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Designing antimicrobial active packaging films based on chitosan plus fungus comb ethyl acetate extract from Indo-Malayan termite mounds

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Abstract. *Witasari LD, Lizar DP, Florencia OM, Ramadhan A, Fiana NAO, Supriyadi S, Pratiwi SUT, Nandika D, Karlinasari L, Arinana A, Batubara I, Santoso D, Sudiana IK, Hertanto DM, Rachmayanti Y, Firmansyah D. 2023. Designing antimicrobial active packaging films based on chitosan plus fungus comb ethyl acetate extract from Indo-Malayan termite mounds. Biodiversitas 24: 5947-5955.* Antimicrobial active packaging systems incorporate antimicrobial compounds into polymer films, i.e., chitosan, thus suppress spoilage microorganism growth in foods. Chitosan films itself cannot prevent microbial growth, thus other antimicrobial agent must be added. Fungus comb extract from Indo-Malayan termite (*Macrotermes gilvus* (Hagen, 1858)) mounds is a potential active organic antimicrobial compound, which can be incorporated into chitosan films. The purpose of this study was to develop and characterize active packaging films based on chitosan plus an antimicrobial fungus comb ethyl acetate extract. Films contained 1% chitosan and fungus comb ethyl acetate extract (0.5, 1, 2, and 5% w/v). Notably, films containing 2 and 5% extracts inhibited *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), and *Pseudomonas aeruginosa* (ATCC 27853), but not fungi. Films plus 2% extract showed a thickness, tensile strength, and strain of 0.13 mm, 2.15 MPa, and 32.55%, respectively. Films had a moisture content of 0.26%, water solubility of 2.68%, swelling property of 2.37%, and water vapor permeability (WPV) of 3.31×10^{-10} $g.m^{-1}s^{-1}.Pa^{-1}$. They displayed smooth morphology but no new functional groups. Thus, chitosan plus 2% of fungus comb ethyl acetate extract can be used in antimicrobial active packaging films for food application. It could be applied as a green alternative to prolong the shelf-life of food products.

Keywords: Active packaging, chitosan, films, fungus comb, *Macrotermes gilvus*

INTRODUCTION

Active packaging technologies incorporate additives into packaging systems with a view to maintaining food quality and extending shelf life. Active packaging inhibits spoilage and pathogen growth, prevents contaminant migration, and identifies package leaks, thus ensuring food safety (Biji et al. 2015). Antimicrobial packaging is a promising form of active food packaging. It can be fabricated using antimicrobial packaging materials or agents inside package spaces or foods. Antimicrobial packaging can be used for sensitive foods, including meat, fish, poultry, baked goods, cheese, fruit, and vegetables (Al-Tayyar et al. 2022). Different biopolymers have been used to develop packaging films and include active substrates, such as polysaccharides, lipids, and proteins. Carbohydrate polymers such as starches, cellulose derivatives, chitosan and pectin that are edible and biodegradable, have been utilized as packaging materials (Espitia et al. 2014). Chitosan is a popular polysaccharide due to its potential as an active packaging-based material (Flórez et al. 2022) and is composed of deacetylated chitin, which is found in crab residues, shrimp shells, cell walls, fungal cell membranes, insect exoskeletons, and arachnids (Bakshi et al. 2018). Chitosan is a linear cationic polysaccharide consisting of d-glucosamine and N-acetyl-d-glucosamine units linked by β-(1 → 4) linkages (Dash et al. 2011). Chitosan is a weak base so that it can dissolve in acidic solutions, but it remains insoluble in water. Chitosan has good packaging-based properties, which exert antimicrobial, biocompatible, non-toxic, biodegradable, and excellent film-forming characteristics (De Carli et al. 2022; Gan et al. 2022). Although having several advantageous antimicrobial properties, chitosan itself

cannot prevent microbial growth, so other antimicrobial properties must be introduced to ensure food safety levels for sustainable active packaging (De Carli et al. 2022).

Chemical preservatives in antimicrobial release systems consist of organic acids (e.g., benzoates, propionates, and sorbates), nitrites, sulfites, parabens, chlorides, phosphates, epoxides, alcohols, ozone, hydrogen peroxide, diethyl pyrocarbonate, bacteriocins, and antibiotics (Coban 2020). Potassium sorbate and sodium benzoate have a generally recognized as safe (GRAS) status and can inhibit bacterial and fungal growth. Furthermore, Potassium sorbate had been incorporated as antimicrobial agent in biopolymers films of chitosan (Remedio et al. 2019). Green tea extract was incorporated into a chitosan film to develop active packaging for pork sausages which showed enhanced antioxidant and antimicrobial properties (Siripatrawan and Noipha 2012). Fungus comb is a potential active organic antimicrobial compound which can be incorporated into biopolymeric matrices. The fungus comb is a unique structure made by termites from the subfamily *Macrotermitinae* (Isoptera: Termitidae) in their nests as a substrate. The only species of fungus of *Termomyces* sp. lives in the fungus comb, which means it might have contained substances to eliminate another fungus (Arinana et al. 2016). Fungus combs extracted from Indo-Malayan, *Macrotermes gilvus,* termite mounds using ethyl acetate exhibited antimicrobial and antifungal activities against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria, and *Aspergillus flavus* and *Aspergillus niger* fungi (Witasari et al. 2022). Fungus comb extraction generated dominant compounds, including glycerol, phenolic compounds (phenol, 2-methoxy, and phenol, 2, 6-dimethoxy), and Bis(2-ethylhexyl) phthalate, which demonstrated antimicrobial, antifungal, antioxidant and immunomodulator (inflammation-modulating) activities (Nandika et al. 2021; Rachmayanti et al. 2022; Caesario et al. 2023; Nandika et al. 2023). Besides, the fungus comb extracts considered as preservative for controlling wood-decaying fungi (Nandika et al. 2023). Therefore, these unique fungus comb characteristics render it a suitable natural resource for incorporation with chitosan into active film production.

To date, chitosan films incorporated with fungus comb extracts (hereafter extracts) from Indo-Malayan termite (*M. gilvus*) mounds have not been reported. In this study, the microstructural, physical, and functional properties of composite active antimicrobial packaging systems based on chitosan-incorporated extracts were examined. We provide insights on these characteristics with a view to generating potential applications for the food packaging industry.

MATERIALS AND METHODS

Preparing antimicrobial active packaging films

Films were prepared according to Butler et al. (1996) with some modifications. Films were made by dissolving 1% chitosan (w/v) in 0.1 M acetic acid and adding the extracts in dimethyl sulfoxide at different concentrations (0.5, 1, 2, and 5% b/v) and stirring. Mixtures were poured into 12×15 cm^2 trays and dried at 50°C for 15-18 h in a cabinet dryer incubator (Aneka Mesin, Yogyakarta, Indonesia).

Active packaging film characterization

In vitro antimicrobial activity

Antibacterial susceptibility tests

Antibacterial activity was determined using the Kirby-Bauer disc diffusion method (CLSI 2015). *Escherichia coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), and *S. aureus* (ATCC 25923) culture stocks from Remel, Thermo Scientific, Santa Fe Drive were prepared using a streak plate method on Mueller-Hinton agar (MHA, OXOID, Basingstoke, England) and plates incubated at 37°C for 18 h. Single colonies were diluted in 2 mL of 0.85% NaCl and adjusted to approximately 1.5×10^8 CFU/mL using 0.5 McFarland turbidity units with the Wickerham card approach. The cell suspension was then inoculated onto MHA medium using a sterile cotton swab (Onemed, Indonesia). Films were cut into rounds (diameter = 6 mm) and placed onto agar surfaces. Films without extracts were used as negative controls. Petri dishes were left at room temperature for 1 h and then incubated at 37°C for 18 h. Clear zones around the samples were determined as the diameter of the inhibition zone (DIZ). Samples were tested in triplicate.

Antifungal susceptibility tests

Stock *A. niger* (FNCC 6114) and *A. flavus* (FNCC 6181) cultures were adjusted to 10^6 CFU/mL and resuspended in 5 mL 0.05% Tween 80. Film antifungal susceptibility to both fungal cultures was determined using the Kirby-Bauer disk diffusion method (CLSI 2015). Single colony was inoculated onto potato dextrose agar medium (Merck, Germany) using a streak plate technique. Film rounds (diameter = 6 mm) were placed onto agar surfaces. Films without extracts were used as negative controls. *A. flavus* and *A. niger* susceptibility was determined by measuring DIZ values around films after 48 h at 30°C.

Film thickness

Films were cut into hourglass shapes (100×25 mm^2) using a cutter (Dumddel, China) and stored for 24 h at 25°C in 80% relative humidity (RH). Packaging thickness was then measured using a micrometer screw (Coolant Proof, Mitutoyo, Japan).

Mechanical properties

Film tensile strength and strain levels were measured using the Universal Testing Machine (Zwick BL-GR-500N), ZwickRoell Pte. Ltd., Banten, Indonesia, at test rates = 50 mm/s (Contessa et al. 2021).

Moisture content

Moisture content measurements were performed by weighing packaging samples (2.5 × 2.5 cm) (m0) and drying at 100°C for 24 h (Rachtanapun et al. 2021). Samples were reweighed until a constant weight was

obtained (m1) and the moisture content calculated using equation below:

$$Moisture\ Content\ (\%) = ((m0 - m1)/m0) \times 100$$

Measurements were performed three times and averaged.

Water solubility and swelling properties

Water solubility and swelling properties were examined according to Zhang et al. (2022), with modifications. Films (2.5 × 2.5 cm) were dried at 100°C for 24 h, weighed (m1), and soaked in 10 mL aquadest for 24 h. Then, the samples were placed on filter paper, vacuum filtered, and then reweighed (m2). Finally, the samples were dried at 100°C for 24 h and reweighed (m3). The final mass was estimated by weighing the samples (m3). Water solubility and swelling properties were calculated as follows:

$$Solubility\ (\%) = (\ m1 - m3/m1) \times 100$$

$$Swelling\ properties\ (\%) = (m2 - m1)/m1 \times 100$$

Color properties

A Chroma Meter CR-400 (Konica Minolta, Japan) was used to measure sample color. Films were placed on a white plate and L* (brightness), a* (redness + or greenness), and b* (yellow + or bluish) light values from film surfaces were calculated using CIELAB values.

Scanning Electron Microscopy (SEM)

Film surface morphology was analyzed using SEM (SU3500, Hitachi, Tokyo, Japan) at 5 kV voltage acceleration and magnification = 100×, 500×, and 1000× (Riaz et al. 2018). Prior to analyses, films were coated with gold (thickness <50 nm) to prevent damage during scanning.

Fourier-transform infrared spectroscopy (FTIR)

Film functional groups were examined using FTIR (Thermo Scientific iS10, New York, USA) with spectra obtained between 4000-400 cm^{-1}. Samples were analyzed using transmittance mode and baseline correction.

Water Vapor Permeability (WVP)

Water vapor transmission rates were examined according to Song et al. (2022), with some modifications. Packaging (diameter = 4 cm) was placed in a cup plus 10 g silica gel at the bottom. Samples were attached to the cup and stored in a desiccator at 80% RH. Samples were then weighed over a 0-8 h period and WPV percentages calculated as follows:

$$\%WVP = ((W(g) \times X(m))/(t(s) \times A(m2) \times P0 \times \Delta RH)) \times 100$$

Where: W = weight change (g), X = film thickness (m), t = weight gain time (s), A = permeation area (m^2), P_0 = water vapor pressure at the test temperature, ΔRH = difference in RH between two sides of the sample.

Statistical analysis

One-way analysis of variance was used to analyze the data. Mean and standard deviation values were calculated and a $p<0.05$ significance level was used. All assays were performed in triplicate.

RESULTS AND DISCUSSION

Active packaging films

Film-based chitosan samples plus extracts were successfully produced (Figure 1). Higher extract concentrations generated darker film colors as indicated by color analyses (Table 4). Color has a significant impact on consumer acceptance of packaged foods. Films plus 2% extract had a lower brightness (L*) when compared with the control, but significantly increased a* and b* values ($p<0.05$). Therefore, 2% extract addition increased redness and yellowness traits in films (Figure 1D).

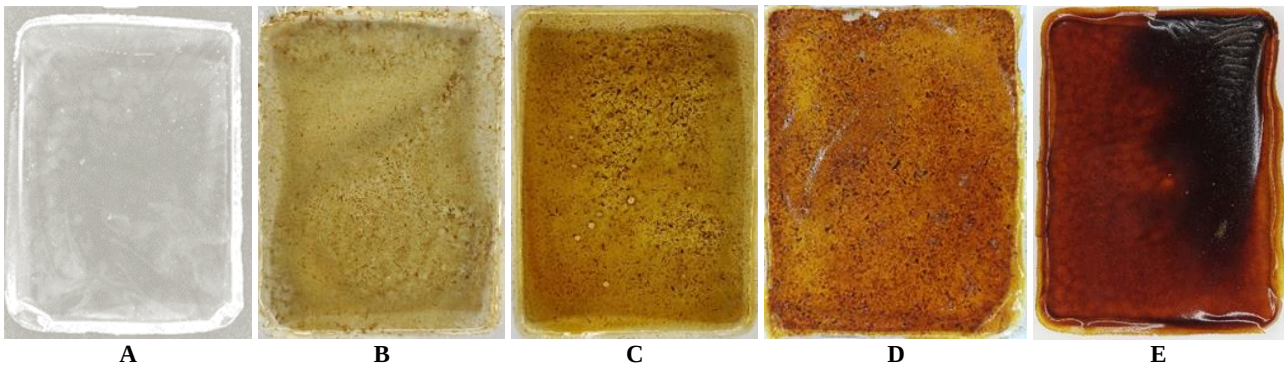

Figure 1. Film color appearance. Control film (A) and films plus extracts at 0.5% b/v (B), 1% b/v (C), 2% b/v (D), and 5% b/v (E)

Antibacterial and antifungal activity

Higher extract concentrations in chitosan films significantly increased DIZ values (Table 1). No clear zones were detected in control and films plus 0.5 and 1% extracts (Figure 4). Gram-positive *S. aureus* (ATCC 25923) bacteria were the most sensitive bacteria. Unfortunately, chitosan films plus extracts (all concentrations) displayed no inhibitor zones against *A. flavus* (FNCC 6181) and *A. niger* (FNCC 6114) (Table 1 and Figure 4). Thus, the films exhibited no antifungal activity. From our antimicrobial activity data, chitosan films plus 2% extract were selected for physical and chemical characterization. Results are expressed as the mean ± standard deviation in columns and followed by different superscripts denoting significant differences ($p<0.05$); ND, Not Detected. C1F0.5, C1F1, C1F2, C1F5 represent active packaging films (6 mm) plus extracts at 0.5, 1, 2, and 5% b/v, respectively.

Physical properties of packaging films

The addition of 2% extract to films significantly increased the film thickness and strain when compared with the control (Table 2), whereas the tensile strength was lower. Results were expressed as the mean ± standard deviation in columns and followed by different superscripts denoting significant differences ($p<0.05$); C1F2, packaging films plus extract (2% b/v).

Barrier and chemical properties of packaging films

From Table 3, films plus extract (2% b/v) displayed higher moisture content and WPV levels when compared with the control. However, the samples exhibited lower water solubility and swelling properties. Results were expressed as the mean ± standard deviations in columns and followed by different superscripts denoting significant differences ($p<0.05$); C1F2, active packaging film plus extract (2% b/v).

FTIR and SEM analyses

FTIR was used to determine functional group properties in negative control (C1) and C1F2 samples and identify the interactions between chitosan and extract (2%). From the spectral analyses, no new peaks were formed, indicating no new functional groups were created (Figure 2). SEM analyses revealed that film surfaces plus extracts (2% b/v) were smoother when compared with controls (Figure 3). White spots indicated less smooth film texture.

Table 2. Thickness and mechanical properties of packaging films

Sample	Thickness (mm)	Tensile (MPa)	Strain (%)
C1	0.05 ± 0.00^{a}	42.58 ± 3.10^{b}	21.86 ± 3.35^{a}
C1F2	0.13 ± 0.00^{b}	2.15 ± 0.76^{a}	32.55 ± 5.72^{b}

Results were expressed as the mean ± SD and followed by different superscripts denoting significant differences ($p<0.05$); C1, negative control; C1F2, Active packaging biofilm plus fungus comb ethyl acetate extract (2% b/v)

Table 1. Antibacterial and antifungal susceptibility assays

Sample	Diameter of the inhibition zone (DIZ) (mm)				
	E. coli (ATCC 25922)	*P. aeruginosa* (ATCC 27853)	*S. aureus* (ATCC 25923)	*A. flavus* (FNCC 6181)	*A. niger* (FNCC 6114)
C1 (Negative control)	ND	ND	ND	ND	ND
C1F0.5	ND	ND	ND	ND	ND
C1F1	ND	ND	ND	ND	ND
C1F2	7.75 ± 0.65^{a}	8.93 ± 1.03^{a}	8.70 ± 1.13^{a}	ND	ND
C1F5	13.10 ± 1.75^{b}	13.86 ± 1.49^{b}	15.91 ± 3.03^{b}	ND	ND

Note: Results were expressed as the mean ± SD and different superscripts denoting significant differences ($p<0.05$); C1F0.5, C1F1, C1F2, C1F5 were active packaging biofilm plus fungus comb ethyl acetate extract 0.5, 1, 2, and 5% b/v, respectively

Table 3. Moisture content, water solubility, swelling properties, and water vapor permeability (WVP) of packaging films

Sample	Moisture content (%)	Water solubility (%)	Swelling properties (%)	WVP (10−10g.m−1s−1.Pa−1)
C1(Negative control)	0.04 ± 0.02^{a}	2.94 ± 0.21^{a}	2.80 ± 0.19^{a}	1.09 ± 0.02^{a}
C1F2	0.27 ± 0.11^{b}	2.68 ± 0.48^{a}	2.37 ± 0.45^{a}	3.31 ± 0.04^{b}

Table 4. Color parameters of packaging biofilms

Sample	L* (Lightness)	a* (Red/Green Value)	b* (Blue/Yellow Value)
C1 (Negative control)	88.56 ± 0.25^{b}	0.28 ± 0.08^{a}	11.22 ± 0.43^{a}
C1F2	51.87 ± 0.83^{a}	18.08 ± 0.35^{b}	43.94 ± 2.55^{b}

Note: Results were expressed as the mean ± SD and followed by different superscripts denoting significant differences ($p<0.05$); C1F2, Active packaging biofilm plus fungus comb ethyl acetate extract (2% b/v)

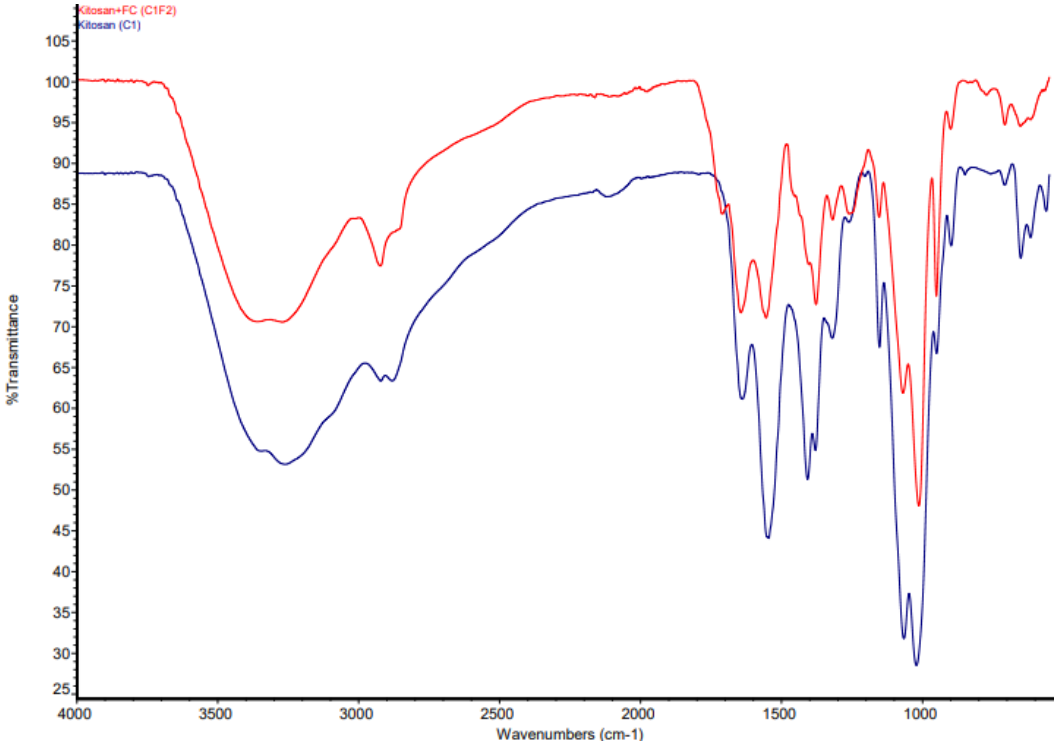

Figure 2. FTIR spectrum showing the negative control (C1), chitosan (blue), and film (C1F2) plus extract (2% b/v) (red)

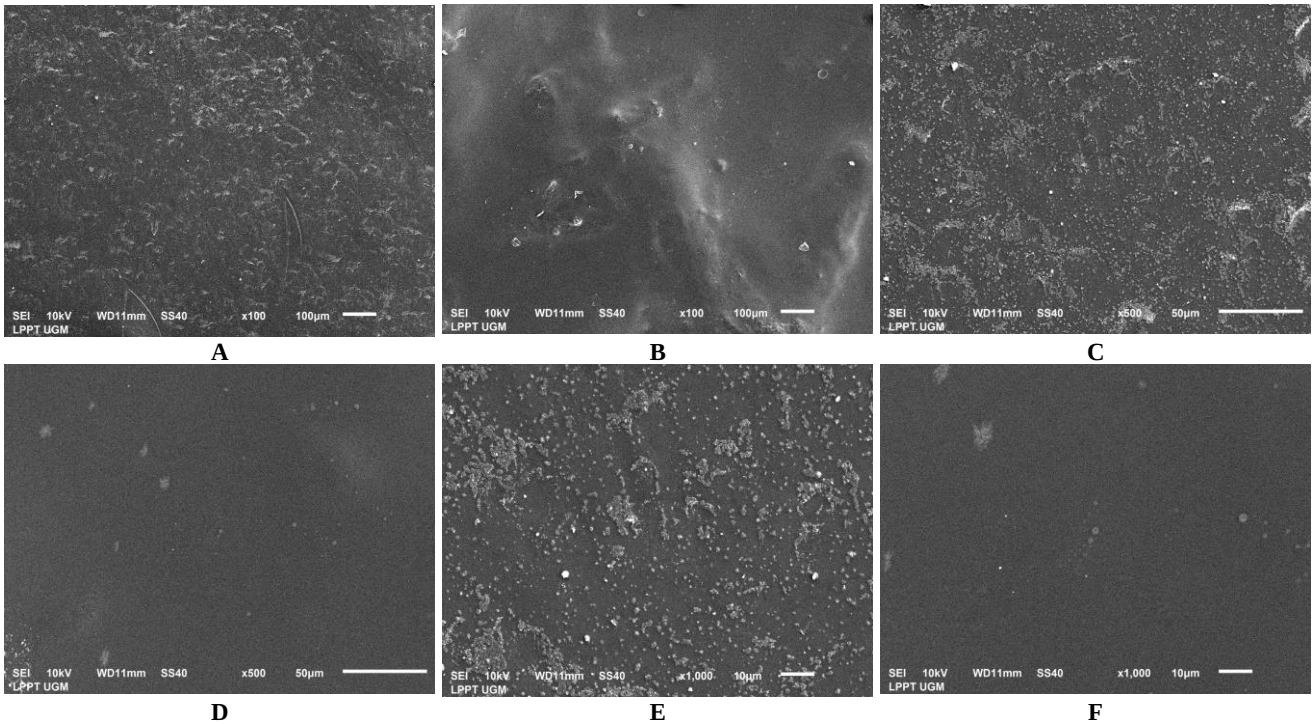

Figure 3. Scanning electron microscopy (SEM) analysis of films. A, C, E were negative control films and b, d, f were packaging films plus fungus comb ethyl acetate extract (2% b/v). Microscopy magnification: 100× (A and B), 500× (C and D), and 1000× (E and F)

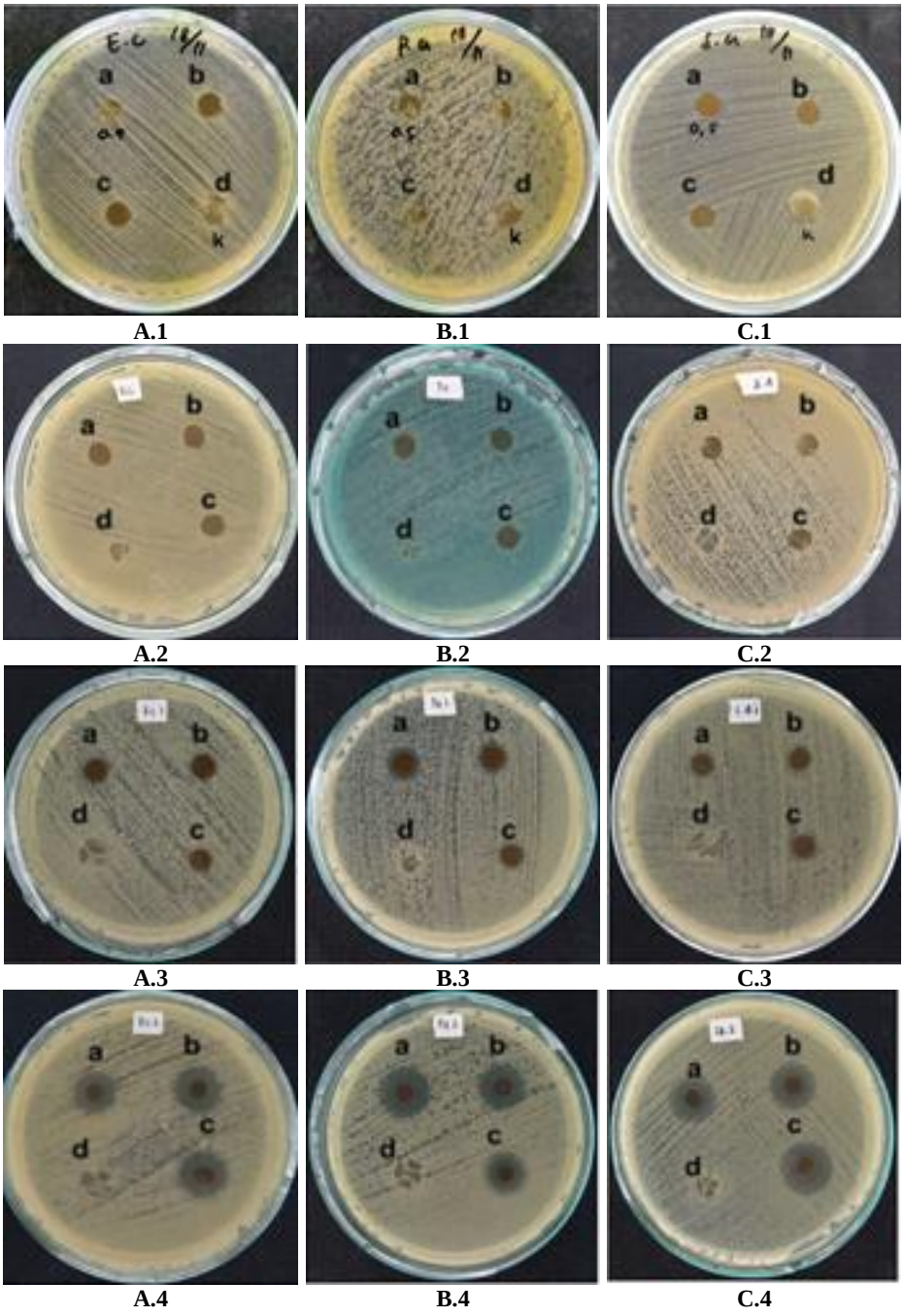

Figure 4. Antibacterial susceptibility assay on active biofilms. A. *E. coli* (ATCC 25922); B. *P. aeruginosa* (ATCC 27853); C. *S. aureus* ATCC 25923; Number 1, 2, 3, 4 are films added with 0.5, 1, 2, and 5% fungus comb ethyl acetate extract, respectively

Discussion

To investigate antibacterial film properties against Gram-negative and Gram-positive bacteria, we used the Kirby-Bauer disc diffusion method. Inhibition zones indicate how antimicrobial compounds in samples can inhibit microbial growth (Balouiri et al. 2016). As indicated (Table 1), the highest concentration of the extract (5%) showed the highest DIZ values for all bacteria. By reducing the extract concentrations, smaller inhibition zones were generated. The 2% concentration was the lowest concentration which inhibited bacterial growth. In contrast, no inhibition zones were observed in samples plus 0.5 and 1% extracts (Table 1). Thus, the antimicrobial compounds in fungus combs were not completely released into chitosan films. Chitosan and fungus comb antimicrobial compounds are polar and form hydrogen bonds due to interactions between amino acid residues in chitosan polymer chains and aldehyde groups in fungus comb extracts. This observation was supported by FTIR data, which indicated a higher transmittance for C1F2 films at 2922.46 cm^{-1} when compared with the negative control, thereby indicating that O-H bond numbers were increasing (Figure 2). Hydrogen

bonds have the greatest intermolecular forces, making it difficult for antimicrobial agent release from samples (Wu et al. 2017).

Staphylococcus aureus (ATCC 25923) was more sensitive when compared with the other bacteria (Table 1). Similar data were reported by Witasari et al. (2022). Gram-positive bacterial wall structures allow for more rapid hydrophobic antibacterial agent penetration into the cytoplasm and cell. In contrast, Gram-negative bacterial cell walls contain thin peptidoglycan, which is covered by an outer lipopolysaccharide and lipoprotein membrane, rendering it impermeable to hydrophobic molecules (Nazzaro et al. 2013). After contact with films, *S. aureus* (ATCC 25923) cell surfaces became rougher and cell structures were not intact due to cell leakage. Such structural damage occurred due to interactions between positive charges in the samples, which bound to negative charges in teichoic acid. These interactions caused cell wall brittleness, thereby disrupting cell membrane stability. This occurred with Gram-negative bacteria; *P. aeruginosa* (ATCC 25922) and *E. coli* (ATCC 25922) cell walls were damaged due to cell leakage (Hao et al. 2022).

A similar study by Wai et al. (2019) reported that DIZ values for *P. aeruginosa* (ATCC 27853), from edible chitosan films plus lime extracts (2 and 5%), were 7.13 mm and 8.32 mm, respectively. Remya et al. (2016) also examined the inhibitory activity of chitosan-based packaging films plus ginger essential oil extracts and reported inhibition zones of 8.5-14.5 mm for *S. aureus* (ATCC 25923) and 2.5-4.5 mm for *E. coli* (ATCC 25922) at different extract concentrations. Therefore, our chitosan films plus extracts were better at inhibiting *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), and *P. aeruginosa* (ATCC 27853) growth (Table 1).

Witasari et al. (2022) showed that fungus comb ethyl acetate extract displayed antifungal activity against *A. niger* and *A. flavus*. Extracts contained dominant antifungal compounds, including phenol, guaiacol, and syringol (Nandika et al. 2021). Our observation that *A. niger* and *A. flavus* growth was inhibited by packaging films plus extracts (up to 5%) may have been due to the low concentrations (Table 1), thus higher concentrations may inhibit fungal growth. In a study by Nandika et al. (2021), 6% extract addition inhibited *Aspergillus foetidus* growth.

From antimicrobial analyses, no clear zones around chitosan films without extracts were observed (Figure 4). Chitosan is a natural antimicrobial agent, however, according to Contessa et al. (2021), its antimicrobial activity in films was negligible because it was not adequately dispersed in culture medium. Based on antimicrobial activity data, packaging samples plus 2% extract (C1F2) successfully inhibited microbial growth. Therefore, we examined C1F2 physical, barrier, and chemical properties.

To determine chitosan antimicrobial active packaging quality and utility, we analyzed film thickness, tensile strength, and strain characteristics. Film thickness can represent resistance to water vapor, gas, or other volatile compounds passing through it (Pagella et al. 2002). Extract addition to chitosan films induced significant differences in thickness due to increased total solids (Table 2). Both tensile strength and strain mechanical properties are important for determining food packaging material strength. Tensile strength is the maximum tensile force that a film can withstand before breaking. The lower the tensile value, the weaker the film. We observed that films plus extract (2% b/v) exhibited lower tensile strength when compared with controls (Table 2). Thus, C1F2 samples were more easily broken. Decreased tensile values are associated with changes in intermolecular bonds which cause polymer chains to weaken and induce film deformation (Sogut and Seydim 2018). Strain values indicate the film ability to stretch to its maximum. In our study, films plus extracts exhibited higher strain values when compared with controls (Table 2). Increased strain was caused by extract interaction with chitosan, which formed an elastic matrix.

In terms of film color, 2% extract addition reduced the brightness values. This addition induced color changes because the extract had a brownish yellow color. Therefore, the resultant films had higher a* and b* values when compared with controls. According to Tien et al. (2006), increased viscosity can darken films as they are thicker. Depending on the application, film discoloration to brownish/yellow can be either desirable or undesirable. Dark antimicrobial active packaging can be used to wrap light-sensitive products, such as foods high in fat and vitamins (Rokilah et al. 2018).

Moisture content, swelling properties, and water solubility percentages were used to determine water sensitivity effects on film properties and may be used as reference points for film applications in high humidity environments (Zhang et al. 2022). As indicated (Table 3), we observed a significant increase in moisture content after 2% extract addition to chitosan films (25.94%) (Nandika et al. 2021). Other studies reported that edible chitosan films, incorporating levan or pullulan and enriched with ε-polylysine, had increased moisture content (Gan et al. 2022). However, in our study, after 2% extract addition, the film swelling and water solubility properties decreased. In other research, chitosan/zein bilayer films plus curcumin/nisin-loaded pectin nanoparticles decreased water solubility and swelling properties in films when compared with chitosan films (Zhang et al. 2022).

WVP properties in films act as barriers to water vapor in the environment. Film WVP levels should be kept as low as possible to protect products from moisture transfer from surrounding environments to extend shelf life (Riaz et al. 2018). WVP values were also improved by adding extracts to chitosan films (Table 3). The dominant compound in the extracts was glycerol (28.93%) (Nandika et al. 2021) which is a polar hydrophilic compound, so its presence in films can increase hydrophilicity by reducing intermolecular forces in biomaterials (Pinto et al. 2018). Interactions between hydrophilic extracts may have decreased water resistance in films and increased WVP levels. Similar data were recorded for chitosan-hydroxypropyl methylcellulose blend films enriched with nettle leaf extracts (Bigi et al. 2021).

The addition of 2% extract induced no bonding effect in packaging films (Figure 2). Both compounds simply accumulate due to mixing processes (Kusumawati and Putri 2013). However, we observed transmittance changes in films treated with 2% extract at 2922.46 cm^{-1} and 1554.09, indicating that O-H and C=O (amide 1) bonds were increasing, respectively. This was possibly due to interactions between the extract and chitosan, which increased hydrogen bonds (Kaya et al. 2018). Increased amide group levels also indicated that films could be degraded and suggested the generation of environmentally friendly plastic (Handayani and Wijayanti 2015).

SEM analysis showed that 2% extract addition to chitosan films smoothed the film morphology as evidenced by fewer white spots when compared to controls (Figure 3). A rougher texture on control films indicated a low density of insoluble chitosan compounds. Due to the presence of cross-links between materials, extract addition smoothed and homogenized the film morphology. Additionally, smooth surfaces indicated that hydrogen bonds were uniform throughout the films (Setiani et al. 2013)

In conclusion, this study showed that chitosan film development, incorporating 2% fungus comb ethyl acetate extract, inhibited *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), and *P. aeruginosa* ATCC 27853. Unfortunately, these films failed to inhibit *A. flavus* (FNCC 6181) and *A. niger* (FNCC 6114). Chitosan films plus 2% extract exhibited lower tensile strength but higher strain, moisture content, and water vapor quality when compared with controls. Moreover, the solubility and swelling power levels in chitosan films plus 2% extract were not significantly different to controls. Thus, films based on chitosan plus extracts may be used as antimicrobial active packaging films for food. Further research on film applications in the food packaging industry requires more study.

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