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# Bioactive cassava starch-chitosan film incorporated with vanillin for active packaging applications

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ABSTRACT

Incorporating natural compounds into starch-based films has been widely explored to enhance their bioactive properties. In this study, an active food packaging approach was developed using starch-based films supplemented with vanillin as an antimicrobial component. Vanillin concentrations of 2 and 4 mg/mL were first identified as the minimum inhibitory concentrations effective against food-contaminating bacteria, and these concentrations were subsequently incorporated into the films. The films were active against *Escherichia coli* SZMC 0582, *Staphylococcus aureus* SZMC 0579, *Salmonella enterica* SZMC 23289, *Bacillus subtilis* SZMC 0209 and *Listeria monocytogenes* SZMC 21307, with growth inhibition of 12-44%. Fluorescence microscopy showed reduced adhesion of *E. coli* and *S. aureus* cells on films containing vanillin. Chitosan in 5-10 mg/mL concentrations enhanced film hardness and elongation time. The biopolymer films were thermally stable up to 250 °C and the vanillin became amorphous during the casting. Raman spectroscopy confirmed Schiff base formation, while scanning electron microscopy showed that chitosan improved film smoothness. Vanillin- and chitosan-containing films demonstrated increased antioxidant activity, biodegradability in compost, and reduced the bacterial counts on coated and packaged strawberries after 8 and 16 days of storage. The bioactive films show strong potential as functional food packaging materials.

## 1. Introduction

The extensive use of petroleum-based plastics since the 20th century has raised serious environmental concerns due to their pollution aspect. As of 2019, global plastic production has reached approximately 460 million tons, with this figure continuing to rise steadily (Xu et al., 2024). In response to these issues, increasing efforts have focused on the development of environmentally friendly thermoplastics derived from renewable biopolymers. Among these biopolymers, starch stands out as the most abundant and renewable polysaccharide synthesized by plants. Starch is a sustainable substance and well-suited for film-forming applications with modifiers and other polymers, particularly in food packaging (Khalid & Arif, 2022). Structurally, starch is composed of amylose and amylopectin, linked by α-1,4 and α-1,6 glycosidic bonds, respectively. Amylose content plays a crucial role in film formation via intermolecular hydrogen bonding, contributing to the transparency and integrity of the resulting films (Cui et al., 2021). Cassava starch has been widely used in previous research as a primary matrix for films suitable for food packaging applications (Arias et al., 2024; Krümmel et al., 2024).

Starch-based films possess inherent limitations, including hydrophilicity, lack of antimicrobial properties, and poor mechanical strength. To overcome these drawbacks, natural additives such as plant extracts, essential oils (EOs) and phenolic compounds have been

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incorporated to enhance bioactive properties, broadening potential applications, thereby, support the film functionality. For example, prior studies have demonstrated that the inclusion of EOs from oregano and rosemary can significantly improve the physical and antimicrobial properties of starch-based films (Araya et al., 2022; Hernández et al., 2023). Additionally, phenolic compounds have been reported to impart antioxidant, antibacterial, and antifungal activity to biopolymer matrices (Pan et al., 2024). Fang et al. (2022), for instance, investigated the effect of chitosan-based films supplemented with tea polyphenols on the preservation of broccoli using conventional dipping methodology. Another study tested films containing rosemary EO and gallic acid to maintain freshness in storage experiments (Valentino et al., 2020). The studies summarized in the recent review of El Alami El Hassani, Baraket, and Alem (2025) also demonstrate that antioxidant phenols can be used for food preservation purposes by embedding them in films.

Vanillin, a naturally occurring phenolic aldehyde originally extracted from *Vanilla planifolia* and other plants from the *Vanilla* genus, has received attention for its broad applicability in food, pharmaceuticals, and medicine (Olatunde et al., 2022). Its molecular structure, comprising a benzene ring with aldehyde (-CHO), methoxy (-$OCH_3$), and hydroxyl (-OH) groups, confers notable antioxidant and antimicrobial potential. Vanillin has been shown to inhibit the growth of food-contaminating bacteria such as *Escherichia coli* and *Listeria monocytogenes* by disrupting cellular membranes, modification of cellular permeability, and inhibition of respiration activity (Cava-Roda et al., 2021).

Blending starch with other biopolymers, such as chitosan, can enhance the mechanical properties of the films (Aguirre-Loredo et al., 2023), while the incorporation of clay has been associated with reduced water absorption rate (Mansour et al., 2020). Although vanillin is frequently applied in biopolymer films (Matar & Andac, 2025), including as a cross-linking agent in chitosan-based composites (Westlake et al., 2023; H. Yu, Ge, Ding, Yan, & Wang, 2023), it is worth further investigating the effect of stabilizing fillers such as clay additives and chitosan on mechanical and microbiological properties of starch-vanillin films. In addition, bacterial adhesion to films and biofilm formation have been comparatively less investigated for such biocomposites. Therefore, the present study aimed to: (i) evaluate the antimicrobial efficacy of vanillin against nine foodborne pathogens, (ii) prepare cassava starch-based films incorporating montmorillonite clay and vanillin, (iii) evaluate the antimicrobial action of starch-vanillin films against planktonic and biofilm cells of foodborne pathogens, (iv) characterize the influence of vanillin and chitosan on film mechanical properties, surface morphology, antioxidant activity and degradability, and (v) assess the application of the developed films in extending the shelf life of strawberries. The present assay provides useful information and a promising approach for the development of a consumer-friendly, biologically active, non-synthetic polymer composite food preservative.

## 2. Materials and methods

### 2.1. Microorganisms and growth conditions

The bacteria *Escherichia coli* SZMC 0582, *Staphylococcus aureus* SZMC 0579, methicillin-resistant *Staphylococcus aureus* (MRSA) SZMC 6270, *Salmonella enterica* SZMC 23289, *Bacillus cereus* SZMC 0042, *Bacillus subtilis* SZMC 0209, *Listeria monocytogenes* SZMC 21307, *Pseudomonas aeruginosa* SZMC 0568 and *Pseudomonas putida* SZMC 6010 used in the research were obtained from the laboratory collection of Department of Biotechnology and Microbiology, University of Szeged (Szeged Microbiology Collection, SZMC, Szeged, Hungary). The media employed in the experiment were lysogeny broth (LB; tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 5 g/L) for *P. aeruginosa* SZMC 0568, *P. putida* SZMC 6010, *E. coli* SZMC 0582, *S. enterica* SZMC 23289, *S. aureus* SZMC 0579 and MRSA SZMC 6270, tryptone glucose yeast extract (TGE; glucose, 1 g/L; yeast extract, 3 g/L; tryptone, 5 g/L) for *B. cereus* SZMC 0042 and *B. subtilis* SZMC 0209 and tryptic soy broth (TSB; casein peptone, 17 g/L; $K_2HPO_4$, 2.5 g/L; glucose, 2.5 g/L; NaCl, 5 g/L; soya peptone, 3 g/L) for *L. monocytogenes* SZMC 21307. Solid media were prepared by addition of 20 g/L agar-agar. All medium ingredients were purchased from VWR International (Debrecen, Hungary).

Prior to each assay, fresh bacterial cultures were obtained by incubating the strains for 24 h at either 37 °C (for *E. coli* SZMC 0582, *S. aureus* SZMC 0579, MRSA SZMC 6270, *S. enterica* SZMC 23289 and *L. monocytogenes* SZMC 21307) or 30 °C (for *B. cereus* SZMC 0042, *B. subtilis* SZMC 0209, *P. aeruginosa* SZMC 0568 and *P. putida* SZMC 6010), depending on the specific growth requirements of the bacteria. Subsequently, the bacterial cell suspensions were adjusted to a concentration of $10^5$ CFU/mL in the respective growth medium followed by counting in a Bürker-chamber under a light microscope.

### 2.2. Screening of antimicrobial activity of vanillin

#### 2.2.1. Preparation of vanillin solution

Vanillin (Sigma-Aldrich, Munich, Germany) solution was prepared by dissolving in 10% (v/v) of ethanol aseptically as a stock solution at 8 mg/mL concentration. Afterwards, the solution was used for antimicrobial screening against the selected microorganisms.

#### 2.2.2. Determination of minimum inhibitory concentration (MIC)

The MIC of vanillin was determined by the microtiter broth dilution method in sterile flat-bottom 96-well polystyrene plates (Sarstedt, Nümbrecht, Germany) (Kimani et al., 2021). The optical density (OD) of the bacteria was then confirmed with a spectrophotometer. To determine the MIC, firstly, serial dilution of vanillin was performed in eight steps from 4 to 0.125 mg/mL concentrations in 10% (v/v) ethanol. A volume of 100 μL of bacterial suspension ($10^5$ CFU/mL) prepared in double concentrated medium was transferred to the wells containing 100 μL of diluted vanillin sample or corresponding stock solution. Thus, the final concentration of vanillin in each well ranged from 4 to 0.0625 mg/mL. A volume of 100 μL of bacterial suspension supplemented with 100 μL of 10% (v/v) ethanol was considered as the positive control, while the negative controls contained 100 μL of double concentrated growth medium and 100 μL of vanillin solution. Absorbance measurements were made for each plate at 620 nm (SPECTROstar Nano spectrophotometer, BMG Labtech, Offenburg, Germany) before incubation. Afterwards, the polystyrene plates were incubated for 24 h at 37 °C or 30 °C depending on the culturing requirements of the used bacteria (see 2.1.), and absorbance was measured again at the end of the incubation. The concentration of the phenolic compound that caused 90% or higher growth inhibition, i.e., where the absorbance was 10% or lower than that of the positive control was considered as the MIC.

#### 2.2.3. Determination of minimum bactericidal concentration (MBC)

The MBC was determined by inoculating 5 μL of the suspension contents from the microtiter wells which have concentration similar to or higher than the MIC value, and subsequently transferring them to the agar plates and incubated them for 24 h at 37 °C or 30 °C depending on the tested bacteria (see 2.1.). The MBC was identified as the absence of bacterial growth on the agar plates.

### 2.3. Starch-based film preparation

The preparation of the film was conducted by a casting method described in the study of Perdana et al. (2021) with slight modification. Briefly, cassava starch (3% w/v; BiOrganik Kft., Budapest, Hungary) was dissolved in distilled water at 80 °C for 20 min until gelatinized. Then, the gelatinized cassava starch was supplemented with glycerol (20 wt% of starch). Afterwards, montmorillonite clay (4 wt% of starch; Sigma-Aldrich, Munich, Germany) was added, and the mixture was stirred (400 rpm) for 15 min at 80 °C. Finally, vanillin suspended in 10% ethanol solution was added to achieve a final concentration of 2 mg/mL

(designated as SGMV2) or 4 mg/mL (designated as SGMV4) in the film solution. A vanillin-free biopolymer film containing only starch, glycerol and montmorillonite was considered as the control (designated as SGM). The prepared solutions then cast onto a Petri dish and dried at 50 °C in oven for 2 days. Traces of ethanol solutions were evaporated during the final casting procedure.

The effect of chitosan on the mechanical, physical, and chemical properties of the SGMV2 films was also studied. Firstly, a chitosan (low molecular weight 75-85% deacetylated chitin; Sigma-Aldrich, Munich, Germany) stock solution was prepared using 1% (v/v) acetic acid solution. Then, combination of chitosan in the starch films was also made by incorporating the chitosan stock solution with the final concentration of 2.5 mg/mL (designated as SGMV2-CHIT2.5), 5 mg/mL (designated as SGMV2-CHIT5) or 10 mg/mL (designated as SGMV2-CHIT10), during film preparation. Fig. S1 shows a sample of approximately 6 $cm^2$ cut from the prepared SGM, SGMV2 and chitosan-containing SGMV2 (designated as SGMV2-CHITS) films and illustrates the transparency of the film.

### 2.4. Antimicrobial activity assay for the films

Antimicrobial activity of the prepared SGM, SGMV2 and SGMV4 films was tested in a 24-well microtiter plate (Sarstedt, Nümbrecht, Germany). Equal diameter (5 mm) of disks was prepared from the films, then, the disks were transferred to each well of the plate. A volume of 250 μL of bacterial suspension ($10^5$ CFU/mL) was transferred to the wells containing 250 μL of respective medium and film. The plates were then incubated for 24 h at 37 °C or 30 °C depending on the culturing requirements of the tested bacterium (see 2.1.). Absorbance was measured at 620 nm using a SPECTROstar Nano spectrophotometer (BMG Labtech, Offenburg, Germany) before and after incubation. A volume of 250 μL of bacterial suspension added to 250 μL respective medium was considered as the positive control, while the negative controls contained growth medium and the corresponding films.

### 2.5. Fluorescence microscopy assays

Fluorescence microscopy was conducted to visualize the bacterial adhesion and biofilm formation on the surface of the SGM, SGMV2 and SGMV4 films. Fresh *E. coli* SZMC 0582 and *S. aureus* SZMC 0579 cell cultures were prepared by incubating the bacteria for 24 h at 37 °C in LB medium. Then, cell suspension with a final concentration of $10^5$ CFU/mL was prepared by diluting the initial culture. The cell suspension was homogenized by a gentle vortexing, and a volume of 2 mL of the suspension was dispensed to the wells of a sterile 12-well microtiter plate (Sarstedt, Nümbrecht, Germany), into which a 5 mm diameter disk made from the films had previously been placed. The microtiter plate was then incubated for 5 h at 37 °C allowing the cells to adhere. Afterwards, the films were taken from the wells and incubated for 24 h at 37 °C. After the incubation, film was fixed with absolute methanol for 10 min and stained with 20 μL of 0.01% acridine orange for 15 min. The excess stain was removed by washing with distilled water, and the films were dried for 10 min. The dyed films were visualized by AxioLab (Carl Zeiss, Oberkochen, Germany) fluorescence microscope equipped with an Axiocam 503 mono (Carl Zeiss, Oberkochen, Germany) camera. The images were captured and analyzed by the software Axio Vision 4.6 (Carl Zeiss, Oberkochen, Germany). Excitation and emission wavelengths of 500 and 526 nm, respectively, were used to examine the acridine orange staining.

### 2.6. Assay of mechanical properties

The mechanical properties of the SGM, SGMV2 and SGMV2-CHITS films were tested according to the film hardness and time of elongation. The hardness of the prepared films, and the time of elongation until the breaking point were measured with a texture analyzer developed at the Institute of Pharmaceutical Technology and Regulatory Affairs, University of Szeged. The film was cut to 1 $cm^2$ pieces, and the film thickness was measured by a screw micrometer (Mitutoyo, Tokyo, Japan) prior to hardness test. For measuring the film hardness, the sample was fixed in a sample holder equipped with 5 mm aperture, and a needle-like probe with 3 mm diameter was moved downward at a constant speed of 2 mm/s against the film. The test was finished when the film was pierced and the time of the test and force (in a range of 0-50 N) required to break the film were recorded. The output was presented in the form of a force-time curve displayed by a computer connected to the equipment. Nine parallel measurements were performed on 1 $cm^2$ pieces of each casted sample, and the mean values and standard deviations were calculated.

### 2.7. Thermal analysis

Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were performed using a DSC 3+ and a TGA/DSC 1 instrument, respectively (Mettler-Toledo GmbH, Gieβen, Germany). For both analyses, SGM, SGMV2 and SGMV2-CHITS films stored at room temperature and ambient relative humidity were cut into small pieces without additional drying prior to measurement.

For DSC measurements, samples of 5 ± 0.3 mg were placed into non-hermetically sealed 40 μL aluminum pans to prevent pan rupture caused by gas evolution during heating. The pans were crimped and inserted into the furnace, and the samples were heated from 25 to 300 °C at a rate of 10 °C/min under a constant argon flow of 150 mL/min to prevent oxidation. Each measurement was normalized to sample mass.

For TGA measurements, samples of 10 ± 0.3 mg were placed into non-hermetically sealed 100 μL aluminum pans, which were then crimped and inserted into the furnace. The measurements were performed over a temperature range of 25-275 °C at a heating rate of 10 °C/min under a constant nitrogen flow of 50 mL/min. The results were analyzed using STARe Software (Mettler-Toledo GmbH, Gieβen, Germany).

### 2.8. X-ray powder diffraction (XRPD)

XRPD diffractograms were recorded using a Bruker D8 Advance X-ray diffractometer (Bruker AXS GmbH, Karlsruhe, Germany) with Cu K λI radiation (λ = 1.5406 Å) and a VÅNTEC-1 detector (Bruker AXS GmbH, Karlsruhe, Germany). A voltage of 40 kV and an amperage of 40 mA were used during the measurements. The angular range was 3° to 40° 2θ with a step time of 0.1 s and a step size of 0.007°. The sample was placed on a quartz holder and measured at ambient temperature and ambient relative humidity. All manipulations, including Kα 2-stripping, background removal and smoothing of the diffractograms were performed using the DIFFRACTPLUS EVA software (Bruker AXS GmbH, Karlsruhe, Germany) (Katona et al., 2016).

### 2.9. Raman spectroscopy

Raman spectroscopic investigation was performed using a Thermo Fisher DXR Dispersive Raman Instrument (Thermo Fisher Scientific, Inc., Waltham, MA, USA) equipped with a CCD camera and a diode laser (780 nm). Raman measurements were carried out with a laser power of 24 mW at 50 μm split aperture size with an exposure time of 4 s and acquisition time of 6 s, for a total of 32 scans per spectrum in the spectral range of 3500 to 200 $cm^{-1}$ with cosmic ray and fluorescence corrections. The manipulations and evaluations were carried out using the OMNIC 8.1 Software (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

### 2.10. Scanning electron microscopy (SEM)

The surface morphology of the prepared SGM, SGMV2 and SGMV2-CHITS films was examined by SEM. Samples were mounted on

aluminum stubs using highly conductive silver cement (EM-Tec AG42). For cross-sectional imaging, films were fractured after immersion in liquid nitrogen and positioned in the holder at a 90° tilt. Samples were sputter-coated with a 12 nm gold layer. Imaging was performed using a Zeiss Sigma 300 field-emission SEM (Carl Zeiss, Oberkochen, Germany) under high-vacuum conditions at an accelerating voltage of 2 kV.

### 2.11. Total antioxidant activity of the films

Total antioxidant activity of the SGM, SGMV2 and SGMV2-CHITS films was determined via the 2,2-diphenylpicrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays referred to Chang et al. (2024) and de Sousa Silva et al. (2025) with modifications.

For DPPH assay, 5 mg of film was dissolved in a volume of 1 mL of ethanol, which was then placed in dark condition for 24 h. Afterwards, a volume of 40 μL of film extract was dissolved in 100 μL of 0.1 mmol/L of DPPH solution. The control sample contained 100 μL of 0.1 mmol/L DPPH and 40 μL ethanol. The mixtures were incubated in a dark place at room temperature for 30 min, then the absorbance was measured at 517 nm (SPECTROstar Nano spectrophotometer, BMG Labtech, Offenburg, Germany). The following equation was used to calculate percentage (%) DPPH radical scavenging capacity:

$$\text{DPPH}\ (\%) = \frac{\text{A(control)} - \text{A(extract)}}{\text{A(control)}} * 100$$

where A(control) is the absorbance of the control sample, and A(extract) is the absorbance of the reaction mixture containing film extract.

For the FRAP measurement, 5 mg of film was soaked in 1 mL of ethanol, and the solution was kept in dark condition for 24 h. Afterwards, the film extract (6 μL) was dissolved in 200 μL of FRAP reagent contained 10 mmol/L 2,4,6-tris(2-pyridyl)-*s*-triazine diluted in 40 mmol/L hydrochloric acid, 300 mmol/L sodium acetate buffer and 20 mmol/L iron (III) chloride solutions (Kovács et al., 2024). The reaction solution was then incubated at 37 °C for 30 min, and the absorbance was measured at 593 nm using a SPECTROstar Nano microplate reader (BMG Labtech, Offenburg, Germany). The standard curve was made using 1 mmol/L iron (II) sulfate solution in the concentration range of 0.1-1.0 mmol/L. Results were expressed as μmol Fe(II)/100 g film.

### 2.12. Solubility assay

For solubility tests, the SGM, SGMV2 and SGMV2-CHITS films stored at room temperature and relative humidity were cut into approximately equal size and immersed in distilled water at room temperature for 24 h with continuous stirring (115 rpm) (Arias et al., 2024). After incubation, the samples were left to dry at room temperature for 24 h, and their weights before and after immersion in water were compared. Percent solubility (%) was calculated according to the following equation:

$$\text{Percent solubility}\ (\%) = \frac{\text{A} - \text{B}}{\text{A}} * 100$$

where A and B are the weights of the film samples before and after immersion in water, respectively.

### 2.13. Application of films for strawberry preservation

The film containing 2 mg/mL vanillin and 5 mg/mL chitosan (SGMV2-CHIT5) was applied for strawberry preservation test by coating and supplemented packaging methods. For the experiments, fresh strawberry samples were obtained from a local market in Szeged, Hungary. In coating assay, strawberry samples were washed under tap water and dried at room temperature. A total of 36 strawberry samples were selected, 18 of them were dipped into the film solution, then dried at room temperature (25 °C). Remaining strawberries were considered as uncoated control samples. In supplemented packaging approach, a previously prepared film layer was placed on the bottom of a plastic packaging box frequently used for storage of grab-and-go strawberry products in markets. A total of 15 strawberries were placed in a packaging box which contained film at the bottom, and 15 samples were placed in a film-free control plastic box. The strawberry samples were arranged in such a way as to ensure contact with or close proximity to the film. The weight loss and the total bacterial count were monitored at the 0th, 4th and 8th day of the room temperature (25 °C) incubation for the coated strawberry samples, while at the 0th, 4th, 8th, 12th and 16th day of the 4 °C temperature incubation for the supplemented packaging approach. The percentage of weight loss was measured using analytical balance with unit of grams based on the weight differences between every four days and divided by initial weight. The total bacterial count of strawberry samples was determined by the pour plate method. One gram of strawberry was homogenized into 9 mL of the 0.1% peptone water. The suspension (100 μL) was then spread into LB medium and incubated for 24 h at 37 °C.

### 2.14. Biodegradation assay

Biodegradability of SGMV2-CHIT5 film was assessed using a compost-based burial test system. Samples measuring approximately 1 × 1.5 cm were cut from the prepared film and weighed to determine their initial mass. The film samples were then placed into open 200 $cm^3$ plastic cups filled with compost obtained from Szeged Environmental Management Nonprofit Ltd, and the cups were kept at ambient temperature (20 ± 5 °C) condition. An initial volume of 30 mL water was added to each cup at the beginning of the experiment and again after one week of incubation to maintain moisture content. The films were carefully removed from the compost every two weeks during the six-week incubation period, gently cleaned, and weighed to determine mass loss.

### 2.15. Statistical analysis

Experiments were performed in at least three independent measurements and data were expressed as an average of the replicates ± standard deviation. Statistical analysis of the data was carried out by paired *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test in the GraphPad Prism 8.00 software (GraphPad Software Inc., San Diego, CA, USA). A $p$ value less than 0.05 was considered statistically significant.

## 3. Results and discussion

### 3.1. Minimum inhibitory (MIC) and minimum bactericidal concentration (MBC) of vanillin

The antimicrobial efficacy of vanillin against the tested bacterial

**Table 1**
Minimum inhibitory concentration (MIC, in mg/mL) and minimum bactericidal concentration (MBC, in mg/mL) of vanillin against food-contaminating bacteria.

| Bacteria | Vanillin | |
|---|---|---|
| | MIC (mg/mL) | MBC (mg/mL) |
| *Escherichia coli* SZMC 0582 [a] | 2 | 4 |
| *Pseudomonas aeruginosa* SZMC 0568 | 4 | 4 |
| *Pseudomonas putida* SZMC 6010 | 4 | 4 |
| *Salmonella enterica* SZMC 23289 | 2 | 4 |
| *Bacillus cereus* SZMC 0042 | 4 | 4 |
| *Bacillus subtilis* SZMC 0209 | 2 | 2 |
| *Listeria monocytogenes* SZMC 21307 | 2 | 4 |
| *Staphylococcus aureus* SZMC 0579 | 2 | 4 |
| methicillin-resistant *Staphylococcus aureus* (MRSA) SZMC 6270 | 4 | 4 |

[a] SZMC = Szeged Microbiology Collection.

strains varied, as indicated by the MIC and MBC values presented in Table 1. Vanillin exhibited higher effectiveness against *E. coli* SZMC 0582, *S. aureus* SZMC 0579, *S. enterica* SZMC 23289, *B. subtilis* SZMC 0209, and *L. monocytogenes* SZMC 21307, all of which showed relatively low MIC values of 2 mg/mL. In contrast, *P. aeruginosa* SZMC 0568, *P. putida* SZMC 6010, *B. cereus* SZMC 0042, and MRSA SZMC 6270 demonstrated reduced sensitivity to vanillin, as reflected by higher MIC values (Table 1). Vanillin exhibited an MBC of 2 mg/mL for *B. subtilis* SZMC 0209, whereas this value was 4 mg/mL for the other bacteria tested (Table 1 and Fig. S2). The observed differences in susceptibility among bacterial strains may be attributed to variations in surface characteristics, including extracellular polymeric substances, flagella, surface hydrophobicity, and other extracellular structures, all of which are known to affect bacterial responsiveness to antimicrobial agents (Dzigbor et al., 2024). Vanillin was active against the growth of various other bacterial strains as well. For instance, *P. aeruginosa*, *Aeromonas hydrophila* and *Mycobacterium smegmatis* were effectively inhibited by vanillin at concentrations of 50, 160 and 15-125 μg/mL, respectively (Arya et al., 2019; Ponnusamy et al., 2013; Sharma et al., 2016). In *Cronobacter* strains, mean MICs of 0.5-3 mg/mL were identified for vanillin which varied depending on the temperature and pH set in the experimental system (Yemis et al., 2011). Wang et al. (2024) reported MICs of 1.25 mg/mL for. *E. coli* and *S. typhimurium* and 2.5 mg/mL for *S. aureus* pathogenic bacteria.

Vanillin's antimicrobial mechanism is primarily associated with its interaction with bacterial membranes, particularly through disruption of surface hydrophobicity. This interaction leads to the leakage of essential intracellular components, such as nucleic acids and proteins, which are critical for maintaining cellular homeostasis and viability. Previous studies have reported that vanillin acts as a membrane-active agent in bacteria such as *E. coli*, *Lactobacillus plantarum*, and *Listeria innocua*, causing disturbances in ion gradients and inhibition of respiration (Yang et al., 2021). In *E. coli*, for instance, vanillin readily penetrates membrane pores, disrupting potassium ion balance and intracellular pH homeostasis, which ultimately leads to the inhibition of ATP synthase activity and energy metabolism (Chen et al., 2023). Due to the varying sensitivity of the bacteria involved in the test, both 2 mg/mL and 4 mg/mL concentrations of vanillin were incorporated into the starch-based biopolymer films produced.

### 3.2. Antimicrobial activity of starch-vanillin films

#### 3.2.1. Growth inhibitory effect

In this study, biopolymer films were successfully developed using cassava starch, glycerol, and montmorillonite (SGM), with the addition of vanillin at concentrations of 2 mg/mL (SGMV2) and 4 mg/mL (SGMV4) during the film-forming process. Since all tested bacterial strains were shown to be sensitive to vanillin, we considered it worthwhile to examine the extent of growth inhibition exerted by vanillin incorporated into the starch-based film matrix. For this purpose, equal-sized portions of SGM, SGMV2, and SGMV4 films were placed into the wells of a microtiter plate, inoculated with the respective bacterial suspensions, and microbial growth was assessed. The experimental setup is demonstrated in Fig. S3. The bacteria selected for testing, namely, *E. coli* SZMC 0582, *S. enterica* SZMC 23289, *L. monocytogenes* SZMC 21307, *S. aureus* SZMC 0579 and *B. subtilis* SZMC 0209, were those for which we identified an MIC value at a concentration of 2 mg/mL for vanillin.

The results demonstrated that the incorporation of vanillin at both 2 mg/mL (SGMV2) and 4 mg/mL (SGMV4) concentrations significantly ($p < 0.05$) reduced bacterial growth compared to the control (SGM) (Fig. 1), indicating that vanillin retained its antimicrobial activity within the starch film matrix. It is worth mentioning, however, that the antimicrobial effect of the vanillin-containing films (SGMV2 and SGMV4) was moderate (from 12 to 44%; Fig. 1) considering the >90% growth inhibition presented by the individual vanillin compound at MIC against the tested bacteria (see Table 1). Although the release of vanillin into the culture medium has not been investigated, the moderate inhibitory effect can be attributed to the low vanillin content (0.2-0.4%) of films. In Kurabetta et al. (2025), films containing vanillin at concentrations of 1-3% exhibited notable antimicrobial activity against foodborne microorganisms in disk-diffusion assay, and the increase in activity was attributed to the effect of the vanillin component diffusing into the medium. A chitosan composite film containing vanillin and green tea polyphenol extract also demonstrated effective activity against the growth of *S. aureus* (Westlake et al., 2023).

The most sensitive bacterium towards both the SGMV2 and SGMV4 films was the *L. monocytogenes* SZMC 21307, with percentage inhibitions of 40 and 44%, respectively (Fig. 1). The SGMV4 sample exhibited potent inhibitory activity even against the growth of *S. enterica* SZMC 23289. In line with this, a concentration-dependent change in inhibitory effect was observed. Namely, the 11% growth-inhibiting activity identified against *S. enterica* SZMC 23289 with the SGMV2 sample increased to 38% in the presence of the SGMV4 film ($p < 0.05$). For the other bacteria tested, there was no significant difference in the inhibitory activity between the SGMV2 and SGMV4 films ($p > 0.05$). The slight variation in growth inhibitory effects observed depending on the bacterial strain can be attributed to different sensitivity to film components. In the case of vanillin, it is not possible to clearly determine whether it has a greater inhibitory effect on the growth of Gram-positive or Gram-negative bacteria. The effect generally varies depending on the bacterial strain across different studies (Kafali et al., 2024).

The antimicrobial efficacy primarily depends on the diffusion of the active compound from the polymer matrix, making the release profile a

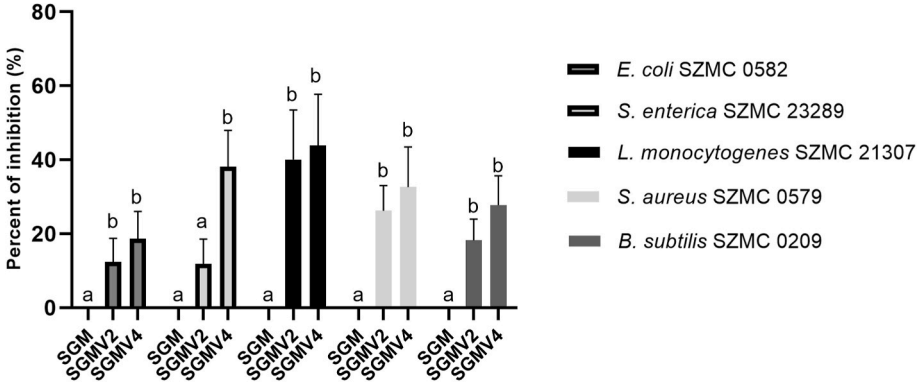



**Fig. 1.** Percentage inhibition of growth of food-contaminating bacteria by vanillin-free (SGM) and 2 mg/mL (SGMV2) and 4 mg/mL (SGMV4) vanillin containing starch films in microtiter plate tests. Average values from three tests ± standard deviation. The different letters above the columns of respective bacterium indicate significant differences according to one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

critical parameter. Additionally, vanillin's hydrophobic nature enhances its antimicrobial properties by interacting with membrane lipids and proteins, leading to disruption of plasma membrane integrity (Kafali et al., 2024). Previous studies have also shown that vanillin can disrupt potassium ion gradients in bacterial cells, inhibiting respiration and disturbing ionic homeostasis (Olatunde et al., 2022). Compared to general plant extracts, vanillin exhibits distinct physicochemical characteristics, including its specific ability to interact with starch molecules, which may enhance the bioactive functionality of the film. Overall, the incorporation of vanillin into biopolymer films offers significant potential for improving their antimicrobial properties. Its ability to inhibit the growth of foodborne pathogens can contribute to enhanced food safety and extended shelf life, while maintaining overall food quality (Upadhyay et al., 2024).

### 3.2.2. Bacterial adhesion to the film surface

Biofilms are bacterial communities that adhere to surfaces and become embedded within a self-produced polymeric matrix. Their formation is a major concern in food contexts as well; however, both cell adhesion and the formation of biofilms can be controlled by bioactive additives (Samrot et al., 2021). In this experiment, the adhesion of *E. coli* SZMC 0582 and *S. aureus* SZMC 0579 to the surfaces of SGM, SGMV2, and SGMV4 films was assessed, and the attached cells were visualized using acridine orange staining under fluorescence microscopy. Both bacteria were sensitive to vanillin in MIC tests, and the planktonic growth of *S. aureus* SZMC 0579 exhibited more sensitivity to SGMV2 and SGMV4 films compared to *E. coli* SZMC 0582 (Fig. 1). In addition, the cell wall structures of the two bacteria differ, therefore, these microorganisms were considered suitable for the adhesion test. The SGM structure in Fig. 2 showed higher adhesion for *S. aureus* SZMC 0579 than *E. coli* SZMC 0582. This is due to the fact that bacterial colonization can be influenced by multiple factors, such as cell surface charge and adhesion structures, hydrophobicity, culture medium, pH, age of the bacterial culture, and surface roughness (Zhao et al., 2023). However, the results showed that SGM films containing vanillin (SGMV2 and SGMV4) reduced biofilm formation by both bacterial isolates on the film surface (Fig. 2), indicating that vanillin retains antimicrobial activity against biofilm development in *E. coli* SZMC 0582 and *S. aureus* SZMC 0579. The inhibitory effect on *E. coli* SZMC 0582 biofilm formation was found to be stronger, which is consistent with the data reported in our previous study where the phenolic compounds generally inhibited biofilm formation to a greater extent in *E. coli* than *S. aureus* (Zambrano et al., 2019). In addition, our findings showed that SGMV4 film exhibited stronger antibiofilm activity than SGMV2, consistent with the concentration-dependent growth inhibition efficacy of vanillin (see Fig. 1).

Biofilm formation directly on composite films containing vanillin has been studied less extensively than the antimicrobial effect of the components in solutions. In the study of Das et al. (2021), the alginate-vanillin components effectively inhibited the formation of biofilm of food related pathogens such as *E. coli*, *B. cereus*, *Shigella flexneri*, *S. aureus* and *Salmonella typhi* both at MIC (2% alginate, 1% vanillin) and MIC/2 concentrations. In the presence of vanillin, the biofouling and biofilm formation of *Agrobacterium tumefaciens* (A136) and *Chromobacterium violaceum* (CV026) showed marked decrease at 62.5-375 mg/L concentrations (Nam et al., 2015). Since biofilm formation contributes to bacterial spoilage, food ripening, and reduced shelf life, the incorporation of vanillin into starch-based films offers a promising strategy for food packaging applications aimed at preserving product quality during storage.

## 3.3. Physicochemical and mechanical properties

Film thickness, solubility, hardness and elongation time until the breaking point were analyzed to assess changes in the physicochemical and mechanical properties following the addition of vanillin. Detailed data of hardness and elongation time and the breaking curves are presented in Fig. 3, and Fig. S4, while Table S1 summarizes thickness data. The results showed that incorporating vanillin at 2 mg/mL concentration into starch films (see SGMV2) reduced both film hardness (Fig. 3A) and elongation time (Fig. 3B) compared to the vanillin-free SGM. These findings are consistent with previous studies on the effect of phenolic

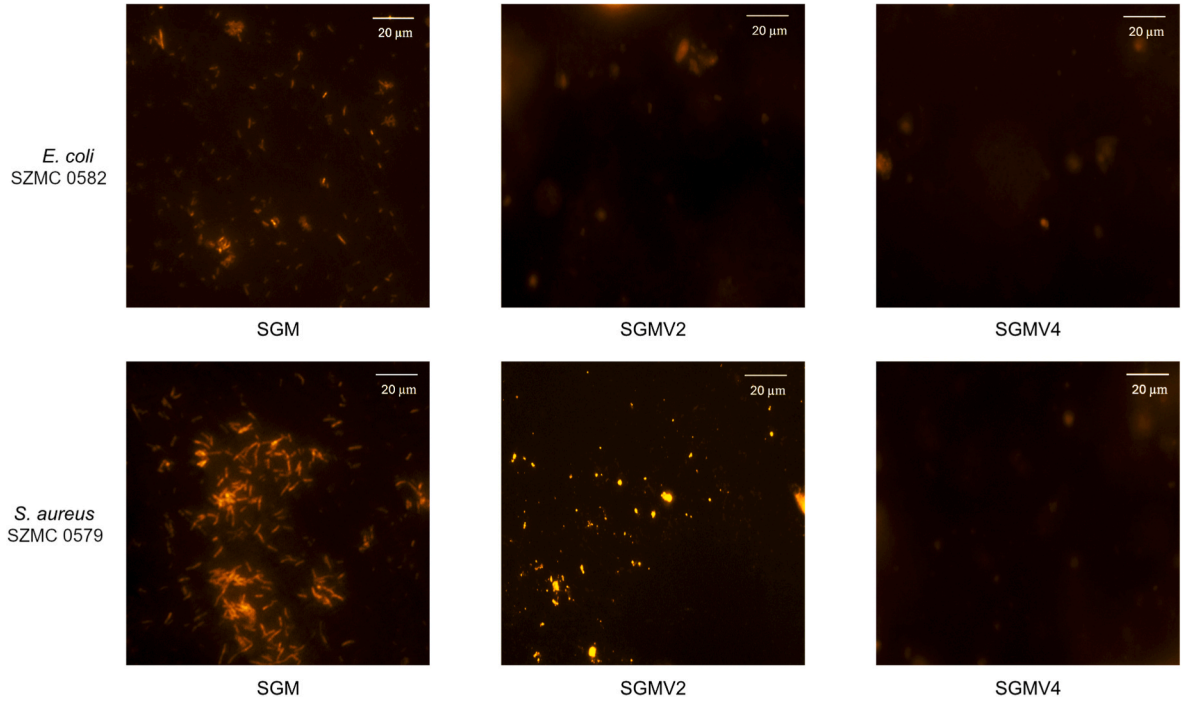



**Fig. 2.** Biofilm formation of *E. coli* SZMC 0582 and *S. aureus* SZMC 0579 on vanillin-free (SGM) and 2 mg/mL (SGMV2) and 4 mg/mL (SGMV4) vanillin containing starch films. After an incubation at 37 °C for 5 h (cell adhesion) and 24 h (biofilm formation) biofilms were stained with acridine orange and examined by fluorescence microscopy with magnification of 40x.

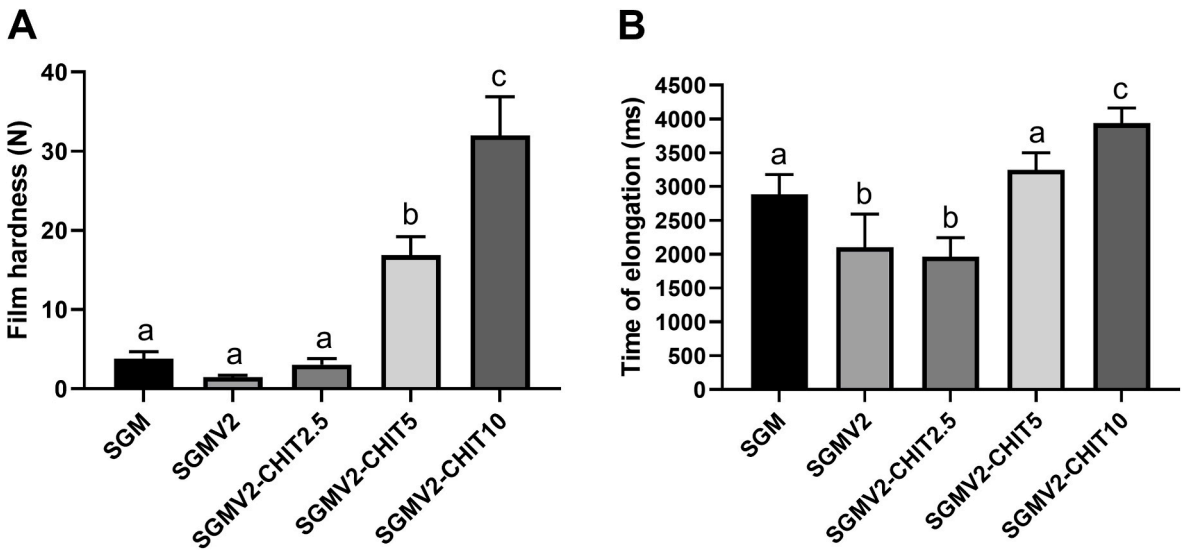



**Fig. 3.** The hardness (A) and time of elongation (B) of the produced films (SGM: starch-glycerol-montmorillonite film; SGMV2: SGM with 2 mg/mL vanillin; SGMV2-CHIT2.5, SGMV2-CHIT5 and SGMV2-CHIT10: SGMV2 with 2.5 mg/mL, 5 mg/mL or 10 mg/mL chitosan, respectively). Results shown are the mean of three replicates; error bars represent standard deviation. The different letters above the columns indicate significant differences according to one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

compounds from potato peel (Lopes et al., 2021) and fungal extract of *Aspergillus niger* endophytic fungus (de Souza Falcão et al., 2024) on starch film mechanical properties. The reduction in mechanical performance can be attributed to the plasticizing effect of phenolic compounds, which increase the mobility of starch chains and weaken intermolecular hydrogen bonding (do Nascimento et al., 2024). Furthermore, our findings agreed with previous studies in which the addition of vanillin alone could reduce the mechanical properties of polyvinyl alcohol-based films, including the tensile strength and elongation at break (He et al., 2025). On the other hand, previous studies have reported that the incorporation of vanillin into polyvinyl alcohol-based films can decrease film transparency. This reduction in transparency may be attributed to decreased inter-chain spacing within the polymer matrix, which limits the transmission of light through the film (Kurabetta et al., 2025). The reduction of mechanical properties and transparency may become a disadvantage compared to synthetic packaging. However, these films may offer advantages such as antimicrobial properties. It is also important to note that in our study, film thickness increased after the addition of vanillin compared to SGM (Table S1) but in varying degrees for the SGMV2 film due to the uneven distribution of vanillin.

Chitosan is known to influence the mechanical parameters of polymer films. In this context, chitosan was incorporated into starch-vanillin films containing 2 mg/mL vanillin (SGMV2) at concentrations of 2.5 mg/mL (SGMV2-CHIT2.5), 5 mg/mL (SGMV2-CHIT5), and 10 mg/mL (SGMV2-CHIT10). Increasing chitosan concentration did not significantly affect film thickness ($p > 0.05$) (Table S1) but resulted in a more uniform structure for the chitosan-containing SGMV2 films (SGMV2-CHITS) compared to SGMV2 (see SEM investigations in 3.7). However, the addition of 5 mg/mL chitosan to SGMV2 (SGMV2-CHIT5) significantly ($p < 0.05$) improved both hardness and elongation time from 1.49 to 16.87 N (Fig. 3A) and from 2100 to 3246 ms (Fig. 3B), respectively. Further enhancement was observed for films containing the chitosan at 10 mg/mL concentration (SGMV2-CHIT10, $p < 0.05$) (Fig. 3A and B). This effect can be explained by increased cohesive interactions between glycerol (acting as a plasticizer) and chitosan (Pamlényi et al., 2022). There was also a change in the visible appearance and transparency of the film after the addition of vanillin and chitosan shown in Fig. S1.

Chitosan also functions as a matrix material in the film, reinforcing structural integrity and strength. Previous work by Jiménez-Regalado et al. (2021) demonstrated that corn starch films without chitosan had lower tensile strength and reduced elongation at break, while chitosan incorporation markedly improved these parameters. During blending, gelatinization between chitosan and starch disrupts the crystalline starch structure, promoting molecular interactions and forming intermolecular hydrogen bonds between the $NH_3^+$ groups of chitosan and the OH groups of starch (Hao et al., 2023).

### 3.4. Thermal properties of films

The thermal behavior of the films was characterized using differential scanning calorimetry (DSC) and thermogravimetry (TG). DSC analysis showed that the biopolymer films were thermally stable at elevated temperatures (Fig. 4A). A sharp endothermic peak at 83.5 °C was observed for vanillin, corresponding to its melting point and confirming its crystalline nature. No other sharp endothermic peaks were detected for the remaining components, indicating that they were predominantly amorphous (Fig. 4A). In chitosan-based formulations, no sharp peak attributable to vanillin was detected (Fig. 4A), suggesting that vanillin transitioned to an amorphous state during the film casting/solvent evaporation process. Similar reductions in crystallinity have been reported for ethyl vanillin-chitosan blends (Narasagoudr et al., 2021) and vanillin loaded into chitosan-coated silica (Fan et al., 2018). Minor endothermic changes between 50 and 120 °C in the DSC curves of the starting materials and film formulations (SGMV2, SGMV2-CHITS, SGM) were attributed to moisture loss (Fig. 4A).

TG analysis supported the DSC findings. At 83.5 °C, vanillin showed no weight loss, confirming that this temperature corresponds to melting rather than degradation (Fig. 4B). This result aligns with earlier reports placing the melting range of vanillin between 81 and 83 °C (Gomes & Rodrigues, 2020). The onset of thermal degradation for vanillin occurred at approximately 160 °C, consistent with previous work indicating complete degradation at this temperature during lignin valorization (Zhu et al., 2018). In addition, Fig. 4B shows a marked weight loss for vanillin above 225 °C, which may be attributed to the oxidation of vanillin to vanillic acid at this condition (Mourtzinos et al., 2009). The starch and chitosan film components, as well as the SGM, SGMV2 and SGMV2-CHITS polymers, exhibited significant weight loss at temperatures above 250 °C (Fig. 4B).

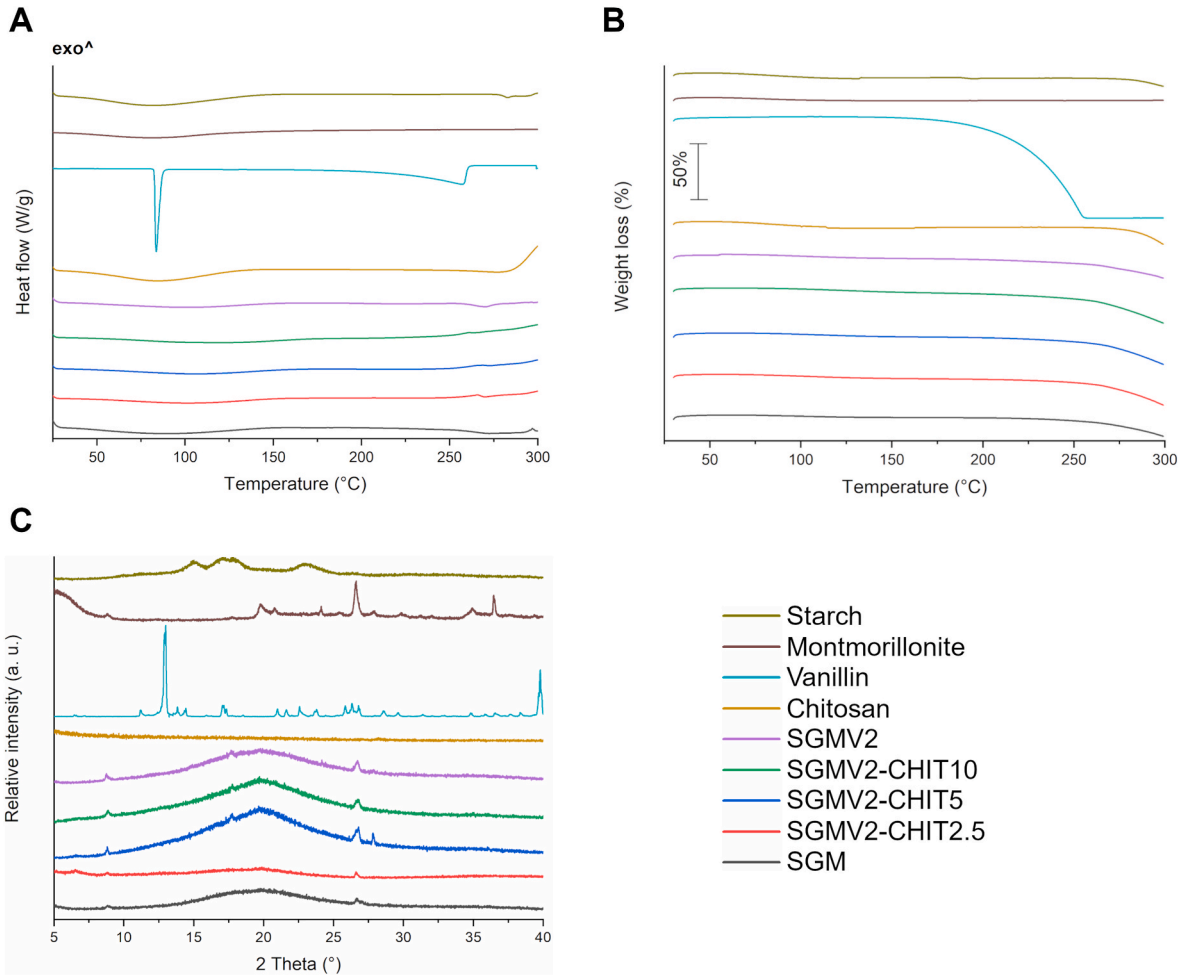



**Fig. 4.** Differential scanning calorimetry (A), thermogravimetry (B) and X-ray powder diffraction (C) curves of film formulations prepared, and their components (SGM: starch-glycerol-montmorillonite film; SGMV2: SGM with 2 mg/mL vanillin; SGMV2-CHIT2.5, SGMV2-CHIT5 and SGMV2-CHIT10: SGMV2 with 2.5 mg/mL, 5 mg/mL or 10 mg/mL chitosan, respectively).

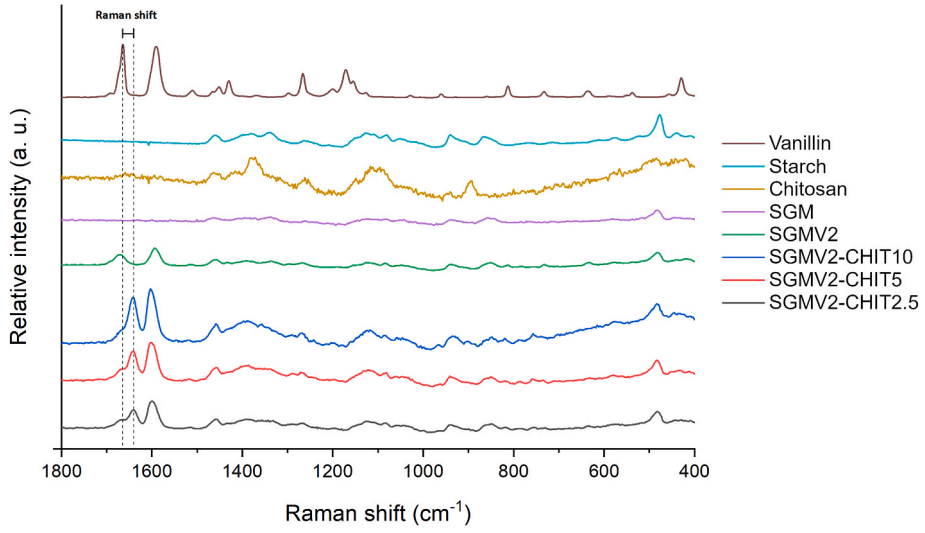



**Fig. 5.** Raman spectra of film formulations prepared, and their components (SGM: starch-glycerol-montmorillonite film; SGMV2: SGM with 2 mg/mL vanillin; SGMV2-CHIT2.5, SGMV2-CHIT5 and SGMV2-CHIT10: SGMV2 with 2.5 mg/mL, 5 mg/mL or 10 mg/mL chitosan, respectively). The Raman shift of aldehyde group peak at 1670 cm$^{-1}$ in vanillin to 1641 cm$^{-1}$ in SGMV2-CHITS referring to the formation of Schiff base.

### 3.5. X-ray diffraction

The crystallographic structure of the composite films was determined by X-ray powder diffraction (XRPD) analysis. The XRPD diffractograms confirmed the crystalline nature of vanillin and montmorillonite, as indicated by their characteristic peaks (Fig. 4C). Starch and chitosan exhibited amorphous structures, and the characteristic peaks of montmorillonite (at 8.8°, 17.7°, 26.7°, and 27.8°) were visible on the diffractograms of the SGMV2, SGMV2-CHITS and SGM formulations. Notably, no characteristic peaks for vanillin were detected, indicating that vanillin transformed from a crystalline to an amorphous state during the film casting process, in agreement with the result obtained from DSC analysis. The amorphous transformation may increase the dissolution rate of molecules supporting their bioavailability (Jog & Burgess, 2017).

### 3.6. Raman spectroscopy

Raman spectroscopy was used to investigate structural changes in the polymer films. The Raman spectrum of SGMV2-CHITS (Fig. 5) displayed characteristic features, including a band at 1670 $cm^{-1}$ corresponding to the aldehyde group of vanillin. For vanillin, additional characteristic peaks were observed at 1588 $cm^{-1}$ [ν(C=C)], 1510 and 1429 $cm^{-1}$ [ν(benzene ring)], and 1267 $cm^{-1}$ [δ(CH) and ν(benzene ring)], where ν denotes stretching and δ denotes bending vibrations. Remarkably, in the SGMV2-CHITS, a pronounced Raman shift of the aldehyde group peak from 1670 $cm^{-1}$ to 1641 $cm^{-1}$ was observed (Fig. 5). This shift indicates a Schiff base crosslinking, formed between the aldehyde moiety in vanillin and the amine groups in chitosan, resulting in an imine bond (Li et al., 2025; Liu et al., 2024), which is clearly visible in the spectra (see Raman shift in Fig. 5).

The spatial distribution of Schiff base was further assessed using Raman mapping (Fig. S5). For localization, the Raman peak at 1641 $cm^{-1}$ attributed to the aldehyde group vibration in Schiff base was used as a reference, as unique spectral vibrations in the SGMV2-CHITS in comparison to other samples. The chemical maps show the relative intensity of Schiff base occurrence: red areas indicate strong presence, green represents mixed composition, and blue denotes regions dominated by other components. Results revealed negligible Schiff base signals (0.01-0.09) for the SGM (Fig. S5A) and SGMV2 films (Fig. S5B). In contrast, intense Schiff base distribution (0.88-0.92) was identified even in the SGMV2-CHIT2.5 film (Fig. S5C, yellow-red areas), demonstrating that chitosan is essential for Schiff base formation. The increased chitosan concentration in SGMV2-CHIT5 (Fig. S5D) and SGMV2-CHIT10 (Fig. S5E) further elevated Schiff base frequency. Previous studies have reported that the formation of resistant starch-based Schiff base structures in starch gels enhances material properties via cross-linking interactions (Liu et al., 2024). Moreover, such interactions can improve antimicrobial activity, UV resistance and radical scavenging activity, attributed to the compact structure formed by strong covalent bonding (Li et al., 2025). Vanillin Schiff bases exhibited notable inhibitory effects against the growth of *E. coli*, *P. aeruginosa*, and *Klebsiella pneumoniae* in the study of Khan et al. (2024). In addition, growth of *E. coli*, *B. subtilis* and *S. aureus* strains was effectively inhibited by *o*-vanillin Schiff bases at 0.6 and 6 mg/mL concentrations (Cheng et al., 2009).

### 3.7. Film morphology

The morphology of the prepared films is shown in Fig. S6, based on SEM images that include both surface and cross-sectional views. The SGM film exhibited a smooth, crack-free and thin surface (Fig. S6A). Upon the incorporation of vanillin, the film surface became slightly rougher and more compact (see SGMV2 in Fig. S6B). The absence of visible porous structures indicates good compatibility between starch and vanillin, consistent with previous observations (Z. Zhang, Han, Zeng, Xiong, & Liu, 2015).

Vanillin incorporation into starch films resulted in aggregate formation (Fig. S6B), which may be derived from amylopectin aggregation or unreacted vanillin (Ke et al., 2023; Liu et al., 2024). The presence of nano agglomerates within the matrix may enhance the antimicrobial activity of the films. These morphological changes can be attributed to interactions between the functional groups of vanillin and the hydrogen bonds in starch.

The addition of chitosan to the SGMV2 film resulted in smoother surfaces and improved film compatibility (see SGMV2-CHITS in Fig. S6C–E). This improvement is likely due to the formation of interfacial adhesion between chitosan and starch, which enhances the compactness of the microstructure (L. Cheng, Hao, Song, & Chang, 2021). Moreover, chitosan incorporation reduced surface roughness and increased film density through the creation of a more homogeneous blend (Dang & Yoksan, 2016). Understanding surface roughness characteristics is essential for optimizing material properties and developing effective surface modifications for packaging applications.

### 3.8. Evaluation of antioxidant activity of the films

Both vanillin and chitosan possess notable antioxidant properties. In line with this, the next step was to assess the antioxidant activity of films prepared, as this characteristic is important for developing packaging materials that can prolong the shelf life of food products. Antioxidant properties of the films were assessed using DPPH and FRAP assays to monitor their action in two different techniques. Application of different antioxidant methods for a sample has advantages and is advisable, but it is important to note that the comparison of the activity results obtained might be difficult due to the different antioxidant reactions that occur (Munteanu & Apetrei, 2021). The DPPH assay was employed to determine the free radical scavenging capacity of the bioactive compounds within the starch-modified films, while the FRAP assay measured their ferric-reducing power.

In the DPPH assay, vanillin incorporation (SGMV2) did not significantly affect free radical scavenging activity compared to the SGM control ($p > 0.05$) (Fig. 6A). In contrast, the addition of chitosan (SGMV2-CHITS) significantly enhanced the antioxidant activity compared to both the SGM and SGMV2 films ($p < 0.05$) (Fig. 6A), likely due to its hydroxyl groups disrupting free radical chain reactions (Chen et al., 2024). The FRAP values of SGM more than doubled with vanillin incorporation (SGMV2) and were further elevated by chitosan ($p < 0.05$), reaching a maximum of 0.035 μmol Fe(II)/100 g film at 10 mg/mL chitosan (SGMV2-CHIT10, Fig. 6B). This observation aligns with previous findings, where the incorporation of chitosan into melatonin-based formulations for preserving broccoli cell integrity also enhanced FRAP antioxidant capacity (Tan et al., 2025). The inconsistency between the trends in the DPPH and FRAP results may be due to differences in the measurement mechanisms, as observed in previous study as well (Kovács et al., 2024). Taken together, the results highlight the contribution of both vanillin and chitosan to the antioxidant potential of starch-based films, supporting their use in active food packaging applications.

### 3.9. Film solubility

Solubility of the SGM, SGMV2 and SGMV2-CHITS films was tested by incubating them in water under gentle agitation for 24 h. Although there was no statistically significant difference between the solubility characteristics of films produced ($p > 0.05$), data suggest that the addition of chitosan may slightly reduce the degradation of the films in aqueous surroundings. The mean percent solubility of SGMV2-CHITS varied between 11 and 14% (Fig. S7), that are in the range (3-18%) described for edible films formulated from cassava starch (Arias et al., 2024). Polymer films with good solubility can degrade easily in environments with high relative humidity, which is beneficial from an environmental

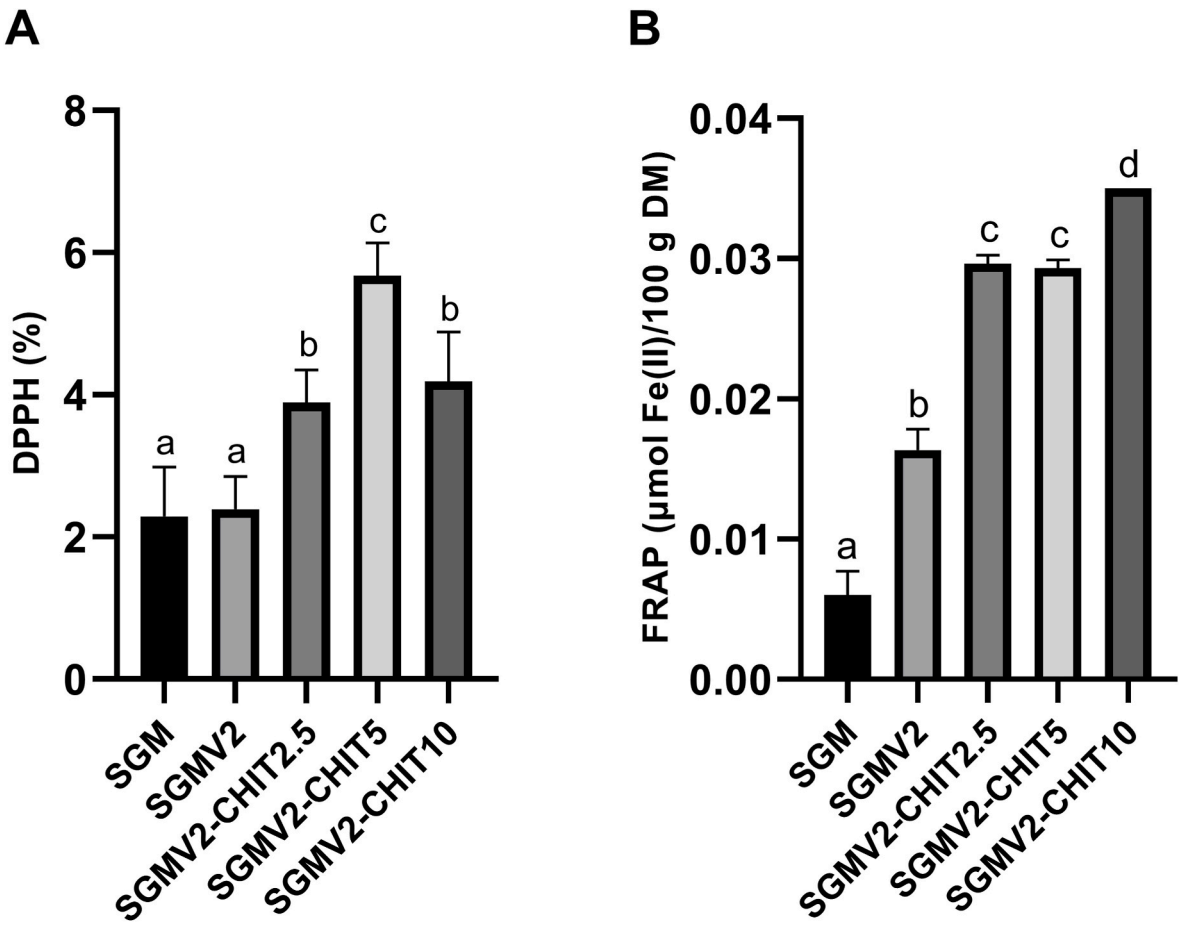



**Fig. 6.** Comparison of DPPH radical scavenging capacity (A) and FRAP activity (B) of prepared films (SGM: starch-glycerol-montmorillonite film; SGMV2: SGM with 2 mg/mL vanillin; SGMV2-CHIT2.5, SGMV2-CHIT5 and SGMV2-CHIT10: SGMV2 with 2.5 mg/mL, 5 mg/mL or 10 mg/mL chitosan, respectively). Results are means of data of three replicates; error bars represent standard deviations. The different letters above the columns indicate significant differences according to one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

perspective. However, this property limits its suitability for preserving foods stored in high-humidity conditions. In SGMV2-CHITS films, the addition of chitosan appears to further reduce the solubility characteristics resulting from low hydrophilicity of cassava under the test conditions used in this study.

### 3.10. Application of films in strawberry preservation

Considering its physical properties and bioactivity, the SGMV2-CHIT5 film appears to be an ideal candidate for the development of active packaging additives for food products. In line with this, the practical applicability of SGMV2-CHIT5 was evaluated using fresh

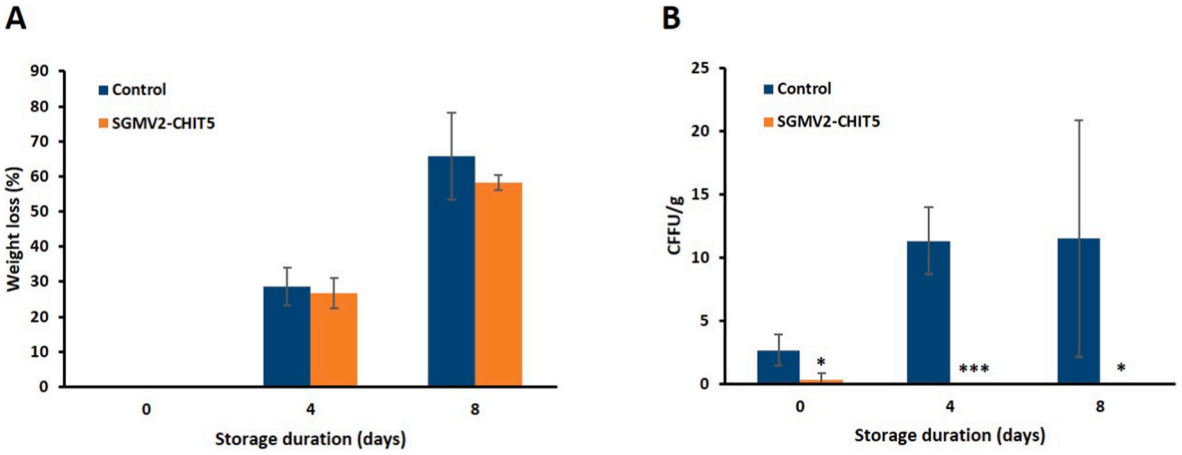



**Fig. 7.** Weight loss (A) and total bacterial count (B) of uncoated (Control) and SGMV2-CHIT5 film coated strawberry samples. Storage test was performed during 8 days at room temperature condition. Results shown are the mean of three replicates; error bars represent standard deviation. Asterisks indicate significant differences between the respective control and coated samples according to a paired *t*-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.0001$.

strawberries as a model system, chosen due to their high perishability and compatibility with the aroma properties of vanillin. Two approaches were tested: (i) a direct coating method, and (ii) a supplemented packaging approach, where the bottom of plastic boxes was lined with solid film pieces.

In the coating method, the weight loss of strawberries treated with SGMV2-CHIT5 did not differ significantly ($p > 0.05$) from uncoated controls during 8 days of room temperature storage (Fig. 7A). Nevertheless, slightly lower values in coated samples suggested reduced water migration through the thin coating layer. Such coatings may act as barriers over fruit stomata, balancing internal-external pressure and slowing transpiration (Chettri et al., 2023). Similar findings were reported by Q. Yang, Ren, Yu, Zhang, & Chen (2025), where chitosan-based films reduced strawberry water loss by limiting molecular diffusion. In contrast, uncovered control samples exhibited higher weight loss due to intense respiration and transpiration during storage.

Microbiological analyses further demonstrated the protective effect of SGMV2-CHIT5 coatings. Total microbial counts were consistently lower compared to uncoated controls (Fig. 7B), and visual assessments revealed an extension of shelf life up to the 4th storage day: molds first appeared in controls by day 4 and spread extensively by day 8, while coated fruits remained largely uninfected (Fig. S8). This aligns with findings by Araújo et al. (2024), where chitosan-seed oil coatings prevented strawberry mold infection for up to 6 days at room temperature. A chitosan-based film supplemented with tomato extract was also effective coating material in strawberry preservation (J. Yu, Xu, Chen, & Shao, 2024). Among the film components used in our study, both chitosan and vanillin contributed to antifungal efficacy. Chitosan, for instance, can disrupt fungal cell wall through electrostatic interactions with negatively charged components, leading to the degradation of intracellular components (Zhang et al., 2021). Vanillin can inhibit fungal growth by impairing membrane integrity and ergosterol biosynthesis (Q. Li, Zhu, Zhao, & Xie, 2022).

In supplemented packaging tests under refrigeration (4 °C), strawberries stored with SGMV2-CHIT5 films showed a slightly greater loss of weight on day 12 compared to the film-free control ($p < 0.05$) (Fig. 8A), which may be due to membrane moisture absorption (Kruk et al., 2023). This implies that further experiments are needed to test the membrane and adjust the formulation for optimal water absorption. However, no significant differences were observed on other storage days (Fig. 8A). In addition, after four days of storage, weight loss remained below 5% (Fig. 8A), which is similar to the findings reported by L. Xu, An, Hu, & Ren (2025) for blueberry preservation in plastic container supplemented with bio-based antimicrobial film. More importantly, the package contained SGMV2-CHIT5 film markedly reduced bacterial growth between days 4 and 16 compared to film-free controls (Fig. 8B). No visible mold infections on strawberries were detected until day 8 in the supplemented packaging system (Fig. S9). These results suggest that incorporation of SGMV2-CHIT5 into packaging effectively suppresses microbial proliferation and extends the postharvest life of strawberries. The antimicrobial activity of the Schiff base complexes formed in these composites may have contributed to the reduction of microbial counts observed with the SGMV2-CHIT5 film (Ceramella et al., 2022).

So far, polylactic acid-based and cinnamaldehyde-containing chitosan films have proven to be effective for strawberry preservation in packaging tests (Crescente et al., 2024; Wang et al., 2019). Taken together, our findings also highlight the dual role of SGMV2-CHIT5 films as both direct coatings and active packaging inserts, offering promising potential for extending the shelf life of highly perishable fruits. It is worth mentioning, however, that our research did not extend directly to the effects of the developed films on the organoleptic properties of strawberry fruit tested. Despite its strong aroma, vanillin has been used in foods, such as bread (Chen et al., 2025), apple (Wang et al., 2022) and litchi (Javed et al., 2023) as a preservation additive to enhance antimicrobial and/or antioxidant activity and extend shelf life. The odor threshold for vanillin in water is 100 μg/L (de-la-Fuente-Blanco & Ferreira, 2020), which is a lower concentration than those use in the developed SGMV2-CHITS films. In addition, a previous study reported that vanillin incorporated in the chitosan/methyl cellulose films may undergo the migration into food (pineapple juice) if the storage temperature is relatively high or increasing. It was found that the migration of vanillin is also influenced by the pH of the environment (Sangsuwan et al., 2009). For these reasons, future research should examine the migration of vanillin into the fruit, as well as the sensory properties of the treated samples after storage.

### 3.11. Film biodegradability

The initial biodegradability of the SGMV2-CHIT5 film was evaluated using a compost burial system over a 6-week incubation period. During the experiment, sufficient moisture was maintained to support microbial degradation, and the residual weight of the films was measured at 2-week intervals. The results demonstrated measurable degradation of the film under the applied composting conditions. A weight loss exceeding 5% relative to the initial sample weight was observed after 4 weeks of incubation ($p < 0.05$) (Fig. S10A). Although the film appeared visually intact even after 6 weeks of incubation (Fig. S10B), the measured mass loss indicates that degradation had already begun during

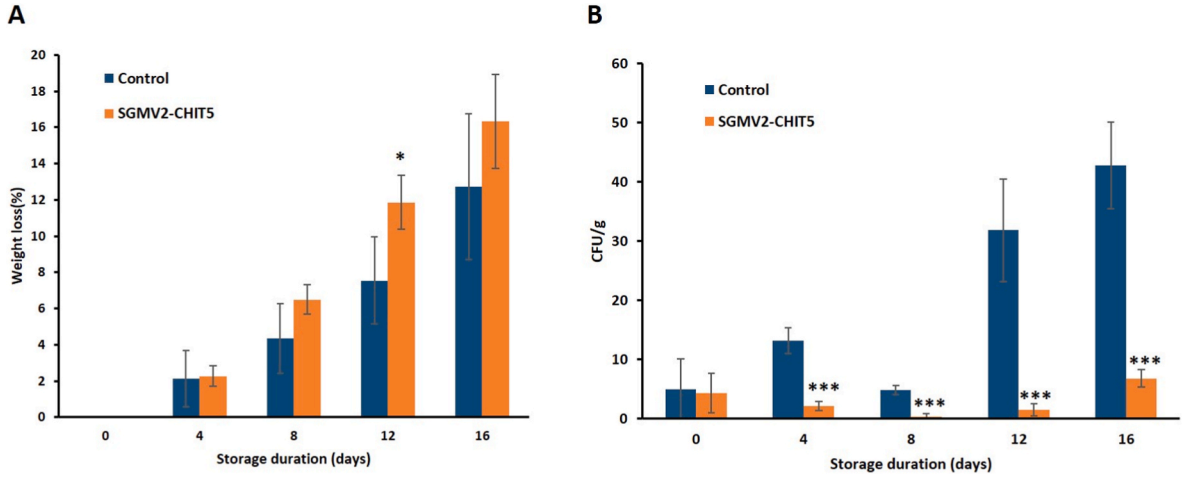



**Fig. 8.** Weight loss (A) and total bacterial count (B) of strawberry samples placed in film-free (Control) or SGMV2-CHIT5 film contained packaging box. Storage test was performed during 16 days at refrigeration (4 °C) condition. Results shown are the mean of three replicates; error bars represent standard deviation. Asterisks indicate significant differences between the respective control and coated samples according to a paired *t*-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.0001$.

the incubation period. Previous studies investigating the biodegradability of vanillin-containing films generally reported significant degradation within 2-12 weeks (Mohamadinezhad & Shekarchizadeh, 2026; Westlake et al., 2023). Overall, the present results suggest that the film may be suitable for incorporation into composting systems; however, longer incubation periods are required for a more comprehensive evaluation of its biodegradability.

## 4. Conclusion

This study demonstrated the production, mechanical and functional characterization, and preservative potential of biopolymer films based on cassava starch and containing montmorillonite clay, vanillin and chitosan as active ingredients. In initial tests, the vanillin in soluble form exhibited notable antimicrobial activity against foodborne pathogens and spoilage bacteria. The starch-glycerol-montmorillonite films with 2 and 4 mg/mL vanillin (SGMV2 and SGMV4) prepared subsequently exerted a moderate inhibitory effect on the planktonic growth of most of the bacteria tested, and inhibited the adhesion of *E. coli* SZMC 0582 and *S. aureus* SZMC 0579 in biofilm formation assays. Both DSC and XRPD analyses revealed a crystalline to amorphous state transformation for vanillin during casting, which suggests improved bioavailability of this active ingredient. The chitosan biopolymer, when added as an additive to SGMV2, resulted in Schiff base crosslinking formation, smooth surface, and improved mechanical compactness and antioxidant capacity to the membranes.

In coating and packaging assays with strawberries, the SGMV2-CHIT5 film, which contained chitosan at 5 mg/mL, reduced microbial growth, delayed fungal infection, and moderately decreased water loss, extending fruit shelf life under room temperature or refrigerated storage. These findings show that the SGMV2-CHIT5 film may be a suitable composite for the development of active packaging systems in fruits, as it combines antimicrobial and antioxidant effects with biodegradability and improved physicochemical and mechanical properties. The water solubility of the film may influence its applicability under high-humidity storage conditions while also contributing to its environmental degradation behavior. Further experiments are needed to investigate the effect of prolonged storage conditions on the mechanical, physical, or chemical properties of the films, to test the composites for optimal water absorption, and to assay the organoleptic properties of fruit samples after treatments. Additionally, solubility and biodegradability studies should be continued, for which long-term incubation of films under high humidity conditions and in various soil burial test systems are the appropriate methods. Finally, it is important to note that all compounds used in the developed films are classified as generally recognized as safe, underscoring their suitability for food-related applications.

## CRediT authorship contribution statement

**Muhammad Iqbal Perdana:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Gábor Katona:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Tamás Sovány:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Attila Farkas:** Investigation, Formal analysis. **Ferhan Ayaydin:** Resources, Investigation, Formal analysis. **Bernard Gitura Kimani:** Methodology. **Nermin Tahboub:** Investigation. **Tamás Kovács:** Methodology. **Csilla Szebenyi:** Methodology, Investigation. **Tamás Papp:** Resources, Funding acquisition. **Csaba Vágvölgyi:** Supervision, Resources, Project administration, Funding acquisition. **Miklós Takó:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Muhammad Iqbal Perdana, Gábor Katona, Tamás Sovány, Attila Farkas, Ferhan Ayaydin, Bernard Gitura Kimani, Nermin Tahboub, Tamás Kovács, Csilla Szebenyi, Tamás Papp, Csaba Vágvölgyi, Miklós Takó.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2026.119654.

## Data availability

Data will be made available on request.

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