EVALUATING MICROBIAL COMMUNITY AND BIOGEOCHEMICAL CHARACTERISTICS DURING POULTRY LITTER COMPOSTING

A Dissertation Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

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December 2021

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Acknowledgements

I would like to express my appreciation to my supervisors, Dr. Jennifer M. DeBruyn and Dr. Shawn Hawkins, they give me this fantastic opportunity to join this program. I also would like to thank my committee members, Dr. Sindhu Jagadamma, Dr. Rebecca Tiffany Trout Fryxell and Dr. Forbes R. Walker, they helped me to find the right direction and encourage me throughout my Ph. D study. Thanks also to Dr. Jie Zhuang and all Dr. DeBruyn lab members for their kindness and encouragement.

Abstract

Composting is a widely used method for the treatment of animal waste, but improper management leads to a poor performance. Benefits of biochar have been identified in animal waste composting including reduced odor and greenhouse gas emissions, and improved heating performance. However, the benefits of low amendment rates of biochar addition for composting are unclear. Our research goal was to evaluate the effects of composting, with and without biochar addition, on poultry litter microbial community structure, N-cycling, and pathogens in commercial poultry farm and further determine the effects of temperature and moisture on the presence of pathogens through a laboratory simulated composting experiment. Our research indicates that composting changed bacterial and fungal community structures in poultry litter. Across all the samples from two compost trials, Firmicutes and Acidobacteria were the dominant bacterial phylum. Ascomycota was the predominant phyla in fungal community. Abundance of N-cycling functional genes did not change in the first trial, but three denitrification genes (*nir*K, *nir*S and *nos*Z) and *amo*A were increased in the second trial. Low amounts of biochar (1%) did not influence microbial community structures or the relative abundance of selected taxa in either of two compost trials, but it increased the abundances of denitrification genes in the first trial.

Marker genes of *Salmonella*, *E. coli* O157:H7, *C. jejuni* and *C. perfringens* were used to evaluate the pathogen removal in windrow composting and simulated composting. High moisture content and low moisture content poultry litter were tested in a simulated composting experiment. Decreased abundances of *Salmonella*, *E. coli*

O157:H7 and *C. jejuni* after composting indicated the benefits of composting for pathogen removal. Biochar addition (1%) did not improve the windrow composting performance. Thermal inactivation decreased total bacterial abundance in simulated composting at 50 °C for high moisture content litter and 62 °C for low moisture content litter. In summary, changes in poultry litter microbial community structure and N-cycling functions were detected during in-house composting, but low amounts of biochar did not increase compost performance or pathogen removal. This study also highlights the importance of initial litter moisture content and heating temperature for pathogen removal.

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CHAPTER 1 INTRODUCTION

Composting is a widely used method for the treatment of organic wastes, including green waste (Ventorino et al., 2016), animal waste (Bernal et al., 2009) and urban waste (Takaku et al., 2006). As a self-heating process, it effectively reduces waste volume and breaks down organic matter using microbial decomposition. High temperature generated by microbial activity also serves to eliminate or reduce potential pathogenic microorganisms, parasites, and weed seeds (Grundy et al., 1998). The end-product of compost also can be used to improve soil nutrient content and decrease chemical fertilizer application (Evanylo et al., 2008, Agegnehu et al., 2016).

The intensity of the livestock industry generates extraordinary amounts of animal waste (USEPA, 2013), including manure, carcasses and slaughter waste. Improper management causes environmental problems, including odor, greenhouse gas emissions (Bong et al., 2017), and leachates (Hill et al, 2005). However, the benefits of composting have been identified, especially in the event of disease outbreaks or disasters. For example, during the outbreak of highly pathogenic avian influenza (HPAI) in the U.S., which killed large amounts of poultry in a very short period (2014- 2015), composting was applied as an efficient and economic waste treatment method (Peer and Flora, 2010).

The potential value of animal waste has been always recognized. In addition to the application as biogas (Sorathiya et al., 2014). The residue of composted animal waste can be applied as fertilizer to supply crop nitrogen requirements (Evanylo et al., 2008), since composting decreases the risk of transition of potential pathogens, parasites and

weed seeds associated with the agricultural land application and produces a stabilized product which can be used to improve and maintain soil fertility (Larney and Hao, 2007). Composting is not a new or advanced technology, but this treatment method has attracted attention as an advisable choice for animal waste with economic and environmental benefits. In general, windrow composting is the preferred method for large-scale composting applied on farms. During windrow composting, large amounts of organic wastes are mounded in long piles, and regular turning is often applied to maintain aeration and homogenize the organic wastes.

This dissertation presents two experiments that were conducted to investigate the changes of microbial communities and functions during poultry litter composting. The first experiment is presented in chapter 3, which focuses on the in-house windrow composting and low amounts of biochar addition on poultry litter microbial communities and microbial nitrogen cycling. Chapter 4 describes the results of the second experiment, focused on the presence of pathogens in poultry litter. In-house windrow composting and biochar addition was applied to evaluate the removal of pathogens from poultry litter. To determine the effects of environmental factors, a simulated poultry litter composting experiment was designed to evaluate the effects of temperature and initial moisture content on the presence of pathogens in poultry litter.

CHAPTER 2 LITERATURE REVIEW

2.1 Poultry litter

As the largest poultry producing country in the world, U.S. produced 890 million heads of birds in 2017 and 922 million in 2020 (USDA National Agricultural Statistical Services, 2014-2020). Increased poultry production necessarily generates huge amounts of poultry litter, with about 1.07 to 2 tons of litter produced per 1000 birds (Paterson et al., 1998, Chamblee and Todd, 2002). Poultry litter is a mixture of bedding materials and feces excreted by birds. Improper management of poultry litter can lead to environmental problems, such as air and water contamination (Wheeler et al., 2006, Crippen et al., 2021) and the risk of spreading of pathogenic microorganisms (Bouzo et al., 2019). Thus, appropriate poultry litter management is required for the poultry industry, and recycling of poultry litter is an effective way to reduce the cost and environmental impacts (Roll et al., 2011; Wei et al., 2013; Wang et al., 2016).

2.2 Animal waste composting

During composting, organic materials are degraded by microbes resulting in microbial growth which releases heat. Composting usually has two stages with different characteristics, including different temperatures, oxygen content, moisture content and nutrient levels (Zhang et al.,2014). In the first stage, microbes in raw materials use labile nutrients to grow, including primarily soluble carbon and organic acids. This stage is defined as the thermophilic stage or active composting stage, the blooming of microbial activity increases the microbial biomass, which further increases the temperature in the composting piles. The high temperatures of this thermophilic stage

act as a “screening function”, in which microbial taxa which can tolerate high temperature can survive while other taxa may be eliminated or inactivated by high temperature, especially the potential pathogenic microbial taxa (Duan et al., 2019). The second stage is the maturation stage. In this stage, temperature declines and the microbial activity is reduced to a steady stage due to the inhibition caused by limiting nutrient concentrations and high temperature (Mukhtar et al., 2005). This stage is also referred to as the curing stage or mesophilic stage.

Composting systems can be affected by various environmental factors, including the ratio of carbon and nitrogen, oxygen content, moisture content, pH, and temperature. Based on USDA guidelines (USDA-NRCS, 2000), to effectively eliminate the potential pathogenic microbes, the target temperature inside the compost should be 55℃ sustained for 3 days in in-vessel composting and at least 15 days for windrow composting. The recommended condition for C: N ratio is about 25:1 to 30:1. Because animal waste is a nitrogen-rich material, adding carbon-rich material is necessary to provide the ideal stoichiometry for the growth of microorganisms. Wood chips, sawdust, pine litter, and dry leaves are commonly used carbon-rich materials in animal waste composting systems. The ideal moisture content of composting piles is 40% to 60%. If the composting is conducted where the rainfall is high, a shelter is often necessary to limit moisture in the compost system. Composting is an aerobic process, but some oxygen will be depleted in the core of composting piles. For composting materials, particle size also influences composting performance; the ideal particle size is about 0.6

to 5 cm, which provides porosity for replacement with oxygen, but also helps hold the appropriate temperature. In most conditions, the temperature distribution in composting pile is uneven. Thus, turning is necessary to homogenize the organic waste and provide oxygen for the growth of microbes, as well as ensuring that the particles of compost are exposed to the high temperature in the inner of compost pile. However, it should be noted that turning is not used for large carcass composting, because turning is not feasible.

2.2.1 C: N ratio

To achieve the best decomposition performance, a suitable C: N ratio is required for the growth of microorganisms. A high C: N ratio will decrease the decomposition rate because a low nitrogen supply limits microbial activity, but a low C: N ratio will generate odor because high nitrogen supply will generate excess ammonia. To avoid issues caused by low C: N ratio, carbon-rich materials can be added to balance the nitrogen-rich animal waste composting. A previous study proposed that a sawdust: poultry manure ratio of 1:4 generates higher poultry carcass degradation rates and lower NH_3 emission during composting (Wang et al., 2016). In a laboratory pig manure composting, three C: N ratios were selected to test the effect, including 15: 1, 18: 1 and 21: 1. Results indicated that composting with low C: N ratio produces more ammonia (Jiang et al., 2011). A previous study investigated the effects of swine manure and sawdust addition on swine carcasses, suggesting that the ratio of sawdust and manure of 1:1.5 could better induce decomposition (Won et al., 2016).

C: N ratio not only affects ammonia emissions but also changes the mobility of heavy metals and pathogen removal. Heavy metals accumulate in animal manure and enter into environment (Wang et al., 2017). Pig manure composting with 25: 1 C: N ratio reduced the mobility of Zn and Cu compared to compost with 15:1 and 20:1, which suggested 25: 1 was the optimal ratio to produce low risk and high quality composted material (Wu et al., 2017). In a poultry manure bioreactor composting, when the C: N ratio was 10: 1, it had faster reductions of *E. coli* than the 40:1 and 20:1 C: N ratio (Thomas et al., 2020). A similar result was indicated in a simulated poultry manure composting, 20: 1 C: N ratio removal more pathogens than the compost with 30: 1 and 40: 1 (Erickson et al., 2010).

2.2.2 Moisture content

Previous studies have discussed the effects of moisture content on composting. In a study of in-vessel poultry manure composting, five initial moisture contents, 45%, 49%, 53%, 57%, and 61% were tested, with the 53% moisture content resulting in the highest temperature (61 °C), highest nutrient content (TN, P_2O_5, and K_2O), and longest heating duration time (15 days) during composting; however, the composting with low moisture had reduced leaching (Li et al., 2021). In a laboratory pig manure composting experiment, three different moisture contents, 65%, 70% and 75% were evaluated, but produce similar methane and ammonia emissions (Jiang et al., 2011). The importance of moisture was also shown in other types of feedstock composting. For example, mixed municipal waste with 75 % moisture content had the highest temperature and longest

heating duration time, which suggested the best degradation performance (Makan et al., 2013). Moisture content of the animal waste influences composting in two ways: first, moisture is important for microbial growth. Liang et al. (2017) found microbial activity increased when moisture content increased from 30% to 60%, suggesting that moisture content has a bigger effect on microbial activity than the temperature in biosolid composting. The second aspect is the effect of moisture on the oxygen content in compost piles: a high moisture content may reduce oxygen diffusion. Aerobic degradation is the dominant process when moisture is low. High moisture content (>64%) results in more anaerobic degradation conditions in composting (Zavala et al., 2005) because the pore spaces were filled by water instead of air (Kim et al, 2016).

2.2.3 Aeration

To maintain the efficiency of composting, turning is necessary during composting to keep the oxygen content high. Stanford et al. (2009) designed an experiment to evaluate the effects of turning technology and composting environment on cattle carcass decomposition, and found no significant differences among two different turning methods (tractor-loader bucket and grinding bucket). When the aeration rate was kept from 0.21 to 0.72 L/kg DM min^{-1} in pig manure composting, methane emissions were reduced, but N_2O and NH_3 emissions increased. A reduction of NH_3 emission can also be achieved with low aeration rates (Jiang et al., 2011). During a study of pig feces and corn stalks composting, an air flow rate of 0.48 L/ kg DM min^{-1} had the best stability and maturity of compost. It also suggested aeration influenced the concentration of

NH_4^+ -N, low aeration had the highest increase in NH_4^+ -N and the lowest nitrogen losses as NH_3 (Guo et al., 2012). In vegetable and fruit waste composting with an aeration rate from 0.37 to 0.99 L/kg DM min^{-1}, the lowest C: N was detected in the composted material with 0.62 L/kg DM min^{-1}, suggesting that the composted material was stabilized (Arslan et al., 2011). In barley straw and hen manure composting, NH_3 emissions decreased with the decreased aerate rate but the CH_4 emission increased, suggested low aeration (0.17 -0.23 L/kg DM min^{-1}) can be used to reduce NH_3 emission (Chowdhury et al., 2014). All the results highlight the importance of aeration or turning in the degradation of animal waste.

2.3 Other animal waste management approaches

Composting is not the only method in animal waste management, other management approaches included anaerobic treatment, aerobic treatment, traditional on-farm burial, and burning. Each management may be applied specifically based on the purpose and types of animal waste. Anaerobic digestion is a process that can be used to produce CH_4, an renewable biogas energy (Karim et al., 2005). Aerobic treatment is a process that requires the involvement of oxygen to ensure microbial activity, and is widely used in the treatment of wastewater and livestock sludge. The aerobic treatment also can be applied in anaerobic treatment to reduce the N content in target waste by denitrification (Miner et al., 2000). In livestock farming systems, the other unavoidable animal waste is animal mortality, the inevitable carcasses generated from livestock farming systems that cannot be used for food. Animal carcasses not only contain biological tissues, like

feathers, manure, bone, and muscle but also contain numerous microorganisms, including bacteria, viruses, and parasites (Mukhtar et al., 2005). In addition to composting, there are several other ways to dispose of livestock carcasses in the U.S. First, on-farm burial is a relatively simple process, but only works in areas with deep enough soils, and should be conducted away from wells, public use area, and groundwater table. Previous studies suggested improper burial may lead to the introduction of containments in groundwater; including bacteria, nitrogenous compounds (Hseu and Chen, 2017), and even steroids and pharmaceuticals (Yuan et al., 2013). Based on the contaminant concerns, European Union has banned animal carcass burial since 2002 (Anon, 2002). A second disposal method is burning, which has a minor effect on the groundwater, but incomplete burning can lead to other environmental problems, including the emission of carcinogens like dioxins and furans (Kogevinas, 2001). A third method is incineration, which can destroy infective agents at high temperatures and produce inorganic ash, but has similar side effects as burning (Chen et al., 2003; Chen et al., 2004). Compared to the other disposal methods, composting is economical and effective. A proper mortality composting system also provides nutrient-rich organic matter which can be applied in crop farming as an environment-friendly method to reduce artificial fertilizer application. Previous studies have suggested that qualified finished composting residue from animal carcass composting can be used as fertilizer (Senesi, N. 1989, Kalbasi et al., 2005).

In summary, composting is an efficient method to manage different waste types, including green waste (Ventorino et al., 2016), animal carcasses and manure. Many studies have shown that well-designed compost systems do result in a safe and efficient method of disposing of carcasses and animal waste with minimal environmental impacts, especially the potential pathogenic microorganisms and environmental contamination. Compared with other studies related to composting, including gas emission, leachate and N contamination, microbial processes and associated risks of disease transfer still required more research.

2.4 Microbial ecology of composting

Composting is a natural biological process, which does not require extra heat or other additives. The benefits of composting, such as high temperature and reduced waste volume, are caused by microbial growth and succession during the composting process. Thus, studies about microbial ecology are considered to be helpful for a good understanding of composting process. Although many studies have focused on poultry litter microbial communities, including pathogens, most microbial analyses relied on culture-based methods which only detect the culturable microorganisms (Terzich et al., 2000; Roberts et al., 2013; Skóra et al., 2016). Only a few research projects have used molecular approaches to characterize poultry litter bacterial communities (Oakley et al., 2013; Wei et al., 2013; Wang et al., 2016), and these have been limited to bacterial communities, providing us very little information about fungi in composting systems.

Microbial community compositions in waste composting have been reported in several studies. A previous study applied DGGE (denaturing gradient gel electrophoresis) to assess the variation of microbial communities during composting of food waste and rice hulls. In this system, the dominant bacterial taxa changed from Firmicutes in the thermophilic stage to Bacteroidetes in the maturation stage (Takaku et al., 2006). Proteobacteria presented in a higher proportion in the initial composting microbial community (days 30-60), high abundance of Firmicutes was also shown in the intermediate period (days 120-180) of swine carcass composting, as other dominant bacterial taxa, (Yang et al., 2018). Throughout the whole composting process, *Bacillus*, a genus belonging to Firmicutes, is widely reported to be present in various raw materials composting, including chicken manure-straw composing (He et al., 2013) and human feces (Li et al., 2013). Yang et al. (2018) added an exogenous bacterium, *Bacillus*, to speed up the decomposition process in carcass composting; during the composting process, the three most dominant bacteria phyla were Proteobacteria (34.5%), Firmicutes (31.0%), Actinobacteria (21%) (Yang et al.,2018). It is worth mentioning that Acidobacteria is usually detected as a dominant phylum in soils, but is generally not detected in composting systems (Jones et al., 2009), due to the high pH during composting. The additional *Bacillus* in composting not only changed microbial community composition but also increased functional gene abundances , such as genes involved in carbohydrate and amino acid metabolism, further increase the composting efficiency (Yang et al., 2018). More lipid-degrading microorganisms also were detected

after *Bacillus* inoculation, included *Pseudomonas* and *Bacillus* (Yang et al., 2018). Consistent with Yang et al. (2018), Actinobacteria, Firmicutes and Proteobacteria were detected as high abundance phyla during composting. Once temperatures reached 55 ℃, the relative abundance of Firmicutes increased, but the relative abundance of Proteobacteria decreased (Tkachuk et al., 2014). The above-mentioned studies indicated a major role of Firmicutes in the composting process.

Concerning fungal communities in composting, a dominance of Ascomycota had been reported in many studies, included the composting of poultry manure (Duan et al., 2019a), green waste (Bru-Adan et al., 2009), and agricultural waste with feedstock manure (De Gannes et al., 2013). The thermophily of Ascomycota has been shown, including orders belong to Ascomycota, such as *Saccharomycetales* and *Eurotiales* (Natvig et al., 2015). Ascomycota also produce cellulase which can degrade organic matter in composting (Singh et al., 2010). The presences of other fungi were also identified, including Basidiomycota (Bru-Adan et al., 2009, Wang et al., 2018), Chytridiomycota and Mucoromycota (Duan et al., 2019b, Wang et al., 2018). Meng et al., (2018) and Li et al, (2020) reported the presence of Basidiomycota in cow manure composting (10%) and pig manure-pine leaf composting (6.65 -76.24%), respectively. Chytridiomycota was detected in the early stage of cow manure composting (0.05%) (Wang et al., 2018), chicken manure composting (76.57-97.97%) (Duan et al., 2019b), and sewage sludge composting (12%) (Robledo et al., 2020). This phylum can tolerate

the changes in temperature and moisture, also can degrade organic matter as a saprotrophic microbe (Krings et al., 2009).

2.5 N-cycling in composting

Nitrogen transformations during composting control the fate of N and final N content of the composted litter (Figure 1-1). Composting produces NH_3 and greenhouse gases (CO_2, CH_4, and N_2O) due to microbial activity. Nitrogen content in the compost material usually decreases due to ammonia volatilization, which leads to the high NH_3 content in the adjacent air and decreases the fertilizer value of composted waste. A NH_3 concentration not only inhibits bacterial activity, leading to reduced decomposition rates in composting system (Kuok et al., 2013) but also influences the growth and production of chickens (Williams Ischer et al., 2017). In a combined soil burial-composting experiment, a positive correlation between a chemolithoautotrophic bacteria, *Halothiobacillus* and high NH_3 emission was found (Ki et al., 2018). Carbonyl sulfide pathway processed by *Halothiobacillus* can produce NH_3 which could explain the positive correlation between *Halothiobacillus* and NH_3 (Sorokin et al., 2014).

Nitrification and denitrification are the two important N cycling processes in animal waste composting. Ammonia-oxidizing bacteria (AOB) are the dominant nitrifiers throughout composting (Maeda et al., 2011) and the stages of thermophilic and cooling in agriculture composting (Zhong et al., 2020). Even an abundance of ammonia-oxidizing archaea (AOA) was also found in agriculture waste composting,

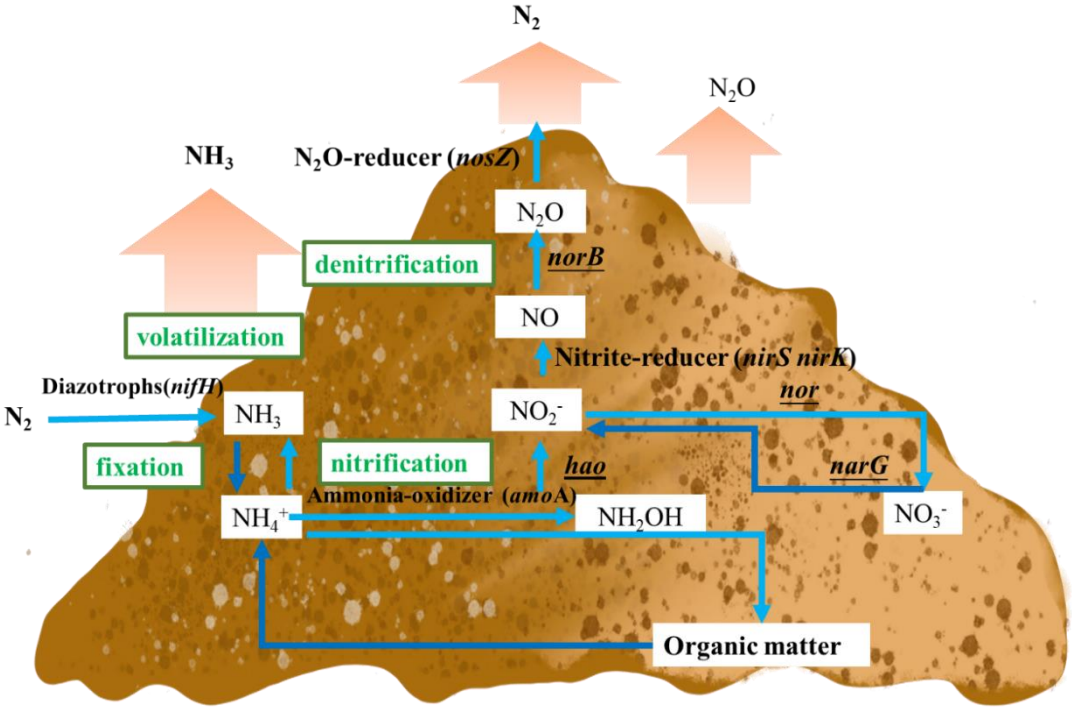


Figure 1-1 Nitrogen cycle and related functional genes during composting.

but typically in lower abundances compared to AOB (Zhong et al., 2020). NH_3 volatilization is not the only method of N loss in composting. Zhong et al., (2020) suggested that denitrification is also a dominant reason for N loss in composting. Nitrous-oxide reductase and nitrite reductase are two nitrite reductases in denitrification, these nitrite reductases with different structures, copper-containing nitrite reductase and cytochrome cd_1 containing nitrite reductase which are coded by *nir*K and *nir*S genes, respectively. Previous studies suggested the importance of *nir*K rather than *nir*S denitrifiers in N_2O production during cow manure composting because different environmental conditions favor different denitrifiers populations (Maeda et al., 2017, Meng et al., 2019). The last step in denitrification is processed by nitrous oxide reductase, which is coded by *nos*Z. N_2 is the product of full denitrification. A previous study has shown that these three denitrification genes, *nir*S, *nir*K, and *nos*Z, increased abundance during the thermophilic stage and decreased in the maturation stage of composting (Zhang et al., 2015).

Even though N loss is a popular question in the research area of composting, N input also influences N content during composting. Diazotrophs are the dominant N input as well as animal waste during composting which may also affect N content in composting. The abundance of marker genes encoding for a subunit of nitrogenase (*nifH*), an enzyme involved in nitrogen fixation, was higher in the early stage of sewage sludge composting (Pepe et al., 2013) and agriculture waste-cattle manure composting

(Sun et al, 2016), which was attributed to potential low oxygen content caused by the high moisture content in the early stage.

2.6 Pathogens in composting

Contamination of manure left by the previous poultry flock has been implicated as the potential risk factor for following flocks (Roberts et al., 2013). Some Gram-negative bacteria contain pro-inflammatory and allergenic lipopolysaccharide complexes, known as endotoxins, which are released after the death of the bacterial cell. Gram-positive bacteria showed high abundance in poultry litter microbial communities (Lu et al., 2003). Based on the survey of potential pathogens throughout the U.S., poultry litter contains bacteria that have the potential to cause illness, such as *Salmonella* and *Staphylococcus* (Martin et al., 1998; Terzich et al., 2000). The load of microbes generally increases over time in poultry houses, both in the air (Witkowska et al., 2017) and in poultry litter (Chen et al., 2014). A high growth rate was reported for aerobic mesophilic bacteria, which account for the majority of known pathogenic bacteria (Olonitola et al., 2015). Pathogens in poultry litter may influence chicken health, for example, the bacteria *Salmonella* and *E. coli* O157:H7 are a common cause of infections in poultry (Bavaro, 2021). Fungi also cause poultry diseases, including *Candida albicans* (Osorio et al., 2007), *Aspergillus fumigatus,* and *Paecilomyces variotii* (Skóra et al., 2016).

Composting has been shown as an effective method to eliminate the transmission of bacterial pathogens and viruses, particularly with enteropathogenic bacteria

(Erickson et al., 2015), since aerobic composting of animal waste is a beneficial treatment that inactivates these pathogens due to high temperature (Erickson et al., 2010 and 2014). Vitosh-Sillman et al., (2017) demonstrated a reduction of viral load in carcasses infected with a porcine epidemic diarrhea virus in composting piles when the temperature was held at 55 ℃ for 4 h. For the removal of typical pathogens, the reduction of *C. perfringens* was found in poultry litter composting (Macklin et al., 2008). Chen et al.(2018) also reported the decrease of *Salmonella* and *E. coli* O157:H7 in poultry manure composting when the temperature reached 55 °C for 8 days.

2.7 Biochar as an additive in composting

Biochar is obtained when organic feedstock is pyrolyzed under high temperature with low oxygen concentration or without oxygen (Lehmann et al., 2011). Different types of biochar have different nutrient contents, pH, and physical-chemical properties. It has recently gained attention as a soil additive because of its soil quality-enhancing properties (Lehmann et al., 2011). It has been shown that the physico-chemical properties and reactivity of biochar in soil can help decrease nutrient leaching, greenhouse gas emissions and increase crop production (Lehmann et al., 2011, Dong et al., 2013). Biochar addition to agricultural soils is used to mitigate greenhouse gas emissions, such as nitrous oxide (N_2O) and CH_4 emissions (Lehmann et al., 2011). Studies emphasize the importance of N_2O and CH_4 because their warming potential is dramatically higher than CO_2 (Energy Information Administration, 2003). However, the impacts of biochar addition on CH_4 emissions from soil are not consistent, included

no response (Kammann et al., 2012), increased (Zhang et al., 2010) or decreased emissions (Feng et al., 2012; Dong et al., 2013).

When added to soil, biochar can change abiotic factors which may give a competitive advantage to some microbial populations over others, such as soil pH and soil organic carbon (Novak et al., 2009; Jeffery et al., 2011) and moisture content (Novak et al., 2012). Biochar can influence nutrient transformation (Lehmann et al., 2011) and rapid labile carbon compound turnover (Farrell et al., 2013) which may change the microbial community composition. Biochar additions also modify the communication between and among soil fungi by adsorbing important signaling molecules (Masiello et al., 2013). Biochar may affect the soil food web by changing carbon source quality and its availability to soil microbes (Rahman et al., 2014; Coban et al., 2016). Biochar additions introduce carbon with different degrees of stability, which may change the amount of carbon to be mineralized by microorganisms. In short-term incubations, positive priming effects caused by biochar addition also increases the turn-over of the soil organic matter (SOM) (Farrell et al., 2013). Meanwhile, the presence and bioavailability of toxic compounds can be changed by biochar addition, leading to a significant decrease in Cd, Zn, polycyclic aromatic hydrocarbons (PAH) (Beesley et al., 2010), and NH_3 (Taghizadeh-Toosi et al., 2012).

Recent studies suggest that biochar amendment can enhance composting performance, also retaining nutrients and improving the quality of the end-product (Sánchez-García et al., 2015), improve the heating performance (Czekała et al., 2016),

reduce nitrogen loss in poultry manure compost (Dias et al., 2010) and weaken the volatile gas emissions in sewage sludge compost (Malińska et al., 2014). Biochar amendment had positive effects on composting heating performance, 5% (Steiner et al., 2010) and 10% (Jindo et al., 2012) biochar additions increased the peak temperatures in poultry manure composting. Biochar addition also has been shown to affect microbial community composition during composting (Duan et al., 2019). The reasons why biochar affects microbial processes still not consistent, with studies suggesting different mechanisms, including increased nutrients, moisture content, or biochar providing a more reactive surface and serves as a shelter. Biochar has been shown to absorb nutrients because of its porous structure. After nutrients are absorbed, the biochar can be a hot-spot with a higher process rate and interactions compared to the adjacent area (Kuzyakov, 2015). Although biochar is recalcitrant, the labile carbon stored in biochar could be used by microorganisms (Farrell et al., 2013). In compost systems, studies found that a higher labile carbon content was detected in biochar, suggesting that biochar served as a nutrient-rich niche to provided different types of labile carbon and improved the microbial ability to use carbon source (Sun et al., 2016). In addition to the absorption ability of biochar, biochar can provide nutrients to microorganisms directly. Many studies observed higher CO_2 emissions after biochar addition, a result which may be caused by the priming effect and the mineralization of the labile carbon in biochar (Cross and Sohi, 2011).

Biochar application changes the microbial community composition and diversity in soil and composting (Kolton et al., 2017, Duan et al., 2019). In general, Proteobacteria, Acidobacteria, Actinobacteria, Chloroflexi, Bacteroidetes, Firmicutes, and Gemmatimonadetes are common bacterial phyla in soil. Acidobacteria was reported to be very sensitive to biochar addition to soil (Xu et al., 2014; Abujabhah et al., 2016; Xu et al., 2016; Yao et al., 2017) because biochar addition typically increases soil pH. Low abundance of Chloroflexi also was detected after biochar was added to soil (Xu et al., 2014; Xu et al., 2016). In contrast with Acidobacteria, the relative abundance of Proteobacteria and Actinobacteria have been shown to increase after biochar addition (Xu et al., 2014; Xu et al., 2016). The diverse morphologies and metabolisms of Actinobacteria showed this phylum can consume different nutrient sources, included recalcitrant carbon forms (Lehmann et al., 2011). Gemmatimonadetes has shown different responses to biochar addition. For example, Xu et al. (2016) suggested biochar amendment reduced the relative abundance of Gemmatimonadetes in soil because they prefer drier conditions (DeBruyn et al., 2011). The opposite response, an increase in Gemmatimonadetes with biochar addition, was detected in crop cover land in Europe (Jenkins et al., 2016) and forest soil (Khodadad et al., 2011). Firmicutes showed no response to biochar addition in soil (Chen et al., 2013).

Biochar amendments affect the abundance of microbes, which can influence microbial biogeochemical cycling, including N transformations. Previous studies in soil showed that 2% and 10% biochar addition to water-saturated soil did not affect the

abundance of bacterial or archaeal *amoA*, a gene that encoded an enzyme related to the key step in the nitrification process. Increased relative abundances of *Bradyhizobium* (Phylum: Proteobacteria) and *Mycobacteriaceae* (Phylum: Actinobacteria) were found after biochar addition in pasture soil. *Bradyhizobium* can produce nitrous oxide reductase (NosZ) enzymes which convert N_2O to N_2. *Mycobacteriaceae* also play an important role in reducing N_2O to N_2, and the increased abundance of *Mycobacteriaceae* also can help explain the N_2O reduction seen after biochar addition (Anderson et al., 2011). Similar results were observed in a biochar-amended soil, where copy numbers of *nifH*, *nosZ*, *nirS,* and *nirK* genes increased. Notably, the initial copy number of *nirS* was much lower than *nirK*, but after incubation, *nirS* copy numbers exceeded *nirK* copies. Harter et al (2013) suggested that high ratio of denitrification gene copies, *nosZ*/(*nirS*+*nirK*), imply the growth of *nos*Z-type denitrifies, which means N_2O is converted to N_2. While there has been less research on the microbiology of N-cycling in biochar-amended compost, compared other studies have investigated biochar as a soil amendment. During biochar-manure composting, the *nirK* abundance decreased but *nosZ* abundance increased which suggested biochar addition induced less N_2O producing and more N_2O consuming microorganism (Wang et al., 2013). The other important process in N-cycling is nitrogen fixation. One study suggested biochar addition to compost increased the abundance of *nifH* which lead to increased nitrogen content in compost (Liu et al., 2019).

Different types of biochar may result in different microbial community responses to biochar addition. Biochar characteristics are determined by the feedstock and carbonization temperature. Carbonization temperature can affect the characteristic of biochar, including surface area, pore structure, and absorption ability (Lu et al., 2013). Biochar produced at high temperature processes has higher surface areas (Ahmad et al., 2012) and therefore high recalcitrance and good adsorption properties. Low temperature processes generate more biochar, but the biochar may more phytotoxic because of the partial carbonization and the stability of the aromatic backbone. Ameloot et al. (2015) detected the effects of different biochar derived from two carbonization temperatures on soil, the biochar which was produced at relatively low temperature induced the rapid carbon mineralization and high CO_2 emissions. A greater abundance of bacteria was detected in biochar produced under low temperatures, but no effects were found on specific phylum such as Actinobacteria. Dong et al. (2013) showed that straw biochar reduced more CH_4 emissions than bamboo biochar because straw biochar can effectively increase soil pH and aeration. In an antibiotic resistance genes (ARG) removal experiment, straw biochar effectively reduces the abundance of ARGs during poultry manure composting, but mushroom biochar increased the abundance of bacteria with ARGs (Cui et al., 2016).

Study Objectives

Studies have evaluated the change in microbial community composition in many types of composting processes. However, few studies investigate how biochar addition affects

microbes and nitrogen-cycling in animal waste composting. Due to the increased production of poultry farming, a deeper understanding of how to treat animal waste effectively and safely is required. Knowledge of the variation of microbial community change and their potential role in biological composting is necessary to improve efficiency and safety. This research aims to provide biological information about animal waste-biochar composting and the mechanisms of how biochar influences microbial activities and functions during composting.

The overall goal of the first study (Chapter 3) was to evaluate the variations of function and diversity in poultry litter microbial community during windrow composting. In our study, we tested the effects of windrow composting and biochar as an amendment on microbial community structure and N-cycling functional gene abundances, because biochar has been suggested as an additive for composting poultry litter (Steiner et al., 2010). The objectives were to 1) test if in-house windrow composting and a low dosage of biochar as an additive changed poultry litter physico-chemical properties, bacterial and fungal community composition, diversity, and relative abundance of dominant microbial taxa; 2) determine the changes in N-cycling functional gene abundance, included *nifH*, *amoA*, *nirS*, *nirK,* and *nosZ*; and 3) identify the dominant environmental factors driving the changes in microbial communities and N-cycling functional genes. We hypothesized that: 1) windrow composting would reduce moisture and water extractable carbon (WEC), but biochar amendment would improve moisture content and reduce nitrogen loss. Further, windrow composting

would decrease the diversity of bacterial and fungal communities, and specifically increase the relative abundance of Firmicutes and decrease the relative abundance of Acidobacteria; 2) biochar addition influences the nitrification and denitrification process, and the abundance of *nosZ, nifH, nirS,* and *nirK*, which will increase, while the abundance of *amoA* decreases; and 3) poultry litter temperature, pH and nutrient concentration, such as NO_3^- -N and NH_4^+ -N, would be the dominant driving factors in shaping microbial community compositions and abundances of N-cycling functional genes.

The overall goal of the second study (Chapter 4) was to assess the removal of bacterial pathogens during poultry litter composting. We combined an in-house windrow composting experiment with a simulated poultry litter composting experiment in the laboratory to evaluate the presence of pathogens. In this research, we have four objectives: 1) evaluate the change of selected pathogens in poultry litter windrow composting with or without low amounts of biochar addition (1%); 2) detect the impacts of different temperatures on the presence of pathogens in poultry litter simulated composting; 3) evaluate the effects of moisture content on the presence of pathogens in poultry litter simulated composting; 4) compare traditional plate counting methods with quantitative PCR for the evaluation of total bacteria in poultry litter. We hypothesized that 1) the abundances of pathogens would decrease in poultry litter windrow composting, and biochar addition would increase the pathogen removal in composting; 2) high temperature with long heating time would remove more pathogens in simulated

poultry litter composting; 3) suboptimal moisture content would decrease the effectiveness of composting; 4) traditional plate counting and qPCR would show similar results with respect to the change of total bacteria in simulated poultry litter composting.

References

Arslan, E.I., Ünlü, A., and Topal, M. (2011). Determination of the Effect of Aeration Rate on Composting of Vegetable–Fruit Wastes. CLEAN – Soil, Air, Water 39(11), 1014-1021.

Abujabhah, I.S., Bound, S.A., Doyle, R., and Bowman, J.P. (2016). Effects of biocha and compost amendments on soil physico-chemical properties and the total community within a temperate agricultural soil. *Applied Soil Ecology* 98**,** 243-253.

Agegnehu, G., Bass, A.M., Nelson, P.N., and Bird, M.I. (2016). Benefits of biochar, compost and biochar–compost for soil quality, maize yield and greenhouse gas emissions in a tropical agricultural soil. *Sci Total Environ* 543**,** 295-306

Agyarko-Mintah, E., Cowie, A., Van Zwieten, L., Singh, B.P., Smillie, R., Harden, S., et al. (2017). Biochar lowers ammonia emission and improves nitrogen retention in poultry litter composting. *Waste Manage* 61**,** 129-137

Ahmad, M., Lee, S.S., Dou, X., Mohan, D., Sung, J.-K., Yang, J.E., et al. (2012). Effects of pyrolysis temperature on soybean stover- and peanut shell-derived biochar properties and TCE adsorption in water. *Bioresour Technol* 118**,** 536-544.

Ameloot, N., De Neve, S., Jegajeevagan, K., Yildiz, G., Buchan, D., Funkuin, Y.N., et al. (2013). Short-term CO2 and N2O emissions and microbial properties of biochar amended sandy loam soils. Soil Biol Biochem 57, 401-410.

Anderson, C.R., Condron, L.M., Clough, T.J., Fiers, M., Stewart, A., Hill, R.A., et al. (2011). Biochar induced soil microbial community change: implications for biogeochemical cycling of carbon, nitrogen and phosphorus. *Pedobiologia* 54(5-6)**,** 309-320.

Beesley, L., Moreno-Jiménez, E., and Gomez-Eyles, J.L. (2010). Effects of biochar and greenwaste compost amendments on mobility, bioavailability and toxicity of inorganic and organic contaminants in a multi-element polluted soil. *Environ Pollut* 158(6)**,** 2282-2287.

Bernal, M.P., Alburquerque, J.A., and Moral, R. (2009). Composting of animal manures and chemical criteria for compost maturity assessment. A review. *Bioresour Technol* 100(22)**,** 5444-5453.

Bong, C.P.C., Lim, L.Y., Ho, W.S., Lim, J.S., Klemeš, J.J., Towprayoon, S., et al. (2017). A review on the global warming potential of cleaner composting and mitigation strategies. Journal of Cleaner Production 146, 149-157.

Bru-Adan, V., Wéry, N., Moletta-Denat, M., Boiron, P., Delgènes, J.-P., and Godon, J.-J. (2009). Diversity of Bacteria and Fungi in Aerosols During Screening in a Green Waste Composting Plant. Curr Microbiol 59(3), 326-335.

Chowdhury, M.A., de Neergaard, A., and Jensen, L.S. (2014). Potential of aeration flow rate and bio-char addition to reduce greenhouse gas and ammonia emissions during manure composting. Chemosphere 97, 16-25.

Crippen, T.L., Sheffield, C.L., Singh, B., Byrd, J.A., Beier, R.C., and Anderson, R.C. (2021). Poultry litter and the environment: Microbial profile of litter during successive flock rotations and after spreading on pastureland. Sci Total Environ 780, 146413.

Chen, J., Liu, X., Zheng, J., Zhang, B., Lu, H., Chi, Z., et al. (2013). Biochar soil amendment increased bacterial but decreased fungal gene abundance with shifts in community structure in a slightly acid rice paddy from Southwest China. Applied Soil Ecology 71, 33-44.

Coban, H., Miltner, A., Centler, F., and Kästner, M. (2016). Effects of compost, biochar and manure on carbon mineralization of biogas residues applied to soil. *Eur J Soil Sci* 67(2)**,** 217-225.

Cross, A., and Sohi, S.P. (2011). The priming potential of biochar products in relation to labile carbon contents and soil organic matter status. *Soil Biol Biochem* 43(10)**,** 2127-2134.

Cui, E., Wu, Y., Zuo, Y., and Chen, H. (2016). Effect of different biochars on antibiotic resistance genes and bacterial community during chicken manure composting. Bioresour Technol 203, 11-17.

Czekała, W., Malińska, K., Cáceres, R., Janczak, D., Dach, J., and Lewicki, A. (2016). Co-composting of poultry manure mixtures amended with biochar – The effect of biochar on temperature and C-CO2 emission. *Bioresour Technol* 200**,** 921-927..

DeBruyn, J.M., Nixon, L.T., Fawaz, M.N., Johnson, A.M., and Radosevich, M. (2011). Global biogeography and quantitative seasonal dynamics of Gemmatimonadetes in soil. *Appl Environ Microbiol* 77(17)**,** 6295-6300.

Dias, B.O., Silva, C.A., Higashikawa, F.S., Roig, A., and Sánchez-Monedero, M.A. (2010). Use of biochar as bulking agent for the composting of poultry manure: Effect on organic matter degradation and humification. *Bioresour Technol* 101(4)**,** 1239-1246.

Dong, D., Yang, M., Wang, C., Wang, H., Li, Y., Luo, J., et al. (2013). Responses of methane emissions and rice yield to applications of biochar and straw in a paddy field. *J Soils Sed* 13(8)**,** 1450-1460.

Duan, Y., Awasthi, S.K., Chen, H., Liu, T., Zhang, Z., Zhang, L., et al. (2019). Evaluating the impact of bamboo biochar on the fungal community succession during chicken manure composting. Bioresour Technol 272, 308-314.

Duan, Y., Awasthi, S.K., Liu, T., Chen, H., Zhang, Z., Wang, Q., et al. (2019). Dynamics of fungal diversity and interactions with environmental elements in response to wheat straw biochar amended poultry manure composting. *Bioresour Technol* 274**,** 410-417

De Gannes, V., Eudoxie, G., and Hickey, W. (2013). Insights into fungal communities in composts revealed by 454-pyrosequencing: implications for human health and safety. *Frontiers in Microbiology* 4(164)..

Erickson, M.C., Liao, J., Boyhan, G., Smith, C., Ma, L., Jiang, X., et al. (2010). Fate of manure-borne pathogen surrogates in static composting piles of chicken litter and peanut hulls. *Bioresour Technol* 101(3)**,** 1014-1020.

Erickson, M., Liao, J., Jiang, X., and Doyle, M. (2014). Contribution of chemical and physical factors to zoonotic pathogen inactivation during chicken manure composting. *Agricult. Food Anal. Bacteriol* 4**,** 96-108.

Erickson, M.C., Smith, C., Jiang, X., Flitcroft, I.D., and Doyle, M.P. (2015). Manure source and age affect survival of zoonotic pathogens during aerobic composting at sublethal temperatures. *J Food Prot* 78(2)**,** 302-310.

Evanylo, G., Sherony, C., Spargo, J., Starner, D., Brosius, M., and Haering, K. (2008). Soil and water environmental effects of fertilizer-, manure-, and compost-based fertility practices in an organic vegetable cropping system. Agric, Ecosyst Environ 127(1), 50-58.

Grundy, A., Green, J., and Lennartsson, M. (1998). The effect of temperature on the viability of weed seeds in compost. Compost Sci Util 6(3), 26-33.

Guo, R., Li, G., Jiang, T., Schuchardt, F., Chen, T., Zhao, Y., et al. (2012). Effect of aeration rate, C/N ratio and moisture content on the stability and maturity of compost. Bioresour Technol 112, 171-178.

Gurtler, J.B., Doyle, M.P., Erickson, M.C., Jiang, X., Millner, P., and Sharma, M. (2018). Composting To Inactivate Foodborne Pathogens for Crop Soil Application: A Review. *J Food Prot* 81(11)**,** 1821-1837.

Han, I., Congeevaram, S., Ki, D.-W., Oh, B.-T., and Park, J. (2011). Bacterial community analysis of swine manure treated with autothermal thermophilic aerobic digestion. Appl Microbiol Biotechnol 89(3), 835-842.

He, Y., Xie, K., Xu, P., Huang, X., Gu, W., Zhang, F., et al. (2013). Evolution of microbial community diversity and enzymatic activity during composting. Res Microbiol 164(2), 189-198.

Farrell, M., Kuhn, T.K., Macdonald, L.M., Maddern, T.M., Murphy, D.V., Hall, P.A., et al. (2013). Microbial utilisation of biochar-derived carbon. *Sci Total Environ* 465**,** 288-297.

Feng, Y., Xu, Y., Yu, Y., Xie, Z., and Lin, X. (2012). Mechanisms of biochar decreasing methane emission from Chinese paddy soils. *Soil Biol Biochem* 46**,** 80-88.

Harter, J., Krause, H.-M., Schuettler, S., Ruser, R., Fromme, M., Scholten, T., et al. (2013). Linking N2O emissions from biochar-amended soil to the structure and function of the N-cycling microbial community. *The Isme Journal* 8**,** 660.

Jeffery, S., Verheijen, F.G.A., van der Velde, M., and Bastos, A.C. (2011). A quantitative review of the effects of biochar application to soils on crop productivity using meta-analysis. *Agric, Ecosyst Environ* 144(1)**,** 175-187.

Jiang, T., Schuchardt, F., Li, G., Guo, R., and Zhao, Y. (2011). Effect of C/N ratio, aeration rate and moisture content on ammonia and greenhouse gas emission during the composting. Journal of Environmental Sciences 23(10), 1754-1760.

Karim, K., Thomas Klasson, K., Hoffmann, R., Drescher, S.R., DePaoli, D.W., and Al-Dahhan, M.H. (2005). Anaerobic digestion of animal waste: Effect of mixing. Bioresour Technol 96(14), 1607-1612.

Kammann, C., Ratering, S., Eckhard, C., and Müller, C. (2012). Biochar and Hydrochar Effects on Greenhouse Gas (Carbon Dioxide, Nitrous Oxide, and Methane) Fluxes from Soils. *Journal of Environmental Quality* 41**,** 1052-1066.

Kolton, M., Graber, E.R., Tsehansky, L., Elad, Y., and Cytryn, E. (2017). Biochar-stimulated plant performance is strongly linked to microbial diversity and metabolic potential in the rhizosphere. *New Phytol* 213(3)**,** 1393-1404.

Krings, M., Dotzler, N., Galtier, J., and Taylor, T.N. (2009). Microfungi from the upper Visean (Mississippian) of central France: Chytridiomycota and chytrid-like remains of uncertain affinity. Rev Palaeobot Palynol 156(3), 319-328.

Kuok, F., Mimoto, H., and Nakasaki, K. (2013). Reduction of ammonia inhibition of organic matter degradation by turning during a laboratory-scale swine manure composting. *International Journal of Waste Resources*.

Kuzyakov, Y., and Blagodatskaya, E. (2015). Microbial hotspots and hot moments in soil: Concept & review. *Soil Biology & Biochemistry* 83**,** 184-199.

Lehmann, J., Rillig, M.C., Thies, J., Masiello, C.A., Hockaday, W.C., and Crowley, D. (2011a). Biochar effects on soil biota - A review. *Soil Biology & Biochemistry* 43(9)**,** 1812-1836.

Lehmann, J., Rillig, M.C., Thies, J., Masiello, C.A., Hockaday, W.C., and Crowley, D. (2011b). Biochar effects on soil biota – A review. *Soil Biol Biochem* 43(9)**,** 1812-1836

Li, Q., Wang, X.C., Zhang, H.H., Shi, H.L., Hu, T., and Ngo, H.H. (2013). Characteristics of nitrogen transformation and microbial community in an aerobic composting reactor under two typical temperatures. Bioresour Technol 137, 270-277

Li, M.-X., He, X.-S., Tang, J., Li, X., Zhao, R., Tao, Y.-Q., et al. (2021). Influence of moisture content on chicken manure stabilization during microbial agent-enhanced composting. Chemosphere 264, 128549.

Lu, J., Sanchez, S., Hofacre, C., Maurer, J.J., Harmon, B.G., and Lee, M.D. (2003). Evaluation of Broiler Litter with Reference to the Microbial Composition as Assessed by Using 16S rRNA and Functional Gene Markers. Appl Environ Microbiol 69(2), 901-908.

Lu, H., Zhang, W., Wang, S., Zhuang, L., Yang, Y., and Qiu, R. (2013). Characterization of sewage sludge-derived biochars from different feedstocks and pyrolysis temperatures. *Journal of Analytical and Applied Pyrolysis* 102**,** 137-143.

Makan, A., Assobhei, O., and Mountadar, M. (2013). Effect of initial moisture content on the in-vessel composting under air pressure of organic fraction of municipal solid waste in Morocco. Iranian Journal of Environmental Health Science & Engineering 10(1), 3.

Peer, R.W., and Flory, G.A. (2010). Verification of Poultry Carcass Composting Research through Application during Actual Avian Influenza Outbreaks. ILAR Journal 51(2), 149-157.

Macklin, K.S., Hess, J.B., Bilgili, S.F., and Norton, R.A. (2006). Effects of In-House Composting of Litter on Bacterial Levels. *The Journal of Applied Poultry Research* 15(4)**,** 531-537

Malińska, K., Zabochnicka-Świątek, M., and Dach, J. (2014). Effects of biochar amendment on ammonia emission during composting of sewage sludge. *Ecol Eng* 71**,** 474-478.

Masiello, C.A., Chen, Y., Gao, X., Liu, S., Cheng, H.-Y., Bennett, M.R., et al. (2013). Biochar and Microbial Signaling: Production Conditions Determine Effects on Microbial Communication. *Environ Sci Technol* 47(20)**,** 11496-11503.

Miner, J.R., Humenik, F.J., and Overcash, M.R. (2000). *Managing livestock wastes to preserve environmental quality.* Iowa State University Press.

Natvig, D.O., Taylor, J.W., Tsang, A., Hutchinson, M.I., and Powell, A.J. (2015). Mycothermus thermophilus gen. et comb. nov., a new home for the itinerant thermophile Scytalidium thermophilum (Torula thermophila). *Mycologia* 107(2)**,** 319-327.

Novak, J.M., Busscher, W.J., Laird, D.L., Ahmedna, M., Watts, D.W., and Niandou, M.A. (2009). Impact of biochar amendment on fertility of a southeastern coastal plain soil. *Soil science* 174(2)**,** 105-112.

Novak, J.M., Busscher, W.J., Watts, D.W., Amonette, J.E., Ippolito, J.A., Lima, I.M., et al. (2012). Biochars impact on soil-moisture storage in an ultisol and two aridisols. *Soil Science* 177(5)**,** 310-320.

Rahman, L., Whitelaw-Weckert, M.A., and Orchard, B. (2014). Impact of organic soil amendments, including poultry-litter biochar, on nematodes in a Riverina, New South Wales, vineyard. *Soil Research* 52(6)**,** 604-619..

Robledo, M., Gómez-Silván, C., Andersen, G.L., Calvo, C., and Aranda, E. (2020). Assessment of bacterial and fungal communities in a full-scale thermophilic sewage sludge composting pile under a semipermeable cover. *Bioresour Technol* 298**,** 122550.

Rui, J., Peng, J., and Lu, Y. (2009). Succession of Bacterial Populations during Plant Residue Decomposition in Rice Field Soil. *Appl Environ Microbiol* 75(14)**,** 4879-4886.

Sánchez-García, M., Alburquerque, J.A., Sánchez-Monedero, M.A., Roig, A., and Cayuela, M.L. (2015). Biochar accelerates organic matter degradation and enhances N mineralisation during composting of poultry manure without a relevant impact on gas emissions. Bioresour Technol 192, 272-279.

Sorathiya, L.M., Fulsoundar, A.B., Tyagi, K.K., Patel, M.D., and Singh, R.R. (2014). Eco-friendly and modern methods of livestock waste recycling for enhancing farm profitability. International Journal of Recycling of Organic Waste in Agriculture 3(1), 50.

Sorokin, D.Y., Abbas, B., van Zessen, E., and Muyzer, G. (2014). Isolation and characterization of an obligately chemolithoautotrophic Halothiobacillus strain capable of growth on thiocyanate as an energy source. *FEMS Microbiol Lett* 354(1)**,** 69-74.

Sun, D., Lan, Y., Xu, E.G., Meng, J., and Chen, W. (2016). Biochar as a novel niche for culturing microbial communities in composting. *Waste Manage* 54**,** 93-100.

Sun, J., Qian, X., Gu, J., Wang, X., and Gao, H. (2016). Effects of oxytetracycline on the abundance and community structure of nitrogen-fixing bacteria during cattle manure composting. Bioresour Technol 216, 801-807.

Takaku, H., Kodaira, S., Kimoto, A., Nashimoto, M., and Takagi, M. (2006). Microbial communities in the garbage composting with rice hull as an amendment revealed by culture-dependent and-independent approaches. J Biosci Bioeng 101(1), 42-50.

Thomas, C., Idler, C., Ammon, C., and Amon, T. (2020). Effects of the C/N ratio and moisture content on the survival of ESBL-producing Escherichia coli during chicken manure composting. Waste Manage 105, 110-118.

USEPA (2013) Literature review of contaminants in livestock and poultry manure and implications for water quality. EPA 820-R-13–002. Office of Water, Washington, DC

Ventorino, V., Parillo, R., Testa, A., Viscardi, S., Espresso, F., and Pepe, O. (2016). Chestnut green waste composting for sustainable forest management: Microbiota dynamics and impact on plant disease control. J Environ Manage 166, 168-177.

Wang, C., Lu, H., Dong, D., Deng, H., Strong, P.J., Wang, H., et al. (2013). Insight into the Effects of Biochar on Manure Composting: Evidence Supporting the Relationship between N2O Emission and Denitrifying Community. *Environ Sci Technol* 47(13)**,** 7341-7349.

Wang, L., Lilburn, M., and Yu, Z. (2016). Intestinal Microbiota of Broiler Chickens As Affected by Litter Management Regimens. *Frontiers in Microbiology* 7(593).

Wu, H., Zeng, G., Liang, J., Chen, J., Xu, J., Dai, J., et al. (2016). Responses of bacterial community and functional marker genes of nitrogen cycling to biochar, compost and combined amendments in soil. *Appl Microbiol Biotechnol* 100(19)**,** 8583-8591.

Wu, S., Shen, Z., Yang, C., Zhou, Y., Li, X., Zeng, G., et al. (2017). Effects of C/N ratio and bulking agent on speciation of Zn and Cu and enzymatic activity during pig manure composting. Int Biodeterior Biodegrad 119, 429-436.

Xu, H.-J., Wang, X.-H., Li, H., Yao, H.-Y., Su, J.-Q., and Zhu, Y.-G. (2014). Biochar Impacts Soil Microbial Community Composition and Nitrogen Cycling in an Acidic Soil Planted with Rape. *Environ Sci Technol* 48(16)**,** 9391-9399.

Xu, N., Tan, G.C., Wang, H.Y., and Gai, X.P. (2016). Effect of biochar additions to soil on nitrogen leaching, microbial biomass and bacterial community structure. *European Journal of Soil Biology* 74**,** 1-8.

Yamamoto, N., Otawa, K., and Nakai, Y. (2009). Bacterial Communities Developing during Composting Processes in Animal Manure Treatment Facilities. *Asian-Australas J Anim Sci* 22(6)**,** 900-905.

Yang, X., Hu, Q.Q., Han, Z.Z., Ruan, X.Y., Jiang, S.W., Chai, J., et al. (2018). Effects of exogenous microbial inoculum on the structure and dynamics of bacterial communities in swine carcass composting. Canadian Journal of Microbiology 64(12), 1042-1053.

Yao, Q., Liu, J., Yu, Z., Li, Y., Jin, J., Liu, X., et al. (2017). Three years of biochar amendment alters soil physiochemical properties and fungal community composition in a black soil of northeast China. *Soil Biol Biochem* 110**,** 56-67.

Zhang, A., Cui, L., Pan, G., Li, L., Hussain, Q., Zhang, X., et al. (2010). Effect of biochar amendment on yield and methane and nitrous oxide emissions from a rice paddy from Tai Lake plain, China. *Agric, Ecosyst Environ* 139(4)**,** 469-475.

Zhang, L., and Sun, X. (2014). Changes in physical, chemical, and microbiological properties during the two-stage co-composting of green waste with spent mushroom compost and biochar. Bioresour Technol 171, 274-284.

Zhang, L., Zeng, G., Zhang, J., Chen, Y., Yu, M., Lu, L., et al. (2015). Response of denitrifying genes coding for nitrite (nirK or nirS) and nitrous oxide (nosZ) reductases to different physico-chemical parameters during agricultural waste composting. *Appl Microbiol Biotechnol* 99(9)**,** 4059-4070.

Zhong, X.-Z., Zeng, Y., Wang, S.-P., Sun, Z.-Y., Tang, Y.-Q., and Kida, K. (2020). Insight into the microbiology of nitrogen cycle in the dairy manure composting process revealed by combining high-throughput sequencing and quantitative PCR. *Bioresour Technol* 301**,** 122760.

CHAPTER 3 – CHANGES IN MICROBIAL COMMUNTIES AND NITROGEN CYCLING FUNCTIONAL GENES DURING POULTRY LITTER IN-HOUSE WINDROW COMPOSTING

ABSTRACT

Composting is a self-heating biological process driven by microbial activity, which effectively reduces pathogens and relatively low cost. Microbial activities are vital to the composting performance and nitrogen loss. Thus, this study focused on the changes in microbial communities and nitrogen cycling capacity during poultry litter in-house windrow composting. In addition, the effects of biochar addition were evaluated as a composting additive. Two in-house windrow composting trails (growouts) were conducted in a commercial poultry farm, and the abundances of N-cycling functional genes (*nif*H, *amo*A, *nir*S, *nir*K and *nos*Z) were measured using quantitative PCR (qPCR). Bacterial and fungal community composition in poultry litter was evaluated using 16S and ITS amplicon sequencing. Our results indicated that composting decreased moisture content in two growouts, but did not show any significant influence on other physical-chemical properties except for elevated nitrate (NO_3^--N) concentration in growout 3. In-house windrow composting significantly influenced poultry litter bacterial community structure and richness in both growouts; while fungal community structure was only affected in growout 3. Alpha diversity of fungal communities did not change during either growout. Low amounts of biochar amendment did not influence microbial community structure, diversity nor the relative abundances of dominant microbial taxa, including *Bacillales* (Phylum Firmicutes), *Actinomycetales* (Phylum Actinobacteria), and *Saccharomycetales*, *Microascales*, and *Eurotiales* (Phylum Ascomycota). Abundances of all measured N-cycling functional genes did not change in growout 1, but in growout 3, *amo*A, *nir*S, *nir*K and *nos*Z

increased while *nif*H decreased. Low amounts of biochar addition did not affect microbial community structure, but increased abundances of denitrification genes (*nir*K, *nir*S and *nos*Z) in growout 1. In addition, NO_3^--N, total nitrogen (TN), total carbon (TC) and temperature affected bacterial communities. Fungal community structure was most related to water extractable carbon and nitrogen (WEC/WEN), NO_3^--N, NH_4^+-N, TN and temperature. N-cycling functional gene abundances were related to NO_3^--N and temperature. Our analysis revealed the importance of temperature and nitrogen on driving microbial community structure and N-cycling functions during poultry litter composting.

INTRODUCTION

Composting is a self-heating biological process driven by microbial activity. This process decomposes complex organic matter waste into simpler molecules. Windrow composting is a large-scale composting in which large amounts of organic waste are built up as long piles. Composting results in changes in physical and chemical properties, including increased pH and temperature in the thermophilic stage, and decreased moisture content (Wang et al., 2018). Studies have shown that these physical and chemical factors shape microbial community structure, diversity and functions in compost systems (He et al., 2013; Wang et al., 2018). Specifically, bacterial community diversity in composted poultry litter was affected by the incubation time, pH, moisture, and NH_4^+-N concentration (Bucher et al., 2020). He et al. (2013) suggested the bacterial community structure is positively correlated with pH, total nitrogen (TN), and carbon to nitrogen ratio (C: N), but the diversity has a negative correlation with organic carbon

content in chicken manure and rice-mushroom residue composting. In a poultry and cow manure composting, the microbial community was most shaped by the temperature, C: N ratio, and bulk density (Smith et al., 2014). During green waste composting, the changes in bacterial communities were attributed to humic acid contents which can be degraded by microbes and temperature (Liu et al., 2018). Therefore, even though environmental factors affect microbial community, the dominant driving factors in shaping microbial community are inconsistent between studies. Thus, it is necessary to have a deep understanding of the relationship between microbial community and environmental factors.

Previous studies have described the succession of bacterial communities in various types of composting, including poultry manure (Cui et al., 2016; Duan et al., 2019; Duan et al., 2019), pig manure (Awasthi et al., 2020) and household waste (Liu et al., 2018). However, changes in microbial communities were not consistent between studies. In green waste composting, the dominant phyla were Firmicutes, Actinobacteria, Chloroflexi, and Proteobacteria; over the course of composting the relative abundance of Firmicutes was reduced while Chloroflexi and Proteobacteria increased (Liu et al., 2018). However, the dominance of Chloroflexi has not been confirmed in other studies. Firmicutes and Proteobacteria were the dominant bacteria in poultry manure composting (Cui et al., 2016) and pig manure composting (Awasthi et al., 2020). The dominance of Bacteroidetes also was identified in the poultry manure composting (Cui et al., 2016). Successions of fungal communities during manure composting has also been described. Ascomycota was the dominant fungal phyla in

poultry manure composting (Duan et al., 2019a) and cow manure composting (Wang et al., 2018). Duan et al. (2019b) indicated that Chytridiomycota was the predominant phylum in poultry manure composting, followed by Mucoromycota, Ascomycota and Basidiomycota. Awasthi et al. (2021) suggested Basidiomycota was the dominant fungi, followed by Ascomycota, in poultry manure and wheat straw composting. A similar result was shown by Li et al. (2020) for pig manure and sawdust composting. The different results are likely due to the different feedstock types (animal waste vs. green waste) and composting methods (general composting vs. composting with amendments).

One side effect of composting is nitrogen (N) loss, such as NH_3 and N_2O emissions. High NH_3 emissions during composting lead to N loss that reduced the quality of final composted products as fertilizer, and also negatively influences the growth and production of chickens (Williams Ischer et al., 2017). Nitrification and denitrification are the two important N cycling processes in animal waste composting that can control the fate of N. Ammonia-oxidizing bacteria (AOB) are the dominant nitrifiers in composting (Maeda et al., 2011), and ammonia monooxygenase which is coded by *amo*A gene is the rate limiting enzyme in nitrification. Denitrifiers perform denitrification via nitrate reductases; two common marker genes for denitrifiers are *nir*S and *nir*K, encoding for subunits of two types of nitrite reductases containing heme iron and copper in the structures, respectively. Previous studies suggested the importance of *nir*K rather than *nir*S denitrifiers in N_2O production during cow manure composting

(Maeda et al., 2017). The last step in the denitrification is processed by nitrous oxide reductase (*nos*Z), which transforms N_2O to N_2 (Maeda et al., 2011).

Biochar has been suggested as an additive for composting. Biochar may absorb NH_3 and $NH4^+$-N, further reducing N loss (Steiner et al., 2010). Biochar amendment may have positive effects on compost heating performance, which may remove more pathogens and degrade more organic wastes. Steiner et al. (2010) observed that the peak temperature in poultry litter-pine chips biochar composting (20%, w/w) is 65. 1 °C, which is higher than the composting without biochar (58.2 °C). Peak temperature in poultry manure and green waste composting did not exceed 55 °C, but the highest temperature was over 65 °C in the composting with 2% (v/v) biochar addition (Jindo et al., 2012). While there has been less research on microbial communities in biochar-amended compost, other studies have investigated biochar as a soil amendment. Previous studies in soil showed that 2% and 10% (w/w) biochar addition to water-saturated soil did not affect either bacterial or archaeal *amo*A abundance, a gene encoding ammonium monooxygenase which catalyzes a key step in the nitrification process. In contrast, biochar addition increased the copy numbers of *nos*Z, a gene coded nitrous-oxide reductase in denitrification (Harter et al., 2013). The other important process in N-cycling is nitrogen fixation. A prior study suggested *nifH* gene abundance decreased in the later stage of cattle manure composting (Sun et al., 2016). Liu et al. (2019) suggested an increased abundance of *nifH* would increase the nitrogen content in composting. Previous studies have suggested that biochar addition to compost

decreased diazotroph community diversity (Wu et al., 2020) but increased *nifH* abundance in soil (Harter et al., 2013).

Although composting has long been used as a waste management approach for poultry litter, and microbes are a key to waste conversion and nutrient cycling, most previous studies focus on microbial communities in simulated composting and pilot-scale composting, and there is limited research on microbial communities during poultry litter composting at field scale (*i.e.*, in an operational poultry house). In this study, we investigated the effects of in-house windrow composting on microbial communities and N-cycling functional genes in the poultry litter from a commercial farm. We also evaluated the addition of biochar (1%) to windrow composting as an additive. The main research goals of this study were to 1) evaluate the changes in microbial communities and N-cycling functional genes during poultry litter in-house windrow composting; 2) determine if biochar amendment changes the poultry litter properties and microbial community dynamics; and 3) identify the effects of physical and chemical properties of the poultry litter on microbial community structures, relative abundances of dominant taxa and N-cycling functional genes. The understanding of microbial communities and functions will provide more information about how to improve the performance of in-house windrow composting.

MATERIALS AND METHODS

Site Description and Sampling

The study was conducted at four commercial broiler houses (17 m * 152 m) in Cleveland, Tennessee, U.S.. Each house was divided into brood and non-brood (grow) ends. During the first 7-10 days, birds were raised in the brood end then the house was opened up, so that bird also filled the non-brood end. Between flocks, poultry litter was subject to windrow composting. Two or three turns were conducted during each windrow composting; the first turn was 5-7 days after windrows were formed and the following occurred every 3-5 days later. Two types of biochar were added to house 2 and house 3, respectively (Table 3-1). For each biochar-added house, 4000 lbs biochar (dry mass) was added to the top of poultry litter, which was the equivalent of about 1% addition before the first flock was placed. The remaining two houses (house 1 and house 4) did not receive biochar as an amendment. Four flock growouts followed by windrow composting occurred in each house, samples were collected during windrow composting between flocks 1 and 2 (growout 1) and flock 3 and 4 (growout 3).

Poultry litter samples were collected at four different dates: 1) October 25th, 2017, at the end of growout but a few days before composting, 2) November 15th, 2017 after the first composting was completed and before new birds were placed, 3) February 19th, 2018, after flocks left and acidifier added to brood end; 4) March 8th, 2018 after the third composting was completed. Two individual samples were collected from the brood and non-brood end separately. Each sample was a composite of poultry litter taken from the piles. Poultry litter moisture content, pH, water extractable carbon (WEC)

and nitrogen (WEN), nitrate-N (NO_3^--N), ammonium-N (NH_4^+-N), total carbon (TC) and total nitrogen (TN) were measured. Fresh poultry litter was oven-dried at 105 °C for at least 12 h until the weight was constant. Poultry litter pH was measured using a pH meter (poultry litter: Milli-Q water=1:3). A 2M KCl extraction was applied to measure inorganic nitrogen (NO_3^- -N and NH_4^+-N) in air-dried poultry litter (poultry litter: KCl solution=1:10), then the extractions were measured using Skalar automated analyzer (Sun Plus; Skalar‑Analytical, Breda, the Netherlands). To measure WEC and WEN, air-dried poultry litter and Milli-Q water were mixed as a ratio of 1:4, shaken for 4 h, and filtered using filter paper, then measured using a vario TOC cube (Elementar, Langenselbold, Germany). Dry poultry litter samples were ball-milled using TissueLyser LT system (Qiagen, USA), then the concentrations of poultry litter TC and TN were measured using the Vario Max cube CN elemental analyzer (Elementar, Hanau, Germany).

Poultry litter DNA extraction and sequencing

To improve extraction efficiency, 10 g poultry litter samples were first ground using blender. Four grindings were applied for 5 s each to avoid excessive heat generation. DNA was extracted using DNeasy PowerLyzer PowerSoil Kit (Qiagen, USA) according to the user's guide. Amplicon libraries were prepared for sequencing on Illumina MiSeq platform, targeting the fungal Internal Transcribed Spacer 2 (ITS2) region and bacterial V4 region. Two-step PCR was used for amplicon libraries. In the first step, DNA samples were amplified using target primers in KapaHifi Master Mix

with 20 cycles. In the second step, Illumina-compatible adapters were added to index. Magnetic beads were applied to purify PCR products. For fungi, a pool of forward primers was used to amplify fungal ITS2: ITS3NGS1 5'-CATCGATGAAGAACGCAG-3', ITS3NGS2 5'-CAACGATGAAGAACGCAG-3', ITS3NGS3 5'- CACCGATGAAGAACGCAG-3', ITS3NGS4 5'-CATCGATGAAGAACGTAG-3', ITS3NGS5 5'- CATCGATGAAGAACGTGG-3', ITS3NGS10 5'- CATCGATGAAGAACGCTG-3', and reverse primers ITS4NGR 5'-TCCTSCGCTTATTGATATGC-3', ARCH-ITS4 5'- TCCTCGCCTTATTGATATGC-3' (Tedersoo et al., 2014). A set of primers, 515F (5'- GTGCCAAGCAGCCGCGGTAA-3') and 806R (5'- GGACTACHVGGGTWTCTAAT-3'), was used to amplify the bacterial V4 region. Finally, sequencing was performed using 300 PE (pair-end) at the University of Tennessee Genomics Core laboratory on the Illumina MiSeq platform (Illumina, USA) and 250 PE at HudsonAlpha Institue for Biotechnology, Huntsville, AL. Raw data was processed in mothur v.1.42.3 based on the mothur MiSeq SOP (Kozich et al., 2013). Make.contigs command was used to merge forward and reverse reads. Sequences were filtered to remove the overhangs at the ends. To denoise sequences, two nucleotide differences were set as the threshold to pre-cluster sequences. Sequences were filtered Chimeras were removed using VSERACH. Operational taxonomic units (OTUs) were clustered at 97% similarity using aligned sequences and classified against using SILVA132 and UNITE v.8 reference database for bacteria and fungi respectively. Sequences are available in the NCBI SRA under BioProject PRJNA702156.

Table 3- 1 Basic properties of biochar L and biochar P applied in this study (Wang, 2019)

Properties	Biochar L	Biochar P
Moisture content (%, wet basis)	54.1 ± 2	9.8 ± 1
Ash content (%, wet basis)	3.0 ± 0.4	7.3 ± 1
TC (%, wet basis)	83.1 ± 5.6	85.3 ± 1.7
TN (%, wet basis)	1.1 ± 0.1	0.8 ± 0.1
BET surface areas (m^2/g)	278.9 ± 0.6	295.1 ± 0.44
PV (cm^3/g)	0.079 ± 0.002	0.83 ± 0.002

*TC: total carbon; TN: total nitrogen; BET surface area: Brunauer, Emmett and Teller surface area; PV: pore volume.

Quantitative PCR

Quantification of bacterial 16S rRNA genes (Femto Bacterial DNA Quantification Kit), fungal ITS rRNA genes (Femto Fungal DNA Quantification Kit) were performed according to the manufacturer's directions on CFX96 Optical Real-Time Detection System (Bio-Rad, Laboratories Inc., Hercules, CA, USA). The abundance of *amo*A, *nif*H, *nir*K, *nir*S, and typical *nos*Z harbored in microbial organisms were used as indicators for the potential ability to consume and produce different forms of nitrogen. Functional marker genes related to nitrogen cycling (*amo*A, *nir*K, *nir*S, *nif*H, and *nos*Z) were quantified using qPCR with gene-specific primers (Table 3-2) in Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific, USA) as described in Hu et al. (2021). PCR cycling conditions for *amoA* were: 3 min at 95℃, followed by 1 min at 94℃, 45 s at 56℃ and 1 min at 72℃ with 40 cycles, and 72℃ for 10 min as extension step. *amo*A reactions contained 10 µl Master Mix, 0.5 µl (10 µm) for each primer, 3 µl DNA and 6 µl nuclease-free water, for a final volume of 20 µl. PCR cycling conditions for *nif*H were: 10 min at 95℃, followed by 30 s at 95℃, 1 min at 58℃ and 1 min at 72℃ with 40 cycles, and 72℃ for 10 min as extension step. *nif*H reactions contained 10 µl Master MIx, 1 µl (10 um) for each primer, 2.5 µl DNA and 5.5 µl nuclease-free water, for a final volume of 20 µl. PCR cycling conditions for *nir*S were: 10 min at 95℃, followed by 30 s at 94℃, 1 min at 57℃ and 1 min at 72℃ with 40 cycles, and 72℃ for 10 min as extension step. PCR cycling conditions for *nir*K were 2 min at 94℃, followed by 30 s at 94℃, 1 min at 58℃ and 1 min at 72℃ with 40 cycles, and 72℃ for 10 min as extension step. PCR cycling conditions for *nos*Z were: 5 min at 94℃, followed by 40 s at 94℃, 40 s at 60℃ and 1 min at 72℃ with 40 cycles, and 72℃ for 10 min as extension step. TOPO TA Cloning Kit (Thermo Scientific, USA) was used to make the plasmids with target genes to use as standards. The standard curves for each

assay were generated by preparing 10-fold serial dilutions (10^2-10^8 copies μl^{-1}) of plasmid containing cloned target genes. The copy number of each functional gene was be converted to per fresh weight litter.

Statistical Analysis

Pearson's correlation analysis was performed to examine the relationships between environmental factors and abundance of functional genes and relative abundance of dominant taxa. Bonferroni method was used to adjust the *P* values. The effects of biochar treatment and composting on poultry litter physico-chemical properties, the abundance of functional genes, and the relative abundance of dominant microbial taxa were examined using one-way ANOVA by R software (version 3.6.1). Multiple regression on distance matrices (MRM) was applied to identify the dominant factors driving the bacterial and fungal communities. Variance partitioning analysis (VPA) was used to determine the relative contribution of the environmental factors to microbial communities. Non-metric Multi-dimensional Scaling (NMDS) based on Bray-Curtis distances between the abundance of functional genes and the relative abundance of dominant microbial taxa were visualized using “vegan”, “ggplot2” and “phylotype” packages, respectively. Redundancy analysis (RDA) was used to test the relationship between the abundance of N-cycling functional genes and environmental factors by “vegan” in R. Additionally, canonical correspondence analysis (CCA) was conducted to identify the abiotic factors that are significantly related to poultry litter communities, then combined the detected data with temperature to variation portioning analysis in R environment. FUNGuild was used to determine the trophic mode of fungi during poultry litter composting.

Table 3- 2 Primers used for quantitative PCR

Gene	oligo	reference
*nif*H	DVV:5’- ATIGCRAAICCICCRCAIACIACRTC-3’ IGK3: 5’- GCIWTHTAYGGIAARGGIGGIATHGGIAA-3’	Gaby and Buckley, 2012
*amo*A	amoA-1F: 5′-GGGGTTTCTACTGGTGGT-3′ amoA-2R: 5′-CCCCTCKGSAAAGCCTTCTTC-3′	Zhang et al., 2011
*nir*K	FlaCu: 5'-ATCATGGTSCTGCCGCG-3' R3Cu: 5'-GCCTCGATCAGRTTGTGGTT-3'	Henry et al., 2004
*nir*S	nirS4F: 5'-GTSAACGTSAAGGARACSGG-3' R3cd: 5'-GASTTCGGRTGSGTTTGA-3'	Throback et al., 2004
*nos*Z (typical *nos*Z)	nosZ-I-F(1840F) : 5’-CGC RAC GGC AAS AAG GTS MSS GT-3’nosZ-I-R(2090R): 5’-CAK RTG CAK SGC RTG GCA GAA-3’	Henry et al., 2006

RESULTS

Effect of windrow composting on poultry litter physico-chemical properties

To evaluate the effects of composting on poultry litter physico-chemical properties, samples were assigned as groups without the consideration of biochar treatment and control group (Table 3-3). In this study, composting did not change the poultry litter pH. Poultry litter moisture significantly decreased in each growout ($P < 0.01$, *T*-test). The much higher moisture content in February (the beginning of growout 3) may have been caused by water addition that occurred in growout 2. The NO_3^--N concentration significantly increased as a result of composting in growout 3 ($P < 0.05$). No significant changes were detected in the other measured environmental factors. In both growout 1 and 3, reductions of mean poultry litter NH_4^+-N concentration were found but without a significant decrease. Poultry litter NH_4^+-N concentrations were over 20000 mg kg^{-1} in poultry litter from growout 3, which was approximately four times higher than growout 1(< 5000 mg kg^{-1}). The properties of poultry litter did not show any significant changes under biochar addition with the exception of a slight reduction in TC in growout 3 in the biochar amendment composting ($P < 0.05$) (Table S3-1).

Effects of composting on poultry litter microbial communities

Bacterial and fungal diversity showed different trends during in-house windrow composting. The richness of bacterial community significantly decreased as a result of growout 1 composting but increased after growout 3 composting ($P < 0.05$) (Figure 3-1A). Bacterial diversity (Shannon index) significantly decreased in growout 1 (Figure 3-1B). Composting did not have a significant influence on fungal diversity (Figure 3-1C and D). Biochar addition did not influence bacterial and fungal alpha diversity indices (Table S3-2).

Table 3- 3 Poultry litter physical and chemical properties during in-house windrow composting. Shown are the mean values ± standard error (n =8). Statistically significant difference was described using *T*-tests, asterisk indicates a significant difference between before composting and after composting ($P < 0.05$). Bond fonts indicate significant values during composting.

		pH	moisture %	NO_3^--N mg kg^{-1}	NH_4^+-N mg kg^{-1}	TC g kg^{-1}	TN g kg^{-1}	C/N	WEC mg kg^{-1}	WEN mg kg^{-1}	WEC/WEN
Growout1	Before composting (Oct)	8.09±0.03	0.40±0.01	167.77±13.95	4320.23±125.14	331.43±2.83	33.12±0.22	10.02±0.11	27171.56±1542.03	5261.31±281.14	5.15±0.03
	After composting (Nov)	8.30±0.0	**0.30±0.01***	144.47±7.39	2772.50±77.27	327.54±1.69	33.11±0.33	9.91±0.11	20645.55±1169.81	3901.11±207.43	5.26±0.04
Growout3	Before composting (Feb)	8.12±0.06	0.58±0.01	69.28±574	28079.50±746.92	318.19±2.53	35.87±0.41	8.89±0.12	54672.14±568.42	9477.50±99.77	5.78±0.09
	After composting (Mar)	7.95±0.13	**0.35±0.01***	**164.19±15.3***	23754.09±1193.36	297.99±4.78	33.61±0.43	8.93±0.24	56040.22±925.56	9069.47±169.19	6.23±0.16

Pearson correlation was applied to test the alpha diversity and the environmental factors in poultry litter composting. In growout 1 (Table S3-5), bacteria richness and Shannon indexes showed significantly positive correlations with WEC, WEN, and NO_3^--N, but showed significantly negative correlations with temperatures ($P < 0.05$). Fungal alpha diversity indexes showed significantly negative correlations with WEC and WEN which were opposite to bacterial diversity ($P < 0.05$) (Table S3-5). In growout 3 (Table S3-6), poultry litter NO_3^--N showed a significantly positive correlation with bacterial richness index ($P < 0.05$), but showed a significantly negative correlation with bacterial Shannon index. Moisture content was significantly positive correlated with bacterial Shannon index ($P < 0.05$) but significantly negative correlated with richness ($P < 0.05$). Fungal Shannon index did not have any significantly correlations with any poultry litter properties in growout 3. When growout 1 and 3 were combined together, WEC, WEN, WEC: WEN, and NO_3^--N showed significantly positive correlations with bacterial richness ($P < 0.05$), but temperature and heating duration showed significantly negative correlations with bacterial Shannon index ($P < 0.05$). Fungal richness only had a significantly positive correlation with WEC: WEN ($P < 0.05$). Shannon index did not have any significant correlation with poultry litter properties (Table S3-4).

Composting significantly changed bacterial community structure in each growout (Figure 3-2) (ADONIS, $P < 0.05$). The fungal community structure was significantly altered in growout 3 only (Figure 3-2) (ADONIS, $P < 0.05$). Before composting, the initial bacterial and fungal communities in each growout were significantly different (Oct in growout 1 vs. Feb in growout 3). However, after composting, the microbial communities became much more similar, especially in the fungal community (Nov in growout 1 vs. Mar in growout 3), indicating the composting influenced the microbial

community structure. Low biochar addition did not significantly alter the microbial communities during poultry litter composting (Table S3-3).

Across all the samples, the dominant bacterial phyla were Firmicutes (average relative abundance 68.57%), Actinobacteria (28.58%), Bacteroidetes (1.02%) and Proteobacteria (1.00%) (Figure S1). The relative abundance of Actinobacteria significantly decreased in each growout ($P < 0.0.5$). Composted poultry litter had a higher relative abundance of Firmicutes ($P < 0.05$). Dominant bacterial classes were *Bacilli* (65.53%) and *Actinobacteria* (28.58%), followed by *Clostridia* (2.52%), *Gammaproteobacteria* (0.73%) and *Flavobacteria* (0.49%). Further evaluations of the dominant order showed a lower abundance of *Actinomycetales* (Figure 3-3B), *Lactobacillales* (Figure 3-3C), and *Clostridials* (Figure 3-3D) after poultry litter composting ($P < 0.01$), but a significant increase ($P < 0.01$) in the relative abundance of *Bacillales* in each growout (Figure 3-3A). Across all the samples, the fungal communities were dominated by Ascomycota (> 98%), followed by Basidiomycota (0.72%) and Mucoromycota (0.22%). No significant differences were observed among poultry litter composting at the phylum level with respect to the relative abundance of Ascomycota and Basidiomycota across the two growouts. Within this phylum, *Eurotiomycetes* was the dominant class, followed by *Sordariomycetes*, *Saccharomycetes*, and *Dothideomycetes* (> 1%). At the order level, the relative abundance of *Saccharomycetales* (Figure 3-4A) significantly decreased ($P < 0.05$), but the relative abundance of *Euritiales* (Figure 3-4B) significantly increased in growout 3 ($P < 0.05$). The rest of the other dominant fungal taxa included *Microascales* (Figure 3-4C), *Glomerellales* (Figure 3-4D), *Hypocreales* (Figure 3-4E), and *Onygeriales* (Figure 3-4F), however, these taxa remained unchanged in relative abundance in the growouts. FUNGuild results suggested species *Diutina_catenulata* (Order:

Saccharomycetales), *Microascus_verrucosu* and *Microascus_brevicaulis* (Order: Microascales), *Aspergillus_ruber, Aspergillus_insolitus,* and *Aspergillus_awamori* (Order: Eurotiales) are potential saprotrophic fungi (Table S3-7).

Multiple regression on distance matrices (MRM) was applied to identify the dominant factors explaining the variation in bacterial and fungal community structures. Considering growout 1 and 3 together, WEC was the shared factor significant for both bacterial and fungal communities ($P < 0.05$). NH_4^+-N and moisture also had significant effects on bacterial community structure ($P < 0.01$), while TN and temperature showed significant effects on fungal community structure ($P < 0.05$) (Table 3-4). However, the important environmental factors were different in each growout. NO_3^--N and temperature were the factors that influence the bacterial community in growout 1 and 3 (Table 3-5). No shared factor was found for in fungal communities between the two growouts. Fungal community structure in growout 1 was shaped by NO_3^--N and TN ($P < 0.05$). In growout 3, NH_4^+-N ($P < 0.01$), WEC/WEN, and temperature ($P < 0.05$) were the factors that had significant effects on the fungal community structure (Table 3-6).

To further quantify the contribution of poultry litter properties and environmental factors on bacterial and fungal community structures, variation partitioning analysis (VPA) was conducted with a subset of poultry litter properties that had significant relationships ($P < 0.05$) with the poultry litter microbial community structure that were identified using CCA. For the all poultry litter bacterial communities (growout 1 and growout 3), pH and moisture content were the remaining factors after variable selection. Temperature was the only selected factor that has a significant influence on bacterial community in growout 1 ($P < 0.01$). In growout 3, the total contribution of properties and temperature was 81.0%. Temperature and heating duration explained 37.0% variation ($P < 0.01$) and selected poultry litter properties (WEC, WEN, WEC: WEN

and pH) explained about 14.98% of the variation ($P < 0.01$) of bacterial community. For the fungal community (growout 1 and growout 3), the combination of heating duration and the selected poultry litter physico-chemical properties (WEC, WEN, NO_3^--N, C: N) explained 33.53% of the variation in fungal communities ($P < 0.01$), while the selected properties and heating duration explained 18.57% ($P < 0.01$) and 27.09% ($P < 0.01$) respectively. In growout 1, the combination of selected properties (NO_3^--N, TC, TN, C: N), temperature and heating duration explained 32.79% ($P < 0.05$) of the variation of fungal community. In growout 3, temperature and WEC, the only selected property, showed a significant influence on fungal community structure and explained 31.33% of the variation ($P < 0.01$), while temperatures and WEC explained 22.08% ($P < 0.01$) and 11.45% ($P < 0.01$) respectively (Figure S2).

Pearson correlation results showed that the dominant bacterial taxa, Firmicutes and *Bacillales* were significantly positive correlated with temperature ($P < 0.05$), heating duration ($P < 0.01$), and pH ($P < 0.05$), but negative correlated with NH_4^+-N ($P < 0.05$), and WEN ($P < 0.05$) in growout 1 (Table S3-8) In growout 3, the same taxa were positive correlated with NO_3^--N instead of NH_4^+-N (Table S3-9). For the abundance of dominant fungi, Ascomycota showed significantly positive correlations with WEC, WEN, and NH_4^+ -N ($P < 0.05$), but negative correlations with temperature and heating duration ($P < 0.01$) in growout 1 (Table S3-10). *Saccharomycetales* (phylum: Ascomycota) was negatively correlated with WEC: WEN ratio and pH ($P < 0.01$), but was positive correlated with NH_4^+-N ($P < 0.05$) in growout 1. *Eurotiales* had a significantly negative correlation with NO_3^--N ($P < 0 .01$). In growout 3, *Saccharomycetales* and *Eurotiales* showed significantly negative and positive correlations with temperature respectively ($P < 0.05$) (Table S3-11). These results

indicated that temperature, heating duration, and nitrogen formation had significant correlations with the relative abundances of the dominant microbial groups.

Abundances of bacteria, fungi, and N-cycling functional genes in poultry litter composting

Total bacterial abundance was not significantly changed in each growout (Figure 3-5 A and B), but composting significantly decreased fungal abundance (Figure 3-5 C and D) ($P < 0.05$). For N-cycling functional genes, the copies of *amo*A (Figure 3-6A), *nos*Z (Figure 3-6F), *nir*K Figure 3-6E), *nir*S (Figure 3-6D) and *nos*Z/(*nir*S+*nir*K) (Figure 3-6C) increased as a result of composting for growout 3. A *nos*Z/(*nir*S+*nir*K) ratio was used to reflect the status of denitrification, increased ratio implied the increased conversion from N_2O to N_2. N-cycling functional genes did not show any significant changes as a result of composting in growout 1 (Figure 3-6).

To test the effects of biochar addition, the changes in abundance of N-cycling functional genes and total bacteria (16S rRNA genes) were calculated as final abundance minus initial abundance. In these two growouts, the effects of biochar addition on the changes of microbial abundances and N-cycling functional genes were different. In growout 1, biochar addition significantly decreased *amo*A gene copies (Figure 3-8D), but increased the gene copies of denitrification genes (Figure 3-8A, B and C), including *nos*Z, *nir*S and *nir*K ($P < 0.05$), compared to no biochar addition. In growout 3, there was a greater decrease in *nif*H (Figure 3-8E) and fungal abundance (relative to ITS) ($P < 0.01$) in composting without biochar addition (Figure 3-7D).

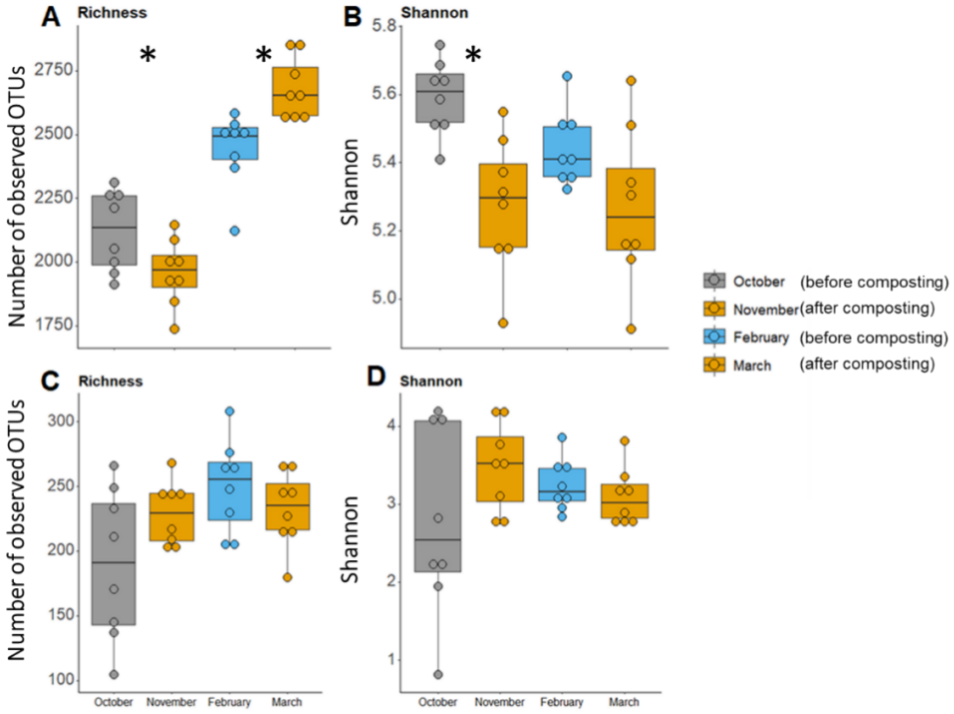


Figure 3- 1 Alpha diversity in poultry litter bacterial (upper panel, A: bacterial richness, B: bacterial Shannon index) and fungal communities (lower panel, C: fungal richness, D: fungal Shannon index) from growout 1 (October and November) and 3 (February and March). Statistically significant differences were determined between before and after composting samples using *T*-tests. Asterisk indicates significant difference ($P < 0.05$) between before and after composting.

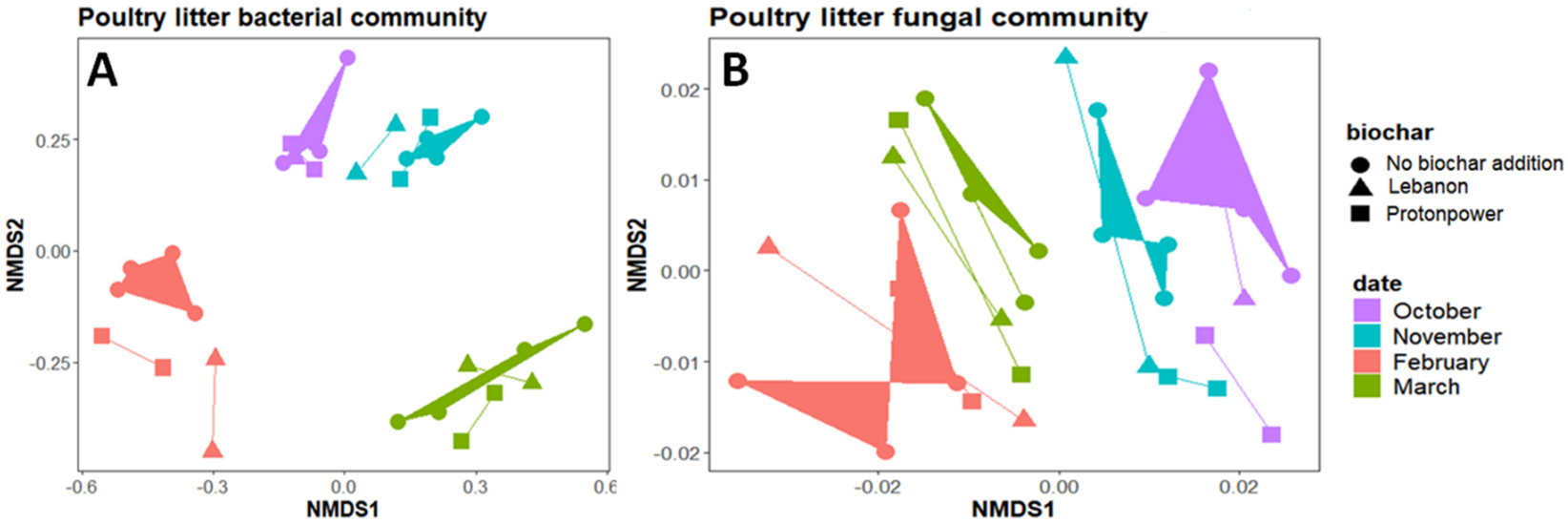


Figure 3- 2 Variation in bacterial (A) and fungal (B) community structure in growout 1 and 3, as revealed by Non-Metric Multidimensional Scaling (NMDS) of Bray-Curtis distances. Lines indicate paired samples from the same house. Filled areas connect the samples from the no biochar addition houses.

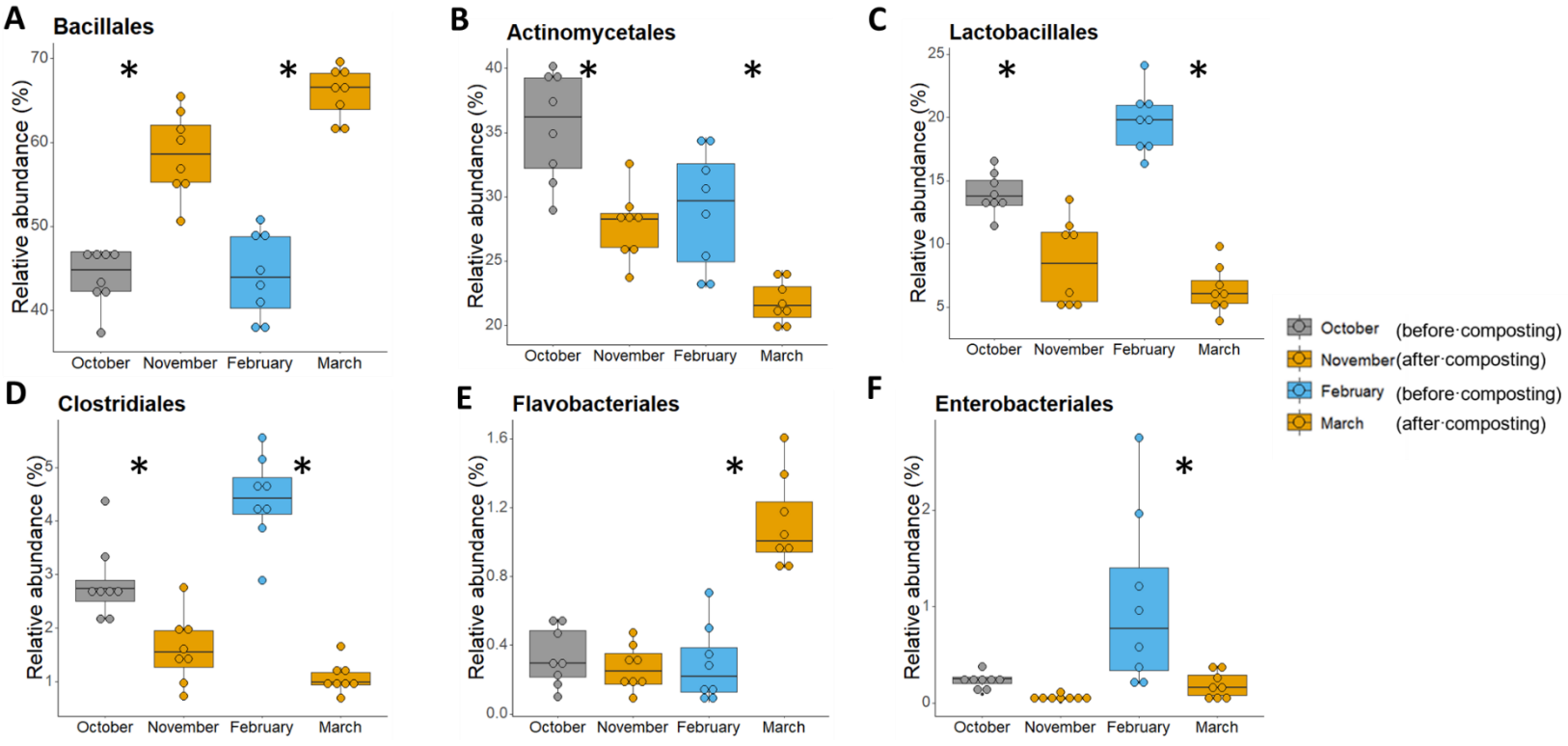


Figure 3- 3 Changes of the relative abundance of dominant bacterial orders in poultry litter as a result of composting. Growout 1: October and November. Growout 3: February and March. Statistically significant difference was determined using *T*-tests, asterisk indicates significant differences ($P < 0.05$) between before (gray and blue) and after composting (gold).

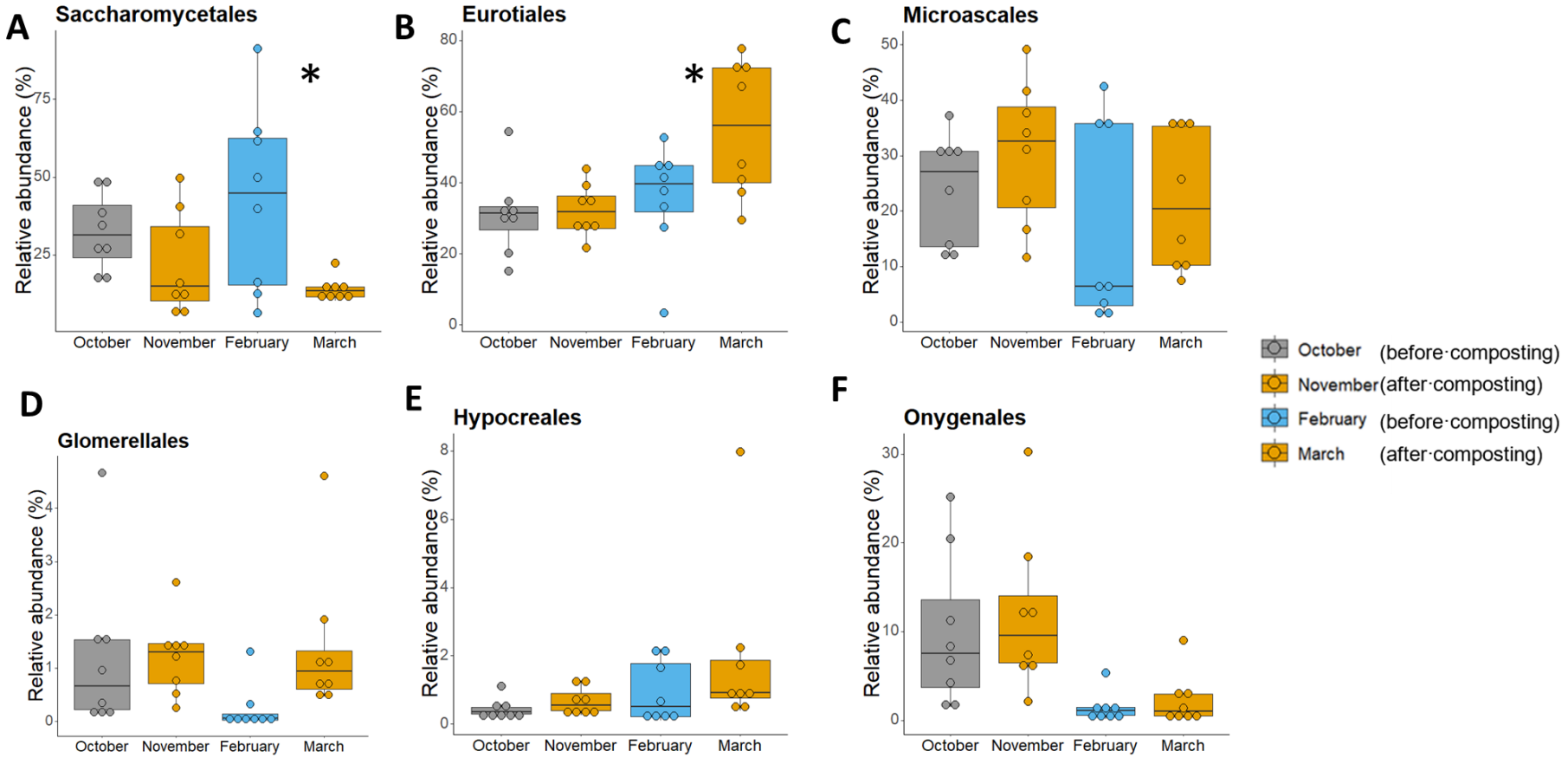


Figure 3- 4 Changes of the relative abundance of dominant fungal orders in poultry litter as a result of composting. Growout 1: October and November. Growout 3: February and March. Statistically significant difference was decided using T-test. Asterisk indicates significant differences ($P < 0.05$) between before (gray and blue) and after composting (gold).

Table 3- 4 Multiple regression with distance matrices (MRM) was applied to evaluate the importance of poultry litter properties and environmental factors on bacterial and fungal community structures in two growouts (growout 1 and 3 combined). Values indicated the matrix with regression coefficients and associated *P* values from the permutation test. Bold fonts and asterisks indicate significant effects. *: $P < 0.05$, **: $P < 0.01$

	Bacteria		**Fungi**	
	(R^2= 0.5103, F= 50.54, *P*= 0.001)		(R^2= 0.323, F= 25.17, *P*= 0.001)	
	Coefficient	*P*	Coefficient	*P*
WEC	1.60×10^{-6}	**0.005****	1.19×10^{-6}	**0.010***
WEN	-3.56×10^{-6}	0.305	-1.42×10^{-7}	0.962
WEC/WEN	1.08×10^{-2}	0.084	-3.01×10^{-3}	0.553
NO_3^--N	1.69×10^{-5}	0.707	-1.21×10^{-5}	0.735
NH_4^--N	2.36×10^{-7}	**0.001****	-1.60×10^{-8}	0.834
TN	-1.43×10^{-3}	0.521	-4.21×10^{-3}	**0.014***
TC	3.75×10^{-4}	0.096	-9.05×10^{-5}	0.615
C/N	-4.33×10^{-3}	0.354	7.02×10^{-3}	0.071
pH	3.51×10^{-2}	**0.001****	1.18×10^{-2}	0.098
moisture	1.95×10^{-4}	**0.001****	-1.85×10^{-4}	0.996
temperature	-4.84×10^{-5}	0.268	1.12×10^{-4}	**0.005****
duration time	-1.72×10^{-3}	0.469	9.65×10^{-4}	0.508

Table 3- 5 Multiple regression with distance matrices (MRM) was applied to evaluate the importance of poultry litter properties and environmental factors on bacterial community structures in growout 1 and 3. Values indicated the matrix with regression coefficients and associated P values from the permutation test. Bold fonts and asterisks indicate significant effects. *: $P < 0.05$, **: $P < 0.01$

	Bacteria_Growout1		Bacteria_Growout3	
	(R^2= 0.5855, F= 12.59, *P*= 0.001)		(R^2= 0.82, F= 40.32, *P*= 0.001)	
	Coefficient	*P*	Coefficient	*P*
WEC	-3.80×10^{-6}	0.171	1.48×10^{-6}	0.061
WEN	1.74×10^{-5}	0.238	-3.81×10^{-6}	0.339
WEC/WEN	5.61×10^{-3}	0.791	-2.83×10^{-3}	0.555
NO_3^--N	2.079×10^{-4}	**0.004****	-9.95×10^{-5}	**0.005****
NH_4^--N	-2.17×10^{-7}	0.967	-2.77×10^{-9}	0.936
TN	7.32×10^{-3}	**0.037***	4.58×10^{-4}	0.645
TC	3.63×10^{-4}	0.370	3.52×10^{-4}	**0.018***
C/N	-2.78×10^{-4}	0.694	-4.87×10^{-4}	0.122
pH	-1.66×10^{-2}	0.540	5.81×10^{-4}	0.200
moisture	1.95×10^{-1}	0.078	-5.63×10^{-2}	0.138
temperature	4.22×10^{-4}	**0.001****	1.16×10^{-4}	**0.001****
duration time	-2.47×10^{-4}	0.234	3.38×10^{-4}	0.060

Table 3- 6 Multiple regression with distance matrices (MRM) was applied to evaluate the importance of poultry litter properties and environmental factors on fungal community structure in growout 1and 3. Values indicated the matrix with regression coefficients and associated P values from the permutation test. Bold fonts and asterisks indicate significant effects. *: $P < 0.05$, **: $P < 0.01$

	Fungi_Growout1		Fungi_Growout3	
	(R^2= 0.5855, F= 12.59, *P*= 0.001)		(R^2= 04133, F= 6.28, *P*= 0.001)	
	Coefficient	*P*	Coefficient	*P*
WEC	-2.49×10^{-6}	0.480	-6.07×10^{-7}	0.645
WEN	1.21×10^{-5}	0.527	-1.01×10^{-5}	0.098
WEC/WEN	6.67×10^{-3}	0.810	1.64×10^{-2}	**0.044***
NO_3^--N	1.61×10^{-4}	**0.046***	6.12×10^{-5}	0.296
NH_4^--N	6.39×10^{-6}	0.408	2.56×10^{-7}	**0.003****
TN	-9.22×10^{-3}	**0.038***	-1.26×10^{-3}	0.553
TC	-4.30×10^{-4}	0.377	3.84×10^{-6}	0.984
C/N	1.48×10^{-3}	0.114	1.90×10^{-3}	0.765
pH	-1.93×10^{-3}	0.946	1.13×10^{-2}	0.105
moisture	-6.67×10^{-2}	0.251	-1.92×10^{-2}	0.756
temperature	1.83×10^{-5}	0.842	3.09×10^{-4}	**0.046***
duration time	4.98×10^{-3}	0.062	2.17×10^{-3}	0.360

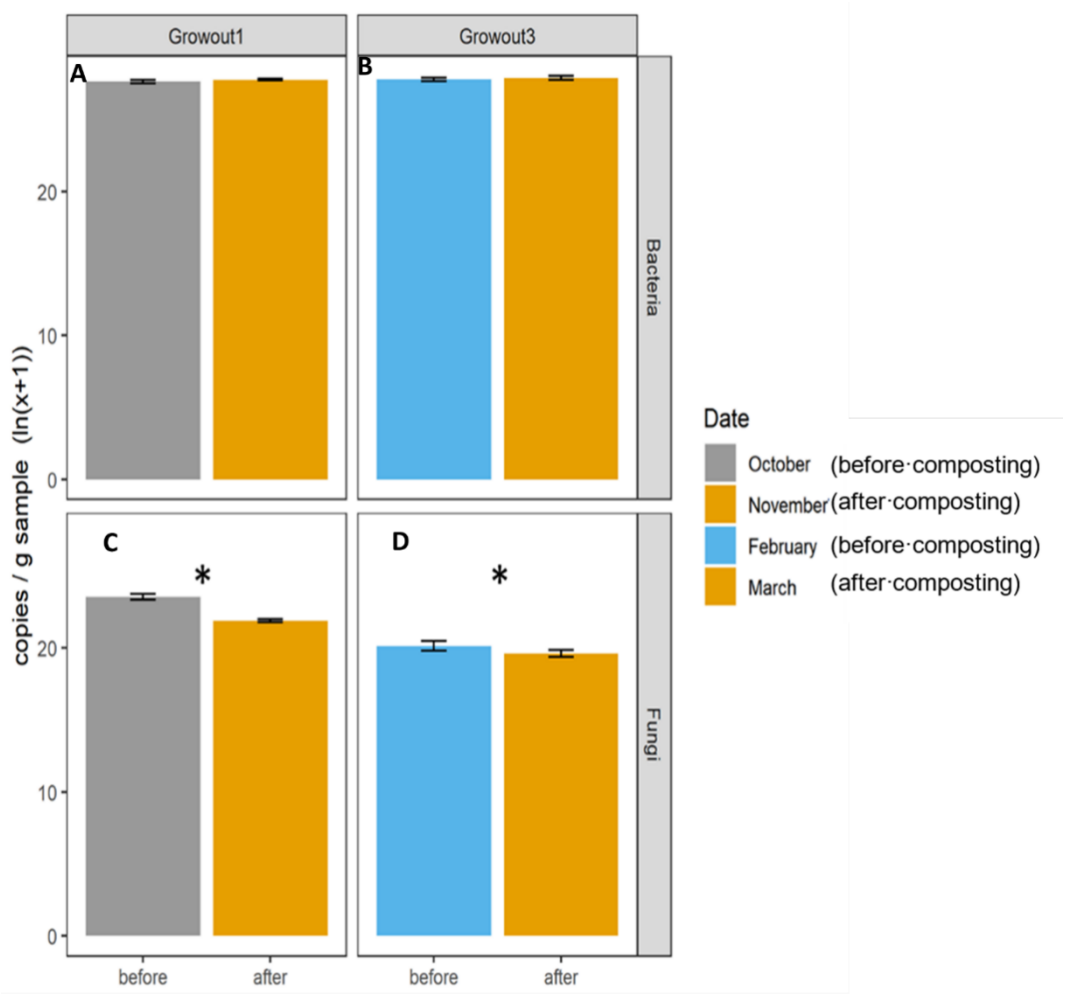


Figure 3-5 Total bacterial 16S rRNA gene (A and B) and fungal ITS gene (C and D) copy abundances in poultry litter before and after in-house windrow composting for two growouts. The bars are the mean normalized copies of genes ± standard error (n=8). Statistically significant difference was decided using *T*-test, asterisk indicates significant differences ($P < 0.05$) between before (gray and blue) and after composting (gold).

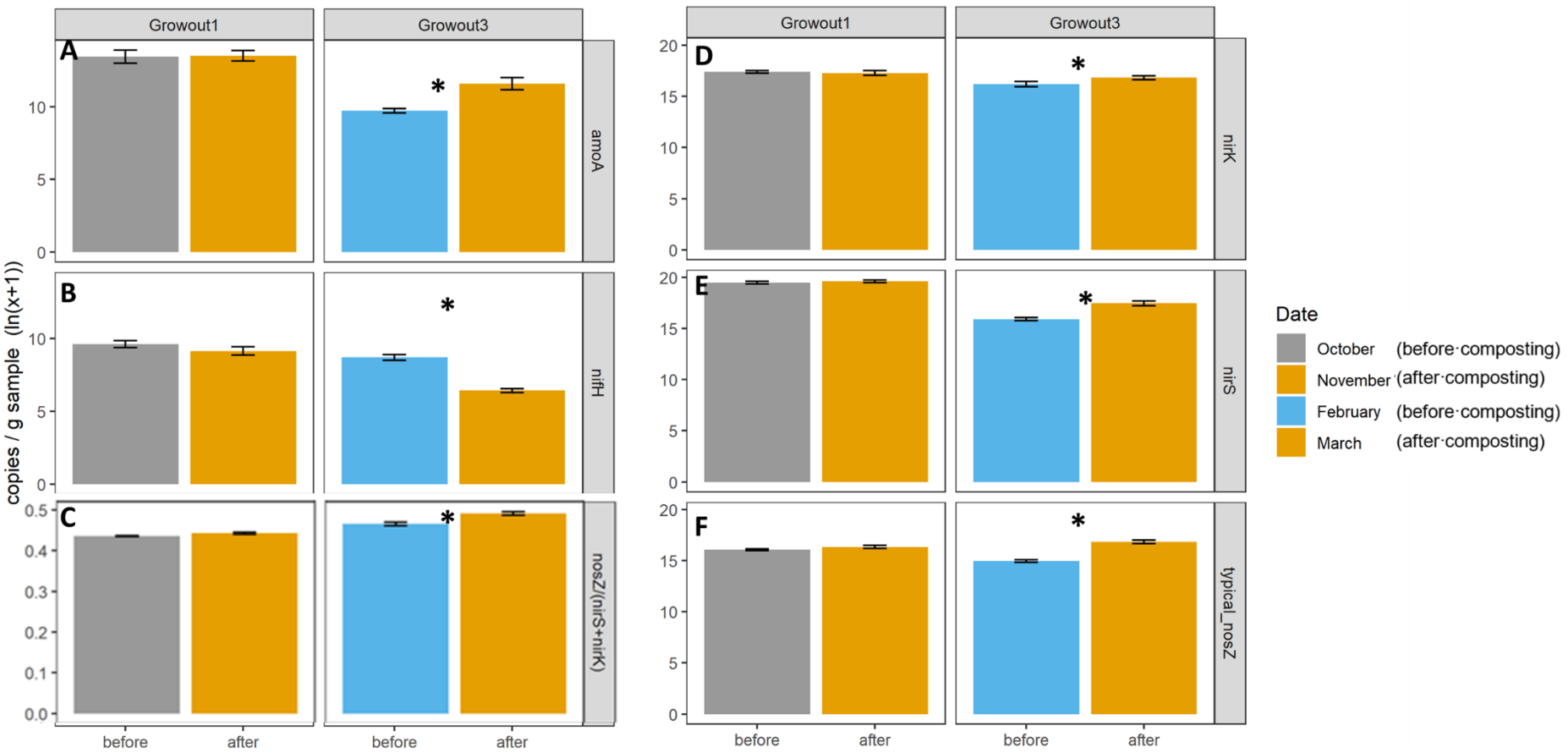


Figure 3- 6 Abundance of N-cycling functional genes (A: *amo*A, B: *nif*H, C: *nos*Z/(*nir*S+*nir*K), D: *nir*K, E: *nir*S, F: *nos*Z) in poultry litter before and after in-house windrow composting for two growouts. Error bars are standard error. The bars are the mean normalized copies of genes ± standard error (n=8). Statistically significant difference was evaluated with *T*-test. Asterisk indicates significant differences ($P < 0.05$) between before (gray and blue) and after composting (gold)

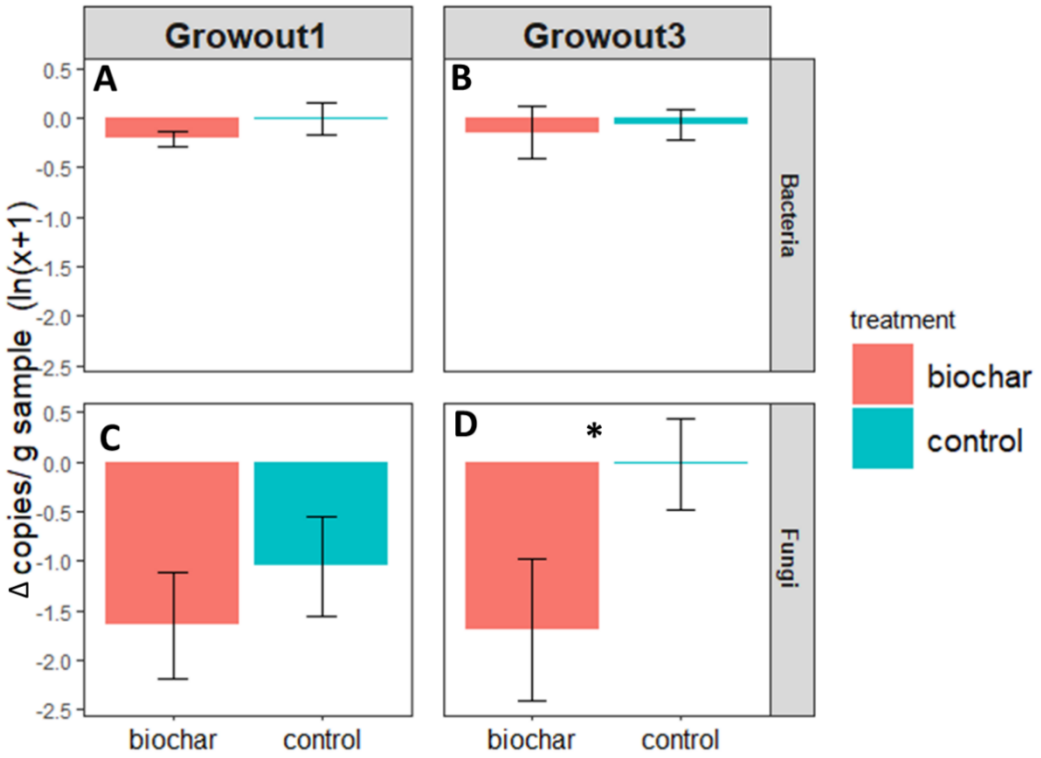


Figure 3- 7 The changes (delta) in bacterial 16S rRNA gene copy (A and B) and fungal ITS copy gene (C and D) abundances in poultry litter before and after in-house windrow composting for two growouts. Error bars are standard error. The bars are the delta mean normalized copies of genes ± standard error (n=4). Statistically significant difference was evaluated with *T*-test. Asterisk indicates significant differences ($P < 0.05$) between biochar treated (red)and control composting (cyan).

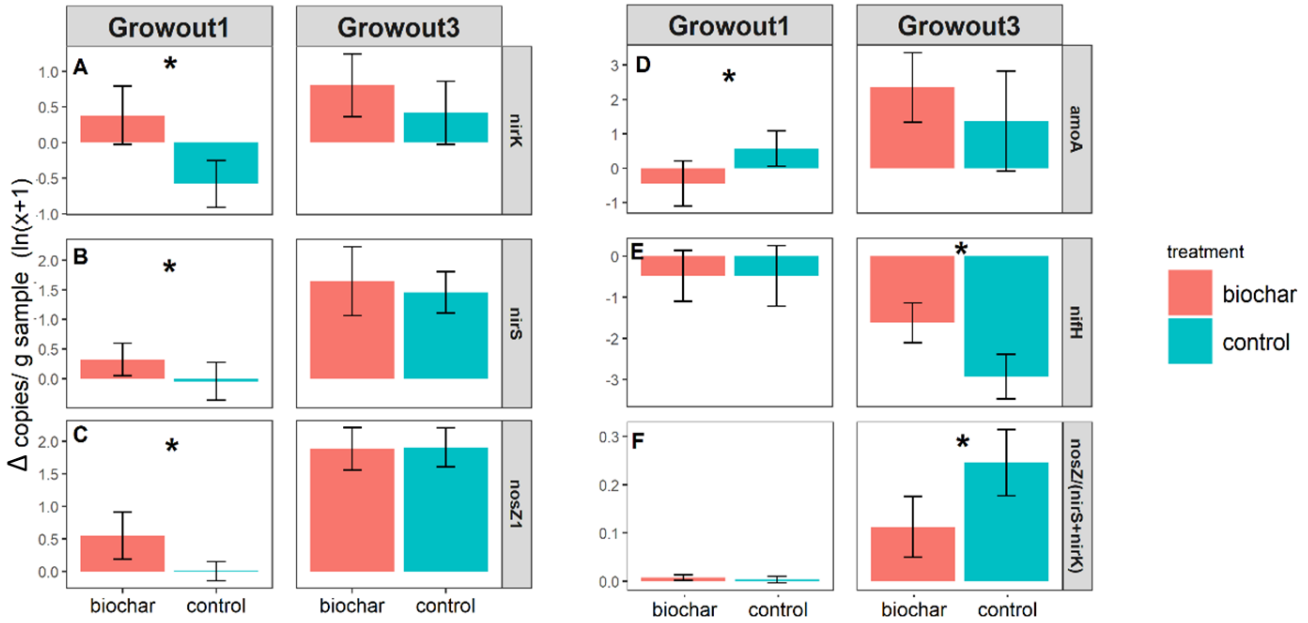


Figure 3- 8 Abundances of N-cycling functional genes in poultry litters before and after in-house windrow composting for two growouts. Error bars are standard error. The bars are the delta mean normalized copies of genes ± standard error (n=4). Statistically significant difference was decided using *T*-test. Asterisk indicates significant differences ($P < 0.05$) between biochar treated (red)and control composting (cyan).

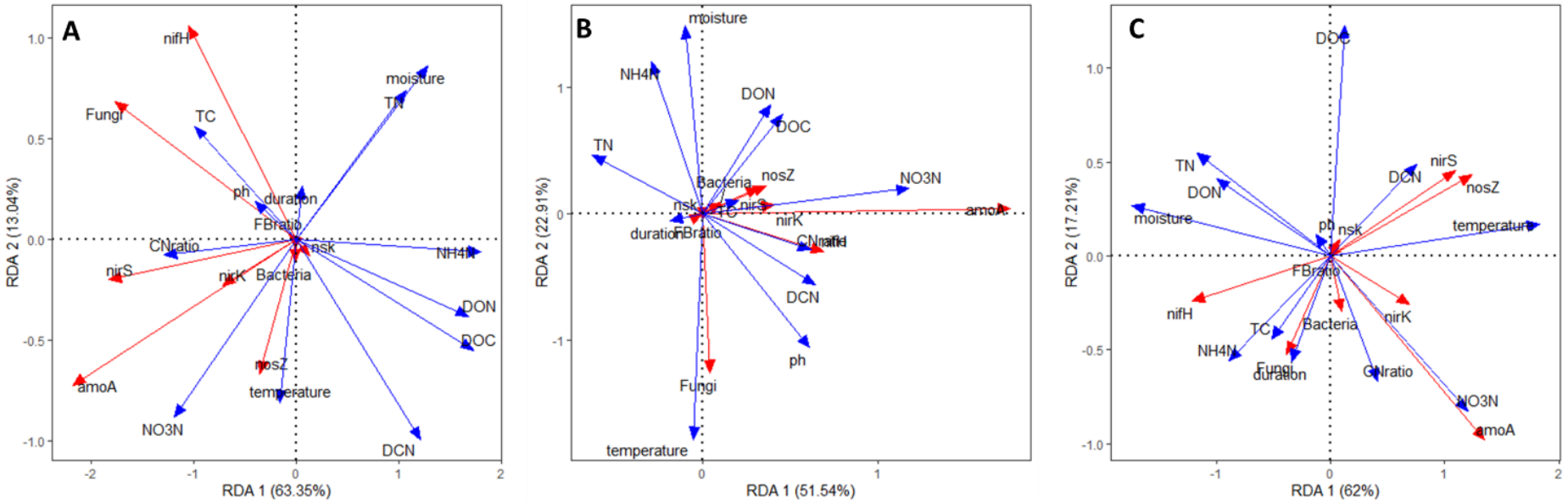


Figure 3- 9 Redundancy analysis (RDA) plot of the abundances of bacteria, fungi and N-cycling functiona genes in relation to poultry litter properties and environmental factors in (A) growout 1 and 3; (B) growout 1 and (C) growout 3. Blue lines indicate the poultry litter physico-chemical properties and environmental factors. Red lines indicate the abundances of bacteria, fungi and N-cycling functional genes.

Poultry litter physico-chemical properties and environmental factors were used for redundancy analysis (RDA) to evaluate the relations between microbial abundances and N-cycling functional gene abundances (Figure 3-8). RDA results indicated in growout 1, *amo*A abundances showed a significant positive relationship with NO_3^--N ($P < 0.05$), but the abundance of *nir*S showed a significant negative relationship with NO_3^--N ($P < 0.05$). In growout 3, *amo*A was negatively related to moisture, WEN and TN, but positively related to temperature and NO_3^--N. Abundance of *nif*H was negatively related to temperature. Genes associated with denitrification, *nosZ, nirS* and *nirK,* showed a positive relationship to temperature ($P < 0.05$), but negatively related with moisture ($P < 0.05$). Abundance of *nir*K also showed a significantly negative relationship to TN.

DISCUSSION

Changes in poultry litter physico-chemical properties as a result of composting were observed in our study. High poultry litter pH is common in poultry houses (Coufal et al., 2006). Even though the poultry litter in growout 3 was treated with an acidifier before the flock, the poultry litter still became alkaline during composting. In our study, poultry litter pH did not show significant increase as a result of composting, which may be neutralized by organic acids produced by the organic matter decomposition. The slight but not significant decrease in growout 3 was caused by the acidifier application. In this study, in-house windrow composting significantly reduced the moisture content, which is consistent with previous studies (Liang et al., 2014), because the heating of composting promotes evaporation. A higher poultry litter NH_4^+-N concentration was found in growout 3, because the applied acidifier reduced NH_3 production by inhibiting ammonia volatilization (Kai et al., 2008). Although composting reduced poultry litter

NH_4^+-N concentrations by 35% and 15% in growout 1 and 3, respectively, these were not significant reductions.

Biochar has been suggested as an ideal bulking agent for composting poultry litter (Steiner et al., 2010) because biochar can reduce NH_3 through the adsorption process (Ro et al., 2015), micro-pores absorb NH_3, NH_4^+-N and decrease DOC (Agyarko-Mintah et al., 2017) in composting. Biochar amendment to soil has been found to increase soil water and nutrient retention, and raise soil pH (Clough and Condron, 2010). In this study, biochar addition did not show any effects on poultry litter physico-chemical properties, except for TC in growout 3. This is likely due to our addition rate (1%), which was a logistically feasible amendment rate at the farm scale, but is low compared to other lab scale studies on biochar amendement. Higher amounts of biochar addition may influence the physico-chemical properties; previous studies have shown the addition of 20% biochar significantly reduced the NH_4^+-N concentrations in poultry litter composting. (Steiner et al., 2010). Hen manure mixed with 10% biochar also resulted in a higher composting temperature and NO_3^--N than unamended composting (Chen et al., 2017).

Xu et al. (2014) showed that biochar application to acidic soil increased bacterial alpha diversity, and also changed the relative abundance of carbon and nitrogen-cycling related taxa, but the effects of biochar on microbial diversity were not seen in our study. Our study suggested low amounts of biochar did not change bacterial and fungal diversity in poultry litter composting. Studies indicated the succession of microbial communities and species in composting are related to the change of environmental factors (He et al., 2013). In this study, the relative abundance of dominant microbial taxa significantly changed during composting. *Actinomycetales, Lactobacillales* and *Clostridiales* showed high relative abundance prior to the windrow composting but

decreased as a result of composting. An OTU belonging to family *Bacillaceae_2* represented the highest abundance (40.12%) among all the poultry litter samples, which is consistent with a finding in reused poultry litter (Bucher et al., 2020).

The poultry litter fungal community was dominated by Ascomycota, which was consistent with previous studies of fungal communities in food and garden waste composting (Langarica-Fuentes et al., 2014). Even though the relative abundance of Ascomycota as a phylum did not change, but the abundance of the Ascomycota orders did. *Saccharomycetales* decreased and *Eurotiales* increased in growout 3. The dominant orders of *Euritiales* and *Saccharomycetales* have also has been shown in the tomato plant waste and pine woodchips composing (López-González et al., 2015). High relative abundance of *Saccharomycetales* was found before composting, but it was replaced by the *Eurotiales*. *Eurotiales* can live in extreme environmental conditions, such as the high temperature during windrow composting (Neher et al., 2013; Wang et al., 2018). Fungi can utilize amino acids and carbohydrates, and thermophilic fungi have a strong potential to degrade organic matter during composting, which could improve the efficiency of the composting process (Wang et al., 2018). Based on the results of FUNGuild, several dominant genera belonging to *Saccharomycetales* and *Eurotiales* were saprotrophic fungi, included *Diutina*, *Microascus*, and *Aspergillus*. Saprotrophic taxa can degrade stubborn organic matter such as wood shavings in poultry litter and were the dominant fungal classes in composting.

The proper treatment of reused litter is important for the growth of poultry, because chickens live on the litter and consume some litter as a minimal part of their daily intake, and certainly influence the poultry litter microbial community through excretions. Previous studies have identified *Bacillaceae* as a dominant family in reused litter, which was consistent with our study (Davitt, 2014; Bucher et al., 2020). This bacterium can

be assigned as a biological indicator of reused litter. Previous study also suggested *Nocardiopsis* can be used as biological indicators of reused litter, because *Nocardiopsis* is an antibiotic producer and had negative effect on a pathogenic bacteria, *Salmonella* (Bucher et al., 2020). However, in our study, the abundance of *Nocardiopsis* decreased in each growout which suggested similar trends with *Salmonella* (Chapter 4). *Aspergillus,* a common opportunistic pathogen of humans, birds and other animals, was found in our study. The presence of *Aspergilli* has been found in poultry farms (Viegas et al., 2012). Although Aspergillus is an opportunistic pathogen, this fungi can degrade organic matter, producing enzymes capable of breaking down keratinous matter like chicken feathers in poultry farms (Mazotto et al., 2013).

Increased abundance of denitrification genes *nos*Z*,* *nir*S and *nir*K suggested an increase in the potential for microbial denitrification during composting. In growout 1, the *nirK* abundance decreased but *nosZ* abundance increased in general composting which suggested less N_2O producing and more N_2O consuming microorganisms (Wang et al., 2013). Many denitrifiers which can convert the nitrate to nitrogen gas belong to Proteobacteria (Ji et al., 2015). The average abundance of Proteobacteria in growout 3 (1.29%) was higher than growout 1 (0.72%) which may explain the increase the denitrifiers abundance in growout 3. A high *nosZ*/(*nirS*+*nirK*) in soil was suggested to be due to the growth of microorganisms capable of complete denitrification to reduce N_2O emission (Harter et al., 2013). In our study, the increased *nos*Z/(*nir*S+*nir*K) in growout 3 may lead to decreased N_2O emission. The abundance of *amo*A genes was much lower than denitrification genes which suggested the relatively low ammonification activity during poultry litter composting. Decreased *nif*H abundance as a result of composting in our study suggested a potentially low nitrogen storage ability (Liu et al., 2019). Previous research has documented that biochar addition in soil

increased copies of *nifH* and *nosZ* which encode enzymes involved in N_2 fixation and denitrification, respectively (Harter et al., 2013, Xu et al., 2014). Biochar addition decreased or did not influence the abundance of *amo*A in this study. In contrast, other studies have reported that biochar amendment in composting increased *amo*A abundance and decreased NH_3 emission, proposing that biochar absorbs NH_4^+-N and makes it suitable for the growth of ammonia-oxidizing bacteria (Pratscher et al., 2011, Yang et al., 2020). These results indicated that low biochar addition did not reduce potential N loss in this study from the perspective of microbes.

The effects of environmental factors on bacterial and fungal communities have been identified in waste composting, such as temperature (Liu et al., 2018, Wang et al., 2018), moisture content (Wang et al., 2018), NH_4^+-N and NO_3^--N (Meng et al., 2019), C: N ratio (He et al., 2012, Smith et al., 2014), pH (He et al., 2012), and TN (He et al., 2012). In our study, low dosage (1%) of biochar did not change the temperatures in composting and most physico-chemical properties. Nevertheless, biochar addition reduced TC loss in poultry litter possibly, because the carbon in biochar was recalcitrant (Khan et al., 2014). Temperature was an important indicator to reflect the composting process. High temperatures during composting accelerate the decomposition of organic matter during composting. The decreased bacterial Shannon index indicated that a few species became dominant in poultry litter bacterial community after composting, which may be caused by the limited nutrients and high temperature (Meng et al., 2019). Our study also identified that bacterial and fungal communities responded differently to in-house windrow composting. Similar result also was found in other animal manure composting (Meng et al. 2019). Multiple regression on distance matrices (MRM) results indicated a significant relationship between NO_3^--N, temperature, and bacterial communities, which suggested bacterial communities are sensitive to the change of

NO_3^--N concentrations. No shared environmental drivers were found in two growouts fungal communities. Fungal community structures were affected by N-related factors, such as NO_3^--N and TN in growout 1 and WEC: WEN ratio and NH_4^+-N in growout 3. The relationship between different types of N and microbial community structure suggested the importance of N in shaping microbial community during composting (Meng et al., 2019, John and John, 2018). The effects of NO_3^--N, WEN, and TN on the change of N-cycling functional gene abundances also suggested the importance of microbes in the transformation of nitrogen in composting. The importance of C:N was reported in composting studies because C:N was related to the organic matter degradation and maturity of compost (Wang et al., 2015), but the effects of C:N were not detected in our study due to the a consistent C: N across in-house windrow composting.

CONCLUSION

In this study, we evaluated the effects of in-house windrow composting and biochar addition on poultry litter properties, microbial communities and N-cycling functional genes. This study indicated different responses of microbial community and functions to in-house windrow composting. Temperature and NO_3^--N concentrations were the main predictive factors related to microbial community structure and N-cycling functional genes. The addition of low amounts of biochar (1%) did not have much impact on compost performance, poultry litter physico-chemical properties, or microbial communities. Further study is necessary to determine the effects of high amounts of biochar addition on microbial community structure and N-cycling functional genes. In summary, our study suggested that physico-chemical properties of poultry litter and temperature shape microbial community structures and abundances of

N-cycling functional genes, but that there is considerable variation in the important environmental drivers.

REFERENCES

Awasthi, M.K., Duan, Y., Liu, T., Awasthi, S.K., and Zhang, Z. (2020). Relevance of biochar to influence the bacterial succession during pig manure composting. *Bioresour Technol* 304, 122962.

Bucher, M.G., Zwirzitz, B., Oladeinde, A., Cook, K., Plymel,C., Zock, G., et al. (2020). Reused poultry litter microbiome with competitive exclusion potential against Salmonella Heidelberg. *Journal of Environmental Quality* 49(4), 869-881.

Cui, E., Wu, Y., Zuo, Y., and Chen, H. (2016). Effect of different biochars on antibiotic resistance genes and bacterial community during chicken manure composting. *Bioresour Technol* 203, 11-17.

Chen, W., Liao, X., Wu, Y., Liang, J.B., Mi, J., Huang, J., et al. (2017). Effects of different types of biochar on methane and ammonia mitigation during layer manure composting. *Waste Manage* 61**,** 506-515.

Coufal, C., Chavez, C., Niemeyer, P., and Carey, J.B. (2006). Effects of top-dressing recycled broiler litter on litter production, litter characteristics, and nitrogen mass balance. *Poultry science* 85(3)**,** 392-397.

Davitt, J.L. (2014). An assessment of broiler litter bacterial diversity using next generation DNA sequencing.

Duan, Y., Awasthi, S.K., Chen, H., Liu, T., Zhang, Z., Zhang, L., et al. (2019). Evaluating the impact of bamboo biochar on the fungal community succession during chicken manure composting. *Bioresour Technol* 272, 308-314.

Duan, Y., Awasthi, S.K., Liu, T., Chen, H., Zhang, Z., Wang, Q., et al. (2019). Dynamics of fungal diversity and interactions with environmental elements in response to wheat straw biochar amended poultry manure composting. *Bioresour Technol* 274, 410-417.

Harter, J., Krause, H.-M., Schuettler, S., Ruser, R., Fromme, M., Scholten, T., et al. (2013). Linking N2O emissions from biochar-amended soil to the structure and function of the N-cycling microbial community. *The Isme Journal* 8, 660.

He, Y., Xie, K., Xu, P., Huang, X., Gu, W., Zhang, F., et al. (2013). Evolution of microbial community diversity and enzymatic activity during composting. *Res Microbiol* 164(2), 189-198.

Hou, D., Huang, Z., Zeng, S., Liu, J., Wei, D., Deng, X., et al. (2017). Environmental factors shape water microbial community structure and function in shrimp cultural enclosure ecosystems. *Frontiers in microbiology* 8, 2359.

He, Y., Xie, K., Xu, P., Huang, X., Gu, W., Zhang, F., et al. (2013). Evolution of microbial community diversity and enzymatic activity during composting. *Res Microbiol* 164(2)**,** 189-198.

Ji, B., Yang, K., Zhu, L., Jiang, Y., Wang, H., Zhou, J., et al. (2015). Aerobic denitrification: a review of important advances of the last 30 years. *Biotechnology and Bioprocess Engineering* 20(4)**,** 643-651.

Jindo, K., Suto, K., Matsumoto, K., García, C., Sonoki, T., and Sanchez-Monedero, M.A. (2012). Chemical and biochemical characterisation of biochar-blended composts prepared from poultry manure. *Bioresour Technol* 110**,** 396-404.

Kai, P., Pedersen, P., Jensen, J.E., Hansen, M.N., and Sommer, S.G. (2008). A whole-farm assessment of the efficacy of slurry acidification in reducing ammonia emissions. *European Journal of Agronomy* 28(2)**,** 148-154

Khan, N., Clark, I., Sánchez-Monedero, M.A., Shea, S., Meier, S., and Bolan, N. (2014). Maturity indices in co-composting of chicken manure and sawdust with biochar. *Bioresour Technol* 168**,** 245-251.

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., and Schloss, P.D. (2013). Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. *Appl Environ Microbiol* 79(17), 5112-5120.

Li, J., Bao, H., Xing, W., Yang, J., Liu, R., Wang, X., et al. (2020). Succession of fungal dynamics and their influence on physicochemical parameters during pig manure composting employing with pine leaf biochar. *Bioresour Technol* 297**,** 122377..

Liu, L., Wang, S., Guo, X., Zhao, T., and Zhang, B. (2018). Succession and diversity of microorganisms and their association with physicochemical properties during green waste thermophilic composting. *Waste Manage* 73, 101-112.

Liu, T., Kumar Awasthi, M., Kumar Awasthi, S., Duan, Y., Chen, H., and Zhang, Z. (2019). Effects of clay on nitrogen cycle related functional genes abundance during chicken manure composting. *Bioresour Technol* 291, 121886

Langarica-Fuentes, A., Zafar, U., Heyworth, A., Brown, T., Fox, G., and Robson, G.D. (2014). Fungal succession in an in-vessel composting system characterized using 454 pyrosequencing. *FEMS Microbiol Ecol* 88(2)**,** 296-308

Liang, Y., Payne, J.B., Penn, C., Tabler, G.T., Watkins, S.E., VanDevender, K.W., et al. (2014). Systematic evaluation of in-house broiler litter windrowing effects on production benefits and environmental impact. *Journal of Applied Poultry Research* 23(4)**,** 625-638.

López-González, J.A., Vargas-García, M.d.C., López, M.J., Suárez-Estrella, F., Jurado, M.d.M., and Moreno, J. (2015). Biodiversity and succession of mycobiota associated to agricultural lignocellulosic waste-based composting. *Bioresour Technol* 187**,** 305-313.

Maeda, K., Toyoda, S., Philippot, L., Hattori, S., Nakajima, K., Ito, Y., et al. (2017). Relative Contribution of nirK- and nirS- Bacterial Denitrifiers as Well as Fungal Denitrifiers to Nitrous Oxide Production from Dairy Manure Compost. *Environ Sci Technol* 51(24), 14083-14091

Mazotto, A.M., Couri, S., Damaso, M.C., and Vermelho, A.B. (2013). Degradation of feather waste by Aspergillus niger keratinases: comparison of submerged and solid-state fermentation. *Int Biodeterior Biodegrad* 85**,** 189-195.

Neher, D.A., Weicht, T.R., Bates, S.T., Leff, J.W., and Fierer, N. (2013). Changes in Bacterial and Fungal Communities across Compost Recipes, Preparation Methods, and Composting Times. *Plos One* 8(11).

Pratscher, J., Dumont, M.G., and Conrad, R. (2011). Ammonia oxidation coupled to CO2 fixation by archaea and bacteria in an agricultural soil. Proceedings of the National Academy of Sciences 108(10), 4170-4175.

Steiner, C., Das, K.C., Melear, N., and Lakly, D. (2010). Reducing Nitrogen Loss during Poultry Litter Composting Using Biochar. *Journal of Environmental Quality* 39**,** 1236-1242.

Smith, A.M., Sharma, D., Lappin-Scott, H., Burton, S., and Huber, D.H. (2014). Microbial community structure of a pilot-scale thermophilic anaerobic digester treating poultry litter. *Appl Microbiol Biotechnol* 98(5), 2321-2334.

Steiner, C., Das, K.C., Melear, N., and Lakly, D. (2010). Reducing Nitrogen Loss during Poultry Litter Composting Using Biochar. *Journal of Environmental Quality* 39, 1236-1242. doi: 10.2134/jeq2009.0337.

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N.S., Wijesundera, R., et al. (2014). Global diversity and geography of soil fungi. *science* 346(6213), 1256688.

Teixeira, L.M., and Merquior, V.L.C. (2014). "The Family Moraxellaceae," in *The Prokaryotes: Gammaproteobacteria,* eds. E. Rosenberg, E.F. DeLong, S. Lory, E. Stackebrandt & F. Thompson. (Berlin, Heidelberg: Springer Berlin Heidelberg), 443-476.

Viegas, C., Carolino, E., Malta-Vacas, J., Sabino, R., Viegas, S., and Veríssimo, C. (2012). Fungal contamination of poultry litter: a public health problem. *J Toxicol Environ Health, A* 75(22-23)**,** 1341-1350.

Wang, D. (2019). Biochar amendment and moisture effects on poultry litter windrow heating performance. 2019. University of Tennessee, Knoxville, Master thesis

Wang, K., Yin, X., Mao, H., Chu, C., and Tian, Y. (2018). Changes in structure and function of fungal community in cow manure composting. *Bioresour Technol* 255**,** 123-130. doi: 10.1016/j.biortech.2018.01.064.

Wang, K., Mao, H., and Li, X. (2018). Functional characteristics and influence factors of microbial community in sewage sludge composting with inorganic bulking agent. *Bioresour Technol* 249, 527-535.

Wang, X., Cui, H., Shi, J., Zhao, X., Zhao, Y., and Wei, Z. (2015). Relationship between bacterial diversity and environmental parameters during composting of different raw materials. *Bioresour Technol* 198**,** 395-402.

Williams Ischer, S., Farnell, M., Tabler, G., Moreira, M., O'Shaughnessy, P., and Nonnenmann, M. (2017). Evaluation of a sprinkler cooling system on inhalable dust and ammonia concentrations in broiler chicken production. *J Occup Env Hyg* 14(1), 40-48.

Xue, P.-P., Carrillo, Y., Pino, V., Minasny, B., and McBratney, A.B. (2018). Soil properties drive microbial community structure in a large scale transect in South Eastern Australia. *Scientific reports* 8(1), 1-11.

Yang, Y., Kumar Awasthi, M., Wu, L., Yan, Y., and Lv, J. (2020). Microbial driving mechanism of biochar and bean dregs on NH3 and N2O emissions during composting. *Bioresour Technol* 315**,** 123829.

APPENDIX

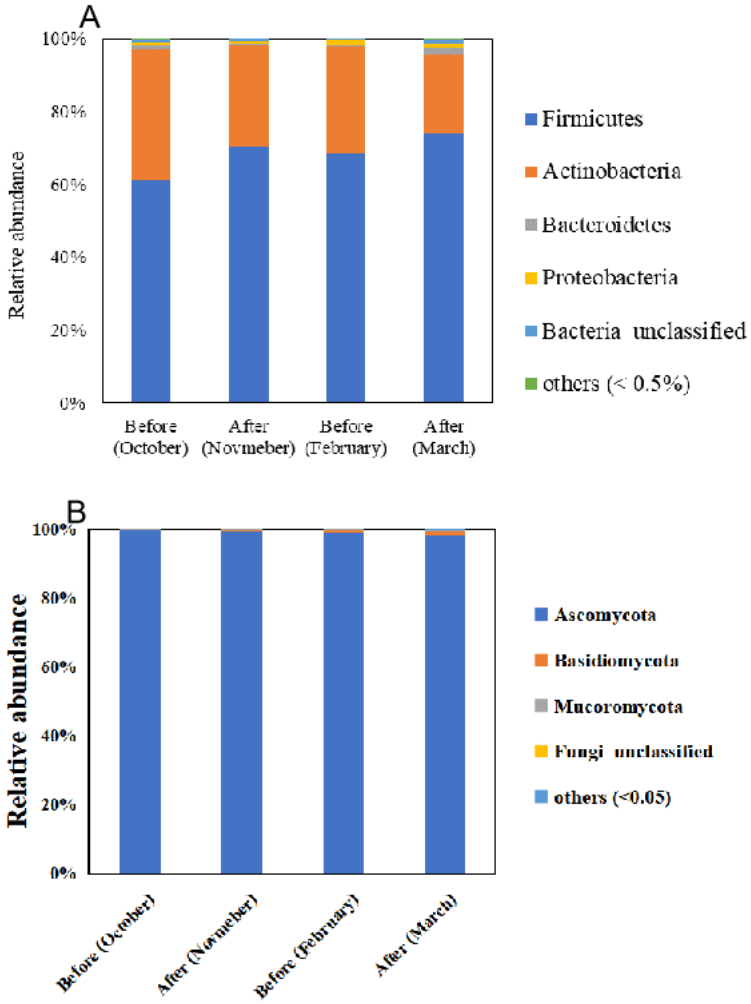


Figure S3-1 The relative abundances of bacterial (A) and fungal (B) phyla in poultry litter composting

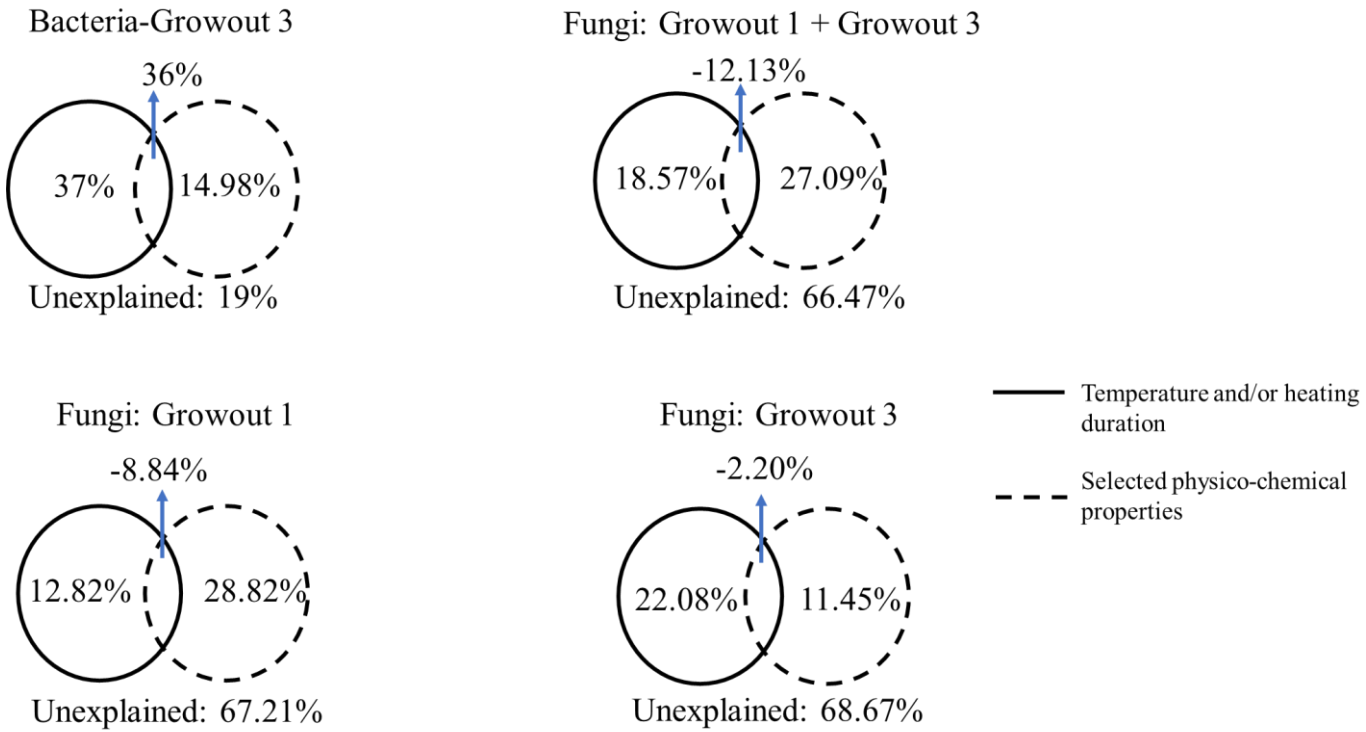


Figure S3-2 Variation partitioning analysis (VPA) differentiating the effects of selected poultry litter physico-chemical properties and environmental factors in poultry litter in-house windrow composting. Values marked by blue arrows indicated the explained proportions by temperature and/or heating duration x selected physico-chemical properties.

Table S3-1 Physico-chemical properties of poultry litter with and without biochar amendment in in-house windrow composting. Shown are the mean values ± standard error (n =4).

		pH	moisture	NO_3^--N	NH_4^+-N	TC	TN	C/N	WEC	WEN	WEC/ WEN
			%	mg kg^{-1}	mg kg^{-1}	g kg^{-1}	g kg^{-1}		mg kg^{-1}	mg kg^{-1}	
Growout 1	Before composting Amended (Oct)	8.16±0.03	0.38±0.02	169.64±14.82	4349.19±189.28	340.17±4.57	32.79±0.27	10.37±0.09	25069.98±1800.05	4837.12±131.74	5.18±0.02
	Unamended	8.00±0.03	0.42±0.03	165.89±29.07	4291.27±222.72	322.69±1.97	33.45±0.40	9.66±0.14	29273.15±9589.42	5685.50±608.19	5.12±0.07
	After composting Amended (Nov)	8.37±0.04	0.30±0.01	158.25±10.85	2758.88±101.06	327.70±3.05	32.54±0.53	10.09±0.19	23789.81±5608.01	4267.75±361.82	5.41±0.02
	Unamended	8.22±0.04	0.31±0.01	130.68±11.54	2986.11±138.61	327.37±2.51	33.68±0.47	9.74±0.14	18145.94±3990.49	3534.47±266.07	5.12±0.04
Growout 3	Before composting Amended (Feb)	8.26±0.03	0.59±0.01	52.37±6.12	28105.99±995.95	326.10±3.04	34.87±0.07	9.35±0.10	55858.35±3144.74	9280.88±122.05	6.02±0.012
	Unamended	7.97±0.11	0.58±0.03	86.18±8.42	28053.02±1434.86	310.28±3.16	36.87±0.82	8.44±0.14	53485.92±1196.96	9674.12±172.84	5.54±0.13
	After composting Amended (Mar)	7.96±0.21	0.35±0.01	127.33±12.33	23539.09±2083.24	316.41±5.33	33.07±0.70	9.63±0.36	56513.18±2371.62	9142.68±314.04	6.25±0.28
	Unamended	7.94±0.23	0.35±001	201.044±29.92	23969.94±1853.83	279.56±3.43	34.15±0.68	8.22±0.22	55567.26±5604.22	8996.27±237.88	6.20±0.26

Table S3-2 Alpha diversity index of bacterial and fungal community in poultry litter with and without biochar amendment in in-house windrow composting. Shown are the mean values ± standard error (n =4).

		Bacteria		Fungi	
		Richness (Numbers of observed OTUs)	Shannon	Richness (Numbers of observed OTUs)	Shannon
Growout1	Before composting (Oct) Amended	2134.00±50.30	5.57±0.04	230.50±10.00	3.00±0.06
	Unamended	2109.75±43.87	5.62±0.03	234.25±6.0	3.19±0.13
	After composting (Nov) Amended	1995.50±50.03	5.30±0.08	254.50±12.42	3.14±0.05
	Unamended	1925.00±17.30	5.25±0.03	246.50±7.26	3.36±0.12
Growout 3	Before composting (Feb) Amended	2357.75±46.73	5.40±0.02	199.50±13.23	3.17±0.29
	Unamended	2526.75±12.15	5.49±0.04	179.75±19.41	2.43±037
	After composting (Mar) Amended	2682.50±32.69	5.29±0.05	223.25±6.12	3.29±0.12
	Unamended	2682.00±38.52	5.24±0.09	235.00±7.25	3.67±0.17

Table S3-3 Differences of microbial communities between biochar amendment composting and general composting based on ADONIS analysis. Values in the table are the *P* value. Significant difference level was set at $P < 0.05$.

		Growout1		Growout3	
		Before composting (October)	After composting (November)	Before composting (February)	After composting (March)
Biochar amendment vs. General composting	Bacteria	0.794	0.406	0.345	0.975
	Fungi	0.505	0.318	0.679	0.701

Table S3-4 Pearson correlations between poultry litter properties, environmental factors and bacterial and fungal alpha diversity in all growouts (growout 1 and growout 3 combined). Values indicated the coefficients. One asterisk indicates $P < 0.05$, two asterisks indicate $P < 0.01$. NS means no significant.

	Bacteria		Fungi	
	Richness	Shannon	Richness	Shannon
WEC	0.858**	NS	NS	NS
WEN	0.808**	NS	NS	NS
WEC: WEN	0.651**	NS	0.384*	NS
NO_3^--N	0.405*	NS	NS	NS
NH_4^+-N	NS	NS	NS	NS
TN	NS	NS	NS	NS
TC	-0.49*	NS	NS	NS
C: N	-0.424*	NS	NS	NS
pH	NS	NS	NS	NS
moisture	NS	0.378*	NS	NS
temperature	NS	-0.610**	NS	NS
heating duration	NS	-0.370*	NS	NS

Table S3-5 Pearson correlations between poultry litter properties, environmental factors and bacterial and fungal alpha diversity in growout 1. Values indicated the coefficients. One asterisk indicates $P < 0.05$, two asterisks indicate $P < 0.01$. NS means no significant.

	Bacteria		Fungi	
	Richness	Shannon	Richness	Shannon
WEC	0.512*	0.538*	-0.621*	-0.646**
WEN	0.552*	0.581*	-0.611*	-0.637**
WEC: WEN	NS	NS	NS	NS
NO_3^--N	0.781**	0.569*	NS	NS
NH_4^+-N	NS	NS	NS	NS
TN	NS	NS	NS	NS
TC	NS	NS	NS	NS
C: N	NS	NS	NS	NS
pH	NS	NS	NS	NS
moisture	NS	0.578*	NS	NS
temperature	-0.506*	-0.721**	NS	NS
heating duration	NS	NS	NS	NS

Table S3-6 Pearson correlations between poultry litter properties, environmental factors and bacterial and fungal alpha diversity in growout 3. Values indicated the coefficients. One asterisk indicates P <0.05, two asterisks indicate P < 0.01. NS means no significant.

	Bacteria		Fungi	
	Richness	Shannon	Richness	Shannon
WEC	NS	NS	NS	NS
WEN	NS	NS	NS	NS
WEC: WEN	NS	NS	NS	NS
NO_3^--N	0.507*	-0.708**	NS	NS
NH_4^+-N	NS	NS	NS	NS
TN	NS	NS	NS	NS
TC	NS	NS	NS	NS
C: N	NS	NS	NS	NS
pH	NS	NS	NS	NS
moisture	-0.711**	0.576*	NS	NS
temperature	0.703**	NS	NS	NS
heating duration	NS	-0.503*	NS	NS

Table S3-7 FUNGuild results showing the trophic mode classification of the dominant fungal OTUs in poultry litter.

	Phylum	Class	Order	Famiy	Genus	Trophic Mode
Otu0001	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales_ fam_Incertae_sedis	Diutina	Saprotroph
Otu0003	Ascomycota	Saccharomycetes	Microascales	Microascaceae	Microascus	Pathotroph-Saprotroph -Symbiotroph
Otu0002	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Aspergillus	Saprotroph
Otu0004	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Aspergillus	Saprotroph
Otu0005	Ascomycota	Eurotiomycetes	Onygenales	Arachnomycetaceae	unclassified_ Arachnomycetaceae	-
Otu0006	Ascomycota	Sordariomycetes	Microascales	Microascaceae	Microascus	Pathotroph-Saprotroph -Symbiotroph
Otu0008	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Aspergillus	Saprotroph
Otu0007	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Aspergillus	Saprotroph
Otu0009	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Aspergillus	Saprotroph
Otu0010	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales_ fam_Incertae_sedis	Saccharomycetales_ fam_Incertae_sedis _ unclassified	Saprotroph
Otu0012	Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	unclassified_ Plectosphaerellacea e	Pathotroph-Saprotroph -Symbiotroph

Table S3-8 Pearson correlations between poultry litter properties, environmental factors and bacterial taxa in growout 1. Values indicated the coefficients. One asterisk indicates $P < 0.05$, two asterisks indicate $P < 0.01$. NS means no significant

	Firmicutes	Actinobacteria	Bacteroidetes	Proteobacteria	*Bacillales*	*Actinomycetales*	*Lactobacillales*	*Clostridiales*	*Enterobacteriales*
WEC	NS	NS	NS	NS	NS	NS	NS	NS	NS
WEN	-0.499*	NS	0.498*	0.539*	-0.530*	NS	NS	NS	0.518*
WEC: WEN	NS	NS	NS	NS	NS	NS	NS	NS	NS
NO_3^--N	NS	NS	0.864**	0.857**	NS	NS	NS	NS	NS
NH_4^+-N	-0.575*	0.581*	NS	NS	-0.670**	0.581*	0.620*	-0.718**	0.781**
TN	NS	NS	NS	NS	NS	NS	NS	NS	NS
TC	NS	NS	NS	NS	NS	NS	NS	NS	NS
C: N	NS	NS	NS	NS	NS	NS	NS	NS	NS
pH	0.588*	-0.558*	-0.504*	NS	0.580*	-0.558*	NS	0.635**	-0.646**
moisture	-0.712**	0.696**	NS	NS	0.689**	0.697**	NS	-0.536*	NS
temperature	0.769**	-0.753**	-0.578*	NS	0.873**	-0.753**	-0.742**	0.852**	-0.952**
heating duration	0.780**	0.765**	-0.568*	NS	0.889**	-0.765**	-0.737**	0.771**	-0.914**

Table S3-9 Pearson correlations between poultry litter properties, environmental factors and fungal taxa in growout 1. Values indicated the coefficients. One asterisk indicates $P < 0.05$, two asterisks indicate $P < 0.01$. NS means no significant

	Ascomycota	Basidiomycota	Mucoromycota	*Saccharomycetales*	*Eurotiales*	*Microascales*	*Onygenales*	*Glomerellales*	*Hypocreales*
WEC	0.510*	-0.554*	NS	NS	NS	NS	NS	NS	NS
WEN	0.560*	-0.599*	NS	NS	NS	NS	NS	NS	NS
WEC: WEN	NS	NS	NS	-0.714**	NS	0.572*	0.618*	NS	NS
NO_3^--N	NS	-0.552*	NS	NS	-0.697**	NS	NS	NS	NS
NH_4^+-N	0.647**	-0.600*	NS	0.530*	NS	-0.500*	NS	NS	NS
TN	NS	NS	NS	NS	NS	NS	NS	NS	NS
TC	NS	NS	NS	NS	NS	NS	NS	NS	NS
C: N	NS	NS	NS	NS	NS	NS	NS	NS	NS
pH	NS	NS	NS	-0.644**	NS	0.641**	NS	NS	NS
moisture	NS	NS	NS	NS	NS	NS	NS	NS	NS
temperature	-0.668**	0.652**	NS	NS	NS	NS	NS	NS	NS
heating duration	-0.696**	0.677**	NS	NS	NS	NS	NS	NS	NS

Table S3-10 Pearson correlations between poultry litter properties, environmental factors and bacterial taxa in growout 3. Values indicated the coefficients. One asterisk indicates $P < 0.05$, two asterisks indicate $P < 0.01$. NS means no significant.

	Firmicutes	Actinobacteria	Bacteroidetes	Proteobacteria	*Bacillales*	*Actinomycetales*	*Lactobacillales*	*Clostridiales*	*Enterobacteriales*
WEC	NS	NS	NS	NS	NS	NS	NS	NS	NS
WEN	NS	NS	NS	NS	NS	NS	NS	NS	NS
WEC: WEN	NS	NS	NS	NS	NS	NS	NS	NS	NS
NO_3^--N	0.711**	-0.627**	0.596*	NS	0.692**	-0.625**	-0.600*	-0.655**	NS
NH_4^+-N	NS	NS	NS	NS	NS	NS	NS	NS	NS
TN	-0.514*	0.507*	NS	NS	-0.508*	.505*	NS	0.517*	NS
TC	-0.623**	0.614*	-0.526*	NS	-0.607*	.615*	0.566*	NS	NS
C: N	NS	NS	NS	NS	NS	NS	NS	NS	NS
pH	NS	NS	NS	NS	NS	NS	NS	NS	NS
moisture	-0.784**	0.825**	-0.805**	NS	-0.931**	0.825**	0.893**	0.943**	NS
temperature	0.688**	-0.735**	0.837**	NS	0.937**	-0.737**	-0.951**	-0.952**	-0.549*
heating duration	0.643**	-0.630**	NS	NS	0.889**	-0.630**	-0.819**	NS	-0.710**

Table S3-11 Pearson correlations between poultry litter properties, environmental factors and fungal taxa in growout 3. Values indicated the coefficients. One asterisk indicates $P < 0.05$, two asterisks indicate $P < 0.01$. NS means no significant

	Ascomycota	Basidiomycota	Mucoromycota	*Saccharomycetales*	*Eurotiales*	*Microascales*	*Onygenales*	*Glomerellales*	*Hypocreales*
WEC	NS	NS	NS	NS	NS	0.611*	NS	NS	0.525*
WEN	NS	NS	NS	NS	NS	NS	-0.553*	NS	NS
WEC: WEN	NS	NS	NS	NS	NS	0.711**	0.562*	NS	0.626**
NO_3^--N	NS	NS	NS	NS	NS	NS	NS	0.572*	NS
NH_4^+-N	NS	NS	NS	NS	NS	-0.645**	-0.589*	NS	-0.524*
TN	NS	NS	NS	NS	NS	NS	NS	NS	NS
TC	NS	NS	NS	NS	-0.564*	NS	NS	NS	NS
C: N	NS	NS	NS	NS	NS	NS	NS	NS	0.603*
pH	NS	0.542*	NS	NS	-0.761**	NS	NS	NS	NS
moisture	NS	NS	NS	NS	NS	NS	NS	-0.520*	NS
temperature	NS	NS	NS	-0.574*	0.504*	NS	NS	0.522*	NS
heating duration	NS	NS	NS	-0.505**	NS	NS	NS	NS	NS

CHAPTER 4 – IMPACTS OF COMPOSTING AND HEATING ON THE PRESENCE OF PATHOGENS IN POULTRY LITTER

ABSTRACT

Pathogens in poultry litter pose potential risks to human and animal health. Composting is an ideal poultry litter management method, due to its relatively low cost and efficiency in reducing pathogen loads. To evaluate the effects of composting on poultry litter pathogen removal, an in-house windrow composting in a commercial poultry farm along with a laboratory simulated composting were conducted, and the persistence of pathogens in poultry litter was measured using quantitative PCR (qPCR). Biochar amendements were also evaluated as a compost additive to determine effects on pathogen removal during poultry litter composting. Four pathogens, *Clostridium perfringens, Salmonella, E. coli* O157:H7 and *Campylobacter jejuni*, were selected as target pathogens to determine the efficiency of composting and biochar amendment in terms of reducing pathogen loads. In-house windrow composting significantly reduced the abundances of *Salmonella*, *E. coli* O157:H7, and *C. jejuni*, but not *C. perfringens*. Biochar addition did not significantly affect pathogen abundance. A simulated composting study was conducted to determine the effects of temperatures and moisture on pathogen removal. In the simulated composting study, poultry litters with different moisture contents (35% and 45%) were incubated at temperatures ranging from 30 °C to 62 °C to determine the effect of temperature on pathogen abundances. Total bacterial abundance decreased at 50 °C (45% moisture) and 62 °C (35% moisture). Abundances of pathogens showed a decreasing trend with heating but were not significantly reduced. Abundance of *E. coli* O157:H7 decreased after 62 °C when the poultry litter moisture content was high (45%). 58 °C was found to be a sufficient temperature for pathogen removal. This study demonstrated the advantage of composting in reducing the abundance of pathogens in poultry litter. This study also evaluated the impacts of temperature and initial moisture in poultry litter management, suggesting that poultry

litter with low moisture only needs to reach 58 °C to achieve pathogen removal instead of the highest temperature (62 °C) in this study.

INTRODUCTION

Poultry litter contaminated by pathogens may increase the outbreak of disease in poultry industry. Chickens consume poultry litter as a minimal part of their daily intake (Wazuru et al., 2017), thus the poultry litter can be a source of pathogenic bacteria, spreading disease between birds (Wang et al., 2016). For example, the economic loss caused by *Clostridium perfringens*-induced necrotic enteritis in the global poultry industry is estimated at $6 billion per annum (Choct and Kocher, 2008; Wade and Keyburn, 2015). The hazards of *Salmonella* and *E. coli* O157:H7 to poultry are also well documented. *Salmonella* infections cause delayed growth and high mortality, resulting in considerable economic losses (CDC 2011, Gast and Porter Jr, 2020). The presence of pathogens in poultry litter not only affects the health of the birds but also may contaminate poultry products and produce when poultry litter is applied as fertilizer (Delgado et al., 2012; Chen and Jiang, 2014; Atidégla et al., 2016). Furthermore, when contaminated chickens were consumed by humans, these pathogens have impacts on human health. Foodborne illness caused by *Salmonella*, *C. perfrigens*, and *Campylobacter* affects about 3 million people in the United States annually and are the cause of 34% of foodborne illness deaths (Elaine et al., 2011). In U.S., the human illness cases of *E. coli* O157:H7 are about 75000 per year (Bavaro, 2012).

To reduce the presence of pathogens in poultry litter, low-cost methods are needed for the industry. Several methods for controlling pathogens have been reported in poultry industry, including quicklime (Roll et al., 2011), alum additions to poultry litter (Rothrock et al., 2008), and dietary antibiotic bacitracin (Wei et al., 2013). In-house windrow composting, which heats the litter, has been used in the poultry industry to

reduce microbial content in poultry litter (Macklin et al., 2008). The United States Department of Agriculture (USDA) suggests that compost piles should maintain temperatures of 55-77 °C for three days in in-vessel compost and 15 days for windrow compost (USDA, 2011) for pathogen reduction. Because of the high cost of fresh bedding materials, many poultry producers perform in-house windrow composting between flocks as a low-cost method to reduce pathogen loads so that they can re-use the litter. Composting poultry manure has been shown to effectively reduced the levels of fungal (Langarica-Fuentes et al., 2014) and bacterial pathogens (Cui et al., 2016). A reduction of *C. perfringens* by poultry litter composting also was reported (Macklin et al., 2008). To limit pathogens in re-used litter, poultry litter at the end of each flock should be tested for the presence and level of pathogens before it is recycled for the new flock or other agriculture applications. Although composting will not destroy all pathogens, it is still considered a useful and cost-effective method of reducing pathogen loads in poultry litter (Cui et al., 2016). Thus, composting is considered to be an effective method that adds value to poultry waste (Burton and Turner, 2003; Chen and Jiang, 2014).

Despite the potential of composting as a pathogen control practice, incomplete pathogen removal has been reported in several studies. For example, *Salmonella* and *E. coli* O157:H7 survived for more than 8 days in poultry manure composting when exposed to temperature above 55 °C (Chen et al., 2018). The survival of *E. coli* O157:H7 also was reported in a 50 °C dairy manure composting after 7 days (Shepherd et al., 2007). In a simulation of dairy composting, *E. coli* O157:H7 was still detected after 240 h heating at 50 °C (Singh et al., 2011). Composting effectively reduced the load of *E. coli* O157:H7, but did not remove all the *E. coli* O157:H7 because of regrowth in the late stage of bovine manure composting (Berry et al., 2012). These

studies suggested that the temperature and duration time standard (55 °C for at least three days) set by USDA may not always be effective enough to ensure the complete removal of pathogens in animal waste composting.

Temperature is not only the only abiotic factor affecting pathogens; other environmental factors such as moisture content may also play a role in their persistence. The optimal moisture content for active composting is 40% to 60%. A simulated poultry manure composting study suggested that the extended survival of *Salmonella* was longer at 40% moisture content than 50% (Singh et al., 2012). Thus, variations in moisture and temperature may influence the abundance and survival of pathogens (Chen and Jiang, 2017). Therefore, the relative importance of temperature and moisture content should be considered together to ensure the efficiency of composting.

Biochar is a carbon-rich material that can be added during composting to improve the decomposition of organic matter and reduce nitrogen loss (López-Cano et al., 2016; Godlewska et al., 2017). Previous studies found a shift in microbial communities in pig manure (Awasthi et al., 2020) and poultry manure composting with biochar addition (Duan et al., 2019). Different types of biochar also led to variations in bacterial community structures during pig manure composting (Awasthi et al., 2020). Bacterial community composition may influence the abundance of pathogens during animal waste composting, because indigenous microbes adapted to the waste environment may outcompete pathogens for nutrients (Singh et al., 2012; Chen et al., 2018). The addition of biochar in composting may be another strategy to reduce the abundance of pathogens. Biochar amendments to soil have been shown to reduce soilborne plant pathogens, via electron transfer disruption, increased soil pH and adsorption of signaling compounds (Graber et al., 2014).

Even though the effects of composting on pathogen removal have been shown, most studies have been conducted in containers, and it is unclear if these results are relevant at the farm scale. The objectives of this research were to: 1) evaluate the efficiency of in-house windrow composting on the reduction in the abundance of four key pathogens (*Clostridium perfringens*, *Salmonella*, *Campylobacter jejuni*, and *E. coli* O157:H7) in poultry litter; 2) investigate the effects of low addition of biochar on the reduction of pathogens; 3) determine if changes in pathogen abundances will mirror changes in the total bacterial abundance; and 4) assess the effects of temperature and initial moisture content on pathogen removal in poultry litter simulated composting. We hypothesized that 1) in-house windrow composting would reduce the abundances of four key pathogens; 2) low amendment rates of biochar in poultry litter would decrease pathogen abundances during in-house windrow composting; 3) the abundances of pathogens would decrease along with the decrease of total bacterial abundance; and 4) suboptimal litter moisture content would decrease the effectiveness of composting in terms of reduction of pathogens. To test these hypotheses, in-house litter composting was conducted at a commercial poultry farm, with poultry litter amended with two different types of biochar. Additionally, a simulated composting laboratory experiment was conducted to test the effects of temperatures and initial moisture on the presence of pathogens in poultry litter. Poultry litter with low (35%) and high moisture (45%) contents were used in this simulated composting as a complementary experiment with in-house windrow composting.

MATERIAL AND METHODS

Site description and sampling

The study was conducted at four poultry houses (17 m * 152 m) in Cleveland, Tennessee, USA. Each house was divided into brood and non-brood (grow) ends. During the first 7-10 days, birds were raised at brood end then the house was opened up, so that birds also filled the non-brood end. Between flocks, windrow composting was applied on poultry litter. Two or three turns were conducted during each windrow composting, the first turn was 5-7 days after piles were formed and the following turnings occurred 3-5 days later. Two types of biochar were added to house 2 and house 3, respectively (Table 4-1). For each biochar-added house, 4000 lbs biochar (dry mass) was added to the top of poultry litter, which was the equivalent of about 1% addition before the first flock was placed. The remaining two houses (house 1 and house 4) did not receive biochar as an amendment. There were four growouts in each house, samples were collected from growout1 (Octiber and November) and growout 3 (February and March).

Poultry litter samples were collected before composting and after composting. Before collected the samples, composting piles were mixed well. For growout 1, samples were collected on October 25, 2017, at the end of growout few days before windrowing and November 15, 2017, after the first composting was completed and before new birds were placed. For growout 3, samples were collected on February 19, 2018, after acidifier was added to brood end before the third flock was introduced; and March 8, after the third composting was completed. Based on the number of windrows in the house, four or five samples were collected from both brood and non-brood ends and composited into a representative sample for the house. Samples were collected using shovels and stored in zip-log bags then transported to the lab in a cooler. Poultry litter samples were stored in a -20 °C freezer for subsequent analyses.

Table 4- 1 Basic properties of biochar L and biochar P applied in this study (Wang. 2019).

Properties	Biochar L	Biochar P
Moisture content (%, wet basis)	54.1 ± 2	9.8 ± 1
Ash content (%, wet basis)	3.0 ± 0.4	7.3 ± 1
TC (%, wet basis)	83.1 ± 5.6	85.3 ± 1.7
TN (%, wet basis)	1.1 ± 0.1	0.8 ± 0.1
BET surface area (m^2/g)	278.9 ± 0.6	295.1 ± 0.44
PV (cm^3/g)	0.79 ± 0.002	0.83 ± 0.002

*TC: total carbon; TN: total nitrogen; BET surface area: Brunauer, Emmett and Teller surface area; PV: pore volume.

Simulated laboratory composting

Thermal inactivation of pathogens in poultry litter was tested using a simulated composting laboratory experiment (48 - 72 h duration time). Poultry litter was mixed well to homogenize and 60 g of litter was placed into autoclaved glass Mason jars (16 oz). The target temperatures selected for the thermal inactivation experiment were 30°C, 45°C, 50°C, 58°C, 62°C (45% high moisture content poultry litter) and 35°C, 50°C, 58°C and 62°C (35% low moisture content poultry litter). Each treatment had three replications (three jars), jars were initially incubated at 30°C for high moisture poultry litter and 35°C for low moisture poultry litter. Then temperatures were raised to the target temperature for 48 h (high moisture) or 72 h (low moisture), followed by 48 h cooling at 30°C for high moisture litter and 35°C for low moisture litter.

To determine the effect of moisture content on pathogen survival, a second simulated composting experiment was set up using litter with three different moisture contents (35%, 40% and 45%). Three replicate jars for each treatment were incubated at 35°C for 48 h, then heated to 35°C, 50°C, 58°C and 62°C, and cooled at 35°C for 48 h. Gravimetric moisture content of the litter was determined by drying 2 g poultry litter in an oven at 105°C for 12 h until the weight was constant.

In the thermal deactivation study, poultry litter samples were collected after preincubation, thermal deactivation and the end of recovery stage. Collected samples were frozen in the liquid nitrogen for DNA extraction. To evaluate the abundance of viable microbes in poultry litter, 0.1 g portion of poultry litter sample was mixed with 0.9 ml PBS (phosphate buffered saline) solution (pH= 7.4). Serial dilutions of the mixture were plated in triplicate on Plate Count Agar (Table S4-1) and incubated at 35°C for 48 h. Visible colonies were counted and colony-forming units (CFUs) were calculated based on the selected dilutions and the numbers of colonies.

DNA extraction and quantitative PCR

To improve DNA extraction efficiency, poultry litter samples were first ground using a blender. Approximately 10 g poultry litter from each sample was ground in the blender four times for 5 secs each time. DNA was extracted using DNeasy PowerLyzer PowerSoil Kit (Qiagen, USA) according to the manufacturer's guide. Four sets of primers were used to analyze the effects of in-house windrow composting on the abundances of four pathogenic bacteria: *C. jejuni*, *Salmonella*, *C. perfringens,* and *E. coli* O157:H7 (Table 4-2).

Total bacterial abundance was quantified using Femto Bacterial DNA Quantification Kit (Zymo Research, Irvine, CA, USA). To prepare the standard curves for the quantification of pathogens, target genes were PCR-amplified using the primers list in Table 4-2 from poultry litter DNA. PCR products were purified using QIAquick PCR Purification Kit (QIAGEN Inc. Valencia, CA, USA), then cloned using TOPO TA cloning kit for sequencing (Invitrogen Inc., Carlsbad, CA, USA). Recombinant plasmids were cleaned using PureLink Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, USA). Plasmids were sequenced to confirm the correct insertion. The concentration of each recombinant plasmid was measured using NanoDrop One Spectrophotometer (ThermoFisher Scientific Inc., USA). Concentrations of target gene copies were calculated based on the plasmid concentration and the length of the target sequence. Serial dilutions (10^2 - 10^8 copies μL^{-1}) of the standard plasmids were made prior to each qPCR assay. The conditions for total bacterial abundance (16S rRNA) qPCR were 10 min at 95°C, followed by 40 cycles with 30 s at 95°C, 30 s at 50°C and 1 min at 72°C. 7 min at 72°C for the final step. The conditions for *C. jejuni* (*flaA*) qPCR were 5 min at 95°C, followed by 40 cycles with 30 s at 95°C, 30 s at 55°C and 3 min at 72°C. The conditions for *C. perfrigens* (*cpa*) were 5 min at 94°C, followed by 40

cycles with 94°C for 30 s, 57°C for 30 s, 72°C for 1 min. The conditions for *Salmonella* (*inv*A) were 95°C for 3 min, followed by 38 cycles of 95°C for 30 s, 63°C for 30 s and 72°C for 30 s. The conditions for *E. coli* O157:H7 (*uid*A) were 94°C for 10 min, followed by 40 cycles of 94°C for 20 s, 63°C for 25 s and 72°C for 30 s. All the qPCR cycles contained a step melting curve in the end. All the qPCR assays were performed with three replicate reactions for each poultry litter template DNA sample. Each qPCR assay also included a no template control. The amplification was performed using CFX96 Optical Real-Time Detection System (Bio-Rad, Laboratories Inc., Hercules, CA, USA). The copies of target genes in each reaction were calculated based on the standard curves (which all had a PCR efficiency of $R^2 > 0.984$) and expressed as copies per gram fresh weight poultry litter. The detection limits of all the qPCR assays were calculated based on lowest target gene copies in standard curves. All the detection limits were 10^3 copies per gram fresh weigh poultry litter (Table 4-2).

Statistical analysis

The qPCR results were ln (x +1) normalized to improve normality. The change of pathogen abundances was calculated as Δ = normalized copies after composting – normalized copies before composting. *T*-tests were applied to test the effects of composting and biochar on the abundance of pathogens in composting. One-way ANOVA (with posthoc Tukey test) was applied to test the difference of pathogen abundance in simulated composting. Pearson correlation with Bonferroni correction was used to evaluate the relationship between the abundances of pathogens and temperatures and moisture contents. The statistical significance level was set at $P < 0.05$. The creation of figures and statistical analyses were performed using ggplot2 and corrr packages in R (v 3.6.2).

Table 4- 2 **Primers used in this study for the quantification of pathogens.**

Target gene		Primer	Reference	Detection limits (copies g^{-1} fresh weight litter)
Campylobacter	*flaA*	flaAF: 5'-ATGGGATTTCGTATTAACAC-3'	Wieczorek et al., 2019	1.72 x 10^3
jejuni		flaAR:5'-CTGTAGTAATCTTAAAACATTTTG-3'		
Clostridium	*cpa*	Cpalpha-F: 5;- TCATCCTGCTAATGTTACTG-3'	Wei et al., 2013	1.03 x 10^3
perfringens		Cpalpha-R:5'-CCACTAGTTGATATGTAAGC-3'		
Salmonella	*invA*	invA F: 5'-GTGAAATTATCGCCACGTTCGGGCAA--3'	Malorny et al., 2003	1.43 x 10^3
		invA R: 5'-TCATCGCACCGTCAAAGGAACC-3'		
Escherchia.	*uidA*	uidAF241: 5'-CAGTCTGGATCGCGAAAACTG-3'	Elizaquível et al., 2012	2,02 x 10^3
coli O157:H7		uidAR383: 5'-ACCAGACGTTGCCCACATAATT-3'		

RESULTS

In-house windrow composting performance

Poultry litter moisture significantly decreased from 0.40 ± 0.01 to 0.30 ± 0.01 in growout 1 and also decreased from 0.58 ± 0.01 to 0.35 ± 0.01 in growout 3 as a result of composting ($P < 0.05$, *T*-test). Biochar addition did not have a significant effect on moisture contents in poultry litter composting (Table 4-3). Composting significantly increased temperatures in poultry litter ($P < 0.01$), from an initial temperature around 14°C to 66°C in growout 1 and stayed above 55°C for 7-8 days. Temperatures also increased in growout 3 from around 20.5°C to 66°C and heated for a similar duration (Table 4-3). Low biochar addition did not increase the temperatures during poultry litter composting. Heating duration (temperatures ≥ 55°C) also did not show significant differences among treatments. More details about the temperature change were reported by Wang (2019).

Composting reduced the abundance of total bacteria and pathogens in poultry litter

In growout 1, total bacterial abundance (16S rRNA gene copies) in poultry litter increased from 27.62 ln (1+ 16S rRNA copies g^{-1}) to 27.73 ln (1+ 16S rRNA copies g^{-1}) as a result of composting, but this difference was not significant (P =0.16) (Figure 4-1A). All four selected pathogens (*E. coli* O157:H7, *Salmonella*, *C. jejuni* and *C perfringens*) were detected in poultry litter (Figure 4-1). In growout 1, in-house windrow composting significantly decreased the abundance of *C. jejuni* by 2.88 ln (1+ *camp* copies g^{-1}) (Figure 4-1B), *E. coli* O157:H7 by 1.32 ln (1+ *uid*A copies g^{-1}) (Figure 4-1D) and *Salmonella* by 18.02 ln (1+ *inv*A copies g^{-1}) (Figure 4-1E) ($P < 0.05$). Meanwhile, the abundance of *C. perfringens* significantly increased by 1.96 ln (1+ *cap* copies g^{-1}) as a result of composting ($P < 0.01$) (Figure 4-1C). The abundance of

Salmonella showed a significant positive correlation with moisture content ($P < 0.05$), but negative correlations with temperature and effective heating duration (> 55°C) were detected in poultry litter composting ($P < 0.05$). Abundance of *C. perfringens* showed significant positive correlations with temperature and heating duration ($P < 0.01$). Abundances of total bacterial abundance, *C. jejuni* and *E. coli* O157:H7 did not show significant correlations with moisture content, temperature and heating duration.

In growout 3, the change in total bacterial abundances (16S rRNA gene copies) was similar to growout 1, and increased from 27.77 ln (1+ 16S rRNA copies g^{-1}) to 27.87 ln (1+ 16S rRNA copies g^{-1}), and did not change as a result of composting, similar to growout 1 ($P = 0.56$) (Figure 4-1F). Composting significantly reduced the abundances of *C. jejuni* by 6.87 ln (1+ *camp* copies g^{-1}) (Figure 4-1G) and *E. coli* O157:H7 by 2.51 ln (1+ *uid*A copies g^{-1}) (Figure 4-1I) ($P < 0.01$). No significant changes were found in the abundances of *C. perfringens* (Figure 4-1H) and *Salmonella* (Figure 4-1J). Poultry litter moisture content was not significantly correlated to total bacteria nor selected pathogen abundances. The abundances of *C. jejuni* ($P < 0.05$) and *E. coli* O157:H7 ($P < 0.01$) showed significant negative correlations with temperature in poultry litter windrow composting. Abundance of *E. coli* O157:H7 also showed a significant negative correlation with heating duration ($P < 0.01$).

Biochar addition did not influence the abundance of pathogens

To test the effects of biochar addition, the samples collected from different biochar-treated houses were assigned as a biochar-treated group. The change of pathogen gene copy numbers was calculated as Δ = normalized copies after composting – normalized copies before composting. Negative values indicated a decreased abundance, while positive values reflected an increase in composting. Biochar addition did not influence the reductions between control and biochar addition treatments. Compared to the

unamended composting, the addition of biochar did not result in a higher removal rate of pathogens (Figure 4-2B-E and G-J) or change in total bacterial abundance (Figure 4-2A and F). There was no statistical difference in the abundance of total bacteria and tested pathogens between the two types of biochar (Table S4-2).

Impacts of temperature on the presence of pathogens in simulated poultry litter composting

Two laboratory scale simulated composting experiments were conducted to test the influence of temperature on the presence of pathogens. In the first experiment, high moisture litter was incubated at various temperatures (30°C to 62°C) for 48 h. The 50°C treatment resulted in a significant reduction of total bacterial 16S rRNA gene copy abundance by 0.50 ln (1+ 16S rRNA copies g^{-1}) ($P < 0.01$), followed by an increase of 0.21 ln (1+ 16S rRNA copies g^{-1}) in recovery stage (30°C for 48 h) ($P < 0.05$) (Figure 4-3B). Bacterial colony forming units (CFUs) in the high moisture poultry litter was about 10^7 g^{-1} poultry litter. Significant reductions of CFUs were detected in high moisture content poultry litter in the 30°C and 45°C treatments. CFUs significantly decreased after 48 h incubation at 30°C, from 5.13 x 10^7 CFU g^{-1} to 1.68 x 10^7 CFU g^{-1}. In treatments that were heated to 45°C, CFUs significantly decreased from 3.20 x 10^7 CFU g^{-1} to 3.36 x 10^6 CFU g^{-1} (Figure 4-3A). Bacterial CFUs showed different results than total bacterial 16S rRNA gene copy abundances detected with qPCR: Bacterial CFUs decreased with incubation time and did not increase in the recovery stage. However, no significant trends were found in the 30°C, 50°C and 58°C treatments based on total bacterial 16S rRNA gene copy abundance. Three pathogens were detected in the high moisture poultry litter simulated composting experiments, included *C. perfringens*, *E. coli* O157:H7 and *C. jejuni* (Figure 4-3C-E). The abundance of *C. perfringens* was significantly reduced in the 62°C treatment, decreasing by 0.17 ln (1+

cpa copies g^{-1}). No significant decrease was found in *E. coli* O157:H7 or *C. jejuni* at any temperature.

In the second heating experiment with a low moisture (35%) poultry litter, total bacterial 16S rRNA gene copy abundance was reduced in the 62°C treatment, decreasing by 0.93 ln (1+ 16S rRNA copies g^{-1}) ($P < 0.01$). The abundance of total bacteria 16S rRNA gene copies did not change significantly in other treatments during this experiment (Figure 4-3G). As with the first experiment, plate counting methods showed different results than qPCR. Low moisture poultry litter contained about 10^5-10^6 CFU g^{-1} poultry litter, and CFUs in low moisture litter were significant reduced after 72 h heating treatment at 50°C, 58°C and 62°C (Figure 4-3F). In the 35°C treatment, CFUs did not show a significant decrease after 72 h heating period, but were significantly reduced after the additional 48 h incubation at 30°C (recovery stage), decreasing from 7.85 x 10^5 CFU g^{-1} to 3.03 x 10^5 CFU g^{-1} ($P < 0.05$) (Figure 4-3G). This was not consistent with total bacterial 16S rRNA gene copy abundance. Total bacterial abundance showed a slightly increasing, but not significant, trend in the recovery stage of 50°C, 58°C and 62°C treatments. Three pathogens were detected in litter used in the second experiment: *E. coli* O157:H7, *C. jejuni*, and *Salmonella* (Figure 4-3H-J). *C. perfringens* was the only pathogen significantly affected by the heating treatments. The abundance of *C. perfringens* decreased by 5.74 ln (1+ *cpa* copies g^{-1}) after 72 h heating at 62°C ($P < 0.01$) and increased by 4.16 (1+ *cpa* copies g^{-1}) at 35°C after the 48 h recovery stage ($P < 0.05$). For the other detectable pathogens, *E. coli* O157:H7 and *Salmonella* were not significantly changed in four selected temperature treatments (35°C, 50°C, 58°C and 62°C).

Table 4- 3 Poultry litter moisture content, temperatures and heating duration at >55 °C in biochar-amended and unamended poultry litter composting. Statistically significant difference was evaluated with *T*-test.

		Moisture (%)		Initial temperature (°C)	Maximum temperature achieved (°C)	Duration at >55 °C (d)
		Before composting	After composting			
Growout1	Amended	0.38±0.02	0.30±0.01	14.80±1.44	66.07±0.37	7.84±1.84
	Unamended	0.42±0.03	0.31±0.02	13.75±1.00	66.02±0.50	6.94±0.54
Growout3	Amended	0.59±0.02	0.35±0.02	20.50±1.97	65.75±0.95	6.93±1.44
	Unamended	0.58±0.03	0.35±0.02	20.49±1.81	66.06±0.67	7.81±0.67

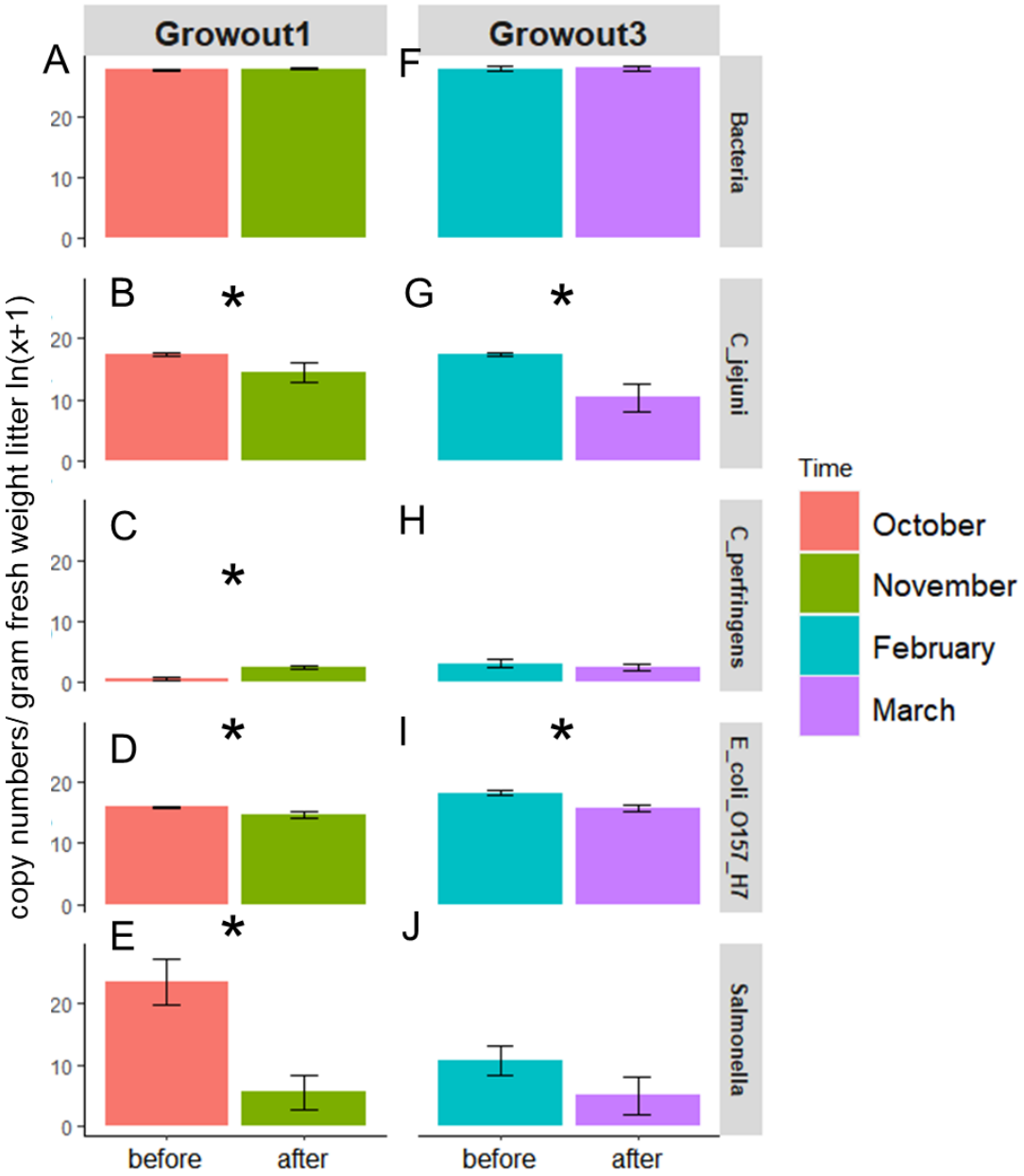


Figure 4- 1 Abundance of total bacteria (A and F) and four pathogen marker genes (*C. jejuni*: B and G, *C. perfringens*: C and H, *E. coli* O157:H7: D and I, and *Salmonella*: E and J) in poultry litter before and after in-house windrow composting for growout 1 and 3. The bars are the mean copies of pathogen genes ± standard error (n = 8) based on 2 samples from each of the four houses (includes both biochar amended and unamended control). *T*-tests were applied to determine statistically significant differences in pathogen abundance in each growout. Asterisks indicate statistically significant differences between before and after composting (*T* tests, $P < 0.05$).

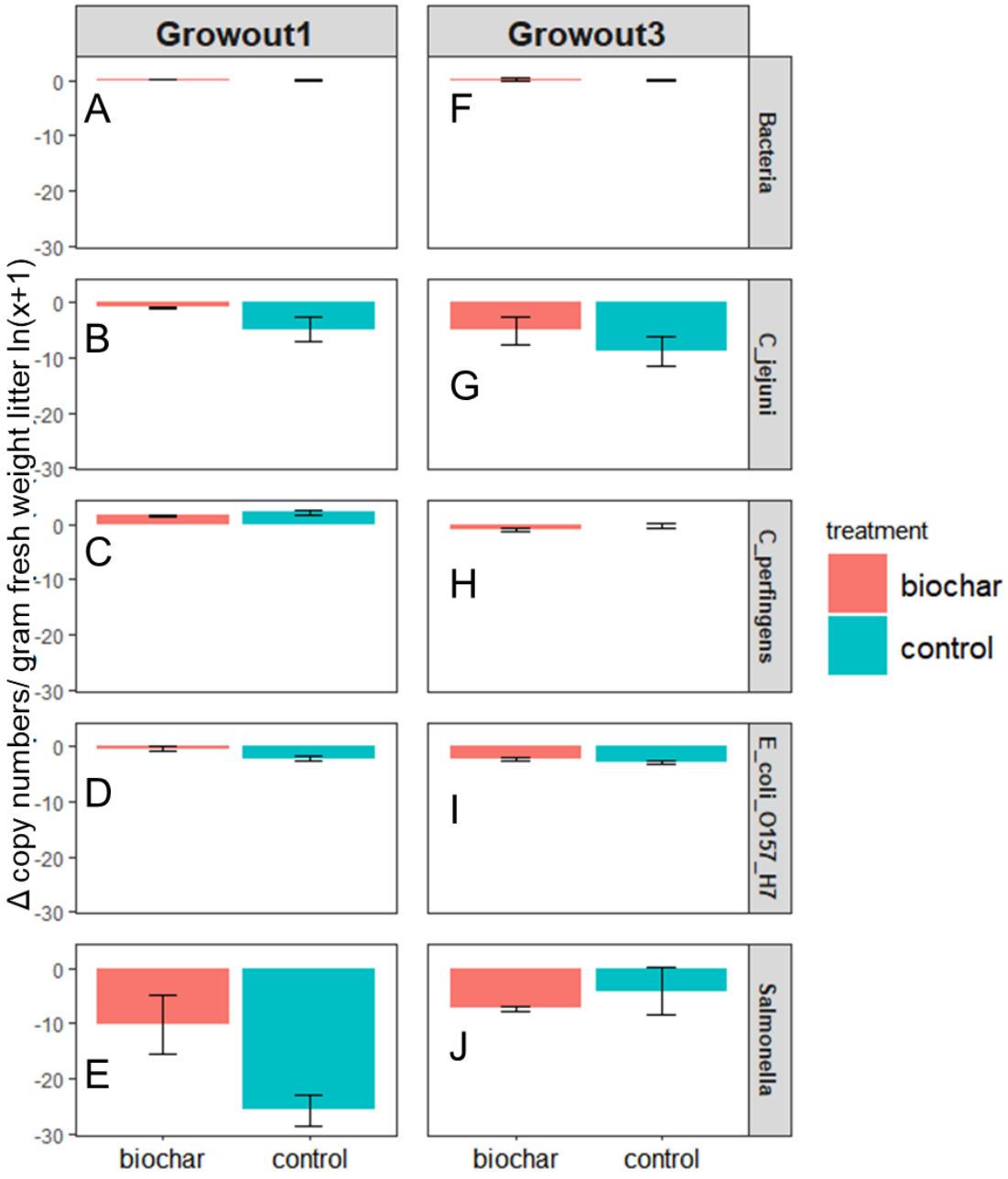


Figure 4- 2 Changes in total bacterial abundance (A and F) and pathogen gene copy abundances (*C. jejuni*: B and G, *C. perfringens*: C and H, *E. coli* O157:H7: D and I, and *Salmonella*: E and J) as a result of in-house windrow composting in biochar-amended and unamended poultry litter. The changes of gene copies were calculated as Δ = normalized initial copies after composting - normalized copies before composting. The bars are mean delta value ± standard error (n = 4).

Effects of initial moisture content on the presence of pathogens in poultry litter

To test the effects of initial litter moisture on pathogen abundance, poultry litter with three different moisture contents (35%, 40% and 45%) were used in a third simulated composting experiment. Four temperatures, 30°C, 50°C, 58°C and 62°C, were selected as the incubation temperatures. Poultry litter moisture content significantly decreased after heating to 50°C, 58°C and 62°C ($P < 0.01$), with no change during the recovery stage (Figure 4-4A-C). No significant change in moisture occurred in the 35°C treatment. In the simulated composting with 35% moisture, poultry litter moisture decreased by 9.85%, 8.8% and 8.83% after heating at 50°C, 58°C and 62°C treatments, respectively (Figure 4-4A). When initial moisture was 40%, poultry litter moisture decreased by 14.00%, 25.07% and 24.07% after heating at 50°C, 58°C and 62°C treatments (Figure 4-4B). In the 45% moisture treatments, poultry litter moisture decreased by 15.87%, 24.78%, and 28.95% at 50°C, 58°C and 62°C temperatures (Figure 4-4C).

In the 40% moisture content treatment, total bacterial 16S rRNA gene copy abundances decreased by 2.19 ln (1+ 16S rRNA copies g^{-1}), 0.5 ln (1+ 16S rRNA copies g^{-1}) and 1.89 ln (1+ 16S rRNA copies g^{-1}) following heating to 50°C ($P < 0.01$), 58°C and 62°C ($P < 0.05$), respectively (Figure 4-4H). In the treatments heated to 50°C and 62°C, bacterial 16S rRNA gene copy abundances increased during the recovery stage ($P < 0.05$), by 1.67 ln (1+ 16S rRNA copies g^{-1}) and 0.40 ln (1+ 16S rRNA copies g^{-1}), respectively (Figure 4-4H). When initial moisture content was 45%, total bacterial 16S rRNA gene abundances decreased by 1.65 ln (1+ 16S rRNA copies g^{-1}), 0.55 ln (1+ 16S rRNA copies g^{-1}) and 1.63 ln (1+ 16S rRNA copies g^{-1}) at 50°C ($P < 0.01$), 58°C and 62°C ($P < 0.05$) (Figure 4-4I). Following that, total bacterial 16S rRNA gene abundances increased during the recovery stage, increasing by 0.84 ln (1+ 16S rRNA

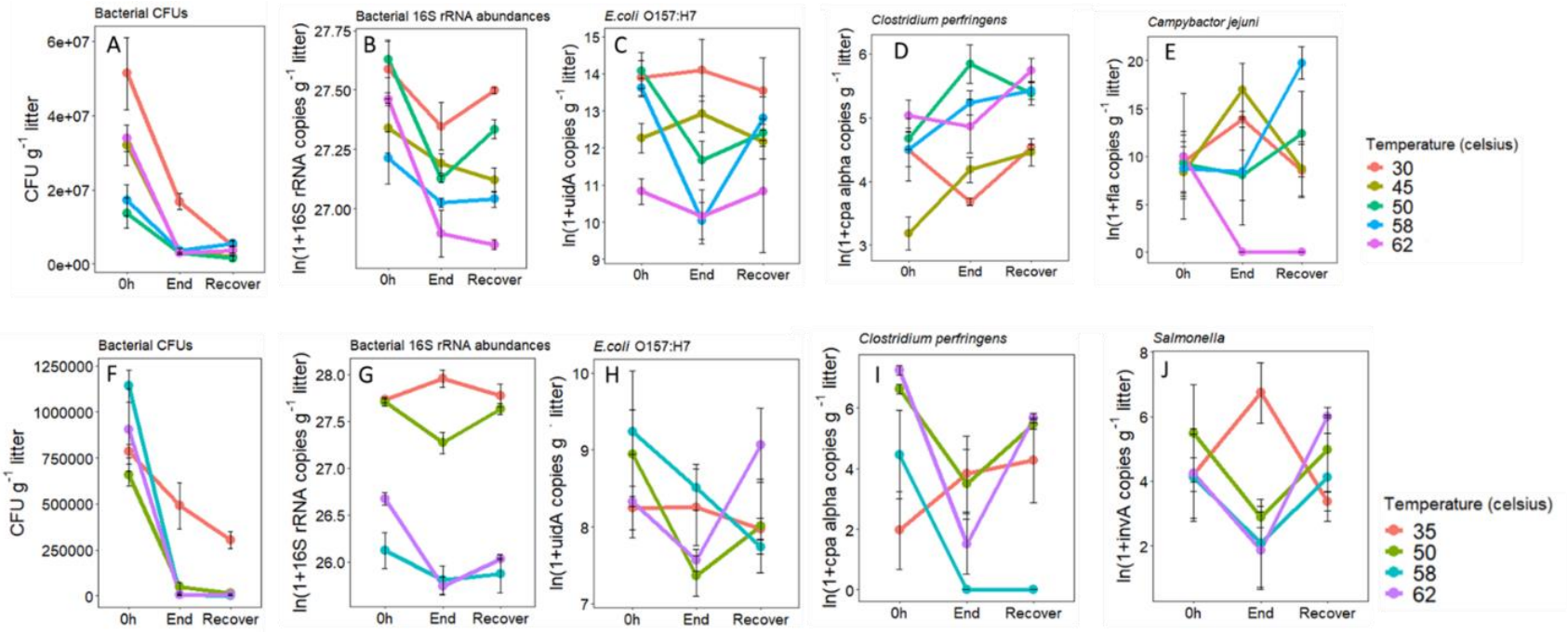


Figure 4- 3 Changes of bacterial CFUs (Figure 3A and F), total bacterial 16S rRNA gene copy abundance (Figure 3B and G), and the abundance of detectable pathogens in high moisture content (45%) poultry litter simulated composting (Figure 3C-E) and low moisture content (35%) poultry litter simulated composting (Figure 3H-J). Panels A-E show results from the first experiment with high moisture content (45%) poultry litter. Panels F-J show the results from the second experiment with low moisture content (35%) poultry litter. Points in Figure 4-3A and F are the actual value of CFUs. Points in the other figures are the average normalized value ± standard error calculated based on 3 replicate treatments.

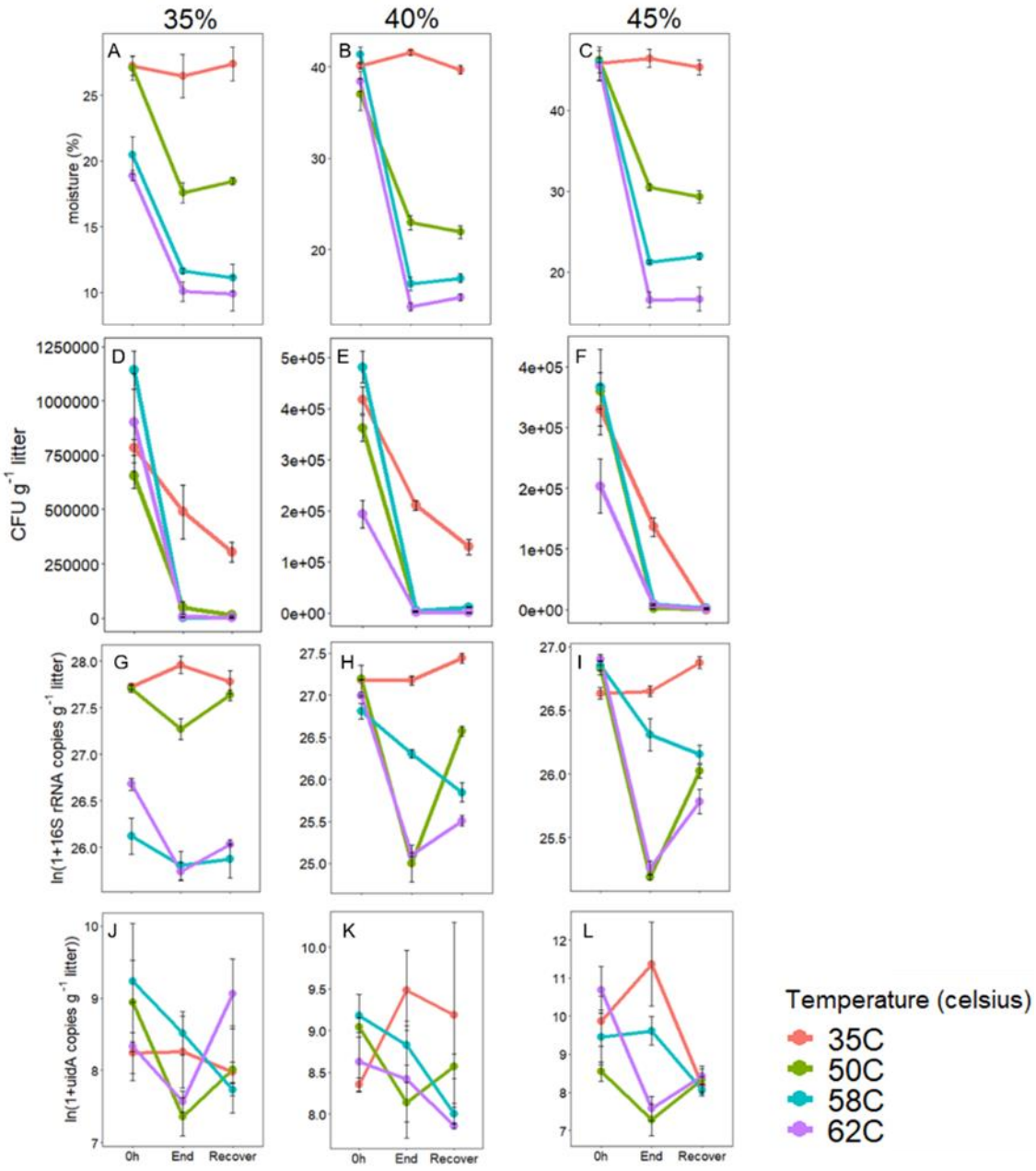


Figure 4- 4 Changes of moisture content (A-C), bacterial CFUs (D-F), total bacterial 16S rRNA gene abundances (G-I), and the abundance of *E. coli* O157:H7 (J-L) in three initial litter moisture contents (35%, 40%, and 45%) simulated poultry litter composting. Points in D-F are the actual values of CFUs.The points in G-L are the mean normalized gene copies ± standard error calculated based on 3 replications.

copies g^{-1}) and 0.48 ln (1+ 16S rRNA copies g^{-1}) at 50°C and 62°C, respectively. Bacterial CFUs in low moisture poultry litter indicated that microbes decreased with incubation time in all four temperatures and no regrowth was detected in the recovery stage (Figure 4D-F). *E. coli* O157:H7 was the only detectable pathogen in poultry litter for this experiment. In the 35% moisture content treatment, the abundance of *E. coli* O157:H7 decreased by 1.59 ln (1+ *uid*A copies g^{-1}), 0.73 ln (1+ *uid*A copies g^{-1}) and 0.67 ln (1+ 16S rRNA copies g^{-1}) at 50°C ($P < 0.01$), 58°C and 62°C ($P < 0.05$) after heating but then increased during recovery by 0.66 ln (1+ *uid*A copies g^{-1}) and 1.49 ln (1+ *uid*A copies g^{-1}) at 50°C and 62°C, respectively (Figure 4-4J). A similar change was detected in other temperatures in the 40% moisture treatments (Figure 4-4K). The abundance of *E. coli* O157:H7 significantly decreased at 62°C when initial moisture was 45%, decreasing by 3.11 ln (1+ *uid*A copies g^{-1}) ($P < 0.05$) (Figure 4-4L).

DISCUSSION

Understanding the impacts of in-house windrow composting is essential to guide proper poultry litter management and poultry health. In this study, in-house windrow composting did not significantly change total bacterial 16S rRNA gene copy abundance in poultry litter. Meng et al. (2019) found total bacterial 16S rRNA gene copy abundances increase in first 49 days and decrease in the last 10 days in cow manure-corn straw composting. In a 65-day poultry litter and lignite composting study, samples were collected at day 0, 8, 28 and 65. Total bacterial 16S rRNA gene copy abundance on day 28 was higher than day 0, but the abundance on day 65 was similar with day 0 (Cao et al., 2020). Based on the reported studies, the increased total bacterial abundance in the early stage was likely due to the availability of nutrients and aeration from turning which provided an ideal condition for microbial growth. In later stages, the total

bacterial abundance decreased because of higher temperatures and limited availability of nutrients compared to early stage. The stable bacterial abundance in our studies may be caused by only two sampling dates in our study instead of multiple sampling dates in other studies, which could reflect a complete trend of microbial abundance during windrow composting.

Enumerating bacteria by a plate counting method and qPCR gave different estimates of bacteria abundances in our simulated composting. Cultivation-based methods suggested total bacteria decreased in composting in our study which was consistent with previous studies. Chandan et al. (2013) indicated that in-house windrow composting is an effective method to reduce total bacterial numbers in animal waste and green waste composting. Green waste composting also decreased mesophilic amylolytic and caseinolytic bacteria (Ryckeboer et al., 2003). The inconsistent results in our results may be caused by different detection methods; cultivation-based methods miss unculturable microbes and molecular-based study would capture more unculturable, but viable microbes. Some microbes may be viable but cannot be cultured in general conditions. Culture-based methods are estimated to culture about 1% to 15% bacteria and so can be a heavily biased method (Wagner et al. 1993). Some pathogens have been documented as viable but unculturable in some cases, such as *C. jejuni*, *Salmoenlla* and enterohemorrhagic *E. coli* (Oliver, 2010), which could be used to explain the inconsistent results in our study.

Successful composting generates high temperatures which help eliminate the pathogens in animal waste. In this study, composting reduced the abundance of three pathogens, *Salmonella*, *C. jejuni* and *E. coli* O157:H7, showing that these pathogens were sensitive to the elevated temperatures, which reached maximum temperatures around 66 °C inside the compost piles and hold for 7-8 days (> 55°C). *Campylobacter*

has been shown to survive in large scale windrow composting of bovine, but high temperatures (55°C) decreased the abundance of *C. jejuni* rapidly after 2 h (Inglis et al., 2010). The reduction of *C. jejuni* observed in our study was in good agreement with previous cattle manure composting (Klein et al., 2011). Abundance of *Salmonella* also significantly reduced during poultry litter composting (Macklin et al., 2008; Singh et al., 2012). Although composting reduced the abundance of *Salmonella* in growout 1 and growout 3, the lower reduction in growout 3 may be due to the enhanced thermal-resistance in aged poultry litter (Chen et al., 2018). Longer survival of *Salmonella* in poultry litter composting may be caused by the higher resistance of *Salmonella* to heat-temperature stress (Singh et al., 2012). However, we observed a greater reduction in *Salmonella* compared to *E. coli* O157:H7 in this research, which may be *Salmonella* was more sensitive to temperature than *E. coli* O157:H7. Lung et al. (2019) reported that *Salmonella* is not detectable after 2 days of composting at 45°C, but *E. coli* O157:H7 was detectable until day 3.

In contrast to the other three pathogens, the abundance of *C. perfringens* significantly increased in growout 1 and decreased in growout 3. Like other *Clostridium* spp., *C. perfringens* forms resistant endospores which may explain their persistence under high temperature (Doyle, 2002; Qian et al., 2016). The different patterns of *C. perfringens* observed in growout 1 and growout 3 maybe be due to the following reasons: first, the highest germination temperature of *C. perfringens* is about 46°C (Labbe and Chang, 1995). High temperature can injure spores, but appropriate environmental conditions and nutrient level may promote the germination of injured spores. Second, it could be due to differences in the starting litter between growout 1 and 3: for example, if *C. perfringens* infected the previous flock, the recycled litter might become a source of pathogens further influencing the concentration of *C.*

perfringens and the health of following flocks (Wei et al., 2013). The third reason may be due to differences in aeration between the two growouts. Even though turning the piles helps aerate the piles, depleted oxygen is unavoidable due to the large size of the composting pile. As an obligate anaerobic bacterium, the depleted oxygen in composting piles may lead to the proliferation of *C. perfringens*.

Low biochar addition did not enhance pathogen removal during poultry litter composting

In our study, 1% biochar addition in poultry litter had no significant effect on pathogen removal during in-house windrow composting. Previous research has suggested that the percentage of biochar is a key factor for driving bacterial community composition, diversity and abundance, and a high biochar dosage leads to the high variation in bacterial diversity (Awasthi et al., 2020). Compared to the 1% biochar addition in this research, higher amendment rates of biochar (3 to 10%) has been showed to influence the abundance of fungi (Duan et al., 2019) and bacteria (Zhou et al., 2019), bacterial community structure, metabolism, total nitrogen (Zhou et al., 2019), NH_3 emission (Khan et al., 2014), reduce C: N and pathogens (Ravindran et al., 2019). Lower C: N ratios (8.12:1) deactivated the *Salmonella* and *E. coli* more effectively than high C: N ratios (18.58:1) (Chen et al., 2018), but C: N ratio did not significantly change in our study (chapter 3). Biochar addition also increased the peak temperature or accelerate the composting process (Jia et al., 2016), and temperature is the primary factor influencing composting due to its control on microbial activities (Jiang et al., 2017), but biochar amendment did not increase peak temperature in our study.

Inoculation studies have suggested that the indigenous microbial community in composting can suppress the growth of pathogens. Although we did not use spiked pathogens in this study, the presence of a stable and bio-diverse microbial community

might influence competition for nutrients, resulting in antagonistic effects on pathogens (Cui et al., 2016). Feed supplementation with 2% biochar has been shown to significantly reduce the abundance of *Campylobacter* in chicken guts, but 4% biochar caused a slight increase, suggesting that low biochar addition may be effective in pathogen removal (Willson et al., 2019). However, in our study, low amounts of biochar addition in windrow composting were not enough to influence the physico-chemical properties of the litter nor improve compost heating performance and therefore had limited effects on microbial activity and pathogen persistence and microbial diversity (Chapter 3).

Presence of pathogens in poultry litter windrow composting and simulated composting

In this study, we tested the effects of temperature and initial moisture levels on the presence of pathogens in low moisture and high moisture poultry litter at different selected temperatures in laboratory simulated composting. The poultry litter with high moisture content lost more moisture than the litter with low moisture content. For example, poultry litter with 45% moisture lost the highest moisture in three moisture adjustment simulated composting. Based on our results, the initial moisture content influenced the abundance of *E. coli* O157:H7: the reduction of *E. coli* O157:H7 in poultry litter with 45% was greater than 35% and 40% at 62°C treatment. Previous studies have also reported the effects of moisture on the presence of pathogens and bacteria. Singh et al. (2011) reported a longer survival of *Salmonella* in poultry compost with 40% moisture content compared to an optimal content of 50%. They suggested that the pathogens in the compost with low moisture content may adapt to the heat stress compared to the compost with high moisture content. High moisture content in composting leads to lower airflow and temperature rise, low moisture results in reduced

microbial activity and heating performance (Singh et al., 2011). Compared to the composing in the field, our laboratory study was conducted under controlled environmental conditions. The treatment conditions in this study were designed around the USDA recommendations for composting temperatures. The presence of *E. coli* O157:H7 suggested that suboptimal heating may lead to the persistence of pathogens in composting. Inefficient heating may increase pathogen resistance: In a field study, the presence of heat-shocked *E. coli* O157:H7 was detected at the bottom of the dairy compost after 5 days, while non-heat shocked *E. coli* O157:H7 only survived for 1 day (Shepherd et al., 2010). Thus, it is necessary to consider the effects of heat-shock on the pathogen removal in field composting, because the large volume of composting pile may cause non-uniform heat distribution. Suboptimal temperatures may induced the heating resistance of pathogens, further increase the survival when the pathogens are later exposed to inactivation temperatures (Singh et al., 2010).

The persistence of pathogens was different between the in-house windrow experiment and the controlled lab simulated composting, even though both used the same poultry litter. The main difference between simulated composting and in-house windrow composting is the source of heat. Although the temperatures in each experiment reach the recommended temperatures, the heating was produced through different aspects. The physical heating in the simulated composting experiments was from the incubators while the high temperature in windrow composting was from the microbial activity in composting piles. Microbial activities in windrow composting consumed nutrients and degraded organic matter, then increased temperature in composting, physico-chemical properties also changed during composting. However, simulated composting only had a thermal inactivation stage and did not have a stage of high microbial activities. Furthermore, it's necessary to consider the heating treatment

methods and the initial moisture content of poultry litter, as all these factors may influence the effectiveness of heating treatment on pathogen removal.

CONCLUSION

We demonstrated that in-house windrow composting in a commercial farm setting successfully reduced abundances of three of the four tested pathogens: *E. coli* O157:H7, *Salmonella* and *C. jejuni,* but not *C. perfringens*. Treatment with 1% biochar did not improve the reduction of pathogens during windrow composting, and higher rates of biochar addition may be required to have effects at the farm scale. Our laboratory poultry litter simulated composting experiments suggested that the abundance of total bacteria and detected pathogens were lower in the low moisture (35%) litter compared to high moisture (45%). Moisture adjustment simulated composting demonstrated that incubation at the highest temperature in this study (62 °C), significantly reduced the abundance of total bacteria and *E. coli* O157:H7 in high moisture content (45%) poultry litter. Even though plate count method and qPCR did not show a similar result in evaluating the change of total bacteria, the co-application of two detection methods should be applied as a complementary method due to their respective advantages. Our study highlighted the efficiency of in-house windrow composting in the removal of pathogens and the importance of temperature and initial moisture of poultry litter in controlling pathogen persistence.

REFERENCES

Atidégla, S.C., Huat, J., Agbossou, E.K., Saint-Macary, H., and Glèlè Kakai, R. (2016). Vegetable contamination by the fecal bacteria of poultry manure: case study of gardening sites in Southern Benin. *International journal of food science* 2016.

Awasthi, M.K., Duan, Y., Liu, T., Awasthi, S.K., and Zhang, Z. (2020). Relevance of biochar to influence the bacterial succession during pig manure composting. *Bioresour Technol* 304**,** 122962..

Burton, C.H., and Turner, C. (2003). *Manure management: Treatment strategies for sustainable agriculture.* Editions Quae.

Bavaro, M.F. (2012). E. coli O157:H7 and Other Toxigenic Strains: The Curse of Global Food Distribution. *Current Gastroenterology Reports* 14(4)**,** 317-323.

Chen, Z., and Jiang, X. (2017). Microbiological Safety of Animal Wastes Processed by Physical Heat Treatment: An Alternative To Eliminate Human Pathogens in Biological Soil Amendments as Recommended by the Food Safety Modernization Act. *J Food Prot* 80(3)**,** 392-405.

Chen, Z., Kim, J., and Jiang, X. (2018). Survival of Escherichia coli O157:H7 and Salmonella enterica in animal waste-based composts as influenced by compost type, storage condition and inoculum level. *J Appl Microbiol* 124(5)**,** 1311-1323.

Chandna, P., Nain, L., Singh, S., and Kuhad, R.C. (2013). Assessment of bacterial diversity during composting of agricultural byproducts. *BMC Microbiol* 13**,** 99-99.

Chen, Z., and Jiang, X. (2014). Microbiological Safety of Chicken Litter or Chicken Litter-Based Organic Fertilizers: A Review. *Agriculture* 4(1)**,** 1-29.

Chen, Z., Kim, J., and Jiang, X. (2018). Survival of Escherichia coli O157:H7 and Salmonella enterica in animal waste-based composts as influenced by compost type, storage condition and inoculum level. *J Appl Microbiol* 124(5)**,** 1311-1323.

Choct, M., and Kocher, A. (2008). Dietary factors influencing necrotic enteritis outbreaks in broilers. *World's Poult. Sci. J* 64**,** 159.

Cooper, K.K., and Songer, J.G. (2009). Necrotic enteritis in chickens: A paradigm of enteric infection by Clostridium perfringens type A. *Anaerobe* 15(1)**,** 55-60.

Cui, E., Wu, Y., Zuo, Y., and Chen, H. (2016). Effect of different biochars on antibiotic resistance genes and bacterial community during chicken manure composting. *Bioresour Technol* 203**,** 11-17.

Delgado, M., Rodríguez, C., Martín, J.V., Miralles de Imperial, R., and Alonso, F. (2012). Environmental assay on the effect of poultry manure application on soil organisms in agroecosystems. *Sci Total Environ* 416**,** 532-535.

Doyle, M.E. (2002). *Survival and growth of clostridium perfringens during the cooling step of thermal processing of meat products: a review of the scientific literature.* Food Research Institute, University of Wisconsin--Madison.

Duan, Y., Awasthi, S.K., Chen, H., Liu, T., Zhang, Z., Zhang, L., et al. (2019). Evaluating the impact of bamboo biochar on the fungal community succession during chicken manure composting. *Bioresour Technol* 272**,** 308-314.

El-Hack, M.E.A., Samak, D.H., Noreldin, A.E., El-Naggar, K., and Abdo, M. (2018). Probiotics and plant-derived compounds as eco-friendly agents to inhibit

microbial toxins in poultry feed: a comprehensive review. *Environmental Science and Pollution Research* 25(32), 31971-31986.

Elaine, S., Robert, M.H., Frederick, J.A., Robert, V.T., Marc-Alain, W., Sharon, L.R., et al. (2011). Foodborne Illness Acquired in the United States—Major Pathogens. *Emerging Infectious Disease journal* 17(1), 7.

Elizaquível, P., Sánchez, G., and Aznar, R. (2012). Quantitative detection of viable foodborne E. coli O157:H7, Listeria monocytogenes and Salmonella in fresh-cut vegetables combining propidium monoazide and real-time PCR. *Food Control* 25(2), 704-708.

Gast, R.K., and Porter Jr, R.E. (2020). Salmonella infections. *Diseases of poultry*, 717-753.

Graber, E.R., Frenkel, O., Jaiswal, A.K., and Elad, Y. (2014). How may biochar influence severity of diseases caused by soilborne pathogens? *Carbon Management* 5(2), 169-183.

Galitskaya, P., Biktasheva, L., Saveliev, A., Grigoryeva, T., Boulygina, E., and Selivanovskaya, S. (2017). Fungal and bacterial successions in the process of co-composting of organic wastes as revealed by 454 pyrosequencing. *PloS one* 12(10), e0186051-e0186051.

Godlewska, P., Schmidt, H.P., Ok, Y.S., and Oleszczuk, P. (2017). Biochar for composting improvement and contaminants reduction. A review. *Bioresour Technol* 246, 193-202.

Graber, E.R., Frenkel, O., Jaiswal, A.K., and Elad, Y. (2014). How may biochar influence severity of diseases caused by soilborne pathogens? *Carbon Management* 5(2), 169-183.

Inglis, G.D., McAllister, T.A., Larney, F.J., and Topp, E. (2010). Prolonged Survival of Campylobacter Species in Bovine Manure Compost. *Appl Environ Microbiol* 76(4), 1110-1119.

Jia, X., Wang, M., Yuan, W., Ju, X., and Yang, B. (2016). The influence of biochar addition on chicken manure composting and associated methane and carbon dioxide emissions. *Bioresources* 11(2), 5255-5264.

Jiang, C., Wu, Y., and Cheng, Y. (2017). Bacterial and fungal communities and contribution of physicochemical factors during cattle farm waste composting. *MicrobiologyOpen* 6(6), e00518.

Jones, P.J., Niemi, J., Christensen, J.-P., Tranter, R.B., and Bennett, R.M. (2019). A review of the financial impact of production diseases in poultry production systems. *Animal Production Science* 59(9), 1585-1597.

Khan, N., Clark, I., Sánchez-Monedero, M.A., Shea, S., Meier, S., and Bolan, N. (2014). Maturity indices in co-composting of chicken manure and sawdust with biochar. *Bioresour Technol* 168, 245-251.

Klappenbach, J.A., Dunbar, J.M., and Schmidt, T.M. (2000). RRNA operon copy number reflects ecological strategies of bacteria. *Appl Environ Microbiol* 66(4), 1328-1333.

Klein, M., Brown, L., Ashbolt, N.J., Stuetz, R.M., and Roser, D.J. (2011). Inactivation of indicators and pathogens in cattle feedlot manures and compost as determined by molecular and culture assays. *FEMS Microbiol Ecol* 77(1)**,** 200-210.

Kuok, F., Mimoto, H., and Nakasaki, K. (2012). Effects of turning on the microbial consortia and the in situ temperature preferences of microorganisms in a laboratory-scale swine manure composting. *Bioresour Technol* 116**,** 421-427.

Labbe, R., and Chang, C.A. (1995). *Lett Appl Microbiol* 21(5)**,** 302-306.

Langarica-Fuentes, A., Zafar, U., Heyworth, A., Brown, T., Fox, G., and Robson, G.D. (2014). Fungal succession in an in-vessel composting system characterized using 454 pyrosequencing. *FEMS Microbiol Ecol* 88(2)**,** 296-308.

López-Cano, I., Roig, A., Cayuela, M.L., Alburquerque, J.A., and Sánchez-Monedero, M.A. (2016). Biochar improves N cycling during composting of olive mill wastes and sheep manure. *Waste Manage* 49**,** 553-559.

Lung, A., Lin, C.-M., Kim, J., Marshall, M., Nordstedt, R., Thompson, N., et al. (2001). Destruction of Escherichia coli O157: H7 and Salmonella enteritidis in cow manure composting. *J Food Prot* 64(9)**,** 1309-1314.

Macklin, K.S., Hess, J.B., and Bilgili, S.F. (2008). In-House Windrow Composting and Its Effects on Foodborne Pathogens. *The Journal of Applied Poultry Research* 17(1)**,** 121-127.

Malangu, N. (2017). *Poisoning: From Specific Toxic Agents to Novel Rapid and Simplified Techniques for Analysis.* BoD–Books on Demand.

Malorny, B., Hoorfar, J., Bunge, C., and Helmuth, R. (2003). Multicenter validation of the analytical accuracy of Salmonella PCR: towards an international standard. *Appl Environ Microbiol* 69(1)**,** 290-296.

Meng, Q., Yang, W., Men, M., Bello, A., Xu, X., Xu, B., et al. (2019). Microbial Community Succession and Response to Environmental Variables During Cow Manure and Corn Straw Composting. *Frontiers in Microbiology* 10(529).

Oakley, B.B., Morales, C.A., Line, J., Berrang, M.E., Meinersmann, R.J., Tillman, G.E., et al. (2013). The Poultry-Associated Microbiome: Network Analysis and Farm-to-Fork Characterizations. *PLOS ONE* 8(2)**,** e57190.

Oliver, J.D. (2010). Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol Rev* 34(4)**,** 415-425.

Qian, X., Sun, W., Gu, J., Wang, X.-J., Sun, J.-J., Yin, Y.-N., et al. (2016). Variable effects of oxytetracycline on antibiotic resistance gene abundance and the bacterial community during aerobic composting of cow manure. *J Hazard Mater* 315**,** 61-69.

Ravindran, B., Nguyen, D.D., Chaudhary, D.K., Chang, S.W., Kim, J., Lee, S.R., et al. (2019). Influence of biochar on physico-chemical and microbial community during swine manure composting process. *J Environ Manage* 232**,** 592-599.

Roll, V., Dai Prá, M., and Roll, A. (2011). Research on Salmonella in broiler litter reused for up to 14 consecutive flocks. *Poultry science* 90(10)**,** 2257-2262.

Rothrock, M.J., Jr., Cook, K.L., Warren, J.G., and Sistani, K. (2008). The effect of alum addition on microbial communities in poultry litter. *Poult Sci* 87(8)**,** 1493-1503.

Ryckeboer, J., Mergaert, J., Coosemans, J., Deprins, K., and Swings, J. (2003). Microbiological aspects of biowaste during composting in a monitored compost bin. *J Appl Microbiol* 94(1)**,** 127-137.

Singh, R., Kim, J., and Jiang, X. (2012). Heat inactivation of Salmonella spp. in fresh poultry compost by simulating early phase of composting process. *J Appl Microbiol* 112(5)**,** 927-935.

SHEPHERD, M.W., JR., LIANG, P., JIANG, X., DOYLE, M.P., and ERICKSON, M.C. (2007). Fate of Escherichia coli O157:H7 during On-Farm Dairy Manure–Based Composting. *J Food Prot* 70(12)**,** 2708-2716.

Shepherd, M.W., Singh, R., Kim, J., and Jiang, X. (2010). Effect of heat-shock treatment on the survival of Escherichia coli O157:H7 and Salmonella enterica Typhimurium in dairy manure co-composted with vegetable wastes under field conditions. *Bioresour Technol* 101(14)**,** 5407-5413.

Singh, R., Jiang, X., and Luo, F. (2010). Thermal inactivation of heat-shocked Escherichia coli O157:H7, Salmonella, and Listeria monocytogenes in dairy compost. *J Food Prot* 73(9)**,** 1633-1640.

Singh, R., Kim, J., and Jiang, X. (2012). Heat inactivation of Salmonella spp. in fresh poultry compost by simulating early phase of composting process. *J Appl Microbiol* 112(5)**,** 927-935.

Singh, R., Kim, J., Shepherd, M.W., Luo, F., and Jiang, X. (2011). Determining Thermal Inactivation of Escherichia coli O157:H7 in Fresh Compost by Simulating Early Phases of the Composting Process. *Appl Environ Microbiol* 77(12)**,** 4126-4135.

Su, J.-Q., Wei, B., Ou-Yang, W.-Y., Huang, F.-Y., Zhao, Y., Xu, H.-J., et al. (2015). Antibiotic Resistome and Its Association with Bacterial Communities during Sewage Sludge Composting. *Environ Sci Technol* 49(12)**,** 7356-7363.

Sun, D.-L., Jiang, X., Wu, Q.L., and Zhou, N.-Y. (2013). Intragenomic Heterogeneity of 16S rRNA Genes Causes Overestimation of Prokaryotic Diversity. *Appl Environ Microbiol* 79(19)**,** 5962-5969.

Větrovský, T., and Baldrian, P. (2013). The Variability of the 16S rRNA Gene in Bacterial Genomes and Its Consequences for Bacterial Community Analyses. *PLOS ONE* 8(2)**,** e57923.

Wang, D. (2019). Biochar amendment and moisture effects on poultry litter windrow heating performance. 2019. University of Tennessee, Knoxville, Master thesis.

Wagner, M., Amann, R., Lemmer, H., and Schleifer, K.H. (1993). Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. *Appl Environ Microbiol* 59(5)**,** 1520-1525.

Waziri, M.I., and Kaltungo, B.Y. (2017). "Poultry Litter Selection, Management and Utilization in the Tropics," in *Poultry Science*. InTech).Wade, B., and Keyburn, A. (2015). The true cost of necrotic enteritis. *World Poult* 31(7)**,** 16-17.

Wei, S., Gutek, A., Lilburn, M., and Yu, Z. (2013). Abundance of pathogens in the gut and litter of broiler chickens as affected by bacitracin and litter management. *Vet Microbiol* 166(3-4)**,** 595-601.

Wieczorek, K., Wołkowicz, T., and Osek, J. (2019). flaA-SVR Based Genetic Diversity of Multiresistant Campylobacter jejuni Isolated From Chickens and Humans. *Frontiers in microbiology* 10**,** 1176-1176.

Willson, N.-L., Van, T.T.H., Bhattarai, S.P., Courtice, J.M., McIntyre, J.R., Prasai, T.P., et al. (2019). Feed supplementation with biochar may reduce poultry pathogens, including Campylobacter hepaticus, the causative agent of Spotty Liver Disease. *PloS one* 14(4)**,** e0214471-e0214471.

Zhou, G., Xu, X., Qiu, X., and Zhang, J. (2019). Biochar influences the succession of microbial communities and the metabolic functions during rice straw composting with pig manure. *Bioresour Technol* 272**,** 10-18

APPENDIX

Table S4-1 Plate count agar recipe

	Weight/volume
Tryptone	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
Agar	15.0 g
Mill-Q water	1000 ml

pH: 7.0±0.2

Table S4-2 Abundances of total bacteria and four pathogens in two types of biochar-amendment composting and unamended poultry litter windrow composting.

			Total bacteria	*Salmonella*	*E. coli* O157:H7	*Campylobacter jejuni*	*Clostridium perfringens*
Growout 1	Before composting (Oct)	control	27.59±0.03	21.04±0.89	15.62±0.19	17.11±0.27	0.28±0.15
		Biochar L (Lebanon)	27.73±0.03	22.63±4.26	15.89±0.03	17.19±0.05	0.80±0.30
		Biochar P (ProtonPower)	27.58±0.04	9.733±0.33	16.35±0.22	17.80±0.01	0.99±0.16
	After composting (Nov)	control	27.61±0.05	5.28±2.82	13.39±0.36	12.28±2.19	2.52±0.33
		Biochar L (Lebanon)	27.92±0.03	0	15.62±0.34	16.30±0.03	2.87±0.33
		Biochar P (ProtonPower)	27.81±0.00	11.8±0.04	15.83±0.22	16.85±0.03	2.28±0.16
Growout 3	Before composting (Feb)	control	27.63±0.11	12.92±1.82	18.05±0.36	17.18±0.32	2.30±0.56
		Biochar L (Lebanon)	27.76±0.06	9.98±0.16	18.14±0.39	17.07±0.15	5.22±0.45
		Biochar P (ProtonPower)	28.02±0.01	7.53±0.54	18.73±0.18	17.72±0.05	2.78±0.45
	After composting (Mar)	control	27.70±0.12	8.78±2.64	15.23±0.62	8.34±2.57	2.08±0.50
		Biochar L (Lebanon)	27.94±0.01	2.84±0.01	15.84±0.06	7.93±2.99	4.25±0.29
		Biochar P (ProtonPower)	28.140.11	0	16.23±0.13	16.65±0.10	1.85±0.70

CHAPTER 5 CONCLUSIONS AND FUTURE DIRECTIONS

Two types of experiments were set up in this study: in-house windrow composting in a commercial poultry farm and simulated poultry litter composting in the laboratory. The overall goal of this dissertation research was to evaluate the changes in physico-chemical properties, microbial community structures, N-cycling functional genes and pathogens in poultry litter composting.

In chapter 3, in-house windrow composting was conducted in a commercial farm and low amounts of biochar (1%) was added as composting additive. Two growouts (1 and 3) were selected for study. Basic poultry litter physical- and chemical properties, bacterial and fungal community and N-cycling functional genes were measured to evaluate the effects of in-house windrow composting and biochar amendment. Moisture contents decreased in each growout, NO_3^--N concentration increased in growout 3, the remaining physico-chemical properties did not have significant changes in each growout. Bacterial community and diversity significantly changed in each growout: dominant bacterial orders were *Bacillales* and *Acinomycetales* which belong to Firmicutes and Actinobacteria, respectively. Fungal community diversity did not change as a result of composting, and the community structure only changed in growout 3. *Sarcharomycetales*, *Microascales* and *Eurotiales* which belong to Ascomycota were the dominant fungal orders. N-cycling functional genes did not change in growout 1, but increased in growout 3, including *amoA*, *nirS*, *nirK* and *nosZ*. Low amounts of biochar did not influence microbial community structure, diversity and abundance of dominant taxa, but increased the abundances of *nirS*, *nirK* and *nosZ* in growout 1 and decreased total carbon in growout 3. These results indicated the effects of in-house windrow composting on N-cycling functional gene abundances, bacterial and fungal community compositions. Even though low amounts of biochar addition (1%) did not change bacterial and fungal community structure, biochar addition may have the

potential to increase denitrification in composting because of the increased abundance of denitrification genes in growout 1.

To assess the reduction of pathogens in poultry litter management, four indicator pathogens, *Salmonella*, *E. coli* O157:H7, *C. jejuni* and *C. perfringens* were selected to evaluate the effects of in-house windrow composting, low amounts of biochar addition, different temperatures and initial moisture contents. In-house windrow composting significantly decreased the abundances of *Salmonella*, *E. coli* O157:H7 and *C. jejuni*, not *C. perfringens.* The effects of biochar on pathogen removal were not detected during in-house windrow composting. As a complementary experiment, simulated poultry litter composting was conducted to test the effects of temperature (30°C, 45°C, 50°C, 58°C, 62°C in high moisture content poultry litter, 35°C, 50°C, 58°C, 62°C in low moisture content poultry litter) and initial moisture contents (35%, 40%, and 45%) on the presence of pathogens. Two types of poultry litter were selected in this simulated composting experiment, including the high moisture content poultry litter (45%) and low moisture content poultry litter (35%). Our results indicated that high temperature decreases total bacterial abundance in poultry litter, but *Salmonella*, *E. coli* O157:H7 and *C. jejuni* did not significantly change in the temperature adjustment experiment. *E. coli* O157:H7 significantly decreased after 62 °C when initial moisture was 45%. Our results indicated that 1) in-house windrow composting is beneficial for pathogen removal; 2) low amounts of biochar addition (1%) do not affect pathogen removal in poultry litter in-house windrow composting; 3) high temperature reduced total bacteria in poultry litter composting, but plate count method and qPCR showed different results in estimating bacterial abundance, which suggests use of both these methods to evaluate the change in bacterial abundance; 4) when poultry litter moisture is low, no need to reach the highest temperature to remove pathogens; and 5) changes in total bacteria and

pathogens had different responses to composting, which suggests total bacterial abundance cannot be used as an indicator of poultry litter pathogenic safety.

Even though our study provides some information on microbial communities, N-cycling functions, and presence of pathogens in poultry litter composting, further experiments would be beneficial to understand the biological process during windrow composting: 1) Measure NO_2 and NH_3 emissions during windrow composting with and without biochar amendments. It would be helpful to understand the N-related odor and greenhouse gas emissions in large-scale windrow composting instead of the prediction based on molecular biology. The reduction of odor emission has been identified in a bioreactor composting, such as H_2S and NH_3, where the biochar amendment rate was about 5 to 20%. However, their results suggested the reductions were caused by improved aeration rather than the sorption capacity of biochar (Steiner et al., 2010). Thus, the further experiments should focus on the relative importance of biochar's aeration and sorption in windrow composting; 2) To have a better understanding of the persistence of pathogens in the poultry litter, it is necessary to elucidate the ecological relationships between pathogens and the indigenous microbial community in poultry litter. As only four pathogenic bacteria in poultry litter were evaluated in this study, a more comprehensive study contains more different pathogens in poultry litter is necessary. This type of investigation should focus on the antagonistic microbial interactions and nutrient competition between pathogens and microbial communities in poultry litter. Further, it would be helpful to verify our findings in a poultry farm with disease outbreak history instead of conducing pathogen inoculation in poultry litter in the laboratory; 3) To capture the dynamics of composting microbial communities, it would be beneficial to monitor the continuous change of microbial process and environmental properties during windrow composting. In our study, samples were

collected on two dates, before composting and after composting. More sampling dates would give a better understanding of the succession of microbial communities and functions during composting. 4) The efficiency of biochar amendment may relates to the scale of composting. Even though large scale in-house windrow composting is widely used in poultry industry, studies at the farm scale are still lacking. Thus, it's necessary to evaluate the efficiency of biochar amendment in large-scale windrow composting instead of pilot composting, in-vessel composting and laboratory simulated composting. This suggested that future studies should be designed by scientific organizations and the livestock industry together. 5) Improve the recycling of poultry litter in the farm, including the application of poultry litter as fertilizer, feedstock of biochar production and anerobic digestion. The benefits of biochar amendment on agricultural lands and composting have been identified, but the efficiency of biochar amendment are related to the biochar dosage rates. The initial aim of our study was to use logistically feasible amounts of biochar during windrow composting to reduce the biochar cost. As a available biochar feedstock, poultry litter-based biochar would reduce the cost for biochar Pyrolysis produces biogas and biochar, so if the poultry litter biochar can be applied in the farm, it would not only reduce the cost for biochar but also supply biogas energy to the farm. Future studies should enhance the recycling of poultry litter in the farm and test the efficiency of poultry litter-based biochar in poultry litter composting and adjacent agricultural fields.

In summary, this study revealed the profile of microbial community and N-cycling functional genes during windrow composting, which will help us to better understanding the microbial ecology in animal waste composting. The efficiency of in-house windrow composting on pathogen removal in poultry litter was identified. This

study also emphasized the importance of proper temperature and initial moisture content in poultry litter management.

VITA

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