ABSTRACT

Title of Thesis: THE EFFICACY OF OOLITIC ARAGONITE FOR AMMONIA CONTROL AND MICROBIAL MODULATION IN BROILER LITTER

Luke Patrick Feeney, Masters of Science, 2025

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Ammonia (NH_3) emissions from broiler production raise environmental, agronomic, and animal welfare concerns. This study evaluated oolitic aragonite (OA), a calcium carbonate-rich Bahamian sand, as a broiler litter amendment to reduce NH_3 emissions, improve nitrogen retention, and shape microbial communities. Three consecutive 6-week broiler flocks were raised in environmentally controlled chambers with litter prepared from wood shavings and OA sand at 100:0, 80:20, and 60:40 (shavings:OA) weight ratios. Chamber environmental conditions were continuously monitored, and weekly composite litter samples were analyzed for pH, uric acid, moisture, and water activity. At the end of each flock, litter was tested for total nitrogen, ammonia-N, ammonium-N, and total phosphorus (as P_2O_5). Despite initial hypotheses that OA would regulate NH_3 volatilization through reducing water activity and promoting nitrifying/denitrifying pathways, no significant differences were observed between OA and control litter in NH_3 emissions, total nitrogen, uric acid, or water activity. The NH_3 flux rose during flock cycles and peaked after windrow composting across all treatments. Microbial community analysis (16S rRNA sequencing) of start and end samples from each flock showed alpha and beta diversity were driven mainly by temporal succession, with shifts from gut-associated taxa (e.g., *Enterococcus*, *Escherichia/Shigella*, *Clostridium_sensu_stricto*) to litter-adapted genera (e.g., *Corynebacterium*,

Brachybacterium, *Salinicoccus*). While differential abundance testing found few consistent treatment effects, *Lysobacter*, a genus associated with antimicrobial activity, was persistently enriched in OA treated litter. Observed effects on microbial diversity were subtle and did not result in meaningful functional shifts in the broader community composition between treatments. While OA as a standalone amendment did not significantly affect NH_3 emissions, its selective enrichment of *Lysobacter* warrants further investigation.

THE EFFICACY OF OOLITIC ARAGONITE FOR AMMONIA CONTROL AND MICROBIAL MODULATION IN BROILER LITTER

by

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A Thesis submitted to the Faculty of the Graduate School of the
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Dedication

To my parents, whose endless love, encouragement, and unwavering belief in me have been my foundation throughout this journey.

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Chapter 1

1.1 Literature Review

Background

The broiler industry in the United States has expanded significantly in the past three decades, growing from 7.4 billion birds in 1995 to 9.33 billion birds in 2024 (USDA Economic Research Service, 2014; USDA NASS, 2024). This rapid increase has positioned chicken as the most consumed meat in the U.S. since the mid-1990s, surpassing both pork and beef (USDA Economic Research Service, 2018). The Delmarva Peninsula, comprising Delaware, the Eastern Shore of Maryland, and the Eastern Shore of Virginia, has historically been a foundational region for commercial broiler production and continues to be a critical contributor to the national supply. In 2024, the Delmarva region alone raised over 613 million chickens, yielding 4.6 billion pounds of processed chicken with a wholesale value of $4.8 billion (Delmarva Chicken Association, 2025). This intensive scale of production inherently generates substantial volumes of broiler waste in the form of litter.

Broiler litter is a mixture of manure (excreta) and bedding material (e.g. wood shavings, sawdust, straw), along with spilled feed, feathers, and moisture from drinking water spillage (Rahman et al., 2022; Omeira et al., 2006). This diverse composition makes broiler litter characteristically rich in nutrients such as nitrogen (N), phosphorus (P), potassium (K), and other trace minerals, making

litter a valuable agricultural fertilizer, but also a significant concern as a source of nutrient pollution when mismanaged (Cook et al., 2011; Naseem & King, 2018).

Inadequate litter pH and moisture control, insufficient ventilation, and prolonged reuse can lead to many environmental and production challenges (Bolan et al., 2010; Naseem & King, 2018). Ammonia (NH_3) emission from litter is one such challenge. As microbial activity in litter increases, nitrogenous waste materials within the litter get broken down and volatilized in the form of NH_3 gas (Burt et al., 2017; Schefferle, 1965; Kim & Patterson, 2003; Joerger et al., 2020). High NH_3 levels within poultry houses (> 25ppm) harm broiler health and performance, causing respiratory tract damage, eye irritation, and increased susceptibility to diseases. Prolonged exposure at high levels has been shown to inhibit growth and feed efficiency in broilers (Sheikh et al., 2018; Kristensen & Wathes, 2000). These high NH_3 levels also pose an occupational health risk to the farm workers who are consistently exposed (Padappayil & Borger, 2025; Naseem & King, 2018). In terms of negative environmental implications, NH_3 volatilization contributes to atmospheric pollution when being exhausted from poultry houses. Once released, NH_3 can react with other atmospheric compounds to form fine particulate matter, which poses risks to both environmental and human health (Bist et al., 2023; Naseem & King, 2018). Especially in poultry-dense regions like the Delmarva Peninsula, atmospheric deposition of NH_3 can lead to soil acidification and nutrient imbalances in nearby ecosystems like the Chesapeake Bay watershed, contributing to issues such as eutrophication and biodiversity loss (Larsen et al., 2001; Baker et al., 2020; Malone, 1992). Another environmental issue related to poor litter management is nutrient runoff that occurs when broiler litter is routinely utilized as fertilizer for fields. Litter is valuable for growing crops because of its high N and P content, but improper or over-application can lead to loss of these nutrients in the form of leaching or runoff (Bolan et al., 2010; Moore et al., 1995).

Additionally, broiler litter can harbor many pathogenic microorganisms such as *Salmonella*, *Campylobacter*, and *E.coli* (Rothrock et al., 2008a; de Toledo et al., 2020; Lu et al., 2003). When litter conditions are not properly managed, higher pathogenic load can result in increased risks to broiler health and food safety (Oladeinde et al., 2025; Liang et al., 2014; Bailey et al., 2022). Proper litter management is essential in modern commercial production to maintain bird health and welfare, optimize fertilizer value, and minimize negative environmental impacts.

Ammonia Production

NH_3 in poultry houses originates primarily from the microbial breakdown of uric acid that has been deposited into the bird litter (Kim & Patterson, 2003; Joerger et al., 2020). Uric acid and are the main nitrogenous waste components in bird manure, and they are quickly degraded through a series of enzymatic steps in a process called mineralization (Nahm, 2005). Mineralization is the process whereby organic N compounds such as uric acid, urea, or proteins are converted into inorganic NH_3 and ammonium (NH_4^+). NH_3 readily volatilizes, escaping the litter profile as a gas whereas NH_4^+'s charge helps to keep it much less mobile (Cook et al., 2011). Bacteria and fungi possessing the enzyme uricase initiate the process of mineralization, converting uric acid into allantoin. Allantoin is then further metabolized by enzymes such as allantoinase and allantoicase in a degradation pathway that leads to the production of urea and/or NH_3 (Rothrock et al., 2010; Pustake et al., 2022). Likewise, any urea or amino acids present in the litter can be hydrolyzed by microbial urease enzymes, adding to the NH_3 pool (Schefferle, 1965). Under typical broiler litter conditions with warm temperatures and moderate moisture, these microbial processes proceed rapidly. Studies have demonstrated that untreated broiler litter harbors a high abundance of genes

associated with nitrogen decomposition, with the microbial conversion of uric acid and urea into NH_3 occurring predominantly within the first two weeks of accumulation (Rothrock et al., 2010; Cook et al., 2008).

The volatilization of NH_3 from litter is dependent on multiple physicochemical factors which influence the equilibrium between NH_4^+ to NH_3. The amount of NH_3 being volatilized is highly pH dependent. In alkaline litter NH_3 release is accelerated due to NH_4^+ being progressively deprotonated to free NH_3 gas. Fresh poultry droppings initially have a mildly acidic to neutral pH, but rapidly increasing microbial activity and NH_3 accumulation can raise litter pH into the alkaline range (often pH 8–9), creating a cycle where more NH_3 leads to higher pH, which in turn favors further NH_3 volatilization. However, in low pH nitrogen tends to remain in the NH_4^+ form as NH_3 acts as a base and accepts protons. Additionally, the temperature of the litter and house environment influences NH_3 production. Higher temperatures not only increase the vapor pressure of NH_3, facilitating its release, but also stimulate microbial metabolism rates. For example, Miles *et al.* (2011) observed that raising litter temperature from ~ 18 °C to ~ 40 °C resulted in up to a seven-fold increase in the rate of NH_3 emissions. The intentional heating of poultry litter through a process known as "windrowing" is a widely used management practice in the poultry industry. Windrowing enhances litter quality by reducing moisture content, accelerating the decomposition of organic matter, and suppressing pathogenic microbes through elevated temperatures. However, this process also intensifies NH_3 volatilization by stimulating microbial activity and increasing litter temperature. Burgess *et al.* (1998) points out that simply drying litter without pH adjustment can still result in major nitrogen loss, since drying often involves a rise in pH that encourages NH_3 evaporation. Moisture content of the litter exerts a more complex, non-linear influence on NH_3 emissions. Some moisture is necessary for microbial activity and for NH_4^+ to be in solution. If pH

is controlled, dry litter can slow NH_3 generation by desiccating microbes and immobilizing uricase enzymes. On the other hand, excessively wet litter also tends to reduce NH_3volatilization, as shown in controlled studies by Miles *et al.* (2011). They found that increasing litter moisture led to greater NH_3 release up to a point, but beyond a critical moisture level, NH_3 emissions plateaued or even declined as moisture continued to rise. This effect is likely because extremely wet litter can create a physical barrier to gas exchange and may dilute or carry away some NH_4^+. In practical terms, however, litter moisture in commercial operations is usually well below such saturation levels to protect bird welfare, and because high moisture is correlated with higher NH_3 in most field situations (Miles et al., 2011). To help regulate these physicochemical factors in the litter, most commercial operations facilitate the use of amendments to help maintain optimum litter quality. To manage these physicochemical conditions and mitigate NH_3 emissions, poultry producers commonly apply litter amendments. These are materials specifically designed to improve litter quality by influencing pH, moisture, and microbial activity.

Amendment Strategies for Ammonia Mitigation

Considering the relationships discussed, mitigation strategies typically involve either chemically binding or transforming NH_3, or altering the litter's microenvironment to reduce microbial N decomposition and volatilization. In modern broiler production, litter amendments are a widely used approach. Amendments are substances added to the bedding and litter to chemically or physically reduce NH_3. The two major categories are acidifying agents and adsorbent or mineral agents. Acidifying amendments are often chemical effectors, and mineral agents typically function

by sorbing NH_4^+ or moisture. Many of these amendments have been developed and tested in the last two decades.

Acid-based litter amendments are among the most effective, and as a result, the most commonly used NH_3 controls in the poultry industry. By applying acids or acid-generating compounds to litter, the pH is driven downward, converting NH_3 into NH_4^+ salts that remain in the litter. One prominent example is aluminum sulfate (alum), which has been extensively studied and adopted in poultry houses. When alum is applied to litter (usually before a new flock is placed or during a clean-out), it reacts with water to produce aluminum hydroxide and sulfuric acid (Eugene et al., 2015; Moore et al., 1995; Sims & Luka-McCafferty, 2002). This pH drop curtails ammonia volatilization and prevents N loss in the litter. In addition to its chemical effects, alum exhibits strong antimicrobial properties. Cook et al. (2008) and Rothrock et al. (2008a) reported that alum application reduced total bacterial populations by approximately 50%, with ureolytic bacteria declining by as much as 90% over time. Interestingly, this bacterial suppression was accompanied by an eventual increase in fungal populations, highlighting the importance of evaluating the broader and long-term ecological impacts of litter amendments on microbial community dynamics.

Other acids or acid-producing compounds have shown similar benefits. Sodium bisulfate ($NaHSO_4$), commercially named Poultry Litter Treatment (PLT) is another widely used amendment with a similar mode of action. PLT dissolves to release hydrogen sulfate (HSO_4^-), an acid that drives down pH by releasing hydrogen ions in water. Similar to alum treatment, PLT is usually applied to litter prior to bird placement and has been proven to reduce NH_3 emissions in the range of 70-90% compared to untreated litter (Mohammadi-Aragh et al., 2025; Choi & Moore, 2008). The industry-standard practices involve spreading alum or PLT on the house floor or litter, which effectively curbs ammonia emissions for several weeks (Li et al., 2013). These treatments

are particularly valuable in the brooding period of a flock's grow-out, when chicks are young and ventilation is often kept low to conserve heat, a combination that can otherwise lead to high ammonia accumulations (USDA APHIS & Iowa State University, 2022). By reducing initial ammonia release, acidifiers improve bird health and growth and allow producers to ventilate less aggressively.

Aside from alum and PLT, research has been done to evaluate other chemical amendments. For example, ferrous sulfate ($FeSO_4$) and phosphoric acid (H_3PO_4) were found to dramatically reduce ammonia volatilization in experimental settings. Moore *et al.* (1996) showed $FeSO_4$ could bind with ammonia or alter microbial activity, achieving large NH_3 reductions comparable to alum. However, some of these chemicals are less commonly used in practice due to undesirable side effects. Phosphoric acid application to litter can harm its value as an agricultural fertilizer by adding too much P to the litter. On the other hand, ferrous sulfate has been associated with extreme cases of bird mortality due to iron toxicity (Wallner-Pendleton et. al., 1986; Pescatore and Harter-Dennis, 1989). A key point with any acidifier is that while they effectively "capture" ammonia, it is important to fully understand their side benefits or drawbacks. Alum, for instance, significantly reduces greenhouse gas production and P runoff potential (Eugene et al., 2015; Sims & Luka-McCafferty, 2002). On the other hand, dry acidifiers can have a corrosive effect on farm equipment and can create dusty conditions which can create health concerns (Barrientos, 2025). Furthermore, as discussed pH alterations to the microbial community can have potentially significant effects in the long-term if not managed properly. Commercially, both alum and PLT are established amendments. Alum has been used in broiler and turkey houses for over two decades, and PLT is favored for its ease of application and immediate action.

Beyond acidifying treatment options, researchers have explored mineral-based amendments and other porous sorbents that do not necessarily rely on acidification. These include clays, zeolites, gypsum, and carbon materials that can immobilize ammonium or reduce moisture. The mode of action for these materials is typically through adsorption/ion exchange, moisture absorption, or chemical reactions that stabilize ammonium. One class of such amendments is natural clays, like bentonites and montmorillonites, which have high cation-exchange capacity and can bind ammonium ions from the litter. In a laboratory study, Redding (2013) compared standard alum to a bentonite clay and found that, at sufficiently high application rates, bentonite can outperform alum in retaining ammonia. The clays essentially sequester ammonium on their exchange sites keeping the litter nitrogen in a stable state. However, natural clays alone require high inclusion rates with the litter to achieve the best results, making them less cost effective as a standalone amendment. Evidence points to utilizing natural clays in combination with other amendments being a more efficient method of NH_3 treatment. For example, a study done by Szymula et al. (2021) showed that even a small mixture addition of 2% bentonite and 1% clinoptilolite zeolite (by weight) mixed into the litter was able to cut ammonia emissions by roughly 30% over the course of their experiment. This finding is valuable because it suggests that relatively small additions of certain aluminosilicate minerals can make a measurable difference in ammonia release. It also reinforces the idea that amendments can have significant synergistic effects on mitigation even if they are not cost-effective as standalone treatments. Zeolites have attracted interest as NH_3 sorbents. Zeolites are porous aluminosilicate minerals with a high affinity for NH_4^+ and even NH_3 gas (Szymula et al., 2021). Zeolites are widely studied for agricultural research and can exchange native cations for NH_4^+, making them effective at preventing N loss from broiler litter (Ellersdorfer et al., 2020; Sheng et al., 2015). Another benefit of zeolites and clays is that

they tend to be non-corrosive and nutrient-neutral, meaning they do not add undesirable elements to the litter. Additionally, clay or zeolite bound NH_4^+ can still serve as fertilizer when the litter is applied to crops.

Another mineral amendment of interest is gypsum (calcium sulfate dihydrate, $CaSO_4 \cdot 2H_2O$). Flue-gas desulfurization gypsum (FGDG), a byproduct from power plant scrubbers, has been studied as an in-house litter amendment and as an alternative bedding material. Gypsum's effect on ammonia is not through strong pH reduction, but through moisture management and ionic reactions. Gypsum is a moderately hygroscopic salt and it can absorb and hold water. Burt *et al.* (2017) found that mixing 20–40% FGDG into broiler litter significantly dried the litter and imposed osmotic stress on microbes, suppressing the populations of uric acid degrading bacteria by 38–71%. Although only having modest impact on emissions compared to using other amendment types, gypsum offers other benefits. Gypsum has been shown to tie up soluble P by forming calcium phosphate in litter, and because it is a neutral salt it does not pose the same corrosion risk as acid amendments (Le & Y, 2004).

Carbon-based adsorbents like biochar have also been tested as alternative amendments. Biochar is highly porous and can sorb ammonia. Additionally, if the biochar is made from high-carbon materials, it can temporarily immobilize nitrogen by adsorption or by providing surfaces for microbial biofilms. Recent studies have shown that surface-applying certain biochars or activated carbons to poultry litter can reduce ammonia emissions on the order of 10–30%, though results vary with the type of char (Agyarko-Mintah et al., 2017; Ritz et al., 2011).

Current ammonia mitigation strategies in litter management rely heavily on litter amendments. Acidifiers such as alum and bisulfates are proven to be an effective means to instantly reduce ammonia emissions. Alongside these, alternative mineral amendments provide additional or

complementary strategies by targeting moisture and ammonium retention rather than pH alone. Often, promising results are achieved by a combination of approaches. It is within this context that oolitic aragonite has been proposed as a novel litter amendment, offering a potentially unique mix of properties.

Oolitic Aragonite

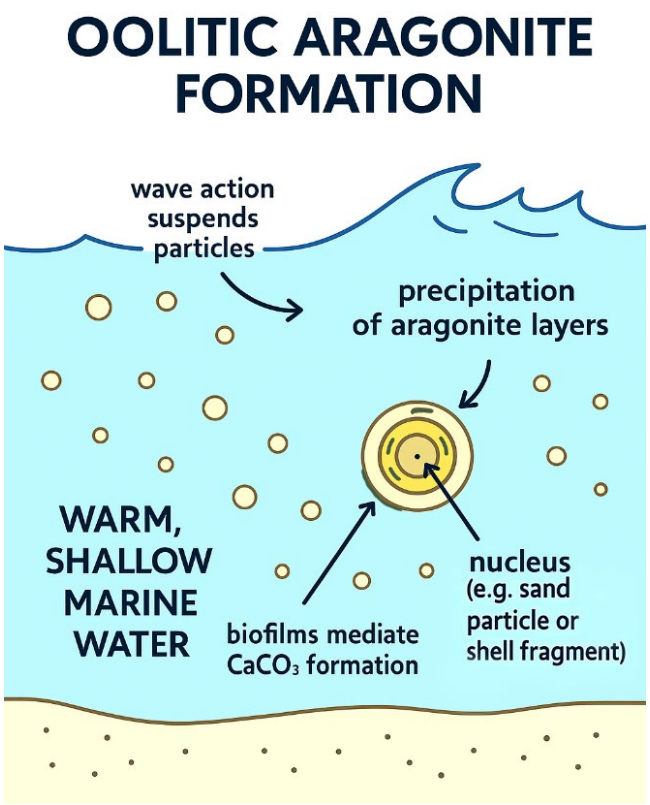

Figure 1.1 Diagram showing how oolitic aragonite forms. Ooids grow in warm, shallow, moving seawater where tiny particles (such as sand grains or shell fragments) are suspended. Layers of aragonite (a crystalline form of calcium carbonate, $CaCO_3$) gradually build up around these particles, forming concentric layers. This process happens through both chemical precipitation and the activity of microorganisms.

Oolitic aragonite (OA) sand is a naturally occurring form of calcium carbonate ($CaCO_3$) characterized by its aragonite crystal structure and egg-shaped grain morphology. While much about the formation process of OA is still unknown, it has been shown that OA is formed by concentric precipitation of $CaCO_3$ in warm, high energy, shallow marine environments, producing

roughly spherical “ooids” of varying size (Trower et al., 2018; Newell et al., 1960; Lincoln et al., 2022). These ooids develop around a nucleus, typically a sand grain, shell fragment, or other microparticle, that is coated with successive laminae of aragonite. As these laminae of tangentially or radially arranged aragonite form, they create multiple concentric cortical layers. The layers create intricate internal structuring that result in high microporosity and surface area. Organic matter often gets trapped within the cortex layers, making ooids grains an ideal niche for microbial growth (Diaz et. al., 2017; Lion et al., 2004). In fact, recent studies suggest that the formation of OA is, at least in part, a biogenic process. Biogeochemical markers indicate that ooid grains can serve as a host environment for a wide range of bacterial metabolisms such as denitrification, nitrification photosynthesis, sulfate reduction, and sulfide oxidation (Folk and Lynch, 2001; O’Reilly, 2017; Diaz et al., 2013, 2014) These various microbial activities and their byproducts, such as extracellular polymeric substances (EPS), are theorized to play key part of ooid development. Intragranular biofilms may directly cause minerals to precipitate, forming new ooid layers, or create the ideal conditions for further mineral growth and increased grain complexity (Diaz et. al., 2017).

The distinctive structure and composition of OA suggest potential functional advantages for use as an amendment to broiler litter systems. Its high surface area and microporosity, resulting from its concentric cortical layering, are likely to influence moisture dynamics by capturing “available” water in the litter microenvironment. These attributes are relevant for managing the microbial processes which are critical to ammonia generation and nitrogen retention in broiler production systems (Cook et al., 2008; Rothrock et al., 2010).

As discussed previously, ammonia volatilization from poultry litter is closely tied to pH, moisture content, and microbial activity, conditions that OA may help to moderate. As a primarily $CaCO_3$

mineral, aragonite can contribute to the buffering capacity that stabilizes litter pH, potentially reducing the shift to more alkaline conditions that favor NH_3 formation and N loss (Moore et al., 1995; Choi & Moore, 2008). Unlike other amendments that rely on acidification, OA may offer a more neutral pH adjustment while still inhibiting volatilization, depending on its dissolution dynamics and local microenvironmental conditions. Aragonite's relatively high solubility compared to calcite may enhance its reactivity in the moist and biologically active conditions of used poultry litter (De Choudens-Sánchez & González, 2009). Perhaps more notably, the microbiological affinity of ooid grains supports the hypothesis that OA could shape microbial communities within poultry litter. As previously mentioned, the internal cortex of ooid grains likely hosts diverse microbial metabolisms, including those involved in nitrogen transformations such as denitrification and nitrification. Given that uric acid/urea degradation and ammonia production are microbial-mediated processes in litter, introducing a mineral substrate capable of fostering beneficial or competitive microbial assemblages may alter the balance of ammonia-cycling pathways and prevent N loss (Ritz et al., 2004; Rothrock et al., 2008a). This raises the possibility that OA might not only modify physicochemical litter conditions but also create ecological pressure that shifts the composition or function of the litter microbiome.

These properties present a scientifically grounded rationale for assessing OA's efficacy as a litter amendment. An evaluation of OA is warranted considering industry interest in alternative, low-cost strategies for ammonia control that avoid the drawbacks of acidifiers or synthetic additives. Testing OA in broiler litter systems can provide important data on its ability to influence physicochemical conditions and microbial succession as well as its impact on NH_3 emissions and nutrient retention.

Chapter 2

2.1 Introduction

Ammonia (NH_3) in the poultry production industry is a problematic source of environmental pollution and a commercial management concern. Confined animal operations or CAOs, such as broiler houses with high-density flocks, generate a significant output of NH_3 following the decomposition of litter. Once volatilized and released, NH_3 contributes to air quality degradation by forming fine particulate matter, which leads to environmental problems such as acid rain and eutrophication (US EPA, 2013; Brink et al., 2022). Elevated atmospheric NH_3 also represents a direct loss of nitrogen from the production system, reducing the value of litter as a potential agricultural fertilizer (Moore et. al., 1995; Agyarko-Mintah et al., 2017). Inside poultry houses, it is recommended that NH_3 concentrations be maintained below ~20–25 ppm to protect the health of the birds and workers. Prolonged exposure to >25 ppm of NH_3 impairs broiler chick welfare and performance, causing respiratory irritation, ocular burns, reduced feed intake, poorer growth, and greater susceptibility to disease (Kristensen & Wathes, 2000; Sheikh et. al. 2018). Volatilized NH_3 is a caustic gas that can damage the respiratory epithelium (e.g. destroying cilia and inflaming air sacs). Damage to these respiratory mechanisms leads to birds being unable to clear mucus accumulation, which promotes bacterial proliferation and increased risk of infections (Kristensen & Wathes, 2000; US EPA, 2013; Sheikh et al., 2018; Padappayil & Borger, 2025). Controlling

NH_3 emissions in broiler houses is critical both for animal welfare and to minimize environmental impacts of poultry production.

Nitrogenous waste is the main source of NH_3 in poultry litter. These nitrogenous waste products like uric acid and urea are present in avian excreta and undigested protein and provide a substrate for diverse bacterial communities to grow. Uric acid usually constitutes a large percentage of the total nitrogen in poultry litter, and its hydrolysis is the first step in the pathways that lead to NH_3 generation (Naseem & King, 2018). This process is primarily mediated by microbes possessing the enzyme *uricase*, which decomposes uric acid into urea and NH_3. Litter moisture availability and pH are key factors in litter management practices because they strongly influence the rate of NH_3 production. Damp, warm litter with a moderate-to-high pH creates ideal conditions for microbial activity and NH_3 volatilization (Miles et al., 2011). In general, wetter litter leads to greater NH_3 volatilization because water supports microbial metabolism and can solubilize ammonium (NH_4^+), making it prone to evaporation as NH_3 gas. Likewise, litter pH influences the $NH_4^+ – NH_3$ equilibrium. More specifically, a higher pH favors conversion of NH_4^+ to gaseous NH_3. Effective NH_3 mitigation in poultry systems therefore is reliant on proper litter pH, moisture, and microbial management. (Kristensen & Wathes, 2000; Miles et al., 2011; Naseem & King, 2018).

Poultry producers use both feed strategies and litter treatments to reduce NH_3 levels in chicken houses. Managing dietary protein and adding ingredients like Yucca extract can lower nitrogen excretion, but much nitrogen still ends up in the litter (Ayoub et al., 2019). To control NH_3 after excretion, producers use methods like better ventilation, moisture control, and manure removal, though these can be costly or challenging in practice. Chemical litter amendments, especially

acidifiers like alum, are widely used because they lower litter pH and trap NH_3, significantly cutting emissions and boosting the litter's fertilizer value (Moore et al., 1995). However, these treatments need frequent reapplication, can be potentially harmful to workers and chicks, and may have side effects like equipment corrosion or soil buildup (Shah et al., 2006; Oğuz et al., 2012; USALCO, 2020; Barrientos, 2025). Furthermore, many of these amendment types are not considered organic compliant, limiting amendment options for organic poultry farming (Barrientos, 2025). Because of these drawbacks, commercial poultry managers and researchers are interested in testing alternative mineral-based amendments that might control NH_3 more safely, effectively, and affordably (Bist et al., 2023; Kim & Patterson, 2003; Swelum et al., 2021).

The objective of this study was to investigate the effectiveness of OA as a broiler litter amendment for NH_3 mitigation and nutrient retention. Primarily composed of microcrystalline calcium carbonate ($CaCO_3$) (about 98%), OA has an egg-shaped structure that is referred to as an ooid. The ooid consists of a nucleus, often encased in a concentrically laminated envelope. It is formed in warm, shallow, turbulent, and high-salinity waters, so typically in tropical marine environments (Sipos et al., 2018). OA contains high amounts of organic matter incorporated into its structure (Lincoln et al., 2022; Trower et al., 2018; Newell et al., 1960). The study was designed to assess OA's impact on broiler litter NH_3 emissions and to investigate any underlying mitigation mechanisms, particularly concerning moisture control and microbial activity.

We specifically tested if OA is capable of lowering water activity (a_w) and moisture content of broiler litter by limiting free water within pore spaces. This process would create an environment unsuitable for NH_3-producing microbial processes and as a result, suppress the overall microbial decomposition rate of uric acid and other nitrogenous compounds (Dunlop et al., 2016; de Toledo

et al., 2020; Ismael & Ismail, 2021; Jiao et al., 2023). The second proposed control mechanism posited that OA's extensive surface area and organic matter load, stemming from its overlapping concentric layers, could create microsites favorable for alternative nitrogen cycling processes like nitrification or immobilization (Lincoln et al., 2022; Trower et al., 2018; Newell et al., 1960; Simone, 1980). This could effectively lock nitrogen into non-volatile forms (Swelum et al., 2021; Iwata et al., 2010). By promoting nitrifying bacteria or facilitating nitrogen incorporation into microbial biomass, OA-treated litter could retain a higher proportion of nitrogen as nitrate or organic N, consequently reducing NH_3 gas loss. We therefore anticipated that OA would improve the N:P ratio in litter by increasing its nitrate content or total Kjeldahl nitrogen.

These hypotheses were tested through a combination of laboratory analyses and in situ measurements in simulated broiler house conditions within environmental control chambers. Emission fluxes for NH_3 were monitored from OA-amended and control litter, litter water activity and moisture were measured, microbial community composition and uric acid concentrations were assessed, and litter nitrogen forms were quantified. The following sections of this thesis detail the results of these experiments and evaluate whether OA has potential as a sustainable litter amendment for NH_3 mitigation in poultry production.

2.2 Materials and Methods

Chamber Design and Environmental Control

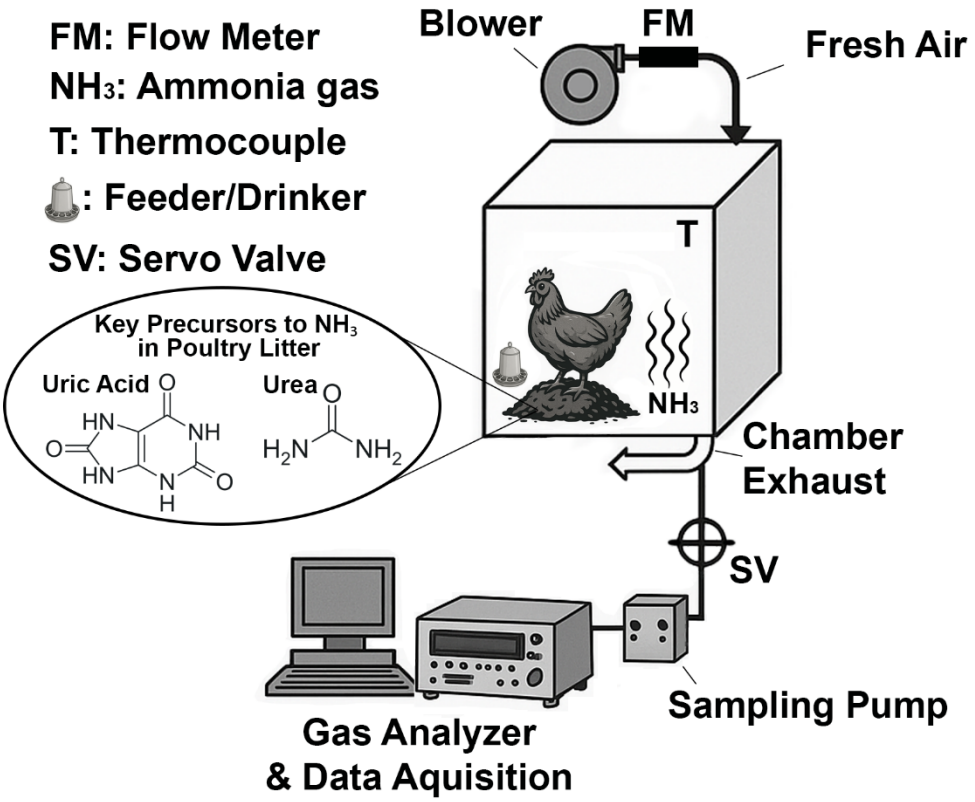

Figure 2.1 Environmental chamber system used for ammonia monitoring. Each chamber received fresh, NH_3-free air via a blower and flow meter (FM), with airflow adjusted based on bird body weight. A thermocouple (T) monitored internal temperature, and chambers were equipped with feeders/drinkers and bedding for broilers. Exhaust air containing NH_3 was routed through a servo valve (SV) and directed to a sampling pump and photoacoustic gas analyzer for real-time NH_3 quantification. Data acquisition modules collected flow, temperature, and gas concentration. Uric acid and urea, shown as molecular structures, are the primary precursors of ammonia in poultry litter.

Experiments were conducted using a block design in six environmentally controlled emission chambers **(Figure 2.1)**, each designed to mimic commercial poultry production settings. Each chamber measured 74 × 72 × 74 cm (29 × 28 × 29 in) and was housed within an environmentally controlled room. An air blower mounted on the top of each chamber delivered fresh, NH_3-free air via PVC ducts. The airflow rate in each chamber was continuously monitored with an air mass

flow meter installed in the supply stream, ensuring an airflow range of 0.1 to 5 cubic feet per minute (cfm) per bird, adjusted according to bird body weight.

Ventilation and Air Sampling

Airflow and NH_3 concentrations were continuously measured through a sequential sampling system. Exhaust air from each chamber, along with room supply air, was sampled at 5-minute intervals using six solenoid valves that directed the flow sequentially. Concentrations of NH_3 were quantified with a Chillgard RT photoacoustic NH_3 analyzer (MSA Safety). Ventilation within the chambers was managed to maintain relative humidity below 60% during the first flock, with the rate further adjusted based on an NH_3 threshold of 25 parts per million (ppm) in the control chamber.

Data Acquisition and Environmental Monitoring

Temperature in each chamber was maintained through a combination of room temperature adjustments, variable airflow rates, and the use of individual chamber heat lamps. Thermocouples provided real-time temperature data, while portable relative humidity sensors measured ambient moisture levels. Analog signals from mass flow meters and thermocouples, along with digital outputs from the gas analyzer, were collected via a data acquisition module at 1-second intervals, although only 1-minute averaged values were recorded for subsequent analysis.

Bird and Litter Management

Chamber Assignment

Three flocks of female broiler chickens (Ross 708) were utilized for this study, with each flock raised over a 6-week period in the six environmental chambers. For each flock, the chambers were allocated with two chambers per treatment and two chambers serving as controls. Each chamber housed six birds, and all birds were fed commercial diets *ad libitum*. The chambers were maintained under identical temperature and lighting conditions. Bedding treatments consisted of softwood shavings mixed with OA sand at three different ratios: 100:0, 80:20, and 60:40 (shavings:sand, w/w). Two chambers were randomly allocated to each treatment. The total bedding load per chamber was standardized at 2.3 kg. For mixed treatments, an initial 0.3 kg of shavings (representing a one-inch layer) was set aside, and the remaining shavings were thoroughly mixed with the sand before being placed into the bedding tray. The reserved shavings were then layered on top of the mixture.

Flock Cycles, Litter Reuse, and Ventilation Protocol

Three complete 6-week flock cycles were performed. After each cycle, the top or "cake" layer (approximately 5-7cm deep) was removed using a spatula, while the remaining litter was reused in subsequent flocks. Windrowing, a composting and drying process performed during flock downtime, is commonly practiced in commercial poultry operations to improve litter quality for reuse. In this study, a 3-week windrowing period was implemented between flocks. The litter was turned twice, 7 and 14 days after bird removal, and the chambers were continuously ventilated for the 21 day downtime to reduce moisture content to 13–15%. Live performance metrics (body

weight, average weekly gain, feed conversion, and footpad dermatitis scores) were recorded throughout the study.

Sample Collection and Analytical Procedures

Air and Litter Sampling

Air samples were collected continuously from the exhaust of each chamber and from the room supply, while key parameters (NH_3 concentration, ventilation rate, temperature, and relative humidity) were monitored throughout the study. Litter samples (approximately 50 g) were collected weekly from five locations within each chamber, encompassing both cake (two locations) and non-cake (three locations) areas. Composite samples were prepared for moisture, water activity, and pH analyses. At the conclusion of each flock, additional composite litter samples were analyzed for total Kjeldahl nitrogen (TKN), NH_3-N, uric acid, pH, water activity, moisture content, and litter microbiome.

Litter Water Activity Measurement

Water activity was determined using an Aqualab 4TE dew point instrument (METER Group, Inc., USA). The chilled mirror dew point method was chosen for its measurement speed and accuracy (±0.003 aw), with each measurement completed in approximately 5 minutes. Calibration was verified before and after each series of analyses using a series of standard solutions (13.41 kg/mol LiCl, 8.57 mol/kg LiCl, 6.00 mol/kg NaCl, 2.33 mol/kg NaCl, and distilled water). Litter samples were homogenized by crushing and grinding in a mortar and pestle before being transferred to

sample cups to ensure complete coverage of the base. All measurements were performed in triplicate.

Uric Acid Extraction and HPLC Analysis

For uric acid quantification, 0.1 g of each homogenized litter sample was placed into a 50 mL centrifuge tube, and 25 mL of 0.1 M sodium acetate was added. After vigorous shaking for 10 seconds, the samples were incubated in an oven at 50 °C for 2 hours, followed by 1 minute of sonication. The mixture was then centrifuged at 1,000 rpm, and 1 mL of the supernatant was transferred into an amber HPLC vial.

Uric acid analysis was conducted on a Hewlett-Packard Series 1100 HPLC system (Palo Alto, CA) equipped with a degasser and autosampler. The UV/VIS detector was set at 290 nm as demonstrated by Finlayson and Smith (1974), which allows for high specificity for uric acid, effectively eliminating potential interfering peaks from compounds unrelated to the study. Separation was achieved using a C8 column (250 × 4.6 mm, 5 µm particle size) maintained at 35 °C. The mobile phase consisted of 10 mM ammonium acetate (pH 4.5) delivered at a flow rate of 1.0 mL/min. An injection volume of 50 µL was used, and the analysis time was reduced to 15 minutes per sample without any observed carry-over. Column pressure ranged from 8.3 to 9.4 bar, with elution times between 8.5 and 10 minutes. Uric acid standards were prepared by dissolving 20 mg of reference compound weighed on an analytical balance (Mettler ME22, Mettler-Toledo, Columbus, OH), in 0.002 mol/L ammonium hydroxide.

Statistical Analysis

Data were analyzed on a per-chamber basis, with each chamber serving as an independent replicate (n = 2 per treatment). One-way ANOVA followed by Tukey–Kramer HSD tests was performed using R (v4.4.1) with emmeans() package to determine significant differences among treatment groups. All statistical tests were conducted at a significance level of 5% ($P < 0.05$).

DNA Extraction and 16S rRNA Gene Sequencing

DNA was extracted from a total of 84 samples for the microbiome analysis using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Germantown, MD, USA). A total of 72 poultry litter samples were collected during the flock periods. For each of the three flocks, 24 samples were collected across all six chambers, with 12 during the first week and 12 during the final week. An additional 12 samples were collected during windrowing, with six samples taken during each of the two windrow events.

The V3-V4 region of the bacterial 16S rRNA gene was amplified using the primer pair 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′), selected for their broad coverage across bacterial taxa as evaluated by Klindworth et al. (2013). Libraries were prepared using the Nextera DNA Flex kit (Illumina, San Diego, CA, USA), beginning with 100 ng of extracted DNA per sample. Paired-end sequencing (2 × 250 bp) was performed on the Illumina MiSeq platform according to the manufacturer's instructions.

Microbiome Data Processing and Analysis

Microbial community analysis was conducted using a custom pipeline in R (v4.4.1) adapted from the Bioconductor workflow described by Callahan et al. (2016), implemented in R (v4.2.2). Quality control, denoising, taxonomic assignment, and downstream analyses were conducted using the following core packages: DADA2, phyloseq, DECIPHER, and phangorn described in detail below.

Sequence Preprocessing and Denoising

The raw forward and reverse reads were visually inspected using plotQualityProfile. Due to poor overlap and quality in reverse reads, only forward reads were retained for downstream analysis. Forward reads were filtered using DADA2's filterAndTrim() function, which removed PhiX contaminants, truncated low-quality tails, and discarded reads exceeding expected error thresholds (maxEE = 2, truncQ = 2). Adapter and primer sequences were trimmed from the 5′ end. Denoising was performed using pooled inference across all samples to improve detection of rare variants, followed by chimera removal with removeBimeraDenovo(). The resulting amplicon sequence variants (ASVs) were compiled into a sequence table.

Taxonomic Assignment and Phylogenetic Tree Construction

Taxonomy was assigned using a naive Bayesian classifier trained on the RDP reference database (v18) with a minimum bootstrap confidence of 80. A multiple sequence alignment of the non-

chimeric ASVs was performed using DECIPHER, and a phylogenetic tree was constructed using phangorn with the maximum likelihood (GTR+Γ+I model).

Phyloseq Object Construction and Filtering

The ASV table, taxonomic annotations, sample metadata, and phylogenetic tree were combined into a phyloseq object. Taxa with uncharacterized phylum-level annotations or belonging to contaminant or irrelevant phyla (e.g., Chloroflexi, Cyanobacteria/Chloroplast, Verrucomicrobia) were excluded. Low-prevalence taxa were filtered using a 5% sample prevalence threshold. For community-level summaries, taxa were agglomerated at the genus and order levels using tax_glom().

Community Structure and Ordination

Microbial community composition was assessed using Bray–Curtis dissimilarity and visualized with non-metric multidimensional scaling (NMDS). PERMANOVA was used to test for differences in community structure among treatments using the adonis2() function from vegan, stratified by flock and windrow periods. Homogeneity of multivariate dispersion was confirmed using betadisper().

Differential Abundance Testing

Differential abundance of microbial taxa was assessed using DESeq2. Analyses were conducted separately for flock and windrow periods, and for order and genus-level taxonomic ranks. DESeq2 models included treatment as a primary factor, adjusted for confounding variables such as week or sequencing depth where applicable. The taxa with adjusted p-values (padj) < 0.05 were considered significantly differentially abundant.

2.3 Results

Environmental Factors

Chamber temperature and relative humidity were monitored daily throughout the experiment across all flock periods (**Figure 2.2**). Average chamber temperatures followed a consistent declining trend over the course of each flock, aligning with expected management practices. Relative humidity increased steadily during each flock period, reaching peak levels by the end of each flock period. Measurements were not collected during the windrow composting periods. Statistical analysis revealed no significant differences in either temperature or RH among the three bedding treatments (Control, Low, High; $p > 0.1$), indicating that environmental chamber conditions were consistently maintained across all groups.

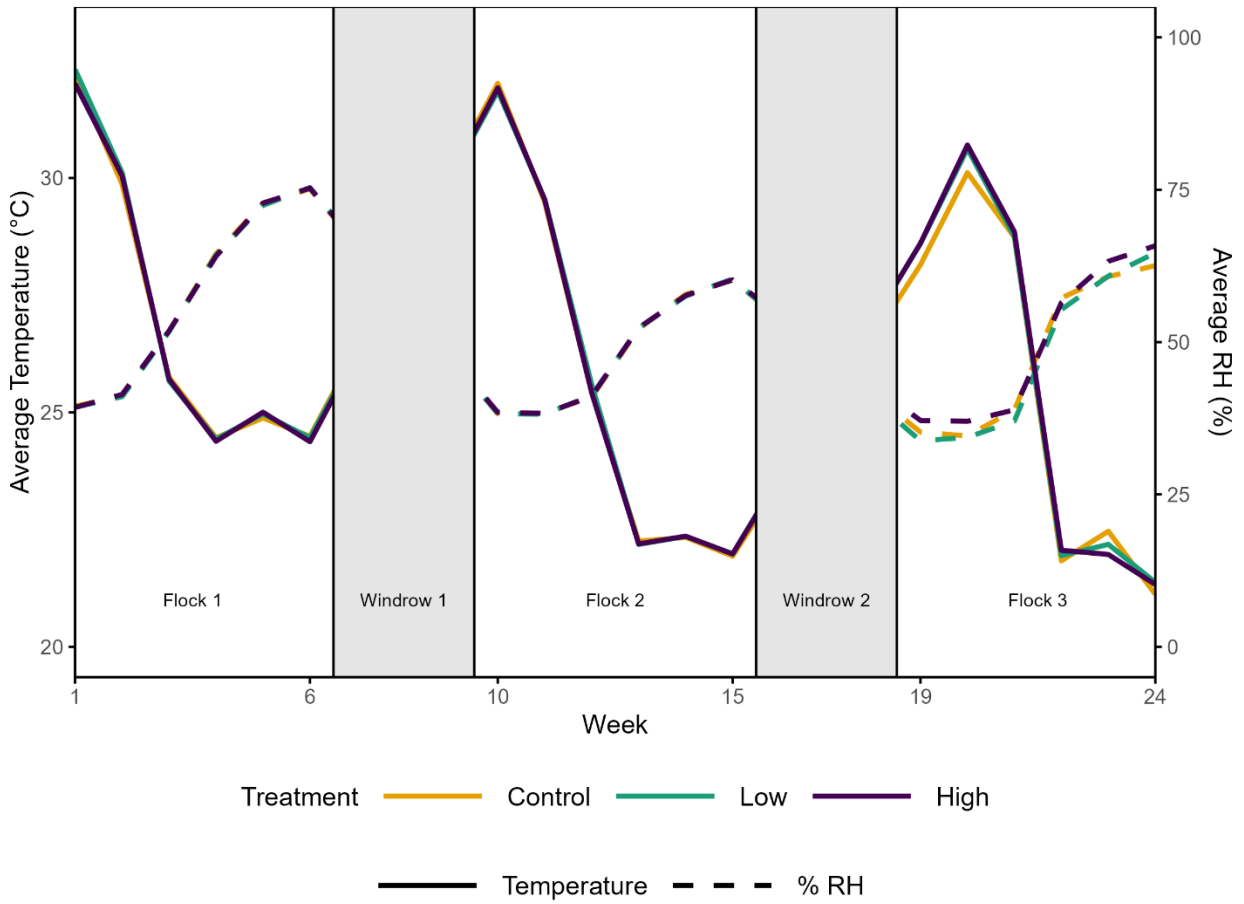

Figure 2.2 Environmental conditions within the controlled chamber system across three broiler flocks and two windrow composting phases. Average weekly temperature (°C) and average weekly relative humidity (RH, %) are shown for each treatment group (Control, Low, and High OA). Lines represent treatment means, and shaded regions indicate the standard deviation across replicate chambers. Vertical black lines mark transitions between flock and windrow periods; shaded grey areas indicate windrow composting phases.

End-of Flock Bird Performance

Growth performance and welfare outcomes were summarized at the completion of each flock **(Figure 2.3, Table 2.1)**. Final live weights were statistically similar between the OA treatments and the control, indicating that the litter amendment had no significant impact on bird growth.

Both groups reached approximately the same market weight (~2.5 kg per bird on average for all treatments, $p > 0.4$), and feed conversion ratios (FCR) were comparable at ranging roughly from 1.5-1.9 in all treatments ($p > 0.1$) **(Table 2.1)**.

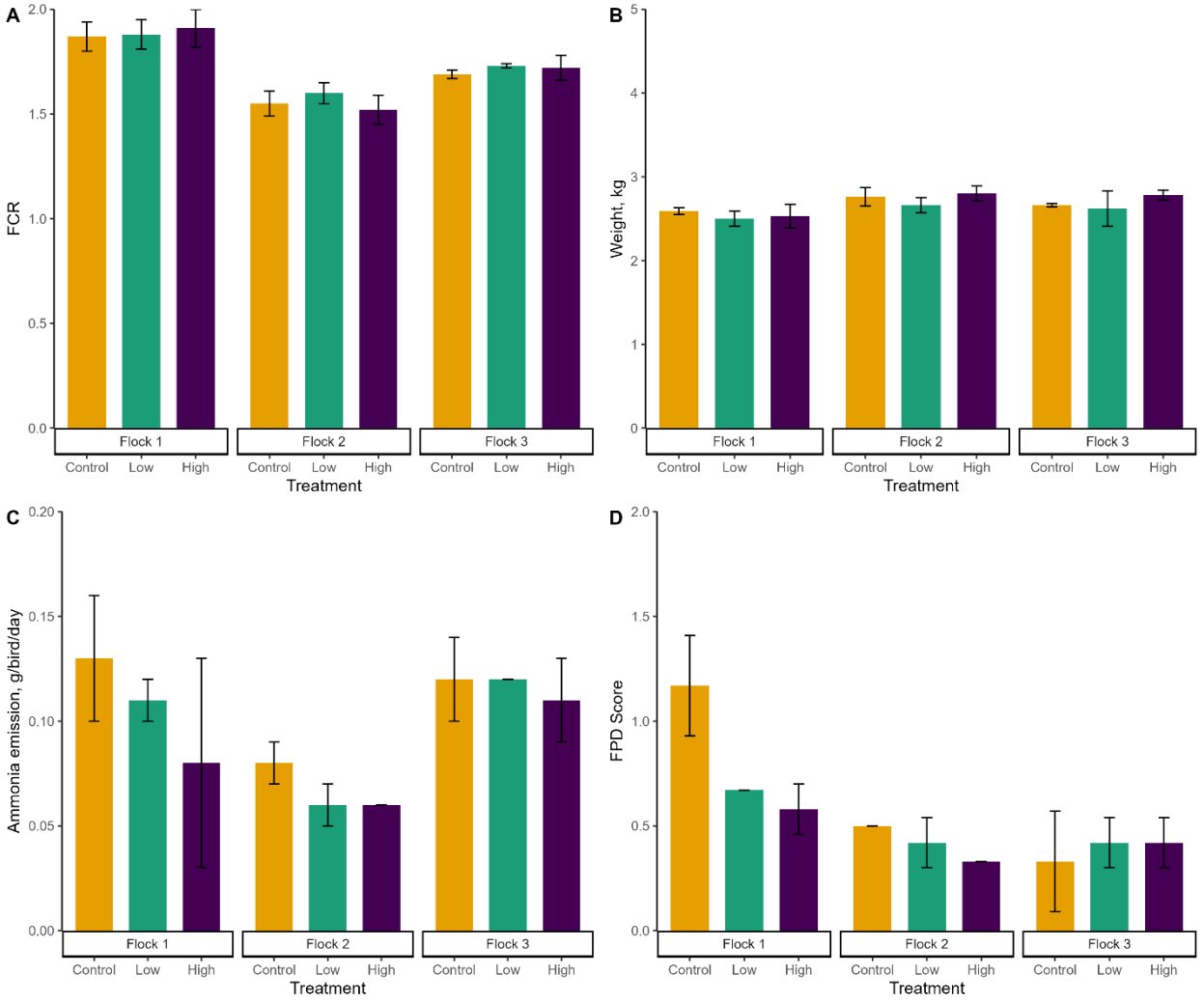

Figure 2.3 Broiler production and welfare outcomes by treatment and flock. Bars represent means ± standard deviation. Panels display (A) feed conversion ratio (FCR), (B) final body weight (kg), (C) average daily NH_3 emissions per bird (g/bird/day), and (D) average footpad dermatitis (FPD) score across litter treatments. FPD scores were assigned on a 3-point scale:0 = no lesion, 1 = lesion <¼ inch, and 2 = lesion >¼ inch. Measurements were taken at the end of each flock to assess treatment impacts on broiler performance. Values represent the chamber-level mean based on 6 birds per chamber, with two replicate chambers per treatment per flock (n = 2).

Welfare metrics over three flocks likewise showed no major differences between treatments. The average footpad dermatitis (FPD) score in OA-amended chambers did not differ significantly from the control (all $p > 0.6$) **(Table 2.1)**. The average daily NH_3 emission rates per bird **(Figure 2.3 C)** were not significantly affected by the treatment either ($p > 0.05$). Although there was a reduction in some OA treated chambers compared to the control, the treatment effects were not consistent over replicate chambers throughout the length of the study.

Table 2.1 Mean (± SD) broiler performance and welfare metrics over the three flocks.

Treatment	FCR	Weight (kg)	Ammonia Emission (g/bird/day)	FPD Score
Control	1.70 ± 0.16	2.67 ± 0.09	0.11 ± 0.03	0.67 ± 0.44
Low	1.74 ± 0.14	2.59 ± 0.08	0.10 ± 0.03	0.50 ± 0.14
High	1.72 ± 0.20	2.70 ± 0.15	0.08 ± 0.03	0.44 ± 0.13

End-of-Flock Litter Chemistry

End-of-flock litter analyses **(Figure 2.4, Table 2.2)** revealed that OA had a limited impact on most litter chemical properties. The litter N:P ratio, an important indicator for agronomic productivity and environmental stewardship, remained essentially the same between treatments and control at ~1.2. This ratio showed no significant change (all pairwise tests $p > 0.4$).

The total nitrogen content of the litter did not differ significantly between the treated and untreated groups by the end of the flock (all pairwise tests $p > 0.2$). On a content basis, all groups retained a similar amount of nitrogen in the litter. Furthermore, the partitioning of nitrogen into organic

(insoluble) N and NH_4^+-N was not significantly altered. The OA-treated litter had nearly the same NH_4^+-N concentration as control litter, and similar overall total N content.

The final litter moisture content was comparable between the two treatments and control (all $p > 0.2$), indicating that aragonite did not dry the litter or absorb moisture. Litter pH at the flock end remained in the slightly basic range for both groups; any pH increase from the $CaCO_3$ was modest and not statistically significant. Both control and treated litter had pH values around 8.5–8.8.

The most pronounced chemical difference observed was in litter calcium content. Litter from the aragonite-amended pens had a substantially higher Ca concentration at flock-end compared to control litter ($p < 0.01$). For instance, the Low OA treated litter contained roughly double the calcium (e.g. ~6.2% Ca) of the control litter (~3.3% Ca) on a dry matter basis **(Table 2.2)**. Aside from Ca enrichment, no significant changes in macro-nutrient litter content were attributable to OA treatments.

Table 2.2 Mean (± SD) of litter composition parameters measured at the end of each flock period.

Treatment	Ammonium (%)	Calcium (%)	Moisture (%)	N/P	Organic N (%)	Phosphorus (%)	Total N (%)	pH
Control	0.16 ± 0.08	3.31 ± 0.09^a	45.0 ± 6.15	1.18 ± 0.08	3.60 ± 0.19	3.22 ± 0.22	3.77 ± 0.26	8.77 ± 0.03
Low	0.16 ± 0.06	6.24 ± 0.66^b	43.4 ± 6.07	1.35 ± 0.03	3.82 ± 0.35	2.96 ± 0.22	3.98 ± 0.38	8.85 ± 0.09
High	0.14 ± 0.06	9.36 ± 0.84^c	39.6 ± 3.83	1.25 ± 0.10	3.29 ± 0.33	2.76 ± 0.36	3.44 ± 0.38	8.67 ± 0.21

Superscript letters indicate significant differences (Tukey HSD, $p < 0.05$).

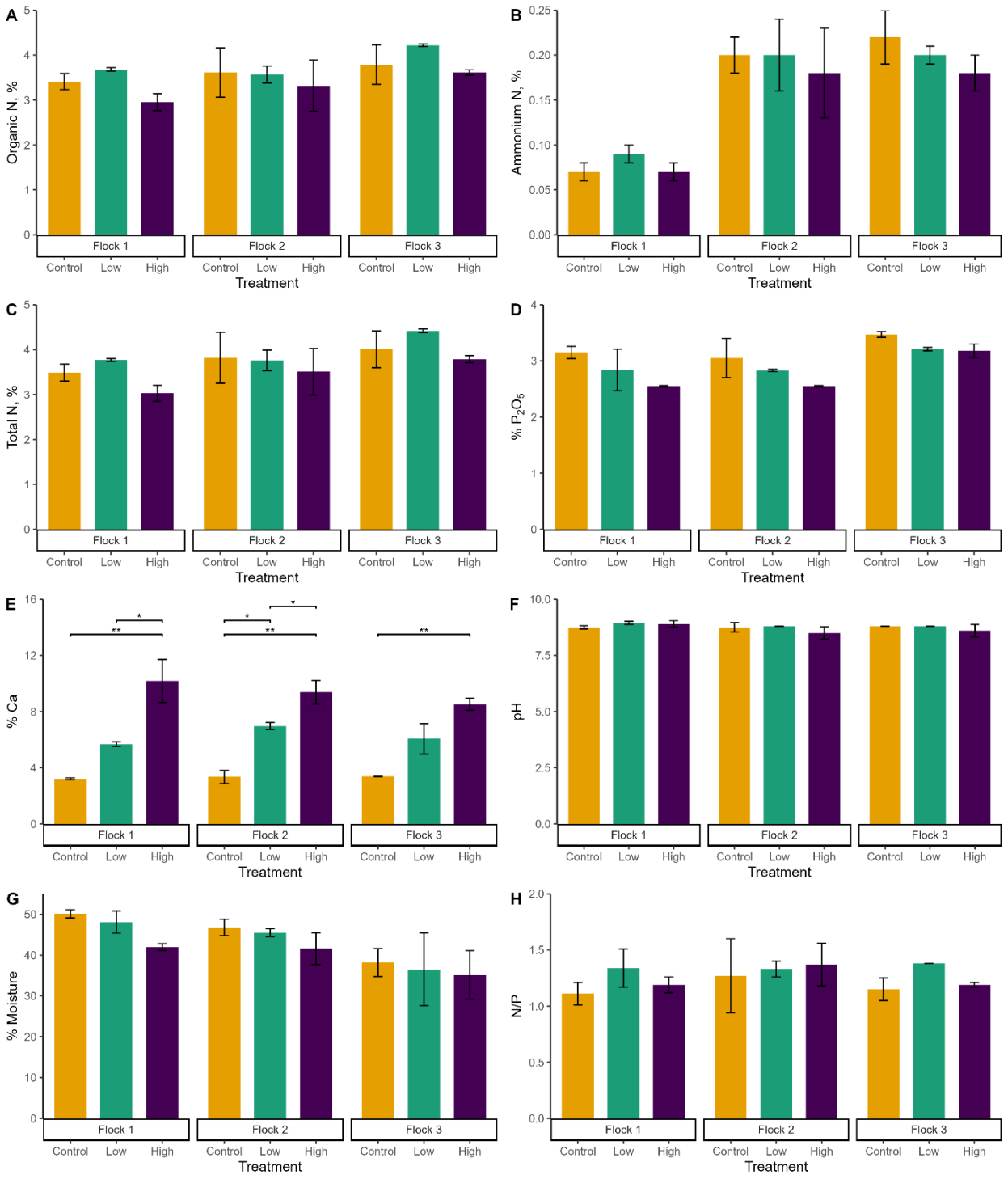

Figure 2.4 End-of-flock litter chemistry by treatment and flock cycle. Bars represent means ± standard deviation. Panels show (A) organic nitrogen (%), (B) NH_4^+ nitrogen (%), (C) total nitrogen (%), (D) phosphorus as % P_2O_5, (E) calcium (%) with brackets and asterisks identifying significant differences, (F) pH, (G) moisture content (%), and (H) nitrogen-to-phosphorus (N/P) ratio across Control, Low, and High treatments for Flocks 1–3. Values reflect composite litter samples collected at the end of each flock with two replicate chambers per treatment (n = 2).

Ammonia Emission Dynamics

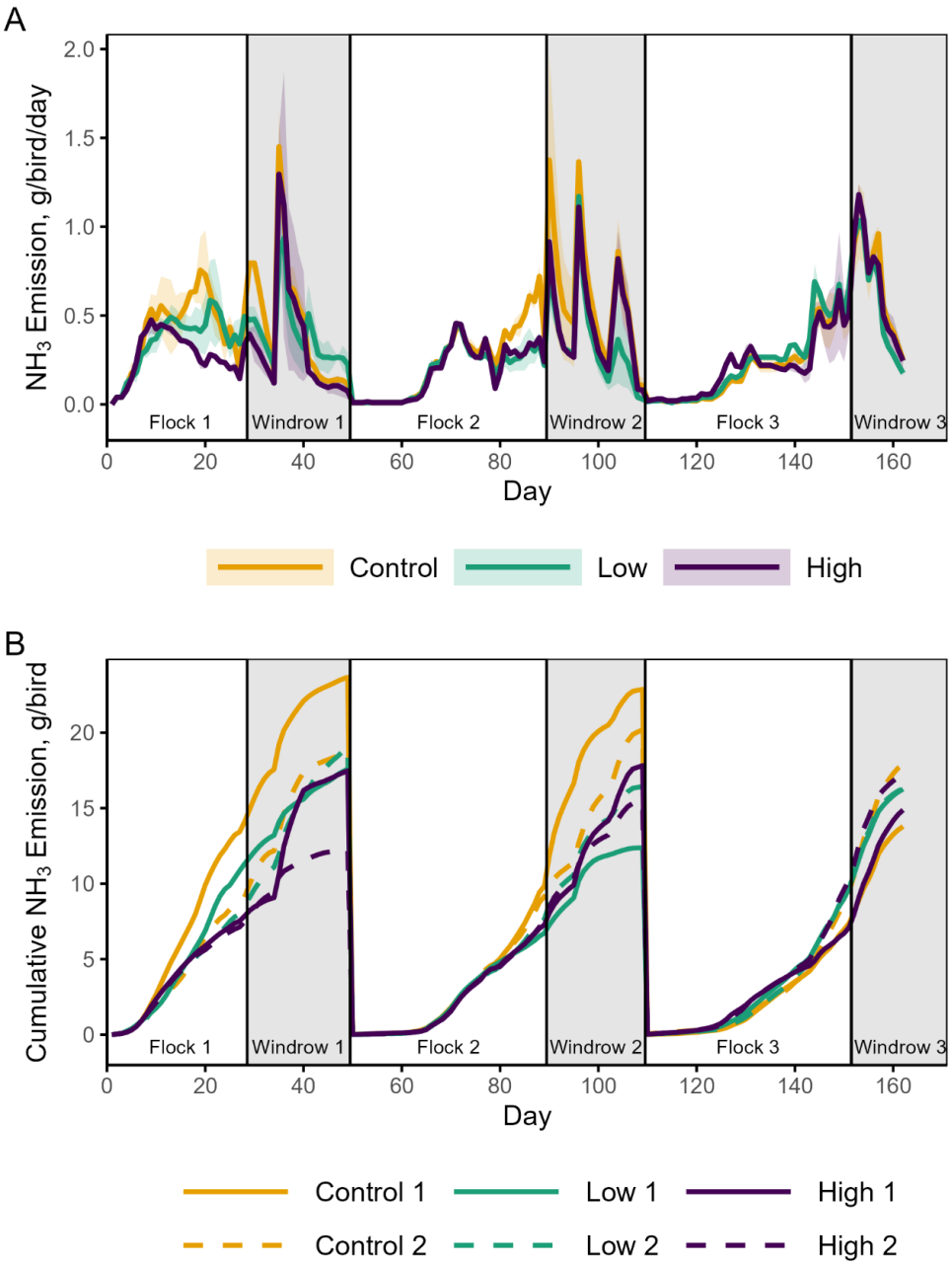

Figure 2.5 Daily NH_3 emissions from poultry litter across three flocks and associated windrow composting periods under three treatment conditions over the 160-day study. **(A)** Mean daily NH_3 emissions per bird. Lines represent averaged daily NH_3 emission rates (g/bird/day) for each treatment group over replicate chambers. Shaded bands indicate variability between replicates. Vertical black lines mark transitions between flock and windrow periods. Shaded grey regions indicate windrow composting periods. **(B)** Cumulative NH_3 emissions per bird over time, separated by treatment replicate (solid vs. dashed lines).

Daily NH_3 emission rate per bird **(Figure 2.5 A)** and cumulative NH_3 emitted per bird **(Figure 2.5 B)** over three consecutive flock cycles were visualized in time series. In each flock, daily NH_3 emissions increased over time and peaked during litter windrowing events, then dropped back to low levels at the start of the next flock cycle. All treatments followed similar emission trajectories throughout the trial.

Results of ANOVA across the three flock cycles indicated no significant treatment effect on either daily emission rates or cumulative NH_3 per bird. Tukey's HSD post-hoc tests likewise found no significant pairwise differences between any treatment means.

Another ANOVA was also used to test for within flock cycle treatment effects (Cycle 1, $p > 0.2$; Cycle 3, $p > 0.9$). Treatment effects only approached significance in the second flock cycle ($p = 0.095$), where the two treated groups showed a consistent trend toward lower emissions when compared to the control in both flock and windrow periods. However, this difference did not reach the 5% significance threshold. By the end of the third flock, the total cumulative NH_3 loss per bird was similar in all treatment groups.

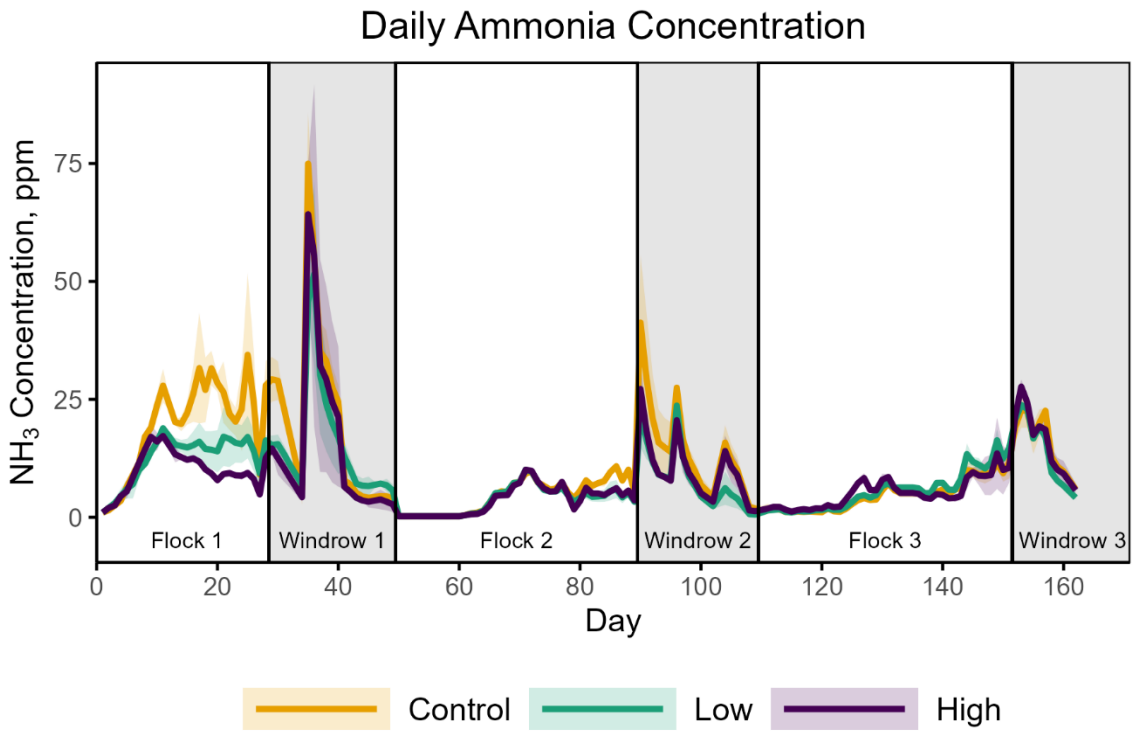

Figure 2.6 Daily NH_3 concentration (ppm) measured in poultry litter across three flock cycles and subsequent windrow composting periods for Control, Low, and High litter treatments. Lines represent treatment means, and shaded areas indicate standard deviation (n = 2 chambers per treatment). Vertical black lines mark transitions between flock and windrow periods. Shaded grey regions indicate windrow composting periods.

Daily NH_3 concentration data collected from each chamber **(Figure 2.6)** followed similar patterns, remaining low in early weeks and rising as the flock aged, with the only discernible treatment separations during Flock 1 (~Days 10–28) and the end of Flock 2 (Day 80-90). Across the three flock periods, statistical analysis inferred no significant differences in daily NH_3 concentrations between the OA-amended chambers and the controls (all $p > 0.7$). $CaCO_3$

Litter Properties Time Series

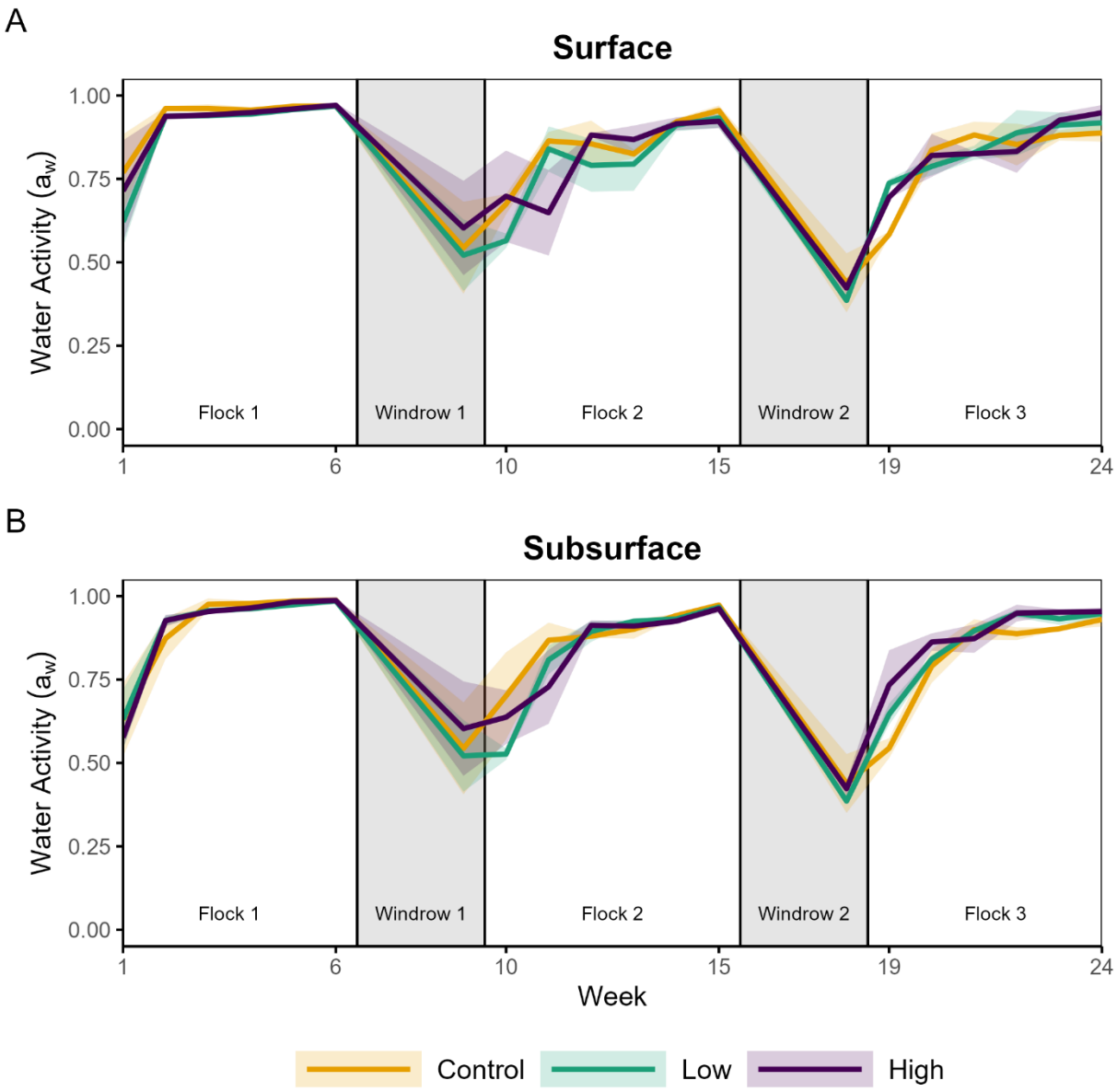

Figure 2.7 Water activity (a_w) in surface and subsurface layers of poultry litter across flock and windrow composting periods. **(A)** Mean water activity in surface litter collected from 1–2 cm depth. **(B)** Mean water activity in subsurface litter ("cake") collected from 5–7 cm depth. Vertical black lines mark transitions between flock and windrow periods. Shaded grey regions indicate windrow composting periods. Colored lines indicate treatment group means, with ribbons representing standard deviation. Note that the first two weeks of each windrow composting period were not sampled; data shown represent only the final week of windrowing.

The litter water activity (a_w) measured at the litter surface **(Figure 2.7 A)** and subsurface **(Figure 2.7 B)** over time. Water activity exhibited a cyclical pattern, remaining near saturation ($a_w \approx 1.0$) during each flock cycle and then dropping during the windrow periods between flocks after the removal of the broilers/drying of the litter. This pattern was consistent across all treatments. ANOVA across the three flocks detected no significant differences in water activity among the Control, Low, and High treatments at either the surface or subsurface ($p > 0.90$).

As an exploratory approach, a GAMLSS model was also used to examine effects of treatment, depth, and week. This model indicated significant effects of slightly lower mean water activity under the Low treatment on the litter surface.

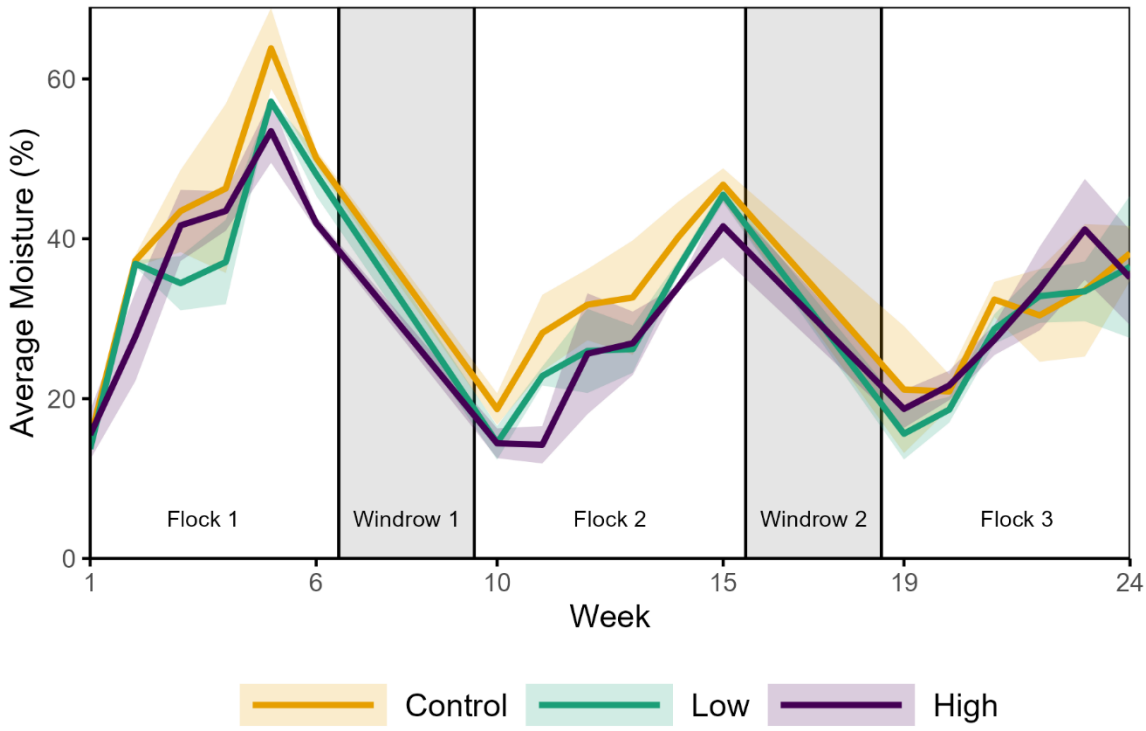

Figure 2.8 Weekly average litter moisture (%) across three flock periods for Control, Low, and High treatments. Lines represent mean values for each treatment, with shaded ribbons indicating standard deviation (n = 2 chambers per treatment). Vertical black lines mark transitions between flock and windrow periods. Shaded grey regions indicate windrow composting periods. Note that the windrow composting period was not sampled.

Time series of average litter moisture content (%) was plotted over the three flock periods **(Figure 2.8).** Litter moisture increased steadily throughout each flock, reaching 40–60% by the end of a flock and then declined to around 15–20% following each windrow period after the litter was stirred and dried. This moisture pattern was visually similar in all treatment groups but appeared to be especially elevated in Flock 1. Furthermore, moisture levels appeared to be slightly more elevated in one control chamber compared to the rest, although the difference was not pronounced enough to be statistically significant. Statistical analysis indicated that the treatments did not significantly affect litter moisture. ANOVA across flock periods found no significant differences in moisture content among treatments ($p > 0.6$).

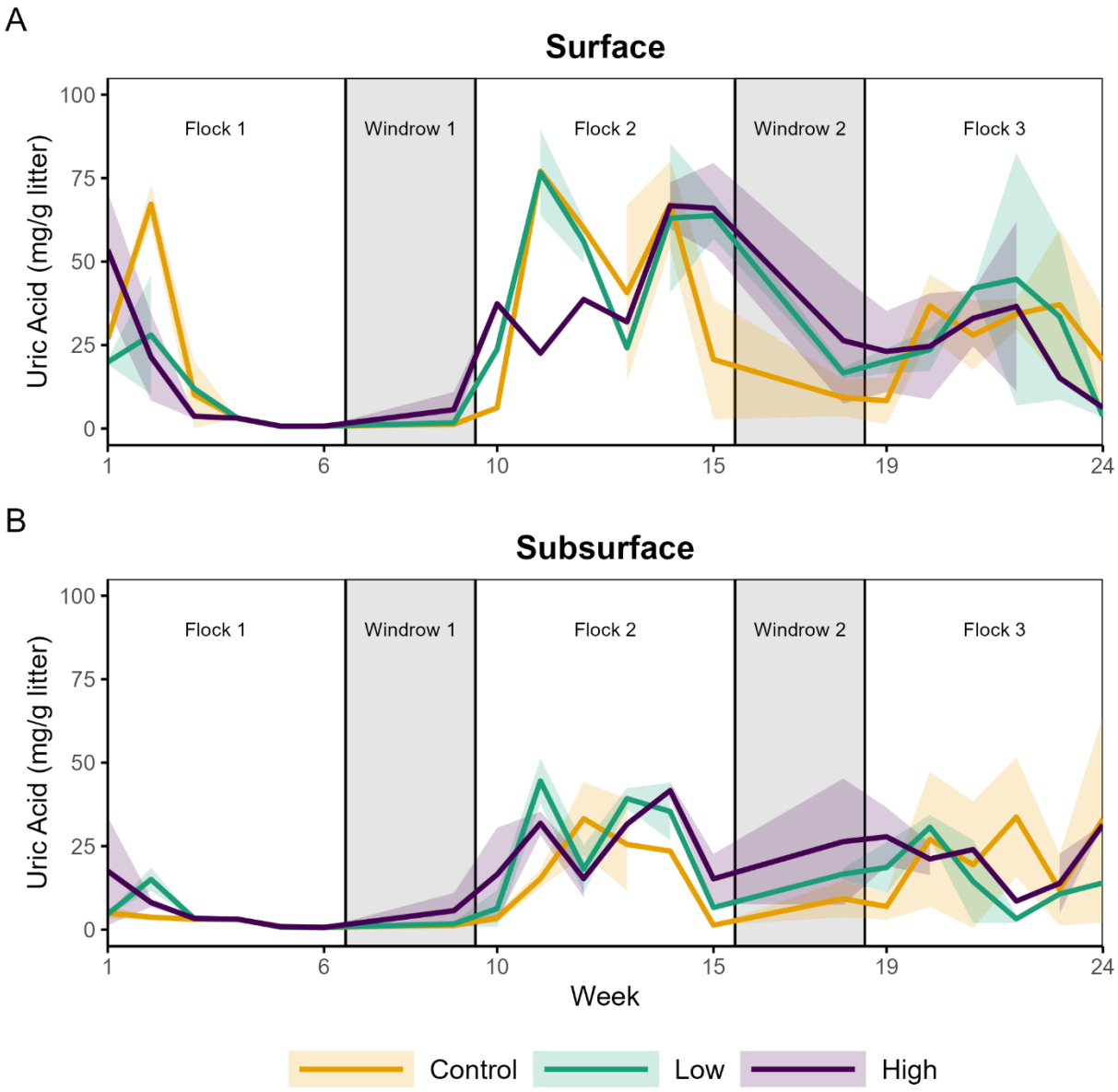

Figure 2.9 Temporal trends in mean uric acid concentrations in poultry litter across treatment groups and flock cycles. **(A)** Surface litter samples 1–2 cm depth and **(B)** subsurface litter samples 5–7 cm depth were collected weekly to assess uric acid content (mg/g litter) across three cycles. Vertical black lines mark transitions between flock and windrow periods. Shaded grey regions indicate windrow composting periods. Error ribbons represent standard deviation. Note that the first two weeks of each windrow composting period were not sampled; data shown represent only the final week of windrowing.

Temporal patterns of uric acid concentration varied across treatment groups and litter depth **(Figure 2.9)**. Surface litter samples (1–2 cm depth) exhibited higher uric acid concentrations and greater week-to-week variability compared to subsurface litter samples (5–7 cm depth). Peaks in uric acid concentration varied flock-to-flock, while concentrations decreased for the final week of windrow period.

Statistical analysis of mean uric acid concentrations across flock periods showed no significant treatment effects. One-way ANOVA indicated no differences among treatments in the surface layer ($F(2,6) = 0.037$, $p = 0.964$) or the subsurface layer ($F(2,6) = 0.089$, $p = 0.916$). Tukey's HSD post hoc comparisons similarly revealed no significant pairwise differences between treatment groups for either litter depth.

Overview of Sequencing and Quality Filtering

Of the initial 18.9 million raw reads, approximately 39.5% were retained after primer removal, quality filtering, denoising, and chimera removal. The largest reductions occurred during filtering and denoising (**Table 3**). After denoising and chimera removal, 3,744 unique ASVs were retained across all samples, with richness per sample ranging from 106 to 1,834 ASVs (**Table 4**).

Table 2.3 Read retention throughout 16S rRNA data processing pipeline.

Processing Step	Total Reads	% of Previous Step	% of Raw Reads
Raw reads	18,900,929	-	100.0%
After primer removal	18,151,849	96.0%	96.0%
After filtering	14,730,088	81.2%	77.9%
After denoising	9,511,229	64.6%	50.3%
After chimera removal	7,471,510	78.5%	39.5%

Table 2.4 Amplicon sequence variant (ASV) counts detected after processing steps

Processing Step	Number of ASVs
After denoising	12,149
After chimera removal	3,744
Per-sample richness range	106–1,834

Taxonomic classification of non-chimeric ASVs revealed that the dominant bacterial phyla across all samples were Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes, which together accounted for most of the reads. Phyla with low prevalence or of non-bacterial origin, including Cyanobacteria/Chloroplast, Plantae, and others, were excluded from downstream analyses to focus

on ecologically relevant bacterial taxa. At the order level, sequences were dominated by members of the Bacillales, Lactobacillales, and Micrococcales. Among the top genera, *Enterococcus*, *Brachybacterium*, *Corynebacterium*, and *Salinicoccus* were the most abundant (**Table 5**).

Table 2.5 (A) Top 10 most abundant phyla detected across all samples, (B) Top 10 most abundant bacterial orders detected across all samples, (C) Top 10 most abundant bacterial genera detected across all samples based on total read counts.

A.

Phylum	Total Reads
Firmicutes	4,331,496
Actinobacteria	2,114,068
Proteobacteria	811,157
Bacteroidetes	92,512
Cyanobacteria/Chloroplast	32,651
Plantae	2,117
Verrucomicrobia	1,955
Tenericutes	390
Chloroflexi	65
Deinococcus-Thermus	36

B.

Order	Total Reads
Bacillales	2,199,002
Lactobacillales	1,513,516
Micrococcales	1,262,497
Mycobacteriales	689,948
Clostridiales	540,771
Enterobacterales	443,256
Xanthomonadales	259,571
Streptosporangiales	53,676
Erysipelotrichales	52,619
Burkholderiales	38,572

C.

Genus	Total Reads
Enterococcus	764,287
Brachybacterium	642,126
Corynebacterium	619,198
Salinicoccus	609,199
Escherichia/Shigella	438,358
Staphylococcus	405,871
Atopostipes	288,449
Clostridium_sensu_stricto	241,629
Brevibacterium	233,655
Jeotgalicoccus	199,018

Rarefaction curves indicated that most samples reached asymptotic levels of observed ASV richness with increasing sequencing depth, suggesting sufficient coverage for diversity estimation (**Figure 2.10**). Based on these curves, a rarefaction threshold of 20,000 reads was selected for flock samples, and 16,000 reads for windrow samples. Two windrow samples representing the second biological replicates for the Control and Low treatments in the first windrow period were excluded from alpha diversity analysis due to low read counts below this threshold. Samples with the highest observed richness were generally associated with Flock 3, while lower richness values were more common in early timepoints.

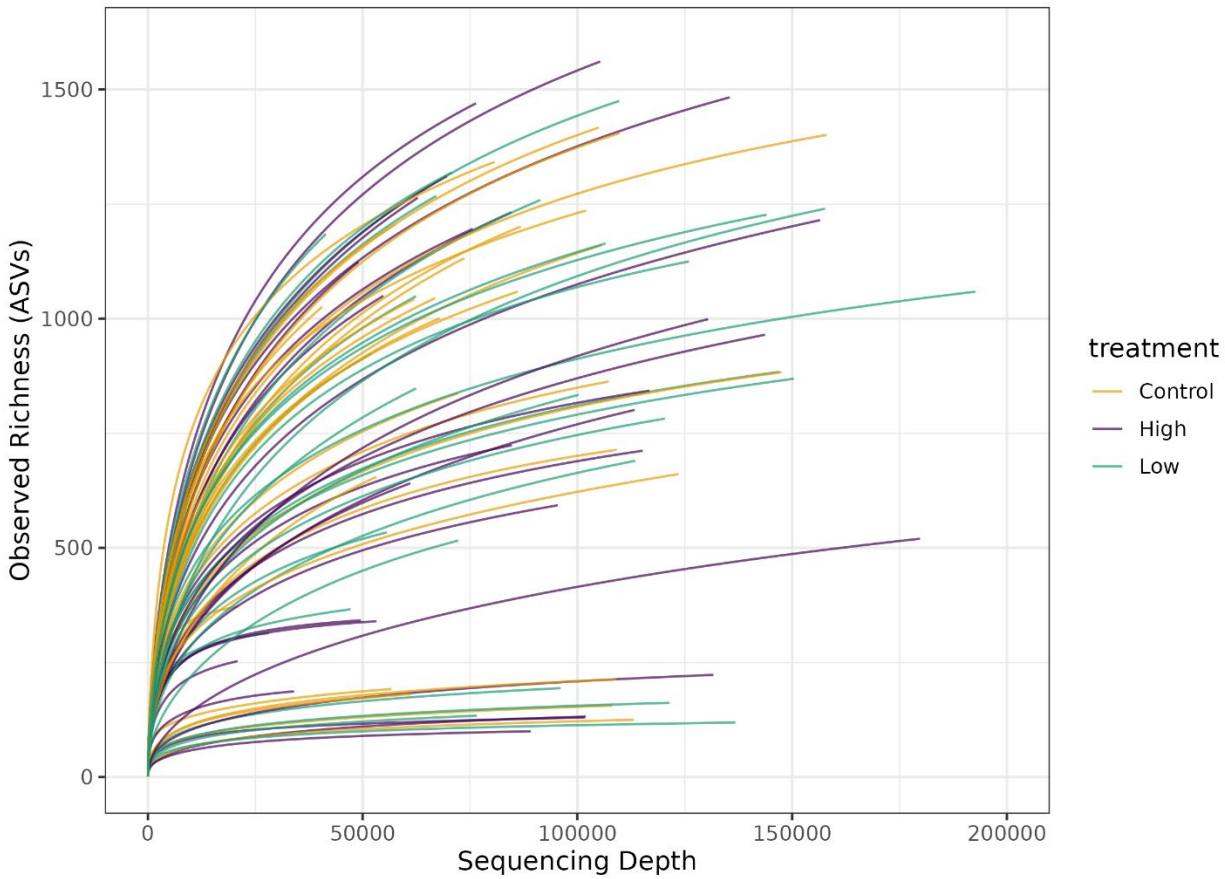

Figure 2.10 Rarefaction curves showing the relationship between sequencing depth and observed amplicon sequence variant (ASV) richness across all samples. Each line represents an individual sample, colored by treatment.

Alpha Diversity Patterns Across Flocks and Windrow Periods

Alpha diversity did not differ significantly among litter treatments during the flock periods based on observed species richness, Shannon diversity, or Simpson's diversity (Kruskal-Wallis tests, $p > 0.05$ for all metrics; **Figure 2.11**). Diversity metrics exhibited substantial variation, particularly in observed richness, but this variability was not dependent on treatment. Several low-diversity

outliers were associated with samples taken early in the study (The first week of Flock 1), while samples with higher richness and diversity were generally observed in Flock 3 (Weeks 19&24).

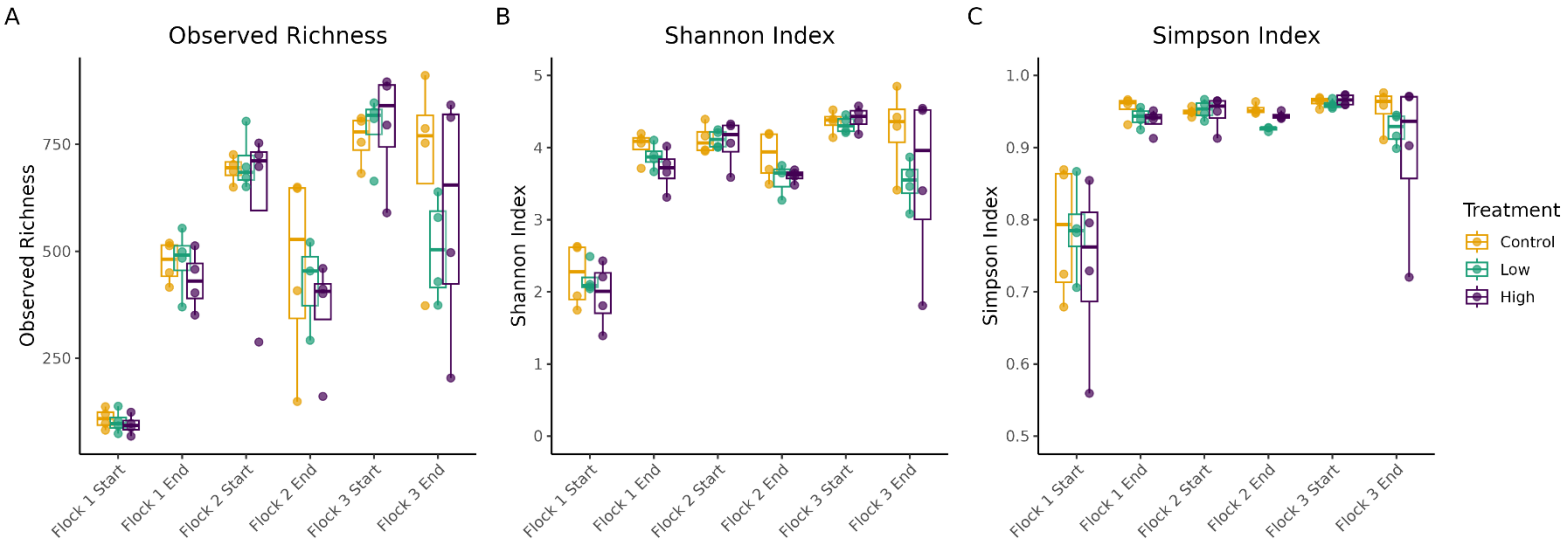

Figure 2.11 Alpha diversity metrics across litter treatments during flock periods. (A) Observed species richness, (B) Shannon diversity index, and (C) Simpson's diversity index. Samples were rarefied to 20,000 reads.

Alpha diversity during windrow composting did not differ significantly across litter treatments based on observed species richness, Shannon index, or Simpson's index (Kruskal-Wallis tests; $p > 0.05$ for all metrics; **Figure 2.12**). A wide range of richness values was observed within each treatment, but there were no consistent treatment-specific patterns. Samples with the highest observed species richness were primarily from the Control windrow chambers.

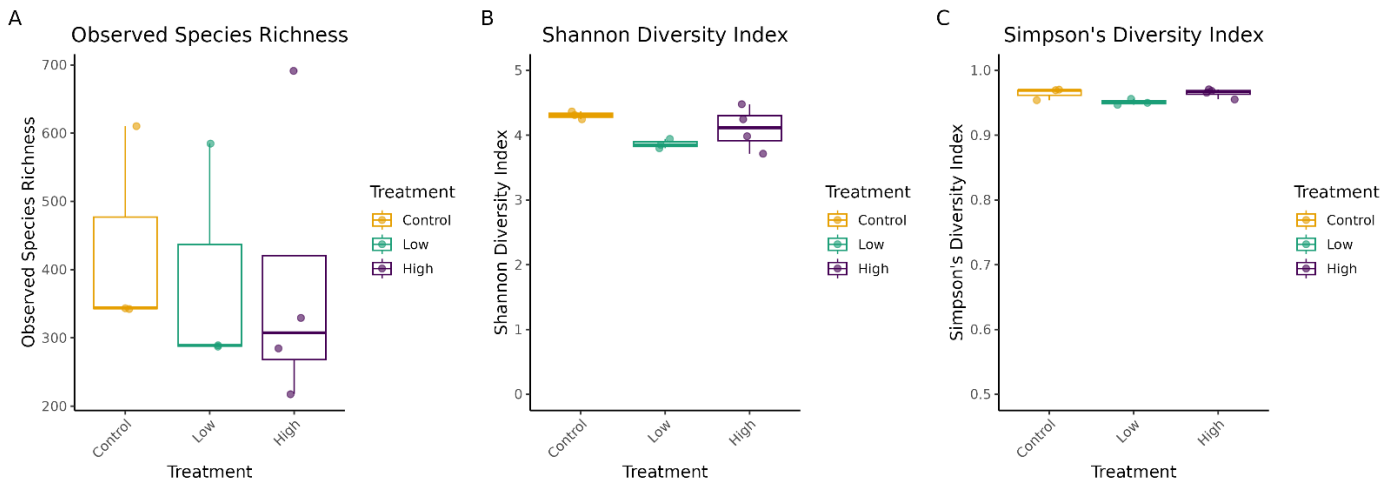

Figure 2.12 Alpha diversity metrics across litter treatments during windrow composting. (A) Observed species richness, (B) Shannon diversity index, and (C) Simpson's diversity index. Windrow samples were rarefied to 16,000 reads.

Beta Diversity and Temporal Dynamics of the Litter Microbiome

Based on the Bray-Curtis dissimilarities, PERMANOVA analyses revealed no significant differences in microbial community composition across litter treatments during either the flock or windrow periods (**Table 6**; $p > 0.05$). Homogeneity of multivariate dispersions was confirmed, supporting the validity of the PERMANOVA results.

Table 2.6 Results of PERMANOVA and beta dispersion tests assessing the effect of litter treatment on microbial community structure.

Subset	PERMANOVA R^2	F-value	p-value	Dispersion F	Dispersion p-value
Flocks	0.030	1.070	0.135	0.430	0.652
Windrows	0.076	0.330	0.611	0.011	0.989

NMDS ordinations provided further visualization of community structure. In flock samples, microbial communities clustered primarily by collection week rather than by treatment, with a stress value of 0.095 indicating good representation of the dissimilarity space **(Figure 2.13A, 13B)**.

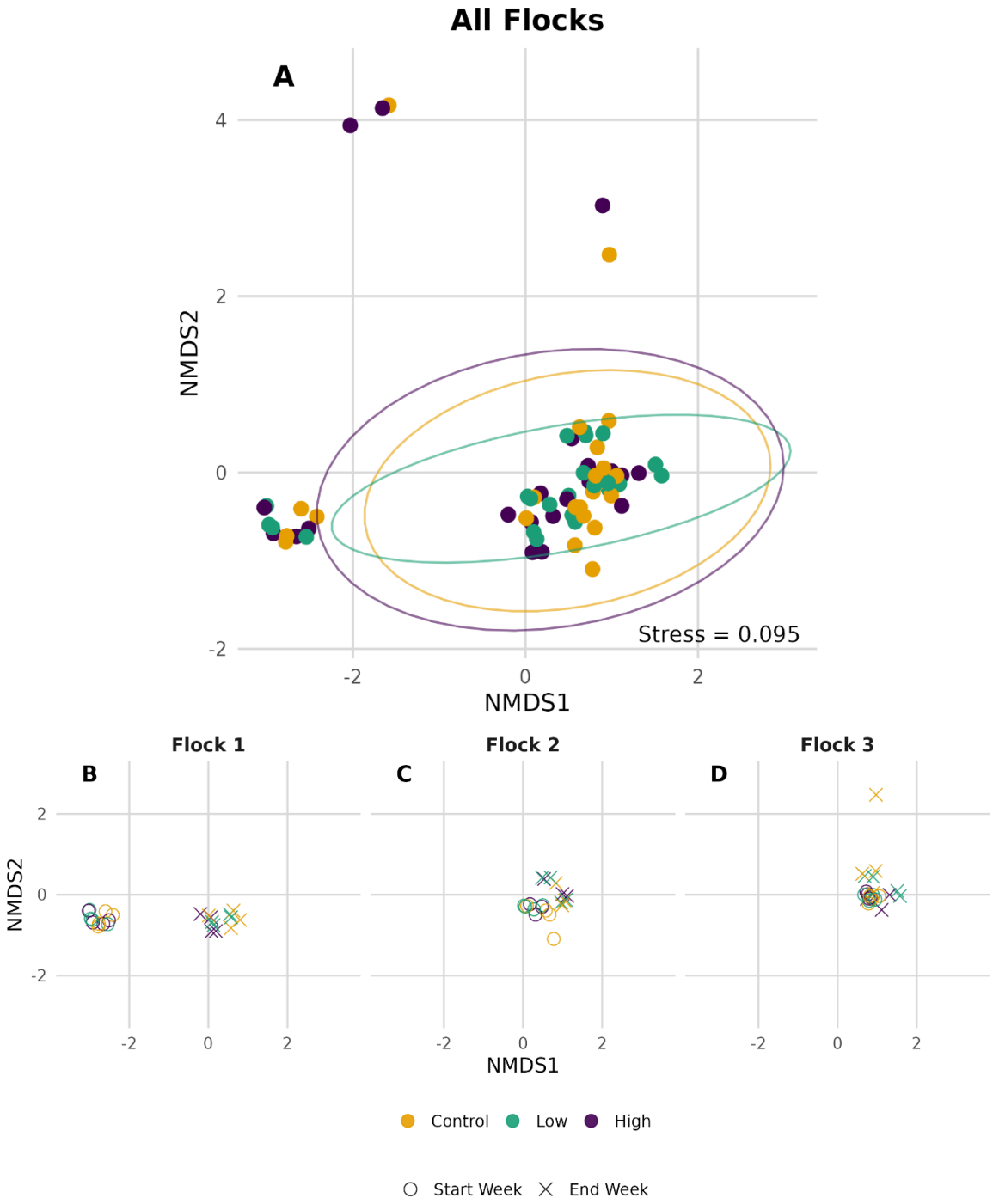

Figure 2.13 Non-metric multidimensional scaling (NMDS) ordinations of microbial community structure in broiler litter samples based on Bray–Curtis dissimilarities. **(A)** NMDS plot of all flock samples (Flocks 1, 2, and 3), colored by treatment group. Each point represents an individual litter sample. 95% confidence ellipses are shown for each treatment group. **(B)** NMDS ordinations faceted by flock and shaped by collection week to show temporal progression of microbial communities during each flock cycle. Symbols represent sampling at start and end week time points.

Panels B–D in **Figure 2.13** are derived from the single ordination in Panel A, allowing direct comparison of temporal and treatment influences within a common beta diversity framework. Panels B–D are faceted by flock and differentiate between start versus end sampling weeks. These subpanels reveal distinct temporal shifts within flocks, but treatment effects remained subtle.

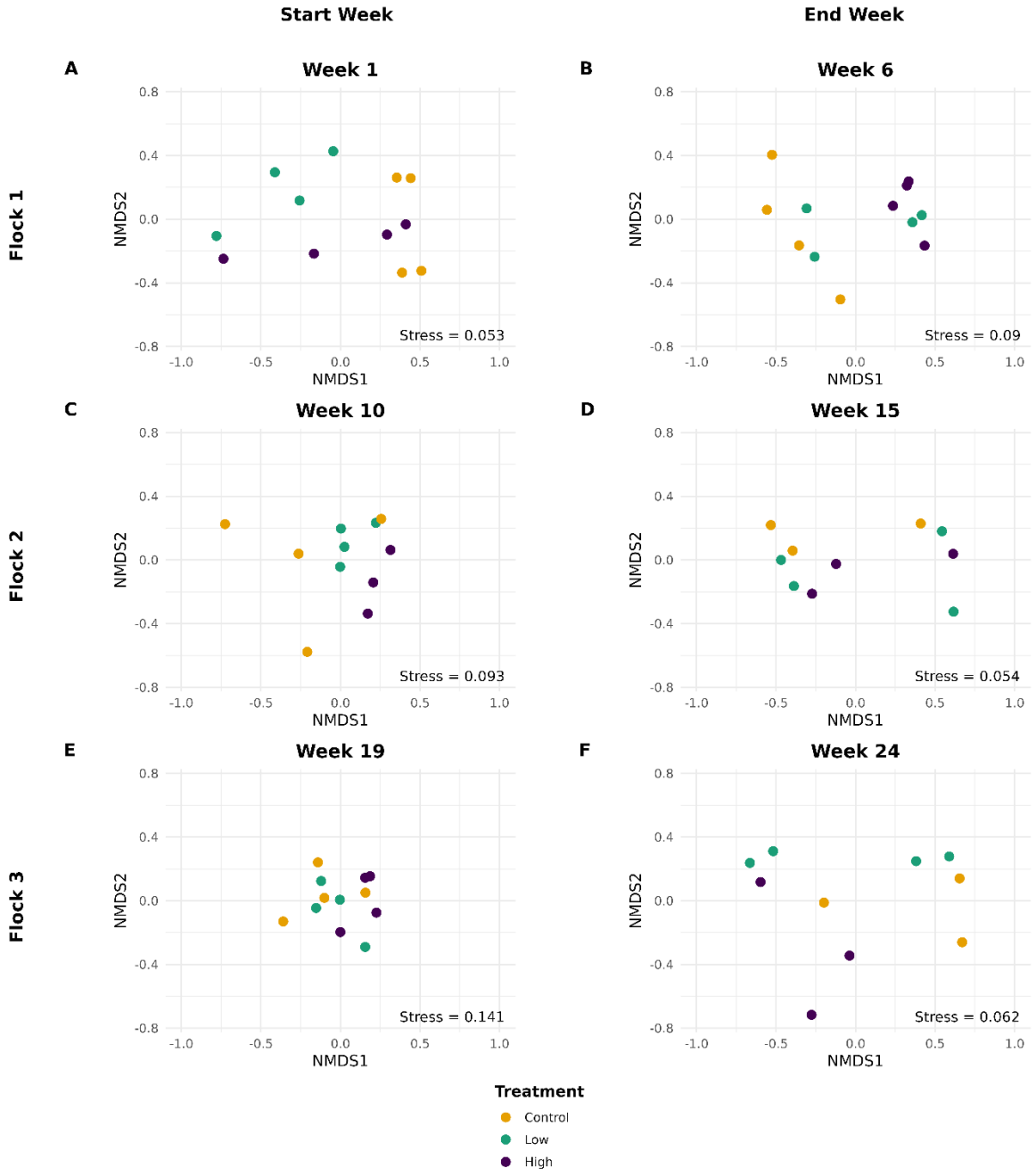

Figure 2.14 Non-metric multidimensional scaling (NMDS) plots showing Bray–Curtis dissimilarities of microbial communities in broiler litter across six sampling weeks. Points are colored by treatment group. Each panel represents a different time point within the study: Weeks 1 and 6 (Flock 1), Weeks 10 and 15 (Flock 2), and Weeks 19 and 24 (Flock 3).

To explore potential treatment effects that were not clearly resolved in the **Figure 2.13** unified NMDS ordination, **Figure 2.14** presents separate NMDS plots for each of the six individual

sampling timepoints. By isolating communities at each timepoint, this figure removes temporal variation as a factor, to improve the ability to detect subtle treatment-related differences in community composition. While some panels (e.g., Weeks 1 and 6) suggest modest clustering by treatment, overall separation between treatment groups remained limited. Treatment groups frequently overlapped within the NMDS space, especially at later timepoints, and no consistent pattern of divergence was observed.

Bacterial Community Composition at the Order Level

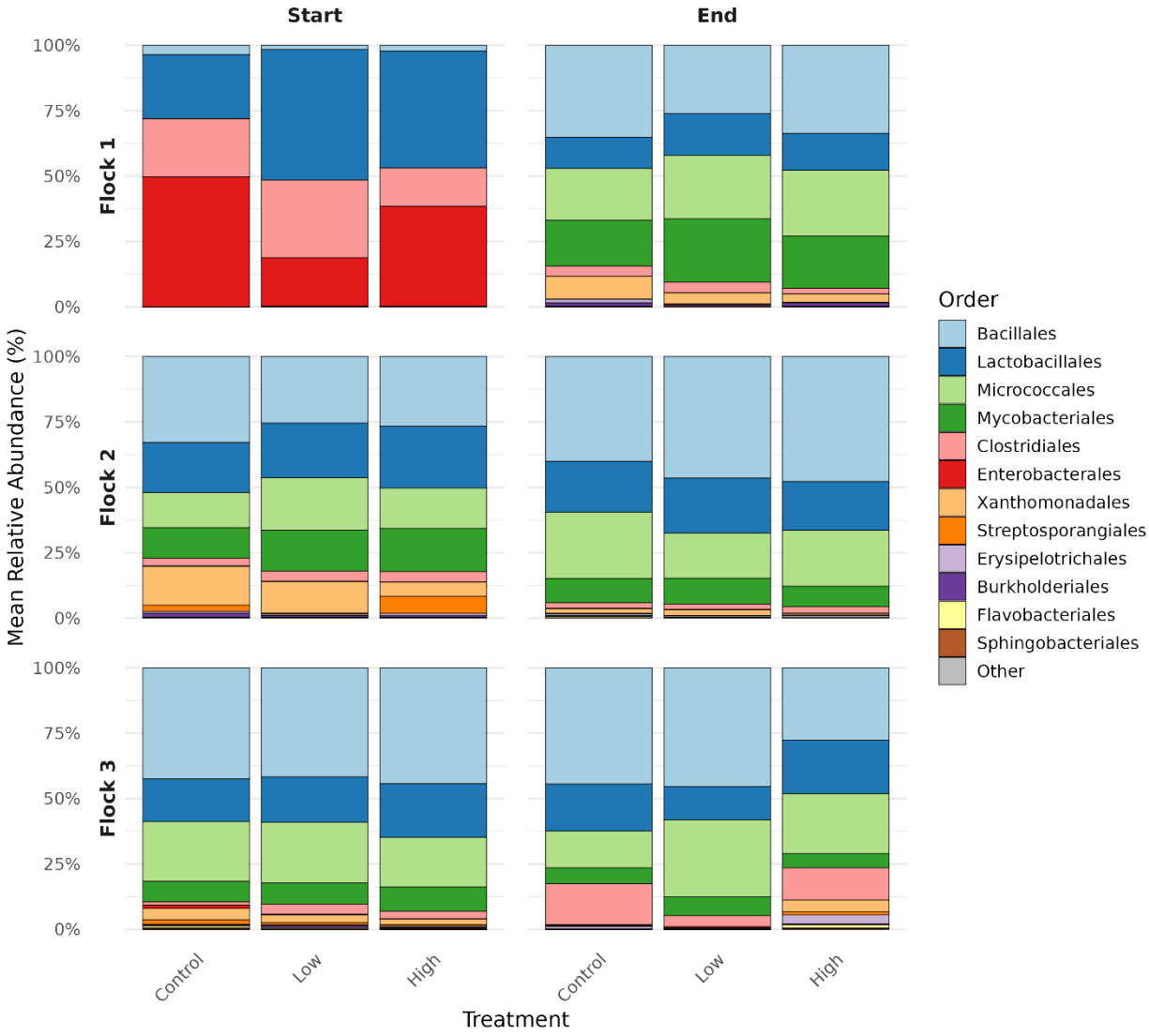

Figure 2.15 Mean relative abundance of bacterial orders in poultry litter, grouped by treatment and stratified by flock and sampling timepoint. Stacked bar plots display the mean order-level relative abundance for each treatment group (Control, Low, High) at the beginning ("Start") and end ("End") of each flock cycle. Only the top 12 most abundant orders are shown; less abundant orders are grouped as "Other."

The mean relative abundance of bacterial orders revealed consistent dominance of Bacillales, Lactobacillales, and Micrococcales across treatments and flock cycles (**Figure 2.15**). Stratifying

by both flock and sampling timepoint highlighted dynamic shifts in community composition over time. Notably, Flock 1 started with a higher relative abundance of Clostridiales and Enterobacterales, which declined by the end of the cycle. In later flocks, particularly Flocks 2 and 3, microbial communities showed increased representation of orders such as Xanthomonadales, Streptosporangiales, and Burkholderiales, especially at the start of each cycle.

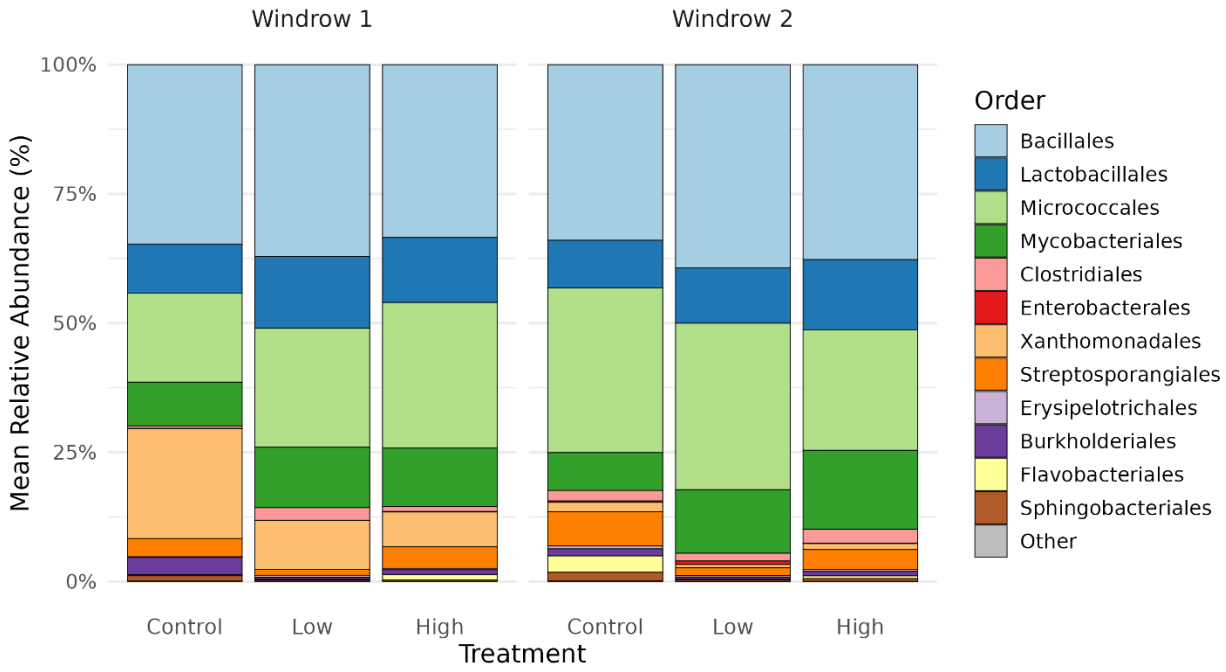

Figure 2.16 Mean order-level relative abundance of bacterial communities in poultry litter samples collected during Windrow 1 (Week 9) and Windrow 2 (Week 18) periods across treatment groups. Bars represent the mean relative abundance of bacterial orders within each treatment (Control, Low, and High). Only the top 12 most abundant orders are shown; less abundant orders are grouped as "Other." Two samples representing the second biological replicates for the Control and Low treatments were excluded from Windrow 1, results should be interpreted with caution, particularly for that timepoint.

During both windrow periods, the poultry litter microbiome remained dominated by Bacillales, Lactobacillales, and Micrococcales, across all treatments (**Figure 2.16**). However, windrowing appeared to temporarily enrich certain lower-abundance bacterial orders, particularly Xanthomonadales, Streptosporangiales, and Burkholderiales, which were more prominent during

Windrow 1 and Windrow 2 but declined following the start of new flock periods as seen in **Figure 2.15**. Meanwhile, Mycobacteriales showed increased relative abundance during windrow periods and persisted to some extent into the following flocks.

Bacterial Community Composition at the Genus-Level

The genus-level profiles revealed clear temporal shifts in bacterial community composition over the course of each flock cycle **(Figure 2.17)**. In Flock 1, the litter microbiome was initially dominated by *Enterococcus, Clostridium_sensu_stricto* and *Escherichia/Shigella*, which declined were reduced by the end of the cycle, coinciding with an increase in genera such as *Corynebacterium* and *Brachybacterium*. This trend continued in Flocks 2 and 3, with a decline in gut-associated anaerobes and opportunistic pathogens, and a corresponding increase in Gram-positive, litter-adapted genera not typically associated with the gut. Notably, *Salinicoccus*, *Staphylococcus*, and *Atopostipes* became increasingly dominant during these later flock cycles. At the end of Flock 3 we see a degree of increase in *Weissella* and *Romboutsia*, which we don't see in any of the other timepoints.

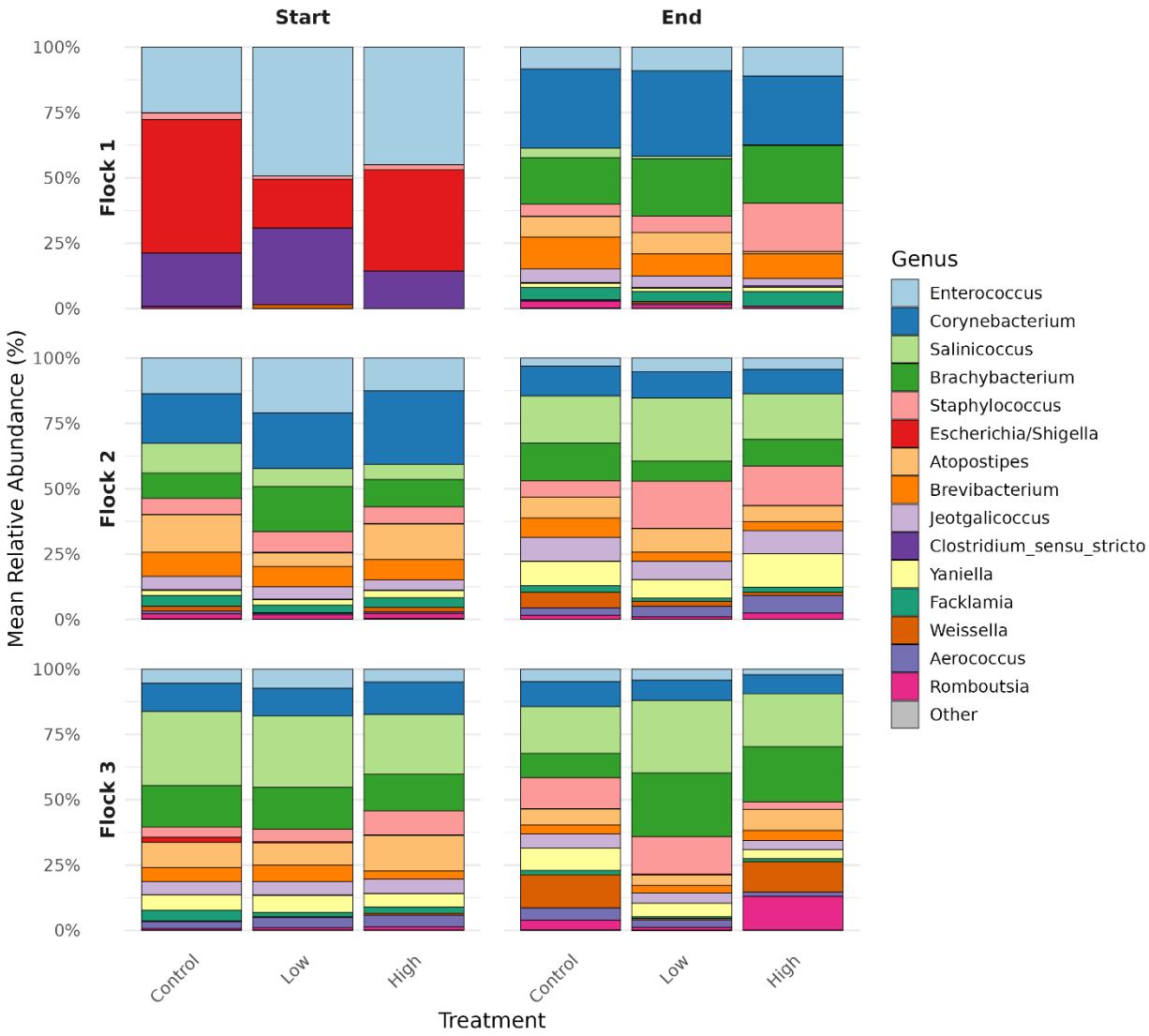

Figure 2.17 Mean relative abundance of dominant bacterial genera in poultry litter across treatment groups and flock timepoints. Stacked bar plots display the average genus-level relative abundance for each treatment (Control, Low, High) at the beginning ("Start") and end ("End") of each flock cycle (Flock 1, Flock 2, and Flock 3). Genera shown represent the most abundant taxa across all samples, with less prevalent genera grouped under "Other."

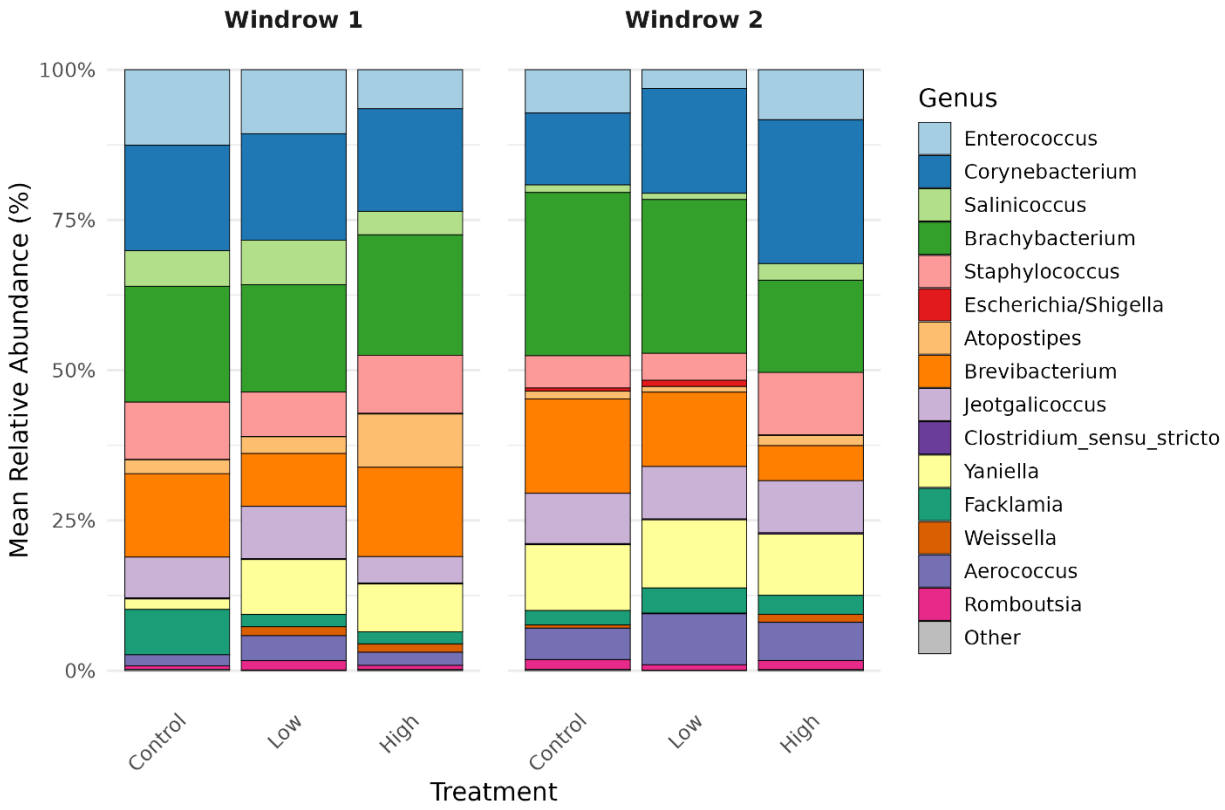

Figure 2.18 Mean genus-level relative abundance of bacterial communities in poultry litter during Windrow 1 (Week 9) and Windrow 2 (Week 18) following flock periods. Stacked bar plots represent the average relative abundance of the top 15 most abundant genera across treatment groups (Control, Low, High), with less abundant genera grouped as "Other." Relative abundances were averaged across replicate chambers within each treatment. Two samples representing the second biological replicates for the Control and Low treatments were excluded from Windrow 1, results should be interpreted with caution, particularly for that timepoint.

The bacterial community structure during Windrow 1 (Week 9) and Windrow 2 (Week 18) in **Figure 2.18** showed a distinct shift compared to the flock-associated timepoints in **Figure 2.17**. Several genera that were previously less prominent became more abundant during windrowing. *Brevibacterium*, *Jeotgalicoccus*, *Yaniella, Facklamia,* and *Aerococcus* increased across all treatment during windrow. *Corynebacterium* and *Brachybacterium* remained the most dominant across windrow phases and appeared to increase compared to flock timepoints. Meanwhile, *Enterococcus* and *Staphylococcus* remained stable at similar levels to flock-associated timepoints.

DESeq2-Based Differential Abundance Analysis Across Timepoints

Genus-level differential abundance analysis was conducted using DESeq2 to assess microbial shifts among treatments during the start and end flock period timepoints. DESeq2 analysis was not performed for the Windrow periods due to insufficient sample retention following filtering steps.

Week 1 – Initial Treatment Effects

Table 2.7 Differentially abundant genera (padj < 0.05) in Week 1 sampling during Flock 1, one week post treatment exposure. Results are based on DESeq2 differential abundance testing comparing the treatments to the Control. Columns include $\log_2$ fold change ($\log_2$FC), standard error (SE), normalized mean abundance (baseMean), and adjusted p-values (padj).

Comparison	Genus	$\log_2$FC	SE	baseMean	padj
Low vs Control	Weissella	26.59	3.68	490.34	1.91e-11
Low vs Control	Bacteroides	24.27	3.68	1687.83	8.17e-10
Low vs Control	Romboutsia	-10.41	1.80	221.88	1.03e-07
Low vs Control	Kineothrix	-7.61	2.55	61.97	2.73e-02
Low vs Control	Corynebacterium	-6.15	2.23	15.16	4.49e-02
High vs Control	Bacteroides	27.86	3.68	1687.83	1.39e-12
High vs Control	Romboutsia	-4.93	1.58	221.88	3.47e-02
High vs Control	Bacillus	-5.37	1.80	162.22	3.63e-02

One week after treatment application, multiple genera were differentially abundant between treatment groups and the control (**Table 7)**. Genera such as *Weissella* and *Bacteroides* were

significantly enriched in the Low treatment, while *Romboutsia*, *Kineothrix*, and *Corynebacterium* were depleted. In the High treatment, *Bacteroides* showed further enrichment, with reductions in *Romboutsia* and *Bacillus*

Week 6 – End of Flock 1

By the end of the first flock, *Lysobacter* was significantly enriched in both Low and High treatments. Several genera, including *Alicoccus*, *Aerococcus*, and *Lactobacillus*, were reduced in the High group. *Staphylococcus* and *Luteimonas* were enriched, while *Salinicoccus* and *Amphibacillus* decreased (**Table 8**).

Table 2.8 Differentially abundant genera (padj < 0.05) in Week 6 sampling during Flock 1, at the conclusion of the first flock period and prior to windrowing.

Comparison	Genus	log_2FC	SE	baseMean	padj
Low vs Control	Lysobacter	9.06	1.67	640.82	5.17e-06
High vs Control	Lysobacter	10.70	1.67	640.82	1.30e-08
High vs Control	Aliicoccus	-8.77	1.91	522.87	1.77e-04
High vs Control	Aerococcus	-4.14	0.91	219.74	1.77e-04
High vs Control	Lactobacillus	-6.60	1.82	153.37	6.78e-03
High vs Control	Staphylococcus	2.83	0.84	6821.48	1.36e-02
High vs Control	Salinicoccus	-3.92	1.21	914.51	1.93e-02
High vs Control	Amphibacillus	-1.85	0.60	926.79	2.67e-02
High vs Control	Luteimonas	4.61	1.56	88.43	3.60e-02

Week 10 – Post-Windrow 1 and Flock 2 Start

Immediately after Windrow 1 and flock restocking, *Lysobacter* remained enriched in both treatments. *Acinetobacter* was depleted across both Low and High treatments, while *Sporosarcina* and *Mycobacterium* were significantly reduced in the High treatment (**Table 9**).

Table 2.9 Differentially abundant genera (padj < 0.05) in Week 10 sampling during Flock 2, immediately following windrow treatment and flock restocking.

Comparison	Genus	log_2FC	SE	baseMean	padj
Low vs Control	Acinetobacter	-8.85	2.09	42.84	1.23e-03
Low vs Control	Lysobacter	6.60	1.39	176.89	2.04e-04
High vs Control	Lysobacter	6.30	1.39	176.89	5.99e-04
High vs Control	Acinetobacter	-7.89	2.05	42.84	5.95e-03
High vs Control	Sporosarcina	-4.12	1.25	61.92	2.46e-02
High vs Control	Mycobacterium	-3.89	1.15	12.79	2.46e-02

Week 15 – End of Flock 2

Enrichment of *Lysobacter* persisted into week 15 in both treatments, while *Oceanobacillus* showed modest enrichment in Low and *Sphingobacterium* declined in High (**Table 10**).

Table 2.10 Differentially abundant genera (padj < 0.05) in Week 15 sampling during Flock 2, at the conclusion of the second flock period and prior to the second windrowing.

Comparison	Genus	log_2FC	SE	baseMean	padj
Low vs Control	Lysobacter	8.94	1.70	137.06	1.21e-05
Low vs Control	Oceanobacillus	2.05	0.57	121.21	1.50e-02
High vs Control	Lysobacter	5.96	1.71	137.06	2.49e-02
High vs Control	Sphingobacterium	-6.18	1.80	18.52	2.49e-02

Week 19 – Post-Windrow 2 and Flock 3 Start

Following Windrow 2 and the onset of Flock 3, *Gracilibacillus* was enriched in High, while *Lysobacter* and *Luteimonas* remained elevated. In contrast, several genera, including *Virgibacillus*, *Anaerotignum*, and *Negativibacillus*, were depleted (**Table 11**).

Table 2.11 Differentially abundant genera (padj < 0.05) in Week 19 sampling during Flock 3, following the second windrow event and flock restocking.

Comparison	Genus	log_2FC	SE	baseMean	padj
Low vs Control	Gracilibacillus	-4.03	1.08	115.44	2.05e-02
High vs Control	Aquihabitans	-5.14	1.42	13.73	3.21e-02

Week 24 – End of Flock 3 and Study Conclusion

At the final sampling point, a large reduction in *Pediococcus* and *Halomonas* was observed in the Low group. *Weissella* abundance was also reduced in Low, contrasting with its early enrichment in the first week. The High treatment again showed significant enrichment of *Lysobacter* and *Luteimonas*.(**Table 12**)

Table 2.12 Differentially abundant genera (padj < 0.05) in Week 24 sampling during Flock 3, at the end of the final flock and study period.

Comparison	Genus	log_2FC	SE	baseMean	padj
Low vs Control	Pediococcus	-25.25	2.59	680.92	1.99e-20
Low vs Control	Halomonas	-22.34	2.85	35.82	2.12e-13
Low vs Control	Weissella	-7.99	1.91	19083.91	9.32e-04
High vs Control	Gracilibacillus	7.67	1.70	50.31	4.13e-04
High vs Control	Lysobacter	8.87	2.24	23.95	2.56e-03
High vs Control	Luteimonas	5.20	1.72	119.39	4.24e-02
High vs Control	Enteractinococcus	3.02	1.08	565.01	4.74e-02
High vs Control	Virgibacillus	-5.14	1.70	7001.29	4.24e-02
High vs Control	Anaerotignum	-4.08	1.44	15.43	4.74e-02
High vs Control	Negativibacillus	-3.97	1.36	10.86	4.74e-02

Lysobacter was the most consistently enriched genus in both Low and High treatments, appearing in every flock and persisting through windrow phases. It had moderate to high baseMean values and low standard error in most instances where it appeared significant. Other genera like *Gracilibacillus*, *Luteimonas*, and *Bacteroides* also showed repeated significant changes but varied by treatment and timepoint.

A subset of significant genera had very low normalized abundance (baseMean < 20), including *Mycobacterium*, *Negativibacillus*, *Aquihabitans*, *Corynebacterium*, and *Anaerotignum*. Also, several taxa showed extremely large log_2 fold changes (e.g., *Weissella* and *Pediococcus*) accompanied by relatively high standard errors.

Functionally Relevant Genera

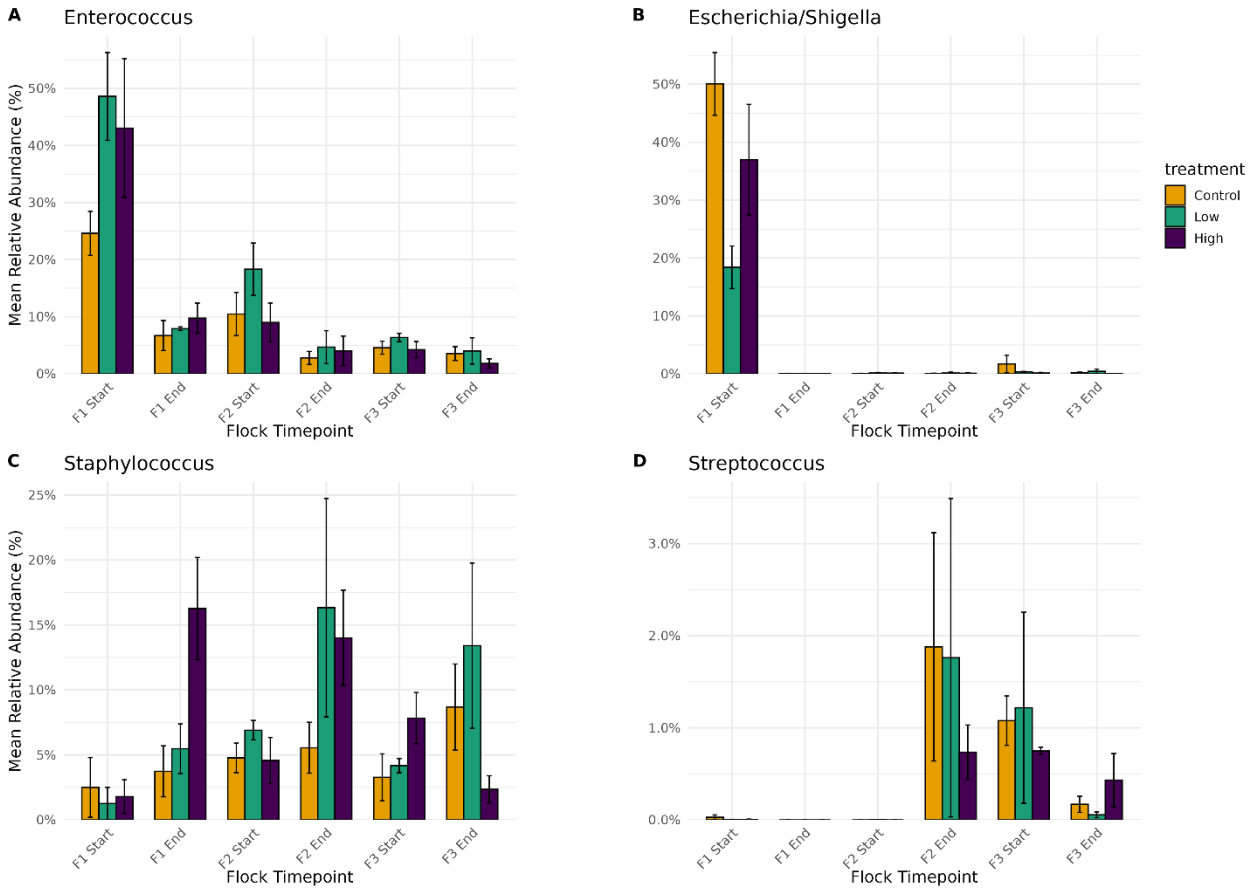

Figure 2.19 Mean relative abundance of potentially pathogenic genera across broiler flocks. Bar plots show the mean (± SE) relative abundance of Enterococcus (A), Escherichia/Shigella (B), Staphylococcus (C), and Streptococcus (D) in poultry litter samples collected during each flock period (Flock 1–3). Each bar represents a treatment group with error bars indicating standard deviation among replicates.

The relative abundance of several genera associated with potential pathogenicity in poultry markedly over time and between treatment groups (**Figure 2.19**). *Enterococcus* (**Figure 2.19A**) was dominant at the start of Flock 1 across all treatments, with the Low and High groups exhibiting the highest mean relative abundance (~50%). Abundance declined sharply by the end of Flock 1 and remained low and relatively stable through Flocks 2 and 3, with no clear treatment-associated differences in later timepoints. *Escherichia/Shigella* (**Figure 2.19B**) also showed high relative abundance at the beginning of Flock 1, particularly in the Control group, but rapidly declined to

near-undetectable levels by the end of the same flock period and stayed low throughout the remainder of the study. *Staphylococcus* (**Figure 2.19C**) displayed the opposite pattern. Initially low in all groups, its abundance steadily increased across timepoints, particularly in the High and Low treatment groups, with the most notable elevation at the end of Flock 2. *Streptococcus* (**Figure 2.19D**) was present at low relative abundance throughout, with a small uptick at the end of Flock 2 and start of Flock 3 (~1-2%) in all treatments. Variability among replicates was high for this genus, and no consistent treatment-associated pattern emerged. Sequences corresponding to *Salmonella* and *Campylobacter* were not detected in any samples, while *Pseudomonas* were present only at low abundances (<0.1%) for most of the study.

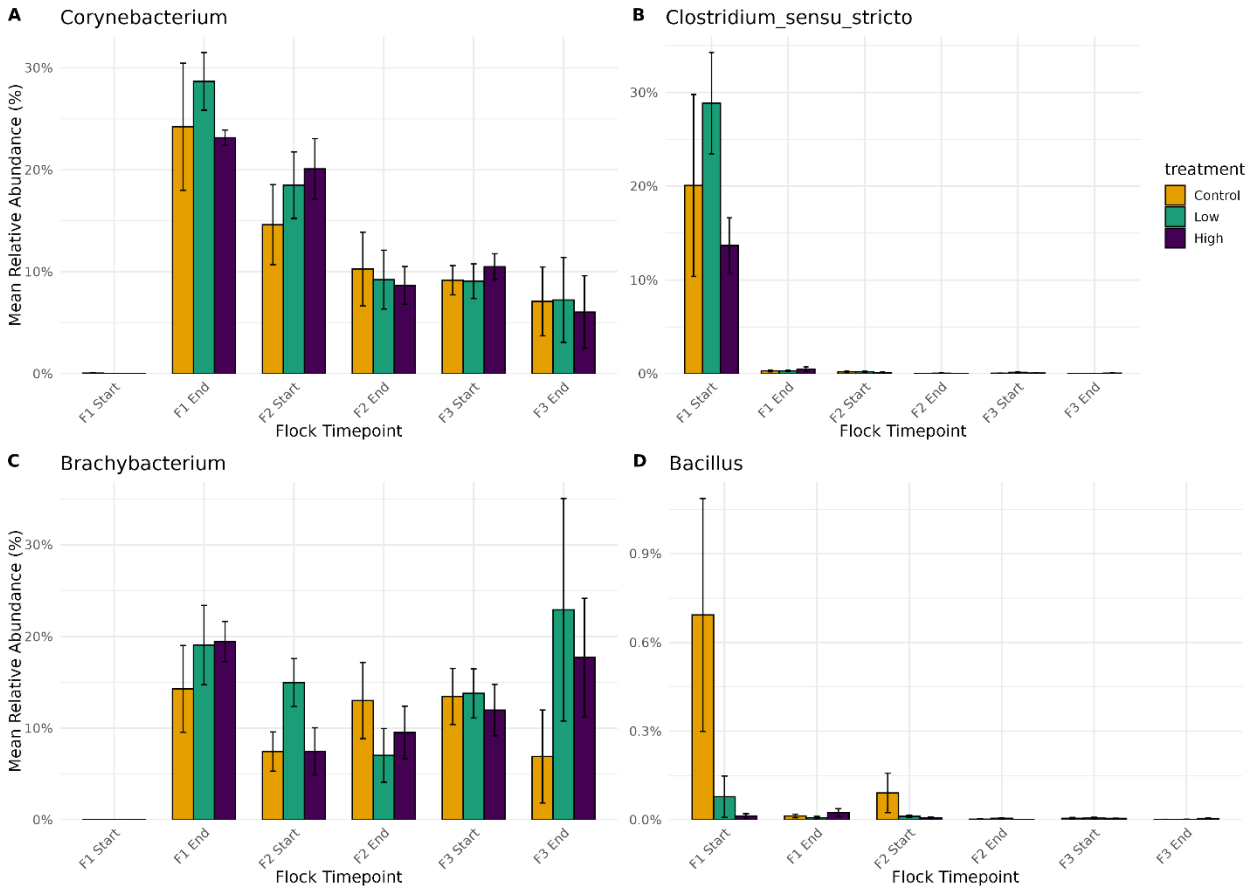

Figure 2.20 Mean relative abundance of dominant litter-associated genera across broiler flocks with known roles in uric acid/urea decomposition. Bar plots show the mean (± SE) relative abundance of Corynebacterium (A), Clostridium sensu stricto (B), Brachybacterium (C), and Bacillus (D) in poultry litter during each flock period (Flock 1–3), grouped by treatment.

Several genera associated with nitrogen metabolism or litter adaptation exhibited dynamic abundance patterns across flocks and treatment groups (**Figure 2.19**).

At the start of Flock 1 *Corynebacterium* (**Figure 2.20A**) was nearly absent and below 1% mean relative abundance, but increased sharply particularly in the Low treatment, peaking at the end of that flock. At the start of Flock 2 it displayed elevated abundances in the treatment groups. Its abundance declined gradually in later flocks but remained consistently present across all

treatments at similar levels. Like *Escherichia/Shigella, Clostridium sensu stricto* (**Figure 2.20B**) was highly abundant at the start of Flock 1 but dropped to near-zero levels by the end of that flock and remained at even lower levels in subsequent timepoints across all treatments. *Brachybacterium* (**Figure 2.20C**) exhibited an increase in abundance after Flock 1 start and fluctuated between ~10-20% mean abundance over time, with marked peaks by the end of Flock 1 and 3 in the Low and High treatments. The genus *Bacillus* (**Figure 2.20D**) exhibited low mean relative abundances across all flock timepoints, consistently remaining below 1% for the study duration. The most notable presence was observed at Flock 1 Start, particularly in the control treatment (~0.7%) and to a lesser extent in the High and Low OA treatments (~0.1% and ~0.05%, respectively). However, this was followed by a substantial decrease in all groups to near zero by Flock 1 End. Subsequent timepoints (Flock 2 Start, Flock 2 End, Flock 3 Start, and Flock 3 End) consistently showed very low mean relative abundances of *Bacillus* across all groups, indicating a temporally driven reduction and sustained low levels after the initial timepoint.

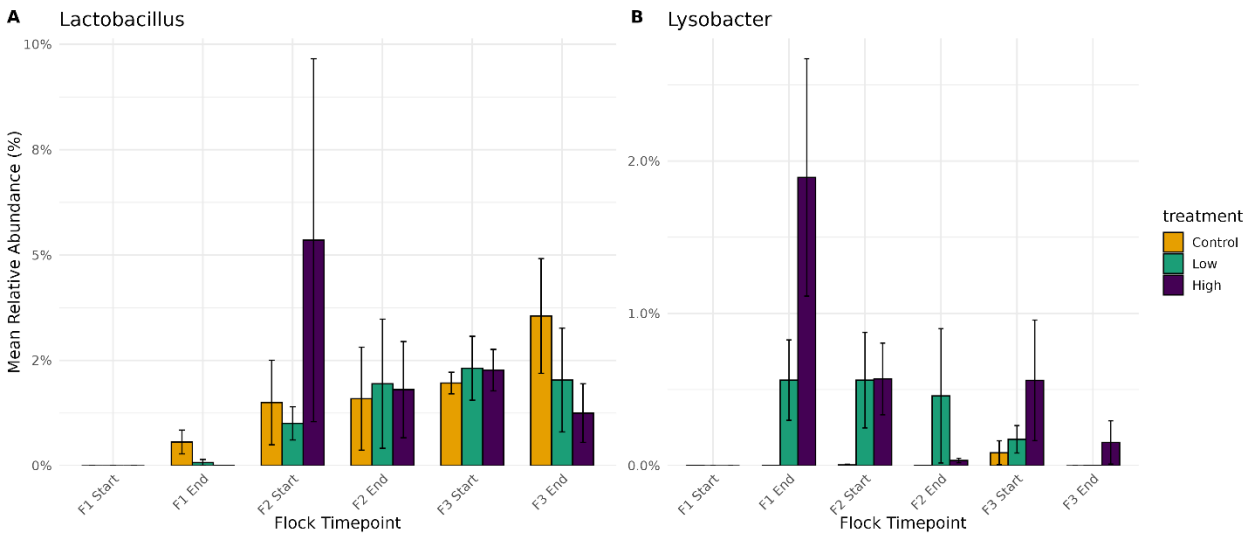

Figure 2.21 Mean relative abundance of Lactobacillus and Lysobacter across broiler flocks and windrow periods. Bar plot shows the mean (± SE) relative abundance of Lactobacillus and Lysobacter in poultry litter samples collected during each flock/windrow period (Flock 1–3), grouped by treatment.

Figure 2.21 shows the mean abundance timepoint and treatment distribution of *Lactobacillus* and *Lysobacter,* two genera of interest due to their potential involvement in pathogen suppression. *Lactobacillus* (**Figure 2.21A**) was initially rare in all groups during Flock 1, but increased over time, particularly in the High treatment during Flock 2, though variability was quite high. This peak in mean abundance at the start of Flock 2 was followed by a general stabilization across groups in the rest of the later timepoints. *Lysobacter* (**Figure 2.21B**) was not present during early litter development either but later showed a pronounced and sustained increase in the High treatment group, with the highest abundance at the end of Flock 1 and continued elevation in Flocks 2 and 3. In contrast, *Lysobacter* remained low in the Control group and moderately elevated in the Low treatment.

2.4 Discussion

Broiler Growth Performance

The inclusion of OA sand as a litter amendment did not significantly impact broiler growth performance in this study relative to control. Final live weights and FCR did not differ significantly across treatments. This suggests that any observed variation was within the range of normal biological variability among birds.

Across the study period, however, differences in performance emerged between flocks. FCR was highest in Flock 1, compared to Flock 2 & 3, indicating reduced production efficiency during the initial cycle. One plausible explanation for this is the elevated litter moisture observed during Flock 1, which exceeded 60% int chamber and remained well above 30% for much of the Flock 1 period. Excessive moisture can impair bird performance by fostering microbial growth, increasing NH_3 levels, and compromising litter quality (de Toledo et al., 2020; Brink et al., 2022; Gençoğlan & Gençoğlan, 2017; de Jong et al., 2014).

Ventilation during Flock 1 was later determined to be insufficient to maintain optimal litter conditions and was adjusted across all chambers before Flock 2. These changes coincided with improved FCR in the later flocks. In addition to elevated moisture, the litter sampled in Flock 1 also showed greater relative abundance of potentially pathogenic bacterial genera, including *Enterococcus*, *Escherichia/Shigella*, and *Clostridium sensu stricto.* These taxa are usually associated with negative health outcomes in broilers and may have contributed to the elevated FCR and FPD scores observed in Flock 1. Persistently wet litter is also a known risk factor for causing

FPD, due to skin maceration and microbial irritation (Rashid et al., 2020; Mayne et al., 2007). FPD scores were highest in Flock 1 and declined in subsequent flocks, following improvements in ventilation and litter conditions. Birds from OA treated chambers showed numerically lower average footpad scores, but these differences were not statistically significant. The greatest apparent reduction in footpad severity occurred in Flock 1, where High OA birds exhibited milder lesions than controls. However, this result may be partially confounded by one of the Control chambers exhibiting particularly high litter moisture during Flock 1, potentially exaggerating the apparent advantage of the OA treatments.

Despite a slightly higher FPD score in the first flock control, scores across all treatments and flocks remained consistently low, suggesting that baseline welfare was well-maintained in the experimental system. Regular litter windrowing, post-Flock 1 ventilation adjustments, and precise control of temperature and relative humidity likely contributed to this outcome.

Overall, OA did not significantly improve broiler growth performance, NH_3 emissions, or footpad dermatitis outcomes under the conditions tested. Importantly, there were also no negative performance effects connected with OA. This indicates that incorporating up to 40% OA sand in litter is feasible without impairing growth. If OA did have some subtle positive effects on performance, they could have been masked by the relatively high-moisture conditions of this study. Some moisture likely originated from unavoidable drinker spillage, because of this the ventilation system drying was not always adequate. If OA's primary mode of action is moisture mitigation, its benefits may be more pronounced under drier baseline conditions.

Oolitic Aragonite Amendment Effects on Litter Chemistry

End-of-flock litter analyses revealed that inclusion of OA in broiler litter had minimal impact on most measured chemical properties, aside from a pronounced increase in calcium. As expected, OA amendments substantially elevated calcium concentrations in the litter, most likely a result of OA being primarily composed of $CaCO_3$ (Keener et. al., 2014). OA-treated and control litters showed similar nitrogen and phosphorus profiles, suggesting a lack of treatment effect in terms of nitrogen retention in the litter.

The pH of the broiler litter remained consistently alkaline (~8.5-8.8) across all treatments. This pH range is typical for broiler litter that has not been treated with acidifiers. Microbial degradation of uric acid and associated NH_3 production raises litter pH from neutral pH in fresh litter to ~8.5 in used litter (Rashid et al., 2017; Gençoğlan et al., 2017). Moisture content also remained elevated across all groups, indicating that OA did not provide any notable drying effects. These results suggest that the addition of OA sand did not disrupt or enhance the litter's nitrogen dynamics, moisture retention, or NH_3 volatilization. Chemically, the OA treated litter remained largely similar to untreated litter, apart from a higher calcium content due to enrichment from $CaCO_3$.

Implications for Agricultural Use

From an agronomic perspective, the primary goal of broiler litter application is to supply essential nutrients such as nitrogen (N) and phosphorus (P) in ratios aligned with crop requirements. A desirable N:P ratio for litter-based fertilizer is generally at least 2:1 and often as high as 8:1, depending on the target crop (G. S. Toor et al., 2007; Roswall et al., 2022). However, in this study, the N:P ratios remained near the 1:1 across treatments, suggesting a phosphorus-rich litter profile

with high potential for over-application if used as agricultural fertilizer. As a result, OA addition did not enhance the fertilizer value of the litter in terms of nutrient balancing.

The increase in calcium offers limited direct fertilizer benefit, as calcium is rarely a limiting nutrient in most cropping systems and inexpensive agricultural lime is readily available for soil pH correction. While the additional calcium may provide minor secondary benefits, such as a slight liming effect in acidic soils or increased soil calcium levels upon land application, these benefits are likely marginal. Calcium can also react with phosphate to form less soluble Ca–P compounds, potentially reducing the immediate risk of phosphorus runoff (Wang et al., 2012). However, this immobilization effect is limited; Moore et al. (1994) demonstrated that ground calcitic limestone, which is chemically similar to OA, has minimal efficacy in reducing water-soluble phosphorus compared to more reactive calcium amendments. Moreover, any reduction in phosphorus solubility from Ca–P precipitation is temporary and limited by the soil's phosphorus sorption capacity (Castro & Torrent, 1998). Long-term application of phosphorus-rich litter, regardless of calcium content, can still lead to phosphorus accumulation and saturation in soils. Therefore, while OA may slightly buffer phosphorus mobility through calcium interactions, it does not resolve the underlying issue of excess phosphorus loading. In neutral or calcareous soils, overapplication could even pose a risk of unnecessary liming (Cole et al., 1953).

In summary, OA amendments did not significantly enhance the fertilizer nutrient profile of broiler litter. Although calcium levels were elevated, this provided limited agronomic value and did not meaningfully improve nitrogen availability or phosphorus management. Thus, from a soil fertility

standpoint, OA's role appears to be restricted to serving as a calcium additive with minimal impact on the primary fertilizing effects of poultry litter.

Ammonia Emissions in OA-Amended Litter

The time-series analysis of emissions data indicated that adding OA sand to broiler litter had no statistically significant effect on NH_3 emissions, across three flock cycles. All treatment groups (0%, 20%, 40% OA by litter weight) followed similar NH_3 emission trajectories during each flock, with only minor transient separation. For instance, a brief separation in mean daily NH_3 concentration with high variability was observed during early Flock 1 (with OA-amended litters showing slightly lower NH_3 on average), but this difference quickly dissipated after windrowing. Overall, NH_3 concentrations were similar across treatments. Higher NH_3 emissions in Flock 1's control group were largely driven by a single chamber, while other chambers followed comparable emission patterns. This elevated emission corresponded with higher moisture content in that same control chamber, suggesting that excessive moisture may have driven the increased NH_3 release. The only indication of treatment effect appeared in the last 10 days of Flock 2, where the OA-treated groups emitted slightly less NH_3 than both control chambers. While this trend suggested some mitigation, it did not reach statistical significance. These findings suggest that any NH_3-suppressing effect of OA is likely subtle, transient, and susceptible to being obscured by environmental factors such as litter moisture.

Litter Moisture and Water Activity

Litter moisture dynamics followed an expected cyclic pattern in all treatments, increasing during each flock (as birds added water via excreta and respiration) and then dropping during the windrow composting periods when the litter was turned and aerated after broiler removal. Measurements of water activity(a_w) showed that during active flocks, the litter was nearly water-saturated (surface a_w often near 1.0), indicating extremely high relative humidity in the litter pore space. A paper that studied water activity in poultry litter by Dunlop et. al. (2016) showed that increases in water activity followed non-linear patterns and could reach near saturation values even when moisture content was in the low range of 22-33%. Throughout most flock periods, both control and OA-treated chambers showed high moisture levels and water activity, providing ample water for microbial growth. In the context of our study, at these elevated moisture levels, there is no evidence that supports the idea that OA helps to control water activity. No significant treatment differences in moisture content or water activity were detected. If the slightly higher moisture content of the control treatment litter is not due to drinker leakage or other spurious chamber effects in one of the control chambers, and due to treatment effects from OA, then it is not through the mechanism we expected, which was water activity regulation.

Uric Acid Degradation

Uric acid measurements in the litter provide further insight into the nitrogen cycling under each treatment. Uric acid (UA) concentrations were consistently higher in surface litter than in subsurface samples, across all groups, especially in the early stages of the flocks. This makes sense, uric acid enters the litter via bird droppings deposited on the top; thus the surface layer always contains the freshest uric acid input. The subsurface, in contrast, is older litter where some of that

uric acid has already been metabolized or leached down. The data showed UA levels fluctuating week-to-week during Flocks 2 and 3, reflecting the balance between inputs (ongoing excretion) and decomposition. Interestingly, we don't see this weekly variation occurring in Flock 1, which could be a reflection of Flock 1's different microbial community structure than Flock 2 and 3 and its influence on uric acid breakdown. For instance, the first week of Flock 1 had much higher mean abundances of *Enterococcus*, *Escherichia/Shigella, Clostridium sensu stricto* than other flocks.

At the end of each windrow composting, uric acid concentrations were low in both surface and subsurface layers. This indicates that the composting process was effective at breaking down uric acid in the litter. Microbial degradation of uric acid is known to accelerate at elevated temperatures and aerated conditions achieved during windrow composting (Dittoe et al., 2018). In fact, a substantial portion of nitrogen loss during composting is attributed to rapid decomposition of organic nitrogen and subsequent NH_3 volatilization by aerobic bacteria (Mahimairaja et al., 1994). In our experiment, the spikes in NH_3 emissions observed during the windrow periods correspond to this change in environment, as the litter piles are aerated and heated up, microbial enzymes (uricases, ureases, and amidases) quickly convert uric acid and other nitrogenous residues into NH_3, causing a surge of emissions. During our windrow phase, we also see the corresponding change in community structure where the activity of genera such as *Corynebacterium*, *Brachybacterium,* and *Brevibacterium* start to take prominence. These groups contain species that thrive in these aerobic conditions created by the composting of the litter (Funke et al., 1997; Schefferle, 1965; Joerger et al., 2020). Windrowing is intended to facilitate the drying of litter as well as reducing the microbial load, but if the litter is not supplemented with an effective treatment method, the side effect is a significant increase in NH_3 volatilization (Liang et al., 2014).

There were no significant differences in uric acid concentration between control and OA treatments. At both surface and subsurface layers, uric acid levels varied over time but showed overlapping patterns across all treatments. This suggests that OA had no consistent or statistically measurable impact on uric acid decomposition in the litter. There were hints of transient uric acid retention in OA-treated chambers during early Flock 1 and Flock 2, but these effects were not sustained. In contrast, it was difficult to discern any clear treatment-related differences in the surface litter, potentially due to greater exposure to environmental fluctuations and continual exposure to fresh bird waste. If the aragonite amendment were altering microbial activity in a way that reduced nitrogen loss (e.g., by inhibiting uricase-producing microbes or diverting N into other forms), we would expect to see persistently higher residual uric acid in OA treatments. Instead, any potential effects appeared to be temporary and were not reflected in a sustained shift in uric acid profiles. Overall, uric acid decomposition appeared more influenced by composting schedule and microbial succession than by OA treatment.

Proposed Nitrification Enhancement Potential

This finding is important when interpreted alongside the litter nutrient data (**Figure 2.4**). One of the proposed benefits of OA was its potential to retain nitrogen in the litter by limiting uric acid breakdown. Preliminary investigations suggested that OA might improve the nitrogen-to-phosphorus (N:P) ratio in litter by promoting nitrogen retention relative to phosphorus (Hesselgrave, 2023). Technically, this would imply that OA somehow conserved nitrogen in non-volatile forms such as organic nitrogen or potentially nitrate via enhanced nitrification rather than allowing it to be lost as NH_3.

However, our results did not support this mechanism. We observed no consistent increases in retained nitrogen forms in OA treatments. Daily NH_3 emissions were not significantly reduced, and uric acid levels were not consistently elevated in OA-treated chambers. While it remains possible that oxidized N forms such as nitrate were increased (which we did not directly measure), the TKN and NH_4^+-N data suggest no meaningful shift in nitrogen partitioning.

If OA had enhanced nitrification or served as a niche for nitrifying microbes, we might expect either increased TKN or reduced NH_4^+-N levels, neither of which was observed. It is plausible that the environmental conditions in the litter were not conducive to sustained nitrifier activity. While the MIPS project at UMCES hypothesized that OA could act as a biofiltration substrate for nitrifiers and denitrifiers (University of Maryland Center for Environmental Science, 2018), Such effects likely depend on maintaining specific environmental conditions, particularly a stable pH in the slightly acidic to neutral range and adequate moisture content, to support sustained nitrifier and denitrifier activity (Salim et al., 2014; Prakasam & Loehr, 1972; Anthonisen et al., 1976). Broiler litter is often alkaline and subject to shifting environmental conditions, which can suppress nitrification processes. Therefore, the environmental variability across our three-flock study may not have provided the stability or conditions necessary for nitrifier-based biofiltration mechanisms to strongly establish or persist within granule microsites.

While OA clearly increased calcium content, its impact on nitrogen retention appears minimal. The overall lack of difference in NH_3 emissions, TKN, and uric acid levels suggests that total nitrogen losses and retention were comparable across treatments. Any slight improvements in N:P ratio or reduced phosphorus likely stemmed from calcium-phosphate interactions rather than nitrogen conservation.

Alpha and Beta Diversity Patterns

Alpha diversity analyses indicated that microbial richness and diversity were not significantly affected by OA treatments during either the grow-out or windrow periods. All treatment groups (Control, Low OA, High OA) exhibited similar ranges of observed species richness, Shannon index, and Simpson index values within a given period, suggesting that adding OA did not measurably alter overall diversity. This was true for both the active flock phases and the interim windrowing phases, consistent with the idea that the amendment did not disrupt the broader ecology of the litter microbiome. However, clear temporal patterns in alpha diversity were observed over the course of the experiment, with species richness generally increasing with each successive flock and peaking by Flock 3. Early in Flock 1, many samples reflected low diversity, likely due to initial colonization when the litter was still fresh. As the litter aged and accumulated microbial input from bird excreta and the environment, microbial diversity increased. Many values within the upper quartile range were reflections of high diversity samples from flocks 2 and 3, an expected trend as reused litter develops more ecological niches and resource availability (Kubasová et al., 2022). While OA amendment did not appear to influence alpha diversity, the progression of time and litter reuse did, with older litter supporting a richer and more even bacterial community. Beta diversity patterns reflected in NMDS indicate a clear successional trajectory in community structure over time as well. The microbial community changed significantly from one flock to the next, reflecting the strong influence of temporal dynamics and litter maturation, whereas any treatment-induced shifts across the six sampled timepoints were either not present or very subtle relative to this overall succession.

Successional Dynamics Across Flock Phases

Consistent with these beta diversity results, we observed pronounced shifts in the taxonomic makeup of the litter microbiome across successive flocks, with early communities dominated by enteric bacteria and later communities increasingly comprised of litter-adapted taxa, but no consistent differences attributable to OA treatment at this level. In the first flock, the litter's microbial community was dominated by a relatively narrow set of bacteria typically associated with fresh poultry litter and chicken gastrointestinal origin. Specifically, genera such as *Enterococcus*, *Escherichia/Shigella*, and *Clostridium sensu stricto* were highly abundant in the first week of Flock 1, typical of early colonization patterns in fresh poultry litter (Kubasová et al., 2022). Their dominance in Flock 1 indicates the inoculation of initially sterile bedding by fecal microbes. While certain visual treatment patterns in relative abundance were observed among these enteric taxa, none were found to be differentially abundant.

Clostridium sensu stricto garnered interest not only for its pathogenic species such as *C. perfringens* but also for members like *C. acidurici,* which can degrade uric acid into NH_3 (Hartwich et al., 2012; Dürre & Andreesen, 1983). This early-community profile is in line with previous studies showing that fresh broiler litter (within the first few weeks) is characterized by an influx of enteric bacteria and opportunists from the birds' gut and environment (Kubasová et al., 2022). The relatively low NH_3 levels early in the production cycle and a lack of established microbial competitors are conditions that likely allowed *Enterococcus* and the other fecal bacteria to flourish. In practical terms, Flock 1 had the simplest community structure, which may have implications for chick health and performance. For instance, earlier in the trial, it was observed that birds on fresh litter had slightly worse growth and health metrics compared to later flocks like

lower footpad health scores. The lower litter NH_3 during Flock 1 (due to minimal nitrogen buildup initially) would also favor acid-tolerant fermentative bacteria like *Enterococcus*, which could partly explain their dominance under those early conditions. Indeed, prior work has shown that enteric bacteria such as *Enterococcus* and coliforms become only minor constituents of the litter microbiome once the litter ecosystem develops, accounting for <0.2% of cultivable bacteria in reused litter (Lu et al., 2003). This suggests that the overwhelming initial dominance of those gut-derived taxa in Flock 1 is a temporary state that dissipates as the litter microbiome diversifies and matures. This transitory phase reported in the literature is also reflected in our study, where all three early bacterial genera are only a fraction of their original relative abundance in the litter sampled from the final week of Flock 1.

By the later flocks, the microbial community had shifted dramatically toward taxa adapted to the aged litter environment. In Flock 2 and 3 the early fecal colonizers were largely supplanted by Gram-positive, litter-associated genera that can tolerate the high-solids, high- NH_3, and nutrient-enriched conditions of built-up litter. Genera such as *Salinicoccus*, *Corynebacterium*, and *Brachybacterium* rose to prominence in the second and third flocks. These organisms are characteristic of well-used poultry litter and other manure-rich, drying habitats. Many are halotolerant or alkaliphilic: *Salinicoccus* (family Staphylococcaceae) for example, is known to thrive in saline or high-pH environments, and *Brachybacterium* and *Corynebacterium* (family Corynebacteriaceae) are Actinobacteria that prosper in nutrient-rich organic waste and produce enzymes to degrade uric acid, feathers, and other litter components (Lu et al., 2003).

Staphylococcus, although typically part of the normal skin, respiratory, and intestinal microbiota of chickens, includes opportunistic pathogens such as *S. aureus*, which poses a public health

concern, and *S. agnetis*, a significant broiler pathogen known to cause bacterial chondronecrosis with osteomyelitis (BCO), lameness, endocarditis, and septicemia (Assumpcao et al., 2024; Szafraniec et al., 2022). Unlike other pathogen-associated genera, *Staphylococcus* showed a gradual increase as the litter developed. *Staphylococcus*, initially a minor component, expanded across successive flock cycles, in a few cases appearing more prominently in OA-treated litters, although it was only found to be differentially abundant in the High treatment during the end of Flock 1. This pattern suggests that staphylococci, likely continually introduced via bird skin, feathers, or excreta, establish and proliferate as the litter system stabilizes and other gut-derived bacteria decline. Kubasová et al., 2022 reported that some species of *Staphylococcus* appeared to favor conditions in older litter.

Streptococcus showed a temporary rise during Flock 2 before declining again by Flock 3. There were no clear or consistent treatment-driven differences between the Control, Low, or High OA groups, suggesting that OA amendments did not influence *Streptococcus* dynamics. This genus is of interest because certain streptococcal species are opportunistic pathogens in poultry, associated with conditions like septicemia, arthritis, or endocarditis, and their presence in litter raises potential bird health concerns (Morishita, 2023).

Lactobacillus was also consistently present in aged litter in low relative abundance. *Lactobacillus* species are important litter health indicators because they competitively exclude litter pathogens and aid in NH_3 reduction (Liu et al., 2023; Naseem & King, 2018).

The increased relative abundance of these bacteria in later flocks indicates a successional replacement of the initial gut flora with a “litter-adapted” microbiota. This successional trend aligns with reports by Kubasová et al. (2022), who found that by 3–4 months into litter reuse,

genera like *Brevibacterium*, *Brachybacterium*, *Dietzia*, *Salinicoccus*, and *Yaniella* became dominant, whereas enteric taxa were largely reduced or absent. Our findings reflect that timeline, Flock 3 (approximately 4–5 months of litter age in our study) showed a community heavily composed of Gram-positive Actinobacteria and Firmicutes, analogous to what others have observed in mature litter communities. The rise of *Corynebacterium*, a genus containing known uric acid hydrolyzers such as *C. ammoniagenes* and *C. urealyticum,* likely contributed to the higher NH_3 emissions observed in later flock and windrow phases. (Lu et al., 2003). *Brachybacterium*, was enriched in later flocks and plays a role in decomposing poultry waste products such as uric acid, containing members such as *B. faecium* (Lapidus et al., 2009). While *Bacillus* was typically less than 0.1% relative abundance during our study, early differentially abundant enrichment in control litter compared to OA treatments suggests a potential treatment effect. *Bacillus pasteurii*, now often referred to as *Sporosarcina pasteurii* is a key urea hydrolyzer (Rashid et. al., 2017; Carter et al., 2023). The strong ureolytic potential of *B. pasteurii* could have contributed to increasing NH_3 levels in the control litter chambers even as a less prominent constituent.

As the litter microbial community evolved, so did its functional outputs. Early in the study, the litter pH was around 6, a range more conducive to fermentation processes dominated by lactic acid bacteria such as *Enterococcus*, leading to lower NH_3 production and a predominance of lactic fermentation. By week 3, the litter environment became more alkaline, favoring proteolysis, urease activity, and ammonification. This pH shift coincided with a microbial community transition toward taxa associated with protein degradation and NH_3 production, consistent with the elevated NH_3 levels and odor observed in older litter. From a management perspective, this succession may also correlate with improved litter inoculation and competitive exclusion in later flocks. By Flock 3 the litter microbiome was more complex and possibly more effective at suppressing enteric

pathogens, which is a known benefit of reused litter identified in previous studies (Wang et al., 2016). Notably, the transition toward uricase-associated genera such as *Corynebacterium* and *Brachybacterium* across all treatments during these later timepoints suggests that both OA treatments were ineffective at regulating their ideal growth conditions.

Impacts of Windrow Composting on Microbial Communities

In between the flock grow-out periods, the process of windrow composting markedly altered the microbial community composition, speeding up succession toward an even more specialized microbiota. Windrowing created a high-temperature, high- NH_3 environment that only certain microbes can withstand. After each windrow event, we observed enrichment of thermophilic and halotolerant taxa that were less prominent during the flock periods. Genera like *Brevibacterium*, *Jeotgalicoccus*, and *Yaniella* became much more abundant following windrowing. These three genera are all Gram-positive and are well known to inhabit protein-rich, saline environments. *Brevibacterium* and *Brachybacterium*, for instance, are famously associated with the curing of cheeses and manure where they produce NH_3 and volatile compounds, and *Jeotgalicoccus* and *Yaniella* are halotolerant bacteria often isolated from salted fish or meat fermentations (Asai et al., 2019; Park et al., 2011; Yoon et al., 2003; Li et al., 2004). The conditions during windrow composting likely select for exactly such organisms (Liang et al., 2014; Turan, 2008). Our findings agree with prior literature that noted *Brevibacterium*, *Yaniella*, *Staphylococcus*, and *Salinicoccus* as signature genera of reused or composted litter, in contrast to fresh litter communities (Wang et al., 2016) Notably, OA treatments did not induce significant changes in the abundance of these

major genera after windrowing. These windrow-associated genera were present in all treatment groups, reinforcing that time and the conditions of the composting were the primary drivers.

Treatment-Based Differential Abundance Testing

Despite the clear successional trends described above, relatively few statistically significant differences in specific taxa could be attributed to the OA amendment when examined at each timepoint. Differential abundance testing identified some genera that were enriched or depleted in OA-amended litter at specific points, but most differences were modest and inconsistent over time. For example, at the end of Flock 1, the Low and High OA treatments showed higher abundance of *Bacteroides* (a genus of Gram-negative gut bacteria) and *Weissella* (a lactic acid bacterium) relative to the Control, whereas the Control litter had higher levels of *Romboutsia* and *Bacillus* (both Firmicutes originating from the chicken gut or environment). These early differences might indicate that OA-amended litter slightly favored certain fermentative or gut-associated taxa initially. However, by the end of Flock 2 and Flock 3, those particular differences had disappeared or even reversed. After the first windrow, *Acinetobacter* was more abundant in control litter, while *Lysobacter* was enriched in OA-amended litter. *Acinetobacter* are common soil and environment bacteria that perhaps persisted more in the Control condition, but the fact that they were suppressed in OA treatments suggests a possible effect of the amendment on nitrogen cycling since some *Acinetobacter* can be involved in nitrification or denitrification, but this remains speculative (Zhan et al., 2022; Kim et al., 1997). By the end of Flock 3, OA treatments showed shifts in several genera, including reduced *Pediococcus/Weissella* both lactic acid bacterial groups and increased *Gracilibacillus/Luteimonas*. *Gracilibacillus* is a halophilic, sporulating bacterium (Carrasco et al.,

2006) and *Luteimonas* is a Gammaproteobacterium often found in compost (Young et al., 2007), their enrichment in High OA suggests that higher mineral content or pH might benefit certain species adapted to such conditions by the end of the trial. On the other hand, the lower levels of *Pediococcus/Weissella* in OA treatments by Flock 3 could indicate that OA slowed the proliferation of some fermentative bacteria, possibly due to pH buffering effects of $CaCO_3$ making the litter less favorable for acid-producing microbes (Deutch, 2024; Seleshe & Kang, 2021; Fusco et al., 2015). It's important to note that several of the genera flagged as "significant" in the differential analysis had quite low base mean abundances or high variability (standard errors), which makes their biological significance uncertain. For example, some taxa like *Halomonas* showed large fold-changes despite low abundance. Some genera exhibited contradictory changes at different times. *Weissella* being relatively enriched in OA treated litter early on but depleted in OA treated litter later. These changes underscore that differences might not result directly from the treatment, but rather from indirect or transient effects. Notably, nitrifying groups such as *Nitrosomonas* and *Nitrobacter* were not found to be significantly enriched in OA-treated litter, suggesting that the amendment did not strongly promote known nitrification pathways (Salim et al., 2014; Nodar et al., 1990). In aggregate, the DESeq2 results suggest that while a few bacterial taxa responded to the OA amendment at certain points, there was no single taxonomic group (other than *Lysobacter*) that was persistently shifted by the treatment across all stages. The lack of strong, consistent differences in specific microbes between Control and OA groups is in line with the alpha and beta diversity findings, reinforcing that OA had a subtle impact on the community composition.

Lysobacter Enrichment in OA-Amended Litter

One genus, *Lysobacter*, did stand out as consistently enriched in OA-amended litter. This pattern was observed across multiple flock and windrow phases, with Lysobacter showing significantly higher abundance in OA treatments compared to controls. In other words, while most genera fluctuated without a clear treatment association, *Lysobacter* maintained a treatment-linked difference. The genus *Lysobacter* comprises Gram-negative bacteria in the family Xanthomonadaceae that are widely distributed in soil, water, and other environments. *Lysobacter* spp. are notable for their production of extracellular enzymes and antibiotic compounds. *Lysobacter* spp. have a "predatory" lifestyle, secreting chitinases, proteases, lipases, and antimicrobial metabolites that can lyse other microorganisms. This genus has gained attention in agricultural microbiology as a potential biocontrol agent against plant pathogens, as certain species can suppress fungal diseases by producing antifungal antibiotics. Their ability to thrive in competitive, nutrient-rich niches including some extreme environments is also well documented (Ahmed et al., 2003; Allpress et al., 2002; Au et al., 1991; Chohnan et al., 2002; Weon et al., 2006; Sullivan et al., 2003). The consistent enrichment of *Lysobacter* in the OA treatments suggests that something about the OA amendment or the conditions it created gave *Lysobacter* a competitive edge. Although we did not examine the functional consequences in this study, the proliferation of *Lysobacter* in treated litter could have beneficial implications. Given *Lysobacter*'s known antagonism toward other microbes, its presence might help inhibit potential pathogens in the litter even at low relative abundances. It might also affect NH_3 production dynamics. *Lysobacter* has been reported as a genus capable of nitrogen fixation and could influence nitrogen cycling under certain conditions, although its role in litter is unclear (Iwata et al., 2010). It is worth noting that

previous surveys of poultry litter microbiota have occasionally detected *Lysobacter* as a minor member of the community (Lu et al., 2003). This finding links the microbial community shift to an OA amendment effect, suggesting that even if overall community structure was unchanged, specific functional taxa like *Lysobacter* can be stimulated.

2.5 Conclusion

This study set out to test two primary hypotheses regarding the use of OA as a broiler litter amendment: (1) that OA would reduce water activity (a_w) and moisture content, thereby suppressing NH_3-producing microbial processes, and (2) that OA would enhance alternative nitrogen pathways, such as nitrification or nitrogen immobilization, ultimately improving nitrogen retention in the litter.

Based on the data collected, these hypotheses were only partially supported and the treatment effects observed were generally subtle. Regarding water activity, OA-treated litter showed no significant reductions in either moisture content or a_w compared to control, all treatments showed persistently high-moisture conditions in the litter throughout the flock cycles. This suggests that OA's physical addition did not meaningfully restrict water availability within the litter matrix, and thus did not limit microbial uric acid decomposition or NH_3 production via the expected moisture-suppression pathway.

In terms of nitrogen retention, OA-amended litter showed no consistent improvements in total Kjeldahl nitrogen, NH_4^+-N, or other measured nitrogen forms. Emissions of NH_3 followed similar trajectories across all treatments, and uric acid levels, the primary precursor to NH_3, were largely comparable between OA and control groups, apart from minor, transient differences early in

Flocks 1 and 2. The predicted enhancement of alternative nitrogen pathways, such as nitrification, was not strongly supported by the data; environmental conditions (alkaline pH, fluctuating moisture, and temperature) likely limited sustained nitrifier establishment or activity. Key nitrifying groups *Nitrosomonas* and *Nitrobacter* were not found to be significantly enriched in OA treated litter.

At the microbial community level, OA did not significantly alter overall diversity or successional dynamics, as alpha and beta diversity patterns were overwhelmingly shaped by time and litter aging rather than treatment. However, one notable and consistent treatment effect was the enrichment of *Lysobacter* in OA-amended litter. While subtle in relative abundance, this enrichment points to the possibility that OA creates microsites or chemical niches favoring specific, functionally relevant taxa, even if the broader community structure remains unchanged.

The findings indicate that under the experimental conditions tested, OA did not produce strong or consistent effects on NH_3 mitigation, nitrogen retention, or microbial succession. While certain functional shifts, such as *Lysobacter* enrichment, suggest some biological responsiveness to the amendment, these effects were modest.

From an agricultural feasibility standpoint, the limited and context-dependent benefits observed here suggest that OA, as a standalone litter amendment, may not provide sufficient return on investment for producers. This is particularly true given the added material and labor costs, coupled with no significant improvements in NH_3 control, nitrogen retention, or bird performance over baseline. Its practical value may lie more in combination with other management strategies rather than as a primary solution.

Chapter 3

3.1 Future Directions

The present study offers novel insights into using oolitic aragonite (OA) as a litter amendment for ammonia mitigation. However, further research is needed to fully understand OA's effects on litter and explore its practical application in commercial broiler production and other agricultural systems.

Functional and Species-Level Microbial Characterization

While the 16S rRNA amplicon sequencing done in this study provided a useful overview of microbial community shifts, a more comprehensive functional assessment is warranted. Metagenomic sequencing and qPCR assays of the litter and OA would enable direct characterization of functional gene abundance related to nitrogen cycling, including genes encoding uricase and urease, as well as antimicrobial genes that may influence microbial community structure and ammonia production (Rothrock et al., 2008b; Rothrock et al., 2010; Diaz et al., 2014). Enzyme activity assays for uricase and urease should be done as well to provide functional confirmation of shifts inferred from metagenomic or qPCR analyses. Understanding how these functional pathways differ between OA-amended and control treatments should offer greater insight into how OA might impact litter NH_3 emission and microbial dynamics. Additionally, deeper species-level resolution through whole-genome metagenomics or targeted

amplicon approaches could clarify the taxonomic identity of key nitrogen-transforming bacteria whose roles remain unknown at the genus level in this study.

The genus *Lysobacter* emerged as consistently enriched under OA treatments across multiple flocks and windrow phases. Further investigation into the specific *Lysobacter* species present is warranted due to its well-documented production of extracellular enzymes and antimicrobial metabolites (Xu et al., 2022; Zhang et al., 2014). Isolation and cultivation of *Lysobacter* strains from OA-treated litter would allow for functional characterization, including assessments of their enzyme activities, nitrogen cycling capabilities, and potential contribution to ammonia reduction. Whole-genome sequencing of *Lysobacter* isolates would help to reveal relevant metabolic pathways and ecological roles within the litter microbiome.

Commercial Scale Evaluation

The chamber-based experimental design used here offers important insights under controlled conditions but cannot fully capture the complexities of commercial-scale broiler production houses. Long term field-scale trials are needed to evaluate the sustainability and repeatability of OA performance across multiple production cycles under commercial conditions. These trials should assess not only NH_3 emissions but also the nutrient retention characteristics of OA-amended litter, including N speciation and fertilizer value when applied to croplands. Evaluating potential environmental trade-offs such as nitrate leaching, nitrous oxide emissions, and water quality impacts is also critical for assessing the broader agronomic and environmental viability of OA amendments.

Windrow Analysis

Windrowing represents a unique phase of litter management where ammonia volatilization intensifies due to elevated microbial activity and temperature (Liang et al., 2014). Unfortunately, the number of litter samples processed representing windrow phases was more limited compared to the flock sampling periods, and the available samples yielded lower 16S sequencing depth, further constraining downstream analysis. Expanding sample size, sequencing depth, and temporal resolution during windrowing would provide a clearer picture of microbial succession and functional dynamics under OA treatment during this critical stage.

Expanded Nitrogen Cycling Measurements

Future work should incorporate more measurements of nitrogen species within the litter system including nitrate, nitrite, and microbial N. Measuring nitrate and nitrite concentrations in the litter could help clarify whether OA amendments are influencing nitrogen transformations primarily through enhancement of nitrification or denitrification processes. Direct assessments of microbial nitrogen transformation rates could provide a more comprehensive understanding of how OA amendments affect nitrogen fluxes within the litter system.

Combined Amendment Strategies

Evaluating the performance of OA in combination with established chemical amendments such as alum or other acidifiers may reveal synergistic or additive effects on ammonia mitigation and

microbial community modulation (Moore et al., 1995; Szymula et al., 2021; de Toledo et al., 2020). However, due to the highly acidic nature of amendments like alum and PLT, co-application with OA may lead to chemical neutralization and reduced effectiveness. Staggered application by using acidifiers at flock placement and OA later in the production cycle (e.g., after 2 to 3 weeks) may offer a more strategic approach that preserves the functional properties of both amendments while enhancing overall litter management outcomes.

Experimental Design Changes

Increasing biological replication would enhance the statistical power of future experiments and allow for more robust treatment comparisons. Furthermore, evaluating the effects of repeated OA application across successive flocks may clarify whether cumulative or adaptive microbial responses occur with long-term use.

Conclusion

While the effects of OA on ammonia emissions and nitrogen retention were limited under the conditions tested, the consistent enrichment of specific microbial taxa like *Lysobacter* suggests that OA may exert selective pressures on the litter microbiome. These findings point to the potential of OA to create localized chemical or physical niches that influence microbial composition or activity, even in the absence of broader shifts in community structure or diversity.

Importantly, OA did not significantly reduce water activity or litter moisture content, indicating that its mode of action is unlikely to involve broad suppression of microbial activity via moisture regulation. This underscores the need to investigate alternative mechanisms such as chemical and biogenic interactions with OA that might explain the observed microbial responses.

Rather than serving as a standalone solution, OA may offer value as part of an integrated litter management strategy, particularly when used in combination with other amendments or timed strategically during the production cycle. Further research using functional genomics, enzyme activity assays, and commercial-scale trials will be essential to clarify the mechanisms by which OA influences microbial processes and to assess its practical utility under field conditions.

Bibliography

1. Agyarko-Mintah, E., Cowie, A., Van Zwieten, L., Singh, B. P., Smillie, R., Harden, S., & Fornasier, F. (2017). Biochar lowers ammonia emission and improves nitrogen retention in poultry litter composting. *Waste Management*, *61*, 129–137. https://doi.org/10.1016/j.wasman.2016.12.009
2. Ahmed, K., Chohnan, S., Ohashi, H., Hirata, T., Masaki, T., & Sakiyama, F. (2003). Purification, bacteriolytic activity, and specificity of β-lytic protease from Lysobacter sp. IB-9374. *Journal of Bioscience and Bioengineering, 95*(1), 27–34. https://doi.org/10.1016/S1389-1723(03)80144-5
3. Allpress, J. D., Mountain, G., & Gowland, P. C. (2002). Production, purification and characterization of an extracellular keratinase from Lysobacter NCIMB 9497. *Letters in Applied Microbiology, 34*(5), 337–342. https://doi.org/10.1046/j.1472-765x.2002.01093.x
4. Aluminum sulfate, Solid; Safety Data Sheet CAS-No.: 10043-01-3; USALCO, LLC: Baltimore, MD, August 21, 2020, https://www.cvear.com/wp-content/uploads/2016/07/USALCO-Aluminum-Sulfate-Solid-dry-alum.pdf (accessed June 2, 2025).
5. Anthonisen, A. C., Loehr, R. C., Prakasam, T. B. S., & Srinath, E. G. (1976). Inhibition of Nitrification by Ammonia and Nitrous Acid. *Journal (Water Pollution Control Federation), 48*(5), 835–852.
6. Asai, N., Suematsu, H., Yamada, A., Watanabe, H., Nishiyama, N., Sakanashi, D., … Mikamo, H. (2019). Brevibacterium paucivorans bacteremia: Case report and review of the literature. *BMC Infectious Diseases, 19*, 344. https://doi.org/10.1186/s12879-019-3962-y

7. Assumpcao, A. L. F. V., Arsi, K., Asnayanti, A., Alharbi, K. S., Do, A. D. T., Read, Q. D., … Alrubaye, A. A. K. (2024). Electron-Beam-Killed Staphylococcus Vaccine Reduced Lameness in Broiler Chickens. *Vaccines, 12*(11), 1203. https://doi.org/10.3390/vaccines12111203
8. Au, S., Roy, K. L., & Von Tigerstrom, R. G. (1991). Nucleotide sequence and characterization of the gene for secreted alkaline phosphatase from Lysobacter enzymogenes. *Journal of Bacteriology, 173*(15), 4551–4557. https://doi.org/10.1128/jb.173.15.4551-4557.1991
9. Bailey, M. A., Bourassa, D. V., Krehling, J. T., Munoz, L., Chasteen, K. S., Escobar, C., & Macklin, K. S. (2022). Effects of Common Litter Management Practices on the Prevalence of Campylobacter jejuni in Broilers. Animals : An Open Access Journal from MDPI, 12(7), 858. https://doi.org/10.3390/ani12070858
10. Baker, J., Battye, W. H., Robarge, W., Pal Arya, S., & Aneja, V. P. (2020). Modeling and measurements of ammonia from poultry operations: Their emissions, transport, and deposition in the Chesapeake Bay. Science of The Total Environment, 706, 135290. https://doi.org/10.1016/j.scitotenv.2019.135290
11. Barrientos, R. (2025, March 24). *Litter amendment could be compromising health, safety and productivity.* https://www.thepoultrysite.com/articles/litter-amendment-could-be-compromising-health-safety-and-productivity
12. Bist, R. B., Subedi, S., Chai, L., & Yang, X. (2023). Ammonia emissions, impacts, and mitigation strategies for poultry production: A critical review. *Journal of Environmental Management, 328*, 116919. https://doi.org/10.1016/j.jenvman.2022.116919

13. Brink, M., Janssens, G. P. J., Demeyer, P., Bağci, Ö., & Delezie, E. (2022). Reduction of dietary crude protein and feed form: Impact on broiler litter quality, ammonia concentrations, excreta composition, performance, welfare, and meat quality. *Animal Nutrition, 9*, 291–303. https://doi.org/10.1016/j.aninu.2021.12.009
14. Brink, M., Janssens, G., & Delezie, E. (2022). How do moisture content, friability, and crust development of litter influence ammonia concentrations in broiler production? *Livestock Science*. https://doi.org/10.1016/j.livsci.2022.105109
15. Bolan, N. S., Szogi ,A.A., Chuasavathi ,T., Seshadri ,B., Rothrock Jr.,M.J., & and Panneerselvam, P. (2010). Uses and management of poultry litter. World's Poultry Science Journal, 66(4), 673–698. https://doi.org/10.1017/S0043933910000656
16. Burgess, R. P., Carey, J. B., & Shafer, D. J. (1998). The impact of pH on nitrogen retention in laboratory analysis of broiler litter. Poultry Science, 77(11), 1620–1622. https://doi.org/10.1093/ps/77.11.1620
17. Burt, C. D., Cabrera, M. L., Rothrock, M. J., & Kissel, D. E. (2017). Flue-gas desulfurization gypsum effects on urea-degrading bacteria and ammonia volatilization from broiler litter. Poultry Science,96(8), 2676–2683. https://doi.org/10.3382/ps/pex044
18. Callahan, B. J., Sankaran, K., Fukuyama, J. A., McMurdie, P. J., & Holmes, S. P. (2016). Bioconductor workflow for microbiome data analysis: From raw reads to community analyses. *F1000Research, 5*, 1492. https://doi.org/10.12688/f1000research.8986.2
19. Carter, M. S., Tuttle, M. J., Mancini, J. A., Martineau, R., Hung, C.-S., & Gupta, M. K. (2023). Microbially Induced Calcium Carbonate Precipitation by Sporosarcina pasteurii: A Case Study in Optimizing Biological CaCO3 Precipitation. *Applied and Environmental Microbiology*, *89*(8), e01794-22. https://doi.org/10.1128/aem.01794-22

20. Carrasco, I. J., Márquez, M. C., Yanfen, X., Ma, Y., Cowan, D. A., Jones, B. E., … Ventosa, A. (2006). Gracilibacillus orientalis sp. Nov., a novel moderately halophilic bacterium isolated from a salt lake in Inner Mongolia, China. *International Journal of Systematic and Evolutionary Microbiology, 56*(3), 599–604. https://doi.org/10.1099/ijs.0.63971-0

21. Castro, B., & Torrent, J. (1998). Phosphate sorption by calcareous Vertisols and Inceptisols as evaluated from extended P-sorption curves. *European Journal of Soil Science, 49*(4), 661–667. https://doi.org/10.1046/j.1365-2389.1998.4940661.x

22. Chohnan, S., Nonaka, J., Teramoto, K., Taniguchi, K., Kameda, Y., Tamura, H., … Sakiyama, F. (2002). Lysobacter strain with high lysyl endopeptidase production. *FEMS Microbiology Letters, 213*(1), 13–20. https://doi.org/10.1111/j.1574-6968.2002.tb11279.x

23. Choi, I. H., & Moore, P. A. (2008). Effect of Various Litter Amendments on Ammonia Volatilization and Nitrogen Content of Poultry Litter1. Journal of Applied Poultry Research, 17(4), 454–462. https://doi.org/10.3382/japr.2008-00012

24. Cole, C. V., Olsen, S. R., & Scott, C. O. (1953). The Nature of Phosphate Sorption by Calcium Carbonate. *Soil Science Society of America Journal, 17*(4), 352–356. https://doi.org/10.2136/sssaj1953.03615995001700040013x

25. Cook, K. L., Rothrock Jr., M. J., Warren, J. G., Sistani, K. R., & Moore Jr., P. A. (2008). Effect of Alum Treatment on the Concentration of Total and Ureolytic Microorganisms in Poultry Litter. *Journal of Environmental Quality, 37*(6), 2360–2367. https://doi.org/10.2134/jeq2008.0024

26. Cook, K. L., Rothrock, M. J., Eiteman, M. A., Lovanh, N., & Sistani, K. (2011). Evaluation of nitrogen retention and microbial populations in poultry litter treated with chemical,

biological or adsorbent amendments. *Journal of Environmental Management, 92*(7), 1760–1766. https://doi.org/10.1016/j.jenvman.2011.02.005

27. de Toledo, T. dos S., Roll, A. A. P., Rutz, F., Dallmann, H. M., Dai Prá, M. A., Leite, F. P. L., & Roll, V. F. B. (2020). An assessment of the impacts of litter treatments on the litter quality and broiler performance: A systematic review and meta-analysis. *PLoS ONE, 15*(5), e0232853. https://doi.org/10.1371/journal.pone.0232853
28. De Choudens-Sánchez, V., & González, L. A. (2009). Calcite and Aragonite Precipitation Under Controlled Instantaneous Supersaturation: Elucidating the Role of CaCO3 Saturation State and Mg/Ca Ratio on Calcium Carbonate Polymorphism. Journal of Sedimentary Research, 79(6), 363–376. https://doi.org/10.2110/jsr.2009.043
29. Deutch, D. C. E. (2024). Salt-tolerant extracellular enzymes from the genus Gracilibacillus: A review. *Journal of Advances in Microbiology Research, 5*(2), 25–36. https://doi.org/10.22271/micro.2024.v5.i2a.159
30. Diaz, M., Piggot, A., Eberli, G., & Klaus, J. (2013). Bacterial community of oolitic carbonate sediments of the Bahamas Archipelago. Marine Ecology Progress Series, 485, 9–24. https://doi.org/10.3354/meps10359
31. Diaz, M. R., Van Norstrand, J. D., Eberli, G. P., Piggot, A. M., Zhou, J., & Klaus, J. S. (2014). Functional gene diversity of oolitic sands from Great Bahama Bank. Geobiology, 12(3), 231–249. https://doi.org/10.1111/gbi.12079
32. Diaz, M. R., Eberli, G. P., Blackwelder, P., Phillips, B., & Swart, P. K. (2017). Microbially mediated organomineralization in the formation of ooids. Geology, 45(9), 771–774. https://doi.org/10.1130/G39159.1

33. Dittoe, D. K., McDaniel, C. D., Tabler, T., & Kiess, A. S. (2018). Windrowing poultry litter after a broiler house has been sprinkled with water. *Journal of Applied Poultry Research, 27*(1), 1–15. https://doi.org/10.3382/japr/pfx034

34. Dunlop, M. W., McAuley, J., Blackall, P. J., & Stuetz, R. M. (2016). Water activity of poultry litter: Relationship to moisture content during a grow-out. *Journal of Environmental Management, 172*, 201–206. https://doi.org/10.1016/j.jenvman.2016.02.036

35. Dürre, P., & Andreesen, J. R. (1983). Purine and glycine metabolism by purinolytic clostridia. *Journal of Bacteriology, 154*(1), 192–199. https://doi.org/10.1128/jb.154.1.192-199.1983

36. E, W.-P., Dp, F., & O, H. (1986). Identification of ferrous sulfate toxicity in a commercial broiler flock. Avian Diseases, 30(2). https://pubmed.ncbi.nlm.nih.gov/3729890/

37. Ellersdorfer, M., Pesendorfer, S., & Stocker, K. (2020). Nitrogen Recovery from Swine Manure Using a Zeolite-Based Process. Processes, 8(11), Article 11. https://doi.org/10.3390/pr8111515

38. Eugene, B., Moore, P. A., Li, H., Miles, D., Trabue, S., Burns, R., & Buser, M. (2015). Effect of Alum Additions to Poultry Litter on In-House Ammonia and Greenhouse Gas Concentrations and Emissions. Journal of Environmental Quality, 44(5), 1530–1540. https://doi.org/10.2134/jeq2014.09.0404

39. Folk, R., & Lynch, L. (n.d.). Organic matter, putative nannobacteria and the formation of ooids and hardgrounds—Folk—2001—Sedimentology—Wiley Online Library. Retrieved June 6, 2025, from https://onlinelibrary.wiley.com/doi/10.1046/j.1365-3091.2001.00354.x

40. Funke, G., Von Graevenitz, A., Clarridge, J. E., & Bernard, K. A. (1997). Clinical microbiology of coryneform bacteria. *Clinical Microbiology Reviews, 10*(1), 125–159. https://doi.org/10.1128/cmr.10.1.125

41. Fusco, V., Quero, G. M., Cho, G.-S., Kabisch, J., Meske, D., Neve, H., … Franz, C. M. A. P. (2015). The genus Weissella: Taxonomy, ecology and biotechnological potential. *Frontiers in Microbiology, 6*, 155. https://doi.org/10.3389/fmicb.2015.00155

42. Gençoğlan, S., & Gençoğlan, C. (2017). The effect of the litter materials on broiler chickens welfare and performance. *Turkish Journal of Agriculture: Food Science and Technology, 5*(12), 1660–1667. https://doi.org/10.24925/TURJAF.V5I12.1660-1667.1736

43. Hartwich, K., Poehlein, A., & Daniel, R. (2012). The Purine-Utilizing Bacterium Clostridium acidurici 9a: A Genome-Guided Metabolic Reconsideration. *PLoS ONE, 7*(12), e51662. https://doi.org/10.1371/journal.pone.0051662

44. Hesselgrave, B. (2023, March 3). Research aims to bring sustainable green solutions to Chesapeake Bay. *Stormwater*. https://www.stormwater.com/green-infrastructure/article/53026909/research-aims-to-bring-sustainable-green-solutions-to-chesapeake-bay

45. Ismael, E., & Ismail, E. M. (2021). Effectiveness of Sodium bisulfate and Calcium carbonate litter amendments on the Microbial load of Broiler Built-up Litter. *SVU-International Journal of Veterinary Sciences, 4*(2), 1–10. https://doi.org/10.21608/svu.2021.56458.1095

46. Iwata, K., Azlan, A., Yamakawa, H., & Omori, T. (2010). Ammonia accumulation in culture broth by the novel nitrogen-fixing bacterium, Lysobacter sp. E4. *Journal of*

Bioscience and Bioengineering, 110(4), 415–418. https://doi.org/10.1016/j.jbiosc.2010.05.006

47. Jiao, Y., Su, T., Chen, Y., Long, M., Luo, X., Xie, X., & Qin, Z. (2023). Enhanced Water Absorbency and Water Retention Rate for Superabsorbent Polymer via Porous Calcium Carbonate Crosslinking. *Nanomaterials*, *13*(18), 2575. https://doi.org/10.3390/nano13182575
48. Joerger, R. D., Ganguly, A., de Los Santos, M., & Li, H. (2020). Effect of sodium bisulfate amendments on bacterial populations in broiler litter. *Poultry Science, 99*(11), 5560–5571. https://doi.org/10.1016/j.psj.2020.08.013
49. Jong, I., Gunnink, H., & Harn, J. (2014). Wet litter not only induces footpad dermatitis but also reduces overall welfare, technical performance, and carcass yield in broiler chickens. *The Journal of Applied Poultry Research, 23*, 51–58. https://doi.org/10.3382/JAPR.2013-00803
50. Keener, H., Wicks, M., Michel, F., & Ekinci, K. (2014). Composting broiler litter. *World's Poultry Science Journal, 70*(4), 709–720. https://doi.org/10.1017/S0043933914000798
51. Kim, M. H., Hao, O. J., & Wang, N. S. (1997). Acinetobacter isolates from different activated sludge processes: Characteristics and neural network identification. Retrieved May 23, 2025, from https://dx.doi.org/10.1111/j.1574-6941.1997.tb00404.x
52. Kim, W. K., & Patterson, P. H. (2003). Effect of minerals on activity of microbial uricase to reduce ammonia volatilization in poultry manure. *Poultry Science, 82*(2), 223–231. https://doi.org/10.1093/ps/82.2.223
53. Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and

next-generation sequencing-based diversity studies. *Nucleic Acids Research, 41*(1), e1. https://doi.org/10.1093/nar/gks808

54. Kristensen, H. H., & Wathes, C. M. (2000). Ammonia and poultry welfare: a review. *World's Poultry Science Journal, 56*(03), 235–245. doi:10.1079/wps20000018
55. Kubasova, T., Faldynova, M., Crhanova, M., Karasova, D., Zeman, M., Babak, V., & Rychlik, I. (n.d.). Succession, Replacement, and Modification of Chicken Litter Microbiota. *Applied and Environmental Microbiology, 88*(24), e01809-22. https://doi.org/10.1128/aem.01809-22
56. Lapidus, A., Pukall, R., LaButtii, K., Copeland, A., Del Rio, T. G., Nolan, M., … Klenk, H.-P. (2009). Complete genome sequence of Brachybacterium faecium type strain (Schefferle 6-10T). *Standards in Genomic Sciences, 1*(1), 3–11. https://doi.org/10.4056/sigs.492
57. Larsen, R. K., Steinbacher, J. C., & Baker, J. E. (2001). Ammonia exchange between the atmosphere and the surface waters at two locations in the Chesapeake Bay. Environmental Science & Technology, 35(24), 4731–4738. https://doi.org/10.1021/es0107551
58. Le, de-B., & Y, B. (2004). Recent advances in removing phosphorus from wastewater and its future use as fertilizer (1997-2003). Water Research, 38(19). https://doi.org/10.1016/j.watres.2004.07.014
59. Li, H., Lin ,Chongyang, Collier ,Stephen, Brown ,William, & and White-Hansen, S. (2013). Assessment of frequent litter amendment application on ammonia emission from broilers operations. Journal of the Air & Waste Management Association, 63(4), 442–452. https://doi.org/10.1080/10962247.2012.762814

60. Li, W.-J., Chen, H.-H., Xu, P., Zhang, Y.-Q., Schumann, P., Tang, S.-K., … Jiang, C.-L. (2004). Yania halotolerans gen. Nov., sp. Nov., a novel member of the suborder Micrococcineae from saline soil in China. *International Journal of Systematic and Evolutionary Microbiology, 54*(2), 525–531. https://doi.org/10.1099/ijs.0.02875-0

61. Liang, Y., Payne, J. B., Penn, C., Tabler, G. T., Watkins, S. E., VanDevender, K. W., & Purswell, J. L. (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. https://doi.org/10.3382/japr.2014-00960

62. Lincoln, T. A., Webb, S. M., Present, T. M., Magyar, J. S., & Trower, E. J. (2022). Microbial Activity and Neomorphism Influence the Composition and Microfabric of Ooids From Great Salt Lake, UT. *The Sedimentary Record*, *20*(1). https://doi.org/10.2110/001c.56183

63. Lion, M., Skoczylas, F., & Ledésert, B. (2004). Determination of the main hydraulic and poro-elastic properties of a limestone from Bourgogne, France. International Journal of Rock Mechanics and Mining Sciences, 41(6), 915–925. https://doi.org/10.1016/j.ijrmms.2004.02.005

64. Liu, X., Cao, G., Qiu, K., Dong, Y., & Hu, C. (2023). Lactobacillus plantarum Decreased Ammonia Emissions through Modulating Cecal Microbiotain Broilers Challenged with Ammonia. *Animals: An Open Access Journal from MDPI, 13*(17), 2739. https://doi.org/10.3390/ani13172739

65. Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology, 15*, 550. https://doi.org/10.1186/s13059-014-0550-8

66. Lu, J., Sanchez, S., Hofacre, C., Maurer, J. J., Harmon, B. G., & Lee, M. D. (2003). Evaluation of Broiler Litter with Reference to the Microbial Composition as Assessed by Using 16S rRNA and Functional Gene Markers. *Applied and Environmental Microbiology, 69*(2), 901–908. https://doi.org/10.1128/AEM.69.2.901-908.2003

67. Mahimairaja, S., Bolan, N. S., Hedley, M. J., & Macgregor, A. N. (1994). Losses and transformation of nitrogen during composting of poultry manure with different amendments: An incubation experiment. *Bioresource Technology, 47*(3), 265–273. https://doi.org/10.1016/0960-8524(94)90190-2

68. Malone, G. W. (1992). Nutrient Enrichment in integrated Broiler Production Systems1. Poultry Science, 71(7), 1117–1122. https://doi.org/10.3382/ps.0711117

69. Mayne, R. K., Else, R. W., & Hocking, P. M. (2007). High litter moisture alone is sufficient to cause footpad dermatitis in growing turkeys. *British Poultry Science, 48*(5), 538–545. https://doi.org/10.1080/00071660701573045

70. Miles, D. M., Rowe, D. E., & Cathcart, T. C. (2011a). High litter moisture content suppresses litter ammonia volatilization. Poultry Science, 90(7), 1397–1405. https://doi.org/10.3382/ps.2010-01114

71. Miles, D. M., Rowe, D. E., & Cathcart, T. C. (2011b). Litter ammonia generation: Moisture content and organic versus inorganic bedding materials1. Poultry Science, 90(6), 1162–1169. https://doi.org/10.3382/ps.2010-01113

72. Mohammadi-Aragh, M. K., Norris, K. L., Chesser, G. D., Lowe, J. W., Evans, J. D., Purswell, J. L., & Linhoss, J. E. (2025). Comparison of biochar and Poultry Litter Treatment (PLT) amendments on broiler litter quality and bird performance. Journal of Applied Poultry Research, 34(1), 100499. https://doi.org/10.1016/j.japr.2024.100499

73. Moore, P. A., Daniel, T. C., Edwards, D. R., & Miller, D. M. (1996). Evaluation of Chemical Amendments to Reduce Ammonia Volatilization from Poultry Litter1. Poultry Science, 75(3), 315–320. https://doi.org/10.3382/ps.0750315

74. Moore, P. A., Daniel, T. C., Edwards, D. R., & Miller, D. M. (1995). Effect of Chemical Amendments on Ammonia Volatilization from Poultry Litter. *Journal of Environmental Quality, 24*(2), 293–300. https://doi.org/10.2134/jeq1995.00472425002400020012x

75. Moore, P. A., & Miller, D. M. (1994). Decreasing Phosphorus Solubility in Poultry Litter with Aluminum, Calcium, and Iron Amendments. *Journal of Environmental Quality, 23*(2), 325–330. https://doi.org/10.2134/jeq1994.00472425002300020016x

76. Morishita, T. (2023). Streptococcosis in Poultry. *Merck Veterinary Manual*. Retrieved May 26, 2025, from https://www.merckvetmanual.com/poultry/streptococcosis/streptococcosis-in-poultry

77. Nahm, K. H. (2005). Factors influencing nitrogen mineralization during poultry litter composting and calculations for available nitrogen. World's Poultry Science Journal, 61(2), 238–255. https://doi.org/10.1079/wps200455

78. Naseem, S., & King, A. J. (2018). Ammonia production in poultry houses can affect health of humans, birds, and the environment—techniques for its reduction during poultry production. *Environmental Science and Pollution Research International, 25*(16), 15269–15293. https://doi.org/10.1007/s11356-018-2018-y

79. Newell, N. D., Purdy, E. G., & Imbrie, J. (1960). Bahamian Oölitic Sand. The Journal of Geology, 68(5), 481–497. https://doi.org/10.1086/626683

80. Nodar, R., Acea, M. J., & Carballas, T. (1990). Microbial populations of poultry pine-sawdust litter. *Biological Wastes*, *33*(4), 295–306. https://doi.org/10.1016/0269-7483(90)90133-D

81. Oğuz, E. O., Enli, Y., Şahin, B., Gönen, C., & Turgut, G. (2012). Aluminium sulphate exposure increases oxidative stress and suppresses brain development in Ross broiler chicks. *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research*, *18*(3), BR103–BR108. https://doi.org/10.12659/MSM.882515

82. Oladeinde, A., Chung, T., Mou, C., Rothrock, M. J., Li, G., Adeli, A., Looft, T., Woyda, R., Abdo, Z., Lawrence, J. P., Cudnik, D., Zock, G., Teran, J., & Li, X. (2025). Broiler litter moisture and trace metals contribute to the persistence of Salmonella strains that harbor large plasmids carrying siderophores. Applied and Environmental Microbiology, 91(4), e01388-24. https://doi.org/10.1128/aem.01388-24

83. Omeira, N., Barbour, E. K., Nehme, P. A., Hamadeh, S. K., Zurayk, R., & Bashour, I. (2006). Microbiological and chemical properties of litter from different chicken types and production systems. Science of The Total Environment, 367(1), 156–162. https://doi.org/10.1016/j.scitotenv.2006.02.019

84. O'Reilly, S. S., Mariotti, G., Winter, A. R., Newman, S. A., Matys, E. D., McDermott, F., Pruss, S. B., Bosak, T., Summons, R. E., & Klepac-Ceraj, V. (2017). Molecular biosignatures reveal common benthic microbial sources of organic matter in ooids and grapestones from Pigeon Cay, The Bahamas. Geobiology, 15(1), 112–130. https://doi.org/10.1111/gbi.12196

85. Padappayil, R. P., & Borger, J. (2025). Ammonia Toxicity. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK546677/

86. Park, S.-K., Roh, S. W., Whon, T. W., & Bae, J.-W. (2011). Genome Sequence of Brachybacterium squillarum M-6-3T, Isolated from Salt-Fermented Seafood. *Journal of Bacteriology, 193*(22), 6416–6417. https://doi.org/10.1128/jb.06183-11

87. Pescatore, A. J., & Harter-dennis, J. M. (1989). Effects of Ferrous Sulfate Consumption on the Performance of Broiler Chicks. Poultry Science, 68(8), 1063–1067. https://doi.org/10.3382/ps.0681063

88. Prakasam, T. B. S., & Loehr, R. C. (1972). Microbial nitrification and denitrification in concentrated wastes. *Water Research, 6*(7), 859–869. https://doi.org/10.1016/0043-1354(72)90038-3

89. Pustake, S. O., Bhagwat, P., Pillai, S., & Dandge, P. B. (2022). Purification and characterisation of uricase from Bacillus subtilis SP6. *Process Biochemistry, 113*, 55–61. https://doi.org/10.1016/j.procbio.2021.12.010

90. Rahman, Md. M., Hassan, A., Hossain, I., Jahangir, M. M. R., Chowdhury, E. H., & Parvin, R. (2022). Current state of poultry waste management practices in Bangladesh, environmental concerns, and future recommendations. Journal of Advanced Veterinary and Animal Research, 9(3), 490–500. https://doi.org/10.5455/javar.2022.i618

91. Rashid, A., Banday, T., Hamid, S., & Khan, A. (2020). Effect of chemical treatment of litter on its quality and footpad lesions of broiler chicken. *The Journal of Poultry Sciences, 33*, 331–336.

92. Rashid, A., Banday, T., Hamid, S., Khan, A., Saim, M., Madeeha, A., & Pal, M. (2017). Effect of Chemically Treated Litter on Ammonia Emission, Performance and Carcass Characteristics of Broiler Chicken. *Journal*, 7, 88–93.

93. Redding, M. R. (2013). Bentonite can decrease ammonia volatilisation losses from poultry litter: Laboratory studies. Animal Production Science, 53(10), 1115–1118. https://doi.org/10.1071/AN12367

94. Ritz, C. W., Fairchild, B. D., & Lacy, M. P. (2004). Implications of Ammonia Production and Emissions from Commercial Poultry Facilities: A Review. Journal of Applied Poultry Research, 13(4), 684–692. https://doi.org/10.1093/japr/13.4.684

95. Ritz, C. W., Tasistro, A. S., Kissel, D. E., & Fairchild, B. D. (2011). Evaluation of surface-applied char on the reduction of ammonia volatilization from broiler litter. Journal of Applied Poultry Research, 20(2), 240–245. https://doi.org/10.3382/japr.2010-00327

96. Roswall, T., Haggard, B. E., & Toor, G. S. (2022). Fate and transformations of dissolved phosphorus forms in runoff: Effect of poultry litter and products extracted with variable water extraction ratios. *Chemosphere, 308*, 136220. https://doi.org/10.1016/j.chemosphere.2022.136220

97. Rothrock, M. J., Cook, K. L., Lovanh, N., Warren, J. G., & Sistani, K. (2008a). Development of a Quantitative Real-Time Polymerase Chain Reaction Assay to Target a Novel Group of Ammonia-Producing Bacteria Found in Poultry Litter. *Poultry Science*, *87*(6), 1058–1067. https://doi.org/10.3382/ps.2007-00350

98. Rothrock, M. J., Cook, K. L., Warren, J. G., & Sistani, K. (2008b). The Effect of Alum Addition on Microbial Communities in Poultry Litter. Poultry Science, 87(8), 1493–1503. https://doi.org/10.3382/ps.2007-00491

99. Rothrock, M. J., Cook, K. L., Warren, J. G., Eiteman, M. A., & Sistani, K. (2010). Microbial mineralization of organic nitrogen forms in poultry litters. Journal of Environmental Quality, 39(5), 1848–1857. https://doi.org/10.2134/jeq2010.0024

100. Salim, H. M., Patterson, P. H., Ricke, S. C., & Kim, W. K. (2014). Enhancement of microbial nitrification to reduce ammonia emission from poultry manure: A review. *World's Poultry Science Journal, 70*(4), 839–856. https://doi.org/10.1017/s0043933914000890

101. Schefferle, H. E. (1965). The Decomposition of Uric Acid in Built Up Poultry Litter. *Journal of Applied Bacteriology, 28*(3), 412–420. https://doi.org/10.1111/j.1365-2672.1965.tb02171.x

102. Seleshe, S., & Kang, S. N. (2021). Effect of Different Pediococcus pentosaceus and Lactobacillus plantarum Strains on Quality Characteristics of Dry Fermented Sausage after Completion of Ripening Period. *Food Science of Animal Resources, 41*(4), 636–649. https://doi.org/10.5851/kosfa.2021.e21

103. Sheikh, I., Nissa, S., Zaffer, B., Bulbul, K., Akand, A., Ahmed, H., … Hussain, S. (2018). Ammonia production in the poultry houses and its harmful effects. https://www.semanticscholar.org/paper/Ammonia-production-in-the-poultry-houses-and-its-Sheikh-Nissa/5c1065a692cbdcd2e388bb9af1c9f6626edfb7bf

104. Sheng, J., Adeli, A., & Miles, D. M. (2015). Effects of N and P Immobilizing Agents on Ammonia Emissions and Nutrient Contents of Broiler Litter. JSM Environmental Science and Ecology, 3(2), Article 2. https://doi.org/10.47739/1017

105. Simone, L. (1980). Ooids: A review. *Earth-Science Reviews*, *16*, 319–355. https://doi.org/10.1016/0012-8252(80)90053-7

106. Sims, J. T., & Luka-McCafferty, N. J. (2002). On-farm evaluation of aluminum sulfate (alum) as a poultry litter amendment: Effects on litter properties. Journal of Environmental Quality, 31(6), 2066–2073. https://doi.org/10.2134/jeq2002.2066

107. Sipos, A. A., Domokos, G., & Jerolmack, D. J. (2018). Shape evolution of ooids: A geometric model. *Scientific Reports*, *8*(1), 1758. https://doi.org/10.1038/s41598-018-19152-0

108. Sullivan, R. F., Holtman, M. A., Zylstra, G. J., White, J. F., & Kobayashi, D. Y. (2003). Taxonomic positioning of two biological control agents for plant diseases as Lysobacter enzymogenes based on phylogenetic analysis of 16S rDNA, fatty acid composition and phenotypic characteristics. *Journal of Applied Microbiology, 94*(6), 1079–1086. https://doi.org/10.1046/j.1365-2672.2003.01932.x

109. Swelum, A. A., El-Saadony, M. T., Abd El-Hack, M. E., Abo Ghanima, M. M., Shukry, M., Alhotan, R. A., … El-Tarabily, K. A. (2021). Ammonia emissions in poultry houses and microbial nitrification as a promising reduction strategy. *Science of The Total Environment, 781*, 146978. https://doi.org/10.1016/j.scitotenv.2021.146978

110. Szafraniec, G. M., Szeleszczuk, P., & Dolka, B. (2022). Review on skeletal disorders caused by Staphylococcus spp. In poultry. *The Veterinary Quarterly, 42*(1), 21–40. https://doi.org/10.1080/01652176.2022.2033880

111. Szymula, A., Wlazło, Ł., Sasáková, N., Wnuk, W., & Nowakowicz-Dębek, B. (2021). The Use of Natural Sorbents to Reduce Ammonia Emissions from Cattle Faeces. Agronomy, 11(12), Article 12. https://doi.org/10.3390/agronomy11122543

112. Trower, E. J., Cantine, M. D., Gomes, M. L., Grotzinger, J. P., Knoll, A. H., Lamb, M. P., … Fischer, W. W. (2018). Active Ooid Growth Driven By Sediment Transport in a High-Energy Shoal, Little Ambergris Cay, Turks and Caicos Islands. *Journal of Sedimentary Research, 88*(9), 1132–1151. https://doi.org/10.2110/jsr.2018.59

113. Turan, N. G. (2008). The effects of natural zeolite on salinity level of poultry litter compost. *Bioresource Technology, 99*(7), 2097–2101. https://doi.org/10.1016/j.biortech.2007.11.061

114. University of Maryland Center for Environmental Science. (2018, September 5). Science-industry partnerships awarded for Maryland research. *Horn Point Laboratory*. https://www.umces.edu/news/science-industry-partnerships-awarded-maryland-research

115. USDA APHIS & Iowa State University. (2022, August 26). US Poultry Industry Manual – Broilers: Brooding. The Poultry Site. https://www.thepoultrysite.com/articles/fad-broilers-brooding

116. U.S. Department of Agriculture, Economic Research Service. (2014, December 16). *Livestock, Dairy, and Poultry Outlook: December 2014* (LDPM-246). https://www.ers.usda.gov/publications/pub-details?pubid=37593

117. U.S. Department of Agriculture, Economic Research Service. (2018, December 17). *Livestock, Dairy, and Poultry Outlook: December 2018* (LDP-M-294). https://www.ers.usda.gov/publications/pub-details?pubid=91042

118. U.S. Department of Agriculture, National Agricultural Statistics Service. (2024). *Broilers: production by year, U.S.* Retrieved June 21, 2025, from https://www.nass.usda.gov/Charts_and_Maps/Poultry/index.php

119. US EPA, O. (2013, March 12). Sources and Solutions: Agriculture [Overviews and Factsheets]. https://www.epa.gov/nutrientpollution/sources-and-solutions-agriculture

120. US Poultry Industry Manual - Broilers: Brooding. (n.d.). Retrieved June 5, 2025, from https://www.thepoultrysite.com/articles/fad-broilers-brooding

121. Wang, L., Lilburn, M., & Yu, Z. (2016). Intestinal Microbiota of Broiler Chickens As Affected by Litter Management Regimens. *Frontiers in Microbiology, 7*. https://doi.org/10.3389/fmicb.2016.00593

122. Wang, L., Ruiz-Agudo, E., Putnis, C. V., Menneken, M., & Putnis, A. (2012). Kinetics of Calcium Phosphate Nucleation and Growth on Calcite: Implications for Predicting the Fate of Dissolved Phosphate Species in Alkaline Soils. *Environmental Science & Technology, 46*(2), 834–842. https://doi.org/10.1021/es202924f

123. Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology, 73*(16), 5261–5267. https://doi.org/10.1128/AEM.00062-07

124. Weon, H.-Y., Kim, B.-Y., Baek, Y.-K., Yoo, S.-H., Kwon, S.-W., Stackebrandt, E., & Go, S.-J. (2006). Two novel species, Lysobacter daejeonensis sp. Nov. And Lysobacter yangpyeongensis sp. Nov., isolated from Korean greenhouse soils. *International Journal of Systematic and Evolutionary Microbiology, 56*(5), 947–951. https://doi.org/10.1099/ijs.0.64095-0

125. Xu, S., Zhang, Z., Xie, X., Shi, Y., Chai, A., Fan, T., Li, B., & Li, L. (2022). Comparative genomics provides insights into the potential biocontrol mechanism of two Lysobacter enzymogenes strains with distinct antagonistic activities. *Frontiers in Microbiology*, *13*, 966986. https://doi.org/10.3389/fmicb.2022.966986

126. Yoon, J.-H., Lee, K.-C., Weiss, N., Kang, K. H., & Park, Y.-H. (2003). Jeotgalicoccus halotolerans gen. Nov., sp. Nov. And Jeotgalicoccus psychrophilus sp. Nov., isolated from the traditional Korean fermented seafood jeotgal. *International Journal of Systematic and Evolutionary Microbiology, 53*(2), 595–602. https://doi.org/10.1099/ijs.0.02132-0

127. Young, C.-C., Kämpfer, P., Chen, W.-M., Yen, W.-S., Arun, A. B., Lai, W.-A., … Chou, J.-H. (2007). Luteimonas composti sp. Nov., a moderately thermophilic bacterium isolated from food waste. *International Journal of Systematic and Evolutionary Microbiology, 57*(4), 741–744. https://doi.org/10.1099/ijs.0.64701-0

128. Zhan, Y., Zhang, B., Xie, J., & Liu, W. (2022). Bio-enhanced contact oxidation process using a heterotrophic nitrifying-aerobic denitrifying strain. *Biotechnology & Biotechnological Equipment*. https://www.tandfonline.com/doi/abs/10.1080/13102818.2022.2101383

129. Zhang, J., Du, L., Liu, F., Xu, F., Hu, B., Venturi, V., & Qian, G. (2014). Involvement of both PKS and NRPS in antibacterial activity in Lysobacter enzymogenes OH11. *FEMS Microbiology Letters*, *355*(2), 170–176. https://doi.org/10.1111/1574-6968.12457

R Packages

1. Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods, 13*(7), 581–583. https://doi.org/10.1038/nmeth.3869
2. Lenth, R. (2020). emmeans: Estimated marginal means, aka least-squares means. R package version 1.5.3. https://CRAN.R-project.org/package=emmeans
3. McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE, 8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

4. Schliep, K. P. (2011). phangorn: Phylogenetic analysis in R. *Bioinformatics, 27*(4), 592–593. https://doi.org/10.1093/bioinformatics/btq706

5. Wright, E. S. (2016). Using DECIPHER v2.0 to analyze big biological sequence data in R. *The R Journal, 8*(1), 352–359.