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Biodegradable Chitosan–Casein Films Derived From Dairy Waste for Sustainable Applications

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ABSTRACT

Increasing dairy waste presents significant challenges for sustainable disposal. In this study, expired milk was utilized as a casein source to produce biodegradable films, thereby valorizing dairy by-products. Recovered casein, chitosan as a bio-reinforcer, and glutaraldehyde (GA) were combined via solution casting to fabricate the films. Casein blended with chitosan to form continuous, self-supporting films with uniform thickness and stable morphology. The film properties depended on the mixing ratio. Films with a 3:5:2 weight ratio (casein/chitosan/GA) exhibited a smoother surface, higher tensile strength (10.18 ± 0.04 MPa), and lower swelling compared to those with the 4:5:1 formulation. These results indicate that chitosan reinforces the composite matrix structure. The films demonstrated significant degradation under high humidity, indicating good biodegradability. However, none of the films exhibited antimicrobial activity against *Escherichia coli*. These films may serve as potential eco-friendly packaging materials for low-moisture foods, thereby supporting sustainable food processing and packaging.

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1. Introduction

Petroleum-based plastic packaging contributes to environmental harm and microplastic pollution [1]-[3]. To mitigate these risks, researchers are developing biodegradable alternatives with functional properties suitable for food applications [4]-[6]. Casein is a particularly promising protein source due to its film-forming properties and excellent oxygen-barrier properties [7]-[9]. However, its practical use is hindered by poor mechanical strength and high moisture sensitivity [10].

These limitations are often addressed by combining casein with biopolymers such as chitosan or by using chemical crosslinkers [11], [12]. Chitosan, a polysaccharide derived from crustacean shells, reinforces protein matrices through hydrogen bonding and covalent crosslinking, improving structural integrity [13]-[16]. When combined, chitosan acts as a structural skeleton while glutaraldehyde facilitates the formation of a penetrating polymer network via Schiff base reactions [17], [18].

Managing expired dairy products is a global issue [19]. Recovering casein from this waste for non-food applications supports the circular economy by reducing raw material costs and environmental burdens [20], [21]. Despite this potential, there is a lack of systematic research comparing casein recovered from expired milk with commercial virgin casein.

The present study aims to fabricate biodegradable composite membranes using recycled chitosan as the biopolymer matrix and casein recovered from expired milk. We systematically investigated the impact of formulation ratios on the membranes' chemical structure, mechanical properties, and biodegradability. Furthermore, recycled casein films were compared to virgin casein films to assess the feasibility of utilizing dairy waste for sustainable food packaging.

2. Materials and Methods

2.1. Materials

Expired liquid milk was used as the raw material for casein recovery. Before extraction, milk samples were visually inspected to confirm the absence of visible mold or abnormal odor. The pH of each sample was measured, and only those within the typical acidic range of natural spoilage were selected for casein precipitation. Samples were stored at 4°C and processed within a defined time frame to limit further degradation. Chitosan (a deacetylation degree of 90-92% and an Mw of 500 kDa) was prepared from squid pen, following our previous work [22], [23]. Glutaraldehyde (GA, 50%), acetic acid (CH_3COOH, 99%), hydrochloric acid (HCl, 36%), and NaOH (99%) were purchased from Sigma-Aldrich and used as received.

For antibacterial evaluation, *Escherichia coli* was used as the test microorganism. Agar plates were used for the diffusion-based antibacterial assay, and all film samples were prepared as circular discs (D = 6 mm) for testing.

2.2. Methods

2.2.1. Recovery of casein from expired milk

Casein was extracted from expired milk by isoelectric precipitation [24], [25]. The milk was first heated to 85°C for 15 min under gentle stirring to inactivate endogenous enzymes and partially denature whey proteins, which facilitates subsequent protein separation. The milk was then cooled to room temperature. The pH of the milk was gradually adjusted to 4.6–4.8 using 1 M HCl, corresponding to the isoelectric point of casein, resulting in visible coagulation. The precipitated casein was separated by filtration and washed several times with deionized water until the pH reached neutrality to remove residual acids and soluble impurities such as lactose and whey proteins. The purified casein curd was then dried at 45°C for 48 h, mechanically ground into a fine powder, and stored in airtight containers for further use.

2.2.2. Preparation of casein/chitosan films

Films were prepared by solution casting (Figure 1).

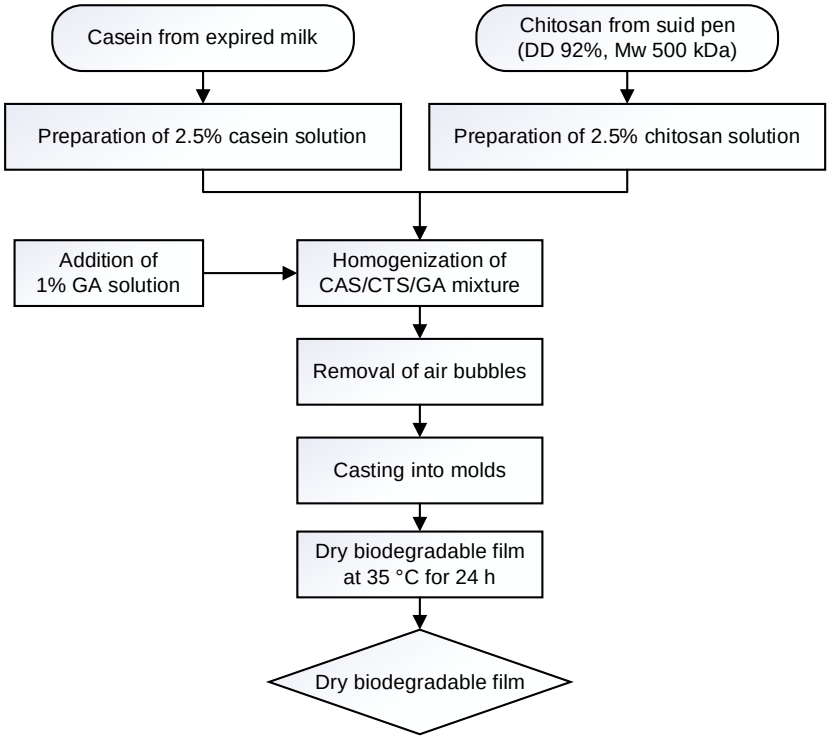

Figure 1. *Preparation of casein/chitosan films by solution casting.*

To prepare the solutions, dissolve 2.5% (w/v) chitosan in 1% (v/v) acetic acid. Disperse 2.5% (w/v) casein in distilled water under stirring. Make a 1% (w/v) GA solution by diluting the GA stock with distilled water. Blend the solutions in casein/chitosan/GA ratios of 4:5:1, 3:5:2, 3:3:4, and 4:3:3, based on previous studies with changes [26], [27]. Homogenize the casein-chitosan mixture for 5–10 min, and then add GA as a crosslinker and mix. Cast the mixture onto a mold, dry at 35°C for 24 h, and store the films over silica gel in controlled humidity until characterization.

2.2.3. Characterization of the casein/chitosan films

The prepared films were characterized to evaluate their structural, mechanical, physicochemical, and functional properties. The film thickness was measured using a digital Panme (Mitutoyo 0–25 mm) with a precision of 1 µm at five random locations, and the average value was recorded. Surface morphology and cross-sectional structure were examined using SEM (Cube II, Tabletop, EMCrafts). The chemical structure was determined via FTIR (ALPHA, Brucker) from 500 to 4000 cm^{-1} at a resolution of 16 cm^{-1} for 32 scans. Tensile strength was tested in accordance with ASTM standards using a rheometer (CR-500DX, SUN). Rectangular film specimens were cut and conditioned at ambient laboratory conditions before testing. Tensile strength was calculated from the stress–strain curve as the maximum force at break divided by the initial cross-sectional area of the specimen.

To assess swelling behavior, pre-weighed dry film samples were immersed in distilled water at room temperature for predetermined time intervals. After immersion, the samples were removed, gently blotted with filter paper to remove surface water, and weighed again. The swelling capacity (%) was calculated based on the percentage increase in sample mass relative to the initial dry mass.

Biodegradation behavior was evaluated by exposing membrane samples to high-humidity conditions (relative humidity > 80%) for 4 weeks. To track degradation over time, the films were periodically observed for physical changes, including fragmentation, surface damage, and color changes.

2.2.4. Evaluation of the antibacterial activity of casein/chitosan films

The antibacterial activity of the fabricated casein/chitosan films was evaluated by agar diffusion according to commonly reported procedures for polymer membrane materials [17]. *Escherichia coli* bacteria were selected as the test microorganism and cultured on nutrient agar plates under standard conditions. The bacterial suspension was spread uniformly on the surface of agar plates to form a microbial lawn. Membrane samples were cut into 6-mm circular discs and carefully placed on the surface of agar plates inoculated with a bacterial suspension. Each agar plate contained five membrane discs to ensure repeatability of observations.

The inoculated discs were incubated at 25°C for 24 hours in a humid environment. After incubation, the discs were visually inspected for the presence or absence of clear inhibition zones around the membrane discs. Antimicrobial activity was qualitatively assessed by the formation of inhibition zones; the absence of such zones was taken to indicate the absence of a detectable antimicrobial effect under the test conditions. This method was used to provide a comparative assessment of the antimicrobial response of membranes prepared with different formulation ratios.

2.2.5. Statistical analysis

All experiments were carried out at least in triplicate unless otherwise stated. Experimental data were expressed as mean ± standard deviation (SD). Statistical analysis was performed to evaluate the effects of formulation ratios on the properties of the fabricated films. Differences among samples were analyzed using one-way analysis of variance (ANOVA). A significance level of $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Recovery of casein from expired milk by isoelectric precipitation

Casein was successfully recovered from expired milk by isoelectric precipitation. The recovered casein product and its FTIR spectrum are shown in Figure 2. After fat removal, the expired milk was heated to 85°C for 15 minutes to inactivate endogenous enzymes and promote partial denaturation of

whey proteins, thereby facilitating subsequent separation. The sample was then cooled to room temperature before acidification. The pH of the milk was gradually adjusted to 4.6 using 1 M HCl, corresponding to the isoelectric point of casein. At this pH, electrostatic repulsion among casein micelles is minimized, resulting in rapid aggregation and visible coagulation. The precipitated casein was separated by filtration, washed repeatedly with deionized water until neutral pH was reached, and subsequently dried at 45°C for 48 h. The dried material was mechanically ground to obtain a fine powder (Figure 2a–c). The recovered casein powder was slightly lighter yellow than commercially refined casein. This was possibly due to differences in particle size distribution caused by the mechanical grinding process.

The recovery yield of casein from expired milk was approximately 2.5 ± 1 % (w/w). Cow's milk typically contains about 2.7% (w/v) casein [7]. Thus, the recovery efficiency of the isoelectric precipitation process is estimated at around 90–93%. These results show that the precipitation and purification steps increased the concentration of the main protein fraction. They also minimized losses during filtration and washing. The recovered casein powder was light yellow. It remained well dispersed in the aqueous solution after hydration. This suggests that isoelectric precipitation preserved physicochemical properties suitable for later membrane preparation.

The FTIR spectrum of the recovered casein, as illustrated in Figure 2d, demonstrates that the protein's chemical structure remains intact following the recovery process. A prominent absorption band is observed at approximately 1628 cm^{-1}, which is characteristic of the Amide I band and corresponds to the C=O stretching vibrations of the peptide bonds in the protein backbone. Additionally, the spectrum also shows strong absorption in the 1300–1200 cm^{-1} range. A distinct peak occurs between 1233 and 1240 cm^{-1}. This signal is linked to the Amide III band and to P=O stretching of phosphate groups, both of which are signature features of casein micelles. Because these functional groups are retained, the results confirm that the recovery method preserved casein's primary chemical identity and molecular integrity.

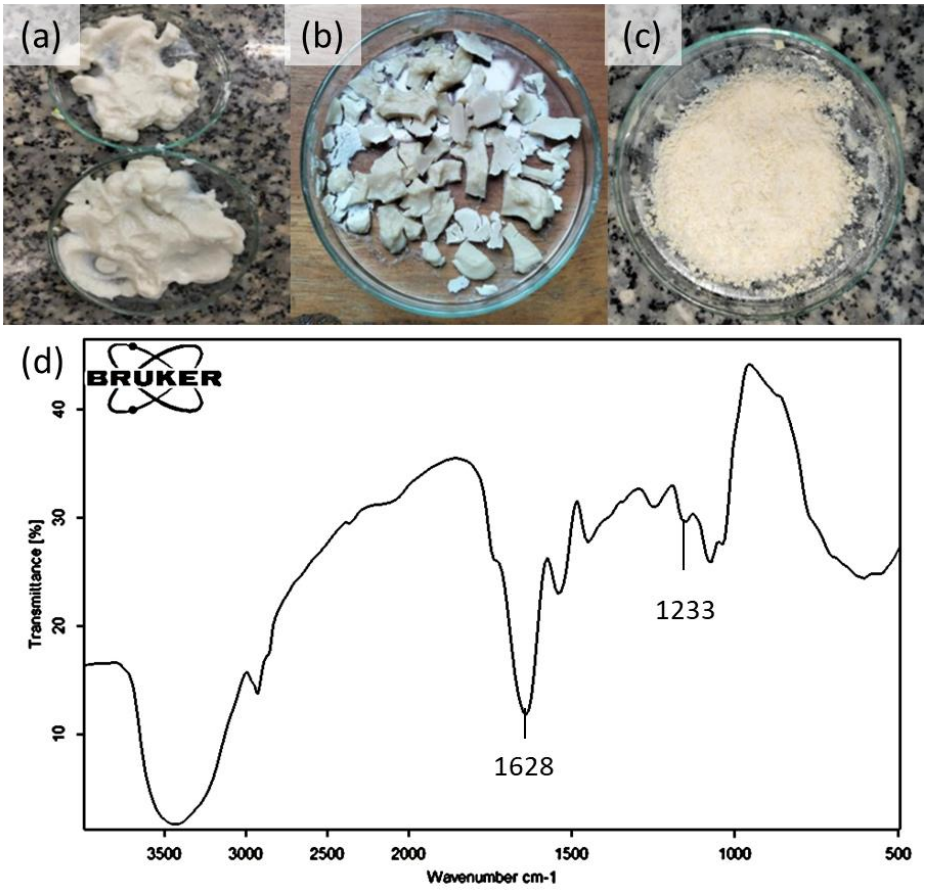

Figure 2. *(a-c) Photos of recovered casein from expired milk and (d) its FTIR spectrum.*

3.2. Characteristics of biodegradable casein/chitosan films

Casein/chitosan films were successfully fabricated by solution casting. As shown in Figure 3a, the resulting films were continuous, self-supporting, and uniformly yellow, with no cracks or apparent phase separation, indicating an efficient film-forming process. Of the four initial formulations, films with CAS/CTS/GA ratios of 4:5:1 and 3:5:2 showed superior structural integrity and flexibility. In contrast, those with higher GA contents (3:3:4 and 4:3:3) were more brittle and had greater microstructural heterogeneity during preliminary evaluation. Detailed morphological characterization was therefore focused on the two most stable and representative films.

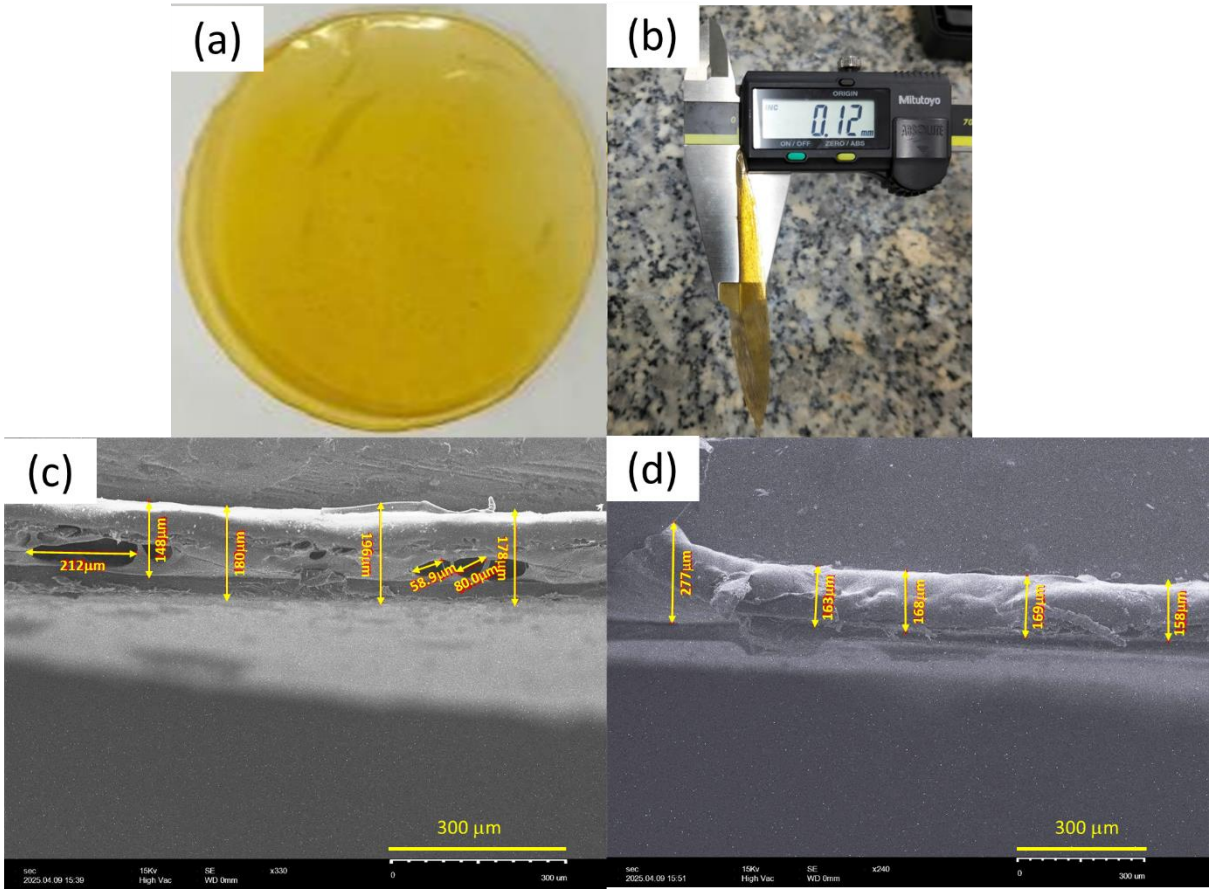

Figure 3. *(a) Photo of the CAS/CTS film; (b) thickness measurement using a digital caliper; (c,d) cross-sectional SEM images of the prepared films.*

The film thickness measured by an electronic caliper (Figure 3b) was approximately 0.12 mm. Cross-sectional SEM images (Figures 3c,d) showed a compact, continuous internal structure with a thickness of 150–200 µm, consistent with caliper measurements. No delamination or large pores were observed in the cross-section of the film. These results confirm that the solution-casting process enables the fabrication of uniform casein/chitosan films with controlled thickness and a coherent internal structure, suitable for further characterization studies.

In addition to macroscopic appearance and thickness characteristics, the surface morphology and chemical structure of the prepared films were further examined, as shown in Figure 4. SEM surface images reveal that the 3:5:2 (wt.%) casein/chitosan film exhibits a relatively smooth and homogeneous surface without visible cracks or large pores (Figure 4a). In contrast, localized surface irregularities and micro-defects were observed for the 4:5:1 (wt.%) formulation (Figure 4b), indicating that the component ratio influences surface uniformity and microstructural stability.

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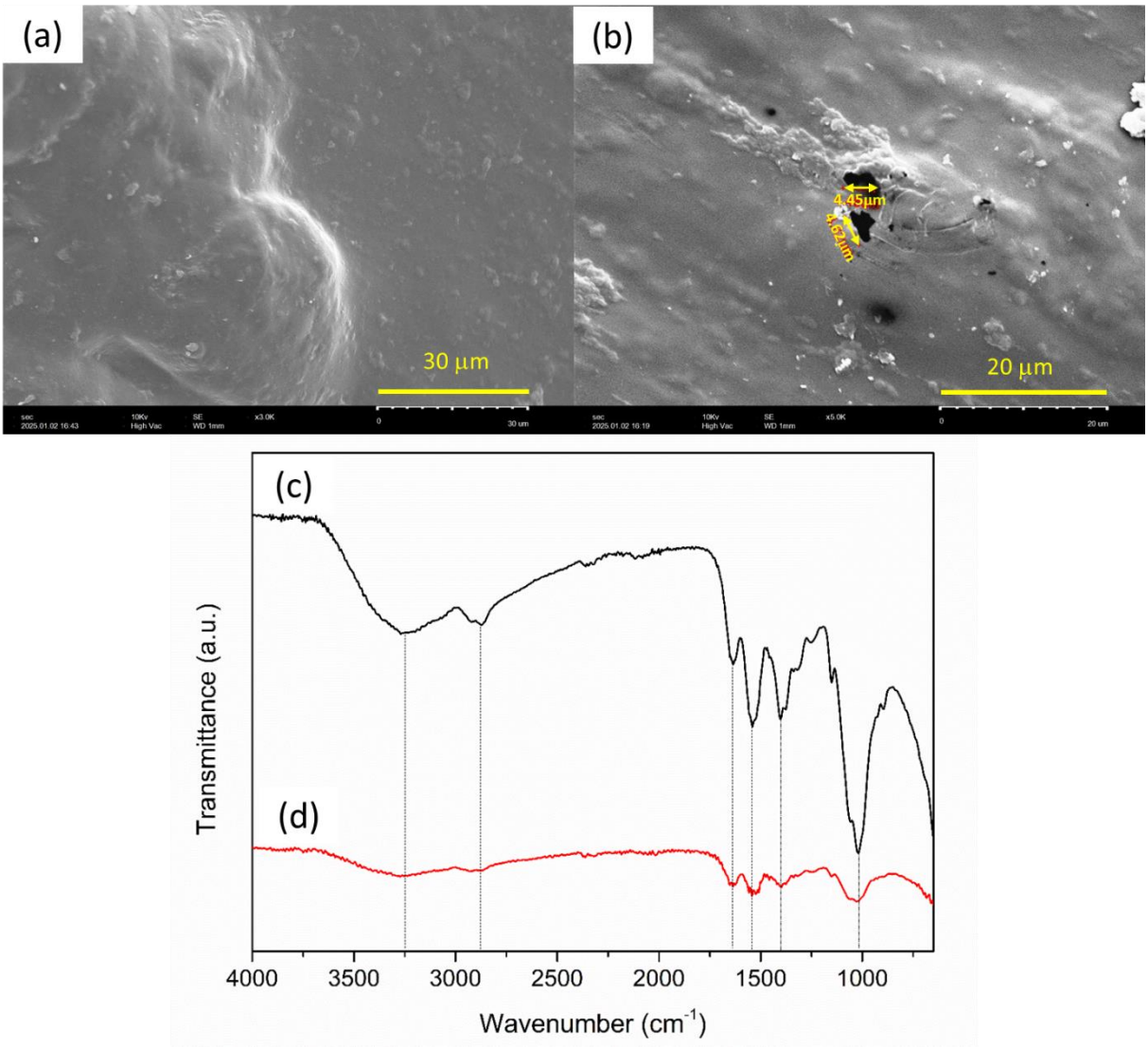

Figure 4. *(a,b) SEM images and (c,d) FTIR spectra of the prepared films at CAS/CTS/GA mass ratios of (a,c) 3:5:2 and (b,d) 4:5:1.*

The FTIR spectra of the prepared films are presented in Figures 4c and 4d. Both spectra show broad absorption bands in the 3300–3000 cm^{-1} range, corresponding to overlapping O–H and N–H stretching vibrations from casein and chitosan. Characteristic amide bands associated with the protein backbone and chitosan structure were retained in the prepared films, confirming the successful incorporation of both biopolymers into the film matrix. These broad bands are typical for protein–polysaccharide systems and indicate the presence of hydrogen bonding interactions between hydroxyl and amino groups, which contribute to the stability of the composite network [28], [29]. Differences in band intensity between the two formulations suggest variations in intermolecular interactions and crosslink density with the CAS/CTS/GA ratio. These observations, combined with thickness and cross-sectional analyses, demonstrate that the 3:5:2 (wt.%) formulation yields films with more uniform surface morphology and coherent structural characteristics, providing a suitable basis for subsequent mechanical and physicochemical analyses. evaluations.

3.3. Mechanical properties, swelling behavior of CAS/CTS films

Table 1 summarizes the tensile properties of the films. The 3:5:2 (wt.%) casein/chitosan film shows higher tensile strength (10.18 ± 0.04 MPa) than the 4:5:1 (wt.%) film (6.12 ± 0.08 MPa). Both samples have the same cross-sectional area (1.8 ± 0.01 mm^2). The higher tensile strength of the 3:5:2 film indicates greater structural integrity.

Table 1. *Tensile properties of different casein/chitosan films.*

Sample (CAS/CTS/GA)	Parameter	(1)	(2)	(3)	Mean ± SD
3:5:2	Maximum tensile force before rupture (N)	18.38	18.25	18.36	18.33 ± 0.07
	Cross-sectional area (mm²)		1.8 ± 0.01		
	Tensile strength (MPa)	10.21	10.14	10.20	10.18 ± 0.04
4:5:1	Maximum tensile force before rupture (N)	11.03	10.88	11.15	11.02 ± 0.13
	Cross-sectional area (mm²)		1.8 ± 0.01		
	Tensile strength (MPa)	6.13	6.04	6.19	6.12 ± 0.08

The swelling behavior results (Table 2) show that both films absorbed water progressively over the 10-day immersion period. However, the 3:5:2 (wt.%) film consistently exhibited lower swelling ratios than the 4:5:1 (wt.%) film. After 10 days, the swelling ratio reached 12.5% for the 3:5:2 film, compared to 13.658% for the 4:5:1 film.

Table 2. *Swelling behavior of different casein/chitosan films.*

Sample (CAS/CTS/GA)	Swelling capacity (%)				
	2 days	4 days	6 days	8 days	10 days
3:5:2	3.37 ± 0.05	5.29± 0.03	7.21± 0.06	10.58± 0.11	12.50± 0.12
4:5:1	5.00± 0.04	5.37± 0.12	8.78± 0.10	10.73± 0.03	13.66± 0.05

3.4. Biodegradation and antibacterial behavior of casein/chitosan films

The biodegradability of the casein/chitosan films was evaluated under high-humidity conditions for four weeks. As shown in Figure 5a and 5b, the film exhibited visible physical changes after exposure. The biodegradability of the casein/chitosan films was evaluated under high-humidity conditions for four weeks. As shown in Figure 5a and 5b, the film exhibited visible physical changes after exposure, including fragmentation and a color change from yellow-orange to brownish tones. These changes indicate progressive degradation and can be attributed to the natural polymer components, casein and chitosan. Protein- and polysaccharide-based materials are known to undergo hydrolytic and microbial degradation under humid conditions, leading to structural breakdown of the polymer matrix [29], [30]. The observed behavior, therefore, confirms the films' biodegradable nature.

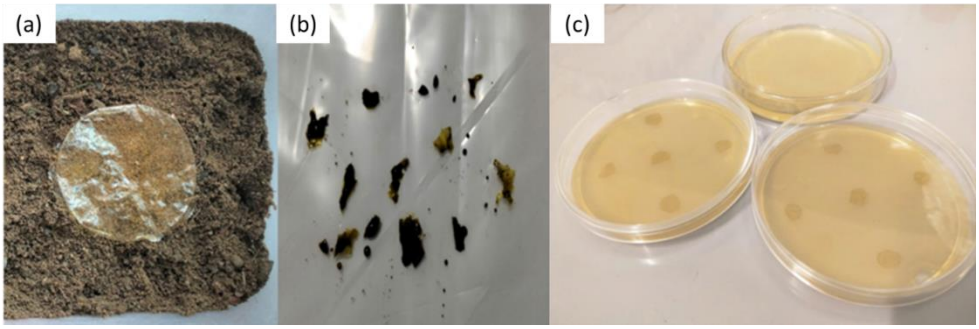

Figure 5. *Visual appearance of the prepared film (a) before and (b) after a four-week biodegradation test under high-humidity conditions. (c) Antibacterial activity of the film against Escherichia coli, evaluated by the agar diffusion method.*

The antibacterial activity of the obtained films against *Escherichia coli* was assessed using an agar diffusion method. Chitosan is widely reported to exhibit antimicrobial activity due to its protonated

amino groups. These groups can interact with negatively charged bacterial cell membranes [31]. Therefore, composite films containing chitosan are expected to exhibit some antibacterial activity. However, as shown in Figure 5c, no clear inhibition zones appeared around the film discs after 24 h of incubation. Bacterial growth continued in direct contact with and under the film samples. This result shows that the prepared films did not exhibit detectable antibacterial activity under the tested conditions. The film composition and structure may explain this outcome. In the present formulation, chitosan was embedded within a cross-linked casein matrix and further reacted with GA. This process may reduce the availability of free amino groups needed for antimicrobial interactions. Also, the relatively low chitosan content and the limited release of active components from the dense film matrix into the agar could further weaken the inhibitory effects. Similar results have been reported for protein–polysaccharide composite films. In these cases, chitosan's antimicrobial activity was reduced by immobilization within cross-linked polymer networks [30], [32]. Overall, these results show that the fabricated casein/chitosan films are clearly biodegradable but primarily serve as structural materials rather than antimicrobial films.

4. Conclusions

In this study, biodegradable films were successfully fabricated from casein recovered from expired milk, chitosan, and glutaraldehyde via solution casting. The recovered casein retained its characteristic chemical structure and was suitable for film formation. The resulting films were uniform, self-supporting, and exhibited consistent thickness. Film properties were strongly influenced by the formulation ratio. Among the investigated compositions, the 3:5:2 (CAS/CTS/GA, wt.%) formulation exhibited the most favorable performance, including higher tensile strength, lower swelling, and a more homogeneous microstructure compared to the 4:5:1 formulation. All films showed clear biodegradability under high-humidity conditions. This behavior can be attributed to the hydrophilic nature of the casein–chitosan matrix, where moisture penetration promotes hydrolysis of peptide and glycosidic bonds, gradually weakening the polymer network and leading to structural degradation. Such moisture-sensitive degradation is characteristic of protein–polysaccharide biodegradable films reported in previous studies. No detectable antibacterial activity against Escherichia coli was observed under the tested conditions. This result indicates that the fabricated films function primarily as biodegradable structural materials. Overall, this work demonstrates the potential of valorizing expired milk as a sustainable protein source for the development of biodegradable films. This process contributes to waste reduction and the circular use of materials.

Conflict of Interest

The authors declare no conflict of interest.

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