Microbial diversity observed during hemp retting

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Abstract Historically used in textile and paper industry, hemp fibres have started to find new applications in composite materials with important economic and ecological advantages. However, their applications are limited since manufacturers have some difficulties to standardise fabrication processes. This study is a first step before selection and isolation of strains that could later be used to optimise microbial retting efficiency and hence fibre quality. We studied six samples harvested on different ground types, at different dates and with different retting durations on field to obtain an exhaustive representation of the process. After DNA extraction, total bacteria and fungi associated with stems during retting were specifically quantified using real-time PCR. Then, using sequence analysis of randomly cloned 16S and 18S ribosomal RNA (rRNA) genes, a phylogenetic characterisation of the dominant microorganisms was carried out. Quantitatively, we showed that there were 8.1–9.5 $\log_{10}$ 16S rRNA gene copies per gram of hemp straw for bacteria and 8.6–9.6 $\log_{10}$ 18S rRNA gene copies per gram for fungi. Qualitatively, we noticed a higher bacterial diversity in comparison to fungi. This work showed that in the different samples, the same species were present but in significantly different proportions according to ground type, harvest dates and retting durations on field. The most frequent bacterial sequences were affiliated to species *Escherichia coli*, *Pantoea agglomerans*, *Pseudomonas rhizosphaerae*, *Rhodobacter* sp., *Pseudomonas fulva*, *Rhizobium huautlense* and *Massilia timonae*, whereas fungal sequences were principally related to the genera *Cladosporium* and *Cryptococcus*.

Keywords Hemp retting · Microbiota · Molecular inventory · qPCR

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Introduction

Bast fibre plants such as flax, hemp, and nettle produced valuable sclerenchymatous fibres which can be used in various products like textiles and high-quality papers (McGovern 1983). Hemp fibre plant (*Cannabis sativa* L.) has been recently used in bio-based composite materials for the automobile, the building, the household electrical appliances and the packaging (Pickering et al. 2007). Glass fibres have been the most used fibres to reinforce moulds due to their low production cost. Nevertheless, the incorporation of plant fibres in composite materials instead of glass fibres is an already marketed concept (Pickering et al. 2007).

The substitution of the plant fibres in mineral or synthetic fibres presents numerous advantages, such as the renewable character of the resource, the biodegradability and the reduction in the environmental impact and costs (Tahir et al. 2011). Nevertheless, certain drawbacks can put a brake on the industrial development, in particular the heterogeneity of the quantity and quality of the plant fibres. In plant stem, elementary cellulose fibres are glued together to form bundles located between the epidermis and the xylem core and parallel to the longitudinal axis of the stem. In these bundles, fibres are embedded in a pectic polysaccharidic network (Cronier et al. 2005; Vignon and Garcia-Jaldon 1996). The variability of hemp fibres is due to the preliminary retting, indispensable stage to the later use of fibres, which depends on environmental factors such as temperature, moisture, duration, etc. Stem retting is a process which facilitates the separation of fibres from other stem tissues. The main idea is to degrade the pectins (backbone of galacturonic acid residues with side chains of L-rhamnose, arabinose, galactose and xylose) and other cementing compounds that bind the bast fibres and fibre bundles to other tissues and thereby separate fibres and nonfibre materials ((Akin 2013) for a review). Enzymes hydrolysing pectins are broadly known as pectinases and include polygalacturonases, pectin esterases, pectin lyases and pectate lyases (Alkorta et al. 1998). A wide variety of microorganisms produce pectinases. Retting is thus a fermentation process involving several bacteria (e.g., *Bacillus*, *Clostridium*) and fungi (e.g., *Aspergillus*, *Penicillium*) which degrade the bark and release fibres (Sharma and Robinson 1983). To achieve anaerobic retting, straw sheaves are submerged in water pits, in concrete tanks or in running fresh water. The first microorganisms able to grow belong to the genus *Bacillus* in the aerobic phase; at that point, tank or other stagnant retts become depleted of dissolved oxygen encouraging the development of a different microbiota, notably *Clostridium acetobutylicum* and *Clostridium felsineum* for hemp water retting (Tamburini et al. 2004). *Achromobacter parvulus*, several *Clostridia* and *Pseudomonas aeruginosa* were identified as bacterial retting agents implicated in the water retting of Brazilian flax (Rosemberg 1965). Pollution due to this fermentation process as well as important costs linked to labour and drying lead this anaerobic retting to be replaced by dew retting (an aerobic process) (Henriksson et al. 1997). In dew (or field) retting, plant straw is thinly spread on the ground and exposed to the action of fungi and aerobic bacteria for 2–10 weeks. Species of *Cladosporium*, *Penicillium*, *Aspergillus* and *Rhodotorula* have been isolated from dew-retted plants (Fogarty and Ward 1972). Fungi such as *Cladosporium herbarum*, *Epicoccum nigrum*, *Alternaria alternata*, *Fusarium* sp., *Aureobasidium pullulans*, *Phoma* sp., *Mucor* spp., *Rhizomucor pusillus* and *Rhizopus* sp. were also identified during flax dew retting (Akin et al. 1998; Henriksson et al. 1997; Sharma 1986a). Since hemp is more lignified than

flax, different enzymes should be needed. A mechanical step is then used to clean the separated fibres. The main disadvantage of the whole microbial process is the risk that cellulolytic enzymes secreted by the microbiota damage fibres. The duration is thus extremely important.

For the industries, it is necessary to master the retting to obtain value-added fibres with a constant quality and a high yield. Furthermore, there are only few data in the literature concerning the dew retting of hemp. The objective of this study was to gather information on the dynamics of the retting, especially on the identification of microorganisms involved during this primary process. On the one hand, quantitative information will permit the estimation of the colonisation level of the various microorganisms present using real-time PCR. On the other hand, qualitative information will characterise the retting profile by identifying the microbial species involved. The phylogenetic analysis of bacterial 16S and fungal 18S ribosomal RNA (rRNA) genes, amplified directly from complex communities, provides an efficient strategy for exploring the biodiversity and had been applied to many ecosystems (Borneman and Hartin 2000; Renault et al. 2012; Song et al. 2001; Suau et al. 1999), notably to kenaf (Visi et al. 2013) and to jute (Munshi and Chattoo 2008) water retting. Therefore, in order to take into account cultivable as well as non-cultivable microorganisms, molecular tools have been chosen to investigate the hemp retting community. Detailed phylogenetic inventories (bacterial and fungal) of six hemp samples cultivated on two types of ground, with two harvested data, and two retting durations on field have been carried out. The results are expected to obtain the best representation of the process. The long-term objective is to optimise retting efficiency and control the process through new strategies. Recently, the water retting process and fibre quality of hemp were improved by simultaneously inoculating water tanks with two selected pectinolytic strains (Di Candilo et al. 2010). Four pectinolytic strains were also selected to improve water retting of jute (Das et al. 2012). These strains belonged to *Clostridium* and *Bacillus* genera. Microbial water retting of kenaf was also optimised by adding *Bacillus* and *Paenibacillus* strains in experimental tanks (Visi et al. 2013). Development of specific starter strains selected from the molecular inventories could be envisaged for improving dew retting efficiency of hemp and hence the fibre quality.

Materials and methods

Plant material and growth conditions

Hemp (*C. sativa* L. cv Fedora 17) was grown in 2011 by professional hemp producers in field under standards conditions: sowing in April at 50 kg/ha in a soil fertilised with 120 kg nitrogen/ha (Institut Technique du Chanvre; CETIOM,

2007, Le chanvre industriel, 26 p, http://www.cetiom.fr/). Field trials were performed in the South of Champagne region in France on two types of ground characteristic of this hemp production area: a white "chalky soil" corresponding to the field located at Echemines (48° 38′ N, 3° 83′ E) and a "red soil" corresponding to the field located at Marcilly le Hayer (48° 35′ N, 3° 63′ E). Soil in these sectors is sandy clay of homogeneous texture with less than 1-m depth before the limestone. The clay level is the major difference between these two types of soils; "red soils" are more clayish resulting in their "red colour" compare to "white soil". These fields being 20 km away from each other, the climatic conditions were similar.

Straw harvest and samples preparation

Two dates of harvest were tested surrounding the seed maturity: the early date (D1) corresponds to the end of flowering stage and the late date (D2) corresponds to the seed maturity stage. From an agronomical point of view, D2 was the normal stage to harvest hemp in this area, farmers harvesting seeds and straw. Considering stem anatomy, tissue organisation was achieved at D1. Whatever the date, harvesting corresponds to stem cut at their base and their disposal on ground. Hemp straw was then swathed to form a long, narrow strip in field, 1 week after cutting. Straw cutting and swathing were mechanically performed. Straw remains on ground during the retting period. According to farmer experience, two levels of retting were tested: a mid-retting corresponding to 3 weeks and a full retting corresponding to 5 or 6 weeks. All these conditions were resumed in Table 1.

After harvest in field, samples of straw were immediately stored in a −20 °C cool box. Five gram of each stem sample were cut into 2-cm pieces in sterile conditions to allow their grinding in the Stomacher® (Lab-blender 80, England) with 45 mL of NaCl 0.9 % for 5 min. Liquid fractions were then recovered in 50-mL sterile tubes and centrifuged during 15 min at 4,000×g, at 4 °C. Pellets were resuspended in 250 μL of NaCl 0.9 %, and total DNA was extracted for each sample. This procedure was repeated twice, the one for culture, the second for total DNA extraction.

Table 1 Hemp sampling conditions

Culture conditions		Length of the retting process (weeks W)						
Ground type R=red C=chalky	Harvesting data D1=end of flowering stage D2=mature seed stage	0	1	2	3	4	5	6
R2	D1				+			+
R6	D2				+			
C2	D1				+	+		
C6	D2				+			

Hemp fibre quality control

The quality of hemp fibre was estimated through a measure of linear density of scutched tow. To extract fibre, hemp straw was parallelized and dried then scutched which consists of breaking straw by breaker rollers and "beating" out short fibre and wooden parts into scutched tow. Drying and scutching were proceeded using industrial equipment under the expertise of FRD companies.

Linear density of hemp tow was measured according to Müssig et al. (2006). Hemp tow was cut into 2-cm pieces, then weighed to obtain a measure in grams per kilometre.

Quantification of viable microorganisms

Each sample was analysed using cultivation. Tenfold serial dilutions were carried out on tryptone-salt medium, after which 0.1 mL of each dilution was inoculated onto different media and incubated at the appropriate temperatures. Total aerobic bacteria were cultured at 30 °C in tryptic soy agar (BioMérieux SA, Marcy l'Etoile, France). Anaerobic bacteria were cultured at 30 °C in Brain Heart Infusion agar (BioMérieux SA, Marcy l'Etoile, France) supplemented with yeast extract (5 g/L) and hemin (5 mg/L), under anaerobic conditions (85 % N_2, 10 % H_2, 5 % CO_2). Total fungi were cultured in Sabouraud chloramphenicol agar (BioMérieux SA, Marcy l'Etoile, France) at ambient temperature. Bacteria were numerated after 3 days of incubation, and fungi were quantified after 5 days.

Extraction and purification of total DNA

Total DNA was extracted from 250 μL sample aliquots as previously described (Godon et al. 1997). To extract both bacteria and fungi DNA, a mix of sterile glass beads (diameter 0.1 mm and 0.5 mm) for mechanical extraction was used. The purification was performed using one wash with saturated phenol (pH 8.0) followed by two washes with chloroform: isoamyl alcohol (24:1). Sodium acetate (3 M) and isopropanol 100 % were used to precipitate DNA. Cleaning was achieved with a 70 % ethanol wash. Dried pellets were then resuspended in sterile water.

Clone library construction, screening and sequencing

Twelve libraries of about 50 clones were constructed from the DNA extracts from the bacterial and fungal communities isolated from each hemp sample. Bacterial 16S rRNA genes were amplified with the forward primer S-D-Bact-0339-a-S-20 (5′ CTC CTA CGG GAG GCA GCA GT 3′ (Amann et al. 1990)) and the universal reverse primer S-*-Univ-1385-a-A-18 (5′ GCG GTG TGT ACA AGR CCC 3′ (Magne et al. 2006a)). Fungal 18S rRNA genes were amplified with the forward

primer S-D-Fung-0817-a-S-24 (5′ TTA GCA TGG AAT AAT RRA ATA GGA 3′) and the reverse primer S-D-Fung-1536-a-A-20 (5′ ATT GCA ATG CYC TAT CCC CA 3′) (Borneman and Hartin 2000). PCR amplifications were carried out with a standard PCR mix (0.5 U of *Taq* DNA polymerase (Ampli*Taq* Gold; Perkin-Elmer Corporation, Foster City, Calif.), 1 x reaction buffer II, 2.5 mM MgCl$_2$, 200 μM of each dNTP, 0.4 μM of each primer and sterile water added to a final volume of 50 μL). Three PCR reactions were made for each of the six samples for both inventories. Initial enzyme activation step was performed at 94 °C for 10 min in a Mastercycler® thermocycler (Eppendorf AG, Hamburg, Germany), followed by 30 cycles of denaturation at 96 °C for 15 s, annealing at 59 °C for bacteria or at 55 °C for fungi for 1 min and elongation at 72 °C for 4 min, which were followed by a final elongation at 72 °C for 15 min. The three PCR products were pooled according to sample, purified and concentrated with a QIAquick spin PCR purification kit (Qiagen, S.A., Courtaboeuf, France) and eluted with 50 μL of sterilised, nuclease-free water. The purified products were ligated into pGEM®-T vector (Promega Corporation, Madison, USA) and used to transform competent *Escherichia coli* DH10b cells. White recombinant cells were selected on Luria-Bertani medium (Difco, Detroit, USA) supplemented with ampicillin (100 μg/mL), isopropyl-β-D-thiogalactopyranoside (IPTG, 500 μmol/L) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 80 μg/mL). Plasmidic inserts were amplified with appropriate primers then sequenced using primers Bact-0339 or Fung-0817 (Beckman Coulter Genomics, Takeley, UK). The sequences were corrected using Chromas Pro version 1.5. A search of the GenBank nucleotide database was conducted using the BLAST algorithm (www.ncbi.nlm.nih.gov/blast) to determine the closest relative of partial 16S or 18S rRNA gene sequences (Maidak et al. 1999). The sequences were aligned using Clustal Omega 1.1.0. with the profile alignment option in SeaView 4.5.2 (Gouy et al. 2010). A representative of each operational taxonomic unit (OTU or molecular species) was submitted to the GenBank database with the accession numbers KM507203 to KM507290. An OTU or a phylotype consisted of all sequences (clones and reference strains) with less than 2 % divergence (Suau et al. 1999) from 695 to 800 aligned homologous nucleotides and was also generally consistent with the results of the comparison of 16S homology and DNA-DNA re-association values (Stackebrandt and Goebel 1994). The same rate was used for divergence of 18S rRNA gene. Maximum-likelihood trees were built using PhyML 3.1 (Guindon et al. 2010). The stability of phylogenetic relationships was assessed using the bootstrap method based on 100 replications. Chimeric sequences were detected and removed. Generated during PCR, these sequences formed from two or more sequences joined together, branched differently in trees based on 200 aligned bases at the 5′ and at the 3′ ends. Find chimeras from software

DECIPHER confirmed the first assessment (Wright et al. 2012). Coverage values were calculated by Good's method (Good 1953), according to which the percentage of coverage was calculated with the formula $[1 − (n/N)] \times 100$, where *n* is the number of molecular species represented by one clone (single-clone OTUs) and *N* is the total number of sequences. This calculation estimates how well the clones accounted for the biodiversity.

Quantification of bacterial and fungal populations by real-time PCR

Reactions were performed in duplicate in a volume of 25 μL within 96-well twin-tech PCR plates, using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Cergy Pontoise, France). The forward and reverse primers used were Bact-0339 and Bact-0788 for total bacteria (Magne et al. 2006b) and Fung-0817 and Fung-1196 for total fungi (Borneman and Hartin 2000). Amplifications were performed in a Mastercycler ep Realplex4 (exCitation source 470 nm, emission 520/550 nm) (Eppendorf AG, Hamburg, Germany) with the following temperature profile: one cycle at 50 °C (2 min) for uracil-DNA glycosylase action, one cycle at 96 °C (2 min), 40 cycles of denaturation at 96 °C (15 s), primer annealing at 55 °C for bacteria and fungi (1 min) and elongation step at 68 °C (2 min) with fluorescence measure. Finally, the melting curve was made by slowly heating the PCR mixtures from 60 to 96 °C (20 min) with simultaneous measurements of the SYBR Green I signal intensities. A standard curve made from known amounts of plasmid DNA containing a 16S rRNA gene insert from *E. coli* or a 18S rRNA gene insert from *Saccharomyces cerevisiae* allowed quantifications.

Data analysis

Quantitative data were presented as $\log_{10}$ 16S (or 18S) rRNA gene copies per gram of straw. Each replicate was determined by the average value of two samples. Viable counts were transformed in logarithmic values and then expressed as the mean of the two replicates.

Results

Quality of fibre extract from retted straw

Linear density (weight of a theoretical 1-km strip of tow) gives an estimation of the fineness of tow. Among the parameters used to estimate fibre quality, the fineness of scutched tow seems to be the most global. This parameter has a great impact for textile, composite and building industries. For example,

the quality of yarn appears closely linked to the thinness of scutched tow (Sharma and Van Sumere 1992); furthermore, the fineness of fibre added to thermoplastic resin could explain the mechanical properties of composite (Bourmaud 2013).

Retting have an impact on the fineness of hemp scutched tow: a reduction of near 50 % of the linear density could be observed between hemp straw after 6-week retting and raw hemp straw (Fig. 1). No significant difference could be observed between chalky and red soils. The improvement of hemp tow fineness during retting appeared progressive; nevertheless, it was more regular in chalky soil. The substantial standard deviation at 3 weeks for both chalky and red soils came from the heterogeneity of tow and could be an evidence of an unfinished retting process. Finally, these results confirmed that the retting process lead to more thin hemp tow, so the final quality of hemp fibre for technical application was enhanced.

Quantification of total bacteria and total fungi using culture

CFU counts ranged for anaerobic bacteria from 5.1 to 8.1 $\log_{10}$ per gram and for aerobic bacteria from 5.5 to 8.7 $\log_{10}$ per gram (Table 2a). Total fungi counts ranged from 5.9 to 7.0 $\log_{10}$ per gram. Red ground had higher counts of total bacteria and fungi after 6 weeks of retting process than after 3 weeks. The same was not observed with chalky ground. Indeed, higher counts of bacteria were found after 3 weeks of retting process than after 5 weeks. Early or normal harvesting data did not change the number of total bacteria or fungi after 3 weeks of retting process on red ground (R2W3 versus R6W3). The number of total bacteria was lower on chalky ground compared to red ground in the same conditions (C6W3 versus R6W3), with similar counts of fungi. Moreover, bacteria and fungi increased on chalky ground between normal harvesting data after 3 weeks of retting process and early harvesting data after 5 weeks of retting process, as
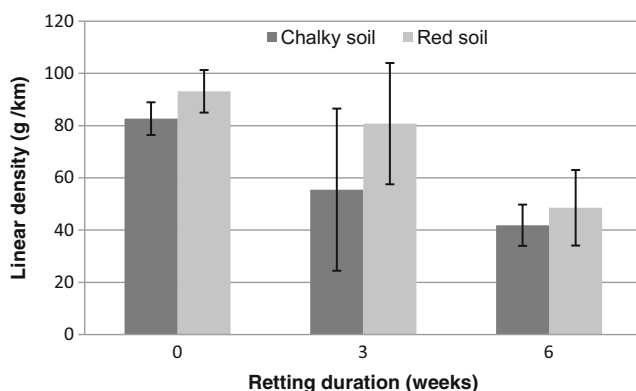

Fig. 1 Linear density (g/km) of hemp tow in function of retting. *Bars* represent standard deviation

Table 2 Quantification of bacterial and fungal counts per gram of straw hemp using

a) culture

$\log_{10}$ CFU/g

	Anaerobic bacteria	Aerobic bacteria	Fungi
R2W3	6.4	6.5	5.9
R2W6	7.1	7.0	7.0
R6W3	6.0	6.2	6.0
C2W3	8.1	8.7	6.7
C2W5	6.4	6.7	6.8
C6W3	5.1	5.5	6.0

b) qPCR

$\log_{10}$ 16S or 18S rRNA gene copies/g

	Bacteria	Fungi
R2W3	8.1	8.6
R2W6	9.4	9.6
R6W3	9.0	8.6
C2W3	9.4	8.9
C2W5	9.5	8.8
C6W3	8.6	8.7

attended (C6W3 versus C2W5). These data tend to show that the high bacterial level observed for C2W3 sample is unexpected and may be artefactual.

Quantification of total bacteria and total fungi using qPCR

Quantitatively, we showed that there were 8.1–9.5 $\log_{10}$ 16S rRNA gene copies per gram of straw hemp for bacteria and 8.6–9.6 $\log_{10}$ 18S rRNA gene copies per gram for fungi (Table 2b). From red ground, the bacteria and fungi quantified after 6 weeks of retting were more numerous than after 3 weeks. However, the microorganisms quantified from chalky ground at 3 and 5 weeks were not different and equivalent to the counts observed on R2W6 sample for bacteria. Fungi counts were higher in R2W6 sample. The number of total bacteria was lower from chalky ground compared to red ground in the same conditions (C6W3 versus R6W3), with similar counts of fungi.

Clone library screening

Analysis of the 16S rRNA clone libraries

To assess bacterial diversity, 300 16S rRNA genes from the six samples (cultivated on two types of ground, with two harvested data, and two retting durations on field, Table 1), were sequenced and analysed (about 50 per sample). Four sequences were considered as chimera and were removed

from analyses. The other sequences were grouped into 66 different phylotypes according to the phylogenetic uniqueness of the closest relative (Supplemental Table S1). The percentages of similarities based on sequence comparison varied from 97 to 100 %. Among the 66 phylotypes, 65 (98.5 %) presented more than 98 % of similarity with previously identified sequences such as *Rhizobium huautlense* (99–100 %), *Pseudomonas graminis* (99 %) and *Rhodococcus fascians* (99 %) (see Supplemental Table S1). The most represented phylum was *Proteobacteria* (77 % of sequences). Among *Proteobacteria*, there were mainly γ-*Proteobacteria* (48 %), α-*Proteobacteria* (21.5 %) and β-*Proteobacteria* (7.5 %). Other dominant phyla were *Actinobacteria* (11 %) and *Bacteroidetes* (11 %). *Firmicutes* phylum was represented by only two sequences (1 %). At the phylotype level, the most abundant phylotypes were related to *E. coli* (JQ907529) (rank 1, 15 % of sequences), *Pantoea agglomerans* (FJ357815) (rank 2, 12 % of sequences), *Pseudomonas rhizosphaerae* (GU396285) (rank 3, 10 % of sequences), *Rhodobacter* sp. CCBAU (5 %), *Pseudomonas fulva* (4 %), *R. huautlense* (4 %), *Rhodobacter* sp. JA745 (3 %) and *Massilia timonae* (2 %) (Supplemental Table S1). These species were retrieved in almost all libraries.

Analysis of the R2W3 16S rRNA clone library (red ground, early harvesting data, 3 weeks of retting process)

The 49 sequences from the R2W3 library were distributed into 27 phylotypes and three phyla (Supplemental Table S1, Fig. 2). *Proteobacteria* was the most abundant phylum (80 % of the sequences); they were exclusively γ-*Proteobacteria* (43 %), α-*Proteobacteria* (31 %) and β-*Proteobacteria* (6 %). Other phyla including *Actinobacteria* (12 %) and *Bacteroidetes* (8 %) were also identified. Using a threshold of 98 % sequence similarity in 16S rRNA gene to define a molecular species, all sequences were identified to the species level (Supplemental Table S1). Most of them were *Pseudomonas* species (*Pseudomonas psychrotolerans*, *P. rhizosphaerae*, *P. fulva*, *Pseudomonas fluorescens*; 12 sequences), *Rhodobacter* species (eight sequences), *E. coli* species (five sequences), *Pantoea* species (*P. agglomerans* and *Pantoea* sp.; three sequences) and *Rhizobium* species (three sequences). The other species were classified within the following genera: *Methylobacterium*, *Rhodococcus*, *Sphingobacterium* and *Leifsonia* (two sequences each) and *Xanthomonas*, *Massilia*, *Variovorax*, *Frigoribacterium*, *Pedobacter*, *Chryseobacterium*, *Brevundimonas*, *Nocardioides*, *Achromobacter* and *Roseomonas* (one sequence each). The percentage of coverage ($[1 − (n/N)]$ x 100) was low (65 %). That means that, for this sample, the probability for the next sequence to represent a new phylotype was 35 %.

Analysis of the R2W6 16S rRNA clone library (red ground, early harvesting data, 6 weeks of retting process)

The 46 sequences from the R2W6 library were related to 18 unique phylotypes and 4 phyla (Supplemental Table S1, Figs. 2 and 3). The most abundant were *Proteobacteria* (74 % of the sequences), mostly γ-*Proteobacteria* (48 %), α-*Proteobacteria* (24 %) and β-*Proteobacteria* (2 %). Two other phyla found in abundance were *Bacteroidetes* (13 %) and *Actinobacteria* (9 %). *Firmicutes* phylum was represented only by two sequences (4 %). All sequences had a sequence similarity equal or greater than 98 % with a sequence present in database and were identified as *P. agglomerans* species (15 sequences), *Rhodobacter* species (seven sequences), *E. coli* species (five sequences) and *Rhizobium* species (*Rhizobium huautlense* and *Rhizobium soli*; four sequences). *Pseudomonas*, *Chryseobacterium*, *Epilithonimonas*, *Paenibacillus* (two sequences), *Massilia*, *Pedobacter*, *Sphingobacterium*, *Rhodococcus*, *Microbacterium*, *Pseudoclavibacter* and *Aeromicrobium* (one sequence) genera were also found. The percentage of coverage (78 %) was higher than for the sample R2W3.

Analysis of the C2W3 16S rRNA clone library (chalky ground, early harvesting data, 3 weeks of retting process)

The 52 sequences from the C2W3 library corresponded to 21 phylotypes distributed among three phyla (Supplemental Table S1, Fig. 2). The sequences were mainly affiliated to *Proteobacteria* (77 % of the sequences), mostly γ-*Proteobacteria* (60 %), α-*Proteobacteria* (13 %) and β-*Proteobacteria* (4 %). As previously, *Bacteroidetes* and *Actinobacteria* (11.5 % each) were found. Forty-six sequences were identified to cultured species affiliated to species *E. coli* (13 sequences), *P. agglomerans* (six sequences), *Pseudomonas* (*P. fulva*, *P. fluorescens*; six sequences), *Stenotrophomonas* (*Stenotrophomonas chelatiphaga*, *Stenotrophomonas maltophilia*; four sequences), *Rhizobium* (*Rhizobium cellulosilyticum*, *Rhizobium* sp.; three sequences), *Nocardiopsis* (*Nocardiopsis synnemataformans*, *Nocardiopsis prasina*; three sequences), *Rhodobacter*, *Achromobacter* (two sequences), *Aurantimonas*, *Brevundimonas bullata*, *Erwinia billingiae*, *Xanthomonas*, *Microbacterium oxydans*, *Agrococcus jejuensis* and *Saccharopolyspora* (one sequence). The six remaining sequences had a sequence similarity equal to 97–98 % with a sequence identified as uncultured *Sphingobacterium* sp. The percentage of coverage was 77 %.

Analysis of the C2W5 16S rRNA clone library (chalky ground, early harvesting data, 5 weeks of retting process)

The 45 sequences from the C2W5 library represented 25 phylotypes distributed among the already three described phyla (Supplemental Table S1, Fig. 2). *Proteobacteria* was always the most abundant phylum with 62 % of sequences (*γ-Proteobacteria* and *α-Proteobacteria* (26.5 % each), *β-Proteobacteria* (9 %)). *Actinobacteria* (20 %) and *Bacteroidetes* (18 %) phyla were also found in significant abundance. All sequences were identified to the species level and corresponded to *E. coli* species (nine sequences), *Rhizobium* species (*R. huautlense, R. soli, Rhizobium* sp.; six sequences), *Rhodobacter* sp. (four sequences), *Flavobacterium* species (*Flavobacterium johnsoniae, Flavobacterium* sp.; four sequences), *Massilia* species (*M. timonae, Massilia aurea*; three sequences), *Leifsonia* sp. (three sequences), *Pseudomonas* species *(P. rhizosphaerae, P. fulva), Frigoribacterium faeni, R. fascians* (two sequences each), *Sphingomonas* sp., *Methylobacterium* sp., *Achromobacter* sp., *P. agglomerans, Pedobacter hartonius,*

Chryseobacterium piscicola, Epilithonimonas lactis, Dyadobacter alkalitolerans, Agrococcus jejuensis and *Aeromicrobium erythreum* (one sequence each). The percentage of coverage was 67 %.

Analysis of the R6W3 16S rRNA clone library (red ground, normal harvesting data, 3 weeks of retting process)

The 52 sequences from the R6W3 library (13 phylotypes) fell into the same three phyla (Supplemental Table S1, Fig. 2). The sequences were mainly affiliated to *Proteobacteria* (96 % of the sequences), mostly *γ-Proteobacteria* (82 %), then *α-Proteobacteria* (8 %) and *β-Proteobacteria* (6 %). *Actinobacteria* and *Bacteroidetes* were only represented by one sequence (2 % each). All sequences could be identified and were affiliated to *Pseudomonas* species (*P. rhizosphaerae, P. graminis, Pseudomonas syringae*; 23 sequences), *E. coli* (11 sequences), *P. agglomerans* (nine sequences), *R. huautlense, M. timonae* (two sequences each), *Aurantimonas* sp., *Methylobacterium* sp., *Variovorax paradoxus, Hymenobacter* sp. and *Aeromicrobium*

Fig. 2 Phylogenetic distribution in the six clone libraries constructed from bacterial communities of hemp samples
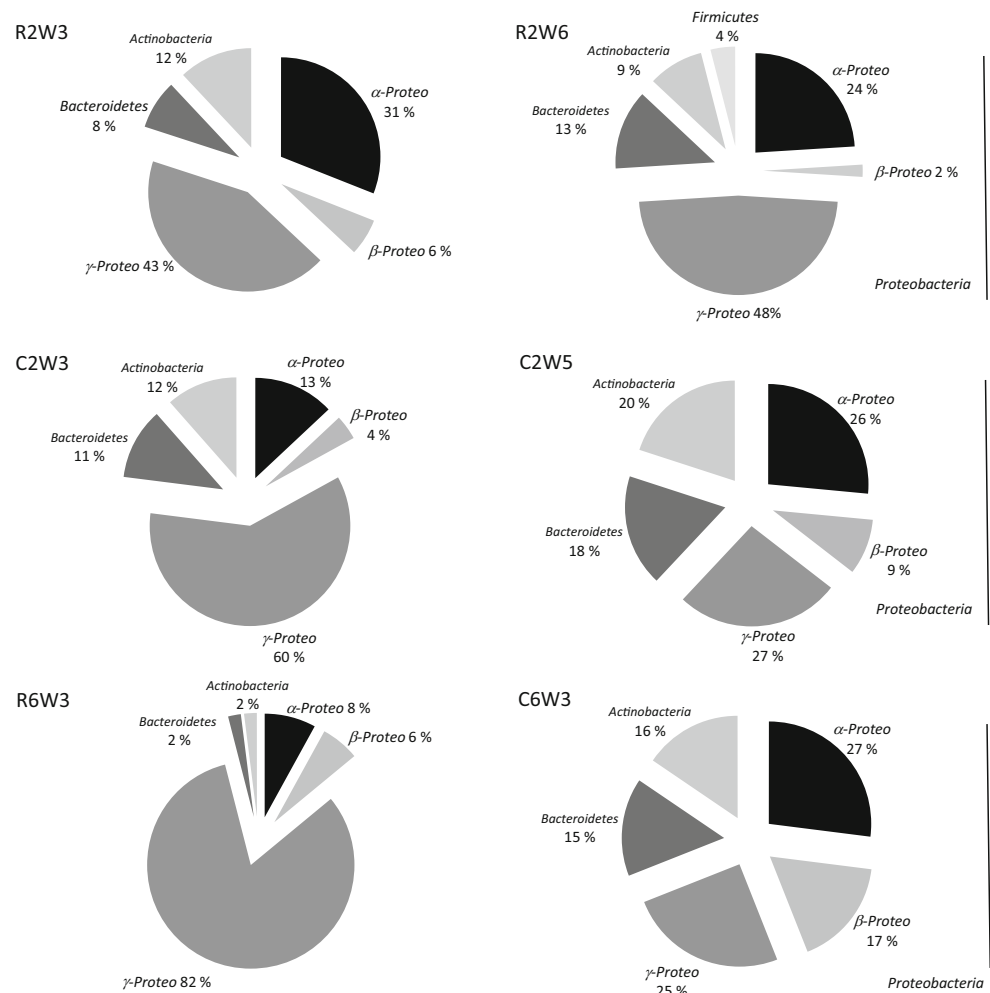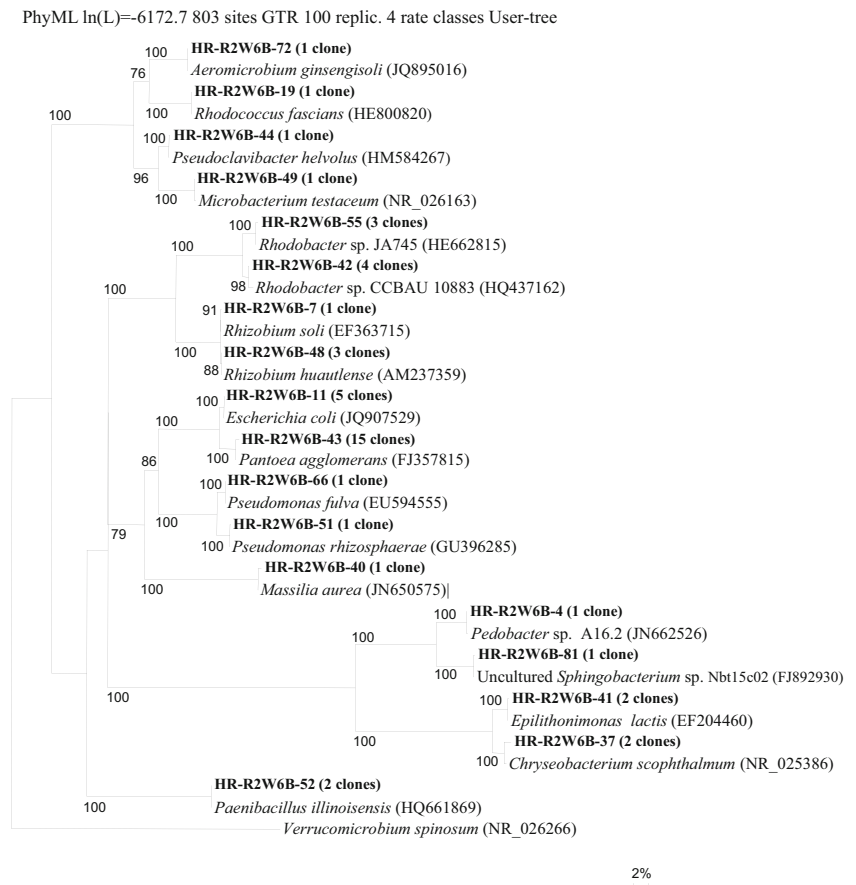
Fig. 3 Phylogenetic tree constructed using bacterial partial SSU rDNA sequences retrieved from R2W6 retting hemp

PhyML ln(L)=-6172.7 803 sites GTR 100 replic. 4 rate classes User-tree

2%

ginsengisoli (one sequence each). The percentage of coverage was high (89 %).

Analysis of the C6W3 16S rRNA clone library (chalky ground, normal harvesting data, 3 weeks of retting process)

Fifty-two sequences were also analysed, corresponding to 26 phylotypes and three phyla (Supplemental Table S1, Fig. 2). *Proteobacteria* (69 % of sequences) were represented by γ-*Proteobacteria* (25 %), α-*Proteobacteria* (27 %) and β-*Proteobacteria* (17 %). *Actinobacteria* and *Bacteroidetes* (15.5 % each) were also found in this library. At the species level, species belonging to the *Pseudomonas* genus were the most abundant (*P. rhizosphaerae*, *P. graminis*, *P. fulva*; eight sequences); then, the following were found: *Methylobacterium* sp. and *Massilia* (*M. timonae*, *M. aurea*; seven sequences each), *Pedobacter* (*P. hartonius*, *Pedobacter agri*) and *F. faeni* (six sequences each), *E. coli* (three sequences), *P. agglomerans*, *Rhodobacter* sp., *Sphingomonas* sp. (two sequences each) and *Paracoccus marcusii*, *Rhizobium* sp., *Aurantimonas* sp., *V. paradoxus*, *Acidovorax* sp., *Hymenobacter* sp., *Flavobacterium rivuli*, *Kineococcus* sp., *Geodermatophilus* sp. (one sequence each). The percentage of coverage was 65 %.

Analysis of the 18S rRNA clone libraries

Three hundred and six 18S rRNA genes were also sequenced and analysed (about 50 per sample). Three sequences were considered as chimera and were removed from analyses. The sequences were grouped into 22 different phylotypes (Supplemental Table S2). The percentage of sequence similarity ranged from 97 to 100 %. Among the 22 phylotypes, 21 (95 %) presented more than 98 % of similarity with previously identified sequences. The best-represented phylum was *Ascomycota* with 70 % of sequences. The other sequences (30 %) were all affiliated to phylum *Basidiomycota*. The most abundant phylotypes were related to *Cladosporium* species (rank 1, 47 % of sequences). However, it was not possible to differentiate species *Cladosporium bruhnei* (JN397376) from *C. herbarum* (EU343080) and *Cladosporium cladosporioides* (JX470336). The second most abundant phylotypes were affiliated to *Cryptococcus* species (rank 2, 28 % of sequences), notably to *Cryptococcus carnescens* (24 % of sequences). These phylotypes were shared by all libraries except the C2S3 one, extremely different from the others and dominated by species *Stilbella fimetaria* (FJ939395).

Analysis of the R2W3 18S rRNA clone library

The 49 sequences from the R2W3 library were distributed into eight phylotypes within two phyla (Supplemental Table S2 and Fig. 4). *Ascomycota* was the most abundant phylum (61 % of sequences) represented principally by the genus *Cladosporium* (29 sequences). The second phylum found was *Basidiomycota* (39 % of sequences) represented principally by the genus *Cryptococcus* (*C. carnescens*, *Cryptococcus psychrotolerans*, *Cryptococcus* sp., 18 sequences). The other species found belong to *Plectosphaerella cucumerina* and *Rhodotorula marina* (one sequence each). The percentage of coverage (94 %) was high. This means that the probability for the next sequence to be already detected was 94 %.

Analysis of the R2W6 18S rRNA clone library

The 48 sequences from the R2W6 library were related to five unique phylotypes within two phyla (Supplemental Table S2,

Figs. 4 and 5). The sequences were mainly affiliated to *Ascomycota* (90 %), notably to *Cladosporium* species (40 sequences), then to *Basidiomycota* (10 %), mostly to *C. carnescens* (five sequences). Another species belonging to *Phoma* genera was also retrieved (one sequence). The last species was phylogenetically close to species *Endosporium aviarium* and *Elsinoe* sp. (one sequence). The percentage of coverage was 96 %.

Analysis of the C2W3 18S rRNA clone library

The 50 sequences from the C2W3 library corresponded to six new phylotypes distributed among the *Ascomycota* phylum (Supplemental Table S2, Fig. 4). Species *S. fimetaria* was found in dominance (35 sequences) followed by *Aspergillus* species (*Aspergillus versicolor*, *Aspergillus glaucus* (renamed *Eurotium herbariorum*), (three sequences each)). The other sequences were affiliated to *Cordyceps chlamydosporia*,

Fig. 4 Phylogenetic distribution in the six clone libraries constructed from fungal communities of hemp samples

R2W3

Basidiomycota
39 %

Ascomycota
61 %

R2W6

Basidiomycota
10 %

Ascomycota
90 %

C2W3

Ascomycota 100 %

C2W5

Basidiomycota
14 %

Ascomycota
86 %

R6W3

Basidiomycota
59 %

Ascomycota
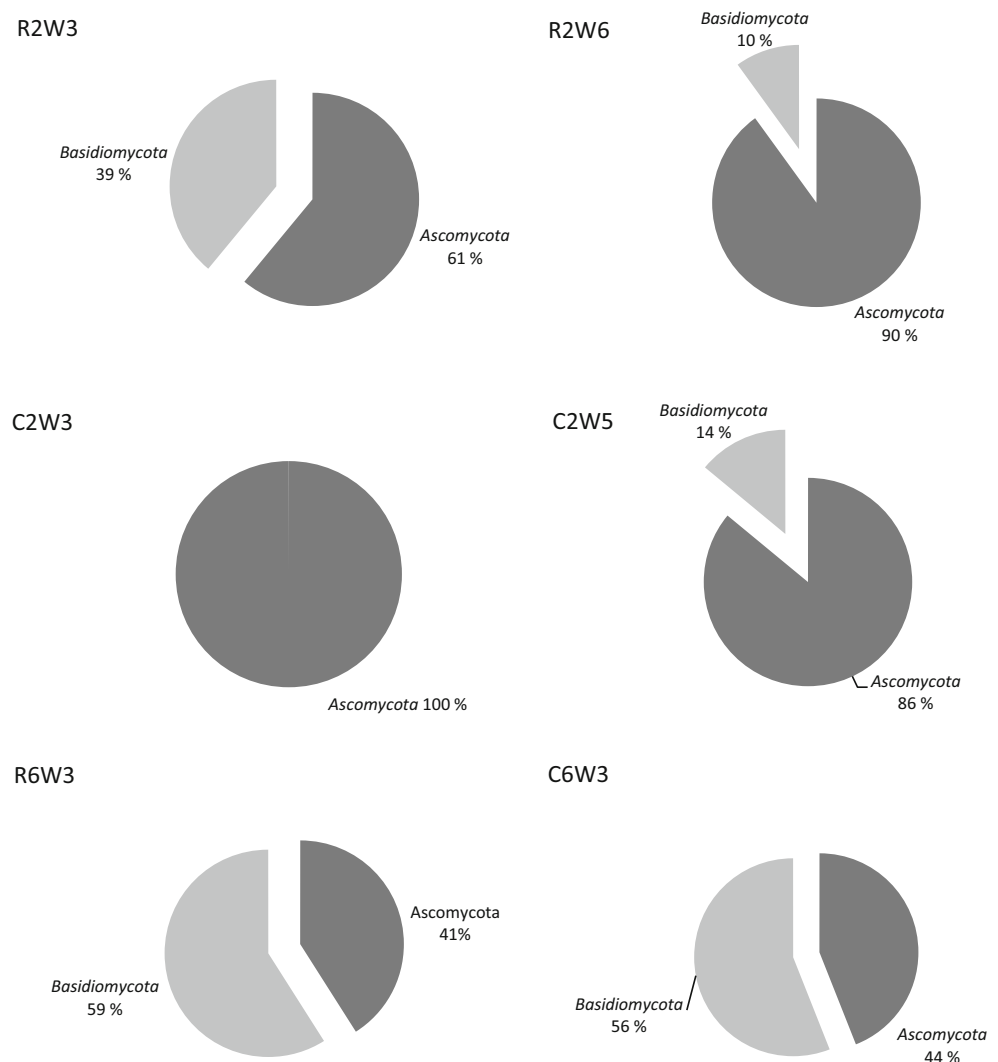41%

C6W3
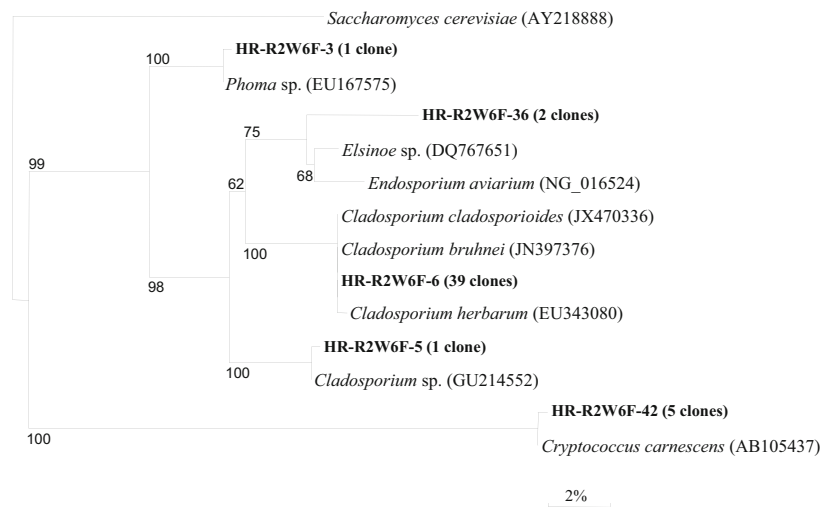
Basidiomycota
56 %

Ascomycota
44 %

Fig. 5 Phylogenetic tree constructed using fungal partial SSU rDNA sequences retrieved from R2W6 retting hemp

PhyML ln(L)=-1741.9 695 sites GTR 100 replic. 4 rate classes User-tree

Saccharomyces cerevisiae (AY218888)

HR-R2W6F-3 (1 clone)

Phoma sp. (EU167575)

HR-R2W6F-36 (2 clones)

Elsinoe sp. (DQ767651)

Endosporium aviarium (NG_016524)

Cladosporium cladosporioides (JX470336)

Cladosporium bruhnei (JN397376)

HR-R2W6F-6 (39 clones)

Cladosporium herbarum (EU343080)

HR-R2W6F-5 (1 clone)

Cladosporium sp. (GU214552)

HR-R2W6F-42 (5 clones)

Cryptococcus carnescens (AB105437)

2%

Acremonium sp. and to *Penicillium brevicompactum* (three sequences each). The percentage of coverage was 100 %.

Analysis of the C2W5 18S rRNA clone library

The 51 sequences were recovered into six phylotypes, affiliated to two phyla already identified (Supplemental Table S2 and Fig. 4). *Ascomycota* was always the most-represented phylum (86 % of sequences); then, *Basidiomycota* was found (14 % of sequences). The most important phylotype was attributed to *C. herbarum* species (33 sequences). Two other important phylotypes corresponded to species *P. cucumerina* and *C. carnescens* (seven sequences each). The remaining sequences were attributed to species *Elaphocordyceps ophioglossoides* (two sequences) and to *Geosmithia* sp. and *E. aviarium/Elsinoe* (one sequence each). The percentage of coverage was 96 %.

Analysis of the R6W3 18S rRNA clone library

The 51 sequences from the R6W3 library corresponded to eight phylotypes distributed among two phyla (Supplemental Table S2, Fig. 4). The sequences were mainly affiliated to *Basidiomycota* (59 % of sequences), particularly to *Cryptococcus* species (28 sequences). Twenty-one of these sequences were identified as *C. carnescens*. *Ascomycota* (41 % of sequences) was abundantly represented by *C. herbarum* species (19 sequences). Two sequences were phylogenetically close to the species *E. aviarium* and *Elsinoe* sp. The remaining sequences were attributed to the species *R. marina* and *Dioszegia hungarica* (one sequence each). The percentage of coverage was 94 %.

Analysis of the C6W3 18S rRNA clone library

Fifty-four sequences were also analysed, corresponding to eight phylotypes within two phyla (Supplemental Table S2, Fig. 4). *Basidiomycota* (56 % of sequences) was the best-represented phylum, and then, *Ascomycota* was found (44 % of sequences). Species *Cryptococcus* were found in dominance (*C. carnescens*, 26 sequences, *Cryptococcus* sp., one sequence), followed by species *Cladosporium* (23 sequences). Species represented by only one sequence were also found, *E. ophioglossoides*, *R. marina*, *Leucosporidium scottii* and *Dioszegia crocea*. The percentage of coverage was 93 %.

Discussion

Industrial use of plant fibres is closely linked to the quality of these fibres (Martin et al. 2013). Several parameters during industrial process from seed to final use could impact on fibre quality. For fibre extracts from plant stems like flax, hemp, jute, kenaf and ramie, the retting process appears to be critical (Tahir et al. 2011). The improvement of hemp fibre tow due to the retting was in line with previous works on flax (Sharma and Van Sumere 1992). According to professional hemp users (farmers, scutching and hackling industrials, composite makers), the quality of the 6-week retted straw and tow obtained in this study was appropriate to thermoplastic composites industries. This quality contrasted with the important heterogeneity of 3-week retted tow which had been described as rough.

Traditionally, hemp retting has been carried out by autochthonous microorganisms present in soil and on plants. However, an industrial application needs standardisation and efficiency of the process. Our goal was to characterise the

microbiota implicated in this process. This study represents the first molecular characterisation of bacterial and fungal microbiota in the dew retting of the hemp. Previous studies using culture-dependent methods identified bacterial and fungal species of flax retting (Akin et al. 1998; Henriksson et al. 1997; Sharma 1986b; Sharma et al. 1992). These methods only permitted a partial characterisation of the microbiota, due to specific media utilisation. Employment of culture-independent methods could improve knowledge on microbial composition of an ecosystem, as it is estimated that a large proportion of microorganisms could not be cultivated (Hugenholtz et al. 1998). Changes in bacterial communities in different stages of jute retting were visualised by PCR-denaturing gradient gel electrophoresis from water samples (Das et al. 2013). This method permits a rapid high throughput comparison of bacterial communities over time, but not their identification. Knowledge of the microbial composition is necessary to identify the species that may play a key role in degrading the stems of hemp.

In our study, the 16S or 18S rRNA gene copy number of total bacteria or fungi per gram of hemp straw was very close for the same sample. They were ranging from 8.1 $\log_{10}$ to 9.6 $\log_{10}$ according to sample. It has been shown that bacterial genomes can contain between 1 and 15 ribosomal operons according to the species (Acinas et al. 2004; Klappenbach et al. 2000) whereas it was estimated to be between 50 and more than 200 for fungi (Howlett et al. 1997). A few examples of the number of rRNA operons for microorganisms present in retting: *E. coli* (seven operons), *Pseudomonas putida* (six or seven), *P. syringae* (five), *P. fluorescens* (five), *R. fascians* (four or five), *Xanthomonas campestris* (two), *Rhodobacter sphaeroides* (three), *Variovorax* sp. (two), *Sphingomonas* sp. (one), *Aspergillus fumigatus* (38 to 91). In consequence, in our samples, since the numbers of rRNA gene were equivalent for bacteria and fungi, there were more bacteria than fungi on straws. This was consistent with the results obtained using culture techniques. From red ground, the bacteria and fungi quantified after 6 weeks of retting process were more numerous than after three. However, the microorganisms quantified from chalky ground after 3 and 5 weeks of retting process were sensibly equivalent together and close to the counts obtained for R2W6. Except for the sample C2W3, higher counts of bacteria and fungi were recovered after 5 or 6 weeks of retting process. And whatever was the method used, fungi were found in higher proportion from red ground after 6 weeks of retting process. It could explain why retting is more efficient on red ground; nevertheless, we have to be cautious since we lack data for chalky ground.

The 296 bacterial clones fell into the phyla *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Firmicutes*. *Proteobacteria* was the most abundant phylum with 62–96 % of sequences (mean 77 %). A high redundancy within molecular species was observed between the six samples. This work showed that in each case, the same core of species were present but in different proportions according to ground type, harvest dates and retting duration on field. The most represented bacteria were related to species *E. coli*, *P. agglomerans*, *P. rhizosphaerae*, *Rhodobacter* sp., *P. fulva*, *R. huautlense* and *M. timonae*. Other *Rhizobium* species were also found depending on samples (*R. soli*, *R. cellulosilyticum*), as well as many other *Pseudomonas* species (*P. psychrotolerans*, *P. graminis*, *P. syringae*, *P. fluorescens*, *Pseudomonas viridiflava*). Genera *Methylobacterium*, *Frigoribacterium* and *Sphingobacterium* were also often retrieved from libraries. *Pseudomonas* species are particularly important in the decomposition of pectin in plant fibres, both in aerobic and in anaerobic conditions (Betrabet and Bhat 1958; Rosemberg 1965). Two 16S rRNA gene libraries from jute retting in water tanks were recently constructed and analysed. The bulk of clones belong to *Proteobacteria* phylum (41 %) and a comparatively smaller proportion of clones belonged to the divisions *Firmicutes* (12 %), *Cytophaga-Flexibacter-Bacteroidetes* group (7 %), *Verrucomicrobia* (5 %), *Acidobacteria* (5 %), *Chlorobiales* (5 %) and *Actinobacteria* (2 %) (Munshi and Chattoo 2008). In our study, *Proteobacteria* represented 77 %, *Firmicutes* 1 %, *Cytophaga-Flexibacter-Bacteroidetes* 11 % and *Actinobacteria* 11 %. In jute retting, *Achromobacter*, *Zoogloea*, *Erwinia*, *Pseudomonas*, *Bacilli*, *Clostridia*, *Cytophaga* and *Verrucomicrobia* were found to be common between the two libraries. In hemp retting, *P. agglomerans*, *E. coli*, *Rhizobium* and *Pseudomonas* species were present in the six libraries. *Rhodobacter* and *Massilia* species were present in five libraries and *Methylobacterium* species in four libraries. In jute retting, 32 bacterial species were isolated on culture media from the two retting environments and affiliated to the attended *Bacillus* and *Clostridium* species and some species and genera described in our own libraries like *Rhodococcus*, *Flavobacterium*, *Chryseobacterium*, *Achromobacter*, *P. agglomerans*, *E. coli*, *Pseudomonas* and *Erwinia* (Munshi and Chattoo 2008). Similarly, *A. parvulus*, *Aerobacter cloacae*, *Bacilli*, *Clostridii*, *E. coli*, *P. aeruginosa* and *Serratia* were isolated from flax retting (Rosemberg 1965). *Achromobacter*, *Erwinia* and various species of *Clostridii* and *Pseudomonas* are known to act as retting agent (Munshi and Chattoo 2008). The species promoting the liberation of flax fibres in the shortest time was *P. aeruginosa* (Rosemberg 1965). Therefore, the abundance of such species in our libraries is in accordance with these previous studies. Concerning kenaf, the best water retting communities are dominated by members of the order *Clostridiales* (Visi et al. 2013). In our work, OTU ranged from 13 to 27 depending on samples. The OTU numbers found on chalky ground were not significantly different from those found on red ground (*t* test, 24±1.5 versus 19±4.1).

The 303 fungal clones fell into the phyla *Ascomycota* and *Basidiomycota*. The most represented fungi were mainly identified to *Cladosporium* and *Cryptococcus* genera, except for one library (C2W3F), dominated by species *S. fimetaria*, *A. versicolor*, *A. glaucus*, *C. chlamydosporia*, *Acremonium* sp. and *P. brevicompactum*. However, the total fungi counts were not different from the other libraries. The length of cloned sequence did not allow to differentiate species *C. bruhnei* from *C. herbarum* and *C. cladosporioides*. Species of *Cladosporium*, *Penicillium*, *Rhodotorula* and *Aspergillus* had been previously isolated from flax in the aerobic dew retting process (Fogarty and Ward 1972). These species are known to be retting agents. *C. herbarum* was notably identified in flax dew retting (Sharma 1986a). It was equally shown that *Arthrobacter*, *Aspergillus*, *Penicillium* genera and *S. fimetaria* possess a disaccharide specific hydrolase (Mazzaferro et al. 2010). Fungal OTU ranged from five to eight depending on samples. The OTU numbers found on chalky ground were not significantly different from those found on red ground (*t* test, 6.7±0.7 versus 7±1.7).

In conclusion, the 16S and 18S rRNA gene clone libraries provided a comprehensive sampling of the diversity and clearly demonstrated that a large diversity is present within the examined hemp retting environment. These results indicated that many microorganisms could have a proper role in retting process. The next step would be to study individually the identified species in order to understand their individual impact. For example, successful penetration of plant tissues by *Pseudomonas* sp., found in all libraries, may require the synergistic action of both pectin and pectate lyases (Hayashi et al. 1997). *Aspergillus niger* (*Aspergillus* genus found in one library) is the most commonly used fungal species for industrial production of pectinolytic enzymes (Naidu and Panda 1998). In parallel to molecular techniques, numerous strains have been isolated after culture and stocked. After 16S or 18S rRNA gene identification, and retting tests, combinations of these species which should secrete cellulases, hemicellulases, pectinases and other polysaccharidases should be performed to improve retting efficiency and fibre quality. Enzymatic retting had been initiated with flax but further developments are needed to make it competitive with traditional methods (Hoondal et al. 2002). The enzyme systems used by microorganisms for metabolising and for complete breakdown of pectin are important tools for elaborating the economical, ecofriendly and green chemical technology to use pectin polysaccharides in nature (Hoondal et al. 2002). It could also be carried out for hemp. Identification of pectinolytic and xylanolytic enzymes which are able to degrade specifically the pectin of hemp have to be isolated from the strains selected earlier for their efficiencies. Moreover, since hemp does differ in composition from the most studied plants, such as flax, the enzyme search should be expanded to proteinases, phenolic acid esterases, or even lignin-degrading enzymes. Indeed,

some bacteria belonging to *Xanthomonadaceae* and *Rhizobium* have been found to be permanently associated with decaying wood, regardless of fungus presence (Tian et al. 2014). These authors described numerous bacteria possessing lignin-degrading enzymes and present in our libraries: the genus *Sphingomonas* is universally distributed in the biosphere and plays an important role as decomposer. *Pseudomonas* strains were among the first lignolytic bacteria to be characterized. For example, *P. fluorescens* was able to produce extracellular lignin peroxidase, a major enzyme involved in lignin degradation. *R. fascians* was capable of secreting protocatechuate-3, 4-oxygenase, an important downstream enzyme in the lignin degradation pathway. The genus *Paenibacillus*, which was first known for xylanase production, also includes strains capable of degrading lignocellulosic biomass. Thus, further studies will be needed to select consortia of bacteria and fungi among our libraries to improve dew retting of hemp. In vitro tests could be carried out on the selected strains to determine the best developmental conditions and the effect on retting process. The fibre quality could be estimated with other strategies such as spectrometric approaches (Himmelsbach et al. 2002) or mechanical analysis (Marrot et al. 2013). These methods could be helpful to assess the impact of strain on the retting process.

These studies will help to improve microbial growth in field (depending on pile density of plant stems swathed in the field, on oxygen, on luminosity, on pH, on sugars and water needs), in order to shorten the process. Correlations with harvesting should also be analysed (early/normal) as well as techniques (haying/swathing).

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