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Endophytic Bacterial Communities in Apple Leaves Are Minimally Impacted by Streptomycin Use for Fire Blight Management

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ABSTRACT

In apple fruit, streptomycin is routinely used to manage fire blight, a devastating disease that greatly affects commercial apple production. Applications of streptomycin may affect the endophytic bacterial communities, which are increasingly recognized for their valuable roles in plant hosts. Altering these communities may have implications for plant health and selection for streptomycin resistance in off-target bacteria. In a previous study, we found rapid selection of streptomycin-resistant epiphytes following season-long streptomycin use, and now sought to investigate the season-long impact of streptomycin applications (one, three, or nine postbloom applications) on endophytic bacterial communities. Studies were conducted in two orchards, and both culture-dependent and independent methods were used to assess the bacterial endophytic community. Streptomycin programs were compared with untreated trees and two programs approved for organic production. Treatments minimally affected abundance of culturable endophytic bacteria as determined by culture-dependent methods. Similarly, there were no significant differences in relative

bacterial abundance or community composition between treatments as determined by 16S ribosomal RNA high-throughput sequencing (culture independent). There were greater differences between orchards than between treatments, suggesting a stronger influence of locality. Communities were dominated by the families *Pseudomonadaceae*, *Enterobacteriaceae*, *Desulfovibrionaceae*, *Bacillaceae*, and *Burkholderiaceae*, which are commonly described in other investigations of microbial communities of apple orchards. In addition, we found a high abundance of *Amoebophilaceae*, a family dominated by arthropod parasites. Our results provide evidence that season-long applications of streptomycin in apple orchards have minimal impact on endophytic bacterial communities, which is in contrast to some reports on epiphytic communities.

Keywords: agriculture, antibiotics, bacteriology, biological controls, biopesticides, copper, *Erwinia amylovora*, *Malus × domestica*, microbiome, phyllosphere, phytobiome, streptomycin

The aminoglycoside antibiotic streptomycin is routinely used to manage fire blight, one of the most devastating diseases of apple and pear worldwide. Streptomycin is applied at bloom and post-bloom (PB), when epiphytic populations of the bacterial pathogen *Erwinia amylovora* are predicted to rapidly increase and cause infection (Norelli 2003). Since its introduction in 1955,

streptomycin has been the most common and effective means of managing fire blight during bloom in apple production in the eastern United States (Cox et al. 2013, Sundin et al. 2009). Despite this utility, antibiotic use in agriculture has increasingly come under scrutiny due to the potential for development of resistance in pathogen and off-target bacterial populations, especially in light of the increasing prevalence of antibiotic resistance in both agricultural and clinical settings (McManus et al. 2002; Stockwell and Duffy 2012). In some agricultural markets, antibiotics are already limited or prohibited. For example, antibiotics have been prohibited in organic production in the United States, following regulation by the National Organic Standards Board in October 2014; this is consistent with organic regulations in other locations, including Canada and the European Union (Granatstein et al. 2015; Johnson and Temple 2013). Alternatives to streptomycin exist, including other antibiotics oxytetracycline and kasugamycin (McGhee et al. 2011), plant defense inducer compounds (systemic acquired resistance or induced systemic resistance products), and numerous biopesticides (Aldwinckle

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et al. 2002; Sundin et al. 2009). Unfortunately, the utility of these products is limited due to lower efficacy in wet temperate climates and higher application costs (Aldwinckle et al. 2002; Sundin et al. 2009). Although streptomycin remains the most important management tool for fire blight in many commercial orchards in the eastern United States, it is becoming increasingly important to understand the lasting repercussions of its use so that informed management recommendations can be made to producers.

Streptomycin-resistant (SmR) *E. amylovora* has been documented in most regions where apple fruit are produced, including California (Moller et al. 1972), Oregon and Washington (Coyier and Covey 1975), Israel (Manulis et al. 1998), New Zealand (Thomson et al. 1993), Michigan (McGhee and Sundin 2011), and New York (Russo et al. 2008). Two mechanisms of resistance have been described: (i) a point mutation in the *rpsL* gene, resulting in the structural alteration of the binding site for streptomycin (Chiou and Jones 1995a), and (ii) presence of the gene pair *strA* and *strB* located within the transposable element *Tn5393*, which code for aminoglycoside-modifying enzymes APH(3'')-Ib and APH(6)-Id (Chiou and Jones 1995b). The former mechanism of resistance (mutation in the *rpsL* gene) is likely conferred by spontaneous mutation whereas, for the latter (presence of the *strA/B* gene pair), horizontal transfer from SmR bacteria in the phyllosphere of apple orchards has been proposed as the main mechanism by which resistance was acquired, in *E. amylovora* (Chiou and Jones 1993) as well as other pathogens in diverse locations around the world (Sundin and Wang 2018).

Repeated applications of streptomycin are believed to contribute to the development of SmR *E. amylovora*, and may dramatically influence the bacterial epiphyte communities in a commercial orchard setting. In some cases, higher incidence of resistance genes *strA* (*aph(3'')-Ib* or *orfH*) and *strB* (*aph(6)-Id* or *orfI*) has been found in soils with a history of antibiotic use (Tolba et al. 2002). In other work, proportions of SmR bacteria were not necessarily related to history of streptomycin use in orchard soil, or on leaves or flowers (Shade et al. 2013a; van Overbeek et al. 2002). However, Yashiro and McManus (2012) found higher proportions of culturable SmR endophytes from streptomycin-treated trees compared with untreated trees but found that environmental variables had a greater impact on bacterial community structures. More recently, we found a rapid increase to 100% culturable SmR epiphytic bacteria after three consecutive applications of streptomycin in Gala apple (Tancos and Cox 2017). These results suggest that the extent to which streptomycin programs influence SmR in overall bacterial communities in the environment is still unclear. More importantly, little is known about the impacts to the bacterial community within the plant where *E. amylovora* primarily resides.

More broadly, antibiotic use may influence the microbial community colonizing the host plant (i.e., the microbiome). There is mounting evidence that the microbiome plays a dramatic role in plant phenotype, and offers services ranging from yield and growth promotion to health and protection (Hardoim et al. 2015; Kandel et al. 2017; Ryan et al. 2008; Sundin and Wang 2018; Turner et al. 2013). Application of antibiotics has the potential to influence these microbial communities by changing community composition (Lin et al. 2016), reducing microbial diversity (Liu et al. 2009), increasing antibiotic resistance (Ding et al. 2014), and causing disturbances in ecological functions (Cycon et al. 2019). Research investigating microbial communities of apple systems has shown that these communities can be influenced by factors such as field treatments (i.e., organic versus conventional management) (Ottesen et al. 2009), leaf or flower age (Afandhi et al. 2018; Shade et al. 2013b), and host genotype (i.e., rootstock) (Liu et al. 2018). However, other

research efforts investigating the impact of streptomycin on epiphytic communities in apple orchards found that the antibiotic had no measured effect on apple leaves or flowers (Rodríguez-Sánchez et al. 2008; Shade et al. 2013a; Yashiro and McManus 2012; Yashiro et al. 2011). In some research, samples collected at different time points indicate that communities may be differently affected at different parts of the season (Ottesen et al. 2009). Including multiple sampling time points has been especially important for elucidating changes in specific communities such as the impact of streptomycin on the floral niche, where the pathogen preferentially colonizes the stigmatic surface prior to entering the host plant (Shade et al. 2013b). However, in studies investigating the effects of antibiotics over the course of a season, differences were not usually detected at different time points (van Overbeek et al. 2002). Therefore, the cumulative impact of streptomycin on bacterial communities or antibiotic resistance genes is most appropriately informed by samples collected at one time point, typically the end of the season (Rodríguez-Sánchez et al. 2008; Shade et al. 2013a; Tancos and Cox 2017; Tolba et al. 2002; Yashiro and McManus 2012). The season-long effects of streptomycin applications on the microbial communities in an orchard environment remain unclear and warrant further investigation.

Although many studies investigating plant microbiomes have focused on the total microbial community, a more specific community deserving scientific investigation in the context of fire blight management may be endophytic bacterial communities of the orchard canopy. Endophytes are generally defined as microorganisms residing inside plant tissues for at least part of their life cycle without causing detrimental effects to their host (Brader et al. 2017; Hardoim et al. 2015). The entire phytobiome (endophytes and epiphytes) is influenced by numerous factors, including both environmental conditions such as temperature, moisture, and UV radiation, as well as host factors, including host species, age or developmental stage, and plant health status (Dastogeer et al. 2020; Lindow and Brandl 2003). Although epiphytes, or microorganisms colonizing the exterior surfaces of plants, are dramatically affected by environmental conditions, endophytes are protected by plant membranes and may be more resilient. In addition, they form close, complex relationships with their hosts without eliciting a defense response, although the mechanisms of these relationships are still poorly understood, and provide services to their hosts ranging from plant growth to pathogen protection (Dastogeer et al. 2020; Lindow and Brandl 2003; Reinhold-Hurek and Hurek 2011; Ryan et al. 2008).

In the apple–fire blight pathosystem, endophytic communities may be especially important. *E. amylovora* is well known to travel and survive endophytically, colonizing the plant well ahead of symptom development (Momol et al. 1998; Rosen 1929; van der Zwet 2012), whereas investigations of leaf and flower surfaces have found *E. amylovora* to be an unsuccessful epiphyte (Bonn 1981; Manceau et al. 1990; Ockey and Thomson 2006; Tancos and Cox 2017). Streptomycin, when applied to the canopy for fire blight management, penetrates both leaf and dermal tissues of spurs (Crosse and Garrett 1958; Dye 1956; van der Zwet and Buskirk 1984). In addition, it is frequently applied with an adjuvant that increases its absorption such as the nonionic surfactant Regulaid (KALO, Overland Park, KS, U.S.A.). This locally systemic movement is considered responsible for most of the disease protection capabilities, including providing protection against blossom and shoot blight infection up to 24 h postinfection event (Agnello et al. 2019; Crosse and Garrett 1958; Dye 1956). However, because of this local systemic activity, streptomycin may also have detrimental effects to the entire endophytic community and, therefore, on plant health. The interaction between

streptomycin, *E. amylovora*, and other apple foliar endophytes warrants more attention in order to understand the downstream effects on the apple host and orchard environment.

Previously, we conducted a study examining the impact of successive PB application of streptomycin and found a rapid increase in the proportion of culturable SmR epiphytic bacteria after three consecutive applications of streptomycin when as many as 10 applications can be applied in nonbearing orchards (Tancos and Cox 2017). This finding, combined with the fact discussed above that *E. amylovora* is a more successful endophyte (Momol et al. 1998; Rosen 1929; van der Zwet 2012) and that streptomycin is locally systemic (Crosse and Garrett 1958; Dye 1956; van der Zwet and Buskirk 1984), led us to become concerned that season-long antibiotic use may also have a detrimental impact on the endophytic community of apple fruit. In this regard, we decided to investigate the cumulative impacts of season-long streptomycin use (one, three, or nine PB applications) on the endophytic bacterial communities of apple leaves in two orchards over 2 years. Regardless of any impacts on *E. amylovora*, results have the potential to improve our understanding of the impacts of antibiotics on the endophytic bacterial communities of the orchard canopy and inform more sustainable management recommendations for commercial producers.

MATERIALS AND METHODS

Study site. To evaluate the relative effect of fire blight disease management programs on endophytic bacterial communities, field trials were conducted at Cornell AgriTech in Geneva, NY (42°87'70.19" N, -77°02'99.15" W) over two field seasons in 2018 and 2019. Experiments were performed in a semidwarf planting of Gala on B.9 rootstock, planted in 2000 at 1.8-m in-row spacing (orchard A), and a high-density planting of Gala on G.935 rootstock planted in 2016 at 1-m in-row spacing (orchard B). Trees were managed following commercial standards according to the Cornell Pest Management Guidelines (Agnello et al. 2019).

Experimental design, application of treatments, and sample collection. Treatments and sample collection were performed using methods previously described by Tancos and Cox (2016) in research investigating the impact of streptomycin applications on epiphytic communities of the apple phyllosphere. A randomized complete block design with four single-tree replications or a panel of four trees surrounded by buffer trees within and across rows was used to evaluate the management programs in the semidwarf (orchard A) and high-density (orchard B) orchards, respectively. All applications were made using a Solo 475-B gas-powered mist blower (Solo Incorporated, Newport News, VA, U.S.A.) calibrated

to deliver water at 935.3 liters ha⁻¹, a standard volume for high-density apple plantings in the northeastern United States. Beginning at bloom, trees were subjected to one of three antibiotic programs (Table 1). Antibiotic programs included an application of streptomycin at 80% bloom, followed by one, three, or nine PB applications of streptomycin on a weekly basis beginning at the “petal fall” phenological stage, approximately 1 week after bloom application (1PBStrep, 3PBStrep, and 9PBStrep). Such antibiotic programs were meant to represent a typical commercial standard program (1PBStrep = positive control) and two programs representing nonrecommended practices, whereby a grower might make excessive streptomycin applications, attempting to avoid shoot blight in a young planting or nursery block (3PBStrep and 9PBStrep). The commercial product used was Firewall 17 (Nufarm, Morrisville, NC, U.S.A.), at the label rate of the product at 1.68 kg ha⁻¹. These streptomycin programs were compared with two other bactericides commonly used to manage fire blight in organic commercial apple production (positive controls). These management programs included either an Organic Materials Review Institute (OMRI)-listed formulation of *Bacillus subtilis*, a biopesticide, or an OMRI-listed fixed copper product, formulated as a soap, which both function as general antimicrobials. Both were applied at 80% bloom and petal fall, to constitute one bloom and one PB application. The commercial products used were Serenade Opti (Bayer, Institute, WV, U.S.A.) and Cueva (Certis, Wasco, CA, U.S.A.), applied at the label rates of 1.40 kg ha⁻¹ and 4.67 liters ha⁻¹, respectively (treatments will be referred to Serenade and Cueva, respectively). All treatments were compared with untreated trees (negative controls) which received no chemical applications for fire blight management (untreated). All replicated treatments were applied to the same trees in subsequent years of the study in order to observe cumulative effects and interaction between treatments.

Sample collection and processing. Foliar tissue samples were collected in late August 2018 and 2019, approximately 2 weeks after the final treatment date. Five mature leaves were randomly collected from each replication using gloved hands, placed in Ziploc bags, and transferred to the lab on ice. Samples were processed within 12 h. Using sterile forceps and scalpel, petioles were removed and leaf blades were surface sterilized, as described by Afandhi et al. (2018) and Muresan (2017). Leaves were surface sterilized by gently washing with sterile water to remove dirt and debris, sequentially submerging in 5% sodium hypochlorite solution, 70% ethanol, and two rinses of sterile water for 30 s each. Removal of potentially contaminating epiphytic bacteria was verified by imprinting leaves on Luria-Bertani agar (LB) and incubating at 28°C for 7 days. Leaf blades were placed in extraction

TABLE 1
Fire blight disease management treatments applied to apple orchards in Geneva, NY

Treatment	Active ingredient	Product (rate per ha)	Timing ^y	Rationale ^z
Untreated	None	None	None	Control
1PBStrep	Streptomycin	FireWall 17 (1.68 kg) + Regulaid (1.4 liter)	B, PF	One PBStrep
3PBStrep	Streptomycin	FireWall 17 (1.68 kg) + Regulaid (1.4 liter)	B, PF, 1C	Three PBStrep
9PBStrep	Streptomycin	FireWall 17 (1.68 kg) + Regulaid (1.4 liter)	B, PF, 1C, 2C	Nine PBStrep
Serenade	<i>Bacillus subtilis</i> strain QST713	Serenade Optimum (1.40 kg) + Regulaid (1.4 liter)	B, PF	Biopesticide
Cueva	Copper soap	Cueva (4.67 liters)	B, PF	General antimicrobial

^y B = bloom, PF = petal fall, 1C = first cover (approximately 1 week after petal fall), and 2C = second cover (approximately 2 weeks after petal fall).
^z PBStrep = one, three, or nine postbloom applications of streptomycin.

bags (BioReba, Reinach, Switzerland) with 3 ml of extract buffer (sodium chloride at 160 g liter⁻¹, potassium monophosphate at 4 g liter⁻¹, sodium phosphate dibasic at 23 g liter⁻¹, potassium chloride at 4 g liter⁻¹, sodium azide at 4 g liter⁻¹, Tween 20 at 0.5 ml liter⁻¹, and polyvinyl pyrrolidone at 20 g liter⁻¹; pH 7.4) according to Muresan (2017). Each sample was macerated for approximately 30 s using a Homex 6 semiautomated homogenizer (BioReba). A 1-ml aliquot of the maceration liquid from each sample was frozen for culture-independent methods. The rest of the maceration liquid was processed immediately for culture-dependent investigations.

Culture-dependent investigations of bacterial communities.

In order to determine relative differences in culturable bacterial endophytes, bacteria were quantified as previously described by Tancos and Cox (2017). Maceration liquid was serially diluted and spread on Crosse Goodman (CG) media (Crosse and Goodman 1973) for identification of *E. amylovora* and on LB agar for enumeration of total endophytic populations, at appropriate dilutions for visual CFU enumeration. Maceration fluid was also spread on CG and LB amended with streptomycin at 100 mg liter⁻¹ for selection of SmR isolates. All media were amended with cycloheximide at 10 mg liter⁻¹ in order to inhibit eukaryotic growth. Plates were incubated at 28°C for 48 h and the resulting colonies were enumerated to calculate total endophytic bacteria per sample. Growth on CG plates was observed using a dissecting microscope to identify *E. amylovora* colonies based on characteristic cratering morphology (Crosse and Goodman 1973; Tancos and Cox 2016).

In order to characterize the most prevalent bacteria isolated, in 2019, a subset of bacteria with the most common unique colony morphologies was isolated and identified by sequencing the 16S ribosomal RNA (rRNA) gene using the primers 515f and 806r, as previously described by Walters et al. (2015) and Wasimuddin et al. (2019). PCR was performed in 25-μl volumes consisting of 12.5 μl of EmeraldAmp GT PCR Master Mix (Takara Bio USA, Mountain View, CA, U.S.A.), 1 μl each of forward and reverse primer, 2 μl of bacterial suspension, and 8.5 μl of nuclease-free H₂O. Cycling parameters were 3 min at 94°C; 35 cycles of 45 s at 94°C, 60 s at 50°C, and 90 s at 72°C; followed by a final extension of 10 min at 72°C. Resulting PCR products were verified by gel electrophoresis and the presence of an approximately 290-bp amplicon (Walters et al. 2015; Wasimuddin et al. 2019). Amplified DNA was purified using ExoSAP-IT Express PCR cleanup reagent (Thermo Fisher, Waltham, MA, U.S.A.) and sequenced at the Cornell Biotechnical Resource Center in Ithaca, NY using an ABI 3730xl capillary electrophoresis instrument (Applied Biosystems, Waltham, MA, U.S.A.). Nucleotide sequences were analyzed using the Basic Local Alignment Search Tool (NCBI) against the entire GenBank database, and identified to genus or species level.

Culture-independent investigations of bacterial communities.

For extraction of bacterial DNA, the reserved 1 ml of maceration fluid was centrifuged for 10 min at 13,000 × g to pelletize bacterial cells, and extraction was carried out on the pellet using the DNeasy PowerLyzer PowerSoil Kit (QIAGEN, Germantown, MD, U.S.A.) according to the manufacturer's instructions. Preliminary experiments investigating bacterial endophytic communities in the applied phyllosphere resulted in a high proportion of reads from plant plastids and mitochondria (>99% of reads). Therefore, extracted DNA samples were first processed with an initial round of thermocycling utilizing locked nucleic acid (LNA) clamps in order to maximize amplification of bacterial 16S rRNA and minimize amplification of plant plastids and mitochondria, as performed in other recent investigations of plant microbial communities (Braasch and Corey 2001; Ikenaga and Sakai 2014; Ikenaga et al. 2015; Liu et al. 2018; Lundberg et al. 2013; Pellestor and Paulasova 2004). The 16S region was first amplified as described by Ikenaga et al. (2015,

2018) and Lundberg et al. (2013) using the primer pair 63f/1492r (approximately 1,400-bp amplicon), including the addition of a pair of LNA clamps to block the amplification of off-target apple mitochondria and plastid sequences. PCR assays were conducted in triplicate in 25-μl volumes consisting of 12 μl of EmeraldAmp GT PCR Master Mix (Takara Bio USA), 1 μl each of forward and reverse primer, 3 μl each of forward and reverse LNA clamps (4 μM concentration), 3 μl of bacterial suspension, and 2 μl of nuclease-free H₂O. Cycling parameters were 3 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 70°C, 1 min at 54°C, and 2 mins at 72°C; followed by a final extension of 10 min at 72°C. Subsequently, samples were processed for 16S high-throughput sequencing according to the Earth Microbiome Project recommended protocols (Caporaso et al. 2011, 2012). PCR was performed in one step to amplify the V4 region of 16S rRNA gene using barcoded primers 515f and 806r, with Illumina adapters (Walters et al. 2015). PCR assays were conducted in 25-μl volumes consisting of 10 μl of Platinum Hot Start PCR Master Mix (Thermo Fisher), 2 μl of forward primer and reverse primers (5 μM), 2 μl of product from first PCR, and 9 μl of nuclease-free H₂O. Cycling parameters were 3 min at 94°C; 35 cycles of 45 s at 94°C, 60 s at 50°C, and 90 s at 72°C; followed by a final extension of 10 min at 72°C. The products of each PCR step were confirmed using gel electrophoresis. Triplicate PCR products were pooled and aggregate samples were purified using Agencourt Ampure XP bead (Beckman Coulter Inc., Brea, CA, U.S.A.). Purified amplicons were quantified using a Qubit 1× dsDNA HS Assay Kit (Invitrogen, Eugene, OR, U.S.A.) and TapeStation 2200 (Agilent, Santa Clara, CA), and pooled in equal concentration. Pooled DNA library was prepared as described previously (Gu et al. 2019) and sequenced on a MiSeq platform using 500-cycle v2 kits (Illumina, San Diego, CA, U.S.A.).

Following sequencing, 16S sequences were demultiplexed using deML (v 1.1.3). The demultiplexed sequences were then filtered to remove low-quality sequences using Trimmomatic (Bolger et al. 2014). Taxonomic abundance profiles and assignments were classified using the Kraken 2 classification tool (Wood and Salzberg 2014) and Bracken abundance estimator (Lu et al. 2017), with the SILVA database (Pruesse et al. 2007). Kraken 2 is an updated version of Kraken, released in 2019, that supports 16S rRNA analyses. Kraken and Bracken were chosen for our analysis because these tools are estimated to be up to 300 times faster and more accurate than other pipelines at 16S profiling (Lu et al. 2017; Wood and Salzberg 2014). Mitochondria and plastid sequences were removed using a Python script (extract_kraken_reads.py) which takes in a kraken-style output, kraken report, and taxonomy level to extract reads matching that level. Once the bacterial reads were extracted for each sample, they were subjected to the Kraken 2 classification tool with SILVA database and Bracken abundance estimator. Operational taxonomic units (OTUs) were defined by clustering with a 97% similarity threshold (3% divergence), OTUs with <0.01% abundance were filtered out, and the remaining OTUs were classified to the family level to create an OTU table for downstream analyses.

Data analysis. Culture-dependent investigations. Within each field in each year, bacterial abundance was calculated for each treatment by determining the average CFU per milliliter. The effects of treatment on bacterial abundance and percentage of antibiotic-resistant bacteria were determined by a mixed-effects model using the lmer command in the lme4 package in R (Bates et al. 2015), in which treatments were considered fixed effects and replications were considered random effects. All analyses were carried out in R version 3.6.3 (R Core Team 2020). Bacterial abundance values (CFU counts) were log transformed prior to analysis to normalize abundance values.

Culture-independent investigations. The dataset was analyzed in R version 3.6.3 (R Core Team 2020) using the vegan package version 2.5-6 (Oksanen et al. 2019). The α diversity was evaluated using Shannon and Simpson diversity indices, normality of data was evaluated using the Shapiro-Wilks test, and differences were determined using the Kruskal-Wallis nonparametric test and Dunn's test for pairwise comparisons. To determine whether bacterial communities differed by field or treatment, an OTU table was used to construct a Bray-Curtis dissimilarity matrix that was used to evaluate β diversity. The β diversity was further evaluated using modified Gower to reveal fold-changes between communities and the Morisita-Horn metric of overlap to reveal differences in the most prevalent taxa. To further investigate differences between field and treatment, nonmetric multidimensional scaling (NMDS) was conducted based on the Bray-Curtis dissimilarity matrix. NMDS analyses were implemented using the 'metaNMDS' function of the R package (Oksanen et al. 2019). Differences between sites or treatments were determined using a permutational multivariate analysis of variance, with the 'adonis' function using 'method = "bray", = "horn", or "gower"' as appropriate. Data were visualized using the ggplot2 program for R (Wickham 2016).

RESULTS

Culture-dependent investigations of bacterial communities. Total endophytic bacterial abundance, as determined by CFU counts on LB agar, ranged from 0 to 2.16 log(CFU) ml⁻¹ (Table 2). In orchard A, bacterial abundance was not significantly affected by treatment in 2018 ($P = 0.1430$) or 2019 ($P = 0.0503$). Across all treatments, higher bacterial abundance was observed in 2019 than in 2018. In orchard B, in 2018, culture-dependent investigations of bacterial communities were not possible due to insufficient number of CFU (i.e., <2) recovered from leaf tissues in many treatment replicates on the lowest dilution plates. In 2019, in orchard B, bacterial abundance was affected by treatment ($P = 0.0151$), with lower CFU counts for trees that received 3PBStrep or 9PBStrep treatments (the two higher doses) than untreated trees, but other

treatments were not significantly different ($P > 0.05$). *E. amylovora* was only detected in two samples across all treatments, fields, and years. These were both in 2019, in one sample from the untreated treatment in orchard A and one sample from the Cueva treatment in orchard B. Therefore, abundance of *E. amylovora* was too low to draw meaningful conclusions about treatment effects. Similarly, bacterial CFU were observed on media amended with streptomycin for only six total samples, and also too low in abundance to draw meaningful conclusions about treatment effects on streptomycin sensitivity in bacterial endophyte communities. These were in 2019, in one sample each from the untreated, 1PBStrep, 3PBStrep, and Serenade treatments from orchard A, and the untreated control and Serenade treatments from orchard B. Along these lines, none of the streptomycin treatments resulted in samples with a high number (>10) of SmR CFU.

The most commonly recovered bacterial genera included *Pseudomonas*, *Bacillus*, and *Pantoea*. Most common were *Pseudomonas* spp., which were present in 64.6% of all samples (66.7 and 62.5% of samples from orchard A and B, respectively), followed by *Bacillus* in 35.4% of all samples (33.3 and 37.5%) and *Pantoea* in 27.1% of all samples (25.0 and 29.2%). Two other genera that were less frequently detected included *Curtobacterium* and *Fronihibitans* in 4.2 and 2.1% of all samples, respectively.

Culture-independent investigations of bacterial communities. 16S rDNA high-throughput sequence data were submitted to NCBI under the Bioproject PRJNA663546 with Sequence Read Archive accession numbers SRR1270323 to SRR12708369. After quality control, in total, 4,332,792 bacterial reads were retained, ranging from 34,938 to 178,302 per sample. After mitochondrial and plastid sequences were filtered out, reads present in > 0.01 relative abundance in all samples were assigned to 37 and 59 families for 2018 and 2019, respectively, (Supplementary Datasets S1 and S2).

The most abundant families detected in 2018 included *Amoebophilaceae*, *Desulfovibrionaceae*, *Bacillaceae*, *Enterobacteriaceae*, *Burkholderiaceae*, and *Pseudomonadaceae*, representing 61.0, 6.3, 5.7, 4.8, 4.8, and 4.1% of the total dataset, respectively (Fig. 1A). In 2019, the most abundant families were similar and included *Amoebophilaceae*, *Enterobacteriaceae*, *Pseudomonadaceae*, and *Desulfovibrionaceae*, making up 33.4, 15.8, 11.5, and 11.1% of the total dataset (Fig. 1B). Other families were present in 3.5% or less. The 10 most common families made up 94.9 and 86.3% of the total dataset in 2018 and 2019, respectively, and were present in all samples.

The α diversity, as measured by Shannon diversity index (Fig. 2), in 2018, was affected by treatment ($P = 0.0054$). Lower diversity was observed in trees receiving the 3PBStrep treatment than in untreated trees ($P = 0.0066$) but was not affected by other treatments ($P > 0.05$). In 2019, α diversity was unaffected by treatment ($P = 0.7545$). Orchards A and B did not differ in α diversity in 2018 ($P = 0.0815$) but, in 2019, there was greater diversity in orchard A than orchard B ($P = 0.0005$). Simpson diversity index analysis indicated similar trends. In 2018, diversity was influenced by treatment ($P = 0.0478$). Lower diversity was observed in trees receiving the 3PBStrep treatment than Serenade ($P = 0.0476$) and untreated trees ($P = 0.0059$). In 2019, diversity was not affected by treatment ($P = 0.7734$). In both 2018 and 2019, Simpson index indicated that there was higher diversity in orchard A than orchard B ($P = 0.0478$ and 0.0014 for 2018 and 2019, respectively). In 2018, 37 families were detected, with 16 (42.1%) detected in all treatments in both orchards. In 2019, 60 families were detected, and 20 (33.3%) were detected in all treatments in both orchards. Families were seldom limited to a single treatment–orchard combination. In 2018, all families were found in at least two treatment–orchard combinations and, in 2019, four families (0.07%) were only detected in one treatment–orchard combination.

TABLE 2 Abundance of bacterial endophytes in apple leaves treated with different fire blight management programs for two orchards in Geneva, NY in 2018 and 2019 ^y				
Treatment ^z	Orchard A		Orchard B	
	2018	2019	2018	2019
Untreated	0.99 ± 0.70 a	0.98 ± 0.07 a	NA	2.16 ± 0.39 b
1PBStrep	0.50 ± 0.67 a	0.78 ± 0.39 a	NA	0.87 ± 0.34 ab
3PBStrep	0.35 ± 0.26 a	0.00 ± 0.00 a	NA	0.45 ± 0.27 a
9PBStrep	0.35 ± 0.26 a	0.69 ± 0.30 a	NA	0.26 ± 0.26 a
Cueva	1.25 ± 1.18 a	0.56 ± 0.33 a	NA	0.87 ± 0.34 ab
Serenade	0.35 ± 0.35 a	0.91 ± 0.35 a	NA	1.16 ± 0.30 ab
P value	0.1430	0.0503	NA	0.0151
^y Abundance was measured as the log of CFU per milliliter on Luria-Bertani media. Within a column, different letters indicate significantly different mean CFU ml ⁻¹ based on Tukey honestly significant difference test ($P < 0.05$). NA = CFU counts were insufficient for enumeration (<2 CFU on lowest dilution plates).				
^z 1PBStrep = 1 postbloom application of streptomycin, FireWall 17 (1.68 kg) + Regulaid (1.4 liter); 3PBStrep = three postbloom applications of streptomycin; 9PBStrep = nine postbloom applications of streptomycin; Serenade = the biopesticide formulation of <i>Bacillus subtilis</i> strain QST713, Serenade Optimum (1.40 kg) + Regulaid (1.4 liter); and Cueva = the copper soap product Cueva (4.67 liters).				

NMDS ordination and all β -diversity metrics (Bray-Curtis, Morisita-Horn, and Gower) indicated strong separation between orchard A and B in both 2018 and 2019, as determined by the permutational analysis of variance using the 'adonis' function in R ($P < 0.001$ for all metrics in both years; $R^2 = 0.1258, 0.1651$, and

0.1262 in 2018 and $R^2 = 0.1213, 0.1987$, and 0.0935 in 2019 for Bray-Curtis, Morisita-Horn, and Gower, respectively) (Table 3; Fig. 3). Separation by treatment was never detected for any of the β -diversity metrics evaluated in 2018 (Bray-Curtis: $P = 0.061$, $R^2 = 0.1446$; Morisita-Horn: $P = 0.098$, $R^2 = 0.1492$; Gower: $P = 0.091$,

Fig. 1. Relative abundance of the 10 most dominant bacterial families in endophytic bacterial communities of leaf samples in **A**, 2018 and **B**, 2019, collected from apple trees treated with different fire blight management programs, in two Gala apple orchards in 2018. Colors indicate bacterial families.

$R^2 = 0.1377$) or in 2019 (Bray-Curtis: $P = 0.069$, $R^2 = 0.1290$; Morisita-Horn: $P = 0.348$, $R^2 = 0.1149$; Gower: $P = 0.062$, $R^2 = 0.1322$) (Table 3).

DISCUSSION

In this work, we investigated the effects of PB streptomycin applications used for fire blight management on the endophytic bacterial communities of the apple canopy. A growing understanding of the impacts of antibiotic applications on microbial communities in agricultural landscapes, including apple orchards as one of the greatest users of antibiotics in agriculture, has been facilitated by continuously improving and more available technologies in both culture-dependent and independent techniques. Numerous reports have scrutinized the use of antibiotics in agriculture, citing the potential for selecting antibiotic resistance in pathogen populations, the subsequent loss

of invaluable crop protection tools, and potential off-target effects to the agroecosystem and other environments (McManus et al. 2002; Stockwell and Duffy 2012).

In our culture-dependent investigations, a higher bacterial abundance was observed in 2019 than in 2018, regardless of treatment. This discrepancy may have been caused by environmental conditions, in particular rainfall, which would have facilitated greater bacterial growth and higher degree of biofilm formation. In New York in 2018, average precipitation accumulated from April through August (the time of active tree growth and application of treatments) was 50.52 cm, 1.63 cm greater than the 30-year average in the state. However, in 2019, average precipitation accumulated from April through August was 56.57 cm, a 7.7-cm departure from the 30-year average (reported by the Northeast Regional Climate Center, <http://www.nrcc.cornell.edu/regional/tables/tables.html>). The significantly greater precipitation in 2019 would have likely contributed to the greater observed bacterial

Fig. 2. The α diversity (Shannon Diversity Index) values displayed as box plots for endophytic bacterial communities in **A**, 2018 and **B**, 2019 of leaf samples collected from apple trees treated with different fire blight management programs, in two different Gala apple orchards in 2018. 1PBStrep, 3PBStrep, or 9PBStrep = one, three, or nine applications of streptomycin postbloom, respectively; Cueva = a fixed copper bactericide, formulated as a soap, applied at bloom and petal fall; and Serenade = a formulation of a strain of *Bacillus subtilis*, applied at bloom and petal fall.

abundance in this year. However, it did not seem to affect the composition of community differences between the 2 years.

In the present study, both culture-dependent and independent methods described similar communities of bacterial endophytes in apple foliage in both orchards. Members of the *Pseudomonadaceae* family were most commonly isolated in culture and most abundant in 16S sequencing analyses. The common detection of *Pseudomonas*, *Bacillus*, *Enterococcus*, and *Pantoea* spp. in culture was consistent with community members found in most abundance in 16S high-throughput sequencing. *Curtobacterium* and *Fronihibitans* were also expected members of this community because they are common Gram-positive inhabitants of the soil and foliar environments, respectively. These results are consistent with other studies of the apple phyllosphere, in which the most common genera isolated or detected from foliar and floral tissues included *Pseudomonas*, *Pantoea*, *Bacillus*, *Clavibacter*, *Curtobacterium*, *Erwinia*, and *Micrococcus* (McGhee and Sundin 2011; Tancos and Cox 2017; Yashiro and McManus 2012).

The high abundance of *Amoebophiliaceae* detected in all samples in both years was interesting and unexpected. A large part of this family is composed of the genus *Candidatus Cardinium*, which comprises a group of Gram-negative bacteria commonly found as parasites within arthropods and nematodes (Bergey and Holt 2015; de Vienne 2016; Erban et al. 2020; Nakamura et al. 2009). Detection of these bacteria in such high abundance can be regarded as evidence for horizontal transfer of endosymbionts between insect and plant hosts. This is an actively growing area of investigation, with implications for the life cycles of both the bacterial parasites and their hosts (Chrostek et al. 2017; Gonella et al. 2015). These bacteria would be undetected by culture-based methods, which underlines the importance of using sequencing technologies to characterize microbial communities. The high abundance of *Desulfovibrionaceae* was unexpected in apple leaf samples. This family is composed of five described genera, which are desulfonating bacteria most typically found in anoxic marine environments (Kuever et al. 2015).

The high abundance of the remaining organisms was not unexpected. The *Burkholderiaceae* family is composed of ecologically extremely diverse organisms, including environmental saprophytic organisms, phytopathogens, opportunistic pathogens, and mammalian pathogens (Coenye 2014, Garrity et al. 2015). Similarly, members of *Microbacteriaceae*, *Sphingomonadaceae*, *Microscillaceae*, and *Rhizobiaceae* families are diverse and commonly isolated from environmental samples, including soil, water, and the plant phyllosphere and rhizosphere (Alves et al. 2014; Bergey and Holt 2015; Evtushenko 2015; Glaeser and Kämpfer 2014). *Beijerinckiaceae* spp. are metabolically diverse aerobic bacteria, which fix nitrogen (Marin and Arahall 2014). Overall, the bacterial endophytic communities identified through both culture-dependent and independent methods were largely expected in the environmental niche we investigated, and commonly found in microbial communities described in other investigations of the apple microbiome.

Applications of streptomycin for fire blight management have minimal effects on endophytic bacterial communities.

We found little evidence of PB streptomycin applications affecting endophytic bacterial communities. In only a few comparisons, a greater number of antibiotic applications led to a small reduction in microbial abundance. Specifically, in only one orchard and in 1 year, trees receiving higher numbers of streptomycin applications (3PBStrep and 9PBStrep) had lower culturable bacteria than untreated trees. Additionally, in 2018, bacterial abundance (α diversity) was lower for 3PBStrep than untreated trees. However, this was not corroborated by results for the 9PBStrep treatment, for which diversity was not affected. If an increasing number of streptomycin applications was responsible for this community shift, we should have expected to see a greater impact on trees receiving the 9PBStrep treatment, and greater impact in 2019 after two consecutive years of treatments. Community composition, as indicated by β -diversity metrics, was never different for treatments in our analyses. Based on these results, we found minimal evidence to support the notion that substantial decrease in endophytic bacterial communities can be caused by streptomycin programs. This was different than our previous work, in which increasing number of streptomycin applications resulted in rapid selection for SmR in epiphyte communities, with an increase in number of SmR *Pseudomonas* and reduction in the number of SmR *Pantoea agglomerans* (Tancos and Cox 2017). The difference in observations between our current and previous work is that the former study examined epiphytic bacterial communities, which would have more direct exposure to treatments and, therefore, be less resilient than the endophytic communities examined here.

There is also concern that antibiotic use may have greater impact on microbial communities than organic or biopesticide-based programs. We found little evidence supporting this notion. Bacterial communities of trees receiving Serenade, and OMRI-listed formulation of antimicrobial metabolites produced from fermentation of a strain of *B. subtilis*, generally were not different than that with other streptomycin programs, with the one exception of 3PBStrep in 2018. Again, this trend was not consistent with the 9PBStrep treatment or the observations after the second consecutive year of treatment. Previous work examined the difference in microbial communities of orchards under conventional and organic management (Ottesen et al. 2009). Streptomycin was used in both management programs, because this study was carried out prior to the prohibition of antibiotics in organic agriculture in 2014 (Granatstein et al. 2015). Therefore, differences in bacterial communities observed in this earlier study could be attributed to other management factors such as fungicides, insecticides, fertilizers, or herbicides. Moreover, the study by Ottesen et al. (2009) also

TABLE 3 Results of permutational multivariate analysis of variance showing the effects of fire blight management program and orchard block on β diversity of endophytic bacterial community composition in the phyllosphere of Gala apple fruit for various β -diversity metrics ^y				
Metric	2018		2019	
	P value	R ² value	P value	R ² value
Treatment ^y				
Bray-Curtis	0.061	0.14459	0.069	0.12901
Morisita-Horn	0.098	0.1492	0.348	0.11493
Gower	0.091	0.13765	0.062	0.13219
Orchard ^z				
Bray-Curtis	<0.001	0.12584	<0.001	0.12128
Morisita-Horn	<0.001	0.16512	<0.001	0.19869
Gower	<0.001	0.12622	<0.001	0.09353
^y Gala trees at Cornell AgriTech in Geneva, NY received one of the following treatments: 1 = untreated; 2 = one postbloom application of streptomycin (PBstrep); 3 = three PBstrep; 4 = nine PBstrep; 5 = application of Cueva (a copper soap); or 6 = application of the biopesticide Serenade optimum (<i>Bacillus subtilis</i> strain QST713).				
^z Trees were located in one of two orchard blocks. Orchard A = Gala on B.9 rootstock, planted in 2000 at 1.8-m in-row spacing, and orchard B = a high-density planting of Gala on G.935 rootstock planted in 2016 at 1-m in-row spacing.				

examined only epiphytic communities, which would have more direct contact with orchard sprays and other inputs than endophytic communities. Our findings suggest that conventional fire blight management with antibiotics and biopesticide-based programs are likely similar in their effects on endophytic bacterial communities. In addition, we observed very little change in endophytic bacterial communities, even when the number of streptomycin applications was far greater than the number typically applied under responsible commercial practices, demonstrating a very high level of resiliency of these communities.

Impact of orchard site on endophytic bacterial communities.

Greater differences in microbial community composition and diversity were observed between orchards than among the management programs. This could be related to the resident bacterial

communities in the environment at each site (soil, air, and water). Orchards A and B were located in very close proximity (<0.25 miles), indicating that these communities are extremely locality specific. Host factors such as cultivar, rootstock, and tree history could play a large role in shaping the microbial communities. Liu et al. (2018) found that endophytic communities varied by different rootstock and scion combinations, suggesting a genotype-specific influence, and Steven et al. (2018) found evidence for cultivar and floral anatomy influencing the microbial communities. Additionally, studies of the floral microbiome provided strong evidence that this niche selected for specific communities in a successional manner, demonstrating the profound effect that hosts could play in shaping their microbial communities, even when challenged with *E. amylovora* (Cui et al. 2020; Shade et al. 2013b). In our study, the

Fig. 3. Nonmetric multidimensional scaling (MDS) ordination of Bray-Curtis dissimilarity measures in **A and C**, 2018 or **B and D**, 2019 of leaf samples collected from apple trees treated with different fire blight management programs, in two different Gala apple orchards in 2018. Colored by treatment (A and B) or by orchard (C and D). Ellipses define the upper 95th percentile limit of the respective distribution.

same cultivar was investigated but orchards included different rootstocks, year of establishment, and planting systems. Future work is warranted to investigate these host- and orchard-specific communities and the factors that shape them. Improved understanding of these dynamics could result in better recommendations for plant health and disease resistance.

In this work, we investigated the season-long effects of streptomycin application for fire blight management on the endophytic bacterial community of the apple canopy. Using both culture-dependent and independent methods, we described communities consistent with other investigations of the apple tree tissues, dominated by the families *Amoebophilaceae*, *Pseudomonadaceae*, *Enterobacteriaceae*, *Desulfovibrionaceae*, and *Bacillaceae*. Our results provided evidence that management programs had minimal effect on these communities and that communities were more greatly influenced by the orchard blocks than by antibiotic applications. This work adds to the growing body of evidence that current commercial practices are sustainable solutions for fire blight management.

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