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1 **Incorporation of commercially-derived antimicrobials into gelatin-based films and**
2 **assessment of their antimicrobial activity and impact on physical film properties**

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16 **KEYWORDS:** Active packaging; Antimicrobials; Heat treatment; Antimicrobial coatings;
17 Food Packaging, Gelatin films

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29 **Abstract**

30 Four antimicrobials, namely; Articoat DLP 02 (AR), Artemix Consa 152/NL (AX), Auranta
31 FV (AFV) and sodium octanoate (SO) were examined for their effectiveness, both before and
32 after heat treatments, against bacterial strains *Bacillus cereus*, *Pseudomonas fluorescens*,
33 *Escherichia coli*, *Staphylococcus aureus* and the microflora obtained from commercial beef
34 steaks. Minimum inhibitory concentrations (MIC) using AR, AX, AFV and SO against these
35 microbes were then obtained using the 96-well plate method. SO was the most effective
36 against all bacterial strains, demonstrating the lowest MIC compared to the other
37 antimicrobials. These antimicrobials were then successively incorporated into beef-derived
38 gelatine films and these films were subsequently tested for structural, mechanical and barrier
39 properties. Significantly ($p < 0.05$) enhanced water vapour barrier properties were determined
40 only for antimicrobial films containing AX or SO when compared to control films. On the
41 basis of FTIR spectra, significant changes in the structure of SO-containing films were
42 determined when compared with control gelatin films. It was shown that active antimicrobial
43 agents could potentially serve as commercial antimicrobial coatings for application onto
44 conventional plastic-based food packaging.

45 **1. Introduction**

46 Microbial contamination has been recognised as the main source for spoilage of food.
47 Common methods used today to preserve foods include fermentation, freezing, drying,
48 modified atmosphere packaging (MAP), vacuum packaging, the addition of preservatives and
49 the use of antimicrobial agents such as salts, organic acids and plant extracts (Quintavalla &
50 Vicini, 2002).

51 When controlling food spoilage using antimicrobial agents, such substances are typically
52 introduced to the food by direct contact. While this approach results in immediate reduction
53 of bacteria, cells which have been sub-lethally damaged will recover and those which were
54 not impacted upon at all will continue to grow. However, if the same antimicrobial
55 substances could be incorporated into packaging materials to create antimicrobially-active
56 packaging, such materials might be more effective in inhibiting the growth of
57 microorganisms in food. Depending on how these antimicrobials react with the matrix, release
58 rate and possible degradation a continuous antimicrobial effect can be observed, thereby,
59 extending product shelf-life (Appendini & Hotchkiss, 2002).

60 The development of biodegradable and edible films used for food packaging has received
61 much interest in recent years due to growing concerns about environmental pollution caused
62 by non-biodegradable packaging materials (Bravin et al, 2006). Newly developed
63 biodegradable forms of active packaging materials could provide for niche antimicrobial
64 applications. Polymers derived from gelatin have unique and interesting properties due to its
65 low melting point (Bergo & Sobral, 2007). Films formed using gelatin sources might be
66 desirable to manufactures due to their edible or biodegradable nature, low cost and wide
67 availability of gelatin sources (Wang et al, 2007 ; Hanani et al, 2012; Park et al, 2008).
68 Gelatin has a unique sequence of amino acids, coupled with a high content of proline, glycine
69 and hydroxyproline, thereby assisting in polymer production.

70 Irrespective of their make-up or composition, antimicrobial films are designed to allow
71 migration or contact of the incorporated antimicrobial agent to the surface of the food in a
72 number of ways, thereby, delivering a continuous antimicrobial effect during exposure.
73 Compounds such as essential oils, organic acids and enzymes have been assessed for
74 antimicrobial activity in food packaging (Malhotra et al, 2015).

75 While the introduction of active substances such as essential oils and plant extracts can
76 improve the antimicrobial and antioxidant properties of the films, thus improving stability
77 and quality during storage (Gómez-Estaca et al, 2007; Kim et al., 2006)., the addition of such
78 extracts could also potentially alter the mechanical and barrier properties of such films
79 (Gómez-Estaca et al, 2009; Gómez-Estaca et al, 2007). Any substance to be introduced into a
80 food product must be pasteurised at a minimum of 70°C in order to be deemed acceptable for
81 food contact. Packaging processes and treatments at the point of manufacture and post-
82 manufacture (such as hot filling at ~100°C, UHT at ~ 135°C etc.) might affect the properties
83 of incorporated antimicrobials (Manfredi & Vignali, 2015; Deeth, 2010). There may be a
84 potential in adding antimicrobial agents into the packaging during the processing stage.
85 However, antimicrobials are sensitive to film process conditions. Packaging materials such as
86 casted Polypropylene (CPP) and Polyethylene terephthalate (PET) can be treated by
87 antimicrobial agents will occur (Jari et al, 2003).

88 Antimicrobials AR and AFV used in this trial where composed of a mixture organic acids
89 such as lactic acid, citric acid and caprylic acid have long been generally recognised as safe
90 (GRAS) food preservatives, with a demand for such natural acids to be incorporated into
91 foods today (Burt, 2004; Cruz-Romero et al., 2013).

92 The objective of this experiment was to evaluate the effectiveness of antimicrobials, as food
93 coatings, incorporated into gelatin-based films and to assess the effectiveness of the film
94 forming solutions at different processing temperatures, as well as determining the impact of
95 these coatings on film properties.

96 **2. Materials and methods**

97 **2.1. Materials and microbiological media**

98 The antimicrobials used in this trial were Articoat –DLP-02 (sodium diacetate, lactic acid,
99 acetic acid, citric acid, in pectin and water), Artimex 152/NL (sodium citrate, sodium
100 ascorbate, sodium metabisulphite) were both obtained from Chemital (Chemital, Barcelona,
101 Spain), Sodium octanoate (Caprylic acid salt) obtained from (Sigma Aldrich, Kent, England)
102 and Auranta FV (Citric acid, malic acid, lactic acid and caprylic acid in water) was obtained
103 from (Envirotec, Ireland). These solubilisates were diluted with sterile distilled water to 2%,
104 3%, 4.5%, 6%, 9% and 12% of the active antibacterial content. Beef gelatin 100 bloom
105 (Helan ingredients UK) was used as the basal material for all film matrices, glycerol (KB
106 Scientific limited) was used as plasticizer, Muller Hilton agar (Oxoid LTD, Basingstoke,
107 England), Plate count agar, Muller Hilton Broth (Oxoid LTD, Basingstoke, England) where
108 used as general-purpose media, and Maximum recovery diluent (Oxoid LTD, Basingstoke,
109 England) was used as an isotonic diluent for maximum recovery of microorganisms.

110 **2.2. Bacterial strains and sample preparation**

111 The following bacterial strains were chosen to evaluate the antimicrobial effectiveness of the
112 chemical agents selected for use in this study: *Escherichia coli* (*E. coli*): (NCIMB 11943),
113 *Staphylococcus aureus* (*S. aureus*): (NCIMB 13062), *Bacillus cereus* (*B. cereus*): (NCIMB
114 9373) and *Pseudomonas fluorescens* (*Ps. fluorescens*): (NCIMB 9046). A microflora isolated
115 from raw beef sourced from a local retail outlet was also used. To prepare these samples, 10 g
116 samples of beef fillets were placed in Stomacher bags along with 90 ml of Mueller Hinton
117 broth and homogenised for 3 minutes in a Colworth Stomacher 400 (Seward Ltd., England).
118 A total of 10 ml of the resulting homogenate was then incubated for 18 hr at 37°C prior to
119 testing. For all tests, Mueller-Hinton broth (MHB) and maximum recovery diluent (MRD)
120 was used as growth media and for any culture dilution, respectively.

121

122 **2.3. Heat treatment of Antimicrobials**

123 Antimicrobials undiluted were aseptically weighed out and placed into sterile 50 ml Pyrex
124 glass bottles and heat-treated at five different temperatures using a binder oven, namely;
125 75°C, 100°C, 135°C, 170°C and 270°C for 15 min in accordance with published guidelines
126 (Hsieh et al, 2011). A sterile thermometer was used to determine that the required
127 temperature had been reached. The antimicrobials were allowed to cool to below 40°C at
128 room temperature before application.

129 **2.4. Determination of MIC**

130 Antimicrobial activity of AFV, AX, AR, and SO was assessed by determining the MIC in a
131 96 flat-bottomed-well tissue culture microplate using an alpha numeric coordination system
132 (Columns 1–12 and Rows A-H) (Sarstedt Inc., NC, USA) according to the NCCLS broth
133 microdilution method (NCCLS, 2000) using a cation-adjusted Mueller Hinton Broth. Briefly,
134 a standardised inoculum for the antimicrobial assay was prepared by making serial dilutions
135 of an overnight grown culture using sterile Mueller Hinton broth in order to obtain a
136 concentration of 5×10^5 CFU/ml. The final concentration of the microbial load was then
137 determined by using the spot plate method according to Gaudy et al, (2011). The culture
138 suspension was used within 15 min of preparation. A 200 µL volume of the culture
139 suspension was added to wells in Row H, Columns 1–11 and 200 µL volume of sterile
140 Mueller Hinton broth added to Column 12. In each well of Row G, 200 µL of the
141 antimicrobial substances diluted in sterile water were dispensed. Using a 12-channel pipette,
142 50 µL of each antimicrobial substance were serially transferred from the well in row G into
143 the corresponding wells through to row B. After mixing, 50 µL was removed from each well
144 in row B and discarded. Positive and negative growth controls were included in each assay
145 plate. Lastly, using a 12-channel pipette, after mixing the standardised inoculum culture in
146 wells in row H, 15 µL of the test microorganisms were carefully pipetted from each well in
147 Row H to the parallel wells in Row A, followed by Rows B to G. Afterwards the inoculated
148 plates were incubated in a wet chamber for 24 hr at 30°C (*Pseudomonas fluorescens* and *B.*
149 *Cereus*) and 37°C (*E. coli*, *S. aureus* and beef microflora). The lowest concentration of
150 antimicrobial agent presenting an inhibition of growth was considered to be the MIC for the
151 test organism. This procedure was repeated with different percentages of antimicrobials in
152 solution. In order to obtain an anaerobic MIC, 96-well plates were placed into an anaerobic
153 jar during incubation.

154 **2.5. Preparation of film forming solutions**

155 The film forming solutions were prepared according to Wang et al, (2007), with minor
156 modifications. In particular, 100 ml 5% beef gelatine solutions were formulated with the
157 addition of glycerol (33% w/w dry matter). For the control, the solution was heated at 90°C in
158 a shaking water bath at 90 rpm for 30 min and allowed to cool to 40°C at room temperature
159 before use. The antimicrobial solutions of AFV, AX, AR, and SO were formulated as above.
160 After cooling to a temperature of 40°C, antimicrobials were added into gelatine solutions at a
161 concentration of two times the MIC obtained (AFV 3g, AX 6g, AR 9g and SO 2g) and stirred
162 for 15 min using a sterile magnetic stirrer before application. This method was repeated for
163 the heat treated antimicrobials AFV, AX, AR, and SO; again at twice the MIC obtained at
164 each temperature and after a cooling temperature of 40°C was reached.

165 **2.6. Agar diffusion method**

166 The sensitivity of the bacterial strains and native microflora derived from beef to different
167 antimicrobial films was determined by the diffusion-type assay according to Zivanovic et al.,
168 (2005), with minor adjustments. Twice the MIC of antimicrobials was chosen in order to
169 determine the impact that this might have on the antimicrobial, mechanical and barrier
170 properties of beef-derived gelatin films. AR, AX, SO and AFV solutions were prepared as
171 described previously. The solutions were poured into petri dishes (10ml), placed in an
172 equilibrium chamber at 23°C & 50% relative humidity (R.H.) and stored for 48 hr in order to
173 produce films. The films were then aseptically cut into 1 ×1 cm squares and placed on the
174 surface of a previously inoculated Muller Hilton Agar (MHA) agar plate with a culture of the
175 indicator microorganism which had been incubated for 24hr previously (0.1 mL of
176 inoculum: microbial load of 5×10^5 CFU/ml.). After a 24hr incubation of bacteria strains at
177 37°C, antimicrobial effectiveness of films and the inhibition zone area developed around each
178 film square were measured.

179 **2.7. Heat treatment of films**

180 Pre-conditioned films containing untreated antimicrobials were then heat treated at five
181 different temperatures using a binder oven; 75°C, 100°C, 135°C, 170°C and 270°C. A sterile
182 thermometer was used in order to determine that the required temperature was reached. The
183 antimicrobial films were allowed to cool to 40°C at room temperature before testing.

184

185 **2.8. Assay of antimicrobial effectiveness of film forming solutions**

186 The sensitivity of the bacterial strains and native microflora on beef to film-forming solutions
187 without heat treatment was determined by the agar diffusion method. An inhibition zone
188 assay was conducted by inoculating MHA with a culture of the indicator microorganism
189 which had been incubated for 24hr previously (0.1 mL of inoculums: microbial load of $5 \times$
190 10^5 CFU/mL.). The method was conducted according to the methodology previously described
191 by Ponce et al. (2003) with minor modifications. Disks (6mm) were aseptically placed onto
192 the surface of inoculated MHA plates. A 20 μ L volume of the different antimicrobial
193 solutions were placed aseptically onto 6 mm disks on MHA agar using a sterile pipette. The
194 petri-dishes were incubated at 37°C for 24hr and the effectiveness and inhibition zones, if
195 present, were measured. This assay was repeated for twice and three-times the MIC obtained.
196 The microbial sensitivity to the different antimicrobial solutions was classified as: not
197 sensitive, sensitive by contact or diameter of halo sensitive. Each assay was performed in
198 triplicate in 3 independent experimental runs. For each test, growth controls without adding
199 the film-forming solutions were inoculated to ensure that viable organisms were present. This
200 method was repeated for the treated antimicrobial solutions AFV, AX, AR, and SO after a
201 cooling temperature of 40°C was reached.

202 **2.9. Film Characterisation**

203 Before measuring, films were pre-conditioned for at least 48 hr in an environmental chamber
204 set at 23° C and 50% relative humidity.

205 **2.9.1 Film thickness**

206 Thicknesses of all gelatine films with antimicrobials was measured using a digital micrometer
207 (Käfer Digital Thickness gauge, Käfer Messuhrenfabrik GmbH & Co., Villingen-
208 Schwenningen, Germany). Results are based on a mean of ten replicates at three central and
209 seven edge locations on each film.

210 **2.9.2 Structural analysis**

211 FTIR (Fourier Transformed Infrared) analysis of the films was carried out using a Varian 660
212 FTIR spectrometer ATR Golden Gate (Soecac). Spectra were taken with 32 scans at 4cm^{-1}
213 resolution in a wavenumber range of 4000 to 500cm^{-1} . Results are based on a mean of five
214 replicates.

215 **2.9.3 Colour**

216 A Chromameter CR-300 (Minolta Camera Co., Osaka, Japan) was used in order to determine
217 the surface colour of films. Before each measurement, the chromate was calibrated using a

218 white ceramic ($L^* = 97.15$, $a^* = -5.28$, $b^* = +7.82$). L^* -, a^* - and b^* - values represent
219 lightness, redness and yellowness respectively. Total colour difference between the treated
220 sample and control (ΔE^*) where calculated as follows:

221 Eq. 1
$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

222 The classification scale for total colour difference reported by Cruz-Romero et al, (2007) was
223 used to classify the films according to the total colour difference. Results are based on a mean
224 of ten replicates.

225 **2.9.4 Transmittance and transparency**

226 Barrier to ultraviolet (UV) and visible light of films was carried out using a UV-2501PC UV–
227 visible Recording Spectrophotometer (Shimadzu, Japan) at wavelengths ranging from 200 to
228 750 nm. The absorbance values at 600, were recorded using the software UV Probe v2.31.

229 Transparency of the films was calculated using the following equation (Han & Floros, 1997):

230 Eq.2
$$\text{Transparency} = A_{600}/X$$

231 Where A_{600} and X are the absorbance at 600 nm and the film thickness (mm), respectively.

232 Results based on a mean of ten replicates.

233 **2.9.5 Mechanical properties**

234 Tensile strength (TS) and elongation at break (EB) tests of the beef gelatin films with
235 antimicrobials were performed at room temperature using a universal testing machine
236 (Mecmesin imperial 2500, Mecmesin, UK) with a 250 N load cell according to the standard
237 testing method (ASTM D 882-01). Ten specimens from each film were tested.

238

239 **2.9.6 Oxygen transmittance rate (OTR) measurements**

240 MOCON OX-TRAN ML module and WinPerm™ software permeability software was used
241 to determine OTR properties of beef gelatin films in accordance to ASTM D 3985-05 at 50%
242 relative humidity using 100% oxygen as a test gas (Convergence by Cycles, No individual
243 zero modes were used). Aluminium foil masks with the area 1 cc were used to fix all films in
244 the test cells. The samples were equilibrated at $50 \pm 1\%$ relative humidity ($25 \pm 1^\circ\text{C}$) during
245 5 days after preparation in Petri dishes before installation into the MOCON.

246

247 **2.9.7 Water vapour permeability**

248 WVTR (water vapour transmission rate) of films was determined gravimetrically according
249 to the ASTM E-96 standard method as described by (Wang et al, 2009) with some minor
250 adjustments. Anhydrous calcium chloride (CaCl₂) was used as a desiccant instead of distilled
251 water. Briefly, 7g of CaCl₂ was placed in each PerspexTM test cup in order to maintain a 0%
252 R.H. The gelatin film samples containing antimicrobials were then mounted across the cup
253 opening with vacuum silicone grease. The films were then sealed into test cups with four
254 screws symmetrically located around the circular cup. The weight gain of test cells will be
255 monitored over 18 hr and recorded every 2 hr intervals. Results are shown as an average of
256 three replicates.

257 **2.10 Statistical analysis**

258 One-way analysis of variance of data was carried out using the SPSS 22 for Windows (SPSS
259 Statistical software, Inc., Chicago, IL, USA) software package Tukey's HSD test was used to
260 compare treatment means when significant differences were found with the ANOVA. The
261 significance level was always set to 0.05.

262 **3. Results and discussion**

263 **3.1. MIC of commercial antimicrobials**

264 All MIC results pertaining to assessment of all antimicrobial agents assessed against test
265 microorganisms in this study is shown in Figure 1. SO demonstrated the highest antimicrobial
266 activity (highest MIC 1%) against all bacterial cultures tested. AR exhibited a broad spectrum
267 of activity, against both Gram (+) and Gram (-) bacteria, with highest antimicrobial activity
268 against *S. aureus*, *Ps. fluorescens* and *E. coli* but reduced activity against *Bacillus cereus* and
269 the mixed beef microflora (with mean MIC values of 4.5% w/v). These results are in
270 accordance with Cruz-Romero et al, (2013) where AR showed higher antimicrobial activity
271 against individual bacterial strains tested but demonstrated less effectiveness against a mixed
272 chicken microflora. In fact, these authors showed that a doubling of the MIC was required to
273 inhibit the raw chicken microflora. These findings are also in agreement with Holley & Patel
274 (2005) who demonstrated that Gram-positive bacteria are more sensitive to antimicrobials
275 based on organic acids than Gram- negative. AFV also demonstrated good antimicrobial
276 activity against all bacterial cultures tested, with beef microflora having the highest MIC
277 value of 1.5% w/v.

278 Sodium octanoate, a sodium salt of Caprylic acid (octanoic acid), occurs naturally in the
279 milk of ruminants, human breast milk, coconut oil, and palm kernel oil and has been
280 described as a saturated medium chain fatty acid (Hulankova et al, 2013). Caprylic acid and
281 its salts have been shown to have an antimicrobial effects against a wide variety of both Gram
282 + and Gram – pathogens (Boyen et al, 2008). Hulankova et al. (2013) demonstrated that an
283 application of 0.5% caprylic acid combined with 0.2% oregano essential oil and 0.1% citric
284 acid reduced counts of lactic acid bacteria by 1.5 log CFU/g and counts of psychrotrophic
285 bacteria by more than 2.5 log CFU/g in inoculated vacuum packed minced beef.

286 Organic acids have long been recognised for their ability to extend the shelf-life of food, they
287 have been shown to exhibit bactericidal and bacteriostatic properties depended on the
288 physicochemical characteristics of the environment and the physiological status of the
289 bacterial species in question (Ricke, 2003). It is generally agreed that the antimicrobial
290 properties of organic acids are due to the weak acid nature of these compounds. It has been
291 hypothesised that undissociated forms of organic acids penetrate the lipid membranes of
292 bacterial cells and once assumed into the neutral pH of the cells cytoplasm, dissociate into
293 anions and protons (Davidson, 2001, & Cherrington et al., 1990). A rise in excess protons
294 then requires consumption of cellular adenosine triphosphate which may then result in a
295 reduction of energy within the cell (Davidson, 2001, & Cherrington et al., 1990).

296 The variation in MIC's observed in this study can be attributed to the different concentrations
297 and compositions of the antimicrobials tested.

298

299 **3.2. MIC of heat treated antimicrobials**

300 The application of various heat treatments on the antimicrobial compounds assessed had a
301 considerable effect on the originally determined MIC levels for these compounds (Fig.2).
302 While the MIC of AFV, AX and SO against bacterial strains was significantly affected (P
303 <0.05) after heating at 75°C, 100°C and 130°C, the MIC of AR was significantly affected (P
304 <0.05) by heating at 75°C and 170°C.

305 A higher concentration of each antimicrobial agent, at each heat treatment, was required in
306 order to determine a MIC at all. At 270°C, a loss of activity for all antimicrobial agents was
307 observed due to the high processing temperatures used.

308 Interestingly MIC values dropped for AFV after 100°C heat treatment in comparison to 75°C.
309 It is possible due to the high water content of AFV (49.5%) that a heat treatment of 100°C,

310 evaporation of this water may have taken place possibly making the antimicrobial
311 components more concentrated, and thus, more potent.

312 Similarly, a loss of antimicrobial activity at higher processing temperatures were also shown
313 by Ha et al. (2001) in which a high temperature profile 160-190°C was used to extrude
314 antimicrobial Linear low-density polyethylene based films. This resulted in a significant loss
315 of grape fruit seed extract, consequently resulting in no antimicrobial activity at all. At
316 temperatures of 100°C, 130°C and 170°C no MIC was obtained for aerobic and anaerobic
317 microflora derived from beef. This can be attributed to the initial concentration of
318 antimicrobial agents (12%) failing to produce a MIC.

319 AX failed to produce an MIC after a heat treatment of 170°C. High concentrations of AX
320 were required at 100°C and 130°C in order to obtain an MIC, which is probably attributed to
321 a loss of the antimicrobial agent. Suppakul et al. (2002) experienced a loss of antimicrobial
322 agents at about 96.7% weight after blown film extrusion of linear low density polyethylene
323 (LLDPE); similarly Marino et al. (2012) showed the remaining weight of antimicrobial
324 agents, thymol and carvacrol to be 25-44% in a polypropylene film at temperatures of 190°C
325 for 18 min in a hot press process. Consequently, an initial high concentration (8% w/w) of the
326 antimicrobial agent was needed in order to show antimicrobial effectiveness towards *S.*
327 *aureus*. Thus it can be postulated that it would not be feasible to utilise AX in cast and blown
328 film extrusion, however, it might be possible to employ SO, AFV and AR in extrusion
329 processes due to the resilience of these antimicrobials.

330

331 **3.3. Transparency and colour**

332 In this study, thickness of the control film (value 0.051 ± 0.006 mm) was significantly lower
333 as compared to antimicrobial films (See Table 1). Rocha et al. (2013) reported that an
334 increase in the concentration of organic acids significantly ($p < 0.05$) increased the thickness
335 of films manufactured from protein isolate, incorporating sorbic acid and boric acid. The
336 thickness of a beef-derived gelatin films, with and without antimicrobials, has an important
337 influence on the optical qualities of packaging films. The thickness of the finally produced
338 antimicrobial films is highly dependent on film preparation methods and the concentration of
339 antimicrobials used (Akhtar et al., 2010).

340 The results of our study showed significant differences ($P < 0.05$) in total colour differences
341 between all antimicrobial films, compared to the control (Table 1). There was also a

342 significant difference ($P < 0.05$) between films AFV and SO compared to AR and AX films.
 343 SO films visually appeared milky in colour compared to control films, a similar effect was
 344 observed by Jipa et al(2012) where the formation of crystals was observed in PVA films
 345 incorporated with sorbic acid adding to a significant increase of the white index and decrease
 346 of yellowness index showing that films containing antimicrobial agent become less
 347 yellowish. It is possible that the amine acid NH_2 groups of gelatine can be protonated by
 348 organic acids to form salt crystals, which may precipitate after gelatinisation, thereby
 349 resulting in low solubility.

350 The results show a decrease in transmittance for antimicrobial films at the wavelength shown
 351 in Table 1. Transparency was significantly affected ($P < 0.05$) by the addition of SO
 352 (5.196 ± 0.041 A600/mm) compared to the control (0.881 ± 0.110 A600/mm). There was no
 353 significant difference in transparency between the other antimicrobial films and when
 354 compared to the control. These results suggest that the use of antimicrobials or organic acid
 355 salts block the passage of light through the films, hence increasing the opacity.

356 **Table 1.** Thickness, transparency, and total colour difference values for control and antimicrobial films.

Films	Thickness (mm)	A600/mm	ΔE
Control	0.051 ± 0.006^a	0.881 ± 0.110^b	0.000^a
AFV	0.056 ± 0.008^b	1.041 ± 0.191^b	0.978 ± 0.02^b
SO	0.057 ± 0.004^b	5.196 ± 0.041^a	1.253 ± 0.06^b
AR	0.056 ± 0.004^b	1.014 ± 0.074^b	2.859 ± 0.08^c
AX	0.056 ± 0.006^b	1.124 ± 0.082^b	2.154 ± 0.08^c

357 Values of thickness, transparency, and total colour are a mean \pm standard error. Different letters within the same
 358 column indicate significant differences ($P < 0.05$).

359 3.4. Agar diffusion and film forming solution

360 All antimicrobial compounds showed inhibition against Gram-positive, Gram-negative and a
 361 mixed bacterial microflora derived from beef samples (Table 2). SO produced the largest
 362 inhibition zone against *B.cereus* (22.46 ± 1.6 mm), as well as inhibiting the growth of all other
 363 bacteria tested. The control film showed no reduction in growth against any bacterial culture,
 364 with the exception of *P. Fluorescens*. This may have occurred as a result of the gelatin film
 365 depriving this microorganism of the necessary levels of oxygen required for respiration,
 366 growth and survival owing to its strict aerobic nature. An inhibition zone was also presented
 367 for AR against *S. aureus* (15.71 ± 0.46 mm) and functioned similarly by contact for all other
 368 bacterial strains. With the exception of *S. aureus*, AX films reduced bacterial growth only
 369 when the surface of the treated film was in direct contact with the bacterial culture of choice.

370 The impact of antimicrobial film forming solutions against Gram +, Gram- and a mixed
 371 bacterial microflora derived from beef samples is shown in Table 3. Similarly, SO exhibited
 372 the largest inhibition zone against *B. cereus* and *S. aureus* (12.14±0.23mm, 10.11±0.86mm)
 373 respectively, while also being the most effective antimicrobial agent against the growth of all
 374 bacteria tested. AR showed inhibition zones against *S. aureus* (9.63±0.51mm) and again, was
 375 effective against all bacterial cultures upon direct contact. AX again proved itself to be the
 376 least effective of all the antimicrobials assessed, showing only a capacity to reduce bacterial
 377 growth.

378 Numerous mechanisms have been suggested for the antibacterial effects exhibited by
 379 antimicrobial agents which contain organic acids and essential oils, such as their; capacity to
 380 attack the phospholipid bilayer of the cell membrane, ability to disrupt enzyme systems, role
 381 in causing coagulation of cellular cytoplasm, propensity to damage proteins and lipids, and
 382 disturbance of the proton motive force (Burt, 2004).

383 The Gram-positive microorganisms (*S. aureus* & *B. cereus*) were more susceptible to the
 384 action of the antimicrobials employed in this study than their Gram-negative counterparts.
 385 Gram-positive microorganisms lack the outer hydrophilic membrane that Gram-negative
 386 bacteria possess and which can act as a barrier to antimicrobial agents. The antimicrobial
 387 agent can penetrate the Gram-positive bacterial cell membrane easier, thereby providing the
 388 antimicrobial with a greater inhibitory effect. Similar effects as have been reported in this
 389 study have also been observed by Ojagh et al., (2010) who assessed biodegradable films
 390 made from chitosan and cinnamon essential oil and Peng & Li (2014) who studied the
 391 combined effects of two kinds of essential oils on physical, mechanical and structural
 392 properties of chitosan films.

393 After heating to 75°C, AX failed to reduce the growth of all bacteria tested, while the other
 394 antimicrobials showed a reduction of growth, or inhibition of growth, by direct contact (Table
 395 4). After applying a 100°C heat treatment, it was determined that all films melted to varying
 396 degrees, with the exception being noted for SO films. It was subsequently shown that this SO
 397 film inhibited bacterial growth by direct contact.

398 **Table 2.** Antimicrobial effects of active films on bacterial growth by direct contact following no heat
 399 treatment

Inhibition zone (mm)*	Film	<i>E.coli</i>	<i>B.cereus</i>	<i>P.fluorsecens</i>	<i>S.aureus</i>	Beef aerobic	Beef anaerobic
	Control	-	-	+-	-	-	-
	AR	+	+	+	+	+	+

AX	+ -	+ -	+ -	+	+ -	+ -
AFV	+ -	+	+	+	+	+ -
SO	+	+	+	+	+	+

400 Data reported are average values \pm standard deviations, - represents no reduction +- reduced
 401 growth and + worked by contact

402

403 **Table 3.** Antimicrobial effects of active agents on bacterial growth by film forming solution following
 404 no heat treatment

Inhibition zone (mm)*	Film forming solution	<i>E.coli</i>	<i>B.cereus</i>	<i>P.fluorsecens</i>	<i>S.aureus</i>	Beef aerobic	Beef anerobic
	Control	-	-	-	-	-	-
	AR	+	+	+	+	+	+
	AX	+	+ -	+ -	+	+ -	+ -
	AFV	+ -	+	+	+	+	+ -
	SO	+	+	+	+	+	+

405 Data reported are mean values \pm standard error, - represents no reduction +- reduced growth and +
 406 worked by contact

407

408 **Table 4.** Antimicrobial effects of active agents on bacterial growth by film forming solutions
 409 following heat treatment at 75°C

Inhibition zone (mm)*	Film	<i>E.coli</i>	<i>B.cereus</i>	<i>P.fluorsecens</i>	<i>S.aureus</i>	Beef aerobic	Beef anerobic
	Control	-	-	-	-	-	-
	AR	+	+	+	+	+	+
	AX	-	-	-	-	-	-
	AFV	-	+	+ -	+	+	+ -
	SO	+	+	+	+	+	+

410 Data reported are mean values \pm standard error, - represents no reduction +- reduced growth and +
 411 worked by contact

412 **Table 5.** Antimicrobial effects of active agents on bacterial growth by film forming solutions
 413 following heat treatment at 100°C

Inhibition zone (mm)*	Film	<i>E.coli</i>	<i>B.cereus</i>	<i>P.fluorsecens</i>	<i>S.aureus</i>	Beef aerobic	Beef anerobic
	Control	x	x	x	x	x	x
	AR	x	x	x	x	x	x
	AX	x	x	x	x	x	x
	AFV	x	x	x	x	x	x

414 Data reported are mean values \pm standard error, x represents melting of films and + worked by
 415 contact

416

417 **3.5. FTIR studies of the films with antimicrobial agents**

418 The FTIR spectra results show changes in the structure of gelatin films which contained
 419 antimicrobials (Fig.3). The distinctive methyl and methylene stretches are evident at 2940,
 420 2925, 2831 and 2850 cm^{-1} in the FTIR spectra for SO (Sigman et al, 2003).

421 The main absorption bands at characteristic peak frequency values corresponds to amide A,
 422 amide I, amide II and amide III. Bands centred around 3500 cm^{-1} are present mainly due to
 423 an extension of the group N-H of amide A. This broad band may be attributed to bound free
 424 O-H and N-H groups that may form hydrogen bonding with the carbonyl group of the peptide
 425 linkage located in the protein (Guerrero et al, 2012).

426 The amide I bands located between 1700 and 1600 cm^{-1} are a result of C=O extension. These
 427 peaks mainly belong to collagen in gelatin and can contain parts of CO group bands found in
 428 the acids in the antimicrobials used (Tiong et al. 2008 & Vidal et al 2001).

429 In numerous studies, it is stated that Amide I absorption band are due almost entirely because
 430 of CO stretch vibrations of peptide linkages and is useful for determination of secondary
 431 structure of proteins (Jackson & Mantsh, 1995). Intense bands located between 1600 and
 432 1500 cm^{-1} are known as amide II and are present as a result of C-N extension and angular
 433 deformation of the N-H ligation (Mantsch & Chapman, 1996). Amide II bands are related to
 434 hydration. A peak shift to greater frequencies specifies increasing levels of protein backbone
 435 hydration (Yakimets et al., 2005), as was evident for SO films $\sim 1556 \text{ cm}^{-1}$ compared to
 436 control films $\sim 1545 \text{ cm}^{-1}$ (Fig.4). A small increase was also noted for AR films $\sim 1546 \text{ cm}^{-1}$
 437 compared to control films. In this study a small decrease was observed between A.FV films \sim
 438 1544 cm^{-1} compared to the control $\sim 1545 \text{ cm}^{-1}$ indicating a slight decrease in the level of
 439 protein hydration and an increase in polypeptide chain interactions (Jakobsen et al., 1983).

440 The above statement can also be confirmed for the results shown for the amide III band, a
 441 slight increase was observed between $\sim 1229\text{-}1301 \text{ cm}^{-1}$ compared to the control peak ~ 1238
 442 cm^{-1} . A slight banding increase was also observed for AX and SO films $\sim 1239 \text{ cm}^{-1}$ and
 443 1240 cm^{-1} respectively, however, a decrease was observed for AFV films $\sim 1236 \text{ cm}^{-1}$. FTIR
 444 results showed a decrease in amplitude of peaks for AX films $\sim 1551 \text{ cm}^{-1}$, thereby

445 indicating a decrease in hydration. A decrease in amplitude these peaks may be related to
446 molecular recording of the triple helix as a consequence of uncoupling of intermolecular
447 cross-linkage (Kittiphonabawon et al., 2010).

448 The broad band location between the ranges 3500-3000 cm^{-1} FTIR results showed a decrease
449 in amplitude of peaks for AX films $\sim 3296 \text{ cm}^{-1}$ and SO films $\sim 3280 \text{ cm}^{-1}$ compared to the
450 control $\sim 3296 \text{ cm}^{-1}$, representing a decrease in the amount of free water available (Bergo &
451 Sobral, 2007).

452 These changes in FTIR bands, which were observed for Amide I, II and III, provide evidence
453 with respect to increases or decreases in polypeptide chains interactions, thereby leading to a
454 toughening or weakening of the film structure, respectively.

455

456 **3.6. Mechanical properties**

457 The mechanical properties of untreated active antimicrobial films are shown in Figure 5.
458 Differences in TS and EB may be attributed to addition and presence of antimicrobials
459 interacting with the gelatin, thereby forming new linkages which affected the film structure.
460 Interaction of hydrocolloids, protein and additives, plasticisers, water and antimicrobial
461 agents can impact the mechanical properties of packaging films (Park et al, 2001). The TS
462 and EB results for beef-derived gelatin films containing different antimicrobials are shown
463 (Fig 5). The results showed an increase in TS for antimicrobial active films compared to the
464 control, with the only exception being that for AFV. This decrease may be due to the
465 polyphenol compounds found in AFV which may have resulted in the formation of covalent
466 and hydrogen bonds with the amino and hydroxyl groups of the polypeptides present in
467 gelatin, which would then weaken the protien-protien interactions to destabilise the protein
468 network (Orliac et al, 2007). Moreover, high amounts of this extract might also induce the
469 development of a heterogeneous structure and a presence of a discontinuous area, producing a
470 low tensile strength (Bravin et al, 2004). AFV is composed of organic acids (malic and citric
471 acids). Cao et al, (2009) reported that with the addition of malic acid into gelatin films, EB
472 increased while the TS and Young's Modulus (YM) decreased as shown by the results for
473 AFV compared to the control. It was suggested that the malic acid had a plastizing effect on
474 gelatin.

475 Significant differences were observed in TS and EB ($P < 0.05$) between SO and AX
476 antimicrobial films compared the control. It is possible that the addition of antimicrobials
477 may interfere with film cross-linkages, thereby resulting in films with a better EB and TS. At
478 a molecular level, these active agents may potentially insert themselves between protein
479 chains and subsequently increase flexibility (Guilbert, 1986). An increase in TS may also be
480 attributed to an increase in concentration in the film forming solution, thereby causing
481 molecules to become more closely packed together. This may serve as a probable explanation
482 for the behaviour of AX and AR films which required a concentration of 3% w/v and 4.5%
483 respectively in order to be effective against all Gram + and Gram – bacteria. Respect to the
484 Control, an increase in TS and a decrease in EB can be observed for AR (Fig. 5), similar
485 results were reported by Erdohan & Turhan. (2005) were an increase in solid content
486 demonstrated a similar effect on methylcellulose–Whey Protein Films.

487 As discussed for the FTIR results, changes observed in bands for Amide I, II and III may be
488 associated with formation of hydrogen bonds, along with a decrease in protein hydration.
489 This may explain the increase in EB observed for SO films. The hydrophilic nature of SO
490 favours absorption of water molecules within the film matrix, thereby creating hydrogen
491 bonds within gelatin matrices, thus increasing the moisture content. It has been reported that
492 within hydrophilic biopolymers, water molecules can act as a plasticizer that weakens
493 existing hydrogen bonds between polymer chains (Xiao et al, 2012) and as shown in Table 5.
494 SO showed the greatest EB value (36.109 ± 3.34 %) compared to other films, suggesting that
495 films containing SO had been plasticized.

496 Films containing AR showed the lowest EB (6.373 ± 1.47 %) of all films. As highlighted
497 previously, AR is composed of organic acids, including citric acid. Olivato et al, (2012)
498 reported similar results where they showed an increase in TS and remarkable reduction in
499 elongation results in starch/polyester blown films incorporating citric acid. This increase in
500 TS and reduction of EB was attributed the formation of cross-linking reactions that
501 interconnected polymeric molecules, thereby restricting their mobility. Martucci et al. (2015)
502 reported similar results after the inclusion of oregano and lavender essential oils in gelatin
503 based films.

504 **3.7. Water vapour permeability (WVTR)**

505 The results for all experimental gelatin films employed in this study are shown in Figure 6. In
506 summary, a decrease in WVTR was observed for all gelatin films containing antimicrobials

507 when compared to control gelatin films. One of the main functions of a food packaging
508 material is to decrease or avoid the moisture transfer between the product and the surrounding
509 atmosphere. Consequently, the WVTR should be as low as possible (Gontard et al, 1992).

510 The decrease in WVTR although not significant in films containing organic acids such as
511 AFV and AR may be attributed to a reduction in mobility of the polymeric chains, due to
512 cross-linking reactions thus making diffusion of water across the film matrices more difficult.
513 In addition to cross-linking, a decrease in WVTR may have been contributed to decreasing
514 pore size due to the addition of antimicrobials, thereby increasing the solid content also
515 evident in the mechanical properties observed for AX and previously described. The addition
516 of the active agents to gelatin may have simply blocked pores or channels running throughout
517 the film matrix, thereby increasing resistance to the free movement of water throughout
518 various locations within the film structure.

519 A similar result was observed by Olivato et al. (2012) when they incorporated citric acid and
520 tartaric acid in small quantities into starch/polyester blown films, this decrease was attributed
521 to insufficient contribution to cross-linking reactions.

522 The results showed a significant difference ($P < 0.05$) in WVTR values between the
523 antimicrobial films AX and SO compared to the control, this decrease in WVTR properties of
524 films with SO is in agreement with Park (1994). WVTR properties decreased as chain length
525 and concentration of fatty acids increased. No similar significant difference was observed
526 between the other antimicrobial films and the control.

527

528 **3.8 OTR properties of the gelatine films**

529 The OTR properties for all gelatin films studied are shown in Figure 7. Significant
530 differences ($P < 0.05$) in oxygen barrier properties were observed between all active coated
531 films compared to the control. AR and AX films demonstrated the best barrier properties
532 (40.7 and 44.3 cc/m² day, respectively). A higher value of 79.7 cc³/m² per day was reported
533 for SO containing films. This dramatic elevation in permeability could have been brought
534 about by possible formation of salt crystals or by changes in the film structure, which is
535 supported by data derived from both FTIR and mechanical property testing. It must be noted
536 that the permeant concentration had an effect on OTR properties. For instance, SO films
537 measured at 50% oxygen gas flow had a reported value of 124.58 cm³/m² per day. This value

538 is comparable to the results reported by Nassiri & Nafchi. (2013). At the permanent
539 concentration of 100% OTR, values slightly decreased to 79.70 cm³/m² per day. The same
540 difference was observed between AR and AX films. AR films had OTR properties of 32.69
541 cm³/m² per day using 50% oxygen as a test gas and 40.82 cm³/m² per day using 100%
542 oxygen as the test gas, while AX films had OTR properties of 52.92 cm³/m² per day using
543 50% oxygen and 44.33 cm³/m² per day using 100% oxygen. To the best of our knowledge
544 there is no information in literature supporting these results.

545 **4. Conclusion**

546 The antimicrobials tested demonstrated different degrees of antimicrobial activity against all
547 bacterial species evaluated. Heat treatments applied to gelatin film forming solutions
548 containing active antimicrobial agents significantly inhibited their effectiveness. SO films
549 demonstrated the best efficiency against all aerobic and anaerobic bacteria tested between 20°
550 and 170°C. For SO, AFV and AR antimicrobials, it is possible to find appropriate conditions
551 for different extrusion processes in order to manufacture these films commercially. Beef-
552 derived gelatin films containing SO demonstrated good mechanical properties and better
553 WVTR barrier (150 g/m² per day) compared to control gelatin films (330 g/m² per day). FTIR
554 analysis showed that films containing SO demonstrated the most significant differences in
555 terms of film structure. OTR analysis demonstrated that antimicrobials significantly enhanced
556 the oxygen barrier properties compared to control films for all antimicrobials except for SO
557 films. In conclusion, beef-derived gelatine containing active antimicrobial agents could
558 potentially serve as commercial antimicrobial coatings for application onto conventional
559 plastic-based food packaging or for use as compostable or biodegradable packaging
560 materials.

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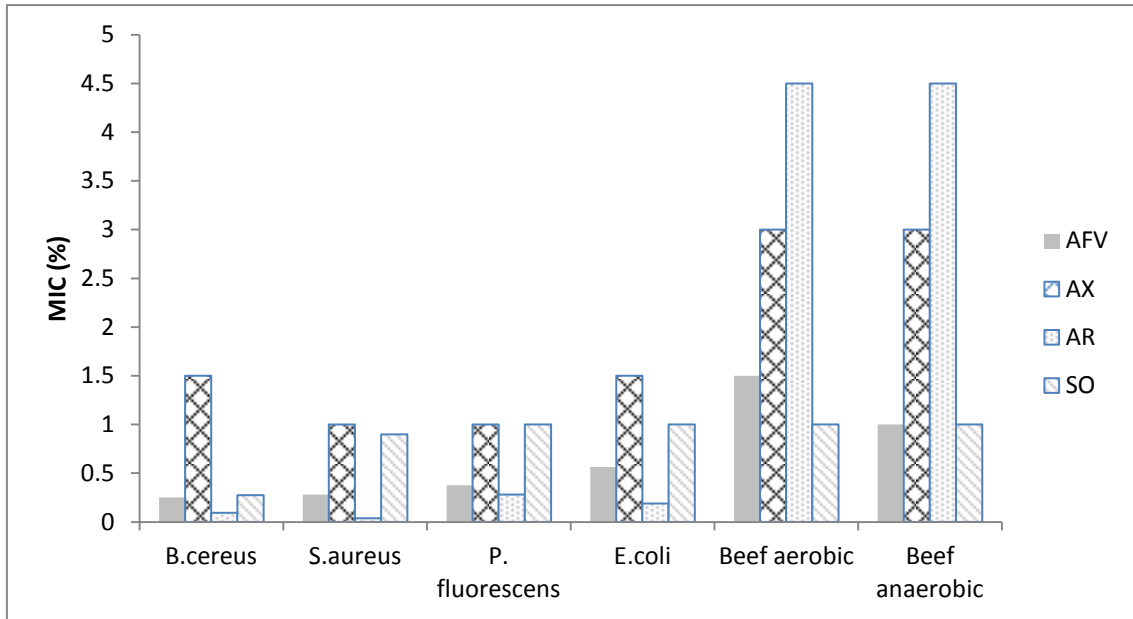
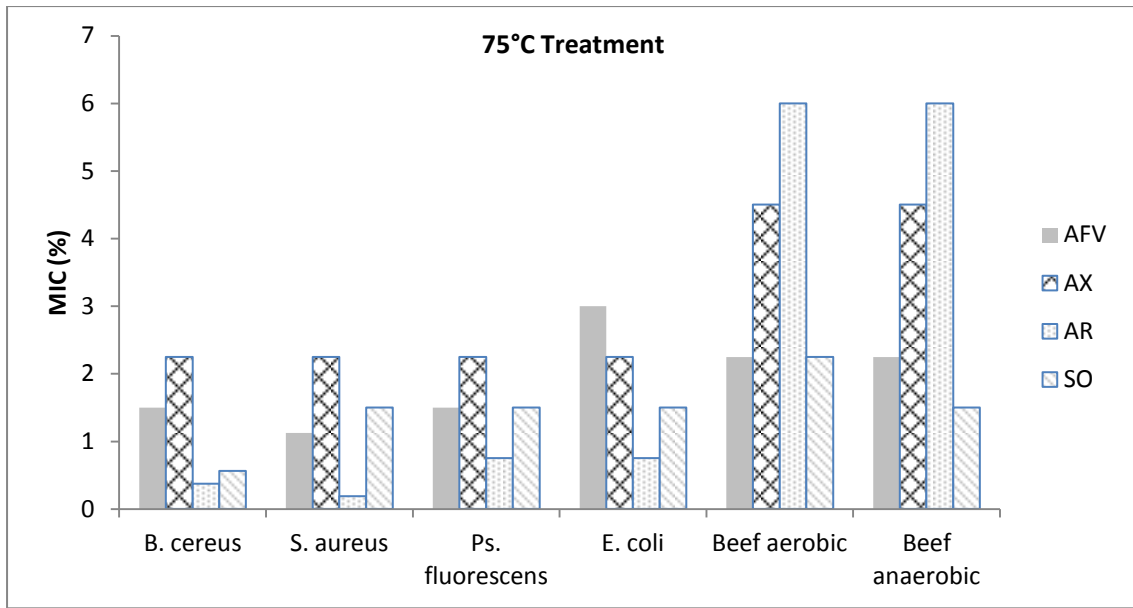
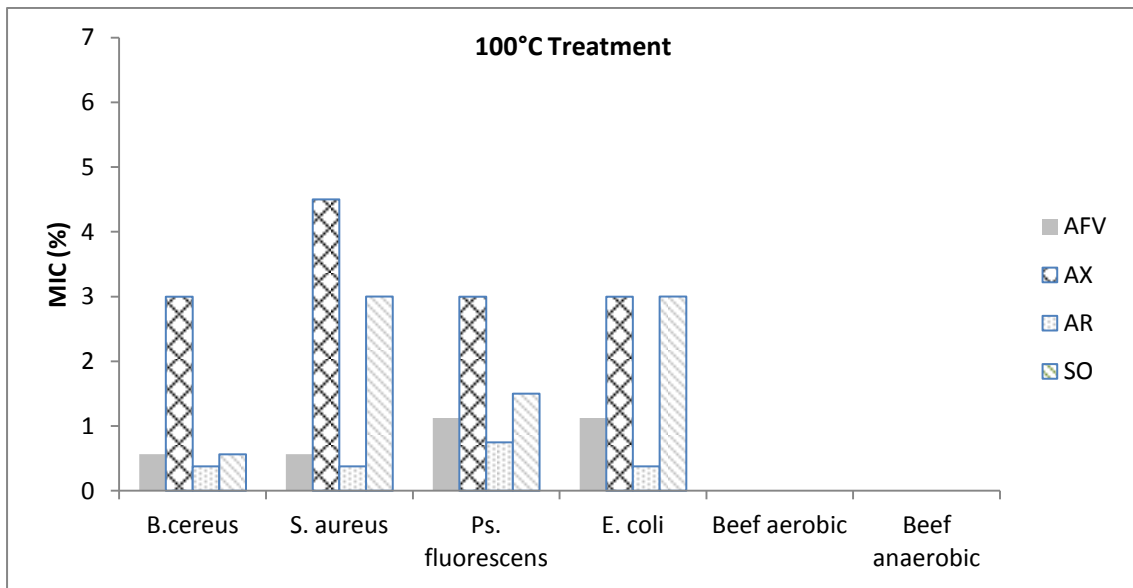


Fig. 1. Antimicrobial activity of AFV, AX, AR, and SO on bacterial strains and beef aerobic and anaerobic micro flora.

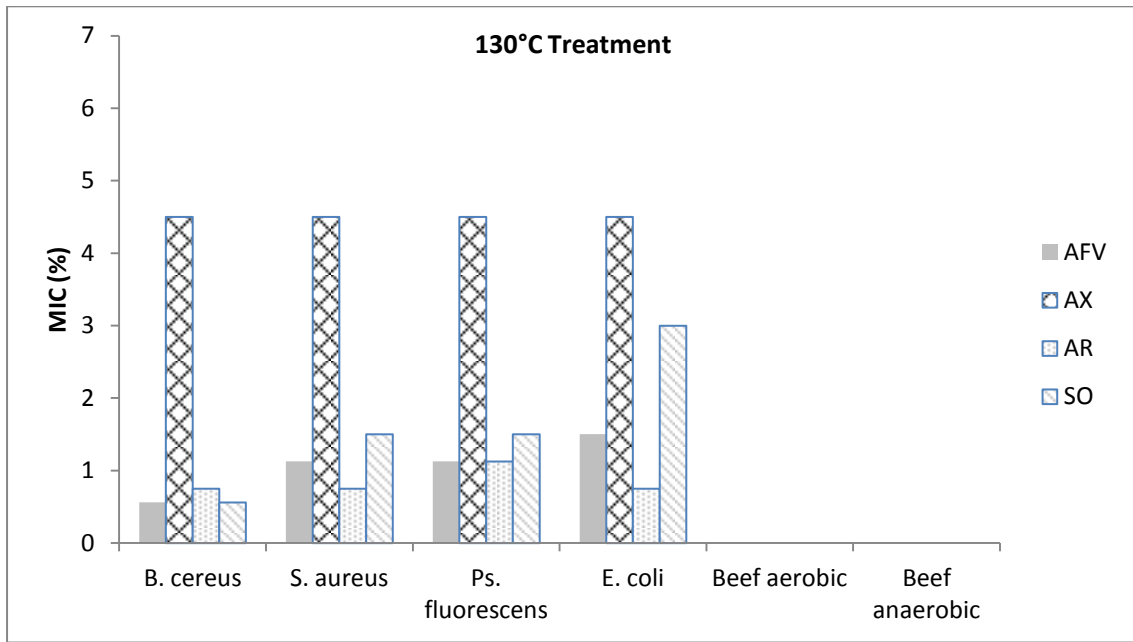
A



B



C



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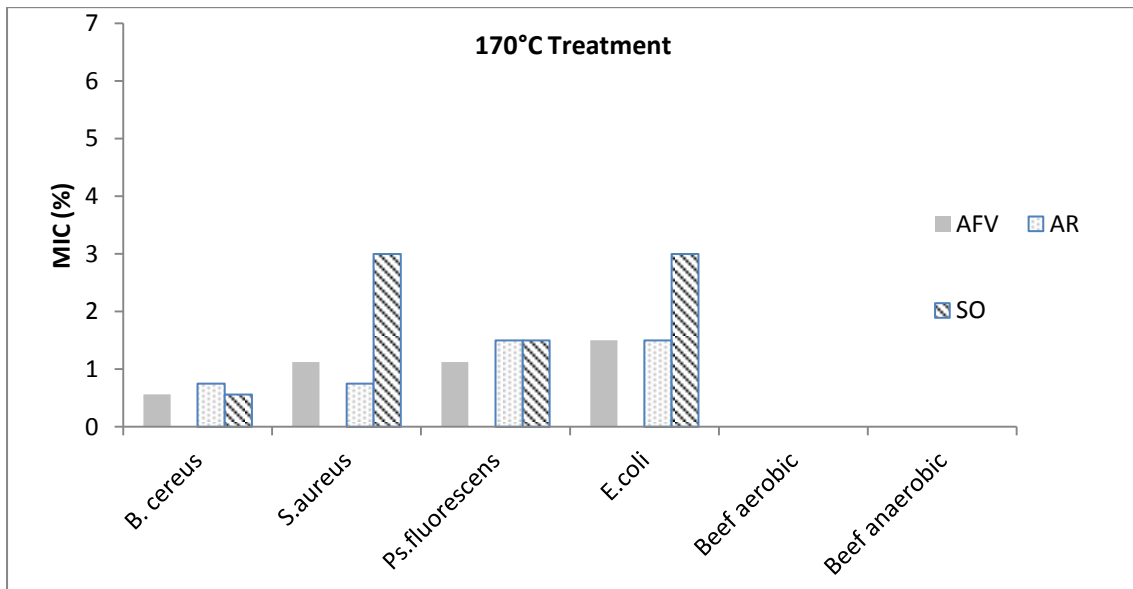


Fig. 2. Antimicrobial activity of heat-treated AFV, AX, AR, and SO on pure and mixed bacterial cultures.

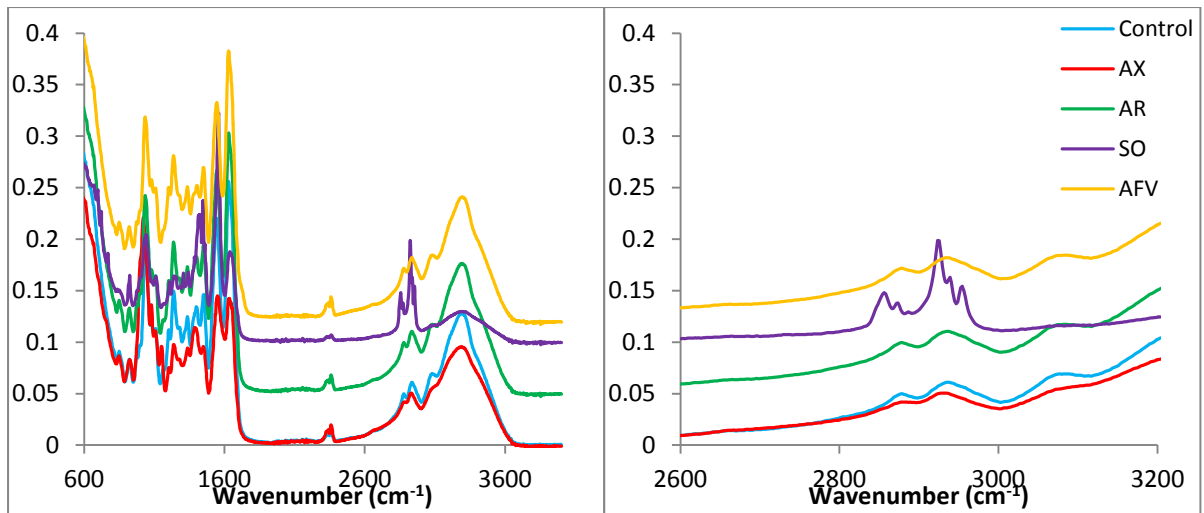


Fig. 3. FTIR spectra of beef gelatin control film and beef gelatin films with antimicrobials (AFV, AX, AR, and SO).

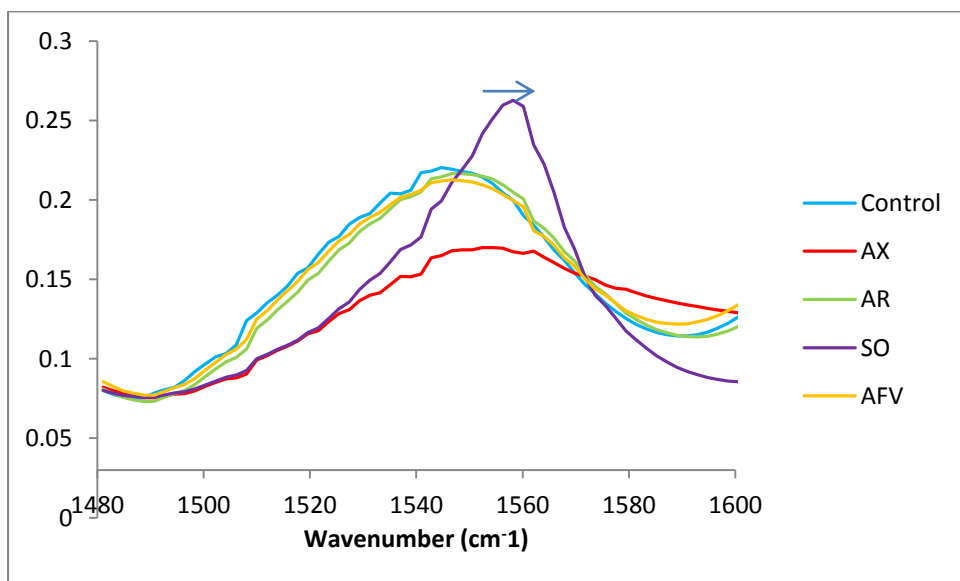


Fig. 4. FTIR spectra of beef gelatin control and beef gelatin with antimicrobials namely AFV, AX, AR, and SO.

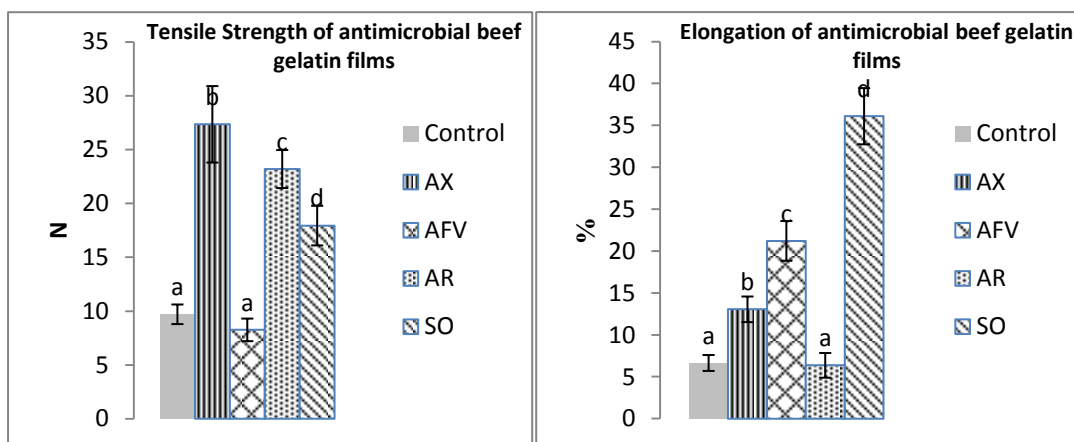


Fig. 5. Mechanical properties of antimicrobial films, Different letters indicate significant differences ($P < 0.05$).

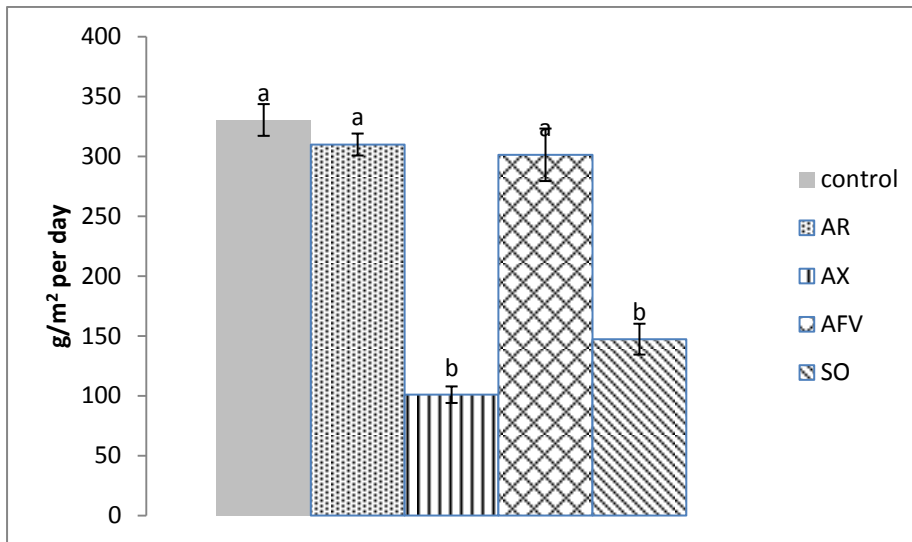


Fig. 6. WVTR (g/m^2 per day) of beef gelatin films with antimicrobials in comparison with a control, Different letters indicate significant differences ($P < 0.05$).

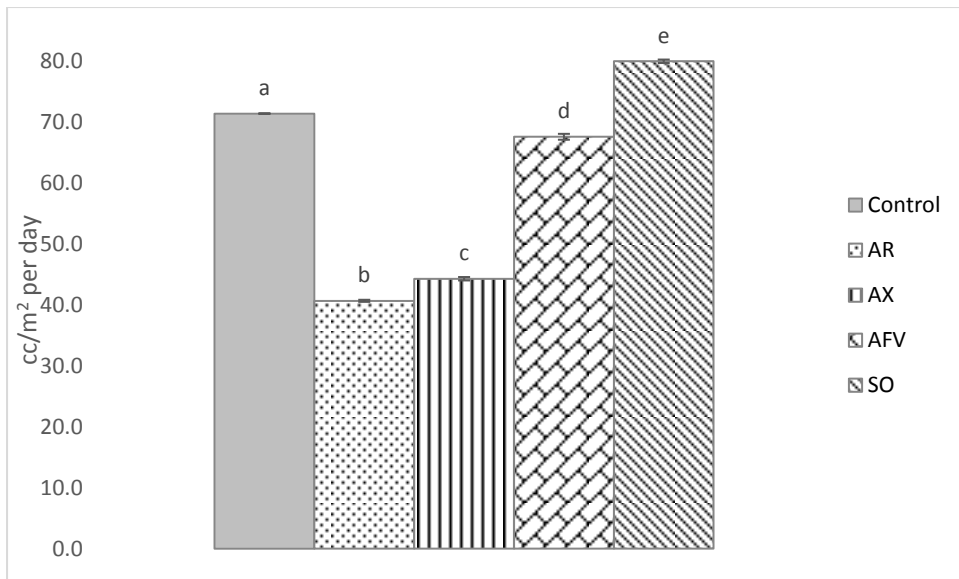


Fig. 7. Oxygen barrier properties ($\text{cc}^3/\text{m}^2 \text{ day}$) with 100% gas permanent concentration of beef gelatin films at 50% relative humidity. Different letters indicate significant differences ($P < 0.05$).

- Four antimicrobials, namely; Articoat, Artemix Consa, Auranta FV and sodium octanoate were examined for their effectiveness, both before and after heat treatments, against bacterial strains and beef microflora.
- Sodium octanoate was the most effective against all bacterial strains according to 96-well plate method.
- These antimicrobials were then successively incorporated into beef-derived gelatine films and tested for structural, mechanical and barrier properties.
- It was shown that active antimicrobial agents could potentially serve as commercial antimicrobial coatings for application onto conventional plastic-based food packaging.