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Mixing plant-based proteins: Gel properties of hemp, pea, lentil proteins and their binary mixtures

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ABSTRACT

One of the challenges in substituting dairy products by alternative proteins is that the properties of mixed protein gels cannot necessarily be predicted by those of single protein gels, whereas the need of mixing is often driven by nutritional aspects. However, mixing plant proteins could also open a door to new textures.

The main goal of this study was to investigate the impact of binary mixing of hemp (H), yellow pea (P), and brown lentil (L) protein concentrates/isolates on their gel and water-holding properties. Dispersions of reconstituted proteins and mixtures thereof were gelled using glucono-δ-lactone (GDL), transglutaminase (TG), and temperature (T) at a protein content of 12% (w/w).

Mixtures of pea and lentil proteins showed gel strengths for TG- and T-induced gels that are proportional to the ratio of the mixture constituents (linear mixing behavior), whereas synergistic effects were observed for GDL-induced gelation. In contrast, all mixtures containing hemp exhibited a non-linear mixing behavior for the three gelation methods, usually resulting in lower gel strengths compared to theoretically expected values.

The study showed that mixing plant-based proteins of different protein sources can lead to very different mixing behaviors in terms of gel properties, showing either a reinforcing, an indifferent or a weakening effect compared to the theoretically expected properties. The results can help developing more targeted plant protein-based soft gel products such as yogurt alternatives with specific techno-functional properties, while adjusting the nutritional characteristics.

1. Introduction

The demand of consumers for alternatives to dairy products has significantly increased due to ethical, ecological, or allergy-related reasons. It is recognized that dairy products can be replaced with plant-based alternatives and a wide range of vegetable protein sources have recently emerged (Grasso et al., 2020). Plant-based products can be categorized in cereal- (oat, rice, corn, and wheat), legume- (soy, peanut, lupin), nut- and drupes- (almond, pistachios, walnut, hazelnut, coconut), seed- (sunflower, sesame, hemp, flax) and pseudo-cereal- (amaranth, quinoa) based products (Sethi et al., 2016). These sources are often commercialized as milk alternatives or, further processed and gelled, for example as yoghurt- or quark-like products. However, due to a limited protein content in such products, most of these soft matter gels include thickening ingredients such as starches and/or further gelling agents like agar-agar, pectin, or natural gum (Grasso et al., 2020; Guo & Yang, 2015). Alternatively, proteins can be extracted to obtain protein concentrates or isolates which can also be used for the generation of milk alternative products.

Gelation of proteins requires either a three-dimensional conformational change (correlating with a change in surface hydrophobicity), an alteration of electrostatic interactions, formation of cross-links or a combination thereof (Totosaus et al., 2002). Glucono-δ-lactone (GDL) is often used to reduce electrostatic interactions by steadily decreasing the pH which, in turn, reduces the net charge of the proteins to approach the isoelectric point (i.e. where the protein net charge is zero), and changes the protein–protein and protein-solvent interactions (Banerjee & Bhattacharya, 2012). GDL could also be replaced by lactic acid fermentation in case of a food product application but it could also impact the specimen texture by protein degradation and/or exopolysaccharide

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Abbreviations: GDL, Glucono-δ-lactone gels (pH-induced gels); H, Hemp protein; H-P, Mix of hemp and pea proteins; L, Lentil protein; L-H, Mix of lentil and pea proteins; P, Pea protein; PDB, RCSB Protein Data Bank; pI, Isoelectric point; P-L, Mix of pea and lentil proteins; T, Temperature gels (heat-induced gels); TG, Transglutaminase gels (enzyme-induced gels); WHC, Water holding capacity.

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formation. Transglutaminase (TG) can induce covalent cross-links between proteins or within the protein by chemically linking the amino group of lysine and the carboxamide group of a glutamine residue (Banerjee & Bhattacharya, 2012). In the food industry, transglutaminase is used to catalyze the cross-linking of plant proteins such as soy, gluten or myosin. It can also be used to encapsulate lipids, form heat resistant films, improve elasticity or other functional properties (Zhu et al., 1995). Thermal treatment (T) at high temperatures is often used to gel proteins due to altered tertiary structures. Once unfolded, the proteins expose additional non-polar residues which can interact with other non-polar residues resulting in the formation of a non-covalent gel network (Munialo et al., 2018). Heat treatment can also induce the formation of cross-links via disulfide bonds, often in combination with the unfolding and exposure of thiol groups to the surface of proteins (Wang & Damodaran, 1990; Zha et al., 2021).

Regarding the gelling properties of plant protein mixes, Lin et al (2019) showed that a combination of rice protein isolate and peanut protein isolate formed a stronger gel than rice protein isolate alone. The authors showed that the driving forces were hydrophobic interactions and disulfide bonds. In contrast, nonspecific associations, ionic and hydrogen bonds showed a minor contribution to gelation (Lin et al., 2019). It was hypothesized that the molecular weight of proteins played an important role due to not only their three-dimensional structure, but also the physical constraint as a result of their size and shape (Wang & Damodaran, 1990). The approach of mixing different protein sources was already investigated with animal and vegetal proteins together, and some gel attributes such as gel strength, gel stiffness and water holding capacity (WHC) were affected (Alavi et al., 2020; Ben-Harb et al., 2018; Jose et al., 2016; Wong et al., 2013). In another study, a mixture of pea and whey proteins showed a synergistic interaction at pH 6. The authors hypothesized that repulsive and attractive forces within the system were in an optimal balance for co-gelation at this particular pH (Wong et al., 2013). Most reports support that the gelation technique plays a crucial role in the mechanical stability of the gels.

The extraction of proteins can also have an impact on their gelling capacity. For example, Yang et al. (2021) showed that the extraction methods (alkaline extraction followed by isoelectric precipitation or ultrafiltration; salt extraction followed by ultrafiltration or dialysis; micellar precipitation; air classification) had a strong impact on the protein composition of the pea protein extracts, leading to different techno-functionalities (e.g. solubility or gelling capacity) what is probably the case for most protein sources. Another way to tune some of the protein techno-functionality during extraction is to perform a mixed extraction as Alu'datt et al. (2012) showed with their combined extraction of whey protein and soy protein. It led to improved WHC as well as higher gel strength compared to the isolates alone. Macroscopic properties as well as the microstructure and aggregation dynamics were also found to correlate with the physicochemical and mechanical characteristics, inherent to each gelling method and plant protein. A recent study showed that whey protein mixed with rapeseed protein could either exhibit mutual protein interactions, causing a mixed microstructure, or they can form two protein structures independent from each other within one gel, depending on the gelling conditions (pH, concentration, ratio of the proteins) (Ainis et al., 2018).

Such mixtures could serve as basis for soft gel milk alternative products such as yoghurt, fresh cheese or panna cotta. Eventual synergistic interaction could be economically beneficial if they would lead to a reduction of the required amount of proteins in a product. For the present study, hemp (*Cannabis sativa*), yellow pea (*Pisum sativum*), and brown lentil (*Lens culinaris*) protein isolates were selected as they can well grow in Switzerland. In addition, hemp protein has a beneficial nutritional quality and can be extracted from hemp meal, a side product of the hemp oil extraction, thus giving a revalorization to this side stream. Lentil was selected due to its high protein content and pea as it is rather cheap protein source.

The protein quality, in particular the amino acid profile, differs strongly between animal and plant-based proteins. The use of animal proteins leads to products generally having a higher proportion of essential amino acids compared to plant-based proteins (32–44 %, compared to 20–35 %) (Gorissen et al., 2018). A centuries-old strategy to improve the nutritional value of a product is to mix proteins of different sources in order to balance out deficiencies. For example, it is reported to be beneficial to combine peas and lentils, which proteins are lysine-rich, with cereal grains, which have a complementary essential amino acid profile (i.e. poor in lysine but with high levels of sulfur amino acid) (Lam et al., 2018). Hemp protein has been described to have a superior nutritional amino acid profile with its wide range of essential amino acid profile and with a considerable amount of glutamine and arginine (Khazaei et al., 2019; Wang & Xiong, 2019). Peas and lentils contain relatively low amounts of sulfur-containing amino acids and of all the essential amino acids, except lysine and leucine (Khazaei et al., 2019).

In this work, we investigate binary mixes of hemp, pea and lentil proteins (H, P, L, respectively) at different ratios and with three different gelation strategies (thermal treatment, acidification and enzymatic crosslinking; T, GDL and TG, respectively) and investigate the impact of mixing on color, gel strength and water holding capacity. Fig. 1 depicts the process from the protein extraction to the analysis performed.

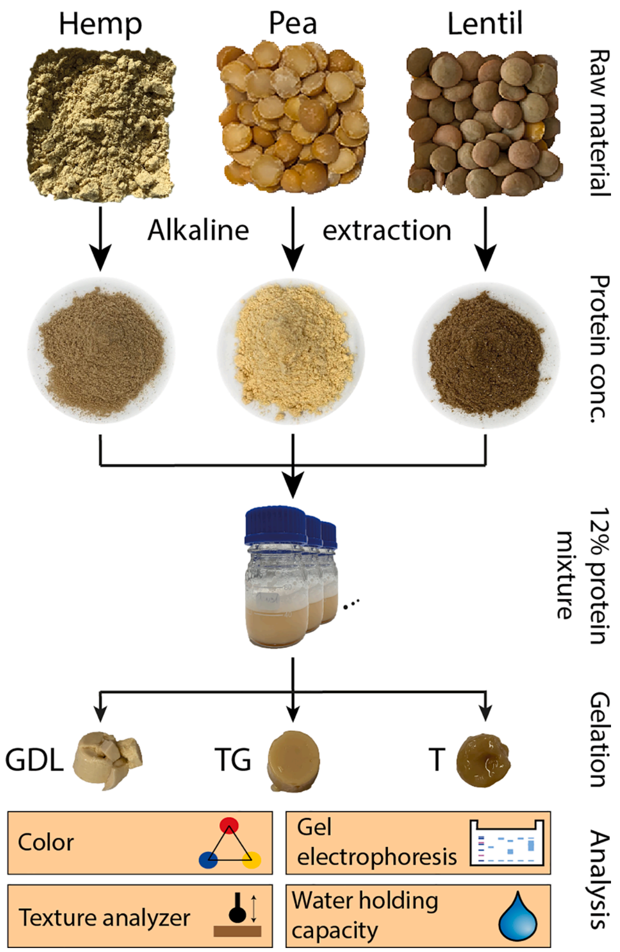

Fig. 1. Representation of the experimental workflow performed in this study. Protein extraction from dried material (pea, lentil) and from hemp meal by alkaline solubilization followed by isoelectric precipitation. Preparation of 12% (w/w) protein mixtures, acid-, enzyme- and heat-induced gelation. Gel characterisation by color, gel electrophoresis, texture analysis and water holding capacity.

2. Materials and methods

2.1. Material

Yellow peas and brown lentils were purchased from Coop (NaturaPlan, Switzerland). The dried peas and lentils were ground in a knife mill (GM 200, Retsch, Germany) for 40 s at 8,000 rpm followed by 30 s at 10,000 rpm. The powder was then passed through a 0.5 mm sieve. Hemp meal was obtained from SwissTop GmbH (Zurich, Switzerland) after oil pressing and grinding. Chemicals were purchased at Sigma-Aldrich, Buchs, Switzerland unless stated otherwise.

2.2. Defatting

Hemp meal was mixed with heptane at a 1:5 powder to heptane ratio (w/w) in a glass bottle. The bottles were placed on a shaking table at 180 rpm overnight at room temperature. The defatting conditions were set based on preliminary experiments (data not shown). The defatted meal was recovered on a Buchner filter with 20–25 µm pore size and dried in a 37 °C oven for 16 h. Peas and lentils were not defatted before protein extraction because the fat content was below 2 %, according to the suppliers.

2.3. Extraction

Pea and lentils were extracted according to Jarpa-Parra et al. (2014), the extraction of hemp was based on Malomo et al. (2014) with slight modifications as indicated in the following.

In general, the raw materials were mixed with demineralized water at a dry matter of 10 % (w/w). The solutions were placed in a water bath at 37 °C (hemp) or 45 °C (pea, lentil) under mechanical stirring for 2 h (hemp) or 1 h (pea, lentil), their pH-values were adjusted to 10.0 ± 0.1 (hemp) or 9.0 ± 0.1 (pea, lentil) and kept constant during the extraction process by eventually adding further 1 M NaOH. 90 min (hemp) or 45 min (pea, lentil) after the start of the hydration, the solutions were sonicated at 50 % amplitude for 2 min in 800 mL batches with a CS4d4OL1 sonotrode and a B4-1.8 booster-horn. 2 h (hemp) or 1 h (pea, lentil) after the start of the hydration, the dispersions were centrifuged for 10 min at 5000*g* (hemp, pea) or 15 min at 10,000*g* (lentil) to remove non-dissolved material, including starch. The pellets were discarded, and the supernatants were adjusted to pH 5.0 (hemp) / 4.5 (pea, lentil) with HCl to precipitate the proteins. The dispersions were centrifuged again for 10 min at 5000*g*. The supernatant was discarded, the pellets resuspended into demineralized water and thoroughly dispersed (Polytron PT-MR 6100 D, Kinematika A.G., Malters, Switzerland) at 8000 rpm for 1 min. The dispersions were neutralized to pH 7.0 and sonicated for 2 min at an amplitude of 50 % with a CS4d4OL1 sonotrode and a B4-1.8 booster-horn to disintegrate protein aggregates. Finally, the solutions were freeze-dried (Alpha 2–4 LSCplus, Martin Christ, Germany). The resulting flakes were ground to a powder and stored in plastic bags under vacuum at 4 °C until further use. The protein content of the protein isolates was determined using the Dumas method (FP828 P, Leco, Michigan, USA) which bases on the quantification of the nitrogen by combustion. The combustion was done at 950 °C, under argon atmosphere, EDTA was used as reference and for calibration purposes. The standard nitrogen-to-protein conversion factor of 6.25 was used to convert nitrogen into protein quantity. When “protein” or “protein of a source” (or terms used mutatis mutandis) are referred to in the following, this refers to the totality of all protein fractions that are present together after the extraction process of a protein source.

2.4. Hydration

Protein dispersions at 12 % (w/w) (based on the nitrogen content of each powder) were prepared with demineralized water. The concentrations of 12 % was based on preliminary experiments where concentration between 8 % and 18 % were tested (data not shown). The focus was to allow gelation for ideally all proteins and their mixtures for the applied gelation methods, and using as little amount of protein as possible. The powders for the mixed protein dispersions were mixed together before hydration at different ratios (3:1; 1:1; 1:3; w/w). The solutions were homogenized and placed in a water bath at 45 °C under magnetic stirring for 1 h for powder hydration. After 45 min, samples were sonicated at 30 % for 30 s, while being kept in an ice bath to prevent the temperature form rising above 45 °C.

2.5. Gelation

Gels were prepared in custom-made hollow cylindrical wells with a diameter of 20 mm and a height of 30 mm. To avoid the attachment of gels to the walls, these were coated with sunflower oil before adding 5 g of a foam-free protein dispersion. If necessary, foam on top of each sample was gently removed using a pipette. Sunflower oil (between 1 and 2 mL) was placed on top of each gel to avoid evaporation. The heat-induced gels (T) were then incubated for 1 h at 90 °C.

For acid- and enzyme-induced gelation (GDL and TG, respectively), 20 g of a hydrated dispersion were placed in a plastic tube and cooled to 15 °C ± 2 °C on an ice bath. Then 408 mg of GDL (corresponding to a final 2 % (w/w)) or 121 mg of TG (0.6 % (w/w) meat glue, sku: 5060341114533, Special Ingredients ltd, Chesterfield, UK) were added to the respective tubes. Gelation parameters for the gelation methods were chosen according to Ben-Harb et al. (2018) with slight modifications for GDL and TG. Solutions were vortexed three times for 10 s before being poured into the wells and incubated at 45 °C for 1 h (GDL) or 3 h (TG). The amount was sufficient for triplicates in the above-described cylindrical wells, with a height of the gels of 18–22 mm.

After gelation, the samples were kept at 4 °C for a minimum of 2 h. Before performing mechanical analyses, gels were allowed to equilibrate to room temperature for approx. 15 min. Each gelation experiment was performed twice in triplicate.

2.6. Water holding capacity

The water holding capacity of the different gels was measured according to the method of Wu et al. (2009) with slight modifications. Briefly, 5 mL of each protein dispersion were placed in 50 mL centrifuge tubes and were gelled according to the procedure described above. Gels were immediately cooled, kept at 4 °C and taken out of the fridge 15 min before the centrifugation test to allow for equilibration at room temperature. The tubes were centrifuged at 8,000 *g* for 10 min at 20 °C. Each tube was weighed, the supernatant was carefully removed with absorbing tissue and the tubes were weighed again. WHC [%] was calculated as follows:

$$WHC[\%] = \frac{W_t - W_r}{W_t} \cdot 100 \tag{1}$$

where W_t is the total weight of the sample [g] and W_r the amount of water [g] removed.

2.7. Texture analysis

Gel strength was measured with a texture analyzer (TA.HDPlus, Stable Micro Systems, Godalming, UK) with a P/0.25S spherical probe of 6.25 mm diameter in stainless steel. The indentation tests were done directly in the sample holder with 0.5 mm/s test speed, a trigger force of 0.3 g, and a target distance of 10 mm into the samples. The gel strength was defined as the average load on the last 1.5 mm of the measurement (i.e. depth between 8.5 and 10.0 mm) in order to avoid superficial skin effects to impact the measurements. The gel strength was therefore defined as the near-plateau value after the probe had completely penetrated the sample. As described in section 2.5, the gels had an diameter of 20 mm and a height of 20 ± 2 mm.

2.8. Color measurements

The CIE-L*a*b* value of each gel was measured with a spectrophotometer (CM-700d, Konica Minolta, Japan). The spectrophotometer was calibrated with a white and a black value, the light source was a pulsed xenon lamp with a UV filter set on D_{65} (daylight temperature, color temperature: 6504 K), the observer angle was 10°. The gels were taken out of their support and placed on a white plate. The measurements were performed on each gel with a large opening covered with plastic.

The whiteness index (WI) was measured according to the following equation (Vargas et al., 2008):

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

2.9. Gel electrophoresis

For the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) the samples were prepared from 0.1 % (w/v) freeze-dried powder solutions hydrated as previously described. Samples were prepared by mixing 20 μL of sample with 19 μL of Laemmli loading buffer and 1 μL of β-mercaptoethanol. Then 20 μL of each sample were loaded onto the gel (Criterion Precast Gel, 12.5 % TRIS-HCl; 1.0 mm, 18 well Comb) previously rinsed with TRIS/Glycine/SDS buffer and a prestained protein standard (Bio-Rad Precision Plus Protein Dual Color, art. 1610374). The gel ran for 45 min at constant 200 V and was then washed three times with distilled water.

Native gel electrophoresis was performed using the same samples and apparatus, with tris–glycine gels (Novex Tris-Glycine 4–12 %, ThermoFisher Scientific) and native running buffer. In order to evaluate the impact of heat on the samples, the samples were subjected to a heat treatment (0.1 % (w/v), 1 h at 90 °C) and compared to the native, untreated samples.

The gels were staining with Coomassie R-250 in water/ethanol/acetic acid 5:4:1 using a standard microwave-assisted protocol (ThermoFisher, 2020).

2.10. Protein visualization

The 3D structures of the proteins were visualized with Chimera (UCSF Chimera, version 1.15) with the structure from PDB (RCSB Protein Data Bank). Since the protein structures of the studied plants were not available, proteins with the highest analogies were used. However, it should be noted that the similarity between the analogs and the original primary sequences was only about 50 %. This should be considered when interpreting the protein 3D structure.

In order to select the closest characterized protein structures, a sequence search was performed using the (hemp/pea/lentil) primary structure available on the Uniprot database (https://www.uniprot.org) and the analysis tool available on the RCSB Protein databank (https://www.rcsb.org/search/advanced). This tool uses the mmseqs2 software to find similar protein and nucleic sequences (Steinegger & Söding, 2017). The output of the search leads to a PDB file and a similarity index.

2.11. Statistical analysis

All data were obtained from six different replicates (two sets of experiments with three replicates) unless mentioned otherwise. Data is expressed as mean ± standard deviation (shaded areas). The statistical analysis in Fig. 5 and Fig. 6 was performed with RStudio (v 1.4.1106) using t-tests (two-tailed) between measured and expected values. The expected values are obtained by interpolating gel strengths or water holding capacities of unmixed gels according to the mixing ratio, which corresponds to a linear behavior. In order to quantify the effect, the data was normalized by calculating the quotient of experimental divided by expected values. In Fig. 5b and Fig. 6b, the single gels have, by definition, a value of 1.0 whereas a value above 1.0 of the mixture represents a synergistic, constructive interaction between the proteins and a value below 1.0 either an antagonistic,destructive interference or no interactions between proteins. A p-value < 0.05 was considered to be statistically different to arithmetically calculated values for linear mixing behavior (ratio 1.0) (*); $p < 0.01$: **; $p < 0.001$: ***.

3. Results and discussion

3.1. Protein extraction

Fig. 2 shows the extraction yield as well as protein concentration in the samples (on a dry matter basis). The extraction yield was higher for pea and lentil (Fig. 2, 55 % and 66 %, respectively) compared to hemp (23 %). This difference usually is a consequence of a variation in the solubility of the proteins during the extraction process under the conditions chosen. In general, the solubility of different plant proteins can range between 20 and 90 % and is highly specific on the species (Sari et al., 2015).

The extraction yield of hemp protein is low with 23 % compared to the yield reported by Malomo et al. (2014) who realized 37 % with the same method. However, according to Hadnađev et al. (2018) the differences in the yield could be a result of the previous material processing history, which is unknown. Although hemp had the lowest extraction yield, the purity of this protein isolate was the highest. Pea and lentil may have had small fractions of fat in the protein powder since no defatting was performed. The iodine test showed no blue coloration, indicating that all protein powders were free of starch after extraction. The amount of protein contained in the powders was determined by quantifying the nitrogen content (Fig. 2) and were found to be between 79 % and 97 % applying the standard conversion factor of 6.25. However, for legumes, a conversion factor of 5.24 to 5.64 may be more appropriate (Mariotti et al., 2008) and 5.21 to 5.52 should be used for hemp (Gosukonda et al., 2020). Since the exact value was not known for each source, the standard conversion factor was used. The solutions were finally freeze dried to avoid significant protein losses and high temperatures implied by spray-drying. In preliminary work, the proteins were both spray-dried and freeze-dried, but no significant difference in terms of gels strength were observed (data not shown).

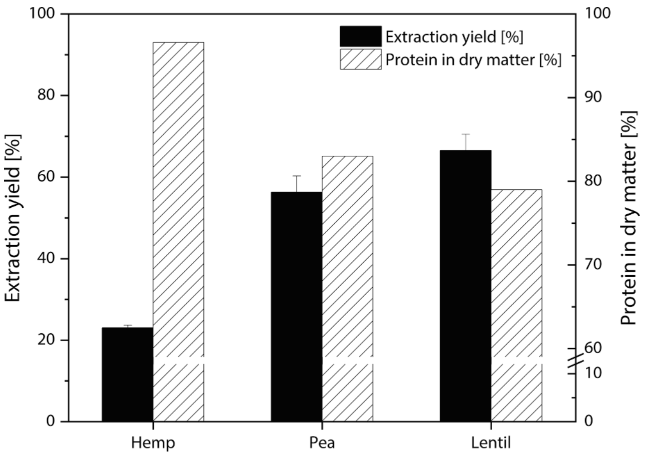

Fig. 2. Protein extraction yield (solid bars, left Y-axis) and protein content (dashed bars, right Y-axis) in the freeze-dried protein concentrates/isolates. Although the extraction procedure was performed three times, the protein content was only quantified once. The standard conversion of 6.25 was used for calculation.

3.2. Protein composition

The differences in gelling behavior may be referred to protein composition pattern/molecular weight distribution shown in the SDS-PAGE (Fig. 3a). The objective of analyzing the protein composition alone and in mixtures (only at a ratio of 1:1) was to exclude eventual irreversible reactions between proteins during hydration. Using native running conditions, we assumed to gain insights in eventual formation of new aggregates or complex during hydration/heating. Although some proteins were predominant, plant protein profiles remain complex. The main protein constituents of hemp were reported to be albumin and edestin (Callaway, 2004; Tang et al., 2006). Edestin is composed of an acidic subunit (with an acidic isoelectric point (pI)) (Staswick et al., 1981) which is about 33 kDa as well as a basic subunit (with a basic pI) which is heterogeneous (i.e. several bands on the gel) and about 20 kDa (Tang et al., 2006). In the gel, different subunits of edestin could be found at the band labeled “a” for the acidic subunit and at the two bands “b” for the basic ones (Fig. 3a). However, the albumin fraction (specifically hemp 2S albumin, 14 kDa) was probably not extracted with this chosen method. The protein profile is very similar to reports in literature using salt extraction, which in turns led to a higher protein concentration in the extract than simply using alkaline extraction and acid precipitation (Sun et al., 2021).

Three different protein subfractions of pea are part of the globulin group: legumin, vicilin, and convicilin. Each of the six subunits of legumin had acidic (40 kDa) and basic (20 kDa) polypeptides (Lu et al., 2020). They likely corresponded to the bands “*e*” and “*f*” in Fig. 3a, respectively. The band labeled “*d*” probably corresponded to the vicilin subunit of 50 kDa (Khazaei et al., 2019). Gel electrophoresis indicated that the protein subfractions present in lentils have a similar molecular weight profile compared to those in pea. However, their relative concentrations differ significantly (Khazaei et al., 2019; Lu et al., 2020). Assuming the bands “*e*” and “*d*” around 30 kDa represented legumin proteins, small difference in molecular weights indicated variations in the primary sequence between pea and lentil. Without deeper analysis such as western blotting, mass spectrometry or X-ray crystallography, further analysis is difficult.

To identify interactions between different proteins upon heating, samples were first analyzed with a standard denaturing electrophoresis (Fig. 3a), which highlighted significant differences between hemp and the two other protein sources. In order to identify interactions caused by heating to 90 °C (T gelation), a second set of experiments was performed where non-denaturing conditions were used. The samples loaded on the gel were both heat-treated and non-heat-treated (untreated). A strong interaction or agglomeration between proteins could be observed due to the disappearance of existing bands (white arrow) while a new array of band appeared at a higher molecular weight (black arrow). This was particularly intense for L and the mixture H-L and L-P (Fig. 3b). By heating proteins/protein mixtures, proteins are unfolded and aggregate into larger clusters. The size of the agglomerates is difficult to predict as it is dependent on many factors such as surface hydrophobicity or the amino acid distribution, which are characteristic of each protein source. It should be noted that the formation of agglomerates could be either beneficial or detrimental regarding the gel’s mechanical stability. Mechanisms underlying plant protein aggregation are still to be unraveled and seem to depend both on the extent of aggregation and the geometry of the aggregates (Hinderink et al., 2021). In addition, the effects of heat and pH were studied on mixtures made of plant proteins and casein, which were shown to give diverse structures, attributed to a different gelation kinetics of two proteins (Hinderink et al., 2021).

3.3. Color

The visual evaluation of the samples consisted in measuring the color and calculating the WI, which is an important parameter for consumer acceptance. Ideally, WI should be as high as possible, to allow mixing into matrices without influencing the visual appearance and, on the other hand, to avoid non-appealing colors in gels such as dark brown (Vargas et al., 2008). In this study, the protein concentrates/isolates had a variety of colors, ranging from dark brown to light ochre. In addition to the materials themselves, the type of gelation and the corresponding final pH values (pH 7.0 for TG and T gels and pH 4.5 for GDL gels) also had an impact on the color, depending on the pigments present.

As observable in Fig. 4, lentil protein led to the darkest protein gels with a brown color, compared to yellow-beige pea and light beige hemp. The color of the lentil-based gels was also dependent on the pH. Specifically, an important class of pigment are anthocyanins, are commonly found in many plants. Anthocyanins’ colors typically range from red to purple to brown and their stability as well as chroma is known to be dependent on pH (Khoo et al., 2017). In GDL gels, in which the final pH was around 4.5, the WI of the lentil protein was significantly higher (55) compared to TG or T gels at a pH of 7.0 (31 and 26, respectively). Pea and hemp were overall whiter (T, TG, GDL: 52, 54, 71 for pea and 65, 66, 69 for hemp, respectively). The increase of WI with decreasing pH is

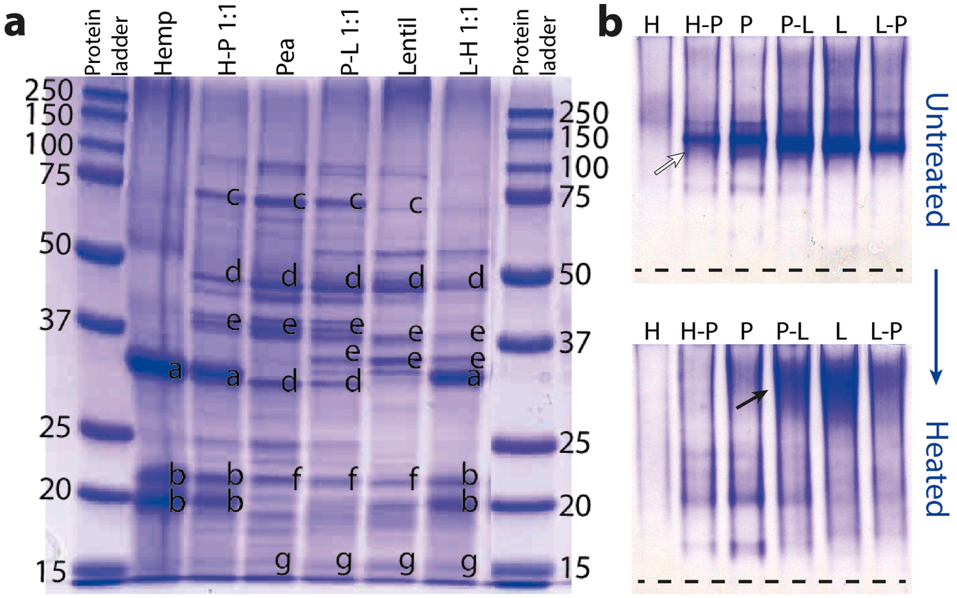

Fig. 3. a) Gel electrophoresis of the different proteins, alone and in combination. The samples were analyzed after hydration, hence before gelation. a = Edestin acid; b = Edestin basic; c = Convicilin; d = Vicilin; e = Legumin acid; f = Legumin basic; g = Albumin; b) Native gel electrophoresis (without both SDS and β-mercaptoethanol) of untreated/temperature-treated samples. The order of the samples is identical to subfigure a) The proteins shown by a white arrow disappear upon heating to form larger agglomerates indicated by the black arrow.

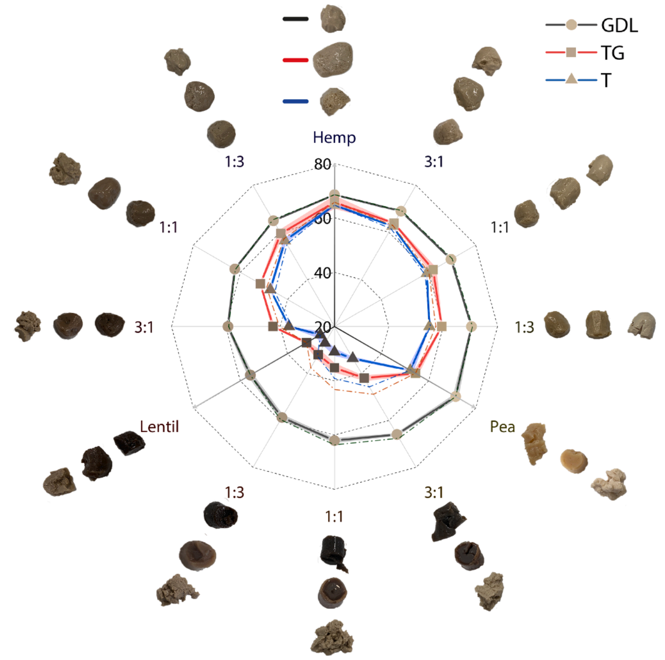

Fig. 4. Visual representation and whiteness index of the different gels. Each single protein source is represented on an axis, separated by a 120° angle, the protein mixtures can be found in between, each separated by a 30° angle. Each symbol color represents the color of the gel converted to RGB values (using a L*a*b* to RGB converter) and the distance from the center represents the whiteness index (WI, calculated with equation (2), the higher the index, the whiter the sample). The three gelation methods, glucono-delta-lactone (GDL), transglutaminase (TG) and temperature (T) are represented with three different colors, black, red and blue for the connecting lines, respectively. Dot-dashed lines represent the expected WI values. Around the graph: photographs of the different gel mixtures and ratios for each type of gel. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

advantageous for yoghurt products, as they typically exhibit a low pH of approximately 4.6 (Sodini et al., 2006; Vargas et al., 2008).

The whiteness analysis in Fig. 4 also showed that the colors of the mixtures correlated often linearly with the ratio of the mixed protein. An exception was observed in pea/lentil mixtures, specifically for T and TG gels. The dark color of lentil protein extracts dominated the yellow-beige color of pea. From a consumer's perspective, this drawback could be improved by choosing other lentil species having lighter colors or by focusing on decoloring during protein processing whereas the latter is not a standard step in the usual downstream process.

3.4. Gel strength

In this study, gel strength is defined as the average load (resistance again penetration) at a penetration depth between 8.5 mm and 10 mm. This was chosen to allow a comparison between the gelation methods which lead to contrasting penetration-resistance curve morphologies (Supplementary Fig. 1). In particular, heat- and enzyme-induced gels showed a peak during the first part of the penetration what does not exist in the acid-induced gels.

3.4.1. Acid-induced gelation (GDL)

Glucono-δ-lactone (GDL gelation) allows a retarded, steady pH decrease during gel setting, resulting in an undisturbed formation of a gel network. Gelation is induced due to the reduction of the proteins' net charges caused by the compensation of negative charges, thus resulting in reduced repulsion forces which, in turn, leads to the unfolding and aggregation of the proteins. The modification of protein–protein and protein-solvent interactions induces gel network formation (Banerjee & Bhattacharya, 2012).

Acid-induced gelation led to the formation of gels of all proteins, alone and in mixtures. Significant differences were observed for single proteins where lentil-based gels showed the highest gel strength compared to the others (Fig. 5 and Supplementary Fig. 1). The pI of the proteins is known to be a determining factor for gelation and gel strength of gels prepared with a pH-induced gelation. Specifically, the pI of hemp is reported to be around 5.0 (Hadnađev et al., 2018; Tang et al., 2006), which was higher than the other proteins, i.e., 4.3 for pea (Doan & Ghosh, 2019) and 4.5 for lentil (Joshi et al., 2017). The latter value could vary significantly between the numerous lentil species (for example red lentils have a pI of 5.2 (Lee et al., 2021)). In the single protein GDL gels, the final pH measured was 4.3 ± 0.2 for hemp, 4.6 ± 0.1 for pea and 4.7 ± 0.2 for lentil. The differences in the final pH between the protein is probably related to different buffering capacities (Ebert et al., 2021). This means that hemp protein gels show a final pH-value below its pI-value which could affect its gel strength or WHC. However, solubility/precipitation curves indicate that the influence of small pH-value deviations is rather small beyond a pH-value of 5.0

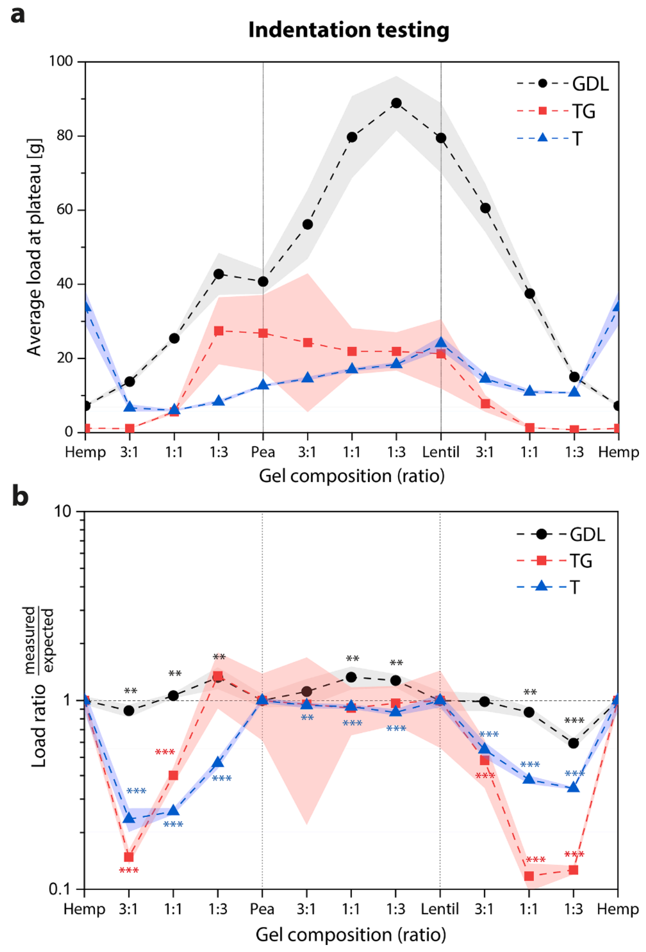

Fig. 5. Gel strengths of the single and binary gels. a) Average load during penetration test at plateau (i.e. the last 1.5 mm) as a function of the ratio, the mixtures, and the gel type. The curves are the average of triplicate measurements for each process, which was itself repeated two times (n = 6). The symbols represent the average while standard deviation is shown using shadows. b) Ratios between the measured value and theoretical value (assuming that the gel strength of a protein mixture is proportional to the gel strengths of the proteins composing it, meaning values < 1 represent a dilution effect or antagonistic, destructive interactions and values > 1 represent synergistic, constructive interactions). Statistical differences to a linear mixing behavior (ratio = 1.0) are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

(Supplementary Fig. 2).

Regarding binary blends, for P-L, synergistic interactions are very likely as the gel strengths are consistently higher than theoretically expected. The highest gel strength was found at a ratio of 1:3 (P-L) which was also higher than the gel strength of lentil protein gels alone (Fig. 5). As a result, lentil protein in gels can be substituted with pea protein up to 50 % without falling below the gel strength of lentil protein alone. For H-P, a synergistic effect seemed to appear at smaller proportions of hemp protein in the mixture, while a gel weakening effect occurred at higher hemp protein proportions. Since hemp protein alone formed only a comparatively weak GDL-induced gel at 12 %, a dilution effect may have occurreddisproportionately weakening the hemp protein gel, even with the addition of minor amounts of pea protein. For H-L, on the other hand, there was no evidence of a synergistic effect, whereas a mutual dilution effect was again likely to occur when hemp proteins were dominant in the mixture. Specifically, it can be seen that GDL gels do not have the same characteristic penetration-resistance curve as the two other gel types (Supplementary Fig. 1). Indeed, the TG and T gels have a peak representing the breaking point of the top layer by the probe, showing the formation of a skin, which GDL gels do not have. Also, a correlation between the gel elasticity of the TG and T gels compared to the breakability of the GDL ones is likely.

3.4.2. *Enzyme-induced gelation (TG)*

TG is an enzyme that crosslinks lysine and glutamine residues of amino acid side chains (Dube et al., 2006). Standard deviations were much higher for TG than for GDL and T gels (Fig. 5). Some TG gels fracture only at the top layer while some others had different fracturing events thus inducing differences in the penetration-resistance curves. Interactions between two gel networks (i.e. if they are independent or dependent) can dictate fracture properties. For example, a large variability of fracture properties could be attained by mixing soy protein isolate and gelatin, which was attributed to micro phase separation (Ersch et al., 2015). Pea and lentil proteins alone formed stiffer gels (Fig. 5, 26.8 g and 21.3 g respectively) compared to hemp protein (1.1 g), which behaved like a viscous fluid. Very soft, gel-like viscous structures, as it was the case for hemp-containing gels exhibited load values close to zero, quickly leading to a very low ratio value, as it can be observed in Fig. 5b. These differences may result from the fraction and location of lysine and glutamine residues within the 3D protein structure of each source. A hypothesis can be formulated based on the analog structures depicted in Supplementary Fig. 3: the amount of glutamine residues in edestin is exceptionally high. However, edestin contains only few lysine residues, which are mainly placed in the core of the protein, thus limiting transglutaminase activity. Albumin, on the other hand, contains mostly lysine residues on its surface and makes it an ideal candidate to react with the glutamine-rich edestin. Therefore, mixing both protein sources (i.e., hemp with lentil or pea) could favor transglutaminase-induced cross-linking. On the contrary, legumin A and B had a more homogeneous distribution of both amino acids as well as vicilin (Supplementary Fig. 3). These results are consistent with those from Dube et al. (2006) who showed that pea, soy, lupin, rice and sunflower, respectively, were good substrates for TG. It should be underlined that the structures used in Supplementary Fig. 3 only share about 50 % similarity with the actual proteins (the tridimensional structure of which being unavailable) but are useful to emphasize and visualize the importance of amino acids residues respective to each gelation method.

Binary mixtures of P-L did not show synergistic effects in TG-induced in contrast to GDL-induced gels but a linear mixing behavior, i.e., the proteins of both sources can obviously largely replace each other in mixed gels. In contrast, a synergistic effect was observed in H-P blends at low hemp protein concentrations, i.e., very likely lower concentrations of hemp proteins can be profitably integrated into a pea protein gel. This means that about 25 % of the pea protein can be replaced by hemp protein without any significant loss in gel strength. At higher concentrations of hemp protein, and knowing that hemp alone produced weak gels, a dilution effect of the pea protein probably dominated again. The shape of the curve for increasing hemp concentrations indicated that there may have been weak interactions between lentils/peas and hemp proteins, but these did not seem to be predominant.

3.4.3. *Heat-induced gelation (T)*

Heat-induced gelation is often a two-step process. Proteins are denatured and unfolded leading to the exposure of non-polar regions and subsequently to aggregation and formation of a protein network (Totosaus et al., 2002). Sun and Arntfield (2012) reported that the initial structure of a gel is developed during heating due to electrostatic and hydrophobic interactions while, in a second phase, the hydrogen bonds stiffen the gel during cooling.

In contrast to GDL- and TG-induced gelation, the strength of gels prepared with hemp protein alone was the highest for heat-induced gelation (hemp: 33.7 g, compared to pea 12.7 g, lentil 24.1 g, Fig. 5a). The pea protein gel was perceived as a viscous solution rather than a gel. The transition from viscous to gel-like structures cannot be defined by a precise value, based on our observations we estimate values below 5–10 g representing rather a viscous, liquid-like than gel-like behavior. Sun and Arntfield (2011) showed that between pH 6.0 and 8.0, the denaturation temperature of pea proteins was > 95 °C. This could explain why pea proteins did not form a solid gel at the applied temperature herein (90 °C), since the proteins could be in a partially unfolded state. Since the method was based on the literature (Ben-Harb et al., 2018) and for practical reasons, the temperature of 90 °C was chosen for gelation even if it was lower than the denaturation temperature of pea proteins. Hemp and lentil proteins have a lower denaturation temperature as pea with 87.5 °C (Wang et al., 2008) and 78 °C (Shnyrov et al., 1996), respectively. This suggests that they probably reached their unfolded state which allowed them to aggregate to a stronger extent.

P-L mixed gels showed an almost linear mixing behavior. The gel strength decreased nearly proportionally from lentil protein gels to pea protein gels, which suggested that proteins likely formed a joint gel network and could replace each other in a network. In contrast, both H-P and H-L gels showed a non-proportional behavior. The strength of H gels decreased significantly at a fraction of 25 % of the second protein. We hypothesize that this reduction in gel strength is probably caused by a dilution effect of the pea and lentil proteins and at the same time the lack of ability to form a common network. However, at higher ratios (50 % and 75 % of lentil and pea protein, respectively), at least subfractions of the different proteins seemed to exhibit interactions, as the gel strength decreased towards higher hemp protein proportions, but not in such a way that one could assume a dilution effect. Therefore, these results would indicate that subfractions of proteins from different sources could form a combined gel network, at least to a certain extent, and that this seemed to be highly dependent on the ratios of the mixing partners. In a study on a soy-whey protein system using thermally induced gelation, it was hypothesized that the two protein sources form two distinct networks whereas whey protein seemed to form a primary network while soy acted as particulate fillers. In addition, hydrophobic interactions between the proteins contributed to the elasticity of the whole system which varied depending on each constituent's concentration and ratio (McCann et al., 2018).

3.5. *Water holding capacity*

The water holding capacity (WHC) represents the capacity of a material to retain water. In particular, gelled soft matter products like yogurts with a high water content should have a high WHC to avoid high degrees of syneresis, which is unappealing for the consumers (Afoakwa, 2014; Stone et al., 2015).

In this study, it was observed that the WHC of a gel was strongly depending on the gelation method as well as on the protein/protein mixture. Acid-induced gels showed a much lower WHC (Fig. 6a black

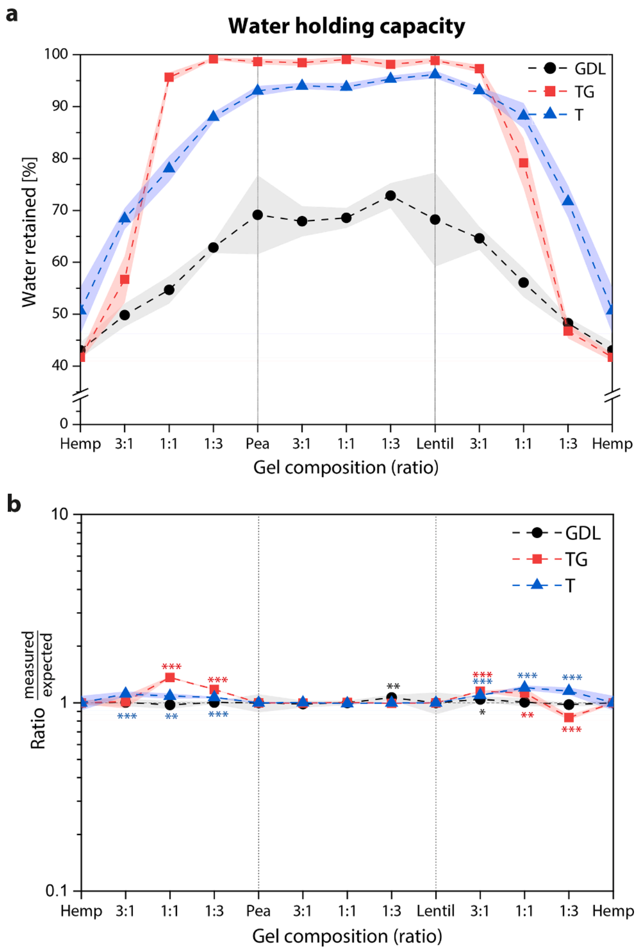

Fig. 6. Water holding capacity of the different gels. a) average of the water holding capacity depending on the ratio, the mixture and the gel type. The curves are the average of six measurements coming from two hydration processes (the symbols representing the average and the shaded areas, the standard deviation; n = 6). b) ratios between the measured value and theoretical value (assuming that the gel strength of a protein mixture is proportional to the gel strengths of the proteins composing it, meaning values < 1 represent a dilution effect or antagonistic, destructive interactions and values > 1 represent synergistic, constructive interactions). Statistical differences to a linear mixing behavior (ratio = 1.0) are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

curve, between 43 % and 69 %) than those prepared with TG (Fig. 6a red curve, between 41 % and 99 %) or T (Fig. 6a blue curve, between 50 % and 96 %). It appeared that hemp protein gels had a lower WHC (between 41 % and 50 %) than pea (between 69 % and 99 %) and lentil protein (between 68 % and 99 %) independently of the gelation technique. According to Tang et al. (2006), the low WHC of hemp may be due to its strong protein aggregation at pH 7 what traps polar amino acids residues inside the aggregates. Interestingly, the WHC of pea protein gels was shown to be higher than its oil holding capacity, in opposition to hemp protein gels (Liu et al., 2022; Stone et al., 2015). The WHC of TG-gels of mixtures of pea protein and lentil protein seemed to be independent of the mixing ratio. In contrast, the maximum fraction of hemp protein in mixture with pea or lentil protein was limited to 50 % (H-P) and 25 % (H-L), Larger quantities of hemp protein in the mixtures led to a considerable decrease of the WHC (Fig. 6b red curve, H-P 3:1 and L-H 1:3). GDL-induced gels showed nearly full proportional mixing behavior for mixtures with hemp protein with a ratio very close to 1.0 for each mixture (Fig. 6b black curve) whereas T-induced gels seemed to exhibit a synergistic behavior (Fig. 6b blue curve, for H-P and L-H). This is supported by the findings in section 3.4 where we assumed synergistic interaction at medium to low ratios of hemp protein in mixtures. By comparing results between gelation and WHC it can also be revealed that the gel strength was not a major factor determining the WHC. This is in opposition to what Li et al. (2020) found with peanut protein isolate, where a high WHC (>75 %) was correlated with a strong gel. The highest WHC measured in this study (>90 % for the mixture between pea and lentil for TG and T) is not linked to the highest gel strength. On the other hand, the WHC was apparently also independent of the extent and type of gelation in the case of hemp protein, so that the WHC of the proteins themselves is likely to be the decisive factor in this case.

Comparing the mixing behavior in terms of WHC with that in terms of gel strength implies parallels. This suggests that the WHC depends mainly on the microstructural immobilization of water and less strongly on the resistance of a gel network to compression forces. However, adhesion effects (gel network to the wall of the centrifuge tube) cannot be excluded, which can have a considerable influence on the quantification using a centrifugation-based method. Overall, the data suggested that especially pea and lentil proteins are well suited to form water-holding gel networks, alone or in mixtures.

3.6. *Considerations of mechanistic reasons for the mixing behavior*

In literature, different explanations and models are proposed for protein mixing behavior with respect to techno-functionalities. However, such propositions remain speculative in the present context, especially since most of the findings in literature based on experimental work on mixtures of animal with plant proteins.

As previously mentioned, authors suggest that in (binary) protein mixtures the continuous network is composed of one protein while the other protein is incorporated into it as a filler (Comfort & Howell, 2002; McCann et al., 2018). The idea that a protein dominates the network formation could indirectly explain mixture-induced weakening effects as mixing proteins at constant protein content inevitably leads to a protein dilution effect. Sterically induced/segregative phase separation, due to different sizes of proteins/structures, could obviously largely compensate for this dilution effect in the case of a soy/whey protein mixture (Comfort & Howell, 2002). Other authors proposed independent gel network structures that resulted in higher elasticity moduli when mixed (Ersch et al., 2015), whereas hydrophobic associations between mixed proteins were assumed to contribute to the overall properties (McCann et al., 2018). Also suggested as an influencing factor was the protective function of some proteins, which can interact with other proteins and thus, for example, prevent them from thermal network formation (Wu et al., 2021). While beta-conglycinin and glycinin were mentioned as examples, it is imaginable that new chaperone-like protein combinations are formed by mixing proteins. On the other hand, the formation of aggregates/coacervates is conceivable, especially between oppositely charged protein subfractions, with far-reaching effects on the gelling ability of the mixture.

It is striking that a similar molecular weight distribution of the proteins involved (P-L) tends to lead to linear or synergistic mixing behavior, while different distributions (P-H; L-H) tend to favor strongly under-proportional mixing behavior. Conceivable would be here, on one hand, the effect of segregative phase separation, which, however, could not be verified, at least not microscopically (data not shown). On the other hand, the presumed dilution effect seems to be explainable: if one assumes that proteins involved form individual networks, then the gel strength would depend on the individual protein concentrations rather than total protein concentration (i.e. when the concentration of both proteins is insufficient, the gel strength remains low). In contrast, linear mixing behavior can be expected if the proteins form a common network or synergistic mixing behavior if further interactions of the proteins in mixture are added. All of this remains speculative based on the present experimental data, however, we assume that this is the mechanistical framework for the investigated protein mixtures in this work.

4. Conclusion

The study showed very different techno-functional properties for three plant-based protein concentrates/isolates, be it sole or in binary blends, at the same concentration. Gels of unmixed proteins exhibited considerably different gel strengths depending on the protein source and on the gelation method. However, it was shown that in some mixed protein gels (e.g., GDL gel for all ratios of P-L), synergistic effects can compensate to a limited extent for the theoretically expected decrease in gel strength, if gel strengths of the individual gels are different and assuming a proportional contribution of the proteins to gel strength in a mixture. Thus, pea protein gels could contain up to 25 % of hemp protein for GDL- and TG-based gelation without loss of gel strength, whereas lentil protein gels could not. On the other hand, lentil protein gels based on GDL could contain up to 50 % pea through synergistic interactions without the gel strength of the mixture falling below the gel strength of the lentil protein gels alone. For TG-induced gels, pea and lentil appeared to be unrestrictedly miscible without appreciable loss of gel strength, strongly favored by the fact that gel strengths of mixing partners are already in similar orders of magnitude. In the case of reduced gel strengths caused by mixing, it remains open whether the absolute gel strengths can be re-adjusted by increasing the total protein concentrations at the same mixing ratio (limit: too high viscosity).

In binary mixtures of pea or lentil with hemp protein, the proteins involved in the gel network apparently could not substitute for each other; even lower proportions of one or the other led to a strong decrease in gel strength. The molecular reason for this under-proportional mixing behavior remains unknown, but it is conceivable that at least the main protein subfractions cannot establish a joint gel network. Nevertheless, based on the characteristic curve shape (e.g. in TG or T-based gelation for H-L as well as H-P) there are indications of interactions between the proteins, even at a low level of gel strength. It is conceivable that some, but not all, protein subfractions may have interacted with each other and form a weak gel network. Insights into gel structures/gel networks (joint network formation, individual binary gelation) cannot be obtained based on the available experimental data. Thus, it remains unclear whether partial protein interaction, antagonistic or dilution effects of the individual protein in mixtures occur in case of strongly under-proportional mixing behavior (i.e. gel strength when hemp protein is added).

Overall, this study showed that the techno-functional properties of the plant protein gels could be tuned by mixing proteins. Acid-induced gels showed the best gel strength, however, the poorest water binding capacity. Combinations of different gelation methods were not investigated in this study, but it is suggested that a combination of enzymatic- and acid-induced gelation could lead to gel properties suitable for soft gels like yogurts. On the other hand, the addition of hemp protein to pea or lentil protein could be used to reduce/inhibit gelation what could be of interest for thermally treated products that should remain liquid (e.g. high protein plant-based drinks requiring pasteurization). Further research is needed to investigate whether the described mixing behavior also applies to other protein concentrations or types.

CRediT authorship contribution statement

Sarah Guidi: Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Florian A. Formica:** Validation, Formal analysis, Writing – review & editing, Visualization, Supervision. **Christoph Denkel:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111752.

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