1- and β-caseins by hydrophobic effects [31]. In contrast, when plant-dairy protein mixtures involve whey proteins, the resulting networks generally exhibit firm textures and homogeneous microstructures with no phase separation. Heat-induced gels made with mixtures of milk and pea proteins (1/1, w/w) were, for instance, as firm as pea protein gels and firmer than gels made with skim milk powder alone [40]. In acid-induced gels, as the major type of cold-set gels, preheated mixtures of casein micelles, whey proteins and soy proteins gelled at higher pH and yielded firmer networks than when only the casein micelles and soy proteins were present [36]. Unheated mixtures, or separately heated globular proteins and casein micelles, rather make coarse and soft gels with little water holding capacity, although the presence of soy or pea proteins promotes an early onset of gelation [6,41]. It has been proposed that in the presence of the heat-sensitive and thiol-containing β-lg (and possibly also of bovine serum albumin) promotes thiol/disulfide exchanges with the other globular proteins and with κ-casein upon heating, thus favouring protein aggregation and the formation of firm homogeneous gels [36]. The size and interactions involved in the aggregates depend on the protein composition (respective hydrophobicity and content in cysteine), heating temperature (respective denaturation temperatures of the individual proteins) and ionic strength. The presence of casein micelles may interfere with the aggregation of the globular proteins, possibly through a preferred pathway involving β-lg and κ-casein [36] and/or through the already mentioned chaperone activity of caseins.

Compared to the current knowledge on the gelation of either milk or plant proteins, research on the gelation mechanisms and gel properties of their mixtures is only emerging. The wide variety of ingredients, especially on the plant side, calls for systematic studies that would help identify the required properties to be sought when selecting a plant protein ingredient for a given application. The denaturation temperature, surface hydrophobicity when uncoiled, presence of thiols or cystines, interactions with calcium or other minerals, occurrence of pre-aggregates in the used concentrate or isolate, are all factors of interest. A promising research perspective lies in the development and expansion of a detailed database regarding how the physico–chemical properties of plant proteins, especially the denaturation behaviour as well as the presence and reactivity of thiols, influence their interactions with animal-derived proteins upon heating, which could support rational choices upon benchmarking [42]. Thorough investigation of the heat–aggregation pathways in plant-dairy protein mixtures are also needed, for example, by using thiol-blocking agents [43,44], reduction of disulfide bonds [45], or modified proteins with additional or deleted cysteines, in order to better understand the relative action of different globular proteins upon interactions and gelling.

As previously mentioned, the plant protein composition, structures and concentration may largely differ depending on the isolation process, especially in the wet fractionation steps where organic solvents and/or acidification are involved. For instance, uncontrolled aggregation of the plant ingredient was shown to prevent co-gelation and rather promote phase separation of co-heated whey and soy proteins [36,46]. Furthermore, the possible role of some of the components of flours or concentrates that are usually eliminated upon isolate purification, such as some proteins, polysaccharides and fibres, on the textural properties of mixed gels, has thus been under-investigated so far. Studies relating plant-protein fractionation and plant–dairy gel properties recently emerged [43,44]. It was shown that the use of moderately purified plant fractions obtained through mild processing opens perspectives for the conception of plant–dairy protein-based gels with good gelation and textural properties. Depending on the fractionation process and purity of the pea proteins, 50wt.% substitution of the whey proteins by a pea protein fraction can lead to heat-set gels with similar gel strength compared to that of the pure whey protein gels [43]. A greater gel strength can even be achieved when using an albumin fraction of pea proteins or a less purified pea protein concentrate [44]. It appears that fractionation of pea proteins by using high temperature or pH values far from the neutrality leads to reduced protein solubility and to uncontrolled pre-aggregation that impairs the gelling capacity [43].

Enzymatically-induced gels

Enzymatically-induced mixed gels have comparatively been only scarcely investigated, in spite of the potentially wide biodiversity of both substrates and enzymes. As a

derivative of the cheese-making process, chymosin (or rennet), largely used as a coagulant of the caseins, combined with acidification yielded higher gel firmness and lower syneresis when applied on a mixture of preheated soy and unheated milk, compared to milk alone. Interestingly, Corredig et al. showed that chymosin could hydrolyse soy protein particles in a non-specific way and increase the elastic modulus of acid-soy protein gels. However, the renneting process alone, that is, hydrolysis of the κ-casein, at the specific bond Met_{105}-Phe $_{106}$, and aggregation of the resulting para-κ-casein (κ-casein f(1–105)) micelles is unlikely to support the formation of a mixed gel, because the reduced concentration of casein micelles compromises the gel's connectivity [36]. Therefore, chymosin may be used in combination with a cross-linking enzyme. For example, chymosin and transglutaminase jointly applied to unheated and non-acidified mixtures of pea or soy proteins, added to casein micelles or skim milk, yielded gels that were softer than when only the individual protein components were used [40] but could exhibit finer texture with smaller pores and improved water holding capacity [47]. Transglutaminase has been shown to crosslink whey and soy proteins, provided that protein structures have been opened by an ultrasonic pre-treatment, and lead to dense and uniform protein gels [48]. Such an approach seems thus useful to overcome technological limitations, but it should be pointed out that the use of transglutaminase, which has not been traditionally applied in dairy technology, may raise questions regarding consumer acceptability. Other physical treatments, such as high-pressure processing, microwaves and ultraviolet light are emerging tools prior to the action of transglutaminase [49] and other enzymes [50]. These treatments trigger the unfolding and thus the degree of exposure of reactive sites in protein molecules, which in turn, reinforces the strength of the gels, their elasticity, density and homogeneity of the gel microstructure after the reaction with transglutaminase [49]. They have the advantage to be environmentally friendly and eco-efficient methods, and to avoid the use of additional chemicals to form the gels. They can also be applied to decrease protein allergenicity [51], to increase water holding capacity and sensory attributes (appearance, colour and flavour) of protein-based food products [49]. Although such physical methods, and notably high pressure treatment, in combination with transglutaminase have up to now been applied to individual animal or plant protein ingredients (typically, whey and soy proteins). It is very likely that they will be used on mixed systems in the near future. Other food-grade enzymes, such as laccase, peroxidase or tyrosinase, have been tested on plant or dairy proteins, but not yet on their mixtures, to seek new or improved gelation properties [52,53].

Microorganisms can also provide food-grade enzymes of interest to ferment raw materials of animal and plant origin. In particular, proteolytic enzymes can largely affect the texture of processed gels, for example cell-envelope proteinases. The peptides produced can then alter the overall gel texture [54]. In addition, since microorganisms such as lactic acid bacteria can acidify the medium, the changes in physico–chemical conditions during fermentation tend to also favour cold gelation of proteins. By involving many different enzymes, fermentation can be used as a toolbox to provide a broad range of proteolysis reactions, as well as other relevant reactions. For instance, some enzymes are able to decrease the content in phytates or polyphenols during plant protein isolation or product processing, as a means to avoid undesirable side effects, such as uncontrolled formation of globular protein and/or protein-quinones aggregates during pH cycles [52,53]. Other bacterial enzymes (e.g. enzymes implied in the vitamin synthesis, endo- or amino-peptidases and amino-transferase) can enhance the nutritional properties of the products by increasing the vitamin content. Furthermore, they can produce sapid molecules such as peptides and free amino acids and reduce the well-known off-flavours of plant protein ingredients, that is, beany, green or chalky markers (due to e.g. alcohol dehydrogenase), which is one of the main issues for the implementation of large amounts of such ingredients in food products. This strategy has recently been identified as useful to mitigate sensory defects in mixed systems including dairy and legume proteins (from pea or lupin) [55,56].

Protein fibrils

As an alternative pathway, producing amyloid fibrils from plant and milk globular proteins could open perspectives for the design of new food gels. Assembling proteins into amyloid fibrils has been achieved for animal proteins such as whey or egg proteins, and many plant proteins, including zein, rice albumin and globulin, wheat gluten and gliadin, soy and pea protein isolates, β-conglycinin and glycinin, and many vicilins [57]. These fibrils produced by prolonged heat treatment, mostly in strongly acidic conditions with a low-salt content, are assemblies of peptides derived from protein hydrolysis in a cross-β pattern, in which β-sheets and β-strands lay parallel and perpendicular to the fibril axis, respectively [57,58]. Depending on conditions such as temperature, pH, protein concentration, stirring, heating time, and protein origin, size, morphology and rigidity of the fibrils can be tuned. Owing to a high aspect ratio and high stiffness and stability, they can be used in dispersions for their techno–functional properties such as foaming, emulsifying, gelling or thickening [57,58]. Whey, soy, rice proteins or caseins form fibrils with specific rheological properties in solutions or gels at extremely low concentrations [57]. Promising results could be achieved in this field, in future prospective studies that

would mix animal and plant proteins to form mixed fibrils. Systematic investigation may be needed to review and evaluate the properties of proteins that facilitate fibrillation, such as their structural order or flexibility, their surface hydrophobicity or other possible structural requirements to achieve compatibility between the plant and the animal protein to be co-fibrillated. Although some authors reported a high resistance of amyloid fibrils to acidic pH values, heat treatment and to some chemicals [57], aggregation and collapse of the fibrils near their iso-electric point and their resistance to disintegration at pH values above 6 is probably a point to be addressed for their potential use in food products [58]. Last but not least, the question of the digestibility, safety and health risks of amyloid fibrils, through *in vitro* and above all, *in vivo* studies are also one of the key subjects of upcoming studies [58,59].

Foams and emulsions

Foams and emulsions are interface-dominated systems, where the microstructure and properties of the interfacial layer control to a great extent the physical stability and macroscopic physical properties of the systems. In foams and oil-in-water (O/W) emulsions stabilised by protein mixtures, besides the adsorbed proteins that have a direct effect on interface stabilisation, the non-adsorbed proteins (present in the continuous phase) may also have a significant impact on their physical and chemical stability.

Air–water interface and foams

Many proteins form viscoelastic layers at fluid interfaces and provide physical stabilisation to bubbles and emulsion droplets. Dilatational rheology, performed with a drop tensiometer or Langmuir trough, provides valuable insights in the viscoelastic properties of adsorbed protein layers. For plant–dairy protein mixtures, the interfacial dilatational rheological properties deviate from what would be expected when simply assuming additivity of those of the individual proteins. For instance, mixing pea proteins with sodium caseinate led to air–water interfacial films with the same interfacial stiffness as for pea proteins only, but with increased stiffness compared to sodium caseinate-based films only. This was explained by the ability of pea proteins to retain the disordered sodium caseinate at the interface upon compression [60]. Mixing pea proteins with whey proteins hindered the formation of a stiff interconnected network, typical for whey protein-based interfaces [60], and thus reduced the stiffness compared to whey protein films only, but did improve the interfacial layer's strength compared to that of the pea protein-stabilised interfaces. Another study showed that at pH 7.0, soy β-conglycinin in combination with β-lg resulted in a synergistic behaviour where the mixture outperformed its individual constituents (i.e. enhanced interfacial stiffness, smaller foam bubbles and increased physical stability) [61]. In contrast, the combination of soy glycinin with β-lg led to an intermediate functionality between that of the individual proteins. It was suggested that strong associative interactions with β-lg, as observed in the bulk for conglycinin, but not for soy glycinin, are responsible for the synergistic behaviour. This points out the importance of characterising and understanding the behaviour of such protein mixtures in aqueous solutions/suspensions, to be able to understand their compatibility when it comes to applications in multiphase colloidal systems.

In the previous examples, plant and dairy protein solutions were simply mixed together. Recently, an alternative approach used co-precipitation of protein dispersions at their isoelectric point to produce mixed protein isolates, which were subsequently used in further experiments [25,62]. When comparing simple whey pea protein mixtures versus co-precipitates, the protein mixtures were superior in foaming capacity (foam overrun) compared to that of the co-precipitated proteins, which is probably due to the higher solubility of pea proteins when they have not been subjected to co-precipitation [62].

Overall, it seems that whey proteins and plant–dairy protein mixtures have better abilities to stabilise air–water interfacial films compared to those of the plant proteins isolates alone (typically, globulin-containing soy or pea fractions). Plant protein isolates contain non-proteinaceous components such as polyphenols, carbohydrates and lipids, which is inherent to the biological origin and functions of these different proteins and typically leads to protein ingredients with a much lower extent of purification compared to dairy proteins. These non-proteinaceous materials may interfere with the interfacial behaviour of proteins and decrease the resulting interfacial network strength. For example, mixing phenolic components and whey proteins, the presence of sinapic acid reduced the interfacial stiffness and foam stability [63]. Furthermore, residual lipids were shown to reduce the interfacial stiffness of films made of rapeseed proteins [64], but did not decrease the foam stability, which illustrates that non-protein components may not be necessarily detrimental when it comes to macroscopic product properties and applications.

Oil–water interface and emulsions

Physical stability

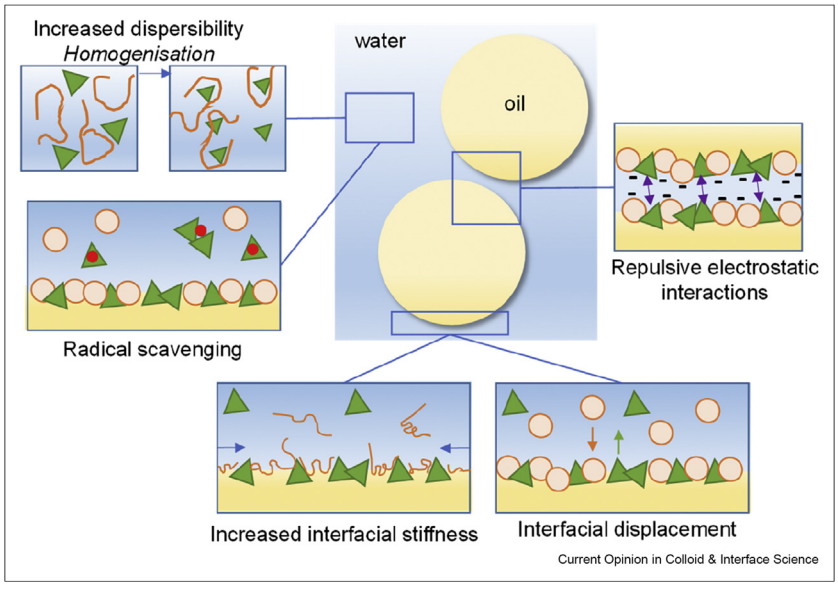
Whereas only little is known about the stabilising effect of plant–dairy protein mixtures at the air–water interface, more information is available regarding their behaviour at the oil–water interface. Using model interfaces, it was shown that WPI-stabilised interfaces had superior stiffness compared to pea protein-stabilised interfaces [60,65]. The strength of the layer decreased

when whey proteins were mixed with pea proteins. Sodium caseinate, a disordered protein, forms mechanically weak interfacial layers, but mixing it with pea proteins improves the mechanical strength of the layer. Furthermore, it was shown that mixing sodium caseinate and pea protein isolate increased droplet resistance against coalescence compared to that of the sodium caseinate only [60,65,66]. A synergistic behaviour was found when using plant–dairy protein mixtures (caseins or whey proteins with pea proteins) for emulsion stabilisation. This was attributed to various mechanisms such as decreased depletion interactions due to enhanced plant protein solubility [67], increased electrostatic repulsions between the adsorbed proteins [66] and increased stiffness of the interfacial layer [65,66,68] as depicted in Figure 3.

For mixtures of pea or soy proteins with sodium caseinate, an antagonistic behaviour was observed regarding the physical stability of emulsions [65]. In this study, the protein concentrations were chosen such that the non-adsorbed concentrations were low. The authors hypothesised that the interface was dominated by caseins that prevented further adsorption of plant proteins, which resulted in weak interfacial layers. This means that in protein blend-stabilised emulsions, interfacial composition studies are needed to verify the composition of the interface and thus the contributions of the individual proteins. When using a binary mixture of soy or pea proteins with caseins or whey proteins, both dairy and plant proteins co-adsorb at the interface in the protein-rich regime (i.e. protein concentration largely higher than the amount needed to cover the oil-water interfacial area), and thus contribute to interface stabilisation [62,66,67,69]. Yet, in time, the ageing of the blend-stabilised interfaces led to interfacial rearrangements and to displacement of some of the initially adsorbed proteins [66,70]. More specifically, whey proteins were able to partly displace pea proteins from the interface, which were themselves able to displace caseins. This displacement seemed to be driven by the increase in interfacial elasticity after displacement rather than by a decrease in the interfacial tension [70]. This was explained by the removal of water from adsorbed proteins and in-plane protein–protein interactions, responsible for the high interfacial elasticity, that would be thermodynamically more favourable compared to interfacial tension changes. Clearly, such emulsions are strongly non-equilibrated systems; although the applied fabrication process determines their physical organisation to a large extent, this organisation may evolve over long time scales (in the order of magnitude of at least days).

At high continuous phase protein concentrations, adsorption and protein–protein interactions are facilitated, which promotes the establishment of an interconnected protein network. A prerequisite for interfacial displacement is thus a sufficiently high concentration of proteins with displacement capacity

Figure 3



Schematic representation of mechanisms relevant for ensuring the physical and oxidative stability of emulsions stabilised with plant–dairy protein mixtures: increased dispersibility of the plant proteins [67], radical scavenging ability of plant proteins [72], increased interfacial stiffness [60] and increased repulsive interactions [66]. Interfacial displacement may impair stabilisation mechanisms of protein blend-stabilised emulsions [70]. Plant proteins (green triangles), whey proteins (orange spheres), caseins (orange loops), free radicals (red spheres).

in the continuous phase. This criterion is often met in food-relevant emulsion formulations, where the protein concentration greatly exceeds what is strictly needed to stabilise the oil–water interface – mostly to comply with product specifications. Interfacial rearrangements may thus be ubiquitous in most food emulsions. So far, there is limited work available on this topic for plant–dairy protein blend-stabilised systems, and on the properties of the compositionally complex interfaces. Interfacial rearrangements in such blend-based emulsions are probably important to consider, since the integrity of the interfacial network could be compromised, possibly leading to physical destabilisation of emulsions over time.

In the protein-rich regime, emulsions with small droplet sizes and high long-term stabilities can be formed using plant–dairy protein mixtures, suggesting that droplets are efficiently stabilised during the homogenisation process, which typically occurs within short timescales. However, in the protein-poor regime (i.e. when the protein concentration is limiting, and thus determines the droplet size), larger droplets were observed for blend-stabilised emulsions, compared to emulsions stabilised with dairy proteins only, suggesting rapid droplet recoalescence during homogenisation or very shortly thereafter [66]. Probing emulsion stability at relevant timescales (typically 10^{-3} to 10^{-1} s) is inherently difficult because most conventional methods allow for studying only longer-term stability. Using microfluidic devices is a promising approach in that respect. For instance, experiments were conducted with microfluidic chips including a cross-flow junction to form oil droplets in a protein solution as continuous phase. This was followed by a channel comprising constrictions that allowed the characterisation of the deformation and relaxation of droplets, or a larger channel where droplets may collide and possibly coalesce. This work showed that whey proteins were able to rapidly adsorb (within 0.16 s) at the surface of oil droplets, providing sufficient interfacial stiffness to protect them against coalescence. Conversely, when using the same total protein concentration with a whey–pea protein mixture, adsorption was slower and is not complete enough to lead to sufficiently strong in-plane interactions between adsorbed proteins to prevent droplet coalescence [71].

To design physically stable emulsions using plant–dairy protein mixtures, the total protein concentration and the distribution of the different proteins between the available environments of the system need to be considered. At high enough protein concentration, high surface loads and densely packed interfacial layers can be formed with a high resistance against deformation, which thereby protect emulsion droplets against rapid and long-term coalescence. However, proteins present in excess in the continuous phase may lead to depletion interactions and interfacial rearrangements may occur, which can alter the interfacial stiffness and thus droplet stability.

Oxidative stability and bioavailability of hydrophobic components

Next to the physical stability of emulsions, their oxidative stability (i.e. oxidation of polyunsaturated fatty acids or of other bioactive hydrophobic ingredients) are important topics when it comes to emulsion design. For example, emulsions stabilised by a whey–soy protein blend led to a higher stability of lycopene (a carotenoid) compared to emulsions stabilised with either of the proteins used alone [65]. For sodium caseinate–pea protein-stabilised emulsions containing curcumin [73] or lycopene [65], chemical degradation was lower compared to that of the emulsions stabilised with sodium caseinate only, which was related to the high antioxidant properties (e.g. metal chelating activity) of caseins. When present in excess in the continuous phase, proteins can chelate metal ions and scavenge free radicals, which improves the chemical stability of the emulsions. This was illustrated by Feng et al[72], who showed that excess soy proteins reduced lipid oxidation in whey protein-stabilised emulsions more compared to excess whey proteins. This may be attributed to different properties of the proteins themselves (metal-chelating and free radical-scavenging activities), or to the presence of non-protein antioxidant molecules in soy protein isolate (such as traces of phytic acid and phenolic compounds). This, again, illustrates that not only the interfacial structure needs to be considered, but also the continuous phase protein concentration and composition. Finally, when targeting food applications, it is important to also consider the fate of emulsions in their ultimate environment, i.e., the consumer's gastrointestinal tract. For instance, the in *vivo* bioaccessibility of lipophilic bioactive compounds may be reduced by the presence of plant proteins, as suggested for emulsions based on a sodium caseinate–pea protein blend, due to the lower digestibility of the plant proteins [73]. Such validations will probably be important for future applications, and plant-dairy protein mixtures could be investigated as a potential means to circumvent specific drawbacks related to the use of plant proteins only.

Emulsion-based matrices

Protein mixtures have been used to formulate emulsions that are subjected to macroscopic post-emulsification structural changes, for instance spray-dried infant formula [74] or emulsion gels [37,39,40]. In infant formula, when whey proteins were partly (50wt.%) replaced by pea or faba bean proteins, larger powder particles were formed upon spray drying, with low dispersibility, resulting in a higher degree of flocculation in the reconstituted formula compared to the control sample (whey proteins only [74]). The poor solubility was assumed to be a result of the high-thermal load during

the process leading to extensive plant protein aggregation [74]. In all infant formulas tested, the emulsion droplets were stable upon processing and storage, which may be explained by the high concentration of whey proteins present, which is sufficient to stabilise the interface without the contribution of the plant proteins.

Micellar casein can be successfully replaced by plant proteins in emulsion-filled gels without decreasing the gel stiffness, which was not the case for gels formed in the absence of oil [37]. In pea, soy (individual or mixed with dairy) protein blend-stabilised emulsion gels (acid- or enzymatically-induced), the protein-stabilised droplets behaved as active fillers and reinforced the gel firmness [39,40]. The adsorbed proteins at the oil–water interface interact with the protein network in the continuous phase, and thus contribute to the gel strength. Similar observations were recently made in emulsion-filled gels prepared with various plant proteins (from pea, lupin or oat) in combination with dairy proteins (skimmed milk, whey proteins), where extensive bridging between the oil droplets occurred [75]. In another study, it was shown that whey protein microgels (i.e. protein particles) were able to adsorb at the oil–water interface in emulsions and to thereby bridge neighbouring lipid droplets, leading to flocculation and the formation of a continuous network [76]. Plant proteins can often be regarded as particles owing to the combination of large native supramolecular structures, and thermo-aggregation upon the processes applied to make protein isolate. This feature may also lead to droplet flocculation and formation of an emulsion gel, which could be purposely used and tuned in food product design.

Conclusions and future outlook

Plant–dairy protein mixtures have been gaining interest in food research lately, especially for systems based on pea or soy proteins blended with dairy proteins to form gels and emulsions. We highlighted the importance of understanding protein–protein interactions in aqueous media as a result of the molecular and supramolecular properties of proteins. The subsequent assembly of proteins in an aqueous phase into aggregated structures can be linked to their functional properties in more complex applications (in particular, gels and interface-dominated systems). Furthermore, additional effects such as interfacial rearrangements need to be considered for the stability upon ageing of the products.

Besides pea- or soy-based protein fractions, there is also a need for increased knowledge on plant proteins from oilseeds, as a means to valorise extraction meals, preferably with a nonsolvent-based extraction, and thereby produce plant protein isolates from by-products. In general, the use of minimal transformation-based processes has been gaining interest for plant protein extractions, resulting in less purified plant protein ingredients. Therefore, when it comes to plant protein functionality, the contribution of non-proteinaceous components should not be overlooked in the design of plant–dairy protein mixed systems. The components that interact with proteins (e.g. polyphenols, lipids) or with calcium (e.g. phytates) seem quite essential in that respect; for instance, in the latter example, calcium is essential in dairy products for both functional (structuring, texture) and nutritional reasons.

To conclude, despite some intrinsic limitations for using mixtures of dairy and plant proteins (and notably the fact that they are out of scope for e.g. the vegan market), such mixtures still seem to hold a great potential in the food science field. In particular, they may be instrumental in mitigating many issues currently encountered when using plant proteins only to form and stabilise colloidal systems, such as physical destabilisation, off-flavours, oxidative stability, and potentially lower nutritional quality. Future work to thoroughly characterise the composition of the selected plant protein ingredients (in particular, regarding non-protein components), and their interactions with dairy proteins, will be important next steps from research and application perspectives.

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Declaration of competing interest

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