High-Throughput Coating With Biodegradable Antimicrobial Pullulan Fibres Extends Shelf Life and Reduces Weight Loss in an Avocado Model

High-throughput coating with biodegradable anti-microbial pullulan fibres extends shelf-life and reduces weight loss in an avocado model

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Abstract

Food waste and food safety motivate the need for improved food packaging solutions. However, current films/coatings addressing these issues are often limited by inefficient release dynamics that require large quantities of active ingredients. Here, we developed antimicrobial pullulan fiber (APFs) based packaging that are biodegradable and capable of wrapping food substrates, increasing their longevity and food safety. APFs were spun using a high-throughput system termed focused rotary jet spinning (FRJS) with water as the only solvent, allowing the incorporation of nature-derived antimicrobial agents. Using avocados as a representative example, we demonstrate that APF-coated samples had their shelf life extended by inhibited proliferation of natural microflora, as well as reduced weight loss compared to uncoated control samples. This work offers a promising technique to produce scalable, low cost and environmentally friendly biodegradable antimicrobial packaging systems.

Introduction

Each year, microbial contamination accounts for more than 600 million cases of foodborne illness and 420,000 deaths worldwide[1], with at least 17.6B dollars in economic cost due to foodborne illness in the U.S. alone[2]. Active antimicrobial food packaging systems, with release kinetics tailored to inhibit microbial proliferation[3,4], have the potential to simultaneously reduce foodborne illnesses and spoilage, while saving money in lost revenue. Although antimicrobial films/coatings have been developed that can help reduce contamination [5,6], they are often limited by poor release kinetics, requiring the use of large quantities of active ingredients[7,8]. For example, antimicrobial zein films released essential oil but only initially reduced bacterial counts[7]. In parallel to these efforts, biodegradable materials and environmentally friendly processing methods are also desired[9]. Collectively, the development of sustainable food packaging materials to enhance food safety and quality has become a major societal priority.

Fibrous materials are an attractive alternative to films for producing antimicrobial food packaging systems, as their high surface-to-volume ratios allow for more efficient release of antimicrobial agents. This means that fibrous polymers have the potential to reduce the quantity of antimicrobial agents for food safety, while minimizing the negative effects on the organoleptic properties of foods. To fabricate micro/nanofibers for food packaging applications, electrospinning is one of the most popular techniques[10]. Electrospun antimicrobial zein-based composite nanofibers in miniscule quantities were reported to reduce *Escherichia coli* and *Listeria innocua* populations after 24 hours of exposure[11,12]. However, due to low production rates[11] and the requirement for high-voltage electrical fields, electrospinning has yet to gain practical applications in food packaging.

The aforementioned challenges motivate the development of a cost-effective and high-throughput fiber-based food packaging system capable of rapidly wrapping food. In this study, we introduce an approach to antimicrobial packaging, directly coating food products using non-toxic, biodegradable antimicrobial fibers. Here, a high-throughput system termed focused rotary jet spinning (FRJS)[13] was used to produce antimicrobial fibers containing naturally-derived antimicrobial agents. To achieve direct packaging onto food, we use water as the only solvent, and pullulan as the fibrous backbone, where pullulan is a naturally occurring extracellular polysaccharide that is Generally Regarded as Safe (GRAS) by the United States Food and Drug Administration[14]. Using this process, we show that antimicrobial packaging can be produced in an

environmentally safe and sustainable manner. To validate this approach, antimicrobial pullulan fibers (APFs) were deployed against common food pathogens such as *Escherichia coli (E. coli),* *Listeria innocua, (L. innocua)* and *Aspergillus fumigatus* (*A. fumigatus),* showing a reduction in bacterial and fungal populations. Additionally, we demonstrate APFs can be directly deposited on avocados to extend their shelf life through inhibiting proliferation of microorganisms and reducing weight loss. Using avocados as a proof of concept, these results indicate that FRJS produced APFs offers a promising approach to produce scalable, low cost and environmentally friendly antimicrobial food packaging systems.

Results

Direct coating of avocados with pullulan fibers

To achieve direct coating of fresh food products, focused rotary jet spinning (FRJS) was used to manufacture pullulan fibers (PFs) with water as the only solvent. Using FRJS, fibers can be conformally deposited onto a target surface, by using centrifugal force to push dissolved polymer solutions through a small orifice in the spinneret to form fibers and utilizing a focused air stream to control fiber deposition (**Fig. 1a and S1**)[13]. Here we used FRJS to synthesize PFs and directly deposit PFs onto the surface of avocados in a layer-by-layer fashion to form a stable coating (Fig. 1b). Avocados were selected as an exemplar fruit in this study, as they are prone to postharvest deterioration, including uneven ripening and decay[15], making them a suitable candidate for testing this direct coating approach to food packaging.

Using FRJS, the entirety of an avocado could be coated in 2-4 minutes with no further processing required, as shown in **Fig. 1c-d** and **Video S1**. First, individual pullulan fibers were obtained by applying a heat gun near the spinneret to quickly evaporate water solvent during fiber formation (**Fig. S2**). Next, as an initial test to determine if these PFs could act as a carrier system for various antimicrobials agent, a green food dye was then incorporated into the polymer solution. This yielded a bright green fiber coating (**Fig. 2d-f**), suggesting a capacity for carrying antimicrobial agents, while also providing visual decoration that may be of consumer interest. This packaging was easily removed as shown in Fig. 1e-f and Video S2, rinsing in water for at least 20 seconds resulted in the complete dissolution of PFs. Additionally, PFs were also completely degraded in soil after 3 days (Fig. S3a). It is worth noting that we also tested the chemically crosslinked pullulan fibers in soil and as expected they show a relatively slower degradation rate (> 14 days) as compared to pure PFs (Fig. S3b)

Morphological and physico-chemical characterization of antimicrobial pullulan fibers

We asked if PFs could act as a carrier system for antimicrobial agents, without disrupting fiber formation or subsequent fiber morphology. To test this, antimicrobial pullulan fibers (APFs) were synthesized, incorporating naturally derived antimicrobial agents[11], including thyme oil (1%, w/v), citric acid (5% , w/v) and nisin (0.005%, w/v). Confirming that these dopants caused minimal morphological changes, PFs and APFs were examined using SEM (**Fig. 2a&c**). Here, we observed similar morphology for both conditions as shown in **Fig. 2b&d**, with fiber diameters of 1.4 ± 0.7 μm and 1.5 ± 0.6 μm for PFs and APFs respectively, suggesting that the incorporation of antimicrobial agents into the PFs had minimal effect on the fiber formation process. Additionally, the specific surface area, average pore diameter, and total pore volume of PFs and APFs determined by Brunauer−Emmett−Teller (BET) surface area analysis (**Table S1**) are similar. This further indicates that the fiber formation process was not disrupted by the addition of antimicrobials.

Given these minimal changes in morphology, we sought to ensure that antimicrobial agents were successfully incorporated into APFs using Fourier-transform infrared spectroscopy (FTIR) (**Fig. 2e**). For PFs, the characteristic peaks between 1500–650 cm^{-1} were observed for pullulan, with accompanying C-O stretches at 1130-1180 cm^{-1} and 1070–1090 cm^{-1}, which were consistent with previous literature reports[16]. For APFs, the presence of each antimicrobial ingredient was observed in the spectra, with characteristic peaks at ~1721 cm^{-1} belonging to the C-O stretch of citric acid and the characteristic peak at 1653 cm^{-1} belonging to amide groups of nisin[11,17]. Thyme oil is less well resolved but exhibited characteristic peaks at 1627, 1360, 1250, and 800 cm^{-1}, due to the aromatic C-C stretching, isopropyl group, C−O stretching and aromatic C−H bending vibrations respectively[18]. This suggested that the resulting peak observed at 1250 cm^{-1} in the spectra of APFs belonged to the vibrational C−O stretch of thyme oil. In summary, the C−O 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} suggested the incorporation of these antimicrobial agents into APFs.

To confirm that these antimicrobial components were well distributed throughout the body of the fiber, we then performed X-ray diffraction (XRD) to check the presence or absence of crystalline domains, which could indicate incomplete dissolution (**Fig. 2f**). Nisin and citric acid displayed their characteristic crystalline peaks, while PFs and APFs displayed broad peaks at 2θ = 10°−25°, which are characteristic of amorphous polymers. This confirmed that crystalline domains

were not present inside the fibers, suggesting the antimicrobial agents were likely to be uniformly dissolved throughout the body of fibers.

Antimicrobial and antifungal efficacy of APFs

To assess the antimicrobial and antifungal efficacy of APFs, fiber substrates were put into tight contact with *E. coli* (~ 5 log colony-forming unit (CFU)/sample), *L. innocua* (~ 5 log CFU/sample) and *A. fumigatus* (~3 log CFU/sample) for 5 minutes, 1 hour and 24 hours periods using a direct contact assay method[11] (**Fig. 3a**). Here, aluminum foil and PFs without antimicrobial agents were used as controls, with fibers coated at a surface density of 2.5 mg/cm^2. The antimicrobial and antifungal efficacy of APFs is summarized in **Fig. 3b-d**, with fold changes in inhibition being normalized by the initial sample concentrations (on aluminum foil after 5 minutes contact time). As shown, APFs achieved a ~2 and ~5 log CFU/mL population reduction of *E. coli* after 5 minutes and 1 hour of contact time, respectively (**Fig. 3b**), while the aluminum foil substrate and PFs controls showed minimal influence on the growth of *E. coli*. For *L. innocua* (**Fig. 3c**), a 3.25 and ~5 log reduction was obtained after 5 minutes and 1 hour of contact time with the APFs, respectively. It was also notable to see that both PFs and aluminum foil controls resulted to ~1 log reduction of *L. innocua* after 24 hours of contact time. In terms of antifungal efficacy, a ~1 log CFU/mL population reduction of *A. fumigatus* was obtained for APFs after 24 hours of contact time (**Fig. 3d**). Similarly, aluminum foil and PFs controls had no impact on the growth of fungal spores, with a less than 1 log population fluctuation after 24 hours of contact time. Taken together, this indicated that the inclusion of antimicrobial agents can lead to significantly reduced levels of contamination by microorganisms, in turn leading to enhanced food safety and potentially reduced food spoilage and extended viable storage times.

Effect of APF coating on the preservation and quality of avocados

To assess whether APFs can reduce food spoilage, we measured the number of rotten avocados, amongst the APF, PF, and uncoated experimental groups after seven days of storage at 22°C (**Fig. 4a-b**). Packaging with fiber surface density of 5.0 mg/cm^2 was used due to lower weight loss and higher antimicrobial activity as compared to fiber coating of 2.5 mg/cm^2 as shown in **Figs. S5-6**. APF coatings were able to reduce the percentage of rotten avocados from 90% to 50% over a seven-day storage period (**Fig. 4c**). The uncoated and PF-coated avocados started to visibly decay by Day 7 and visible rotten aeras were observed on 90% of the avocados (**Figs. 4d,e**). In contrast, only 50% of APF coated avocados showed obvious rotting (**Fig. 4f**). The weight loss and the

natural microflora on the exocarp of avocado were measured as shown in **Fig. 4g,h**. APF coated avocados displayed less weight loss and the least amount of natural microflora after seven days in comparison with the uncoated and PF-coated avocados. We note that natural microflora level of the PF-coated avocados was lower than that of the control on Day 7, which may have resulted from natural microflora colony differences among avocados, as well as the physical barrier caused by fiber coating or the potential removal of microflora from exocarp before recovery.

To determine how APFs density might preserve fruit over time, metrics of avocado quality were quantitatively defined as weight loss, natural microflora, color change, pH, and firmness (**Fig. 5 and Figs. S5-7**). Two groups of avocados coated with different surface densities of APFs (2.5 mg/cm^2 and 5.0 mg/cm^2) were prepared. The weight loss and natural microflora in two cases was also measured. As shown **Fig. S5**, both APF-coated avocados show less weight loss as compared to the control, but the fiber density has minimal effect on weight loss. For total aerobic bacteria (**Fig. S6 a,c**), both APF-coated avocados groups showed a lower amount of total aerobic bacteria as compared with the control starting at Day 4. A relative larger reduction of total aerobic bacteria was observed with higher surface density (5.0 mg/cm^2) as compared to 2.5 mg/cm^2 at Day 11. For yeasts and molds **(Fig. S6 b,d**), higher APFs density resulting lower amount of yeasts and molds is more obvious in the earlier period (Days 4 and 7). In summary, higher density APFs is more efficient to inhibit the population of natural microflora and fungi.

To assess discolorations in the avocados, the exocarp and mesocarp were measured for shifts in green discoloration (**Figs. 5a-c and S7**) based on the CIELAB color space[19], where a^* is used as a measure of color preservation (green value). In the first 4 days of storage, a sharp shift in exocarp a^* was observed, indicating a discoloration of the avocado from green. After the initial shift, minimal discoloration of the exocarp was observed, both in the presence or absence of APF coatings. With respect to mesocarp discoloration, APF-coated avocados displayed lower a^* as compared to uncoated controls, indicating a preservation of the avocado's internal green coloration, which was also consistent across different fiber surface densities (2.5 mg/cm^2 versus 5.0 mg/cm^2).

The firmness and pH change of avocados with and without APF coating were also measured during storage (**Fig. 5d-g)**. The mesocarp displayed a rapid decline in firmness over a four-day storage period, while no significant changes were noted in the exocarp. We observed that APF-coated avocados did not show significant differences in the degree of firmness as compared to the control samples, regardless of fiber surface density (**Fig. 5d,e**). With regards to pH, avocados

with APF coatings show a difference, with the exocarp yielding a lighter acidity, likely because of miniscule amounts of citric acid present on the surface (**Fig. 5f,g**). However, mesocarp pH was maintained at natural levels, with no difference between coating and control samples.

FRJS-produced APFs in active food packaging applications

Active antimicrobial food packaging systems are a promising approach to enhance safety and extend the shelf life of foods[4,20]. To date, the use of micro/nanofibers for food packaging has been limited due to the low experimental throughput and their reliance on non-GRAS materials and chemical processes. This study demonstrates a scalable fiber spinning system for sustainable food packaging technology that allows for the one step synthesis and direct coating of antimicrobial fibers onto fresh foods without further treatment. In FRJS, centrifugal force was used to form fibers while compressed air was used to control fiber deposition, which is distinct from other fiber production methods such as solution blow spinning where compressed air was used to both fiber formation and deposition processes. In addition, the scalability of the FRJS -based approach is evident by the higher fiber production rate (0.2 g/min) as compared to electrospinning (0.01 g/min)[21]. Therefore, FRJS can potentially be used directly in the field to deposit antimicrobial packaging onto fresh foods. The FRJS can also be used at other various critical control points across the farm to the fork to package food substrates to enhance their food safety and quality.

With respect to environmental effect, pullulan is an attractive biopolymer for biodegradable food packaging applications[16,22-24]. Our study shows that pullulan fibers are dissolved in liquid environment and biodegraded in soil environment. It is worth noting that the chemically crosslinked PFs show a relatively slower degradation rate as compared to pure PFs (Fig. S3). The commercial use of these materials for food packaging have been hampered by high cost (ranging between 25 and 30 USD/kg)[25]. Here, micro/nanofiber-based coating, with their high surface-to-volume ratio, has allowed us to achieve effective antimicrobial activities using only limited surface treatments, with surface densities of 5.0 mg/cm^2. This suggests that fresh fruits, such as avocado, can be coated with antimicrobial fibers inexpensively (for only a few cents per fruit), even without accounting for further cost reductions when scaling up production. The concern for environmental effect also extends to the selection of antimicrobial agents, polymers and organic solvents used in the synthesis process. In this case water was used as a solvent to synthesize fibers from a non-toxic, GRAS, biodegradable polymer.

In regard to food safety, fresh fruits and vegetables, such as avocados[26,27] and apples[28], can be contaminated with pathogens during post-harvest processing. This suggests a need for improved food packaging, which can be rapidly deployed so as to protect against a wide range of potential contaminants. Here, APFs were shown to have broad antimicrobial and antifungal functionality by incorporating multiple naturally derived agents. The successful incorporation of these naturally derived antimicrobials in APFs is evident in the FTIR spectra as shown in **Fig 2e**. Additionally, we confirmed the high surface to volume ratio of APFs using BET surface data analysis (**Table S1**). These data suggested that reduced quantities (5.0 mg/cm^2) of antimicrobial pullulan fibers should be sufficient to inactivate pathogens as compared to film-based packaging systems. More specifically, APFs showed a strong antimicrobial efficacy against Gram-negative and Gram-positive bacteria (**Fig. 3b-c**). Compared with conventional techniques such as dip coating to preserve avocados[29], APF coatings show a similar result where both antimicrobial packaging methods can prolong their shelf life under ambient conditions for up to 7 days. Additionally, APFs also offer a strong antifungal efficiency, with the population of *A. fumigatus* being significantly reduced (>90%) after a 24-hour contact time. Fungal spoilage is often considered a more challenging problem to address, with fewer studies having reported efficient antifungal food packaging systems, especially in fiber form[30,31]. In addition, APFs may be recommended to reduce spoilage during transportation, where simple rinsing can be used to remove the packaging prior to consumption.

The effects of APFs on shelf-life were also explored and APFs can significantly reduce the percentage of rotten avocados as compared to the control and PFs coated avocados (**Fig. 4**). This suggests the ability of APFs to reduce decay can primarily be attributed to the addition of antimicrobial agents in the PFs. For avocados, postharvest disease is common and is believed to be the result of bacteria contaminating, resulting in further degradation[32]. As shown in **Figs. 4f** and **S5**, the natural microflora of avocados coated with APFs were continuously lower than those of the control samples, suggesting that bacteria were unable to initiate this infiltration process, in turn, significantly reducing the natural decay of avocados. Additionally, reductions in the natural microflora on the exocarp may also reduce the chance for surface-to-surface cross contamination, further potentiating gains in food safety and quality. It is worth noting that in order to assess pathogen populations on avocado exocarp, we inoculated avocados with precise numbers of pathogens and thereby accurately estimated fold-reduction with or without APF coatings.

To test the practical applications of APFs, APF coated avocados were stored in the refrigerator. Though the majority of APF coating are fibrous structure, we observed some fibrous coatings in the contact areas started to dissolve in Day 3 and gradually changed to a transparent layer due to water accumulation either from metabolic activity or humidity in the refrigerator. An additional layer of crosslinked pullulan fiber coating on the avocados significantly can improve the durability of the fiber coating but need a longer rinse time to remove (Fig. S8).

Conclusions

Recently, fiber-based active food packaging systems have gained increased attention due to their potential to enhance food safety and quality. Here, a scalable, sustainable, and cost-effective approach, termed focused rotary jet spinning (FRJS), has been deployed to synthesize environmentally friendly and rinsible pullulan fibers containing naturally derived antimicrobial agents. Such fibers can be directly coated on food substrates (i.e., avocado). The APFs show high antimicrobial efficacy against food pathogens such as *E. coli* and *L. innocua*. By using avocados as a case study, APF coatings were shown to be able to reduce the percent of rotten avocados with lower natural microflora populations, less weight loss and reduced discoloration of the mesocarp during storage. The water-based synthesis process, along with the edible and washable nature of pullulan, and high-throughput fiber technology combine to present a promising method to package perishable food products to enhance food safety and quality while reducing food waste.

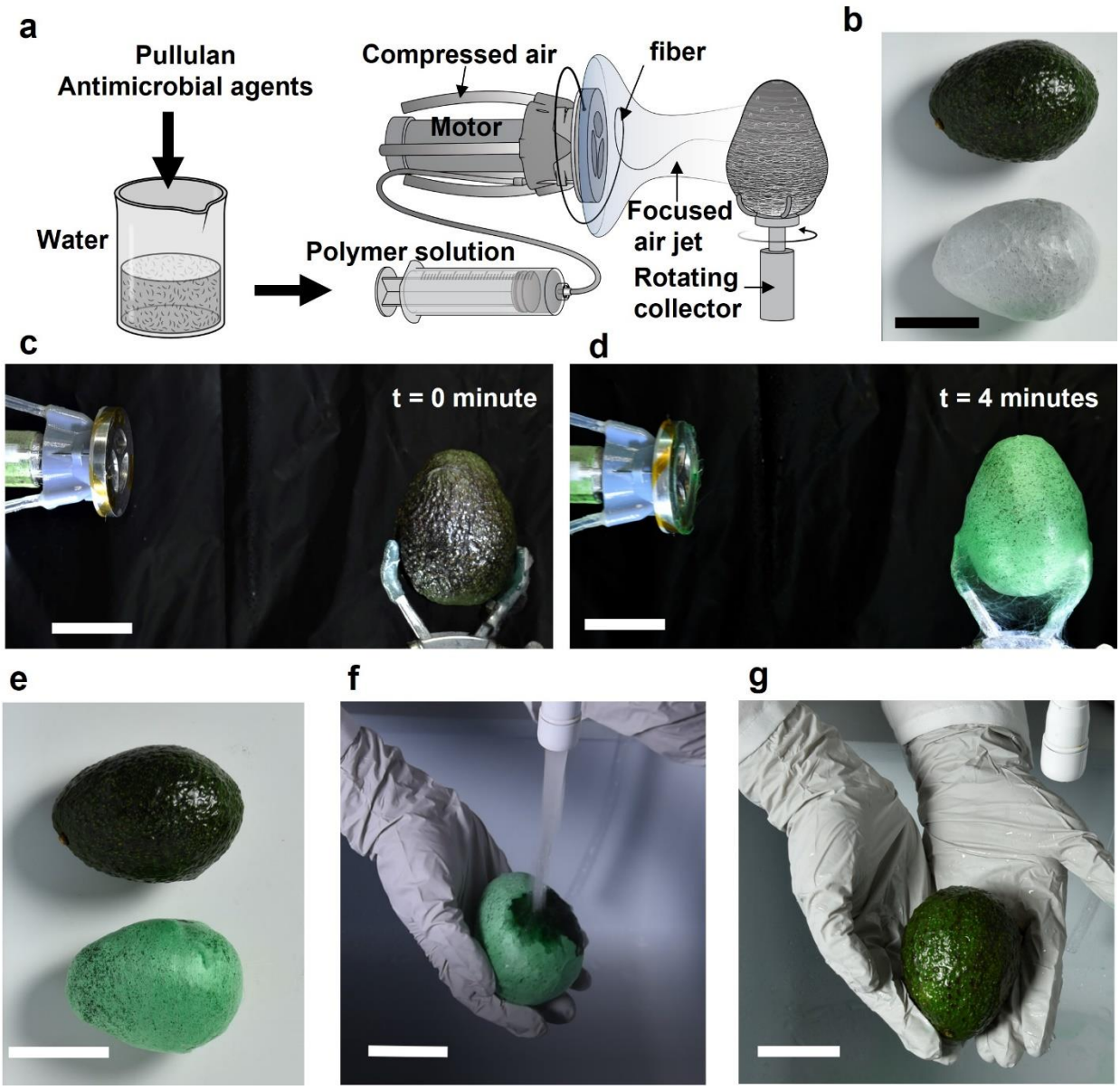


Figure 1. Direct coating of avocados with rinsible pullulan fibers (PFs). a, Schematic of fiber spinning system termed as focused rotary jet spinning (FRJS). **b**, Avocado with and without PF**. c-d,** Setup of FRJS before and after PF coating. **e,** PFs color can be changed by adding food dye. **f,** Removal of the coating by water rinse. **g,** Avocado after the PF coating removal. Scale bars are 5 cm.

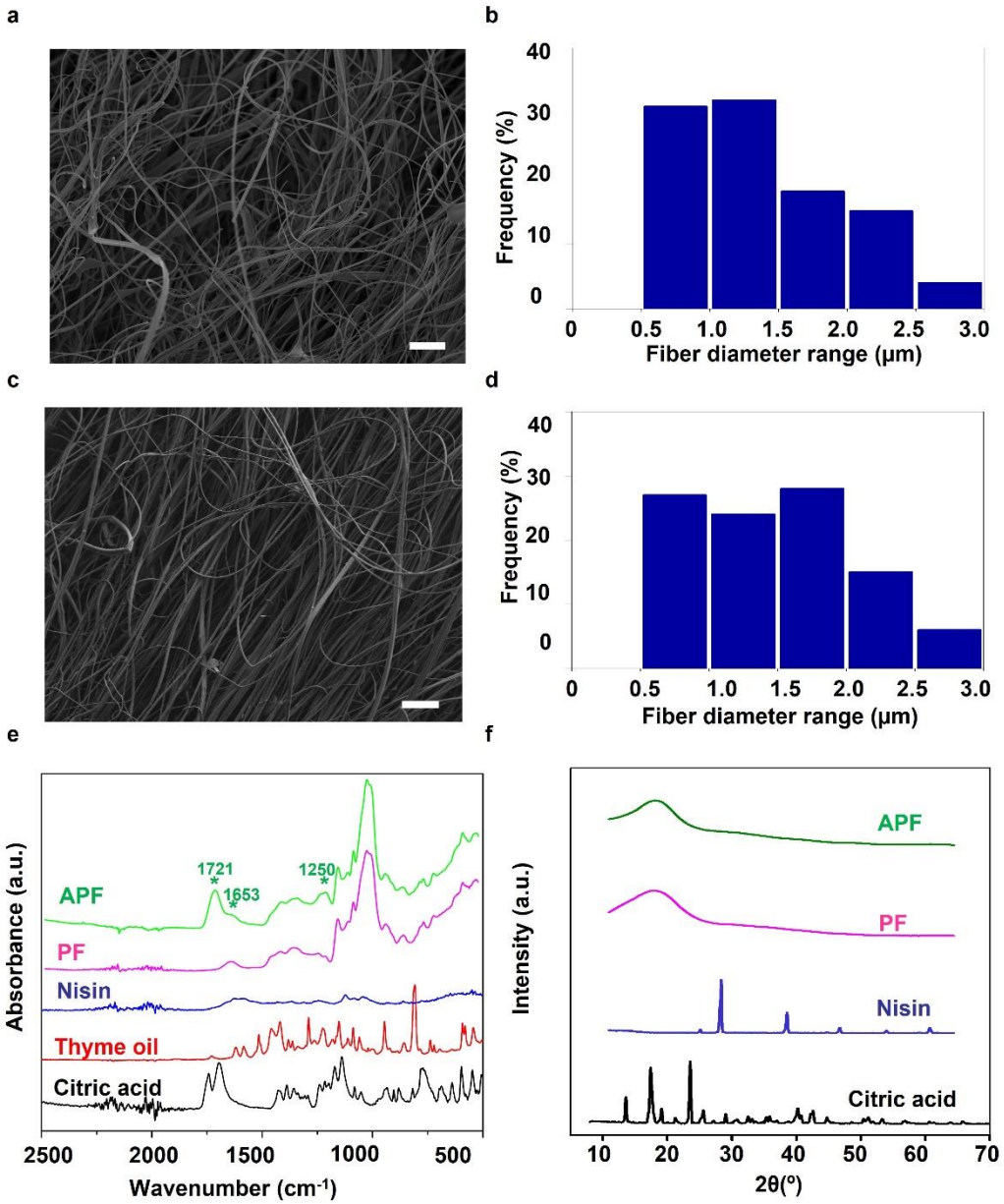


Figure 2. Morphology and chemical composition of pullulan fiber containing antimicrobial agents. **a-b**, Representative SEM micrographs of pullulan fibers, both in the absence (**a,b**, PFs) and presence of naturally derived antimicrobial agents (**c,d**, APFs), with histograms (**b**, PFs and **d**, APFs) depicting the corresponding distribution of fiber diameters (n=100). Scale bars are 20 μm. **e**, FTIR spectrograph of PFs, and APFs with corresponding reference spectra for thyme oil, citric acid, and nisin, indicating their inclusion in APFs. **f**, X-ray diffraction of PFs, and APFs, with reference peaks for crystalline nisin and citric acid, indicating minimal crystalline domains in the fibrous materials.

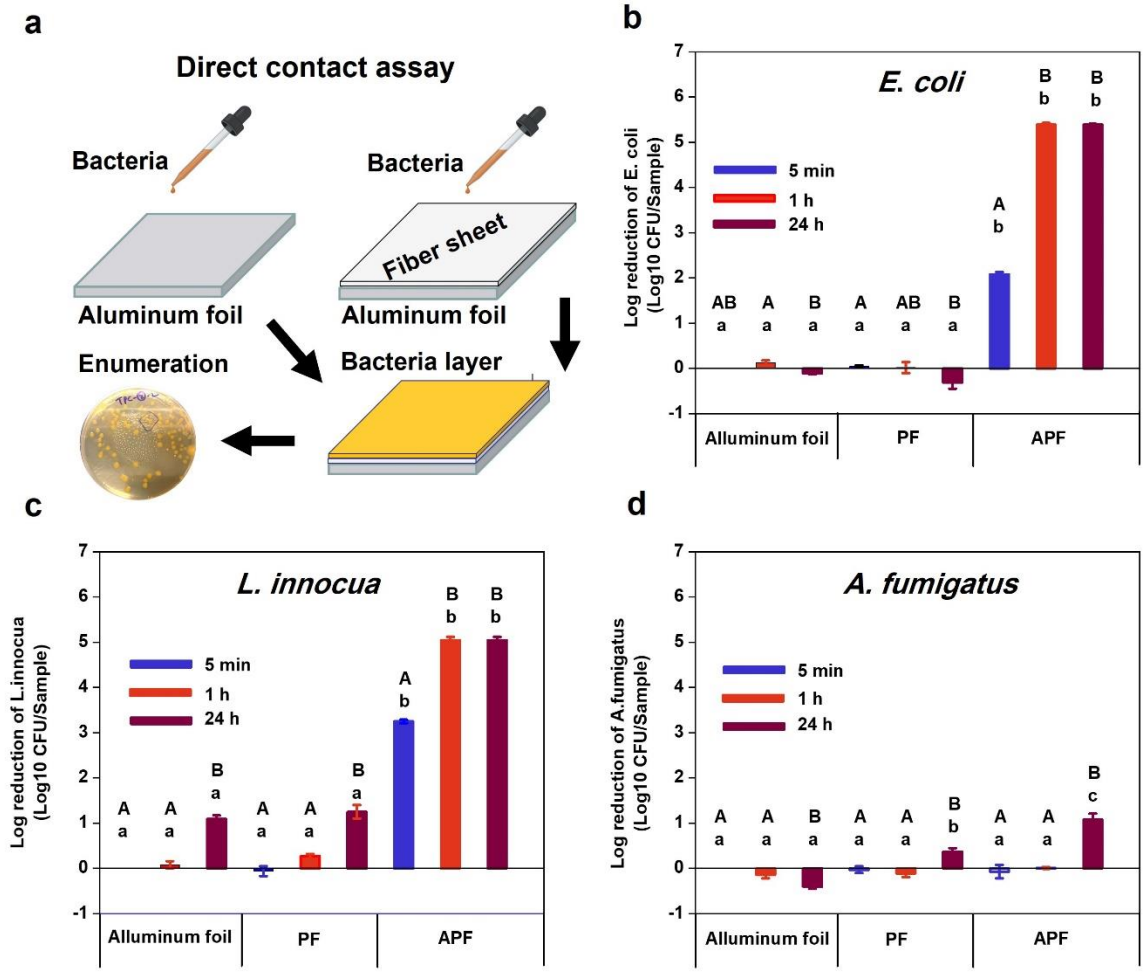
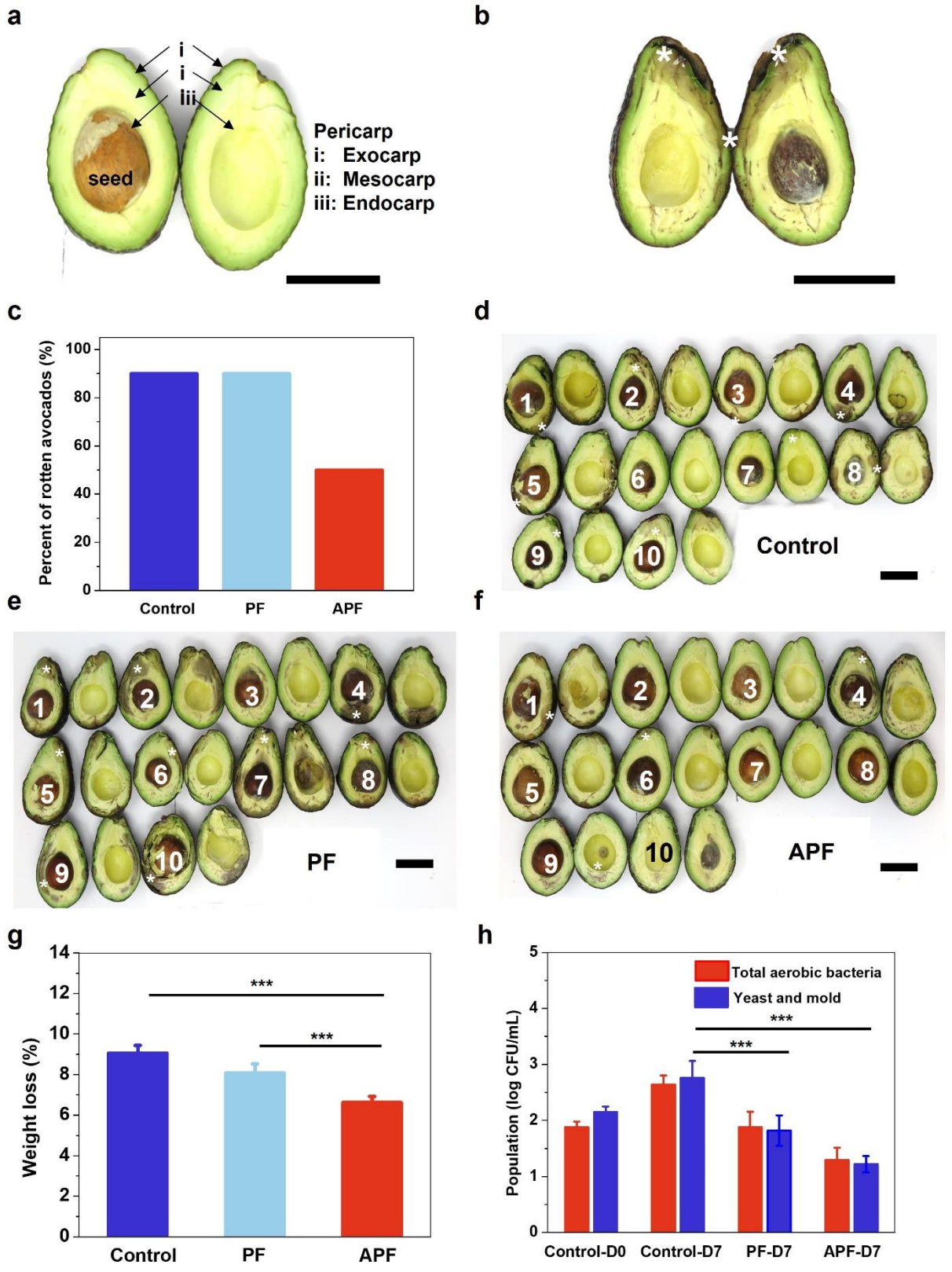


Figure 3. Direct contact assay of antimicrobial and antifungal activity of APFs. a, Schematic of the direct contact assay. **b,** *E. coli* **c,** *L. innocua*, and **d**. *A. fumigatus* were directly contacted with aluminum foil, pullulan fibers (PFs), and antimicrobial pullulan fibers (APFs) (2.50 mg/cm^2) at a 2 × 2 cm dimension and incubated at 37 °C for 5 minutes, 1 hour, and 24 hours. (n=3, error bars are standard error of mean). Data in the same material group labeled with different uppercase letter are significantly different (p-value < 0.05). Data in the same contact time group labeled with different lowercase letter are significantly different (p-value < 0.05).


a
i
i
iii
seed
Pericarp
i: Exocarp
ii: Mesocarp
iii: Endocarp
b
c
Percent of rotten avocados (%)
100
80
60
40
20
0
Control
PF
APF
d
1
2
3
4
5
6
7
8
9
10
Control
e
PF
f
APF
g
Weight loss (%)
14
12
10
8
6
4
2
0
***
***
Control
PF
APF
h
Population (log CFU/mL)
5
4
3
2
1
0
Total aerobic bacteria
Yeast and mold
***
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Control-D0
Control-D7
PF-D7
APF-D7

313

Figure 4. Effect of APF coating (5.0 mg/cm^2) on avocado rotting. a, Diagram of avocado. **b**, Representative rotten mesocarp areas of avocados indicated by asterisk. **c.** Percentage of rotten avocados after storage at 22℃ for 7 days, under various fiber coating conditions. Ten avocados were examined for each of the three conditions (control with no coating, PFs = pure pullulan fibers, APFs = antimicrobial pullulan fibers), each of which is shown in panels d-f at Day 7 (D7). **d**, Images of cut avocados from the uncoated control group. **e**, Pullulan fiber (PF)-coated avocados without antimicrobial agents. **f**, APF coated avocados. Mesocarp rot areas are indicated by white asterisk. Scale bars in panels d and f are 5 cm. **g,** Weight loss of avocados (n=10 avocados)**. h.** Natural microflora (total aerobic bacteria, yeast and mold) of avocados. (n=3 avocados, Day 7: D7). All error bars are standard error of mean. Data labeled with *** symbol are significantly different (p-value < 0.05).

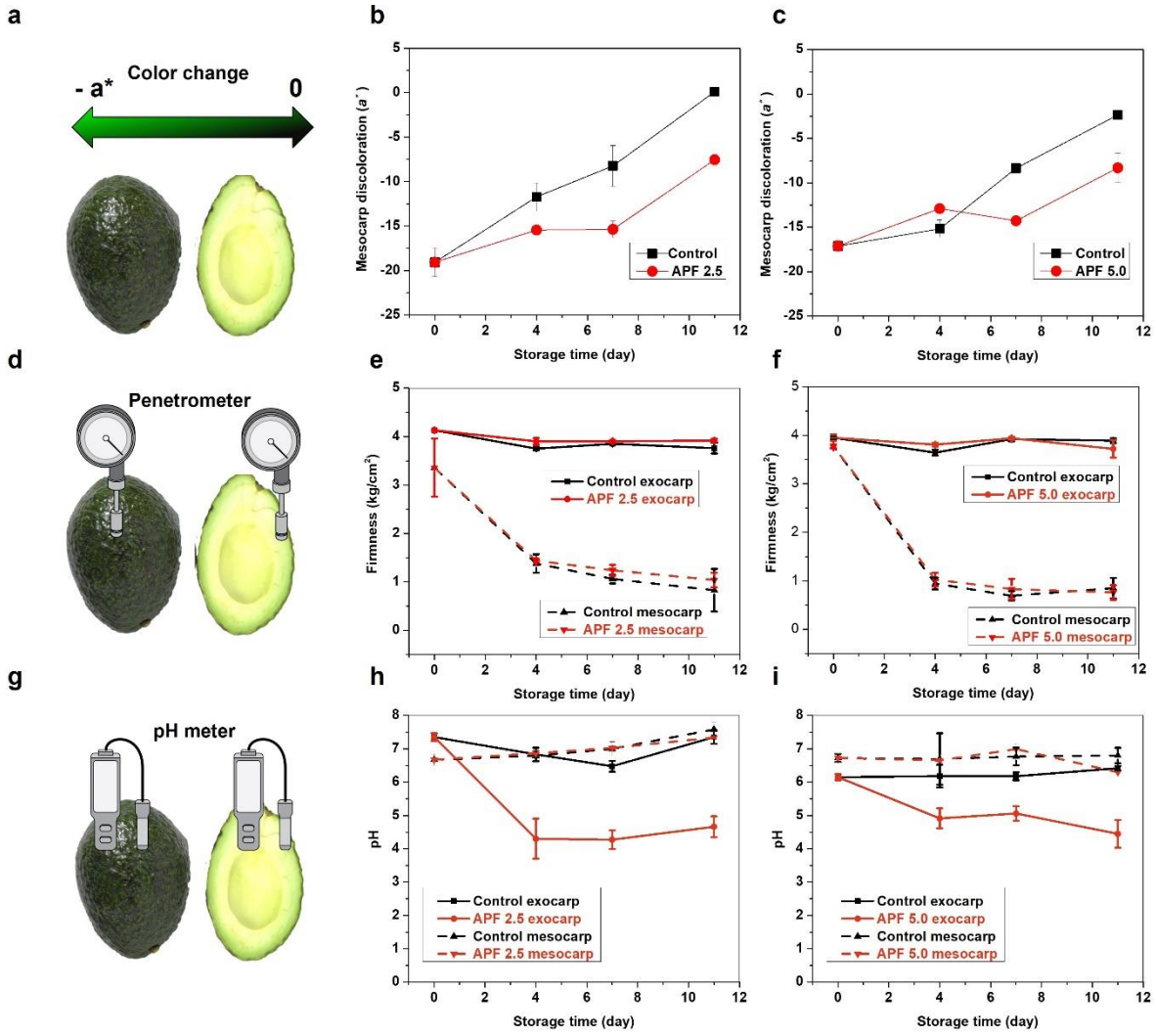

Figure 5. The effect of APF coating on avocado's color, firmness and pH during storage at 22℃. a-c Exocarp discoloration as denoted by *a**. **d-f**, Firmness of mesocarp and exocarp, **g-i**, pH of mesocarp and exocarp. control: avocados without fiber coating. APF 2.5: APF coating of 2.5 mg /cm^2 and APF 5.0: APF coating of 5.0 mg/cm^2. (n=3 and three areas were measured for each avocado. Error bars are standard error of mean)

Materials and methods

Materials

Pullulan (Lot No. 0E2732, Hayashibara Co. Ltd.) was purchased from DKSH North American Inc (NJ, USA). Sodium trimetaphosphate (STMP, Lot No. SLCD1552), sodium hydroxide (NaOH, pellets) and active agents including citric acid (251275-100G, Lot No. MKCH1340), nisin (N5764-5G, Lot No. 019M4063V) and thyme oil (W3066509-1KG-K, Lot No. MKCJ4106) were purchased from Sigma-Aldrich (MO, USA). Food Color & Egg Dye were purchased from McCormick Inc. (MD, USA).

Direct coating of food substrates with focused rotary jet spinning system

Focused rotary jet spinning (FRJS) was used for fiber production as shown in Fig S1. The FRJS system itself consists of a high-speed motor spindle, a custom-made spinneret to extrude polymer solutions, a syringe pump (Lot No. 703007, Harvard Apparatus) to transfer precursor solutions to the spinneret, a 3D printed air blower with 3 air nozzles to focus the ejected fiber stream, and a stand to hold the motor and spinneret in place. A motor with ~200 revolutions per minute speed was also used to rotate the food substrate, avocados, during fiber coating to ensure uniform deposition across the food surface. Additionally, A heat gun (Steine HL1502S) was used to rapidly evaporate water from the pullulan fibers prior their impact with food substrate, thereby ensuring an efficient pullulan fiber production. The custom-made spinneret was composed of stainless steel and contains three orifices drilled into the side walls (400 μm in diameter), to allow for the extrusion of polymer solutions while under rotation[13].

An aqueous solution of pullulan and a cocktail of antimicrobial agents was prepared for the synthesis of antimicrobial pullulan fibers (APFs). Various pullulan concentrations ranging from 10 to 30% (weight/volume) were used as part of a parametric analysis to synthesize the fibers. The antimicrobial cocktail used here was composed of three agents: 1% (w/v) thyme oil, 5% (w/v) citric acid, and 0.2% nisin mixture (w/v, 0.005% pure nisin), with the formulation of the antimicrobial cocktail[11]. The solutions were then loaded in a plastic syringe (60 mL BD Luer-Lock tip) and the syringe pump was used to transfer solutions to the spinneret. Pullulan fibers were deposited on the collection mandrel or directly onto avocados, and the mass per surface area was adjusted by varying the collection time.

After a systematic investigation of each parameter, the final parameters used to spin continuous pullulan fibers were as follows: 20% (w/v) pullulan solution in water, 1 mL/min solution flow rate, 10,000 revolutions per minute rotating speed, 0.2 MPa compress air flow, and a 15-20 cm distance from spinneret to the collector. To make chemically crosslinked pullulan fibers, STMP was added into the pullulan solution at concentrations of 0.5 wt% of pullulan polymer and then mixed for at least 2 hours. Before spinning, 10 wt% NaOH aqueous solution was added at a volume ratio of 1:10 (NaOH/pullulan solution) to activate crosslinking[33]. The spinning parameters are same as pure pullulan solution. It takes about 2 minutes to produce fibers with 2.5 mg/cm^2 on an avocado and about 4 minutes to produce 5 mg/cm^2 on an avocado.

Morphology and physico-chemical characterization of fibers

Fiber samples were mounted on a stub using double-sided carbon tape and then coated by Pt/Pd (Denton Vacuum, Moorestown, NJ) to minimize charging. Fiber morphology was observed by scanning electron microscope (SEM, Zeiss FESEM Ultra Plus). The average diameter of fibers was measured from SEM images using ImageJ Software (n =100).

The specific surface area (m^2/g) of fibers in addition to average pore radius (nm), and total pore volume (cc/g) were investigated by Brunauer−Emmett−Teller (BET, Quantachrome NOVA touch LX4) surface area analyzer. Prior to the analysis, fibers were degassed in cells at 323.15 K for 12 h. Then, low temperature (77.35 K) nitrogen adsorption isotherms were obtained at relative pressures from 0.005 to 1.00.

The Fourier transform infrared (FTIR) spectra of the fibers and antimicrobial agents were measured using attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR; Lumos, Bruker, MA, USA). For each sample, the recorded spectrum was collected from a total of 32 scans.

The inclusion of nisin and citric acid in the pullulan fibers were investigated by X-ray diffraction (XRD, Bruker D2 Phaser) in the 2θ range of 8°−70°. Due to the liquid state at room temperature, XRD was not performed for thyme oil.

Biodegradability test

To evaluate the biodegradability of the pullulan fibers in soil, a soil degradation test was performed in organic soil (100% organic soil, Organic Plant Magic) containing 10 volume/volume %

water. Four fibrous sheets (~ 0.5 × 2 cm) were cut and stored on the surface of organic soil in a Petri dish at 22 °C. The dimensions of the pullulan fiber sheets were recorded daily to assess their biodegradability

Antimicrobial efficacy of APFs against food related microorganisms

Strain information

Escherichia coli ATCC 25922 (*E. coli*), *Listeria innocua* ATCC 33090 (*L. innocua*), and *Aspergillus Fumigatus* ATCC 96918 (*A. fumigatus*) were used in these studies as representatives for Gram-negative and Gram-positive microorganisms, and fungus, respectively.

E. coli and *L. innocua* were resuscitated and streak plated from the stock and maintained on tryptic soy agar (TSA; Hardy Diagnostic, Santa Maria, CA) at 4 °C. A single colony from TSA was transferred into 10 mL of tryptic soy broth (TSB; Hardy Diagnostic, Santa Maria, CA). After incubation at 37 °C for 24 h, bacterial broth was centrifuged at 1462 g for 20 min (Allegra 6R, Beckman Coulter, Indianapolis, IN). After discarding the supernatant, 2 mL Phosphate-Buffered Saline (PBS 1×) buffer was used to resuspend the pellet. The cell density was adjusted to ~10^{8} colony-forming unit (CFU/mL) by PBS. Freeze-dried *A. fumigatus* was rehydrated in sterile deionized water and further transferred onto Malt Extract Agar (MEA) and incubated at 30 °C for 3 days. To produce spores, single colonies were further transported onto MEA and incubated at 30 °C for 7 days until the conidia became dark green. Mature spores were harvested from the lawn and then diluted by deionized water. The final concentration of spores is about ~10^{7} measured by a manual hemocytometer (Diagnocine, Hackensack, NJ).

Direct contact assay

To test the antimicrobial efficacy of fibers, 100 μL of bacterial culture was diluted in a 10 mL agar slurry (0.85% NaCl, 0.3% agar). Inoculated agar slurry (300 μL) was transported onto a 2 x 2 cm^2 fiber sheet placed onto a similar sized piece of aluminum foil as a substrate (Figure 3a). After a five minutes contact time, the agar slurry formed a gel layer with a thickness of less than 1 mm. The treated fiber sheets were then transported into an incubator at 37 °C for 1 hour and 24 hours, respectively. To maintain gel hydration, an open water reservoir was placed in incubator the to keep the relative humidity at ~80%. Since aluminum foil was used as a substrate to deposit the fibers, the bacteria survivors on aluminum foil and pullulan fibers (PFs) without antimicrobial agents were used as controls. The population reduction shown in Figure 3 was the absolute

population difference with the starting concentration of bacteria or fungus on aluminum foil with 5 minutes contact time.

Enumeration

After the desired exposure time, each test fiber sheet together with aluminum foil substrate was transferred into a sterile Whirl-Pak bag with 2.7 mL of PBS to reach a 10-fold dilution. Then, the sample bag was homogenized with a stomach for 2 min at a normal speed. The elute was then serially diluted to a proper level. For *E. coli* and *L. innocua*, 100 μL of proper dilution was pour plated onto TSA and incubated at 37 °C for 24 hours. For *A. fumigatus*, 100 μL of proper dilution was pour plated onto MEA and incubated at 30 °C for 48 hours.

Statistical analysis of antimicrobial efficacy testing

Three independent replicates were conducted for each condition. The number counts of *E. coli*, *L. innocua* and *A. fumigatus* were converted into log CFU/sample. Statistical analysis to illustrate difference within the same materials (upper case letter) and within the same contact time (lower case letter) was performed by one-way ANOVA within the confidence interval of 95% ($P < 0.05$) (IBM SPSS Statistics for Windows, version 19.0, IBM Corp., Armonk, NY).

Shelf-life study of APFs coated avocados

Sample preparation

Hass avocados were purchased from local stores on the same day of experiment. Avocado samples were selected with similar color, size, shape, firmness, and no obvious bruises or fungal infections by visual observation. Avocados were transported to the laboratory in paperboard boxes with support to avoid mechanical damage. All avocados were grouped and labeled properly before further usage.

Avocado coating and storage

Pullulan fibers with and without antimicrobial agents were directly deposited on the avocados. During this process, avocados were held and rotated using a clamp and motor system, enabling a uniform fiber coating across the entire avocado surface where surface density (mg/cm^2) is controlled by coating time. The surface density of each fiber sheet was adjusted to 2.5 or 5.0 mg/cm^2 by measuring the weight and area of fiber sheets.

After coating, all avocados were stored in a board box at room temperature (22°C) with relative humidity approximately 30%. The experimental design of the avocado shelf-life study is illustrated in **Fig. S4**. To get the rotting rate in percentage, 10 avocados from control, pullulan fibers without antimicrobial agents (PFs, 5.0 mg/cm^2), and antimicrobial pullulan fibers (APFs, 5.0 mg/cm^2) were used and withdrawn on Day 0 (the same day after coating) and Day 7. The natural microflora and weight loss of each group was analyzed. For the remaining test parameters (weight loos, color change, firmness and pH), 3 avocados from control, APF (2.5 mg/cm^2 and 5.0 mg/cm^2) coating groups were used. Sample analysis was conducted on Day 0, Day 4, Day 7 and Day 11. On each analysis date, three avocados were randomly selected from each group and the PF or APF coatings was removed gently by hand to avoid bruising avocados. The data from three independent samples were used.

To test the practical applications of PFs, PFs coated avocados and chemically crosslinked PF coated avocados are stored in a refrigerator (~ 4°C and 50 ± 5 % humidity). The fiber coated avocados were digitally photographed by camera every day to assess the durability of fiber coatings.

Natural microflora analysis

For natural microflora analysis, each avocado was put into a 500 mL stomacher® bag, mixed with 100 mL of maximum recovery diluent (MRD) and hand massaged gently for 2 minutes. The solution was then serially diluted and plated on selective media plates. The following categories of microorganisms were analyzed: total aerobic bacteria, yeast and mold. For total aerobic bacteria, diluted samples were enumerated on plate count agar (PCA) and incubated at 35 °C for 2 days. For yeast and mold, diluted samples were enumerated on PDA (acidified with 10% tartaric acid to pH 3.5) and incubated at room temperature (22°C) for 5 days. To reach the detection limit 10 CFU/mL, 1 mL of solution from the stomacher bag was separated into three plates and the total number of colonies from the three plates were combined and reported.

To assess the effect of surface density on the natural microflora of avocados, fiber coatings with different surface densities (2.5 and 5.0 mg/cm^2) are completed by two separate studies. To ensure APF-coated, PF-coated and uncoated controls samples are under same experimental conditions, uncoated controls samples are used for each set of experiments.

Quality analysis

For quality analysis, weight, color (exocarp and mesocarp), pH (exocarp and mesocarp), and firmness (exocarp and mesocarp) of avocados were measured as a function of storage time. Before analysis, the pullulan fibers on the surface of coated avocados were manually rubbed away by hand until no observable fiber remained.

The percent of rotting avocados was calculated by rotted avocados over the total number of avocados tested (n=10), which is expressed by equation 1:

$$Percent\ of\ rotting\ avocados\ \ (\%) = \frac{n_{rotted}}{n} \times 100 \quad (1)$$

Where n_{rotted} is the number of avocados that is defined as rotted, n is the total number of avocados tested. Avocado was regarded as rotted when there was an any observed of decay area on the cut regions, regardless the decay size.

For weight measurements, avocados were weighed using an Ohaus SCOUT® balance scale. The weight of all avocados from each group at different days was measured. The weight loss on each measured day is expressed by equation 2:

$$Weight\ loss\ (\%) = \frac{Weight_{Day0} - Weight_{Dayx}}{Weight_{Day0}} \times 100 \quad (2)$$

Avocado color analysis was achieved by photographing each avocado using a digital camera (Canon PowerShot G1X; 50 mm lens; aperture of F/4.0; exposure time of 1/125 second; ISO of 400) in a shooting light tent with controlled lighting (two LED lights; balanced daylight at 5600 K). Three representative marked areas of each avocado picture were segmented, and the average RGB values were calculated by ImageJ (NIH, Bethesda, MD). Then, the RGB values were transferred into a^* values (greenness) by using CLELAB color space to define the green-red axis. After cutting the avocado half, the mesocarp image of each avocado was captured and analyzed by the same manner.

The exocarp pH of avocados was measured with a pH meter and a flat-head pH probe (Sper Scientific, Scottsdale, AZ). For exocarp pH, 100 μL of neutral deionized water (pH=7.3) was added onto the marked circle area of each avocado. The pH probe was pressed onto the liquid droplet and the pH value was recorded. After cutting the avocado into half, the mesocarp pH was also measured in the same manner. Three representative locations of each avocado were selected for both exocarp and mesocarp pH measurement.

Avocado firmness was measured with a fruit Sclerometer (Beslands). During the measurement, the fruit Sclerometer was perpendicular to the measurement surface and evenly pressed into the avocado. When the test head reached the scale line (10 mm), the measurement was recoded as the firmness of the avocado. Avocado firmness is expressed by kg/cm^2. Exocarp firmness was measured by placing the fruit Sclerometer on the avocado surface. After cutting the avocado in half, mesocarp firmness was measured on the cutting side in a similar manner. Three representative locations were measured for each avocado for exocarp or mesocarp, separately.

For statistical comparisons for weight loss and nature microflora, a one-way ANOVA was performed to determine statistically significant differences between the groups. Statistical significance was assumed with a p-value<0.05 for all tests. We then used an F-test to determine differences in variances. For groups that possessed equal variances, we performed a t-test with two samples assuming equal variance. Groups with significantly different results are indicated with a ******* symbol in the plots. All statistical analyses were performed using Microsoft Excel Version 2111 with the Analysis ToolPak.

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Author contributions

K.K.P. and P.D. supervised the research. K.K.P., P.D. H.C. J.X. and L.A.M. designed the study. H.C., J.X. Z.A. and M.M.P. conducted the experiments and analyzed the data. L.A.M, T.X. and J. F. Z. provide the support to perform experiments and data analysis. All authors discussed the results and contributed to the writing of the final manuscript.

Competing interests

Harvard University filed for intellectual property relevant to this manuscript, listing K.K.P., P.D. H.C. and L.A.M. as inventors.

Data availability statements

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Additional information

Materials and Methods

Figs. S1 to S8

Table S1

Movies S1-S2

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Supplementary Information

High-throughput coating with biodegradable anti-microbial pullulan fibres extends shelf-life and reduces weight loss in an avocado model

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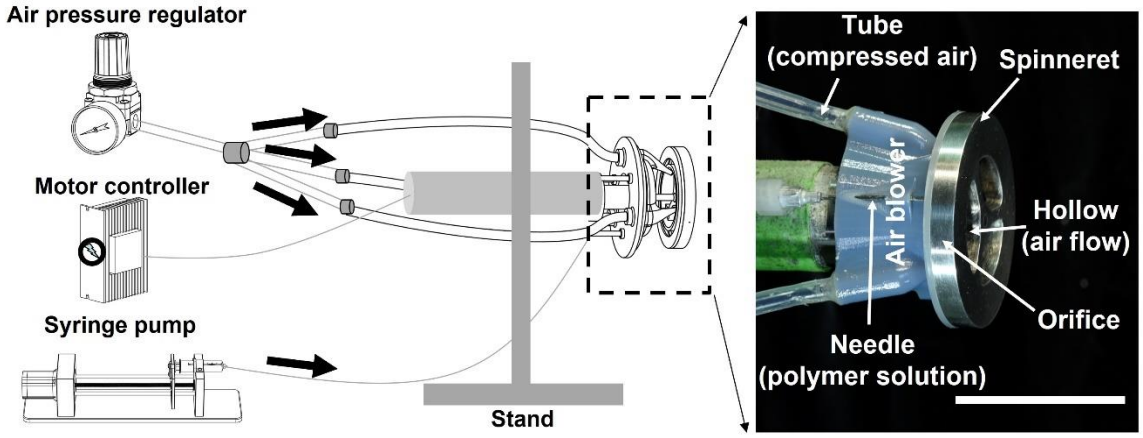

Fig. S1 Focused rotary jet spinning system components. An air pressure regulator is used to control the rate of air flow through a branching network of tubing that converges within a custom manufactured air blower mounted on a central motor shaft located immediately upstream from a spinneret, which is attached to the motor spindle. A motor controller is used to control the motor and spindle rotation rate, while a syringe pump is used to control the rate at which polymer solutions are fed through tubing and a terminal needle to the spinneret. Polymer jets are ejected from orifices in the side walls of the rotating spinneret, forming fibers by solvent evaporation, which are then focused and directed downstream of the spinneret assembly by focused air. (Scale bar is 5cm)

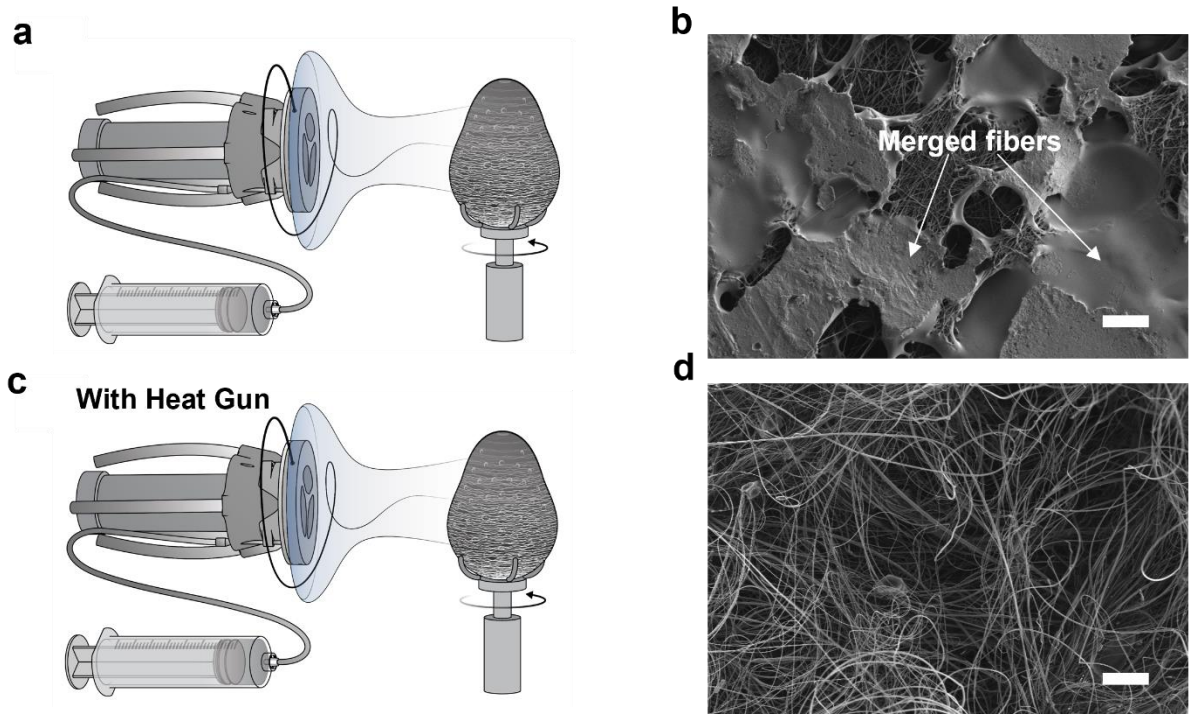

Fig. S2 Morphology of pullulan fibers spun from focused rotary jet spinning (FRJS). a-b, FJRS without a heat gun. **c-d**, FRJS with a heat gun. b, Merged pullulan fibers are observed in SEM. **d,** Individual pullulan fibers are obtained by using a heat gun to quickly remove the water. (Scale bars are 100 μm)

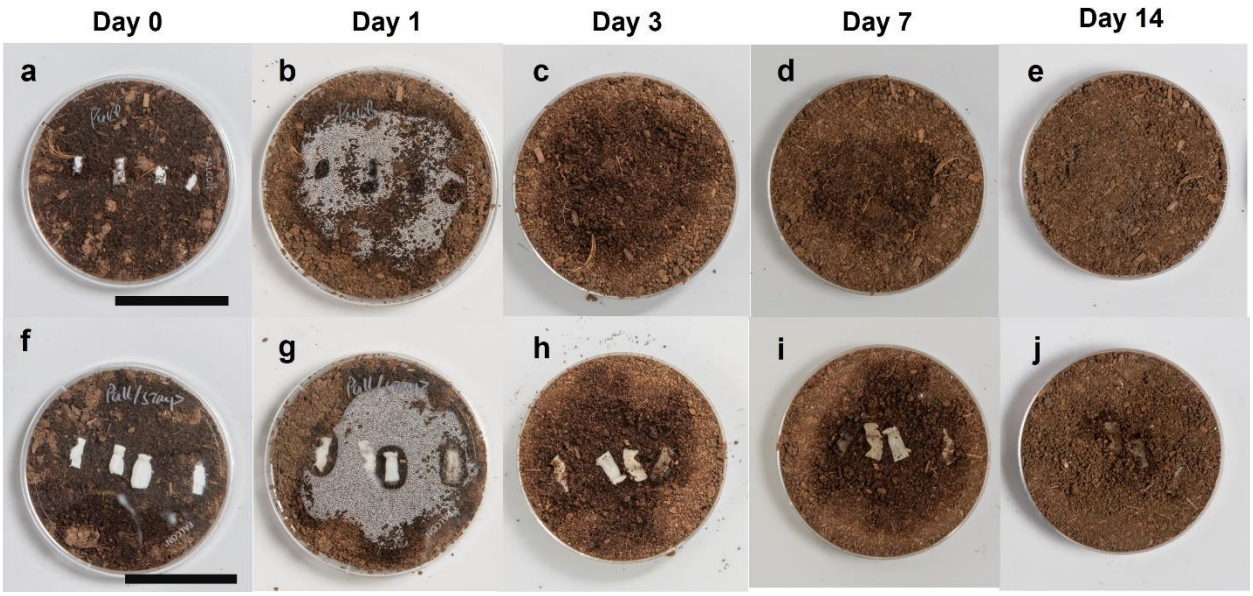

Fig. S3 Biodegradability test of pullulan fibers (a-e) and crosslinked pullulan fibers (f-j) in soil. The dimensions of the pullulan fiber sheets were digitally photographed daily to assess their biodegradability. (Scale bars are 5 cm)

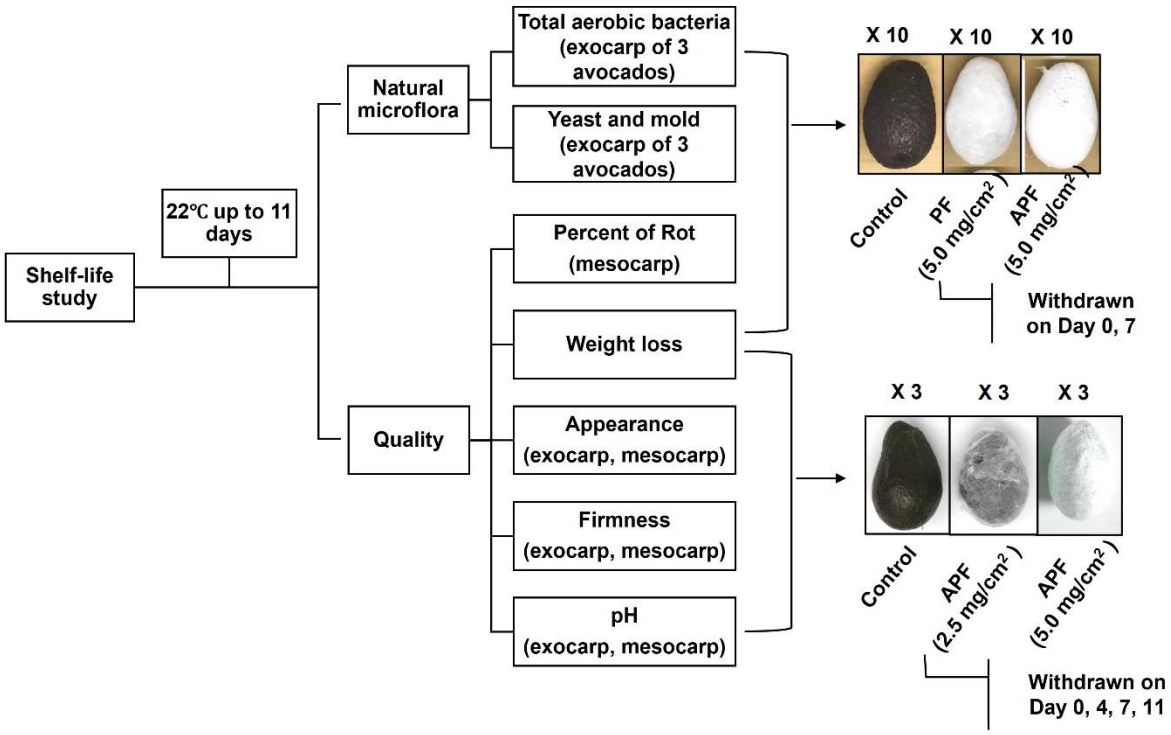

Fig. S4 Effect of APF coating with different surface density (2.5, 5.0 mg/cm^2) on the shelf-life of avocados. Each parameter is tested by using three or 10 avocados from each group.

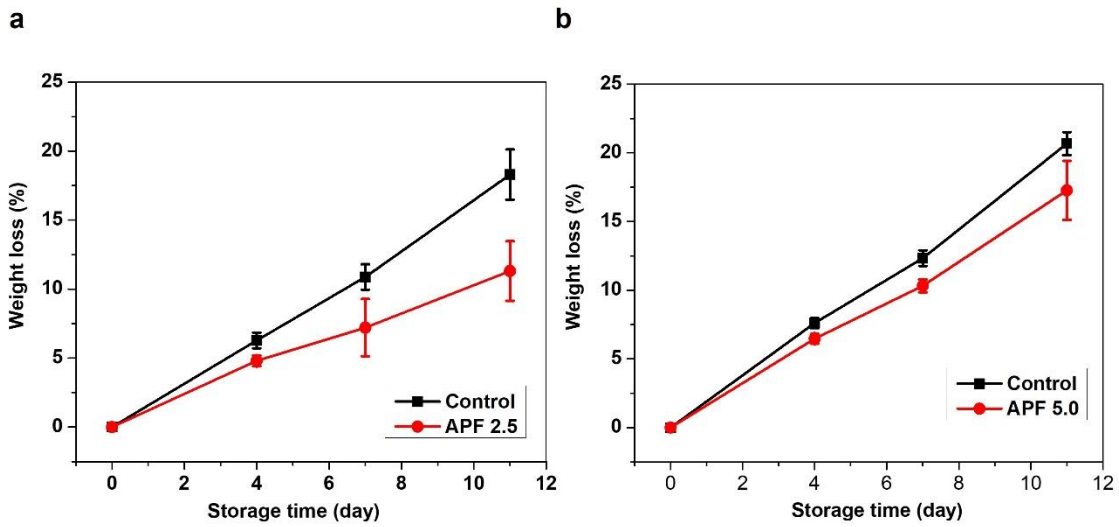

Fig. S5 The effect of APF surface density (2.5, 5.0 mg/cm^2) on the weight loss of avocados a, APF 2.5: APF coating of 2.5 mg/cm^2 and **b**, APF 5.0: APF coating of 5.0 mg/cm^2. Avocados without any fiber coating are used as control. (n=3, error bars are standard error of mean)

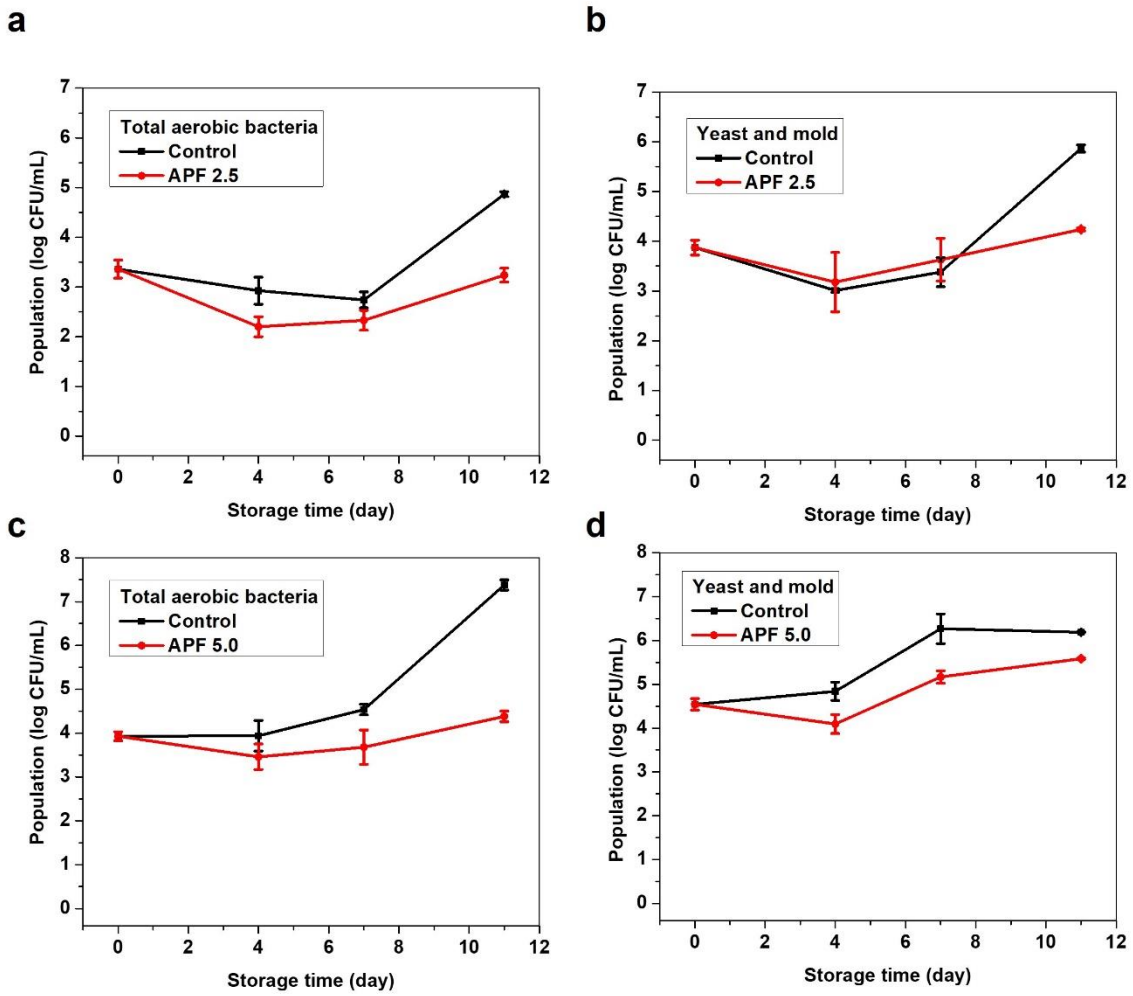

Fig. S6 The effect of antimicrobial pullulan fiber (APF) coating on avocados' natural microflora during storage at 22°C. a, c, Total aerobic bacteria count, **b, d,** Yeast and mold count. APF 2.5: APF coating of 2.5 mg/cm^2 and APF 5.0: APF coating of 5.0 mg/cm^2. (n=3, error bars are standard error of mean).

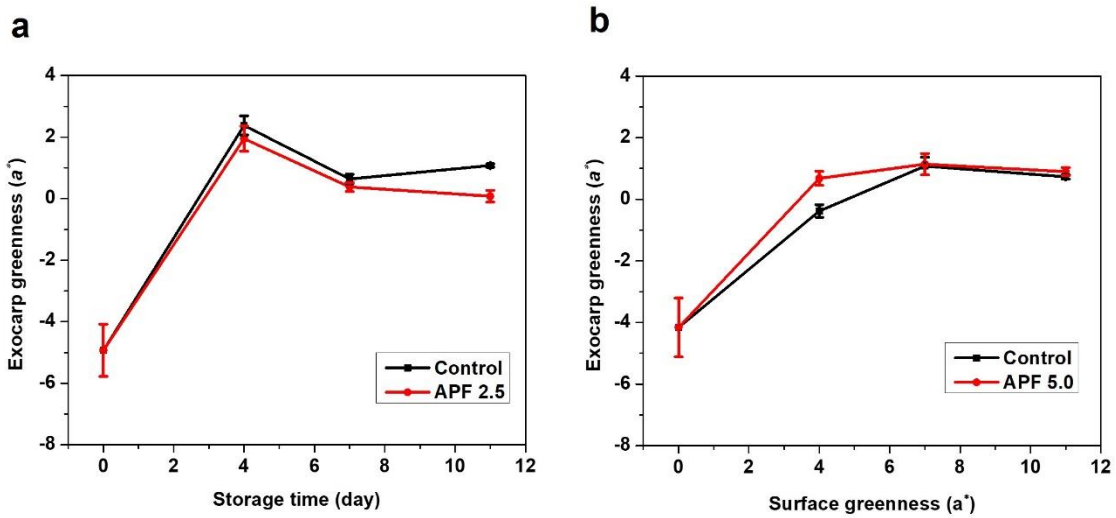

Fig. S7 The effect of antimicrobial pullulan fiber (APF) coating on the exocarp discoloration of avocados. a, APF 2.5: APF coating of 2.5 mg/cm^2 and **b**, APF 5.0: APF coating of 5.0 mg/cm^2. Avocados without any fiber coating are used as control. (n=3, error bars are standard error of mean).

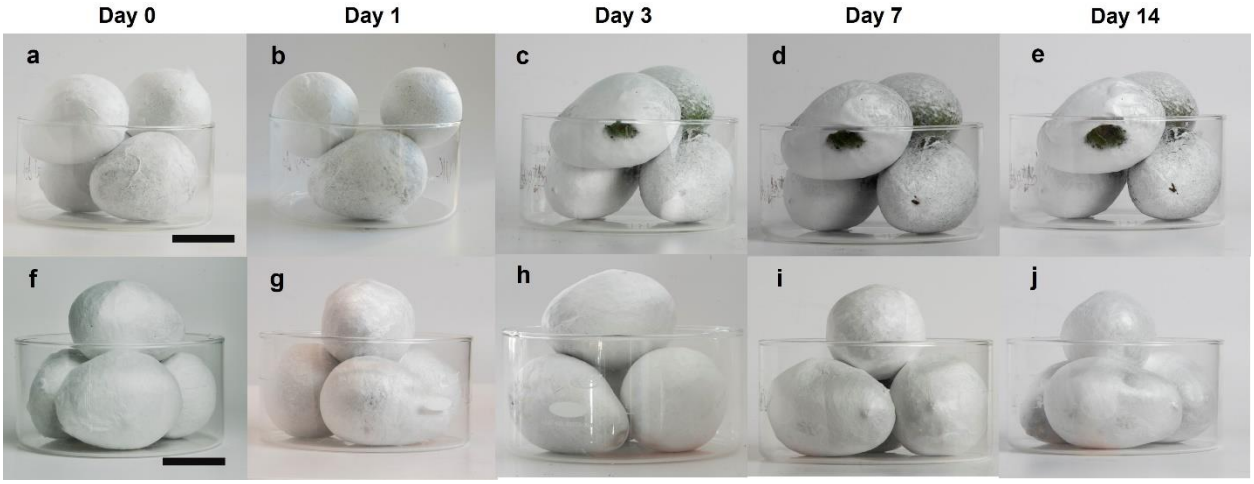

Fig. S8 Morphology change of pullulan fiber coating (a-e) and chemically crosslinked pullulan fibers coating (f-j) on avocados stacked in a refrigerator. The morphology change of the pullulan fiber coatings was digitally photographed daily. (Scale bars are 5 cm)

87 Table S1. Specific surface area, average pore diameter, and total pore volume of pullulan fiber (PF)
88 and antimicrobial pullulan fiber (APF).
89

Fibers	Multipoint BET surface area (m^2/g)	Average pore diameter (nm)	Total pore volume (cc/g)
PF	10.38	1.01	5.23×10^{-3}
APF	9.53	1.86	8.85×10^{-3}

90

Video S1: Direct coating avocados with pullulan-based fibers.

Video S2: Pullulan fiber coating on avocado was removed by rinsing in water.