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# Livestock−Crop−Mushroom (LCM) Circular System: An Eco-Friendly Approach for Enhancing Plant Performance and Mitigating Microbiological Risks

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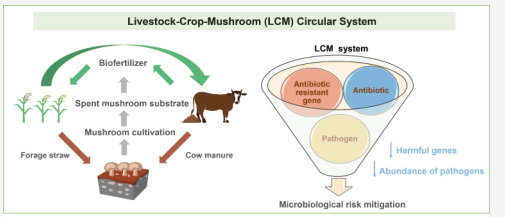
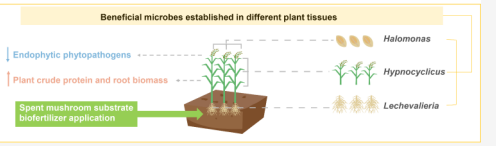
**ABSTRACT:** Mushroom production using agroforestry biowaste is a great green cycling agriculture alternative. Therefore, the current study explored the Livestock−Crop−Mushroom (LCM) circular production model, starting with co-composting of straw and cow manure as a'St' biofertilizer further used for mushroom cultivation that ultimately produced a'StM' biofertilizer. The two biofertilizers were tested for their impacts on plant growth and potential microbial risks. The results show significant growth of oats stimulated by biofertiliser use. Both'St' and'StM' increased plant biomass, while with the latter, the crude protein content (+5.1%) and root biomass were also higher. Reduced abundances of resistome genes (30%) and pathogens (25%) were observed during the oat growth. Further, metagenomics analysis also indicated a reduction in antibiotic-resistance genes by −20% in soils with oats treated by'St' and −46% in'StM' biofertilizer treatment. The'StM' had a three-fold stronger inhibitory effect on oat rhizosphere soil pathogens than'St'. Moreover, compared to'St','StM' suppressed pathogens in seeds and stems, with specific beneficial biomarker microbes in different plant parts. Overall, the antibiotic resistance gene related to oxytetracycline decreased more than three-fold in the LCM system. This study demonstrates the substantial potential and scalability of the LCM circular system within the agricultural domain.




**KEYWORDS:** *mushroom cultivation, antibiotic resistance gene, crop biowaste production, microbiological risk, circular agriculture system*

## INTRODUCTION

Nearly 140 billion tons of crop biomass waste and 125 million tons of livestock nitrogen (N) waste are intensifying global agricultural organic waste production.[1,2] Manures from piggery (27%), poultry (14%), and cow yard (40%) are the dominant fertilizers used in China, indicating intensive animal rearing.[3] However, due to the slow decomposition of recalcitrant materials in these manures, it is difficult to produce easily degradable humus using traditional composting methods.[1,2] Using co-composting techniques to transform this kind of waste is a viable approach, and it also helps in maintaining the quality of the compost.[3,4] In the context of healthy food items, there has been a significant increase in global mushroom consumption. Unfortunately, this increase in mushroom consumption has led to the generation of more than 60 million tons of spent mushroom substrates as waste.[5,6] The mushroom substrate is rich in mycelium and important nutrients,[7] lignocellulose, and diverse microorganisms.[7,8] It can be combined with co-composting byproducts to form a potential Livestock−Crop−Mushroom (LCM) integrated circular system to meet the demands of the mushroom cultivation industry.

Manure-derived biofertilizers contain numerous pathogenic microbes like bacteria, fungi, and yeast, typically originating from livestock food, feed, and solid substrates.[9] Their application on soils can stimulate the proliferation of human pathogenic bacteria, such as *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, and *Pseudomonas aeruginosa*.[9,10] Moreover, these pathogens can be further transferred from the soil to plant tissues, affecting plant growth. For instance, if certain harmful bacteria or fungi are transferred from the soil to the roots, stems, or leaves of a plant, they might interfere with the plant's normal functions, such as absorbing nutrients,

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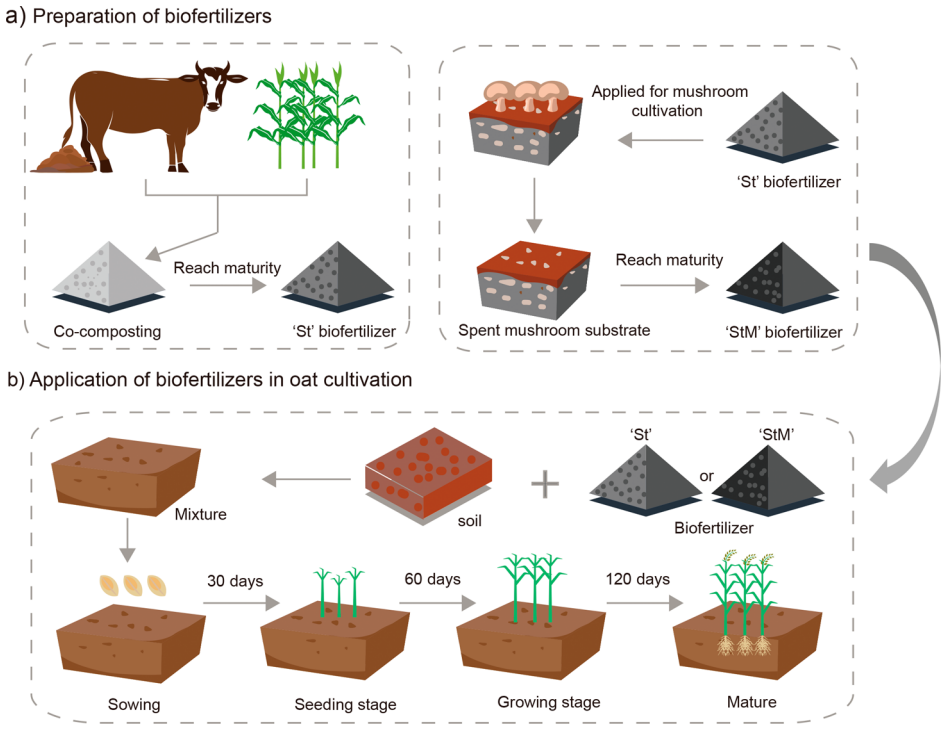


**Figure 1.** Experimental procedure from biofertilizer preparation to its subsequent application in oat cultivation.

conducting water, or carrying out photosynthesis. This interference could result in stunted growth, reduced yield, or even plant death.[11] Moreover, endophytic bacteria colonize plant tissues internally and are known to exhibit a natural variation in diversity across different plant organs. This variation is influenced by multiple factors, including plant species, developmental stage, and environmental conditions.[12,13] Understanding this natural variation is crucial as endophytes can have profound effects on plant physiological processes, such as nutrient metabolism, stress tolerance, and disease resistance. In the context of this study, where the impact of fertilizer application on plant−endophyte interactions is investigated, knowledge of the natural variation of endophytes in plant organs serves as an essential foundation.

However, limited research has been done on a holistic framework for plant−endophyte mutual actions and soil microbiological risk assessment after applying composts from agroforestry biowastes and livestock manure, especially considering the LCM system. Furthermore, antibiotics are extensively utilized in treating livestock diseases, with the global use increasing by 8% until the end of 2023.[14] Among Asian countries, India and China have some of the highest levels of antibiotic usage. Specifically, China's annual consumption of antibiotics can reach up to 8,000 tons.[15] Within China, certain antibiotics, like oxytetracycline, are commonly employed in the livestock farming industry.[15−17] Such extensive use poses risks and contributes to the emergence of antibiotic-resistant bacteria and the spread of resistance genes,[18−22] thereby affecting both animals and human health.[23,24] Oats have been selected owing to their status as a major cattle feed and the fact that their well-developed root system has the potential to interact with soil-dwelling microorganisms. Such interactions can influence the degradation of antibiotics and the prevalence of resistance genes via the'soil-plant-resistant gene' interaction mechanism, which will be comprehensively investigated in this study.

We hypothesize that (i) the spent mushroom compost acquired from the LCM system would have a suppressive effect on the endophytic pathogen due to a higher proportion of intrinsic saprotrophs and a lower pathogen-to-saprotroph ratio; (ii) it would furthermore assist in the establishment of beneficial endophytic microbes and promote plant growth; and (iii) the content of hazardous biological contaminants (virulent and resistant genes, pathogenic microorganisms, and antibiotics) would decrease after composting and its subsequent application in plant growth.

## MATERIALS AND METHODS

**Biofertilizer Preparations.** Within the LCM system, the saprotrophic edible mushroom *Stropharia rugosoannulata* was selected as a representative mushroom for the following reasons: (i) it is widely cultivated using agricultural byproducts as substrates, suggesting a potential market;[25] (ii) the genome of this mushroom highlights its ability to thrive in environments rich in organic matter and decompose complex lignocellulosic materials, indicating its potential for utilizing agroforestry wastes (i.e., crop residues and sawdust);[26] and (iii) a broad set of substrates can be used for its cultivation, as it grows in a substrate composed of agroforestry wastes.

In the current study, two types of biofertilizers were used:'St', made by co-composting straw and cow manure, and'StM', made by composting the spent mushroom substrate (SMS) (Figure 1a). In this study, straw was collected from local agricultural fields in Hongyan Village, Huize County, Yunnan, China (103°29′E; 26°41′N), with the physicochemical properties of total carbon (TC) = 467 mg/g, total nitrogen (TN) = 7.2 mg/g, C/N ratio = 65, pH = 7.5, and moisture content = 48.7%. The straw was chopped into small pieces to ensure uniform mixing with cow manure during composting.


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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

The cow manure, sourced from a nearby state-owned cattle farm, had properties of TC = 281 mg/g, TN = 17.6 mg/g, C/N ratio = 16, pH = 8.2, and moisture content = 53.4%. Through co-composting of straw and cow manure,[3] a mature biofertilizer 'St' was obtained. This biofertilizer was used for cultivating mushrooms (*Stropharia rugosoannulata*), and the spent mushroom substrate (SMS) was collected after harvesting. The SMS had TC = 286 mg/g, TN = 23.3 mg/g, C/N ratio = 12, pH = 7.2, and moisture content = 68.4%. After the biofertilizers were prepared, they were used for oat cultivation (Figure 1b).

**Soil Collection.** The top 30 cm of red soil was collected from an agricultural field (cultivated with corn (*Zea mays* L.), implementing a regime of arable land rotation and following) located near the Kunming Institute of Botany, China Academy of Sciences, Yunnan, China (25°08′56.8″N, 102°44′03.8″E). A 1000 kg portion of homogeneous and sieved (10 mm) soil was prepared for the microcosm experiment. According to the soil classification system of the Food and Agriculture Organization of the United Nations (FAO), the soil is typical of the fluvisol soil group (IUSS Working Group WRB)[27] with a soil texture of 62% sand, 22.5% silt, and 15.5% clay. The soil was typically acidic (pH = 5.2) red soil, having an organic matter content of 2%. The red soil was chosen as it is a prevalent soil type in the vicinity of the study site. The use of biofertilizers in this study aimed to explore their potential to enhance plant growth and soil quality, even in acidic soils with a low organic matter content. Detailed information on comprehensive soil macro- and micronutrients can be found in Supporting Information File 1.

**Plant Cultivation.** Oat (*Avena sativa*) is extensively cultivated in cold and cool regions and plays a significant role in livestock farming in numerous countries.[28,29] As a local cultivar, oat was selected as the plant species for the microcosm experiment since it is an important annual grass forage with good palatability and high yield. Oat seeds were obtained from Qinghai University. The cultivation experiment was conducted in permeable plastic boxes (49 cm length × 36 cm width × 17 cm height) in an open-air setup at the Kunming Institute of Botany. The temperature was maintained at 20−25 °C during the day and 15−20 °C at night. The relative humidity was kept at ∼65%. Irrigation was carried out with tap water, and the soil moisture was maintained at around 60−70% of the field capacity. Cultivating substrates comprised 15 kg of soil and 1 kg of the biofertilizer (homogenized with a miniature mixer); pure soil (set as the control) did not receive any form of fertilization other than the natural background nutrients in the soil. The quantity of seeds used for each box was 6 grams (equivalent to a seeding rate of approximately 200 seeds per square meter).[29] In total, the overall number = 3 (treatments: 2 biofertilizers + 1 pure soil) × 3 (growing stages: seeding (30 days after oat seed sowing), growing (60 days), and maturing (120 days)) × 3 replicates) = 27 soil samples.

**Soil Sampling, Plant Tissue Harvest, and Production Calculation.** In each box, we used a randomized sampling strategy to minimize soil heterogeneity: 5 randomized 0−20 cm (using a mini-type sampler with a 1 cm inner diameter) bulk soil samples were pooled as a representative sample for each replicate. Soil samples were obtained at the three growing stages. Fresh samples were sieved through <2 mm mesh to remove discernible roots and tiny stones and then sealed in sterile plastic bags and stored at −80 °C until DNA extraction. After plant maturation, the obtained total plant samples = (3 (treatments) × 3 (plant tissues) × 3 replicates) = 27. Plant tissues (root, stem, and grain) were sampled, rinsed, and processed for the total of weight for their biomass and plant microbiome analysis, according to reported methodologies.[30,31] Briefly, bulk soil was shaken off the roots, and the roots were washed three times to remove the adhering soil. Soil particles and debris of the root samples were carefully removed with a fine brush, washed with tap water, and rinsed three times with sterile water. The three equations shown below are specific to the oat production calculation:

i) The area of each permeable plastic box = 49 cm × 36 cm = 1764 $cm^2$.

ii) Total forage weight (t/ha) = [(the number of individual oat within the box × the weight of individual fresh oat (g)/box area ($cm^2$)] × 10 (conversion coefficient from g/$cm^2$ to t/ha).

iii) Total grain yield (t/ha) = total forage weight (t/ha)/10 (conversion coefficient between fresh weight and grain weight).

**Crude Protein Content in Grain.** After that, the oats were weighed, and one portion of the grain samples was sent for plant quality determination (total sugar and crude protein) in Nanjing Convinced-test Technology Co., Ltd. In short, to determine the crude protein content in plants by the Kjeldahl method with sulfuric acid-catalyst digestion, the sample is digested with sulfuric acid and a catalyst. Subsequently, the released ammonia is distilled and trapped. Titration is carried out to determine the nitrogen content, from which the crude protein is calculated.[32]

**Determination of Antibiotics.** Measuring oxytetracycline antibiotic residues involves sample preparation, extraction with a 60% acetonitrile and 40% water (v/v) organic solvent mixture, and removal of the substances by purification. The extracted sample was then analyzed by using high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS). The HPLC separates the components, while MS/MS identifies and quantifies the oxytetracycline by comparing it with external standards/calibration. External standards are prepared in a matrix similar to that of the sample. A calibration curve is generated by plotting the detector response against standard concentrations. The sample response is measured, and the concentration is determined. The analytical conditions, the limit of detection, the quantitation of oxytetracycline, and detailed experimental steps followed previously described procedures.[33,34]

**Metagenomic Sequencing and the Analysis of N-Cycling Genes, Resistance and Virulence Genes, and Potential Pathogens.** One portion of the fresh and clean samples of grain, stem, and root was subjected to surface disinfection. The disinfection process involved fully immersing the samples in ethanol for 10 seconds, followed by a 2 min immersion in bleach, and finally a 2 min immersion in 70% ethanol.[35] The samples were processed for DNA extraction using specific kits, with the extracted DNA's quality and concentration determined, DNA libraries constructed, and shotgun metagenomic sequencing performed at Personalbio Company after sample verification, all following detailed procedures (Supplementary File 1). Raw data were trimmed to remove low-quality reads using the NGS toolkit[36] to guarantee the quality of downstream analysis. All the metagenomic sequencing data were searched against the NCycDB (an integrative database specifically designed for profiling nitrogen cycling genes in metagenome sequencing data), VFDB (virulence factor database),[37] ARDB (antibiotic


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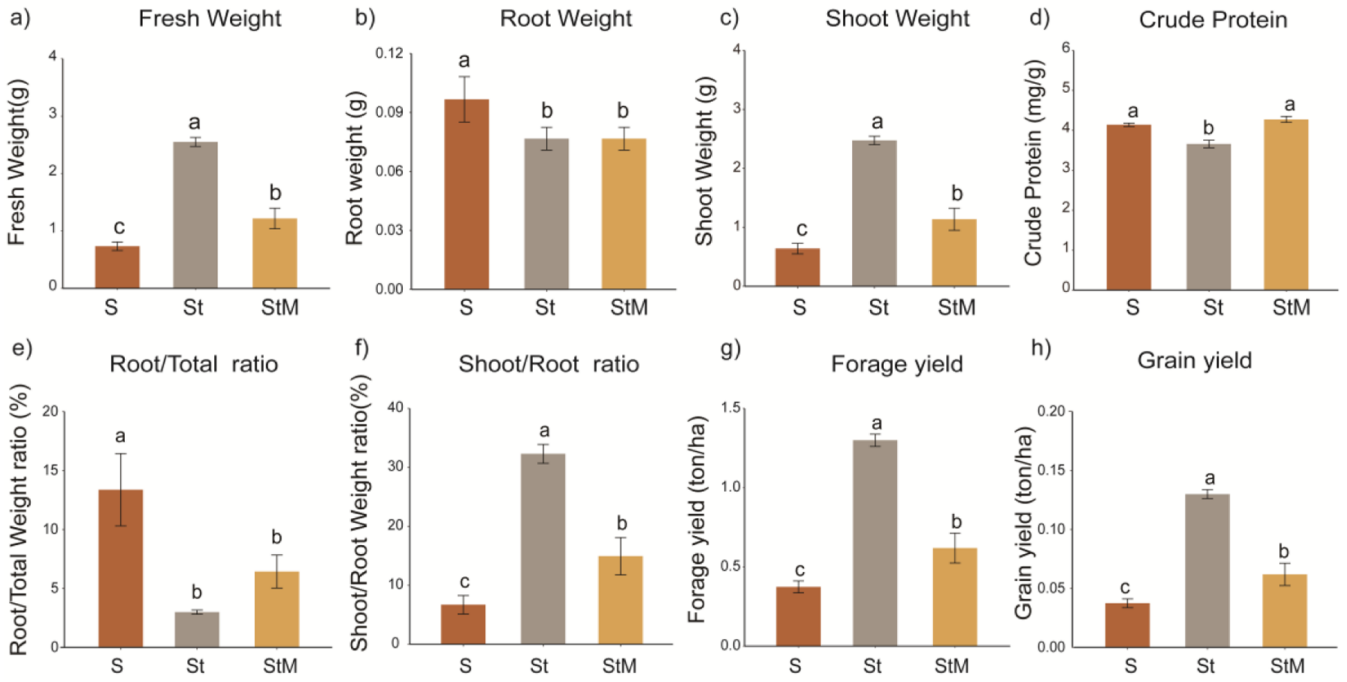


**Figure 2.** Variances in oat characteristics and yield, cultivated in biofertilizers: St, a straw and cow manure co-composted biofertilizer; StM, the'St' biofertilizer incorporated with SMS; and S, pure soil without biofertilizer inputs. For each bar chart, different letters represent significant differences between treatments (S, St, and StM) with $p < 0.05$ (Tukey's multiple comparison). Error bars indicate mean ± standard deviation ($n = 3$).

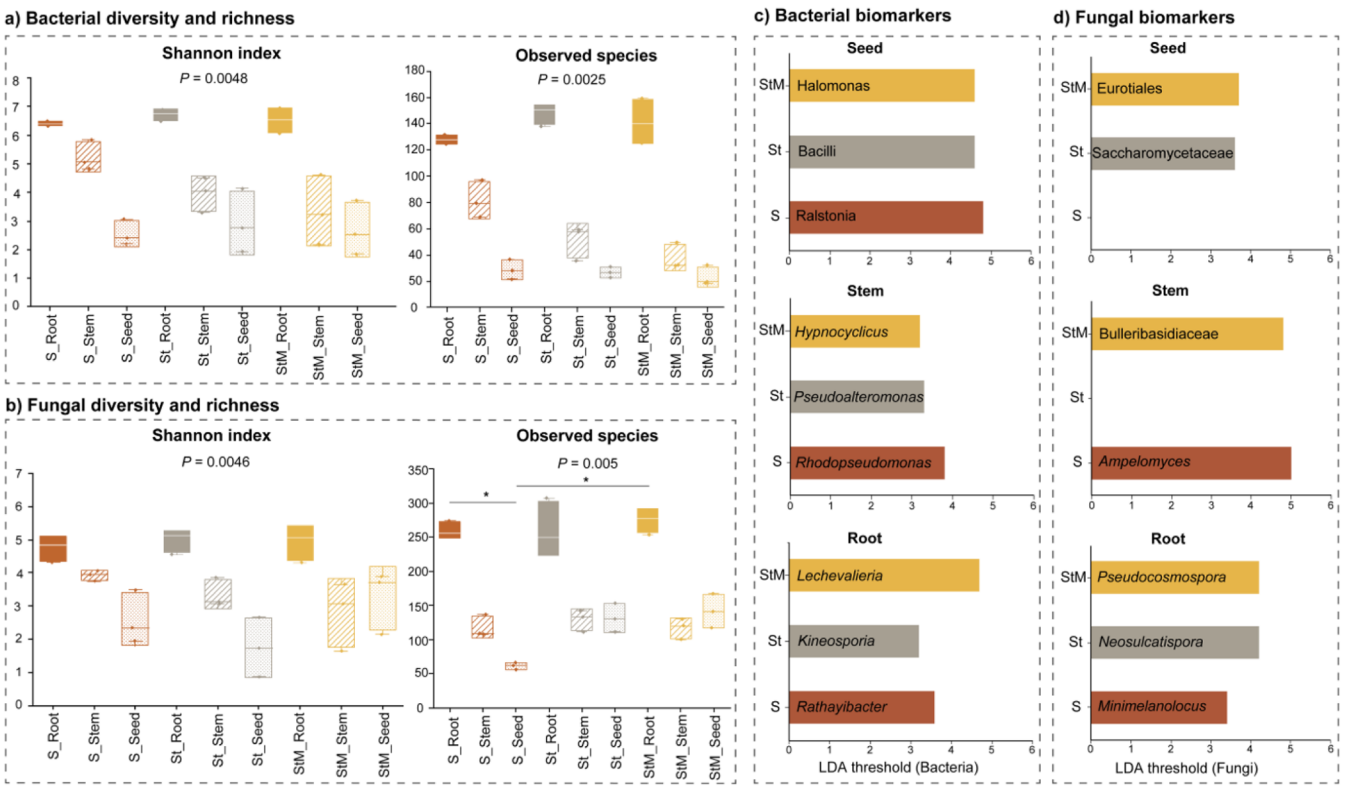


**Figure 3.** Variations in the endophytic microbial community within different tissues of oats grown in biofertilizers: St, a straw and cow manure co-composted biofertilizer; StM, the'St' biofertilizer incorporated with SMS; and S, pure soil without biofertilizer inputs. (a,b) Alpha-diversity indices based on microbial richness (observed species) and diversity (Shannon index). (c,d) Tissue-enriched bacterial taxa were investigated by linear discriminant analysis effect size at five taxonomic levels from phylum to genus.



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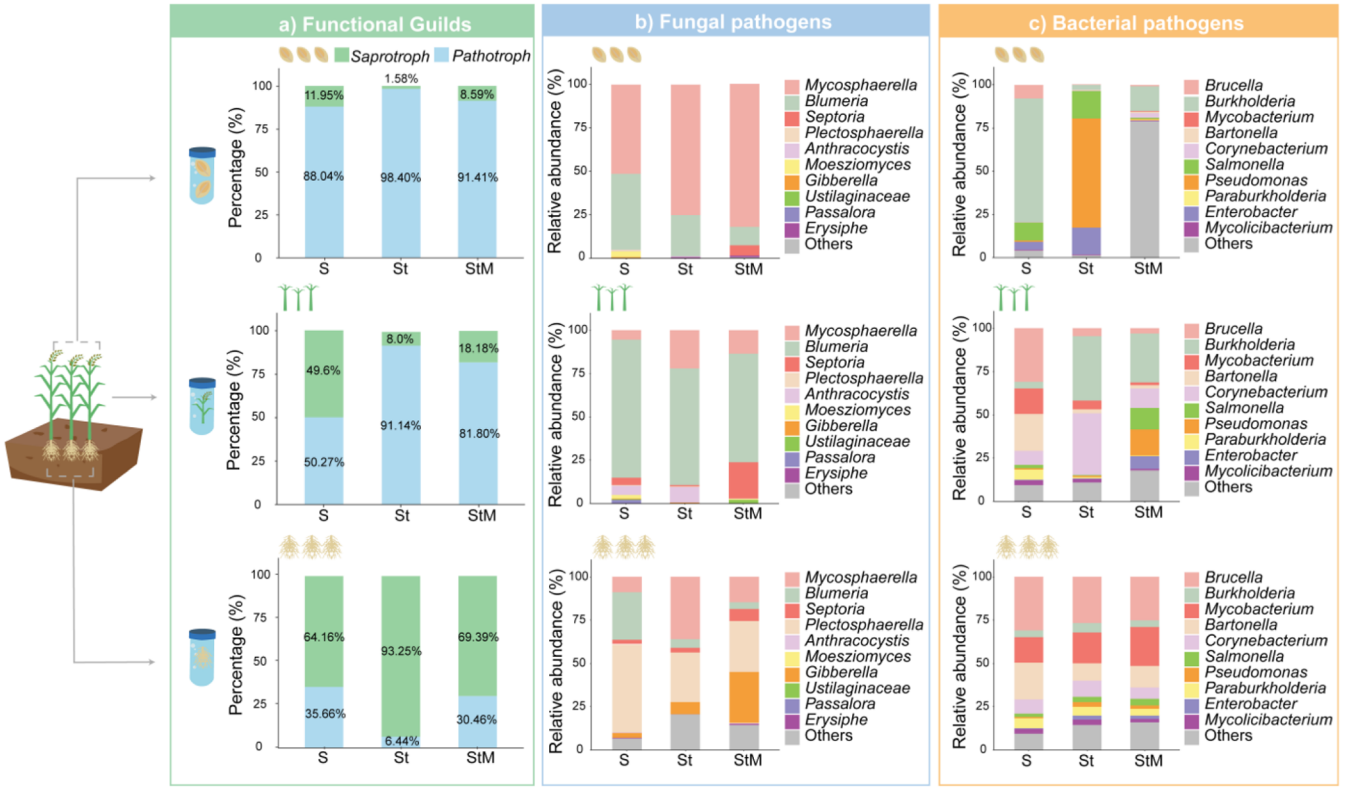


**Figure 4.** Variations in the functional types of endophytic microbes (a), the abundant (top 10 in relative abundance) pathogenic fungi (b), and pathogenic bacteria (c) within different tissues (seed, stem, and root) of oats grown in biofertilizers: St, a straw and cow manure co-composted biofertilizer; StM, the'St' biofertilizer incorporated with SMS; and S, pure soil without biofertilizer inputs.

resistance genes database) , CARD (comprehensive antibiotic resistance database),[38] and PHI (pathogen−host interactions) database. The abundance was defined as the copies of VGs/ARGs/PGs per cell and calculated according to Zhang et al. (2019).[39] Within the selected ARGs and VGs, the highly abundant genes were quantitated by pPCR.

**Plant Endophytic Microbiome Analysis.** We conducted the PCR amplification of V5−V7 and 799F-1193R.[40] Filtered reads were then inferred into amplicon sequence variants (ASVs) at ≥99% similarity via the Greengenes (for 16S rRNA) and UNITE (for ITS) databases, respectively.[41] To understand the variations in the functional types and community composition of endophytic microbes within different plant tissues (seed, stem, and root) of oats grown in biofertilizers. Fungi functional guild analysis was performed to assign different trophic modes (Supplementary File 1) in FUN-Guild.[42] BLASTN software was used to compare the sample 16S rRNA sequence to the HPB database. An *E* value of $<1\times10^{-10}$ and an identity value of ≥99% were used as threshold values.[43] The relative abundance of HPB in the sample is the HPB obtained by a comparison ratio of the number of sequences to the total number of sequences (16S rRNA).[44]

**Statistical Analysis.** All data were tested for normal distribution and homogeneity before statistical analysis using the Shapiro-Wilk ("stats" package in R) and Levene ("car" package in R) tests. The significant variations of oat quality characteristics (i.e., fresh weight, root/shoot weight, and crude protein) and oxytetracycline were examined by one-way analysis of variance (ANOVA; "stats" package in R), followed by Tukey's HSD. Pearson correlation analysis was conducted to assess the relationships between abundant pathogens and genes ("cor.test" function from the "stats" package). The alpha diversity indices for the oat endophytic microbial community (bacteria and fungi) were computed based on normalized microbial ASVs (Amplicon Sequence Variants). In R, the "vegan" package was used to calculate the Shannon index, which considers the number and abundance of microbes and to evaluate the number of observed species for assessing the microbial richness. Linear discriminant analysis (LDA) effect size (LEfSe) was adopted to investigate potential biomarkers at five taxonomic levels ranging from phylum to genus for bacterial and fungal communities that specifically enrich different tissues of oats. Important biomarkers were selected based on a LDA score of >2.0 and $p < 0.05$.

## RESULTS

**The Effect of Biofertilizer on Plant Performance and Quality.** In relation to the control soil, the application of biofertilizers significantly increased the fresh weight of oats (Figure 2a). Specifically, biofertilization (either "St" or "StM" biofertilizer) markedly enhanced the level of shoot weight of the oats (Figure 2c).

Regarding forage quality indices, the "StM" biofertilizer was more effective in boosting the crude protein content in oats by +5.1% compared to the "St" biofertilizer (Figure 2d; $p < 0.05$). Compared with control soil (without any fertilizer), the two biofertilizers (with an addition ratio of ∼7%) significantly increased the root ratio (Figure 2e), the shoot-to-root ratio (Figure 2f), the forage yield (Figure 2g), and the grain yield (Figure 2h).


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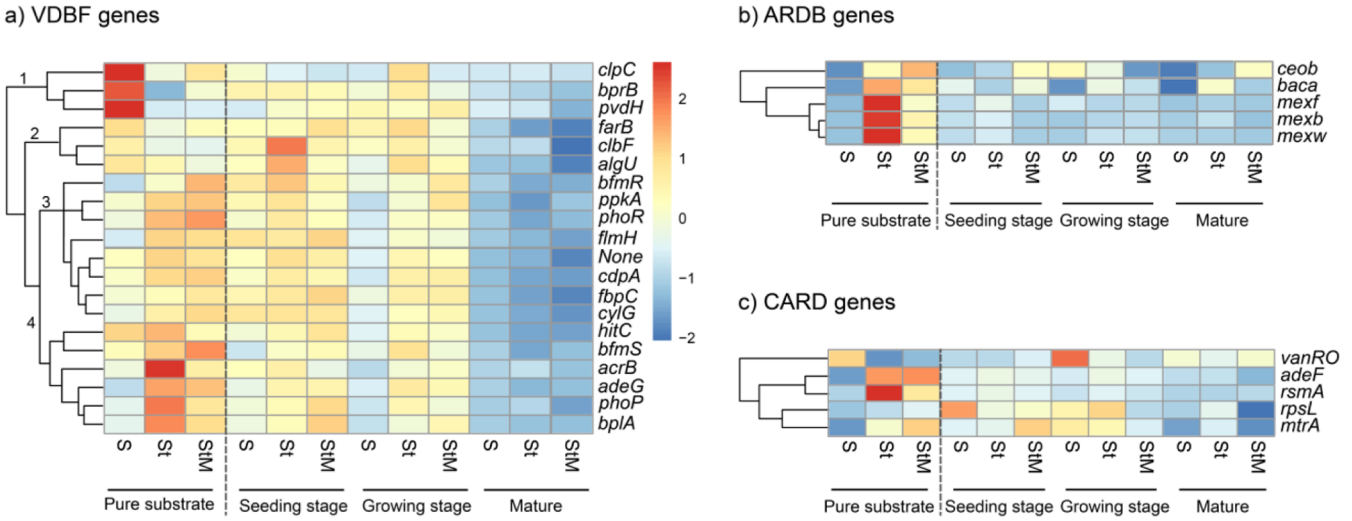


**Figure 5.** Resistant gene variations in the oat growth soil based on VFDB, ARDB, and CARD annotations. Treatments: straw−cow manure biofertilizer (St), St with the spent mushroom substrate (StM), and control soil (S). Samplings at seeding (30d), growing (60d), and mature (120d) stages. Tree clusters for resistome genes; redder shades denote higher gene abundance.

**Changes in Endophytic Microbial Community within Plant Tissues.** Across the three treatments, there was a progressive decline in the diversity (Shannon index) and richness of endophytic bacteria from roots to stems to seeds (Figure 3a). Upon applying a biofertilizer (relative to unfertilized soil, CK), the diversity and abundance of endophytic bacteria in oat stems decreased; however, differences in the seeds were not marked. Compared to the "St" biofertilizer, the application of the "StM" biofertilizer resulted in reduced accumulation of endophytic bacteria in terms of both quantity and diversity in all parts of oat plants (Figure 3a). Throughout the three treatments, there was a gradual reduction in the diversity (Shannon index) and richness of endophytic fungi from roots to stems to seeds. Post-application of biofertilizers (compared to control unfertilized soil) increased the abundance of endophytic fungi in oat stems and seeds. Compared to the "St" biofertilizer, the application of the "StM" biofertilizer increased both the quantity and diversity of endophytic fungi in oat seeds (Figure 3b).

Distinct groups of endophytes were identified across each treatment condition, leading to the formation of characteristic marker groups (Figure 3). Following the application of the "StM" biofertilizer, specific marker groups emerged within the oat's roots, stems, and seeds, characterized, respectively, by the presence of *Pseudocosmospora*, Eurotiales, and Bulleribasidiaceae. Distinct endophytic bacterial groups were identified in each treatment (Figure 3), and characteristic marker groups emerged. Following the application of the "StM" biofertilizer, specific marker groups were established in the roots (*Lechevalieria*),[45] stems (*Hypnocyclicus*), and seeds (*Halomonas*).

**Variations in Pathogenic Microbes within Plant Tissues.** Pathogenic functional groups are predominant in seeds (∼93%) and stems (∼74%), while saprotrophic groups are dominant in roots (∼76%). When two different types of biofertilizers, namely, "St" biofertilizer and "StM" biofertilizer, are applied to oat soil, there is an increase in the proportion of pathogenic functional groups in seeds (by 7%) and stems (by 36%) and an increase in saprotrophic functional groups in roots, which is particularly noticeable with the "St" biofertilizer (by 29%). Compared to the "St" biofertilizer, the "StM" biofertilizer has a lesser effect in elevating pathogenic groups (a decrease of 7% in seeds and 9% in stems)(Figure 4a).

Furthermore, the compositional analysis of endophytic pathogenic fungi in different parts of the oat plant shows the following: (1) in oat seeds and stems, fungal pathogens are predominantly represented by *Mycosphaerella* (∼70%) and *Blumeria* (∼70%), whereas in roots, a co-dominance is observed with *Plectosphaerella* (∼37%), *Gibberella* (∼13%), and *Mycosphaerella* (∼20%). (2) The application of both "St" and SMS biofertilizers led to an increase in the relative abundance of *Mycosphaerella* across all plant parts (stem by 12%, root by 18%, seed by 21%). (3) Following the application of the SMS biofertilizer, there was a notable increase in the proportion of *Septoria* compared to the "St" biofertilizer (Figure 4b). The compositional analysis of endophytic pathogenic bacteria in different parts of oats shows the following: (1) the composition of pathogenic bacteria in oat roots is relatively consistent, predominantly comprising *Brucella* (28%), *Mycobacterium* (18%), and *Bartonella* (15%). Conversely, the composition of pathogenic bacteria in the stems and seeds exhibits considerable variability between treatments (Figure 4c).

**Changes of Harmful Genes in the Livestock−Crop−Mushroom (LCM) System.** The total gene abundances for the three resistome databases were 4431 (VFDB database), 201 (ARDB database), and 38 (CARD database) (Table S4). The total gene numbers were 1066 (VFDB), 38 (ARDB), and 26 (CARD), respectively (Table S4). From ARDB and CARD, the top 5% abundant genes were chosen as they occupied 97% (194/201) and 95% (36/38) of the total gene abundance in each database. Considering the high number (1066) of genes in VFDB, we selected the top 20 genes that represent ∼50% to cover the main changes in total gene abundances.

The VFDB provides up-to-date knowledge of the virulence factors of various bacterial pathogens (http://www.mgc.ac.cn/VFs/). Within VFDB, the top 20 genes relevant to bacterial virulence were grouped into 4 clusters according to their variation during oat growth. Genes of clusters 1 (*clpC, bprB,* and *pvdH*) and 2 (*farB, clbF,* and *algU*) featured their significantly higher abundance in soils than in'St' or'StM' biofertilizers. Other 16 genes from clusters 3 and 4 were


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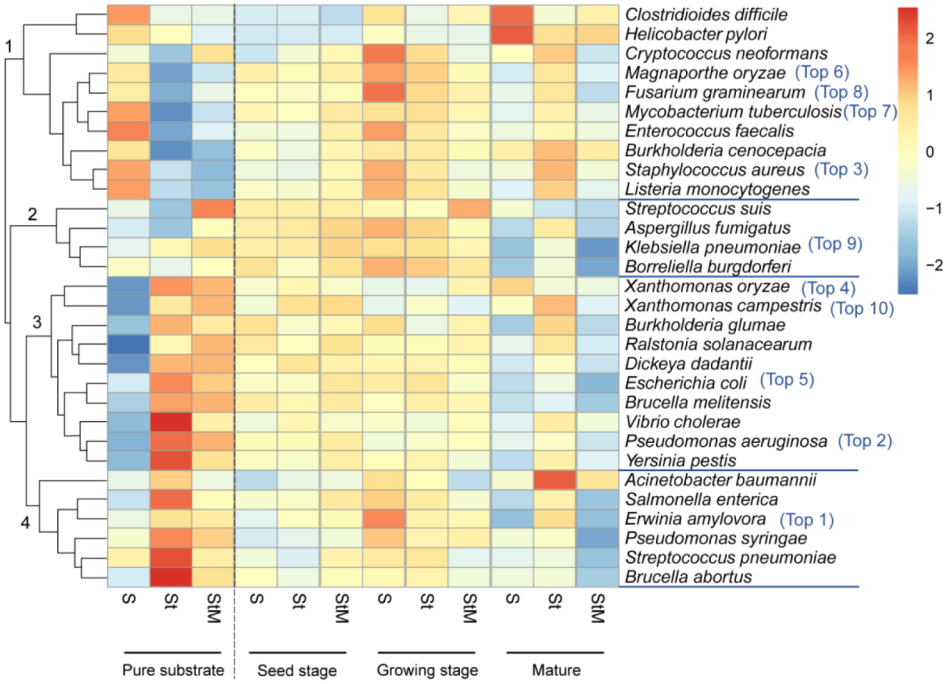


**Figure 6.** Variations in pathogenic species during oat growth in two types of straw and cow manure co-composted biofertilizers. Abbreviations are given in Figure 5. The vertical cluster represents the pathogens in cluster trees. The redder the square color, the higher the abundance of the pathogens among samples.

relatively higher in the two biofertilizers than in soils (Figure 5a). For the most abundant gene (*None*) from VFDB, the qPCR result showed that the absolute abundance of this gene substantially reduced from the initial pure substrate to the soils after oat harvest (mature stage; Figure S1).

The ARDB mainly contains multiple resistance gene data of bacterial pathogens. The most abundant genes (>97% in total genes) were grouped into three clusters, including *ceob* (cluster 1), *baca* (cluster 2), and *mex*-series cluster (*mexf*, *mexb*, *mexw*) (Figure 5b). Among the initial substrates, *mex*-series genes were significantly higher in St than in StM or soil (Figure 5b), and their abundances were decreased during oat growth; however, the abundances of *ceob* and *baca* displayed an opposite trend (Figure 5c). The CARD revealed that abundant genes (>95% of total genes) were grouped into three clusters: *vanRO* (cluster 1) was highly abundant in soil; cluster 2 (*adeF* and *rsmA*) seemed to be rich in the St biofertilizer but generally decreased in abundance after applying it in soil for oat growth; and cluster 3 (*rpsL* and *mtrA*) maintained high abundance during the oat initial and growing stage but also decreased after oat maturation (Figure 5c). Except for *vanRO*, the abundance of all other genes reduced with oat growth.

**Variations in Potential Pathogens and Their Relationships with Harmful Genes.** In total, 1,048,575 genes were annotated from the PHI database, a database of genes interacting with pathogens and hosts. The PHI resources have been verified by experiments, mainly from fungal, oomycete, and bacterial pathogens. The infection hosts include animals, plants, fungi, and insects (available at www.phi-base.org). All the PHI annotated genes were assigned to 245 pathogen species (Table S5). Among those speces, the top 30 (in relative abundance) representing 75% of the total pathogen abundance were selected (Figure 6).

The pathogens were divided into four groups based on their abundance. Pathogens in cluster 1 had higher abundances in the raw soil. Pathogens in clusters 2, 3, and 4 had higher abundances in the initial biofertilizers (Figure 6). It should be noted that in the soil applied with the'St' biofertilizer, the abundance of significant pathogens tends to be stable after oat maturation, with a variation of only ∼1% (Table S6). This denotes the potential risk of applying the "St" biofertilizer to soils. In comparison, the abundance of significant pathogens (except for *Clostridioides difficile* and *Helicobacter pylori*) tends to decrease with oat growth, especially for the ones grown in pure soil and in the "StM" biofertilizer. The abundant animal-hosted pathogens, such as *Salmonella enterica* and *Pseudomonas aeruginosa*, exhibit robust correlations with harmful genes (i.e., *hitC*, *fbpC*, *None*, *vanRO*, and *mtrA*) (Figure S3). In certain instances, multiple pathogens are found to be correlated with the same set of genes, implying a potential co-occurrence or interaction pattern (Figure S3). These results demonstrate the presence of significant correlations between abundant pathogenic species and resistome (and/or virulence factor) genes.

**The Change of Oxytetracycline after Composting and the Subsequent Application for Oat Cultivation.** The measured oxytetracycline concentration in the raw cow manure was 3232 μg/kg (Figure 7). After composting, its content in the maturing stage significantly decreased to ∼1000 μg/kg. Using biofertilizers for forage cultivation showed an oxytetracycline concentration of ∼630 μg/kg after oat-planting soils ($p < 0.05$) (Figure 7).

## DISCUSSION

**Oat Performance after Biofertilization Application.** The application of biofertilizers ('St' and'StM') has significantly increased the fresh weight of oats, indicating essential nutrient supply or growth-promotion in plants. The increase in the shoot weight further supports the role of biofertilizers in promoting plant growth. This could be due to beneficial microorganisms in the biofertilizers that improve nutrient


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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

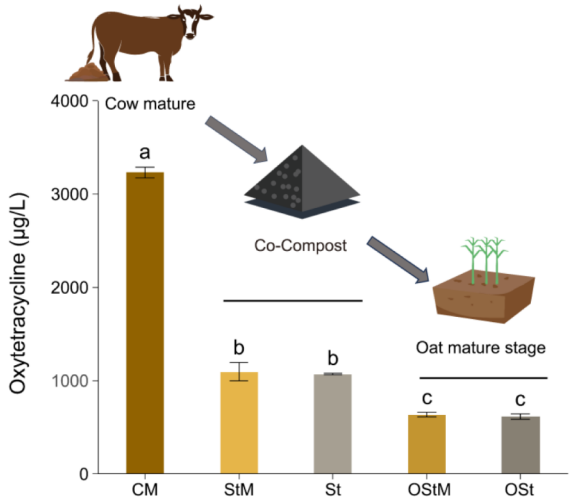


**Figure 7.** Variations of oxytetracycline after composting and subsequent application for oat cultivation. CM, an initial cow manure that is used for co-composting with straw; St, a straw and cow manure co-composted biofertilizer; StM, the "St" biofertilizer involved with the spent mushroom substrate; OStM, the "StM" biofertilizer used for oat cultivation; and OSt, the "St" fertilizer used for oat cultivation. All the soil samples were obtained after oat harvest (120 days after sowing). Different letters represent significant differences between treatments with $p < 0.05$ (Tukey's multiple comparison test). Error bars indicate mean ± standard deviation ($n = 3$).

uptake efficiency, root development, or the production of plant growth hormones.[46−48] When considering forage quality indices, the'StM' biofertilizer stands out with its ability to increase the crude protein content in oats by 5.1% compared to the'St' biofertilizer. Higher crude protein content is highly desirable in forage as it directly impacts the nutritional value of livestock.[49,50] This suggests that the specific composition of the'StM' biofertilizer may contain microorganisms that are more effective in nitrogen fixation or promoting the synthesis of proteins within the plant. The crude protein accumulation in oats was significantly positively correlated with the following important N-cycling genes: *asnB*, *gdh_K00261*, *gs_K00284*, *nosZ*, and *nirD* (Figure S2). Specifically, *asnB* encodes enzymes as precursors for amino acids,[51] *gdh_K00261* sustains nitrogen equilibrium for protein synthesis,[52] and *gs_K00284* connects with inorganic nitrogen assimilation for protein production.[53] *nosZ* and *nirD* are potentially indirectly associated by influencing N-transforming processes and thus affect the overall protein synthesis mechanism.[54] Moreover, the significant increase in the forage yield and grain yield further emphasizes the practical importance of biofertilizer application (Figure 2). Higher yields not only benefit agricultural productivity but also have economic implications for farmers.

Biofertilizer treatments improve soil conditions, as demonstrated by a significantly higher shoot-to-root ratio compared to that of the control soil (Figure 2). A higher shoot-to-root ratio indicates enhanced growth of a plant's above-ground part and suggests better soil fertility.[55,56] Among them, "StM" contributes to developing a stronger root system, and "St" appears to have better soil conditions than StM. In relevance to the total root biomass, we found that the treatment of the "StM" biofertilizer has a higher proportion of root biomass (6.5% in "StM" vs. 3.0% in "St"; Figure 2); this suggests some trade-offs between optimizing above-ground yields and root-derived positive effects. Moreover, "StM" adds an additional economic value through the mushroom cultivation procedures. In the context of life-cycle assessments (LCAs), it has been observed that the economic gain derived from mushroom production is approximately five-fold higher than that from composting manure with straw.

**Changes in Microbial Community Composition and Beneficial Endophytes in Different Oat Tissues.** The observed variation in the diversity of endophytic bacteria within different plant tissues (Figure 3) has important implications for the understanding of the interaction between fertilizer application and plant−endophyte relationships. The natural variation of endophytes in plant organs is a complex phenomenon. The differences in endophytic bacteria diversity among organs could be an important factor contributing to the various responses of plant tissues to fertilizer application. Tissues with more diverse endophytic communities may have a greater capacity to adapt to the changes in nutrient availability induced by fertilizer application. Future research should be centered on elucidating the specific mechanisms by which endophytic bacteria in different plant tissues interact with fertilizers and how this interaction can be optimized to improve plant growth and agricultural productivity. Compared to the "St" biofertilizer, the application of the "StM" biofertilizer led to reduced accumulation of endophytic bacteria (Figure 3). The observation that the application of the'StM' biofertilizer results in a reduction in the accumulation of endophytic bacteria in terms of both quantity and diversity throughout all parts of oats compared to the'St' biofertilizer implies a distinctive impact of this kind of biofertilizer on the bacterial population. Additionally, a connection may arise from fungal hyphae and the bacteria that feed on them. Since bacteria are inclined to consume dead fungal necromass, this could modify the food web depending on the substrate and the fungal necromass that might be present in the spent mushroom substrate.[57] Furthermore, it could also be ascribed to changes in the soil nutrient profile or alterations in the chemical composition of plant tissues, affecting the abundance and diversity of endophytic bacteria.[46] Following the application of the "StM" biofertilizer, specific biomarker microbes were established in the roots (*Lechevalieria*, *Pseudocosmospora*), stems (*Hypnocyclicus*, *Eurotiales*), and seeds (*Halomonas*) (Figure 3). These biomarker microbes are important plant growth promoters and enhance the defense mechanism in plants. For instance, *Lechevalieria*, a rare genus of actinobacteria, harbors secondary metabolites with antibacterial activity and has the potential for pathogen suppression in roots.[45,47] In the stems, *Hypnocyclicus* might be related to structural support or the plant's transportation of nutrients and signals. *Bulleribasidiaceae* could produce enzymes that contribute to cell wall modification and growth.[48] For the seed-existing biomarker *Halomonas*, it can play a role in seed germination regulation and protect the seeds from desiccation and environmental stresses.[48,58] Another bacterial biomarker found in the seed was Eurotiales, which could be involved in maintaining seed viability and defense against pathogens during storage.[59,60]

We found that the roots of the oat predominantly host saprotrophic fungal groups, whereas pathogenic groups are primarily found in the stems and seeds. Moreover, compared to the "St" biofertilizer, the "StM" biofertilizer demonstrated a suppressing effect on pathogens (Figure 4). The information provided offers significant insights into the fungal dynamics within oat plants and the influence of biofertilizers on


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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

pathogenic groups. The observation shows that in terms of fungal functional guilds, fungal saprotrophic groups are mainly found in the roots, while pathogenic groups are prevalent in the stems and seeds. As far as we know, this is the first time that plant compartmentalization of fungal functional communities has been demonstrated. This distribution pattern may be related to each plant tissue's different physiological and environmental conditions. For instance, the stems and seeds might be more exposed to external pathogens due to their role in reproduction and dispersal.[61] The finding that applying different biofertilizers affects the proportion of pathogenic functional groups is crucial for agricultural practices. The increase in pathogenic groups with the "St" biofertilizer and the suppressing effect of the "StM" biofertilizer indicate the specific impact of biofertilizer types on the soil microbiome and pathogen prevalence, demonstrating that certain organic amendments can modify the soil environment to promote or inhibit pathogen growth.[11,62]

**Changes Associated with Resistome in LCM System.** Among VFGs, three VFGs (*clpC*, *bprB*, and *pvdH*) showed high abundance in the pure soils. These abundant VFGs in soils can be related to the soil matrix, a reservoir for diverse microbial habitats, and a source of antimicrobial resistance in pathogenic bacteria.[63,64] There was a putative hosting relationship between VFGs and their host species; for instance, *pvdH* and *bprB* were proved to be hosted in *Pseudomonas aeruginosa* and *Burkholderia pseudomallei*, respectively,[65] and these pathogens were detected in pure- and biofertilizer-applied soils (Figure 6). Moreover, accompanied by the contamination of antibiotics from manure or organic fertilizer application, the risk of microbial pollution is becoming increasingly severe in agricultural soils. Hence, from a biosafety perspective, it is noticeable that microbiological examination of livestock manure compost during composting is necessary.

Our results showed that, compared to soil, most of the VFGs and ARGs remained higher in the two biofertilizers prepared from cow manure and other carbon additives. This is because manure biofertilizers represented one of the abundant sources of antibiotic-resistant bacteria, pathogens, and ARGs.[66,67] Among the initial substrates, *mex*-series genes (*mexf*, *mexb*, and *mexw*) were significantly higher in the'St' biofertilizer than in the'StM' (a biofertilizer involved with the spent mushroom substrate). This is a good indication of SMS processing regarding the control of *mex*-series genes. After the growth of oats, the abundance of *mex*-series genes and other abundant ARGs can be continuously decreased (Figure 5), indicating that above-ground gramineous plant cultivation seems to be a valuable way to eliminate ARGs further. Meanwhile, the ARG-carrying plasmid can spread to plant endophytic bacteria, with soil bacteria serving as carriers.[68] This process has the potential to transfer ARGs into plants via horizontal gene transfer and endophytic bacteria. After the biofertilizer was added to the soil for oat planting, the abundance of most hazardous genes (ARGs and VFGs) decreased with oat growing time (Figure 3), indicating the potential effects of resistance gene reduction in the above-ground herbage planting process. Similarly, studies on the distribution and transfer of ARGs in soil-plant systems involving pakchoi and barnyard grass also found a reduction in ARGs. It highlights the importance of considering plant−soil systems when studying the fate of antibiotic resistance in the environment.[69,70]

The results demonstrated that regardless of gramineous plants (oats) in various matrices, such as pure soil or soil mixed with two biofertilizers ('St' and'StM'), the hazardous gene abundances were reduced overall (averaged 30%; Table S7) from the oat seeding stage to the final mature stage. The possible reasons were as follows: (i) the reduction of hazardous genes in the LCM system can likely be ascribed to improvements in soil properties. In our study, the application of the'StM' biofertilizer, which contains the spent mushroom substrate, led to remarkable changes in soil pH. The initial pH of the control soil was 5.2, whereas in the'StM'-treated soil, it ranged from 5.5 to 5.8 during oat growth. This pH shift is significant as it can profoundly impact the survival and metabolic activity of microorganisms harboring hazardous genes.[71] A change in pH can disrupt the physiological processes of bacteria, altering their ability to thrive and, consequently, reducing the prevalence of genes associated with antibiotic resistance and virulence.[72−74] (ii) Gramineous plants can remove soil antibiotics or reduce their bioavailability through root absorption and root fixation, thus reducing the ecological risk of antibiotic pollution.[75,76] For example, the gramineous plant (ryegrass) has a high ability to degrade tetracycline, floxacin, sulfonamides, and other antibiotics in soil.[77,78] (iii) Oat root exudates contain a wide range of metabolites, including sugars, sugar alcohols, nucleotides, nucleosides, amino acids, organic acids, fatty acids, plant hormones, and compatible solutes. The plant roots release antibacterial active substances (organic acids, phenolic acids, terpenoids, etc.) to kill or inhibit the direct defense of pathogenic bacteria and change the soil environment through secretions to enrich beneficial bacteria to change the soil colony structure and to inhibit the growth of pathogenic bacteria. For instance, an antibacterial plant defensin peptide (*AsDef1*) was successfully identified and characterized from the transcriptome of the oat, which can be used in producing pathogen-resistant transgenic plants and in developing potential antibacterial agents in the future using *AsDef1* from *A. sativa*.[79] Biodegradation mainly uses microorganisms resistant to antibiotics to ingest and degrade antibiotics.[80] However, it should be noted that bioadsorption and biodegradation are microbial-based antibiotic resistance mechanisms, which only have a particular effect on removing antibiotics but cannot effectively prevent the risk of antibiotic resistance from spreading in the environment. Notably, the amount of high-abundance antibiotic resistance genes in the "StM" biofertilizer containing the spent mushroom substrate was lower than that in the "St" biofertilizer. After later biofertilizer application to soils, there is still a sustained effect of reducing resistance genes (Figure 5). Physically, the spent mushroom substrate has high porosity and a fibrous texture. These features contribute to a large surface area and form a network.[80,81] Chemically, it is abundant in organic matter and contains charged functional groups, facilitating interactions. These characteristics enable spent mushroom substrates to be regarded as either fertilizers or soil amendments. This reduces the biological availability of pollutants and prevents the migration and spread of antibiotic-resistant genes in soil systems.[80,81]

**Variations in Pathogens and Oxytetracycline Antibiotic after Composting and Application for Oat Cultivation.** For the initial substrates, we found that the contents of widely existing phytopathogens were significantly higher in soils than those in biofertilizers, for instance, *Magnaporthe oryzae* that hosted in either *Oryza sativa* or *Hordeum vulgare* and *Fusarium graminearum* (hosted in *Zea*


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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

*mays*, *Triticum aestivum*, and *Hordeum vulgare*) that commonly causes the diseases of *Fusarium* head/ear blight, *Fusarium* head blight, and ear rot (Table S8). We also noted that *Staphylococcus aureus* (top 3 in relative abundance), which causes skin infections and respiratory diseases, was the highly present taxon in soils.

When comparing the two biofertilizers,'StM' seems to be a promising choice to suppress pathogens, as shown by the lower pathogenic abundance in'StM' than in'St' (Figure 4). Specifically, the top five pathogens, including four animal-hosted pathogens (*Salmonella enterica*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*) and one phytopathogen *Xanthomonas oryzae* (Table S8), have all been significantly reduced in the StM biofertilizer that composted with the spent mushroom substrate. Animal-hosted pathogens are often of the greatest concern when entering the soil. They are frequently shed in large quantities in animal feces, can survive and persist in the soil environment, and can cause diseases in animals and humans. While plant pathogens are a major concern for agricultural productivity, their impact on human and animal health is usually more indirect. In terms of agricultural fields, beneficial results were also presented by other researchers who found that plants grown in soil amended with the spent mushroom substrate were protected from fungal or bacterial diseases.[82,83] Our results indicated a close relationship between pathogens and harmful genes (Figure 5). This should be associated with the coexistence patterns between genes and their host−pathogen based on the relationships between pathogens and harmful genes.[65] For instance, the high abundance of dangerous human bacterial pathogens usually has robust associations with VFGs. These results suggested that pathogenicity and VFGs will be increased with the enrichment of bacterial pathogens in different manure-amended soils.[65] Additionally, the variations in pathogen abundance throughout the oat growth stages in different biofertilizers distinctly highlight the influential role of the agricultural environment in pathogen dynamics (Figures 4 and 6). These findings offer valuable insights into the intricate relationships between pathogens and resistance genes, which are of great importance for developing strategies to mitigate microbiological risks and guarantee the safety of agricultural and food production systems.

The number of identified pathogenic species (245) was higher than those in previous studies, where more than 150 animal pathogens had been identified in livestock manures.[84,85] This discrepancy can be mainly attributed to the following factors: (i) detection method differences: we used metagenomic sequencing and multiple databases (VFDB, ARDB, CARD, and PHI). These advanced techniques offer a more comprehensive view of the microbial community and can detect more pathogens than less-sensitive methods in prior studies. (ii) Pathogen abundance context: while the raw count of pathogenic species is high, the LCM system's impact on pathogen-related risks is complex and not simply negative, as multiple factors interact to determine the actual risk. However, for the ones grown in the'StM' biofertilizer, the abundance of most pathogens tends to be decreased with oat growth, in agreement with the findings of former studies.[70,71] The protective effect was due to the induction of systemic acquired resistance by fungal components of the spent mushroom substrate.[82,83] Moreover, the spent mushroom substrate amendment can improve microorganisms' activities in soil and comprehensively change the soil physiochemical properties (including pH and nutrient content) and microbial biomass.[86,87] It may also indirectly affect the rhizosphere microbial communities by changing the rhizosphere environment.[87] The concentration of oxytetracycline in cow manure was reduced by 3 times (to 1000 $\mu$g/kg), mainly due to bulk dilution (the mixing of cow manure and straw), as well as the reduction mechanism of oxytetracycline at high temperature,[88,89] since thermophilic composting has been proven to be an essential way in effectively reducing (with an average removal efficiency of 85%) multiple veterinary antibiotics.[90] During composting, the oxytetracycline degradation process can be expedited by the C-mineralization process. This is evidenced by Cheng's finding, which shows a positive correlation between the C/N ratio of the biofertilizer and the enhanced biodegradation of oxytetracycline.[90] The upcycling of livestock manure, which produces organic or biofertilizers, has been regarded as an effective approach to reducing the residual antibiotic load.[91] In our study, after applying the matured composts as biofertilizers for 120 days of oat cultivation (serving as cow forage), the concentration of oxytetracycline in the soil was further decreased by approximately 50%. Farmers and agricultural producers can incorporate livestock manure upcycling into their practices in several ways to benefit the environment and food security.

Overall, the LCM system has the potential for sustainable agriculture, as it reduces risks and promotes plant growth. These results suggest the potential for the broader use of biofertilizers in oat and other crop cultivation. Future research should optimize application rates and combinations of biofertilizers. Understanding the long-term effects of biofertilizer use on soil health and agricultural sustainability is crucial for environmentally friendly farming. The LCM system has great application potential in agriculture, with factors such as waste utilization, mushroom cultivation versatility, growth benefits, and risk mitigation contributing to its scalability.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.4c12517.

Variation of fungal community trophic dataset (Table S1), change of endophytic pathogenic-fungi and pathogenic-bacteria within different compartments (Tables S2 and S3), total number and abundance of gene annotated from the four databases (Table S4), variation of pathogenic abundance (Table S5), percentage change of pathogenic abundance (Table S6), percentage change of virulence genes (from VFDB database) and antibiotic resistance genes (from ARDB and CARD databases) (Table S7), functional annotation of abundant (Top 10 in relative abundnce) pathogenic abundance (Table S8) (XLSX)

Overviews of the plant cultivation conditions; soil macro- and micronutrients measurements; metagenomic sequencing and the analysis of N-cycling genes, resistance and virulence genes, and potential pathogens; antibiotic resistance genes (ARGs) and virulence genes via quantitative real-time PCR analysis; plant endophytic microbiome analysis; and changes of potential function and endophytic microbial communities (PDF)



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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

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### Notes

The authors declare no competing financial interest.

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*Environ. Sci. Technol.* XXXX, XXX, XXX−XXX