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The Microbiology of Hemp Retting in a Controlled Environment: Steering the Hemp Microbiome towards More Consistent Fiber Production

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Abstract: Industrial hemp (*Cannabis sativa* L.) production is increasing dramatically in the US due to recent changes which lift restrictions on the growth and sale of hemp products; however, due to the decades-long prohibition of hemp, there is a lack of current research with respect to varieties and best agricultural practices for the many uses of this versatile crop. Natural fiber production relies on retting, a microbially-mediated process necessary for the separation of fibers from the plant which can occur unevenly in the field environment and result in inconsistent fiber quality and lower processing efficiency. In this study, the microbiome of hemp stalks is investigated throughout the retting process using 16S rRNA gene amplicon sequencing using the Illumina MiSeq platform. Field retting conditions were simulated in a controlled greenhouse environment in order to determine the effects of different moisture levels and soil contact on the retting process. Samples were taken over six time points, reflecting the community of freshly cut stalks to optimally-retted material, and finally over-retted material showing degraded fibers. The results show a very consistent population throughout retting, dominated primarily by *Proteobacteria*, but showing an increase in the abundance of the *Bacteroidetes*, namely *Chryseobacterium*, in time points corresponding to optimally-retted and over-retted stalks in treatments receiving higher moisture levels, but not in the low-moisture treatment. Soil application did not appear to influence the microbial community throughout retting, indicating a resilient population present in and on the hemp stalks at harvest.

Keywords: hemp microbiome; fiber crops; fiber retting

1. Introduction

Hemp (*Cannabis sativa* L.) is one of the oldest crops continuously cultivated by humans, with hempen cloth found dating to over 6000 years ago [1]. In the United States, hemp was introduced in the mid- to late-18th century when hemp fibers were used to produce fabrics, twine, and paper [2], with Kentucky leading the US in production from the civil war era to WWII [3]. Hemp production would eventually decline due to decreased demand for hemp sails and ropes in modern ships, the rise in demand for fabrics made from cotton and synthetic fibers, and innovations in the wood pulping industry for making paper. Production of hemp was all but eliminated in North America after the passing of the US Marihuana Tax Act and Canadian Opium and Narcotics Act in 1938, which prohibited all cultivation of *Cannabis* without government permission [4]. Currently, as the result of increased demand for renewable and sustainable materials, hemp has experienced a renewed interest as a fiber crop around the world, with Australia planting the first crop in 1990, followed by England in 1993, Germany in 1995, Canada in 1998, and the US in 2014 [1].

Presently, synthetic fibers dominate the market when it comes to industrial applications [1]. Hemp is considered an attractive alternative to synthetics in part due to the versatility of the plant fibers—they can be used in textiles, yarns, paper, construction materials, auto parts, and composites [2]. The automotive industry has been especially influential for the production of industrial hemp due to the lighter weight and improved structural properties when compared to glass and resin-based materials [5]. Additionally, using hemp for paper production could reduce the need for wood pulping due to the decreased cost of pulping hemp and the fact that hemp can be recycled twice as many times as wood paper [5]. The projected profitability of fiber hemp is difficult to assess due to current limitations of processing facilities and uncertain demand in the manufacturing industry; however, estimates from various sources suggest fiber hemp could be comparable to other major field crops [3].

The 2014 US Farm Bill allowed state departments of agriculture to approve hemp pilot programs for farmers, colleges, and universities. The long lapse in hemp cultivation, however, has set the US behind the rest of the world in experience and research. For hemp fiber crops in particular, one of the main problems that has been identified by farmers and fiber processors concerns field retting, also known as dew retting, whereby hemp stalks are cut and left in the field for a period of time before baling. During retting, microbial activity degrades polysaccharides, mainly pectin, that bind the bast fibers to the hurd core such that they can be separated by a mechanical process called decortication [6]. This is distinct from the process of water retting, used mainly for textile quality fibers, in which the stalks are submerged in water which may contain additional enzymes and/or microbial cultures [6]. Field retting is the most common method used by western countries, as it is considerably cheaper and does not produce waste water. The fiber quality from optimally retted hemp is suitable for many industrial uses, though factors such as environmental conditions and grower inexperience can result in less valuable fiber grades and poor uniformity; fibers that are under-retted cannot be decorticated effectively and over-retted fibers are weaker and less valuable [6]. Processors who buy field retted hemp from farmers in order to make the kinds of fiber products that can be used in industry need a steady supply and consistent quality, however the economics of the industrial fiber market are, at this time, prohibitive to practices or inputs that increase costs.

Field retting is the most practical method of hemp fiber production for farmers in the US, but little research has been done on the microbiota associated with hemp stalks during field retting, or on applied practices that improve the quality and consistency of the fiber without substantially increasing cost. Work here describes the microbial communities associated with hemp in a controlled greenhouse retting study. Three varieties were chosen for the study, two common fiber varieties, Futura 75 and Felina 32, and SS Alpha, an experimental variety that is being tested for its suitability for fiber crops in the US. We hypothesize that the bacterial population of the stalk will shift over retting time, correlating with under-retted and optimally-retted, as well as considerably over-retted material, and that treatments manipulating moisture and access to environmental microbiota will influence the population at these time points. Information about how these factors affect the microbial population involved in the retting process and whether or not it differs by variety can lead to improvements in the quality and value of field-retted hemp, thereby increasing profitability for farmers and encouraging the inclusion of more natural fibers in industry and manufacturing.

2. Materials and Methods

Industrial hemp (*Cannabis sativa* L.) varieties Futura 75 (FU), Felina 32 (FE), and SS Alpha (SSa) were planted on 1 July 2016 at Spindletop Farm in Lexington, KY (38.125885, −84.497585) as part of a variety trial. Plots were tilled conventionally and fertilized with 150 lb/ac (168.3 kg/ha) of nitrogen in the form of granulated urea (incorporated pre-planting), and planted with a seed density of 40 lb/ac (44.83 kg/ha). Stalks were cut manually at the base of the plant on 28 September 2016. At this time, varieties FE and FU had flowered and were in the early stages of senescence, which is typically when hemp stalks are cut for field retting; however the SSa variety was still in vigorous vegetative growth and had not flowered. As an experimental variety being tested for suitability for fiber production in

Kentucky, it was discovered in trials that the SSa performed very differently than the other varieties tested, in that vigorous vegetative growth continued throughout the growing season without flowering, up to when the plants were killed by hard frost.

The cut stalks were prepared for retting in the greenhouse by measuring the total length of each stalk and cutting a 4 ft (73.44 cm) section from the center before placing in constructed retting boxes. The retting box consisted of three wooden frames (3 m × 1.5 m), separated into three equal sections (3.33 m), which were set on benches in the greenhouse and overlaid with 4 mil plastic, with 4 layers of burlap lining the bottom of each section. The burlap was intended to absorb and retain moisture from applied treatments as soil would in the field, and to prevent stalks getting uneven amounts of moisture from the pooling of water on the plastic lining. Each frame represented a treatment, and each section within the frame contained 21 stalks of an individual variety.

Three treatments were applied to each variety during greenhouse retting: low moisture (LM), high moisture (HM) and high moisture with soil slurry (SHM). The LM treatment consisted of 200 mL autoclaved deionized water misted over stalks every day using a surface sterilized hand-held sprayer. Additionally, 1 L of water was used to wet the burlap (avoiding stalks) every other day to simulate moisture retained in the soil in a field environment. The HM treatment consisted of 2 L of sterile deionized water showered over stalks from a sterile plastic watering can every two days, and misting with 200 mL sterile deionized water as above on days in between. The SHM treatment was the same as HM, except 1 g L^{-1} of soil collected from the field where the hemp was grown was added to the 2 L of water showered over stalks to simulate exposure to soil microorganisms during heavy rain. Six time points were used for sampling over the course of retting, corresponding to 24 h after placing stalks in the greenhouse, before any applied treatment (T1), and every seven days thereafter until it was determined the majority of stalks were sufficiently retted (bast fiber readily separated from the hurd core, but could not be easily broken by hand), which took between four and five weeks (T4-T5). Stalks were turned over in their boxes once during the study, at T3, as it is typical to turn hemp in the field during retting at least once. An additional sample was taken after seven weeks to examine highly over-retted conditions, in which significant fiber degradation was observed through easy breakage and visible fungal growth on the stalks and fibers (T6). Retting quality was ascertained by observation (color) and mechanical properties (ease with which bast and hurd separate by hand) to mimic what is done in a conventional agricultural field to determine when the majority of the crop is well-retted and ready to be baled for processing.

Three stalks were sampled from the each treatment at each time point. A six inch section was removed from the center of each stalk and cut into 1 cm pieces which were processed with a paddle blender (Bag Mixer 400, Interscience) using BagPage XR filter bags at high speed and closest setting for 5 min in 150 mL 100 mM sodium phosphate buffer, pH 7.0. Buffer filtered from the plant debris was poured into 250 mL sterile centrifuge bottles and centrifuged at 10,000× *g* for 20 min. The supernatant was discarded and the pellet resuspended in 2 mL of the phosphate buffer, then transferred to 2 mL microcentrifuge tubes and centrifuged again at 10,000× *g* for 5 min. The buffer was discarded and the pellet frozen at −20 °C. DNA extraction from frozen pellets and field soil samples used in SHM treatments was done using the MOBIO Power soil kit (Carlsbad, CA, USA).

DNA from hemp and soil samples was shipped to The University of Michigan Microbial Systems Molecular Biology Laboratory core sequencing facility (http://microbe.med.umich.edu/services/microbial-community-analysis) for PCR amplification and sequencing of the V4 region of the 16S rRNA gene on the Illumina Miseq platform (dual-barcoded, paired-end reads, 2 × 250 flow cell) according to Kozich et al. [7].

Sequence data from MiSeq analysis was processed using Mothur software (v1.40.5) following the MiSeq SOP (https://www.mothur.org/wiki/MiSeq_SOP, accessed July 2018) [7,8]. Briefly, paired-end reads were assembled into contigs, sequences were filtered for length, ambiguous bases, and homopolymer regions, then aligned to a SILVA reference alignment of the V4 region (SSU Silva 132). Pre-clustering to merge highly similar (2 bp or less mismatch) sequences was followed

by the removal of chimeras and 16S rRNA sequences derived from mitochondrial and chloroplast DNA. Operational taxonomic units (OTUs) were defined using a cutoff of 0.03 (97% similarity) and taxonomic classification was assigned based on Ribosomal Database Project (RDP) reference sequences (version 16, February 2016). The hemp data set was normalized to 2234 sequences per sample, which resulted in 2629 OTUs. This sampling depth resulted in at least 90% coverage using Good's Non-parametric Coverage estimator, with most samples greater than 97% [9,10]. Soil samples used in the SHM treatment were processed separately and normalized to 10,737 sequences per sample, resulting in 8661 OTUs. Two sampling depths were chosen for hemp and soil sequences because at 2234 sequences per sample, the soil samples were below 80% coverage. The soil and hemp samples were not statistically compared in this study, therefore different cutoffs for each of the data sets were chosen for maximum data retention. Raw sequence reads for all samples in this study were uploaded to the NCBI BioProject database under accession number PRJNA494847.

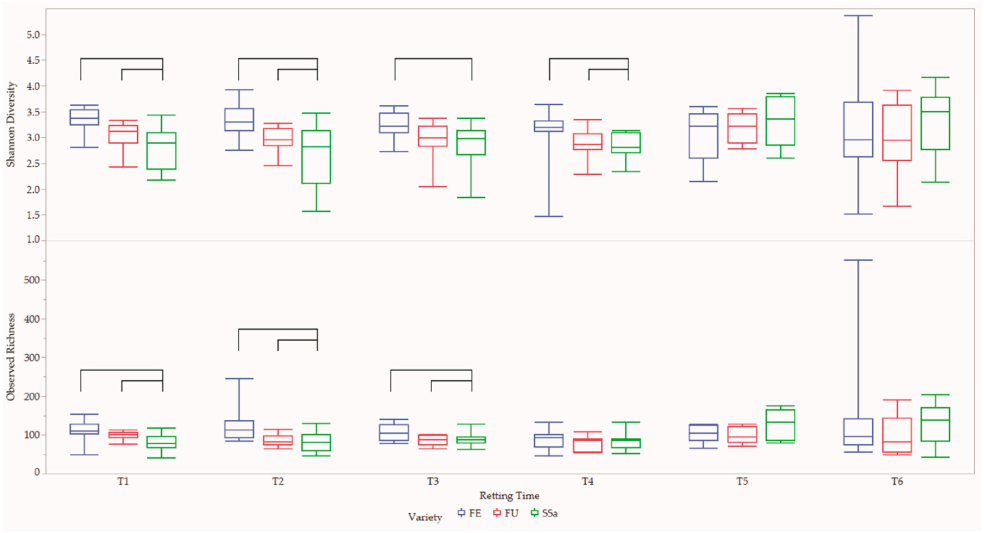
Statistical analysis was performed using programs in Mothur [8], Phyloseq package for R [11], and LEfSe (Linear discriminate analysis Effect Size) [12]. In Mothur, analysis of similarities (ANOSIM) [13] was used to compare bacterial community structure, and Indicator Species Analysis (ISA) [14] was used to identify individual OTUs whose presence was strongly indicated according to sample groups. The LEfSe program is a tool used to detect features that are most likely to explain differences between two or more sample groups. The Phyloseq program for R was used to calculate alpha diversity measures of Observed Richness and Shannon diversity. Jmp Pro software (version 13.2) was used to compare alpha diversity measures and abundance using the Kruskal-Wallis rank sum test, with significant results followed by Wilcoxon each-pair signed-rank test. The threshold used to determine statistical significance is $\alpha = 0.05$ unless otherwise indicated.

3. Results

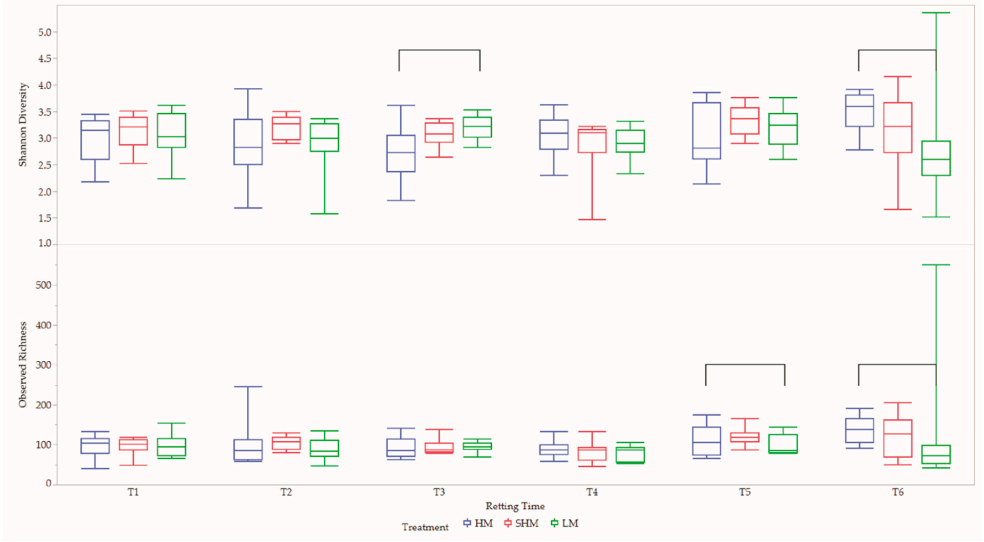
3.1. Diversity/Community Similarity

The three varieties (FE, FU, SSa) were compared at each time point. At T1, the SSa variety showed significantly lower alpha diversity values than FE and/or FU (Figure 1). Observed richness of variety SSa was lower than FE and FU for the first three time points, and showed lower Shannon diversity at T4, but generally, significant differences by variety decreased at each time point, with no further varietal differences after T4. ANOSIM comparisons between all varieties at T1-T2 were significant—at time points T3 and T4, SSa still showed significant differences from the other varieties, and by T4-T5 no significant differences between varieties remain (Table 1).

Treatments (LM, HM, SHM) within each variety (FE, FU, SSa) and among all varieties were compared over time and at each time point. Within each variety, all treatments compared at each time point and over time, showed no significant differences in alpha diversity measures and ANOSIM analysis (not shown). When treatments were compared at each time point including all varieties, no differences were seen for time points T1 and T2. At T3, the LM treatment was significantly lower than HM for measures Shannon diversity, and at T6 the LM treatment was lower for observed richness as well as Shannon diversity compared to the HM treatment (Figure 1b). ANOSIM global values were significant for T3, T5 and T6, with pair-wise comparisons showing significance between LM and HM treatments (Table 1), although R values were small (<0.3).



(**a**)



(**b**)

Figure 1. Alpha diversity (**a**) Observed richness and Shannon diversity by variety (**b**) Observed richness and Shannon diversity by treatment. Lines connecting box plots indicate significant difference using Wilcoxon each-pair signed-rank text, $\alpha = 0.05$.

Table 1. ANOSIM comparisons over time by variety and treatment [1].

	Retting Time					
	T1	**T2**	**T3**	**T4**	**T5**	**T6**
Variety	**0.337**	**0.263**	**0.228**	**0.228**	0.064	0.045
FE-FU	**0.220**	**0.206**	**0.152**	**0.152**		
FE-SSα	**0.486**	**0.317**	**0.308**	**0.308**		
FU-SSα	**0.376**	**0.302**	**0.235**	**0.235**		
Treatment	0.031	−0.017	**0.104**	0.031	**0.208**	**0.154**
D-HR			**0.220**		**0.353**	**0.345**
D-SS			0.098		**0.209**	0.088
HR-SS			0.000		0.021	0.015

[1] Global tests with *p*-values greater than 0.05 were followed by pair-wise comparisons. R-values in red bold indicate significance at $\alpha = 0.001$, R-values in black bold indicate significance at $\alpha = 0.05$.

3.2. Taxonomic Distribution and Community Profile

A total of 20 Phyla were present in the dataset; the most abundant were Proteobacteria (85.55%), Bacteroidetes, (7.58%), Bacteria_unclassified (2.78%), Actinobacteria (2.27%), and Firmicutes (1.13%), with Deinococcus-Thermus, Verrucomicrobia, Planctomycetes, Acidobacteria, candidate_division_WPS-1, Armatimonadetes, Candidatus_Saccharibacteria, Gemmatimonadetes, Fusobacteria, Chloroflexi, Tenericutes, Chlamydiae, SR1, candidate_division_WPS-2, and Nitrospirae making up the remaining 0.71%. Both HM and SHM treatments show a slight but significantly higher abundance in Bacteroidetes and a decrease in Proteobacteria at T5 and T6 (Figure 2). The Bacteroidetes phylum contained 319 OTUs including 40 genera; only one genus from the 20 most abundant OTUs (approximately 75% of the total abundance in the dataset) was a Bacteroidetes (Chryseobacterium), the others were comprised of 13 genera within Proteobacteria (Figure 3). While lower in abundance, and not statistically significant, several other OTUs in the Bacteroidetes phylum classified as Sphingobacteriaceae, Flavobacterium, Pedobacter, and Mucilaginibacter also increased in abundance from T4 to T6 (not shown).

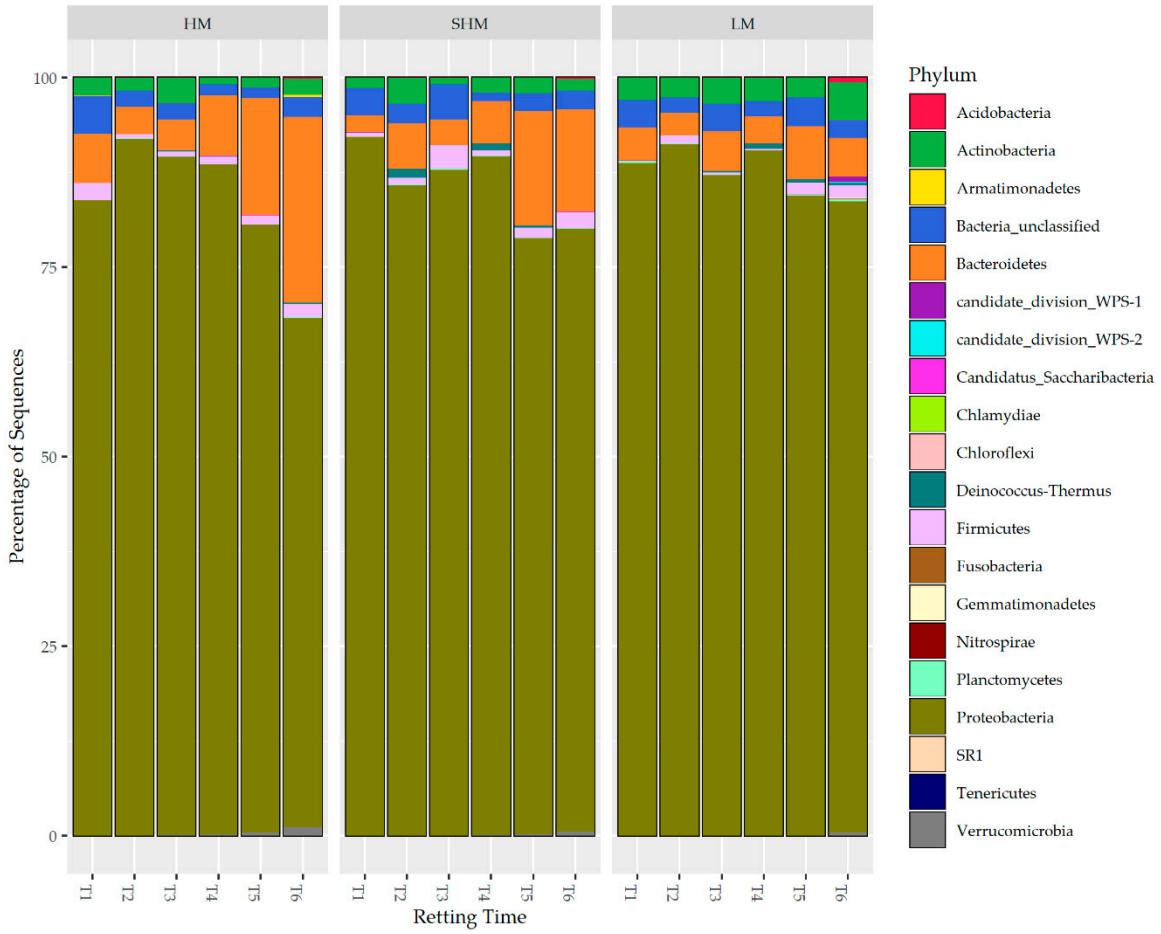


Figure 2. Relative abundance of Phyla according to treatment over time.

The SHM treatment was exposed to field soil, in the form of 1 g L^{-1} added to the water it received. We hypothesized that the field environment could contribute to the microbial profile by splashing onto stalks during rain and contact with the ground. While the soil slurry profile showed many of the same phylum groups as the hemp stalks, only three of the 20 most abundant genera were shared with hemp samples: *Massilia*, *Pseudomonas*, and *Sphingomonas*.

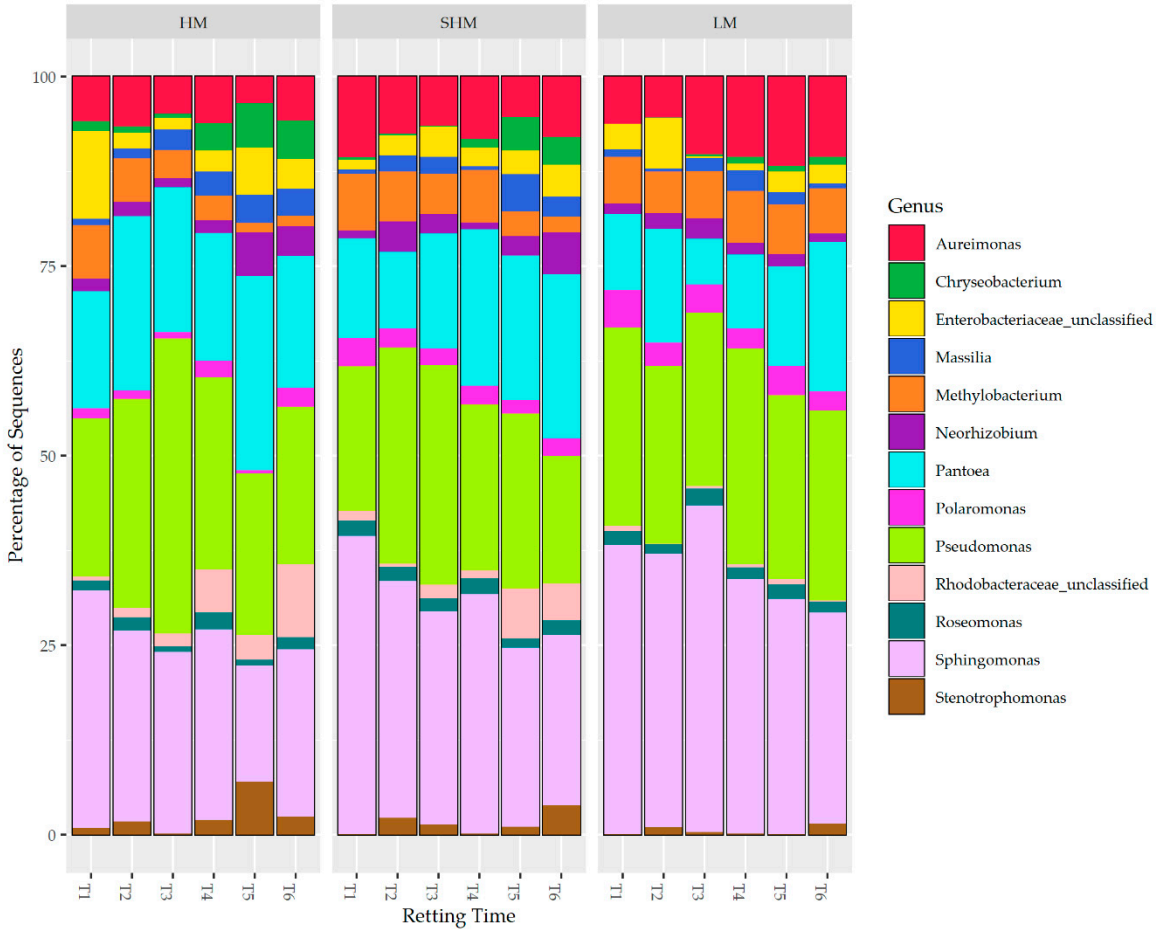


Figure 3. Relative abundance of genera composed of the 20 most abundant OTUs, according to treatment over time.

4. Discussion

Improving hemp fiber crop practices in order to make the resulting products more competitive for industrial use remains a goal that is largely unfulfilled at this time. This study, along with previous research, looks to the microbial population as a means to manipulate and/or control the retting process, with the intent to provide a cost effective means to increase consistency of the product. This experiment was conducted in order to learn how the microbial population responds to environmental conditions such as moisture variability and soil exposure, but controlled within a greenhouse rather than variable conditions in the field. Initially, we hypothesized that the bacterial population of the stalk at cutting would shift over time, correlating with under-retted and optimally-retted, as well as considerably over-retted material, and that treatments manipulating moisture and access to environmental microbiota would influence the population at these time points; however, the results indicate that changes in the retting population happen slowly, and significant treatment effects began to emerge only in the final time points.

The LM treatment, which received the least amount of water during retting, resulted in significant differences compared to HM and SHM, including lower richness and evenness at T6 and ANOSIM community similarity comparisons showing increasing R-values over time. While specific OTUs did not show strong associations in ISA or LeFse analysis by treatment, the HM and SHM samples showed a significant increase in the abundance of the phylum *Bacteroidetes* at T5 and T6, whereas the LM treatment did not show significant differences in abundance of phyla or genera over time. The addition of soil in the SHM treatment did not significantly impact the microbial community of the stalk compared to the other treatments, nor did it increase the speed of retting. Analysis of the soil used in the treatment

showed some overlap with respect to the most abundant bacteria found associated with the samples, but as there was little change from T1, in which no treatment had yet been applied to samples cut and removed from field, it seems likely that organisms associated with the hemp stalks were already present in/on the stalks at the time of cutting, and were not influenced greatly by exposure to soil throughout the retting process in this study. Varietal differences seen initially decreased over time—a possible explanation for the initial difference between SSa (lower alpha diversity) and the other two varieties is the stage of growth at harvest. While FE and FU varieties had flowered and were beginning senescence at harvest, the SSa had not flowered and was still in a stage of vegetative growth—a peculiarity of this experimental variety which was found not to flower at all under conditions from this field trial.

Our results indicate that a resilient microbial community associated with the plant stalks at harvest remained throughout the retting process and changed little according to the treatments applied and over the five weeks it took to ret the stalks completely. While the results do not support large shifts in the population according to time or treatment, a notable increase in the phylum *Bacteroidetes*, especially the genus *Chryseobacterium,* was observed in the final time points for HM and SHM treatments, but not LM, indicating moisture levels may influence this population over the course of retting. These results are in agreement with field studies of hemp and flax dew-retting, where similar microbial profiles were reported, and *Bacteroidetes* was found to increase toward the end of the retting process [15–17]. Previous analysis of the bacterial population changes that occur within retting hemp stalks showed an increase in the ratio of *Bacteroidetes* from mid-retting to full-retting conditions [18]. Flax, another natural fiber plant that relies on retting, also showed an increase in *Bacteroidetes* during the dew-retting process [15]. This phylum has previously been implicated in cellulolytic activity in both agricultural soils as well as gut microbiomes, and thus an increase in abundance of these organisms may signal a shift towards increased cellulose degradation and over-retting of fibers, following the depletion of pectin [18,19]. More research is needed to determine if higher abundance of *Bacteroidetes* correlates with fiber quality post-retting, nevertheless these results suggest a relationship between moisture levels during retting and abundance of this phylum at a crucial time in the retting process where the fiber is optimal, but before over-retting resulting in fiber degradation occurs. The relatively minor treatment effects on microbial diversity and community composition in this study did not appear to have affected retting time; all samples regardless of treatment were well-retted by T4/T5, and over-retted at T6.

Since the passing of the 2014 Farm Bill, opportunities for both growers and researchers interested in the potential of industrial hemp have increased in the US, although many obstacles remain. Specifically for fiber use, problems hinge around issues of scale and economy, in which the retting and decortication process has been described as a "bottleneck" for supplying consistent product to industry manufacturers [2,4,20,21]. Research addressing this problem has focused broadly on two areas: alternatives to field-retting such as solid-state fermentation with specific organisms and post-harvest treatments that focus on improving the end product without regard to the logistical and economic considerations, or adjusting agronomic practices such as timing of planting and harvest, turning in the field, and variety selection [6,15,17,22–24]. Fungal species are predicted to play a role in retting, by both their own degradation abilities and the process by which they facilitate entry of surface microorganisms past the cuticle, and have been a target for attempts to manipulate retting outcomes [17,25]. These efforts have all contributed useful information, but moving towards cost-effective retting practices that result in consistent fiber production with the specific qualities required by industry will require substantially more research from a variety of directions, such as traditional plant breeding for desirable field retting qualities (high cellulose, low lignin and pectin), molecular research into genes that affect fiber yield and quality, and microbiological investigations into specifically how the microbial profile associated with bast fiber crops is recruited and maintained throughout the plant's life as well as the retting process [20,26–29].

5. Conclusions

This work, together with past and recent studies of bast fiber retting, indicate that hemp (as well as other common bast fiber plants) harbor a resilient cohort of microorganisms that appear to be present at harvest and continue to persist throughout the retting process. To our knowledge, research into which microorganisms may be endophytes living within the stalks and which are surface dwelling has not been done. Endophytes are likely to be present in relatively low abundances at the time of harvest, and while significant patterns among the low abundance microorganisms were not detected, their contribution to the retting process cannot be discounted, as they are already "on-site" within the stalk when the plant is cut and the degradation begins. The resiliency of the bacterial community itself is ecologically interesting, and may provide a platform on which to study the concepts of resistance and resiliency in retting communities according to environmental disturbance and/or attempts to alter the community thorough applications of specific materials such as fungal/bacterial inoculum [30].

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