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Development of a low-temperature extrusion process for production of GRAS bioactive-polymer loaded compounds for targeting antimicrobial-resistant (AMR) bacteria

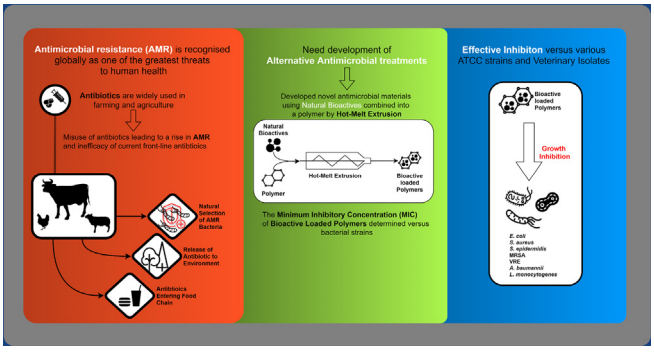
Kevin Masterson [a,*], Elaine Meade [d], Mary Garvey [d], Mark Lynch [a], Ian Major [b], Neil J. Rowan [a,c]

[a] Bioscience Research Institute, Athlone Institute of Technology, Ireland
[b] Materials Research Institute, Athlone Institute of Technology, Ireland
[c] Centre for Disinfection, Sterilization and Biosecurity, Athlone Institute of Technology, Ireland
[d] Department of Life Science, Sligo Institute of Technology, Ash Lane, Sligo, Ireland

HIGHLIGHTS

- New copolymer process for delivery of heat-sensitive antimicrobial bioactives
- Efficacy against a range of veterinary antimicrobial resistant bacterial isolates
- Process appropriate for delivery of next-generation bioactives
- A new 'One-health' approach for potentially addressing priority AMR challenges

GRAPHICAL ABSTRACT



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ABSTRACT

Antimicrobial resistance (AMR) is recognised globally as one of the greatest threats to human and animal health; thus, discovery of alternative antibacterial agents to address AMR is a priority challenge. This study constitutes the first report of a low-melting temperature, polymer- extrusion process for the smart delivery of thermally-sensitive antimicrobial bioactives, including generally-regarded-as-safe (GRAS) bioactives derived from various sources. Bioactives were assessed before and after extrusion by determining their respective minimum inhibitory concentrations (MIC). WHO-priority AMR-bacterial isolates causing zoonotic infections were evaluated along with use of standard ATCC strains. Findings revealed that this copolymer method was capable of delivering thermally-sensitive bioactives with varying degrees of growth inhibition against the AMR-bacterial strains. The extrusion process was found to increase the effect of nisin against MRSA (4-fold increase) and *L. monocytogenes* (6.4-fold increase), silver nitrate ($AgNO_3$) against *E. coli* (3.6-fold increase) and *S. epidermidis* (1.25-fold increase), and chitosan against *S. aureus* (1.25-fold). Findings show the potential applicability of this polymer extrusion process for developing future bioactive-loaded polymer compounds; thus, highlighting the potential of converging bio-based industry with novel materials for enabling 'One-Health' solutions.

1. Introduction

Antimicrobial resistance (AMR) is recognised globally as one of the greatest threats to human health (Interagency Coordination Group on

* Corresponding author.
E-mail address: k.masterson@research.ait.ie (K. Masterson).

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Antimicrobial Resistance, 2019). The World Health Organisation (WHO) has called for immediate action to counteract what has become an "antibiotic resistance crisis" (World Health Organistaion, 2020). The emergence and spread of AMR is directly related to misuse and overuse of antibiotics across various sectors (Talebi Bezmin Abadi et al., 2019). WHO have developed the antibiotic classification framework, Access, Watch, Reserve (AWaRe), for aiding antibiotic stewardship (WHO report on Surveillance of Antibiotic Consumption, 2018). Access antibiotics are classified as having the lowest potential for resistance, Watch antibiotics are those that are prone to AMR, while Reserve antibiotics are classed for use as "last resort" only. It was reported that between 2000 and 2015, global consumption of Watch antibiotics increased by 90·9% per-capita, while consumption of Access antibiotics increased by 26·2% (Klein et al., 2021). On any given day, about one in every three hospital patients in Ireland are being treated with antibiotics (Plachouras et al., 2018). This is further complicated by antibiotic use in the agricultural sector, which has also been identified as a major contributor to AMR on a global scale (Economou and Gousia, 2015; Manyi-Loh et al., 2018).

In collaboration with other major World Organisations, the WHO has designed an approach, known as "One-Health", which aims to combine resources across multiple sectors particularly converging public health and animal health (FAO et al., 2008). The concept of One-Health is not new; however, in recent times it has become increasingly important such as in the area of AMR (Evans and Leighton, 2014). Studies of plasmid-encoded colistin resistance (mcr-1), initially found in pigs in China, have shown its presence in humans and animals worldwide; thus highlighting the one health link between humans, animals and the environment in the context of AMR (Caniaux et al., 2017). Additionally, there are various types of antimicrobial resistant organisms (ARO), some of which are resistant to "last resort" antibiotics (Tacconelli et al., 2018). As such, there is a pressing need to develop new or alternative antimicrobial compounds to combat the global threat of AMR for human and animal intervention (Aminov, 2010; Interagency Coordination Group on Antimicrobial Resistance, 2019; Ventola, 2015).

There are a number of potential alternatives currently under investigation, such as phages and various organic and inorganic compounds; however, such alternatives hold limitations in treating bacterial infections (Fenton et al., 2013; Gill et al., 2006; Kwiatek et al., 2012; O'Flaherty et al., 2005; Wilson and González, 2003; Wittebole et al., 2014). Bacteriophages have a narrow spectrum of effect and are generally tailored per bacterial species, leading to increased costs and higher probability of inefficacy versus infections with more complex aetiology (Breyne et al., 2017; Gomes et al., 2016; Porter et al., 2016). Biofilms also limit the efficacy of therapeutics by reducing or preventing interaction with their targets (Marques et al., 2017). For example, certain biomedical devices are complex in design with narrow lumen diameters and are prone to unwanted biofilm formations as are challenging to clean (Bhattacharya et al., 2015; Wagner et al., 2011). However, alternative interventions include using polymer-based materials in order to prevent a build-up of bacterial cells in such devices; thus, stopping initial attachment and preventing or disrupting the formation of biofilm (Costerton, 2005; Konai et al., 2018). Development of alternative bio-based therapeutics and smart materials also has implications for efficacy of reprocessing and sterilization (Chen et al., 2019).

Polymer-based therapeutic delivery solutions are plausible through the use of smart manufacturing approaches, such as hot-melt extrusion (HME), which necessitates the capacity of the active compound to withstand thermal processing to maintain bioactivity (Simões et al., 2019). However, traditional antibiotics have low thermal stability, excluding them as possible candidates for HME applications (Wylie et al., 2021). There is growing interest in exploiting bio-based ingredients as potential antimicrobial therapies; however, there is a gap in knowledge as to effective non-thermal processing and delivery approaches (Rowan and Galanakis, 2020; Rowan and Casey, 2021). HME has seen growing use in the area of pharmaceuticals as it allows the production of biologically active polymers with various formulations, dosage forms, and can enhance the physical properties of the bioactive component (such as increased water solubility, improved stability and shelf-life) (Patil et al., 2016).

This constitutes the first study to report on use of low-temperature polymer extrusion process using four bioactive compounds for one health applications. Silver nitrate ($AgNO_3$), Zinc Oxide (ZnO), nisin and chitosan were assessed similarly to that of antibiotics, by determining their minimum inhibitory concentration (MIC) against three standard, ATCC bacteria strains. These bioactives were assessed for their processing potential by incorporating them into a polymer by HME and then re-assessing their antibacterial capabilities. By assessing their potential as antibiotic alternatives, further studies can be conducted to determine suitable areas they can be utilised, replacing traditional antibiotics; thus, potentially lowering their overall use and mitigating against occurrence of AMR development as a consequence of antibiotic misuse.

$AgNO_3$ is the most commonly used and documented silver salt derivative, and has raised great interest recently for its revival as an antimicrobial (Atiyeh et al., 2007; Balazs et al., 2004; Gao et al., 2018; Prabhu and Poulose, 2012; Russell and Hugo, 1994). While previous studies have shown that $AgNO_3$ incorporation into hydrogels and liquid-based polymers can increase it's antimicrobial efficacy (dos Santos et al., 2012; Wu et al., 2018), this present study represents the first to incorporate the compound into a solid-form polymer by HME and assess its antimicrobial abilities.

ZnO is a commonly-used ingredient in cosmetics and other various areas, categorised generally recognised as safe (GRAS) by the FDA; it has recently elicited interest due to its antimicrobial abilities, particularly when used as a nano-particle (Beyth et al., 2015; Padmavathy and Vijayaraghavan, 2008; Pasquet et al., 2014; U.S. Food and Drug Administration, 2019). ZnO and ZnO nanoparticles (ZnO-NPs) have shown numerous successes in their ability as antimicrobials against bacteria and fungi. A number of studies in the food packaging area have shown ZnO-NPs suitability for polymer incorporation, demonstrating their resilience to processing, holding their antimicrobial abilities while in polymer form (Espitia et al., 2012; Silvestre et al., 2016).

Nisin, a bacteriocin, has been studied for use in food packaging to reduce spoilage. While recent studies have focused primarily on its use as a general antibacterial agent, previous works have shown its active properties within polymer preparations (Kawada-Matsuo et al., 2019; Lewies et al., 2018; Tong et al., 2014). Chitosan has been also studied in food packaging, drug delivery and as a synergistic carrier of antimicrobials (Kim et al., 2017; Kravanja et al., 2019). Both nisin and chitosan are recognised GRAS compounds, where they were previously investigated for use in active food packaging due to their known antimicrobial abilities and established non-toxicity in mammalian studies (Asli et al., 2017; Bastarrachea et al., 2015; Cardozo et al., 2014; Moon et al., 2007; J. Wu et al., 2007; Yang et al., 2014).

While the antimicrobial efficacy of these bioactives have been previously reported, this constitutes the first study to report on use alone and combined by way of incorporation into a solid polymer by HME for antimicrobial use. While their efficacy toward bacteria is important, the ability of these bioactives to be utilised in polymer forms potential creates opportunities for their adaptability across a number of important sectors such as biomedical devices (implants, catheters etc.) and in veterinary products. The HME process was chosen as it allows for complex formulations of the active ingredients, and incorporation into various polymer carriers allowing for refinement of the final product's physical characteristics. In addition, this supports and potentially enables incorporation of smart bioactive products that have been biorefined from difference sources for antimicrobial use; thus, providing a process for current markets and practises. The HME process will also enable combinations of individual bioactives that will support broad-spectrum antimicrobial properties. Specifically, this study assesses the efficacy of

these bioactive-polymer combinations against wild-type and AMR bacteria, including pathogenic veterinary strains.

2. Materials & methods

2.1. Isolation and characterization of veterinary zoonotic strains

Bacterial isolates including *Escherichia coli*, Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant Enterococci (VRE), *Listeria monocytogenes* and *Acinetobacter baumannii* were obtained from diagnostic testing of canine, equine and farm animals manifesting with conditions such as bacteraemia, renal infection, open wound infections, and mastitis. Collected samples of infection or disease in the form of urine, blood material, milk and swabs were provided by registered veterinary personnel in sterile containers (Cruinn Diagnostics, Dublin, Ireland). Liquid samples were immediately inoculated onto nutrient agar and incubated at 37 °C for 24 h. Swabs were inoculated in nutrient broth and incubated at 37 °C for up to 24 h under rotary conditions (125 rpm) before streaking onto nutrient agar plates.

Individual colonies were re-streaked for isolation and pure isolated colonies inoculated into nutrient broth for further biochemical characterization. Colonies were identified based on their morphological characteristics, biochemical profile, and growth on selective agars, specifically CHROMagar™ Acinetobacter (CHROMagar™, Paris, France), Harlequin™ *E. coli*/Coliform Medium, Harlequin™ Listeria Chromogenic Agar, Baird Parker agar (LabM, Cruinn Diagnostics Ltd., Dublin, Ireland) and BBL™ Enterococcosel™ Agar (Becton, Dickinson and Company, Dublin, Ireland). Identity was confirmed via polymerase chain reaction (PCR). Specifically, a single colony of each bacterial test isolate was subcultured in nutrient broth and incubated overnight at 37 °C. Genomic DNA was directly extracted using the GenElute™ Bacterial Genomic kit (Sigma Aldrich, Dublin, Ireland) according to the manufacturer's instructions. The bacterial primers ITS_8F (5′- AGAGTTTGATCCTGGCTCAG -3′) and ITS_U1492R (5′- GGTTACCTTGTTACGACTT -3′) (Sigma Aldrich, Dublin, Ireland) were used for amplification of 16s rRNA gene. PCR was performed in a total reaction volume of 20 μL, containing 17 μL red Taq 1.1× master mix (VWR, Dublin, Ireland) 1 μL ITS_8F, 1 μL ITS_U1492R and 1 μL of pure genomic DNA eluate. DNA amplification was performed in a thermo cycler (VWR, Dublin, Ireland) using the recommended parameters. Following DNA amplification, the PCR products were examined by electrophoresis on a 1% w/v agarose gel run at 120 V for 50 min. Successful reactions were sent to Source Bioscience (Waterford, Ireland) for clean-up and gene sequencing of products. Strains were stored long term in 20% glycerol at −20 °C and short term in nutrient broth at 5 °C. Identity of strains was confirmed via Gram stain and selective agars prior to each experimental set up.

2.2. Antibiotic resistance profile of veterinary isolates

Antibiotic resistance profiles were established using CHROMagar™ agars selective for Extended Spectrum Beta-Lactamase (ESBL), vancomycin-resistant enterococci (VRE) and Methicillin-resistant *Staphylococcus aureus* (MRSA) (CHROMagar™, Paris, France) and a range of antibiotic susceptibility disks (ThermoFisher Scientific, Ireland) as per European Committee for Antibiotic Susceptibility Testing (EUCAST) guidelines (EUCAST, 2020). Specifically, colonies of an overnight bacterial culture suspended in sterile saline at a density of 0.5 McFarland (ca. 1×10^8 cfu/mL) were overlaid on to Mueller-Hinton agar (MHA) (4 mm) in 90 mm circular petri plate as per the EUCAST disk diffusion method (EUCAST, 2020). An antibiotic inoculated disk was placed in the centre of the plate and incubated inverted for 18 h at 37 °C. Antibiotics used during profiling include Streptomycin, Vancomycin, Chloramphenicol, Erythromycin, Ampicillin, Amoxicillin/Clavulanic acid, Cefpodoxime, Cefotaxime, Aztreonam, Doripenem, Meropenem, Ciprofloxacin, Levofloxacin, Colistin, Doxycycline.

Zones of inhibition were measured and used to determine the bacterial species resistance profile. The absence of a zone of inhibition denotes complete resistance (R) of the species against the tested antibiotic. Susceptible species were graded as being completely susceptible (S), or as having intermediate susceptibility (I), based on the ability of the test drug to produce a zone diameter according to EUCAST zone diameter guidelines. MRSA and VRE, which are listed as high importance, were assessed for resistance to vancomycin and quinolones amongst other therapeutics. Isolates that tested positive on CHROMagar™ ESBL and displayed resistance to the extended-spectrum cephalosporin group of antibiotics were selected for phenotype confirmation of ESBL production. ESBL detection and characterization are recommended for public health and infection control purposes (EUCAST, 2020). This was carried out by placing cefpodoxime (10 μg) and cefpodoxime/clavulanate (10 μg/1 μg) discs on an inoculated MHA plate, 30 mm apart. Plates were then incubated overnight at 37 °C. A zone diameter of ≥5 mm is considered positive for ESBL production.

2.3. Hot-melt extrusion (HME) co-polymer process

The bioactive compounds, nisin (2.5% w/w, SKU: N5764, CAS: 1414-45-5), Chitosan (low molecular weight, SKU: 448869, CAS: 9012-76-4), Zinc Oxide (ZnO, nanopowder: <100 nm particle size, SKU: 544906, CAS: 1314-13-2) and Silver Nitrate ($AgNO_3$, SKU: S8157, CAS: 7761-88-8) were purchased from Sigma Aldrich (Sigma-Aldrich Ireland Limited, Wicklow, Ireland), and incorporated into a polymer by hot-melt extrusion (HME) process using a PRISM Twin Screw Extruder-16-TC (Twin bore diameter: 16 mm, screw diameter: 15.6 mm, channel depth: 3.3 mm, barrel length: 384 mm). Polymers were processed using an upper barrel temperatures 140 °C and screw speed of 100 RPM. The HME process was selected to match material stability of bioactives such that it enabled processing without affecting material functionality or activity post-processing. Poly-vinyl-pyrrolidone/vinyl acetate (PVPVA) was purchased from BASF (BASF SE Headquarters, Ludwigshafen, Germany) and chosen as the copolymer carrier due to its low melting temperature, which would ensure minimal thermal damage to the test bioactives. PVPVA is a hydrophilic polymer matrix that dissolves readily in water; thus, forming bioactive polymer solution when loaded with drugs. Processed PVPVA also has a very solid yet brittle composition allowing it to be ground to a fine powder, allowing for easier solution preparation.

2.4. Bioactive solution preparation

Chitosan was dissolved in 1% (v/v) acetic acid and then adjusted to pH 5.5 with 0.4 M sodium hydroxide (NaOH). ZnO was suspended in dH_2O. Nisin was dissolved in a solution of 400 mM sodium chloride (NaCl), pH 3.25. These solutions were then sterilised by autoclaving. Nisin concentrations are reported in terms of active nisin content, where 1 g of commercial nisin powder contains 25 mg of active nisin. $AgNO_3$ was put to a solution of 28% (v/v) Poly (ethylene glycol), average molecular weight 400 (PEG-400) and 26% (w/v) d-sorbitol This solution was then filter sterilised by use of a 0.2 μm syringe filter tip. The bioactive polymers were ground into a fine powder using a mortar and pestle and prepared as per their respective bioactive, apart from $AgNO_3$-ploymer, which was dissolved in dH_2O and sterilised by autoclaving.

2.5. Antibacterial assay

The minimum inhibitory concentration (MIC) was determined for each standard bacterial and veterinary isolate strain by use of the broth microdilution method, adapted from previous literature (Wiegand et al., 2008). The standard strains, *Escherichia coli* (ATCC 25922, NCTC 12241) and *Staphylococcus aureus* (ATCC 29213, NCTC 12973) were purchased from Public Health England (Culture

Collections, Public Health England, Salisbury, UK). *Staphylococcus epidermidis* (ATCC 35984) was purchased from ATCC (LGC Standards, Middlesex, UK). Briefly, microdilution assays were carried out in untreated 96-well plates using Mueller-Hinton broth (MHB). Bacterial strains were subcultured twice from frozen stocks before use. Working stock dilutions of the bioactives and bioactive polymers were prepared in MHB, aliquoted into the first column of a 96-well plate and serial diluted along the plate. Treatment vehicles (T_v) were included to assess any effects that the solutions may have (excluding the bioactive itself). Two columns were left untreated, with one of these left uninoculated to act as a sterility and positive control (SC) and the other as a growth and negative control (GC). Colonies of the test bacteria were taken from an overnight streak plate used to prepare a 0.5 MacFarland bacterial suspension (1×10^8 cfu/mL). This suspension was adjusted and aliquoted into the wells of the plate (excluding the SC) giving a final in-well bacterial concentration of 5×10^5 cfu/mL. Plates were incubated in a shaker incubator (120 rpm) for 18 h at 37 °C. Wells were observed for growth as determined by visible turbidity and absorbance readings (625 nm). The MIC was determined as the lowest concentration of a treatment to prevent cell growth (i.e. broth turbidity).

2.6. Statistical analysis

Experiments were carried out in replicates of three or four. Significance between MIC values of the bioactives before and after polymer processing was determined by use of either a paired *t*-test (one-tailed), or a two-way ANOVA model with Bonferroni post-hoc test. A P value <0.05 was considered significant.

3. Results

3.1. Resistance profile of veterinary isolates

Resistance profiles were established in accordance with the WHO priority pathogen list for veterinary isolates MRSA, VRE, *L. monocytogenes*, with critically important *E. coli*, and *A. baumannii* also being assessed for resistance to 3rd generation cephalosporins and carbapenems, amongst other drug classes (Table 1). *A. baumannii* and *E. coli* both exhibited resistance to vancomycin and ampicillin, with ESBL activity, which was confirmed, with the bacteria exhibiting zones of 15 mm and 20 mm vs Cefpodoxime discs, and 23 mm and 28 mm vs Cefpodoxime + clavulanic acid discs, respectively. *E. coli* also demonstrated resistance to amoxicillin/clavulanic acid. *E. coli* and *A. baumannii* displayed intermediate susceptibility to an array of antibiotics including streptomycin (10 mm, 15 mm), erythromycin (9 mm, 9 mm), and chloramphenicol (21 mm, 9 mm) respectively.

MRSA give clear indication of resistance to streptomycin and 3rd generation cephalosporins, with intermediate susceptibility to vancomycin (16 mm), tetracycline (11 mm), and erythromycin (22 mm). The high priority pathogen VRE demonstrates clear resistance to streptomycin, cephalosporins and quinolones, with low susceptibility to the macrolide erythromycin (7 mm) and full susceptibility to tetracyclines (doxycycline, 30 mm). *L. monocytogenes* displays resistance to ampicillin, erythromycin, and cephalosporins.

Table 2
Antibiotic Minimum Inhibitory Concentrations for Veterinary Isolate strains. Table shows the minimum inhibitory concentration (MIC) of various traditional antibiotics versus veterinary bacterial isolates.

		Bacterial species			
		A. baumannii	*E. coli (93)*	*MRSA*	*L. monocytogenes*
Antibiotic (μg/mL)	Streptomycin	8	4	R	16
	Vancomycin	R	256	1	0.5
	Erythromycin	32	R	1	16
	Azithromycin	4	8	8	32
	Amoxicillin	256	128	256	4
	Ceftazidime (3rd)	64	16	32	R
	Cefotaxime (3rd)	128	16	32	R
	Ceftriaxone (3rd)	64	4	32	R
	Cefepime (4th)	64	2	32	16
	Aztreonam	256	8	–	–
	Meropenem	0.5	0.125	32	16
	Doxycycline	16	64	128	8
	Tetracycline	128	128	128	16
	Ciprofloxacin	0.125	0.25	0.25	2
	Levofloxacin	0.25	0.25	0.25	2

3.2. Minimum inhibitory concentration of antibiotics

The MIC of an array of antibiotics was determined against each veterinary isolate of MRSA, VRE, *E. coli* and *A. baumannii* (Table 2).

3.3. Minimum inhibitory concentration of bioactives

The MIC values of the bioactive compounds were determined for each ATCC bacterial strain and veterinary isolate. Results of the MIC assays gave positive indication to the bacterial inhibitory properties of $AgNO_3$, chitosan, ZnO and nisin versus the three ATCC bacterial strains, *E. coli*, *S. aureus* and *S. epidermidis*. The $AgNO_3$ preparation exhibited

Table 1
Resistance profile of veterinary isolates to a range of antibiotics as determined by zones of inhibition with reference to EUCAST cut off points.

			Zone diameter (mm) of bacterial species				
Drug class	Antibiotic	Conc. (μg/disc)	*A. buamannii*	*E. coli (93)*	MRSA[a]	VRE[a]	*L. monocytogenes*
Aminoglycoside	Streptomycin	10	15	10	R	R	11
Glycopeptide	Vancomycin	30	R	R	16	R(12)	13
Chloramphenicol	Chloramphenicol	30	9	21[24]	22(18)[24]	20	27
Macrolide	Erythromycin	15	9	9	15(18)[26]	7	15(25)
Penicillin	Ampicillin	10	R	R	R	15	10(16)
Penicillin-like	Ampicillin/clav	20:10	25	14(19)	11	24	25
Cephalosporins	Cefpodoxime	10	10	20(21)	R	15	R
	Cefotaxime	5	16	16(17)	R	R	R
Monobactam	Aztreonam	30	32	25(21)	–	–	–
Carbapenems	Doripenem	10	29	22[9]	16	25	38
	Meropenem	10	25(15)	23(16)	30	12	30(26)
Quinolones	Ciprofloxacin	5	30(21)	36(22)	28(24)	R(15)	10
	Levofloxacin	5	32(20)	31[33](19)	29(22)	R(15)	26
Polymyxin	Colistin	10	13	12[9]	–	–	–
Tetracycline	Doxycycline	30	10	12	11	30	40

R donates complete resistance to antibiotic.
( ) EUCAST 2020 cut-off zone diameter (mm) for antibiotic resistance for certain species and antibiotic. Zones below this are deemed resistant.
[ ] EUCAST 2019 cut-off zone diameter (mm) for antibiotic resistance for certain species and antibiotic. Zones below this are deemed resistant.
[a] WHO high priority pathogens.

Table 3
Bioactive Minimum Inhibitory Concentrations versus *ATCC Bacterial Strains*. Table shows the mean minimum inhibitory concentration (MIC) of silver nitrate ($AgNO_3$), chitosan, zinc oxide (ZnO) and nisin against the ATCC bacterial species, *E. coli*, *S. aureus* and *S. epidermidis*, as determined by use of broth microdilution assays. Bioactives were assessed before and after extrusion with the co-polymer PVPVA via hot-melt extrusion (HME). Significance changes of MIC were determined by use of a 2-way ANOVA and shown in terms of a P value. N = 4.

		Bacterial species		
		E. coli (ATCC 25922)	*S. aureus* (ATCC 25913)	*S. epidermidis* (ATCC 35984)
Bioactive MIC (μg/mL)	$AgNO_3$	31.25	42.97***	19.53
	$AgNO_3$ -PVPVA	8.789	109.4***	15.63
	Chitosan	156.3	390.6	156.3
	Chitosan-PVPVA	175.8	312.5	208.3
	ZnO	203.125*	156.25	97.6625
	ZnO-PVPVA	562.5*	312.5	140.625
	Nisin	No MIC[a]	6.833**	4.885
	Nisin-PVPVA	No MIC[a]	19.53**	8.3

[a] Up to 0.125 mg/mL tested.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

effective growth inhibitory effects, as determined by the absence of broth turbidity, against *all tested bacterial strains* at concentrations between 15 and 62.5 μg/mL (Tables 3 & 4). The $AgNO_3$ T_v, consisting of PEG-400 and d-sorbitol, exhibited no effects upon bacterial growth.

Chitosan showed efficacy versus all test strains at concentrations between 78 and 625 μg/mL except for VRE which required up to 1250 μg/mL (Tables 3 & 4). The Chitosan T_v only held noticeable effect at the higher concentrations of 0.125–0.25% acetic acid (AcOH), which is equivalent to a chitosan concentration of 1250–2500 μg/mL. The AcOH concentration in the MIC range would be between 0.0137 and 0.0235% AcOH. This indicates that the T_v has no effect on the acquired MIC values as they are much too low.

The ZnO suspension held varying degrees of efficacy, with a mean concentration range of 78.125–312.5 μg/mL versus ATCC while the MRSA vet isolate required up to 7500 μg/mL and VRE vet isolate showed no inhibition, even with testing up to 30 mg/mL (Tables 3 & 4). The ZnO suspension was effective in inhibiting growth of the three ATCC strains, being least effective against *E. coli* and most effective against *S. epidermidis*. This can be accounted for by the bacteria's intrinsic resistance and pathological strengths. Gram-negative bacterium, such as *E. coli*, have multiple, thin layers of membrane combined with an inner peptidoglycan cell wall-layer, which present formidable barrier for therapeutics. Gram-positive bacteria, such as *S. aureus* and *S. epidermidis*, comprise of a single, outer cell membrane under a thick peptidoglycan layer that has actually been shown to enable therapeutics by aiding their absorption into the cell. Furthermore, *S. epidermidis* is a well-known opportunistic, biofilm forming bacteria recognised as an etiological agent in complex device-mediated infection in healthcare and in veterinary practice. It generally exhibits low resistance to antimicrobials while in planktonic forms, requiring biofilm formation to become more resistant, thus justifying its lower MIC compared to *E. coli* and even *S. aureus*. The resistance of VRE to ZnO may be accounted for by its alternative peptidoglycan synthesis, which alters the bonding potential of its outer peptidoglycan layer, preventing ZnO from interacting and penetrating the bacteria cell (Ahmed and Baptiste, 2018).

Nisin was effective in inhibiting the growth of the ATCC strains of *S. aureus* and *S. epidermidis*, with MIC values between 3.9 and 7.81 μg/mL. Effective inhibition was also seen against the veterinary isolates MRSA, VRE and L. *monocytogenes*, with MICs ranging between 12.5 and 15.6 μg/mL. Nisin held no inhibitory effect against *E. coli* (both ATCC and veterinary isolates) or *A. baumannii*, even with concentrations up to 125 μg/mL. This finding is appreciated given that nisin cannot carry out its mechanisms of growth inhibition versus Gram negative bacteria due to outer cell membrane preventing this bioactive from interacting with its target, the intramembrane molecule lipid II.

3.4. Minimum inhibitory concentration of bioactive loaded polymer compounds

The bioactives were extruded into a polymer and assessed using the broth microdilution assay to determine their MIC versus both ATCC and veterinary isolates. Samples of the stock polymer, PVPVA, were also included at concentrations up to 60 mg/mL to assess their effects upon the bacteria. The MIC values of the bioactives before and after polymer incorporation versus the ATCC bacterial strains are presented together for comparison (Tables 3 & 4).

$AgNO_3$ held notable inhibitory effects against tested strains, before and after polymer processing (Tables 3 & 4). MIC values against *E. coli* and *S. epidermidis* obtained after extrusion into the PVPVA polymer were lower than those obtained from $AgNO_3$ pre-hot melt extrusion, noting an increase in its efficacy. $AgNO_3$ proved the most consistently effective of the four compounds, displaying consistently low MIC values against each test bacterial strain while also exhibiting lowered MIC values following polymer incorporation. This showing that not only does $AgNO_3$ still hold function following the HME process but appears to have its mechanism of actions enhanced. Additional observations have shown the bioactive to have greater stability when incorporated into the polymer, denoted by a lack of colouration after long-term air exposure, an occurrence normally seen with unmodified $AgNO_3$.

Chitosan was quite effective against tested bacterial strains, with very little interference seen from its T_v. Following HME, chitosan exhibited no notable change in efficacy (Tables 3 & 4). Although the MICs versus *S. aureus*, *L. monocytogenes* and *E. coli (isolate)* were lowered 1.25-fold (390.6 → 312.5 μg/mL), 1.5-fold (234.38 → 156.25 μg/mL) and1.5-fold (234.38 → 156.25 μg/mL) respectively, showing an increase in efficacy, these were not considered significant ($P > 0.05$).

Table 4
Mean Minimum Inhibitory Concentrations versus *Veterinary Isolates*. Table shows the minimum inhibitory concentration (MIC) of silver nitrate ($AgNO_3$), nisin, zinc oxide (ZnO) and chitosan, before and after incorporation with the polymer PVPVA against the veterinary isolates, *MRSA*, *VRE*, *L. monocytogenes*, *E. coli* and *A. baumannii*. Significant changes in MIC were determined by use of either a paired *t*-test (nisin) or a two-way ANOVA with Bonferroni post-test, and shown in terms of a P value. N = 3.

		Veterinary Isolate				
		MRSA	VRE	*L. monocytogenes*	*E. coli (isolate)*	*A. baumannii*
Bioactive (μg/mL)	$AgNO_3$	20.83	20.83	15.63	15.63**	13.02
	$AgNO_3$-PVPVA	31.25	31.25	15.63	31.25**	13.02
	Nisin	15.6**	15.6**	12.5**	No MIC	No MIC
	Nisin-PVPVA	3.9**	1.95**	1.95**	No MIC	No MIC
	Chitosan	208.33	1250	234.38	234.38	260.42
	Chitosan-PVPVA	260.42	1666.67	156.25	156.25	416.67
	ZnO	4583.33**	No MIC[a]	937.5	390.63	364.58
	ZnO-PVPVA	25000**	No MIC[a]	937.5	1562.5	208.17

[a] Up to 30 mg/mL tested.
** $P < 0.01$.

ZnO appeared to have its antimicrobial abilities greatly hindered by the polymer processing, as all MIC values were observed to increased. *E. coli* and *S. aureus* MIC values saw a 2.8-fold (203.13 → 562.5 μg/mL) and a 2-fold (156.25 → 312.5 μg/mL) increase, respectively (Table 3). MRSA and *E. coli (isolate)* MICs were seen to increase 5.5-fold ($P < 0.01$) and 4-fold respectively (Table 4). The MIC against *S. epidermidis* demonstrated the lowest effect from the polymer process, with a 1.4-fold increase (97.66 → 140.63 μg/mL). While it is unlikely that the heat from the polymer process caused this loss of potency (as ZnO has a melting temperature of 1975 °C), it is possible that the shearing effect of the twin-screw extruder may have denatured it. Although it is probable that the polymer itself bound too strongly to the ZnO molecules; thus preventing it from freely interacting with bacterial cells.

Incorporation of nisin into a PVPVA polymer through HME exhibited a significant decrease in efficiency, as denoted by an increase in MIC values against the ATCC strains *S. aureus* (6.83 → 19.53 μg/mL) and *S. epidermidis* (4.89 → 8.3 μg/mL), which represents a 2.9-fold ($P < 0.01$) and 1.7-fold increase in MIC values respectively (Table 3). However, the opposite was observed in assays against the veterinary isolates, which resulted in nisin-PVPVA achieving lower MIC values versus MRSA (15.6 → 3.9 μg/mL), VRE (15.6 → 1.95 μg/mL) and *L. monocytogenes* (12.5 → 1.95 μg/mL); thus, representing 4-fold, 8-fold and 6-fold decreases, respectively (Table 4). This increase in potency is noteworthy as was found to be statistically significant ($P < 0.01$). Another noted characteristic of the nisin-PVPVA polymer was its increased shelf life in solution. Many of the additional components of the commercial nisin powder are intentionally left within its composition in-order to increase the compounds shelf life. However once in solution, it loses potency after 7–10 days. The nisin-PVPVA were observed to hold same level of potency for up to 30 days following solution preparation, which could be accredited to the polymer supporting the nisin's polycyclic structure stability.

Overall, the four bioactives exhibited satisfactory bacterial growth inhibition against *E. coli*, *S. aureus* and *S. epidermidis*. In terms of effective treatment dose, $AgNO_3$ and nisin held the greatest efficiency to lowest concentrations. Chitosan had equally consistent results across bacterial strains and, along with $AgNO_3$, was the least negatively affected following HME with PVPVA. All bioactives have demonstrated their suitability for HME as they have retained sufficient activity post-processing, with enhanced solubility and potency lifetime.

4. Discussion

There has been a pressing need for alternative therapies in the treatment of bacterial infections and diseases. With the current antibiotic resistance pandemic, stringent restrictions and management are being implemented to hinder its impact nationally and globally. The use of antibiotics in medicine has taken a hesitant approach in recent years due to their misuse being observed in various sectors worldwide, including agriculture, which having a huge influence on the increasing levels of AMR bacteria. The monitoring and documenting of AMR is a vital phase in controlling its emergence and instigating future actions. Simply differentiating which drugs bacteria susceptible to, and which they are resistant to, is essential to clinicians, where susceptibility results guide treatment options. The emergence of *S. aureus* strains displaying intermediate susceptibility (VISA) or full resistance (VRSA) to vancomycin, is currently a significant threat to public health and safety where they are associated with hard-to-treat nosocomial infections globally. The evidence of varying levels of resistance in these veterinary isolates contributes to the association between the veterinary use of antibiotics and emergence/proliferation of antibiotic resistance. With the increasing demand on livestock production, there will undoubtedly be an increase in the veterinary use of antibiotics with proliferation of AMR and environmental pollution with resistant genes, which can impact upon the human health sector. In conjunction with monitoring resistance profiles, research on the sensitivity of treated bacteria to antibiotic alternatives is important for monitoring and controlling the threat of AMR.

Here, four bioactive compounds, silver nitrate ($AgNO_3$), nisin, chitosan and zinc oxide (ZnO), were presented and assessed for their antibacterial abilities under conditions equal to that of antibiotic testing. Studies have been conducted to evaluate and compare $AgNO_3$ and silver nanoparticles (AgNPs), with reported $AgNO_3$ MIC values ranging between 31 and 140 μg/mL against *E. coli* and 31–80 μg/mL against *S. aureus*, which closely resemble MIC values determined the in the present study (Lima et al., 2019; Salman, 2017). Another study investigated the antibacterial and cytotoxic properties of $AgNO_3$, reporting MIC values of 6 μg/mL versus *E. coli* and *S. aureus*, which are slightly lower than those described in the present study (31.25 and 42.97 μg/mL respectively) (Mulley et al., 2014). The present study has found that $AgNO_3$ not only holds antibacterial ability following HME, but exhibited an increased efficacy against ATCC *E. coli* and *S. epidermidis* strains.

While nisin has been extensively studied for its antibacterial abilities, there appears to be large variation between studies in terms of reported MIC values (Kawada-Matsuo et al., 2019; Lewies et al., 2018; Tong et al., 2014). Kawada-Matsuo et al. (2019) reported on a similar solution prepartion method where determined MIC values (3.2–6.4 μg/mL) against *S. aureus*, were similar to what were observed in this present study (6.8 μg/mL). Tong et al. (2014) reported MIC values of 1000 μg/mL against *Enterococcus* species, which is much greater than those reported here (15.6 μg/mL versus VRE). The variation may be a result of the preparations used in its testing, where the current study utilises a set pH and salt content for preparing the nisin solution; where some literature reports using a dilute acid in-order to dissolve the nisin after which the solution is sterilised by autoclaving or filtration. Other studies have reported the need of a specific salt content in-order to stabilise nisin in solution, without which would increase its susceptibility to the damaging effects of the autoclaving process (Rollema et al., 1995; Yamazaki et al., 2000). Results from this study show nisin to inhibit bacterial species in a similar capacity as before in relation to *S. aureus*, or, in some cases, to even higher efficacy in terms of *Enterococcus* species tested.

The majority of studies that focus on use of nisin are concerned with its incorporation into food packaging materials, either as an active component or as a coating on processed polymer films. There are numerous polymer materials examined as a carrier for nisin, such as polylactic acid (PLA), nitrocellulose (NC), methylcellulose (MC), polyethylene oxide (PEO), and even natural polymers such as chitosan (Cutter et al., 2001; Cha et al., 2003; Jin and Zhang, 2008; Jin et al., 2009; Imran et al., 2010; Imran et al., 2014; Han et al., 2017). Many of these studies have reported increased antimicrobial activity following nisin incorporation. However, many of these methods involve numerous and time-consuming steps for the preparation of the nisin/polymer, requiring mixing of the components in a liquid form and then allowing them to dry. The incorporation of nisin into a polymer by extrusion has also been documented, although is reported that the heat and shear forces of the process have negative effects upon the antimicrobial abilities of nisin (Gharsallaoui et al., 2016). The present study represents the first known to extrude nisin using the PVPVA co-polymer. Results show successful incorporation of the nisin into the polymer, retaining activity after HME and even showing increased efficacy against the veterinary isolate strains, MRSA, VRE, *L. monocytogenes*. Additionally, several advantages over previous studies can be noted in that the preparation was combined using dry stocks, the product was processed in a much shorter time-span with a prolonged shelf.

The reported antimicrobial abilities of chitosan can vary greatly, with studies by Aliasghari et al. (2016) and Zaghloul (2015) showing MIC values much higher than those presented here (ranging between 625 and 1250 μg/mL). While a separate study by Shanmugam et al. (2016) reported lower MIC values of 100 μg/mL versus *E. coli* and *S. aureus*. The lower MIC reported by Shanmugam et al. (2016) is quite significant in comparison to the values presented here (156.3–390.6 μg/mL), however it should be noted that the study extracted and produced its own form of chitosan, whereas the present study utilised

commercial chitosan, similarly to the two other mentioned studies. Modifications of chitosan to increase its antimicrobial potential have also shown success by reporting lowered MIC values (Hassan et al., 2018); however, these were not superior to the present study (Hassan et al., 2018). Chitosan itself being a polymer, commonly used in HME and other processes, owes for its resilience. Notably, the HME process alone without bioactives, as reported in this study, had no significant effect upon antibacterial effect. This present study also reported that ZnO can be effectively incorporated into a polymer for antimicrobial action. Pantani et al. (2013) noted that ZnO extrusion with the polymer polylactic acid (PLA) hindered diffusion of ZnO within the polymer, which would interfere with its antimicrobial abilities and may explain the reduced antimicrobial efficacy exhibited during this study.

5. Conclusion

The present study has demonstrated the potential application of four GRAS bioactives, namely $AgNO_3$, chitosan, ZnO and nisin, as alternative antibiotic compounds. The bioactives were able to inhibit bacterial growth of various standard ATCC strains and antibiotic resistant veterinary strains under similar conditions to that used in traditional antibiotic testing. Furthermore, the bioactives gave a clear indication to their suitability for polymer processing and incorporation, opening the way for their application as potential alternatives in many areas that include antibiotic use. In addition, the bioactive-polymer incorporation supports their use where traditional antibiotics were not previously appropriate (due to over/under-exposure, leading to AMR development) such as general disinfectants, antimicrobial-infused wound treatments, antimicrobial-embedded polymer devices and biofilm-based infection treatments. This constitutes the first study to report on the novel use of GRAS bioactives that have been embedded in a polymer carrier with view to use as potential, alterative antimicrobial therapeutic for challenging zoonotic and human infections. Future studies merited include determining biofilm disruptive capabilities of these bioactive-polymer combinations along with commensurate biocompatibility. The relationship between use of nonthermal reprocessing and sterilization modalities, such as electron beam and vaporized hydrogen peroxide, and maintenance of bioactive-polymer functionaty post treatment are also merited (McEvoy and Rowan, 2019; McEvoy et al., 2021). Also, studes revealing molecular and cellular mechanisms underpinning the effectiveness of this novel GRAS bioactive-polymer combintion on targeted AMR bacteria will help advance this platform technology (Farrell et al., 2011). Initial studies surrounding use of this novel co-polymer delivery process shows promise for use of heat-sensitive bioactives, such as for bio-based bioactives mined from food and from the bioeconomy.

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CRediT authorship contribution statement

Kevin Masterson: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. **Elaine Meade:** Data curation, Formal analysis, Methodology, Writing – original draft. **Mary Garvey:** Formal analysis, Investigation, Methodology, Writing – original draft. **Mark Lynch:** Formal analysis, Methodology, Supervision, Writing – original draft. **Ian Major:** Conceptualization, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **Neil J. Rowan:** Conceptualization, Formal analysis, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

There is no conflict of interest for authors.

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