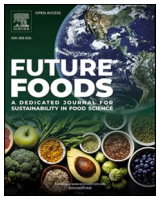
Future Foods 12 (2025) 100835

Contents lists available at ScienceDirect

Future Foods

journal homepage: www.elsevier.com/locate/fufo



Microbiological and physical evaluation of nonfat set-type hybrid yogurts formulated with soy and rice proteins

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ARTICLE INFO

Keywords:
Dairy alternatives
Soy protein
Rice protein
Structure
Fermentation

ABSTRACT

This study aimed to investigate the impact of incorporating soy protein isolate (SPI) and rice protein isolate (RPI) on the microbiological, physicochemical, and structural properties of high-protein, nonfat, set-type hybrid yogurts. Yogurts were formulated to contain 6 % total protein, consisting of 3 % protein from skim milk powder and an additional 3 % from whey protein concentrate, and/or the selected plant proteins in varying proportions. The fermentation process was driven by *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus,* and the yogurt samples were monitored over a 14-day refrigerated storage period. Yogurts containing SPI exhibited the fastest acidification (3.5 h to reach pH 4.5) and the highest bacterial counts at day 0 (~9.8 log CFU/g). Conversely, RPI-containing yogurts required a longer fermentation time (~5 h) and exhibited lower microbial viability (~8.6 log CFU/g). After 14 days, all samples remained above 7 log CFU/g, confirming starter stability during storage. Physicochemical and structural analyses showed that Y-S developed a branched, less dense and more porous protein network than the dairy control (Y-W), whereas Y-WS (with 1.5 % WPC + 1.5 % SPI added proteins) exhibited an intermediate microstructure. SPI-containing formulations displayed texture parameters (firmness, consistency, cohesiveness) closest to the control, while RPI led to a weaker gel and higher syneresis (up to 15 % spontaneous and 64 % forced). Overall, hybrid yogurts containing soy proteins showed superior texture and stability, and faster fermentation. Although both plant proteins can be incorporated into dairy matrices, further optimization is required to achieve the desired texture and stability in high-protein hybrid yogurts.

1. Introduction

Nowadays, there is an increased interest in flexitarian diets, with more and more consumers exploring reducing animal protein consumption for health, environmental or animal welfare reasons (Lang, 2020). Hybrid foods, characterized by integrating plant-based ingredients into traditional animal-based products, are gaining prominence as a sustainable alternative to meet the rising global food demand and address environmental concerns. This novel category of foods combines the nutritional benefits of both plant and animal sources, aiming to enhance dietary diversity while minimizing the ecological footprint (Grasso and Goksen, 2023). Hybrid foods are seen as a practical solution to enhance food security by diversifying protein sources and reducing dependency on traditional livestock systems, which are often resource-intensive and associated with high greenhouse gas emissions (Grasso, 2024).

Yogurt is the oldest fermented milk product and is very popular around the world. Fermentation has been featured as a huge opportunity to enhance the potential of dairy/plant protein combinations (Guyomarc'h et al., 2021). This biological process not only improves the sensory quality, like texture and flavor, but also aids in mitigating undesirable characteristics such as off-flavors typically found in plant-based components (Pua et al., 2022). Consumer acceptance of set-type yogurts is based on their sensory and techno-functional

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https://doi.org/10.1016/j.fufo.2025.100835
Received 30 April 2025; Received in revised form 25 October 2025; Accepted 9 November 2025
Available online 10 November 2025

properties. These yogurts are particularly valued for their stable and firm gel structure, which is essential for a satisfying eating experience. It is critical for this type of yogurt to exhibit robust water-holding capacities to avoid syneresis, thereby extending shelf-life and enhancing sensory appeal. Additionally, a good quality yogurt should maintain a consistent texture during storage, without the formation of grittiness or lumpiness (Ma and Chen, 2025).

The complex molecular structure of dairy proteins, particularly casein and whey, allows for excellent water retention, stable gel formation, and a creamy mouthfeel, which are essential for high-quality set-type yogurts (Lesme et al., 2020). Replicating these results with plant-based proteins presents significant challenges, and, therefore, the selection of the plant protein type and concentration is a key point in the formulation of hybrid yogurts. Fermentation could benefit texture and sensory qualities by transforming the protein structures and improving gelation, which is crucial for set-type yogurts.

Various plant protein ingredients are used in the formulation of food products. Soy and rice proteins are often used in plant-based dairy analogues due to their distinctive nutritional and techno-functional characteristics. Soy protein is renowned for its comprehensive amino acid profile, making it nutritionally equivalent to animal protein. It is particularly effective in emulating the texture and water-binding capacity of traditional dairy products, which enhances the mouthfeel and structural integrity of dairy alternatives. Soy protein's ability to form stable emulsions contributes significantly to the creamy texture and consistency desired in products like yogurts and cheese analogues (Moss et al., 2023). Rice protein is known for its hypoallergenic properties and is therefore ideal for consumers sensitive to other plant or dairy proteins. Techno-functionally, rice protein contributes to a smooth texture and mild flavor in dairy alternatives, although its emulsifying and water-binding capacities are generally lower compared to soy protein (Moss et al., 2023). Hence, including plant proteins may modify the structure and consistency of fermented milk and affect syneresis. Comparative data between the behavior of soy and rice proteins under identical processing and protein content conditions are scarce.

Despite the growing interest in hybrid foods, research on hybrid yogurts is incipient, with only a few recent studies exploring their development and potentialities (Akin and Ozcan, 2017; Batista Silva et al., 2024; Canon et al., 2022; Curutchet et al., 2024). High-protein yogurts are gaining consumer attention due to their numerous health benefits. The growing demand for high-protein yogurt is expected to persist, as protein intake is closely linked to weight management and overall wellness (Mitra et al., 2022). This paper explores how plant and dairy proteins can be combined in hybrid yogurts, adhering to dietary trends towards sustainable food options, without compromising the quality.

The objective of this study is to investigate the effect of different plant proteins, soy protein isolate (SPI) and rice protein isolate (RPI), on the microbiological behavior and the physicochemical and structural properties of high-protein set-type hybrid yogurts.

2. Materials and methods

2.1. Materials

The ingredients used for the yogurt preparation were: skim milk powder (SMP) from CONAPROLE (Uruguay) with 36.8 % (dry basis) protein; whey protein concentrate (WPC) Lacprodan 80 Instant (Arla Foods Ingredients, Denmark) with 81 % (dry basis) protein; soy protein isolate (SPI) Supro® 590 with 92.4 % (dry basis) protein (Solae™, USA) and rice protein isolate (RPI) Remypro N80+ (Beneo, Belgium) with 93 % (dry basis) protein. The fermentative culture used was Lyofast Y450B (Sacco Systems, Italy), comprising *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. Table sugar (sucrose) was purchased at a local supermarket.

2.2. Set-type yogurt manufacture

All set-type yogurts were formulated to contain 6 % (w/w) total protein (3 % protein from SMP + 3 % added proteins) to be considered "high-protein yogurts" according to Uruguayan law (Mercosur, 2012).

First, a milk base was prepared by reconstituting SMP with distilled water to a final concentration of 3 % (w/w) protein. Six formulations were prepared by incorporating proteins from different sources (WPC, SPI, and/or RPI) at different concentrations to achieve 6 % (w/w) protein content along 10 % (w/w) sucrose (Table 1). The mixtures were stirred continuously for 2 h at 40 °C and then refrigerated at 5 °C overnight to ensure complete hydration. After that, the mixtures were heat-treated (85 °C/30 min) in a water bath, cooled to 45 °C and inoculated with the fermentative culture (0.02 %). After inoculation, portions of 100 mL of the mixtures were placed in plastic containers and fermented in an Innova® 44 incubator (New Brunswick Scientific, USA) at 43 °C until reaching pH= 4.5. Then, the samples were placed at 5 °C for 24 h to stabilize the formed gels. The set-type yogurt samples were coded according to the added protein ingredients, as presented in Table 1.

2.3. Physicochemical properties

The pH of the set-type yogurts was measured using a calibrated LAQUA-twin pHmeter (Horiba Scientifics, Japan) and the titratable acidity, expressed as g of lactic acid per 100 g of yogurt, was determined by titration with NaOH 0.1 M until pH 8.2. pH and titratable acidity were measured one day after manufacture (day 0 of storage) and after 7 and 14 days of storage at 5 °C.

Total solids (TS) content was determined by oven drying at 102 °C until constant weight.

The instrumental color parameters L*, a* and b* were evaluated using spectrocolorimeter (LabScan®XE, HunterLab, USA), calibrated with standard black and white tiles. The L* value represents the luminosity, the a* value represents red/green (positive/negative) hues, and b* value represents yellow/blue (positive/negative) hues of the yogurt samples. Additionally, the whiteness index (preferred white and yellow) was calculated according to Eq. (1).

$$\text{Whiteness} = 100 - \sqrt{(100 - L)^2 + a^2 + b^2} \quad \text{(Eq. 1)}$$

2.4. Microbiological behavior

The viable counts of *S. thermophilus* and L. *delbrueckii* ssp. *bulgaricus* were performed by standard plate count method to evaluate the growth behavior during fermentation time and the viability for 14 d at 5 °C. Briefly, 1 g of mixture or set-type yogurt was suspended in 9 mL of peptone water (0.1 %) and subjected to subsequent serial dilution. M-17 and MRS agar (Oxoid Ltd, UK) were used for *S. thermophilus* and L. *delbrueckii* ssp. *bulgaricus*, respectively. The plates were incubated at 37 °C for 48 h under aerobic and anaerobic conditions, respectively. All results were expressed as log of colony-forming unit per g of sample (log CFU/g).

Table 1
Set-type yogurt samples' codification.

Sample code	Protein from SMP (%, w/w)	Protein from WPC (%, w/w)	Protein from SPI (%, w/w)	Protein from RPI (%, w/w)
Y-W	3	3	-	-
Y-S	3	-	3	-
Y-R	3	-	-	3
Y-WS	3	1.5	1.5	-
Y-WR	3	1.5	-	1.5
Y-SR	3	-	1.5	1.5

SMP: skim milk powder; WPC: whey protein concentrate; SPI: soy protein isolate; RPI: rice protein isolate.

2.5. Syneresis

Syneresis or whey separation in the set-type yogurts was measured through siphon (spontaneous syneresis) and centrifugation methods. The siphon method described by Amatayakul et al. (2006) was used. Briefly, a 50-mL Falcon tube containing 30 g of yogurt sample was taken from the cold room, weighed and kept at room temperature (~ 25 °C) at an angle of approximately 45 ° to allow whey collection at the side of the tube. A needle connected to a syringe was used to siphon the whey from the surface, and the tube was weighed again. The centrifugation method was performed according to Naibaho et al. (2022). Ten g of yogurt were weighed and centrifuged (SIGMA 6–16KS, Sigma Laborzentrifugen GmbH, Germany) at 4500 rpm for 15 min at 10 °C and the sediment weighed again after whey removal. Spontaneous and centrifuged syneresis were measured at days 0, 7 and 14 of storage at 5 °C. Syneresis was expressed as the percentage weight of the whey separated from the yogurt sample over the initial weight of the yogurt sample.

2.6. SDS-PAGE

Reducing and non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to identify the protein components. Briefly, stacking and separation gels were prepared with 5 and 12 % acrylamide, respectively. The freeze-dried yogurt samples and the protein ingredients (SMP, WPC, SPI and WPI) were prepared in sample buffer system (0.06 M Tris–HCl buffer pH 6.8, 25 % glycerol, 2 % SDS, 0.1 % bromophenol blue) with or without 0.72 mM β-mercaptoethanol. In reducing conditions, the mixtures were boiled for 5 min and centrifuged for 5 min a 12,000 × *g*. Ten μl of the molecular weight marker (10–250 kDa, #26,619, ThermoScientific, USA) and the protein solutions (0.3 mg/mL) were loaded on the gel. A running buffer system (pH 8.3; 0.025 M Tris base, 0.15 M glycine, and 0.8 % SDS) was used. Electrophoresis was performed using an omniPAGE WAVE Maxi System (Cleaver Scientifics, Rugby, UK) at 200 V. The gels were stained with Coomassie brilliant blue and destained with methanol (30 %) and acetic acid (10 %).

2.7. Texture analysis

One day after manufacture, yogurt texture was evaluated by the backward-extrusion test using the TA.XT Plus Texture Analyser (Stable Micro Systems, UK) equipped with a 5 kg loading cell. A cylinder probe of 35 mm diameter was used to penetrate the containers with 100 mL yogurt samples at a 1.0 mm/s speed with a penetration distance of 30 mm and a surface trigger force of 10 g (Raza et al., 2022). The instrumental texture parameters firmness (N), consistency (N*s), cohesiveness (N), and work of cohesion (N*s) were calculated using the Exponent software. Firmness is the force necessary to attain a given deformation and was defined as the maximum force reached during penetration. Consistency was the positive area under the curve; a higher value indicates a thicker consistency. Cohesiveness was the maximum negative force; the more negative the value the more 'sticky' or 'cohesive' is the sample. Finally, the area of the negative region was referred to as the work of cohesion; the higher the value the more resistant to withdrawal the sample, indicating that the yogurt is more resistant to gradual deformation of shear stress.

2.8. Rheological behavior

Rheological measurements were conducted at 8 °C, to reflect the serving temperature of yogurt (Laiho et al., 2017) using an Anton Paar Physica MCR 301 rheometer (Austria) with a parallel plate geometry of 50 mm diameter and a 1 mm gap. The strain amplitude varied logarithmically from 0.01 % to 70 % at a constant frequency of 1 Hz. Each of the 80 measurement points was recorded over a duration of 10 s to characterize the material's viscoelastic properties across both linear and non-linear regions.

2.9. Microstructural analysis

The yogurt samples for microscopy observation were prepared according to Lesme et al. (2019) with some modifications. Three hundred μL of a 0.2 % rhodamine B solution was mixed with 50 g of yogurt immediately after inoculation of the starter culture. A few drops of the mixture were then placed on a slide and a cover slip was placed over the sample. The slide was wrapped in aluminum foil and incubated at 43 °C until pH= 4.5, in the same conditions as the regular yogurts' preparation. Confocal laser scanning microscopy (CLSM) was conducted using a ZEISS LSM 800 AiryScan confocal microscope (Germany) equipped with a 63 × NA 1.4 Plan Apo objective. The fluorescent dye rhodamine B was excited at 561 nm, with emission collected in the spectral range of 560–700 nm using a GaAsP detector. The Master Gain and laser power settings were kept consistent across all images and adjusted to remain below the thresholds established for controls without rhodamine B.

2.10. Statistical analysis

All measurements were conducted at least in triplicate, and the results are presented as means and standard deviations. Data were analyzed for significant differences, with minimum significance at the 5 % level ($P \leq 0.05$) using Statgraphics Centurion XVII (Statpoint Technologies Inc, Warrenton, VA, USA) statistical software. One-way ANOVA test followed by multiple comparisons of means (Tukey's test) was used to determine the statistical difference.

3. Results

3.1. Fermentation time and microbiological behavior

The rate of fermentation was related to the drop in pH of set-yogurt due to the conversion of lactose into lactic acid by *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* during incubation at 43 °C. All the samples reached the yogurt's typical end-point pH 4.5 (Fig. 1). As shown in Fig. 1, the yogurts containing soy protein (Y-S) and whey and soy protein (Y-WS) reached the shortest endpoint in 3.5 h. By contrast, the yogurt with rice protein (Y-R) had the longest fermentation time (5 h), while the rest of samples (Y-W, Y-WR and Y-SR) had an

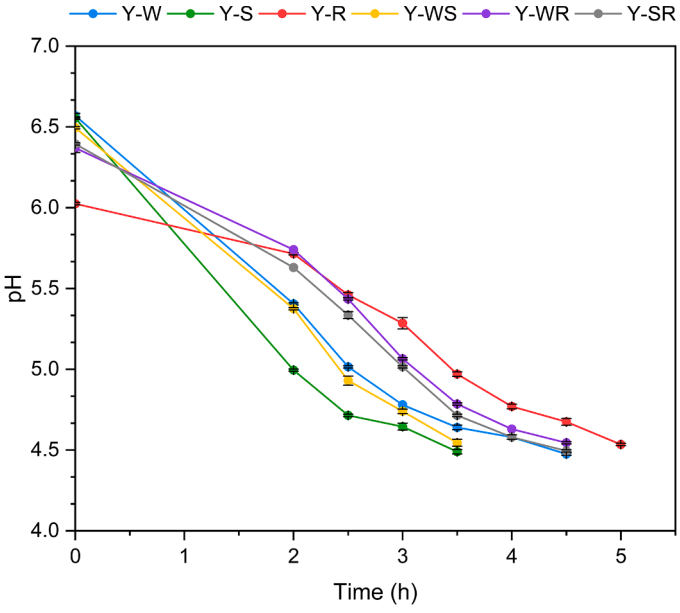

Fig. 1. pH variation during fermentation of the set-type yogurts at 43 °C.

intermediate fermentation time (4.5 h).

Fig. 2A and B show the behavior of the microorganisms *S. thermophilus* and L. *delbrueckii* ssp. *bulgaricus*, respectively, during fermentation time. For *S. thermophilus*, the log count increased between 1.2 and 1.6 log during the first 2 h. Y-S showed the highest bacterial counts (9.8 log CFU/g) at the end of the fermentation process (3.5 h). By contrast, the hybrid yogurts containing rice protein, i.e., Y-R, Y-WS and Y-SR, showed the lowest bacterial counts (~8.6–8.7 log CFU/g) by the end of fermentation (Fig. 2A, Table 2). For L. *delbrueckii* ssp. *bulgaricus*, during the initial 2 h, the growth trend was different from *S. thermophilus* and dependent on the yogurt type. Y-W had the highest log count after 2 h (~2 log), followed by Y-S and Y-WS, while Y-R, Y-WR and Y-SR showed less bacterial growth. At the end of fermentation, Y-S had the highest log count in 3.5 h (9.83 log CFU/g), followed by Y-W (9.24 log CFU/g). In the rest of the samples, L. *delbrueckii* ssp. *bulgaricus* reached 7.88 - 8.35 log CFU/g (Fig. 2B, Table 2).

Table 2 shows the microbiological counts in the yogurt samples, during cold storage. In Y-S, *S. thermophilus* had the greatest depletion after 14 d of storage at 5 °C, decreasing significantly ($P \leq 0.05$) every week. However, Y-S still showed the highest counts by the end of storage. In the rest of the set-type yogurts, the microbial count was almost stable. Indeed, there was a slight difference between the beginning and the end of the storage, which is <1 log and is not considered as a microbiological significant difference. L. *delbrueckii* ssp. *bulgaricus* showed the same behavior in Y-S. Y-R and Y-SR had the lowest microbial count after 14 d at 5 °C.

3.2. Physicochemical properties

The results of the changes in pH and titratable acidity during storage are presented in Table 2. During refrigerated storage, the pH declined significantly in all samples, except Y-WR. After 14 d, the pH in Y-W and Y-S (4.36 and 4.34, respectively) was significantly lower than in the remaining samples, while Y-R had the highest pH (4.63). Consistent with the pH reduction, Y-W and Y-S showed the highest increase in acidity, reaching 1.06 and 1.04 %, respectively, after 14 d Y-R, Y-WR and Y-SR

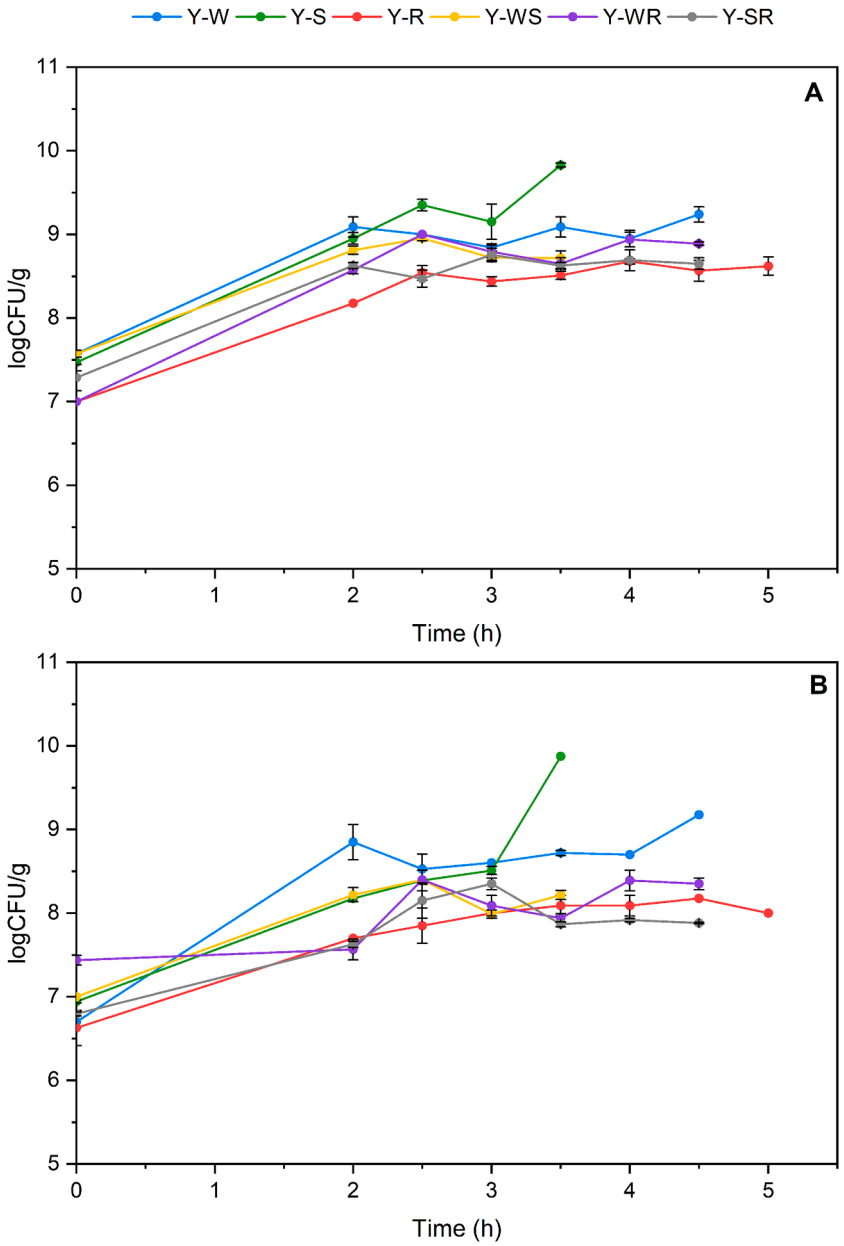

Fig. 2. Changes in the viability of *Streptococcus thermophilus* (A) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (B) during fermentation time.

Table 2
Microbiological counts, pH and titratable acidity of set-type yogurts during storage at 5 °C.

	Sample	Storage time (d)		
		0	7	14
S. thermophilus (log CFU/g)	Y-W	9.24 ± 0.09 ^{B}	9.10 ± 0.02 $^{ab\ BC}$	8.85 ± 0.11 $^{a\ CD}$
	Y-S	9.83 ± 0.02 ^{C}	9.18 ± 0.00 $^{b\ C}$	8.98 ± 0.03 $^{a\ D}$
	Y-R	8.62 ± 0.11 ^{A}	8.67 ± 0.10 ^{A}	8.40 ± 0.00 $^{a\ AB}$
	Y-WS	8.72 ± 0.09 ^{A}	8.84 ± 0.09 $^{a\ ABC}$	8.85 ± 0.11 $^{a\ CD}$
	Y-WR	8.89 ± 0.02 $^{b\ AB}$	8.83 ± 0.07 $^{b\ AB}$	8.18 ± 0.00 ^{A}
	Y-SR	8.65 ± 0.07 ^{A}	8.71 ± 0.15 ^{A}	8.63 ± 0.04 $^{a\ BC}$
L. *delbrueckii* ssp.*bulgaricus* (log CFU/g)	Y-W	9.18 ± 0.00 $^{a\ D}$	9.15 ± 0.21 $^{a\ C}$	8.59 ± 0.16 $^{a\ C}$
	Y-S	9.93 ± 0.07 $^{b\ E}$	8.81 ± 0.05 $^{a\ C}$	8.33 ± 0.21 $^{a\ C}$
	Y-R	8.00 ± 0.00 $^{b\ AB}$	7.39 ± 0.12 ^{A}	7.39 ± 0.13 ^{A}
	Y-WS	8.22 ± 0.06 $^{a\ BC}$	8.38 ± 0.05 $^{a\ B}$	8.25 ± 0.10 $^{a\ BC}$
	Y-WR	8.35 ± 0.07 $^{b\ C}$	8.33 ± 0.04 ^{B}	7.78 ± 0.05 $^{a\ AB}$
	Y-SR	7.88 ± 0.00 ^{A}	7.72 ± 0.03 ^{A}	7.72 ± 0.09 ^{A}
pH	Y-W	4.49 ± 0.02 $^{c\ A}$	4.42 ± 0.01 $^{b\ AB}$	4.36 ± 0.01 ^{A}
	Y-S	4.51 ± 0.01 $^{b\ A}$	4.38 ± 0.03 ^{A}	4.34 ± 0.00 ^{A}
	Y-R	4.74 ± 0.01 ^{C}	4.69 ± 0.01 $^{b\ E}$	4.63 ± 0.00 $^{a\ D}$
	Y-WS	4.57 ± 0.01 ^{B}	4.47 ± 0.01 $^{a\ BC}$	4.43 ± 0.04 $^{a\ B}$
	Y-WR	4.57 ± 0.01 $^{a\ B}$	4.57 ± 0.01 $^{a\ D}$	4.56 ± 0.01 $^{a\ C}$
	Y-SR	4.61 ± 0.01 $^{c\ B}$	4.53 ± 0.01 $^{b\ CD}$	4.43 ± 0.01 $^{a\ B}$
Titratable acidity (g lactic acid %)	Y-W	0.82 ± 0.02 $^{a\ BC}$	0.96 ± 0.00 $^{b\ D}$	1.06 ± 0.01 $^{c\ D}$
	Y-S	0.81 ± 0.01 $^{a\ BC}$	0.92 ± 0.00 $^{b\ C}$	1.04 ± 0.00 $^{c\ CD}$
	Y-R	0.72 ± 0.02 ^{A}	0.83 ± 0.01 ^{B}	0.82 ± 0.02 ^{B}
	Y-WS	0.85 ± 0.00 $^{a\ C}$	0.97 ± 0.01 $^{b\ D}$	0.99 ± 0.02 $^{b\ C}$
	Y-WR	0.82 ± 0.01 $^{ab\ BC}$	0.78 ± 0.01 ^{A}	0.86 ± 0.00 ^{B}
	Y-SR	0.77 ± 0.01 $^{a\ B}$	0.87 ± 0.00 ^{B}	0.76 ± 0.00 ^{A}

Different lowercase letters within rows indicate significant differences ($P \leq 0.05$) among storage times. Different uppercase letters within columns indicate significant differences ($P \leq 0.05$) among yogurt samples.

had the lowest acidity values, which aligns with their lower acidification activity.

The color characteristics of yogurt are essential as they impact consumer appeal and can indicate product quality and freshness. The color parameters of set-yogurt samples are presented in Table 3. The 100 % dairy yogurt (Y-W) presented the highest luminosity (L*), while Y-R was the least luminous yogurt. Similar results were obtained for the WI. Regarding a* and b*, Y-W and Y-WS presented the lowest values. These results indicate a white-creamy color in the dairy yogurt and a slight brownish hue for the hybrid yogurts, especially Y-R.

3.3. Syneresis

The spontaneous syneresis of the set-type yogurts during storage is presented in Fig. 3A. Initially (day 0), The Y-R sample showed the highest level of spontaneous syneresis (15.7 ± 0.5 %), followed by Y-SR

Table 3
Total solids, color parameters (L*, a*, b*) and whiteness index (WI) of different set-type yogurts.

Sample	Total solids (%, w/w)	L*	a*	b*	WI
Y-W	21.56 ± 0.07 ^{a}	91.7 ± 0.0 ^{d}	−1.5 ± 0.2 ^{a}	13.6 ± 0.1 ^{a}	84.0 ± 0.1 ^{e}
Y-S	21.38 ± 0.13 ^{a}	85.0 ± 0.3 ^{b}	0.9 ± 0.1 ^{e}	17.0 ± 0.0 ^{c}	77.3 ± 0.2 ^{b}
Y-R	21.43 ± 0.23 ^{a}	83.8 ± 0.3 ^{a}	0.3 ± 0.1 ^{cd}	17.7 ± 0.0 ^{d}	76.0 ± 0.2 ^{a}
Y-WS	21.59 ± 0.08 ^{a}	87.4 ± 0.4 ^{c}	−0.5 ± 0.2 ^{b}	14.5 ± 0.0 ^{b}	80.8 ± 0.2 ^{c}
Y-WR	22.33 ± 0.02 ^{b}	86.8 ± 0.1 ^{c}	0.1 ± 0.0 ^{cd}	17.1 ± 0.3 ^{c}	78.4 ± 0.3 ^{d}
Y-SR	21.72 ± 0.14 ^{a}	84.5 ± 0.0 ^{ab}	0.8 ± 0.1 ^{de}	18.0 ± 0.0 ^{d}	76.3 ± 0.0 ^{a}

WI: whiteness index. Different lowercase letters in the same column mean significant differences between samples ($P \leq 0.05$).

(4.0 ± 0.4 %), while the rest of the yogurt samples presented ≤ 1 % spontaneous syneresis (Fig. 3A). Although the level of spontaneous syneresis in Y-R significantly ($P \leq 0.05$) decreased after 7 days of storage, it remained the sample with the highest whey separation at day 14 (10.5 ± 0.8 %).

Similarly, on day 0, the 100 % dairy yogurt (Y-W) presented the lowest centrifuged syneresis (26.7 ± 3.4 %), while Y-R and Y-SR presented the highest centrifuged syneresis (64.3 ± 0.5 % and 63.2 ± 0.5 %, respectively) (Fig. 3B). During storage, the centrifuged syneresis level remained stable ($P > 0.05$) in all yogurts except for Y-W, where a significant ($P \leq 0.05$) increase was observed by day 14 (46.4 ± 2.3 %). Despite this increase, the yogurts containing WPC (Y-W, Y-WS and Y-WR) presented the lowest centrifuged syneresis on day 14.

3.4. SDS-PAGE

Under reducing conditions (Fig. 4A), all yogurt samples presented the characteristic milk protein bands, as they were all produced with SMP. In milk, 80 % of the proteins correspond to the casein fraction, which can be separated in four bands α_{s1}-, α_{s2}-, β- and κ-casein in order of increasing electrophoretic mobility (Dupont et al., 2013). The remaining 20 % of milk proteins correspond to whey proteins, which in turn are comprised of 50 % β-Lactoglobulin (18 kDa) and 20 % α-Lactalbumin (14 kDa) and other minor whey proteins, such as bovine serum albumin (66 kDa) (O'Mahony and Fox, 2013). As expected, the β-Lactoglobulin and α-Lactalbumin bands were more intense in the yogurts enriched with WPC (lanes 2, 5 and 7). The SPI ingredient (lane 9), and with less intensity, the hybrid yogurts containing soy (lanes 3, 6 and 7) showed the main soy proteins: glycinin (11S), which represents 34 %, and β-conglycinin (7S), which represents 27 % of the total proteins. Glycinin consists of six subunits: the acidic (37–42 kDa) (AS) and the basic (17–20 kDa) (BS) linked by disulfide bridges. β-conglycinin is composed of three major subunits (α', α and β) linked by non-covalent bonds (Tang et al., 2006). Amagliani et al. (2017) analyzed rice protein concentrates by SDS-PAGE and reported bands at about 13 kDa, 18–20 kDa, 31–33 kDa, and about 58 kDa, attributed to globulin, β-glutelin, α-glutelin and a globulin subunit, respectively. In the current study, the RPI (lane 10) showed only very faint bands, that were therefore undetectable in the yogurts containing rice (lanes 4, 5 and 6) probably due to the very low solubility of rice proteins.

Under non-reducing conditions (Fig. 4B), β-Lactoglobulin and α-Lactalbumin bands were clearly detected in WPC (Fig. 4B, lane 8), indicating the presence of non-aggregated whey proteins. Conversely, these bands were not evident in SMP (Fig. 4B, lane 1), suggesting that the heat treatment applied during milk powder production had already induced whey protein denaturation and aggregation. In the hybrid yogurts, no noticeable β-Lactoglobulin or α-Lactalbumin bands were

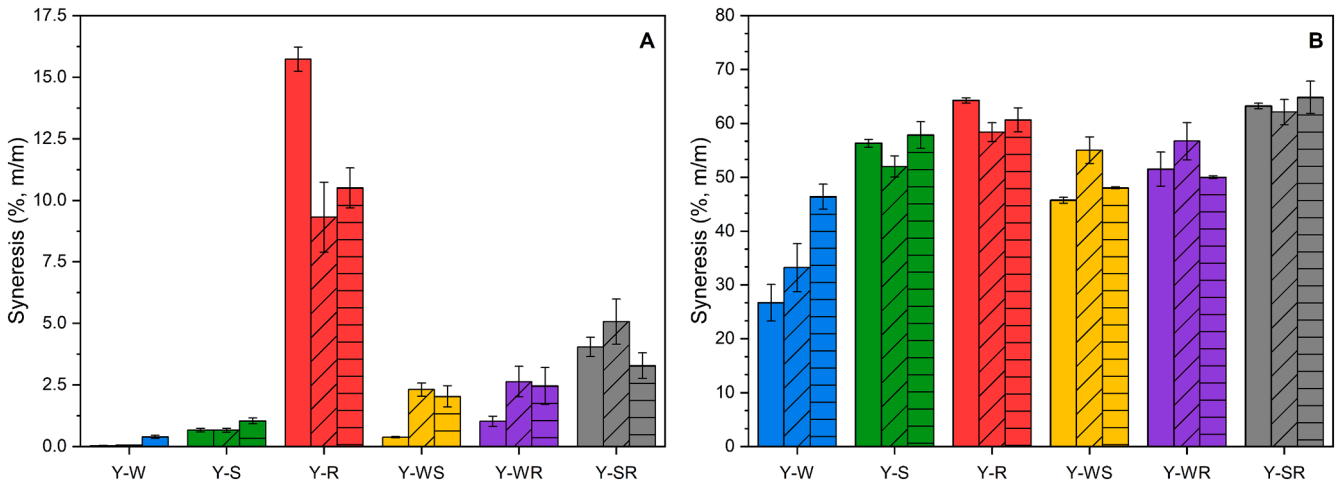

Fig. 3. Spontaneous (A) and centrifuged (B) syneresis of the set-type yogurts at day 0 (solid), 7 (diagonal lines) and 14 (horizontal lines) of storage at 5 °C. Bars represent standard deviation ($n = 3$).

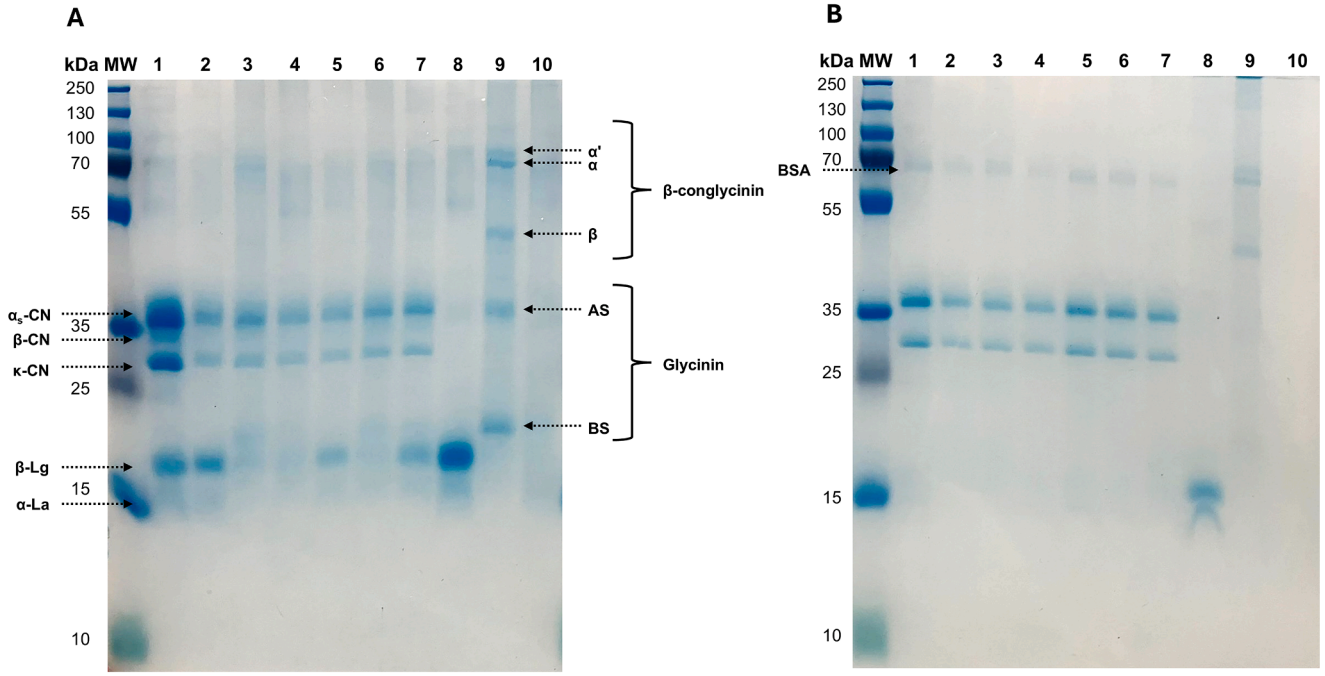

Fig. 4. Reducing (A) and non-reducing (B) protein profiles of the set-type yogurts and their protein ingredients.
Lanes are designated as: 1- SMP; 2- Y-W; 3-Y-S; 4- Y-R; 5- Y-WR; 6- Y-SR; 7- Y-WS; 8- WPC; 9- SPI; 10- RPI; MW, molecular weight ladder; CN, casein; β-Lg, β-Lactoglobulin; α-La, α-Lactalbumin; AS, acidic subunit; BS, basic subunit; BSA: bovine serum albumin.

observed, indicating that these whey proteins formed aggregates during yogurt processing, likely via disulfide bonds.

3.5. Texture analysis

As shown in Fig. 5, the set-type yogurts presented significantly different ($P \leq 0.05$) texture parameters. Such differences can be attributed to their protein profiles, since the total solids levels were nearly constant across samples (Table 3).

The textural attributes evaluated were firmness, consistency, cohesiveness, and work of cohesion. The 100 % dairy set-type yogurt (Y-W) showed the highest firmness, consistency and cohesiveness and the lowest work of cohesion. The yogurts produced by addition of soy protein alone (Y-S), presented the most similar texture profile to Y-W, except for the work of cohesion. The cohesiveness of Y-S showed no significant differences ($P > 0.05$) with Y-W, and Y-S exhibited the second-highest firmness and consistency right after Y-W. By contrast, the addition of rice protein alone markedly affected yogurt texture, with Y-R yielding the lowest values for all texture parameters. Finally, the use of protein blends, whether combining plant proteins or whey with plant proteins, resulted in intermediate texture profiles.

3.6. Rheological behavior

The results of the non-linear rheological characterization of the hybrid yogurts under oscillatory shear deformation are shown in Fig. 6. The rheological behavior varied according to protein formulations. With increasing strain amplitude (γ), the storage modulus (G') first displayed a plateau, representing the linear viscoelastic region (LVR). When γ exceeded a certain value, G' decreased gradually, indicating shear strain

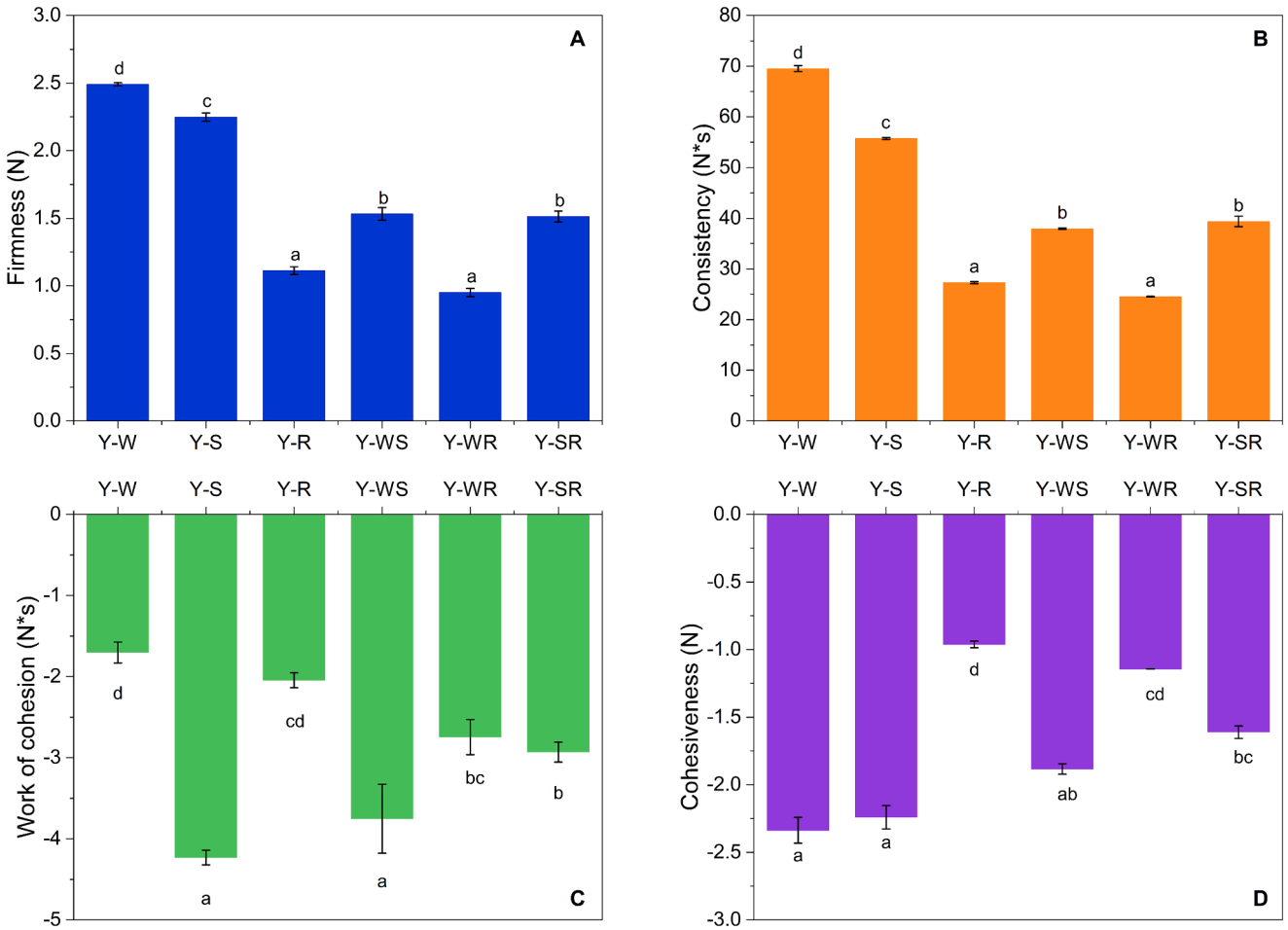

Fig. 5. Instrumental texture parameters: firmness (A), consistency (B), work of cohesion (C) and cohesiveness (D) of the set-type yogurts. Bars represent standard deviation ($n = 3$). Different lowercase letters indicate significant differences ($P \leq 0.05$) among yogurt samples.

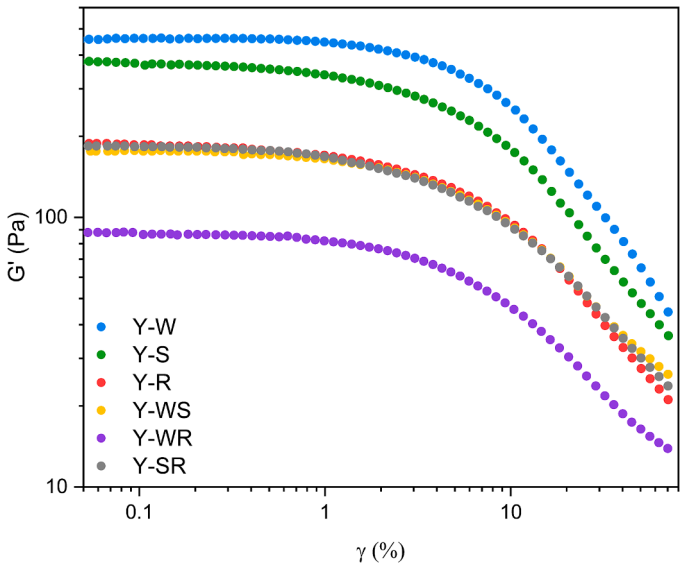

Fig. 6. Strain amplitude (γ) sweep tests of the set-type yogurts.

softening behavior (Xia et al., 2022). All samples exhibited viscoelastic solid-like behavior, as G' exceeded G'' across the entire range of frequencies (data not shown). Y-W had the highest G', followed by Y-S. Y-R, Y-WS, and Y-SR displayed comparable G' and LVRs, although Y-R degraded faster at higher strains. Y-WR showed the weakest structure, being the most susceptible to deformation (lowest G').

3.7. Microstructural analysis

Confocal micrographs of the high-protein yogurts are shown in Fig. 7. The 100 % dairy yogurt (Y-W) exhibited a dense and compact gel network, in agreement with previous reports (Liu et al., 2017; Mahomud et al., 2017). The hybrid yogurts exhibited different microstructure features, highlighting the influence of protein composition on gel formation. Complete substitution of WPC by SPI (Y-S), resulted in the formation of a branched protein network, that was markedly less dense and more porous (black color in Fig. 7) than Y-W. Partial substitution of WPC by SPI (Y-WS), produced an intermediate microstructure between Y-W and Y-S. Replacement of WPC with RPI also altered the gel network. Y-R displayed a loose and branched structure, with large pores distributed throughout the gel. Interestingly, partial replacement of WPC by RPI (Y-WR), did not yield an intermediate structure between Y-W and Y-R, but rather accentuated porosity, with larger pores than Y-R. The mixed soy-rice formulation (Y-SR) showed a structure resembling Y-S but more compact, with large pores similar to those on Y-R and Y-WR.

All hybrid yogurts contained bright yellow particles (indicated with white arrows in Fig. 7), consistent with plant protein aggregates. These aggregates appeared to form mainly through self-association of soy and rice proteins, rather than interactions with milk proteins. The morphology of these particles was different for soy and rice, as observed in the magnified inserts of the images. Both types of morphologies were observed in Y-SR, confirming the coexistence of both types of structures

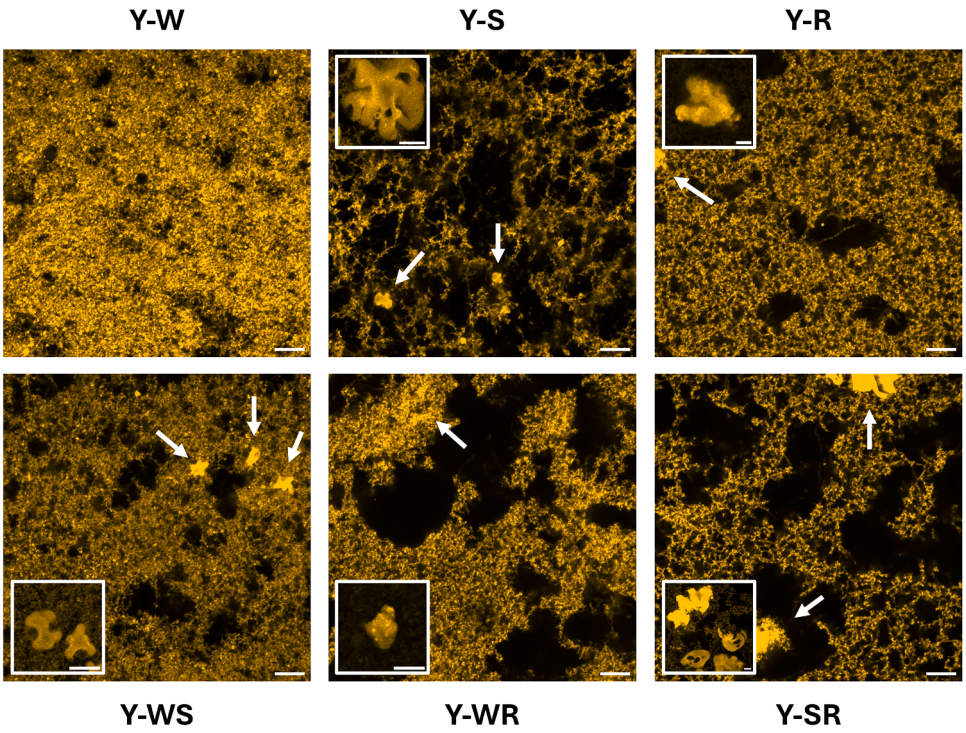

Fig. 7. CLSM images of the set-type yogurts.
The protein networks were stained with rhodamine B and appear in yellow. Scale bars represent 10 μm.

in the mixed system.

4. Discussion

The fermentation behavior of hybrid nonfat set-type yogurts was significantly influenced by the protein composition. The rate of fermentation, measured by the decrease in pH, varied among the different formulations. The addition of plant proteins (soy and rice) significantly affected the fermentation time compared to the 100 % dairy yogurt (Y-W). The inclusion of soy protein increased the fermentation rate, decreasing the processing time required for yogurt manufacture. This effect can be attributed to physicochemical and microbiological factors that enhance the growth and metabolic activity of lactic acid bacteria (LAB), such as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. Soy protein is rich in free amino acids and peptides that can serve as readily available nitrogen sources for LAB, leading to faster bacterial growth and metabolic activity, increasing the production of lactic acid and accelerating acidification. Moreover, the addition of SPI modifies the buffer capacity of the yogurt since soy proteins exhibit lower buffering capacity than casein, causing the pH to drop more rapidly as lactic acid accumulates (Pham and Shah, 2009; Siddiqui et al., 2023; Ziarno and Zar, 2022). This finding has important economic implications for the dairy industry because reduced fermentation times can accelerate processing and reduce production costs.

After 14 days at 5 °C, the yogurts containing rice protein at different levels of substitution (Y-R, Y-WR and Y-SR) had the lowest microbial counts. Higher concentration of *S. thermophilus* and L. *delbrueckii* ssp. *bulgaricus* at the beginning of the storage in the Y-S increased lactic acid production, leading to a decline in bacterial counts. The more pronounced pH drop in Y-S compared to other samples confirms the loss of bacterial viability due to acid accumulation (Table 2). During storage, bacterial survival and proliferation are affected by several factors such as pH changes, nutrient depletion, and the accumulation of metabolic by-products, making the yogurt environment less favorable. As the bacteria continue to metabolize the remaining nutrients, lactic acid production reduces pH, which can inhibit growth and decrease viability (Alsaleem and Hamouda, 2024). However, at the end of the storage, the LAB count was above 7 log CFU/g in all samples, the minimum concentration required to be considered a functional food (Morelli and Capurso, 2012). Although *S. thermophilus* and L. *delbrueckii* ssp. *bulgaricus* are not classified as probiotic organisms, they can improve lactose digestion and may help promote a healthy immune system. Hence, it is desirable that they remain viable and at a high concentration during storage to exert beneficial effects (Paz et al., 2022; Pham and Shah, 2009).

Syneresis denotes the expulsion of liquid whey from yogurt due to contraction of the gel caused by the lowering pH. It is considered a primary defect often related to consumer acceptability and is a quality indicator determining the water holding capacity of the product (Raikos et al., 2018). The siphon method determines the level of spontaneous whey separated on the gel surface while the centrifugation method is also influenced by other factors, such as rigidity and rheological properties of gels (Amatayakul et al., 2006). The results showed that syneresis level was affected by both yogurt formulation and storage time. Y-W presented the lowest syneresis level. This can be attributed to the high water-binding capacity and gel-forming ability of whey proteins, particularly β-lactoglobulin and α-lactalbumin, which form a dense protein network upon heating and fermentation. Whey proteins contribute to a strong, elastic gel that effectively retains water, reducing syneresis. The presence of casein-whey aggregates strengthens the protein matrix, decreasing the likelihood of phase separation (Arab et al., 2023). This is consistent with our SDS-PAGE analysis (Fig. 4), which showed that the whey proteins in all yogurts were aggregated. Nevertheless, the formation of these aggregates alone was insufficient to prevent phase separation in yogurts containing plant proteins.

Throughout the storage period, the weakening of the coagulum causes the structure to release water and serum separation occurs (Akin and Ozcan, 2017). Y-S exhibited slightly higher syneresis than Y-W (control) but remained stable during storage. Although soy proteins have lower gelation properties compared to whey, they still form a relatively structured network through heat denaturation and

acid-induced gelation. SPI has also been reported to retain water effectively through hydrophilic interactions (Jakobson et al., 2023; Zhao et al., 2020). The yogurt with the greater concentration of RPI (Y-R) showed significantly higher spontaneous syneresis. Zhao et al. (2020) reported that rice proteins protein had the lowest solubility across nearly the entire pH range, likely due to the extensive aggregation, disulfide bond cross-linking and glycosylation of glutelins, which account for about 80 % of the total rice protein. This poor solubility negatively influences other functional properties, such as water holding capacity, directly impacting yogurt syneresis (Zheng et al., 2023). When rice protein was combined with whey protein (Y-WR), and hence in lower concentration, the syneresis decreased, reinforcing the idea that whey proteins improve water retention even when combined with lower-quality gel-forming proteins like rice. Conversely, when rice proteins were combined with soy proteins (Y-SR), the syneresis was higher compared to Y-WR, confirming that rice proteins, at this level of substitution, do not contribute to water retention and soy alone (at this concentration) is not sufficient to prevent phase separation. The increase in yogurt syneresis was related to the differences in gel network formation. The isoelectric point, molecular weight, denaturation temperature of the proteins, as well as extrinsic factors such as pH and temperature, influence gel formation its physical properties (Méndez-Galarraga et al., 2025). The presence of plant protein in acid-induced dairy gels, like yogurts, can sterically hinder the development of a strong casein network, resulting in increased syneresis (Lima Nascimento et al., 2023). After 14 days of storage, the spontaneous syneresis in Y-W and Y-WS increased, while interestingly, syneresis in Y-R decreased over time, though remaining the yogurt with the greatest whey separation. Regarding centrifugal syneresis, only Y-W showed a significant decrease during storage. Comparing the two methods, the siphon method would be more appropriate to assess the level of spontaneous whey separation on the surface of set yogurt (Amatayakul et al., 2006).

The acidification during fermentation affects the stability of casein micelles, modifying internal protein interactions, altering their charge, dissolving calcium phosphate crosslinks, and changing protein bonding. These physicochemical interactions promote the aggregation into a cohesive protein network. When plant proteins are incorporated these aggregates could become larger with additional heating or insoluble upon acidification, and the formation of this protein network represents one of the main technological challenges (Lima Nascimento et al., 2023; Martinussi et al., 2024; Rout et al., 2024). Textural properties are an important indicator for the quality assessment of yogurts, influencing the consumers' acceptability. Adding whey proteins provides textural benefits and enhances the gel-forming properties to the yogurt. In fact, there is a relationship between the microstructure of yogurt and the hardness and susceptibility to syneresis. Hardness is primarily influenced by the formation of protein-casein complexes, which increase firmness through the development of a protein network. The interaction between β-lactoglobulin and κ-casein takes place in both the micellar and serum phases, with the extent of this complexation depending on the addition of whey protein. Yogurts with a denser structure and lower porosity exhibit greater water retention capacity (Delikanli and Ozcan, 2014; Nastaj et al., 2019). Among the hybrid yogurts, those including rice proteins had the lowest texture parameters, except for Y-SR. Brückner-Gühmann et al. (2019) reported that oat protein weakened yogurt texture, producing a structure with reduced elasticity and lower water-holding capacity, likely due to no interaction taking place between casein micelles and oat protein, limiting the integration into the yogurt matrix. On the other hand, although soy protein does not interact with caseins in the same way as whey protein, its presence strengthens the gel network. Mitra et al. (2022) showed that 5 % soy protein fortification increased the consistency and stability of the yogurts. The mixed formulations (Y-WS, Y-WR, Y-SR) displayed intermediate values, with whey-containing samples showing improved textural attributes, confirming that the presence of whey proteins enhances gel network formation, whereas rice protein reduces the overall structure stability. These results underscore the role of protein composition (protein type and concentration) in determining yogurt texture and highlight the potential for optimizing hybrid formulations to achieve desirable textural properties. Whey proteins generally form stronger gels than plant proteins, indicating that while substitution is feasible, the overall gel strength may be affected depending on the ratio used (Kornet et al., 2021).

The microstructure and rheological properties of protein gels significantly affect the texture, sensory quality, and storage stability of yogurts. In our study, the protein network density was consistent with the level of syneresis. Pores and gaps in the microstructure hindered water retention, resulting in increased syneresis (Xia et al., 2022). When adding a mix of proteins to the yogurt, the different structural characteristics strongly influence how the mixed protein system is formed. The individual gelling properties of the proteins, as well as the interactions between the different protein fractions, affect the microstructure of the gel in terms of compactness, regular/irregular distribution of the protein network, or gel strength (Méndez-Galarraga et al., 2025). McCann et al. (2018) studied the microstructure of soy-whey gels, showing that 100 % SPI gels exhibited larger clusters, and that the protein network became more uniform as the WPI ratio increased. Li et al. (2025) showed that as the level of soymilk increased, the gaps in the gel network of the hybrid yogurt become larger and more distributed. This suggests that whey proteins, covalently linked, and the caseins from the milk (rigid micelles) form the main structural network, while soy protein (flexible chains) are distributed within protein network and associate through hydrophobic interactions and hydrogen bonding (Li et al., 2025; McCann et al., 2018). Regarding rice proteins, our results showed no interaction between rice and whey proteins and the presence of rice proteins prevented the formation of whey protein aggregates. Wang et al. (2018) found that rice proteins could significantly alter their solubility and structural properties when interacting with soy protein isolates at high pH levels (pH 12), leading to complex formation. These findings highlight the specific influence of environmental conditions and protein types on interaction dynamics, suggesting that under the conditions of our study, rice proteins may stabilize the system differently by disrupting the milk protein gel network rather than forming complexes. Rout et al. (2024) highlighted that dairy–plant protein combinations exhibit both synergistic and antagonistic interactions: while whey proteins may enhance the gelation of plant proteins through hydrophobic and disulfide interactions, structural mismatches can also promote phase separation and heterogeneous network formation.

In our results, the microstructure of the hybrid yogurts exhibited insoluble particles with different shapes. Soy protein particles appeared as wrinkled or shrunk spheres, consistent with the large protein aggregates reported by Gómez-Mascaraque and Pinho (2021) in whey-soy protein heat-set gels. By contrast, rice protein particles showed irregular, undefined shapes. Zhang et al. (2024) reported that rice protein can significantly affect the microstructure of rice starch systems. Their study highlighted that rice protein, when incorporated into starch systems with varying moisture levels, tends to exhibit non-uniform and diverse morphologies, contributing to the irregular appearance of these particles. Wang et al. (2018) studied RP–SPI composites, showing that rice protein formed unstructured agglomerates, whereas the SPI presented smooth surfaces with high integrity. The microstructure of food gels plays a crucial role in defining their mechanical properties, which directly influences consumer perception of texture. Gels with multiple weak or fracture points tend to break into numerous small fragments, unlike homogeneous or protein-continuous gels which are perceived as more spreadable in the mouth, enhancing sensory appeal (Gómez-Mascaraque and Pinho, 2021).

When assessing the rheological behavior of the set-type yogurts, the amplitude sweeps results demonstrated significant variations in storage modulus (G') and linear viscoelastic region (LVR) among yogurt samples with different plant protein integrations. Y-W showed a denser, coarse

and more homogenous structure than the hybrid yogurts (Fig. 7). Yogurts formulated with SPI had a porous, less connected network compared to Y-W. However, soy protein contributed to gel strengthening (Roesch et al., 2004; Xia et al., 2022). As expected, the yogurt made with rice protein formed a less cohesive network, possibly due to limited protein solubility. The poor miscibility between whey and rice proteins likely resulted in an unstable network, which could explain the rapid decrease in G' at higher strains (Y-WR). Variations in yogurt gel structure were influenced by multiple factors, including disulfide bonds, solubility, and composition (Qin et al., 2024). The incorporation of dietary fiber, starch, pectin, and/or other components could fill the voids of protein aggregates and help to reduce the porosity, enhancing the properties of yogurt made with plant proteins.

4.1. Study limitations

The research focused only on soy and rice proteins. Including a broader range of plant proteins could provide a more comprehensive understanding of their potential uses and effects in hybrid yogurt formulations.

Optimizing the formulation is crucial for obtaining a hybrid yogurt with acceptable sensorial characteristics. Flavor profiles play a major role in the success of hybrid yogurts. It is essential that these new formulations not only meet nutritional and textural standards but also satisfy the palate. Taste is key to consumer acceptance of plant-based dairy alternatives, requiring a careful balance of flavors to meet or exceed the sensory quality of traditional dairy products.

The study was conducted on a laboratory scale. Scaling up the production process could introduce new challenges not observed in the controlled small-scale environment.

The microbial viability and other quality parameters were assessed over 14 days. Longer-term studies could provide insights into the stability and shelf life of hybrid yogurts.

Nutritional and health benefits of hybrid yogurt should be studied. Nutritional quality includes compositional parameters such as amino acid composition, but more importantly digestibility needs to be included.

5. Conclusions

The composition of added protein significantly influenced the fermentation behavior, microstructure, and physical properties of the high-protein, non-fat hybrid set-type yogurts. Yogurts containing only added soy protein (Y-S, 1:1 milk protein: SPI) exhibited the shortest fermentation time (3.5 h) and the highest counts of *S. thermophilus* and L. *delbrueckii* ssp. *bulgaricus* (~ 9.8 log CFU/g). In contrast, the formulation containing only added rice protein (Y-R, 1:1 milk: RPI) fermented more slowly (5 h) and showed the lowest microbial viability (~ 8.6 log CFU/g).

Microstructurally, Y-S formed a branched, less-dense, and more porous network than the dairy control (Y-W), whereas the WPC + SPI blend (Y-WS) showed an intermediate structure. Formulations containing RPI exhibited even more open and porous networks with reduced water-holding capacity, which explains the high spontaneous (~ 15 %) and forced syneresis (~ 64 %) and the low firmness and consistency values observed in Y-R yogurts. In contrast, soy protein led to a more cohesive gel network, yielding texture parameters (firmness, consistency, cohesiveness) closer to the dairy control, while the WPC + SPI blend (Y-WS) presented intermediate characteristics.

Overall, soy protein demonstrated the most promising performance among the plant sources evaluated, reducing fermentation time and yielding texture profiles closest to the dairy control. The incorporation of rice protein requires further optimization, particularly to improve its solubility and interaction with milk proteins. These findings provide insights for developing high-protein hybrid yogurts that align with nutritional and sustainability trends while maintaining desirable technological and sensory attributes.

Funding

This work was supported by the postdoctoral fellowship PD_NAC_2023_1_176956, granted to Paula Méndez-Galarraga by the National Agency for Research and Innovation (ANII, Uruguay).

Ethical statement - studies in humans and animals

Not required.

CRediT authorship contribution statement

María Paula Méndez-Galarraga: Writing – review & editing, Writing – original draft, Funding acquisition, Formal analysis, Conceptualization. **Ana Curutchet:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Eliana Budelli:** Writing – original draft, Formal analysis. **Carolina Oliveira-Rizzo:** Writing – review & editing, Writing – original draft, Formal analysis. **Andrés Di Paolo:** Writing – review & editing. **Mariana Rodríguez Arzuaga:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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