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Probiotic Bilayer Edible Films from Pumpkin Seed Flour

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ABSTRACT

Edible films derived from agro-industrial by-products have gained increasing attention as sustainable and functional materials for food applications, particularly as delivery systems for probiotic microorganisms. In this study, mono- and bilayer edible films were developed from shelled and unshelled pumpkin (*Cucurbita moschata*) seed flours as protein-rich, bioprocessed raw materials and enriched with *Lactiplantibacillus plantarum* ATCC 8014. The effects of key formulation variables, including glycerol concentration and the incorporation of a pectin-based outer layer, on the structural, mechanical, barrier, antioxidant, and probiotic-related properties of the films were systematically investigated.

The results demonstrated that formulation-driven modifications significantly altered polymer–plasticizer interactions and matrix organization. Compared with monolayer systems, bilayer films exhibited improved structural integrity and functional performance, highlighting the protective role of spatially separated layers. Films prepared from shelled pumpkin seed flour showed higher protein content and antioxidant activity, whereas those produced from unshelled flour displayed enhanced structural homogeneity and solubility. Probiotic viability was strongly influenced by the glycerol–pectin ratio; however, viable cell counts remained above the recommended threshold of 6 log CFU/g across all film formulations during storage.

This study provides new insights into formulation–structure–function relationships in pumpkin seed flour–based edible films and demonstrates the potential of pumpkin seed by-products as value-added biopolymer matrices for stable probiotic delivery in food bioprocessing and functional food development.

Keywords: Edible films; pumpkin seed flour; *Lactiplantibacillus plantarum*; bilayer films; food packaging; probiotic delivery; antioxidant activity; film characterization

1. Introduction

Food packaging materials, particularly synthetic polymer-based wraps, contribute substantially to environmental pollution due to the generation of millions of tons of non-biodegradable waste each year and their long persistence in landfills [1]. In response to these concerns, edible films and coatings have emerged as sustainable alternatives to conventional packaging materials. These thin, biodegradable layers are applied directly to food surfaces or used as wrapping materials and are intended to be consumed together with the product. Numerous studies have demonstrated the feasibility and effectiveness of edible films across a wide range of food applications, reporting promising functional and environmental outcomes [2]

One of the primary advantages of edible films over synthetic polymers is their biodegradability, which facilitates environmental degradation and aligns with sustainability goals [3]. Materials used for edible film formulation are generally classified into three main categories: lipids (e.g., fatty acids, acylglycerols, and waxes), hydrocolloids (e.g., proteins, polysaccharides, and alginates), and composite systems combining different biopolymers [4]. Beyond their environmental benefits, edible films can improve food quality by acting as carriers for functional additives such as flavorings, colorants, sweeteners, and bioactive compounds. However, their broader industrial application remains limited by relatively weak mechanical and barrier properties compared with conventional synthetic packaging materials [5]. Consequently, current research focuses on the development of novel materials and processing strategies to enhance the functional performance of edible films. A particularly promising advancement is the incorporation of probiotic microorganisms into edible film and coating matrices, offering an effective delivery system capable of improving probiotic stability during processing, storage, and consumption [6].

Probiotics confer health benefits primarily through modulation of gut microbiota composition and activity, as well as through reinforcement of host defense mechanisms. By competing with pathogens for adhesion sites and nutrients, probiotics contribute to microbial balance and enhanced colonization resistance. In addition, probiotic bacteria produce bioactive metabolites, including short-chain fatty acids, organic acids, and bacteriocins, which inhibit pathogen growth and support intestinal barrier function. Beyond antimicrobial effects, probiotics interact with the host immune system by stimulating innate and adaptive immune responses through specific signaling pathways, thereby regulating inflammation, promoting intestinal homeostasis, and supporting metabolic health [7]. When incorporated into edible polymer

matrices, probiotics may therefore function as active or bioactive packaging components, enhancing both the nutritional value and microbiological safety of food products.

Edible films can be formulated from proteins, polysaccharides, lipids, or their combinations, with each biopolymer contributing distinct structural and functional properties. Lipids primarily reduce moisture transfer, proteins generally provide mechanical strength, and polysaccharides regulate gas permeability. Since no single biopolymer can simultaneously fulfill all functional requirements, composite formulations are often necessary [8]. Among advanced fabrication techniques, the layer-by-layer (LbL) approach has proven effective for producing multifunctional films by enabling the sequential deposition and drying of different film-forming solutions, resulting in composite structures with tailored physicochemical properties [9]. To further improve flexibility, elasticity, and mechanical performance, plasticizers are commonly incorporated into edible film formulations. Polyols such as glycerol, propylene glycol, and sorbitol are widely used, while certain fatty acids may also serve as plasticizing agents [10]. Plasticizers disrupt intermolecular interactions within the polymer matrix, increasing chain mobility and free volume; however, this modification may also lead to increased water vapor permeability [11].

The food industry generates substantial quantities of solid waste annually, much of which is rich in biopolymers such as polysaccharides and dietary fibers, as well as bioactive compounds. Agro-industrial by-products, including fruit peels, pomace, and seeds, often exhibit higher antioxidant activity than the edible portions of raw materials [12]. Due to their high carbon content and functional composition, these residues are increasingly recognized as promising resources for upcycling into value-added materials. In particular, fruit and vegetable by-products processed into flours have demonstrated favorable film-forming properties [13; 14]. Pumpkin (*Cucurbita moschata*), cultivated worldwide for culinary and medicinal purposes, represents an underutilized agro-industrial resource [15]. Pumpkin processing generates significant quantities of by-products, especially seeds and peels, with raw pumpkin seeds being rich in proteins, peptides, dietary fiber, and micronutrients [16].

In addition to their nutritional value, plant-based food matrices play an important role in maintaining probiotic viability during processing and storage. Previous studies have shown that the surrounding food matrix strongly influences probiotic survival and functionality, even in the absence of encapsulation. Matrices rich in proteins and complex carbohydrates may provide a protective microenvironment that mitigates environmental stresses and enhances cell stability [17]. In this context, pumpkin seed–based film matrices, characterized by high protein and

dietary fiber contents, represent a promising platform for the direct incorporation of probiotic bacteria into edible films while simultaneously supporting waste valorization.

In the present study, shelled and unshelled pumpkin seeds, commonly regarded as low-value processing residues, were investigated as protein-rich, bioprocessed raw materials for the development of probiotic-enriched mono- and bilayer edible films. The probiotic strain *Lactiplantibacillus plantarum* ATCC 8014 was selected due to its well-documented antimicrobial properties and extensively characterized physiological response to acid stress, making it a reliable reference strain for evaluating probiotic functionality under processing-related conditions [18-20]. Mono- and bilayer film systems were designed to elucidate how raw material characteristics, glycerol concentration, and polysaccharide layer incorporation govern matrix organization, surface properties, and functional performance. In particular, the role of pectin-based bilayer structuring in enhancing film integrity and probiotic protection without direct cell encapsulation was explored. By integrating probiotic microorganisms into multilayer edible film systems derived from pumpkin seed by-products, this study aims to address existing knowledge gaps regarding formulation-driven structure–function relationships and to highlight the potential of agro-food residues as value-added biopolymer matrices for functional food applications.

2. Materials and Methods

2.1 Materials

Pumpkin seeds, both raw shelled and unshelled forms, were purchased from a local market and used as the primary raw material for film preparation. The seeds were ground into flour using a household grinder (Fakir Hausgeräte – Aromatic). Glycerol (Tito, Smart Chemistry Trade and Consultancy Ltd. Co., İzmir, Türkiye) was employed as a plasticizer to impart flexibility and elasticity to the edible films. The probiotic strain *Lactiplantibacillus plantarum* (ATCC® 8014) was acquired in lyophilized form from Microbiologics Inc. (USA; catalog no. 0234L).

2.2 Experimental Methods

Development of Monolayer and Bilayer Films Enriched with Probiotic Microorganisms

Preparation of pumpkin seed flour: To prevent interference of the intrinsic oil content with the film formation and drying processes, a defatting procedure was applied to both shelled and unshelled pumpkin seed flours. Prior to defatting, the seeds were ground using a laboratory-scale grinder (Fakir Hausgeräte – Aromatic). Defatting was carried out by solvent extraction

with n-hexane at a solvent-to-flour ratio of 30:1 (v/w) using a Soxhlet apparatus (Elektro-mag) operated at 85 °C for 4 h. After extraction, the flours were dried in a convection oven at 80 °C for 2 h to ensure complete removal of residual solvent. The dried samples were subsequently sieved through a fine mesh to obtain a uniform powder [12].

Formation of probiotic-enriched monolayer films: Film-forming solutions were prepared from defatted shelled and unshelled pumpkin seed flours at a dry matter content of 14% (w/w). The suspensions were mixed using a magnetic stirrer at 850 rpm for 30 min. To minimize the formation and expansion of air bubbles, which may cause structural defects and fractures in the dried films, the homogenized suspensions were subjected to ultrasonic treatment in a water bath for 45 min. Following sonication, the water-insoluble cellulosic fraction was removed by filtration. Glycerol was incorporated as a plasticizer at concentrations of 0.6% (w/v) for the shelled pumpkin seed solution and 1.0% (w/v) for the unshelled pumpkin seed solution.

The probiotic strain *L. plantarum* ATCC 8014 was activated by inoculating the lyophilized culture into 50 mL of de Man, Rogosa, and Sharpe (MRS) broth (pH 5.6 ± 0.2; Condalab, Madrid, Spain) and incubating at 37 °C for 24 h. Following incubation, the cultures were centrifuged at 4500 rpm for 15 min at 4 °C. The supernatant was discarded, and the resulting cell pellet was washed twice with sterile peptone water (PW, 0.1% w/v, pH 7.0 ± 0.2; Oxoid, Basingstoke, UK). The washed pellet was then re-suspended and incorporated into the pumpkin seed film-forming solution at an inoculation level of 2% (v/v), corresponding to approximately 9 log CFU/g, and homogenized for 5 min to ensure uniform cell distribution.

Subsequently, 20 mL aliquots of the probiotic-enriched solutions were cast into silicone molds and dried using a tray dryer under hot air conditions at 50 °C and an air velocity of 1 m/s. The dry matter content and glycerol concentrations were selected based on preliminary optimization trials.

Preparation of bilayer films: The probiotic-enriched monolayer films were initially dried for 3 h. Following this step, pectin-based coating solutions (15 mL) with concentrations of 1.0%, 1.5%, and 2.0% (w/v), containing glycerol at levels of 6%, 8%, and 10% (w/v), were cast onto the surface of the partially dried monolayer films. The resulting bilayer films were subsequently dried under the same conditions until a constant weight was achieved. A schematic representation of the preparation procedures for mono- and bilayer films is presented in Figure 1.

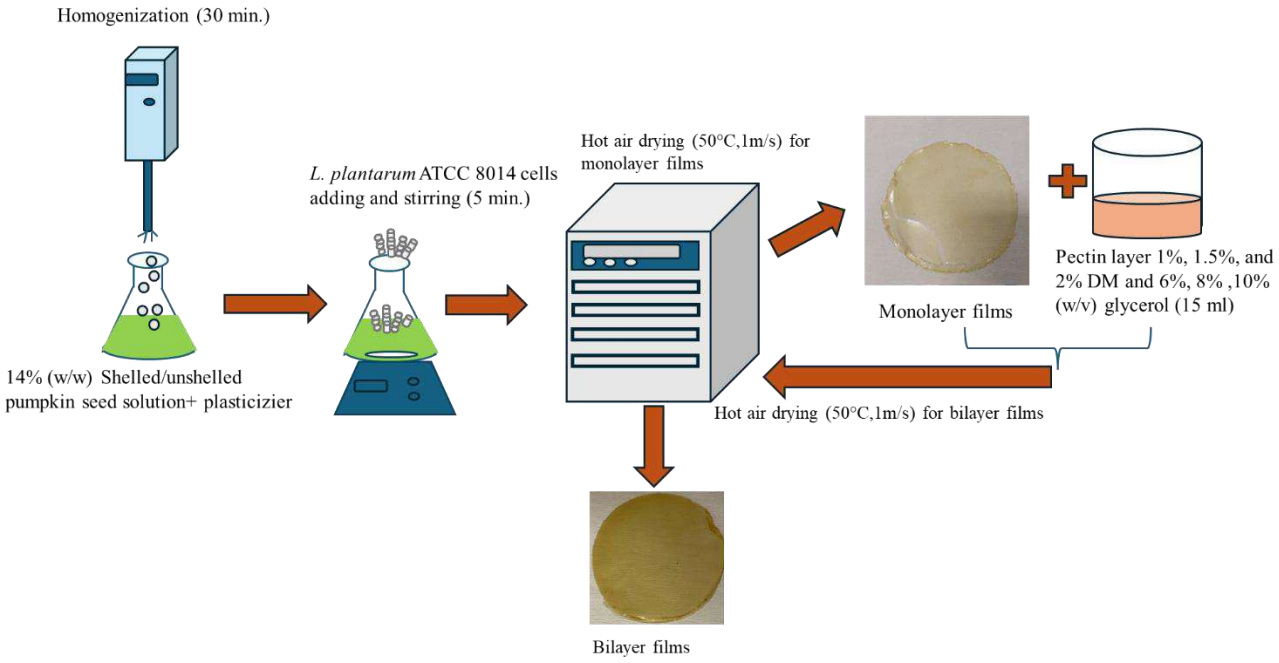


Figure 1. Schematic illustration of the preparation of probiotic-enriched mono- and bilayer edible films using shelled and unshelled pumpkin seed–based solutions.

2.3 Analysis

2.3.1 Water activity and moisture content

According to AOAC 1996, the gravimetric technique was used to determine the moisture content of film samples. Using a water activity (a_w) measuring device (Testo-AG 400, Germany), the sample's a_w was measured. The measurements were performed in triplicate.

2.3.2 Solubility

For the solubility analysis, 1 g of each sample was diluted with 25 mL of distilled water and then mixed using a mechanical stirrer at 3000 rpm for 5 minutes. After that, the mixtures were put into centrifuge tubes and rotated for five minutes at 3000 rpm. Following centrifugation, the supernatant was poured into plates and dried at 105°C in an oven until its weight remained constant. The difference in mass was used to calculate the solubility percentage [9].

2.3.3 Water vapor permeability (WVP)

The water vapor permeability (WVP) of the films was determined using a gravimetric method based on desiccator systems maintained at 75% relative humidity (RH). Desiccators containing a saturated salt solution capable of providing 75% RH were prepared and equilibrated prior to analysis. Silica gel was placed in pre-weighed weighing dishes to act as a desiccant, and the

initial weight of each dish was recorded. Film samples were then carefully sealed over the dishes to prevent edge leakage and placed inside the desiccators. The assembled systems were maintained under controlled conditions for 24 h. Following the incubation period, the dishes were reweighed to determine the amount of water vapor absorbed by the silica gel. Water vapor permeability was calculated using Eq. (1), as described by Fakhouri et al., [21].

$$\text{Water vapor permeability} = \frac{[(m_t - m_0) * z]}{A * t * \Delta P} \quad (1)$$

where, m_o is the initial weight of the silica gel (g), m_t is the weight of the silica gel at time t (g), z is the film thickness (m), A is the exposed area of the film (m^2), t is the duration of exposure (s), ΔP is the partial pressure difference across the film (Pa), calculated as the difference between the vapor pressure of water at 25°C and 75% RH (P_1) and the vapor pressure inside the weighing dish (P_2).

2.3.4 Color properties

The color parameters of the films were determined using a colorimeter (Konica Minolta, CR-300) based on CIE L*, a*, and b* values. The total color difference (ΔE), hue angle (h°), and browning index (BI) of the films were calculated using Eqs. (2)–(5) [22;23]:

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

$$Chroma = \sqrt{a^2 + b^2} \quad (3)$$

$$Hue = arctan\frac{a}{b} \quad (4)$$

$$BI = \frac{100 \times \left(\frac{a + 1{,}75 \times L}{5{,}645 \times L + a - 3{,}012 \times b} - 0{,}31\right)}{0{,}17} \quad (5)$$

2.3.5 Total protein content

The total protein content of the edible films produced from pumpkin seed flour was determined using the Kjeldahl method in accordance with the official procedures of the Association of Official Analytical Chemists [24].

2.3.6 Antioxidant capacity

Extraction procedure

For antioxidant analyses, 0.30 g of each film sample was accurately weighed and extracted with 9 mL of 80% (v/v) ethanol. The mixture was homogenized using an Ultra-Turrax homogenizer (IKA Ultra-Turrax T25) for 1 min. The resulting homogenates were transferred to centrifuge

tubes and centrifuged at 4100 rpm for 18 min. The supernatants were collected and used for subsequent antioxidant assays.

2.3.6.1 DPPH radical scavenging activity

The antioxidant capacity of the extracts was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. Briefly, 0.1 mL of each extract was mixed with a 25 mM DPPH solution and incubated in the dark at room temperature for 30 min. Following incubation, the absorbance (A) was measured at 517 nm using a UV–Visible spectrophotometer. The antioxidant activity was expressed as Trolox equivalent antioxidant capacity (TEAC) and reported as mg Trolox equivalents per 100 g of dry sample [25].

2.3.6.2 FRAP assay (Ferric Reducing Antioxidant Power)

The ferric reducing antioxidant power (FRAP) of the samples was determined by mixing 3.0 mL of freshly prepared FRAP reagent with 0.1 mL of extract. The reaction mixture was incubated at 25 °C for 10 min, after which absorbance was measured at 593 nm using a UV–Visible spectrophotometer (Shimadzu UV-1800). FRAP values were calculated from a calibration curve prepared with ascorbic acid and expressed as mmol ascorbic acid equivalents (AAE) per gram of dry sample [26].

2.3.6.3 ABTS radical scavenging activity

ABTS radical scavenging activity was determined using the $ABTS^{+}\bullet$ [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] assay. The $ABTS^{+}\bullet$ stock solution was prepared and allowed to react in the dark at 4 °C for 24 h. The solution was subsequently diluted with ethanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm. Thereafter, 0.1 mL of extract was added to the diluted $ABTS^{+}\bullet$ solution, and the mixture was incubated in the dark for 6 min. Absorbance was then recorded at 734 nm. The percentage inhibition of $ABTS^{+}\bullet$ radicals was calculated using Eq. (6) [27]:

$$\%\ \mathrm{ABTS} = \frac{(A_0 - A_S)}{A_0} * 100 \qquad (6)$$

where A_0 is absorbance of the control (without sample), A_s is absorbance of the sample.

2.3.7 Total phenolic content

Total phenolic content (TPC) was determined spectrophotometrically using the Folin–Ciocalteu method. For the analysis, 0.1 mL of each extract was mixed with the Folin–Ciocalteu reagent and allowed to react for 3 min. Subsequently, 1.0 mL of a 7.5% (w/v) sodium carbonate solution

was added to the reaction mixture. The samples were then incubated in the dark at room temperature for 60 min, after which absorbance was measured at 765 nm using a UV–Visible spectrophotometer (Shimadzu UV-1800). Total phenolic content was calculated from a calibration curve prepared with gallic acid and expressed as mg gallic acid equivalents (GAE) per 100 g of dry sample [28].

2.3.8 Determination of viable probiotic cell count

The enumeration of viable probiotic cells in the mono- and bilayer films was performed according to the method described by Golowczyc et al. [29], with minor modifications. Briefly, 1.0 g of each film sample was aseptically transferred into 9.0 mL of sterile peptone water (PW, 0.1% w/v) and homogenized using a stomacher (Stomacher Lab-Blender 400, Seward Medical, London, UK). Serial decimal dilutions were prepared using sterile PW. Viable cell counts were determined using the double-layer pour plate technique on MRS agar (pH 6.2 ± 0.2; Merck, VM040261). The inoculated plates were incubated at 37 °C for 3–5 days under aerobic conditions. Following incubation, colonies were counted and the results were expressed as log colony-forming units per gram of sample (log CFU/g), in accordance with ISO 15214 [30].

2.3.9 Statistical analysis

All experiments were conducted in triplicate, and the results were expressed as mean ± standard deviation. Statistical analyses were performed using SPSS software version 22.0 for Windows (SPSS Inc., Chicago, IL, USA). The effects of formulation parameters, including pumpkin seed type (shelled and unshelled), film structure (monolayer and bilayer), and pectin and glycerol concentrations, on the physicochemical, antioxidant, and microbiological properties of the films were evaluated using analysis of variance (ANOVA). One-way ANOVA was applied when a single factor was considered, while two-way ANOVA was used to assess the interaction effects between multiple factors where appropriate. Differences among mean values were determined using Tukey's honestly significant difference (HSD) post hoc test. Statistical significance was accepted at $p < 0.05$. When necessary, data were logarithmically transformed to meet the assumptions of ANOVA. Results showing statistically significant differences were identified using different superscript letters.

3. Results and Discussion

3.1 Bilayer Films Using Shelled Pumpkin Seed Flour

Monolayer edible films were prepared by dispersing shelled pumpkin seed flour in distilled water to obtain a film-forming solution with a dry matter content of 14% (w/w). Glycerol was incorporated as a plasticizer at a concentration of 0.6% (w/v). The resulting dispersion was inoculated with *Lactiplantibacillus plantarum* ATCC 8014 at an inoculation level of 2% (v/v), and the initial viability of the probiotic cells was determined prior to film formation. Subsequently, 20 mL aliquots of the probiotic-enriched suspension were cast into silicone molds and dried under controlled hot air conditions, yielding monolayer probiotic edible films (Altay et al., 2026).

Bilayer films were produced by applying a secondary pectin-based layer onto newly prepared and dried monolayer films. Pectin coating solutions with three different total solid concentrations (1.0%, 1.5%, and 2.0% w/v), containing glycerol at levels of 6%, 8%, and 10% (w/v), were uniformly cast onto the surface of the base films. The coated samples were then dried until a constant weight was achieved to ensure complete film formation and structural stability. All film samples, including both monolayer and bilayer structures, were evaluated in terms of their physicochemical, antioxidant, and microbiological properties. The analyses included determination of viable probiotic cell counts, moisture content, water activity, solubility, water vapor permeability (WVP), total protein content, antioxidant capacity (DPPH, FRAP, and ABTS assays), total phenolic content (TPC), and color parameters. The morphological characteristics of the developed films as influenced by glycerol and pectin concentrations are illustrated in Figure 2. The visual differences observed among the films reflect formulation-dependent changes in matrix organization and surface uniformity induced by glycerol–pectin interactions.

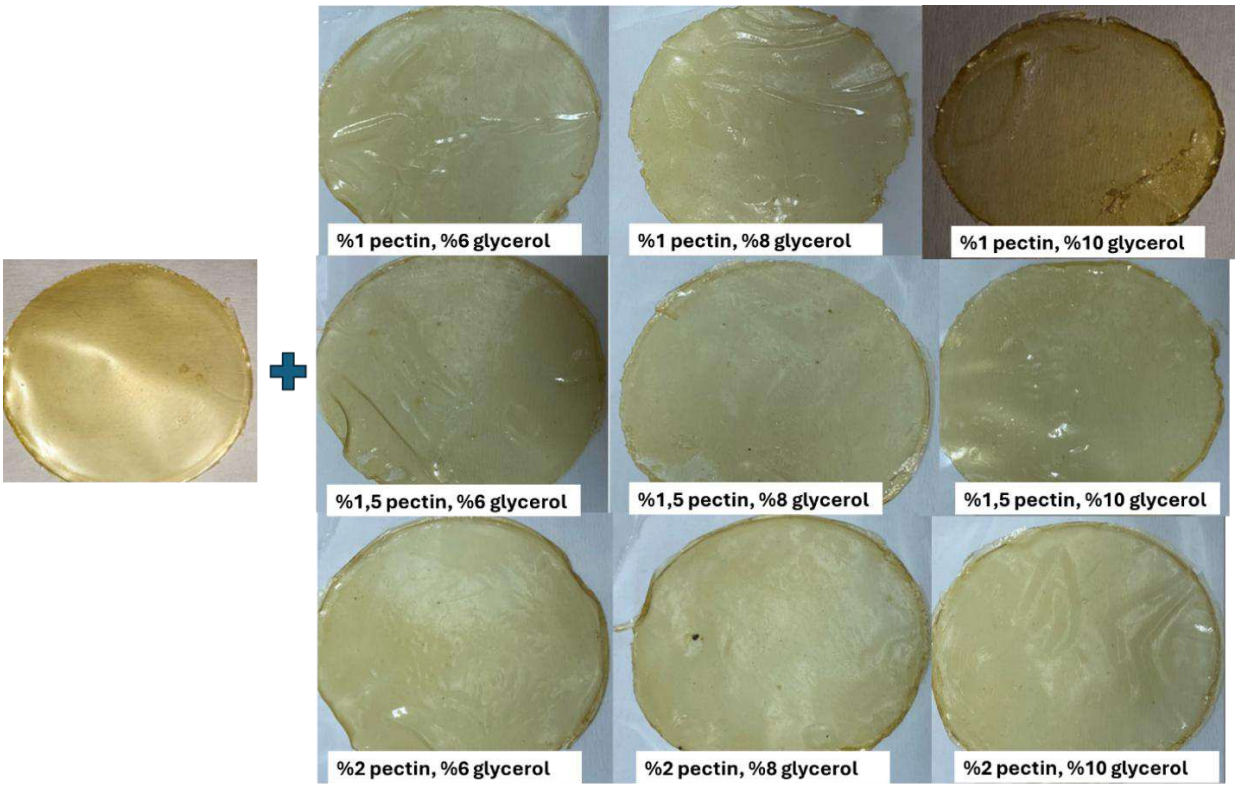


Figure 2. Representative images of bilayer edible films formed by depositing a pectin-based secondary layer onto dried monolayer films prepared from pumpkin seed flour.

Films formulated with moderate levels of glycerol (6%) and pectin (1%) exhibited a uniform, smooth, and semi-matte surface appearance. This surface homogeneity suggests a more balanced plasticization effect, allowing adequate polymer mobility without excessive disruption of the pectin network. In contrast, films containing higher concentrations of glycerol (10%) and pectin (2%) were noticeably thicker and displayed edge curling and increased opacity. The observed edge curling and opacity can be attributed to excessive plasticizer-induced softening, which promotes non-uniform drying and localized stress development within the bilayer structure. A general trend was also observed whereby increasing pumpkin seed flour concentration led to intensified color and opacity of the films. This behavior indicates a denser and more heterogeneous matrix resulting from increased protein and phenolic content, which enhances light scattering within the film structure. This effect may be attributed to the higher protein and phenolic contents of the film-forming solutions, which enhance light scattering and result in a darker film matrix [9]. At elevated glycerol levels, prolonged drying times and undesirable adhesion of the films to the silicone molds were observed. This phenomenon suggests that excessive glycerol interferes with moisture evaporation and delays film solidification by increasing matrix flexibility and water retention. These findings suggest that

excessive glycerol disrupts the carbohydrate-based pectin network, thereby impairing the drying process and compromising the structural integrity of the films. As a consequence, optimal glycerol levels are required to achieve a balance between film flexibility and structural stability in bilayer systems. Similar behavior has been reported for other biopolymer-based edible films, in which excessive plasticizer content weakened polymer–polymer interactions and adversely affected mechanical and barrier properties [31]. The results of probiotic viability (log CFU/g DM), moisture content (%), water activity (a_w), solubility (%), ferric reducing antioxidant power (FRAP; mg AA/100 g DM), and ABTS radical scavenging activity (%) are summarized in Table 1. These parameters collectively provide insight into the functional performance of the bilayer films as probiotic carriers and moisture-sensitive biopolymer systems. The water vapor permeability (WVP) and total protein content of the developed edible films are presented in Figure 3. he observed variations in WVP and protein content further support the role of formulation variables in governing mass transfer behavior and matrix compactness in bilayer edible films.

Table 1. Probiotic viability, moisture content, water activity, solubility, FRAP, and ABTS values of the mono and bilayer edible films.

	Shelled pumpkin seed edible films							
Bilayer edible films	**DM, %**	**Glycerol, %**	**Probiotic counts, log CFU/g DM**	**Moisture Content, %**	**Water Activity, a_w**	**Solubility, %**	**FRAP mg AA/ 100 g DM**	**ABTS, %**
	1	6	$7.72^{e}\pm0.15$	$10.26^{i}\pm0.14$	$0.321^{b}\pm0.027$	$97.92^{b}\pm0.22$	$720.18^{g}\pm2.48$	$95.04^{d}\pm2.13$
		8	$7.35^{d}\pm0.07$	$4.27^{b}\pm0.22$	$0.301^{a}\pm0.035$	$97.91^{b}\pm0.31$	$675.19^{d}\pm2.32$	$94.98^{c}\pm1.90$
		10	$6.92^{b}\pm0.12$	$5.98^{f}\pm0.19$	$0.298^{a}\pm0.022$	$98.20^{bc}\pm0.59$	$670.21^{b}\pm1.01$	$94.60^{b}\pm0.98$
	1.5	6	**$8.44^{i}\pm0.20$**	**$5.47^{d}\pm0.21$**	**$0.305^{a}\pm0.059$**	**$98.01^{bc}\pm0.63$**	**$683.72^{e}\pm2.35$**	**$96.52^{i}\pm2.13$**
		8	$8.16^{h}\pm0.19$	$5.14^{c}\pm0.33$	$0.322^{b}\pm0.063$	$98.47^{bc}\pm0.44$	$761.70^{i}\pm2.62$	$95.95^{h}\pm0.33$
		10	$7.90^{g}\pm0.28$	$8.11^{h}\pm0.52$	$0.360^{d}\pm0.050$	$98.56^{bc}\pm0.10$	$703.36^{g}\pm2.42$	$95.12^{e}\pm0.85$
	2	6	$7.82^{f}\pm0.17$	$5.67^{e}\pm0.10$	$0.345^{c}\pm0.06$	$98.06^{bc}\pm0.07$	$744.33^{h}\pm2.56$	$95.80^{g}\pm0.27$
		8	$7.16^{c}\pm0.09$	$7.29^{g}\pm0.27$	$0.417^{e}\pm0.100$	$98.22^{bc}\pm0.22$	$697.17^{f}\pm2.40$	$95.20^{f}\pm0.52$
		10	$6.50^{a}\pm0.03$	$4.15^{a}\pm0.15$	$0.430^{f}\pm0.149$	$98.44^{bc}\pm0.14$	$674.31^{c}\pm2.32$	$94.55^{a}\pm1.83$
Monolayer edible film			$8.98^{i}\pm0.21$	$13.17^{j}\pm0.35$	$0.585^{g}\pm0.064$	$93.78^{a}\pm0.05$	$379.41^{a}\pm4.07$	$95.12^{e}\pm1.04$
Film solution			$9.19^{i}\pm0.33$					

Different superscript letters (a, b, c, d) within the same column indicate statistically significant differences at $p < 0.05$.

The viability of probiotics in edible films is strongly influenced by the physicochemical characteristics of the film matrix, particularly water activity (a_w), moisture content, and polymer composition. In the present study, the viability of *Lactiplantibacillus plantarum* ATCC 8014 was significantly affected by both the dry matter content and glycerol concentration of the pectin-based outer layer. As shown in Table 1, the bilayer film containing 1.5% pectin and 6% glycerol exhibited the highest viable cell count, whereas the formulation containing 2.0% pectin and 10% glycerol showed the lowest probiotic viability ($p < 0.05$). These results indicate that

increasing glycerol concentration, particularly in combination with higher pectin content, may adversely affect probiotic survival due to increased water permeability and potential structural instability of the film matrix [32]. As also reported in Table 1, a slight reduction in probiotic count was observed following the formation of monolayer films, decreasing from 9.19 to 8.98 log CFU/g, with no statistically significant difference compared to the film-forming solution ($p > 0.05$). This minor reduction may be attributed to stresses associated with mixing and drying processes, such as osmotic and thermal stress, which commonly occur during edible film production [33]. Nevertheless, these stresses were partially mitigated by the pumpkin seed–pectin matrix, which likely provided a protective microenvironment for the probiotic cells. In contrast, an additional reduction of approximately 2 log units was observed following bilayer formation. This further decrease can be attributed to cumulative stresses arising from the secondary rewetting and drying steps, including transient osmotic and pH fluctuations during application of the pectin layer, followed by intensive dehydration to constant weight. Moreover, increased solid loading and film densification at higher pectin concentrations may have further contributed to probiotic viability loss. Although glycerol can function as a membrane-stabilizing plasticizer and lyoprotectant during drying, excessive concentrations may increase osmotic pressure and alter water-binding behavior within the matrix, collectively exacerbating cell damage [34]. Despite these reductions, the final viable cell counts remained at approximately 7 log CFU/g, exceeding the minimum threshold of 6 log CFU/g required to confer probiotic functionality.

Formulation-dependent variations were also observed in the water activity and moisture content of the films. Monolayer films exhibited the highest a_w and moisture content values, which corresponded with lower probiotic viability and antioxidant activity ($p < 0.05$). In contrast, bilayer films demonstrated reduced a_w values (0.298–0.430) and moisture contents (4.15–10.26%), conditions that are more favorable for microbial stability. Water activity and moisture content not only influence immediate probiotic survival during processing but also play a critical role in determining long-term storage stability [17]. Wai et al., [33] reported that optimal moisture contents for maintaining the viability of *Lactobacillus salivarius* subsp. *salivarius* ranged between 2.8% and 5.6%. Similarly, Ying et al. [35] demonstrated that increased environmental relative humidity enhanced water mobility within the matrix, leading to accelerated probiotic viability loss.

The solubility of the bilayer films ranged from 97.91% to 98.56%, which was significantly higher than that of the monolayer film (93.78%, $p < 0.05$). This increase can be attributed to the

presence of the pectin-based outer layer, which enhances water affinity and film disintegration [36]. Although all bilayer films exhibited similarly high solubility values, minor differences were observed among some bilayer formulations, as reflected by the partial overlap of statistical groupings (b and bc). However, these differences were not systematic with respect to pectin or glycerol concentration, suggesting that the bilayer architecture, rather than formulation-level variations, was the primary factor influencing solubility behavior.

Antioxidant activity, as assessed by FRAP and ABTS assays, was markedly higher in bilayer films compared to monolayer films ($p < 0.05$). The formulation containing 1.0% pectin and 6% glycerol exhibited the highest FRAP value, whereas the monolayer film showed the lowest antioxidant capacity. Similarly, ABTS radical scavenging activity in bilayer films increased to 96.52%. The enhanced antioxidant performance of bilayer films may be attributed to improved preservation of bioactive compounds within the less oxidatively stressed inner layer, as well as potential synergistic interactions between pectin and phenolic compounds derived from pumpkin seeds [12]. Furthermore, the lower a_w values of bilayer films likely limited oxidative degradation, thereby supporting antioxidant stability [37]. Bilayer films showed significantly higher FRAP and ABTS values than the monolayer film ($p < 0.05$), while only minor, non-systematic differences were observed among bilayer formulations, as indicated by overlapping statistical groupings.

As illustrated in Figure 3, significant differences were observed in water vapor permeability (WVP) and total protein content between monolayer and bilayer edible films prepared from shelled pumpkin seed flour. These parameters are critical indicators of film structural integrity, barrier performance, and potential applicability in food packaging systems.

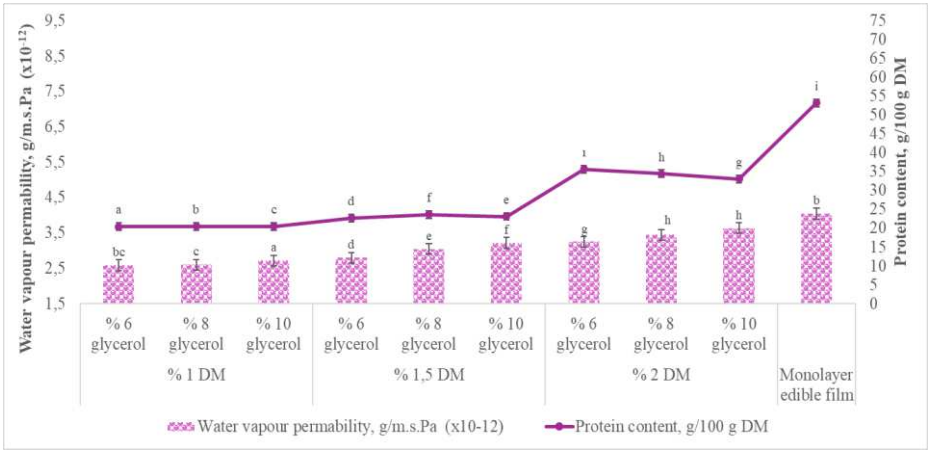


Figure 3. Total protein content and water vapor permeability (WVP) of monolayer and pectin-coated bilayer edible films.

The monolayer film exhibited a significantly higher water vapor permeability (WVP) compared to the bilayer films, indicating a more porous matrix and inferior moisture barrier performance ($p < 0.05$). This behavior can be attributed primarily to the absence of a secondary coating layer. In monolayer systems, the higher glycerol content required to impart sufficient flexibility may have increased the free volume between polymer chains, thereby facilitating water vapor diffusion. Similar effects have been reported by Sothornvit and Krochta [38], who demonstrated that plasticizers such as glycerol enhance polymer chain mobility and consequently reduce resistance to water vapor transmission. Furthermore, the elevated moisture content and water activity observed in the monolayer films support the presence of a less compact structure, which is inherently more susceptible to moisture transfer. These findings are consistent with previous studies indicating that hydrophilic polymer matrices with limited crosslinking exhibit increased WVP due to enhanced water diffusivity [39;40]. In contrast, bilayer films, particularly those containing higher pectin concentrations displayed significantly lower WVP values ($p < 0.05$). The pectin-based outer layer likely improved moisture barrier properties by forming a denser and more cohesive surface network. The intrinsic film-forming ability of pectin, together with intermolecular interactions between polysaccharide chains and glycerol, may have effectively restricted water mobility within the matrix [41].

With respect to protein content, monolayer films exhibited the highest protein concentration, as they were composed exclusively of shelled pumpkin seed flour. In bilayer films, protein content decreased progressively with increasing thickness of the pectin coating due to a dilution effect, as pectin does not contribute protein to the system This decreasing trend was statistically significant ($p < 0.05$), with all formulations belonging to different statistical groups, confirming a clear and systematic dilution effect associated with increasing pectin coating thickness. This observation is in agreement with previous reports indicating that the incorporation of carbohydrate-based outer layers in multilayer film structures results in a proportional reduction in protein content [42;43]. Given the combined effects of barrier properties and compositional changes, their implications for antioxidant performance are discussed in the following section. The antioxidant activity of the edible films, as evaluated by DPPH radical scavenging activity and total phenolic content (TPC), is presented in Figure 4. The antioxidant properties of the monolayer and bilayer films, as assessed by DPPH radical scavenging activity and total phenolic content (TPC), clearly demonstrate the significant influence of film architecture and formulation variables, particularly pectin and glycerol concentrations, on the functional performance of the edible films ($p < 0.05$).

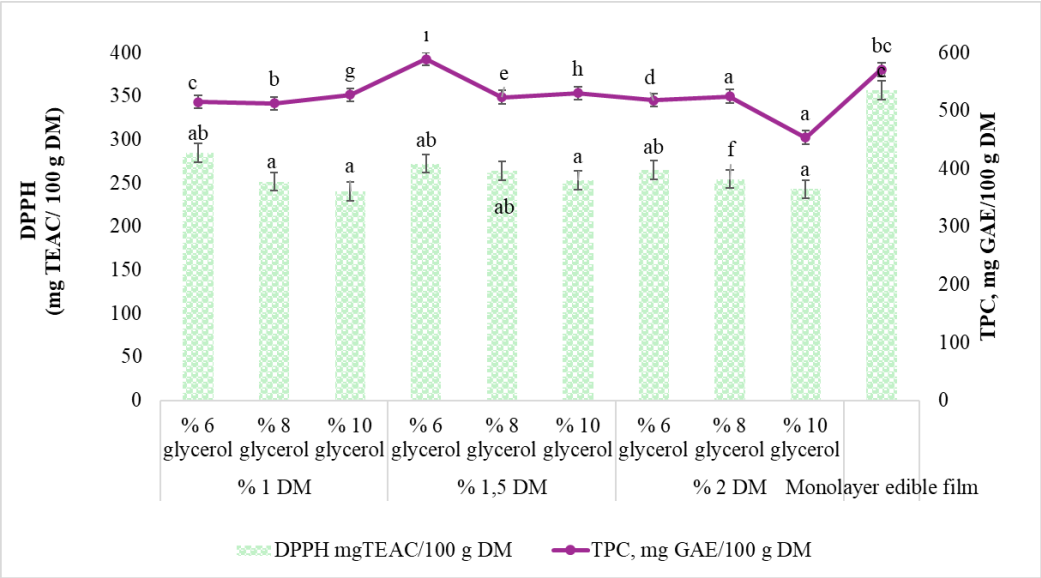


Figure 4. Comparative analysis of DPPH and TPC in monolayer and pectin-coated bilayer edible films.

Monolayer films prepared solely from shelled pumpkin seed flour, without an additional pectin coating, exhibited significantly lower DPPH scavenging activity and TPC values than the bilayer formulations. This behavior can be attributed to the absence of a protective outer layer, rendering phenolic compounds more susceptible to oxidative degradation during drying and handling. In contrast, the pectin-based outer layer in bilayer films likely acted as a physical and diffusional barrier, limiting oxygen penetration and improving phenolic compound retention. Similar protective effects of multilayer structures on antioxidant stability have been reported for chitosan–pectin edible films [44]. An increase in antioxidant activity was observed in bilayer films with increasing glycerol content (6–10%), particularly at intermediate formulations (1.5% pectin and 8% glycerol). This enhancement may be associated with improved matrix flexibility and better dispersion of phenolic compounds within the polymer network, facilitating their interaction with free radicals. However, at the highest pectin (2%) and glycerol (10%) levels, a marked reduction in both DPPH activity and TPC was detected. This decline may be related to excessive plasticization, leading to phase separation, increased hydrophilicity, and possible diffusion or leaching of phenolic compounds during the second drying step. Such adverse effects of over-plasticization on structural integrity and bioactive compound retention have also been reported by Figueroa-López et al. [45]. Overall, these results indicate that bilayer film design, when combined with optimized pectin and glycerol concentrations, can significantly enhance antioxidant functionality, whereas excessive plasticizer and polymer levels may negatively affect bioactive stability. Moreover, bilayer films showed significantly higher

antioxidant activity than the monolayer film ($p < 0.05$), while formulation-level differences were dependent on the balance between pectin content and plasticizer level. Table 2 presents the color analysis results of the edible films.

Table 2. Color results of the mono and bilayer edible films

	DM, %	Glycerol, %	L	a*	b*	BI	ΔE
Bilayer edible films	1	6	$75.91^{h}\pm1.30$	$-0.53^{b}\pm0.12$	$5.70^{a}\pm1.23$	$15.93^{i}\pm3.63$	$58.88^{b}\pm0.60$
		8	$75.25^{g}\pm1.91$	$-0.30^{d}\pm0.09$	$4.70^{a}\pm0.53$	$13.46^{b}\pm1.91$	$59.88^{h}\pm0.93$
		10	$75.13^{e}\pm0.99$	$-0.46^{c}\pm0.15$	$4.50^{a}\pm1.00$	$13.06^{a}\pm1.57$	$59.75^{d}\pm0.24$
	1.5	6	$74.70^{b}\pm1.73$	$-0.17^{h}\pm0.06$	$4.67^{a}\pm0.66$	$13.83^{d}\pm2.49$	$60.14^{i}\pm0.85$
		8	$75.14^{f}\pm0.94$	$-0.27^{e}\pm0.12$	$4.74^{a}\pm0.34$	$13.60^{c}\pm0.88$	$59.87^{g}\pm0.59$
		10	$76.25^{i}\pm1.75$	$-0.48^{b}\pm0.04$	$5.21^{a}\pm0.40$	$14.21^{e}\pm1.04$	$59.04^{c}\pm1.21$
	2	6	$74.92^{c}\pm1.27$	$-0.22^{f}\pm0.09$	$4.97^{a}\pm0.21$	$14.57^{f}\pm0.41$	$59.82^{f}\pm0.82$
		8	$74.26^{a}\pm0.98$	$-0.22^{g}\pm0.09$	$5.17^{a}\pm0.23$	$15.46^{h}\pm0.41$	$60.04^{i}\pm0.68$
		10	$74.99^{d}\pm1.75$	$-0.30^{d}\pm0.16$	$5.06^{a}\pm0.35$	$14.59^{g}\pm0.43$	$59.75^{e}\pm1.18$
Monolayer edible film			$69.88^{i}\pm0.92$	$-3.89^{a}\pm0.14$	$21.26^{b}\pm0.89$	$26.38^{i}\pm1.35$	$13.26^{a}\pm1.06$

Different superscript letters (a, b, c, d) within the same column indicate statistically significant differences at $p < 0.05$.

The L* values were significantly higher in bilayer films than in the monolayer sample, indicating enhanced surface brightness ($p < 0.05$). This brightening effect can be attributed to the presence of a pectin-based outer layer, which increases light scattering and partially masks the intrinsic pigments of pumpkin seed flour. Similar behavior has been reported for other polysaccharide-coated film systems, where surface layering improved optical lightness and visual uniformity [46].

The Browning Index (BI) showed pronounced formulation-dependent variations ($p < 0.05$). The lowest BI value was observed in the bilayer film containing moderate pectin (1.5%) and glycerol (8%), indicating reduced non-enzymatic browning under these conditions. In contrast, the highest BI values were detected in the monolayer film and in bilayer formulations with elevated pectin and glycerol levels, suggesting intensified oxidative and Maillard reactions. These results imply that moderate pectin and plasticizer levels can mitigate surface browning, whereas excessive polymer and plasticizer concentrations may prolong drying and enhance browning reactions. This behavior is consistent with previous reports indicating that pectin-based coatings can reduce color degradation by limiting protein exposure to oxygen and thermal stress during drying [47].

3.2 Bilayer Films Using Unshelled Pumpkin Seed Flour

The production of mono- and bilayer edible films incorporating unshelled pumpkin seed flour followed the same formulation and processing conditions described for films prepared with shelled pumpkin seed flour. Both film configurations were systematically evaluated to assess

their physicochemical, functional, and microbiological properties. Accordingly, all samples were analyzed for probiotic viability, moisture content, water activity, solubility, water vapor permeability (WVP), and protein content. In addition, antioxidant capacity was determined using DPPH, FRAP, and ABTS assays, while total phenolic content (TPC) and color parameters were also measured. The physical appearance and structural characteristics of the resulting mono- and bilayer edible films are illustrated in Figure 5.

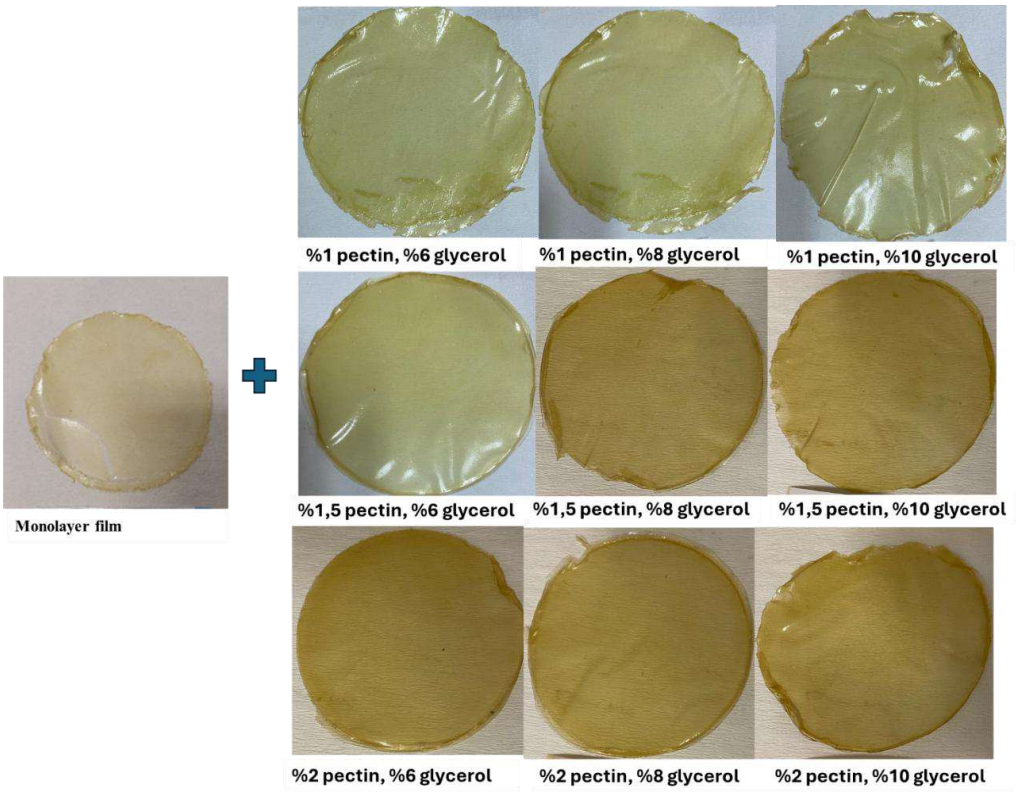


Figure 5. Representative images of bilayer edible films formed by depositing a pectin-based secondary layer onto dried monolayer films prepared from unshelled pumpkin seed flour.

Figure 5 clearly illustrates the morphological variations among the bilayer films formulated with different pectin (1%, 1.5%, and 2%) and glycerol (6%, 8%, and 10%) concentrations in comparison with the monolayer edible film. Bilayer films containing 1% pectin exhibited relatively higher transparency; however, slight surface heterogeneities were observed, suggesting limited interfacial compatibility between the monolayer matrix and the pectin-based outer layer. Increasing the glycerol concentration to 10% resulted in enhanced surface smoothness and flexibility, although a minor loss of clarity was detected, likely due to increased light scattering and potential microstructural phase separation within the film matrix. Films formulated with 1.5% pectin displayed a smoother and more homogeneous surface morphology, which is consistent with their improved visual uniformity and the superior antioxidant activity

observed for these samples. These results indicate that an intermediate pectin concentration promotes improved interlayer adhesion and a well-integrated bilayer structure, yielding balanced optical and functional properties. In contrast, films containing 2% pectin appeared opaquer and more exhibited a noticeable yellowish hue, in agreement with the higher b* and browning index (BI) values (e.g., BI ≈ 40.43 for 2% pectin and 6% glycerol). This increase in opacity is indicative of a denser polymeric network and the contribution of naturally occurring pigments present in the unshelled pumpkin seed flour. Table 3 summarizes the probiotic counts (log CFU/g DM), moisture content (%), water activity (aw), solubility (%), FRAP (mg AA/100 g DM), and ABTS (%) results of the developed edible films, while the water vapor permeability (WVP) and total protein content are presented in Figure 6.

Table 3. Probiotic viability, moisture content, water activity, solubility, FRAP, and ABTS values of the mono and bilayer edible films produced from unshelled pumpkin seed flour.

Bilayer edible films	Unshelled pumpkin seed edible films							
	DM, %	**Glycerol, %**	**Probiotic counts, log CFU/g DM**	**Moisture Content, %**	**Water Activity, a_w**	**Solubility, %**	**FRAP mg AA/ 100 g DM**	**ABTS, %**
	1	6	8.45^{i}±0.33	7.77^{d}±0.22	0.412^{e}±0.045	96.98^{a}±0.16	700.75^{c}±2.41	97.56^{i}±0.11
		8	8.43^{g}±0.17	6.18^{a}±0.27	0.363^{b}±0.029	97.27^{b}±0.21	726.15^{e}±1.57	96.98^{f}±0.20
		10	8.39^{f}±0.15	7.33^{b}±0.19	0.438^{h}±0.101	98.51^{e}±0.33	696.97^{b}±2.91	95.23^{b}±0.05
	1.5	**6**	**8.43^{h}±0.10**	**8.62^{f}±0.05**	**0.430^{g}±0.089**	**97.87^{d}±0.22**	**745.54^{i}±2.68**	**94.46^{a}±0.06**
		8	8.20^{d}±0.27	10.63^{i}±0.17	0.340^{a}±0.012	97.70^{d}±0.30	745.88^{i}±2.89	97.40^{h}±0.09
		10	7.74^{c}±0.30	7.48^{c}±0.34	0.384^{c}±0.035	97.85^{d}±0.17	729.19^{f}±3.64	97.33^{g}±0.21
	2	6	8.31^{e}±0.14	10.04^{h}±0.10	0.416^{f}±0.06	97.50^{c}±0.09	737.45^{h}±2.55	96.29^{d}±0.20
		8	7.68^{b}±0.08	9.98^{g}±0.29	0.377^{d}±0.112	97.95^{d}±0.34	737.24^{g}±8.38	96.49^{e}±0.01
		10	7.60^{a}±0.17	8.47^{e}±0.09	0.435^{i}±0.149	97.09^{ab}±0.14	719.14^{d}±1.41	95.37^{c}±0.04
Monolayer edible film			8.92^{i}±0.30	8.24^{de}±0.66	0.539^{i}±0.002	95.65^{a}±0.75	321.88^{a}±2.33	95.98^{cd}±1.12
Film solution			9.10^{j}±0.05					

Different superscript letters (a, b, c, d) within the same column indicate statistically significant differences at $p < 0.05$.

As shown in Table 3, edible films prepared from unshelled pumpkin seed flour exhibited high probiotic viability throughout both the drying and bilayer formation processes. Soukoulis et al. [32] reported that probiotic viability losses remain limited under low-temperature drying conditions, as applied in the present study [48]. Accordingly, the slight reduction in viable cell counts can be primarily attributed to osmotic stress associated with water removal rather than thermal damage. The viable cell count decreased marginally from 9.10 log CFU/g in the film-forming solution to approximately 8.92 log CFU/g after drying into a monolayer film, indicating that the osmotic stresses induced during dehydration Rahma et al., [49], effectively buffered by the unshelled pumpkin seed flour matrix. Following bilayer formation, probiotic counts remained within the range of 7–8 log CFU/g, confirming that the unshelled matrix provided a favorable protective microenvironment for *L. plantarum* ATCC 8014. The

statistically significant differences observed among formulations ($p < 0.05$) highlight the strong influence of coating thickness and formulation composition on probiotic survival during bilayer formation.

As also summarized in Table 3, increasing the pectin concentration from 1% to 2% generally resulted in elevated moisture content and water activity, particularly at higher glycerol levels. This trend reflects the intrinsically hydrophilic nature of pectin and its strong water-binding capacity [50]. Among the bilayer formulations, films containing 1.5% pectin combined with moderate glycerol levels exhibited relatively higher aw values, reflecting the hydrophilic nature of the pectin-rich matrix. In contrast, film solubility decreased slightly with increasing pectin concentration, indicating enhanced intermolecular cohesion and reduced water diffusivity within the biopolymer matrix—an effect commonly reported in pectin–glycerol systems [51]. Furthermore, antioxidant activity, assessed by FRAP and ABTS assays, was markedly enhanced in bilayer films compared to monolayer counterparts ($p < 0.05$). Notably, the formulation containing 1.5% pectin and 8% glycerol exhibited a FRAP value approximately 2.3-fold higher than that of the monolayer film. This enhancement is likely attributable to improved dispersion, stabilization, and retention of phenolic compounds derived from both pectin and pumpkin seed proteins within the bilayer architecture. These findings are consistent with previous reports highlighting the efficacy of pectin-based films as carriers and protectants of antioxidant compounds [51;52].

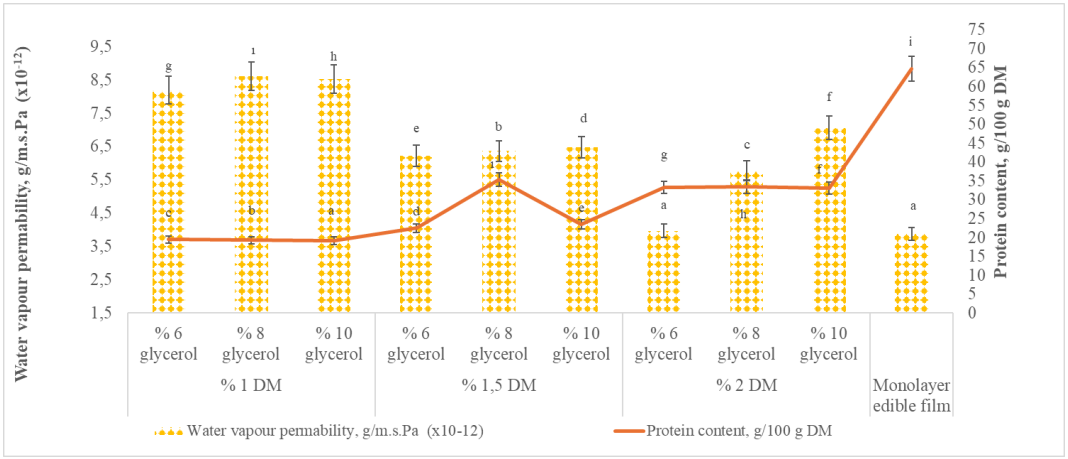


Figure 6. Comparative analysis of water vapor permeability and protein concentration in monolayer and pectin-coated bilayer films produced from unshelled pumpkin seed flour.

The incorporation of a pectin outer layer significantly enhanced the moisture barrier properties of the edible films, particularly in formulations containing 1.5–2% pectin combined with moderate glycerol levels (approximately 8–10%) ($p < 0.05$), as seen in Fig.6. These bilayer films exhibited substantially lower water vapor permeability (WVP) values than the monolayer film, in agreement with previous reports demonstrating that multilayer biopolymer architectures improve moisture resistance by increasing diffusion tortuosity and reducing effective water transport pathways [53-56]. In contrast, bilayer films formulated with higher glycerol content (10%) displayed elevated WVP values despite the presence of a pectin coating ($p < 0.05$). This behavior can be attributed to the pronounced plasticizing effect of glycerol, which increases polymer chain mobility, free volume, and overall matrix permeability. Similar effects have been reported for pectin-based and other hydrophilic biopolymer films containing excessive plasticizer levels [57].

With respect to protein content, monolayer films consistently exhibited higher protein concentrations than bilayer films ($p < 0.05$). This outcome is primarily associated with a dilution effect arising from the addition of the pectin layer, which does not contribute protein and may partially alter protein–polysaccharide interactions during film formation and drying. Comparable trends have been observed in protein–polysaccharide composite and multilayer films, where the incorporation of carbohydrate-based layers reduces protein content on a mass basis [58]. Notably, films containing lower glycerol concentrations (6–8%) showed improved protein retention, suggesting reduced structural disruption and limited protein migration or leaching during drying. In contrast, elevated glycerol levels may promote phase separation and decrease protein detectability in dried films, as reported in recent studies on protein-based edible film systems [59].

Figure 7 presents the comparative antioxidant activity of monolayer and bilayer edible films, as assessed by DPPH radical scavenging capacity and total phenolic content (TPC). Despite the higher TPC values observed in bilayer formulations, a reduction in DPPH radical scavenging activity was detected compared to monolayer films, even in samples with elevated phenolic concentrations ($p < 0.05$). This apparent discrepancy highlights that antioxidant effectiveness in edible film systems is governed not only by the total amount of phenolic compounds but also by their accessibility and reactivity within the polymer matrix. Such divergence between TPC and radical scavenging assays has been frequently reported for composite and multilayer edible films. For example, alginate–pectin films enriched with chokeberry or thyme extracts showed significantly increased TPC, while exhibiting only marginal or reduced DPPH scavenging

activity, attributed to strong matrix–phenolic interactions that restrict radical diffusion and phenolic availability [60]. Similarly, in pectin–polyphenol systems, the mobility, spatial distribution, and exposure of phenolic compounds at the film surface rather than their bulk concentration have been identified as key determinants of functional antioxidant performance [50]. Therefore, the reduced DPPH activity observed in bilayer films can be reasonably attributed to phenolic entrapment within the denser pectin-rich layer and limited radical accessibility, despite enhanced phenolic retention. The color properties of the edible films are presented in Table 4.

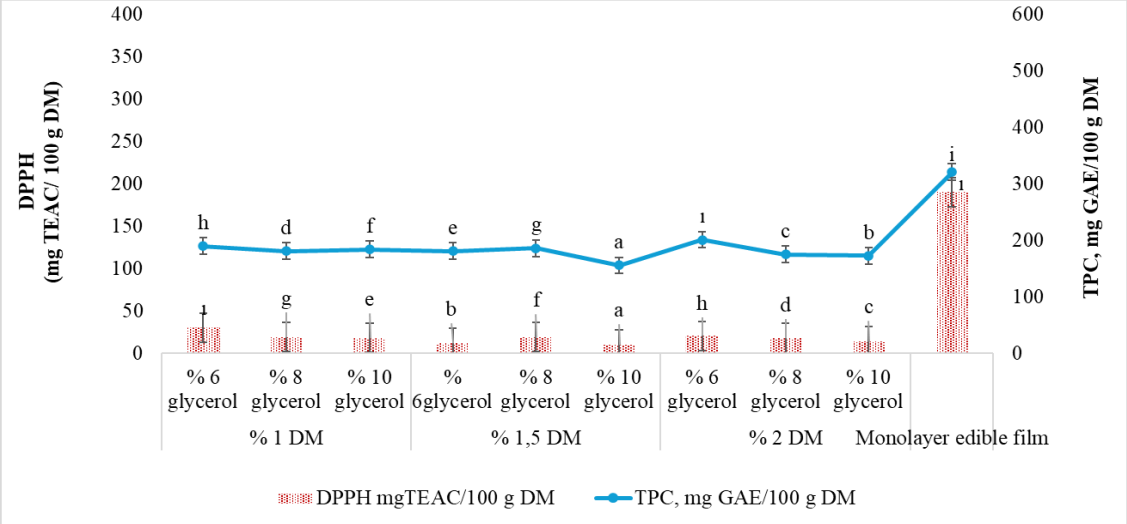


Figure 7. Comparative analysis of DPPH and TPC in monolayer and pectin-coated bilayer edible films produced from unshelled pumpkin seed flour.

Table 4. Color results of the mono and bilayer edible films produced from unshelled pumpkin seed flour.

	DM, %	Glycerol, %	L	a*	b*	BI	Δ E
Bilayer edible films	1	6	$78.50^{i}\pm0.55$	$-4.08^{c}\pm0.05$	$25.03^{e}\pm0.42$	$33.27^{d}\pm0.96$	$38.07^{cd}\pm0.38$
		8	$69.23^{b}\pm1.91$	$-3.99^{d}\pm0.09$	$22.19^{b}\pm0.53$	$35.18^{g}\pm1.91$	$40.84^{g}\pm0.93$
		10	$65.13^{a}\pm0.99$	$-3.46^{f}\pm0.15$	$21.50^{a}\pm1.00$	$34.06^{e}\pm1.57$	$41.75^{h}\pm0.24$
	1.5	6	$77.89^{g}\pm0.50$	$-3.60^{e}\pm0.12$	$23.38^{c}\pm1.03$	$31.21^{b}\pm2.02$	$38.63^{f}\pm0.85$
		8	$76.73^{d}\pm0.18$	$-3.24^{i}\pm0.16$	$24.77^{d}\pm0.42$	$34.20^{f}\pm0.77$	$37.31^{e}\pm0.27$
		10	$77.17^{f}\pm0.63$	$-4.13^{b}\pm0.27$	$25.21^{f}\pm0.40$	$32.84^{c}\pm0.75$	$32.55^{b}\pm0.47$
	2	6	$75.78^{c}\pm0.89$	$-2.94^{i}\pm0.22$	$27.36^{i}\pm0.63$	$40.43^{i}\pm1.91$	$35.10^{c}\pm0.71$
		8	$78.11^{h}\pm0.45$	$-3.35^{h}\pm0.18$	$26.17^{h}\pm3.83$	$36.51^{i}\pm1.33$	$36.83^{cd}\pm2.10$
		10	$77.03^{e}\pm0.49$	$-3.38^{g}\pm1.17$	$25.39^{h}\pm0.56$	$35.42^{h}\pm0.90$	$36.97^{d}\pm0.58$
Monolayer edible film			$78.66^{i}\pm0.39$	$-5.01^{a}\pm0.13$	$23.51^{cd}\pm0.94$	$29.57^{a}\pm1.65$	$14.72^{a}\pm0.71$

Different superscript letters (a, b, c, d) within the same column indicate statistically significant differences at $p < 0.05$.

According to Table 4, the colorimetric parameters revealed a formulation-dependent relationship between pectin concentration and film color characteristics. Increasing pectin levels generally resulted in higher b* (yellowness) and Browning Index (BI) values, with the

most pronounced effect observed in the formulation containing 2% pectin and 6% glycerol ($p < 0.05$). This trend is consistent with previous studies reporting that pectin-based films exhibit increased yellowness and browning due to intrinsic pigments and enhanced susceptibility to thermal reactions during drying [61;62].

In contrast, elevated glycerol content at lower pectin concentration, particularly 10% glycerol combined with 1% pectin, resulted in significantly reduced L* values, indicating decreased lightness ($p < 0.05$). This effect can be attributed to glycerol-induced disruption of matrix compactness, which enhances light scattering within the film structure [51].

Regarding total color difference (ΔE), the monolayer film exhibited the lowest value overall, reflecting minimal deviation from the reference color. Among the bilayer formulations, the lowest ΔE value was observed for the film containing 1.5% pectin and 10% glycerol, suggesting improved color uniformity and a visually homogeneous surface under these conditions ($p < 0.05$). This observation aligns with reports indicating that moderate plasticizer levels can improve optical homogeneity by promoting polymer chain rearrangement [63].

3.3 Comparative Evaluation of Bilayer Edible Films Using Shelled and Unshelled Pumpkin Seed Flour

A comparative evaluation of the functional properties illustrated in Figures 3, 4, 6, and 7 reveals notable differences between bilayer films produced from shelled and unshelled pumpkin seed flours. As shown in Figure 3, all pectin–glycerol bilayer formulations based on shelled pumpkin seed flour exhibited significantly lower water vapor permeability (WVP) values than their unshelled counterparts. This behavior can be attributed to the presence of fibrous and cellulosic components in the shelled flour, which contribute to the formation of a denser and more cohesive matrix. When combined with the pectin coating, this structure establishes a stronger physical barrier, effectively limiting moisture diffusion. Similar reductions in permeability have been reported in pectin-based systems reinforced with cellulose-rich matrices [64;65]. Among all formulations, the bilayer film containing 1.5% pectin and 6% glycerol prepared from shelled flour exhibited the lowest WVP, likely due to optimal network formation and minimal plasticizer-induced free volume.

Regarding protein content, films produced from shelled pumpkin seed flour generally showed higher protein levels than those derived from unshelled flour under comparable conditions. Increasing glycerol concentration was associated with a reduction in measured protein content, particularly in unshelled flour films at 10% glycerol. This effect is likely related to glycerol-

induced dilution of the protein fraction and weakening of protein–pectin interactions, as previously reported for protein–polysaccharide film systems [66].

As illustrated in Figure 4, films prepared with shelled pumpkin seed flour demonstrated markedly higher antioxidant capacity and total phenolic content (TPC) compared to unshelled flour films. This finding is attributed to the richer phenolic composition of the shelled material, which appears to be more effectively retained within the pectin-rich bilayer structure. Notably, the bilayer film formulated with 1.5% pectin and 6% glycerol achieved the highest DPPH radical scavenging activity and TPC values. This formulation likely provided enhanced protection against oxidative degradation due to its lower glycerol content and the formation of a well-organized pectin network that stabilized phenolic compounds, consistent with previous reports on pectin-based antioxidant films [68;69].

Finally, both shelled and unshelled film systems successfully maintained probiotic viability throughout processing, as shown in Tables 1 and 3. However, films produced from shelled pumpkin seed flour particularly those containing 1.5% pectin and 6% glycerol, exhibited the highest viable cell counts. This superior performance can be attributed to the ability of the optimized pectin–glycerol matrix to maintain relatively low water activity and controlled moisture levels, thereby minimizing osmotic and environmental stress on probiotic cells [17] . In contrast, higher glycerol concentrations increased matrix flexibility and water activity, which negatively affected probiotic survival.

4. Conclusion and Future Trends

This study demonstrated the feasibility of developing sustainable bilayer edible films using shelled and unshelled pumpkin seed flours as protein-rich biopolymeric matrices combined with pectin-based outer layers. The successful incorporation of Lactiplantibacillus plantarum ATCC 8014 enhanced the functional value of the films, resulting in a multifunctional system that integrates agro-industrial by-product valorization with probiotic delivery. Throughout processing and storage, probiotic viability consistently remained above the critical threshold of 6 log CFU/g, confirming the suitability of the developed films as functional food packaging materials.

The probiotic cells exhibited notable tolerance to osmotic and mild thermal stresses during drying, which can be attributed to the hydrophilic, protein-rich matrix that preserved localized moisture and mitigated desiccation-induced cellular damage. Comparative evaluation revealed that films produced from shelled pumpkin seed flour consistently outperformed those derived

from unshelled flour in key functional parameters. Specifically, shelled-based films exhibited lower water vapor permeability, higher antioxidant activity (DPPH, FRAP, and ABTS), and greater total phenolic content. These improvements are associated with lignocellulosic and phenolic-rich fractions originating from the shell material, which contributed to matrix densification, enhanced barrier performance, and improved retention of bioactive compounds.

Formulation optimization further identified 1.5% pectin and 6% glycerol as the most effective combination for achieving a balanced performance in terms of moisture barrier properties, mechanical stability, antioxidant functionality, and probiotic survival. These findings are consistent with previous studies reporting synergistic effects between plant-derived polysaccharide coatings and natural fiber inclusions in edible film systems.

From a circular economy perspective, this research provides a promising strategy for upcycling agro-industrial by-products, particularly pumpkin seed residues, into high-value, biofunctional packaging materials. By simultaneously reducing waste streams and delivering nutritional and technological benefits, the developed bilayer films align with sustainability-driven trends in food packaging and functional food design.

Future research should focus on scaling up the film production process and evaluating its economic feasibility and environmental performance through comprehensive life cycle assessment. In addition, film behavior under real food storage conditions, including interactions with different food matrices and potential sensory impacts, should be assessed. The development of advanced multilayer or composite systems incorporating other waste-derived biopolymers or nano-reinforcements may further improve mechanical strength, barrier efficiency, and probiotic protection. Finally, long-term probiotic stability and controlled probiotic release during extended storage and under simulated gastrointestinal conditions should be investigated to substantiate functional and health-related claims.

Overall, the outcomes of this study contribute to the advancement of next-generation active and sustainable packaging systems, supporting global efforts toward food waste reduction, functional food innovation, and environmentally responsible material development.

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Author Statement: Özgül Altay, Ayşegül Kırmızıgül Peker: Conceptualization, Formal analysis, Validation, Investigation, Methodology, Writing and editting original draft.

İlkin Şengün: Conceptualization, Project administration, Supervision, Writing and editting original draft.

Figen Kaymak Ertekin: Conceptualization, Funding acquisition, Project administration, Supervision, Writing and editting original draft.

Data Availability

All relevant data supporting the findings of this study are included within the article. Further information is available from the corresponding author upon reasonable request.

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