Exploring the microbiota dynamics related to vegetable biomasses degradation and study of lignocellulose-degrading bacteria for industrial biotechnological application

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The aims of this study were to evaluate the microbial diversity of different lignocellulosic biomasses during degradation under natural conditions and to isolate, characterise new well-adapted bacterial strains to detect potentially improved enzyme-producing bacteria. The microbiota of biomass piles of *Arundo donax*, *Eucalyptus camaldulensis* and *Populus nigra* were evaluated by high-throughput sequencing. A highly complex bacterial community was found, composed of ubiquitous bacteria, with the highest representation by the Actinobacteria, Proteobacteria, Bacteroidetes and Firmicutes phyla. The abundances of the major and minor taxa retrieved during the process were determined by the selective pressure produced by the lignocellulosic plant species and degradation conditions. Moreover, cellulolytic bacteria were isolated using differential substrates and screened for cellulase, cellobiase, xylanase, pectinase and ligninase activities. Forty strains that showed multienzymatic activity were selected and identified. The highest endo-cellulase activity was seen in *Promicromonospora sukumoe* CE86 and *Isoptericola variabilis* CA84, which were able to degrade cellulose, cellobiose and xylan. Sixty-two percent of bacterial strains tested exhibited high extracellular endo-1,4-ß-glucanase activity in liquid media. These approaches show that the microbiota of lignocellulosic biomasses can be considered an important source of bacterial strains to upgrade the feasibility of lignocellulose conversion for the ‘greener’ technology of second-generation biofuels.

Lignocellulosic biomass includes dedicated energy crops, such as miscanthus, switchgrass, *Arundo donax*, *Populus nigra* and *Eucalyptus camaldulensis*. These plants can easily grow in farmland not suitable for food crops or in soils subjected to accelerated erosion, which affects most of the Mediterranean hilly croplands1-3. Vegetable biomasses represent an inexpensive alternative to fossil sources of fermentable sugars that can be utilised in several industrial applications, including second-generation biofuels and biopolymer production4.

The enzymatic hydrolysis of plant carbohydrates has emerged as the most prominent eco-technology for the degradation of such biomasses. From the microbiological point of view, lignocellulosic biomass represents a complex ecosystem in which environmental conditions influence living organisms. In particular, geochemical (pH and salinity) and physical (temperature, pressure and radiation) factors can have a selective pressure on the biodiversity of microorganisms5. As a consequence, autochthonous microbial communities may prevail over other microorganisms because they possess enzymes that are able to degrade complex molecules such as cellulose and hemicellulose, forming the lignocellulosic biomass that are the most abundant renewable energy source on Earth6.

Generally, at an industrial level, the bioconversion of pretreated cellulose-based materials into fermentable sugars is performed by using a reaction mixture composed of multiple enzymes for complete hydrolysis. However, because the biorefining process is still economically unfeasible, novel biocatalysts from bacteria could
help overcome costly hurdles due to the operative steps of cooling, oxygen pumping, stirring and neutralisation, as well as the intrinsically high cost of hydrolytic enzyme production\(^7\). Different microorganisms producing hemicellulolytic enzymes that are potentially usable as new biocatalysts for hemicellulose hydrolysis have been isolated from different natural environments such as compost\(^8\). They belong to specific groups of microorganisms that are able to synthesise cellulases, xylanases and other biocatalysts necessary to allow a complete hydrolysis of the recalcitrant components of the lignocellulosic biomass\(^9\). Cellulolytic microorganisms can synthesise distinct enzymes such as endoglucanases, exoglucanases, including d-cellodextrinases, cellobiohydrolases and 6-glycosidase, which cooperate in cellulase degradation. Indeed, the hemicellulolysis microorganisms produce xylanases for degrading xylan into xylose, and a variety of enzymes such as endo-\(\beta\)-1,4-xylanases and \(\beta\)-xylidasises as well as auxiliary enzymes such as \(\alpha\)-glucuronidases, \(\alpha\)-arabinofuranosidases, acetyl-esterases and acetyl xylan esterases\(^10\).

The use of culture-independent high-throughput sequencing can potentially reveal uncultivable microbota and enables the study of the microbial ecology and taxonomic diversity at a high resolution. A thorough determination of the microbial diversity in biomass degradation can be fundamental to evaluating potential sources of novel enzymes and activities\(^2,11\).

In the present work, the changes in the microbiota during the natural biodegradation of lignocellulosic biomasses of \(A. \) donax, \(E. \) camaldulensis and \(P. \) nigra were studied. In addition, new well-adapted bacterial strains from the three lignocellulose biomasses were isolated, identified and characterised. This study shows that the microbiota of lignocellulosic biomasses can be considered an important source of bacterial strains to upgrade the feasibility of lignocellulose conversion for the ‘greener’ technology of second-generation biofuels.

### Results

**Physicochemical measurement.** The temperature values observed in the piles were approximately 24°C during the first 45 days. This value increased up to about 29°C after 135 days of biodegradation, before declining up to 25°C at the end of the experiment (180 days). The values of \(a_w\) ranged from 0.91 to 0.99. The environmental temperature increased from April (20.5°C on the average) to August (34.2°C on the average) and declined in September (29.2°C).

**Microbial Diversity of Lignocellulosic Biomasses by High-Throughput Sequencing.** The microbiota of three different lignocellulosic biomasses (\(A. \) donax, \(E. \) camaldulensis and \(P. \) nigra) was characterised by partial 16S rRNA gene sequencing obtained from DNA directly extracted from environmental samples. A total of 238,450 number of reads were obtained by high-throughput sequencing. However, following the removal of short, ambiguous and/or low-quality pyrotag reads, the final data set consisted of 138,336 high-quality reads with an average sequence length of 456 bp. The alpha-diversity was determined by calculating the Shannon diversity index and the Chao1 richness index base on OTUs of 97% identity (Table 1). The results showed that the highest diversity indices were observed in the \(E. \) camaldulensis and \(P. \) nigra biomasses after 180 days of biodegradation in the underground condition (\(P < 0.05\)) but were quite variable in the \(A. \) donax biomass (Table 1). Good’s coverage indicated that more than 90% of the microbial diversity was described in most of the samples.

The relative abundances of bacterial taxa were examined at the level of phyla and class to determine whether there were any significant shifts in the composition of the bacterial communities according to the plant species, degradation conditions and sampling time. In total, twenty-six different phyla were detected in the biomass samples, but only *Actinobacteria, Proteobacteria, Bacteroidetes* and *Firmicutes* were detected in all samples (Fig. 1). These phyla together

### Table 1 | Number of sequences analysed, observed diversity and estimated sample coverage for 16S rRNA amplification from DNA extracted from the chipped ligno-cellulosic biomasses

| Sample | No. reads | No. OTUs | Chao1 | Shannon index* | Good’s coverage (%) |
|--------|-----------|----------|-------|---------------|---------------------|
| A0     | 12,021    | 322      | 432.68| 4.28AB        | 99.24               |
| A11OF  | 6,235     | 477      | 682.92| 6.59DE        | 97.45               |
| A11UW  | 9,713     | 506      | 713.56| 5.51BC        | 98.38               |
| A22OF  | 4,158     | 592      | 955.73| 7.09DE        | 93.70               |
| A22UW  | 2,564     | 286      | 443.81| 6.09C         | 95.44               |
| A32OF  | 1,699     | 404      | 618.70| 7.33C         | 88.99               |
| A32UW  | 5,451     | 441      | 561.42| 6.36CDE       | 97.54               |
| A42OF  | 5,160     | 620      | 1111.02| 6.94D         | 94.46               |
| A42UW  | 4,293     | 501      | 671.50| 6.61DE        | 95.64               |
| E10    | 5,582     | 340      | 555.28| 6.05C         | 97.76               |
| E11OF  | 5,271     | 322      | 485.11| 3.92A         | 97.67               |
| E11UW  | 3,207     | 286      | 430.38| 4.65A         | 96.35               |
| E22OF  | 3,983     | 453      | 656.84| 6.65DE        | 95.81               |
| E22UW  | 1,069     | 228      | 405.60| 6.52DE        | 89.5                |
| E32OF  | 4,520     | 470      | 671.11| 6.52DE        | 96.00               |
| E32UW  | 7,091     | 623      | 978.51| 6.46CDE       | 96.63               |
| E42OF  | 4,271     | 571      | 882.78| 7.49G         | 94.66               |
| E42UW  | 5,309     | 887      | 1367.25| 8.37GH        | 93.60               |
| P0     | 10,116    | 471      | 623.72| 5.94C         | 98.61               |
| P11OF  | 4,292     | 501      | 865.63| 6.18CD        | 94.43               |
| P11UW  | 10,117    | 845      | 1128.45| 8.24G         | 97.68               |
| P22OF  | 3,524     | 728      | 1097.07| 7.89F         | 91.06               |
| P22UW  | 1,226     | 367      | 727.57| 7.58FG        | 84.01               |
| P32OF  | 5,031     | 743      | 1086.64| 6.63DE        | 93.76               |
| P32UW  | 2,188     | 583      | 1169.58| 8.08FG        | 86.15               |
| P42OF  | 2,576     | 654      | 975.19| 7.82FG        | 88.94               |
| P42UW  | 7,669     | 1,629    | 3122.43| 9.48H         | 89.79               |

*Different letters after Shannon index values indicate significant differences (\(P < 0.05\)).
Abbreviations: A. donax; E. camaldulensis; P. nigra; T0: 0 days of degradation; t1: 45 days of degradation; t2: 90 days of degradation; t3: 135 days of degradation; t4: 180 days of degradation; OF: open field degradation condition; UW: underwood degradation condition.
accounted for approximately 98%, 99% and 91% of the total biodiversity in *A. donax*, *E. camaldulensis* and *P. nigra*, respectively.

The native composition of the microbial community in the *A. donax* biomass was strongly dominated by *Proteobacteria* (69.03%), followed by *Actinobacteria* (22.23%) and *Bacteroidetes* (8.61%). During the biodegradation process, the microbial composition remained the same in terms of diversity but varied in terms of abundance. This pattern was primarily observed in the piles processed under the open field condition (Fig. 1). Interestingly, in this environment, an inverse correlation was observed between *Actinobacteria* and *Bacteroidetes*. In particular, *Actinobacteria* decreased from 32.70% after 45 days of degradation under the open field condition to 7.31% after 180 days ($P < 0.05$), whereas *Bacteroidetes* increased from 7.84% after 45 days to 34.30% after 180 days ($P < 0.05$). *Firmicutes* was very low at the initial time (0.02%) and remained roughly constant in the underwood pile (0.77% after 180 days of biodegradation). By contrast, in the open field pile, the relative abundance of this taxon gradually increased from 1.20% after 45 days of biodegradation to 19.28% after 180 days ($P < 0.05$). *Minor phyla included Acidobacteria* and candidate phyla SC4 and TM7 were recovered in all *A. donax* biomass samples, although at an incidence $\leq 1\%$ (Fig. 1).

As in the *A. donax* biomass, *Proteobacteria* was the taxon that heavily dominated the native microbial community in *E. camaldulensis* and *P. nigra* (71.26% and 73.01%, respectively) ($P < 0.05$), remaining high during all degradation processes, followed by *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Acidobacteria* (Fig. 1). In the *E. camaldulensis* pile, *Acidobacteria* increased to 7.35% and 9.02% after 180 days of degradation under the open field and underwood conditions, respectively, whereas a slight variation was detected in the *P. nigra* samples. By contrast, the abundance of *Firmicutes* increased in the *P. nigra* pile after 135 (35.88%, $P < 0.05$) and 180 days (21.20%, $P < 0.05$) of degradation under the open field condition and was relatively stable in the *E. camaldulensis* pile (Fig. 1). Other taxa, including *Armatimonadetes*, *Chloroflexi*, *Planctomycetes*, *Verrucomicrobia*, SC4 and TM7, were also detected. Interestingly, *Armatimonadetes* increased in the *P. nigra* pile after 45 days under the open field condition (26.70%, $P < 0.05$) and then gradually decreased during the degradation process to 1.63% (Fig. 1).

The microbial diversity was also analysed at a deeper taxonomic level. The identification of OTUs at the class level is reported in the heatmap shown in Fig. 2. *Actinobacteria*, $\gamma$-*Proteobacteria*, $\beta$-*Proteobacteria*, $\alpha$-*Proteobacteria*, *Acidobacteria*, *Sphingobacteria*, *Flavobacteria* and *Bacilli* were recovered in all samples, with the exception of *Acidobacteria*, which was not detected in the *A. donax* biomass at the initial time. The abundance of $\alpha$-*Proteobacteria* was high and exhibited the same variations during the biodegradation experiment in all three lignocellulosic biomasses. Interestingly, $\gamma$-*Proteobacteria* was the dominant taxon at the beginning of the experiment, with an abundance of 58.32%, 42.64% and 67.48% in the *A. donax*, *E. camaldulensis* and *P. nigra* samples, respectively; however, a dramatic reduction was recorded in all plant biomasses after 180 days (1.07%, 8.12% and 11.49%, respectively) (Fig. 2). A steady reduction in class *Actinobacteria* during degradation of the *A. donax* biomass under the open field condition was observed. By contrast, *Bacilli* showed an opposite trend, increasing from 0.02% at time zero to 19.26% after 180 days. Under the underwood condition, the middle phase of the biodegradation process was primarily dominated by *Sphingobacteria*, which showed an abundance of approximately 50%. Ever-increasing *Sphingobacteria* and $\beta$-*Proteobacteria* taxa abundance was recorded in the *E. camaldulensis* pile during the degradation process under the open field experiment. *Acidobacteria* showed a similar trend under both the open field and underwood conditions. The *P. nigra* biomass was characterised by an increase in the incidence of *Bacilli* and $\beta$-*Proteobacteria* during the biodegradation process under the open field conditions (Fig. 2). Other taxa were present at a very low incidence and with a great variability in the different
samples. However, as shown in Fig. 2, the highest bacterial diversity was recorded in the *P. nigra* biomass. An interesting finding was the high incidence of the uncultured bacterium CH21 (26.45%) in the *P. nigra* pile during the first phase (after 45 days) and its constant decrease during the other phases of the degradation process in the open field. *Chloroflexi* was detected only in the *P. nigra* biomass and from 90 days to 180 days of biodegradation, showing an abundance ranging from 0.33% to 1.55%.

However, the PCoA of the weighted UniFrac community distances showed a marked difference between the native microbiota of the lignocellulosic biomasses and the microbiota in the analysed samples during the biodegradation process. The three samples of the chipped biomasses of *A. donax*, *E. camaldulensis* and *P. nigra* at harvest time clustered separately (Fig. 3). However, microbial diversity seemed to be influenced by a correlation between the degradation condition and the biomass type. In fact, no difference was observed among the samples during the biodegradation process under the underwood condition (Fig. 3 panel a). By contrast, under the open field condition, *P. nigra* samples at different time points clustered separately from the other biomasses (Fig. 3, panel b).

Moreover, the statistical ADONIS test showed that the composition of bacterial community in the different piles during biodegradation process was significantly influenced by lignocellulosic plant species (*P* < 0.001), by the degradation conditions (*P* < 0.05) and by sampling time (*P* < 0.01). This difference increased combining the three factors. In fact, the analysis performed with ADONIS found significant difference combining lignocellulosic plant species x sampling time (*P* < 0.001), lignocellulosic plant species x degradation condition (*P* < 0.001) and degradation condition x sampling time (*P* < 0.01).

**Multienzymatic Screening and Identification of Isolated Cellulolytic Bacterial Strains.** A culture-dependent approach enables the isolation of putative cellulolytic strains. A total of 366 aerobic endo- and exo-cellulolytic bacteria were isolated from samples of the *A. donax*, *E. camaldulensis*, and *P. nigra* biomasses. In particular, more than one hundred isolates were obtained from each pile, although the *P. nigra* biomass produced the highest number (127 isolates). In total, 95.9% of the isolates that were obtained from Avicel agar plates, exhibited exo-cellulase activity, whereas isolates with endo-cellulase activity on the CMC agar plates were detected at values less than 50%. To establish the number of putative multifunctional-degrading bacteria, all isolates were assayed for different enzymatic activities using the methods described below. Forty aerobic endo- and exo-cellulolytic strains that showed higher I<sub>CMC</sub> values (> 10) were chosen for further characterisation. The polyphasic approach of identification resulted in bacterial isolates with different shapes, dimensions and, in some cases, spore presence (data not shown) and great biodiversity, as twenty genera and twenty-seven different species were found (Table 2). *Curtobacterium* spp. and *Bacillus* spp. were the most representative genera with the species *Curtobacterium flaccumfaciens* and *Bacillus subtilis*.
Curtobacterium citreum as well as Bacillus amyloliquefaciens, Bacillus licheniformis and Bacillus subtilis, respectively. The bacterial strains showed activities for three to six different enzymes. All strains showed endo- and exo-cellulolytic activities and, at same time, expressed cello-biase, xylanase and pectinase (Table 2). The highest endo-cellulase activity was observed in the CE86 strain, identified as Promicromonas sukumoe (I_{CMC} = 34, P < 0.05), in Isoptericola variabilis CA84b (I_{CMC} = 32, P < 0.05) and Staphylococcus warneri CE83 (I_{CMC} = 32, P < 0.05). The first two strains were also able to degrade exo-cellulose, cellobiase and xylan. Moreover, Isoptericola variabilis CA84b produced the highest pectinase activity (I_{PEC} = 22, P < 0.05). Curtobacterium citreum CE711 and Bacillus amyloliquefaciens CA81 represented the most versatile strains because they possessed six different enzymatic activities. Thirty-two bacterial strains showed, in addition to endo- and exo-cellulases, cellobiase and xylanase activities that were missed in only 10% of the screened strains (Staphylococcus warneri CE83, Lysobacter enzymogenes CE710, Lysobacter gummosus CP72, Mycobacterium frederiksbergense

Figure 3 | Principal Coordinates Analysis of weighted UniFrac distances for 16S rRNA gene sequence data of lignocellulosic biomasses during the biodegradation process under the underwood (panel a) and open field (panel b) conditions. Colour label. red: A. donax at harvest time; blue: E. camaldulensis at harvest time; orange: P. nigra at harvest time; green: A. donax; violet: E. camaldulensis; yellow: P. nigra. Abbreviations. A: A. donax; E: E. camaldulensis; P: P. nigra; T0: 0 days of degradation; t1: 45 days of degradation; t2: 90 days of degradation; t3: 135 days of degradation; t4: 180 days of degradation; OF: open field degradation condition; UW: underwood degradation condition. 
| Strain Source | Identification (% identity) | Accession Number |
|--------------|----------------------------|------------------|
| E. camaldulensis | Promicromonaspora sukumae (99%) | KF057947 |
| A. donax | Isotricha variabilis (98%) | KF057948 |
| E. camaldulensis | Staphylococcus warneri (100%) | KF057949 |
| P. nigra | Lysobacter gummus (99%) | KF049972 |
| G. mycis | Mycobacterium frederiksbergense (99%) | KF057950 |
| P. nigra | Microbacterium lansum (99%) | KF057952 |
| L. gummosus | Enterobacter aerogens/Klyvera cryacera (98%) | KF057960 |
| P. nigra | Promicromonaspora atrea (99%) | KF057951 |
| E. camaldulensis | Novosphingobium resinorum (100%) | KF049976 |
| X. campestris | Xanthomonas campestris (99%) | KF049971 |
| P. nigra | Xanthomonas oriza (98%) | KF049975 |
| E. camaldulensis | Pediciococcus acidilactici (99%) | KF057953 |
| P. nigra | Curtobacterium flaccumfaciens (99%) | KF057945 |
| S. warneri | Schumannella luteola (97%) | KF057956 |
| P. nigra | Mycobacterium frederiksbergense (99%) | KF057957 |
| A. donax | Bacillus subtil is subsp. subtilis (99%) | KF049977 |
| E. camaldulensis | Bacillus amyloliquefaciens (99%) | KF057950 |
| A. donax | Bacillus licheniformis (99%) | KF049979 |
| E. camaldulensis | Novosphingobium resinorum (99%) | KF049980 |
| A. donax | Bacillus licheniformis (99%) | KF049981 |
| E. camaldulensis | Pediciococcus acidilactici (99%) | KF057958 |
| A. donax | Curtobacterium flaccumfaciens (99%) | KF057945 |
| A. donax | Aurantimonas altamirensis (99%) | KF057959 |
| E. camaldulensis | Isotricha variabilis (98%) | KF049982 |
| A. donax | Lysobacter enzymogenes (99%) | KF049983 |
| P. nigra | Promicromonaspora sukumae (99%) | KF049984 |
| E. camaldulensis | Curtobacterium flaccumfaciens (99%) | KF049985 |
| P. nigra | Sphingobacterium multivorans (99%) | KF049986 |
| E. camaldulensis | Curtobacterium flaccumfaciens (99%) | KF049987 |
| P. nigra | Curtobacterium flaccumfaciens (99%) | KF049988 |
| E. camaldulensis | Promicromonaspora atrea (99%) | KF049989 |
| A. donax | Curtobacterium flaccumfaciens (99%) | KF049990 |
| E. camaldulensis | Curtobacterium flaccumfaciens (99%) | KF049991 |
| P. nigra | Microbacterium testaceum (99%) | KF049992 |
| A. donax | Microbacterium testaceum (99%) | KF049993 |
| E. camaldulensis | Microbacterium testaceum (99%) | KF049994 |
| P. nigra | Curtobacterium atrea (99%) | KF049995 |
| A. donax | Curtobacterium atrea (99%) | KF049996 |

Enzymatic activities: C = endo-cellulase; A = exo-cellulase; CE = cellulase; X = xylanase; P = peroxidase; AZ = peroxidase; AB = laccase; L = ligninase with guaiacol and lignin alkali; AD = ligninase with guaiacol and Auranda donax; 1-COD or 1-EC index; values represent the means ± SD of three replicates. Different letters refer to values indicate significant differences (P < 0.05). *: growth; +: low intensity; ++: middle intensity; +++: high intensity.
CP710b, Promicromonospora citrea CE73, Novosphingobium resinorum CE77, Bacillus subtilis subsp. subtilis CA816 and Aurantimonas altamirensis SBP73). All the strains belonging to the different Bacillus spp. and Curtobacterium spp. had the highest cellulase activities. In particular, the genus Curtobacterium, represented by the species Curtobacterium citreum and Curtobacterium flaccumfaciens, was able to grow at least on four/five different substrates and, in one case, also showed peroxidase activity. In total, 87.5% of the bacterial strains produced xylanase (Table 2). Similar behaviour was observed for pectinase activity production as only 15% of the bacterial strains produced xylanase (Table 2). Screening of Cellulolytic Microorganisms in Liquid Medium.

Phylogenetic Analysis of Selected Cellulolytic Bacteria. 16S rRNA gene sequences of the forty cellulolytic bacteria identified as described below were grouped by phylogenetic analysis into five different clusters, generating a consensus tree. The clusters were Actinobacteria (n=21; 52.5%), Bacilli (n=8; 20.0%), α-Proteobacteria (n=3; 7.5%), γ-Proteobacteria (n=7; 17.5%), Sphingobacteria (n=1; 4%) (Fig. 4). A phylogenetic tree was generated from the distance data using the Neighbour-Joining method with the Maximum Composite Likelihood model in a MEGA4 Program (Fig. 5). The nucleotide sequences of related type strains of different genera were included in the data set. High bootstrap values were observed and indicated significant branching points in the phylogenetic tree. Strains representative of the dominant class of Actinobacteria were placed in a cluster with bootstrap values higher than 52% and could be differentiated into nine subclusters of different genera. The bacterial strains primarily belonged to Curtobacterium (33.3%) and Promicromonospora (19.0%), whereas other genera (Schumanniella, Labeledella, Cellulomonas, Cellulosimicrobium, Microbacterium, Isopericiola, and Mycobacterium) were represented by one or two strains. The strains Curtobacterium citreum CE711, Curtobacterium citreum CA84 and Curtobacterium citreum CE78 showed a sequence similarity of 100%. Representatives of the Bacilli class were placed in three clusters of the genera Bacillus (62.5%), Staphylococcus (12.5%) and Pedicoccus (25.0%). The strains belonging to Bacillus licheniformis CA82 and CA81b showed a similarity level of 100%, as did Bacillus amyloliquefaciens CA812 and CA81 and Pediococcus acidilactici CE75b and CE73b (Fig. 5). The α-Proteobacteria strains were placed in two different genera, in which two strains of Novosphingobium resinorum (CE77 and CE84) exhibited a sequence similarity of 100% with 100% of the bootstrap analysis. The other strain, belonging to Aurantimonas altamirensis (SBP73), formed a phylogenetically distinct cluster with a low 16S rRNA gene sequence similarity (60%) (Fig. 5). The representative strains of the γ-Proteobacteria cluster belonged to the genera Xanthomonas and Lysobacter and included the species Xanthomonas campestris, Xanthomonas oryzae, Lysobacter gummosus, and Lysobacter enzymogenes. The other genera, identified as the Enterobacter aerogenes/Klebsiella oxytoca group, Raoultella and Pantoaea, were represented by one strain. The strain Sphingobacterium multivorum CA77 formed a phylogenetically distinct cluster with the type strain of the respective species with 100% of the bootstrap analysis (Fig. 5).

Screening of Cellulolytic Microorganisms in Liquid Medium.

Sixteen bacterial strains were selected based on at least one of these characteristics: higher halo dimension on CMC agar, multienzymatic activities, high pectinase activity or high peroxidase activity, and no pathogenicity for humans; these strains were submitted to a quantitative CMCase assay. All the analysed strains achieving the maximum AZO-CMCase level of production between the 8th and 26th hours, corresponding to the exponential and stationary phases of growth, respectively (data not shown). In Table 3, the values of the maximum AZO-CMCase activity measured for each strain and the corresponding time of production are reported. Schumanniella lutella SBP71 showed the highest AZO-CMCase activity, at 0.33 ± 0.09 U mL⁻¹, although no significant differences were found with other strains. In particular, high activity levels ranging from 0.20 ± 0.06 to 0.32 ± 0.08 U mL⁻¹ were also found for Pediococcus acidilactici CE75b, Curtobacterium citreum CA84, Curtobacterium flaccumfaciens CA83, Cellulomonas flavigena CP713 and Pantoaea annanatis SBA76a (Table 3). Mycobacterium frederiksgaardense CP78b, Curtobacterium flaccumfaciens CP77b and Promicromonospora citrea SBP79 (with a maximum value of 0.18 ± 0.02 U mL⁻¹ and 0.16 ± 0.02 U mL⁻¹) exhibited less AZO-CMCase activity than the microorganisms mentioned above but more than Cellulosimicrobium cellulosi CA817 (0.15 ± 0.01 U mL⁻¹) and Isopericiola variabilis CA84b (0.13 ± 0.01 U mL⁻¹). All other bacterial strains tested showed values lower than 0.10 U mL⁻¹.

Figure 4 | Percentage composition of different phyla of eso- and endo-cellulolytic bacteria isolated from lignocellulosic biomasses on the basis of 16S rRNA gene sequence similarity.
Figure 5 | Neighbour-Joining tree based on the comparison of 16S rRNA gene sequences showing the relationships among cellulolytic strains. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are given at the nodes. Strains marked with "(T)" represent type strains. The scale bar estimates the number of substitutions per site.
Proteobacteria

The biodiversity of the microbial community in our study correlated to microorganisms previously characterised as biomass degraders. Recovered in enriched microbial consortia using a medium with lignin depolymerisation since a dominance of this phylum was observed. Cellulolytic microbes have been isolated and characterised from lignocellulosic waste, with cellulases being essential for the conversion of cellulose into fermentable sugars. In this context, particular attention must be given to exploring the biodiversity of natural ecosystems, represented by lignocellulosic biomasses, with the relative abundance of this taxon gradually increasing in the early stages of our experiment and its abundance increased during the later stages. Prokaryotes belonging to the class Acidobacteria, the major taxa involved in lignocellulosic waste declining during the early stages of the process. In the open field experiment, the importance of the phylum Acidobacteria as the primary cellulose decomposers in forest litter.

An interesting finding was the high incidence of the uncultured bacterium CH21 in the P. nigra pile during the first phase of the

**Table 3 | Maximum value of Azo-CMCase activity measured for each strain and the corresponding time of production**

| Bacterial strains                        | Maximum value of Azo-CMCase activity[^1] (U mL⁻¹) | Time (h) |
|------------------------------------------|--------------------------------------------------|----------|
| Schumannella luteola SBP71               | 0.33 ± 0.09⁷                                       | 12       |
| Pedicoccus acidilactici CE75b            | 0.32 ± 0.10⁶                                      | 12       |
| Curtobacterium citreum CA84              | 0.29 ± 0.01⁶                                      | 17       |
| Curtobacterium flaccumfaciens CA83       | 0.24 ± 0.03⁶                                      | 15       |
| Cellulomonas flavigena CP713             | 0.22 ± 0.10⁶                                      | 20       |
| Pantoaea ananatis S8A76a                 | 0.20 ± 0.06⁷                                      | 8        |
| Mycobacterium frederiksenii CP78b        | 0.18 ± 0.02⁹                                      | 26       |
| Curtobacterium flaccumfaciens CP77b      | 0.16 ± 0.02⁶                                      | 20       |
| Promicromonospora citreina SBP79         | 0.16 ± 0.02⁶                                      | 12       |
| Cellulosimicrobium cellulosi CA817       | 0.15 ± 0.01⁶                                      | 12       |
| Fuscobacillus variabilis CA84b           | 0.13 ± 0.01⁶                                      | 12       |
| Bacillus subtilis subsp. subtilis CA816  | 0.09 ± 0.00⁶                                      | 12       |
| Microbacterium testaceum CA818           | 0.09 ± 0.01⁶                                      | 17       |
| Curtobacterium flaccumfaciens CA83b      | 0.08 ± 0.03⁶                                      | 12       |
| Promicromonospora sukumoe CE86           | 0.07 ± 0.02⁶                                      | 14       |
| Bacillus licheniformis CA82              | 0.07 ± 0.00⁶                                      | 15       |

[^1]: The values represent the means ± SD of three replicates of three independent experiments. Different letters after the values indicate significant differences (P < 0.05).

**Discussion**

In recent years, the competitive production of alternative renewable biofuels has stimulated research into new bacteria as a source of lignocellulolytic materials. Analysing the microbial diversity more deeply, different bacteria belonging to the Actinobacteria, β-Proteobacteria, β-Proteobacteria, β-Proteobacteria, Acidobacteria, Actinomycetales, and Acidobacteria were recovered in our study. In particular, Actinobacteria, β-Proteobacteria, Acidobacteria and β-Proteobacteria were also recovered in our study. According to Castillo et al., the dominance of members of the phylum Proteobacteria such as β-Proteobacteria was observed only at the beginning of the biodegradation process, and its strong reduction during the experiment could be due to its involvement in lignocellulosic waste declining during the early stages of the process. In the open field experiment, Bacilli increased in both the A. donax and P. nigra biomasses. Members belonging to this taxon are known to have specific genes encoding enzymes involved in cellulose and hemicellulose degradation.

Different bacteria belonging to the Actinobacteria class are involved in complex glycoside degradation such as chitin and cellulose and are fundamental in lignin and polyphenol degradation. Martins and co-workers reported that biomass degradation in the composting process, including the deconstruction of recalcitrant lignocellulose, is fully performed by bacterial enzymes, most likely by members of the Clostridiales and Actinomycetales orders. β-Proteobacteria and β-Proteobacteria were also recovered in our study. According to Castillo et al., the dominance of members of the phylum Proteobacteria such as β-Proteobacteria was observed only at the beginning of the biodegradation process, and its strong reduction during the experiment could be due to its involvement in lignocellulosic waste declining during the early stages of the process. In the open field experiment, Bacilli increased in both the A. donax and P. nigra biomasses. Members belonging to this taxon are known to have specific genes encoding enzymes involved in cellulose and hemicellulose degradation.

The constant increase in the relative abundance of Sphingobacteria and Acidobacteria is due to the presence of complex glycoside compounds, such as lignin and polyphenols, which are recalcitrant to microbial degradation. Kanokratana et al., using complementary shotgun pyrosequencing, identified different genes encoding glycosyl hydrolases targeting cellulose and hemicellulose degradation in a bagasse pile, most of which were found in orders Clostridiales, Bacteroidales, Sphingobacteriales and Cytophagales. Moreover, the Acidobacteria are known to use a diversity of carbon sources, from simple sugars to complex plant biomass substrates.

Another adapted-lignocellulosic taxon was β-Proteobacteria, which increased in the E. camaldulensis and P. nigra piles. In recent work, Stursova et al. identified β-Proteobacteria, Bacteroidetes and Acidobacteria as the primary cellulose decomposers in forest litter.
degradation process in the open field. CH21 is a member of the phylum *Armatimonadetes*, formerly called candidate division OP10. The phylum *Armatimonadetes* is very poorly studied and its phylogeny is still poorly defined. Members of this phylum are detected in different ecosystems and they are phylogenetically different. This phylum includes species with a wide variety of metabolic potentials. Wang and co-workers reported that their prevalence in plant-fed anaerobic bioreactors indicates a role in degradation of plant material. In our research, CH21 is the only recovered member of this phylum. Since its relative abundance increased in the *Populus nigra* pile after 45 days and decreased during the other phases of the degradation process in the open field, it is possible its involving in the biodegradation of this specific lignocellulosic biomass during the first phase of the process.

The differing trends observed in this study in terms of taxa abundance during the biodegradation process and between the vegetable species used demonstrated a local selective pressure in the lignocellulosic ecosystems.

The culture-dependent methodology used here, which was based on a functional approach of detection and isolation to find new lignocellulose-degrading bacterial strains, provides us with key insight. Special attention to the methodology was required to determine the optimal culture and assay conditions. A comparison with another study revealed that differential substrates containing CMC and Avicel are effective for the enumeration and isolation of putative colonies of cellulolytic microorganisms. According to Soares et al., exo- and endo-cellulolytic bacterial isolates are found at different frequencies, and the number of microorganisms that were able to grow on Avicel as the sole carbon source was high. The cellulolytic strains isolated from the biomasses showed multienzymatic activities useful to perform the hydrolysis of a complex substrate like lignocellulose, an important initial step in many technological applications that require the action of different specific enzymes. All forty bacterial strains submitted to the multienzymatic screening showed both endo- and exo-glucanase activities, confirming that these enzymes act synergistically during the saccharification of celluloses. These observations were reinforced by the fact that many of these bacterial strains also possessed β-cellulase as well as cellulolytic activity. Xylanase activity was also commonly observed, which is unsurprising because a close correlation between cellulase and xylanase activities has been demonstrated and is due to their coexpression in the same operon.

The Neighbour-joining phylogenetic method generated a consensus tree that grouped all 16S rRNA gene sequences of the isolated strains into five different clusters at the class level. Culture-dependent data showed similar predominant bacterial classes detected by high-throughput sequencing in the lignocellulosic biomasses, with an abundance of *Actinobacteria*, *Bacilli*, *α-Proteobacteria*, *γ-Proteobacteria* and *Sphingobacteria*. Moreover, the bacteria isolated are dominant players since these microbial strains were isolated from a high serial dilution (10−2–10−3). According to phylogenetic research on cellulose-decomposing bacteria isolated from soil carried out by Ulrich et al., *Actinobacteria* are the most prevalent bacterial group based on 16S rRNA gene sequences. This cluster included the species *Curtobacterium citerum*, which is able to use up to six different lignocellulose components and that is phylogenetically related to *Microbacterium testacium* and *Microbacterium laxis*. In particular, previous studies reported the production of enzymes involved in cellulose and xylan degradation by *Microbacterium* species. The *Actinobacteria* cluster included other genera involved in lignocellulosic biomass degradation, such as *Cellulomonas*. Akasaka et al. reported that more than 60% of isolates from rice plant residues was closely related to *Cellulomonas* and involved in their degradation. Our study revealed the production of cellulase and pectinase activities in *Actinobacteria* members, such as *Cellulosimicrobium cellulans*, *Isoptericola variabilis*, *Promicromonospora sakumae* and *Promicromonospora citrea*, which belong to the suborder Micrococcineae. Many of the representatives of the *Promicromonosporaceae* and *Corinebacteriaceae* families can degrade polysaccharides such as cellulose and xylan.

The *Bacilli* cluster was primarily represented by the *Pediococcus* and *Bacillus* genera on the basis of 16S rRNA gene sequence analysis. Interestingly, Zhao and co-workers reported the use of a strain of *Pediococcus acidilactici* with high tolerance to temperature and a lignocellulose-derived inhibitor in simultaneous saccharification and fermentation (SSF) for high lignocellulosic lactic acid production. The strains CA812, CA81, CA816, CA82 and CA81b, isolated from the *A. donax* biomass and phylogenetically correlated to the *Bacillus* genus, showed high endo-cellulolytic and multi-enzymatic activities. The capacity of *Bacillus* strains to produce large quantities of extracellular enzymes has placed them among the most important industrial enzyme producers isolated from compost, soil and several other natural habitats. In particular, *Bacillus amyloliquefaciens*, *Bacillus subtilis* and *Bacillus licheniformis*, isolated from soil and compost, are able to hydrolyse cellulose waste material by both cellulolytic activities and multi-enzyme complexes. The selected bacterial strains could have cross-specificity facilitated by specific or non-specific active sites and also distinct catalytic domains binding to different substrates. *Xanthomomas campestris*, known as a phytopathogenic bacterium, and *Lyosphacter gummosus* and *Lysobacter enzymogenes*, potent biocontrol agents that release cellulolytic enzymes such as glucanases, were identified in the phylogenetic group of *γ-Proteobacteria*. Other strains of this class, such as *Enterobacter sp.* and *Pantoea sp.*, although known as insect-associated bacteria that are able to produce bioactive compounds and digestive enzymes that are responsible for lignocellulose degradation, are less attractive for possible biotechnological application since human disease has been reported to be caused by these bacteria as well as *Rhodocetes terrigena* and *Sphingobacterium multivorans*.

The two strains CE77 and CE84, included in the *α-Proteobacteria* cluster and characterised for their peroxidase activity, were closely related to the species *Novosphingobium resinovorum*, previously isolated from soil and studied for its capacity to degrade oil resins. Interestingly, the strain *Aurantimonas altamirensis* SPS73 showed multienzymatic activity. To our knowledge, this study is the first to report the multienzymatic activity of this species.

The bacterial strains screened in liquid medium showed enzymatic activity levels similar to the values reported in previous studies. For example, Ekperigin reported that the maximum activity of the cellulose-degrading enzyme determined for CMC in the culture supernatant of *Branhamella* spp. was 0.34 U mL−1. Even though comparing cellulase production across studies is difficult because there are too many differences in the production of extracellular enzymes (e.g., media composition, fermentation conditions and raw materials), the results demonstrated that the bacterial strains isolated and characterised in this study could represent a very interesting biological source for the conversion of lignocellulose carbohydrates into products of commercial significance. Moreover, their biotechnological performance could be enhanced by modifying the biotechnological parameters of the fermentation process such as the temperature.

In conclusion, in this work, pyrosequencing-based technology increased our knowledge of lignocellulosic-adapted microbiota and the microbial dynamic during the degradation of three different biomass under natural conditions. The dominant taxa found during the biodegradation process were members of classes *Actinobacteria*, *γ-Proteobacteria*, *α-Proteobacteria* and *Sphingobacteria*. However, the abundance of the major and minor taxa retrieved during the process was determined by selective pressure employed by the lignocellulosic plant species and the degradation conditions. In addition, new multifunctional degrading bacteria have been selected and identified that are potential producers of multiple enzymes that have synergistic actions on cellulose and hemicellulose.
This step is fundamental for biotechnological applications of interest to industry because they constitute a microbial source of new multifunctional enzymes that can increase the efficiency of the hydrolysis of lignocellulosic biomass into fermentable sugars for biofuel technology.

Methods

Lignocellulosic Biomasses and Sampling. Chipped wood from A. donax, E. camaldulensis and P. nigra was processed for biodegradation under natural conditions for 180 days. The chipped wood was used to form two piles of approximately 30 kg (length 0.85 m, width 0.85 m and height 0.70 m) for each plant species. From March 22, 2012, the piles were placed under two different environmental conditions in order to increase microbial biodiversity: open field conditions, in which the biomass piles were established on agronomic soil without any coverage shady species; on March 22, 2012, the piles were placed under two different environments (Naples, Italy; 40° 48’ 50.17’ N, 14° 20’ 48.2’ E). The controlled environment (Naples, Italy; 40° 48’ 47.8’ N, 14° 20’ 50.4’ E). Samples of 0.5 kg were collected from the external part (right and left side of the pile) and the internal central part of the biomass immediately after preparation (T0) and at 45, 90, 135 and 180 days (T1, T2, T3 and T4, respectively) of biodegradation process, temperature was measured using specific temperature sensors (VWR International PBI, Milan, Italy) placed directly in the core of the piles at a depth of 30–40 cm. The water activity (aw, water readily available for microbial metabolic activities) was also monitored using a HygroPalm23-AW (Rotenburg AG, Baserden, Germany).

Analysis of the Microbiota by High-Throughput Sequencing of the 16S rRNA Gene. Total DNA recovery from microorganisms adherent to the plant biomass was performed as previously described46. The bacterial diversity was evaluated by pyrosequencing using the primers Gray2F (5’-TGTATTGCTGGTTCGGC-3’) and Gray519r (5’-GTTTACTCGGCGCCTG-3’) spanning the V1-V3 region of the 16S rRNA gene. 454-adaptors were included in the forward primer followed by a 10-bp sample-specific Multiplex Identifier (MD). The PCR mixture (50 μl total volume) included 20 ng of target DNA, 1 x Taq DNA polymerase buffer (Invitrogen, Milano, Italy), 2.5 mM MgCl2, 0.5 mM of each dNTP, 0.4 μM of each primer and 2.5 U of Taq DNA polymerase (Invitrogen). PCR amplification was performed as previously reported48. After agarose gel electrophoresis, PCR products were purified twice by Agencourt AMPure kit (Beckman Coulter, Milano, Italy) and quantified using the QuantFluorTM (Promega, Milano, Italy), and an equinomial pool was obtained prior to further processing. The amplicon pool was used for pyrosequencing on a 454 Junior platform (454 Life Sciences, Roche, Italy) according to the manufacturer’s instructions using Titanium chemistry.

Bioinformatics and Data Analysis. The pyrosequencing data were filtered using the following quality check parameters using the QIIME software package49: a minimum sequence length of 200 bp, maximum homopolymer number of 5, minimum quality score of 25, and maximum number of ambiguous bases and primer mismatch. The QIIME software was also used to denoise, split sequences into the proper samples, and pick operational taxonomy units (OTUs) at 97% sequence identity using UCLUST50. The representative sequences were submitted to the RDP II classifier51 to obtain a taxonomy assignment using the Greengenes 16S rRNA gene database52.

Alpha diversity was evaluated by rarefaction curves, Good’s coverage, Chao1 richness53 and Shannon diversity indices54. To test for significant differences in alpha diversity, QIIME’s compare_alpha_diversity.py script was used to run nonparametric two-sample t-tests. The default number of Monte Carlo permutations (999) was used to calculate P-values in the nonparametric t-tests, and a significance threshold of P < 0.05 was used. Statistical tests for calculating the influence of sample variables on microbial ecology were applied using QIIME’s group_significance.py (ANOVA) and compare_categories.py (ADONIS) scripts. The OTU taxonomy tables generated by QIIME were used to produce heatmaps using the software TmeV v 4.85. Beta diversity was also evaluated by UniFrac55, and principal coordinate analysis (PCoA) was performed by QIIME. Unweighted UniFrac distances were also calculated, but we only report weighted distances here because sample clustering was more informative.

Isolation of Cellulolytic Functional Groups in the Lignocellulosic Biomasses. Different samples were collected from the chipped vegetable biomass piles of A. donax, E. camaldulensis and P. nigra for the isolation of aerobic cellulolytic bacteria. Serial dilutions (0.1 mL) of the samples were spotted on the surface of plates containing basal medium (1 g L-1 [NH4]2SO4, 1 g L-1 yeast extract, 50 mL L-1 standard salt solution, 1 mL L-1 trace element solution and 15 g L-1 agar bacteriological, pH 7.0) with 5 g L-1 ‘Avicel’ (Sigma-Aldrich, Milan, Italy) or 5 g L-1 carboxymethylcellulose (CMC) with 0.1% Congo red (Sigma-Aldrich) for exo- and endo-cellulolytic colonies were isolated, purified again on the same isolation medium and stored at 4 °C until their characterisation.

Screening for Functional Activities on Solid Media. The endo- and exo-cellulolytic activity of all isolates was measured on CMC by a semi-quantitative agar spot method. After adjusting the turbidity of the bacterial suspensions (0.5 x McFarland Turbidity Standard corresponding to approximately 1.5 x 109 CFU mL-1) with 1 mL L-1 carboxymethylcellulose (CMC) with 0.1% Congo red (Sigma-Aldrich) the colonies spotted on CMC agar medium in triplicate. After a 4-day incubation at 28°C, the plates were stained with 0.1% Congo red solution for 30 min followed by washing with 5 mL NaCl. Cellulolytic activities on screening media were recorded as the ‘Indices of Relative Enzyme Activity, IREA = diameter of clearing or halo zone/colony diameter. Cellulolytic activity was similarly estimated by inoculating all the isolates by spotting on the Avicel agar and observing the development of bacterial colonies after incubation at 28°C for 10 days. All isolates were also assayed for xylanase, β-1,4-xylosidase, pectinase and ligninase activities.

Identification and Phylogenetic Analysis of Cellulolytic Strains. Bacterial isolates were identified by molecular method (16S rRNA gene sequencing). Total genomic DNA was extracted and purified by InstaGeneTM (Bio-Rad Laboratories, Hercules, CA, USA) according to the supplier’s recommendations. Approximately 50 ng of DNA was used as the template for PCR assays. Synthetic oligonucleotide primers F1 (5’-AGAGTTTGATCCTGGCTCAG-3’) and R1 (5’-AAGAGGTTGACCTCAG-3’) were used to amplify the 16S rRNA gene. The PCR mixture was prepared as reported by Alfonzo et al.48. The PCR products were purified after visualisation by agarose gel electrophoresis at 100 V for 1 h, purified using a QIAquick gel extraction kit (Qiagen S.p.A., Milan, Italy) and sequenced. The DNA sequences were then analysed and compared with the GenBank nucleotide data library using Blast software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/Blast.cgi).

Multiple nucleotide alignments of nearly full-length 16S rRNA genes of isolated strains and type strains within each of the defined species were performed using the Clustal W program56 from MEGA version 4.07. The nucleotide sequences of the type strains were retrieved from the Ribosomal Database Project (RDP - http://rdp.cme.msu.edu/). The phylogenetic tree was inferred by using the Neighbour-Joining method with the Maximum Composite Likelihood model in MEGA4 program with bootstrap values based on 1,000 replications.

Screening of Cellulolytic Activity in Liquid Media. The bacterial strains were pre-inoculated by dissolving a single colony in 3 mL of liquid medium containing 0.5% CMC, 0.7% yeast extract, 4 g L-1 KH2PO4, 4 g L-1 NaH2PO4, 0.2 g L-1 MgSO4, 7H2O, 0.001 g L-1 CaCl2, 2H2O and 0.004 g L-1 FeSO4. The overnight incubation at 37°C, a volume of the broth culture corresponding to 0.1 O.D. was used to inoculate 100 mL plugged Erlenmeyer flasks, each containing 20 mL of the same medium. During incubation at 37°C on a rotary shaker at 225 rpm, samples of the liquid culture were withdrawn and used to measure the optical density (O.D.590nm) and the extracellular endo-1,4-β-glucanase activity by Azo-CMCase assay (Megazyme, Ireland), following the supplier’s instructions. The analytical determinations correspond to the mean value of three replicates.

Accession Numbers. The 16S rRNA gene sequences obtained were deposited in the GenBank nucleotide database under accession numbers from KF040971 to KF040996.

Statistical Analyses. One-way ANOVA followed by Tukey’s HSD post hoc for pairwise comparison of means (at P < 0.05) was used to assess the difference in the
enzymatic activities of isolated strains such as *I. CMC*, *I. PEC*, and *Azo-CMCase*. Statistical analyses were performed using SPSS 13.0 statistical software package (SPSS Inc., Cary, NC, USA).

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Author contributions

V.V. wrote the main manuscript text and, in particular, microbial diversity of lignocellulosic biomasses by High-Throughput Sequencing and Phylogenetic analysis of selected cellulolytic bacteria. She prepared Figure 1, 2, 3, 4, 5 and table 1. A.Aliberti and A.R. wrote the isolation and identification of cellulolytic bacterial strains by culture-dependent approach and selection by multienzymatic screening. They prepared Table 2. V.F., S.G. and A.Amore wrote the screening of cellulolytic microorganisms in liquid medium and prepared Table 3. D.E. wrote the microbial diversity of lignocellulosic biomasses by High-Throughput Sequencing. M.F. wrote lignocellulosic biomasses and sampling. O.P. is the corresponding author. All authors reviewed the manuscript.

Additional information

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