Compost applications increase bacterial community diversity in the apple rhizosphere

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Abstract
Sustainable practices are key to the improvement of soil fertility and quality in apple (Malus × domestica Borkh.) orchards. Rootstock genotype and fertilizer inputs can alter soil biology, as well as aboveground traits including nutrient acquisition. In this study, a factorial design was used to assess the interaction between two apple rootstocks, ‘Geneva 41’ (‘G.41’) and ‘Malling 9’ (‘M.9’) with four fertilizer treatments [chicken-litter compost, yardwaste compost, fertigation using Ca(NO₃)₂, and an unamended control]. The bacterial community in the rhizosphere was assessed for its impact on both plant and soil properties for each rootstock × fertilizer treatment combination. The bacterial community was dominated by Acidobacteria, Proteobacteria, and Planctomycetes, but Verrucomicrobia and Chloroflexi were the most responsive to the fertilizer treatments. The chicken litter and yardwaste treatments had a greater effect on bacterial community structure than the control. Yardwaste, in particular, was associated with increased relative abundance of Chloroflexi, which was correlated with soil nutrient concentrations. Malling 9 had a greater bacterial diversity than G.41, but the rootstock treatment had no independent effect on the rhizosphere community structure. There was, however, a strong interaction between the rootstock and fertilizer treatments. Carbon cycling was the most prominent functional change associated with the soil bacterial community. These results suggest that compost amendments have a more positive effect on soil bacterial activity and nutrient availability than Ca(NO₃)₂. Our work shows that waste-stream amendments can lead to multiple positive responses, such as increasing aboveground tree biomass, thus potentially improving orchard productivity.

INTRODUCTION

Grafting apple (Malus × domestica Borkh.) scions onto rootstocks is a practice that dates back thousands of years. The technique was originally used to preserve scion genotypes with desirable fruit characteristics. Apple rootstock breeding efforts arose in the 20th century to select and create genotypes that control tree size, fruit bearing habits, and other aboveground characteristics such as cold tolerance, pest and disease resistance.
resistance, and fruit quality (Fazio, 2017). These breeding efforts largely originated at the East Malling Research Station in the United Kingdom where scientists characterized many genotypes, including the ‘Paradis Jaune de Metz’ apple, which they dubbed ‘Malling 9’ (‘M.9’) (Basile & DeJong, 2018). The M.9 rootstock produces a very short (“dwarfed”) tree that has greater yield efficiency and has a reduced juvenile period, which shortens the time between planting and obtaining fruit yields. The M.9 rootstock is largely credited with revolutionizing how apples are grown around the world, since the smaller trees could be planted at high density, which increased the overall orchard light interception and thus yields. Rootstock breeding is now undertaken by several programs throughout the world. The joint USDA and Cornell University rootstock breeding program is among the most active (Fazio et al., 2015). Based in Geneva, NY, the commercially released selections from this program are identified with the initial “G.”

In addition to dwarfing and precocity, the Geneva series of rootstocks were selected for resistance to diseases such as phytophthora and fire blight [Erwinia amylovora (Burrill) Winslow et al.] (Fazio et al., 2015). The ‘Geneva 41’ (‘G.41’) rootstock is a progeny of ‘Malling 27’ (‘Malling 13’ × M.9) and Malus × robusta Rehd. (‘Robusta 5’) and is reported to produce a tree similar in size to M.9 (Fazio et al., 2005, 2015).

More recent work has sought to exploit the potential of apple rootstocks to increase nutrient and water use efficiency. Belowground, nutrient and water acquisition traits may be affected by the soil matrix and the suite of microorganisms that interact with the root system in the rhizosphere (Fazio et al., 2013; Marguerit et al., 2012). Plants affect rhizosphere microbial communities through the production of C-based exudates, rhizodeposits, antimicrobials, and other exudates that alter the rhizoplane (Hartmann et al., 2009). It has been previously reported that the interaction between tree roots and the soil matrix in the rhizosphere is mediated by a large complex of microorganisms, thus it is not surprising that rootstock genotype has been shown to alter the soil microbiome in ways that are associated with improved plant health and productivity (Rumberger et al., 2004; Song et al., 2015; St. Laurent et al., 2008). For example, using terminal restriction length polymorphisms (T-RFLPs), studies have determined that replant disease-resistant rootstock cultivars had similar soil microbial communities (St. Laurent et al., 2010; Rumberger et al., 2004).

Soil characteristics, such as pH and organic matter (OM), are key determinants of rhizosphere microbial communities and can vary in apple orchard soils (Zhang et al., 2018). Regardless of soil type, recent replant status, or rootstock selection, soils have been typically shown to be dominated by three phyla: Acidobacteria, Actinobacteria, and Proteobacteria (Franke-Whittle et al., 2015; St. Laurent et al., 2010; Sun et al., 2014; Zhang et al., 2013). Bacteria belonging to these phyla, which appear to be ubiquitous in the rhizosphere of woody perennial plants, are attracted from neighboring bulk soil to C-based exudates produced by roots (Zarraonaindia et al., 2015).

Furthermore, applications of synthetic N fertilizers may reduce soil respiration, microbial biomass, and enzyme activity and thus shift microbial community composition, resulting in reduced C sequestration compared with unfertilized controls (Ramírez et al., 2012). In contrast, compost applications have been shown to change soil edaphic properties, including increasing soil mineral content, OM, cation exchange capacity (CEC), and microbial biomass in apple orchards (Kramer et al., 2006; Rumberger et al., 2004; Strauss et al., 2014; Yao et al., 2006). Bacterial community composition may also be affected. For example, manure applied to apple trees planted in a sand culture led to an overall increase in soil bacterial diversity (Zhang et al., 2013). This study also found that the relative abundance of Sinobacteraceae and Arthrobacter populations were sensitive to the added OM, whereas Actinobacteria and Proteobacteria populations decreased compared with the unfertilized control. Similarly, 8 yr of cover cropping in an apple orchard altered soil bacterial community structure, specifically with bacteria closely associated with OM degradation (Zheng et al., 2018).

Soil microbial communities may also affect aboveground plant traits and functions. For example, changes in belowground bacterial communities have been found to influence grapevines (Vitis vinifera L.) defense responses, and alter fruit secondary metabolites (López-Fernández et al., 2016; Zarraonaindia et al., 2015). In Brassica rapa L., simplifying the complexity of root microbial communities resulted in smaller plant sizes, reduced chlorophyll content, and fewer flowers (Lau & Lennon, 2011). Liu et al. (2018) found that apple rootstock genotype was involved in influencing the associated endophytic bacterial community composition in the scion. In our previous study, both apple rootstock genotype and fertilizer amendments showed the potential to alter rhizosphere soil microbial communities (Thompson et al., 2019). This presents a gap in knowledge on the connection between improvements in apple growth in response to the amendments, the soil nutrient pool, and predicted associated changes in the rhizosphere bacterial community structure and functions.

Core Ideas

- Organic and inorganic fertilizer treatments supported different rhizosphere bacteriomes.
- Soil bacteriome change and organic composts were positively associated with tree growth.
- Positive feedbacks can explain bacteriome change with greater apple growth.
- Results justify the need to test mechanisms of apple–compost–bacteriome feedbacks.
In a previously published paper that used the same experiment reported herein, we found that after 3 yr, total apple tree biomass was greater when fertilized with chicken litter, chicken litter–Ca(NO$_3$)$_2$, yardwaste, and yardwaste–Ca(NO$_3$)$_2$ treatments than a nonfertilized control, but not different among the five rootstock genotypes (i.e., ‘Budagovsky 9’, G.41, ‘G.214’, ‘G.935’, and M.9) that we tested (Thompson et al., 2019). The Ca(NO$_3$)$_2$, chicken litter, and the two integrated compost–Ca(NO$_3$)$_2$ treatments had greater leaf tissue N than the control and yardwaste treatments. Leaf tissue P and B were consistently greater in the Geneva rootstocks than in ‘B.9’ or M.9. The compost and integrated compost–Ca(NO$_3$)$_2$ treatments had greater soil OM, CEC, potentially mineralizable soil N, and soil microbial respiration than the control. Additionally, bacterial and fungal microbial community compositions, as analyzed by T-RFLP, were affected by rootstock genotype, fertilizer treatment, and time (i.e., number of years after application), and these effects were correlated with changes in tree growth and soil properties. These results suggest that compost and integrated compost–Ca(NO$_3$)$_2$ nutrient applications can be used to increase plant growth, leaf mineral content, soil fertility, and microbial activity in newly established apple orchards. We concluded that the Geneva rootstocks appear to be more effective at acquiring soil minerals than other dwarfing apple rootstock genotypes and, therefore, may be well suited for use in fertility management plans that derive all or part of their nutrients from compost.

The objective of the current study was to identify the rhizosphere bacteria associated with the widely planted M.9 rootstock and the recently released G.41 rootstock when grown using either the grower-standard fertilizer [Ca(NO$_3$)$_2$] or compost treatments. Based on our previous work, both the rootstock and fertilizer treatments were hypothesized to cause significant changes in structure and diversity of the rhizosphere bacterial community. Because of the importance of organic amendments for microbial growth, and the large compositional difference between synthetic and organic fertilizers, it was expected that the fertilizer treatments would drive greater change in bacterial communities than rootstock. How these hypothesized changes are related to altered nutrient pools in apple and soil were assessed to determine possible links between apple tree growth and rhizosphere soil bacterial community diversity and predicted functions.

## MATERIALS AND METHODS

### 2.1 Experimental design and treatments

In 2013, a pot-in-pot experiment was established using a completely randomized design setup as a two-way factorial with two rootstock cultivars and four fertilizer treatments at the Virginia Tech Alson H. Smith, Jr. Agricultural Research and Extension Center in Winchester, VA (39°06’ N, 78°17’ W). Rootstock treatments, M.9 and G.41, were bench grafted with ‘Brookfield Gala’ scions. Fertilizer treatments were 40 kg N ha$^{-1}$ from either chicken litter compost (CL), yardwaste compost (YW), or fertigation with weekly Ca(NO$_3$)$_2$ (Yara) applications for eight consecutive weeks (FERT). Unfertilized trees served as a control (CON). There were four replications of each rootstock × fertilizer combination. These treatments were selected from a larger experiment that included six fertilizer and five rootstock treatments, the results of which are described in Thompson et al. (2019). The chosen treatments were hypothesized to have the greatest impact on the rhizosphere bacterial community.

New compost was acquired yearly and applied on 3 June 2013, 28 May 2014, and 16 May 2015. A hand trowel was used to mix the soil to a depth of 10 cm after compost applications. Similar disturbance was conducted in the non-amended pots. Compost application was based on plant available N (Campbell-Nelson, 2015). Prior to application, compost was analyzed by Pennsylvania State University Agricultural Analytical Services Laboratory (University Park, PA) to ensure N was applied at an equal rate among fertilizer treatments. The chemical properties for the compost are listed in Supplemental Table S1. Application of other mineral nutrients and OM differed among treatments. No other macro- or micronutrient fertilizers were applied in this experiment. Trees were irrigated using a micro-spray irrigation system three times a week throughout the spring and summer, hand weeded when necessary, and uniformly treated for pests and diseases.

Trees were planted in 38 L (33 cm in diameter × 36 cm in height) pots containing Poplimento silt loam orchard soil, a fine, mixed, subactive, mesic Ultic Hapludalf (Soil Survey Staff, 2014), mixed with 10% (v/v) STALITE to improve drainage and aeration. Soil pH, CEC, OM, and Mehlich-1-extractable mineral nutrients were uniform across all pots prior to fertilizer applications. The pots were spaced 1 × 1 m apart in an offset grid pattern. Drain lines under each row of pots below the gravel provided supplemental water drainage. Trees were attached to bamboo stakes and the entire experimental area was covered with shade cloth. A photographic presentation of experimental setup and a schematic of the sampling workflow are provided in Supplemental Figure S1.

### 2.2 Rhizosphere soil sampling

On 26 May 2015, 10 d after the third annual compost application, a total of six roots, measuring 10 cm from the root tip, were harvested from each rootstock × fertilizer combination using a sterile Hori-Hori Japanese soil knife. Rhizosphere soil was removed from three randomly selected roots using a sterile paintbrush and then homogenized. Enough soil for the
DNA extractions was acquired from three roots; thus, in order to process all of the samples in a timely manner, we did not use all six roots. Rhizosphere soil samples were stored at −80 °C until DNA extraction.

### 2.3 Soil collection and analysis

On 19 Aug. 2015, soil samples were collected 15 cm from the tree trunk at a depth of 0–10 cm, as described by Thompson et al. (2019). Bulk soil was sieved using a 2-mm mesh (U.S. no. 10) soil sieve and stored for 4 d at 4 °C for biological and chemical analyses (Thompson et al., 2019).

Soil chemical properties were measured at the Virginia Tech Soil Testing Laboratory (Blacksburg, VA) or the Cornell Nutrient Analytical Laboratory (Ithaca, NY), as described by Thompson and Peck (2017). Briefly, total C and N were measured using a CHN Elemental Analyzer-vario EL (Elementar), mineral nutrients (P, K, Ca, Mg, Zn, Mn, Cu, Fe, and B) were extracted using Mehlich-I solution and analyzed with inductively coupled plasma mass spectrometry at the university soil labs, soil OM was measured using the loss on ignition method, CEC was determined through summation of the non-acid-generating cations, and soluble salts were measured using an electrical conductivity probe.

The direct chloroform fumigation method was used to determine microbial biomass C and N from 10 g of soil (Fierer & Schimel, 2003). Soil was prepared, incubated, and analyzed as described by Thompson and Peck (2017). Soil microbial respiration was measured from 50 g of soil placed in an air-tight jar using the conductimetric method (Rodella & Saboya, 1999), as described by Thompson et al. (2019).

### 2.4 Leaf collection and mineral analysis

Twenty-five leaves were removed from each tree and dried for 3 d at 80 °C in August 2015. Leaf N, P, K, Ca, Mg, B, Cu, and Zn were measured at the Pennsylvania State University Agricultural Analytical Services Laboratory (University Park, PA) using the methods described by Thompson and Peck (2017). Leaf N concentration was measured using the combustion analysis method on a Vario Max N/C analyzer (Elementar). Leaf P, K, Ca, Mg, B, Cu, and Zn were measured using a 730-ES ICP optical emission inductively coupled plasma (OES–ICP) Spectrometer (Agilent Technologies) after dry ashing.

### 2.5 Tree growth

Trunk cross-sectional area was calculated by measuring trunk caliper diameter 10 cm above the planting line once tree growth subsided for the year on 8 Oct. 2015, as described by Thompson et al. (2019). Tree biomass was determined by whole tree destructive harvest on 19–20 Oct. 2015 (Thompson et al., 2019). Briefly, leaves were removed from trees and dried for 3 d at 80 °C. Trees were removed from pots and cut into five segments—roots, belowground rootstock shank, aboveground rootstock shank, central leader, and side branches—before oven drying for 5 d at 80 °C. Total biomass included leaf, root, belowground rootstock shank, aboveground rootstock shank, central leader, and side branches segments.

### 2.6 DNA extraction and amplification

A modified protocol was followed to extract total genomic DNA using the MoBio Lab Power Soil DNA Isolation Kit (MoBio). The manufacturer’s protocol was followed with the following modification: approximately 0.25 g of rhizosphere soil was placed in the bead tube provided by MoBio and heated to 65 °C for 10 min in a water bath (Thompson et al., 2019). DNA purity and concentration were assessed using a Nanodrop spectrophotometer (Thermo Scientific). DNA was stored at −20 °C until polymerase chain reaction (PCR) amplifications were performed in triplicate 25-µl reaction volumes for each sample. The bacterial 16s ribosomal RNA (rRNA) v4 region was amplified using the method described by Caporaso et al. (2011). Polymerase chain reactions contained 2 µl template DNA, 11.875 µl nuclease-free water (MoBio), 1 × 5 Prime HotMasterMix, 10 µM 515f forward primer, 10 µM 806r reverse primer, and 0.1 µg µl⁻¹ bovine serum albumin (BSA). The reverse primer contained a unique Golay barcode sequence (Caporaso et al., 2011). The reaction conditions for this PCR were a 3-min denaturation step at 94 °C, followed by 35 cycles of 94 °C for 45 s, 68 °C for 60 s, and 72 °C for 90 s, followed by a final extension step of 72 °C for 10 min. Reactions were amplified in a Bio-Rad C1000 thermal cycler. The PCR products were visualized on a 1.5% agarose gel in 1× tris borate EDTA buffer (TBE) stained with GelStar (Lonza). The triplicate amplifications were pooled and quantified using a Qubit High Sensitivity DNA quantification system (Invitrogen). A single, pooled sample was formed by adding 250 ng of DNA from each PCR. The Qiagen PCR purification kit (Qiagen) was used to purify the pooled sample. After purification, DNA concentration of the sample was assessed using a Nanodrop spectrophotometer. Sequencing using 250-bp paired-end reads was completed at the Bio-complexity Institute at Virginia Tech (Blacksburg, VA) using the Illumina MiSeq platform. Data has been deposited in the NCBI Sequence Read Archive (SRA) repository under project Accession no. PRJNA623378, with run accessions SRR11505108–SRR11505139.
2.7 | Statistical analyses

Samples were demultiplexed by the sequencing facility. Reads were preprocessed through cutadapt version 1.18 (Martin, 2011) using the following parameters: minimum length = 150 bp, maximum length = 250 bp, phred quality score > 30. Reads were merged using fastq-join. A further filtering step through cutadapt dropped merged amplicons lengths <200 bp and >290 bp. This was followed by removing chimeric reads using USEARCH version 6.1 (Edgar, 2010). Further processing was performed in QIIME version 1.8.0 (Caporaso et al., 2010) using the pick_open_reference_otus.py script. Reads were clustered into OTUs at 97% threshold using UCLUST (Edgar, 2010). Operational taxonomic unit (OTU) taxonomy assignments were based on the Greengenes database version 13.8 (McDonald et al., 2012). The OTUs closely related to mitochondrial and plastidial 16S rRNA genes were removed. To reduce OTU inflation, OTUs matching the following criteria were removed: spurious OTUs that have just one read in all samples, OTUs that were present in less than three samples, and OTUs having less than 10 total reads. The remaining OTUs were retained for downstream analyses.

Statistical significance for OTUs was determined using the Kruskal Wallis test for the fertilizer treatment and interaction between fertilizer and rootstock, whereas the Mann–Whitney U test was used for the rootstock treatment. The P values were adjusted using the Benjamini–Hochberg method. Alpha diversity analyses were assessed using several metrics to provide information on evenness, richness, and phylogenetic and ecological diversity. Shannon, Chao1, observed species, Faith’s diversity, abundance-based coverage estimate (ACE), and Good’s coverage were calculated for the rootstock and fertilizer treatments. Statistical tests for significant differences used were similar to above, except for normally distributed indices, and Welch’s t test for indices following a normal distribution. Beta diversity was evaluated based on the Unweighted and Weighted UniFrac distances between the OTUs. The OTU table was rarefied to an even sampling depth of 40,200 sequences prior to the analysis. The effect of the rootstock and fertilizer treatments, as well as their interactions, on the bacterial community beta diversity was further validated using ADONIS (analysis of variance using distance matrices), ANOSIM (analysis of similarities), and MRPP (multiple response permutation procedure) through QIIME (quantitative insights into microbial ecology). All nonparametric tests were run with 999 permutations.

Mantel tests were used to calculate the correlations between the soil, leaf profiles, soil biological properties presented in Supplemental Tables S2–S5, and rhizosphere communities using the vegan package in R (Oksanen et al., 2019). These data were originally published in Thompson et al. (2019). To identify the relationship between the different physicochemical and biological parameters and the rhizosphere communities, their vectors were fitted into a nonmetric multidimensional scaling (NMDS) ordination of rhizosphere distance matrix using the envfit() function in vegan. Prior to vector fitting, their values were log-transformed and standardized. The P values were calculated using 999 permutations.

To determine the bacterial indicators that are representative for each rootstock and fertilizer treatment, as well as their interactions, indicator species analysis (Dufrêne & Legendre, 1997) was carried out using the indval function in the labdsv package. Only OTUs accounting for at least 0.01% of the total read counts were retained for this analysis. A minimum indicator value of 60 and a false discovery rate (FDR) adjusted p value of .05 were used to select the indicator OTUs.

The biological functional implications of the bacterial communities were assessed using PICRUSt version 1.1.3 (Langille et al., 2013). PICRUSt provides a functional profile by predicting and estimating the metagenome based on the community composition. Initially, all OTUs not present in the Greengenes database (de novo predicted OTUs) were filtered out. The gene content was then predicted and normalized based on the 16s copy numbers. Downstream visualization and statistical analysis were performed in STAMP version 2.1.3 (Parks et al., 2014) and R. The P values were also adjusted using the Benjamini–Hochberg method.

3 | RESULTS

Preprocessing steps and quality filtering resulted in 8,719,524 sequences with a mean length of 248 bp, covering roughly the amplified V4 region. Further filtering of lowly abundant and spurious OTUs produced 10,863 clusters at 97% sequence similarity. One replication of the chicken litter × G.41 had only 138 reads and was consequently discarded from downstream analysis, leaving three replicates. The remaining 31 samples had ~40,200 sequences, which was chosen as the appropriate depth for rarefaction analysis and an even sampling of the community.

3.1 | Soil and leaf physiochemical analyses

Soil nutrients and physiochemical measurements, which were originally published in Thompson et al. (2019), were highly correlated to the changes in the rhizosphere bacterial communities, soil microbial biomass, and leaf nutrients (Table 1). To elaborate further on these effects, soil and tree measurements were individually compared with the rhizosphere bacterial communities using nonmetric multidimensional scaling (Supplemental Tables S2–S5, Figure 1, and Supplemental Figure S2). The set of measured soil nutrients and physiochemical measurements were highly correlated with
FIGURE 1 Nonmetric dimensional scaling (NMDS) ordination of the unweighted UniFrac distances for soil (A) chemical and (B) biological properties for bacterial communities from soil in which ‘Gala’ apple trees grafted onto Geneva 41 or Malling 9 rootstocks were treated with 40 kg N ha\(^{-1}\) from either chicken litter (CL), yardwaste (YW), or Ca(NO\(_3\))\(_2\) fertigation (FERT), or a nonfertilized control (CON) in Winchester, VA. Data represents the mean of the two rootstocks. Vector length represents the \(R^2\) value. False discovery rate (FDR)-corrected \(p\) values \(\leq .05\) of the significant correlations are highlighted in red. Individual correlations for the vectors can be found in Supplemental Tables S2 and S3.

TABLE 1 Mantel correlations between the rhizosphere bacterial community matrix and the overall set of soil and plant physiochemical and biomass measurements from soil in which ‘Gala’ apple trees grafted onto ‘Geneva 41’ or ‘Malling 9’ rootstocks were treated with 40 kg N ha\(^{-1}\) from either chicken litter, yardwaste, or Ca(NO\(_3\))\(_2\) fertigation, or a nonfertilized control in Winchester, VA. Data represent a mean of all rootstock and soil treatments. Correlations of individual measurements can be found in Supplemental Tables S2–S5.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mantel score</th>
<th>(p) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil nutrients</td>
<td>0.6935</td>
<td>.001</td>
</tr>
<tr>
<td>Soil microbial biomass</td>
<td>0.5817</td>
<td>.001</td>
</tr>
<tr>
<td>Leaf nutrients</td>
<td>0.3083</td>
<td>.001</td>
</tr>
<tr>
<td>Tree biomass</td>
<td>0.1156</td>
<td>.053</td>
</tr>
</tbody>
</table>

bacterial community structure \((R^2 > .75, \text{Supplemental Table S2})\). The majority of elements were elevated and associated with the compost treatments. For example, soil K, Mn, OM, P, and Zn were highly correlated with Axis 1, which separated the CL and YW treatments from the FERT and CON treatments. More specifically, OM, K, and Mn were strongly associated with the YW treatment and P and Zn were associated with the CL treatment (Figure 1a). Conversely, Cu was strongly associated with the FERT and CON treatments. Most of the soil nutrients were negatively correlated with the FERT and CON treatments.

Soil microbial biomass also had strong and positive correlations with rhizosphere bacterial community change related to the CL and YW treatments (Figure 1b and Supplemental Table S3). A higher soil microbial respiration was associated with the YW application, whereas microbial biomass N was found in the CL associated bacterial communities. None of these biological assays had significant correlations with the FERT or CON treatments.

Several elements measured in the leaf samples had a significant correlation with the belowground rhizosphere bacteria (Supplemental Figure S2a and Supplemental Table S4). In particular, the macronutrients P and K were strongly correlated with the rhizosphere bacteria in the YW treatment \((R^2 > .6)\). Additionally, N had a vector orientation closer to CL than YW.

Tree biomass measurements were all positively associated with the CL and YW treatments (Supplemental Figure S2b and Supplemental Table S5). Tree size (as measured by trunk cross sectional area), total tree biomass, and root biomass were more significantly correlated to the CL and YW treatments, than the FERT or CON treatments \((R^2 > .2)\).

3.2 Taxonomic analyses

The overall diversity of the samples was rich, even at the higher taxonomic ranks (Figure 2). Approximately 43 bacterial phyla were detected in this study. The most abundant phyla were Acidobacteria (34.1%), Proteobacteria (14.3%), Planctomycetes (12.5%), Verrucomicrobia (12.1%), and Chloroflexi (9.9%). At the class level, Acidobacteria-6 (23.6%) and Chloracidobacteria (8.5%), both belonging to Acidobacteria, dominated the rhizosphere. Spartobacteria (Verrucomicrobia), Anaerolineae (Chloroflexi), and Phycisphaerae (Planctomycetes) were the next most abundant classes with about 8.5, 7.5, and 6% of the total abundance, respectively. Acidobacteria iii-15 was the most abundant at the order taxonomic level, with about 23.2% of the total count. Acidobacteria iii-15 was
The soil fertilizer treatments significantly affected several of these groups. Verrucomicrobia was one of the most abundant phyla that changed significantly (p = 0.025) among treatments, ranging from 9.8% in the YW treatment compared with 15.3% in the CON treatment (Figure 2). Chloroflexi was another relatively abundant phylum that was significantly affected by treatment (p = 0.003). Chloroflexi constituted up to 18.1% of the rhizosphere community when soil was amended with YW to as little as 5.4% in the unamended CON. Firmicutes, which includes plant growth-promoting bacteria, such as Bacilli, was not abundant in these soils; however, there were significant changes in this phylum in response to the fertilizer treatment (p < 0.001). Firmicutes was relatively more abundant in YW (0.67%), compared with the other treatments (0.2–0.3%).

At the class level, some key soil bacteria were found to be significantly affected by the fertilizer treatments. Spartobacteria, a dominant class of Verrucomicrobia, was in greater abundance in the CON (11.6%) than the other treatments (p value = 0.019). Anaerolineae, a class of bacteria with multiple capacities related to C degradation under aerobic and anaerobic conditions, varied from 15.8% in the YW to between 3.2 and 6.1% in the other three treatments (p value = 0.003). Finally, Phycisphaerae, one of the two dominant Planctomycetes classes, was significantly different among treatments (p value = 0.028).

### 3.3 Indicator species analysis

Approximately 9,130 (84%) of the OTUs identified in this study were found in at least three of the four fertilizer treatments, but there were some OTUs that were unique to each treatment (Supplemental Figure S3). Indicator species analysis was used to determine which taxonomic groups were most closely associated with a given fertilizer treatment. The OTUs with a minimum representation of 0.01% of the total abundance were included in this analysis. There were 111 indicator OTUs with a minimum indicator value threshold of 60 and statistical significance (FDR-corrected p values < 0.05). Of these, 86 OTUs could be considered as markers of the YW treatment. The top five OTUs in YW were closely related to the Anaerolineae class from the Chloroflexi phylum (Table 2). The CL treatment had 16 indicator OTUs, with the top two most abundant belonging to the Acidobacteria class. Although five indicator OTUs were detected in FERT, they were low in abundance. The most abundant OTU in the

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**Figure 2** Taxonomy summary of the cumulative relative abundance of the rhizosphere bacterial communities from soil treated with 40 kg N ha$^{-1}$ from soil in which ‘Gala’ apple trees grafted onto Geneva 41 or Malling 9 rootstocks were treated with 40 kg N ha$^{-1}$ from either chicken litter (CL), yardwaste (YW), or Ca(NO$_3$)$_2$ fertigation (FERT), or a nonfertilized control (CON) in Winchester, VA. Data represents the mean of the two rootstocks. Stacked bar plots show the top 10 taxa in three taxonomic ranks: (A) phylum, (B) class, and (C) order. Stars indicate significantly different taxa at the false discovery rate (FDR)-corrected p values ≤ 0.05. Taxa are sorted in ascending order by total abundance. Error bars represent the standard errors.
### TABLE 2
Top five indicator operational taxonomic units (OTUs) from soil in which ‘Gala’ apple trees grafted onto ‘Geneva 41’ or ‘Malling 9’ rootstocks were treated with 40 kg N ha\(^{-1}\) from either chicken litter (CL), yardwaste (YW), or Ca(NO\(_3\))\(_2\) fertigation (FERT), or a nonfertilized control (CON) in Winchester, VA. Data represent the mean of the two rootstock treatments. The OTUs were filtered at a base indicator value score 60. The OTUs are sorted by their total relative abundance. Number of samples per treatment is shown (maximum \(n = 8\)). The \(P\) values were adjusted using the Benjamini–Hochberg method.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Indicator value</th>
<th>(p) value (FDR(^a))</th>
<th>No. of samples</th>
<th>Abundance</th>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
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<tbody>
<tr>
<td>CL</td>
<td>85.9</td>
<td>.017</td>
<td>2</td>
<td>0.12</td>
<td>Acidobacteria</td>
<td>Acidobacteria-6</td>
<td>iii1-15</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>72.9</td>
<td>.017</td>
<td>1</td>
<td>0.06</td>
<td>Acidobacteria</td>
<td>Acidobacteria-6</td>
<td>iii1-15</td>
<td></td>
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<tr>
<td></td>
<td>69.5</td>
<td>.045</td>
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<td>[Chthoniobacteraceae]</td>
<td>Chthoniobacter</td>
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</tbody>
</table>

\(a\)FDR, false discovery rate.
Alpha diversity as determined by the Shannon index for the rhizosphere bacterial communities from soil in which ‘Gala’ apple trees grafted onto ‘Geneva 41’ (‘G.41’) or ‘Malling 9’ (‘M.9’) rootstocks were treated with 40 kg N ha\(^{-1}\) from either chicken litter, yardwaste, or Ca(NO\(_3\))\(_2\) fertigation, or a nonfertilized control in Winchester, VA. Data represents the mean of the four soil treatments. The diversity on M.9 was significantly greater than that found on G.41 according to the Welch \(t\) test (\(p\) value = .012).

The FERT treatment closely resembled members of Sinobacteraceae at the family level. Finally, the CON treatment had just four indicator marker OTUs, which each had low indicator values (<70). A Chthoniobacter species was the most abundant indicator OTU in the CON treatment.

### 3.4 Alpha diversity

Bacterial community diversity in the rhizosphere was also affected by the rootstock genotype. The OTU and phylogenetic diversity were significantly higher in the rhizosphere bacterial communities of M.9 than in G.41 (Figure 3). The Shannon index in M.9 was significantly greater than G.41 (Welch two-sample \(t\) test \(p\) = .012). However, neither the fertilizer treatments nor the interaction between fertilizer and rootstock treatments had an effect on alpha diversity based on any of the metrics used in this study. Mean values for Good’s coverage estimator were approximately 99% for most samples, indicating excellent coverage of the community, even though rarefaction plots did not completely plateau for any of the samples.

Beta diversity

There was a strong fertilizer treatment effect on the abundance of several taxonomic ranks at the class and order level. To further understand the effect of fertilizer and rootstock treatments on the bacterial community structure, we used multivariate tests on the unweighted UniFrac distances between the samples. The ADONIS and ANOSIM analyses revealed a significant fertilizer treatment effect, as well as an interaction effect between fertilizer and rootstock (Figure 4, Table 3). However, the rootstock effect on its own was not significant. The CL and YW treatments separated from the FERT and CON treatments along the first principal component. Additionally, the CL and YW treatments were distinctly clustered away from each other along the second principal component.

The strong interaction between the fertilizer and rootstock treatments indicated that the two rootstock genotypes responded differently to the fertilizer treatments. For instance, the bacterial community structure for G.41 clustered together within the CON and FERT treatments (Supplemental Figure S4A). Conversely, although the treatments resulted in fairly identifiable clusters in the rhizosphere of M.9 rootstock...
TABLE 3  ADONIS and ANOSIM multivariate statistical tests of the beta diversity of the rhizosphere microbial communities from soil in which ‘Gala’ apple trees grafted onto ‘Geneva 41’ (‘G.41’) or ‘Malling 9’ (‘M.9’) rootstocks were treated with 40 kg N ha\(^{-1}\) from either chicken litter, yardwaste, or Ca(NO\(_3\))\(_2\) fertigation, or a nonfertilized control in Winchester, VA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ADONIS (R^2)</th>
<th>ADONIS (P) value</th>
<th>ANOSIM (R^2)</th>
<th>ANOSIM (P) value</th>
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<tr>
<td>Fertilizer</td>
<td>.2</td>
<td>.001</td>
<td>.7</td>
<td>.001</td>
</tr>
<tr>
<td>Fertilizer (\times) rootstock</td>
<td>.35</td>
<td>.001</td>
<td>.67</td>
<td>.001</td>
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<td>Rootstock</td>
<td>.03</td>
<td>.208</td>
<td>.03</td>
<td>.156</td>
</tr>
<tr>
<td>Fertilizer (G.41 only)</td>
<td>.4</td>
<td>.001</td>
<td>.81</td>
<td>.001</td>
</tr>
<tr>
<td>Fertilizer (M.9 only)</td>
<td>.31</td>
<td>.001</td>
<td>.683</td>
<td>.001</td>
</tr>
</tbody>
</table>

samples, there was a fair amount of variability (Supplemental Figure S4B). This was evident using multivariate tests, where the ANOSIM had a greater value for the G.41 than M.9 rootstock, due to there being less variability for the G.41 rootstock communities.

3.6 | Functional prediction

Overall, the fertilizer treatments caused larger functional differences than the rootstock treatments (Figures 5–6). The metabolic profile of the microbial communities associated with the YW treatment was different from the other fertilizer treatments and the CON (Figure 5). After removing pathways related to eukaryotes, 35 pathways were found to be significantly different between the YW bacterial communities and the other fertilizer treatments (Figure 6). Based on the FDR-corrected \(p\) values, C cycling and metabolism-related pathways such as glycolysis were more abundant in YW samples than in other treatments. Nitrogen-related functions, such as amino acid cycling, were also different, and the specific pathways were related to different fertilizer treatments. For example, the secondary metabolite metabolism pathways, such as those related to the herbicide atrazine and pathways associated with antibiotic production or resistance, were greater in YW than in other treatments.

4 | DISCUSSION

Bacterial communities from the rhizosphere of young apple trees were hypothesized to change with rootstock genotype, fertilizer, and the interaction of rootstock and fertilizer. In support of these hypotheses, CL and YW had the greatest influence on bacterial community structure, and these treatments had distinctly different bacterial communities from the FERT and CON treatments. There was also a difference in the diversity of bacterial communities between the two rootstock genotypes, with M.9 having greater diversity than G.41. Additionally, a strong rootstock \(\times\) fertilizer interaction was found for the bacterial communities. The rhizosphere bacterial community differences showed a strong positive association with soil nutrient content, soil microbial biomass and activity, leaf nutrients, and tree biomass measurements. FERT had a similar bacterial community to the CON treatment, and therefore we conclude that this treatment had a weak impact on the bacterial community. However, both CL and YW compost altered the rhizosphere bacterial communities, most likely because of the high carbon and nutrient content of these amendments (Thompson et al., 2019). The rootstock \(\times\) fertilizer interaction
FIGURE 6 Mean functional differences between the bacterial communities in the rhizosphere of soil in which ‘Gala’ apple trees grafted onto ‘Geneva 41’ or ‘Malling 9’ rootstocks were treated with 40 kg N ha$^{-1}$ from either chicken litter (CL), yardwaste (YW), or Ca(NO$_3$)$_2$ fertigation (FERT), or a nonfertilized control (CON) in Winchester, VA. Data compare YW with the other three soil treatments averaged for both rootstocks. Metabolic pathways are color coded according to Level 2 KEGG classification: carbohydrate metabolism (red), metabolism of cofactors and vitamins (green), amino acid metabolism (blue), lipid and peptidoglycan biosynthesis and metabolism (brown), metabolism of secondary metabolites (pink), and other pathways (black). Pathways were significantly different according to false discovery rate (FDR)-corrected $p$ value $\leq .05$. 
points to a possible soil bacterial community to soil amendment feedback that might play a role in the greater above-ground apple tree growth and nutrient acquisition found in the two compost treatments (Thompson et al., 2019). Together, these results suggest that compost amendments have a positive impact on apple tree growth, and that these impacts are partly driven by changes in soil–root communities, decomposition, C cycling functions, and possible plant–microbial feedbacks.

### 4.1 Fertilizer treatment had a strong effect on the rhizosphere bacterial beta and alpha diversity

There was a marked difference between the CL and YW compost (organic) and FERT Ca(NO₃)₂ (inorganic) amendments on bacterial communities (Figure 4, Supplemental Figure S4). This is in agreement with several previous findings for multiple crops and agricultural systems (Hartmann et al., 2015; Ma et al., 2018; Sun et al., 2015). Differences in beta-diversity were perhaps related to a bacterial community shift under the higher carbon content fertilizer treatments (CL and YW) from oligotrophic to more copiotrophic bacterial taxa (Hartmann et al., 2015; Wang et al., 2017; Xun et al., 2016). In our study, Verrucomicrobia were depleted, whereas the Chloroflexi were strongly enriched in the YW compared with CON treatments. Verrucomicrobia have been shown to be slow-growing oligotrophs, a result that is consistent with their greater abundance when inorganic fertilizers are applied to soils. However, both Verrucomicrobia and Chloroflexi are highly abundant in soil and much remains to be discovered about the ecological roles of these indicator taxa (Bergmann et al., 2011). Furthermore, it should be noted that there still remains uncertainty about how to define taxa as copiotrophic or oligotrophic; thus, we are cautious to not overstate our findings.

Anaerolinea–Chloroflexi, the indicator OTU of YW, is reportedly involved in numerous C cycling roles (Hug et al., 2013; Liang et al., 2016) and may thrive under anaerobic conditions where high C availability increases microbial growth, which can then cause O₂ to be limited. Chloroflexi were previously reported to be a rhizosphere indicator OTU in a manure treatment (Ai et al., 2015). These results suggest that these bacteria are supported by greater levels of OM, but the exact mechanism for their high abundance needs further investigation. Our results also suggest that changes in bacterial communities and specific taxa can positively affect apple tree growth through changes in plant–microbial feedbacks and changes in decomposer taxa that aid nutrient cycling. Apple tree growth was likely also affected by nutrients directly acquired from the compost, and both factors are likely at play.

Although the largest difference in bacterial community composition was between the two compost treatments and the FERT and CON treatments, the communities associated with CL and YW treatments were also significantly different from each other. Differences in the composition of compost have been shown to affect rhizosphere soil bacterial communities (Jack et al., 2011; Tanu Prakash & Adholeya, 2004). Top indicator species of the CL treatment belonged to the understudied but ubiquitous subdivision 6 of Acidobacteria and Planctomycetes. Conversely, YW, which had more numerous indicator species, was highly dominated by Chloroflexi. Differences in the dominant taxa may be a reflection of the chemical composition and microbial activities of the applied amendments and could also help explain some of the functional differences in the bacterial communities in CL and YW treatments.

### 4.2 High abundance of planctomycetes

Planctomycetes was the third most abundant phylum (Figure 2A). Although they are ubiquitous and present in aquatic environments, their high abundance is uncommon in the rhizosphere of multiple crops such as those in the Long-term Ecological Research site located at the Michigan State University W. K. Kellogg Biological Station in Michigan and at Cornell University’s research orchards (Buckley et al., 2006; Buckley & Schmidt, 2003). Limited cultivation of representative Plantomycetes taxa has possibly led to underreporting, and thus a lack of their presence in databases that describe their close relatives. Nonetheless, recent studies have shown their importance in soil ecosystems, perhaps related to OM decomposition (Wiegand et al., 2018). For example, an increase of the abundance of Planctomycetes was observed in association with an increase in degradation and decomposition of extracellular DNA in soil (Morrissey et al., 2015). Some plants have been observed to suppresses Planctomycetes, which is purported to improve soil carbon sequestration (Jenkins et al., 2006). Phycisphaera, for example, one of the main Planctomycetes classes, can assimilate and hydrolyze complex heteropolysaccharides secreted by other soil bacteria and degrade wood in marine sediments (Bienhold et al., 2013; Wang et al., 2015). If this functional ability results in a bottleneck in the decomposition process, this could result in numerous changes in C and nutrient cycling. This functional role is also one that many fungi play in soils; hence, our focus on bacterial communities may not tell the full story. Phycisphaera also changed significantly among fertilizer treatments (Figure 2B), suggesting their sensitivity to nutrient inputs. The role of these bacteria in decomposition and potential plant–bacterial feedbacks need further investigation, particularly given the low number of sequenced genomes and potential bias against these bacteria during PCR (Klindworth et al., 2013).
Compost amendments increased OM and respiration in these apple orchard soils (Thompson et al., 2019). These changes were correlated with changing bacterial communities and higher aboveground biomass (Supplemental Figure S2b). Thompson et al. (2019) used a different microbial fingerprinting method, T-RFLP, and similarly found that bacterial community composition was altered due to the fertilizer amendment treatment. Thus, these results provide a more detailed in-depth analyses of these bacterial communities, which further complements findings in our previous report.

At the higher taxonomic levels, some of the main bacterial groups, such as Proteobacteria, were generally stable as their relative abundance was unaffected by the rootstock or fertilizer treatments. This may reflect the fact that many Proteobacteria dominate soil regions with greater OM, such as soil near plant roots (Gómez-Acata et al., 2016; Lundberg et al., 2012; Niu et al., 2017). Indicator species abundance near the apple roots may suggest an ecological and physiological relevance to family-level changes, such as those found for Sinobacteraceae in the FERT treatment (Table 2). Though our results need further confirmation as Sinobacteraceae OTUs were in low abundance (0.04%), our data support Zhang et al. (2013), who reported that Sinobacteraceae were sensitive to manure applications.

The bacterial communities in the amendments were not assessed prior to application, and there is a possibility that they caused a priming effect, but the impact from the bacteria that originated in the compost was likely relatively small compared with the influence of C and other factors from the fertilizer and rootstock treatments.

4.4 Rootstock effects on the rhizosphere bacterial communities

The M.9 rootstock rhizosphere had a greater diversity index than G.41 (Figure 3), which may increase community resilience in the face of environmental change and stress (Griffiths & Philippot, 2013). For example, greater soil diversity potentially allows for a more even and rich bacterial distribution that has more genetic interactions with plant roots. Furthermore, bacteria, such as Gammaproteobacteria, can decompose OM and protect against fungal pathogens (Mendes et al., 2011). Gammaproteobacteria were more abundant in M.9 than in G.41 treatments, potentially indicating a bacterial-conferring disease resistance induced by the rootstock genotype. In maize (Zea mays L.), the interaction between plant genotype and fertilizer amendment was reported to affect root exudates, which in turn strongly affected bacterial rhizosphere community structure and activity (Aira et al., 2010). A similar phenomenon might have occurred in our study. Quantifying and characterizing root exudates should be a focus of future rootstock–fertilizer studies.

Although not as strong of a determinant of the community structure and beta-diversity as the fertilizer treatments on their own, the rootstock treatments did have a strong interaction with the fertilizer treatments (Figure 4). The interaction between apple rootstock genotype and belowground bacterial communities may have ramifications on aboveground traits, such as tree growth and resilience to diseases, and thus be could an important orchard management consideration. For example, Rumberger et al. (2004) found that rhizosphere bacterial community composition changed significantly between apple replant disease-tolerant and -susceptible rootstock genotypes. However, apple rhizosphere diversity does not necessarily always achieve greater disease resistance, especially in the case of apple replant disease, so diversity may be superseded by specific species and/or functional groups (Mazzola et al., 2015). Interestingly, rootstock–scion interactions have been shown to be a determinant of the aboveground apple endophytes, whereas rootstock alone was not a significant factor (Liu et al., 2018). This was also accompanied by variations in aboveground biomass changes. Unraveling the interaction between rootstock genotype and rhizosphere bacteria will continue to be an important research area for developing sustainable orchard management.

4.5 Functional change in bacterial communities to fertilizer and rootstock

Major predicted functional differences were associated with the compost treatments (Figure 5). Both CL and YW showed changes in beta-diversity, as visualized in the ordination plot, but only YW showed strong significant changes in bacterial community function. This is perhaps a result of functional redundancy, where different species within the bacterial community have a similar function, but due to limitations in genomics databases, which are still incomplete and may not adequately represent function across all taxa, this was not a hypothesis we tested in our research.

Based on the 16S normalized predicted-metagenome functional genes content, the YW treatment was different than the other treatments in regards to C metabolism (Figures 5 and 6). Similar observations can be inferred from the PICRUSt data, which predicted that genes related to the tricarboxylic acid cycle (TCA) were more abundant in the compost treatments compared with the FERT and CON treatments (Figure 6). Our findings resemble long-term studies conducted in China that also found that C-related functions were enriched in response to the organic fertilizer inputs (Ling et al., 2016).
Regardless of location or cropping system, C5-branched dibasic acid metabolism, C fixation, and CH$_4$ metabolism were important functional terms that differentiated organic and inorganic fertilizer amendments. A possible explanation is that the large amounts of C added to the soil supported an increase in microbial activity and TCA cycling relative to an inorganic amendment. This may have relevance to the relative priming of organic decomposition of soil C. A second explanation is that inorganic N inputs reduced soil microbial respiration and, thus, C cycling (Lazcano et al., 2013; Ramirez et al., 2010; Söderström et al., 1983). For example, (Söderström et al., 1983) found that inorganic N amendments suppressed microbial respiration for at least 3 mo after application, and long-term effects lasted for 3 yr. Assessing the relative contributions of soil priming and repression are not possible with our data, yet both phenomena possibly occurred in our experiment. Overall, there were significant changes in KEGG pathways, particularly related to C cycling, that could change the way the apple trees and orchard soils function in response to high C inputs, such as the YW treatment.

Other predicted functional changes, such as beta-lactam resistance and atrazine degradation, were difficult to explain for our study. For example, beta-lactam resistance may be an important response to changes in microbial competition that affects community structure, but this was not a hypothesis that we are able to confirm with our experimental design.

5 | CONCLUSION

It was confirmed that the bacterial communities associated with the widely planted M.9 rootstock and the recently released G.41 rootstock were dependent on the type of fertilizer that was applied to the soil. The fertilizer by rootstock interaction effects were smaller than those between the compost and Ca(NO$_3$)$_2$ (FERT) or control treatments, but still suggest the potential for changes in belowground root–bacterial community interactions that could translate into aboveground consequences. Though major predicted functional differences were associated with compost amendments, and largely with the YW treatment, these results point to the strong influence compost has to drive changes in soil ecosystem functioning. Hence, the use of compost amendments can have positive effects on the apple orchard ecosystem while reducing synthetic fertilizer applications. Consideration of other impacts of waste-stream amendments, such as the potential for enhanced CH$_4$ production, and support of apple rootstock–microbial interactions should be further investigated and weighed for their environmental benefits.

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AUTHOR CONTRIBUTIONS

Hazem Sharaf: Data curation; Formal analysis; Investigation; Methodology; Software; Validation; Visualization; Writing-original draft; Writing-review & editing. Ashley A. Thompson: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Resources; Validation; Visualization; Writing-original draft; Writing-review & editing. Mark A. Williams: Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Software; Supervision; Writing-review & editing. Gregory M. Peck: Conceptualization; Data curation; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Writing-original draft; Writing-review & editing.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AND MATERIAL AVAILABILITY


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