

Article

Development of pH-Indicative and Antimicrobial Films Based on Polyvinyl Alcohol/Starch Incorporated with Ethyl Lauroyl Arginate and Mulberry Anthocyanin for Active Packaging

Nannan Li ¹, Zeguang Zhou ¹, Fuqi Wu ^{1,2}, Yanyue Lu ¹, Dongyang Jiang ¹, Lei Zhong ^{1,*} and Fengwei Xie ^{3,*} 

- ¹ Guangxi Key Laboratory for Polysaccharide Materials and Modifications, Guangxi Higher Education Institutes Key Laboratory for New Chemical and Biological Transformation Process Technology, School of Chemistry and Chemical Engineering, Guangxi Minzu University, Nanning 530006, China
- ² School of Science, Tianjin Chengjian University, Tianjin 300384, China
- ³ School of Engineering, Newcastle University, Newcastle upon Tyne NE1 7RU, UK
- * Correspondence: leiwin@gmail.com (L.Z.); david.xie@newcastle.ac.uk or fwhsieh@gmail.com (F.X.)

Abstract: Antibacterial and pH-responsive composite films for active food packaging were fabricated based on polyvinyl alcohol (PVA), cassava starch, ethyl lauroyl arginate (LAE), and mulberry anthocyanin. With the incorporation of LAE and mulberry anthocyanin, the PVA/starch blend films exhibited a less compact and more heterogeneous surface structure. The tensile strength and elongation at break of the active films were not significantly affected when the mulberry anthocyanin content was less than 20%. Moreover, the incorporation of mulberry anthocyanin effectively improved the UV barrier property of the blend films. Notably, while mulberry anthocyanin showed obvious color changes in buffer solutions with different pH values, the changes were indistinguishable for the PVA/starch/mulberry anthocyanin films. By contrast, the color changes of the PVA/starch/LAE/mulberry anthocyanin films were more noticeable, indicating the addition of LAE increased the pH sensitivity of the blend films. Furthermore, the PVA/starch/LAE/mulberry anthocyanin films efficiently inhibited the growth of both *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) due to the strong antibacterial activity of LAE. According to the spoilage test, the active films containing 5% mulberry anthocyanin and 5% LAE effectively indicated and slowed down the spoilage process of dairy milk. Our results demonstrate that PVA/starch/LAE/mulberry anthocyanin films have high potential as bioactive packaging materials applied in the food industry.

Keywords: polyvinyl alcohol (PVA); starch; lauroyl arginate (LAE); mulberry anthocyanin; active food packaging



Citation: Li, N.; Zhou, Z.; Wu, F.; Lu, Y.; Jiang, D.; Zhong, L.; Xie, F. Development of pH-Indicative and Antimicrobial Films Based on Polyvinyl Alcohol/Starch Incorporated with Ethyl Lauroyl Arginate and Mulberry Anthocyanin for Active Packaging. *Coatings* **2022**, *12*, 1392. <https://doi.org/10.3390/coatings12101392>

Academic Editor: Ruchir Priyadarshi

Received: 30 August 2022

Accepted: 19 September 2022

Published: 23 September 2022

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1. Introduction

In recent years, considerable research efforts have been focused on using biopolymers to develop novel biodegradable food packaging films to meet the requirements for environmental sustainability in the food industry [1,2]. Moreover, extensive studies have been conducted by incorporating various functional bioactive substances to monitor the freshness and prolong the shelf life of food products [3,4].

Polyvinyl alcohol (PVA) is one of the most used biodegradable polymers in the food industry because of its non-toxicity and biocompatibility [5]. PVA is obtained by the hydrolysis of polyvinyl acetate and possesses high mechanical strength, low protein absorption, high chemical resistance, and excellent film-forming properties, which make it suitable as a food packaging material [6–8]. Since the price of PVA is relatively high compared to other synthetic plastics, it has often been mixed with other cheaper renewable materials to produce low-cost and high-performance composites [9,10]. In previous studies, Singha et al. [11] prepared PVA/starch composite films and found that the maximum tensile strength of the obtained films reached 45.6 MPa. Garavand et al. [12] fabricated composite

films based on PVA and corn starch enriched with chitosan nanoparticles, and the obtained films exhibited superior physical, mechanical, and antibacterial properties.

Among various biopolymers, starch has received considerable attention for food packaging because of its low costs, abundance, edibility, and biodegradability. In general, starch consists of two glucose polymers, amylose and amylopectin [13]. The properties of starch-based materials are closely related to their natural sources, such as corn, wheat, rice, potato, and cassava [14]. Cassava starch is considered to be an excellent film-forming material due to its paste-like clarity, low gelation temperature, and high gel stability [15]. However, pure cassava starch films usually have low mechanical properties, poor barrier properties, and a lack of antibacterial activity [11,15]. To overcome these shortcomings, it is essential to blend cassava starch with other polymers and functional agents to develop composite materials with improved physicochemical properties and desired functions [10].

Novel packaging materials made by incorporating antibacterial agents into a polymer matrix can efficiently inhibit the activity of target microorganisms, thus extending the shelf life of food products [16]. Ethyl lauroyl arginine (LAE) has been recognized as an effective antimicrobial agent, mainly extracted from natural compounds such as lauric acid, L-arginine, and ethanol [17–19]. LAE has strong antibacterial activity, which can inhibit the growth of microorganisms by destroying cell membranes and changing cell permeability as a surfactant. *Via in vitro* experiments, Becerril et al. [19] indicated a strong antibacterial effect of LAE against *Salmonella*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Staphylococcus aureus*. Furthermore, LAE can be rapidly metabolized into lauric acid and arginine after being absorbed by the human body [20–22]. LAE has been authorized as a food preservative by the European Food Safety Agency (EFSA), the Food and Drug Administration (FDA), and Food Standards Australia New Zealand (FSANZ) [23]. The physical, chemical, and antibacterial properties of active packaging films containing LAE have been investigated. According to Pornpun et al. [24], a polylactic acid (PLA) film containing LAE at only a 0.07% loading (*w/w*) effectively inhibited the growth of *Listeria monocytogenes* and *Salmonella typhimurium*, and increasing the amount of LAE could increase the antibacterial activity of the film. Haghighi et al. [25] fabricated a chitosan/PVA/LAE composite film by solution casting and found that the addition of LAE increased the water vapor transmission rate and solubility of the film, and the addition of LAE generated inhibitory zones for the growth of bacteria *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella typhimurium*. Our previous work suggested that PVA/cassava starch/LAE blend films exhibited enhanced mechanical and antimicrobial properties, and only 1% LAE had strong antibacterial activity against both *E. coli* and *S. aureus* [10].

Anthocyanins are natural pigments widely found in plant petals, fruits, and rhizomes. Anthocyanins appear purple, blue, and red in various vegetables and fruits [26]. Anthocyanins can be added to foods as antioxidants to prevent cardiovascular diseases, diabetes, cancer, and atherosclerosis [27]. The colors of anthocyanins are sensitive to pH changes, and therefore, they have been used as colorants to blend with polymers to prepare smart food packaging films that can indicate the freshness of foods [28]. Chen et al. [29] prepared starch/PVA films containing curcumin and purple sweet potato anthocyanin, and they found that the color changes of the composite films were closely related to the process of fish deterioration in a freshness detection experiment. Luchese et al. [30] developed smart films based on starch and blueberry residue anthocyanins and evaluated their color changes in different food products. For the obtained films, anthocyanin powders with smaller particle sizes displayed a more uniform color change. In addition, the introduction of anthocyanins might greatly affect the functional properties of blend films. Qin et al. [31] reported that the incorporation of anthocyanins and betacyanins into starch and PVA improved the light barrier property, water resistance, and oxidation resistance of the blend films.

It is expected that the introduction of LAE and anthocyanins can provide antibacterial and colorimetric freshness-sensing properties to packaging films. However, to our knowledge, there are few studies on the fabrication and properties of PVA/starch/LAE/anthocyanin

films. The combined effects of LAE and anthocyanins on the physical and chemical properties of the blend films have not been systematically investigated, and the performance of such active films in the detection and inhibition of food spoilage also needs to be evaluated.

In this work, a novel intelligent film based on PVA/starch enriched with LAE and mulberry anthocyanin was prepared by the solution-casting method. LAE was added as an antibacterial agent to delay food decay, and mulberry anthocyanin was included in the film as a pH-sensing agent. This blend film was designed for packaging fresh foods, such as meat and milk, to extend their shelf life. The microstructure of the obtained film was characterized, and the effects of mulberry anthocyanin and LAE contents on the mechanical, optical, and water barrier properties of the blend films were evaluated. The colorimetric pH-sensing performance of both anthocyanin and blend films was evaluated in buffer solutions. To clarify the antibacterial activity of LAE and anthocyanin, an *in vitro* antibacterial experiment on the films with and without LAE against *E. coli* and *S. aureus* was conducted. Moreover, the blend films were applied to indicate and slow down milk spoilage. The purpose of this study was to develop a novel intelligent film with a sensitive freshness detection capability, effective antibacterial activity, and excellent physicochemical properties. Such a film has high potential in fresh-food packaging applications.

2. Materials and Methods

2.1. Materials

PVA (degree of polymerization 1750 ± 50) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Cassava starch (food grade) was supplied by Guangxi Hongfeng Starch Co., Ltd. (Nanning, China). Ethyl lauryl arginine hydrochloride (LAE, 98%) was provided by Shanghai Bide Medical Technology Co., Ltd. (Shanghai, China). Mulberry anthocyanin (25%) was purchased from Xi'an Four Seasons Technology Co., Ltd. (Xi'an, China). *S. aureus* LWCC 1002 and *E. coli* LWCC 1033 were provided by Shanghai Luwei Microbial Sci. & Tech. Co., Ltd. (Shanghai, China). Casein soya bean digest agar (TSA) and Casein soya bean digest broth (TSB) were obtained from Guangdong Huankai Microbial Sci & Tech. Co., Ltd. (Guangzhou, China). Glycerol (AR, 99+%) and glacial acetic acid (AR) were purchased from Guangdong Guanghua Sci & Tech Co., Ltd. (Guangzhou, China). Fresh milk was purchased from the local fresh market (Nanning, China).

2.2. Preparation of PVA/Starch/LAE/Mulberry Anthocyanin Films

PVA/starch/LAE/mulberry anthocyanin films were fabricated according to the method used by Jiang et al. with modifications [32]. Cassava starch granules were dissolved in distilled water and gelatinized with constant stirring at 80 °C for 30 min (430 rpm) to obtain a 10% (*w/w*) starch suspension. PVA powder was dissolved in distilled water and stirred at 100 °C for 1 h (430 rpm) to prepare a 10% (*w/w*) PVA solution. Then, the PVA solution was mixed with the starch suspension at a ratio of 70:30 (*w/w*), and then glycerin (20% of the PVA/starch mixture, *w/w*) and glacial acetic acid (20% of the PVA/starch mixture, *w/w*) were added with further mixing at 80 °C for 30 min (430 rpm) to form a PVA/starch blend film-forming solution (FFS). The FFS was divided into two groups, Group I and Group II. Four concentration levels of mulberry anthocyanin (5%, 20%, 35%, and 50%, *w/w*) were added to both groups. Then, to evaluate the antibacterial activity of the blend films, LAE (5% of the PVA/starch mixture, *w/w*) was added only to Group II. Afterwards, both groups of FFSs were heated to 80 °C and stirred for 30 min at 430 rpm, followed by ultrasonication treatment at 20 kHz for 5 min to achieve an even distribution.

All of the resulting FFSs were cooled at room temperature and degasified with a vacuum pump to eliminate bubbles before casting. The blend film samples were obtained by pouring 20 g of an FFS onto polymethyl methacrylate plates ($120 \times 120 \text{ mm}^2$) and dried at 25 °C for 24 h. Dried films were stored and conditioned for 5 days at room temperature and 53% relative humidity (obtained by saturated magnesium nitrate) in desiccators before measurements. The samples of Group I (without LAE) were coded as AC films, and the samples of Group II (containing LAE) were coded as LAC films.

2.3. Characterization of the PVA/Starch/LAE/Mulberry Anthocyanin Films

2.3.1. Scanning Electron Microscopy (SEM)

Cross-section images of the sample films were examined by field-emission scanning electron microscopy (FE-SEM) (Supra 55, Carl Zeiss NTS GmbH, Oberkochen, Germany). The samples were fixed on a sample stage and coated with gold under vacuum before imaging. The observation was carried out at an acceleration voltage of 2 kV.

2.3.2. Fourier Transform Infrared (FTIR) Spectroscopy

The infrared (IR) spectra of the blend films were obtained by an FTIR spectrometer (Nicolette Magna 550II, GMI, Ramsey, MN, USA). The interactions between PVA, starch, LAE, and anthocyanin were investigated by measuring the absorbance spectra in a wavenumber range from 4000 to 400 cm^{-1} at a resolution of 8 cm^{-1} .

2.3.3. X-ray Diffraction (XRD)

The XRD patterns of the blend films were recorded by an X-ray diffractometer (Mini-Flex 600, Rigaku, Tokyo, Japan) using Cu $K\alpha$ radiation under the conditions of 40 kV and 15 mA. The samples were cut into 20 \times 20 mm^2 , placed onto a glass plate, and then scanned in the range of 3° to 40° (2 θ) at a step size of 0.02°. The crystallinity index (CI) of the obtained films was determined by Equation (1):

$$CI(\%) = \frac{A_{crystalline}}{A_{crystalline} + A_{amorphous}} + 100\% \quad (1)$$

where $A_{crystalline}$ is the area of the crystalline region, and $A_{amorphous}$ is the area of the amorphous region.

2.3.4. Thickness and Mechanical Properties

The film thickness was measured by a No. 211–101 electronic spiral micrometer (Jingyou Mould Hardware Co., Ltd., Dongguan, China) at six random positions on each sample. The mean values were recorded and used to determine the tensile properties and water vapor permeability.

The mechanical properties of samples were measured by a universal testing machine (JDL-1000N, Tianfa Instruments Co., Ltd., Yangzhou, China) according to the method described by Liu et al. with modifications [33]. The conditioned samples were cut into rectangular strips of 60 mm \times 20 mm. The tests were carried out with an initial grip separation of 50 mm and an extension rate of 10 mm/min. The tensile strength (TS, MPa) and elongation at break (EAB, %) of the samples were calculated according to the recorded stress–strain curves.

2.3.5. UV Barrier and Light Transmittance

The UV barrier and light transmittance results of the blend films were obtained by a UV-VIS spectrophotometer (UV-2600, Shimadzu, Kyoto, Japan). The films were cut into rectangular strips (10 mm \times 40 mm). The UV barrier property was evaluated in a wavelength range from 200 to 400 nm, and the light transmittance in the visible-light region was evaluated in a wavelength range from 400 to 800 nm. The opacity value of the film was calculated using Equation (2) [29]:

$$\text{Opacity value} = \frac{-\log T_{600}}{H} \quad (2)$$

where T_{600} is the light transmittance at a wavelength of 600 nm, and H is the film thickness.

2.3.6. Water Vapor Permeability (WVP) and Moisture Absorption (MA)

WVP was measured gravimetrically according to ASTM E96-00 with some modifications [34]. The film sample (diameter 60 mm) was sealed with Vaseline over a 50 mL beaker

containing 30 mL of deionized water. Then, the beaker was maintained in a closed desiccator containing 1000 g of dried silica at 25 °C. The weight of the test vessel was weighed every 3 h until reaching a stabilized weight. The WVPs of the films were calculated using Equation (3):

$$WVP \left(\text{g} \cdot \text{m}^{-1} \cdot \text{s}^{-1} \cdot \text{Pa}^{-1} \right) = \frac{\Delta W \times H}{\Delta t \times \Delta P \times S} \quad (3)$$

where ΔW is the weight loss of the beaker system (g), H is the thickness of the film (m), Δt is the time change (s) under the partial pressure difference ($\Delta P = 2533$ Pa), and S is the area of water permeation (m^2).

Moisture absorption (MA) was determined according to the method reported by Abrial et al. with modifications [35]. The film strips (20 mm × 20 mm) were dried in a vacuum oven at 50 °C for 24 h, and the weight was recorded as M_1 . Then, the dried samples were conditioned for 24 h at 25 °C at a relative humidity (RH) of 75% (obtained by a saturated NaCl solution), and the weight was recorded as M_2 . The MA of the films was calculated by Equation (4):

$$MA(\%) = \frac{M_2 - M_1}{M_1} \times 100\% \quad (4)$$

where M_1 and M_2 are the initial and final weights of the film samples, respectively.

2.3.7. Color Indication of Anthocyanin and Blend Films

To evaluate the color change of mulberry anthocyanin, 2 g of anthocyanin was mixed with 500 mL of distilled water, and then the pH (values from 2 to 11) of the solution was adjusted by the dropwise addition of a hydrochloric acid or sodium hydroxide solution.

To evaluate the color change of the blend films, the blend films were cut into 20 mm × 20 mm rectangular strips and submerged into the hydrochloric acid solution with pH from 2 to 7 and the sodium hydroxide solution with pH from 8 to 11 for 5 min.

The color changes of anthocyanin solutions and the blend film samples were imaged by a digital camera.

2.3.8. In Vitro Antimicrobial Activity

An antibacterial experiment was carried out according to the agar diffusion method used by Theinsathid et al. with some modifications [24]. Samples were cut from the obtained films into 8 mm discs with a puncher. A 90 mm-diameter Petri dish was used to prepare the TSA plate medium. Both the samples and plates were sterilized by UV radiation on a sterile operating table for 2 h. Afterwards, 0.1 mL of TSB of 10^8 CFU/mL of *E. coli* and 0.1 mL of TSB of 10^8 CFU/mL of *S. aureus* were evenly spread on the TSA plate, respectively. Finally, the samples were put on the surface of the TSA plate and placed in a constant-temperature incubator at 37 °C for 24 h. The diameter of the bacteriostatic zone (mm) around the samples was measured to evaluate the antibacterial activity of the obtained films.

The in vitro antibacterial activity of mulberry anthocyanin was evaluated following the same procedures, except that the samples were prepared by soaking an 8 mm-diameter filter paper in an anthocyanin solution (0.1 g/L) for 2 h.

2.3.9. Milk Spoilage Test

First, 30 mL of fresh milk was poured into a 90 mm-diameter Petri dish, and then AC and LAC films were cut into 20 mm × 20 mm strips and placed in the milk at room temperature separately. The images of the treated milk were taken after 7 days to monitor the milk spoilage and film coloration.

2.3.10. Statistical Analysis

The analysis of variance (ANOVA) was conducted using IBM SPSS Statistics 13.0. The data were expressed as mean ± standard deviation. Differences were considered to be statistically significant in Tukey's range test if $p \leq 0.05$.

3. Results

3.1. Morphology

The cross-section images of the AC and LAC films with different concentrations of mulberry anthocyanin are presented in Figure 1. It can be seen in Figure 1A(a) that the cross-section of the AC film without mulberry anthocyanin was relatively smooth and homogeneous. This could be due to the excellent compatibility between PVA and cassava starch and the hydrogen bonds formed between their hydroxyl groups [10,36]. As shown in Figure 1A(b–e), the cross-sections of the films became rougher with more aggregations as the anthocyanin content increased, indicating reduced compatibility between PVA, cassava starch, and mulberry anthocyanin. When the anthocyanin content was increased to 50%, small holes were observed. Regarding this, the high viscosity of the film-forming solution could make it difficult to eliminate the bubbles during casting, resulting in the formation of pores in the polymer matrix [8]. By comparing Figure 1B(a) with Figure 1A(a), it can be found that the addition of LAE caused a rougher surface, suggesting that the aggregation of LAE disrupted the interaction between PVA and cassava starch [10]. Figure 1B(b–e) displays a similar trend to that observed in Figure 1A(b–e). The cross-sections exhibited less compact and more heterogeneous structures due to an increase in the anthocyanin content and the high viscosity of the casting solutions.

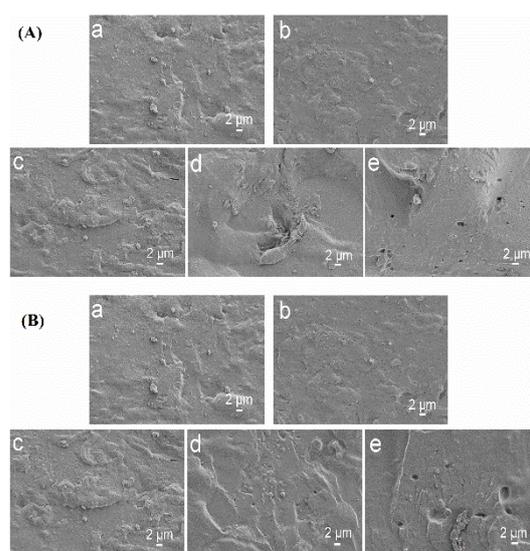


Figure 1. SEM micrographs of AC (A) and LAC (B) films with different anthocyanin contents (a), without anthocyanin; b–e, the anthocyanin contents were 5%, 20%, 35%, and 50% of the polymer matrix, *w/w*).

3.2. FTIR Analysis

Figure 2 shows the FTIR spectra of PVA, starch, and AC and LAC films with different concentrations of mulberry anthocyanin. The bands from 3280 to 3384 cm^{-1} can be attributed to the O–H stretching vibration, and the band at 2938 cm^{-1} is due to C–H bending [25]. The band between 1412 and 1424 cm^{-1} is ascribable to the bending vibration of the $-\text{CH}_2-$ and C–O-specific angular deformation of phenols [36]. The characteristic peak at 1641 cm^{-1} is a feature of tightly bound water present in starch. The characteristic peak at 1656 cm^{-1} becomes wider and more intense with increasing anthocyanin content. This phenomenon may be due to the stretching of the C=C bond of aromatic rings in anthocyanins [34]. The characteristic peak that appears at 1086 cm^{-1} is assigned to the C–O stretching vibration of PVA [36]. The peak at 1156 cm^{-1} results from C–C and C–OH stretching, which confirms the basic carbon skeleton of PVA. The peak at 1008 cm^{-1} is due to the C–O stretching of the glucose ring in starch. Furthermore, the spectra for AC

films and LAC films are similar, indicating that adding LAE did not significantly affect the intermolecular interactions among the components of the blend films.

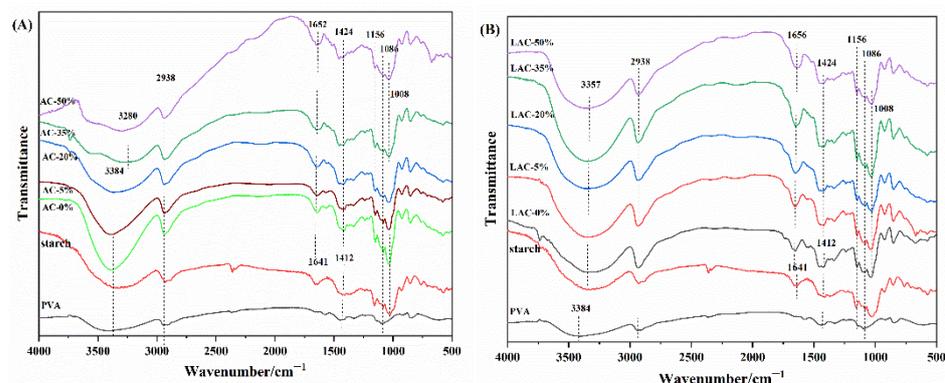


Figure 2. FTIR spectra of AC (A) and LAC (B) films with different anthocyanin contents (0%, 5%, 20%, 35%, and 50% of the polymer matrix, w/w).

3.3. XRD Analysis

The X-ray diffractograms of AC films and LAC films are shown in Figure 3. It is clear that the XRD spectra did not significantly change with the addition of mulberry anthocyanin and LAE. All films showed the strongest diffraction peaks at around 20°, which is the main diffraction peak of PVA. Regarding this, the crystalline structure of the native starch was damaged during processing, and thus, the crystalline structure of PVA dominated the diffraction patterns of the blend films [10].

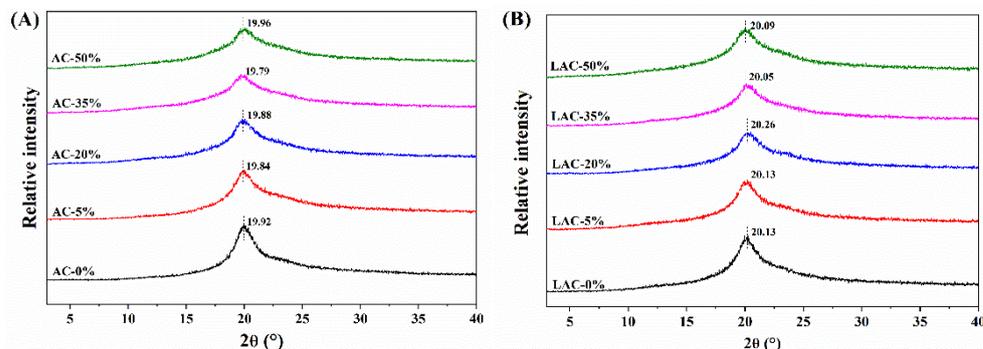


Figure 3. XRD patterns of AC (A) and LAC (B) films with different anthocyanin contents (0%, 5%, 20%, 35%, and 50% of the polymer matrix, w/w).

Furthermore, the crystallinity values of both AC and LAC films showed a slightly decreasing trend with an increase in the anthocyanin content. When the anthocyanin content increased from 0% to 50%, the CI of AC films decreased from 13.61% to 10.27%, and the CI of LAC films decreased from 11.39% to 10.48%, respectively. This might be due to the disruption of the orderly arrangement of PVA and cassava starch by the addition of mulberry anthocyanin. Similarly, Zhai et al. observed that the crystallinity of arbutus anthocyanin–cassava-starch films decreased with an increase in the anthocyanin content [36]. However, Qin et al. found that the crystallinity of *Lycium barbarum*–cassava starch films increased with an increase in the anthocyanin content, which was ascribed to the formation of a strong three-dimensional hydrogen-bonding network [14,37]. These controversial research results indicated that the crystallinity of blend films might be associated with the types of anthocyanins and additives, the preparation process, and the selection of the polymer matrix. The crystallinity is often related to the mechanical properties of polymer materials, especially tensile strength [38]. In our study, the decrease in crystallinity led to a slight decrease in the tensile strength of the active films.

3.4. Thickness and Mechanical Performance

Thickness is a significant factor affecting the WVP, light transmittance, and tensile properties of blend films. Table 1 lists the thicknesses of AC and LAC films with different concentrations of mulberry anthocyanin. It can be seen that the thickness of AC and LAC films increased with an increase in the anthocyanin content. This finding is in agreement with previous reports by Zhai et al. [36] on starch/polyvinyl alcohol films incorporated with roselle anthocyanin. The presence of glycerol and anthocyanin might have a plasticizing effect by lowering the cohesion within the polymer chain network, generating a higher free volume and thus resulting in thicker films [39]. This result is also in agreement with the results of SEM, which show that a higher amount of anthocyanin could create a more complex film matrix, which led to an increase in the film thickness.

Table 1. Thickness and mechanical properties of AC and LAC films with different anthocyanin contents (0%, 5%, 20%, 35%, and 50% of the polymer matrix, w/w).

Film	Anthocyanin Content (%)	Tensile Strength (MPa)	Elongation at Break (%)	Thickness (mm)	Young's Modulus (MPa)
AC	0	13.61 ± 0.07 ^a	19.86 ± 2.81 ^a	0.11 ± 0.004 ^a	343.91 ± 44.91 ^a
	5	10.87 ± 0.84 ^b	17.78 ± 0.56 ^a	0.13 ± 0.002 ^b	312.63 ± 31.74 ^{ab}
	20	11.37 ± 0.46 ^b	17.08 ± 0.27 ^a	0.13 ± 0.002 ^b	292.41 ± 29.53 ^{ab}
	35	9.63 ± 0.46 ^b	14.07 ± 0.64 ^b	0.15 ± 0.003 ^c	233.99 ± 18.62 ^c
	50	10.27 ± 0.16 ^b	12.31 ± 0.68 ^b	0.16 ± 0.004 ^d	163.75 ± 20.19 ^d
LAC	0	11.39 ± 0.14	20.95 ± 6.03 ^a	0.10 ± 0.014 ^a	324.78 ± 31.45 ^a
	5	11.51 ± 1.18	24.43 ± 1.34 ^a	0.11 ± 0.006 ^a	337.84 ± 35.17 ^a
	20	11.23 ± 1.64	19.04 ± 1.63 ^{ab}	0.12 ± 0.003 ^a	295.81 ± 30.11 ^{ab}
	35	10.55 ± 0.18	13.21 ± 0.78 ^{bc}	0.14 ± 0.005 ^b	217.19 ± 20.75 ^c
	50	10.48 ± 0.43	11.25 ± 0.93 ^d	0.18 ± 0.015 ^c	178.25 ± 18.18 ^d

Values are shown as mean ± standard deviation ($n = 3$). The mean values in the same column of the table with different superscript letters (a, b, c, and d) indicate significant differences in Turkey's test at $p < 0.05$.

Figure 4 shows the mechanical properties, including TS and EAB, of AC and LAC films. It can be seen that both the TS and EAB showed a similar trend. When the anthocyanin content was less than 20%, TS and EAB did not show significant changes ($p < 0.05$). This means that a low concentration of mulberry anthocyanin ($\leq 20\%$) had no significant effect on the mechanical properties of the blend films. Regarding this, the evenly dispersed anthocyanin in the polymer matrix probably did not affect the blending of starch and PVA, so the mechanical properties of the blend films remained unchanged [34]. However, when the anthocyanin content was higher than 20%, the TS and EAB of the blend films gradually decreased. The values of Young's modulus are listed in Table 1. It can be found that Young's modulus followed the same trend and gradually decreased with increasing anthocyanin content. Mali et al. [40] reported a similar result in their study. Regarding this, mulberry anthocyanin at a higher content probably formed agglomerates, which disrupted the structural integrity of the polymer matrix [14,34]. In previous studies, Zhai et al. [36] observed that adding roselle anthocyanin could promote the mobility of macromolecular polymer chains and improve the compatibility between PVA and starch, leading to better mechanical properties of the blend films. However, Zhang et al. [34] found that the mechanical properties of the starch/PVA films deteriorated when the concentration of purple sweet potato anthocyanin was high. They considered this phenomenon to be ascribable to the destruction of the polymer network by the excessive anthocyanin [35]. These inconsistent results indicate that the mechanical properties of blend films vary with the type and dosage of anthocyanin added.

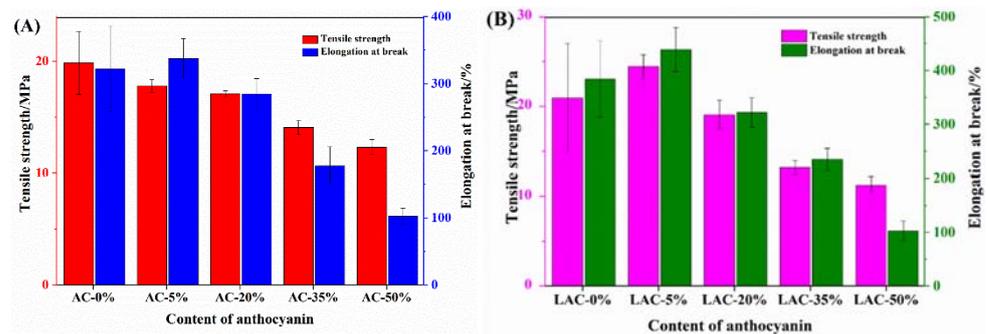


Figure 4. Mechanical properties of AC (A) and LAC (B) films with different anthocyanin contents (0%, 5%, 20%, 35%, and 50% of the polymer matrix, w/w).

3.5. UV-Vis-Light Barrier Properties and Opacity Analysis

Food packaging materials with excellent UV-vis-light barrier properties can help to maintain the quality and prolong the shelf life of foods. Figure 5 shows the light transmittance of AC and LAC films, and the corresponding data are listed in Table 2. As can be seen, AC films with mulberry anthocyanin possessed a higher UV-vis-light barrier effect than the AC film without mulberry anthocyanin, and the performance increased with an increase in the anthocyanin content. At a wavelength of 200 nm, the UV transmittance of the AC film without mulberry anthocyanin was much higher than that of other films. This is because PVA and starch barely possess a UV barrier effect [41,42]. With an anthocyanin content of 5%, the UV light transmittance of the AC film at 400 and 800 nm (T_{400} and T_{800} , respectively) was 22.67% and 47.61%, respectively, whereas the transmittance decreased to 0.35% and 31.45% when the anthocyanin content reached 50%. Given this, it is likely that mulberry anthocyanin possessed a good UV absorption effect, and the agglomeration of anthocyanins hindered the passage of light in the blend films [37]. Moreover, LAC films exhibited an even stronger barrier effect than AC films, which could be ascribed to LAE also inhibiting the light transmittance [22]. Furthermore, an increased film thickness can also decrease the light transmittance [43].

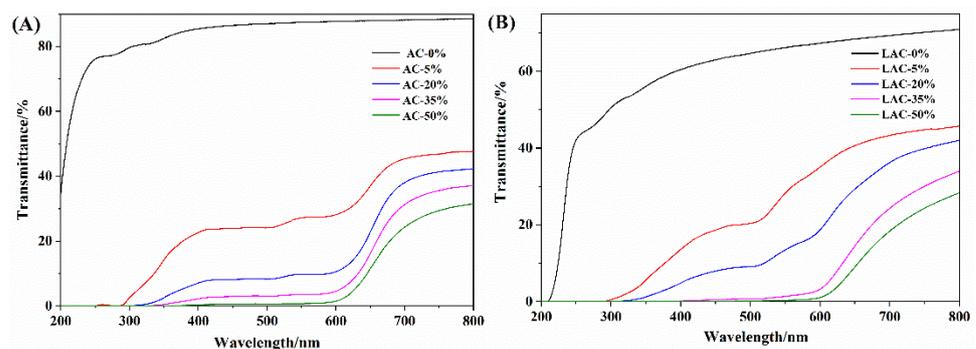


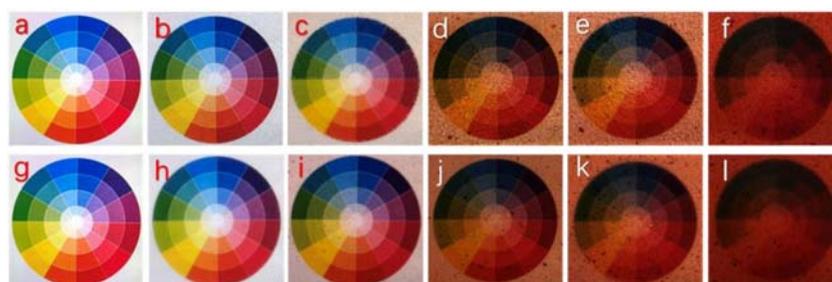
Figure 5. Light transmittance of AC (A) and LAC (B) films with different anthocyanin contents (0%, 5%, 20%, 35%, and 50% of the polymer matrix, w/w).

Table 2. Opacity, moisture absorption (MA), and water vapor permeability (WVP) of AC and LAC films with different anthocyanin contents (0%, 5%, 20%, 35%, and 50% of the polymer matrix, w/w).

Film	Anthocyanin Content (%)	Opacity	MA (%)	WVP ($\text{g}\cdot\text{m}^{-1}\cdot\text{s}^{-1}\cdot\text{Pa}^{-1}$)
AC	0	0.51 ± 0.03^a	16.00 ± 1.31	$4.79 \times 10^{-12} \pm 1.51 \times 10^{-12}$
	5	4.30 ± 0.54^b	18.42 ± 3.72	$1.16 \times 10^{-11} \pm 4.30 \times 10^{-13}$
	20	7.28 ± 0.45^c	18.60 ± 3.29	$1.08 \times 10^{-11} \pm 2.70 \times 10^{-12}$
	35	8.95 ± 0.56^c	19.44 ± 1.96	$8.55 \times 10^{-12} \pm 5.04 \times 10^{-13}$
	50	11.19 ± 0.59^d	20.51 ± 1.81	$9.29 \times 10^{-12} \pm 3.83 \times 10^{-12}$
LAC	0	1.67 ± 0.39^a	14.81 ± 2.62	$1.03 \times 10^{-11} \pm 6.92 \times 10^{-13}$
	5	4.11 ± 0.79^a	15.79 ± 0.30	$1.00 \times 10^{-11} \pm 7.46 \times 10^{-13}$
	20	6.13 ± 0.62^b	15.79 ± 0.77	$1.07 \times 10^{-11} \pm 8.00 \times 10^{-13}$
	35	10.34 ± 0.68^c	15.91 ± 0.37	$1.78 \times 10^{-11} \pm 6.29 \times 10^{-12}$
	50	11.01 ± 0.66^c	17.65 ± 0.10	$1.62 \times 10^{-11} \pm 2.41 \times 10^{-12}$

Values are shown as mean \pm standard deviation ($n = 3$). The mean values in the same column of the table with different superscript letters (a, b, c, and d) indicate significant differences in Turkey's test at $p \leq 0.05$.

Pictures of color charts covered by both AC and LAC films were taken to evaluate the transparency of the blend films against different colors, as shown in Figure 6. It appears that the color difference was substantial, and the transparency decreased with an increase in the anthocyanin content [36]. It is worth noting that the opacity values at 600 nm of both AC and LAC films with 0% and 5% mulberry anthocyanin contents were lower than 5. Therefore, the blend films containing a small amount of anthocyanin (lower than 5%) could be considered transparent [22].

**Figure 6.** Pictures of AC and LAC films with different anthocyanin contents overlaid on a color disk ((a,g), blank; (b–f,h–l), the anthocyanin contents were 0%, 5%, 20%, 35% and 50%, w/w , respectively).

3.6. Water Vapor Permeability (WVP) and Moisture Absorption (MA) Analysis

Water vapor permeability and moisture absorption are two important indicators to evaluate the water resistance of films. As presented in Figure 7 and Table 2, the WVP values of AC films with mulberry anthocyanin were higher than the value of the AC film without mulberry anthocyanin. The highest value obtained was $1.16 \times 10^{-11} \text{ g}\cdot\text{m}^{-1}\cdot\text{s}^{-1}\cdot\text{Pa}^{-1}$ when the anthocyanin content was 5%. Similarly, as shown in Figure 7B, the WVP values of LAC films with mulberry anthocyanin were also mostly higher than the WVP value of the LAC film without mulberry anthocyanin. Regarding this, the agglomeration of anthocyanins probably destroyed the integrity of the film, thereby promoting the transport of water molecules and increasing the WVP [10]. In addition, an increased thickness of hydrophilic films can lead to higher WVP due to their affinity for moisture [44]. Similar findings were reported by Yun et al. [14] and Luchese et al. [30].

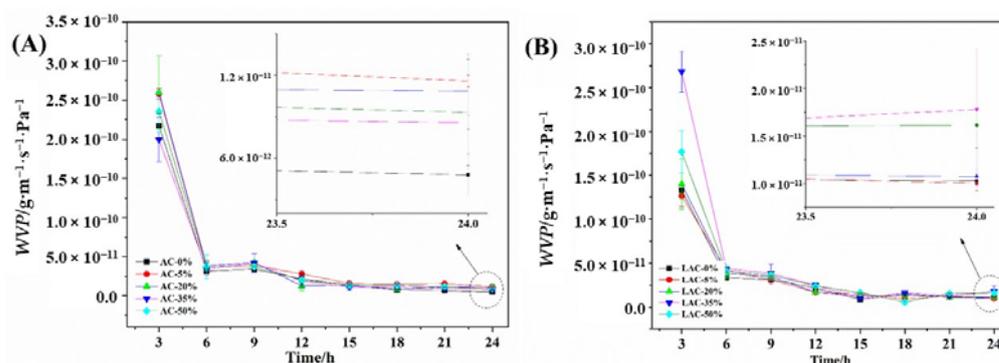


Figure 7. Water vapor permeability (WVP) of AC (A) and LAC (B) films with different anthocyanin contents (0%, 5%, 20%, 35%, and 50% of the polymer matrix, w/w).

The moisture absorption of different films is also listed in Table 2. It can be seen that the MA values slightly increased with an increase in the anthocyanin content. The highest MA values of AC and LAC films were 20.51% and 17.65%, respectively. This phenomenon can probably be ascribed to more hydrophilic groups contained in anthocyanins, which can increase the moisture absorption of the films [34,45].

3.7. In Vitro Antimicrobial Activity

Strong antibacterial activity is essential for food packaging films to decrease microbial contamination. The antibacterial properties of the blend films against two foodborne bacterial pathogens (*E. coli* and *S. aureus*) are shown in Figure 8 and Table 3. It can be seen that there was no inhibition zone around AC films, indicating they had almost no inhibitory effect on either *E. coli* or *S. aureus*. According to the study by Chen et al. [46], the antibacterial behaviors of anthocyanins were dependent on the type and the extraction method. They reported that anthocyanins extracted from black mulberry, rock mulberry, and white mulberry displayed different antibacterial activities. At the same concentration, black mulberry anthocyanins showed excellent antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, while the other two anthocyanins exhibited almost no antibacterial activity. As a comparison, the antibacterial properties of filter paper soaked with a pure mulberry anthocyanin solution are presented in Figure 8C. Similar to the result for AC films, this filter paper showed no inhibition zone, indicating that mulberry anthocyanin is not capable of inhibiting bacterial growth.

Table 3. Inhibition zone of AC and LAC films with different anthocyanin contents (0%, 5%, 20%, 35%, and 50% of the polymer matrix, w/w) on *E. coli* and *S. aureus*.

Film	Anthocyanin Content (%)	Inhibition Zone of <i>E. coli</i> (mm)	Inhibition Zone of <i>S. aureus</i> (mm)
AC film	0	0	0
	5	0	0
	20	0	0
	35	0	0
	50	0	0
LAC film	0	20.65 ± 0.21 ^a	37.30 ± 0.28 ^a
	5	12.40 ± 0.35 ^b	33.55 ± 0.71 ^b
	20	11.55 ± 0.21 ^b	22.10 ± 0.21 ^c
	35	10.20 ± 0.25 ^c	16.45 ± 0.04 ^d
	50	9.70 ± 0.39 ^c	16.25 ± 0.11 ^d

Values are shown as mean ± standard deviation ($n = 3$). The mean values in the same column of the table with different superscript letters (a, b, c, and d) indicate significant differences in Turkey's test at $p \leq 0.05$.

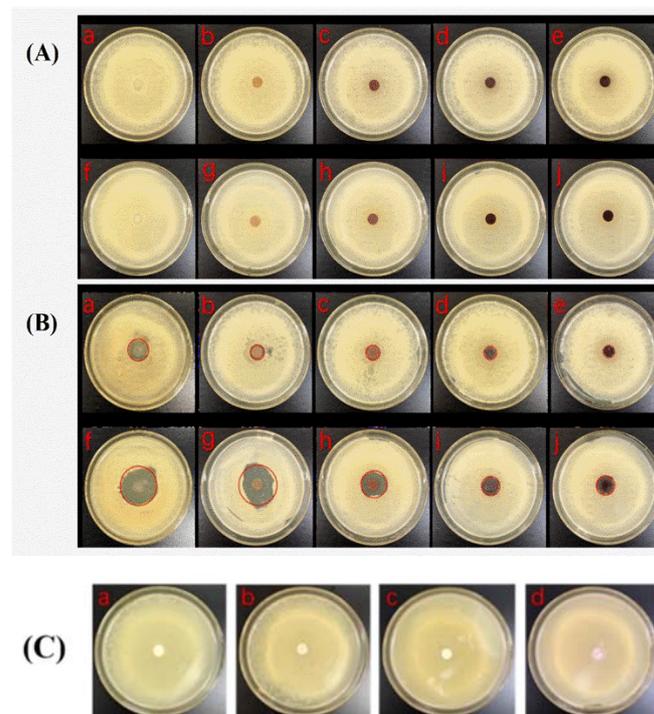


Figure 8. Inhibition zone of AC (A) and LAC (B) films with different anthocyanin contents (0%, 5%, 20%, 35%, and 50% of the polymer matrix, *w/w*) on *E. coli* (a–e) and *S. aureus* (f–j); (C) inhibition zone of anthocyanin (a and c, the blank samples; b and d, samples soaked in an anthocyanin solution) on *E. coli* (a,b) and *S. aureus* (c,d).

By contrast, LAC films generated obvious inhibition zones in both *S. aureus* and *E. coli* culture media. This suggests that the antibacterial function of the obtained blend films was attributed to the inclusion of LAE. It is well known that LAE is an amino-acid-based cationic surfactant with effective antimicrobial behavior against various bacteria. It can damage the cell membranes of bacteria, generating changes in membrane potential and membrane permeability. This subsequently results in the loss of cytoplasm and the rupture of cellular structures, leading to the rapid death of bacteria [23]. The antibacterial effect of LAE was still strong after mixing with anthocyanin in our experiment. It is worth noting that LAC films had stronger inhibitory effects on *S. aureus* than on *E. coli*. The maximum diameters of inhibition zones in the *S. aureus* and *E. coli* culture media were 37.30 mm and 20.65 mm, respectively. This could be due to the structural differences between these two bacteria. *S. aureus* is a Gram-positive bacterium without a lipid membrane in its cell wall, whilst *E. coli* is a Gram-negative bacterium possessing a thick outer lipopolysaccharide membrane. It was more difficult for LAE to break the outer layer of *E. coli* and cause alterations in its cytoplasm [47]. Moreover, the inhibitory zones generated by LAC films on these two bacteria became smaller with an increase in the anthocyanin concentration, suggesting that excessive anthocyanin might weaken the antibacterial activity of LAE in the blend films. Therefore, it is important to adjust the amount and ratio of chromogenic and antibacterial agents when preparing the films. In our study, the film containing 5% LAE and 5% mulberry anthocyanin displayed optimal antibacterial properties.

3.8. Color Analysis of Mulberry Anthocyanin and the Blend Films

Figure 9 shows the visual color changes of mulberry anthocyanin and anthocyanin–LAE solutions in the pH range of 2 to 11. It can be seen that the color changes of the two solutions are distinguishable. Their colors were similar under acidic (pH = 2–6) and neutral (pH = 7) conditions. Both of them appeared peach at pH 2 and gray at pH 7. However, the color differences between these two solutions became obvious in the alkaline environment

(pH = 8–11). As can be seen, the color of the pure mulberry anthocyanin solution was pale yellow-brown at pH 11, and the color of anthocyanin–LAE solutions was dark gray at pH 11. The color characteristics of anthocyanins might be affected when combined with other reagents. Previously, Mozkan et al. [48] reported that xylitol and butyl hydroxyanisole affected the color changes of anthocyanin solutions in their experiments.



Figure 9. Colors of anthocyanin solutions (A) and anthocyanin/LAE solutions (B) at different pH (2–11).

Figure 10 shows the color changes of the AC film and the LAC film with 5% mulberry anthocyanin after being soaked in buffer solutions with pH values ranging from 2 to 11. The AC film and the LAC film gave markedly different responses. AC films were less sensitive within the tested range of pH, all displaying a color close to light orange, which was difficult to differentiate by the naked eye. LAC films exhibited higher sensitivity when exposed to the same pH value range. They appeared light orange at pH 2 and 3, and then the color became intensified and darker as the pH value increased, easily visible to the naked eye. According to this observation, mixing anthocyanin with LAE seems to promote the color changeability of the blend films. Anthocyanins were more likely to interact with pH-inducing compounds due to the addition of LAE. This can be explained by the surfactant effect of LAE, which generated small bubbles in the film-forming solution, leading to a less compact polymer network allowing guest molecules to diffuse easily throughout the film matrix [10,49]. Similarly, Pourjavaher et al. [50] found that the color change response of anthocyanin mixed with nanocellulose was more obvious than that of pure anthocyanin within a pH range of 2 to 10.

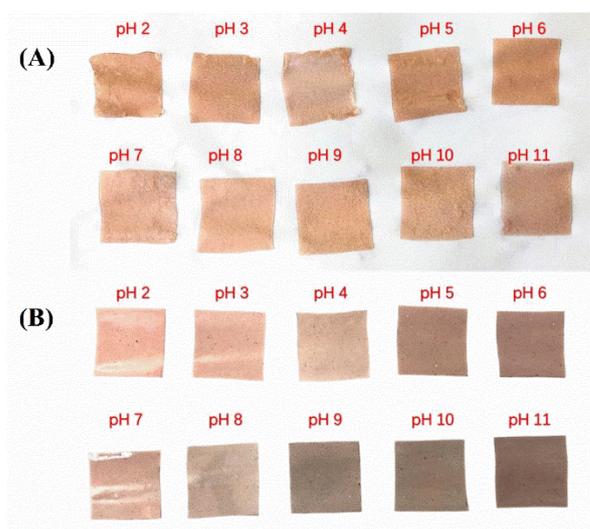


Figure 10. Color of AC (A) and LAC (B) films at different pH with 5% (*w/w*) anthocyanin content.

3.9. Milk Spoilage Test of Active Films

A food spoilage test was performed on milk to investigate the color response and antibacterial behavior of AC and LAC films. Figure 11 shows the changes in the color of AC and LAC films resulting from immersion in milk for a week. It was found that even at a low concentration of anthocyanin (5%), the color alteration of the AC film after being soaked in milk was difficult to detect by the naked eye. With an increase in the anthocyanin content, the color variation became more difficult to distinguish because the film color was much darker. The results of LAC films with high concentrations of mulberry anthocyanin were similar. When the concentration of mulberry anthocyanin was higher than 5%, the visual color variability of the blend films was poor because of their dark and intense color. However, the LAC film containing 5% anthocyanin achieved a profound color response, whose color changed from dark brown to light sanguine after its immersion, easily visible to the naked eye. Thus, this sample shows potential for food-freshness-monitoring applications.

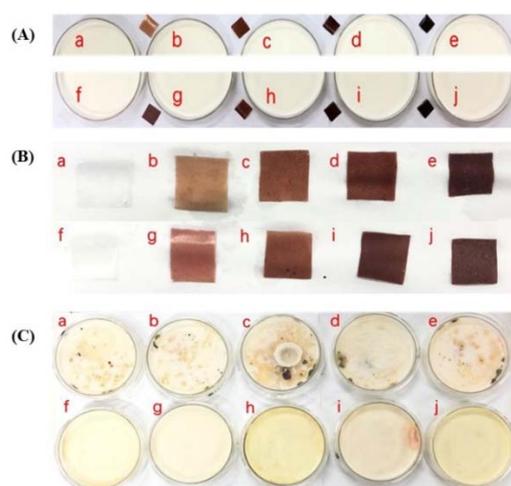


Figure 11. Color of AC films (a–e) and LAC films (f–j) with different anthocyanin contents (0%, 5%, 20%, 35%, and 50% of the polymer matrix, w/w) before (A) and after (B) being soaked in milk; (C) spoilage of milk with AC films and (a–e) and LAC films (f–j) containing different anthocyanin contents (0%, 5%, 20%, 35%, and 50% of the polymer matrix, w/w).

The antibacterial activity results of AC and LAC films for the milk were presented in Figure 11C. It can be observed that though all milk samples were spoiled after 7 days, the degrees of spoilage for the samples with AC films and for those with LAC films were different. The deterioration of milk samples with AC films was more pronounced, with the emergence of many agglutinations and the growth of mold on the samples. By contrast, the samples with LAC films were still in conditions that were relatively close to their initial state, with only a few agglutinations shown. This is consistent with the result of the antibacterial activity test in Section 3.7, which shows that the mulberry anthocyanin used for preparing AC films did not have antibacterial activity, while the antibacterial behavior of LAC films was attributed to the inclusion of LAE.

4. Conclusions

In this study, mulberry anthocyanin and LAE were selected as a colorant and an antibacterial agent, respectively, and added to a PVA/cassava-starch matrix to prepare active food packaging films. The introduction of mulberry anthocyanin and LAE resulted in a less compact microstructure and generated pores on the film cross-sections. No chemical interaction was found between the components, and the crystallinity of the blend films slightly decreased with the addition of mulberry anthocyanin. The concentration of anthocyanin plays an important role in film properties. Films containing less than

20% mulberry anthocyanin did not have obvious effects on the mechanical properties of the blend films, and both TS and EAB decreased with increasing anthocyanin content. Moreover, the blend films showed higher UV-light barrier properties with slightly increased WVP and moisture absorption with the increased anthocyanin concentration. In comparison with AC films, LAC films exhibited more pronounced pH-responsive color changes and excellent antibacterial performance. They showed distinguishable color changes within pH values ranging from 2 to 11 and had good inhibitory effects on the growth of both *E. coli* and *S. aureus*. During the milk spoilage test, the active films containing 5% LAE and 5% mulberry anthocyanin effectively indicated milk deterioration and slowed down the spoilage process. Based on the physical properties, antibacterial behavior, and pH-responsive performance of the active films, we suggest that the film formulation for future applications be a 70:30 PVA/starch blend with 5% LAE and 5% anthocyanin. The PVA/starch/LAE/anthocyanin films can monitor the quality change and extend the shelf life of packaged food, and they demonstrate high potential to be used as an active packaging material in the food industry.

Author Contributions: Conceptualization, N.L. and Z.Z.; methodology, N.L., L.Z. and F.X.; investigation, N.L., F.W. and D.J.; formal analysis, N.L.; data curation, D.J.; writing—original draft preparation, N.L.; writing—review and editing, L.Z. and F.X.; visualization, F.W.; supervision, Y.L.; project administration, L.Z.; funding acquisition, Y.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Natural Science Project of Guangxi Minzu University (No. 2020KJYB004), the Regional Fund of National Natural Science Foundation of China (No. 21968008 and 21566007), and Guangxi Natural Science Foundation (No. 2018GXNSFAA050025).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the authors.

Conflicts of Interest: The authors declare no conflict of interest.

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