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Biocidal Activity of Fast Pyrolysis Biochar against *E. coli* O157:H7 in Soil Varies Based on Production Temperature or Age of Biochar

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ABSTRACT

Fresh produce-growing soils, which become contaminated with foodborne pathogens, are sometimes abandoned/removed from production. The application of biochar has been proposed for bioremediating such pathogen-contaminated soils. The objectives of the present study were to evaluate three fast-pyrolysis-generated biochars (FPBC) (pyrolyzed in-house at 450°C, 500°C and 600°C, in a newly-designed pyrolysis reactor) and ten United Kingdom Biochar Research Center (UKBRC) standard slow-pyrolysis biochars to determine their effects on the viability of four surrogate strains of *Escherichia coli* O157:H7 in soil. Additionally, a previously biocidal fast-pyrolysis biochar was aged two years and tested against *E. coli* to determine changes in antibacterial efficacy over time. While neither the UKBRC slow-pyrolysis biochars or the 450° and 500°C FPBC from the new reactor proved antimicrobial, the 600°C biochar was biocidal ($P < 0.05$) with populations significantly reduced at 3% and 3.5% concentrations (reductions of 5.34 and 5.84 log CFU/g, respectively) compared with concentrations of 0.0-2.0%. The aged 500°C fast-pyrolysis biochar from the older reactor, previously shown to be antimicrobial, demonstrated a loss of efficacy after aging for two years. These results demonstrate that the biocidal nature of FPBC varies based on production temperature and/or age of biochar.

HIGHLIGHTS

- Fast-pyrolysis (FP) (600°C) biochar was biocidal in soil.
- Significant reductions occurred at the 3% level (wt.:wt.) over 7 w.
- Biochars, fast-pyrolyzed at 450 and 500°C, were not biocidal.
- Antimicrobial (FP) biochar that was aged for two years was not biocidal.

Fresh fruits and vegetables have been known to be contaminated with a variety of foodborne pathogens, which has resulted in numerous foodborne illness outbreaks *(83, 88)*. Soils used for growing fresh produce may become contaminated with foodborne pathogens, hence they are sometimes abandoned/removed from production to mitigate fruit and vegetable contamination *(8)*. The application of biochar has been proposed as a means of bioremediating such pathogen-contaminated soils *(43, 44)*. Crop soils sometimes become contaminated with foodborne pathogens such as *Salmonella,* enterohemorrhagic *Eschericia coli,* and *Listeria monocytogenes,* (either from resident bacteria in the soil [in the case of *L. monocytogenes*] or from domestic or wild animal feces), contaminating fresh produce or other seed, grain or legume crops destined for human consumption *(7, 49, 60, 74, 86, 105)*. *E. coli* O157:H7 has been known to survive for up to 217 days in soil amended with compost *(56)*. Ways of mitigating the presence of these pathogens after field soil has become contaminated include chemical fumigation, soil solarization with transparent tarps, soil steaming, antimicrobial drip irrigation (chemigation), soil flaming and biofumigation with crop residues (e.g., cabbage, mustard, radish, canola, etc.), which release antimicrobial isothiocyanates *(43)*. Each of these methods has drawbacks (e.g., use of hazardous and environmentally toxic chemicals, excessive overhead and operational costs, disposal of plastic tarps, and keeping fields out of production with biofumigation), thus biochar has been viewed as an alternative to inactivate bacterial foodborne pathogens in soil. Gurtler et al. *(44)* tested 12 types of slow and fast-pyrolysis-generated biochar to inactivate *E. coli* O157:H7 in soil and found that select biochars were effective at reducing *E. coli* populations in comparison to control (no-biochar) soils, with increasing biocidal activity correlating to lower temperatures and shorter residence times.

Biochar has also been considered a soil ameliorant *(4)* and may be added to soil to accomplish various purposes including sequestering carbon, immobilizing heavy metal and chemical pollutants, improving water retention or influencing saturated hydraulic conductivity *(91)*, increasing disease

resistance, remediating acidic soils, increasing the abundance and diversity of N_2-fixing bacteria, improving soil fertility, changing the relative abundance of select bacteria in soils, and reducing N_2O emissions from soil (a greenhouse gas 300 times more potent than CO_2) *(18, 50)*. Biochar has also been used as a means of remediating contaminated water by soil/biochar filtration, biochar/sand columns, and filters containing biochar and sometimes other components *(2, 6, 13, 54, 68, 84, 85, 86).*

The addition of biochar to animal feed may also affect the microbial community in the gut and subsequent litter or soil *(51)*. Prasai et al. *(93)* added 4% (wt:wt) green woody waste biochar pyrolyzed at 500°C to layer hen poultry feed over a course for 25 weeks. The authors found, by selecting DNA samples from each group and 16S rRNA gene amplicon generation and sequencing, that populations of *Campylobacter jejuni* were reduced in cloacal samples. Further, no other phylum besides Proteobacteria was affected. A number of studies have demonstrated that the addition of biochars to soil have shifted the microbial communities by decreasing the ratio of Gram-negative to Gram-positive bacteria *(82)*. Gram-negative bacteria (including Proteobacteria) are known to be sensitive to certain carbon sources *(16, 70)*. Despite reducing specific classes of bacteria, other studies have revealed that the addition of many types of biochar to soil increases the bacterial activity, diversity and microbial community richness of the soil *(27, 38, 41, 65, 71)*. This may be a result of particular types of biochar serving as a source of microbially-available carbon *(47, 119)*. For example, studies have reported that the addition of biochar can increase the Shannon index of soil microbial communities *(19, 20, 42)*. Chen et al. *(19)* also reported from next-generation pyrosequencing that the addition of 350-550°C pyrolyzed wheat straw to three different rice paddy sites increased overall bacterial gene copy numbers by 25-60%, which is in agreement with other studies in diverse soils *(3, 66, 72)*. Otsuka et al. *(90)* also reported that the biochar added to soil increased the bacterial diversity in soil by 25%. In fact, studies have reported that certain

biochars are amenable to serving as bacterial inoculum carriers *(46, 111)*. It has been reported that certain volatile biochar surface compounds may increase microbial growth by serving as a useable, labile microbial carbon source *(106)*. Increased surface area, produced by biochar added to soil, might also enhance microbial activity *(47, 114)*; however, it has been suggested that pore size should be ≥2 μm in order to provide an adequate environment for microorganisms. Biomass feedstocks with greater lignin content are known to create biochar with larger pores, in contrast to biofeedstocks with greater cellulose content *(55)*. Nevertheless, changes in soil microbiota have been reported as being transient with bacterial populations returning to their original levels within 1-3 years *(101)*.

Biochar is the biologically active, porous, carbon-rich co-product of the incomplete combustion of biomass by pyrolysis under anoxic or low oxygen conditions, described as pyrogenic organic matter *(77, 103)*. Biochar is constitutionally stable and is known to persist in soils for centuries or millennia *(107)*. Biochar is produced by heating any type of biomass feedstock under anoxic or low oxygen conditions, generally at temperatures of between 300 and 750°C, and varies in characteristics according to the type of biofeedstock utilized, biochar interaction with pyrolysis vapors, the kiln residence time, the highest treatment temperature, residence time, etc. *(5, 14, 15, 22, 23, 31, 61, 94, 99)*. Because of the great variation in biochar matrices, various responses of crop output to biochar application have been observed *(9, 39, 48, 57, 102)*. While some studies have reported neutral or negative effects *(36, 62, 97, 108),* others have observed that application of biochar has resulted in enhanced crop yield and productivity *(21, 25, 26, 109)*. Further work is needed to optimize biochar production for increases in crop productivity.

Because there is such a variation in the physical and chemical compositions of biochar, it would be useful to have a standard set of biochars that could be used by various institutions and research teams to cross-reference results. With this in mind, the UK Biochar Research Centre

(UKBRC) at the University Edinburgh, Scotland, created a set of ten standard biochars *(80)*. The UKBRC Standard Biochar is a set of ten biochar produced from five feedstocks (rice husk (RH), oilseed rape straw pellets (OSR), wheat straw pellets (WSP), miscanthus straw pellets (MSP), and softwood pellets (SWP)) using the UKBRC pilot-scale pyrolysis unit at residence temperatures of 550°C and 700°C *(79)*. As of the beginning of 2020, these standard biochars have been used by over 120 research groups around the world and results are continuing to come in and can be cross-referenced based on consistency of materials utilized, although no studies have evaluated the effects of these UKBRC standardized biochars against the viability of foodborne pathogens or their surrogates in cultivable soils. A previous study *(44)* examined the antimicrobial nature of a series of slow and fast pyrolysis biochars (FPBC) against *E. coli* O157:H7 and found them to differ based on biofeedstock materials, as well as pyrolysis time and temperature

Further work, therefore, was warranted to determine whether FPBC retained its antimicrobial efficacy over time, as well as whether decreasing or increasing the temperature during pyrolysis affected survival of the pathogen. The objectives of the present study were to use three fast-pyrolysis-generated biochars (pyrolyzed in-house at 450, 500 and 600°C) and the ten UKBRC standard slow-pyrolysis biochars (SPBC) to determine their effects on the viability of four surrogate strains of *E. coli* O157:H7 in cultivable soil. Additionally, a previously biocidal fast-pyrolysis biochar was aged two years and tested for antimicrobial properties against *E. coli* O157:H7 to determine if there was a difference in antibacterial efficacy over time.

MATERIAL AND METHODS

Fast pyrolysis switchgrass biochar preparation and surface area measurement. Three types of switchgrass biochar were produced by fast pyrolysis at temperatures of 450, 500 and 600°C. Fast pyrolysis biochars were generated in a newly-designed bench-scale fast pyrolysis

reactor through a nitrogen-flushed fluidized silica bed at 450, 500 or 600°C, as described and diagrammed previously *(98)*. This reactor differed from the reactor in our previously published manuscript *(44),* as previously described *(10, 11, 12)*. For the newly-designed reactor, briefly, residence time of switchgrass biomass feedstock (ground to ≤ 2 mm in diameter) was less than 1 second and biochar was separated from accompanying syngas via cyclone and charcoal catch. Two sets of different electric heaters were used to decouple the reactor bed temperature and the freeboard zone temperature. Biochar was separated post freeboard using a Lapple type cyclone (diameter of cyclone = 25 mm) followed by a hot vapor filter with 20 μm mesh.

Surface area measurements on fast pyrolysis bio-chars were obtained from nitrogen adsorption isotherms at 77 K using an Autosorb iQ automated gas sorption analyzer (Quantachrome Corp., Boynton Beach FL). Specific surface areas were determined from adsorption isotherms using the Brunauer, Emmett, and Teller (BET) equation.

Slow pyrolysis biochar preparation. Ten slow pyrolysis biochars were generated in the UKBRC Stage III Pyrolysis Unit (rotary kiln pyrolyser) (only the SWP and WSP feedstocks were processed in Stage I of the pyrolysis unit) *(113)*. Details regarding the processing parameters and characteristics of the slow pyrolysis biochar are listed in Table 1, and production process details can be found in Masek et al., 2018 *(79).*

Two-year-aged 500°C fast-pyrolysis switchgrass biochar preparation. Two-year aged, 500°C fast-pyrolysis switchgrass biochar (with previous antimicrobial activity) *(44)* was pyrolyzed as previously described *(10, 11, 12).*

Bacterial strain preparation. Four strains of non-toxigenic *E. coli* O157:H7 (ATCC #43888, ATCC #700728, Doyle-6980-2, Doyle-6982-2 from Michael Doyle, University of Georgia, Center for Food Safety), were inoculated into 45 ml tryptic soy broth + 100 μg/ml nalidixic acid (TSBN) for 24 h at 37°C. The four 45 ml bacterial suspensions were centrifuged for 10 min at 3,250 × *g,*

decanted and each pellet resuspended with 1.0 ml of 0.1% peptone, to concentrate the population of cells in the inocula. All four concentrated suspensions were then composited into a single four-ml non-toxigenic *E. coli* O157:H7 inoculum with a mean population of 10.26 log CFU/ml.

Soil preparation. Soil was prepared as described earlier *(28, 45)* by combining a 0.75:1:0:1:0:0.75 [V/V] mixture of autoclave-sterilized soil, sand, vermiculite, and turface (calcined clay, Applied Industrial Materials, Corp., Deerfield, IL) (SSVT) for a final carbon content of 0.6% and a final moisture content of 17.25%, prior to the addition of biochar. Moisture was determined with a MA160 moisture analyzer (Sartorius, Göttingen, Germany). SSVT is a material composite representing soils amenable for frequent watering and excellent drainage. Ninety-six grams of SSVT was added to hermetically-sealed 250 ml wide-mouth Nalgene HDPE translucent centrifuge bottles (ThermoFisher Scientific, Fair Lawn, NJ) along with one of six different concentrations (wt:wt) of fast pyrolysis-generated biochars (viz., 1.0, 1.5, 2.0, 2.5, 3.0 or 3.5%). Biochar was added directly to SSVT in bottles and samples were thoroughly mixed on IBI Scientific low-profile bottle rollers (Dubuque, IA) for 1 h. One container with 96 g of inoculated SSVT, only, served as a no-biochar control, for each trial.

Inoculation of soil. The SSVT + biochar treatments in addition to the no biochar controls, were each inoculated with 0.27-0.30 ml (depending on the concentration of biochar added to the sample) of the composited *E. coli* O157:H7 suspension for a final mean population of 7.59 log CFU added per g of SSVT in all samples. All biochar + SSVT treatments, as well as no biochar controls, were adjusted with sterile deionized water to a final moisture content of 16%. Samples were then mixed for 4 hours on bottle rollers and stored in a microprocessor-controlled incubator for 7 weeks at 21±1°C for the fast pyrolysis samples, and 25±1°C for two weeks for the slow pyrolysis samples and the two-year-aged 500°C fast-pyrolysis samples. The conditions differed for the fast and slow pyrolysis samples based on the following factors. For the FPBC, it was hypothesized that the

biocidal nature would be greater, thus we opted for a lower ambient temperature and lower biochar concentrations (1-3.5%), which would purportedly slow down the inactivation curve. For the SPBC, we hypothesized that the biocidal activity would be minimal, thus, to screen the biochar for efficacy, we inoculated at a high concentration of biochar (10%) and a higher temperature in an attempt to increase its antimicrobial action.

Soil sampling for presence of *E. coli* O157:H7. Soil was tested for populations of *E. coli* O157:H7 by cultural sampling methods, as modified from previous studies *(1, 13, 44).* At each weekly sampling time, bottles with SSVT + biochar were mixed on bottle rollers for 1 h, 1 g was removed from the container, added to 9 ml TSBN and vortexed for 3 min. One gram was determined to be an adequate sampling size, based on our previous study *(44).* Sediment was separated from the TSBN suspension in a Nasco Whirl-Pak® filter bag and serial dilutions in 0.1% peptone water were plated onto tryptic soy agar + 0.1% sodium pyruvate + 100 μg/ml nalidixic acid + 175 mg/L 6-Chloro-3-indolyl-β-D-galactopyranoside (salmon-gal) + 100 mg/L IPTG to induce a red/salmon color change in *E. coli* O157:H7 colonies (collectively referred to as TSAPNR). TSAPNR plates were incubated at 37°C overnight prior to counting.

Statistical analysis of the 450, 500 and 600°C fast-pyrolysis samples. The analyses were conducted separately based on the three pyrolysis temperatures. Log CFU/g was analyzed as a three-factor linear repeated measures model using PROC MIXED (SAS Institute) *(104)* for Concentration, Week, a repeated factor, and Rep, a random block. The assumptions of the models were checked. For 450°C, the autoregressive (1) variance-covariance structure was the best fit while for 500°C it was the heterogeneous autoregressive (1) variance-covariance structure that fit best. In the case of 600°C there was large variance heterogeneity. As the Week variance was small compared to the residual variance, log CFU/g was analyzed as a three-factor mixed linear model with no repeated factor, which allowed for the variance grouping technique to be used to correct for

the variance heterogeneity. Population means were separated by use of the Sidak correction method with $P < 0.05$.

Statistical analysis of the 550 and 700°C slow-pyrolysis and two-year aged fast-pyrolysis biochar samples. The variable log CFU/g was modeled as a three-factor mixed linear repeated measures model using PROC MIXED for Concentration and Week, the repeated factor, and Rep a random block. The assumptions of the model were checked. The autoregressive (1) variance-covariance structure was the best fit.

RESULTS AND DISCUSSION

Surrogate *E. coli* O157:H7 strains inoculated into soil. The use of non-toxigenic strains of *E. coli* O157:H7 as surrogates in the place of the virulent bacteria has been reported elsewhere *(53)*. It is to be noted that the sterilized soil we used does not contain the normal background microbiota that would be present in natural soil. This microbiome should have some effect on the survival of *E. coli* O157:H7 inoculated into the soil, but, for now, our purpose was to maximize the bactericidal characteristics of the biochar, saving future experiments for studying the interaction of the pathogen and biochar with innate microbiota.

Fast-pyrolysis samples: 450 and 500°C. The pH values for fast pyrolysis biochar in SSVT were 7.7 for control soils, but addition of fast-pyrolysis biochar increased it up to 7.99 with any of the biochar treatment temperatures. Surface area measured for the 450, 500 and 600°C fast-pyrolysis biochar samples was 10.1 m^2/g, 2.4 m^2/g and 13.0 m^2/g, respectively. Results for the 450 and 500°C fast-pyrolysis biochar samples are included in supplemental material. For samples treated with 450°C FPBC, populations decreased from an initial inoculum level of 7.42 log CFU/g to only 6.40 log CFU/ml in the control and 6.52 log CFU/g for the 3.5% concentration samples. For samples treated with 500°C FPBC, populations decreased from an initial inoculum level of 7.74 log CFU/g to only as low as 6.48 log CFU/g after 7 weeks. When analyzed statistically, for the 450°C

samples, the autoregressive (1) variance-covariance structure was the best fit. Whereas, for 500°C samples, the heterogeneous autoregressive (1) as well as the unstructured were best, so we chose to report only the unstructured model. Within each temperature there was no significant difference based on concentration × week and no difference based on concentration for the 500°C samples. However, there was a difference for the main effects of concentration, over all weeks tested, for the 450°C samples, in which the 3% concentration populations (6.74 log CFU/g) were greater than populations at 1% concentration (6.48 log). While there was a statistically significant difference between the two, this might have little value in terms of bioremediation of pathogen-contaminated soils, as the difference between the two populations was only 0.26 log CFU/g. There was a significant difference in populations over time, for samples at 450 as well as at 500°C at the 7-week sampling time compared to weeks 1-6 sampling times (as the week 0 inoculum levels were not included in the statistical evaluation). Overall, based on the minimal reduction of *E. coli* O157:H7 over 7 weeks (maximum reduction of 0.34 log CFU/g for the 450°C biochar samples) the 450 and 500°C biochars were excluded from further testing to be used as ameliorants for pathogen-contaminated fields. Interestingly, the biochar from the older reactor reported in Gurtler et al. *(44)* was antimicrobial when processed at 500°C, while the 500°C samples from the newly-constructed reactor were not biocidal against *E. coli* O157:H7.

Fast-pyrolysis samples: 600°C. Results for the 600°C fast-pyrolysis biochar samples are presented in Table 2. For the repeated measures model, both the autoregressive (1) and the unstructured variance-covariance structures were good fits. The assumptions of the models were checked and there was large variance heterogeneity. As the repeated factor accounted for only a small amount of the variability (one-third the size of the residual variance), a 3-factor mixed linear model with no repeated measures factor allowed for the variance grouping technique to be used for the variance heterogeneity. The analysis of variance results are given in Table 3. Means and mean

comparisons are in Table 2. Again, similar to the 450 and 500°C samples, there was no significant difference based on concentration × week (Table 3), and, additionally, no difference based on week sampled ($p = 0.168$ by the autoregressive model). However, there was a difference in main effects between concentrations when all weeks were combined (Table 2). Based on the 3-factor mixed linear model, populations were significantly lower at the 3% (1.36 log CFU/g) and 3.5% (0.86 log CFU/g) concentrations than at all other concentrations except for the 2.5% concentration (2.03 log CFU/g). Therefore, the 3% amendment level might be considered a good breakpoint for further testing of bioremediating crop soil for *E. coli* O157:H7. This effect was not due to a change in pH value of the soil as the pH measurements for all three temperatures ranged from 7.72-7.96, values at which *E. coli* O157:H7 can survive. Additionally, the pH measurements were similar at all three temperatures, despite the 450 and 500°C biochar samples showing almost no reduction in bacterial populations in comparison to populations in the 600°C samples.

Slow-pyrolysis and two-year aged fast-pyrolysis biochar samples: 550 and 700°C. Results for the 550 and 700°C slow-pyrolysis and two-year aged fast-pyrolysis biochar samples are presented in Table 4. The repeated autoregressive (1) variance-covariance structure was the best fit. The variability of the repeated factor Week was 93% as large as the residual variability. The analysis of variance is given in Table 5. Time 0 inoculum level was not included in the statistical analysis. Concentration means and mean comparisons are in Table 6. The Week 1 mean (6.95 log CFU/g) was statistically different from Week 2 (6.69 log CFU/g) at less than the 0.02 significance level. Nevertheless, a 0.26 log CFU/g difference is not considered sufficient enough of a reduction to be considered useful as a foodborne pathogen ameliorant. This is especially true considering that the biochar was added at a 10% concentration rate, a level much higher than would be practically applicable for field application *(109)*. Hence, all these biochars were excluded from testing at low amendment concentrations (viz., 1.0-3.5%).

Ten UKBRC slow pyrolysis biochars. When comparing the ten slow-pyrolysis biochars to the no-biochar control (Table 6), one biochar sample (ORP-700°C) caused the recovery of fewer *E. coli* O157:H7 ($p < 0.05$) than the control. However, apparent reductions (Table 4) for the sample recovering the lowest populations (ORP-700°C) over 2 weeks was only 1.61 log CFU/g compared with a 0.78 log CFU/g reduction for the control over the same time period, a difference of only 0.83 logs. This is at a 10% biochar amendment level; therefore, these ten biochar samples may be inappropriate for bioremediation of pathogen-contaminated soils. Because the ORP 700 had a high pH measurement (9.61) it is possible that the decline in *E. coli* O157:H7 may be attributed to a pH effect, at least in part. The ORP 700 also had the second highest content of volatile matter of all biochars (13.18%). This may have accounted for the reduction in *E. coli* O157:H7 except for the fact that ORP 550 had a volatile concentration of 16.38%.

Two-year-aged 500°C fast-pyrolysis switchgrass biochar. For the two-year aged fast-pyrolysis biochar sample (pH 8.26 at 10% biochar in SSVT), there was apparent (Table 4) but non-significant greater survival (Table 6) in pathogen populations when compared with the no-biochar control samples (0.44 log greater survival to the control, Table 4) at the two-week sampling time. This stands in contrast to what we previously reported *(44)* in which the same fast-pyrolysis biochar (aged < 6 mos) was added to soil at 10% concentration and resulted in a 2.42 log reduction over two weeks, which was significantly greater than the 0.05 log reduction in the no-biochar control samples of that study.

From these results, it may be concluded that the biocidal activity of biochar may diminish over time (between 6 and 24 mos), possibly due to loss of key antimicrobial compounds and properties. In fact, studies have demonstrated that, depending on the type of pyrogenic carbon amendments utilized, as well as soil conditions, members of specific microbial communities may increase or decline *(20, 52, 58, 64, 87)*. The increase in microorganisms may be attributed to the availability of

soluble carbon in particular types of biochar, as Hamer et al. *(47)* described experiments in which some types of bacteria were able to use biochar as a sole carbon source. On the other hand, Wu et al. *(115)* found that the addition of 4.8% corncob biochar (produced at 450°C for one h) to soil reduced the relative abundance of phylum Proteobacteria from 47.7% in control soil to 38.6% in biochar-amended soil, as determined by high-throughput sequencing. Dai et al. *(24)* also noted a reduction in populations of Proteobacteria and a 65% increase in Acidobacteria following application of 500°C pyrolyzed wheat straw (residence time not given), which was corroborated in a study by Gregory et al. *(40)* by the application of 10-20 g/kg of 550°C-pyrolyzed chipped willow wood. Gao et al. *(35)* also reported that the addition of rice straw biochar pyrolyzed at 550°C for eight hours reduced the proportion of Actinobacteria with increasing concentrations of biochar added (when compared to crop straw-amended soil) as opposed to the proportions of Proteobacteria and Acidobacteria, which increased. Nevertheless, Xu et al. *(116)* reported a reduction in Acidobacteria populations, proportional to increasing applications of biochar. Others have also demonstrated a reduction in populations of Actinobacteria in biochar-amended versus no-biochar soils *(40, 76)*. On the other hand, studies have demonstrated just the opposite, with Actinobacteria proportions increasing with the addition of biochar *(19, 64, 67, 82, 89, 115)* and Acidobacteria reductions proportional to biochar concentrations *(69, 100)*.

The exact chemical and structural characteristics of biochar necessary for the changes of relative bacterial populations have yet to be elucidated. For example, pH has been shown to be a major factor in increase or decline of some soil bacteria *(34, 100)*, while other studies have demonstrated no correlation between biochar- or fertilizer-based pH changes and phylum-level bacterial abundance *(24, 92)*. Furthermore, some studies have reported that biochar has a negative effect on microbial communities *(73, 81),* while others have contradicted these findings, reporting a neutral affect by biochar on microorganisms *(30, 78)*. Clearly, based on numerous contradictory reports,

the type of biochar utilized, including biofeedstock, processing time and temperature, must be taken into account when considering bacterial pathogen bioremediation of crop soil *(30, 73)*.

This conclusion is borne out in the present study in which biochar produced in our new pyrolysis unit proved biocidal when pyrolyzed at 600°C and at 1.0-3.5% concentrations (Table 2), yet the biochar had a neutral effect on populations of *E. coli* O157:H7 when pyrolyzed at temperatures of 450 or 500°C (see supplemental material). Regarding biochar addition to the soil impacting bacterial populations, a number of components within biochar may be responsible for shaping the microbial community, including volatile organic compounds (VOCs), available carbon, minerals and reactive species *(110)*. The mechanisms affecting the relative abundance of various bacteria is still unclear *(118),* although the generation of reactive oxygen species are known to occur during pyrolysis, which, if persistent, could lend itself to bioremediation of pathogen-contaminated soils *(33, 117)*. Other compounds that may be involved in reducing pathogen populations due to biochar include polycyclic aromatic hydrocarbons *(37, 63, 95, 96, 75)* (especially present in fresh biochar) as well as ketones, benzene, furans, benzoic acids, phenols and methoxyphenols (generated by the pyrolysis of hemicellulose and lignins) *(118)*. VOCs, especially high in fresh biochar, can prove toxic to select bacteria (32) and an array of volatile antimicrobial compounds are known to exist *(17, 29, 59)*. Other persistent free radicals (PFRs) known to be generated and stabilized during anoxic heating of biomass include cyclopentadienyls, semiquinones, and phenoxyls *(112, 118)*.

Further work should be conducted into optimizing biochar for biocidal efficacy as well as determining the chemical components (viz., VOCs, PFRs, and reactive oxygen species, ketones, benzene, furans, benzoic acids, phenols and methoxyphenols, cyclopentadienyls, semiquinones, and phenoxyls). Suggested variables that might be tested could include slow versus fast-pyrolyzing methods, variations in temperatures (e.g., 300-750°C), variations in aging time following biochar

generation (e.g., 0-12 mos), and various biofeedstocks (e.g., various hard and soft woods, green waste, grasses and straws, seed, nut and grain shells and hulls, animal litter, dried sewage, etc.).

In conclusion, in this manuscript we reported the biocidal activity of fast-pyrolysis-generated biochar pyrolyzed at 600°C but not at 450 or 500°C. We also found that fast-pyrolysis biochar aged for 2 years lost its antimicrobial properties that it originally had against *E. coli* O157:H7. The implications of these findings are that the antibacterial nature of biochar changes with processing conditions and age of the finished product.

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SUPPLEMENTAL MATERIAL

Supplemental material associated with this article can be found online at: [URL to be completed by the publisher].

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TABLE 1. *Slow-pyrolysis biochar production parameters and characteristics*

Feedstock	Nominal HTT[a] (°C)	Residence time (min) at HTT	Mean kiln residence time (min)	Heating rate (°C/min)	Volatile Matter (%)	Total surface area (m^2/g)	pH at 10% concentration in SSVT[b]	Biochar yield (%)
Softwood pellets (SWP)	550	3.9	12.0	78	7.48	20.1	8.05	21.80
Softwood pellets (SWP)	700	5.0	12.0	87	6.66	162.3	8.50	17.34
Wheat straw pellets (WSP)	550	5.0	15.0	80	10.55	26.4	9.22	24.11
Wheat straw pellets (WSP)	700	6.0	15.0	79	7.38	23.2	9.72	23.54
Miscanthus straw pellets (MSP)	550	3.9	12.0	65	11.62	33.6	8.87	22.81
Miscanthus straw pellets (MSP)	700	5.0	12.0	80	7.71	37.2	9.49	21.07
Oilseed rape straw pellets (ORP)	550	5.0	12.0	78	16.38	7.3	9.32	28.87
Oilseed rape straw pellets (ORP)	700	5.0	12.0	103	13.18	25.2	9.61	22.62
Rice husk (RH)	550	8.5	15.0	98	7.48	20.1	8.67	37.20
Rice husk (RH)	700	9.0	17.0	92	4.99	42.0	8.74	32.77

[a]Highest treatment temperature
[b]A 0.75:1:0:1:0:0.75 [V/V] mixture of autoclave-sterilized soil, sand, vermiculite, and turface.

TABLE 2. *Survival of* E. coli *O157:H7 (log CFU/g) in soil with varying concentrations of 600°C fast pyrolysis biochar stored at 22°C*[a]

Soil amendment	0[b]	Storage time (weeks)							
		1	2	3	4	5	6	7	Ave.
Soil positive control (0%)	7.64 ± 0.01	6.93 ± 0.11	6.92 ± 0.17	6.69 ± 0.06	6.61 ± 0.12	6.67 ± 0.22	6.64 ± 0.20	6.34 ± 0.43	6.70a[c]
1.0% Biochar	7.64 ± 0.01	5.89 ± 0.62	5.46 ± 0.42	5.02 ± 0.60	4.71 ± 0.75	4.64 ± 0.91	3.48 ± 1.84	3.52 ± 1.87	4.68b
1.5% Biochar	7.64 ± 0.01	3.80 ± 1.92	3.17 ± 1.59	1.87 ± 1.87	1.96 ±1.96	2.85 ± 1.73	2.98 ± 1.71	2.71 ± 1.70	2.75c
2.0% Biochar	7.64 ± 0.01	3.78 ± 1.90	3.41 ± 1.71	2.73 ± 1.51	2.87 ± 1.55	1.79 ± 1.78	3.08 ± 1.59	2.77 ±1.60	2.92c
2.5% Biochar	7.64 ± 0.01	3.25 ± 1.63	2.21 ± 1.34	1.61 ± 1.61	1.67 ± 1.67	1.77 ± 1.80	2.04 ± 2.04	1.80 ± 1.81	2.05cd
3.0% Biochar	7.64 ± 0.01	1.46 ± 1.46	1.03 ± 1.03	1.31 ± 1.31	1.20 ± 1.20	1.56 ±1.56	1.43 ± 1.44	1.51 ± 1.51	1.36d
3.5% Biochar	7.64 ± 0.01	0.00 ± 0.00	1.80 ± 0.93	0.87 ± 0.87	1.21 ± 1.21	1.17 ± 1.17	1.00 ± 1.00	0.00 ± 0.00	0.86d

[a]Data points are means of observations ± SEM.
[b]Day 0 counts consist of original population of inoculum added to soil/biochar mixture.
[a]Concentration means with different letters are different at the 0.05 significance level.

TABLE 3. *Analysis of variance for 600°C pyrolyzed FPBC*[a]

Source	DF	F-value	p-value
Concentration	6	40.97	<.0001
Week	6	1.05	0.3977
Conc × Week	36	0.41	0.9977

[a] Fast pyrolysis biochar.

TABLE 4. *Survival of E. coli O157:H7 (log CFU/g) in soil with 10% slow pyrolysis biochars or two-year aged 500°C FPBC[a] stored at 25°C[b]*

Soil Amendment	Storage time (weeks)		
	0	1	2
Soil positive control (No biochar)	7.57 ± 0.07[c]	6.97 ± 0.01	6.79 ± 0.02
Aged-FPBC	7.57 ± 0.07	7.46 ± 0.23	7.23 ± 0.35
550°C SWP	7.57 ± 0.07	7.49 ± 0.52	6.94 ± 0.00
700°C SWP	7.57 ± 0.07	6.92 ± 0.06	6.76 ± 0.07
550°C WSP	7.57 ± 0.07	7.01 ± 0.06	6.56 ± 0.09
700°C WSP	7.57 ± 0.07	6.44 ± 0.06	6.57 ± 0.31
550°C MSP	7.57 ± 0.07	7.54 ± 0.49	6.70 ± 0.02
700°C MSP	7.57 ± 0.07	7.61 ± 0.42	6.68 ± 0.04
550°C ORP	7.57 ± 0.07	6.38 ± 0.07	6.54 ± 0.16
700°C ORP	7.57 ± 0.07	6.15 ± 0.27	5.96 ± 0.07
550°C RH	7.57 ± 0.07	6.79 ± 0.15	6.84 ± 0.19
700°C RH	7.57 ± 0.07	6.71 ± 0.06	6.70 ± 0.04

[a]Fast pyrolysis biochar.
[b]Data points are means of observations ± SEM.
[c]Day 0 counts consist of original population of inoculum prior to adding to soil/biochar mixture.

TABLE 5. *Analysis of variance for slow pyrolysis biochars and two-year aged 500℃ pyrolyzed FPBC*[a]

Source	DF	F-value	p-value
Concentration	11	4.53	0.0095
Week	1	8.69	0.0122
Conc × Week	11	1.30	0.3295

[a] Fast pyrolysis biochar.

42 TABLE 6. *Log CFU/g means and mean comparisons for slow pyrolysis biochars and two-year aged 500°C pyrolyzed FPBC*[a]

Biochar added	Mean[b]
Control	6.88a[c]
FP-SG	7.34a
SWP 550	7.21a
SWP 700	6.84a
WSP 550	6.79a
WSP 700	6.50a
MSP 550	7.12a
MSP 700	7.15a
ORP 550	6.46a
ORP 700	6.05b
RH 550	6.82a
RH 700	6.70a

43 [a] Fast pyrolysis biochar.

44 [b]Concentration means.

45 [c]Comparisons to the control. Different letters indicate differences at the 0.05 significance level.