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Sustainability spotlight

This research focuses on the development of functional, non-toxic, and sustainable active packaging films made from chitosan blended with polyvinyl alcohol, chitooligosaccharides, and gallic acid. The resulting active films demonstrated strong antioxidant properties against DPPH and ABTS radicals. Additionally, they displayed significant antimicrobial activity against *E. coli*, *L. innocua*, and *S. cerevisiae*, along with strong biocompatibility with HaCaT and Caco-2 cells. These films effectively reduced weight loss in tomatoes and grapes, as well as minimised total volatile basic nitrogen production during the preservation of pearl fish fillets, highlighting their potential as functional packaging solutions for extending the shelf life of perishable foods.

1 **Antioxidant, antimicrobial, and cytotoxicity properties of chitosan-PVA film functionalised with**

2 **chitooligosaccharide and gallic acid for shelf-life extension of perishable foods**

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Abstract

Environment-friendly, non-toxic, and functional packaging films are gaining interest worldwide for extending the shelf-life of perishable food items. Hence, this study explored the antioxidant, antimicrobial, and cytotoxicity patterns of earlier fabricated chitosan films containing polyvinyl alcohol (PVA), chitooligosaccharide (COS), and gallic acid (GA). In addition, the active properties of chitosan films were reconfirmed using tomatoes, green grapes, and pearl fish fillets as model perishable food products. Chitosan films containing COS and GA demonstrated strong DPPH ($99.48 \pm 0.24\%$) and ABTS ($98.06 \pm 0.91\%$) radicals scavenging capacity and robust potentiality in ferric reducing activity (230.93 ± 1.42, equivalent μM Fe^{2+}/g sample). Additionally, the inhibitory rates of the fabricated films towards *Escherichia coli*, *Listeria innocua*, and *Saccharomyces cerevisiae* were $59.49 \pm 9.17\%$, $79.29 \pm 0.94\%$, and $79.55 \pm 8.45\%$, respectively. Further, MTT assay exhibited that chitosan films were biocompatible with viability greater than 90% on HaCaT cells and 75% on Caco-2 cells. The fabricated films demonstrated non-cytotoxicity *in vitro* and show potential suitability for packaging applications. Moreover, the application of chitosan films on tomatoes and green grapes showed the lowest weight loss compared to the control film at room temperature ($23°C$) for up to 7 and 9 days, respectively. Additionally, these films also led to a reduction in total volatile basic nitrogen (TVBN) levels in the preservation of pearl fish fillets, indicating the potential for shelf-life extension.

Keywords: Chitosan films, Bioactive properties, Biocompatible.

1. Introduction

Microbial contamination is a common phenomenon during the storage of perishable foods, leading to degraded food quality and a threat to consumers' health [1]. It is estimated that over 200 million tons of food could be wasted by 2050 due to improper packaging and the short shelf-life of perishable food items [2]. Additionally, the use of petroleum-based materials in food packaging can lead to significant environmental pollution, posing a threat to both terrestrial and aquatic life [3]. Moreover, the use of plastic in food packaging generates microplastics, which accumulate in living terrestrial and aquatic environments through the food chain and enter humans through biomagnification [4]. It is reported that microplastics have been detected in human blood, which can cause severe health risks [5]. In this regard, using biodegradable and functional food packaging materials in the food industry may protect food by preventing contamination, reducing food waste, ensuring food safety, and contributing to the United Nations' Sustainable Development Goals (SDGs) [6, 7].

Several polysaccharides, including chitosan, pectin, starch, and alginate, are considered suitable for developing active food packaging materials [8]. Among them, chitosan is gaining interest in functional packaging materials due to its film-forming capacity, antioxidant and antimicrobial activity, non-toxicity, biodegradability, and biocompatibility properties [9, 10]. Although chitosan has functional properties in the development of packaging film, it still has some drawbacks, including insufficient antioxidant and antimicrobial abilities, which greatly limit the application of neat chitosan in active food packaging [11, 12]. Additionally, the incorporation of polyvinyl alcohol (PVA) is increasing in the development of active chitosan films due to its excellent film-forming ability, biocompatibility, and improved flexibility, although it offers fewer functional properties. Therefore, chitosan derivatives and phenolic compounds are incorporated to fabricate functional chitosan films [13]. Chitooligosaccharide (COS) and gallic acid are highly water-soluble and obtained from the enzymatic depolymerisation of chitosan and secondary metabolites in plant materials (e.g., grapes, apples, blueberries, tea, etc.), respectively [14]. Studies have reported that COS and gallic acid possess several functional activities, such as antioxidant, antimicrobial, and antiviral properties [15-17]. The incorporation of COS in the development of active chitosan films, through conjugation with caffeic acid, showed potential antioxidant activity, particularly in scavenging DPPH radicals [18]. Additionally, the combination of gallic acid with corn starch and pullulan in the production of functional films demonstrated both DPPH radical scavenging properties and antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* [19]. Hence, there has been a growing interest, including COS and gallic acid, in developing active food packaging materials.

90   In general, active food packaging materials come into direct contact with food and release bioactive

91   compounds to extend product shelf life, reducing lipid oxidation and microbial growth [20, 21]. Even though using

92   biomaterials in the development of packaging film is safe, the interaction of raw materials during the film's

93   synthesis process could alter the packaging film's biocompatibility patterns [22]. Therefore, the cytotoxicity patterns

94   of packaging film need to be tested before practical application for extending food shelf-life. In addition, the

95   packaging materials in food applications should not release any toxic compounds that could harm human health,

96   as stated by the Food Safety Act, 1991, in EC 1935/2004 [23]. Thus, the main objective of the current study is to

97   explore the antioxidant, antimicrobial, and cytotoxicity patterns of developed chitosan films containing polyvinyl

98   alcohol, COS, and gallic acid.

99

100   **2. Materials and methods**

101

102   **2.1 Materials**

103   Chitosan (91% degree of deacetylation and 503 kDa molecular weight, Mw) was acquired from Weseta

104   International in Shanghai, China. Chitooligosaccharide (COS) was synthesised following the method of Rajabi et

105   al. [24]. Polyvinyl alcohol ($\geq$ 99% hydrolysed and 89,000-98,000 g/mol Mw) and gallic acid (97.5% and 170 g/mol

106   Mw) were provided by Sigma-Aldrich, New Zealand. TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine), Mw: 312.33, and

107    $\geq$ 98%), ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid, Mw: 548.68 g/mol, and $\geq$ 98%), and

108   DPPH (2, 2-Diphenyl-1-picrylhydrazyl and molecular weight: 394.32),) were obtained from Sigma-Aldrich,

109   Switzerland, Canada, and Germany respectively. Potassium persulfate (Mw: 270.32 and $\geq$99%) was procured

110   from Sigma-Aldrich, methanol (CAS-No: 67-56-1, >99.8%, and Mw: 32.04) supplied by Fisher Scientific, UK,

111   Ltd., India, glacial acetic acid (CAS No: 64-19-7 and >99.7%), sodium acetate trihydrate (Mw: 136.08, and 99%

112   from Biolab (Aust) Ltd.), ferrous sulphate (CAS No: 7782-63-0 and 99%), iron (III) chloride hexahydrate (CAS

113   No: 10025-77-1, Mw: 270.30 g/mol, and 97%) were also obtained from Sigma-Aldrich, USA for the study.

114   *Escherichia coli* (ESR 916), *Saccharomyces cerevisiae* (ICMP 10067), and *Listeria innocua* (ESR 3024) were

115   acquired from the Microbiological Lab culture collection of the Department of Food Science, University of Otago.

116   Luria broth (LB), nutrient yeast peptone dextrose broth (YPD), and tryptic soy broth (TSB) were supplied by

117   Becton, Dickinson, and Company, Le Pont de Claix, France. The Caco-2 cells (P6, human colon cells) and HaCaT

118   cells (P38, human keratinocyte skin cells) were obtained from the American Type Culture Collection (ATCC,

119   Manassas, VA, USA). DMEM (Dulbecco's modified eagle medium), FBS (fetal bovine serum), anti-anti

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120 (Antibiotic-antimycotic), 0.25% trypsin-EDTA, DPBS (Dulbecco's phosphate buffered saline), calcein AM, and

121 propidium iodide were obtained Gibco (Life Technologies Corporation, Grand Island, USA). Additionally,

122 ethylene tetrazolium bromide (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H, MTT) was purchased from Thermo

123 Fisher Scientific (Life Technologies Corporation, Eugene, USA) and dimethyl sulfoxide (CAS No: 67-68-5,

124 molecular weight: 78.13 g/mol, and $\geq$99.7%, DMSO) procured from Sigma-Aldrich, USA. Potassium carbonate

125 anhydrous (K_2CO_3, Mw: 138.21, 99% assay) was purchased from M & B Laboratory Chemicals (May & Baker

126 Ltd., Dagenham, England). Methyl red ($C_{15}H_{15}N_3O_2$), bromocresol green ($C_{21}H_{14}Br_4O_5S$), and phenolphthalein

127 ($C_{20}H_{14}O_4$) were acquired from Hopkin & Williams Ltd., Chadwell Heath, Essex, England. Boric acid (H_3BO_3,

128 Mw: 61.83, 99.5% assay) was supplied by Ajax Chemicals, Auburn, Australia. The analytical grade ethanol

129 (C_2H_5OH, 99.5%) was procured from the Department of Chemistry, University of Otago. Analytical reagent grade

130 hydrochloric acid (HCl, Mw: 36.46, CAS-No: 7647-01-0, and 37%) was supplied by Fisher Scientific, UK.

131 Sodium hydroxide (NaOH, CAS-No: 1310-73-2, Mw: 40, and >98% assay) was provided by Sigma-Aldrich, New

132 Zealand. Fresh tomatoes, green grapes, and pearl fish fillets were purchased from the local supermarkets in

133 Dunedin, New Zealand.

134

135 **2.2 Chitosan film fabrication**

136 The chitosan films were fabricated using the solvent casting method, following the process established by

137 Bhowmik et al. [25]. Briefly, 2% chitosan (CH) solution was obtained by mixing 0.4 g of CH in 2% of 20 mL acetic

138 acid solution. After that, 0.2 g (50% based on CH) of polyvinyl alcohol (PVA), 0.02-0.04 g (5-10% based on CH)

139 of chitooligosaccharide (COS), and 0.02-0.04 g (5-10% based on CH) of gallic acid (GA) were added and stirred

140 for 30 min at room temperature (23°C). Then, the film-forming solution was poured into square Petri dishes (10

141 cm x 10 cm) and kept for 72 h at 23 °C. The fabricated films were manually separated from the Petri dishes. The

142 film containing only chitosan was regarded as CH film, followed by CP2 (CH + 0.2g PVA), CP5 (CH + 0.2g PVA

143 + 0.02 g COS), CP6 (CH + 0.2g PVA + 0.04 g COS), CP7 (CH + 0.2g PVA + 0.02 g GA), CP8 (CH + 0.2g PVA

144 + 0.04 g GA), CP9 (CH + 0.2g PVA + 0.02 g COS + 0.02 g GA), CP10 (CH + 0.2g PVA + 0.04 g COS + 0.04 g

145 GA), CP11 (CH + 0.2g PVA + 0.04 g COS + 0.02 g GA) and CP12 (CH + 0.2g PVA + 0.02 g COS + 0.02 g GA)

146 film based on previous work (**Table S1**), where the physical, mechanical, structural, thermal, and biodegradation

147 properties of films were included [25].

148

149 **2.3 Antioxidant activity of developed chitosan films**

2.3.1 DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The DPPH assay, with some modifications, was used to measure the antioxidant properties of the fabricated chitosan films [26]. In short, 7.88 mg of DPPH powder was dissolved in 100 mL of 99.8% methanol to create 0.2 mM DPPH solution. After that, the film sample (5 mg) was mixed with 1.5 mL of DPPH solution and stored at 22 °C. A UV/Visible Spectrophotometer (Ultrospec 3300 Pro, Biochrom Ltd., Cambridge, England) was then used to test the mixture's absorbance at 517 nm across a range of time intervals (0.5–16 h). The scavenging capacity of DPPH free radicals was determined in triplicate, and the mean values are presented as per equation 1.

$$Scavenging\ ability\ (\%) = [1 - \frac{(A_1 - A_2)}{A_0} \times 100] \ldots\ldots\ldots\ldots(1)$$

Where, A_0 is the absorbance of DPPH solution, A_1 is the absorbance of a film sample with DPPH solution, and A_2 is the absorbance of a film sample with methanol.

2.3.2 ABTS assay

The scavenging property of ABTS free radicals of the developed chitosan films was evaluated using the Božič et al. [27] and Riaz et al. [26] methods with some modifications. In summary, 384 mg of ABTS powder and 66 mg of potassium persulfate were separately dissolved in 100 mL of distilled water to create a 7 mM ABTS solution and 2.45 mM $K_2S_2O_8$ solution. After that, the same volume of ABTS and $K_2S_2O_8$ solution were mixed and left in the dark for 16 h at 22 °C. Subsequently, the combined solution was diluted 1:10 with a 50% methanol solution to create a workable solution. The absorbance of the working solution at 734 nm using a UV/Visible Spectrophotometer (Ultrospec 3300 Pro, Biochrom Ltd., Cambridge, England) was measured and found to be 0.96 (<1). After that, 1.5 mL of working solution and 5 mg of film sample were mixed, and the mixture was left in the dark. At various times (6 min–4 h), the absorbance at 734 nm was measured. Antioxidant ability was calculated in triplicate samples using the following equation: 2.

$$Scavenging\ ability\ (\%) = [1 - \frac{(A_s - A_m)}{A_w} \times 100] \ldots\ldots\ldots\ldots(2)$$

Where, A_w is the absorbance of a working solution, A_s is the absorbance of a film sample with a working solution, and A_c is the absorbance of a film sample with methanol.

2.3.3 FRAP assay

The ferric-reducing antioxidant power (FRAP) assay was conducted following the method of Gulzar et al. [28] with some modifications to measure the capacity of the fabricated chitosan films to transform ferric iron (Fe^{3+}) to ferrous (Fe^{2+}) iron. Concisely, 300 mM acetate buffer (pH < 3.6) was prepared by dissolving $CH_3COONa.3H_2O$

180   in 100 mL ultrapure water containing 1.6 mL CH_3COOH, stored at 22°C using an amber flask. Thereafter, a 10

181   mM HCl solution was prepared by mixing 40 μL 37% HCl in 10 mL ultrapure water; after that, 10 mM 2,4,6-

182   Tris(2-pyridyl)-s-triazine (TPTZ) solution was prepared by adding 31 mg TPTZ in 10 mM HCl solution. A 20

183   mM $FeCl_3.6H_2O$ solution was also prepared by dissolving 54.1 mg $FeCl_3.6H_2O$ in 10 mL ultrapure water.

184   Relatedly, 2000 μM $FeSO_4.7H_2O$ solution was made by mixing 55.6 mg $FeSO_4.7H_2O$ in 100 mL ultrapure water.

185   A 72 mL working solution was prepared, mixing 6 mL $FeCl_3.6H_2O$ and 6 mL TPTZ with 60 mL acetate buffer.

186   Then, 5 mg of film sample was mixed with 0.9 mL of working solution and incubated in a water bath (TECHNE,

187   TE-10A Tempette, Total Lab Systems Ltd.) at 37°C for 15 min. The Fe^{2+} TPTZ complex was assessed by taking

188   absorbance at 593 nm and calculating its activity in triplicate samples by using the equation ($Y = 0.0008x + 0.1303$

189   and $R^2 = 0.99$) from the generated standard curve using $FeSO_4.7H_2O$ (0-2000 μM) and represented as μM of Fe^{2+}

190   equivalents/g of the film sample.

191

192   **2.4 Antimicrobial properties of film**

193   The antimicrobial activities and integrity of microbial cell membranes of chitosan films were assessed towards

194   Gram-positive bacteria (*Listeria innocua)*, Gram-negative bacteria (*Escherichia coli)*, and yeast (*Saccharomyces*

195   *cerevisiae)* following the method of Bi et al. [29] and Sadiq et al. [30] with some modifications. The *E. coli* and *L.*

196   *innocua* were cultured in liquid Luria broth and tryptic soy broth medium at 37 °C to logarithmic phase, and *S.*

197   *cerevisiae* was cultured in yeast peptone dextrose medium at 30 °C. After that, 0.2 mL microbial suspensions

198   (with an initial absorbance of 1 at OD_{600}) of *E. coli, S. cerevisiae,* and *L. innocua* was diluted 50 times using 10

199   mL of LB, YPD, and TSB medium, respectively and incubated with the films (6 mm x 6 mm) with 0.2 mL liquid

200   medium for 24 h. Then, the optical density at 600 nm was measured using a plate reader (CLARIOstar[Plus], BMG

201   LABTECH, Germany) at different time intervals (0-24 h), and the antimicrobial rate was analysed in triplicate

202   samples using the following equation 3.

203    $$Antimicrobial\ rate\ (\%) = (OD_1 - OD_2)/OD_1 \times 100 \ \ldots\ldots\ldots\ldots(3)$$

204   Where, OD_1 is the microbial optical density of the control group (without film), and OD_2 is the microbial optical

205   density treated with developed films.

206   To assess the integrity of microbial cell membranes, 0.15 mL of microbial suspension was obtained after 24 hours,

207   and it was centrifuged (1-16K, SIGMA, Germany) at 11000 × g for five minutes. Utilising 0.1 mL of supernatant,

208   the quantities of cellular constituents were assessed as proposed by Bi et al. [29] by determining the absorbance at

209   260 nm with a plate reader (CLARIOstarPlus, BMG LABTECH, Germany). The control group consisted of the

210   microbial suspension supernatant that had not been film-treated.

211

212   **2.5 Cell assay, viability, and proliferation assays**

213   **2.5.1 Cell assay**

214   The frozen stocks of HaCaT cells (P38, human keratinocyte skin cells) and Caco-2 cells (P6, human colon cells)

215   were removed from liquid nitrogen and warmed in a water bath at 37 °C and thawed for 50 seconds. Then, HaCaT

216   and Caco-2 cells were grown in T-75 cm^2 flasks in complete DMEM (cDMEM) media containing 10% fetal

217   bovine serum and 1% antibiotic-antimycotic (penicillin-streptomycin). Then, the culture flasks were kept in a

218   humidified atmosphere of 95% air and 5% CO_2 using a cell incubator (MCO-19AIC (UV), CO_2 incubator,

219   SANYO Electric Co., Ltd., Japan), maintained at 37 °C. The cDMEM was replaced every three days during the

220   culture periods. Additionally, HaCaT and Caco-2 cells were passaged to maintain their proliferative state when

221   they reached 70-90% confluence through trypsinisation. The cell counting was performed using Septer™ Sensors

222   (60 μm), EMD Millipore Corporation, Burlington, USA.

223

224   **2.5.2 Cell viability assay**

225   The toxicity and biocompatibility of developed chitosan films were evaluated using LIVE/DEAD® assay which

226   determine cytotoxicity/cell viability [31, 32]. Concisely, sterile film samples (6 mm x 6 mm) were immersed in

227   cDMEM, and both the HaCaT and Caco-2 cells were seeded separately per well and incubated at 37 °C for 24 h,

228   48 h and 72 h. The cDMEM with cells but without film was considered a control. The assay staining was prepared

229   using 8 mL DPBS, 12 μL calcein AM, and 24 μL propidium iodide. After 24 h, 48 h, and 72 h, the medium was

230   removed from the well plate, and 100 μL assay solution was added to each well and incubated at 37 °C for 10

231   min. After that, cells were observed under a fluorescence microscope (EVOS M5000, ThermoFisher, Waltham,

232   USA) using EVOS™ M5000 software. The enzymatic conversion of calcein AM to calcein (excitation 494 nm,

233   emission 517 nm) allowed for identifying living cells [33]. The nucleic acids of cells with damaged cell membranes

234   bound to propidium iodide indicate dead cells (excitation 528 nm, emission 617 nm). The number of living and

235   dead cells was counted using Image J software (US National Institutes of Health) with a plugin [34]. The fluorescent

236   images of each cell, captured at different culture periods, were converted to greyscale using ImageJ, resulting in

237   monochromatic 16-bit images. This process transformed the pixels into individual data points. Next, we calculated

238   the number of pixels representing dead (red) cells and live (green) cells. The percentage of live cells was then

239   determined relative to the total number of cells in each image. To calculate the average value, three images of

240   each cell from every culture period and each film were used, maintaining consistent settings applied across all

241   images to ensure uniformity. The percentage of cell viability, which was calculated in triplicate samples using

242   equation 4.

243            $Cell\ viability\ (\%) = Number\ of\ live\ cells/Total\ cell\ number\ \times\ 100$...............(4)

244

245   **2.5.3 Cell proliferation assay**

246   The cell proliferation of HaCaT and Caco-2 cells was measured at 24 h, 48 h, and 72 h calorimetrically using

247   ethylene tetrazolium bromide (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H, MTT) assay [35]. Briefly, 12 mM

248   MTT stock solution was prepared by dissolving 50 mg MTT powder in 10 mL sterile DPBS and kept at -20°C.

249   The HaCaT and Caco-2 cells were seeded separately (5 x 10^3 cells) per well-containing chitosan film samples (6

250   mm x 6 mm) and incubated at 37 °C for 24 h, 48 h and 72 h in quadruplicate. After 24 h, 48 h, and 72 h, the

251   medium was removed from the well plate, and 100 μL MTS reagent (earlier prepared 7 mL by dissolving 1.4 mL

252   MTT solution with 5.6 mL cDMEM) was added to each well and kept in a humidified incubator for 4 h. Then,

253   the medium was removed from the well plate, and 50 μL dimethyl sulfoxide (DMSO) was added to each well.

254   DMSO with HaCaT and Caco-2 cells were transferred to new well plates, and absorbance was taken at 540 nm

255   using a plate reader (CLARIOstar[Plus], BMG LABTECH, Germany). Standard curves (**Fig. S1**) were used to detect

256   the unknown cell numbers of HaCaT and Caco-2 cells treated with films.

257

258   **2.6 Evaluation of storage quality of tomato and green grape**

259   The storage qualities of tomatoes and green grapes were considered model food products and studied to investigate

260   the active properties of the developed films (CH, CP9, and CP10). The prepared tomatoes (3.2-3.5 g) and green

261   grapes (3.1-3.3 g) were kept in a Petri dish (3.5 cm x 3.5 cm) covered with a lid. The films (1.3 cm x 1.3 cm) were

262   inserted inside the Petri dish, and the setup with the tomatoes and grapes, stored for 7 and 9 days, respectively, at

263   room temperature (23 °C, relative humidity 46%). Tomatoes and green grapes in the Petri dish without film were

264   regarded as control, and those without a lid and film were designated as non-packaging (NP). The Petri dish was

265   weighed every day, and the weight was recorded. The weight loss of tomatoes and grapes were measured in

266   triplicate samples using equation 5 [36].

267            $Weight\ loss\ (\%) = (W_1 - W_2)/W_1 \times 100$ ....(5)

268   Where, W_1 is the initial weight of tomatoes and grapes in a Petri dish, and W_2 is the weight on the day of

269   measurement.

270

271   **2.7 Evaluation of storage quality of pearl fish fillet**

272   The purchased pearl fish fillets were cut (20-25 g) and kept in a Petri dish with films (1.3 cm x 1.3 cm) separately

273   at room temperature (23°C, relative humidity 46%). The pH, drip loss, and total volatile basic nitrogen (TVBN)

274   were measured daily in triplicate samples to detect the storage quality of pearl fish fillets. The pearl fish fillet

275   without film was considered the control, and the one without film and lid was regarded as the NP group.

276   The pH of pearl fish fillet was detected, following the method of Pang et al. [37] with some modifications.

277   Five (5) grams of pearl fish fillet were vigorously homogenised in 45 mL of distilled water. After that, the pH of

278   the pearl fish fillet filtrate was measured in triplicate samples using a pH meter (HI 5222, Hanna Instruments,

279   USA). Additionally, the drip loss of packaged pearl fish fillets with films in a Petri dish was assessed using the

280   weighing method [36]. The pearl fish fillets in a Petri dish were weighed daily using an analytical balance, and the

281   following equation 6 was used to calculate the drip loss.

282    $$Drip \, loss \, (\%) = (W_1 - W_2)/W_1 \times 100 \ldots\ldots\ldots\ldots(6)$$

283   Where W_1 is the initial weight of pearl fish fillets in a Petri dish, and W_2 is the weight on the measurement day.

284   The TVBN of pearl fish fillets was measured, following the method of Lee et al. [38] with some

285   modifications. Briefly, 5 g of ground pearl fish fillets were inserted in a 50 mL Falcon tube containing 45 mL

286   distilled water and kept at room temperature (23°C) for approximately 35 min. After that, Whatman No. 1 filter

287   paper was used to filter the samples, and the supernatant was analysed. Before sample analysis, the Conway

288   indicator solution was prepared by dissolving 66 mg of bromocresol green and 66 mg of methyl red in 100 mL of

289   ethanol (99.5%). Additionally, a 0.01 N boric acid solution was prepared by dissolving 0.3 g boric acid in 150 mL

290   of ethanol, and the volume was increased to 500 mL using 350 mL of distilled water. Also, a 50% potassium

291   carbonate solution was made by dissolving 50 g K_2CO_3 in 100 mL of distilled water. In the analysis of TVBN in

292   pearl fish fillets, 1 mL H_3BO_3 and 100 μL of indicator solution were included in the inner part of the Conway

293   unit, and 1 mL of supernatant and 1 mL of K_2CO_3 solution were added in the outer chamber of the Conway unit.

294   Then, the Conway unit was kept at 37°C for 2 h in an oven (THERMOTEC 2000, Lower Hutt, New Zealand).

295   After that, 0.01 N HCl was incorporated into the inner part of the Conway unit until the formation of the pink

296   colour. The content TVBN was calculated in triplicate samples using the following equation 7.

297    $$TVBN \, (mg/100g) = 0.14 \times (b - a) \times f \times d/W \times 100 \ldots\ldots\ldots\ldots(7)$$

298 Where b and a are the titration volumes (mL) of HCl for a sample and blank, respectively, f is the concentration

299 of HCl (0.01 N), d is the dilution factor (45 mL), and W is the pearl fish fillet weight (5 g).

300

301 **2.8 Statistical analysis**

302 The statistical analysis used GraphPad Prism version 9.4.1 (GraphPad Software, Boston, USA). The obtained data

303 from at least three replicates are presented as mean ± standard deviation (SD). To look for significant differences

304 ($P \leq 0.05$) in the generated films, cell viability, and total cell number, Tukey's test and ordinary one-way analysis

305 of variance (ANOVA) were employed.

306

307 **3. Results and discussion**

308

309 **3.1 Antioxidant activity of developed chitosan films**

310 The antioxidant activity of fabricated chitosan films is presented in **Fig. 1**. In the DPPH scavenging assay; the

311 fabricated films showed different activities during 0.5-16 h. Films CP8 (95.37 ± 0.57 – 95.51 ± 0.56%), CP10

312 (91.37 ± 5.52 – 95.38 ± 0.08%), and CP12 (88.30 ± 9.51 – 95.44 ± 0.25%) exhibited the highest DPPH scavenging

313 activities with greater consistency during the time periods, whereas CP7 (46.80 ± 1.44 – 94.12 ± 1.85%), CP9

314 (69.82 ± 5.48 – 96.30 ± 0.04%), and CP11 (41.31 ± 6.14 – 99.48 ± 0.24%) demonstrated linearity in different

315 time intervals. CH (8.31 ± 0.43 – 13.13 ± 4.26%), CP2 (6.88 ± 0.17 – 14.80 ± 2.45%), CP5 (2.76 ± 1.79 – 7.16 ±

316 3.13%), and CP6 (3.13 ± 0.21 – 8.24 ± 2%) films presented the lowest percentage of DPPH scavenging activities.

317 These research findings are consistent with Zhang et al. [39], who reported that neat chitosan film (15%)

318 demonstrated the lowest DPPH scavenging capacity compared to gallic acid-loaded film (50-83%), indicating that

319 the addition of gallic acid in chitosan film enhanced antioxidant activity due to the interaction of native antioxidant

320 properties gallic acid moieties on chitosan backbone. The addition of PVA and COS in the chitosan film did not

321 improve the DPPH radical scavenging activity in the chitosan films. However, the inclusion of gallic acid and

322 COS significantly enhanced the DPPH radical scavenging capacity of the chitosan-PVA films labelled CP9-CP12.

323 These findings align with the research conducted by Yuan et al. [18] who elucidated that COS and caffeic acid-

324 loaded chitosan films significantly increased scavenging ability (93.43%) compared to the control film (10%).

325 Additionally, similar studies reported by Lee et al. [40], indicated that including gallic acid in the preparation of

326 chitosan active film exhibited a strong scavenging property (95.7%) due to the presence of a phenolic hydroxyl

327 group.

11

328   Similarly, in the ABTS scavenging assay, CP10 (97.85 ± 1.01 – 98.06 ± 0.91%), CP12 (97.41 ± 0.69 –

329   97.57 ± 0.53%), CP8 (94.12 ± 5.05 – 95.79 ± 2.63%), and CP11 (94.34 ± 2.87 – 96.68 ± 0.38%) showed the

330   highest percentage of ABTS scavenging properties, whereas CP7 (84.23 ± 9.09 – 96.50 ± 0.38%) and CP9 (85.60

331   ± 5.59 – 93.87 ± 0.67%) demonstrated different activity with linearity in the experimental time, followed by CH

332   (20.76 ± 7.30 – 80.99 ± 1.28%), CP2 (12.80 ± 0.07 – 91.90 ± 4.81%), CP5 (14.23 ± 10.02 – 90.94 ± 4.81%), and

333   CP6 (11.48 ± 8 – 87.06 ± 12.72%). Similar findings were stated by Zhao et al. [11], who described chitosan film

334   (22%) as having the lowest ABTS scavenging activity, whereas adding gallic acid to chitosan film enhanced

335   hydrogen supply capacity, improving ABTS radical scavenging capacity (85%).

336   Likewise, the highest FRAP (equivalent μM Fe^{2+}/g sample) was obtained from CP10 (230.93 ± 1.42)

337   film, followed by CP12 (229.13 ± 4.20), CP8 (228.69 ± 0.49), CP9 (222.88 ± 4.76), CP7 (220.73 ± 1.36) and

338   CP11 (218.11 ± 0.24), whereas the lowest FRAP was exhibited by CP6 (54.66 ± 1.11), CH (33.88 ± 1.79), CP5

339   (25.04 ± 0.06), and CP2 (24.43 ± 4.39) films. Adding COS and gallic acid to the chitosan-PVA film enhanced its

340   ability to reduce Fe^{3+} to Fe^{2+}, potentially indicating synergistic effects. The findings align with Gulzar et al. [28],

341   who reported that adding COS and tannic acid to chitosan film demonstrated the highest FRAP activity, which

342   depends on the amounts of COS and tannic acids in film development. A film with the capacity to convert ferric

343   to ferrous ions and delay the production of free radicals, which might delay the lipid oxidation of foods and avoid

344   oxidative stress [41]. The highest FRAP activities by incorporating COS and gallic acid reconfirmed their active role

345   in developing chitosan films with effective antioxidant properties.
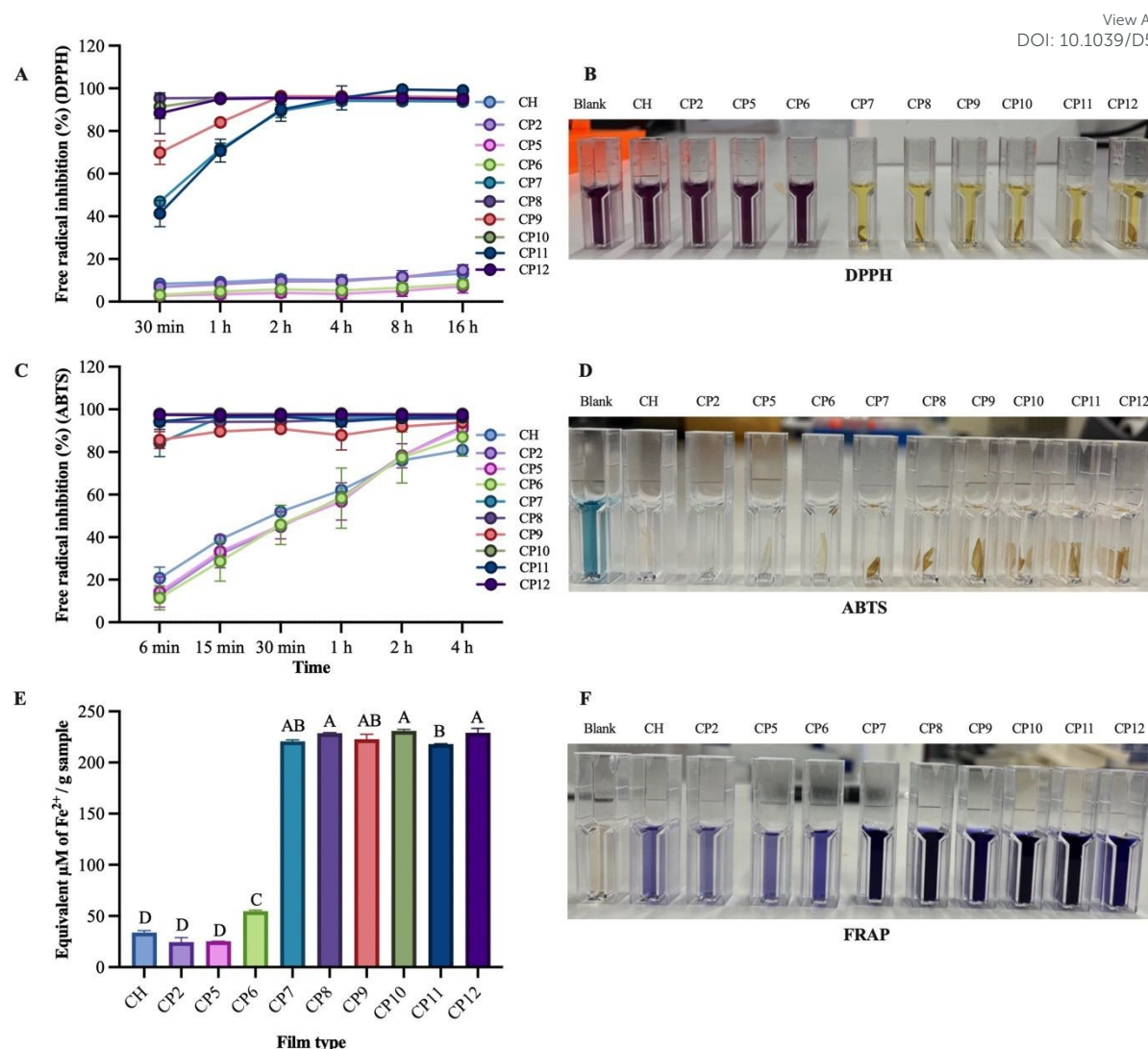
Fig. 1. Scavenging capacity of fabricated films towards DPPH (A), ABTS (C), and FRAP (E) radicals, colour changes of working solution observed during the experiment (B, D, and F). FRAP: Ferric reducing antioxidant power. Bars represent mean ± SD with different letters (A-D) are significantly different at $p < 0.05$, $n = 3$.

### 3.2 Antimicrobial properties of fabricated film

The antimicrobial activity of developed chitosan film towards *Escherichia coli* (Gram-negative bacteria), *Listeria innocua* (Gram-positive bacteria), and *Saccharomyces cerevisiae* (Yeast) is presented in **Fig. 2.** Overall, the COS and gallic acid loaded chitosan films showed higher antimicrobial activity and leakage of greater intracellular substances compared to neat chitosan film. These findings suggested that adding COS and gallic acid effectively improved the antimicrobial properties of chitosan film. The inclusion of COS and gallic acid in chitosan film resulted in 59.49 ± 9.17% (CP10), 79.29 ± 0.94% (CP10), and 79.55 ± 8.45% (CP9) antimicrobial capacity of chitosan film. The findings of the study are in line with Zhao et al. [11] who demonstrated that the addition of gallic

359   acid improved antimicrobial activity towards Gram-positive (*Staphylococcus aureus*) and Gram-negative

360   (*Escherichia coli*) bacteria due to the synergistic effect of the positively charged amino group of chitosan and

361   gallic acid, which disrupts bacterial cell wall and results in bacteria death. Adding PVA and COS alone in chitosan

362   film did not significantly improve the antimicrobial properties of chitosan film. The findings are consistent with

363   Gulzar et al. [28] and Kanatt et al. [42], who reported that elevated concentrations of COS in the film failed to inhibit

364   the growth of microbes (*Listeria monocytogenes* and *Escherichia coli*) effectively, and PVA in chitosan film did

365   not show any significant changes in microbial growth (*Staphylococcus aureus* and *Bacillus cereus*), respectively.

366   However, combining COS and gallic acid in chitosan film improved antimicrobial activity in the current study

367   due to the synergistic interaction with chitosan. Additionally, the release of cellular components is higher in COS

368   and gallic acid-loaded films (CP7-CP12 (*Escherichia coli*), CP8-CP10 (*Listeria innocua*), and CP8, CP9, and

369   CP12 (*Saccharomyces cerevisiae*)) than in chitosan film. This could have happened due to the presence of an

370   abundance of free amino groups, contributed by COS, in the film matrix, leading to electrostatic interactions with

371   the phosphate groups of the microbial cell membrane to damage membrane integrity, resulting in the release of

372   intercellular substances [29, 43]. Additionally, the potential interaction of GA with the chitosan film matrix caused

373   membrane pores of microbes to destroy cell membranes and enhance their cell permeability, resulting in leakage

374   of cell constituents [44]. Also, we hypothesise that the inclusion of COS and gallic acid in the chitosan film acts

375   synergistically to improve membrane permeability and physical disruption of microbial cells, leading to death.

376   These findings are consistent with those of Hou et al. [17], who reported that incorporating gallic acid and tannic

377   acid into a cellulose/chitosan film matrix created an active film with enhanced antimicrobial activity against

378   *Escherichia coli* and *Staphylococcus aureus* due to their synergistic interactions. Therefore, the OD_{260} values of

379   COS and gallic acid-loaded chitosan films were higher than those of the neat chitosan film, reconfirming the

380   enhancement of antimicrobial activity in the fabricated films of the current study.
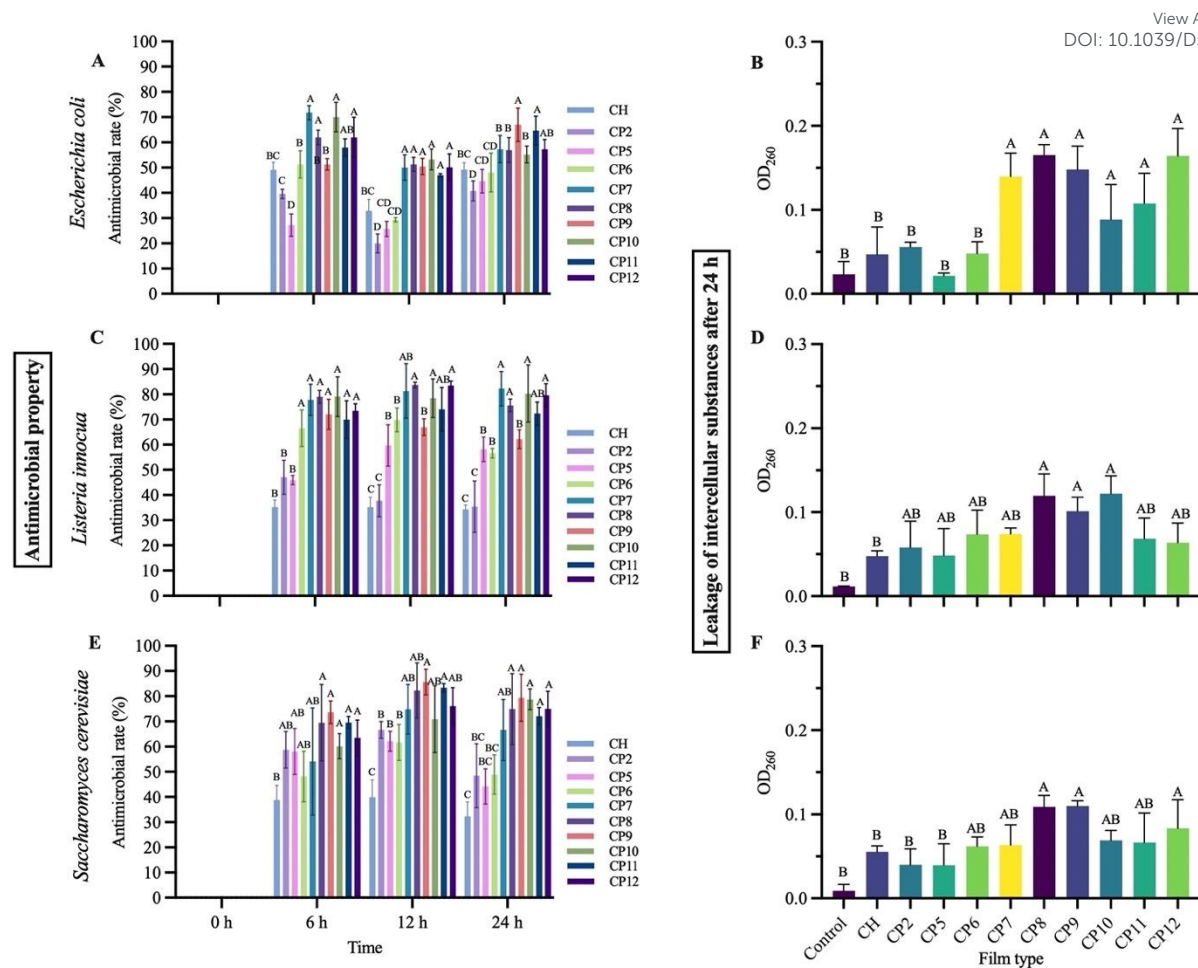
Fig. 2. Antimicrobial rate of fabricated chitosan films towards *Escherichia coli* (A), *Listeria innocua* (C), and *Saccharomyces cerevisiae* (E), and leakage of cell constituents of microbes after 24 h (B, D, and F). Bars represent mean ± SD with different letters (A-D) are significantly different at $p < 0.05$, n = 3.

3.3 Cytotoxicity patterns of chitosan films

3.3.1 Cell viability of HaCaT cells

The cell viability profile of HaCaT cells is presented in **Fig. 3**. The green and red fluorescence demonstrated live and dead cells, respectively. The higher proportion of live cells than dead cells confirmed the positive viability of HaCaT cells toward CH, CP9, and CP10 films. The HaCaT cells exhibited excellent viability, more than 90% in all tested films. There were no significant differences ($p > 0.05$) between control and developed films at 24, 48, and 72 h, except CP10 film at 24 h. Following ISO 10993-5, a biological material is considered non-toxic if the cell survival rate is more than 70% during the culture period. Hence, the film developed with chitosan, polyvinyl alcohol, COS, and gallic acid is considered a safe food packaging material. Yang et al. [45] fabricated packaging film containing polyvinyl alcohol, hexamethylene guanidine, and gallic acid and characterised their cytotoxicity.

396 Research findings of their studies showed that the developed film had good biocompatibility, and the cell viability

397 of mouse fibroblast (L929) cells of the gallic acid-loaded film was greater than 70%. Riaz et al. [46] studied the

398 safety of gallic acid in developing film containing agarose and showed more than 75% cell viability towards L929

399 cells. The current study findings demonstrated that adding GA to the chitosan film is non-toxic to HaCaT cells.

400
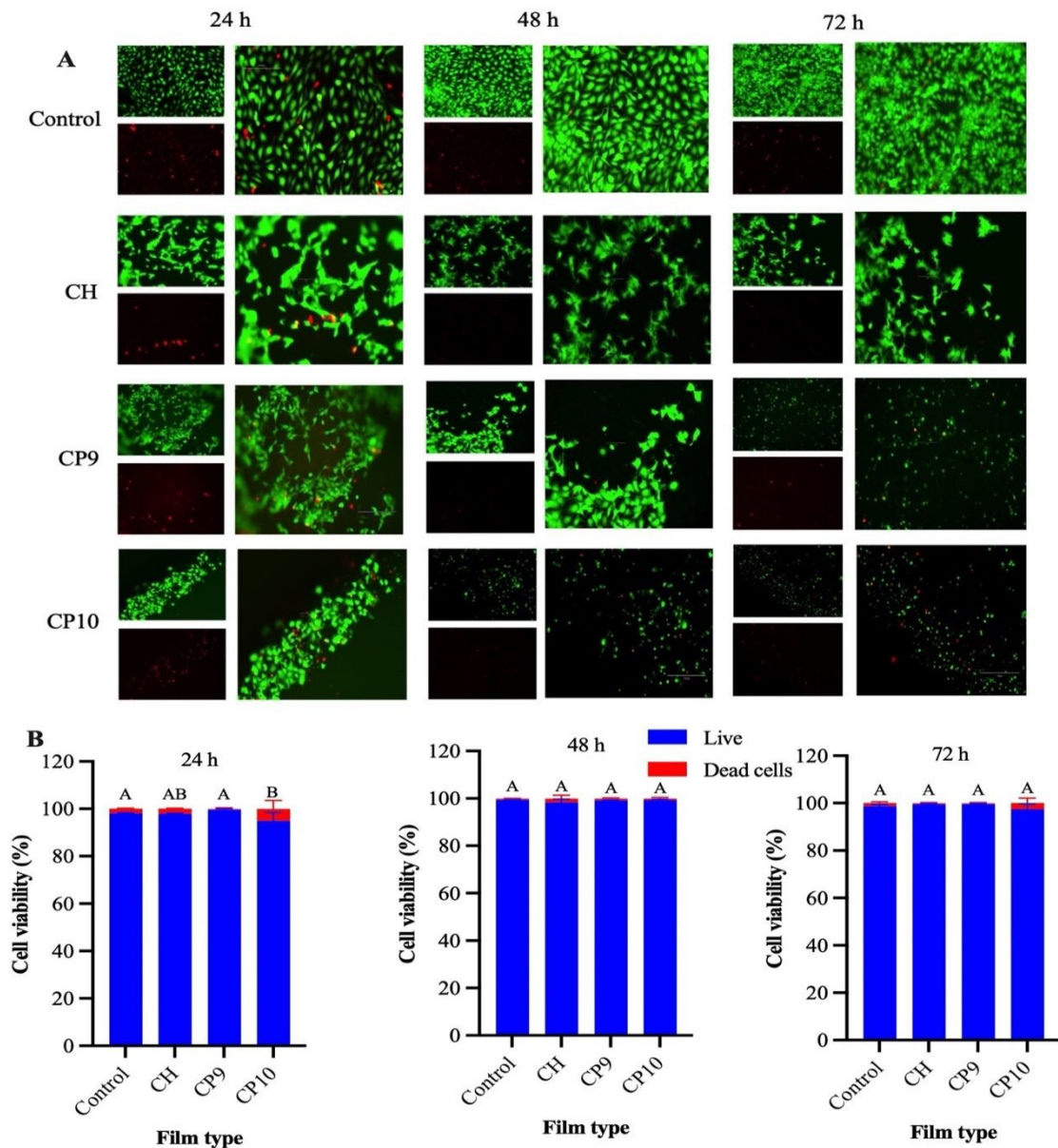
401 **Fig. 3.** Dye-based fluorescent microscopy images (A) and cell viability (B) of HaCaT cells after 24, 48, and 72 h

402 of incubation with CH, CP9, and CP10 films. Bars represent mean ± SD with different letters (A-B) are

403 significantly different at $p < 0.05$, n = 3.

404

405 **3.3.2 Cell proliferation of HaCaT cells**

406 The cell proliferation of HaCaT cells is illustrated in **Fig. 4**. The results showed that the number of HaCaT cells

407 increased from 24 h to 72 h, and no significant differences existed between control and treated HaCaT cells after

408 24, 48, and 72 h, except for the CH film at 72 h. The results suggest that including PVA, COS, and gallic acid

409 does not hinder the growth of cell numbers but rather increases HaCaT cell numbers, reconfirming the non-toxic
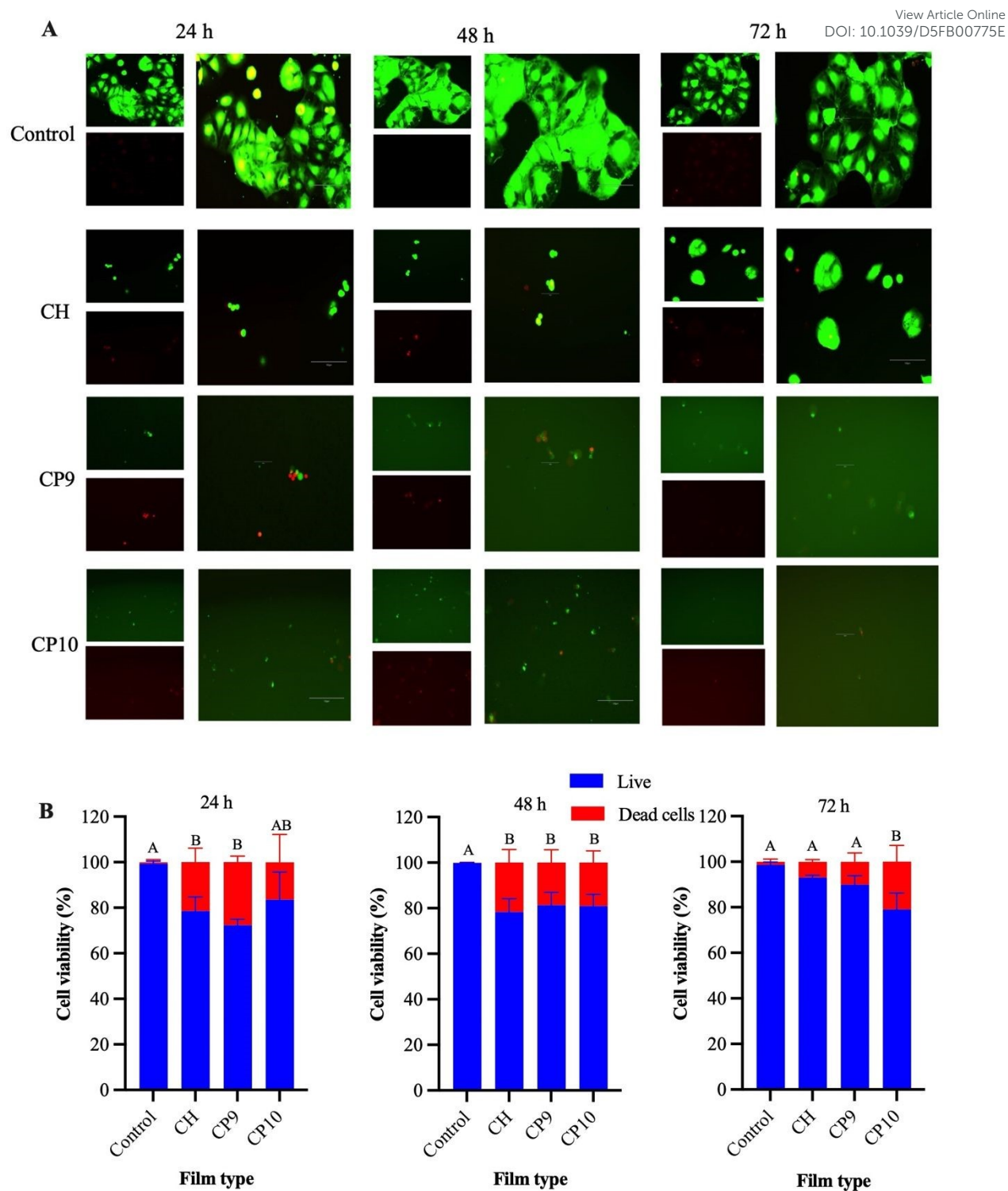
410 properties of the developed films.

411

412
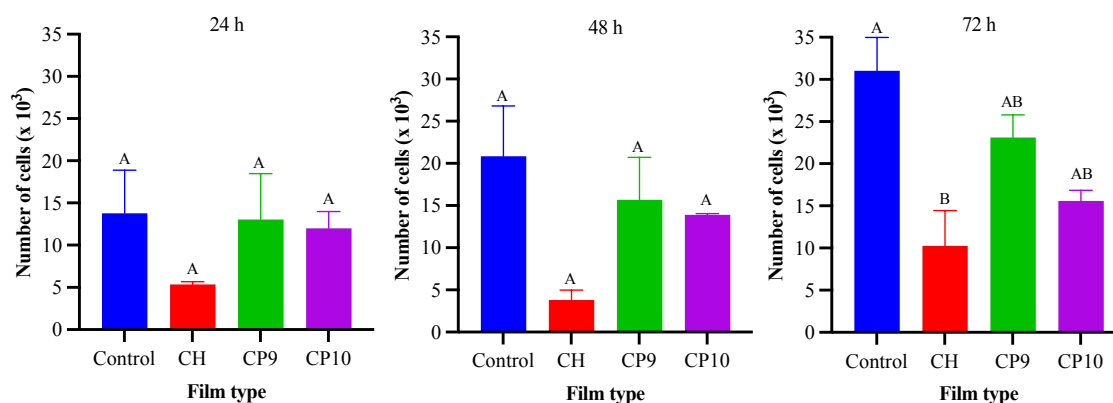
413 **Fig. 4.** Cell proliferation of HaCaT cells after 24, 48, and 72 h of incubation with CH, CP9, and CP10 films. Bars

414 represent mean ± SEM (Standard Error of the Mean) with different letters (A-B) that are significantly different at

415 $p < 0.05$, n = 4.

416

417 **3.3.3 Cell viability of Caco-2 cells**

418 The cell viability of Caco-2 cells is shown in **Fig. 5**. The green and red fluorescence showed live and dead cells,

419 respectively. The higher number of live cells than dead cells in the graph confirmed the positive viability of Caco-

420 2 cells toward CH, CP9, and CP10 films. The Caco-2 cells exhibited good viability, more than 75% after 24 h and

421 80% at 48 and 72 h in all tested films. Additionally, no significant differences ($p < 0.05$) exist between the control

422 and developed films at 72 h, except for the CP10 film. The current research findings are consistent with Liu et al.

423 [47], who reported that adding gelatine and 3-phenyllactic acid in the development of chitosan film demonstrated

424 good biocompatibility on Caco-2 cells. The concentration of gallic acid in the chitosan film could affect the cell

425 viability of Caco-2 cells; therefore, the percentage of live cells was significantly reduced in the CP10-treated film

426 compared to the control, CH, and CP9 films. Forester & Waterhouse [48] reported that the increasing gallic acid

427 concentration significantly inhibits the growth of Caco-2 cells. However, in the present study, all films, including

428 CP10, showed more than 75% cell viability, revealing that the fabricated films are suitable for packaging.
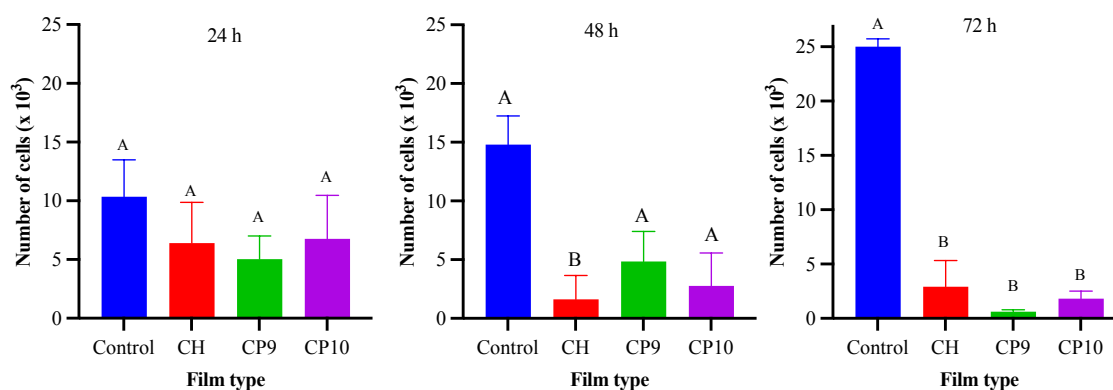
Fig. 5. Dye-based fluorescent microscopy images (A) and cell viability (B) of Caco-2 cells after 24, 48, and 72 h of incubation with CH, CP9, and CP10 films. Bars represent mean ± SD with different letters (A-B) are significantly different at $p < 0.05$, $n = 3$.

3.3.4 Cell proliferation of Caco-2 cells

435 The cell proliferation of Caco-2 cells is shown in **Fig. 6**. The results depict that the number of HaCaT cells

436 increased from 24 h to 72 h in the control compared to film-treated Caco-2 cells. However, no significant

437 differences ($p > 0.05$) were observed between control and film-treated Caco-2 cells at 24 and 48 h, except for the

438 CH film at 48 h. At 72 h, Caco-2 cells treated with films exhibited significantly less proliferation than control.

439 The proliferation of Caco-2 cells depends on the viscosity and molecular weight of chitosan and the film-forming

440 matrix [49, 50]. Additionally, the gallic acid content in the chitosan film could be attributed to minimising the

441 proliferation of Caco-2 cells. Some studies stated that gallic acid demonstrated anti-proliferative effects towards

442 colorectal cancer cell models, including Caco-2 cells [48, 51]. Chitosan film containing gallic acid in DMEM media

443 may generate H_2O_2, increasing cell auto-oxidation and reducing cell proliferation. The findings coincided with

444 Mu & Kitts [52] who described that phenolic acid, especially gallic acid showed the greatest anti-proliferation

445 properties against Caco-2 cells by generating H_2O_2 in DMEM media. Some researchers reported that gallic acid

446 did not affect the proliferation of the normal cell lines compared to cancer cells [53]. Thus, fabricated films could

447 be suitable as food packaging materials. While our current findings suggest suitability for packaging applications,

448 we do not establish a safety margin for oral exposure at this stage. Therefore, the developed material cannot yet

449 be classified as edible packaging, and comprehensive *in vivo* testing together with exposure-based risk assessment

450 is required to confirm its safety for human consumption.

451

452

453 **Fig. 6.** Cell proliferation of Caco-2 cells after 24, 48, and 72 h of incubation with CH, CP9, and CP10 films. Bars

454 represent mean ± SEM with different letters (A-B) are significantly different at $p < 0.05$, n = 4.

455

456 **3.4 Application of film in tomato and green grape packaging**

457 The appearance and weight loss of tomatoes under different pack-film conditions are presented in **Fig. 7A and**

458 **7B**. It was observed that the NP tomatoes gradually lost their appearance, and the surface became wrinkly due to

459   the loss of turgidity in the cells. However, no microbial growth was seen in NP tomatoes compared to other film-

460   packed groups. This observation could be explained by the fact that the NP samples, being without lid and film,

461   did not accumulate moisture droplets from the respiration of the tomatoes. The development of humidity inside

462   the film-packed (Control-CP10), which created a suitable atmosphere for the growth of microbes. In the case of

463   the control group, microbial growth was observed on day 2. In contrast, the development of microbes in the film-

464   packed tomatoes with CH, CP9, and CP10 films was detected on days 3, 4, and 5, respectively. In other words,

465   films such as CP10 delayed the onset of microbial growth. The current research findings confirm the point that

466   the fabricated films had antimicrobial ability that extended the shelf life of the tomatoes. On day 1, all the tomatoes

467   (NK: $3.48 \pm 1.76\%$, control: $2.30 \pm 0.38\%$, CH: $1.64 \pm 0.38\%$, CP9: $1.94 \pm 0.04\%$, CP10: $1.25 \pm 0.07\%$) showed

468   more or less similar weight loss. However, by day 7, the NK group demonstrated the highest weight loss ($37.5 \pm$

469   1.32%), followed by control ($16.57 \pm 1.11\%$), CH ($14.29 \pm 1.25\%$), CP9 ($13.56 \pm 1.5\%$), and CP10 ($9.63 \pm 0.55\%$).

470   The findings are similar to those of Gasti et al. [54] who reported unpacked green chillies demonstrating the highest

471   weight loss compared to chitosan and gallic acid-loaded chitosan film-packed green chillies.

472   A similar observation was made for the grape samples. Non-packaged green grapes demonstrated the

473   highest weight loss ($27.11 \pm 0.62\%$) and shrivelling during the storage periods compared to film-packed green

474   grapes (Control: $15.95 \pm 0.25\%$, CH: $14.77 \pm 1.2\%$, CP9: $13.1 \pm 0.94\%$, and CP10: $13.54 \pm 0.47\%$) (**Fig. 7C and**

475   **7D**). The current research findings align with Zhao et al. [11] who observed the highest percentage of fresh-cut apple

476   weight loss in an unpacked group compared to apple storage with chitosan film and PVA-gallic acid incorporated

477   chitosan film. Another study reported that non-packaged kiwifruit demonstrated shrinking and the highest weight

478   loss compared to packing kiwifruit with polylactic acid film containing chitosan and alizarin [36]. The growth of

479   microbes was not observed with the naked eye in green grapes during the entire storage. This could be possible

480   due to the presence of phenolic compounds, including tannins, anthocyanins, and flavonols, in grapes, which have

481   preventive ability towards microbial growth [55]. Sun et al. [56] reported that unpacked grapes (*Vitis vinifera* L. Kyoho)

482   demonstrated microbial growth and spoilage signs after 14 days of storage periods. In contrast, grapes packaged

483   with chitosan enriched with montmorillonite and lauroyl arginate ethyl showed no microbial growth up to 20 days

484   of storage periods.
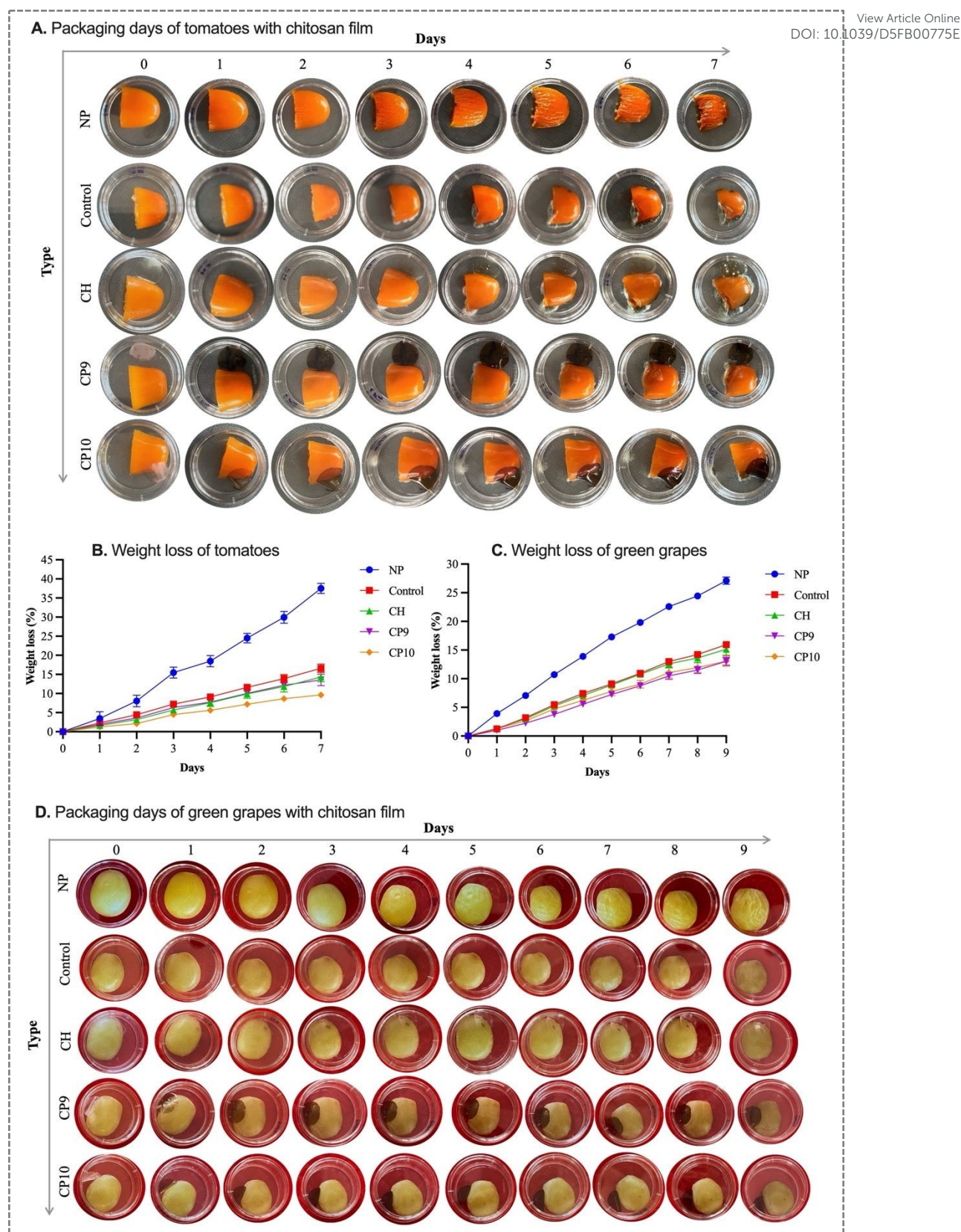
Fig. 7. Application of chitosan films (CH, CP9, and CP10) in freshness evaluation of tomatoes (A) and green

grapes (D) stored at room temperature (23°C) for 7 and 9 days, respectively and their changes of weight loss (B

21

488 and C). Non-packaging (NP): Tomatoes or green grapes without a lid and film. Control: Tomatoes or green grapes

489 in a Petri dish without film. CH: Tomatoes or green grapes with a lid and chitosan film.

490

3.5 Application of film in pearl fish fillet packaging

492 The appearance, drip loss, pH, and TVBN value of pearl fish fillets under film-packed are presented in **Fig. 8**. It

493 can be seen that the pearl fish fillets look shiny and show a 'fresh' colour on day 0 compared to day 2. Also, the

494 shrinkage of pearl fish fillets was observed from day 2, especially in the NP group (**Fig. 8A**). There were

495 significant differences in the drip loss of pearl fish fillets between the NP group and the film-packed group (**Fig.**

496 **8B**). The non-packaging and control pearl fish fillets demonstrated 16.27% and 3.33% drip loss on day 2,

497 respectively, whereas film-packed group showed below 3.22% drip loss. However, there are no significant

498 differences in the drip loss of pearl fish fillets between the control and film-packed groups. This observation could

499 be attributed to the fact that, in the current study, a small portion of the films was used, as compared to other

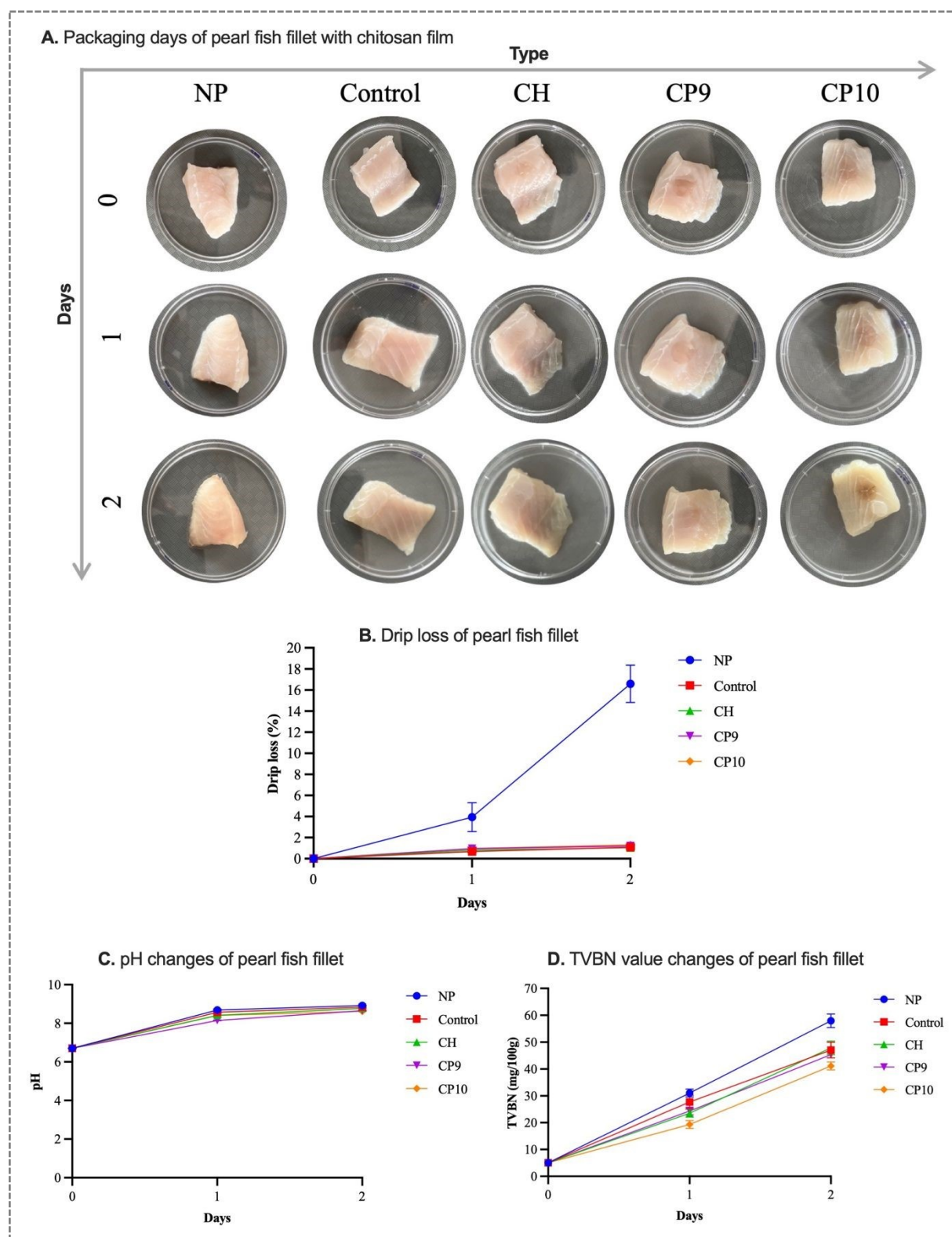500 studies where whole fish fillets were wrapped in the packaging film [36, 57].

501 The pH of fresh pearl fish fillets was 6.7 ± 0.02, and it can be seen from **Fig. 8C** that the pH of both the

502 film-packed and NP groups of pearl fish fillets increased during storage. The pH of the non-packaging group pearl

503 fish was increased to 8.92 ± 0.03 on day 2, followed by control (8.82 ± 0.01), CH (8.76 ± 0.03), CP9 ($8.64 \pm$

504 0.01), and CP10 (8.61 ± 0.01) films. The pH value of pearl fish fillets was significantly lower in the CP9 and

505 CP10 film-packed fish compared to the NP, control, and CH packaging groups. The findings indicate that CP9

506 and CP10 films in packaging pearl fish could minimise the microorganism action and inhibit chemical

507 deteriorative action, resulting in lower pH compared to other groups. The findings are consistent with the work of

508 Yan et al. [58], Amaregouda et al. [59], and Wu et al. [36].

509 The TVBN (mg/100g) of fresh pearl fish fillets was 5.04 ± 0.01. The TVBN of NP and film-packed pearl

510 fish fillets was increased during storage (**Fig. 8D**). The TVBN value of NP (31.08 ± 1.45) and control ($27.72 \pm$

511 2.52) pearl fish fillets was significantly higher than that of CH (23.51 ± 1.44), CP9 (24.36 ± 1.44), and CP10

512 (19.32 ± 1.46) film-packed fish fillets on day 1. However, the TVBN value increased to 57.96, 47.04, and 47.88

513 on day 2 in NP, control, and CH-packed pearl fish fillets, respectively, which is significantly higher than the

514 changes observed in CP9 (45.36) and CP10 (41.16) films. The findings imply that fabricated films could minimise

515 the decomposition of pearl fish fillets by diminishing antimicrobial action and lipid oxidation [60]. The findings in

516 this study align with Chen et al. [61] and Dong et al. [62] develop active packaging film for hairtail fish and shrimp

517 for shelf life extension an identified lowered TVBN compared to control, respectively. Also, several research

518   articles stated that fish are considered spoiled when the TVBN value exceeds 30 mg/100g [63, 64]. In the current

519   study, the TVBN value of pearl fish fillets was much lower in CP9 and CP10 film-packed fish on day 1; however,

520   it exceeded the 30 mg/100g cutoff value on day 2. These findings indicate that the films can potentially extend

521   the shelf life of pearl fish fillets.

A. Packaging days of pearl fish fillet with chitosan film

B. Drip loss of pearl fish fillet

C. pH changes of pearl fish fillet

D. TVBN value changes of pearl fish fillet

522

523   **Fig. 8.** Application of chitosan films in freshness evaluation of pearl fish fillet (A) stored at room temperature

524   (23ºC) for two days, and their changes of drip loss (B), pH (C), and TVBN (D). Non-packaging (NP): Pearl fish

525   fillet without a lid and film, Control: Pearl fish fillet in the Petri dish without film, and CH: Pearl fish fillet with

526   a lid and chitosan film.

527

528   **4. Conclusion**

529   In the present research work, the functional properties of chitosan films containing PVA, COS, and gallic acid

530   were investigated and evaluated. The COS and gallic acid-loaded chitosan-PVA films showed the highest

531   scavenging capacity towards DPPH and ABTS radicals, and the highest FRAP was obtained from the film

532   containing 10% COS and 10% gallic acid (CP10). In addition, CP10 film demonstrated the highest antimicrobial

533   efficacy towards *Escherichia coli* and *Listeria innocua*, and CP9 exhibited the highest leakage of cellular

534   components towards *Saccharomyces cerevisiae*. Also, the highest cell viability of HaCaT and Caco-2 cells was

535   observed on CH and CP9 films compared to CP10. Moreover, the application of CP9 and CP10 films on tomatoes

536   and green grapes showed the lowest weight loss, and pearl fish fillets demonstrated the lowest pH and TVBN

537   compared to CH, control, and no-packaging groups. Based on current research findings, chitosan films containing

538   PVA, COS, and gallic acid could be considered functional and suitable packaging materials to extend the shelf

539   life of perishable foods. Further studies may be necessary to examine the release profile of COS and gallic acid,

540   focusing on their effects on cellular responses, microbial load data, and the quality indices of tomatoes and green

541   grapes. Furthermore, a shelf ageing study of the packaging film could be conducted to evaluate the long-term

542   stability of its functional properties during storage. This research could improve the understanding of the film's

543   functional performance and the synergistic interactions between COS and gallic acid in practical food packaging

544   applications.

545

546   **Data availability**

547   The authors affirm that the data supporting this study's findings are available within the research article and will

548   also be shared upon request.

549

550   **Author contribution**

Sustainable Food Technology Accepted Manuscript

551 **Shuva Bhowmik:** Conceptualization, Investigation, Methodology, Data curation, Visualization, Writing-Original

552 draft. **Dominic Agyei:** Supervision, Writing-review & editing. **Azam Ali:** Supervision, Conceptualization, Project

553 Administration, Writing-review & editing.

554

558

559 **Declaration of competing interest**

560 The authors declare that they have no conflict of interest.

561

562

563 **References**

564 1.   F. Ghaderi, A. Shakerian, Z. Mashak, E. Rahimi and S. M. Jafari, *Journal of Food Measurement and*

565   *Characterization*, 2024, 1–18.

566 2.   V. Guillard, S. Gaucel, C. Fornaciari, H. Angellier-Coussy, P. Buche and N. Gontard, *Frontiers in*

567   *nutrition*, 2018, **5**, 121.

568 3.   Y. Yin and M. W. Woo, *Sustainable Food Technology*, 2024.

569 4.   K. Ma, F. Li, T. Zhe, X. Sun, X. Zhang, P. Wan, H. Na, J. Zhao and L. Wang, *Food Chemistry*, 2024,

570   **435**, 137552.

571 5.   H. A. Leslie, M. J. Van Velzen, S. H. Brandsma, A. D. Vethaak, J. J. Garcia-Vallejo and M. H. Lamoree,

572   *Environment international*, 2022, **163**, 107199.

573 6.   I. Hamed, A. N. Jakobsen and J. Lerfall, *Comprehensive Reviews in Food Science and Food Safety*,

574   2022, **21**, 198–226.

575 7.   J. Paul, J. Jacob, M. Mahmud, M. Vaka, S. G. Krishnan, A. Arifutzzaman, D. Thesiya, T. Xiong, K.

576   Kadirgama and J. Selvaraj, *International Journal of Biological Macromolecules*, 2024, 130850.

577 8.   Q.-b. Yao, F. Huang, Y.-h. Lu, J.-m. Huang, M. Ali, X.-Z. Jia, X.-A. Zeng and Y.-y. Huang, *Trends in*

578   *Food Science & Technology*, 2024, 104390.

579 9.   S. Bhowmik, D. Agyei and A. Ali, *Food Packaging and Shelf Life*, 2022, **34**, 100962.

580   10.   A. A. Hunashyal, S. P. Masti, L. K. Kurabetta, M. N. Gunaki, S. Madihalli, J. P. Pinto, M. B.

581   Megalamani, B. Thokchom, R. B. Yarajarla and R. B. Chougale, *Sustainable Food Technology*, 2025,

582   **3**, 2088–2107.

583   11.   Y. Zhao, L. Yang, M. Xu, H. Wang, X. Gao, B. Niu and W. Li, *International Journal of Biological*

584   *Macromolecules*, 2022, **222**, 2987–3000.

585   12.   J. P. Pinto, M. H. Anandalli, A. A. Hunashyal, Priyadarshini, S. P. Masti, R. B. Chougale, V. Gudihal

586   and R. F. Bhajantri, *Journal of Materials Science: Materials in Electronics*, 2025, **36**, 1035.

587   13.   Q. Zhou, W. Lan and J. Xie, *International Journal of Biological Macromolecules*, 2024, **254**, 127917.

588   14.   A. Singh, A. Mittal and S. Benjakul, *Food reviews international*, 2023, **39**, 2297–2319.

589   15.   Y. Sun, J. Cui, L. Tian, Y. Mi and Z. Guo, *Marine Drugs*, 2023, **21**, 535.

590   16.   W. Weian, Y. Yunxin, W. Ziyan, J. Qianzhou and G. Lvhua, *Biomaterials Science*, 2024.

591   17.   T. Hou, R. Venkatesan, T. Dhilipkumar, V. Mayakrishnan, C. J. Raorane, S. S. Sana, M. A. Ansari and

592   S.-C. Kim, *International Journal of Biological Macromolecules*, 2025, **285**, 138276.

593   18.   Y. Yuan, W. Tan, C. Lin, J. Zhang, Q. Li and Z. Guo, *Food Hydrocolloids*, 2023, **138**, 108431.

594   19.   M. Zhang, B. Yang, Z. Yuan, Q. Sheng, C. Jin, J. Qi, M. Yu, Y. Liu and G. Xiong, *Food Chemistry: X*,

595   2023, 100782.

596   20.   E. Messinese, O. Pitirollo, M. Grimaldi, D. Milanese, C. Sciancalepore and A. Cavazza, *Food and*

597   *Bioprocess Technology*, 2024, **17**, 606–627.

598   21.   M. N. Gunaki, S. P. Masti, L. K. Kurabetta, S. Madihalli, A. A. Hunashyal, R. B. Chougale, V.

599   Holeyannavar and S. kumar Vootla, *Journal of Environmental Chemical Engineering*, 2025, 118397.

600   22.   B. Tian, J. Liu, W. Yang and J.-B. Wan, *Journal of Agricultural and Food Chemistry*, 2023, **71**, 1325–

601   1347.

602   23.   N. Kumar, Pratibha, J. Prasad, A. Yadav, A. Upadhyay, Neeraj, S. Shukla, A. T. Petkoska, Heena and S.

603   Suri, *Food Engineering Reviews*, 2023, **15**, 718–747.

604   24.   M. Rajabi, J. Cabral, S. Saunderson and M. A. Ali, *Carbohydrate Polymers*, 2022, **295**, 119884.
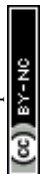
605   25.   S. Bhowmik, D. Agyei and A. Ali, *Cellulose*, 2024, 1–17.

606   26.   A. Riaz, S. Lei, H. M. S. Akhtar, P. Wan, D. Chen, S. Jabbar, M. Abid, M. M. Hashim and X. Zeng,

607   *International journal of biological macromolecules*, 2018, **114**, 547–555.

608   27.   M. Božič, S. Gorgieva and V. Kokol, *Carbohydrate Polymers*, 2012, **87**, 2388–2398.

609   28.   S. Gulzar, M. Tagrida, K. Nilsuwan, T. Prodpran and S. Benjakul, *Food Hydrocolloids*, 2022, **133**,

610   107916.

611   29.   J. Bi, C. Tian, G.-L. Zhang, H. Hao and H.-M. Hou, *Food Chemistry*, 2021, **365**, 130534.

612   30.   M. B. Sadiq, J. Tarning, T. Z. Aye Cho and A. K. Anal, *Molecules*, 2017, **22**, 47.

613   31.   S. S. Sali, M. L. Gould, M. Qasim and M. A. Ali, *Journal of Materials Chemistry B*, 2021, **9**, 1557–1567.

614   32.   D. S. B. Anugrah, G. Delarosa, P. Wangker, R. Pramitasari and D. Subali, *Packaging Technology and*

615   *Science*, 2023.

616   33.   M. L. Gould, J. T. Ratnayake, N. Ramesh, T. J. Powlay, O. J. Curnow, M. P. Staiger and G. J. Dias,

617   *Journal of Polymers and the Environment*, 2023, **31**, 1335–1350.

618   34.   B. Mahaling, N. Pandala, H.-C. Wang and E. B. Lavik, *ACS bio & med Chem Au*, 2022, **2**, 499–508.

619   35.   X. Deng, M. Gould, R. Katare and M. Ali, *Biomedical Materials*, 2024, **19**, 055007.

620   36.   Y. Wu, Y. Ma, Y. Gao, Y. Liu and C. Gao, *International Journal of Biological Macromolecules*, 2022,

621   **214**, 348–359.

622   37.   G. Pang, C. Zhou, X. Zhu, L. Chen, X. Guo and T. Kang, *Journal of Food Safety*, 2023, **43**, e13045.

623   38.   H. Lee, M. S. Kim, W.-H. Lee and B.-K. Cho, *Sensors and Actuators B: Chemical*, 2018, **259**, 532–539.

624   39.   X. Zhang, J. Liu, C. Qian, J. Kan and C. Jin, *Food hydrocolloids*, 2019, **89**, 1–10.

625   40.   C. R. Lee, S. J. Lee, T. I. Kim, K. Chathuranga, J. S. Lee, S. Kim, M. H. Kim and W. H. Park, *Food*

626   *Chemistry*, 2025, **463**, 141322.

627   41.   A. Mittal, A. Singh, S. Benjakul, T. Prodpran, K. Nilsuwan, N. Huda and K. de la Caba, *Food*

628   *hydrocolloids*, 2021, **111**, 106384.

629   42.   S. R. Kanatt, M. Rao, S. Chawla and A. Sharma, *Food hydrocolloids*, 2012, **29**, 290–297.

630   43.   Z. Fang, W. Cong, H. Zhou, J. Zhang and M. Wang, *Journal of Functional Foods*, 2024, **116**, 106219.

631   44.   S. Keyvani-Ghamsari, M. Rahimi and K. Khorsandi, *Food Science & Nutrition*, 2023, **11**, 5856–5872.

632   45.   X. Yang, B. Wang and D. Sha, *Journal*, 2021.

633   46.   Z. Riaz, S. Baddi, F. Gao and C.-L. Feng, *European Polymer Journal*, 2024, **206**, 112778.

634   47.   Y. Liu, R. Wang, D. Wang, Z. Sun, F. Liu, D. Zhang and D. Wang, *Food Hydrocolloids*, 2022, **127**,

635   107546.

636   48.   S. C. Forester and A. L. Waterhouse, *Journal of agricultural and food chemistry*, 2010, **58**, 5320–5327.

637   49.   I. Salcedo, C. Aguzzi, G. Sandri, M. C. Bonferoni, M. Mori, P. Cerezo, R. Sánchez, C. Viseras and C.

638   Caramella, *Applied clay science*, 2012, **55**, 131–137.

639   50.   M. C. Di Santo, A. Alaimo, A. P. D. Rubio, R. De Matteo and O. E. Pérez, *Biochemistry and biophysics*
640   *reports*, 2020, **24**, 100842.

641   51.   I. Y. Ho, A. Abdul Aziz and S. Mat Junit, *Scientific reports*, 2020, **10**, 9987.

642   52.   K. Mu and D. D. Kitts, *Journal of Agricultural and Food Chemistry*, 2023, **71**, 3022–3032.

643   53.   E.-B. Ko, Y.-G. Jang, C.-W. Kim, R.-E. Go, H. K. Lee and K.-C. Choi, *Biomolecules & Therapeutics*,
644   2021, **30**, 151.

645   54.   T. Gasti, S. Dixit, R. B. Chougale and S. P. Masti, *Sustainable Food Technology*, 2023, **1**, 390–403.

646   55.   S. Augustine, V. Kudachikar, V. Vanajakshi and R. Ravi, *Journal of food science and technology*, 2013,
647   **50**, 332–338.

648   56.   Z. Sun, J. Hao, H. Yang and H. Chen, *Food and Bioprocess Technology*, 2018, **11**, 1853–1862.

649   57.   L. Zhang, D. Yu, Y. Xu, Q. Jiang, W. Xia and D. Yu, *Food Bioscience*, 2023, **54**, 102941.

650   58.   J. Yan, R. Cui, Y. Qin, L. Li and M. Yuan, *International Journal of Biological Macromolecules*, 2021,
651   **177**, 328–336.

652   59.   Y. Amaregouda, K. Kamanna and T. Gasti, *International Journal of Biological Macromolecules*, 2022,
653   **218**, 799–815.

654   60.   W.-Y. J. Kam, H. Mirhosseini, F. Abas, N. Hussain, S. Hedayatnia and H.-L. F. Chong, *Food Control*,
655   2018, **90**, 66–72.

656   61.   M. Chen, T. Yan, J. Huang, Y. Zhou and Y. Hu, *International Journal of Biological Macromolecules*,
657   2021, **179**, 90–100.

658   62.   S. Dong, Y. Zhang, D. Lu, W. Gao, Q. Zhao and X. Shi, *Food Packaging and Shelf Life*, 2023, **35**,
659   101022.

660   63.   F. Wang, C. Xie, H. Tang, H. Li, J. Hou, R. Zhang, Y. Liu and L. Jiang, *International Journal of*
661   *Biological Macromolecules*, 2023, **252**, 126423.

662   64.   Y. Liu, J. Chen, H. Li and Y. Wang, *International Journal of Biological Macromolecules*, 2024, **259**,
663   128934.

664

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Data availability

The authors affirm that the data supporting this study's findings are available within the research article and will

also be shared upon request.

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