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Abstract

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Keywords	Gelatin; Coatings; Antimicrobials; Active Food Packaging; Plasma treatment

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Abstract

Two antimicrobial coatings, namely Sodium octanoate and Auranta FV (a commercial antimicrobial composed of bioflavonoids, citric, malic, lactic, and caprylic acids) were used. These two antimicrobials were surface coated onto the inner polyethylene layer of cold plasma treated polyamide films using beef gelatin as a carrier and coating polymer. This packaging material was then used to vacuum pack beef sub-primal cuts and stored at 4°C. A control was prepared using the non-coated commercial laminate and the same vacuum packaged sub-primal beef cuts. During storage, microbial and quality assessments were carried out. Sodium octanoate treated packages significantly ($p<0.05$) reduced microbial counts for all bacteria tested with an increase of 7 and 14 days, respectively compared to control samples. No significant effect on pH was observed with any treatment. The results suggested that these food grade antimicrobials have the potential to be used in antimicrobial active packaging applications for beef products.

1. Introduction

Microbial contamination has long been recognised as the main source of spoilage and reduction of meat quality (Kerry et al., 2012). Prolonging shelf-life, along with delivering a high quality food product in suitable flexible packaging, is essential in order to address issues such as, global supplier demands, economical profit, customer satisfaction, reducing packaging, and more importantly, reduction of food wastage. Smart packaging solutions presented in the form of antimicrobially-active packaging may be a solution, or part of a solution, to these and other issues (Kerry et al., 2008).

Naturally-occurring active antimicrobial substances offer many potential benefits with respect to food packaging applications. Taken from natural food sources, potentially edible, and therefore food contact-friendly films and coatings offer new alternatives in relation to food packaging materials and packaging applications. Edible films and coatings have been used for preservation purposes and can be formulated into active materials with the addition of antimicrobial substances (Cuq et al., 1995; Campos et al., 2011).

Bioactive edible/biodegradable films have limitations with respect to certain mechanical properties such as transparency, and printability issues. Therefore, such limitations mean that these materials will never compete in volume or scale with industrially-based conventional packaging materials for some time to come. However, the development of such materials should be encouraged as they would certainly present novel materials with niche roles and applications, especially when used in combination with conventional plastic-based packaging materials

The use of gelatin to form edible films or coatings has been well documented since the 1960's (Hanani et al., 2012a). Films formed using gelatin sources as a primary biopolymer packaging film are more desirable to manufactures as they are low cost and wide availability (Hanani et al., 2013). Gelatin has been shown to produce films with good transparency, mechanical and barrier properties, and can be manufactured by extrusion or casting processes (Hanani et al., 2012b, Hanani et al., 2013, Molinaro et al., 2015; Wang et al., 2007). There is increased interest in the development and use of edible packaging materials to preserve meat quality for longer storage periods, while at the same time, maintaining food safety based on consumer demands for natural and safe products (Cutter et al., 2006; Ortega et al., 2014). Polymeric matrices, as well as bioactive coatings and films, can be used as carriers for natural antimicrobials. This technique allows for slow diffusion of the antimicrobial agent from the packaging material, thus allowing a continuous antimicrobial effect on the food product surface over time (Han et al., 2014). This approach has been shown to be more effective than spraying, dipping or submerging products with or in solutions of antimicrobial agents (Lu et al., 2010, Muriel-Galet et al., 2012, Quintavalla & Vincini, 2002, Suppakul et al., 2008 & Yingyuad et al., 2006). Numerous publications have reported the effects of activating biopolymer-based materials with antimicrobial substances for packaging applications (Gómez-Estaca et al., 2010, Oussalad et al., 2004, Millette et al., 2007, Gill et al., 2002, & Morsy et al., 2014) to name a few. However, limited studies on bovine gelatin as a polymer carrier of antimicrobial substances to reduce the microbial growth on fresh meat have been reported. Recently our group demonstrated the application of beef gelatin films with incorporated antimicrobials as active packaging. Four commercially derived antimicrobials namely, Articoat DLP 02 (AR), Artemix Consa 4152/NL (AX), Auranta FV (AFV) (a mixture of citric acid, malic acid, lactic acid and

caprylic acid in water) and Sodium octanoate (SO) were 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 food packaging materials (Clarke et al. 2016).

To the best of our knowledge, no studies have reported the effect of the use of cold plasma treatment to improve attachment properties of activated gelatin solutions containing antimicrobials, and the application of the developed coated materials to reduce the microbial growth and extend the shelf life of fresh beef sub-primals. Therefore, the objective of this study was to examine the effectiveness of surface attachment of active beef-derived gelatine antimicrobial (SO and AFV) coatings onto conventional commercial vacuum packaging laminates (polyethylene/polyamide) and assess the performance of these packaging materials on microbial counts of vacuum packaged beef steaks.

2. Materials and methods

2.1. Materials, microbiological media and beef

Antimicrobials used in this study included SO, and AFV. SO was obtained from Sigma-Aldrich, UK and AFVfrom Envirotech Innovative Products Ltd, Ireland. Glycerol (KB Scientific Ltd, UK) was used as a plasticizer and Beef gelatin 100 bloom (Helan ingredients, UK) was used as the basal material for all film matrices. Two beef subprimal striploins were purchased from a local beef supplier and were delivered at 4^{o}C in vacuum-skin plastic packaging formats and used on the same day of delivery. Conventional vacuum polyamide/low density polyethylene (PA/LDPE) pouches (200x300 mm, water vapour transmission rate of 2.8 g/m^2 24 hr and oxygen permeabiltlty rate of 50 cm^3/m^2 24 hr) were supplied by Cryovac (Sealed Air W.R. Grace Europe Inc., Lausanne, Switzerland) and used as industry standard materials for coating and meat packaging trials throughout these studies.

2.2. Plasma treatment

In order to increase the hydrophilicity of the LDPE inner part of the PA/LDPE laminate vacuum pouches, cold plasma treatment was carried out on a Dielectric-

Barrier Discharge (DBD) plasma system prior to the application of the antimicrobial coatings.

Briefly, pouches of 200 x 300 mm size were cut to size 170 x 280 mm and the surface of the LDPE side of the laminate pouches were plasma treated at atmospheric pressure using atmospheric air. A schematic representation of the experimental setup is presented in Figure 1. The plasma source consisted of two circular aluminium plate electrodes (outer diameter = 158 mm). The top dielectric barrier was Perspex dielectric barrier (10 mm thickness) and the bottom dielectric barrier was polypropylene sheet (5 mm thickness). When the potential across the gap reached the breakdown voltage the dielectric barrier prevented the arc transition and also homogenised the micro-discharges. The applied voltage was 80 kV which was obtained from a step-up transformer (Phenix Technologies, Inc., USA) using a variac. The input of 230V, 50 Hz was given to the primary winding of high voltage step-up transformer from the mains supply. At the time of treatment, the atmospheric air condition was 45% relative humidity (RH) and 22°C. The samples were plasma treated for 60 sec to cover the entire film area, leaving only approximately 5 cm from the edge of the film (high voltage electrode was placed 1 cm above the film).

Plasma treatment was carried out on 60 different film samples. Following treatment, plasma treated samples were placed in zip lock plastic bags to protect films from antistatic environments and dust particles.

Static contact angle (Theta Lite Optical Tensiometer, Biolin Scientific, UK) was measured before and after plasma treatment by the sessile drop technique. A drop of each test liquid (water or ethylene glycol) was uniformly placed on the film surface using a needle and the image recorded at 15 frames per sec for 10 sec. Images were analyzed using the OneAttension software (v 2.1). All values reported are the mean of more than 130 data points carried out in triplicate. Surface-free energy was calculated using the Owen-Wendt model, utilising the same software. Water and ethylene glycol were used as the test liquids.

Plasma treated film samples were coated with water-based gelatine coatings containing the test antimicrobials. For plasma treated films, films were gelatine coated after 30 hr of plasma treatment.

2.3. Coatings of edible antimicrobial films and packaging of beef

2.3.1. Preparation of film forming solutions and coatings

Gelatine film forming solutions were prepared according to the procedure outlined by Wang et al, (2007), with minor modifications. Briefly, dry beef gelatine was dissolved in distilled water (5% w/w) by heating at 90° C in a shaking water bath (SW23, Julabo USA INC., Allentown, PA, USA) for 30 min and glycerol was added under constant stirring at a concentration of 33% w/w dry matter. Successively, the solution was cooled down at room temperature to 40° C before the addition of the antimicrobials. The antimicrobials were added at twice the minimum inhibitory concentration (MIC) determined for each of the two antimicrobials used (Clarke et al., 2016). The MIC of AFV and SO was previously determined at levels of 1.5 and 1%, respectively and added at a weight of 3g and 2g respectively (Clarke et al., 2016). The solution was then stirred for 15 min using a sterile magnetic stirrer before casting onto levelled plasma-treated LDPE film surfaces using a Micron II film applicator (Gardco, FL, USA) and dried for 48 hr at 20˚C. Gelatine coating thickness was measured using a digital micrometer - Käfer Digital Thickness gauge (Käfer Messuhrenfabrik GmbH & Co., Villingen-Schwenningen, Germany) at 5 random locations of 10 random films for each sample treatment. The thickness of gelatine coating layer ranged between 11–15 µm.

2.3.2. Vacuum packaging of beef sub-primals using active antimicrobial coated laminate pouches

Plasma-treated conventional PA/LDPE films coated with active gelatin-based coatings were detached from the flat surface on which they were coated, the edges of each laminate sample was cleaned with ethanol to remove unwanted residue. Each pack was heat–sealed to form a pouch (170x290 mm) using Webomatic type D463 (Webomatic Vacuum Packaging Systems, Werner Bonk, Mausegat 59, D 463 Bochum 6, Germany) with the sealing time set at 2.5 s. In order to avoid adhesion between the coated films, sterile food grade aluminium foil was placed between the films prior to sealing.

Beef striploins were cut into 90 steaks (2.5 cm in thickness). A total of 30 steaks were individually vacuum packaged in AFV coated pouches, SO coated pouches or

non-coated pouches without plasma-treatment (control group), respectively using a Webomatic type D463 (Webomatic Vacuum Packaging Systems, Werner Bonk, Mausegat 59, D 463 Bochum 6, Germany) machine (20 sec vacuum process, 2.5 sec heat-seal dwell-time). Vacuum packaged samples were stored under chilled conditions of 4^0C in a walk-in chilled room and sampled at weekly intervals for up to 25 weeks.

2.4. pH

The pH of the beef steaks was measured using a digital pH metre (Mettler-Toledo GmbH, Schwerzenbach, Switzerland) by inserting the glass probe directly into the beef steak. Each value represents the average of three readings.

2.5. Microbiological studies

Microbiological analyses of beef steaks during storage at 4 ^{o}C were performed in triplicate on each measurement day. In order to obtain a representative sample, three steaks from each treatment were randomly selected for analysis. A total of 10g from both the upper and bottom parts of the surface of the steak was aseptically taken using sterile forceps and scalpel, placed into a sterile stomacher bag (Seward, UK) to which 90 ml of sterile Maximum recovery diluent (MRD) (Oxoid, UK) was added as an isotonic diluent and thoroughly mixed for 3 min using a stomacher (Seward, UK) in order to obtain a primary 10-fold dilution. This was then 10-fold serially diluted using MRD and used to enumerate total viable counts (TVC), psychrotrophic bacteria, total anaerobic bacteria (TAB), Lactic acid bacteria (LAB), total coliforms and *Escherichia coli* (*E. coli*).

The TVC and psychrotrophic bacteria were enumerated in Plate count agar (PCA) plates (Oxoid, Basingstoke, UK) after incubation for 48 hr at 30 °C or 7 days at 4° C, respectively. Total coliforms and *E. coli* were enumerated on Compact Dry E.C plates (Nissui Pharmaceutical, Co. Ltd. Japan) (20 cm^2) after incubation at 37^{o}C for 24 hr. LAB were enumerated on overlaid MRSA agar (Oxoid) after incubation for 3 days anaerobically at 30 ^{o}C in anaerobic jars containing Anaerocult® (Merk). TAB were enumerated on Tryptone soy agar (TSA) (Oxoid) agar enriched with 0.6% yeast extract Oxoid (Basingstoke, UK) after 3 days incubation anaerobically at 30^{o}C in anaerobic jars containing Anaerocult®. *Clostridium* spp. were enumerated on

reinforced *Clostridium* agar (Oxoid) after incubation for 3 days anaerobically at 30°C in anaerobic jars containing Anaerocult®. Bacterial numbers were converted to $\log_{10}$ colony-forming units per gram sample (CFU/g sample) prior to statistical analyses.

2.6. 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 discussions

3.1. Surface treatment of PE/PA packs

In a previous study Clarke et al. (2016) demonstrated that SO and AFV displayed the greatest antimicrobial efficacy against the tested bacterial strains; (*Bacillus cereus*, *Pseudomonas fluorescens*, *Escherichia coli*, *Staphylococcus aureus* and aerobic and anaerobic beef microflora) therefore, those antimicrobials were chosen for this study. SO and AFV were incorporated in beef gelatine solutions and coated onto the inner LDPE of the conventional vacuum PA/LDPE pouches

Beef gelatin hydrophilic water solutions cannot be attached properly onto hydrophobic polyethylene surfaces. Polyethylene is by nature a hydrophobic material; therefore, it is necessary to modify the surface of the film prior to coating to improve wettability and achieve good adhesion (Robertson et al., 2014). There are several types of surface modification techniques used in the packaging industry for adhesion improvement of coatings, among others; corona treatment, chemical treatment or priming (Robertson., 2014) and plasma treatment. Cold plasma induces several chemical and physical processes within the plasma volume and on the plasma-polymer interface, which modify surface properties (Pankaj et al., 2014). Therefore DBD plasma treatment of the LDPE surface was applied in order to increase the affinity with the gelatine solution. In order to investigate the surface energy properties of the PE before and after plasma treatment the contact angle was

measured (Yuan and Lee, 2013). The contact angle is defined as the angle Θ formed by the intersection of the liquid interface and the surface interface (fig. 2).

A significant ($p<0.05$) decrease in the contact angle for both test liquids (water and ethylene glycol) was observed after plasma treatment of the surface of LDPE (Table 1A). The untreated LDPE film had a water contact angle Θ of 97.7°, thereby showing the hydrophobic nature of the film. After plasma treatment at 80 kV for 60s the water contact angle decreased to 59.9°, thus demonstrating an increase in the surface hydrophilicity. Contact angle was also measured during film storage to identify if the hydrophobic nature of the film was recovered. A significant ($P<0.05$) increase of the water contact angle was noticed during film storage, reaching a contact angle of 73.6° after 75 hr storage at room temperature. The total surface free energy (γ^{tot}) increased significantly ($P<0.05$) due to a significant increase in the polar component (γ^{p}) and consequently, a decrease in the dispersive component (γ^{d}) (Table 1B). During film storage, an increase in the dispersive component and decrease in the polar component was also observed.

Table 1: Contact angle (A) and surface free energy of DBD plasma treated PE film during storage

A. Contact angle

Sample	Storage time (hr)	Water (°)	Ethylene glycol (°)
Control (LDPE)	0	97.7±1.5	78.2±1.7
80kV-60s	0	59.9±3.0	50.9±2.7
80kV-60s	30	66.9±1.4	37.3±0.4
80kV-60s	53	66.6±0.7	44.1±3.6
80kV-60s	75	73.6±0.2	55.2±0.1

B. Surface free energy

Sample	Storage time (hr)	γ^{tot} [mN/m]	γ^{d} [mN/m]	γ^{p} [mN/m]
Control (LDPE)	0	18.2	14.5	3.7
80kV-60s	0	43.2	5.2	38.0
80kV-60s	30	38.7	23.0	15.7
80kV-60s	53	36.4	16.2	20.2
80kV-60s	75	30.5	13.1	17.4

The results presented in this study clearly demonstrate the importance of DBD plasma treatment of PE films before coating for making a more homogenous antimicrobial coating on the surface of film. However, hydrophobic nature recovery

of the DBD plasma treated film during storage indicated that the coating process must be carried out soon after DBD plasma treatment. In the present study, for optimum adhesion and to obtain a more homogeneous film coating LDPE films were coated with the antimicrobial coating within 24 hr of plasma treatment.

3.2. pH values of packaged beef steaks

The effects of active antimicrobial treatments on the pH values of vacuum packaged beef steaks are shown in Table 2. The initial pH of fresh beef in the control packs was 5.5. Following the immediate vacuum packaging of steaks using both AFV and SO antimicrobial films, steak pH values significantly ($p < 0.05$) increased within 24 hrs of packaging. This increase in pH was most likely due to the initial pH of the antimicrobial coating film forming solutions which were 6.2 and 6.5, respectively. A similar effect was reported by Emiroğlu et al. (2010) who applied soy protein-based edible films (pH 10.0) to beef patty surfaces.

However, in our study, the increased meat pH was only short-lived, possibly due to the initial pH of the beef having a neutralising effect on the overall pH, thus the pH decreased quickly and normalised meat pH values were maintained throughout the storage time. The decrease in steak pH in antimicrobially-treated packs could be attributed to the presence of lactic acid bacteria (LAB). Strains of LAB can produce fermentation products in the form of organic acids which may result in a decrease in pH over time (Jones, 2004; Sakala et al., 2002). LAB strains of bacteria are known to be resistant to many antimicrobial substances (Han et al., 2014; Fik & Leszczyska-Fik, 2007; Emiroğlu et al. 2010). In our study, we observed the successful growth of LAB in our antimicrobially-treated packs (see next section for greater detail).

The opposite trend was observed for the control samples, where a slight increase in pH was observed until day 23 of storage. An increase in pH may have been caused by the high levels of psychrotrophic bacteria present during the initial days of storage. Psychrotrophic bacteria produce volatile amine and ammonia which can increase pH levels and that the accumulation of those metabolites produced by psychotropic bacteria growth may cause pH increase (Cortez-Vega et al., 2012).

Table 2. The changes of pH on vacuum packaged beef steaks held in control and antimicrobially-active packaging during chilled storage

Storage days	Control	AFV	SO
1	5.5 ± 0.01^{a}	6.4 ± 0.02^{b}	6.4 ± 0.01^{b}
7	5.5 ± 0.05^{a}	5.6 ± 0.06^{ab}	5.7 ± 0.03^{b}
14	5.5 ± 0.04^{a}	5.6 ± 0.02^{b}	5.7 ± 0.01^{b}
23	5.7 ± 0.04^{a}	5.6 ± 0.02^{ab}	5.7 ± 0.01^{b}
29	5.6 ± 0.02^{a}	5.6 ± 0.02^{a}	5.7 ± 0.05^{b}
36	5.5 ± 0.02^{a}	5.5 ± 0.02^{a}	5.6 ± 0.01^{b}

^{a,b}Mean values and standard deviations in the same row with different superscripts are significantly different ($p < 0.05$).

3.3. Microbiological analysis

Initial TVC and psychrotrophic bacteria counts for control, AFV and SO packaged beef steak samples after day 1 of storage were reported as 3.18 log, 2.70 log and 3.00 log CFU/g, respectively, indicating that beef steaks were of good microbiological quality (Fig. 3a). In general, during storage independent of the packaging system used, the TVC numbers increased; however, throughout storage higher growth was noticed in control samples compared to samples stored in antimicrobially-coated pouches. Microbiological standards and guidelines give guidance on the types of microorganism and their number that can be considered acceptable or unacceptable or unsafe in a food product. The following recommended microbiological limits are applied for raw meat: Aerobic plate counts: m = 10^{6} (CFU/ g of meat) (acceptable limit) and M= 10^{7} (CFU/ g of meat)) (Unacceptable limit); *E. coli*: m = 50 CFU/g of meat (acceptable limit) and M= 500 CFU/ g of meat), (European Commission, 2007; ICMSF, 1986). For this study the limit of acceptability was set to 6 log (CFU/g of meat) and *E. coli* 50 CFU/g of meat. Microbiological enumeration for TVC, psychrotrophic bacteria, LAB, and TAB bacteria during storage are shown in figure 3.

Throughout storage, significantly (P<0.05) lower bacterial counts were observed in samples that were vacuum packed in packs coated with antimicrobial SO compared to samples that were vacuum packed with control films or films coated with antimicrobial AFV. The results found in this study is in agreement with results reported previously by Clarke et al. (2016) in which SO was the most effective antimicrobial against pure cultures of *B. cereus*, *Ps. fluorescens*, *E. coli*, *S. aureus*

and the aerobic and anaerobic microflora from beef steaks as it had the lowest MIC compared to the other antimicrobials tested. The authors also found that beef-derived gelatin films containing SO enhanced the mechanical properties and water vapour permeability when compared to control gelatin films.

The limit of acceptability was reached at day 27, 35 and 42 for samples that were vacuum packed with control, AFV coated antimicrobial or SO antimicrobially coated films, respectively. Therefore, beef vacuum packed with the antimicrobial coating containing AFV or SO stayed within acceptability levels 33 or 55% longer, respectively compared to samples that were vacuum packed with control films.

Significantly ($P<0.05$) slower growth of psychrotrophic bacteria was noticed in beef steaks that were vacuum packed with SO coated LDPE films compared to beef steaks vacuum packed with AFV coated LDPE films or control films (Fig. 3B). However, at the end of storage, no significant differences were noticed between the samples that were vacuum packed with AFV or SO coated LDPE films.

SO films significantly ($p<0.05$) reduced bacterial counts compared to control samples for psychrotrophic bacteria from day 14 until the end of testing, AFV treated packaging only significantly ($p<0.05$) reduced bacterial counts as compared to control packaging on day 21 of testing (Fig. 3B) No significant differences were observed at the end of the testing period between AFV and SO films for psychrotrophic bacteria.

A similar pattern was observed on TAB and LAB counts. SO coated LDPE films significantly ($p< 0.05$) reduced TAB and LAB bacterial counts compared to steaks that were vacuum packed with AFV coated LDPE films or control films from day 14 onwards until the end of testing and storage period (Fig. 3C, 3D). *Clostridium* spp. was absent throughout the storage period, coliforms were present on day 35 of testing and were slightly higher in treatment samples, however, levels remained within acceptable limits (5×10^2) throughout the testing period. Similar results were reported in a study conducted by Brightwell et al, (2009) who compared the microflora of peroxyacetic acid treated and vacuum-packed beef. Enterobacteriaceae counts were first detected on week 6 of testing and were found to be higher in treated versus untreated samples, with levels of Enterobacteriaceae being 25% and 11%, respectively, of the total microflora present.

4. Conclusion

Beef gelatin as a carrier and coating polymer was used to successfully develop antimicrobial gelatine coated LDPE films by coating the inner PE layer of conventional PA/LDPE laminated films after surface activation using cold plasma treatment. The coated antimicrobials tested demonstrated different degrees of effectiveness against bacterial species evaluated on beef steaks. Based on the limit of acceptability in terms of total aerobic counts and *E. coli*, the antimicrobial AFV and SO coated films significantly prolonged the shelf life of beef steaks by 33 or 55%, respectively, compared to samples that were vacuum packed using control film samples. Overall, SO or AFV have the potential of being used as effective antimicrobials for antimicrobial active packaging applications for muscle-based food products.

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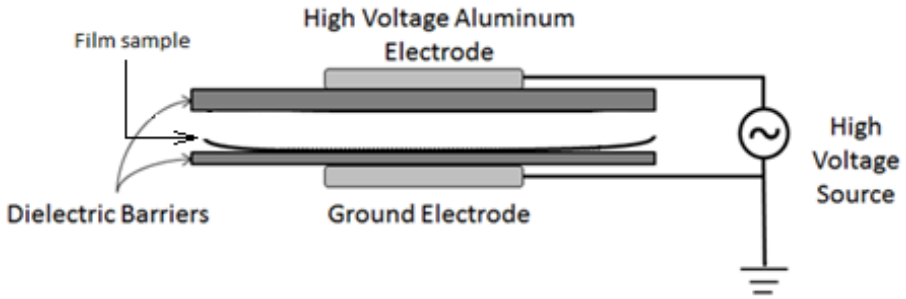


Fig. 1. Schematic representation of the experimental setup for plasma treatment.

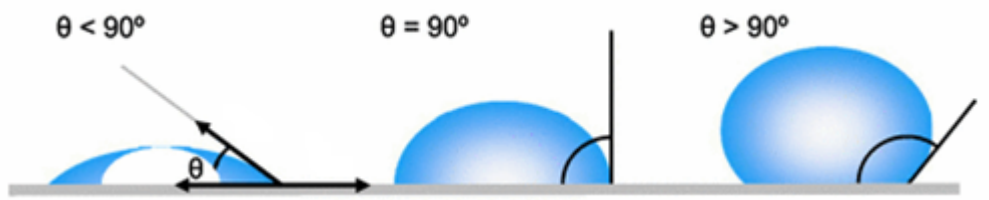
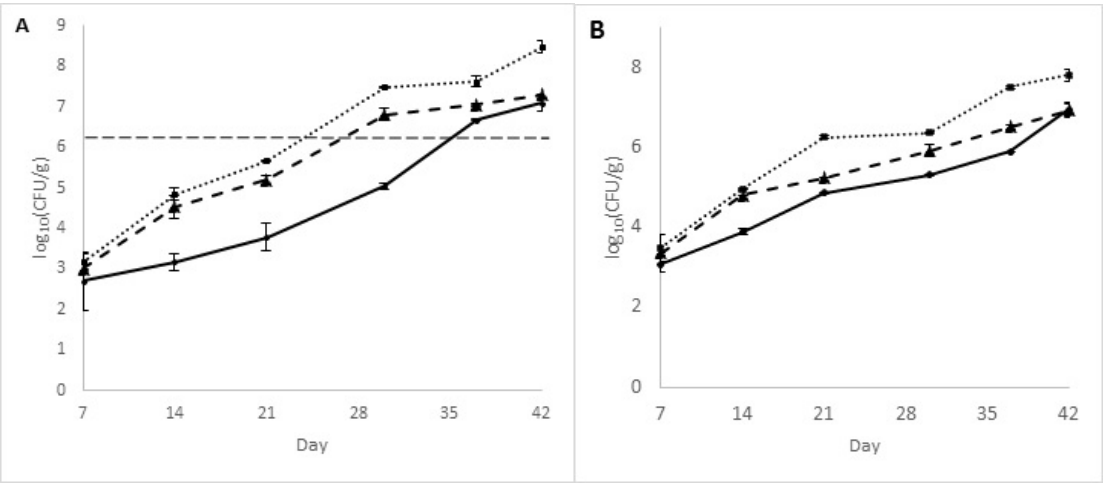


Fig. 2. Contact angle formed by sessile liquid drops on a smooth homogeneous solid surface (hydrophilic surface ($\theta < 90^0$) hydrophobic surface ($\theta > 90^0$), (Yuan and Lee, 2013).



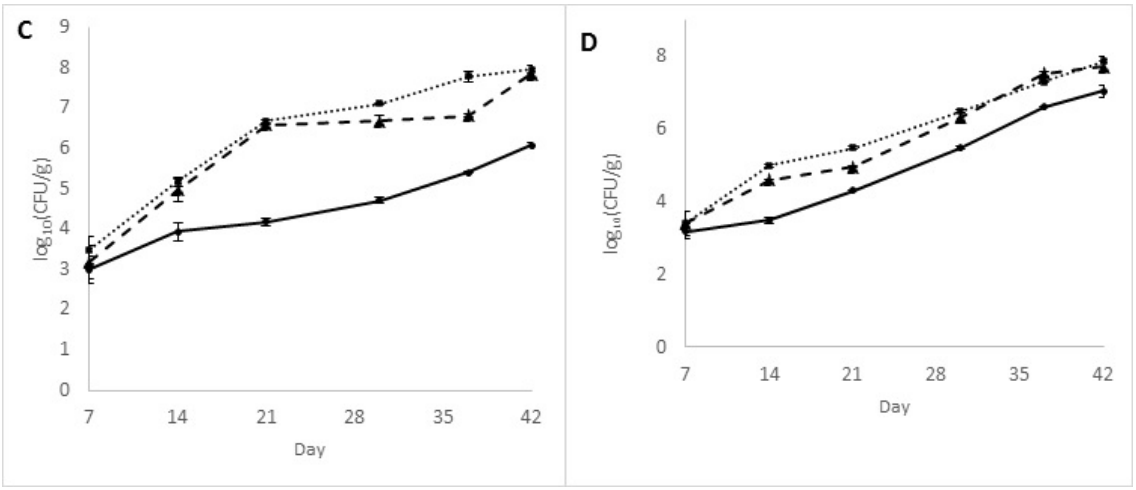


Fig. 3. Changes in (A) Total viable counts, (B) Psychrotrophic bacteria (C) Lactic acid bacteria, and (D) Total anaerobic bacteriaduring chilled storage of beef steaks vacuum-packaged using control (...) or antimicrobially-active (---) AFV or (−) SO packaging films. The dotted line in (A) indicates the limit of acceptability. Bars represent standard deviation of three independent samples and may be hidden by symbols when small.

- Beef gelatin was successfully coated on plasma treated polyethylene surface
- Auranta FV and Sodium octanoate coated films prolonged the shelf life of beef cuts
- AFV and SO potentially can be used in antimicrobial active packaging

Surface attachment of active antimicrobial coatings onto conventional plastic-based laminates and performance assessment of these materials on the storage life of vacuum packaged beef sub-primals.

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