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Analysis of the Cultured Microbiome of Fertilization-Stage Maize Silks (Styles) Reveals Taxonomic Relationships Across North American Maize Genotypes and Heterotic Groups

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Abstract: The style is the female reproductive channel in flowers, receiving pollen and transmitting male gametes through elongating pollen tubes to the ovules during fertilization. In maize/corn, the styles are known as silks. Fertilization-stage silks contain diverse bacteria, possibly originating from pollen. Bacteria were cultured and individually sequenced from the tip and base portions of healthy, fertilization-stage silks of 14 North American maize genotypes, resulting in 350 isolates, spanning 48 genera and 221 OTUs. The objective of this study was to taxonomically analyze these bacteria in the context of the maize host tissue and genotype, taking advantage of long-read (V1–V9) 16S Sanger sequencing. The results suggest that the maize genotype and heterotic breeding group may impact the bacterial diversity of healthy, fertilization-stage silks. Some taxa were relatively conserved across maize genotypes and silk tip/base locations, including *Pantoea*, which may represent part of the core microbiome or form stable, symbiotic relationships with healthy, pollinated silks. We also observed similarities between the silk microbiomes of maize genotypes that were related by plant pedigree; these preliminary results suggest inheritance or the ability of related genotypes to recruit common bacterial taxa. Overall, this study demonstrates that healthy maize silks represent a valuable resource for learning about relationships between plant reproductive microbiomes.

Keywords: style; transmitting silk stage; heterotic group; microbiome; cultured bacteria; pollen tube; vertical transmission; maize genotypes; core taxa; taxonomy

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1. Introduction

In angiosperms, the styles are reproductive channels that male gametes travel through to reach an ovule [1]. Maize (*Zea mays* L. ssp. *mays*, corn) possesses uniquely long style tissues commonly known as silks—they are connected to the cob and emerge from beneath enveloping husk leaves to receive pollen [2,3]. Pollen is released at the top of each plant from the male inflorescence known as the tassel; the pollen is dispersed by wind onto silks on the female inflorescence (ear including its central cob) [2]. When a silk receives pollen, the pollen grain germinates and elongates a pollen tube that grows through the silk channel; the sperm nuclei then migrate toward the ovule, and subsequent double fertilization leads to the formation of a seed [2]. There are hundreds of silks on each cob of maize, and each kernel on a cob of maize marks a successful fertilization event.

The fertilization stage is a critical time point for a silk, because it actively hosts a pollen tube that transmits male gametes; silks at this stage have been called "transmitting silks" [4].

Interestingly, transmitting silks possess culturable microbes. Specifically, microbes have been cultured from open-pollinated, exposed silk tissue from randomly selected cobs from select maize fields in Brazil [5]. In Canada, we have shown that cultured bacteria from healthy, fertilization-stage silks encode genes and express traits that may help promote their survival in or on pollen or silks and also may promote host reproduction [6]. As to the reason that transmitting silks possess microbes, one possibility is that these microbes originate from pollen/pollen tubes and utilize the silks to migrate to developing seeds. Indeed, bacteria that are vertically transmitted into the seed presumably originate from the male parent, i.e., the pollen tube [7,8] or male gametes [9], or the female parent. In hybrid maize, part of the seed microbiome has been shown to be shared with both male and female parents [10]. There is evidence of vertical transmission of microbes from parental plants to their offspring [11], including *Bacillus* where there is some evidence of inheritance in maize via pollen [12], but this is yet to be explored in pollinated silks. Maize genotypes related by pedigree can provide unique insights and further the understanding of reproductive microbiomes.

In addition to culturing, our group has used next generation sequencing (NGS) to show that transmitting silks have a complex microbiome [4]. The study showed that field-grown silks from a diversity panel of North American commercial maize genotypes (11 inbreds and 3 commercial hybrids) contained >1300 bacterial genera and >5000 taxa. The study reported a core microbiome (dominant and prevalent relative abundance) consisting of up to 11 taxa that spanned the majority of the host genotypes sampled but used short-length 16S sequencing (V4 region), which limited taxonomic resolution [13–15]. Additionally, the previous NGS methods used different kits for tip and base samples, which did not allow for comparisons to be made between tip and base portions of the silks.

In the above NGS study [4], fertilization-stage silks were harvested, exposed regions discarded, and then the remaining husk-encased tissue was dissected into tip and base fractions. In one of the field seasons, the tip and base samples were then further split, with a subset used for NGS and another fraction frozen in glycerol to enable systematic culturing [16]. Unlike NGS, which deals with complex templates, culturing allows for longer-read 16S Sanger sequencing from pure cultures, which contributes to better taxonomic resolution [17].

Half of the silks in the above study were treated with the pathogen, *Fusarium graminearum*. Both the NGS analysis [4] and a parallel cultured microbiome study reported shifts in the microbiome based on the *F. graminearum* treatments [16]. However, the untreated, healthy silks lacked in-depth analyses of the culturable microbiome based on long-read 16S Sanger sequencing. Therefore, the objective of this study is to taxonomically analyze microbes cultured from healthy maize silks in the context of their host, including relationships between the host genotypes.

The healthy fertilization-stage silk microbiome likely plays multiple roles which are yet to be explored. For example, in vitro testing and gene mining of 25 bacteria from the healthy silks revealed a potential for members of the healthy fertilization-stage microbiome to promote host reproduction and tolerance to stresses such as drought and nitrogen limitation [6]. Given this knowledge, in-depth taxonomic investigation of the healthy fertilization-stage silk microbiome is warranted to catalog this microbiome in its natural state without the influence of pathogens.

Here, we conducted an in-depth analysis of the bacterial microbiome cultured from healthy fertilization-stage maize silks of a North American modern maize diversity panel to further the understanding of healthy plant reproductive microbiomes. The taxonomic composition was evaluated in the unique context of host maize genotype, heterotic group, silk tip/base location, and potential for shared microbes within maize pedigrees.

2. Materials and Methods

Microbes were cultured from the open pollinated silks of 14 maize genotypes (11 Agriculture and Agri-Food Canada inbred lines and 3 commercial Pioneer hybrids).

These genotypes were selected because they were adapted to Ontario, Canada, varied in their resistance to *Fusarium graminearum*, and were genetically diverse—spanning the heterotic breeding groups that underlie commercial North American maize as described in Thompson et al. [16] (Table 1; Figure 1). Briefly, husk-covered portions of silks were dissected into three segments under sterile conditions, and the exposed silk tips were discarded. Silks from three average-sized cobs were pooled for each plot, and then a subsample of ~10 individual silk segments were randomly selected for culturing from field block 1. The tip and base segments were frozen at −80 °C in 40% glycerol and later ground with 600 µL 0.05 M sodium phosphate buffer (14.425 mL of 1 M Na_2HPO_4, 10.575 mL of 1 M NaH_2PO_4, with autoclaved ddH_2O in a final volume of 500 mL, pH 7) and cultured at 30 °C on potato dextrose agar (PDA, pH 5.6) and LB agar (pH 7.2) in multiple dilutions (full concentration, 1/100, and 1/1000 for tip samples, whereas base samples had a lower number of colony forming units and thus had full concentration and 1/100). The buffer was also plated onto uninoculated LB and PDA plates as controls to ensure that there was no background microbial contamination. As previously described [16,18], unique, individual colonies were restreaked on day 3 and day 5. Isolates were cultured in liquid media, stored as glycerol stocks, and underwent DNA isolation (QIAGEN, Venlo, The Netherlands, QIAamp DNA mini kit, cat# 51306), 16S rDNA sequencing (Figure S1), contig assembly, Basic Local Alignment Search Tool Nucleotide (BLASTn) searching to assign taxonomic predictions, and OTU assignments. Tubes of uninoculated liquid media (LB and potato dextrose broth) were incubated alongside liquid cultures to act as controls. As mentioned previously [16], the primer set was changed from 799F [5′-AACMGGATTAGATACCCKG-3′] and 1492R [5′-GGTTACCTTGTTACGACTT-3′] (V5-V9 regions of 16S rDNA) to 27F [5′-AGAGTTTGATCMTGGCTCAG-3´] and 1492R [5′-GGTTACCTTGTTACGACTT-3′] (V1–V9 regions) midway through the experiment to allow direct comparison between the sequences from cultured bacterial isolates here and the V4-16S MiSeq sequences (~254 bp) previously generated from the silk pools [4] and to provide better taxonomic resolution (~700 bp with 799F/1492R vs. ~1400 bp with 27F/1492R). Every batch of PCR included a negative control, which contained all reagents except genomic DNA. The 16S sequences were filtered for quality by removing sequences that contained <500 base pairs or had >5% N's within the sequence. Taxonomic predictions were assigned using the best estimates based on the 16S sequences. BLASTn searches were performed to predict taxonomy based on best matches to 16S ribosomal RNA sequences in the bacteria and archaea database at NCBI. "Moderate stringency genus" and "moderate stringency species" were recorded as the top hit for genus and species, respectively. In cases where there were two top matches with equal Max Score and %identity values, both were recorded. Additional filtering criteria were applied to ensure sequence quality. Genera were also included in the "high stringency genus prediction" column if they met either of these two criteria: (1) the top hit had 100% identity match; or (2) alternatively, the top hit had at minimum five more base pair matches than the next best predicted genus, and additionally (2a) had at minimum a 98.5% identity match or (2b) the genus was consistent for the top 20 results. If the conditions were not met, a zero was recorded for the "high stringency genus prediction". Species were also included in the "high stringency species prediction" column if they met either of these two criteria: (1) the top hit had 100% identity match; or (2) the isolate had a "high stringency genus prediction", and additionally, (2a) the top hit had at minimum a 98% identity match and at minimum two more base pair matches than the next best predicted species, or (2b) the top hit had at minimum a 99% identity match and at minimum 1 more base pair match than the next predicted species. The cultured OTUs were assigned based on comparisons amongst the 16S sequences, determining which sequences were distinct from one another, and which sequences were conserved (i.e., likely to belong to the same species/strain). Some sequences were assigned multiple cultured OTUs due to short sequences or an unknown base "N" in key locations. The results from the untreated (healthy) plots are reported in the current publication.

Table 1. Summary of the pedigrees of the host maize genotypes used in this study. The seeds were obtained from the Agriculture and Agri-Food Canada breeding program (Ottawa Research and Development Centre, Canada). The red text indicates the parents in pedigree relationships between genotypes. CO462 and CO452 share a parent. CO431 is a parent in the pedigree for CO448. CO432 and CO433 are parents in the pedigree for CO449. The heterotic groups are color-coded to highlight genotypes that share common ancestry. The figure was adapted from Thompson et al., 2023 and Khalaf et al., 2021 with additional notes [4,16,19–21].

Inbred/ Hybrid Line	Year Released	Derivation	Heterotic Group	Heterotic Group Description	Grain Type	Days to Silking
CO462	2016	CO388×W153R	BSSS / Minnesota 13	US Hybrid era stiff stalk/Minnesota US Pre-Hybrid era	Dent	75
CO452	2014	(CO388×CO328)×CO388(4)	BSSS	US Hybrid era stiff stalk	Dent	80
CO444	2007	S1381×CO382	European flint	European flint, Pre-Hybrid era	Flint	79
CO448	2012	CO273×CO431	P3990 / Iodent	Pioneer Hybrid/US Pre-Hybrid era Corn Belt Dent	Dent	70
CO325	1991	(CO256×CO264) CO264 (2)	Early Butler	New York, Pennsylvania US Pre-Hybrid era	Dent	76
CO449	2012	CO432×CO433	Minnesota 13	Minnesota US Pre-Hybrid era	Dent	75
CO441	2002	Jacques 7700×CO298	Lancaster	Pennsylvania US Pre-Hybrid Corn Belt Dent	Dent	72
CO431	1999	Fusarium Resistant Synthetic	Iodent	US Pre-Hybrid Corn Belt Dent	Dent	71
CO433	2000	Pride K127	Minnesota 13	Minnesota US Pre-Hybrid era	Dent	77
CO430	1999	Fusarium Resistant Synthetic	P3990	Pioneer Hybrid	Dent	69
CO432	2000	Fusarium Resistant Synthetic C1	Minnesota 13	Minnesota US Pre-Hybrid era	Dent	74
P35837◊	NA	NA	NA	Pioneer Hybrid	Dent	NA
P38157◊	NA	NA	NA	Pioneer Hybrid	Dent	NA
P9855HR◊	NA	NA	NA	Pioneer Hybrid	Dent	NA

Abbreviations: ◊ = commercial Pioneer hybrid, NA = Not Available.

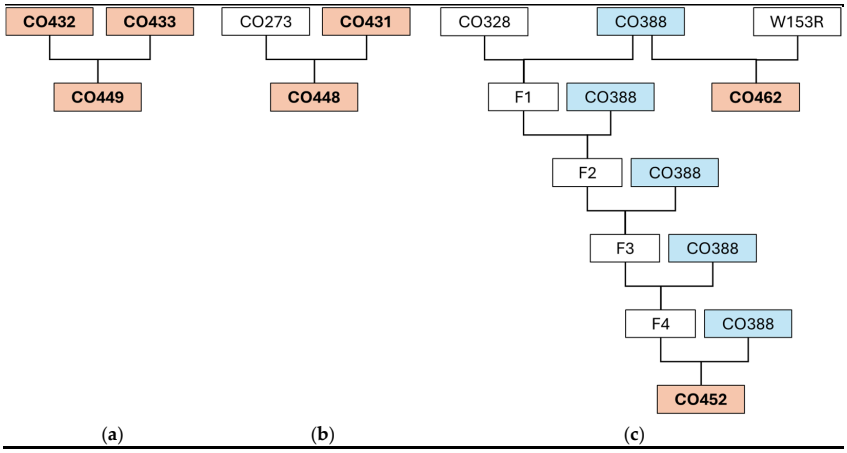

Figure 1. (**a**–**c**) Illustrations of the three pedigrees that connect related host maize genotypes used in this study. The orange boxes indicate maize genotypes that were used in this study. The blue boxes indicate CO388; a cross between CO388 and CO328 was backcrossed to CO388 four times in the development of CO452.

Comparisons were made amongst silk bacteria cultured across heterotic groups, and from the tip and base portions of silks. To identify taxa that might be specific to the silk tip or base, we focused on those that were consistently cultured from only one silk location across host genotypes, given the limitations of culturing. Comparisons were also made amongst silk bacteria cultured from maize genotypes related by pedigree, to investigate whether transmitting silk associated microbiota or host compatibility alleles may be inherited.

3. Results

3.1. Taxonomic Overview of Cultured Bacteria from Silks of Maize Spanning Diverse Heterotic Groups

In total, 367 bacterial isolates from husk-covered silks of 14 maize genotypes spanning 7 heterotic groups were sequenced. Following 16S sequencing and filtering for quality, 350 bacterial sequences remained (Table S1). Of these, 188 isolates were from the silk tip samples and 162 were from base tissues. These isolates spanned 4 phyla, 8 classes, 16 orders, and 28 families (Figure 2; Tables S2–S5). The majority of isolates belong to the phylum Pseudomonadota (232 isolates), the class Gammaproteobacteria (193 isolates), the order Enterobacterales (152 isolates), and the families Erwiniaceae (78 isolates) and Enterobacteriaceae (69 isolates). There were 48 genera based on the moderate stringency criteria, or 42 genera based on high stringency criteria. The most commonly cultured genera included *Pantoea* (range 32–73 isolates: high stringency and moderate stringency criteria, respectively), *Microbacterium* (29–29 isolates), *Klebsiella* (18–27 isolates), *Lactococcus* (24–24 isolates), and *Stenotrophomonas* (21–24 isolates) (Figure S2; Table S6). There were a total of 94 bacterial species based on moderate stringency matches, or 67 species based on high stringency criteria (Table S1). Of the 94 species, 73 were cultured only once or twice, while 21 were cultured 3 to 36 times each (Figure S3a; Tables S7 and S8). The most commonly cultured species in rank order, based on moderate stringency criteria, were *Pantoea agglomerans*, *Lactococcus lactis*, *Pantoea ananatis*, and *Stenotrophomonas pavanii*; or based on high stringency matches, were *L. lactis*, *Microbacterium testaceum*, and *P. agglomerans*.

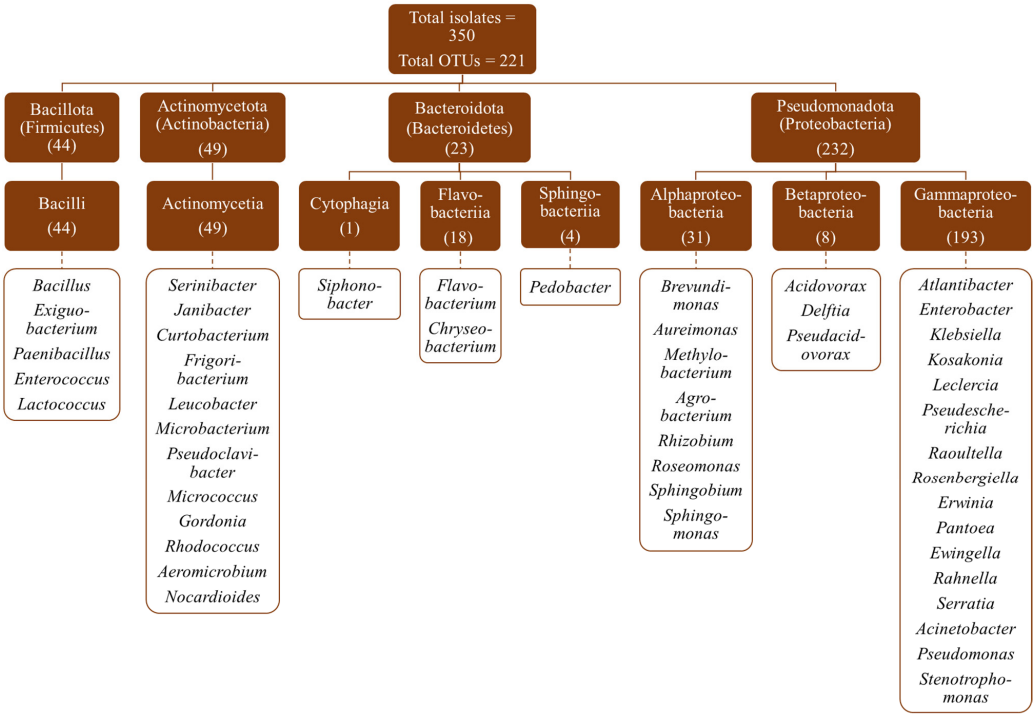

Figure 2. Taxonomic overview tree of bacteria cultured from pollinated maize silks (tip and base combined) at the phylum, class, and genus level. Numbers in brackets indicate the total number of isolates within each phylum and class. Two isolates were unassigned at the phylum level and, thus, not included. The dashed lines indicate that the taxonomy levels between class and genera have been omitted. Genera were assigned here based on moderate stringency criteria. The 16S bacterial sequences from isolates were given taxonomic assignments using the BLASTn tool on NCBI. Isolates that had multiple equal first matches were assigned only to the taxonomic level that was shared by the equal first matches. For example, an isolate with equal first matches being *Rahnella variigena* and

Raoultella terrigena received the following assignments: phylum = Pseudomonadota, class = Gammaproteobacteria, order = Enterobacterales, with all taxonomic levels from family to species = unassigned because *Rahnella* and *Raoultella* belong to different families). Further details are in Supplementary Table S1.

3.2. Conservation and Diversity of Cultured Silk Bacteria Across Heterotic Groups

Some taxa were conserved across all 14 maize genotypes: phylum Pseudomonadota (27/28 samples overall), class Gammaproteobacteria, order Enterobacterales, and family Enterobacteriaceae (Tables S2–S5). The genera *Pantoea* and *Microbacterium* were cultured from 13 and 10 genotypes, respectively (Table S6). *Pantoea* included much diversity (Figure S4).

Though maize hybridization has recently been reported to be associated with changes in the microbiome [22–25], the three commercial Pioneer hybrids had moderate diversity at the phyla, class, order, family, and genus levels compared to the inbreds (Tables S2–S6).

The Minnesota 13 genotypes produced relatively low diversity at higher taxonomic levels (genera to phyla) compared to other heterotic groups, with most isolates belonging to the phylum Pseudomonadota (Proteobacteria) and the class Gammaproteobacteria (Tables S2–S6). The individual maize genotypes of the European Flint and Lancaster heterotic groups hosted relatively high diversity.

Of the 94 bacterial species, 35 were cultured from more than one host genotype, and 18 were cultured from three or more hosts (Table S7). The most prevalent species was *P. agglomerans*, which was cultured from 11 of the 14 genotypes. *P. ananatis* and *S. pavanii* were each cultured from 8/14 maize genotypes, while *L. lactis* was cultured from 5/14 maize genotypes.

Despite some degree of conservation of specific bacterial taxa across maize genotypes, a large number of taxa could only be cultured from a single genotype. Of the 48 genera, 19 (40%) were cultured from only a single genotype: five belonged to CO444 (European Flint), three belonged to CO441 (Lancaster), and three to CO431 (Iodent). Further details can be found in Supplementary Texts S1–S3.

3.3. Conservation and Diversity of Cultured Silk Bacteria at the OTU Level

There were 221 cultured OTUs in the population (Table S1). The most common cultured OTUs were a taxon of *L. lactis* (OTU 158, with 17 isolates) and a taxon of *S. pavanii* (OTU 375, with 15 isolates) (Table 2). There were 153 cultured OTUs that were present in only one maize genotype, 42 were present in two genotypes, and 26 were present in three or more genotypes. For 12/14 genotypes, the number of unique OTUs cultured from each genotype ranged from 23 to 34 (Tables 2, S8 and S9). One of the Minnesota 13 genotypes had only six OTUs, but also had only seven cultured isolates. The three commercial hybrids ranged from 15 to 24 OTUs each.

In terms of conservation, the most prevalent OTU was again OTU 375 (a taxon of *S. pavanii*, as mentioned in [16]), which was cultured from six maize genotypes spanning diverse heterotic groups (E. Butler, BSSS, Minn 13, P3990/Iodent, Lancaster, Pioneer Hybrid) (Table 3). Similarly, OTU 158 (*Lactococcus lactis*, as mentioned in [16]) appeared in every tissue sample containing *L. lactis* across five maize genotypes spanning heterotic groups (BSSS×Minn13, Iodent, Lancaster, Pioneer Hybrids) (Table 2). OTU 44 (*Chryseobacterium* sp.) was cultured from five maize genotypes (spanning European Flint, BSSS, Minn 13, P3990/Iodent, BSSS×Minn13).

Table 2. OTUs identified within bacterial species in the cultured transmitting silk microbiome. Isolates were cultured separately from the tip (T) and base (B) of maize silks spanning diverse host inbred/hybrid lines and heterotic groups. Only species that were cultured 3 or more times (i.e., ≥3 isolates) were included. Yellow highlights indicate the presence of isolate(s). Due to ambiguous bases, some cultured OTUs were shared across genera predictions. Further details are in Supplementary Table S1.

Heterotic Group		**European Flint**	**Early Butler**	**BSSS**	**BSSS/ Minnesota 13**	**Minnesota 13**	**Minnesota 13**	**Minnesota 13**	**P3990**	**P3990/Iodent**	**Iodent**	**Lancaster**	**Pioneer Hybrid**	**Pioneer Hybrid**	**Pioneer Hybrid**
Maize Inbred/ Hybrid Line / **Bacterial Species**	**Tip/Base**	**CO444**	**CO325**	**CO452**	**CO462**	**CO449**	**CO433**	**CO432**	**CO430**	**CO448**	**CO431**	**CO441**	**P35837**	**P38157**	**P9855HR**
Agrobacterium larrymoorei	T	12	0	0	0	0	0	0	393	12, 13, 14, 391, 393	0	12, 14, 391, 393	12	0	0
	B	12	13	0	13	0	0	0	0	0	0	14	12	0	0
Atlantibacter hermannii	T	0	22	0	0	25	0	0	0	0	0	0	0	0	16, 21
	B	0	0	24, 26	0	0	0	31	0	0	0	19	0	0	0
Brevundimonas vesicularis	T	0	0	0	0	0	0	0	39	0	0	0	0	0	0
	B	39	0	0	0	0	0	0	38	0	0	0	0	0	0
Chryseobacterium camelliae	T	0	0	48	0	0	44	0	0	44	0	43	0	0	46
	B	44	0	47	0	0	0	0	0	0	0	0	0	0	0
Chryseobacterium daeguense	T	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	B	44	0	44	44	0	0	0	0	0	0	0	0	0	0
Chryseobacterium hagamense	T	0	0	0	0	0	0	0	0	50	0	49	0	0	0
	B	0	0	0	0	0	0	0	0	0	0	45	0	0	0
Delftia acidovorans	T	0	0	0	0	0	0	64	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	68	0	0	0	61	0
Enterococcus gallinarum	T	0	0	0	0	85	0	0	0	0	0	85	0	85	87
	B	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 2. *Cont.*

Heterotic Group		European Flint	Early Butler	BSSS	BSSS/ Minnesota 13	Minnesota 13	Minnesota 13	Minnesota 13	P3990	P3990/Iodent	Iodent	Lancaster	Pioneer Hybrid	Pioneer Hybrid	Pioneer Hybrid
Bacterial Species \ Maize Inbred/ Hybrid Line	Tip/Base	CO444	CO325	CO452	CO462	CO449	CO433	CO432	CO430	CO448	CO431	CO441	P35837	P38157	P9855HR
Exiguobacterium acetylicum	T	0	0	0	0	0	0	0	0	104	0	106	0	103	105
	B	100	0	0	0	0	0	0	0	0	105	0	0	102	0
Klebsiella aerogenes	T	0	119	0	0	0	0	117, 118, 119, 125, 329	0	0	0	0	0	0	0
	B	0	0	117	0	117, 118, 119, 125, 395	0	0	0	0	0	0	0	0	0
Klebsiella grimontii	T	0	0	0	0	0	0	0	0	130, 135	130	0	0	130	130, 135
	B	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lactococcus lactis	T	0	0	0	158, 159, 161	0	0	0	0	0	158, 159, 164	158	158	0	158, 159, 162
	B	0	0	0	158, 159, 161	0	0	0	0	0	0	158, 161	0	0	158, 159, 161, 162
Leclercia adecarboxylata	T	0	0	0	0	0	0	0	0	167, 168	165	0	0	0	0
	B	0	0	0	0	0	0	166, 167	0	0	0	0	0	0	0

Table 2. *Cont.*

Heterotic Group		European Flint	Early Butler	BSSS	BSSS/ Minnesota 13	Minnesota 13	Minnesota 13	Minnesota 13	P3990	P3990/Iodent	Iodent	Lancaster	Pioneer Hybrid	Pioneer Hybrid	Pioneer Hybrid
Bacterial Species \ Maize Inbred/ Hybrid Line	Tip/Base	CO444	CO325	CO452	CO462	CO449	CO433	CO432	CO430	CO448	CO431	CO441	P35837	P38157	P9855HR
Microbacterium oleivorans	T	0	0	0	0	0	0	0	0	183, 189	0	0	0	0	189
	B	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Microbacterium schleiferi	T	181	0	0	0	0	0	0	0	0	0	0	0	0	0
	B	0	0	0	182	0	0	0	0	188, 189	0	0	0	0	0
Microbacterium testaceum	T	0	173, 175, 184	185	0	0	184	0	0	0	0	186, 388	184	0	0
	B	0	190	0	0	0	0	0	0	0	0	190	175	0	174
Pantoea agglomerans	T	0	259	244, 245, 246, 402	231	206, 209, 210, 218, 245, 246, 251, 252, 253, 402	0	0	0	224	240, 242, 243, 244, 255	228, 235	225, 226, 228, 402	221, 222	0
	B	0	259	226, 239, 259	0	0	0	212, 228, 231, 239	225	0	244, 245, 246, 248, 259, 402	246, 402	0	0	0
Pantoea ananatis	T	0	0	0	0	0	0	271, 276, 290, 292	0	282, 290, 292, 409, 411	0	223	0	264, 274, 411	235, 238
	B	0	0	409	0	265, 271, 276	0	211, 217, 292	283, 286	0	0	282	0	0	0

Table 2. *Cont.*

Heterotic Group		European Flint	Early Butler	BSSS	BSSS/ Minnesota 13	Minnesota 13	Minnesota 13	Minnesota 13	P3990	P3990/Iodent	Iodent	Lancaster	Pioneer Hybrid	Pioneer Hybrid	Pioneer Hybrid
Bacterial Species \ Maize Inbred/ Hybrid Line	Tip/Base	CO444	CO325	CO452	CO462	CO449	CO433	CO432	CO430	CO448	CO431	CO441	P35837	P38157	P9855HR
Pantoea anthophila	T	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	B	215	213	0	0	0	0	238	0	0	0	0	0	0	0
Sphingomonas yabuuchiae	T	354	352	0	0	0	0	0	0	0	0	357	0	0	0
	B	0	0	0	363	0	0	0	0	363	0	357	0	0	0
Stenotrophomonas pavanii	T	0	372	375	0	0	0	375	0	375	0	375	369	374, 375	374
	B	0	0	371, 375	0	0	0	372, 374, 375	0	0	0	0	0	0	0

Table 3. Number of isolates from prevalent cultured OTUs identified in the cultured transmitting silk microbiome. Isolates were cultured separately from the tip (T) and base (B) of maize silks spanning diverse host inbred/hybrid lines and heterotic groups. Only OTUs that were found in 3 or more genotypes were included. The asterisk (*) indicates that the OTU was assigned to isolates which had equal first matches to multiple *Pantoea* species. Yellow highlights indicate the presence of isolate(s). Red-orange highlights indicate cultured OTUs that occurred in five or more host genotypes. Prevalence refers to the number of maize genotypes that gave rise to at least one cultured isolate from that OTU. The colors in the first column (blue, green, etc.) highlight the featured OTUs that share a species prediction (e.g., *Agrobacterium larrymoorei* highlighted in blue). Further details are in Supplementary Table S1.

Heterotic Group		European Flint	Early Butler	BSSS	BSSS/ Minnesota 13	Minnesota 13	Minnesota 13	Minnesota 13	P3990	P3990/Iodent	Iodent	Lancaster	Pioneer Hybrid	Pioneer Hybrid	Pioneer Hybrid	Total isolates	Total Isolates, Merged Tip and Base	Prevalence	Prevalence, Merged Tip Tip and Base
Bacterial Cultured OTUs / Maize Inbred/ Hybrid Line	Tip/Base	CO444	CO325	CO452	CO462	CO449	CO433	CO432	CO430	CO448	CO431	CO441	P35837	P38157	P9855HR				
12 (*Agrobacterium larrymoorei*)	T	1	0	0	0	0	0	0	0	1	0	1	1	0	0	4	6	4	4
	B	1	0	0	0	0	0	0	0	0	0	0	1	0	0	2		2	
13 (*Agrobacterium larrymoorei*)	T	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	3	1	3
	B	0	1	0	1	0	0	0	0	0	0	0	0	0	0	2		2	
393 (*Agrobacterium larrymoorei*)	T	0	0	0	0	0	0	0	1	1	0	1	0	0	0	3	3	3	3
	B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	
44 (*Chryseobacterium daeguense/ Chryseobacterium camelliae*)	T	1	0	0	1	0	1	0	0	1	0	0	0	0	0	4	8	4	5
	B	2	0	1	1	0	0	0	0	0	0	0	0	0	0	4		3	
85 (*Enterococcus gallinarum*)	T	0	0	0	0	1	0	0	0	0	0	1	0	1	0	3	4	3	4
	B	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1		1	
117 (*Klebsiella aerogenes*)	T	0	0	0	0	0	0	5	0	0	0	0	0	0	0	5	8	1	3
	B	0	0	1	0	2	0	0	0	0	0	0	0	0	0	3		2	
119 (*Klebsiella aerogenes*)	T	0	1	0	0	0	0	1	0	0	0	0	0	0	0	2	4	2	3
	B	0	0	0	0	2	0	0	0	0	0	0	0	0	0	2		1	
130 (*Klebsiella grimontii*)	T	0	0	0	0	0	0	0	0	1	1	0	0	2	1	5	6	4	4
	B	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1		1	

Table 3. *Cont.*

Heterotic Group		European Flint	Early Butler	BSSS	BSSS/ Minnesota 13	Minnesota 13	Minnesota 13	Minnesota 13	P3990	P3990/Iodent	Iodent	Lancaster	Pioneer Hybrid	Pioneer Hybrid	Pioneer Hybrid	Total isolates	Total Isolates, Merged Tip and Base	Prevalence	Prevalence, Merged Tip Tip and Base
Bacterial Cultured OTUs / Maize Inbred/ Hybrid Line	Tip/Base	CO444	CO325	CO452	CO462	CO449	CO433	CO432	CO430	CO448	CO431	CO441	P35837	P38157	P9855HR				
158 (*Lactococcus lactis*)	T	0	0	0	1	0	0	0	0	0	1	5	2	0	4	13	17	5	5
	B	0	0	0	1	0	0	0	0	0	0	1	0	0	2	4		3	
159 (*Lactococcus lactis*)	T	0	0	0	1	0	0	0	0	0	3	0	0	0	1	5	7	3	3
	B	0	0	0	1	0	0	0	0	0	0	0	0	0	1	2		2	
161 (*Lactococcus lactis*)	T	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	4	1	3
	B	0	0	0	1	0	0	0	0	0	0	1	0	0	1	3		3	
184 (*Microbacterium testaceum*)	T	0	1	0	0	0	1	0	0	0	0	0	1	0	0	3	3	3	3
	B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	
228 (*Pantoea* sp.*)	T	0	0	0	0	0	0	0	0	0	0	1	1	0	0	2	5	2	5
	B	0	0	0	0	0	0	1	1	0	0	0	0	1	0	3		3	
235 (*Pantoea* sp.*)	T	0	0	0	0	0	0	0	0	0	0	1	0	0	1	2	3	2	3
	B	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1		1	
245 (*Pantoea agglomerans*)	T	0	0	1	0	1	0	0	0	0	0	0	0	0	0	2	3	2	3
	B	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1		1	
246 (*Pantoea agglomerans*)	T	0	0	3	0	1	0	0	0	0	0	0	0	0	0	4	7	2	4
	B	0	0	0	0	0	0	0	0	0	2	1	0	0	0	3		2	
259 (*Pantoea agglomerans*)	T	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	4	1	3
	B	0	1	1	0	0	0	0	0	0	1	0	0	0	0	3		3	
402 (*Pantoea agglomerans*)	T	0	0	3	0	1	0	0	0	0	0	0	1	0	0	5	7	3	5
	B	0	0	0	0	0	0	0	0	0	1	1	0	0	0	2		2	

Table 3. *Cont.*

Heterotic Group		European Flint	Early Butler	BSSS	BSSS/ Minnesota 13	Minnesota 13	Minnesota 13	Minnesota 13	P3990	P3990/Iodent	Iodent	Lancaster	Pioneer Hybrid	Pioneer Hybrid	Pioneer Hybrid	Total isolates	Total Isolates, Merged Tip and Base	Prevalence	Prevalence, Merged Tip Tip and Base
Bacterial Cultured OTUs / Maize Inbred/ Hybrid Line	Tip/Base	CO444	CO325	CO452	CO462	CO449	CO433	CO432	CO430	CO448	CO431	CO441	P35837	P38157	P9855HR				
271 (*Pantoea ananatis*)	T	0	1	0	0	0	0	1	0	1	0	0	0	0	0	3	5	3	4
	B	0	0	0	0	1	0	1	0	0	0	0	0	0	0	2		2	
276 (*Pantoea ananatis*)	T	0	2	0	0	0	0	1	0	0	0	0	0	0	0	3	4	2	3
	B	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1		1	
290 (*Pantoea ananatis*)	T	0	0	0	1	0	0	1	0	3	0	0	0	0	0	5	9	3	5
	B	1	0	0	1	0	0	1	0	0	0	0	1	0	0	4		4	
292 (*Pantoea ananatis*)	T	0	0	0	1	0	0	1	0	4	0	0	0	0	0	6	12	3	5
	B	1	0	0	1	0	0	3	0	0	0	0	1	0	0	6		4	
409 (*Pantoea ananatis*)	T	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	4	1	4
	B	0	0	1	0	0	0	1	0	0	0	0	0	1	0	3		3	
372 (*Stenotrophomonas pavanii*)	T	0	3	0	0	0	0	0	0	0	0	0	0	0	0	3	6	1	3
	B	0	1	0	0	0	0	1	0	0	0	0	0	1	0	3		3	
374 (*Stenotrophomonas pavanii*)	T	0	2	0	0	0	0	0	0	0	0	0	0	1	1	4	8	3	5
	B	0	1	1	0	0	0	1	0	0	0	0	0	1	0	4		4	
375 (*Stenotrophomonas pavanii*)	T	0	2	2	0	0	0	1	0	1	0	1	0	1	0	8	15	6	6
	B	0	1	2	0	0	0	3	0	0	0	0	0	1	0	7		4	

The greatest diversity of cultured OTUs within each maize genotype consistently belonged to the phylum Pseudomonadota (Table S9). For example, within two of the three Minnesota 13 genotypes, 22/23 and 27/28 unique OTUs belonged to Pseudomonadota.

The longer length 16S sequencing also revealed intra-species diversity, i.e., diverse strains within a predicted species. In total, 33 species contained two or more OTUs (Table S1). There was particularly high genetic diversity within *Pantoea*; there were 27 OTUs found within *P. agglomerans,* and 17 OTUs within *P. ananatis* (Table 2; Figure S4). Even amongst isolates cultured from individual host samples, it was common to find multiple cultured OTUs belonging to the same species. The greatest diversity of cultured OTUs within a species and within a single maize sample was in the tip sample of CO449 (Minnesota 13): of the 15 isolates sequenced here, 10 OTUs were closely related to *P. agglomerans* (Table 2).

3.4. Conservation and Diversity of Bacteria Cultured from Maize Silk Tip Versus Base Tissues

Across all samples, ~50% of all genera (21–25 out of 42–48 genera, depending on stringency criteria) appeared in both silk tip and base samples (Tables S1 and S6). *Pantoea* and *Chryseobacterium* were cultured from both tip and base in 10/13 genotypes and 5/7 genotypes, respectively; *Klebsiella*, *Microbacterium*, and *Stenotrophomonas* sp. were also frequently cultured independently from both tip and base samples (Table S6). At the species level, 21–25 out of 67–94 species were cultured from both silk tip and base samples (Table S1). At the OTU level, taxa of *S. pavanii* (OTU 375, as mentioned in [16]) and *L. lactis* (OTU 158 and 159) were cultured from both tip and base across multiple maize genotypes, with some exceptions (Table 2).

The tip samples contained five genera and 21 species (moderate stringency) that did not appear in the base samples; the apparent tip-specific species included *Enterococcus gallinarum*, *Klebsiella grimontii*, and *Microbacterium oleivorans* (Tables S1, S6 and S7). At the OTU level, OTU 184 (taxon of *Microbacterium testaceum*) and OTU 393 (taxon of *Agrobacterium larrymoorei*) were exclusively cultured from silk tips. Conversely, the base samples contained 16 genera and 38 species (moderate stringency) that did not appear in the tip samples; the species included *Chryseobacterium daeguense* and *Pantoea anthophila* (Tables S1, S6 and S7). Interestingly, of the genera that were only found in a single host genotype, 74% (14/19 genera) were only cultured from the base of the silks (Tables S1 and S6).

3.5. Test for Potential Sharing of Microbiota Between Parent and Progeny Within Pedigrees at the Cultured OTU Level

The following observations could be made between bacteria isolated from seven maize genotypes involved in three pedigree relationships (Table 1 and Table S1; Figure 1):

The first relationship involved CO449, which was derived from a hybrid between CO433 and CO432 [26], all of which were in the study. Whereas CO449 (23 OTUs) and CO433 (6 OTUs) did not share any OTUs, CO449 (23 OTUs) and CO432 (28 OTUs) shared 6 OTUs (OTUs 117, 118, 119, 125, 271, 276), many of which had multiple isolates cultured from both maize genotypes but were not prevalent across the 14 maize genotypes. Of these, OTU 118 and OTU 125 were cultured exclusively from CO432 (progeny) and CO449 (parent).

The second pedigree involved CO448, which was derived from a cross between CO273 and CO431 [27], of which CO448 and CO431 were in the study. CO448 (29 OTUs) and CO431 (31 OTUs) shared only a single OTU (OTU 130, a taxon of *Klebsiella grimontii*). Across the study, OTU 130 (and in general, the species *K. grimontii*) was only cultured from two other maize genotypes.

The final pedigree involved CO462 and CO452, both of which were in this study and share the common ancestor CO388 [28]. CO462 (27 OTUs) and CO452 (25 OTUs) shared a single OTU (OTU 44, a *Chryseobacterium* sp.), which appeared in three other genotypes.

In summary, in the first pedigree, the progeny shared 26% of its OTUs (six shared) with one parent, while in the remaining two pedigrees, the related genotypes shared only a single OTU (3–4%).

4. Discussion

4.1. Overview

The fertilization stage at which male gametes are transmitted to the ovule in style/silks (transmitting stage) is a critical stage in the reproduction of angiosperms. Here, microbial culturing allowed for in-depth analysis of the healthy silk microbiome, including the impact of maize heterotic group/genotype, tip/base location, and host pedigree relationships. The findings helped to clarify the taxonomy within the core fertilization-stage silk microbiome.

4.2. Influence of Hybrid Genotype and Heterotic Group on the Taxonomy Within the Transmitting Silk Microbiome

Domestication and the development of hybrids may have altered maize microbiomes, as observed when comparing the rhizosphere of the teosinte ancestors of cultivated maize, maize inbreds, and modern maize hybrids [24]. There has been evidence of heterosis for beta diversity, particularly in the maize rhizosphere microbiome [22]. This microbial heterosis could be due to vertical transmission of bacteria, or both gametes contributing genes that influence the plant's "hospitality" and ability to be colonized by microbes. However, a study by Favela et al. [29] found that modern maize inbreds hosted more diversity in N-cycling microorganisms (taxonomic and functional genes) than hybrids. In the current study, the cultured pollinated silk microbiomes of maize inbreds and commercial hybrids were comparable, but the exact inbred parents of the hybrids were not analyzed.

Genetically related inbreds that result in similar hybrid yield when crossed with a genetically distinct inbred group are defined as a distinct heterotic group [30,31]. In the prior NGS (V4-MiSeq) study by Khalaf et al. [16], the pollinated silk microbiomes of heterotic groups were difficult to compare due to the limited taxonomic resolution [4]. In the current study, Minnesota 13 heterotic group inbreds were found to have low overall diversity at the higher taxonomic levels. Interestingly, silks with a BSSS heterotic group background, either fully BSSS, or BSSS and Minnesota 13 combined, had more diversity than those belonging solely to Minnesota 13. The low diversity in Minnesota 13 genotypes was in contrast to the diversity previously found from *F. graminearum*-treated silks, where two genotypes belonging to the Minnesota 13 heterotic group (CO449 and CO432) produced the greatest bacterial diversity [16]. Whereas *F. graminearum* infection is generally associated with a collapse in microbial diversity, the opposite effect seemed to occur in these two genotypes. Starting in the late 1800s, Minnesota 13 became the dominant cultivar in the U.S. Corn Belt, and the progenitor of later inbreds and hybrids [19]. Today, 13% of the genetic background of U.S. hybrid maize comes from Minnesota 13 [19]. These observations warrant a deeper investigation of the pollinated silk microbiome between specific heterotic groups and hybrids in maize and their potential impacts on silk health and reproductive success.

4.3. Inheritance of the Transmitting Silk Microbiome

It is theorized that the male and/or female gamete carries endophytes to the next generation [9,25]. It has been hypothesized that the pollen tube may be a route for endophyte inheritance [7,8,25]. A study by Liu et al. [10] presented evidence that hybrid maize offspring receive microbes from male and female parents. More recently, Wu et al. [12] used bacterial genome-wide molecular marker profiling as evidence that a *Bacillus* species from pollen could be vertically transmitted in maize.

Pollinated silks potentially contain pollen tubes and migrating male gametes. Inheritance was not analyzed in the previous NGS (V4-MiSeq) study [4], given the limitations of the short 16S sequences analyzed. Here, the longer 16S sequences suggested a low level of sharing of pollinated silk microbiome OTUs between maize genotypes in a pedigree. In two host maize pedigrees, only a single isolate was shared between progeny and close relatives, which could have occurred by random chance. However, in one host pedigree, up to 25% of OTUs were shared between the progeny and one parent. The relationship between these two microbiomes appeared to be non-random, because when comparing CO449 to all other genotypes in the study, the average number of shared OTUs was only 1.6 compared to 6

between CO449 and CO432, the "female parent" in the pedigree. Interestingly, CO449 did not share any OTUs with its male parent, CO433, suggesting that the mode of inheritance was via the eggs/silks, not the pollen. This is in line with a study by Liu et al. [32], which discovered maize hybrid microbiomes to be more aligned with the female parent. The possible similarities between the parent-progeny microbiome suggest either vertical microbial transmission or transmission of host compatibility alleles that promote colonization by these taxa. In terms of the lack of shared taxa in other host pedigrees, this could be due to limitations in culturing. Again, this should be interpreted with caution, noting the likelihood of microbes missed by culturing. Therefore, conclusions should be drawn based on what is present, and not what is lacking. Indeed, in this study, 69% of the OTUs were present in only one maize genotype.

4.4. Influence of Silk Location on the Taxonomy of the Transmitting Silk Microbiome

In plants, the microbiome can differ in different portions of the same organ [33]. This evokes the question of whether the pollinated silk microbiome may differ between the silk tip and the base. The bacteria in this study were from the portions of silks contained within the husk leaves, not the portions that extend into the air but, nevertheless, their origin could be environmental. In the previous V4-MiSeq study, tip and base DNA were isolated using different DNA kits and protocols, and an internal control showed they could not be compared directly. Here, culturing allowed for a higher taxonomic resolution comparison between the microbiome in different portions of pollinated silks, which showed that some species and strains were conserved.

Some cultured isolates appeared exclusively in tip or base samples; however, due to the limitations of culturing, conclusions should only be drawn on what was present, and not what was lacking. With respect to microbes limited to base silk tissue, we speculate these may be involved in pollen tube guidance (see below), or they may be at a preferential location for being transmitted to the seed. With respect to tip-only microbes, logically, some bacteria may appear in the silk tip but not the base if they recently entered from the environment. Additionally, some pathogens entering the silks might be successfully sequestered to the tips. Environmental microbes that are not compatible with the silk environment might only survive a short distance into the silks. Critically, hydraulic flow [3] and the presumed flow of metabolites must occur from base to tip, which may make it more difficult for bacteria to travel in the opposite direction, from tip to base. This suggests that any microbe that originates maternally at the silk base (e.g., ovule, sap) would be more likely to colonize the tip. This may explain why approximately 50% of all pollinated silk microbiome genera and 26–31% of all species were cultured from both the silk tip and base samples. Even at the OTU level, some strains were cultured from both the tip and base across multiple maize genotypes. From the perspective of many members of the pollinated silk microbiome, the silk may represent one continuous niche/habitat without distinct sections.

4.5. Potential of the Pollinated Silk Microbiome to Include Endophytes/Epiphytes Versus Pathogens

We had hypothesized that the pollinated silk microbiome is likely made up of several players including both endophytes/epiphytes and environmental pathogens. The definition between endophytes and pathogens is not as clear as one might expect. For example, *Fusarium verticillioides* is a fungus that acts as a beneficial microbe or a pathogen, depending on the conditions [34]. While there are many known fungal pathogens of maize silks (which would not be detected in this bacterial study) [35], there are six main bacterial pathogens of maize, which are not necessarily silk associated, including *Pantoea ananatis* (white spot foliar disease a.k.a. leaf spot disease [36–39] and brown stalk rot [40,41]), *Pantoea agglomerans* (leaf blight and vascular wilt [42]), *Pantoea stewartii* (Stewart's wilt [43]), *Pseudomonas syringae* pv. *syringae* (Holcus leaf spot [44–46]), *Clavibacter michiganensis* subsp. *nebraskensis* (Goss's wilt [47,48]), and *Erwinia dissolvens* (bacterial stalk rot [49,50]). Of these, *P. ananatis*, *P. agglomerans*, and *P. stewartii* were detected in the healthy pollinated silk

microbiome, albeit *P. stewartii* only once. Critically, however, we have not found literature on these pathogens entering specifically via silks. Sheibani-Tezerji et al. [51] isolated weakly pathogenic *P. ananatis* from maize seeds, but they could have entered via wounds or the maternal vascular system into the ovule. Coutinho and Venter [41] recognize *P. ananatis* causing fruitlet rot in pineapple and post-harvest rot in cantaloupe, but otherwise not infecting the fruit/seed in other plants. Alternatively, studies have shown that strains of *Pantoea* can be beneficial to plants (e.g., growth promotion [51,52], phosphate solubilization [52], nitrogen fixation [53,54], biocontrol [42,55–58], etc.) and some are likely transmitted vertically [11,51]. In support of the *Pantoea* isolates in the current study being non-pathogenic, none of these bacterial species are common pathogens in maize in Ontario, Canada [59]. Furthermore, the pollinated silk isolates originated from host plants that were grown in the field for 2 years without showing visible disease symptoms. Additionally, certified seed was used for the three commercial hybrids, and the remaining seeds came from a public breeding program that would have screened against seed stocks transmitting pathogens. When these observations are combined, it appears unlikely that the bacteria cultured from pollinated silks are pathogenic. Rather, the three *Pantoea* species are also well-known plant endophytes of maize or grasses [51,52,60,61], along with many of the other cultured strains including *Lactococcus lactis* [62], *Stenotrophomonas pavanii* [63,64], and *Microbacterium testaceum* [65]. One intriguing possibility is that silks and pollen host avirulent strains that out-compete pathogenic *Pantoea* for the same niche, similar to biocontrol strains of *Aspergillus flavus* [66]. Indeed, as detailed below, this study showed that pollinated silk isolates corresponding to several of these species encode and/or display traits that are more consistent with them being endophytes rather than pathogens. Thus, these endophytes may have potential to be re-applied to crops to utilize their disease prevention, nutrient acquisition, growth promotion, or yield promotion traits. Additionally, this information could help inform management plans that promote beneficial bacteria within plants and in soil.

4.6. Pollinated Silk Microbiome Taxonomy in the Context of Vertical Transmission

Many of the cultured healthy pollinated silk microbiome species have previously been found in maize seeds [51,67,68], which supports the hypothesis that seed-derived microbes spend part of their life cycle in transmitting silks. Furthermore, Wu et al. [12] recently provided some evidence that a *Bacillus mojavensis* strain carried by maize pollen can be transmitted to seed. Transmitting silks likely contain microbes that originate from the maternal plant and also microbes that originate from pollen/pollen tubes (paternal plant). The hypothesis that the pollinated silk microbiome contains many pollen-derived bacteria is supported by the observation that they are taxonomically related to the pollen microbiomes of other wind-pollinated plants, specifically birch and rye, which also contain many Pseudomonadota and Actinomycetota (Actinobacteria) [69]. Birch and rye pollen microbiomes also have high abundances of Enterobacteriaceae and Microbacteriaceae, respectively [69], which were the second and third most abundant cultured isolates from healthy pollination-stage maize silks. The cultured pollination-stage silk microbiome and these pollen microbiomes share multiple genera including *Rosenbergiella*, *Pseudomonas*, and *Lactococcus* [69]. For bacteria to be vertically transmitted in flowering plants, they must reach the ovule or possibly the seed coat. The cultured microbiome and V4-MiSeq pollination-stage silk microbiome [4] are abundant in Pseudomonadota, as already noted, which interestingly includes many species with flagella-based motility [70].

4.7. Potential Reasons for Diversity Within Pantoea Species in the Pollinated Silk Microbiome

When *Lactococcus lactis* and *Stenotrophomonas pavanii* were isolated, they often belonged to the same OTU (e.g., 17 isolates of OTU 158, and 15 isolates of OTU 375, respectively), suggestive of low rates of DNA exchange. By contrast, when *Pantoea agglomerans, P. ananatis*, and other *Pantoea* species were sampled, they showed tremendous diversity at the OTU level. This reiterates the diversity found within *Pantoea* that were previously cultured

from *F. graminearum*-treated pollinated silks [16]. Indeed, *Pantoea* isolated from many crops contain vast diversity. Particular bacterial species/genera have a proclivity for diversity and adaptability, which may be in part due to ICE components (Integrative and Conjugative Elements) [71,72], the Large *Pantoea* Plasmid (LPP-1) [73], and genetic exchange [74]. There is so much genetic diversity within *Pantoea* that many taxa were historically assigned to different genera and even different families. Additionally, *P. agglomerans* and *P. ananatis* are ubiquitous and diverse in the environment [41,75,76]; these may colonize silks directly and contribute to microbiome diversity.

Diversity can be advantageous, similar to how a farm with diverse crops can build resiliency against crop failure. Different bacteria may be adapted to survive different conditions (e.g., temperature, competing microbes) and offer different traits to the host (e.g., biocontrol against specific pathogens, host reproductive success). This raises the question of whether the diversity of *Pantoea* may have been promoted over time by farmer- or natural selection for host compatibility alleles that favor colonization by *Pantoea*.

4.8. Study Limitations and Future Experiments

As this was a culture-based study, the majority of microbial taxa were likely missed. Based on the literature, culturing captures less than 10% of the bacteria in a microbiome [77]. To improve this rate, this study used two culture media with different pH levels to capture diversity. Rich media were used because silks are one of the fastest growing tissues in nature [3] and, hence, we made the assumption that the silk niche is nutrient-rich. As mentioned above, colony picking occurred at two incubation time points (day 3 and 5, to capture both fast growers and slow growers). A 30 °C incubation temperature was used, typical of field daytime temperatures during the silking interval. Despite these careful considerations, nevertheless, some taxa were expected to be incompatible with in vitro culturing conditions. Additionally, due to the significant level of intra-genus genetic diversity within silks (e.g., *Pantoea*), some strains that appeared morphologically alike may have been missed. Furthermore, this study consisted of one replicate of a single field year, which likely contributed to some discrepancies between the prior MiSeq [4] and cultured results. Future culturing studies should include a wider variety of media and culturing conditions, including media with antibiotics to search for fungi.

In terms of taxonomy, similar to other studies, some 16S sequences from cultured bacteria were incomplete or of poor quality, despite repeated attempts, perhaps due to the universal PCR primers being mismatched to the genomic target (not truly universal) or the isolates containing multiple 16S gene templates [14,15]. Here, to ensure high-confidence taxonomic assignments, transparent and strict sets of threshold criteria were used (see Methods). Additionally, the change from forward primer 799F to 27F midway through the experiment to directly compare amplicons to the MiSeq core OTUs, may have introduced a new primer bias, although it improved taxonomic resolution. However, the five host genotypes that were almost exclusively subjected to the 27F-1492R primer set (CO452, CO441, CO432, CO449, P9855HR, P35837) ranged in OTU level diversity from 15 to 34, which was in the same range as the entire study population (15–34 OTUs), excluding CO443 (which had only 6 OTUs); this suggests the primer change did not affect study interpretations.

Given the limitations of culturing and sequencing, in our analysis, we focused on comparisons based on bacterial presence, not absence. Moving forward, conducting culture-independent microbiome profiling using full length 16S sequencing (e.g., PacBio, Oxford Nanopore) or genome-wide metagenomics [78] will provide a more complete picture of the transmitting silk microbiome. However, an advantage of taxonomic assignment from cultures is that the 16S DNA template is rich and pure, compared to culture-independent profiling where the template represents a diverse microbial community, including organelle DNA which competes for primer hybridization, thus creating biases [17].

Silks are necessary for maize production, but they also are known to contain phytochemicals, have been fed to poultry to alleviate stress, and have been used in herbal or traditional medicine for centuries [79–82]; it is intriguing to consider whether some of the

benefits attributed to silks may be the result of the microbiome [4,6]. Further phenotyping and multi-omics evaluations are recommended to test whether the microbiome may produce some of the phytochemicals found in silks. It would also be interesting to test whether some of the traits associated with maize genotypes could be partially attributed to/explained by the microbiomes.

Multi-omics and culturing should be conducted concurrently; for example, the current study analyzed bacteria that were cultured from a subset of silks that were also used for V4-MiSeq based NGS analyses. Many bacterial genera, such as *Pantoea*, *Pseudomonas*, *Stenotrophomonas*, *Sphingomonas*, *Chryseobacterium* and *Lactococcus*, were common in both the current culture-based study and the previous V4-MiSeq study. However, other bacterial genera were surprisingly common in the cultured results but lacking in the V4-MiSeq results (*Klebsiella* and *Microbacterium*) or less prevalent in the cultured results than expected (e.g., *Acinetobacter, Pseudomonas*), perhaps due to culture bias or limitations of V4-MiSeq primers [13–15]. Culture bias is expected, as many bacteria detected via V4-MiSeq may not be compatible with the specific culture conditions used in this study. Neither method is completely comprehensive, but combining methods creates a more complete picture of the microbiome.

Future studies should also examine the microbiomes of silks and pollen separately, as our group has begun [83,84]; this will allow the roles, origin, and inheritance of microbes to be investigated further. Beneficial microbes should be tested as in-field or seed treatments, and beneficial taxa may be incorporated in breeding programs (possibly via microbial markers).

5. Conclusions

This in-depth analysis of healthy maize silks at the fertilization stage shows the value of culturing paired with long-length 16S sequencing and analyzing the natural microbiome independently without pathogen treatments. The findings suggest that the bacterial diversity in fertilization-stage silks may be impacted by maize host genotype and heterotic group. Some bacterial taxa were relatively conserved across the 14 maize genotypes and tip/base locations in the silks, such as *Pantoea*, suggesting that these taxa may be part of the core microbiome, and/or have long-term, symbiotic relationships with healthy pollinated silks. Lastly, there was some crossover/similarity between the silk microbiomes of maize genotypes that were related via pedigree; these preliminary findings suggest that bacteria could be inherited or related plant genotypes/tissues may recruit/accommodate colonization by common bacterial taxa. The healthy silks, in particular, represent an important resource for discovering relationships between plant reproductive microbiomes. The style microbiomes should be investigated further in maize and a variety of plant species, including detailed functional analysis with the hope that they be used as beneficial, late-season crop treatments such as growth/yield promoters and biocontrols.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/bacteria3040032/s1, Table S1: Spreadsheet of isolate details, accession numbers, and sequences; Figure S1: Summary of the process of sequencing a single culture; Figure S2: Genus level taxonomic identification of the cultured healthy transmitting silk microbiome from maize silks spanning diverse heterotic groups; Figure S3: Species level taxonomic identification of the cultured healthy transmitting silk microbiome from maize silks spanning diverse heterotic groups; Table S2: Number of isolates belonging to the cultured healthy transmitting silk microbiome at the phylum taxonomic level; Table S3: Number of isolates belonging to the cultured healthy transmitting silk microbiome at the class taxonomic level; Table S4: Number of isolates belonging to the cultured healthy transmitting silk microbiome at the order taxonomic level; Table S5: Number of isolates belonging to the cultured healthy transmitting silk microbiome at the family taxonomic level; Table S6: Number of isolates belonging to the cultured healthy transmitting silk microbiome at the genus taxonomic level; Table S7: Number of isolates belonging to the cultured healthy transmitting silk microbiome at the species taxonomic level; Table S8: OTUs identified within bacterial species in the cultured transmitting silk microbiome including summaries; Table S9: Number of unique

OTUs identified within bacterial phyla in the cultured healthy transmitting silk microbiome; Figure S4: Phylogenetic tree of the isolates identified as *Pantoea* from the cultured healthy transmitting silk microbiome [85,86]; Supplementary Text S1: Conservation of cultured silk bacteria across heterotic groups at higher taxonomic levels [22–25]; Supplementary Text S2: Diversity of cultured silk bacteria across heterotic groups at higher taxonomic levels; Supplementary Text S3: Maize host genotype specific bacterial taxa.

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