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

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Abstract

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and methods

2.1. Materials and microbiological media

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

2.2. Bacterial strains and sample preparation

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

2.3. Heat treatment of Antimicrobials

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

2.4. Determination of MIC

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

2.5. Preparation of film forming solutions

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

2.6. Agar diffusion method

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

2.7. Heat treatment of films

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

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 ×
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 μL 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^{o}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^{o}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^o 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

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

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

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

2.9.4 Transmittance and transparency

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

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

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

Where A600 and X are the absorbance at 600 nm and the film thickness (mm), respectively.

Results based on a mean of ten replicates.

2.9.5 Mechanical properties

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

2.9.6 Oxygen transmittance rate (OTR) measurements

MOCON OX-TRAN ML module and WinPermTM software permeability software was used to determine OTR properties of beef gelatin films in accordance to ASTM D 3985-05 at 50% relative humidity using 100% oxygen as a test gas (Convergence by Cycles, No individual zero modes were used). Aluminium foil masks with the area 1 cc were used to fix all films in the test cells. The samples were equilibrated at 50 ± 1% relative humidity (25 ± 1 °C) during 5 days after preparation in Petri dishes before installation into the MOCON.

2.9.7 Water vapour permeability

WVTR (water vapour transmission rate) of films was determined gravimetrically according to the ASTM E-96 standard method as described by (Wang et al, 2009) with some minor adjustments. Anhydrous calcium chloride ($CaCl_2$) was used as a desiccant instead of distilled water. Briefly, 7g of $CaCl_2$ was placed in each Perspex^{TM} test cup in order to maintain a 0% R.H. The gelatin film samples containing antimicrobials were then mounted across the cup opening with vacuum silicone grease. The films where then sealed into test cups with four screws symmetrically located around the circular cup. The weight gain of test cells will be monitored over 18 hr and recorded every 2 hr intervals. Results are shown as an average of three replicates.

2.10 Statistical analysis

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

3. Results and discussion

3.1. MIC of commercial antimicrobials

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

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

Organic acids have long been recognised for their ability to extend the shelf-life of food, they have been shown to exhibit bactericidal and bacteriostatic properties depended on the physicochemical characteristics of the environment and the physiological status of the bacterial species in question (Ricke, 2003). It is generally agreed that the antimicrobial properties of organic acids are due to the weak acid nature of these compounds. It has been hypothesised that undissociated forms of organic acids penetrate the lipid membranes of bacterial cells and once assumed into the neutral pH of the cells cytoplasm, dissociate into anions and protons (Davidson, 2001, & Cherrington et al., 1990). A rise in excess protons then requires consumption of cellular adenosine triphosphate which may then result in a reduction of energy within the cell (Davidson, 2001, & Cherrington et al., 1990).
The variation in MIC's observed in this study can be attributed to the different concentrations and compositions of the antimicrobials tested.

3.2. MIC of heat treated antimicrobials

The application of various heat treatments on the antimicrobial compounds assessed had a considerable effect on the originally determined MIC levels for these compounds (Fig.2). While the MIC of AFV, AX and SO against bacterial strains was significantly affected ($P < 0.05$) after heating at 75^{o}C, 100^{o}C and 130^{o}C, the MIC of AR was significantly affected ($P < 0.05$) by heating at 75^{o}C and 170^{o}C.
A higher concentration of each antimicrobial agent, at each heat treatment, was required in order to determine a MIC at all. At 270^{o}C, a loss of activity for all antimicrobial agents was observed due to the high processing temperatures used.
Interestingly MIC values dropped for AFV after 100^{o}C heat treatment in comparison to 75^{o}C. It is possible due to the high water content of AFV (49.5%) that a heat treatment of 100^{o}C,

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

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

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

3.3. Transparency and colour

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

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

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

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

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]

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

3.4. Agar diffusion and film forming solution

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

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

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

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

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

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

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

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

Data reported are average values ± standard deviations, - represents no reduction +- reduced growth and + worked by contact

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

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

Data reported are mean values ± standard error, - represents no reduction +- reduced growth and + worked by contact

Table 4. Antimicrobial effects of active agents on bacterial growth by film forming solutions following heat treatment at 75^{o}C

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

Data reported are mean values ± standard error, - represents no reduction +- reduced growth and + worked by contact

Table 5. Antimicrobial effects of active agents on bacterial growth by film forming solutions following heat treatment at 100^{o}C

Inhibition zone (mm)*	*Film*	*E.coli*	*B.cereus*	*P.fluorsecens*	*S.aureus*	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

SO	+	+	+	+	+	+

Data reported are mean values ± standard error, x represents melting of films and + worked by contact

3.5. FTIR studies of the films with antimicrobial agents

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

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

The amide I bands located between 1700 and 1600 cm^{-1} are a result of C=O extension. These peaks mainly belong to collagen in gelatin and can contain parts of CO group bands found in the acids in the antimicrobials used (Tiong et al. 2008 & Vidal et al 2001).
In numerous studies, it is stated that Amide I absorption band are due almost entirely because of CO stretch vibrations of peptide linkages and is useful for determination of secondary structure of proteins (Jackson & Mantsh, 1995). Intense bands located between 1600 and 1500 cm^{-1} are known as amide II and are present as a result of C-N extension and angular deformation of the N-H ligation (Mantsch & Chapman, 1996). Amide II bands are related to hydration. A peak shift to greater frequencies specifies increasing levels of protein backbone hydration (Yakimets et al., 2005),as was evident for SO films ~ 1556 cm^{-1} compared to control films ~ 1545 cm^{-1} (Fig.4). A small increase was also noted for AR films ~ 1546 cm^{-1} compared to control films. In this study a small decrease was observed between A.FV films ~ 1544 cm^{-1} compared to the control ~ 1545 cm^{-1} indicating a slight decrease in the level of protein hydration and an increase in polypeptide chain interactions (Jakobsen et al., 1983). The above statement can also be confirmed for the results shown for the amide III band, a slight increase was observed between ~ 1229-1301 cm^{-1} compared to the control peak ~ 1238 cm^{-1}. A slight banding increase was also observed for AX and SO films ~ 1239 cm^{-1} and 1240 cm^{-1} respectively, however, a decrease was observed for AFV films ~ 1236 cm^{-1}. FTIR results showed a decrease in amplitude of peaks for AX films ~ 1551 cm^{-1}, thereby

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

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

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

3.6. Mechanical properties

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

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

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

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

3.7. Water vapour permeability (WVTR)

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

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

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

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

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

3.8 OTR properties of the gelatine films

The OTR properties for all gelatin films studied are shown in Figure 7. Significant differences ($P < 0.05$) in oxygen barrier properties were observed between all active coated films compared to the control. AR and AX films demonstrated the best barrier properties (40.7 and 44.3 cc/m^2 day, respectively). A higher value of 79.7 cc^3/m^2 per day was reported for SO containing films. This dramatic elevation in permeability could have been brought about by possible formation of salt crystals or by changes in the film structure, which is supported by data derived from both FTIR and mechanical property testing. It must be noted that the permeant concentration had an effect on OTR properties. For instance, SO films measured at 50% oxygen gas flow had a reported value of 124.58 cm^3/m^2 per day. This value

is comparable to the results reported by Nassiri & Nafchi. (2013). At the permanent concentration of 100% OTR, values slightly decreased to 79.70 cm^3/m^2 per day. The same difference was observed between AR and AX films. AR films had OTR properties of 32.69 cm^3/m^2 per day using 50% oxygen as a test gas and 40.82 cm^3/m^2 per day using 100% oxygen as the test gas, while AX films had OTR properties of 52.92 cm^3/m^2 per day using 50% oxygen and 44.33 cm^3/m^2 per day using 100% oxygen. To the best of our knowledge there is no information in literature supporting these results.

4. Conclusion

The antimicrobials tested demonstrated different degrees of antimicrobial activity against all bacterial species evaluated. Heat treatments applied to gelatin film forming solutions containing active antimicrobial agents significantly inhibited their effectiveness. SO films demonstrated the best efficiency against all aerobic and anaerobic bacteria tested between 20° and170^oC. For SO, AFV and AR antimicrobials, it is possible to find appropriate conditions for different extrusion processes in order to manufacture these films commercially. Beef-derived gelatin films containing SO demonstrated good mechanical properties and better WVTR barrier (150 g/m^2 per day) compared to control gelatin films (330 g/m^2 per day). FTIR analysis showed that films containing SO demonstrated the most significant differences in terms of film structure. OTR analysis demonstrated that antimicrobials significantly enhanced the oxygen barrier properties compared to control films for all antimicrobials except for SO films. In conclusion, beef-derived gelatine containing active antimicrobial agents could potentially serve as commercial antimicrobial coatings for application onto conventional plastic-based food packaging or for use as compostable or biodegradable packaging materials.

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Figure

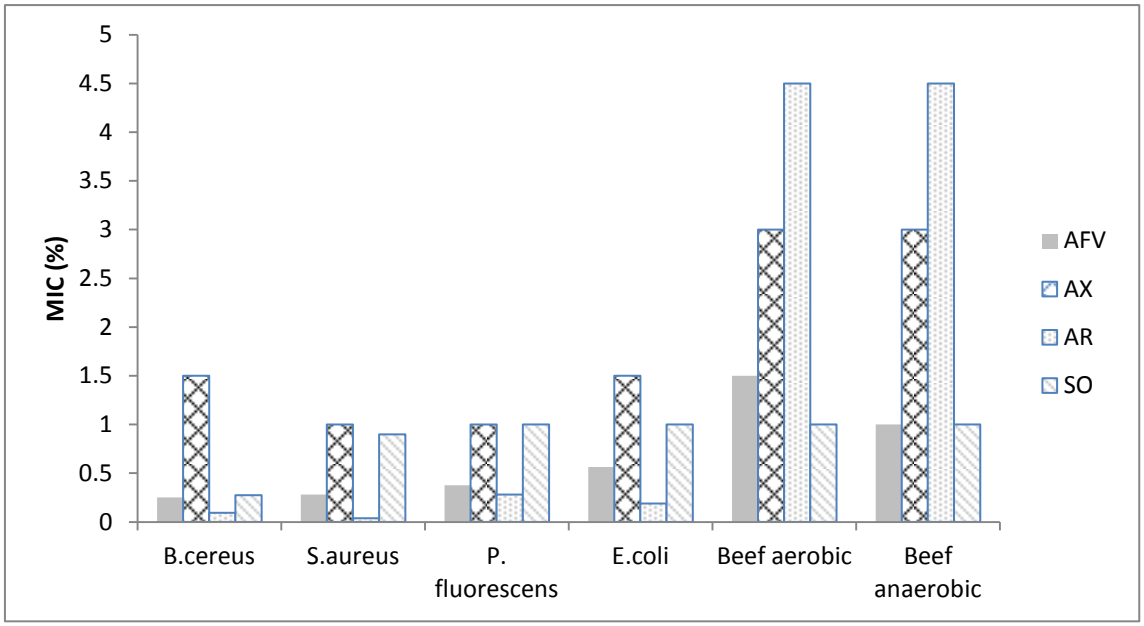
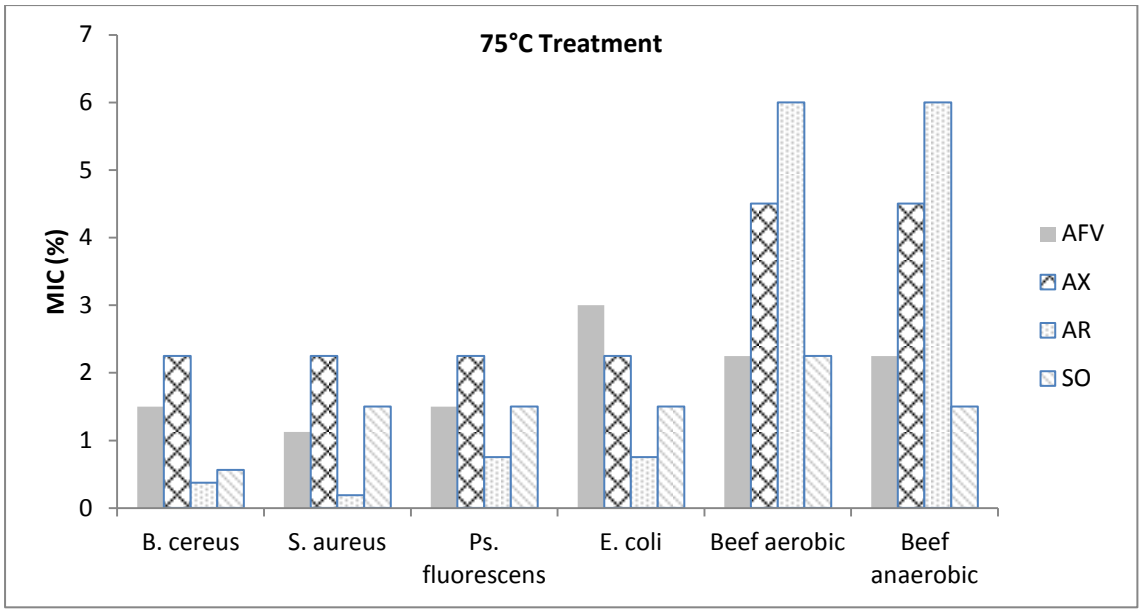
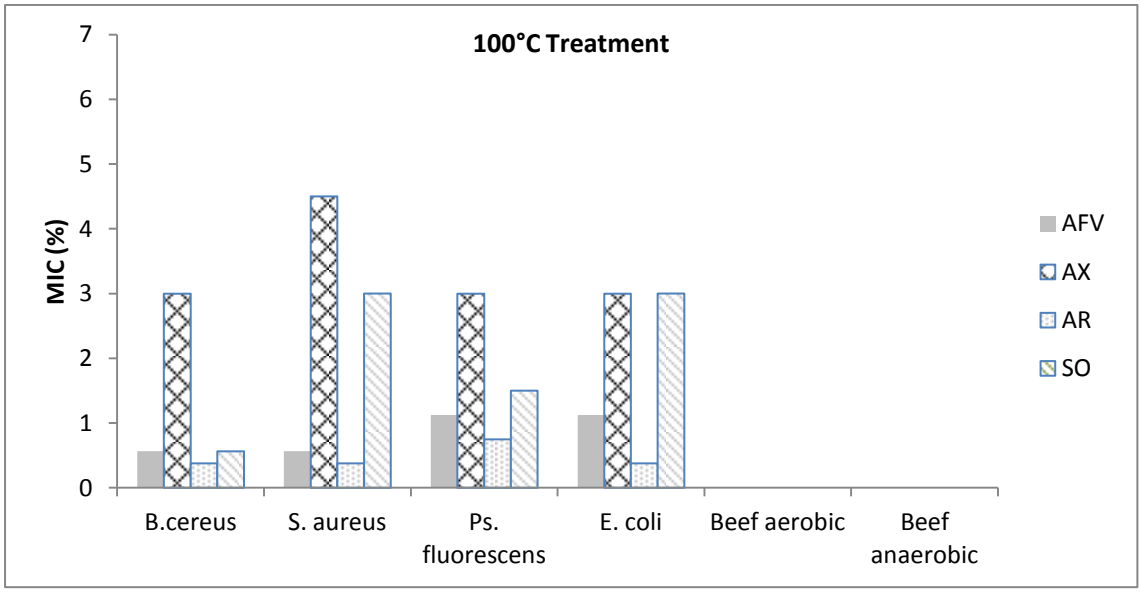


Fig. 1. Antimicrobial activity of AFV, AX, AR, and SO on bacterial strains and beef aerobic and anaerobic micro flora.

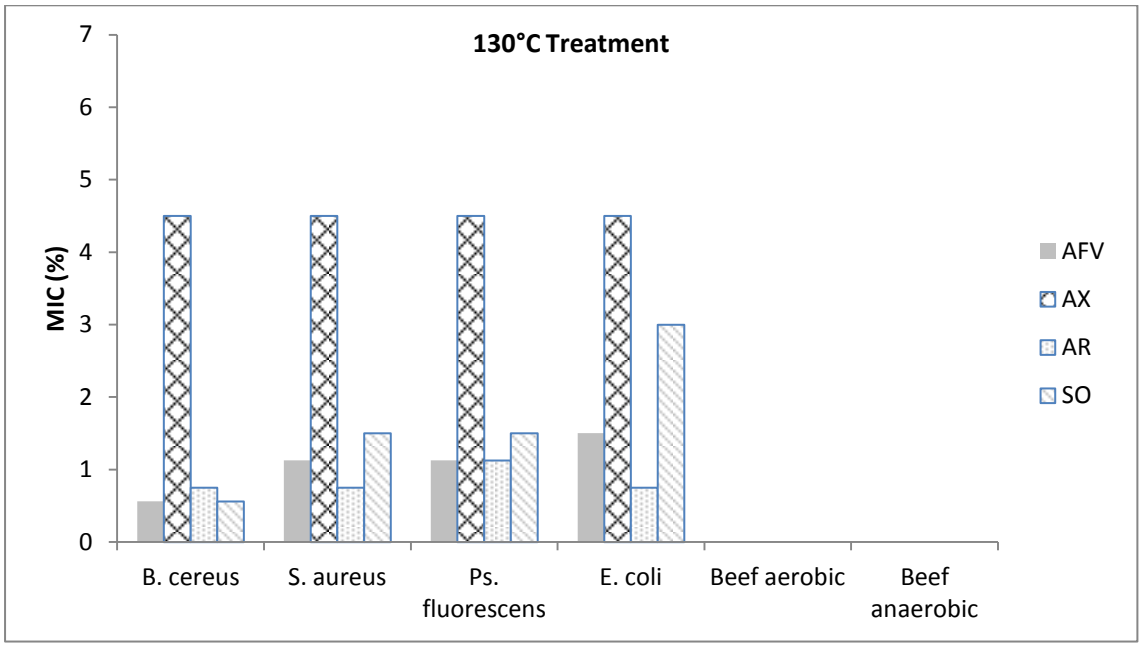
A


75°C Treatment
MIC (%)
0
1
2
3
4
5
6
7
B. cereus
S. aureus
Ps. fluorescens
E. coli
Beef aerobic
Beef anaerobic
AFV
AX
AR
SO

B


100°C Treatment
MIC (%)
0
1
2
3
4
5
6
7
B.cereus
S. aureus
Ps. fluorescens
E. coli
Beef aerobic
Beef anaerobic
AFV
AX
AR
SO

C



D

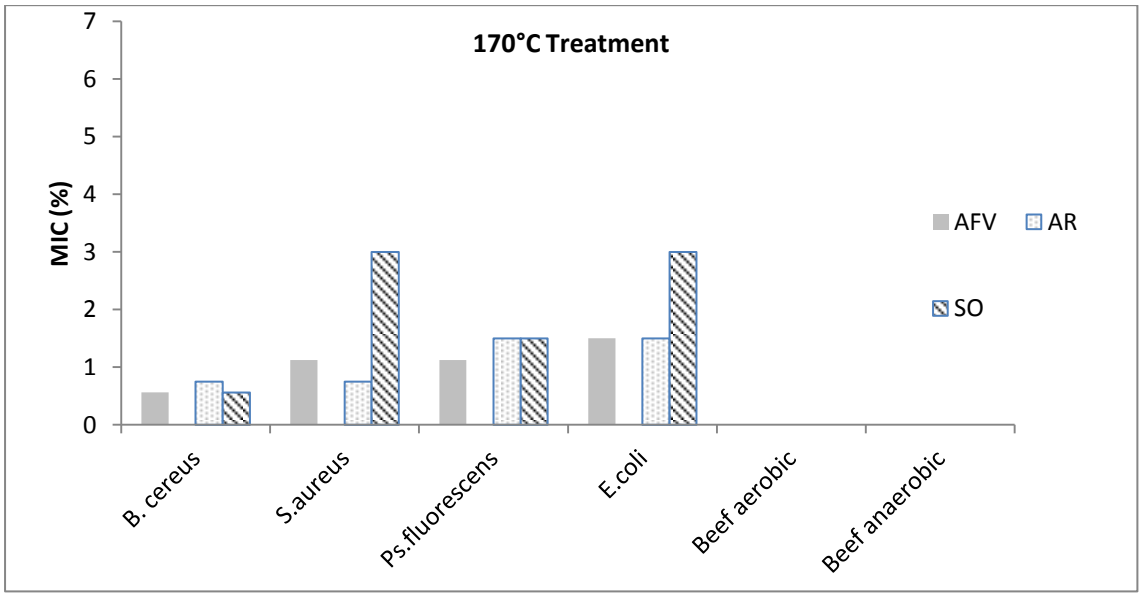


Fig. 2. Antimicrobial activity of heat-treated AFV, AX, AR, and SO on pure and mixed bacterial cultures.

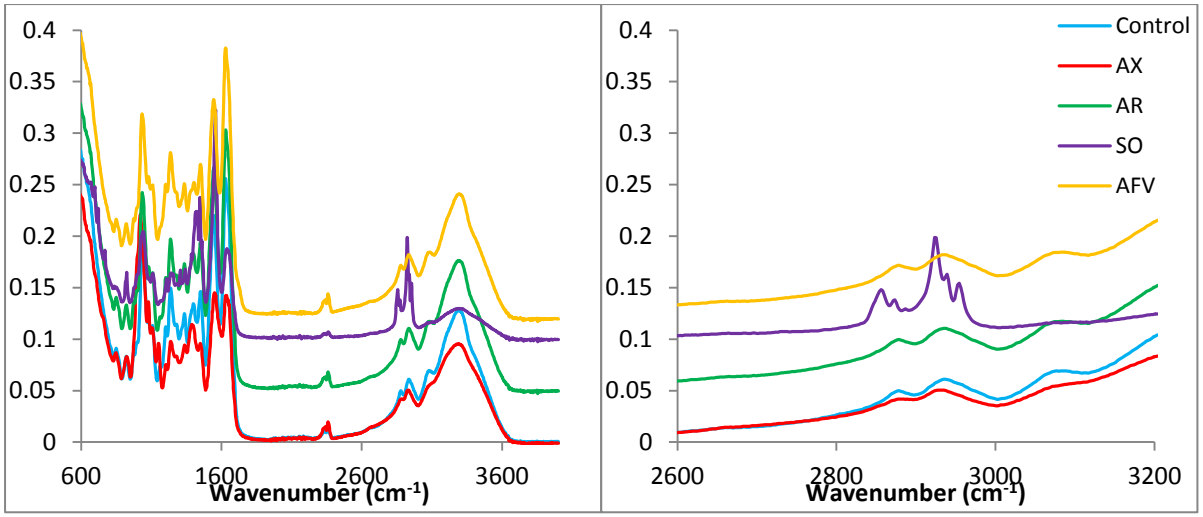


Fig. 3. FTIR spectra of beef gelatin control film and beef gelatin films with antimicrobials (AFV, AX, AR, and SO).

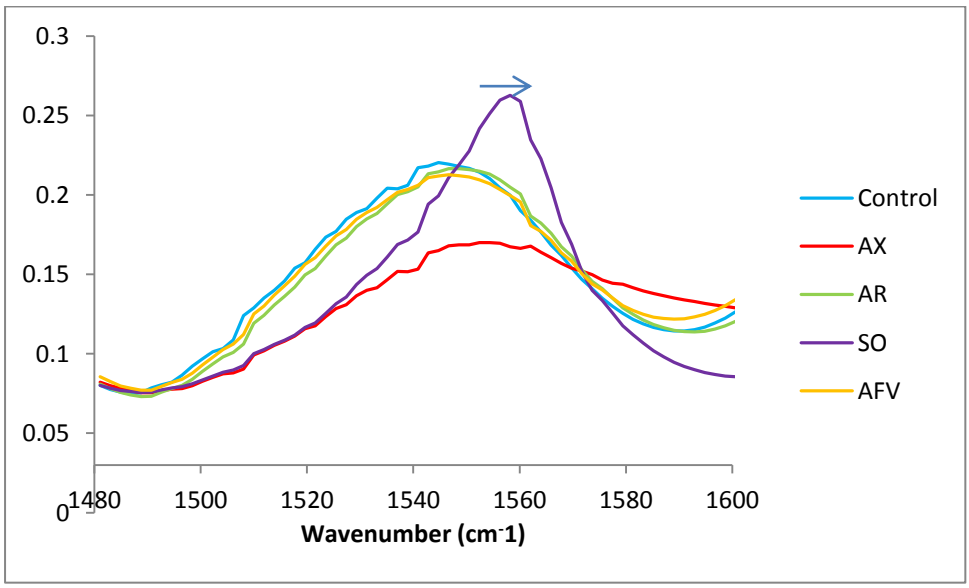


Fig. 4. FTIR spectra of beef gelatin control and beef gelatin with antimicrobials namely AFV, AX, AR, and SO.

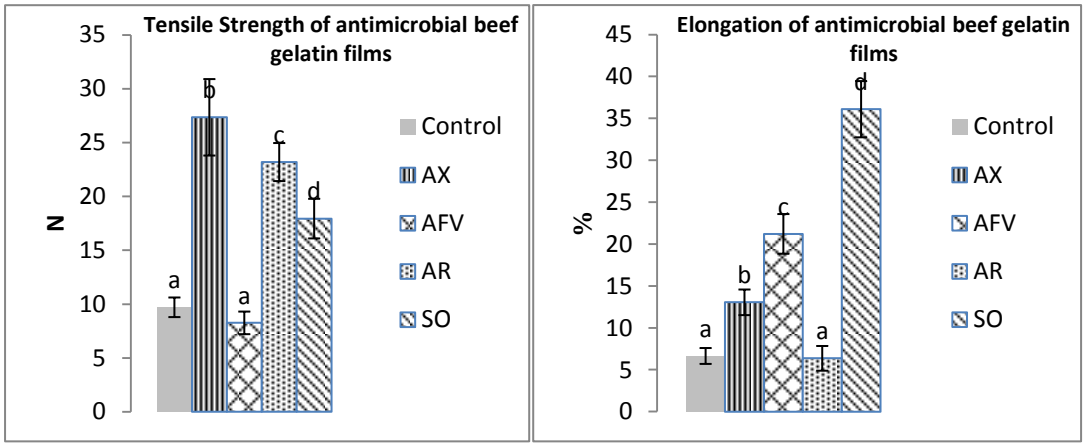


Fig. 5. Mechanical properties of antimicrobial films, Different letters indicate significant differences ($P < 0.05$).

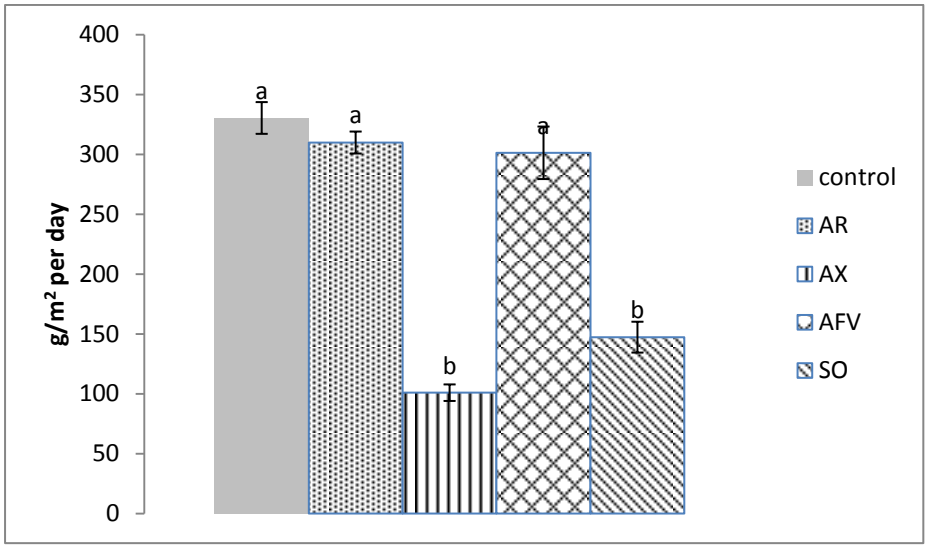


Fig. 6. WVTR (g/m^2 per day) of beef gelatin films with antimicrobials in comparison with a control, Different letters indicate significant differences ($P < 0.05$).

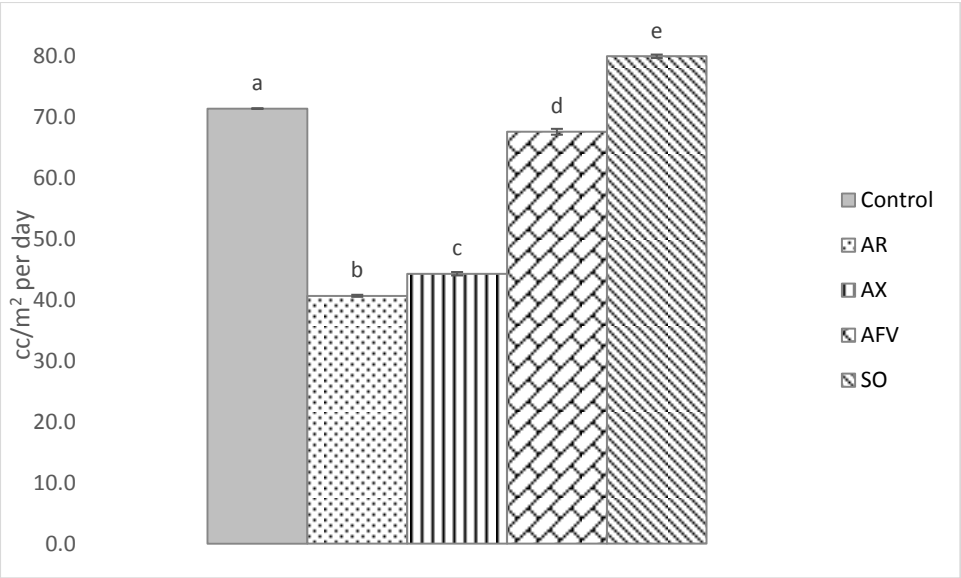


Fig. 7. Oxygen barrier properties (cc^3/m^2 day) with 100% gas permanent concentration of beef gelatin films at 50% relative humidity. Different letters indicate significant differences ($P < 0.05$).

***Highlights (for review)**

- 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 octanote 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.