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Anti-Biofilm Activity of *Ulva prolifera* Polysaccharide and Its Application as a Natural Preservative for Chilled Chicken Breast

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Abstract

Biofilm formation by *Staphylococcus aureus* poses a challenge to food safety and the preservation of chilled meat. This study evaluated the activity of *Ulva prolifera* polysaccharide (UPP) against *S. aureus* biofilms and assessed its potential application as a natural preservative for chilled chicken breast. UPP showed limited antimicrobial activity against *S. aureus* grown as planktonic cells, with a minimum inhibitory concentration higher than 10 mg/mL. However, at sub-inhibitory concentrations of 0.31–1.25 mg/mL, UPP inhibited biofilm formation by *S. aureus* in a concentration-dependent manner. At 1.25 mg/mL, UPP reduced biofilm formation by 50.8% ± 1.1% and disrupted preformed mature biofilms by 57.7% ± 1.9%. Mechanistic investigation showed that UPP decreased surface hydrophobicity of bacterial cells and their early adherence without severe membrane disruption, the crucial first step for biofilm formation. UPP treatment also altered the composition of the biofilm extracellular matrix, leading to a non-compact biofilm architecture as observed by scanning electron microscopy. Furthermore, UPP inhibited *Candida albicans* biofilm formation in a concentration-dependent manner, with a 48.2% reduction at 1.25 mg/mL, indicating that UPP also possesses broad-spectrum anti-biofilm activity. In a non-inoculated, naturally contaminated chilled chicken breast model, immersion with 1.25 mg/mL UPP reduced the total viable count by 1.8 log CFU/g, decreased malondialdehyde (MDA) levels by 34%, maintained lower absolute pH values during storage, and reduced drip loss by 28%. Furthermore, UPP inhibited *C. albicans* biofilms, remained stable at 4 °C for 8 days, and showed low cytotoxicity. These findings indicate that UPP can function as a natural anti-biofilm preservative for improving the microbial safety and quality stability of chilled poultry products.

Keywords: *Staphylococcus aureus*; biofilm; *Ulva prolifera* polysaccharide; food preservation

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1. Introduction

Global climate change and marine pollution have led to the massive proliferation and widespread distribution of large algae in the form of algal blooms, causing devastating impacts on marine ecosystems [1]. *Ulva prolifera*, a dominant seaweed algae found in algal blooms, not only disrupts the balance of nearshore ecosystems through its excessive proliferation but also causes severe economic losses to coastal aquaculture and tourism [2].

The effective utilization of this abundant marine biological resource and its conversion from an environmental burden into a value-added, reusable resource has become a major research focus. *Ulva prolifera* polysaccharide (UPP) are sulfated polysaccharides derived from *U. prolifera* through a range of extraction methods, including hot-water extraction, alkaline extraction, acid extraction, and enzyme-assisted extraction. Due to their unique chemical structure (rich in rhamnose, xylose, and glucose), UPP possesses various biological activities [3], including antioxidant, immunomodulatory, lipid-lowering, antimicrobial, antitumor and gut microbiota-regulating effects [4–6]. Structurally, hot-water-extracted UPP is a high-molecular-weight sulfated heteropolysaccharide, with a weight-average molecular weight on the order of 9.17×10^5 g/mol and a monosaccharide profile dominated by glucose, galactose, xylose, and mannose [7]. *Ulva*n-type polysaccharides from *Ulva* spp. generally have molecular weights of 150–2000 kDa and characteristic disaccharide repeating units composed of glucuronic or iduronic acid linked to rhamnose 3-sulfate [8]. The sulfate content of *Ulva lactuca* ulvan can reach 19.72% and is directly associated with its antimicrobial and antifouling activities. These structural attributes—high-molecular-weight, abundant sulfate esters, and uronic acid carboxyl groups—are the same features commonly associated with the anti-adhesive and anti-biofilm capacity of marine sulfated polysaccharides. However, in-depth research on the role of UPP in regulating the pathogenicity of pathogenic bacteria, particularly their effects on bacterial biofilms, remains to be systematically evaluated.

Staphylococcus aureus is a major Gram-positive foodborne pathogen responsible for a wide range of foodborne illnesses [9]. In China, *S. aureus* accounted for 4.2% of foodborne outbreaks with known etiology in the national surveillance system from 2003 to 2017. Meat is an important vehicle for this pathogen: a meta-analysis estimated a pooled *S. aureus* prevalence of 21.7% in meat, including 22% in chicken [10]. Earlier nationwide surveys in China reported an isolation rate of 73.6% from chicken, and retail raw chicken still showed an overall *S. aureus* prevalence of 24.2% and MRSA prevalence of 1.7%, with provincial rates reaching 39.6%. In the United States, seven animal-product-associated pathogens, including *S. aureus*, cause an estimated 3.3–12.3 million foodborne illnesses and up to 3900 deaths annually, with economic costs of 6.5–34.9 billion [11]. Biofilm formation is a key survival strategy of *S. aureus*, enhancing its tolerance to sanitizers and antibiotics [12–14], facilitating its persistence on meat, food-processing equipment, and packaging surfaces, and serving as a reservoir for cross-contamination, ultimately contributing to the spoilage of chilled poultry products. In addition, *Candida albicans*, a major opportunistic yeast pathogen, is increasingly recognized as a foodborne contaminant and spoilage yeast; its ability to form biofilms on meat and food-contact surfaces promotes persistence and cross-contamination, thereby threatening the microbial safety of chilled poultry products, especially for immunocompromised consumers [15]. Conventional chemical preservatives and disinfectants are facing increasing consumer concern due to potential safety issues and the emergence of antimicrobial resistance. There is an urgent need to develop or seek alternative natural preservatives.

Although a previous study has shown that crude extracts of *U. prolifera* may possess antibacterial activities, capable of disrupting cell membrane permeability and inducing cell lysis [16], it primarily focused on planktonically grown bacterial cells. A comprehensive study of the effects of UPP on biofilms formed by *S. aureus* is still lacking.

This study aimed to evaluate the activity of UPP against *S. aureus* and *C. albicans* biofilms, and to explore its potential application in chilled chicken breast preservation, and to provide scientific evidence supporting the high-value utilization of *U. prolifera* resources, and ultimately developing marine polysaccharide-based natural preservatives for chilled poultry products.

2. Materials and Methods

2.1. Materials and Strains

S. aureus RN6390 was used in this study. The strain was stored at −80 °C in tryptic soy broth containing 20% glycerol and recovered using Tryptic Soy Broth (TSB, Qingdao Haibo Biotechnology Co., Ltd., Qingdao, China) *U. prolifera* was collected from the coast of Qingdao, China. *C. albicans* ATCC 10231 was used for the biofilm inhibition assay and was cultured in Yeast Peptone Dextrose (YPD) medium at 30 °C for 24 h before use. The algal biomass was thoroughly washed, dried at 45 °C, pulverized into powder, and stored at 25 °C for further use. The monosaccharide standards, including fucose (Fuc), rhamnose (Rha), arabinose (Ara), galactose (Gal), glucose (Glc), xylose (Xyl), mannose (Man), galacturonic acid (GalA), glucuronic acid (GlcA), glucosamine hydrochloride (GlcN), D-fructose (Fru), D-ribose (Rib), galactosamine hydrochloride (GalN), L-guluronic acid (GulA), and D-mannuronic acid (ManA), were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China).

2.2. Preparation of UPP

The *Ulva* powder was mixed with deionized water at a ratio of 1:30 (*w*/*v*) and extracted at 90 °C for 3 h. The mixture was centrifuged at 7500 rpm for 10 min to collect the supernatant, which was subsequently deproteinized using the Sevag method [17]. The deproteinized supernatant was then concentrated to one-third of its original volume. Polysaccharides were precipitated by adding three volumes of 95% ethanol, followed by overnight incubation at 4 °C. Finally, the precipitate was collected and lyophilized to yield the UPP as a light-yellow powder. The extraction yield of UPP was calculated as the mass of lyophilized powder relative to the dry weight of the starting *Ulva* powder. Hot-water extraction of ulvan from *Ulva* spp. typically gives yields of approximately 7–20% under comparable conditions [18], and the yield obtained in this study was within this range. The Sevag deproteinization step reduced the protein content to 0.99% (Table S1), and the single, relatively symmetrical elution peak in HPSEC-MALLS-RI (Figure 1) indicated a homogeneous polysaccharide fraction suitable for subsequent bioactivity assays. The lyophilized UPP was readily soluble in water, forming a clear, light-yellow aqueous solution at the concentrations used in this study.

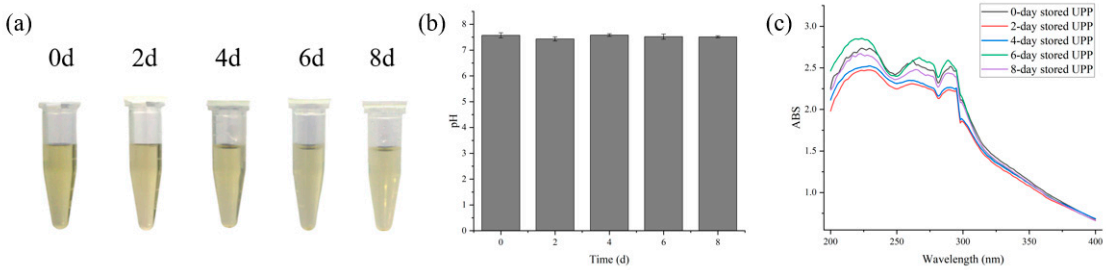

Figure 1. Storage stability of UPP (1.25 mg/mL) at 4 °C for 0, 2, 4, 6, and 8 days. (**a**) Visual appearance of the UPP solution; (**b**) UV–visible absorption spectra (200–400 nm); (**c**) pH values.

2.3. Stability of UPP During Storage

UPP was readily soluble in water and was therefore dissolved in sterile deionized water to a final concentration of 1.25 mg/mL, sterilized by filtration through a 0.22 μm membrane filter, and stored at 4 °C. The storage stability of UPP was evaluated under conditions matching those used in the chicken breast preservation experiments. UPP was dissolved in sterile deionized water to a final concentration of 1.25 mg/mL, sterilized by filtration through a 0.22 μm membrane filter, and stored at 4 °C. At predetermined intervals (days 0,

2, 4, 6, and 8), aliquots were withdrawn and assessed for appearance, pH, and UV–visible absorption. Appearance, including color, clarity, and the presence of precipitate, was recorded under natural light and photographed with a digital camera under fixed lighting conditions. The pH was measured directly using a calibrated pH meter. UV–visible spectra were recorded over the range of 200–400 nm on a TU-1810 UV–visible spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China) using a 1 cm quartz cuvette, with sterile deionized water as the blank. Three independent replicates were performed at each time point.

2.4. Determining the Chemical Composition of UPP

The total sugar content was determined by the phenol-sulfuric acid method [19], using rhamnose as the standard. The uronic acid content was quantified using the *m*-hydroxydiphenyl colorimetric method [20], with D-glucuronic acid as the standard. The sulfate group content was measured using the barium chloride-gelatin method [21], with potassium sulfate as the standard. The protein content was evaluated by the Bradford method, using bovine serum albumin used as the standard [22].

2.5. Determining the Molecular Weight and Polydispersity Index

The molecular weight and polydispersity index (PDI) of UPP were determined using high-performance size-exclusion chromatography coupled with multi-angle laser light scattering and refractive index detection (HPSEC-MALLS-RID) [23]. The detection system consisted of a DAWN HELEOS-II 18-angle laser light scattering detector (Wyatt Technology, Santa Barbara, CA, USA) and an Optilab T-rEX refractive index detector (Wyatt Technology) connected in series. Chromatographic separation was performed on a tandem series of OHpak SB-806M HQ and SB-804 HQ columns (8.0 mm × 300 mm; Showa Denko K.K., Tokyo, Japan). The mobile phase was a 0.1 mol/L $NaNO_3$ solution at a flow rate of 1.0 mL/min, with the column temperature maintained at 40 °C. The operating wavelengths for the MALLS and RID detectors were 662.0 nm and 658.0 nm, respectively. The polysaccharide concentration was determined from the RID response, employing a refractive index increment (dn/dc) value of 0.1380 mL/g. The sample (10 mg/mL) was filtered through a 0.22 μm membrane filter, and a 50 μL filtrate was injected. Molecular weight calculation was performed using the first-order Zimm model, and data acquisition and processing were conducted via ASTRA 6 software (Wyatt Technology).

2.6. Determination of Monosaccharide Composition

The monosaccharide composition of UPP was analyzed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [23]. Briefly, 5.0 mg of the polysaccharide sample was hydrolyzed with 2.0 mL of 3.0 mol/L trifluoroacetic acid (TFA) at 120 °C for 3 h. The hydrolysate was dried under a nitrogen stream, reconstituted with ultrapure water, and centrifuged to collect the supernatant. The resulting supernatant was filtered through a 0.22 μm aqueous membrane filter prior to injection. Separation was achieved on a Dionex CarboPac PA20 anion-exchange column (3.0 mm × 150 mm; Thermo Fisher Scientific, Waltham, MA, USA) at 30 °C. The mobile phase consisted of ultrapure water (A), 15 mmol/L NaOH (B), and a mixture of 15 mmol/L NaOH and 100 mmol/L sodium acetate (C) under gradient elution conditions at a flow rate of 0.3 mL/min.

2.7. Fourier Transform Infrared (FT-IR) Spectroscopy

The UPP were ground with dry potassium bromide (KBr) powder and pressed into a pellet. The FT-IR spectrum was recorded using an IRTracer-100 spectrometer (Shimadzu, Kyoto, Japan) over a wavenumber range of 4000–500 cm^{-1}.

2.8. Inhibitory Effect of UPP on S. aureus Biofilms

S. aureus was cultured in a 37 °C shaking incubator for 24 h, then diluted in TSB medium to a concentration of 1×10^6 CFU/mL. A 100 µL aliquot of the diluted *S. aureus* suspension was mixed with an equal volume of UPP at different concentrations in a 96-well plate. The final concentrations of UPP in the wells were 0.31, 0.63, and 1.25 mg/mL. TSB medium without UPP served as the control. The plates were incubated at 37 °C for 24 h. The formation of biofilms was quantitatively analyzed using the crystal violet staining method [24]. The OD_{590} values of each well were measured using a microplate reader, and the biofilm content was calculated.

$$\text{Biofilminhibitionrate}(\%) = (1 - \text{OD}_{\text{sample}}/\text{OD}_{\text{control}}) \times 100\%$$

2.9. Effect of UPP on the Disruption of S. aureus Biofilms

Following the procedure in Section 2.8, the bacterial suspension was diluted to 1×10^6 CFU/mL. A volume of 100 µL of the diluted bacterial suspension was mixed with an equal volume of TSB, added to a 96-well plate, and incubated in a 37 °C incubator for 24 h to form a biofilm. The supernatant was aspirated, the wells were rinsed with PBS, and 100 µL of UPP at different concentrations (1.25, 0.63, 0.31 mg/mL, and Control) was added. The plates were then returned to a 37 °C incubator for an additional 24 h. Using the crystal violet staining method [25], the absorbance at 590 nm was measured using a microplate reader. The absorbance of the TSB-bacterial suspension mixture served as the blank, and the biofilm content was calculated based on the measured absorbance values.

$$\text{Biofilminhibitionrate}(\%) = (1 - \text{OD}_{\text{sample}}/\text{OD}_{\text{control}}) \times 100\%$$

2.10. Scanning Electron Microscopy (SEM) Analysis of S. aureus Biofilms

SEM was used to observe changes in cell structure before and after treatment of *S. aureus* biofilms with UPP. Following the procedure in Section 2.8, the bacterial suspension was diluted to 1×10^6 CFU/mL. Equal volumes of the bacterial suspension and 1.25 mg/mL UPP were mixed and cultured for 24 h. The supernatant was aspirated, and the biofilm samples were fixed with 2.5% glutaraldehyde for 4 h, followed by sequential dehydration in ethanol solutions of increasing concentration (30%, 50%, 70%, 90%, 95%, and 100%) [26]. The samples were then freeze-dried, mounted on slides, and photographed. Control samples were cultured in TSB medium only.

2.11. Determination of the Minimum Inhibitory Concentration (MIC) of UPP Against Free-Floating S. aureus

Dilute the bacterial suspension to 1×10^6 CFU/mL following the procedure in Section 2.8. Add 100 µL of the bacterial suspension to a 96-well plate, followed by an equal volume of UPP. Incubate in a 37 °C incubator for 24 h, and assess bacterial growth by visually inspecting the turbidity of the liquid in the wells. The MIC is defined as the lowest concentration of UPP capable of completely inhibiting the visible growth of bacteria [18].

2.12. Effect of UPP on the Growth Curve of S. aureus

Dilute the bacterial suspension to 1×10^6 CFU/mL following the procedure in Section 2.8. Mix 500 µL of the diluted *S. aureus* suspension with an equal volume of UPP (1.25, 0.63, 0.31 mg/mL, and Control) respectively, and measure the growth curve of *S. aureus* in a growth curvilinear instrument [27]. Instrument parameters: Chamber temperature: 37 °C; Shaking speed: 800 rpm/min; Measurement interval: 1 h; Measurement duration: approximately 24 h.

2.13. Effect of UPP on S. aureus Cell Integrity

Cell integrity was assessed by measuring DNA content and protein leakage [28]. The bacterial suspension was diluted to 1×10^8 CFU/mL following the procedure in Section 2.8. In separate centrifuge tubes, 1 mL of the diluted bacterial suspension was mixed with an equal volume of UPP (1.25, 0.63, 0.31 mg/mL, Control) and incubated for 24 h. After centrifugation (4 °C, 10,000 rpm, 5 min), the supernatant was collected, and its absorbance was measured at 260 nm to determine nucleic acid content. Protein content was determined using the Coomassie Blue method.

2.14. Effects of UPP on the Morphology of S. aureus Cells

Changes in the cellular structure of *S. aureus* treated with UPP were observed using scanning electron microscopy (SEM). Following the procedure in Section 2.8, the bacterial suspension was diluted to 1×10^6 CFU/mL. The suspension was mixed with 1.25 mg/mL UPP and cultured for 24 h [26]. The samples were then fixed, dehydrated, freeze-dried, mounted, and imaged under the microscope. TSB was used as the control group to observe changes in cell morphology following treatment.

2.15. Determination of Extracellular Matrix Content in S. aureus Biofilms

The extracellular polysaccharide content was determined using the phenol-sulfuric acid method [29]. First, a glucose solution with a concentration of 100.6 μg/mL was prepared. Aliquots of 0, 100, 400, 800, and 1000 μL of the glucose standard solution were pipetted into separate test tubes, and each tube was diluted to 1 mL with deionized water. Next, 500 μL of a 6% phenol solution was added to each test tube, followed by 3 mL of concentrated sulfuric acid. The mixture was reacted in a boiling water bath for 30 min, then cooled to room temperature. A 200 μL aliquot was transferred to a 96-well plate, and the OD value was measured at 490 nm using a microplate reader. A standard curve was plotted based on the glucose concentration and OD values.

Dilute the bacterial culture to 1×10^6 CFU/mL following the procedure in Section 2.8. Add 1 mL of the diluted bacterial suspension and 1 mL of TSB medium to a 48-well plate and incubate for 24 h to form a biofilm. Aspirate the supernatant, then add 1 mL of UPP (1.25, 0.63, 0.31 mg/mL, and Control) to each well, respectively, and incubate for 24 h. Aspirate the supernatant and rinse 2–3 times with PBS. Add 1 mL of sterile water, then sonicate the treated biofilm. Next, add 500 μL of a 6% phenol solution to each tube, followed by 3 mL of concentrated sulfuric acid, and react in a boiling water bath for 30 min. After cooling to room temperature, transfer 200 μL to a 96-well plate and measure the OD at 490 nm using a microplate reader. Calculate the extracellular polysaccharide content in the treated biofilm using a standard curve.

Using the culture method described above, obtain 1 mL of biofilm treatment solution. Centrifuge it (4 °C, 10,000 rpm, 5 min), collect the supernatant, and measure its absorbance at 260 nm to determine the nucleic acid content of the biofilm. Determine the protein content of the biofilm using the Coomassie Blue method.

2.16. Effect of UPP on the Hydrophobicity of S. aureus

Dilute the bacterial suspension to 1×10^6 CFU/mL following the steps in Section 2.8. Add 0.5 mL of the diluted bacterial suspension and an equal volume of UPP (1.25, 0.63, 0.31 mg/mL, Control) to separate centrifuge tubes and incubate for 24 h. Centrifuge (12,000 rpm, 2 min), collect the pellet, resuspend it in 1 mL of PBS buffer, and measure the OD_{600} as OD_1. Mix an equal volume of xylene with the bacterial suspension, vortex the

sample for 3 min, allow it to stand for 30 min after thorough mixing, and measure the OD_{600} of the aqueous phase as OD_2.

$$\text{Hydrophobicity}(\%) = \frac{(OD_1 - OD_2)}{OD_1} \times 100\%.$$

2.17. Bacterial Adhesion

The effect of UPP on the initial adhesion of *S. aureus* was evaluated using a modified crystal violet staining method. Briefly, the bacterial suspension was adjusted to approximately 1×10^6 CFU/mL as described in Section 2.8. Then, 100 µL of the bacterial suspension was mixed with 100 µL of UPP solution at final concentrations of 0.31, 0.63, and 1.25 mg/mL in sterile 96-well polystyrene plates. Wells without UPP treatment were used as the control. After static incubation at 37 °C for 4 h, planktonic cells were removed, and the wells were gently washed three times with sterile PBS to remove loosely attached bacteria. The adhered cells were fixed and stained with crystal violet. After removing excess dye, the wells were observed and photographed using an inverted fluorescence microscope (Nikon ECLIPSE Ti2, Tokyo, Japan). The representative images were used to visually compare the adhesion density among different treatment groups.

2.18. Inhibitory Effect of UPP on C. albicans Biofilms

C. albicans ATCC 10231 was cultured in YPD medium at 30 °C for 24 h. The cells were harvested, washed, and resuspended in RPMI 1640 medium to a concentration of 1×10^6 cells/mL. An aliquot of 100 µL of the suspension was mixed with an equal volume of UPP solution in a 96-well plate to reach final concentrations of 0.31, 0.63, and 1.25 mg/mL. Wells without UPP served as the control. The plates were incubated statically at 30 °C for 48 h. After incubation, the planktonic cells were removed, and the wells were gently washed with PBS. The biofilm inhibition rate was determined as described in Section 2.8.

2.19. Preservation Effect of UPP on Chilled Fresh Chicken Breast

Fresh chicken breast was purchased from a local market and transported to the laboratory under refrigerated conditions. Visible fat and connective tissue were removed under aseptic conditions, and the samples were cut into slices of similar size and weight to minimize variation. The chicken slices were randomly divided into two groups: a control group and a UPP-treated group. The control samples were immersed in sterile distilled water, whereas the treated samples were immersed in 1.25 mg/mL UPP solution. For the in vitro experiments, UPP was dissolved in sterile deionized water to prepare a 1.25 mg/mL stock solution. The stock solution was sterilized by filtration through a 0.22 µm membrane filter, stored at 4 °C protected from light, and used within 3 days. After immersion for 5 min, the samples were removed, placed in Petri dishes, and stored at 4 °C for 8 days.

During storage, samples were collected at predetermined intervals (day 0, 2, 4, 6, and 8) for the determination of appearance, pH, total viable count (TVC), thiobarbituric acid reactive substances (TBARS), and drip loss rate. All experiments were performed in triplicate.

2.19.1. Determination of Appearance Characteristics

At each sampling time, place the chicken sample in a clean Petri dish and observe, under natural light, its color and luster (surface and cut-surface color and sheen) and its tissue condition (firmness, surface moisture, and exudation of juices), smell its odor, and lightly touch the sample surface to assess its elasticity and tackiness. Simultaneously, photograph the samples using a digital camera under fixed lighting conditions to document changes in appearance.

2.19.2. Determination of pH

Take 1 g of the soaked chicken sample, add 10 mL of sterile water, homogenize, and measure the pH using a pH meter. Perform three measurements and calculate the average. During storage, the proteins in chicken meat are gradually degraded by microorganisms and endogenous enzymes, resulting in the formation of alkaline compounds such as amines. This process typically leads to an increase in pH. Therefore, the ability of UPP to slow down the spoilage process was evaluated by comparing pH changes between the control group and the UPP-treated group.

2.19.3. Determination of TVC

Under aseptic conditions, weigh 1 g of chicken meat sample and place it in a sterile homogenization bag containing 9 mL of sterile saline for homogenization. Using a sterile pipette, draw up 1 mL of the homogenized sample and slowly dispense it along the inner wall of a sterile test tube containing 9 mL of diluent. Shake to mix thoroughly, preparing a 1:100 sample homogenate. Based on the estimated level of sample contamination, prepare a 10-fold serial dilution series of the sample solution following the procedure described above. Select 1–3 sample solutions at appropriate dilution levels, draw 100 μL from each using a sterile pipette, and spread them onto agar plates. Then incubate the plates in a 37 °C constant-temperature incubator for 24 h. After incubation, select plates with a colony count between 30 and 300 CFU and no spreading colonies for counting. Colony counts are expressed in colony-forming units (CFU). Results are reported as the common logarithm of the total number of colonies per gram of sample (log CFU/g).

2.19.4. Determination of TBARS

One gram of chicken meat sample was thoroughly mixed and homogenized with 10 mL of trichloroacetic acid solution. The homogenate was then filtered, and 5 mL of the filtrate was mixed with 5 mL of thiobarbituric acid solution. The mixture was heated in a 90 °C water bath for 30 min. After the reaction, the sample was cooled to room temperature, and its absorbance was measured at 532 nm.

2.19.5. Determination of Drip Loss Rate

Weigh the original sample to obtain m_0 and the refrigerated sample to obtain m_1, calculate the juice loss rate using the formula: "driplossrate(%)" $= \frac{(m_0 - m_1)}{m_0} \times 100\%$.

2.20. Cytotoxicity Assay

Cytotoxicity of UPP was evaluated against RAW264.7 using the CCK-8 assay. Cells were seeded in 96-well plates and cultured at 37 °C in 5% CO_2 for 24 h. The medium was then replaced with fresh medium containing UPP at final concentrations of 0, 0.31, 0.63, and 1.25 mg/mL. After 24 h of treatment, CCK-8 reagent was added, and the absorbance was measured at 450 nm. Cell viability was calculated as $(OD_{sample}/OD_{control}) \times 100\%$. Three independent experiments were performed.

2.21. Data Analysis

Data analysis was performed using SPSS 23 statistical software. All data were expressed as mean ± standard deviation (SD) from at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test to determine significant differences between the control and treatment groups. A p-value of <0.05 was considered statistically significant.

3. Results and Discussion

3.1. Storage Stability of UPP

The stability of UPP at the application concentration (1.25 mg/mL) was evaluated under simulated preservation conditions (4 °C for 8 days), and the results are shown in Figure 1. Visually, the UPP solution remained a clear, light-yellow liquid throughout the 8-day storage period, with no observable precipitation, turbidity, or significant color change (Figure 1a), indicating good physical stability at refrigeration temperature.

The pH value of the UPP solution remained highly stable during storage (Figure 1b). The pH fluctuated narrowly around 7.5 with no significant upward or downward trend, demonstrating that the solution maintained a stable acid-base environment.

The UV–visible absorption spectra (200–400 nm) are presented in Figure 1c. All samples exhibited a highly similar spectral profile over the storage period. The strong absorption in the 200–230 nm region and the weak absorption bands around 260–300 nm are characteristic of polysaccharide chromophores (such as sugar rings and uronic acid groups) and trace impurities. Throughout the 8 days, no new absorption peaks appeared, and no significant shifts in peak positions were observed. Although the absolute absorbance values fluctuated slightly among different time points, there was no regular trend of continuous increase or decrease. This suggests that no significant degradation, depolymerization, or accumulation of UV-absorbing small molecular byproducts occurred.

Collectively, these results demonstrate that UPP exhibits excellent physical and chemical stability under the application conditions (4 °C, 8 days). This stability ensures that the anti-biofilm and antioxidant activities of UPP are not compromised by the storage environment, providing a solid basis for its application as a natural preservative for chilled chicken breast.

3.2. Chemical Composition Analysis of UPP

The chemical composition of the UPP is summarized in Supplementary Table S1. The total sugar content was 67.68% ± 1.67%, indicating that carbohydrates were the primary constituents of the extract. The uronic acid and sulfate group contents were 23.25% ± 1.44% and 17.24% ± 1.87%, respectively. This composition is consistent with ulvan, a sulfated anionic heteropolysaccharide found in the cell walls of green seaweeds of the genus *Ulva* [30]. The presence of uronic acid and sulfate groups influences the physicochemical properties and biological activities of these polysaccharides [31]. Additionally, the protein content was 0.99% ± 0.01%, indicating that the Sevag treatment effectively removed protein impurities, yielding a sample suitable for subsequent structural characterization and bioactivity evaluations.

3.3. Molecular Weight Distribution of UPP

Molecular weight and its distribution are key physicochemical parameters influencing the spatial conformation, chain interactions, and biological activities of polysaccharides [32]. As shown in Figure 2, the HPSEC-MALLS-RI chromatogram of the UPP exhibited a single and relatively symmetrical elution peak in both the refractive index (RI) and light scattering (LS) signals, indicating that the extract was a relatively homogeneous polysaccharide fraction. As shown in Supplementary Table S2, the weight-average molecular weight (M_w) and number-average molecular weight (M_n) were 1.017×10^6 g/mol and 7.476×10^5 g/mol, respectively. The polydispersity index (PDI, M_w/M_n) was calculated to be 1.361. This PDI value, slightly above unity, reflects a moderate molecular weight distribution that is commonly observed for naturally extracted polysaccharides and suggests the predominance of a single macromolecular species in the preparation.

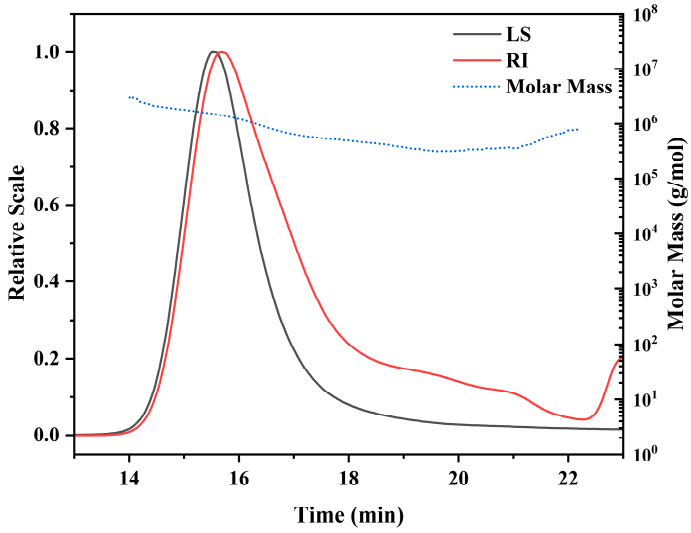

Figure 2. Molecular weight distribution profiles of UPP determined by HPSEC-MALLS-RI.

3.4. Monosaccharide Composition

The monosaccharide composition of the UPP is shown in Figure 3 and Table 1. The polysaccharides consisted of five distinct monosaccharides: rhamnose (Rha), galactose (Gal), glucose (Glc), xylose (Xyl), and glucuronic acid (GlcA). Glucose and rhamnose were the primary monosaccharide constituents, with molar ratios of 0.347 and 0.328, respectively, followed by glucuronic acid (0.158) and xylose (0.141). Galactose was present as a minor component (0.027). The coexistence of rhamnose and glucuronic acid is a characteristic feature of UPP, consistent with the high uronic acid content (23.25%) determined by the colorimetric method (Section 3.1). Additionally, the relatively high glucose content may be attributed to the co-extraction of minor neutral glucans (such as starch-like storage polysaccharides) from the *Ulva* biomass.

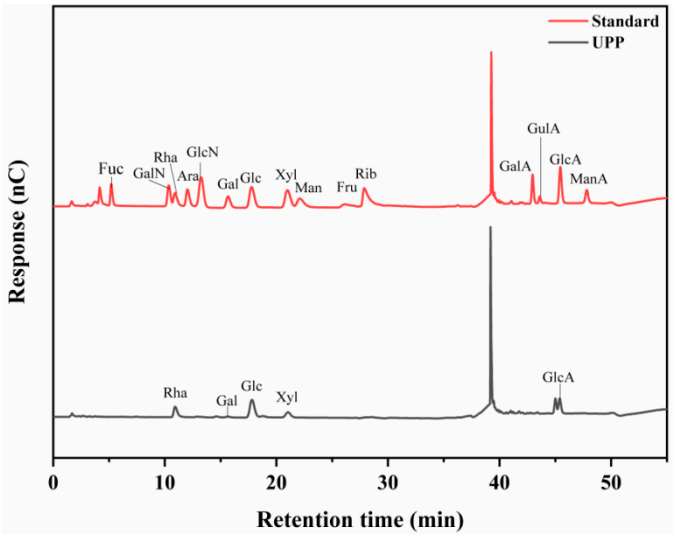

Figure 3. Ion chromatograms of mixed monosaccharide standards and UPP. The peaks at 2.0 min (NaOH solvent) and 40 min (sodium acetate) were excluded from monosaccharide quantification.

Table 1. Monosaccharide composition of UPP.

Monosaccharide	Molar Ratio
Rha	0.328
Gal	0.027
Glc	0.347
Xyl	0.141
GlcA	0.158

3.5. FT-IR Analysis

FT-IR spectroscopy was used to identify the functional groups of the UPP. As shown in Figure 4, the spectrum displayed typical absorption characteristics of sulfated polysaccharides. The broad and intense absorption peak at 3434 cm^{-1} was attributed to the stretching vibrations of intermolecular and intramolecular hydroxyl groups (O–H). The weak peak at 2937 cm^{-1} was assigned to the C–H stretching vibrations of the pyranose ring. The absorption peaks at 1624 cm^{-1} and 1423 cm^{-1} were attributed to the asymmetric and symmetric stretching vibrations of carboxylate groups (COO^-). This provided direct spectroscopic evidence for the presence of uronic acid, which is consistent with the results of chemical composition and monosaccharide analysis. The strong absorption peak at 1049 cm^{-1} was characteristic of the C–O–C and C–O stretching vibrations of the pyranose ring. Additionally, the sharp and intense band at 1254 cm^{-1} was attributed to the asymmetric S=O stretching vibration of sulfate esters. The absorption at 847 cm^{-1} was associated with the C–O–S bending vibration, indicating that these sulfate moieties are covalently linked to the sugar residues, a structural feature consistent with bioactive ulvan [33]. Collectively, the FT-IR analysis confirmed the polyanionic and sulfated characteristics of the UPP.

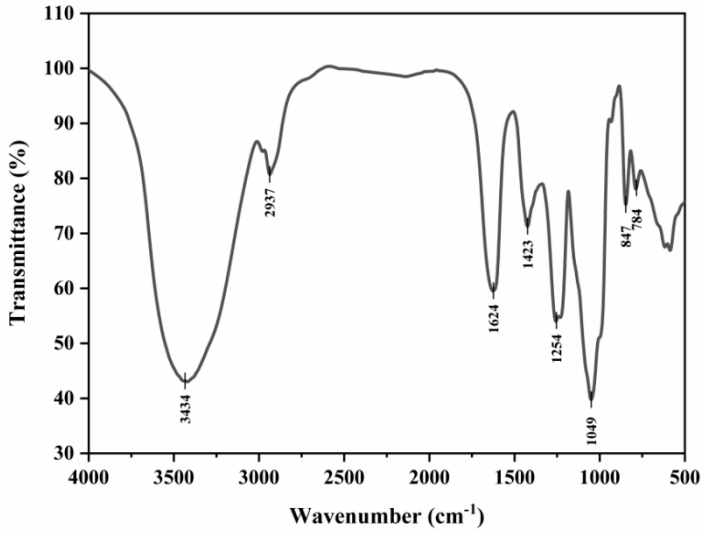

Figure 4. FT-IR spectra of UPP. Characteristic absorption bands are labeled: 3434 cm^{-1} (O–H stretching), 2937 cm^{-1} (C–H stretching), 1624 and 1423 cm^{-1} (COO^- asymmetric and symmetric stretching), 1254 cm^{-1} (S=O asymmetric stretching), 1049 cm^{-1} (C–O–C and C–O stretching), and 847 cm^{-1} (C–O–S bending).

Taken together, the monosaccharide composition and FT-IR data provide preliminary structural clues beyond bulk chemical composition, although these clues are inferential and require confirmation by linkage and NMR analyses. First, the Rha: GlcA molar ratio was approximately 2.1:1 (0.328 vs. 0.158, Table 1), which is compatible with, but does not by itself establish, the 2:1 Rha:GlcA ratio reported for ulvan-type repeating units [8,33]. Second, the bands at 1254 cm^{-1} (S=O asymmetric stretching) and 847 cm^{-1} (C–O–S bending)

are consistent with covalently bound sulfate esters rather than free inorganic sulfate, but the exact sulfation position cannot be assigned from FT-IR alone. Third, the absorptions at 1624 and 1423 cm^{-1} (COO^{-}) and at 1049 cm^{-1} (C–O–C/C–O of the pyranose ring) support the presence of uronic acid and pyranose rings, without resolving whether GlcA is chain-internal or terminal. The relatively high glucose content (0.347) may partly reflect co-extracted neutral glucans, as noted in Section 3.3, and should not be over-interpreted as part of a sulfated repeating unit. Definitive glycosidic linkage patterns, anomeric configurations, and sulfate substitution positions were not determined in this study; methylation analysis combined with 1D/2D NMR (^{1}H, ^{13}C, HSQC, HMBC) is required to establish the repeating-unit structure of UPP and is recommended for future work.

3.6. Inhibitory Effect of UPP on S. aureus Biofilms

The inhibitory effect of UPP on *S. aureus* biofilms formation was evaluated using the crystal violet staining assay. As shown in Figure 5a, UPP inhibited biofilm formation in a concentration-dependent manner. Compared with the control group, UPP at 0.31, 0.63, and 1.25 mg/mL reduced biofilm biomass by 13.3%, 34.2%, and 50.8%, respectively. At 1.25 mg/mL, UPP produced the highest biofilm inhibition among the tested concentrations (50.8% ± 1.1%), which exceeds the ≥50% threshold generally regarded as effective antibiofilm activity [34], indicating that UPP effectively interfered with the biofilm-forming capacity of *S. aureus* at sub-inhibitory concentrations.

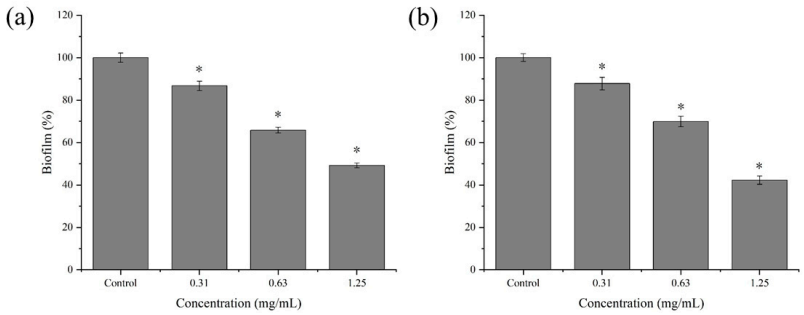

Figure 5. (**a**) Inhibitory effects of different concentrations of UPP on *S. aureus* biofilms. (**b**) The clearance effect of different concentrations of UPP on *S. aureus* biofilms. Data are presented as mean ± SD (n = 3). * $p < 0.05$ compared with the control group.

Biofilm formation is a multistep process involving initial bacterial adhesion, cell aggregation, extracellular matrix production, and biofilm maturation [35]. The concentration-dependent reduction in crystal violet staining suggests that UPP interfered with one or more key stages of this process. Notably, the inhibitory effect occurred at concentrations far below the MIC (results shown as below) against planktonic cells, indicating that the reduction in biofilm formation was not solely attributable to direct bactericidal activity. Instead, UPP may act primarily as an anti-adhesion or biofilm-modulating agent. The antibiofilm activity of marine polysaccharides has been associated with their negatively charged functional groups, high-molecular-weight structure, and ability to alter bacterial surface properties [36]. Sulfated polysaccharides may interact with bacterial cell surfaces or extracellular matrix components, thereby weakening cell-surface and cell–cell interactions. Therefore, the observed inhibition of *S. aureus* biofilm formation by UPP may be closely related to its ability to reduce initial attachment and disturb the structural development of the biofilm [37].

3.7. The Disruption Effect of UPP on S. aureus Biofilms

In addition to inhibiting biofilm formation [27], the ability of UPP to disrupt preformed mature biofilms was further investigated. As shown in Figure 5b, UPP showed a concentration-dependent removal effect on mature *S. aureus* biofilms. The biofilm removal rates were 12.2%, 30.1%, and 57.7% at UPP concentrations of 0.31, 0.63, and 1.25 mg/mL, respectively. These results demonstrate that UPP not only inhibited biofilm formation but also partially disrupted established biofilms [37].

Mature biofilms are usually more difficult to remove than newly forming biofilms because bacterial cells are embedded in a dense extracellular polymeric substance matrix composed mainly of polysaccharides, proteins, extracellular DNA, and other biomolecules [36,37]. The ability of UPP to reduce mature biofilm biomass by 57.7% at 1.25 mg/mL suggests that it may weaken the stability of the biofilm matrix or promote detachment of bacterial cells from the surface. However, the incomplete removal of mature biofilms also indicates that UPP alone may not fully eradicate established *S. aureus* biofilms. This is consistent with the intrinsic tolerance of mature biofilms to antimicrobial or anti-biofilm agents [38]. Therefore, UPP may be more suitable for preventing biofilm development or reducing biofilm accumulation rather than completely eliminating well-established biofilms. In practical food-processing environments, UPP could potentially be used as a natural anti-biofilm additive or in combination with other preservation strategies to reduce persistent contamination by *S. aureus*.

3.8. SEM Analysis of S. aureus Biofilms

SEM was used to further visualize the structural changes in *S. aureus* biofilms after UPP treatment. As shown in Figure 6a, the untreated control group formed compact biofilm aggregates, with bacterial cells densely clustered and closely attached to each other. The biofilm structure appeared continuous and relatively compact, indicating strong bacterial adhesion and aggregation. In contrast, in the treated group, the number of cells is significantly reduced and the biofilm structure became looser and less compact (Figure 6b). Bacterial clusters were less obvious, and the cells were more sparsely distributed on the surface. Some irregular debris or particles were also observed in the treated group, suggesting that UPP treatment disturbed the structural integrity of the biofilm.

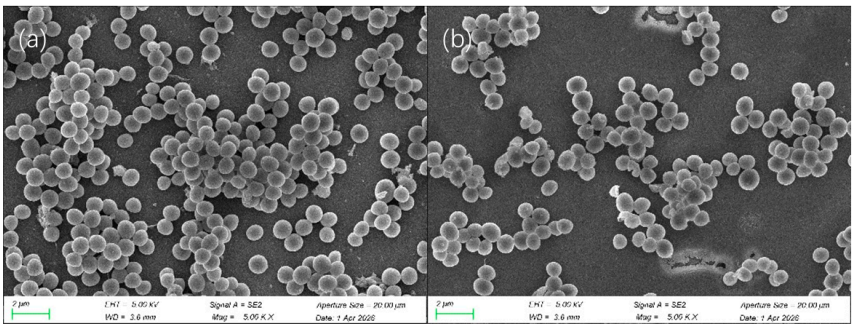

Figure 6. SEM micrographs of *S. aureus* biofilms treated with different concentrations of UPP: (**a**) Untreated control; (**b**) UPP-treated group.

These SEM observations were consistent with the crystal violet staining results [27], confirming that UPP reduced biofilm biomass and impaired the formation of dense biofilm architecture. The looser structure observed after UPP treatment may be associated with reduced bacterial adhesion, altered extracellular matrix composition, and weakened intercellular interactions. Since compact biofilm architecture is essential for bacterial persistence and resistance to environmental stresses [39], the UPP-induced structural disruption may increase the susceptibility of *S. aureus* biofilms to external intervention.

3.9. MIC of UPP Against Free-Floating S. aureus

The MIC of UPP against planktonic *S. aureus* was determined to evaluate its direct antibacterial activity [27]. The MIC of UPP was higher than 10 mg/mL, indicating that UPP had limited inhibitory activity against planktonic *S. aureus* under the tested conditions.

Although UPP did not completely inhibit visible bacterial growth at concentrations below 10 mg/mL, it markedly reduced biofilm formation at 0.31–1.25 mg/mL. This discrepancy between the high MIC value and the clear anti-biofilm effect suggests that UPP does not function primarily as a conventional bactericidal agent. Instead, its anti-biofilm activity may be mainly attributed to interference with bacterial adhesion, surface hydrophobicity, and extracellular matrix organization [37].

This distinction is important for understanding the functional role of UPP. Conventional antibacterial agents usually inhibit or kill planktonic cells, whereas anti-biofilm agents can attenuate bacterial virulence and persistence without necessarily causing strong growth inhibition. Such non-bactericidal or weakly bacteriostatic anti-biofilm strategies may reduce selective pressure for resistance development and have potential value in food preservation and safety control.

Two additional factors help explain this discrepancy. First, the MIC and crystal violet assays measure different endpoints: MIC scores complete suppression of visible growth from planktonic cells, whereas the crystal violet assay records any reduction in adhered biomass. A compound that only slows growth can therefore show a high MIC but a clear anti-biofilm effect. Second, UPP is a high-molecular-weight sulfated polysaccharide that cannot efficiently enter Gram-positive cells, but can act at the cell surface to reduce hydrophobicity and impair initial adhesion-targets absent in planktonic culture. Thus, the high MIC is consistent with a surface-mediated, non-bactericidal mode of action rather than weak activity.

3.10. Effect of UPP on the Growth Curve of S. aureus at Sub-Inhibitory Concentrations

The effect of UPP on the growth kinetics of *S. aureus* was further examined by monitoring bacterial growth curves over 24 h. As shown in Figure 7a, UPP at different concentrations (1.25, 0.63, 0.31 mg/mL, and Control) exhibited slower growth than the untreated control. Compared with the control group, the slopes of the logarithmic growth phase were reduced in all treatment groups, and the OD values at the stationary phase were significantly lower, indicating that UPP slowed bacterial growth rates and reduced the final bacterial population [40].

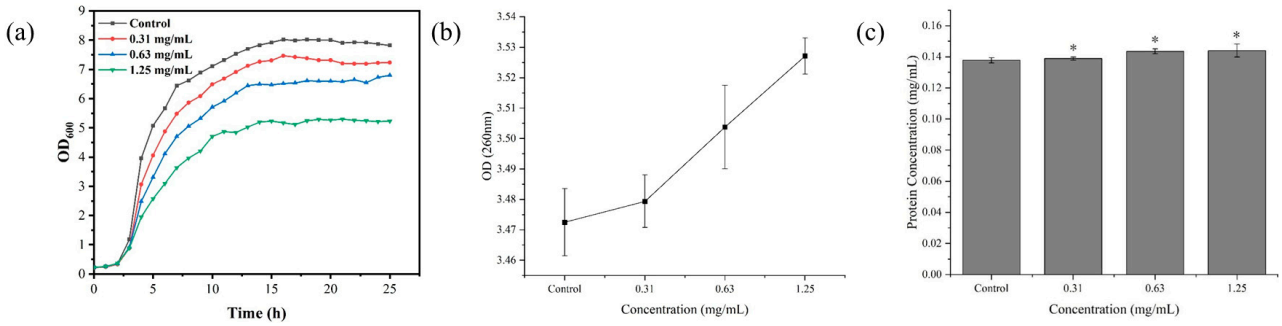

Figure 7. (**a**) Effects of different concentrations of UPP on the growth curve of *S. aureus*. (**b**) DNA content. (**c**) Extracellular protein content. Data are presented as mean ± SD (n = 3). * $p < 0.05$ compared with the control group.

Among the tested concentrations, 1.25 mg/mL UPP showed the most pronounced effect on bacterial growth. However, bacterial proliferation was not completely inhibited even at this concentration, which was consistent with the MIC result showing that the

MIC of UPP was higher than 10 mg/mL. These results indicate that UPP at sub-inhibitory concentrations exerted a moderate growth-suppressive effect but did not fully prevent *S. aureus* multiplication.

The reduction in growth rate may partially contribute to the observed decrease in biofilm formation. Nevertheless, because the anti-biofilm effect was evident at concentrations that did not completely inhibit planktonic growth, the reduction in biofilm biomass cannot be explained by growth inhibition alone. Instead, UPP likely exerts a combined effect by moderately slowing bacterial growth and interfering with adhesion-related events during biofilm development.

3.11. Effect of UPP on the Cell Integrity of S. aureus

To evaluate whether UPP affected bacterial cell integrity, the release of intracellular nucleic acids and proteins into the extracellular environment was measured [28,41]. As shown in Figure 7b,c, the contents of extracellular nucleic acids and proteins showed a slight increasing trend with increasing UPP concentration. Compared with the control group, UPP-treated cells released higher levels of nucleic acids and proteins, especially at 1.25 mg/mL [41].

The leakage of nucleic acids and proteins generally reflects changes in membrane permeability or partial loss of cell envelope integrity [28,41,42]. Therefore, these results suggest that UPP caused mild membrane perturbation in *S. aureus* cells. However, the extent of leakage was relatively limited, indicating that severe membrane disruption was unlikely to be the dominant mode of action at the tested sub-inhibitory concentrations [27].

This result is consistent with the MIC assay and growth curve analysis. Although UPP slightly affected bacterial growth and cell integrity, it did not show strong bactericidal activity against planktonic *S. aureus*. Therefore, the anti-biofilm activity of UPP may be mainly associated with non-lethal mechanisms, such as reducing cell-surface hydrophobicity, inhibiting initial adhesion, and remodeling the extracellular biofilm matrix [27,42].

Importantly, the limited release of nucleic acids and proteins (Figure 7c) indicates that UPP does not cause severe disruption of the cytoplasmic membrane. This is mechanistically distinct from cationic membrane-active agents (e.g., quaternary ammonium surfactants or cationic antimicrobial peptides), which typically disrupt membranes through strong electrostatic interactions with anionic phospholipids. As an anionic sulfated polysaccharide, UPP lacks the strong positive charge required for such membranolytic action. It should also be noted that mild membrane perturbation may indirectly contribute to biofilm inhibition [28]. Changes in membrane integrity and surface properties can affect bacterial adhesion, autolysis, eDNA release, and matrix organization, all of which are closely involved in biofilm development [27].

3.12. Morphological Changes in S. aureus Cells

SEM was used to observe the morphological changes in planktonic *S. aureus* cells after treatment with UPP [36,43]. As shown in Figure 8a, untreated *S. aureus* cells exhibited typical spherical morphology, with smooth surfaces, intact cell structures, and clear boundaries between individual cells [44].

After treatment with 1.25 mg/mL UPP, obvious morphological alterations were observed (Figure 8b). Some cells became irregular in shape, with roughened or wrinkled surfaces. Partial cell collapse and cell aggregation were also visible in the treated group. These morphological changes indicate that UPP affected the surface structure of *S. aureus* cells and caused a certain degree of cell envelope stress [43,45].

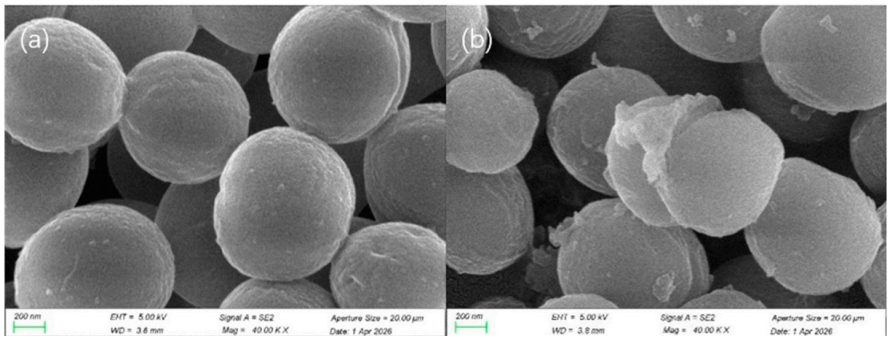

Figure 8. SEM micrographs of *S. aureus* treated with different concentrations of UPP: (**a**) Untreated control; (**b**) UPP-treated group.

The SEM results further support the conclusion that UPP can disturb the physiological state of *S. aureus* cells without necessarily causing complete growth inhibition. Surface deformation and roughening may reduce the ability of bacterial cells to maintain stable interactions with each other and with abiotic surfaces, thereby impairing biofilm development [36,45]. These findings are consistent with the reduced biofilm biomass and decreased bacterial adhesion observed in the UPP-treated groups [44].

However, SEM observations alone cannot directly confirm bacterial death [44]. Therefore, the morphological changes should be interpreted as evidence of cell surface damage or structural stress rather than definitive proof of bactericidal activity [43,45]. Additional assays, such as live/dead staining or membrane permeability analysis, would further clarify the effect of UPP on cell viability [36].

3.13. Determination of Extracellular Matrix Content in S. aureus Biofilms Treated with UPP

The extracellular matrix is a key structural component of bacterial biofilms and plays an essential role in maintaining biofilm stability, adhesion, and environmental resistance [46,47]. To further clarify the anti-biofilm mechanism of UPP, the contents of extracellular polysaccharides, extracellular proteins, and extracellular DNA in *S. aureus* biofilms were determined [48], as shown in Figure 9.

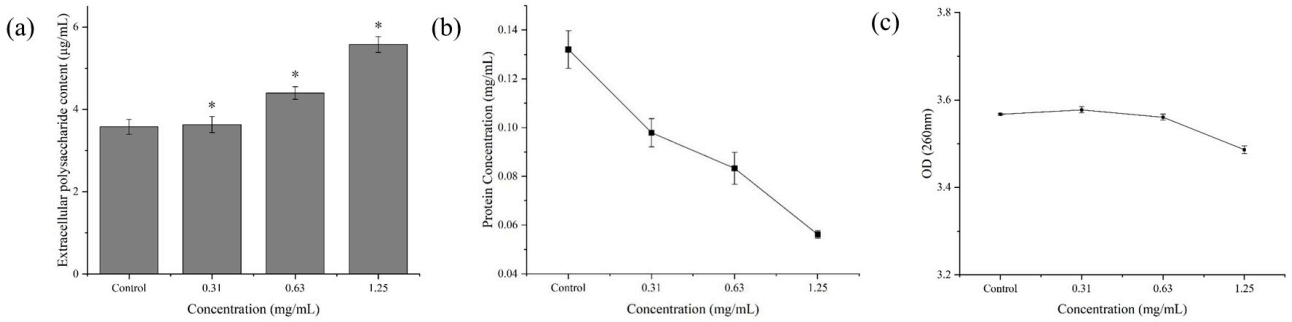

Figure 9. (**a**) Determination of extracellular polysaccharide content of *S. aureus* biofilm by different concentrations of UPP. (**b**) Determination of extracellular protein content in *S. aureus* biofilm treated with different concentrations of UPP. (**c**) Determination of extracellular DNA content in *S. aureus* biofilm treated with different concentrations of UPP. Data are presented as mean ± SD (n = 3). * $p < 0.05$ compared with the control group.

Surprisingly, UPP treatment led to a significant increase in extracellular polysaccharides compared to the control [49,50]. Whereas, the extracellular protein content decreased [48]. Meanwhile, the extracellular DNA content increased to some extent [38,47]. These results indicate that UPP treatment altered the composition of the extracellular matrix rather than simply reducing all matrix components simultaneously.

The increase in extracellular polysaccharides did not correlate with enhanced biofilm formation; instead, it coincided with a looser architecture observed via SEM [49]. We hypothesize that this phenomenon represents a bacterial stress response to UPP exposure [46]. Under the interference of exogenous sulfated polysaccharides, *S. aureus* may upregulate EPS secretion in an attempt to maintain biofilm stability or as a defense mechanism against the perturbation of cell membrane integrity. However, biofilm integrity depends not only on the total amount of extracellular polysaccharides but also on the balanced organization of matrix components [47,50]. Therefore, an increase in polysaccharide content does not necessarily indicate stronger biofilm formation [46,49].

The decrease in extracellular protein content may be particularly important for the weakening of biofilm structure [48,50]. Matrix-associated proteins contribute to bacterial aggregation, surface adhesion, and biofilm maturation in *S. aureus*. A reduction in extracellular proteins suggests that UPP disrupts the protein–polysaccharide interactions essential for a compact matrix structure, which may weaken cell–cell interactions, initial bacterial adhesion and reduce the compactness of the biofilm matrix, ultimately leads to biofilm detachment and inhibition. This could explain the looser biofilm architecture observed by SEM after UPP treatment [50].

The increase in extracellular DNA may be associated with mild cell envelope perturbation or partial autolysis induced by UPP [38]. Although eDNA can contribute to biofilm development in some cases, excessive or disorganized eDNA release may also reflect cellular stress and matrix imbalance [47,49].

Taken together, these results suggest that UPP may inhibit *S. aureus* biofilm formation by remodeling the extracellular matrix and disturbing the balance among polysaccharides, proteins, and eDNA [49,50].

3.14. Effect of UPP on the Hydrophobicity of S. aureus

Initial adhesion is a critical step in biofilm formation [51]. The ability of *S. aureus* to adhere to surfaces is closely associated with its cell-surface hydrophobicity [37]. Therefore, the effect of UPP on the hydrophobicity of *S. aureus* cells was investigated using the microbial adhesion to hydrocarbon assay [36,52].

UPP treatment reduced the cell-surface hydrophobicity of *S. aureus* in a concentration-dependent manner (Figure 10) [52]. Compared with the control group, as the concentration of UPP increased, the hydrophobicity of *S. aureus* gradually decreased, with the most significant decrease observed at a concentration of 1.25 mg/mL [36,37].

The decrease in cell-surface hydrophobicity is primarily attributed to the adsorption of UPP onto the bacterial surface (e.g., wall teichoic acids and surface proteins), which increases surface hydration and masks hydrophobic groups, rather than through disruption of the cytoplasmic membrane [36]. As a sulfated marine polysaccharide, UPP may modify the charge distribution or hydration layer around bacterial cells, thereby reducing cell-surface hydrophobic interactions [37,51]. This effect is one of the key factors influencing the adhesion capacity between bacterial cells and between bacterial cells and the substrate. UPP can reduce the surface hydrophobicity of *S. aureus*, disrupting the hydrophobic adhesion between bacterial cells while simultaneously weakening the ability of bacterial cells to bind to biofilm matrix components [36,37]. Meanwhile, this loss of hydrophobic interaction causes the originally tightly aggregated bacterial cells to dissociate from the biofilm, leading to a loosening of the overall structure of the mature biofilm and ultimately resulting in the gradual clearance of the biofilm [52].

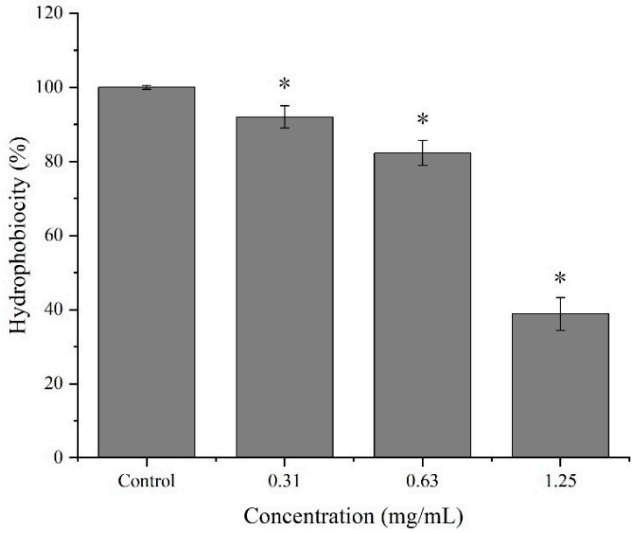

Figure 10. Effects of UPP on the hydrophobicity of *S. aureus*. Data are presented as mean ± SD (n = 3). * $p < 0.05$ compared with the control group.

3.15. Effect of UPP on the Initial Bacterial Adhesion

The bacterial adhesion assay further confirmed this effect (Figure 11). After 4 h of incubation, fewer bacterial cells were attached in the UPP-treated groups than in the untreated control [36,53]. The reduction in early-stage adhesion was consistent with the decreased hydrophobicity [51,54], suggesting that UPP inhibited biofilm formation primarily by impairing the initial attachment of *S. aureus*. This anti-adhesion effect represents a plausible key mechanism underlying the inhibition of *S. aureus* biofilm formation by UPP [51].

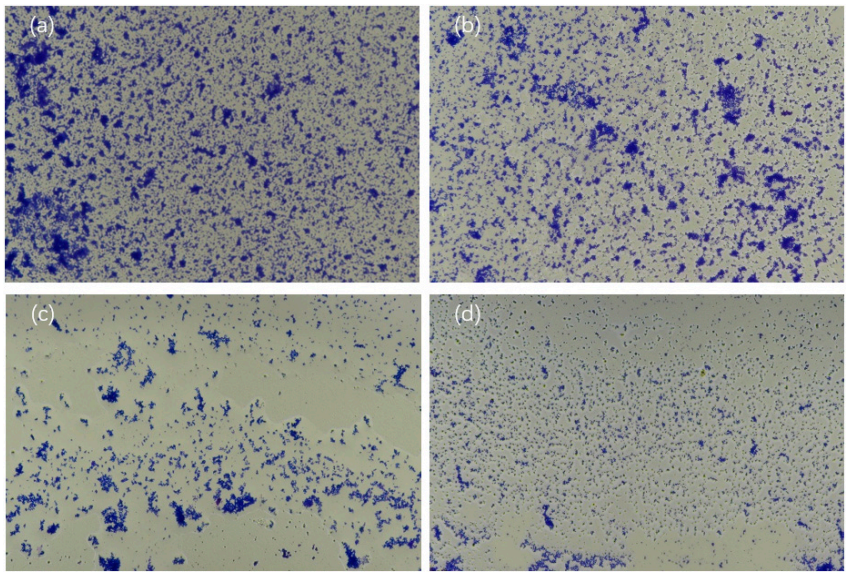

Figure 11. Microscopic observation of crystal violet-stained *S. aureus* cells adhered to the surface after 4 h of incubation. (**a**) Untreated control; (**b**) 0.31 mg/mL UPP; (**c**) 0.63 mg/mL UPP; (**d**) 1.25 mg/mL UPP. Adhered cells appear purple to blue-violet after crystal violet staining; the light gray background is the well surface. Darker, denser staining indicates greater cell adhesion.

Overall, the hydrophobicity and adhesion results provide important mechanistic evidence that UPP acts mainly by interfering with the early stage of biofilm development [51]. This mechanism is consistent with the weak direct antibacterial activity but strong antibiofilm activity observed at sub-inhibitory concentrations [36,54].

Considered together, these findings suggest that UPP interferes with *S. aureus* biofilm development at three interfaces rather than through a single lethal target: the bacterial cell surface, where polysaccharide adsorption lowers cell-surface hydrophobicity and impairs initial adhesion [35]; the extracellular matrix, where a shifted polysaccharide–protein–eDNA balance weakens structural integrity [38,47]; and the cell envelope, where mild perturbation—including interaction with anionic wall teichoic acids—contributes to surface remodeling and limited eDNA release without overt lysis. The relative contribution of these interfaces is likely concentration-dependent, with anti-adhesion dominating at the lowest effective concentrations and matrix disruption becoming more prominent as the concentration increases. This multi-target, sub-inhibitory mode of action is consistent with the weak direct antibacterial activity but pronounced anti-biofilm effect observed here, and is attractive for food applications because it minimizes selection for resistance while reducing the biofilm burden on chilled poultry surfaces.

3.16. Inhibitory Effect of UPP on C. albicans Biofilms

To determine whether the anti-biofilm activity of UPP is specific to *S. aureus* or has a broader spectrum, its inhibitory effect on *C. albicans* biofilm formation was further evaluated. As shown in Figure 12, UPP significantly inhibited *C. albicans* biofilm formation in a concentration-dependent manner. Compared with the untreated control, treatment with UPP at 0.31, 0.63, and 1.25 mg/mL reduced the biofilm biomass by approximately 21.28%, 32.20%, and 48.17%. These results indicate that the anti-biofilm property of UPP is not limited to Gram-positive bacteria, but also extends to pathogenic fungi. This broad-spectrum anti-biofilm activity is highly advantageous for food preservation, as microbial contamination in poultry products often involves a mixed community of bacteria and yeasts. The ability of UPP to interfere with the biofilm formation of both *S. aureus* and *C. albicans* suggests its potential as a versatile natural preservative to improve the microbial safety of chilled meat.

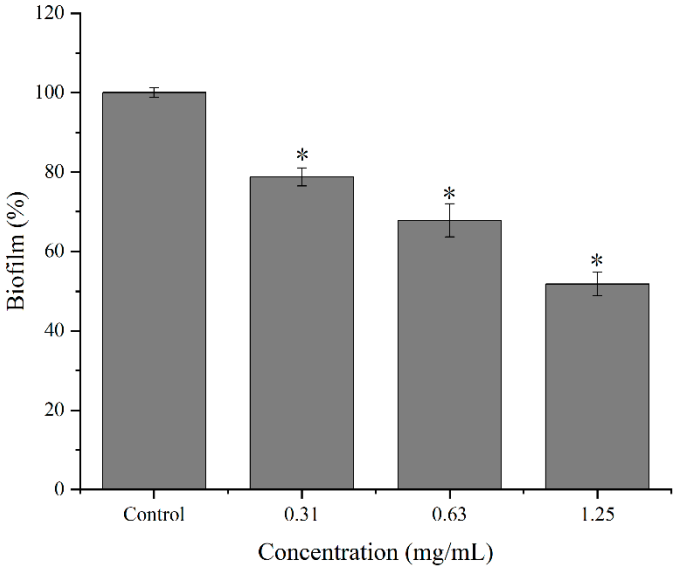

Figure 12. Inhibitory effect of UPP on *C. albicans* biofilm formation. Data are presented as mean ± SD (n = 3). * $p < 0.05$ compared with the control group.

3.17. Preservation Effects of UPP on Chilled Fresh Chicken Breast

The preservation effect of UPP was evaluated using chilled chicken breast stored at 4 °C for 8 days. Changes in appearance, pH, TVC, TBARS value, and drip loss were monitored to assess the effect of UPP on microbial and physicochemical quality deterioration.

3.17.1. Appearance Changes

As shown in Figure 13a, the control samples exhibited progressive quality deterioration during refrigerated storage [55,56]. On day 0, both control and UPP-treated samples showed a fresh appearance [57]. However, with increasing storage time, the control samples gradually became paler and yellowish, accompanied by visible surface dehydration and exudate accumulation [58]. By day 6, the deterioration of the control group became more evident [55,56]. In contrast, the UPP-treated samples retained a more stable color and better surface appearance during storage. These observations suggest that UPP treatment delayed visible spoilage-related changes in chilled chicken breast.

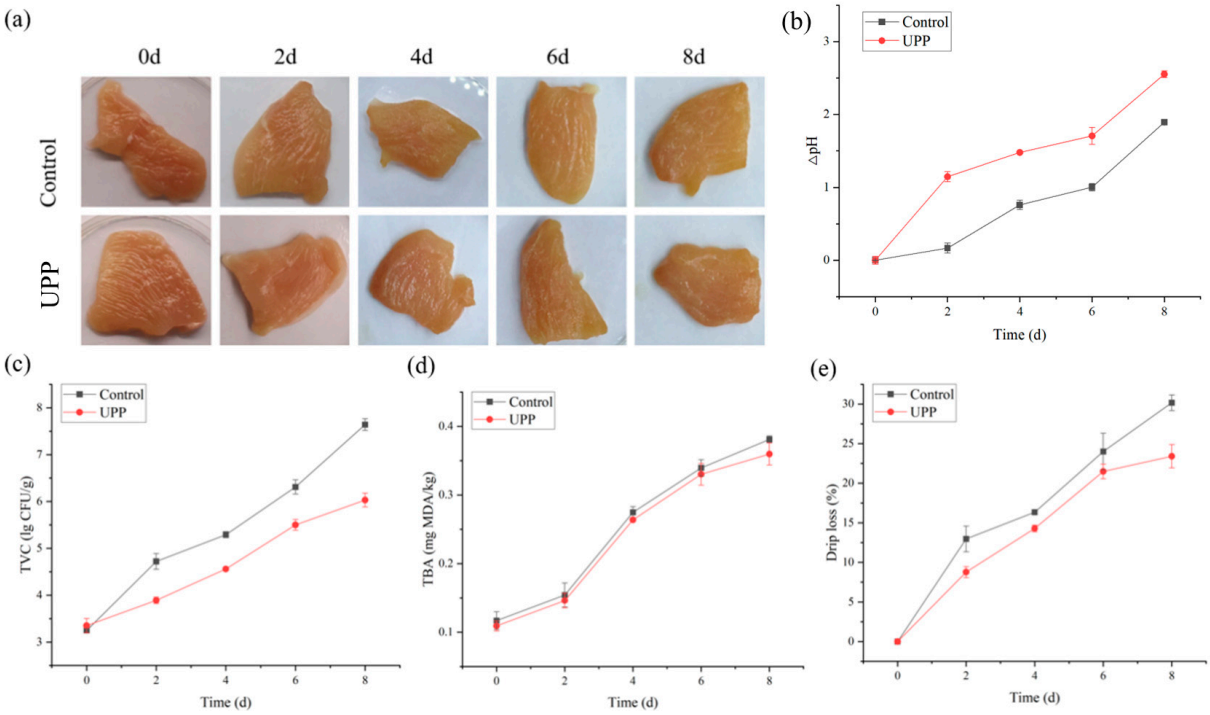

Figure 13. Effects of UPP treatment on the quality changes of chilled chicken breast during storage at 4 °C. (**a**) Appearance; (**b**) Δ pH; (**c**) total viable count; (**d**) TBARS value; and (**e**) drip loss.

3.17.2. pH Changes

The pH value of meat is a key indicator of freshness because microbial metabolism and protein degradation generate alkaline compounds (such as ammonia and amines) during storage [56,58]. In this study, the initial pH differed significantly between the control (5.95 ± 0.02) and UPP-treated groups (4.95 ± 0.02). This difference cannot be attributed to the acidic of the UPP solution, because the UPP solution remained near neutral (pH ≈ 7.5; Figure 1b). Instead, it is likely multifactorial, possibly involving interactions between anionic UPP and muscle proteins/surface charges during dipping, sample-to-sample variation in initial muscle pH and buffering capacity, and possible differences in the initial microbiota among samples [1,59]. Although samples were randomly assigned, such baseline heterogeneity cannot be fully excluded.

Because of this baseline difference, the absolute pH increment ($\Delta\ pH = pH_t - pH_0$) was used to accurately evaluate spoilage progression. As shown in Figure 13b, the Δ pH of the UPP-treated group increased rapidly from day 0 and reached approximately 2.55 by day 8, which was higher than that of the control group. This quantitative analysis indicates that although UPP treatment maintained a lower absolute pH, it did not decelerate the rate of alkalization on the meat surface. The accelerated Δ pH in the UPP group may be partially explained by the higher initial water activity and moisture retention induced by

the polysaccharide coating, which can facilitate the diffusion of microbial metabolites, or by the buffering capacity differences at a lower initial pH [1,3].

Therefore, pH alone is insufficient to reflect the preservation efficacy in this specific experimental design due to the initial baseline difference. The preservative effect of UPP is more reliably evidenced by its significant inhibition of microbial proliferation and lipid oxidation, as discussed in the subsequent sections.

3.17.3. Determination of TVC

As shown in Figure 13c, the TVC increased in both groups during storage, indicating progressive microbial proliferation in chilled chicken breast [57]. However, the bacterial counts in the UPP-treated samples were consistently lower than those in the control group. On day 8, the TVC of the UPP-treated group was approximately 1.8 log CFU/g lower than that of the control group. This result indicates that UPP effectively delayed microbial proliferation in chilled chicken breast.

The microbial inhibitory effect of UPP in the food system may be attributed to multiple factors. First, although UPP showed limited direct antibacterial activity against planktonic *S. aureus*, it moderately affected bacterial growth at the tested concentrations. Second, UPP can inhibit bacterial adhesion and biofilm formation, thereby reducing microbial colonization on the surface of chicken breast. Third, the polysaccharide structure of UPP may form a protective layer on the meat surface, limiting microbial attachment and nutrient accessibility [60]. Although the TVC reflects overall microbial contamination rather than *S. aureus* specifically, the result demonstrates the general preservation potential of UPP in chilled poultry products. Future studies should include an *S. aureus*-inoculated chicken breast model to further confirm the targeted control effect of UPP against this pathogen in real food matrices.

3.17.4. TBARS Value

Lipid oxidation is one of the major causes of quality deterioration in refrigerated meat products [61]. TBARS values were determined to evaluate lipid oxidation in chicken slices during refrigerated storage [62]. In Figure 13d the TBARS value in the control group continued to rise with storage time, whereas the increase in the treated group was lower than that of the control group, indicating slower oxidation. During refrigerated storage, the TBARS value increased gradually in both the control and UPP-treated groups. However, UPP treatment significantly reduced TBARS value. On day 8, the TBARS value of the UPP-treated chicken breast was approximately 34% lower than that of the control group. The lower TBARS value indicates that UPP effectively delayed lipid oxidation in chilled chicken breast.

This effect may be related to the antioxidant activity of UPP [63]. Marine sulfated polysaccharides have been reported to scavenge free radicals, chelate metal ions, and inhibit oxidative chain reactions. These antioxidant properties may contribute to the reduced lipid oxidation observed in the UPP-treated samples. In addition, microbial growth can accelerate lipid oxidation by producing oxidative enzymes and metabolites. Therefore, the reduced microbial load in the UPP-treated group may also indirectly contribute to lower MDA formation. The combined antimicrobial, anti-biofilm, and antioxidant effects of UPP may help maintain the oxidative stability of chilled chicken breast during storage [64].

3.17.5. Determination of Drip Loss Rate

Drip loss is an important quality parameter of chilled meat products and directly affects texture, appearance, nutritional value, and consumer acceptance. As storage time increased, drip loss increased in both control and UPP-treated chicken breast samples. However, the

UPP-treated group showed a lower drip loss throughout storage. On day 8, UPP treatment reduced drip loss by approximately 28% compared with the control group (Figure 13e). The reduced drip loss suggests that UPP helped maintain the water-holding capacity of chicken breast during refrigerated storage.

This protective effect may be associated with the film-forming and water-binding properties of polysaccharides. UPP may form a thin protective layer on the meat surface, reducing moisture migration and exudate loss [65]. In addition, by delaying microbial growth and lipid oxidation, UPP may reduce structural damage to muscle proteins, thereby helping to maintain water retention. The reduction in drip loss, together with the lower pH, lower MDA content, and reduced total viable count, indicates that UPP treatment effectively delayed quality deterioration in chilled chicken breast [66]. These findings demonstrate the practical potential of UPP as a natural preservation agent for refrigerated poultry products.

The results above indicate that UPP delayed microbial proliferation, lipid oxidation, and drip loss during refrigerated storage. These effects indicate that UPP can improve both the microbial safety and quality stability of chilled poultry products.

To further evaluate the competitiveness of UPP as a natural preservative, its preservation efficacy was quantitatively compared with other reported preservatives for chilled poultry products (Table 2).

Table 2. Comparison of the preservation efficacy of UPP other reported preservatives for chilled poultry products.

Preservative	Concentration	Food Matrix	Storage Condition and Time	Reduction in TVC (log CFU/g)	Reduction in Lipid Oxidation
UPP (this study)	1.25 mg/mL	Chicken breast	4 °C, 8 days	1.8	34% (MDA)
Chitosan + oregano essential oil [58]	1% (*w*/*v*)	Chicken breast	4 °C, 6 days	0.82–2.98	Significant decrease
Ulva rigida polysaccharide [57]	0.5% (*w*/*v*)	Chicken breast	4 °C, 20 days	1.5	25.6%
Alginate + protein hydrolysates [60]	2% (*w*/*v*)	Chicken breast	4 °C, 7 days	1.0	Not reported

As shown in Table 2, UPP at a relatively low concentration (1.25 mg/mL) achieved a 1.8 log CFU/g reduction in TVC after 8 days. This antimicrobial efficacy is highly competitive, as it is quantitatively comparable to 8% sodium lactate (2.0 log CFU/g) [57] and 1% chitosan coatings (1.02–3.74 log CFU/g) [58], but requires a significantly lower active dose. Furthermore, UPP effectively inhibited lipid oxidation (34% reduction in MDA), which is comparable to the antioxidant effect of *Ulva rigida* polysaccharide in chicken sausages (25%) [57]. Notably, many conventional treatments only report antimicrobial effects, whereas UPP provides a dual-functional benefit (antimicrobial and antioxidant) within a single natural marine-derived compound. This quantitative comparison highlights the practical competitiveness of UPP as a natural alternative to conventional chemical preservatives.

3.18. Cytotoxicity of UPP

Compared with the control group (100%), cell viability showed a slight decreasing trend after treatment with different concentrations of UPP (Figure 14). At 0.31, 0.63, and 1.25 mg/mL, cell viability was approximately 97%, 96%, and 93%, respectively. Even at the highest tested concentration (1.25 mg/mL), cell viability remained above 90%, and no obvious morphological changes or extensive cell death were observed, indicating that UPP has very low cytotoxicity within the tested concentration range. These results demonstrate that, within the effective antibacterial and anti-biofilm concentration range, UPP exhibits excellent safety toward mammalian cells (cell viability > 90%).

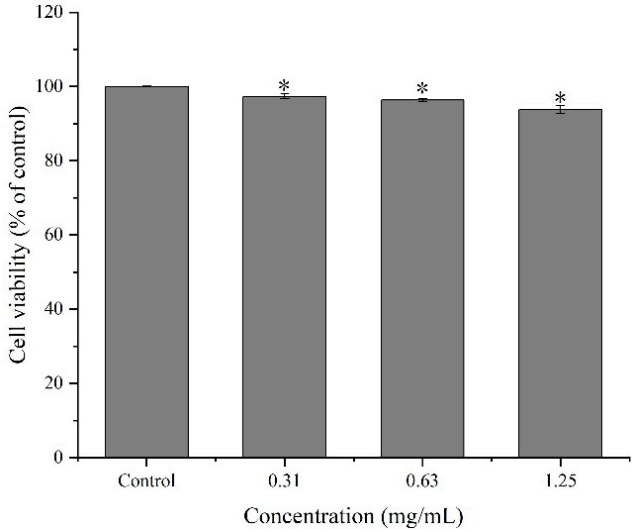

Figure 14. Cytotoxicity of UPP against RAW264.7 determined by CCK-8 assay. Data are presented as mean ± SD (n = 3). * $p < 0.05$ compared with the control group.

This study has several limitations that should be acknowledged. First, the structural interpretation of UPP in Section 3.5 is based on monosaccharide composition and FT-IR spectroscopy; definitive glycosidic linkage patterns, anomeric configurations, and sulfate substitution positions were not determined, because methylation analysis and 1D/2D NMR were not performed. Second, the preservation experiments used naturally contaminated chicken breast rather than a *S. aureus*-inoculated model, so the contribution of UPP specifically to *S. aureus* control in the food matrix remains to be confirmed.

4. Conclusions

In conclusion, UPP exhibited significant anti-biofilm activity against *S. aureus* at sub-inhibitory concentrations. Although UPP showed limited direct antibacterial activity against planktonic cells, it effectively inhibited biofilm formation and partially disrupted mature biofilms. The anti-biofilm effect was closely associated with reduced cell-surface hydrophobicity, impaired early adhesion, and remodeling of extracellular matrix composition via surface adsorption rather than membrane disruption, resulting in a looser and less stable biofilm architecture. Having elucidated the anti-biofilm mechanisms of UPP against *S. aureus*, we further examined its effect on *C. albicans* and found that UPP also inhibited *C. albicans* biofilm formation in a concentration-dependent manner, with a 48.2% reduction at 1.25 mg/mL. This indicates that UPP possesses broad-spectrum anti-biofilm activity against both Gram-positive bacteria and opportunistic fungal pathogens.

In chilled chicken breast stored at 4 °C, UPP treatment effectively reduced microbial proliferation, lipid oxidation, and drip loss. Notably, UPP also exhibited broad-spectrum anti-biofilm activity against *C. albicans*, excellent storage stability at 4 °C, and low cytotoxicity. These results suggest that UPP has promising potential as a natural antibiofilm and preservation agent for improving the quality and safety of chilled poultry products.

The novelty of this study lies in demonstrating that UPP, a marine-derived polysaccharide from green tide biomass, can inhibit *S. aureus* biofilm development mainly by interfering with bacterial adhesion and extracellular matrix organization, while also improving the quality stability of chilled chicken breast during refrigerated storage.

Future studies should further clarify the structure–activity relationship of UPP and evaluate its efficacy against *S. aureus* in inoculated food models and food-processing environments.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/foods15234280/s1, Table S1. Chemical composition analysis of *Ulva prolifera* polysaccharide. Table S2. Molecular weight parameters of UPP.

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