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ABSTRACT

There is an urgent need to develop biodegradable and non-toxic materials from biopolymers and nature-derived antimicrobials to enhance food safety and quality. In this study, electrospinning was used as a one-step, scalable, green synthesis approach to engineer antimicrobial fibers from zein using non-toxic organic solvents and a cocktail of nature-derived antimicrobials which are all FDA-classified Generally Recognized as Safe (GRAS) for food use. Morphological and physicochemical properties of fibers, as well as the dissolution kinetics of antimicrobials were assessed along with their antimicrobial efficacy using state of the art analytical and microbiological methods. A cocktail of nature-derived antimicrobials was developed and included thyme oil, citric acid, and nisin. Its ability to inactivate a broad-spectrum of with food-related pathogens was demonstrated.

Morphological characterization of the electrospun antimicrobial fibers revealed bead-free fibers with a small average diameter of 165 nm, whereas physicochemical characterization showed high surface area-to-volume ratio (specific surface area:21.91 m^2/g) and presence of antimicrobial analytes in the fibers. The antimicrobials exhibited initial rapid release from the fibers in 2 hours into various food simulants. Furthermore, the antimicrobial fibers effectively reduced *E. coli* and *L. innocua* populations by ~5 logs for after 24-hours and 1-hour of exposure, respectively. More importantly, due to the small diameter and high surface area-to-volume ratio of the fibers, only miniscule quantities of fiber mass and antimicrobials per surface area (2.50 mg/cm^2 of fibers) are needed for pathogen inactivation. The scalability of this fiber synthesis process was also demonstrated using a multi-needle injector with production yield up to 1 g/h. This study shows the potential of using nature-derived biopolymers and antimicrobials to synthesize fibers for sustainable food packaging materials.

Keywords: electrospinning, sustainable food packaging, biopolymers, zein, food safety and quality

INTRODUCTION

The global population is expected to reach 10 billion by 2050, and there is an urgent need to find ways to provide the global populace with safe and nutritious food, while at the same time minimizing the significant impact of agriculture on the environment.[1,2,3] The globalization of the food supply has also introduced major challenges associated with food safety and quality. The WHO estimates that microbial contamination causes 584 million foodborne illnesses and 347,000 deaths annually around the world.[2] The monetary loss caused by foodborne illness in the US is estimated to be more than 15.5 billion dollars per year.[4] In addition, it is estimated that 30-40% of the food supply is wasted in the US due to the presence of spoilage microorganisms.[5]

One of the most efficient ways to prevent foodborne disease outbreaks and reduce food spoilage and waste is to develop efficient food packaging materials, as food products spend a considerable amount of time inside packaging while in transit from the farm to the fork.[6] Currently, the majority of packaging is passive, providing only an inert barrier to the external environment.[7] More importantly, the concept of active or intelligent packaging has emerged. These types of packaging aim to extend the shelf life and improve safety and quality of food, not only by protecting the contents from degradation due to oxygen, water vapor, etc., but also by incorporating sensing and other antimicrobial approaches.[8]

Synthetic, petroleum-based polymers such as polyethylene terephthalate (PET), polyethylene (PE), polypropylene (PP), and polystyrene (PS) are widely used as a film for food packaging due to their low cost and useful mechanical and gas barrier properties.[9] However, the use of synthetic non-biodegradable polymers in food packaging and beyond has led to the so-called "micro-nano plastics crisis", which is an emerging issue of major environmental health concern.[10] More importantly, the recycling rate of these polymeric films is quite low.[11] More, recently, interest

has increased significantly in the use of biodegradable biopolymers for sustainable food packaging applications.[12] Such materials include polymeric films made of natural polymers such as starch, zein, and gelatin; polymers synthesized by bacterial fermentation such as polyhydroxy butyrate (PHB) and polyhydroxy butyrate-valerate (PHBV); and polymers synthesized from nature-derived monomers such as polylactic acid (PLA).[12]

An emerging approach for producing antimicrobial active packaging materials is the use of polymer films made of inherently antimicrobial materials such as ε-polylysine and chitosan.[13] However, the antimicrobial activity achieved with these materials is often not adequate.[14] Another approach is the incorporation of antimicrobial agents into the films.[15] However, the main issue of the film-based materials is the low surface-to-volume ratio which requires high quantities of antimicrobial agents for achieving satisfactory antimicrobial effects. This may result in negative sensory effects on the food, such as smell, discoloration, etc.[16]

More recently, nanotechnology has emerged as a promising avenue for the development of novel advanced bio-degradable food packaging materials to enhance food safety and quality.[17,18] For example, nanocellulose-based films have been explored due to their excellent gas barrier properties.[19] Furthermore, electrospun fibers synthesized from both synthetic polymers and biopolymers (e.g. polylactic acid, zein, amaranth, and pullulan) were infused with antimicrobial agents, including carvacrol, thymol, and nisin, to be used for food package materials.[20,21,22] In the study of Altan et al., carvacrol was incorporated into electrospun zein and PLA nanofibers for extending the shelf life of whole wheat bread.[20] Similarly, active food packaging materials for prolonging shelf life of meat was developed from electrospun zein nanofibers incorporated with the cyclodextrin-inclusion complex of thymol by Aytac et al.[21] Soto et al. reported the synthesis of nisin-loaded amaranth protein isolate:pullulan (API:PUL) nanofibers to be used for apple juice and

fresh cheese package.[22] Such fibers can be used to coat widely used polymer-based food packaging materials and provide antimicrobial functionality. The most important advantage of fibers over polymeric films is their high surface-to-volume ratio. This enables almost all antimicrobial agents to be on the surface, especially if the diameter of fibers is in the nanoscale (< 100 nm). However, synthesis of most electrospun fibers used in food packaging materials is not "green" since harsh organic solvents are typically used, and high quantity of essential oils per surface area are widely used to provide satisfactory and broad antimicrobial efficacy which may cause negative effects on the organoleptic properties of food. It is also worth noting that a big challenge of electrospinning is the scalability. However, recent advancements on electrospinning and development of automated multi-injector designs make it possible to increase the production yields.[23] As shown in previous studies by the authors and others, using a cocktail of antimicrobial active ingredients (AIs) rather than a single analyte enhances antimicrobial activity across a spectrum of microorganisms due to synergistic effects.[24,25]

In this study, electrospinning was used as a one-step synthesis approach to engineer functional fibers from zein using non-toxic organic solvents. Zein was selected to synthesize fibers since it is an amphiphilic, edible, bio-degradable, and Generally Regarded as Safe (GRAS) biopolymer by US FDA. A cocktail of nature-derived and US FDA approved as GRAS antimicrobial AIs was explored and antimicrobial activity against a broad spectrum of food pathogens was assessed by microbiological assays. The antimicrobials were incorporated into the chemical structure of the fibers using a direct solution integration method. The morphological and physicochemical properties of fibers, and AI dissolution kinetics as a function of time were assessed, along with their antimicrobial efficacy using a broad range of analytical and microbiological methods.

METHODS

"Green" synthesis of zein fibers using electrospinning: Figure 1a-b illustrates the one-step, green synthesis of fibers using electrospinning. The electrospinning apparatus (DOXA Microfluidics, Málaga, Spain) consists of four main components: syringe pump, injector, high voltage power supply, and collector. When high voltage is applied to a polymer solution, it is electrified. The so-called "Taylor cone" is then formed at the tip of the injector due to the distortion of the drop of polymer solution under the electric field. Once the electric field is high enough to overcome the surface tension of the polymer solution, a jet is formed and moved towards the collector. As the solvent evaporates, fibers are deposited on the collector/substrate.[26] The electrospinning parameters such as flow rate, applied voltage, and needle-collector distance can be modified in order to optimize the morphology and other properties of the fibers.[26]

Synthesis of pristine zein fibers (ZF): Zein (zein from maize, Sigma Aldrich, Z3625) solutions of various concentrations were prepared in acetic acid for synthesizing pristine zein fibers (ZF) in order to assess the effect of zein concentration on fiber morphology and to identify concentration for bead-free electrospun fibers. The zein solutions were then loaded in a plastic syringe (10 mL BD Luer-Lock™ tip) and a syringe pump was used to supply the solution through a stainless-steel single-needle injector (diameter:0.6 mm, 90° blunt end). Voltage was applied from the high voltage power supply to both the needle tip and collector. The electrospinning parameters such as flow rate, applied voltage, and needle-collector distance were modified along with zein concentrations to obtain bead-free zein fibers. ZF were randomly deposited on traditional food package substrates such as aluminum foil (20×20 cm^2) or cellulose nanofibril (CNF) film[27,28] (diameter:10 cm). By varying the collection time, the mass of the fibers per surface area can be adjusted.

Synthesis of antimicrobial zein fibers (AZF): The antimicrobial cocktail, composed of the active ingredients (AIs) selected as described below, was incorporated into zein solution using a direct solution integration method and was then used for the synthesis of fibers by electrospinning. In summary, the cocktail was dissolved in acetic acid and zein was then added into the solution, followed by stirring until complete dissolution was achieved. The solution was then loaded into a plastic syringe (10 mL BD Luer-Lock™ tip) and other electrospinning parameters including flow rate, applied voltage, and needle-collector distance were systematically modified to obtain bead free AZFs as described above for the case of pristine ZFs. AZFs were randomly deposited on traditional food package substrates as above.

Morphological characterization of zein fibers: Morphological characterization of the fibers was performed using scanning electron microscopy (SEM, Zeiss FESEM Ultra Plus). For this purpose, the fibers were peeled off from the substrate and cut into small pieces. The piece of fiber sample was then mounted on a stub using double-sided carbon tape and images were taken. The average diameter of the fibers was calculated from SEM images using Image J Software (n=100) and the results are reported as average ± standard deviation.

Physicochemical characterization of zein fibers: The fiber specific surface area (m^2/g), fiber average pore diameter (nm), and total pore volume (cc/g) of the fibers were measured using a Brunauer-Emmett-Teller (BET, Quantachrome NOVA touch LX4) surface area analyzer. Low temperature (77.35 K) nitrogen adsorption isotherms were measured at relative pressures from 0.005 to 1.00. Prior to measurement, fibers were degassed in 9 mm cell at 323.15 K for 24 hours.

The crystallinity of nisin and the fibers were investigated with Cu Kα radiation by and X-ray diffraction (XRD, Bruker D2 Phaser) in the 2Θ range of 10°-100°. XRD could not be performed for thyme oil and citric acid due to their liquid state at room temperature.

Chemical characterization of AIs and fibers was performed by Fourier transform infrared spectrometry (ATR-FTIR). The infrared spectra of AIs and fibers were obtained by an ATR-FTIR (Thermo Scientific Nicolet IS50). The spectra were recorded between 4000 cm^{-1} and 400 cm^{-1} at the resolution of 4 cm^{-1} and 64 scans were taken per sample.

Development of antimicrobial active ingredient (AI) cocktail: In order to determine the AI cocktail to be used, we examined the antimicrobial efficacy of a range of concentrations of various nature-derived antimicrobials including essential oils, organic acids, peptides[29] and their combinations. This includes thyme oil, citric acid, and nisin. Essential oils are a group of volatile compounds extracted from plants; are effective growth inhibitors against bacteria, fungi, and viruses; and have been studied as antimicrobial agents in food packaging.[30,31] Among essential oils, thyme oil is found to be one of the most effective compounds.[32] Organic acids exhibit their antimicrobial effect by damaging the bacterial membrane and causing stress on intracellular pH homeostasis.[33,34] Citric acid, commonly found in citrus fruits, shows inhibition effect against foodborne pathogens such as *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella*.[35,36] Bacteriocins are antimicrobial peptides showing their effect while inducing permeabilization by disrupting the bacterial membrane or moving across the membrane and interacting with internal targets (i.e. DNA), ultimately killing the cell.[37] Nisin, which is very effective bacteriocin, can inhibit the growth of various gram-positive bacteria, including *Listeria monocytogenes*, *Clostridium botulinum*, and *Staphylococcus aureus.*[32]

The antimicrobial efficacy of AIs against known food pathogen surrogates, *Escherichia coli* (*E. coli*) ATCC#25922 and *Listeria innocua* (*L. innocua*) ATCC#33090, was assessed using the disk diffusion test described in detail by Hudzicki et al.[38] as follows:

Inoculum preparation: To prepare the inoculum of *E. coli* and *L. innocua* for the disk diffusion test, a single colony was selected, transferred into 30 mL of tryptic soy broth (TSB; Hardy Diagnostic, Santa Maria, CA) and incubated at 37°C for 18-24 hours. The culture was then centrifuged at ~2056×g for 20 minutes, and the supernatant was discarded. The culture pellet was resuspended in phosphate buffered saline (PBS; Hardy Diagnostic, Santa Maria, CA), and the concentration was adjusted to ~10^8 CFU/mL using O.D.600 measurement with a UV/Vis spectrophotometer (Beckman Coulter, Brea, CA).

Consequently, dispersion of single AIs and their combinations were prepared fresh in sterile deionized water. It is worth noting that the antimicrobial activity of 0.005% (w/v) nisin could not be investigated following the established antimicrobial assessment protocol which requires dissolution in water since nisin has low solubility at neutral pH. Instead, nisin was dissolved with 1% (w/v) citric acid, which is the minimum amount of citric acid used in the combined AIs and its efficacy assessed following the protocol described below. A positive control of 0.5 mg/mL of ampicillin (VWR, Radnor, PA) and a negative control of deionized water were included. Then, 20 µL of each AI dispersion was added to a blank paper disk (6 mm; Hardy Diagnostic, Santa Maria, CA). *E. coli* or *L. innocua* were then inoculated onto Mueller Hinton Agar (MHA; Hardy Diagnostic, Santa Maria, CA) with a cotton swab. The disks impregnated with AI solutions were placed onto the inoculated MHA plates. The plates were then incubated at 37°C for 24 hours, and the diameter of inhibition zone was measured.

Assessment of antimicrobial efficacy of zein fibers: The antimicrobial efficacy of AZF synthesized as described above was determined against two foodborne pathogen surrogates, *E. coli* and *L. innocua*, and a spoilage-associated fungus (*Penicillium italicum* (*P. italicum*) ATCC#48144) by the direct contact assay based on an ASTM protocol.[39] In more detail:

The inoculum of *E. coli* and *L. innocua* was prepared as described above. To prepare the inoculum of *P. italicum* spores, a published protocol was used with slight modifications.[40] In brief, *P. italicum* was inoculated onto potato dextrose agar (PDA) slants and incubated at 24°C for 7 days. To harvest the *P. italicum* spores, each slant was added with 2 mL of 0.01% Tween-80 aqueous solution, and vortexed for 30 seconds. The solution was then filtered through a two-layer sterile cheese cloth, and the concentration was adjusted to ~10^7 CFU/mL with PBS using manual counting with a hemocytometer (Diagnocine, Hackensack, NJ).

Direct contact assay: The fibers deposited on the aluminum foil substrate were cut into pieces (2×2 cm^2) and disinfected by ultraviolet light in a biosafety hood for 15 minutes on each side and were then placed into 6-well plates. An agar slurry (0.3% agar) and 0.85% sodium chloride) was prepared, autoclaved, and cooled to 45°C. The microorganism inoculum (1 mL) was then added into 100 mL of agar slurry. The inoculated agar slurry (300 µL) were then added onto each test sample to form a thin layer of < 1 mm. The plates were then kept in an incubator for 0 hour (control), 1 hour, and 24 hours. The relative humidity was monitored and maintained at > 75% with an open tray of water. The temperature was maintained at 37°C (for *E. coli* and *L. innocua*) or 24°C (for *P. italicum*). Each sample was mixed with 10 mL of PBS in a sterile 59-mL stomacher bag and homogenized with a stomacher for 2 min. The elute was then serially diluted and plated onto tryptic soy agar (for *E. coli* and *L. innocua*) or PDA (for *P. italicum*). Plates were incubated at 37°C for 24 hours (for *E. coli* and *L. innocua*) or 24°C for 5 days (for *P. italicum*). Typical colonies of microorganisms were counted, and log reduction of microorganisms was calculated by comparing to 0-hour control samples.

Controls and comparator materials: Antimicrobial efficacy of the substrate used to deposit the fibers (aluminum foil), ZF, and comparator samples (aluminum foil coated with AI cocktail)

were also assessed following the direct contact assay described above. The comparator sample was prepared by pipetting AI cocktail solution to uniformly cover the surface of the aluminum foil having the same surface area with the substrate employed to deposit the fibers (20×20 cm^2). The AI cocktail solution, which consists of 1% (w/v) thyme oil, 5% (w/v) citric acid, 0.2% nisin mixture (w/v, 0.005% pure nisin), was also the same in terms of composition (except zein) and the volume used to prepare the fibers. Finally, the comparator sample was kept in hood for 24 hours to evaporate acetic acid; the sample was then cut into 2×2 cm^2 pieces and weighed. The mass per surface area of the samples were found to be 3.63 mg/cm^2 by dividing the total mass of the solution on the foil by the surface area of the samples (2×2 cm^2); the AI loading of the samples was also calculated at 3.63 mg of AI/cm^2 since they were composed of 100% AIs.

Antimicrobial efficacy of the fibers at various fiber mass per surface area: The antimicrobial efficacy of AZF at two mass per surface area ratios of 1.25 and 2.50 mg/cm^2 was assessed.

Antimicrobial efficacy of fibers over time: The antimicrobial efficacy of AZF after storage at 4°C for 4 weeks was also assessed as described above to determine any shelf life effects.

AI release kinetics from zein fibers in various food simulants: The release kinetics of AIs from 6.45 cm^2 of AZF (15 mg) were investigated by immersing fibers into 10 mL of food simulant and the solutions were stirred at room temperature for 10 days.[41] Water, 3% acetic acid, and 50% ethanol were used as food simulants to represent aqueous non-acidic, aqueous acidic, and fatty food, respectively.[41] Aliquot samples (1 mL) were withdrawn from the solutions at 0, 2, 6, 12, 24, and 240 hours and replaced with 1 mL of fresh food simulant. The samples were filtered prior to analysis, and the amount of AIs (thymol and nisin) released was selectively measured using liquid chromatography/high resolution mass spectrometry (LC/HRMS, Dionex Ultimate 3000 LC interfaced to a Thermo Q-exactive HRMS). Thymol was chosen among the various components of

thyme oil since it was the major component that was detected by LC/HRMS. Nisin release can be detected up to 24 hours and this might be likely due to the inactivation of nisin over time.[42] In addition, nisin release in 50% ethanol was not given because 50% ethanol created a matrix effect that greatly altered nisin detection, and reliable quantitation was not possible.

The LC employed an Agilent SB-C18 2.1 x 150 column with a gradient elution. Mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. The LC was held at 5% B for two min, then linearly increased to 95% B for 10 min, where it was held for 3 minutes before returning to 5% B for 3 minutes re-equilibration. The mass spectrometer was operated in the positive ESI mode at 3.5 kV with the capillary and auxiliary gas temperatures set to 300^{o}C, and the gas flows were: sheath 50, auxiliary 15, and sweep 10. The full scan was set to monitor m/z = 120-900 at a resolution of 70,000. Nisin was monitored using the six most intense ions of the cluster created by the $(M+5H)^{+5}$ cluster while thymol used the $(M+H)^{+}$ ion. Quantitation was performed using a series of calibration standards.

The loading efficiency (LE) is defined as the percentage of AI that is loaded in the nanofibers. LE of AIs in AZF was determined by dissolving fiber samples (15 mg) in 10 mL of acetic acid. The solutions were then filtered, and measurements were performed by LC/HRMS as described above. LE is calculated by using the following equation:

$$LE\ (\%) = (\text{concentration of the loaded AI}) / (\text{concentration of the total AI}) * 100 \quad \text{(Equation 1)}$$

The area of each AI peak was first converted to concentration (ppm) using standard calibration curves and then cumulative release (%) was calculated by considering calculated LE for each AI. The release experiments were carried out in triplicate, and the results were reported as average ± standard deviation.

Scalability of fiber synthesis using a multi-needle injector: To demonstrate the scalability of the method, a 20-needle injector was used. The needles on the multi nozzle-injector were arranged 10 mm apart in circular pattern with a total injector diameter of 70 mm ~~(Figure S4a)~~. The solution was prepared as described above and was loaded in a plastic syringe (10 mL BD Luer-Lock™ tip). While feeding the solution through a multi-nozzle injector towards the collector by a syringe pump, the voltage was applied to both the needle tip and collector from high voltage power supply. The electrospinning parameters such as flow rate, applied voltage, and needle-collector distance were then systematically modified for optimization of the cone-jet stability and for the synthesis of bead-free fibers. Fibers were randomly deposited on 20×20 cm^2 aluminum foil substrate.

Preliminary assessment of fibers affinity to attach to the substrates: In order to improve fiber affinity to the substrate surface, pressure was applied on AZF by a hydraulic press machine at room temperature (Carver, Bench top standard automatic lab press, Auto Four/3012-PL, H, 30-ton capacity). AZF deposited on aluminum foil (20×20 cm^2) and CNF film (diameter: 10 cm) was pressed under various pressure and time conditions. To test fiber affinity to the surface, a 200 g weight was applied on fibers deposited on the aluminum foil and removed after 5 minutes. Finally, the detachment of fibers from surface was visually observed to determine qualitatively fiber affinity to the substrate.

Statistical Analysis: Three independent replicates were conducted for each antimicrobial experiment. Microorganism colony counts were converted to log CFU/sample. Statistical analysis was conducted using JMP (Cary, NC). A one-way analysis of variance with Tukey's multiple comparison was used to identify statistically significant difference between treatments ($P < 0.05$).

The statistical analysis for AI release kinetics from zein fibers in various food simulants were performed using one-way ANOVA with a Holm-Sidak multiple comparison test (Student t-test; $p<0.05$).

RESULTS AND DISCUSSION

"Green" synthesis of zein fibers using electrospinning: To achieve green synthesis of antimicrobial electrospun fibers, the biopolymer and solvent were selected by design, as demonstrated below. Zein was chosen as a biopolymer, since it is a protein-based, edible, bio-degradable polymer derived from corn and is a significant byproduct of corn processing for the food and bioethanol industries. Zein has been approved as GRAS by the US FDA. It has both hydrophilic and hydrophobic amino acids in its structure, giving it an amphiphilic character that makes it unique among biopolymers. Consequently, the presence of hydrophobic amino acids such as leucine, alanine and proline in the structure eliminate the need for post treatment such as crosslinking.[43]

Organic solvents that are typically used in electrospinning possess a high dipole moment, dielectric constant, and conductivity, as well as low surface tension. Unfortunately, most solvents with these properties are quite toxic.[44,45,46] Electrospun zein fibers have been produced by various solvent mixtures, including toxic organic solvents such as dimethyl formamide.[47] Since the use of toxic solvents in this study was avoided to enable a "green" synthesis strategy, acetic acid was selected as the solvent. It is worth mentioning that acetic acid is an FDA approved as GRAS and non-toxic compound.[48] The ability of acetic acid to dissolve both polymer and active ingredients (AIs) which results in the synthesis of bead-free and homogenous zein fibers has been demonstrated in our results.

Synthesis of pristine zein fibers (ZF): Bead presence is not desirable during fiber synthesis because they confound fiber homogeneity and integrity. In order to synthesize bead-free zein fibers (ZF), pristine zein solutions were prepared in acetic acid at concentrations ranging from 25-30% (w/v) and electrospinning was then performed as described above in detail. The operational parameters were systematically modified to optimize the cone-jet stability and synthesize bead free fibers. After a systematic modification of each parameter, the final parameters were 0.7 mL/h, 26 kV (25 kV was applied to the needle, -1 kV was applied to the collector), and a 15 cm distance to the collector.

Morphological characterization of zein fibers as a function of electrospinning parameters and zein concentration: Bead-free pristine zein fibers (ZF) were achieved at 30% (w/v) as shown in the SEM image in Figure 2a. The occurrence of bead-free fibers at this concentration is due to sufficient chain entanglement and viscosity of the solution, which result in strong viscoelastic forces that resist axial stretching during electrospinning.[49,50] The fiber diameter distribution graph of ZF in Figure 2b shows diameters reaching up to a maximum of 400 nm, with an average diameter of 140±40 nm. As evident in Figure S1a-b-S2, bead-free fibers cannot be achieved with zein solutions at 25% (w/v) and 27.5% (w/v) concentrations due to insufficient chain entanglement in these solutions.

Synthesis of antimicrobial zein fibers (AZF): Zein at various concentrations in the range 30-37% (w/v) was directly added into the solutions of AI cocktail dissolved in acetic acid and the solutions were then electrospun. In order to optimize the cone-jet stability, the operational parameters such as flow rate, applied voltage, and needle-collector distance were systematically modified. First, the flow rate, applied voltage, and needle-collector distance of the solution were set to 0.7 mL/h, 26 kV (25 kV was applied to the needle, -1 kV was applied to the collector), and

15 cm, respectively. After adjusting the parameters, as shown in Figure 2c, the bead-free fiber parameters were synthesized at zein concentration of 37% (w/v), 0.5 mL/h flow rate, 26 kV voltage (25 kV was applied to the needle, -1 kV was applied to the collector), and 15 cm needle to collector distance.

The fiber diameter distribution graph of bead-free AZF displayed in Figure 2d shows diameters reaching up to a maximum of 400 nm, with an average diameter of 165±35 nm. The slight difference in the average diameters of ZF and AZF is most likely due to the increased viscosity of the zein solution after AI incorporation.[50]

AZFs were deposited on the substrates for 2 and 4 hours and the fibers were then weighed; the masses per surface area were 1.25 mg/cm^2 and 2.50 mg/cm^2, respectively. Since the initial theoretical concentration of AIs in AZF was ~10%, AI loading of each AZF deposited for 2 and 4 hours were calculated as 0.125 mg of AI/cm^2 and 0.250 mg of AI/cm^2, respectively.

Physicochemical characterization of zein fibers: Specific surface area (SSA), average pore diameter, and total pore volume of bead-free ZF and AZF were determined by BET (Table S1-S3). The multipoint BET surface area of ZF and AZF were 16.11 m^2/g and 21.91 m^2/g, respectively. It is worth noting that the SSA of AZF is a bit higher than ZF (21.91 m^2/g for AZF and 16.11 m^2/g for ZF). The incorporation of AIs changes the electric conductivity of the polymer solution and that can result to morphology changes as shown in Figures 2a-d. In order to maintain the bead free and homogenous nature of fibers slight modifications of electrospinning parameters are required as presented in the results section. The average pore diameter is between 4.84 nm and 5.58 nm, which show that the pores in the fibers are in mesoporous range (2 and 50 nm). Total pore volumes were determined to be 1.95 x10^{-2} cc/g and 3.05x10^{-2} cc/g for ZF and AZF, respectively.

These values are consistent with the literature, in which that pore volume increases with surface area.[51]

XRD was performed to determine the crystallinity of nisin, ZF, and AZF (Figure 2e). The most observable nisin peaks were evident at 2θ=31° and 2θ=45°, due to the characteristic diffraction of sodium chloride (NaCl), which is the major component of nisin.[52] ZF and AZF are composed of an amorphous polymer and therefore exhibited amorphous structure, with only a broad peak at 2θ=15°-27°.[53] Since there are no crystalline peaks of NaCl in AZF, NaCl is likely no longer present as a crystalline compound in this fiber, likely being converted into an amorphous state during electrospinning process due to the large elongational force and rapid evaporation of the solvent that may confound crystallization.[54,55]

Chemical characterization was carried out by FTIR (Figure 2f). FTIR spectra of ZF and AZF exhibited peaks at 1653 cm^{-1} and 1540 cm^{-1}, corresponding to the amide I and amide II of C=O stretching of the zein structure.[53] The presence of each of the AIs in AZF was also evident from the spectra. First, thyme oil exhibited peaks at 3500-3300 cm^{-1}, 1627 cm^{-1}, 1360 cm^{-1}, 1250 cm^{-1}, and 800 cm^{-1}, due to the OH stretching, aromatic C=C stretching, isopropyl group, C-O stretching, and aromatic C-H bending vibrations.[56] The peak observed at 1250 cm^{-1} in the spectra of AZF may belong to C-O stretching vibration of thyme oil.[56] Secondly, citric acid has its characteristic peaks at 3290 cm^{-1}, 1721 cm^{-1}, 1105 cm^{-1}, and 778 cm^{-1}, belonging to the O-H stretching, C=O stretching, C-OH stretching, and CH_2 rocking, respectively. Therefore, the peak in the spectra of AZF at 1721 cm^{-1} may be attributed to C=O stretching of citric acid.[57] Lastly, the peaks observed in the spectra of AZF at 1653 cm^{-1} and 1540 cm^{-1}, which were attributed to the amide groups, showed the presence of nisin in AZF.[58,59] However, the extremely low levels of AIs incorporated into the chemical structure of the fibers results to weak signals for those AI in the

FTIR spectra making it difficult to distinguish from the stronger polymer signal (The fibers consists of 90% biopolymer). In the FTIR spectra of AZF, the C-O stretching vibration of thyme oil at 1250 cm^{-1}, C=O stretching of citric acid at 1721 cm^{-1}, and amide groups of nisin at 1653 cm^{-1} and 1540 cm^{-1} were identified indicative of the incorporation of AIs. Finally, the dissolution kinetics of AIs under simulant conditions presented ~~in Figures 4a-b~~ are also confirming the presence of AIs in a more quantifiable manner.

Development of antimicrobial active ingredient (AI) cocktail: Figure S2-S4 summarizes the antimicrobial activity of single AIs (thyme oil, citric acid and nisin) and their combinations at a range of concentrations against *E. coli* and *L. innocua* using the disk diffusion test. Five % (w/v) thyme oil was effective against both *E. coli* and *L. innocua*, showing a zone of inhibition of ~10 mm. Nisin, which was dissolved in 1% (w/v) citric acid because of its poor solubility at neutral pH, was effective against *L. innocua* (zone of inhibition of ~8 mm) but not against *E. coli*. This is not surprising as nisin is known to be effective against gram-positive bacteria by forming pores on cell membrane and disrupting cell wall synthesis.[32,60] On the other hand, the liposaccharide in the outer membrane of gram-negative bacteria protects them from nisin activity.[42,60]

The cocktail of 5% (w/v) thyme oil, 1% (w/v) citric acid and 0.005% (w/v) nisin exhibited the greatest antimicrobial activity against both *E. coli* and *L. innocua*, showing a zone of inhibition of ~10 mm (Figure S2-S4). However, the strong odor of thyme oil in this cocktail will most likely have a negative impact on the organoleptic properties of food. The cocktail of 1% (w/v) thyme oil, 5% (w/v) citric acid and 0.005% (w/v) nisin showed zones of inhibition of ~9 mm for *E. coli* and ~8 mm for *L. innocua*. Therefore, this cocktail, which showed equivalent antimicrobial effects but contains less thyme oil, was chosen for further studies.

It is worth noting that synergistic antimicrobial effects of AIs has been shown previously in studies by the authors and others.[61,62] Zhou et al. (2007) showed that neither thymol nor citric acid inhibited the growth of *Salmonella* Typhimurium, whereas their combination reduced *Salmonella* after 24 hours by 2.8 log.[61] Zhao et al. (2017) showed that nisin did not inhibit the growth of *Listeria monocytogenes* and that citric acid only reduced *Listeria monocytogenes* by ~1.5 log after 10 hours; however, their combination reduced *Listeria* by more than 5 log of after 10 hours.[62]

Assessment of antimicrobial efficacy of zein fibers: Pristine zein fibers **(**ZF) slightly enhanced the growth of *E. coli* by ~1.5 log in 24 hours (Figure 3a); there was no impact on the growth of *L. innocua* (Figure 3b). A possible explanation is that *E. coli* can release extracellular protease and breakdown zein, a protein, in the fiber and thereby release amino acids to support growth. A previous study confirmed that the *E. coli* strain (ATCC 25922) exhibited extracellular protease activity.[63]

Antimicrobial zein fibers (AZF) reduced *E. coli* by 3.0 log and 4.7 log at 1 hour and 24 hours, respectively (Figure 3a). For *L. innocua* (Figure 3b), the reduction caused by AZF was 4.9 log (close to the detection limit) at both 1 hour and 24 hours. Notably, at the 1-hour contact time, the antimicrobial efficacy of AZF against *E. coli* was lower than the case of *L. innocua*. This is likely due to presence of nisin in the cocktail, which is particularly effective against gram-positive bacteria.[32]

Aluminum foil coated with the AI cocktail was used as a comparator sample for the antimicrobial efficacy test. After 1 hour and 24 hours of contact, *E. coli* was reduced by 4.7 log by this comparator sample, in contrast to 3.0 and 4.7 log by AZF, respectively (Figure 3a). The inactivation of *L. innocua* was 4.9 log after 1 hour and 24 hours of contact for both the comparator sample and AZF (Figure 3b). It is worth noting that the comparator sample has more than an order

of magnitude greater AI loading (3.63 mg of AI/cm^2) than AZF (0.250 mg of AI/cm^2). Importantly, the antimicrobial efficacy of AZF after 24 hours of contact was equivalent to the comparator sample. This finding is consistent with the previous studies demonstrating that electrospun fibers showed significantly greater antimicrobial activity than traditional films with similar loadings of antimicrobials.[64,65] As noted above, the unique property of electrospun fibers over AI-infused films is their higher surface-to-volume-ratio, enabling nearly all of the AIs to be on the surface and thereby yielding similar antimicrobial efficacy at much lower AI amount.

The antimicrobial efficacy of AZF against *P. italicum* was also evaluated. AZF did not show inactivation effect against *P. italicum* spores (Figure S3-S5). This is likely due to the greater resistance of fungal spores to antimicrobial agents or perhaps to the loading used; a greater AI loading amount may have yielded control.[66]

In comparison to previous studies with other biopolymer-based fibers, AZF exhibits similar or greater antimicrobial efficacies against *E. coli* and *L. innocua* (~5 log reduction in 24 hours). Lin et al. (2018) showed that electrospun thyme oil/gelatin fibers could inhibit the growth of *Campylobacter jejuni* on chicken by ~1-2 log in 24 hours.[67] Similarly, Lin et al. (2019) reported that electrospun thyme oil/silk fibroin fibers could reduce *Salmonella typhimurium* on chicken and duck meat by ~1-4 log in 24 hours.[68] Aytac et al. (2017) reported that electrospun zein fibers incorporated with thymol, which is the main component of thyme oil, inhibited the growth of *E. coli* and *Staphylococcus aureus* in suspension by ~55% and 67% in 24 hours, respectively.[21]

In addition, as shown in Figure 3a, when the mass per surface area of AZF decreased from 2.50 mg/cm^2 to 1.25 mg/cm^2, the efficacy of *E. coli* inactivation was reduced significantly ($P < 0.05$) from 3.0 to 2.2 log at a contact time of 1 hour. At 24 hours contact time, no significant difference ($P > 0.05$) of *E. coli* inactivation was found between the two AI loadings (Figure 3a).

For *L. innocua*, the antimicrobial efficacy of AZF reached the detection limit in 1 hour for both AI loading levels; there was no significant difference in efficacy between the fibers (Figure 3b).

The antimicrobial efficacy of AZFs after storage at 4°C for 4 weeks was unchanged against *E. coli* at 1 hour and 24 hours of contact (Figure 3a). However, AZF showed slightly reduced inactivation efficiency against *L. innocua* after 4 weeks of storage, with 3.4-log reduction in1 hour compared to 4.9-log reduction prior to storage. It is worth noting that the 4-week storage of the AZF did not affect the 24-hour contact time activity (4.9-log reduction) (Figure 3b). Lower efficacy against *L. innocua* could be due to the loss in nisin activity over time.[69]. Wu et al. (2018) also found that nisin-anchored cellulose film showed less antimicrobial efficacy against a gram-positive bacterium, *Alicyclobacillus acidoterrestris*, after a 3-month storage period.[70] It is also possible that evaporation of thyme oil occurred over time, which was previously reported as a limitation for the use of essential oils.[71]

AI release kinetics from zein fibers in various food simulants: The cumulative release (%) of AIs from AZFs into the selected food simulants over a 240-hour period is shown in Figure 4a-b. The loading efficiency (LE) values, which are the percentage (%) of AIs successfully loaded in the AZFs, were determined as 60% and 57% for thymol and nisin, respectively; these values were used to calculate the percent AI release. As shown in Figure 4a, AZF exhibited a rapid release of thymol in all media, with complete dissolution in 2 hours. Small diameter of fibers results to both high specific surface area (SSA) and surface to volume ratio compared to films. This high SSA results to AIs to be mostly located on the surface of the fiber making them more bioactive when pathogens get in contact with such surfaces.[72] In addition, hydrophilic amino acids in the zein structure facilitated fiber wettability and AI solubility.[43] It is also worth noting that AZF completely dissolved in 50% ethanol and this may have also caused the highest thymol release, along with high

solubility of thymol, in this medium.[73] Lastly, the release of thymol was higher in 3% acetic acid than water due to higher solubility in acidic medium.[74] Nisin release profiles from AZF varied and were also dependent on the medium as shown in Figure 4b. Nisin release was relatively slow in 3% acetic acid compared to water; thus, the release was completed in 12 hours in 3% acetic acid versus 6 hours in water. In addition, the release of nisin in 3% acetic acid was slightly greater than its release in water. This result can be explained by the higher solubility of nisin in 3% acetic acid as compared to water.

The use of miniscule amounts of AIs in the structure of AZF results to miniscule quantities of AIs released in food simulants and as a result not only the microorganisms will be eliminated in contact but potential sensory effects such as taste change, discoloration, etc. will be minimum and less than those of using same AIs in films. It is also worth noting that the AIs used here are non-toxic and classified as safe by US FDA. Finally, in future studies, detailed sensory assessment based on the type of food to be stored using such fibers will be performed.

Scalability of fiber synthesis using a multi-needle injector: Electrospun fibers have a great potential to be used in industrial applications; therefore, it is also important to demonstrate scalability of the proposed synthesis by using a multi-needle injector as described in the method section. It is worth noting that recent advances on electrospinning and development of multi-needle injector enabled scaling up the process and used in biomedical applications.[23] As illustrated in this study, the use of a 20-needle injector enabled us to increase the fiber production yield to 1 g/h which shows that the approach can be scaled up and commercialized and used by the food packaging industry. Here, the operational parameters such as flow rate, applied voltage, and needle-collector distance, are of great importance for the uniform distribution of the electric field throughout the needles.[23,75] The optimized parameters for the multi-needle synthesis were adjusted

and found to be 3 mL/h, 35 kV (27.5 kV was applied to the needle; -7.5 kV was applied to the collector), and 15 cm for flow rate, applied voltage, and needle-collector distance, respectively

Figure S4a-S6 shows the SEM image of AZF synthesized using these optimized electrospinning parameters. The fibers are bead-free and uniform; the diameter distribution graph is given in Figure S4b-S6 and shows values up to 400 nm, with an average diameter of 160±40 nm. Importantly, this is quite similar to the average diameter of AZF (165±35 nm) synthesized by single-needle injector (Figure 2d). It is also worth mentioning that the yield for AZF synthesized by single and multi-needle injectors were calculated to be 0.12 g/h and 1 g/h, respectively. In conclusion, these results showed fiber synthesis scalability, which is important for industrial and food sector applications. It is worth noting that while electrospinning can be scaled up and used in the production of fibers from biopolymers for food packaging, the cost might be prohibitive for some food applications. On the other hand, the use of biodegradable biopolymers will reduce the environmental footprint and more importantly consumers expressed willingness to pay more for those biodegradable materials. [76,77,78]

Preliminary assessment of fibers affinity to attach to the substrates: Since fiber detachment from the AZF surface could confound efficacy, hydraulic pressure was applied to the fibers to improve the affinity to the substrate. SEM images of the pressure-applied AZFs synthesized on aluminum foil are shown in Figure S5a-c-S7. Here, various pressure and time combinations were utilized: 3 MPa for 2 and 4 min, and 12 MPa for 2 min. When the lowest pressure (3 MPa) (Figure S5a-b-S7) was used, the AZF porosity was slightly decreased.[79] The difference in fiber porosity was minimal when the pressure was applied for 2 minutes compared to 4 min. In addition to the change in porosity, applying 12 MPa for 2 minutes also resulted in an overt bunching at certain locations (Figure S5c-S7). Visual observation of fibers showed that an

application of 3 MPa pressure applied for 4 minutes was the optimum condition to increase fiber affinity to the surface.

In order to evaluate fiber affinity to the substrate (aluminum foil), a 200 g weight was applied for 5 minutes and then removed in order to evaluate fiber detachment. Some of the fibers detached from the surface for non-pressure applied fibers, whereas no fiber detached for the hydraulically pressured fibers (3 MPa for 4 min). These results demonstrate that it is possible to coat substrates with fibers that remain intact even after exposure to forces typical of food packaging conditions applied.

CONCLUSIONS

Designing bio-degradable packaging materials has become an area of great interest in the food industry, particularly given that the excess use of synthetic polymers is causing environmental concerns due to the release of micro-/nanoplastics. In addition, the incorporation of antimicrobials into the package is an effective way to enhance food safety and quality while promoting sustainability. Here, a scalable, green, and one-step synthesis approach was developed to synthesize antimicrobial zein fibers containing an AI cocktail composed of nature-derived FDA-classified Generally Recognized as Safe (GRAS) antimicrobials having different mechanisms of action. These antimicrobial fibers are suitable for use in food packaging materials to enhance food safety and quality. Scalability of the platform was also demonstrated using a multi-needle injector system. Finally, the developed fibers were able to efficiently inactivate broad-spectrum of food-associated microorganisms, with reductions of ~5 logs for *E. coli* and *L. innocua* after 24 hours of contact. Additionally, antimicrobial activity at 24-hour contact time was retained after 4 weeks of material storage at 4°C. Further studies are underway to explore sensory evaluation tests such as color, texture, and taste of food that was stored in fiber-containing packaging materials.

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SUPPORTING INFORMATION

The Supporting Information is available free of charge on the ACS Publications website: http://pubs.acs.org.

-SEM images of beaded fibers

-Table showing the surface area analysis of fibers

-Antimicrobial activity of single and combined AIs

- Antimicrobial activity of fibers against *P. Italicum*

- SEM image of fibers synthesized by a multi-needle injector

- SEM images of fibers after applying pressure

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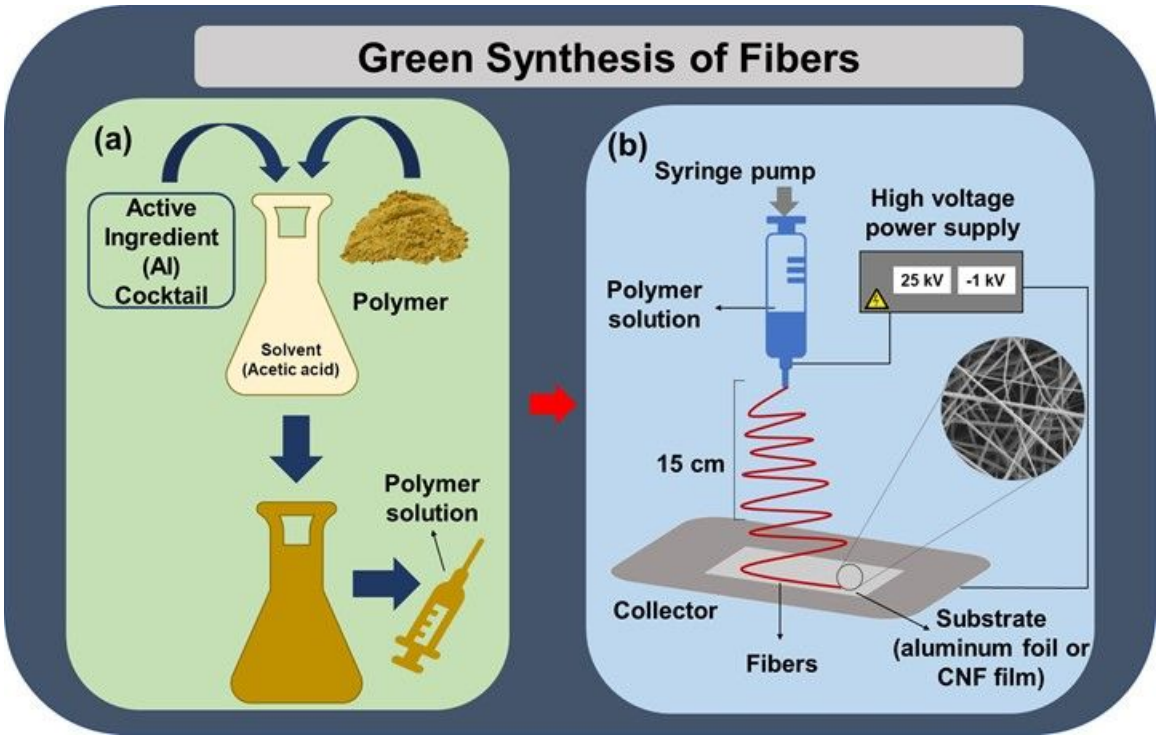

Figure 1. Schematic representation of (a) the preparation of active ingredient (AI) cocktail that is loaded into the polymer solution and (b) the synthesis of fibers incorporated with AI cocktail by electrospinning.

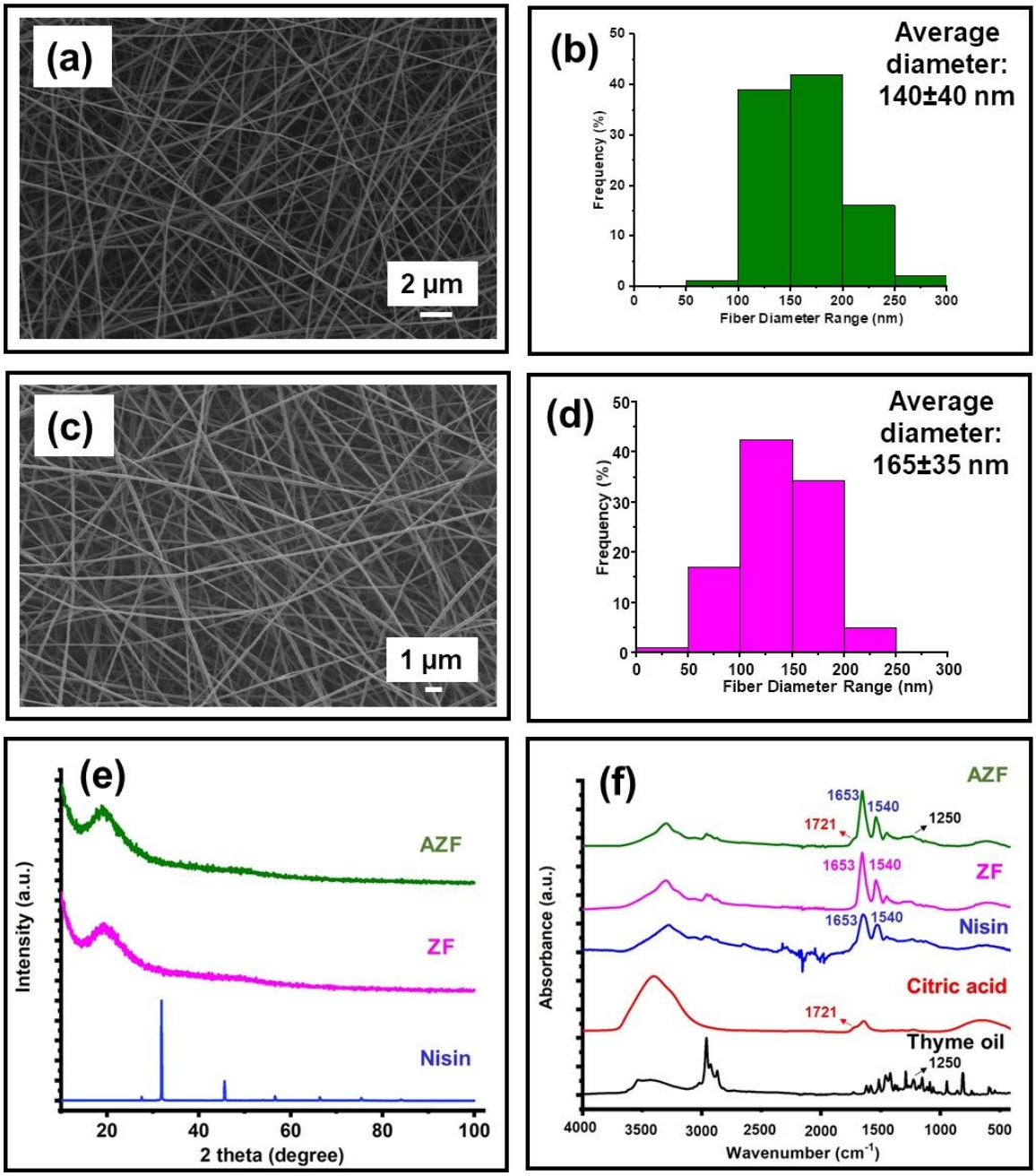

Figure 2. Scanning electron microscopy (SEM) images and fiber diameter distribution graphs of (a,b) ZF and (c,d) AZF; (e) X-Ray diffraction (XRD) patterns of nisin, ZF, and AZF; and (f) Fourier transform infrared spectrometer (FTIR) spectra of thyme oil, citric acid, nisin, ZF, and AZF.

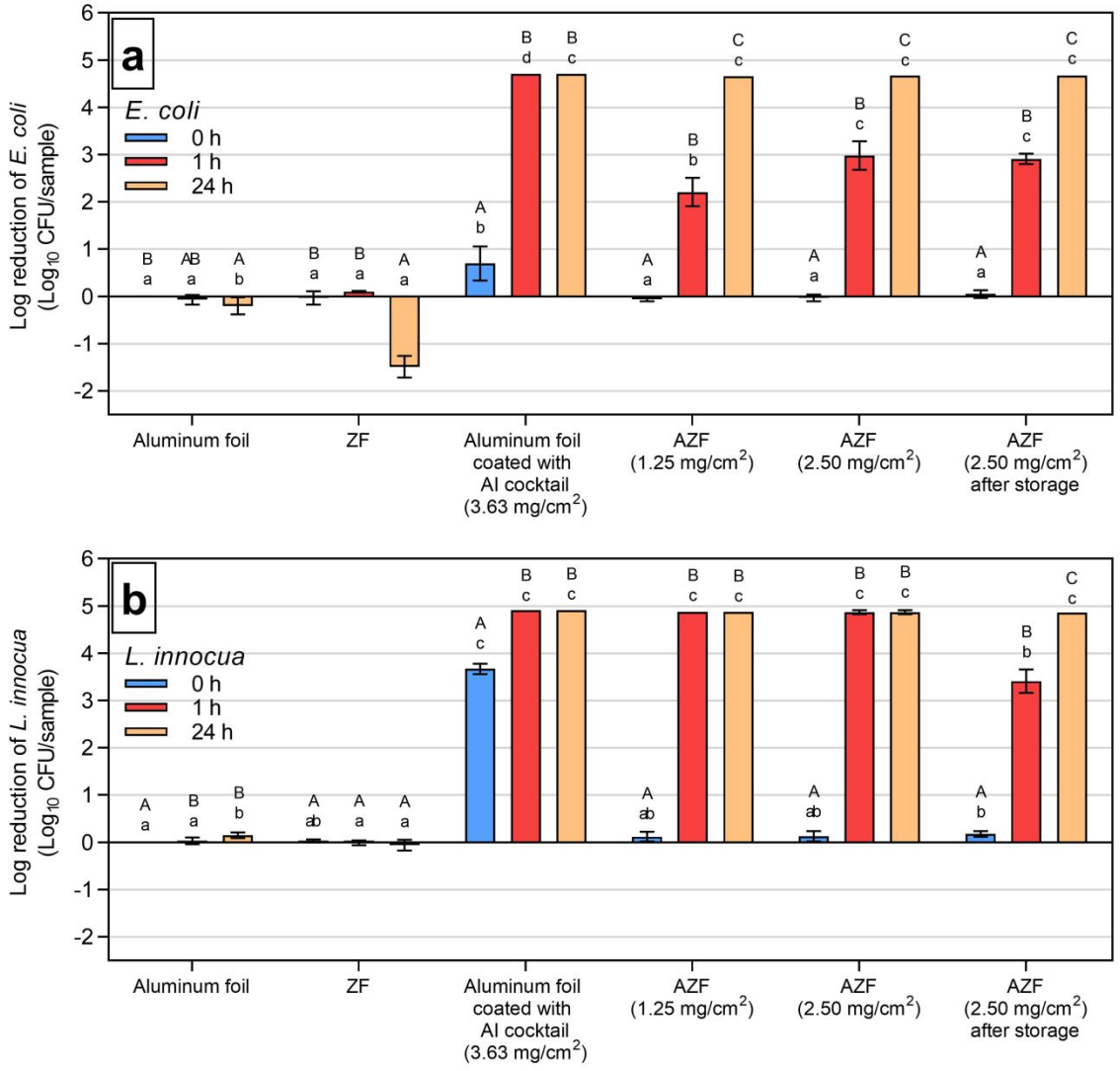

Figure 3. The antimicrobial activity of aluminum foil, zein fibers (ZF), aluminum foil coated with active ingredient (AI) cocktail, antimicrobial zein fibers (AZF) (1.25 mg/cm^2), AZF (2.50 mg/cm^2), AZF after storage at 4°C for 4 weeks against (a) *E.coli* and (b) *L. innocua*. Data in the same material group labeled with the same uppercase letter are not significantly different ($P > 0.05$). Data in the same treatment time group labeled with the same lowercase letter are not significantly different ($P > 0.05$).

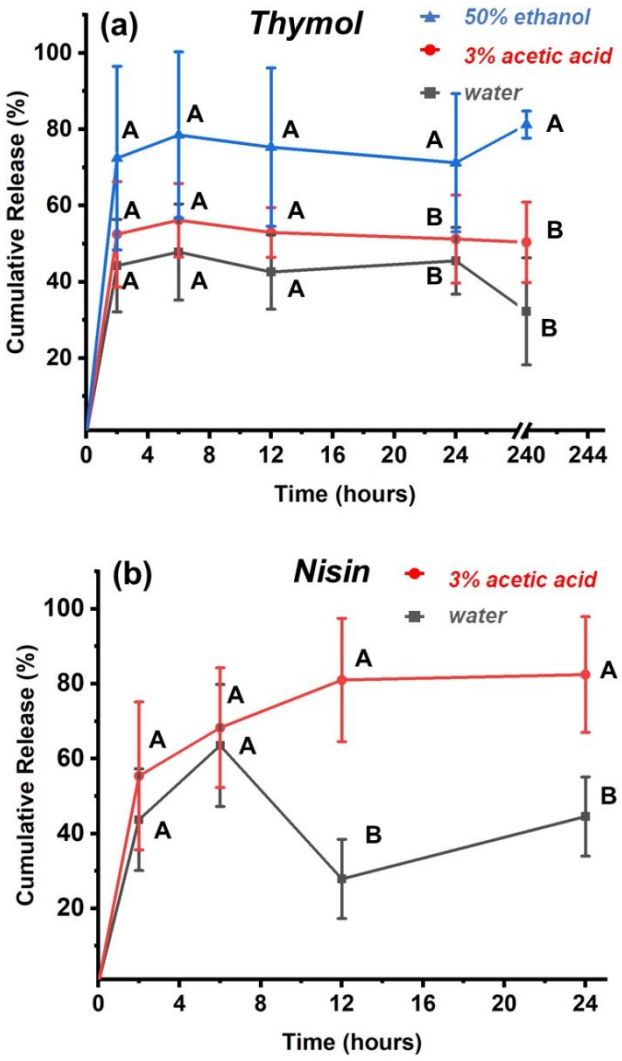

Figure 4. The cumulative release (%) of (a) thymol and (b) nisin from antimicrobial zein fibers (AZF) into food simulants as a function of time. (a) Values at a specific time point that have a different letter are significantly different (One-way ANOVA with a Holm-Sidak multiple comparison test at $p<0.05$). *= significantly different at $p=0.066$. (b) Values at a specific time point that have a different letter are significantly different (Student t-test; $p<0.05$).

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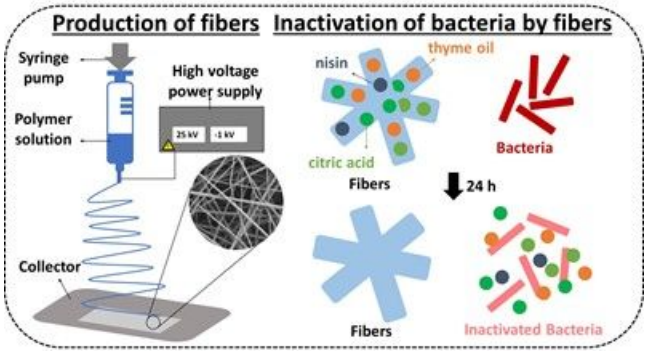

Synopsis

Fiber-based materials for sustainable food packaging were developed from a biodegradable polymer and nature-derived antimicrobial agents by green synthesis approach.