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# Antimicrobial effect of benzoic and sorbic acid salts and nano-solubilisates against *Staphylococcus aureus*, *Pseudomonas fluorescens* and chicken microbiota biofilms

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ABSTRACT

The objective of this study was to evaluate the antimicrobial effects of benzoic and sorbic acid salt and their nano-solubilisates against planktonic and biofilm cultures of *Staphylococcus aureus*, *Pseudomonas fluorescens* and chicken microbiota. The antimicrobial activity was affected by the particle size of the organic acid antimicrobials, the Gram-strain, and the type of culture (planktonic or biofilm) used. The organic acid nano-solubilisates were significantly ($P < 0.05$) more effective compared to their organic acid salt counterpart with respect to both planktonic and biofilm cultures. However, biofilms of *Staphylococcus aureus*, *Pseudomonas fluorescens* and chicken microbiota were significantly ($P < 0.05$) more resistant to both organic acids salts and nano-solubilisates compared to the planktonic cultures. The physicochemical properties of the organic acids antimicrobials was assessed using particle size analysis, Fourier Transform Infrared (FTIR) spectroscopy and Atomic Force Microscopy (AFM) analyses. Biofilm formation after 24 h was quantified using a crystal violet assay and the minimum inhibition concentration against planktonic and biofilm cultures was also determined. The unique physiochemical properties of these nanomaterials may allow for potential applications in the development of naturally derived antimicrobial active packaging materials.

## 1. Introduction

Significant research has been carried out on the effects of naturally derived antimicrobials materials (NAM's) against planktonic bacteria; however, these only account for approximately 0.1% of the global bacterial biomass (Bjarnsholt, Ciofu, Molin, Givskov, & Høiby, 2013). Under appropriate conditions, planktonic bacteria can multiply rapidly; however, their rapid proliferation makes planktonic bacteria more susceptible to pH change, fluctuations in temperature and the presence of antimicrobials (Almasoud, Hettiarachchy, Rayaprolu, Horax, & Eswaranandam, 2015). Food processing and packaging environments can provide ideal conditions for the rapid growth of planktonic microorganism; however, modern cleaning techniques and practices have significantly reduced potential sources of contamination (Simões, Simões, & Vieira, 2010). While planktonic bacteria remain a source of contamination that can result in food spoilage, it has been reported that the majority of food spoilage from microorganisms arises from biofilms which are the source of up to 65% of food contamination (Paraje, 2011). A biofilm is a community of microbes that collate together and attach on a surface and forms a self-produced, protective outer layer made from extracellular polymeric substances (EPS) (Donlan, 2002; Pande, McWhorter, & Chousalkar, 2018). Biofilms can be made up of either a homogeneous or heterogeneous combinations of Gram-positive and Gram-negative bacteria (Kumar & Anand, 1998) and can attach onto surfaces through either a passive mechanisms such as gravity, electrostatic forces or van der Waals interactions or through an active mechanism *via* the pili or flagella structures of these microorganisms. Typically, biofilms will preferentially attach to lipophilic surfaces such plastics, rubbers and polytetrafluoroethylene (PET) (Brooks & Flint, 2008; Chmielewski & Frank, 2003; Simões et al., 2010). Nonetheless, biofilms are also found to form on various materials used in food processing and packaging industry including; stainless steel or glass (Brooks & Flint, 2008; Chae, Schraft, Truelstrup; Hansen, & Mackereth, 2006; Srey, Jahid, & Ha, 2013). Food packaging and processing environments provide suitable conditions for the formation of biofilm such as temperature, pH (Brooks & Flint, 2008), relative humidity (Lee,

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Bae, Lee, & Lee, 2015), and the availability of minerals and nutrients (Donlan, 2002). Upon the formation of a biofilm mass on a substrate, the gene expression of the collated bacteria changes to allow the growth of EPS which protects the biofilm from environmental changes and stressors such as detergents or sanitisers including; hypochlorite's, quaternary ammonium compounds and antibiotics (Chae, Schraft, Truelstrup Hansen, & Mackereth, 2006; Lee et al., 2015; Mah & O'Toole, 2001; Hood & Zottola, 1995). Biofilms can propagate through the release of bacterial EPS fragments that detach from the biofilm mass and can attach elsewhere to start the formation of new biofilm mass (Bridier et al., 2015).

While many sanitising strategies such as clean-in-place have been successfully employed to control the growth of planktonic bacteria, biofilms are more resistant to these types of treatments due to their more complex structure and composition (Bridier et al., 2015). Therefore, novel strategies are required to control biofilm proliferation and studies have shown that biofilm formation and contamination can be significantly reduced through the application of organic acids (Almasoud et al., 2015; Amrutha, Sundar, & Shetty, 2017). Two organic acids widely used in the food industry as preservatives are sorbic and benzoic acid. These organic acids occur naturally in many fruits or plants and are also produced by the gut in millimolar quantities (Pande et al., 2018). Moreover, their application in food products are favourable due to properties including; their Generally Recognized as Safe (GRAS) status from the FDA, clean label, colourless and tasteless properties and a historical use in food preservation (Berger & Berger, 2013; Sieber & Bosset, 1995). Organic acids salts have been previously used on biofilms by Almasoud et al. (2015) who applied lactic and malic acid solutions *via* electro-spraying onto spinach and cantaloupe rinds inoculated with biofilms of *E. coli* O157:H7 and *Salmonella* Typhimurium where it was found that the combined treatment of lactic acid and malic acid (2 + 2% w/v) showed the greatest log reduction of 4.14 and 3.6 (CFU/disk) on spinach and cantaloupe rinds inoculated with biofilm cultures, respectively. Moreover, organic acid preservatives either individually or in combination have been applied on a variety of food products such as buffalo meat (Malik & Sharma, 2014), beef steak microbiota (Clarke et al., 2016), frankfurters (O'Neill, Cruz-Romero, Duffy, & Kerry, 2018), cooked turkey meat (Contini et al., 2014), broccoli sprouts (Chen et al., 2019), and lettuce (Zhao, Zhang, & Yang, 2017).

The recent development of engineered natural antimicrobial (NAM) nanomaterials has shown promising potential for the application of nanotechnology in the food sector (Azlin-Hasim et al., 2015). These NAM materials overcome many of the toxicological and environmental concerns posed by use of metal derived nanoparticles (Gaillet & Rouanet, 2015), while maintaining their enhanced properties such as greater antimicrobial activity over their bulk counterparts (Dutta, Dey, Shome, & Das, 2011; Ngan et al., 2014; Paomephan et al., 2018; Pilon et al., 2015). Studies have reported that biofilms are more susceptible to nanomaterials due to their smaller dimensions which facilitates greater accesses to the internal structures and organs of biofilms, disrupting internal organ functions (Epstein, Pokroy, Seminara, & Aizenberg, 2011). Organic acid salts and nano-solubilisates of sorbic acid and benzoic acid are currently commercially available and Cruz-Romero, Murphy, Morris, Cummins, and Kerry (2013b) applied these materials against *P. fluorescens*, *S. aureus*, *Bacillus cereus*, *Escherichia coli*, chicken microbiota and cheese derived microbiota and found that nano-sized solubilisates of benzoic acid and sorbic acid had significantly ($P < 0.05$) higher antimicrobial properties than their non-nano equivalents.

To the best of our knowledge there is little information regarding the antimicrobial effect of organic acids (sorbic and benzoic acid) and their commercially-available nano-sized solubilisate equivalents against planktonic and biofilm cultures of *Staphylococcus aureus (S. aureus)*, *Pseudomonas fluorescens (P. fluorescens)* and chicken microbiota. Therefore, the aim of this study was evaluate and compare the antimicrobial activity of sorbic and benzoic acid salt and their commercially available nano-sized solubilisates against planktonic and biofilm cultures of *S. aureus*, *P. fluorescens* and chicken microbiota.

## 2. Materials and methods

### 2.1. Materials

Benzoic acid (BAS) and Sorbic acid (SAS) were purchased from Sigma-Aldrich (Ireland). Water- and fat-soluble 4% sorbic acid solubilisate (SASB) and 12% benzoic acid solubilisate (BASB) were obtained from Aquanova (Aquanova, Darmstadt, Germany). Crystal violet (CV) and glacial acetic acid were obtained from Fisher Scientific, Ireland. For all tests, Mueller-Hinton broth (MHB, Oxoid) was used as growth media and for any culture dilution. The bacterial loads were determined using spread plate method on plate count agar (PCA) (Merck, UK). Sterile distilled water was used for any washing process.

### 2.2. Physical characterisation of organic acid salts and nano-solubilisates

Particle size analysis of SASB, and BASB was carried out using a Malvern Zetasizer Nano Series HT (Malvern, U.K.). Solubilisate solutions were loaded into a disposable cell (ZEN0040) and analysis was performed at 25 °C using a scattering angle of 173°. The particle size distribution and polydispersity index (PDI) of the SASB and BASB solutions was determined using the Mark–Houwink method and values are average values of duplicate measurements with 3 replicates per sample. Fourier transform infrared spectroscopy (FTIR) analysis of SAS, BAS, SASB, and BASB was performed on a Varian 660-IR spectrometer (Varian Resolutions, Varian Inc, Victoria, Australia) using a diamond crystal ATR Golden Gate (Specac). Scans were taken with 32 scans at 2 $cm^{-1}$ resolution in a wavenumber range from 4000 to 500 $cm^{-1}$. For the Atomic Force Microscope (AFM, Park systems, XE-100, South Korea) measurement, silicon wafer coupons (2 cm × 2 cm, PI-KEM Limited, Tamworth, UK) were ultrasonicated (Cole-Palmer 8891, IL, USA) for 30 min twice in absolute ethanol and then homogenised diluted solutions of 0.1 w/v % SASB and BASB solutions were spin coated (Specialty Coating Systems, 6800 spin coat series, IN, USA) onto the cleaned Si wafers at 3000 rpm for 30 s and dried under a stream of nitrogen ($N_2$) gas. AFM scans were performed in non-contact mode with high resolution, silicon micro-cantilever tips. Topographic images were recorded at a resonance frequency of 270–300 kHz.

### 2.3. Planktonic and biofilm bacteria growth

The following planktonic and biofilm forming pure bacterial strains were used: *S. aureus* (NCIMB 13062) and *P. fluorescens* (NCIMB 9046). Before use, planktonic cultures of *S. aureus* and *P. fluorescens* were grown for 18 h at 30 °C (*P. fluorescens*) or 37 °C (*S. aureus*) in Mueller-Hinton broth (MHB) (Oxoid, UK) under constant agitation at 170 rpm on an orbital shaker (Innova 2300, New Brunswick™, Germany). A microbiota isolated from raw chicken breast fillets sourced locally was also used where chicken microbiota was isolated from raw chicken breast fillets *via* 10 g of chicken being taken aseptically and placed in a stomacher bag to which 90 ml of sterile MHB was added. The mixture was homogenised for 180 s in a stomacher (Colworth Stomacher 400, Seward Ltd., England) and 10 mL of the resulting homogenate was transferred into a sterile Sterilin™ tube with screw cap (Sterilin, UK) and incubated at 37 °C for 18 h under constant agitation as previously outlined.

### 2.4. Antimicrobial test on planktonic bacteria

The antimicrobial activity of the organic acids and nano-solubilisates against planktonic cultures was tested by determining the minimum inhibitory concentration (MIC) in a 96-well flat bottom plates

(Sarstedt Inc., NC, USA) with alpha numeric coordination system (columns 1–12 and rows A-H) according to the NCCLS broth microdilution method (Wayne, 2002). Briefly, the wells in rows A to F were filled with 100 μL of double strength MHB. A serial 10-fold dilution of overnight grown microorganisms was carried out using MHB as diluent to obtain a final concentration of log 5 CFU $ml^{-1}$. Two hundred microliter of the diluted target microorganisms was added into the wells of row H, columns 1–11 while 100 μL of sterile MHB was added to column 12. In each well of row G, 150 μL of the antimicrobial substance solutions were added (2% w/w). Using a 12 channel pipette, 50 μL of antimicrobial substance solutions was serially transferred from each well in row G into the corresponding wells in row F and the process repeated to row B. After mixing, 50 μL was removed from each well in row B and discarded. Positive (row A) and negative growth controls (column 12) were included in each assay plate. Finally, using a 12 channel pipette, 15 μL of the standardized inoculum were pipetted from each well in row H to the corresponding wells in row A followed by rows B – G. The inoculated plates were incubated for 24 h at 30 °C (*P. fluorescens*) or 37 °C (*S. aureus* and chicken microbiota). The lowest concentration showing inhibition of growth was considered to be the MIC for the targeted microorganism. Each experiment was performed in quadruplicate and with three independently grown cultures. The initial bacterial load (Colony-forming units (CFU)/mL) of *P. fluorescens*, *S. aureus* and chicken microbiota was determined after the appropriate dilution was placed in duplicate on PCA plates and incubated for 24 h at 30 or 37 °C, respectively. After MIC determination, an aliquot of 0.1 mL from all wells without visible bacterial growth was plated onto plate count agar plates. The PCA plates were then incubated for 18 h at 30 °C for *P. fluorescens* or 37 °C for *S. aureus* and chicken microbiota. After incubation, the concentration of BAS, SAS, BASB or SASB at which there was no visible growth of the bacteria on the PCA plates, was noted as the Minimum Bactericidal Concentration (MBC).

### 2.5. Biofilm formation

The biofilm formation was carried out using a procedure outlined by Evaristo et al. (2014) with some modifications. An overnight grown pure culture of bacteria or chicken microbiota isolated as outlined in section 2.3 was 10-fold serially diluted to log 6 CFU $ml^{-1}$ and 100 μL of the diluted target microorganism was added into each well of a 96-well flat bottom plate except in row H and column 12. Then, the 96-well plates were incubated for 24 h at 30 or 37 °C as previously outlined. The formation of biofilm was confirmed by the addition of 100 μL of sterile water without CV present to each well and sonicated for 8 min to remove unattached cells from the surface of the well. To determine the bacterial load of the biofilm, a serial dilution of the biofilm was carried out and enumerated using PCA as the growth medium.

### 2.6. The quantification of the biofilm's formation

After the incubation process, the cultures in the microplates were removed by transferring the liquid into a large empty tray and shaken vigorously to remove the remaining excess liquid. Any unattached bacterial cells were removed by further washing the wells with sterile water from a wash bottle (Azlon Plastics, UK). The washing process was repeated three times and then the 24-well plates turned up-side down into a sterile absorbent paper and tapped vigorously on the sterile absorbent paper towels to remove any remaining water. The 24-well plates were turned upwards and left to dry in a laminar flow (Airclean 600 PCR Workstation STAR LAB) for 2 h. Then, 125 μL of 0.1% (v/v) CV solution was added to each well and left for 15 min at room temperature (~20 °C). The wells were then washed three times using sterile distilled water as outlined above to remove any excess dye and then allowed to dry for 1 h in a laminar flow. In order to quantify the planktonic cells forming the biofilm, 125 μL of 30% acetic acid was added to each well to dissolve the CV and left for 15 min at room temperature. The solubilised CV solution was transferred into an ultra-micro cuvette and the optical density (OD) of the solutions measured at 550 nm in a UV–Vis spectrophotometry (UV Mini 1240, Shimadzu Instruments, Jiangsu, China) using 30% acetic acid as the blank.

### 2.7. Antimicrobial susceptibility of biofilms to antimicrobials

In order to determine the effect of the antimicrobial organic acid materials against biofilm formations of pure culture and chicken microbiota, suspensions were prepared as outlined in section 2.3. A 96-well plate containing biofilms were prepared using a procedure similar to that of formation and staining of the biofilms except no CV dye was added to the well plates. A serial dilution of antimicrobial substances were carried out in another set of sterile 96-well plates using the procedure outlined in the antimicrobial activity test, except no target microorganism were added in the row H. Then, 100 μL diluted antimicrobial substance solutions in the sterile 96-well plates were transferred aseptically to another 96-well plate containing biofilms. The plates were then incubated for 24 h at 30 or 37 °C as outlined above and the susceptibility of the biofilms to the antimicrobial monitored. The lowest concentration showing inhibition of growth was considered to be the MIC for the target biofilms.

### 2.8. Statistical analysis

Statistical analysis was performed using the software STATGRAPHICS® centurion XV (Statpoint, Inc., USA). A difference between pairs of means was resolved by means of confidence intervals using Tukey's test. The level of significance was set at $P < 0.05$.

## 3. Results and discussion

### 3.1. Physical characterisation of organic acid salts and nano-solubilisates

The hydrodynamic particle size of BASB and SASB measured using a zetasizer was found to be 9 and 10.6 nm, respectively (Fig. 1). The polydispersity index (PDI), which is a dimensionless measure of the range of the particle size distribution calculated from the cumulant analysis, was recorded to be 0.247 for BASB and 0.212 for SASB. These PDI results indicate that the nano-solubilisates were monodisperse and had a narrow particle size distribution (Sullivan et al., 2018b). Due to the irregular and large size features of BAS and SAS, topographical imaging was carried out only on the nano-solubilisates. The morphological and topographical features of BASB and SASB measured using the AFM are shown in Fig. 2. Both BASB (Fig. 2a) and SASB (Fig. 2c) had a monodisperse and regular spherical morphology which are features typical of monodisperse micellar emulsion. Moreover, AFM analysis indicated that the average particle diameter of BASB and SASB was 49.5 and 63.85 nm, respectively. In comparison, the particle size diameter of the BASB and SASB obtained using AFM analysis were up to 5.5 and 6 times larger than the particle size results obtained with the zetasizer which was a hydrodynamic light scattering measurement. The larger observed particle size diameter from AFM analysis may perhaps be due to a “pancaking” effect as when removed from aqueous solution, the morphology adopts a disc like shape due to the effects of gravity acting on the micelle. The FTIR spectra of BAS, SAS and commercially available nano-sized BASB and SASB are shown in Fig. 3 a & b, respectively. While both BAS and SAS have similar functional groups their backbone structure is different as sorbic acid is a 6 carbon aliphatic chain monomer with a carboxylic acid group, whereas BAS has a 6 carbon phenol ring with a carboxylic acid attached. Nevertheless, the FTIR spectra of SAS and BAS showed different characteristic peaks. The FTIR spectra of SAS showed peaks at 2500–3500 $cm^{-1}$ due to the O–H of the COOH stretching, 1694 $cm^{-1}$ corresponding to C = O of acid stretching, C = C stretching for alkene at 1613-1638 $cm^{-1}$, bending $CH_3$ at 1377 $cm^{-1}$, stretching COH acid at 1266 $cm^{-1}$ and at 998 $cm^{-1}$

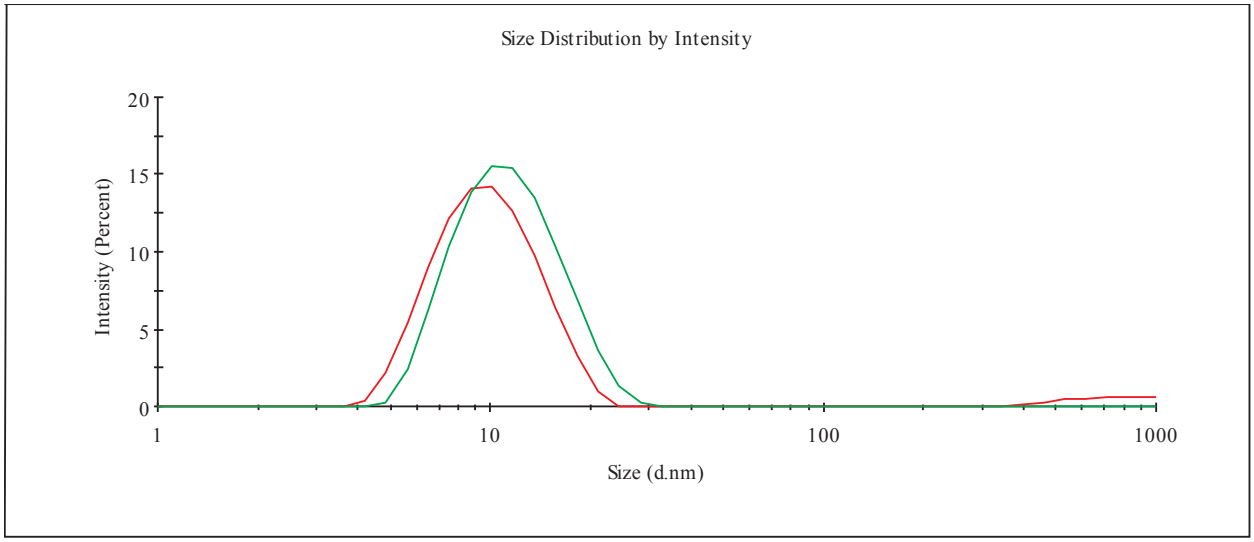


**Fig. 1.** Particle size distribution (a of benzoic acid nano solubilisate (egi10THJBGDZWR) and sorbic acid solubilisate (egi10GP6D7L9ZW) measured using a Malvern Zetasizer Nano Series HT where organic acid solutions were loaded into a ZEN0040 disposable cell and analysis was performed at 25 °C using a scattering angle of 173°.

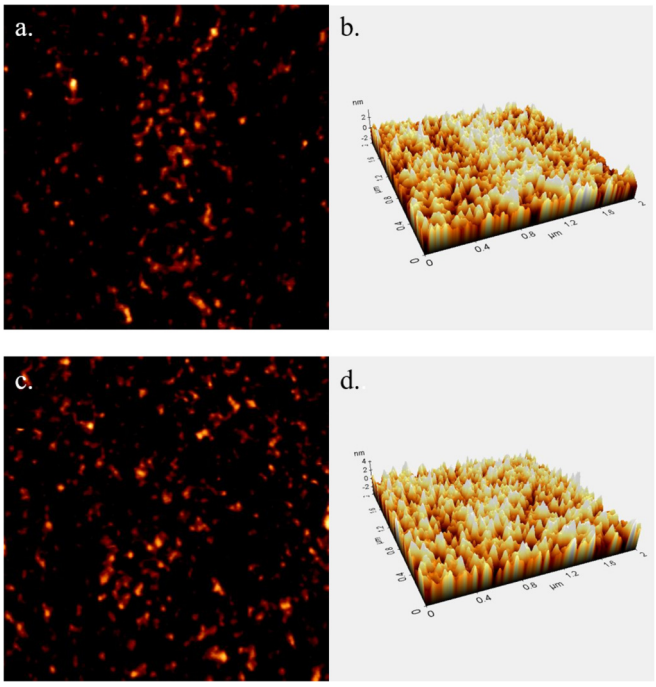


**Fig. 2.** AFM topographical (a.) and 3 D (b.) images of benzoic acid nano-solubilisate and AFM topographical (c.) and 3 D (d.) images of sorbic acid nano-solubilisate.

an out of plane for *trans*-alkene (El-Nemr & Mohamed, 2017). The FTIR spectrum of BAS showed characteristic peaks at 1650 and 2500 - 3500 $cm^{-1}$ which are due to C = C and O–H stretches, respectively. The spectra of the nano-solubilisates showed peaks at 1100, 2900 and 3500 $cm^{-1}$ which are associated with the emulsifying agent. With regards to the overlap of BAS and BASB, a peak can be seen at 715 $cm^{-1}$ which was attributed to BAS; however, none of the SAS peaks are apparent in the SASB spectra.

### 3.2. Quantification of biofilm formation using crystal violet assay

The formation of biofilms was quantified using a crystal violet assay (Fig. 4.). For the crystal violet assay, the OD of *S. aureus, P. fluorescens*, and chicken microbiota biofilms formed on a 24-well plates were taken after 24 h incubation and the OD values were recorded to be 1.760, 0.643 and 0.406, respectively (Fig. 4). Several factors have been reported to effect the growth of biofilms including the material of the microplate well plates which may influence the biofilm growth depending on the affinity of the biofilm forming microbes for either hydrophilic or hydrophobic surfaces (Naves et al., 2008). Typically, microplates are made from polypropylene, a hydrophobic material that allows favourable surface attachment of biofilms through hydrophobic interactions while electrostatic interaction are predominantly seen in hydrophilic surfaces such as stainless steel (Di Ciccio et al., 2015; Dutta et al., 2011). Furthermore, authors such as Peeters, Nelis, and Coenye (2008) and Stepanovic, Vukovic, Dakic, Savic, and Svabic-Vlahovic (2000) have reported that the iodine dye used in crystal violet staining assay can also stain inactive cells and this may increase the observed OD.

### 3.3. Antimicrobial activity of organic acids and nano-solubilisates

The antimicrobial activity of BAS, SAS, BASB and SASB against planktonic and biofilms cultures of *S. aureus*, *P. fluorescens* and chicken microbiota were assessed using a minimum inhibition concentration (MIC) assay (Fig. 5 a & b). Particle size and the type of bacteria (Gram-positive or Gram-negative) affected significantly ($P < 0.05$) the antimicrobial activity of BAS, SAS, BASB and SASB against planktonic and biofilms cultures. As expected, planktonic cultures of *S. aureus, P. fluorescens*, and chicken microbiota were more susceptible to BAS, SAS, BASB and SASB than their respective biofilms. Of the planktonic cultures, chicken microbiota was the least susceptible to the tested antimicrobial agents of which SASB was the most effective antimicrobial while SAS the least effective antimicrobial. The most susceptible culture was *S. aureus* where SASB was the most effective antimicrobial while the least effective was BASB. Furthermore, the MBC assay showed a higher antimicrobial efficacy of nano-solubilisates compared to their respective salt (Fig. 5c). Against *S. aureus,* it was observed that 1.27 and 2.3 times more BAS and SAS was required to have the same bactericidal effect compared to their respective nano-solubilisates. Similarly, 5 and 3.3 times more salt was required than nano-solubilisates against *P. fluorescens* and 4.2 and 3.07 times more salt than their respective nano-solubilisates was required against chicken microbiota.

In comparison of MBC to their MIC values, it was observed that between 1.5 and 2.5 times more antimicrobials were needed to have a

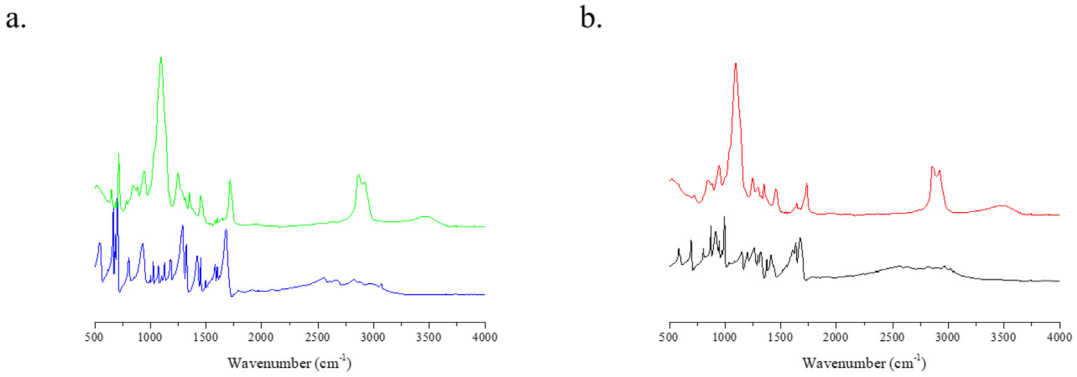


**Fig. 3.** FTIR spectra of a. Sorbic acid (egi1036886MR3R) and sorbic acid nano solubilisate (egi10GP6D7L9ZW) and b. benzoic acid salt (egi10TPTS4L6FR) and benzoic acid solubilisate (egi10THJBGDZWR).

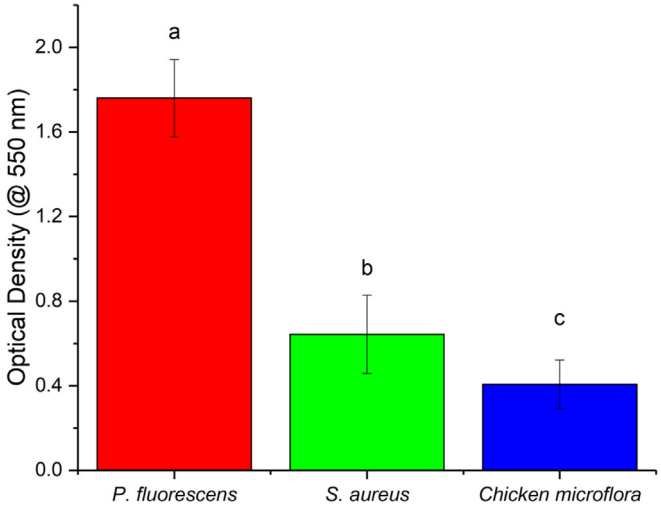


**Fig. 4.** Quantification of biofilm cultures of P. fluorescens (egi10K6NXPVH8B), S. aureus (egi10PK3LPPFXK), and chicken microbiota (egi10G00XR39CC) using optical density assay on biofilms grown in 24 well-plates. [a, b, c] Mean values with different superscripts indicate difference between values are significantly different ($P < 0.05$).

bactericidal effect. Interestingly, when comparing the antimicrobial effect of nano-solubilisates and salts against the Gram-type of pure cultures using an MIC and MBC assays it was observed that Gram-negative *P. fluorescens* was less susceptible compared to Gram-positive *S. aureus* in both instances, suggesting that organic acids are more effective against Gram-positive than Gram-negative planktonic bacteria. These results are in disagreement with Amrutha et al. (2017) who reported that organic acids are more effective against Gram-negative microorganisms. This may be as a result of the proposed mode of antimicrobial action of organic acids which through their ability to dissociate into weak acids which then can cross bacterial membranes as a result of the equilibrium between their ionised and non-ionised forms, the latter of which can freely diffuse cross hydrophobic membranes initiating a collapse in the proton gradients that are necessary for ATP synthesis, as free anions will combine with periplasmic protons pumped out by the electron transport chain, and carry them back across the membrane without passage through the F1Fo ATP synthase (Amrutha et al., 2017; Halstead et al., 2015; Sullivan et al., 2018a). Furthermore, as the pH of the organic acids environment decreases towards the pKa of the acid, its' antimicrobial effectiveness increases, as decreasing pH allows more protonation of the acid (and is in a pH dependant equilibrium). This reduces the polarity of the acid allowing its rapid diffusion through the cell membrane whereupon entering the alkaline internal pH of the cell, the internalised acid dissociates, increasing the concentration of protons and ions in the microbe, acidifying the cytoplasm, which in turn can cause acid-induced protein unfolding, membrane and DNA damage affecting cellular functions such as glycolysis, cell signalling and active transport (Halstead et al., 2015; Mani-López, García, & López-Malo, 2012). However, some studies have suggested that sorbic acid does not release enough protons for this type of inhibition and therefore, it is plausible that its hydrophobicity can interact with the cell membrane affecting its permeability resulting in intracellular leakage (Mani-López et al., 2012). Studies have also reported that the chemical structure of the organic used will also have an effect on the antimicrobial activity (Akbas & Cag, 2016). In addition, it has been proposed that another mechanism is through the ability of organic acids to act as an oxidant by producing hydroxyl free radicals which can interfere with the functionality of components of the cell such as; lipids, proteins and DNA (Zhang & Yang, 2017). Moreover, chelation of macronutrient metals by organic acids has also been shown to reduce the ability of the bacteria to thrive (Mani-López et al., 2012). While the mechanisms of bacterial inhibition was not carried out in this study; however, emerging techniques such as: nuclear magnetic resonance

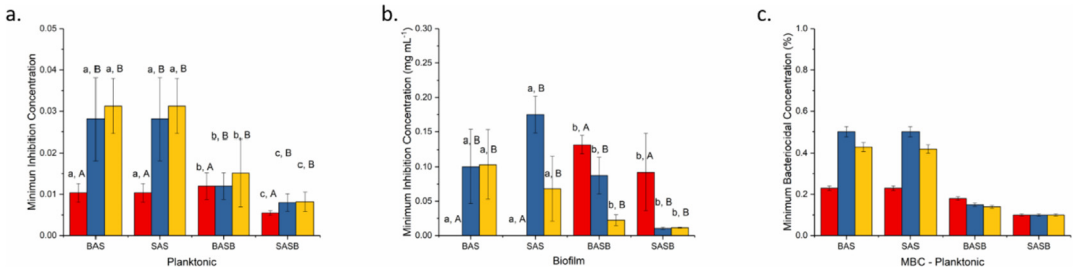


**Fig. 5.** MIC of benzoic acid salt, sorbic acid salt, benzoic acid nano-solubilisate and sorbic acid nano-solubilisate against planktonic (a.) and biofilms (b.) cultures of S. aureus (egi10K6NXPVH8B), P. fluorescens (egi10G00XR39CC) and chicken microbiota (egi10G17VC7KQQ) while in (c.) the MBC is shown. [a, b, c] Mean values with different superscripts indicate difference between BAS, SAS, BASB, and SASB are significantly different ($P < 0.05$). [A, B, C] Mean values with different superscripts indicate difference between S. aureus, P. fluorescens and chicken microbiota are significantly different ($P < 0.05$).

(NMR) spectroscopy or gas chromatography-mass spectrometry (GC-MS) have been successfully used to identify the induced stress effects caused by antimicrobials (Liu et al., 2018; Liu et al., 2017).

Factors such as Gram-strain or organic acid molecular weight (MW) have also been reported to affect the antimicrobial activity of organic acids with Gram-negative bacteria less susceptible than Gram-positive bacteria, potentially due the more complex cell membrane structure (Clarke et al., 2016) and lower MW organic acids predominantly antimicrobial through the "weak acid" preservative theory (Mani-López et al., 2012). Conversely, larger MW organic acids interact with bacterial cell walls through lipophilic interactions with the cell membrane as they are too large to rapidly pass through the cell membrane (Mani-López et al., 2012). Moreover, apparently the physical properties of the organic acids were found to affect the antimicrobial properties. When comparing the antimicrobial properties of the organic salts and nano-solubilisates, it was observed that nano-solubilisates were more effective against both planktonic and biofilm cultures of *S. aureus, P. fluorescens* and chicken microbiota. The greater observed antimicrobial activity of nano-solubilisates may be attributed to its physical properties such as smaller particle size (nano vs non-nano) and the greater surface area of nano-solubilisates compared to their bulk salt materials as reported by Yu, Ang, Yang, Zheng, and Zhang (2017) and Cruz-Romero et al. (2013a).

With respect to the antimicrobial activity against biofilms, BAS and SAS had no antimicrobial activity against biofilms of *S. aureus* due to the concentrations required to inhibit their growth being over their 3400 and 1560 mg $L^{-1}$ solubility parameters in water, respectively. Furthermore, BASB and SASB were found to be significantly ($P < 0.05$) less effective against *S. aureus* biofilms compared to biofilms of *P. fluorescens* and chicken microbiota. This may be due to the more complex structure and composition of biofilms, as outlined previously. Unexpectedly, chicken microbiota biofilms were the most susceptible to the organic acid salts and nano-solubilisates. This may be due to the chicken microbiota being a complex heterogeneous mix of different Gram-positive and Gram-negative bacteria which compete with each other for nutrients and therefore, inhibit optimal growth where as pure isolates of bacteria have no competition and will form a homogenous biofilm (Pande et al., 2018).

When the susceptibility of planktonic and biofilms cultures to organic acid antimicrobials was compared, it was observed that for SASB up to 15.3, 1.37 and 1.5 times more antimicrobial material was required against biofilms of *S. aureus*, *P. fluorescens* and chicken microbiota, respectively. For BASB, the MIC was 11 and 1.64 times higher against *S. aureus* and chicken microbiota biofilms compared to their planktonic counterpart; however, 0.2 times less BASB was needed against biofilms than pure cultures of *P. fluorescens*. Regarding organic acid salts, for BAS 5.26 and 7.29 times more was necessary against *P. fluorescens* and chicken microbiota and for SAS 9.21 and 7.29 more was necessary against *P. fluorescens* and chicken microbiota. These results are in agreement with Surdeau, Laurent-Maquin, Bouthors, and Gellé (2006) who used the disinfectant Oxsil® 320N on planktonic and biofilm cultures of *S. aureus* and *Pseudomonas aeruginosa* and also observed that biofilms were less susceptible than planktonic bacteria. Moreover, it has been reported that biofilms can be up to a thousand times more resistant to antimicrobials than planktonic cells of the same strain (Akbas & Cag, 2016). This may in part be due to the ability of biofilms to form protective EPS layer and in addition their slow rate of growth and metabolism will reduce the absorption of organic acid antimicrobials (Djordjevic, Wiedmann, & McLandsborough, 2002). A proposed mode of antimicrobial activity of nano-solubilisates against biofilms is thorough amphiphilic interactions, facilitating the diffusion of nano-solubilisates into the biofilm matrix and resulting in individual cell membranes death (Dutta et al., 2011).

## 4. Conclusion

Herein we have investigated the antimicrobial effect of organic acid salts and nano-solubilisates on planktonic and biofilm cultures of the food spoilage microorganisms *S. aureus*, *P. fluorescens* and on the microbiota isolated from locally sourced chicken fillets. Results from this study indicate that the assessed organic acid salts and nano-solubilisates showed good antimicrobial activity against planktonic cultures; however, biofilms were found to be significantly more resistant. Moreover, it was found that nano-sized solubilisates of benzoic acid and sorbic acid had significantly ($P < 0.05$) higher antimicrobial properties compared to their non-nano equivalents of which SASB had greatest antimicrobial activity. Overall, the findings of this study indicated that the unique physiochemical properties of organic acid nano-solubilisates show promising potential as an emerging clean label antimicrobial for use in the development of active and smart packing materials to preserve the quality of food products.

## Conflict of interest form

We wish to confirm that there are no known conflicts of interest associated with this manuscript and there has been no significant financial support for this work that could have influenced its outcome.

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