Exotic litter of the invasive plant *Ligustrum lucidum* alters enzymatic production and lignin degradation by selected saprotrophic fungi.

Julieta Mallerman[a*], Raúl Itria[ab], Enrique Alarcón[c], Christian Hernández[c], Laura Levin[a*], Mario Saparrat[de*].

[a] Laboratorio de Micología Experimental, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, INMIBO CONICET, 1428-Buenos Aires, Argentina.

[b] Instituto Nacional de Tecnología Industrial (INTI), Av. Gral. Paz 5445, CC 147,1650-San Martín, Prov. de Buenos Aires, Argentina.

[c] Instituto de Biotecnología y Ecología Aplicada (INBIOTECA), Universidad Veracruzana, Av. de las Culturas Veracruzanas No. 101, Col. Emiliano Zapata, C.P. 910910, Xalapa, Veracruz, México.

[d] Instituto de Fisiología Vegetal (INFIVE), UNLP, CCT-La Plata-CONICET, Diag. 113 y 61, CC 327, 1900-La Plata, Argentina

[e] Instituto de Botánica Spegazzini, Facultad de Ciencias Naturales y Museo, UNLP, 53 # 477, 1900-La Plata, Argentina

***Corresponding authors**: Julieta Mallerman (e-mail: julietamllr@gmail.com); Mario Saparrat (e-mail: masaparrat@yahoo.com.ar); Laura Levin (e-mail: lale@bg.fcen.uba.ar)

Present address (J. Mallerman): Instituto de Investigaciones Fisiológicas y Ecológicas Vinculadas a la Agricultura (IFEVA), Facultad de Agronomía, Universidad de Buenos Aires, CONICET, Av. San Martín 4453, Buenos Aires C1417DSE, Argentina.

ABSTRACT

Chemical changes in leaf input to forest soils have been reported to affect decay processes. In this work, litter mass loss and decomposition constants (*k*) during 200 days in solid-state fermentation of the native tree *Celtis tala* and the exotic one *Ligustrum lucidum* with three common litter saprotrophic basidiomycetes were compared. Alterations in litter quality were characterized by solid-state ^{13}C NMR spectroscopy, pH, soluble sugars, ammonium, proteins and phenol content determination, and were associated with extracellular lignocellulolytic enzyme production. Differences in substrate decomposition related to litter type were observed for *Leratiomyces ceres,* achieving a higher *k* in the exotic *L. lucidum* litter, which might be attributed to the induction of Manganese peroxidase activity. Substrate preference for alkyl C and more degradation of lignified compounds were found in such substrate. Although no statistical differences in mass loss were observed for the rest of the fungi assayed, we detected changes in several of the parameters evaluated. This suggests that exotic invasions may alter ecosystem functioning by accelerating decomposition processes through an increased fungal ligninolytic activity.

Keywords: Basidiomycetes, Extracellular enzymes, Solid-state fermentation, Litter quality, Carbon-13 Cross Polarization Magic Angle Spinning Nuclear Magnetic Resonance.

Introduction

Invasion of exotic plants in pristine terrestrial ecosystems is a major environmental problem due to alterations in the vegetal community with the consequent detrimental impact on the activity of resident organisms (Pyšek *et al*. 2012). Plant-litter provides the main contribution of biomass and energy in several environments such as forests, where the substrate quality strongly influences microbial composition and its related activity in soils (Hättenschwiler *et al*. 2005; Wardle *et al*. 2004). Nevertheless, there are scarce data about the effect of the input of senescent leaves from invasive plants on decomposition and humification processes associated with enzyme activity in soils.

Different decay rates among organic matter kept under identical environmental conditions are mostly associated with changes in the content of its major chemical constituents such as nitrogen, lignin and polyphenol, as well as the relative contribution of carbon: nitrogen and lignin: nitrogen (Wardle *et al*. 2004). Leaf litter consists mainly of polysaccharides cellulose and hemicellulose and aromatic polymer lignin, the latter being considered as the most recalcitrant. Fungi are the dominant decomposers in temperate and boreal forest soils (Hättenschwiler *et al*. 2005) and among fungi, saprotrophic basidiomycetes are capable of producing a wide variety of extracellular oxidoreductases and hydrolytic enzymes that allow them to degrade all these three litter components (Purahong *et al*. 2014). This study was carried out with the aim of comparing the abilities of saprotrophic basidiomycetes in decomposition of leaf-litter of both native and invasive plants, based on the hypothesis that litter quality modifies the lignocellulolytic activity of these fungi and may greatly influence litter decomposition rates.

Pereyra Iraola Park is a Biosphere Reserve declared as such by UNESCO in 2007, located in Buenos Aires Province, Argentina. It constitutes the largest green space (10,248

hectares) of the metropolitan area of the City of Buenos Aires and La Plata, acting as a "green lung" for the urban conglomerate. The area has mixed zones belonging to relictual patches of marginal forest, locally known as Selva Marginal, and forested sites with a noteworthy introduction of exotic species. The former is confined to the coastal strip of the De la Plata river and is considered as a reservoir of an extensive spectrum of biodiversity, on the southern limit of distribution of subtropical forests in South America (Kalesnik *et al*. 2013). While *Celtis tala* Gill. ex Planch. (Cannabaceae) is a typical native tree in the Reserve, *Ligustrum lucidum* Ait. (Oleaceae), also known as Glossy privet, it is in fact a monodominant canopy tree native to China, which has increased its coverage in the region since it was imported for ornamental purposes. In heavily invaded stands, *L. lucidum* can exceed 80% of the tree cover, while elsewhere, it is distributed as solitary individuals adjacent to native trees (Aragón and Groom 2003; Gavier-Pizarro *et al*. 2012). *L. lucidum* is tolerant to a wide range of soils and light conditions and, in addition to vegetative reproduction it produces a large number of seeds, which are readily dispersed by birds. These features contribute to making *L. lucidum* a successful invader with a broad representation at ecosystem level (Marano *et al*. 2013). Previous studies indicated that it influences the species composition and reduces the diversity in invaded plant communities (Lichstein *et al*. 2004). The aggressive invasion of *L. lucidum* was reported to be an important issue in other countries such as Australia and New Zealand (Cronk and Fuller 1995). However, the influence of *L. lucidum* senescent leaves on the decomposing activity of litter saprotrophic basidiomycetes and their role in litter degradation are still unraveled.

The objectives of this work were (i) to evaluate how chemical composition of leaf litter affects mass loss and production of lignocellulolytic enzymes by litter saprotrophic basidiomycete's cultures during solid-state fermentation (SSF) and (ii) to assess if fungi have a

substrate preference for decomposing native *Celtis tala* or exotic *Ligustrum lucidum* litter by comparing their decomposition constants under SSF.

Materials and Methods

Fungal isolates and identification

Fresh basidiomata were collected in the Natural Reserve Pereyra Iraola Park in Buenos Aires Province, Argentina (34°84'S 58°10'W), during the summer of 2014. *Marasmiellus candidus, Leratiomyces ceres* and *Marasmius haematocephalus* were classified based on the morphological characteristics of their basidiomata. These fungi were selected because they were frequently found in both litter assayed (Mallerman 2017). To confirm the identities of fungal isolates, total DNA was extracted from agar cultures with the Microbial DNA Kit (MoBio, USA) and then used for PCR amplification (ABI 3130xl Genetic Analyzer). Amplification reactions used the universal fungal-primers ITS1 (5'- TCCGTAGGTGAACCTGCGG -3') and ITS4 (5'- TCCTCCGCTTATTGATATGC -3') for the ribosomal DNA region (White *et al*. 1990). The PCR products separated through electrophoresis in 1.5% agarose gel were sent to a sequencing facility (University of Buenos Aires). Sequences obtained were analyzed using BLAST, compared for homologous NCBI-GenBank database and deposited under the accession numbers: **KX423791** (*Leratiomyces ceres*), **KX423792** (*Marasmius haematocephalus*) and **KX423793** (*Marasmiellus candidus).* For fungal isolation, a sterile piece of mushroom tissue was collected from the interior of each basidiomata, transferred onto Petri dishes containing 20 mL of malt extract agar medium (MEA: malt extract 1.2%, glucose 1% and agar 2%) and grown at 25°C, in darkness. Strains were maintained at 4°C on MEA slants by periodic transfers and specimens were preserved in the culture collection of Natural and Exact Science Faculty, University of Buenos Aires, Argentina (BAFCcult).

Litter decomposition under SSF conditions

Ligustrum lucidum and *Celtis tala* senescent leaves (just before abscission) were collected from several individuals in the studied area during the summer of 2014. Litter was air-dried at room temperature and stored at 4°C before use. At the beginning of the experiment, leaves were cut into smaller pieces (size > 20 mm) and approx. 2.5 g were placed in 125 mL Erlenmeyer flasks. To calculate the initial litter dry mass, flasks were oven-dried at 80°C until constant weight. The litter was moistened with 20 mL of distilled water, autoclaved for 20 min at 120°C, and inoculated with two 0.6-cm diameter MEA mycelial plugs, taken from the margin of an actively growing colony. Cultures were incubated at 25°C in darkness, sampling them every 30 days, performing the last on day 200. Non-inoculated flasks served as controls and to estimate water loss during the incubation period, in which sterile water was replenished every time a sample was taken. Three replicates per treatment were conducted (3 fungi and 1 control x 2 litter x 3 replicates x 6 sampling times). Decomposition constant (k) was estimated by calculating the percentage of dry mass remaining after each culture incubation period (percentage reduction of inoculated litter in relation to the uninoculated one), using a single exponential decay model (Olson 1963): $M_t = M_0 .e^{-kt}$; where M_0 is the initial dry mass, M_t is the dry mass remaining at time t, k is the decomposition constant and t is time.

Chemical composition of the water soluble and solid fractions from litter

On each sampling time, a water soluble fraction (WSF) and a solid fraction (SF) were obtained from both uninoculated and fungal transformed litter, according to Saparrat *et al.* (2008). For this, the content of each flask was mixed on a rotary shaker at 150 ppm for 1 h with 20 mL of distilled water, filtered through gauze and centrifuged for 10 min at 5000 × g, 4˚C. Supernatants conformed the WSF, while the SF consisted of the resulting pellet collected

132 together with the material retained in the gauze. Dry mass content was measured in the SF by
133 oven-drying at 80°C until constant mass (Saparrat *et al*. 2008).

134 In the WSF, the extracellular proteins were assessed using the Bradford (1976) method
135 with the BioRad protein assay reagent (Bio-Rad, Hercules, CA) and bovine serum albumin
136 (BSA) as standard protein. Ammonium-N was determined following the protocols of Beecher
137 and Whitten (1970). Free phenolic water-soluble compounds were extracted according to Box
138 (1983) and Folin-Ciocalteu reagent was used for analysis of total phenolic content, being results
139 expressed as phenol equivalents.

140 The chemical composition of the SF was analyzed by Cross-Polarization Magic-Angle
141 Spinning ^{13}C Nuclear Magnetic Resonance (^{13}C CPMAS NMR) procedure. The ^{13}C CPMAS
142 NMR spectra were obtained using a 7T Bruker Avance II-300 spectrometer (Bruker Instruments
143 Inc., Germany). Litter samples were dried and ground by a 4-mm zirconium rotor and spun at
144 magic angle at 10 kHz. All the solid-state NMR experiments were performed at room
145 temperature. ^{13}C CPMAS NMR was performed with a ramped ^{1}H pulse during a contact time of
146 3 ms to obtain the best signal-to-noise ratio. ^{1}H decoupling was performed during the acquisition
147 with a TPPM15 sequence to improve the resolution. Recording 1 K transients with a recycle time
148 of 3 s represented standard conditions. The operating frequencies for protons and carbons were
149 300.13 and 75.46MHz, respectively. Glycine was used as an external reference for the ^{13}C
150 spectra and to set the Hartmann-Hahn matching condition in the cross-polarization experiments.
151 Because of the substantial amount of instrument time required, only samples of the initial and
152 ending decomposition time (200 days) were analyzed. Relative areas were calculated as the
153 percentage of total intensity from the integral curves of the following carbon chemical-shift
154 regions: alkyl C (0-45 ppm), O-alkyl C (45-110 ppm), methoxyl C (50-60 ppm), aromatic C

(110-140 ppm), phenolic C (140-160 ppm), and carboxyl C (160-190 ppm). Deconvolution method of the NMR spectra was performed using the DmFit software (Massiot *et al*. 2002). The degree of humification was calculated as the ratio between the corresponding areas (A) of the spectra alkyl C to O-alkyl C (Baldock *et al*. 1997). The alkyl C to carboxyl C ratio was calculated according to Knicker *et al.* (2000).The aromaticity index (AI) was considered as AI = 100 (A (110-160 ppm) /A (0-160 ppm)) (Lorenz *et al*. 2006). Finally, the lignin content was estimated according to Haw *et al*. (1984) as: % lignin = (100%) (183/9.92) l´ lig / ((183/9.92) l´ lig + (162/6) l´ carb). The weighting element in this calculation is the ratio of formula weights for lignin and carbohydrate fractions to the number of carbon atoms in the average formulas assumed for their repeating units [for more details see Haw *et al*. (1984)].

Enzyme activities in the WSF

In order to understand the role of enzymes related to the degradation of the main structural litter components: cellulose, hemicellulose and lignin along time, we evaluated the activity of β-1,4-endoglucanase, β-glucosidase, β-1,4-endoxylanase, laccase and MnP. β-1,4-endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-1,4-endoxylanase (E.C. 3.2.1.8) activities were estimated by measuring the reducing sugars released from carboxymethylcellulose, crystalline cellulose or beechwood xylan, respectively, as substrates in 50 mM sodium acetate buffer, pH 4.8. Liberated reducing sugars were quantified by the DNS method (Miller 1959) using either glucose or xylose as standards. β-glucosidase (E.C 3.2.1.21) activity was determined in 50 mM sodium acetate buffer, pH 4.8, by measuring p-nitrophenol released from p-nitrophenyl-β-D-glucopyranoside (Wood and Bhat 1988). All enzyme activities were determined at 50ºC. Laccase (EC 1.10.3.2) activity was measured by the oxidation of 5 mM 2,6-dimethoxyphenol (DMP) in 0.1 M sodium acetate buffer (pH 3.6), assessing the increase in

absorbance at 469 nm (ε = 27.5/mM cm) (Ben Younes *et al*. 2007). The MnP activity (EC 1.11.1.13) was assayed by the oxidation of phenol red in 0.2 M sodium succinate buffer pH 4.5, with hydrogen peroxide and manganese sulfate at 610 nm (ε = 22/mM cm) according to Paszczynski *et al*. (1988). Both ligninolytic activities were measured at 30ºC. International enzymatic units (U) were used (µmol product/min). Results were expressed in units per gram of dry litter (U/g dry litter). For all spectrophotometric measurements, three technical replicates were performed.

Statistical analysis

Decomposition constants (*k*) among treatments were analyzed using two-way ANOVA, with litter and fungal species as main factors. Data was logarithmically transformed before analysis to meet the necessary assumptions for parametric testing of normality (Shapiro-Wilks test) and equality of variances (Levene's test). All post hoc comparisons were accomplished with a Tukey´s test and α = 0.05 was used for testing statistical significance. When mass loss was compared in a fungus over time, a one-way ANOVA followed by a Tukey's test was performed. The shifts of individual carbon fractions, ratios and indexes calculated in the ^{13}C CPMAS NMR analysis were compared between initial fresh litter types using a pair t test. A one-way ANOVA followed by the Tukey's test was used to compare the chemical categories among fresh and decomposed litter within each litter type. Statistical analyses were performed using Statistica 7.0 software package (Stat-Soft, USA). The correlation structure among chemical variables (based on analytical determinations in the WSF and NMR spectra of the SF from fungal-litter samples at the end of the SSF) and the enzyme activities (detected in the WSF of the same samples) was determined by calculating pairwise Pearson's correlation coefficients. Also, the common structure of these two data tables was examined by Coinertia analysis (CoiA) (Dray *et al*. 2003),

previously used in litter decomposition (Alarcón-Gutiérrez *et al*. 2009). CoiA was performed using the ade4 package in the R environment (R Development Core Team 2008). Principal Component Analysis (PCA) was used as an ordination method in the two data sets and CoiA projected the two PCAs into the same graphical space, in order to visualize variations in individual chemical components among litter types and fungi in relation to enzyme activities. As we had some zero values, a Detrended Correspondence Analysis (DCA) -using *vegan* package in R- was performed first. Because the ordination axes or "gradient lengths" were shorter than two units, the data were linear, and a CoiA with PCA was appropriate for the analysis (Ter Braak 1998). The co-linearity was tested using the vif function with *fmsb* package in R. All variables above a threshold of five were excluded from the analyses. In order to identify which factors significantly corresponded to overall enzyme activities, we used the function envfit in *vegan* package in R. The significant variables were analyzed for variance partitioning with varpart function in *vegan*.

Results

Litter decomposition under SSF

Litter fungal strains *Marasmiellus candidus*, *Leratiomyces ceres* and *Marasmius haematocephalus* were able to grow and colonize native *Celtis tala* and exotic *Ligustrum lucidum* litter, using them as only substrate source in SSF conditions. The percentages of dry mass remaining after each sampling time are shown in Fig. 1. Litter decay rates varied according to the fungus employed in the SSF. In the native litter, fungal degradation began faster than in the exotic one, excepting litter treated by *L. ceres*, a treatment that, however, showed a significant mass loss after 90 days ($P < 0.01$). In the native litter, *Marasmiellus candidus, Leratiomyces ceres* and *Marasmius haematocephalus* caused dry mass loss of 10.97 ± 1.12%,

8.78 ± 3.64% and 24.11 ± 3.19%, respectively, at the end of the fermentation period. In the exotic litter, decay progressed slowly during the first 60 days. Afterwards the rate of substrate decomposition caused by *L. ceres* and *M. haematocephalus* increased till the end of the experiment, reaching 22.90 ± 1.79% and 31.46 ± 2.15% dry mass loss, respectively. On the contrary, in *M. candidus*, a plateau in decomposition rate was detected from day 90 onwards, with 7.52 ± 1.49% final mass loss. Decomposition constants (*k*) followed a decreasing order: *M. haematocephalus* > *L. ceres* growing in the exotic litter > *M. candidus* > *L. ceres* cultivated in the native one (Table 1). Significant differences in *k* values between litter species were found only for *L. ceres*, causing faster daily decay rate in the exotic litter (1.39 $x10^{-3}$ day^{-1}) than in the native one (0.52 $x10^{-3}$ day^{-1}).

Chemical composition of the WSF and SF from litter

The chemical composition of the WSF extracted from native and exotic litter, respectively, was notoriously different. The first one was more alkaline (8.32 ± 0.01 vs. 5.08 ± 0.03; $P < 0.0001$) and also had a lower sugar content (4.03 ± 0.59 mg/g vs. 45.07 ± 1.51 mg/g; $P < 0.0001$), proteins (9.48 ± 0.88 mg/g vs. 11.13 ± 0.32 mg/g; $P < 0.0001$), ammonium-N (0.07 ± 0.01 mg/g vs. 0.25 ± 0.02 mg/g; $P < 0.0001$) and phenolic compounds (4.77 ± 0.31 mg/g vs. 8.30 ± 0.26 mg/g; $P < 0.0001$). Changes in chemical characteristics of leaf litter WSF over time are presented in Fig. 2. The native litter incubated with *Leratiomyces ceres* exhibited a slight increase in pH values. In the exotic litter cultures, strong alkalization of the substrate was observed, especially with *Marasmiellus candidus*, which markedly increased pH within the first 30 days of the experiment. Sugar content in the native litter showed few changes along the SSF with *M. candidus* and *L. ceres*, but when decayed by *Marasmius haematocephalus*, a significant increase was observed after day 60. In the exotic litter, water-soluble sugar content was about 10

247 times higher than in the native one, but it was rapidly consumed and almost exhausted within 60
248 days. Ammonium-N values detected in WSF showed a tendency to increase over time. In the
249 case of *Celtis tala* inoculated with *Marasmius haematocephalus* and *Marasmiellus candidus*, the
250 increment occurred in the initial phase of the SSF, and ammonium levels remained constant once
251 maximum values were attained. In *L. ceres*, a peak at day 90 was detected and after 120 days,
252 ammonium concentration started to rise. For *Ligustrum lucidum* treatments, a first decline in
253 ammonium content was observed. Nevertheless, after 120 days, it started to increase when
254 incubated with *Marasmius haematocephalus* and *Leratiomyces ceres*. Water-soluble protein
255 levels registered a maximum at day 30 in the treated native litter and in the exotic one inoculated
256 with *Marasmiellus candidus* as well, although differences were not statistically significant in the
257 first case. A major increase in protein levels was recorded in the native litter incubated with *M.*
258 *haematocephalus* at initial fermentation stages, remaining constant after day 60. Phenolic
259 compounds in WSF showed no significant changes in the native litter decomposed by *L. ceres*,
260 while incubation with *M. candidus* increased total phenolics in WSF at day 200. On the contrary,
261 total phenolics showed a drastic decrease in the exotic litter and to a lesser extent in the native
262 one when decayed by *M. haematocephalus.*

263 ^{13}C CPMAS NMR spectra shown in Fig. 4 correspond to SF from uninoculated *Celtis*
264 *tala* and *Ligustrum lucidum* litter (initial litter samples) and litter obtained after 200 days-SSF
265 with each of the saprotrophic fungi evaluated. All spectra exhibited the same pattern, differing in
266 the relative intensity of the various chemical shift regions. Important differences were found in
267 spectra signals among uninoculated litter types in all the regions that were considered for
268 integration and in all the ratios and indexes estimated as well, except for the O-alkyl C region (P
269 = 0.15, Table 2, Table 3). A larger proportion of alkyl C was found in *L. lucidum* (33.67%) rather

than in *C. tala* (24.22%) uninoculated litter. The intensity in this region can be attributed to aliphatic components, mostly methylene structures of lipids, cutins and waxes as well as short-chains of acids or amino acids. At the end of the SSF, an increase in their relative content was observed in *Ligustrum lucidum* inoculated with *Leratiomyces ceres* and *Marasmius haematocephalus*. Methoxyl carbons, principally of lignin, were detected at 45-60 ppm, although around 10% were identified as methoxyl groups in some of the less common monomer units of hemicellulose, specifically the glucuronic acid in xylan (Haw *et al*. 1984). Methoxyl carbons content in fresh *C. tala*, was found to be 4 times higher than in *L. lucidum.* In *L. lucidum* a significant increase in methoxyl carbons content was detected by the end of the SSF, only when litter was inoculated with *Leratiomyces ceres*. Signal of the O-alkyl C region constituted the largest proportion in fresh litter and was attributed to polysaccharides, comprising 55.60% in *C. tala* and 48.27% in *L. lucidum.* Mainly, two dominant peaks were found, a wider one at 70-80 ppm, an apparent doublet that was reported to correspond to C-2, C-3, and C-5 carbons of cellulose (I) and a peak at 105 ppm that was generally assigned to the C-1 carbon of the anhydroglucose repeating unit of the cellulose (I) (Haw *et al*. 1984). The relative content declined by the end of the SSF mainly in *Ligustrum lucidum* inoculated with *Leratiomyces ceres* (Fig. 1, Table 2). As the decomposition preceded the alkyl C/O-alkyl C ratio, also known as humification degree, it increased in *M. haematocephalus* mainly growing in the exotic but also in the native litter, and in *L. ceres* it grew in the exotic one. No significant differences in the alkyl C/O-alkyl C index were found in the rest of the fungal-litter combinations (Table 3). The intensity in the spectral region from 110 ppm to 160 ppm was due to the aromatic ring carbons of lignin. The phenolic region, between 140 and 160 ppm, characteristic of oxygen-substituted aromatic ring carbons, was negligible in our litter spectra. Almost twice the content of aromatic

carbons was detected in fresh exotic *L. lucidum* (4.75%) compared to native *C. tala* (2.45%), which is consistent with a higher aromaticity and lignin index. Aromatic C content decreased by the end of the decomposition period as a consequence of lignin-degrading fungal activity and maximal chemical shifts were registered in *M. haematocephalus*, which completely delignified all *L. lucidum* substrate. Finally, signals at 160-190 ppm were attributed to acetate groups known to be present in hemicellulose or carbonyl species also existing in hemicellulose (e.g., CO_2H groups of uronic acids). In *C. tala* inoculated with *Marasmius haematocephalus*, an increment was observed, while in *L. lucidum* the content of carboxyl carbons declined when it was inoculated with *Marasmiellus candidus*.

Enzyme activities in the WSF

Endoglucanase activity was significantly different among substrates in every sample time (Fig. 3A, $P < 0.05$) with higher values detected when using the exotic litter and *Leratiomyces ceres* being the largest producer (7.55 ± 0.99 U/g). In the native litter, *Marasmius haematocephalus* recorded the highest endoglucanase activity. This treatment also showed a β-glucosidase activity that stood out from the rest of fungal-litter combinations, with maximum values of 2.80 U/g approximately recorded upon 120 days (Fig. 3B). Furthermore, *M. haematocephalus* and *L. ceres* growing in the exotic litter were the second and third major β-glucosidase producers, respectively. Endoxylanase activity showed a peak at day 30 when cultivating *M. haematocephalus* in the native litter (Fig. 3C, 6.09 ± 0.31 U/g); while a constantly increasing activity was detected in the other treatments with maximal enzyme activity at day 200 in *L. ceres* growing in the exotic substrate. Ligninolytic activity was represented mostly by *L. ceres* (Fig. 3D, Fig. 3E). When cultivated in the exotic litter, MnP activity was remarkably higher than in the rest of fungal-litter treatments while laccase activity was detected as a first

316 peak produced at day 30. In the native litter, laccase production was greater but the uppermost
317 values were detected at advanced stages of fermentation.

318 ***Coinertia Analysis (CoiA)***

319 Some of the chemical variables evaluated at the end of the SSF were highly correlated
320 with the enzyme activities (Supplementary Table S1[1]). The alkyl C/O-alkyl C ratio (humification
321 degree) positively correlated with endoglucanase (r = 0.82; P < 0.01), endoxylanase (r = 0.65; P
322 < 0.05) and MnP activities (r = 0.62; P < 0.05). The last activity was sensitive to this index
323 despite no significant correlations were observed for its individual components. Endoglucanase
324 (r = -0.76; P < 0.01) and MnP (r = -0.84; P < 0.001) activities were also correlated with pH, with
325 greater activities in the acidic values. β-glucosidase activity was strongly correlated with the
326 content of ammonium in the WSF (r = 0.87; P < 0.001). Nevertheless, laccase activity did not
327 correlate with any of the chemical variables measured. As there was multicollinearity among
328 chemical variables, we decided to select only the non-collinear and "representative" ones to
329 reduce the complexity of the variable matrix for the coinertia analysis. From them, the envfit
330 analysis showed that O-alkyl C proportion, alkyl C/carboxyl C ratio, remaining mass degree, pH,
331 content of phenolics and proteins were the variables that significantly corresponded to overall
332 enzyme activities (Supplementary Table S2). The projected inertia with two axes (Fig. 5A, Ax1
333 and Ax2) represented 90.86% of the total covariance (Fig. 5C). The first axis separated
334 *Leratiomyces ceres* growing on the exotic litter from all the other treatments and responded to a
335 high alkyl C/carboxyl C ratio, more acidic conditions and a major decrease in the phenolic
336 compounds content, principally due to the activities of endoenzymes and MnP (Fig 5B, Fig. 5D).
337 The second axis separated the other fungal species, with *Marasmius haematocephalus* and
338 *Marasmiellus candidus* having opposite scores. *M. haematocephalus* correlated well with β-

339 glucosidase activity and protein content and showed greater mass loss, while *M. candidus* was
340 associated with high chemical scores and low mass loss. Seventy-six percent of the variability
341 was explained jointly by the two sets of variables (Supplementary Fig. S1).

342

343 [1] Supplementary material is available with the article through the journal Web site.

344 **Discussion**

345 Below-ground litter decomposition is hierarchically controlled by climate, chemical
346 composition of the substrate and by soil biota (Swift *et al*. 1979; Coûteaux *et al*. 1995; Cadish
347 and Giller 1997; Hättenschwiler *et al.* 2005). In our study, species-specific responses in litter
348 decomposition as a function of litter chemistry could be detected, because species were
349 separately analyzed in environmentally controlled conditions. Litter decomposition rates varied
350 according to the fungus employed in the SSF and only for *Leratiomyces ceres,* significant
351 differences were observed depending on the litter employed, with higher degradation of the
352 exotic *Ligustrum lucidum* litter.

353 Previous studies recording *Ligustrum lucidum* decomposition detected a *k* of 11.20 $x10^{-3}$
354 day^{-1} in a natural lowland stream from Buenos Aires (Marano *et al.* 2013). This value exceeded
355 the *k* value of the native *Pouteria salicifolia* leaf litter, and it was related to the better quality of
356 the substrate (less lignin and phenolic compounds, lower L:N ratio, similar C:N and higher C:L
357 proportion). Aragón *et al*. (2014) proved that in a native secondary forest *L. lucidum* litter
358 decomposed faster than the litter from the native species *Ocotea porphyria* and *Cupania*
359 *vernalis*, reaching a *k* mean value of 3.018 $year^{-1}$ (8.26 $x10^{-3}$ day^{-1}). Moreover, Fernandez *et al*.
360 (2017) suggested that *L. Lucidum* invasion altered the abundance and composition of fungal
361 decomposers associated with the secondary forest mentioned. Several studies proposed that alien

362 species possess common litter traits that allow them to decompose faster than the native species
363 co-occurring with them, promoting invasion and nutrient cycling (Allison and Vitousek 2004;
364 Liao *et al*. 2008). However, taking into account that other studies showed opposite results
365 (Godoy *et al.* 2010; Knight *et al.* 2007), generalizations concerning the comparative
366 decomposition of native and exotic litters cannot be made. As far as we know, there are no
367 reports on *Celtis tala* decomposition rates and only its degradation by ascomycetes was
368 investigated (Saparrat *et al.* 2008). In comparison, our *k* for *L. lucidum* was considerably lower
369 than *k* values obtained in other studies in natural environments. Such differences are expected
370 since in our experimental approach, degradation was the result of the activity of a single fungus
371 in axenic conditions. In this work, mass loss obtained at day 200 in SSF ranged from 7.50 up to
372 31.50% (Fig. 1), but reached around 20% after 120 days with some litter/fungus combinations i.e
373 *Marasmius haematocephalus* growing in *C. tala* litter and *Leratiomyces ceres* cultivated in
374 *Ligustrum lucidum* litter. An average of 16.60% mass loss was reported for *Chamaecyparis*
375 *obtuse* needle litter degradation by several litter saprotrophic basidiomycetes after 18 weeks in a
376 pure-culture test (Osono *et al*. 2006). Also a mass loss of 16 to 34% was caused by *Hypholoma*
377 *fasciculare* growing in different litter types for 12 weeks under SSF (Voříšková *et al*. 2011).

378 It is assumed that at early stages of decomposition, mass loss is dominated by the
379 degradation of soluble substances and holocellulose that is not protected by lignin (Berg and
380 McClaugherty 2014). A considerable fraction of soluble substances is also leached to the soils.
381 Concurrently, the sugar content in WSF (resulting from easily degradable C compounds) was 10
382 times higher in the exotic *Ligustrum lucidum* than in the native *Celtis tala* (Fig. 2). This larger
383 content of sugars may have supported the initial growth in *L. lucidum* (higher endoglucanase
384 activities responsible for cellulose degradation were detected as well). At the same time, initial

degradation rates are positively affected by an increase in the levels of key nutrients such as S, P and N (Berg 2014), the latter one considered a critical element for litter-decomposing basidiomycetes. Simultaneously, low values of C:N have been generally associated with fast decomposition (Lambers *et al*. 2008). N content in *C. tala* was reported to be around 1% and C:N ratio equal to 27 (Saparrat *et al.* 2008), while Aragón *et al*. (2014) informed 0.82% N content for *L. lucidum* and a C:N ratio of 53.72. During fungal cultivation on *L. lucidum*, ammonium was not detected in the WSF [excluding the end of SSF by *M. haematocephalus* (Fig. 2)] suggesting no net mineralization of the N initially present in the litter (in the form of amino acids and proteins) or a net N immobilization in fungal biomass. It was reported that when C:N is high, a substantial fraction of initial nutrients from organic matter can be immobilized into fungal hyphae and converted to growth (Swift *et al*. 1979). The differences in ammonium-N levels detected may indicate a differential availability of N depending on the type of substrate and therefore, N can be considered limiting in *L. lucidum* compared to *C. tala*. This is in agreement with a greater mass loss in *C. tala* at early stages of SSF. In advanced stages of decomposition, mass loss is more limited and involves the degradation of lignin and lignified compounds (Berg 2014). *L. lucidum* litter showed higher values of relative area of aromatic and aliphatic compounds than *C. tala* as well as a higher content of water-soluble phenolic compounds (Table 2, Fig. 2), compounds that are recalcitrant and are known to delay the decay process (De Marco *et al*. 2012). Lignin, aromaticity indexes and phenolic compounds content of the WSF decreased by the end of culture period in *L. lucidum*, revealing the activity of lignin-degrading fungi (Table 3, Fig. 2). Thus, mass loss was higher in *L. lucidum* during the final stages of SSF. Ammonium detected in *C. tala* at the end of the SSF, derived from organic-N

407 degradation, could affect lignin degradation and decay rate in this substrate. Some litter fungi are
408 known to repress the expression of ligninolytic enzymes when labile N is present (Berg 2014).

409 During the course of decomposition of soil organic matter, a rise in the alkyl C/O-alkyl C
410 ratio has been proposed, as O-alkyl C is converted to alkyl C (Baldock *et al*. 1997). In this work,
411 the index shown to be a reliable predictor as a substantial increment was observed at the end of
412 the SSF in those treatments that recorded the highest percentages of degradation, namely
413 *Marasmius haematocephalus* in both substrates and *Leratiomyces ceres* in the exotic litter (Table
414 3). No significant changes in the alkyl C/O-alkyl C ratio were found in the rest of treatments with
415 respect to the uninoculated litter. Since a loss in the O-alkyl C region is mainly associated to
416 cellulose and/or hemicellulose degradation, our results suggest that at least *M. haematocephalus*
417 and *L. ceres* were active degraders of holocellulose, being the former less selective of substrate
418 type. Therefore, these fungi could have a role in humification due to the increase in the alkyl C
419 domain, which is mostly linked to the accumulation of recalcitrant structural components present
420 in plant litter such as that of waxes, cutin, suberin, lipids and amino acids (Kögel-Knabner *et al*.
421 1992).

422 Changes in the activity of the extracellular enzymes that degrade the main components of
423 soil organic matter have been associated to variations in rates of decomposition and soil carbon
424 storage (Sinsabaugh *et al*. 2008). Extracellular enzyme activities are also sensitive indicators of
425 the effort directed by microorganisms towards obtaining carbon, nitrogen, or phosphorus from
426 specific sources (Veres *et al*. 2015). In our work, we detected a noteworthy difference in β-
427 glucosidase production by *Marasmius haematocephalus* with high titers when growing on *Celtis*
428 *tala* but not on *Ligustrum lucidum* (Fig. 3). The reducing sugars content in the WSF of *L.*
429 *lucidum* at initial SSF stages may suggest a byproduct inhibition mechanism of the β-glucosidase

activity in this substrate (Xiao *et al*. 2004) since when sugar levels declined, at the end of culture period, β-glucosidase activity started to rise. The low levels of sugars present in *C. tala* leaf litter may have allowed *M. haematocephalus* to produce a significant amount of β-glucosidase (high protein levels in the WSF) with a consequent increase in glucose levels after 60 days of cultivation. Accordingly, these variables significantly correlated at the end of the SSF (r = 0.69, Table S1). Saparrat *et al.* (2008) found that β-glucosidase activity was correlated with *C. tala* leaf litter decomposition. On the other hand, while in *M. haematocephalus* cultures developing on *C. tala,* delignification was not significant, this fungus caused a complete delignification on *L. lucidum* substrate, which could compensate k and mass loss values between both litter types. Because laccase and MnP activities were negligible, lignin degradation could involve other peroxidases found, expressed by litter decomposing fungi such as the dye-decolorizing peroxidase (DyP, EC 1.11.1.19) and the unspecific peroxygenase (UPO, EC 1.11.2.1) (Kellner *et al*. 2014) or other mechanisms such as Fenton reaction, in which degradation involves the generation of low molecular highly oxidizing species (Regalado *et al*. 1999). MnP production by *Leratiomyces ceres* increased only when the fungus was growing on *Ligustrum lucidum* litter (Fig. 5). Considering that MnP is one of the key oxidative enzymes in litter degradation and lignin content is the component most often identified as a limiting factor in decomposition (Purahong *et al*. 2014), MnP activity found in this treatment could possibly explain the important mass loss values, as it is shown in the coinertia analysis. Laccase activity could be detected as well, being higher in the native litter than in the exotic one. Laccases are only considered as lignin-modifying enzymes because their redox potential is lower than that of peroxidases, and are thought to be involved in polymerization–depolymerization processes of lignin, melanin and humic substances (Liers *et al*. 2011). Since no specific pattern related with degradation was

observed in our study, the detected activity could be associated with other functions adjudicated to laccase, such as its role in the morphogenesis and differentiation of sporulating and resting structures in basidiomycetes, pigment formation and detoxification (Mayer and Staples 2002).

Environmental pH affects fungal growth and enzyme activity, with the majority of fungi having pH optima in the acidic values (Dix and Webster 1995). Nevertheless, some prefer neutral to slightly alkaline conditions, i.e. some *Coprinus* species. Initial pH of *Celtis tala* was 8.32 and values remained alkaline throughout all SSF. Fungi growing on *Ligustrum lucidum* started with an acidic initial pH of 5.08 but a general tendency to alkalization was observed, mainly by *Marasmius haematocephalus* and *Marasmiellus candidus,* the latter raised the pH to 8.27 within the first 30 days (Fig. 2). Our results suggest that the enzyme system of some of these fungi was highly active even at alkaline pHs. Thus, the stability of *M. haematocephalus* β-glucosidase growing in *C. tala* was tested in a pH range of 4.8-9, retaining more than 50% of its relative activity after 12 h of incubation (Mallerman 2017). Conversely, *Leratiomyces ceres* might be sensitive to alkaline pHs and favored by the introduction of *Ligustrum lucidum*, which provides a lower pH. In agreement a strong correlation between pH and MnP activity was detected, mainly associated with this fungus (r = -0.84, Table S1). Resistant enzymes adapted to alkaline pHs, such of the one found in *M. haematocephalus*, have great potential for basic research and industrial applications. The present work explores the lignocellulolytic abilities of species scarcely investigated until now.

Our study involved a long-term, laboratory, microcosm approach to determine the impact of two sorts of leaf-litter, one native and another exotic, on mass loss and activity of selected lignocellulolytic saprotrophic basidiomycetes. Enzymatic assays were conducted using optimal parameters and reproducible assay conditions that do not occur *in situ* (such as incubation

476 temperature and reaction pH), in order to measure not only the maximum potential enzyme
477 activity (Dick 2011) but also to allow the comparison of enzyme activities among different
478 studies (Nannipieri *et al*. 2012). In addition, the alteration of chemical and physical properties of
479 the litter by autoclaving cannot be ruled out (Berns *et al*. 2008). However, such non-target effects
480 were consistent within a given litter type or fungal culture in our study. Therefore, our findings
481 could not be explained.

482 Litter decay is the result of an active cross-kingdom functional succession and bacteria
483 may also significantly contribute to decomposition (Purahong *et al*., 2016). Enzymes involved in
484 cellulose, hemicellulose, chitin and starch catabolism were detected in litter bacteria proteomes
485 (López-Mondéjar *et al*., 2016). Lignin degradation appears to be limited when compared with the
486 abilities of saprotrophic fungi, nevertheless *Actinobacteria* may add to the solubilisation of
487 phenolics (Větrovský *et al*., 2014). Thus, the lack of bacteria may also contribute to the low *k*
488 rates attained in this work. Bacterial-fungal co-culturing could aid in clarifying their possible
489 interacting roles in lignocellulose complex breakdown during plant litter decomposition.

490 In conclusion, native and exotic litter chemistry differentially affects decomposition rates
491 and enzyme production by common lignocellulolytic saprotrophic basidiomycetes. The
492 introduction of *Ligustrum lucidum* to pristine environments could alter the chemical composition
493 of litter input to forest soils. Preliminary data from our *in-vitro* litter degradation study suggests
494 that an impact on litter decomposition rates should be expected, as some common saprotrophic
495 basidiomycetes such as *Leratiomyces ceres* are present, driving to accelerated litter
496 decomposition through an increased ligninolytic activity. Although *Marasmius haematocephalus*
497 and *Marasmiellus candidus* were less sensitive to alterations in litter quality, the three fungi
498 assayed showed significant changes in their pattern of extracellular lignocellulolytic enzyme

499 production with a concomitant modification in nutrient dynamics. Further research is needed to
500 get a better understanding of the role that basidiomycetous fungi and their extracellular enzyme
501 systems play in degradation of soil organic matter in terrestrial ecosystems, as well as to predict
502 potential effects of plant invasion on ecosystems processes and develop management strategies.

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Table 1. Daily decomposition constants (*k*) in native *C. tala* and exotic *L. lucidum* litter under SSF conditions.

Regression Parameters	*C. tala*			*L. lucidum*		
	M. haematocephalus	***M. candidus***	***L. ceres***	***M. haematocephalus***	***M. candidus***	***L. ceres***
Slope (*k*)	$1.42\times10^{-3}\pm0.10\times10^{-3}$**ab**	$0.6\times10^{-3}\pm0.10\times10^{-3}$**bc**	$0.52\times10^{-3}\pm0.08\times10^{-3}$**d**	$1.98\times10^{-3}\pm0.16\times10^{-3}$**a**	$0.53\times10^{-3}\pm0.06\times10^{-3}$**cd**	$1.39\times10^{-3}\pm0.07\times10^{-3}$**ab**
y-Intercept	$0.12\times10^{-2}\pm1.08\times10^{-2}$	$2.74\times10^{-2}\pm1.04\times10^{-2}$	$-0.74\times10^{-2}\pm0.89\times10^{-2}$	$5.41\times10^{-2}\pm1.74\times10^{-2}$	$1.03\times10^{-2}\pm0.67\times10^{-2}$	$-1.56\times10^{-2}\pm0.95\times10^{-2}$
Adjusted R^2	0.92	0.69	0.70	0.90	0.78	0.93
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Note: Decomposition constants among fungal-litter treatments were tested using a two-way ANOVA ($n = 3$; $P < 0.05$). Different letters indicate significant differences in decomposition rates among fungal-litter treatments (Tukey test; $\alpha = 0.05$).

Table 2. Relative intensities (%) derived from the ^{13}C CPMAS NMR spectra of litter samples at initial (0 days) and final time (200 days) in SSF.

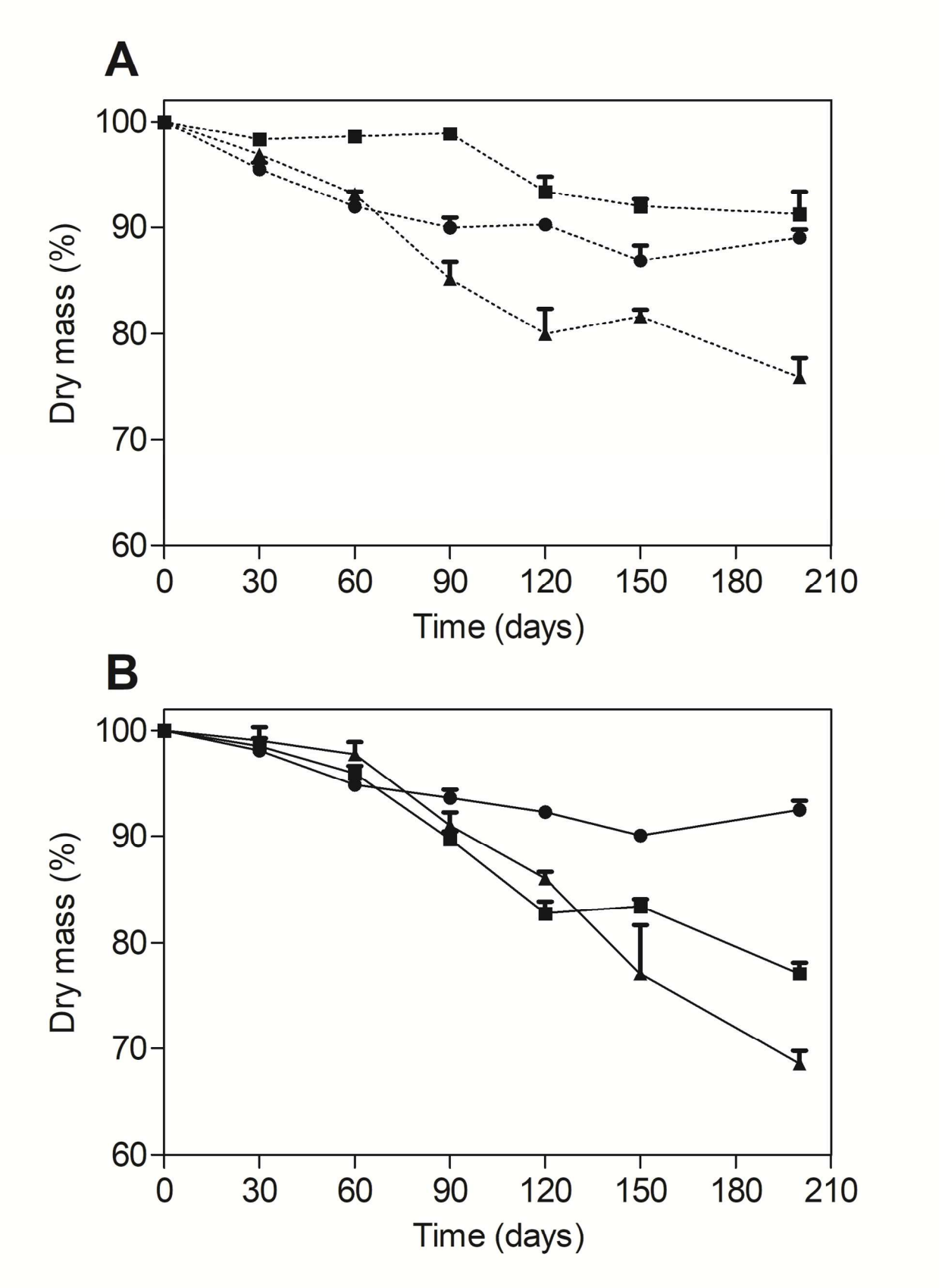
Culture time	Fungi	Litter type	Region of spectra (ppm)				
			Alkyl C 0–45	**Methoxyl 45–60**	**O-alkyl C 45–110**	**Aromatic C 110–140**	**Carboxyl C 160–190**
0 days		*C. tala*	24.22 ± 0.07	7.74 ± 0.18	55.60 ± 0.01	2.45 ± 0.06	17.71 ± 0.03
	M. haematocephalus		26.18 ± 1.00	4.21 ± 2.27	41.24 ± 4.81	2.09 ± 0.71	30.47 ± 3.12
200 days	*M. candidus*		20.73 ± 6.33	5.77 ± 1.94	59.95 ± 10.28	1.78 ± 1.67	17.52 ± 2.26
	L. ceres		23.38 ± 5.08	5.33 ± 2.56	57.55 ± 6.96	1.77 ± 0.85	17.29 ± 1.00
0 days		*L. lucidum*	33.67 ± 1.81	1.65 ± 0.42	48.27 ± 2.51	4.75 ± 0.11	14.30 ± 0.81
	M. haematocephalus		49.95 ± 4.57	2.38 ± 0.13	38.66 ± 4.23	0.00 ± 0.01	11.38 ± 0.34
200 days	*M. candidus*		32.41 ± 3.00	2.60 ± 1.70	56.28 ± 1.50	2.15 ± 0.03	9.16 ± 1.49
	L. ceres		45.87 ± 1.18	6.48 ± 1.19	37.44 ± 1.21	3.61 ± 0.09	13.06 ± 0.12

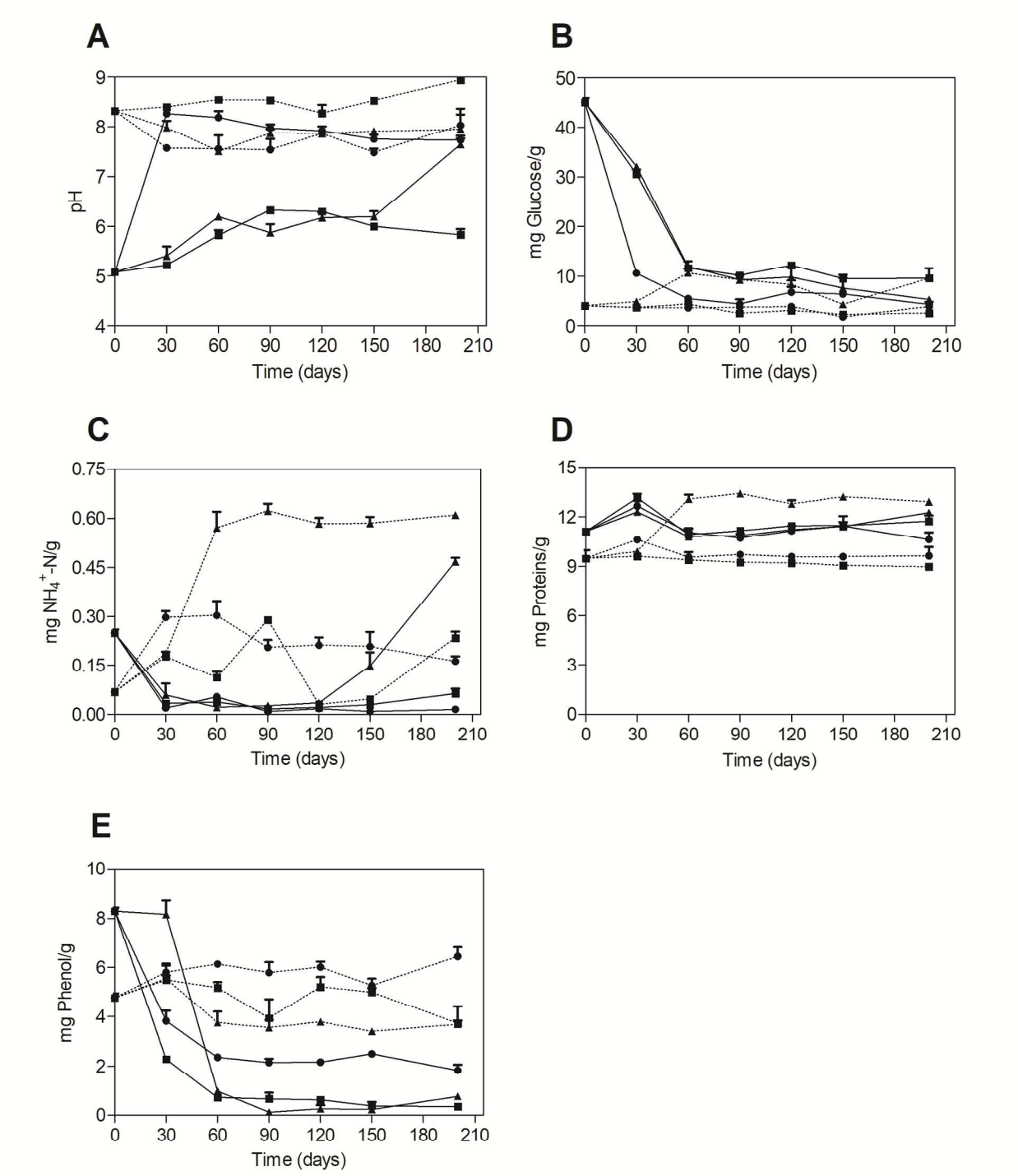
Note: Data are mean (n = 2) ± standard error. Phenolic C region (140-160 ppm) of spectra was negligible

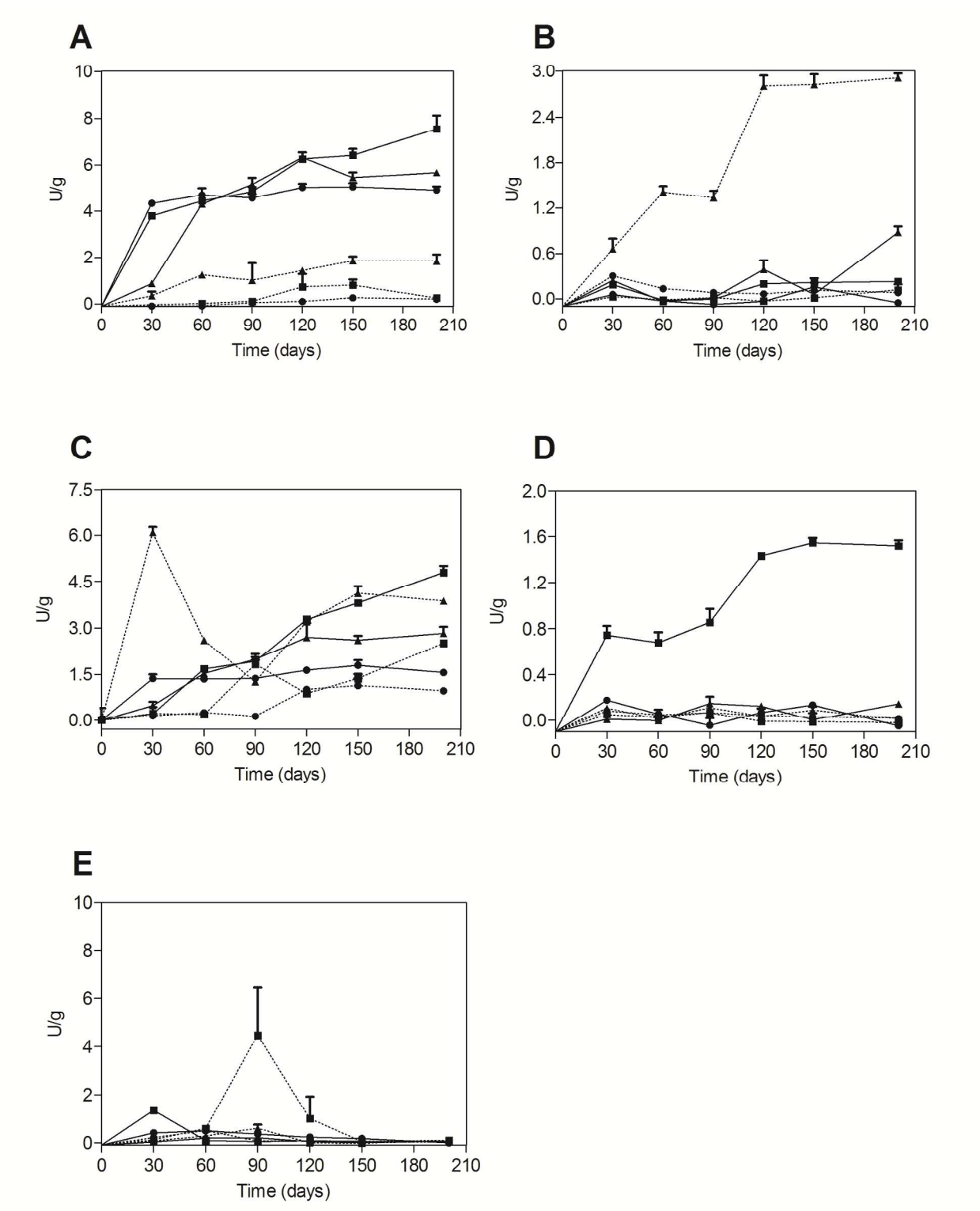
Table 3. Various ratios, lignin content (%), and aromaticities (%) derived from the ^{13}C CPMAS NMR spectra of litter samples at initial (0 days) and final (200 days) culture time in SSF.

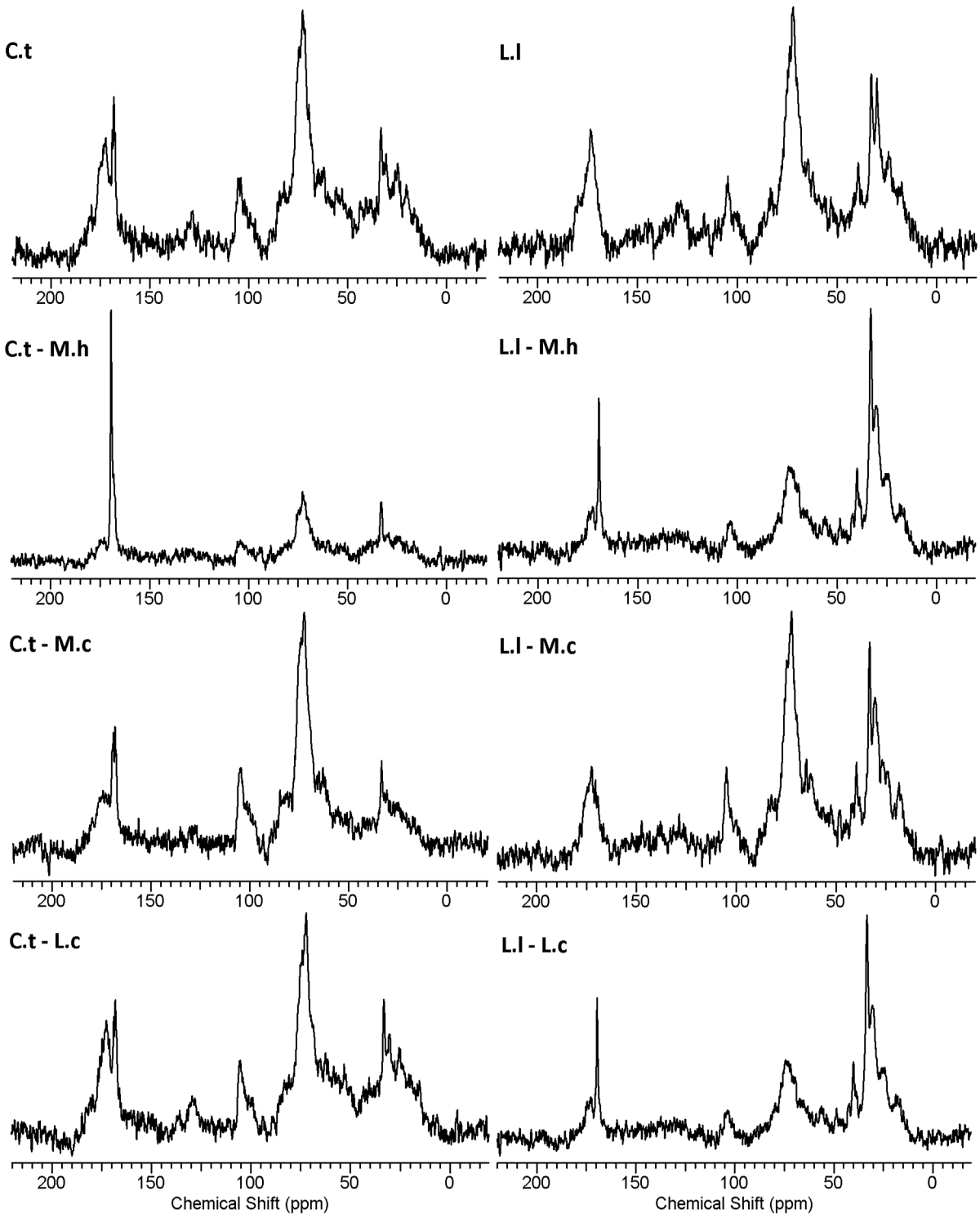
Culture Time	Fungi	Litter Type	Ratios			%	
			Alkyl C/ O-Alkyl C	Alkyl C/ Carboxyl C	O-alkyl C/ Aromatic C	Lignin	Aromaticity
0 days		*C. tala*	0.43 ± 0.01	1.37 ± 0.01	22.65 ± 0.59	4.51 ± 0.13	2.73 ± 0.08
200 days	*M. haematocephalus*		0.64 ± 0.10	0.86 ± 0.06	21.32 ± 9.57	4.71 ± 1.65	2.85 ± 1.00
	M. candidus		0.36 ± 0.17	1.17 ± 0.21	64.89 ± 66.68	3.35 ± 3.15	2.03 ± 1.91
	L. ceres		0.41 ± 0.14	1.35 ± 0.22	37.76 ± 22.12	3.32 ± 1.55	2.01 ± 0.94
0 days		*L. lucidum*	0.68 ± 0.07	2.28 ± 0.01	10.16 ± 0.29	8.99 ± 0.17	5.44 ± 0.10
200 days	*M. haematocephalus*		1.31 ± 0.26	4.40 ± 0.53	ND*	0.00 ± 0.01	0.00 ± 0.01
	M. candidus		0.58 ± 0.07	3.61 ± 0.92	26.17 ± 0.35	3.81 ± 0.18	2.30 ± 0.11
	L. ceres		1.23 ± 0.07	3.51 ± 0.06	10.36 ± 0.07	6.40 ± 0.17	3.87 ± 0.10

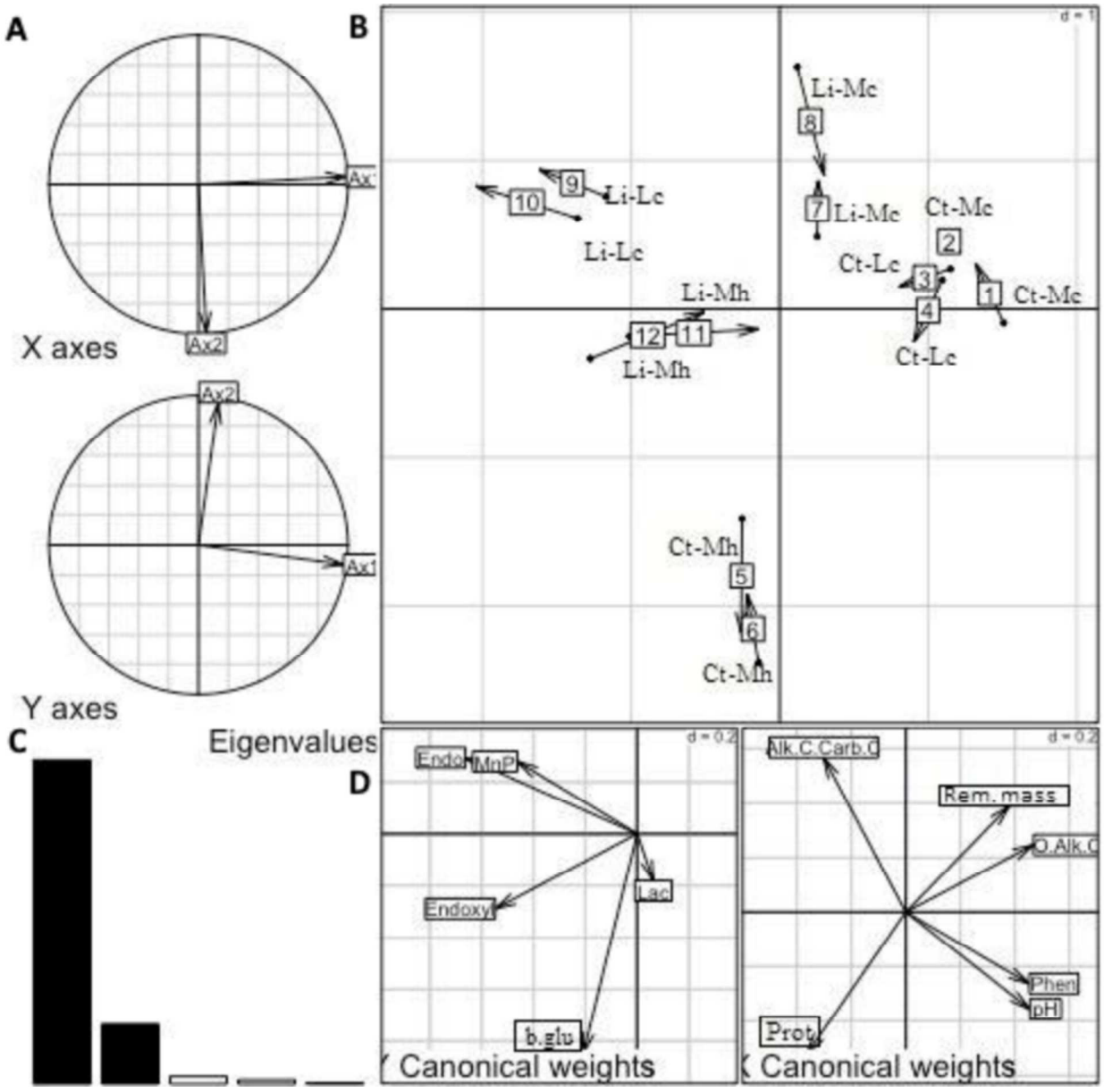
Note: Data are mean (n = 2) ± standard error. ND indicate a ratio value tending to infinite as aromatic C was absent in this treatment.











LEGEND TO THE FIGURES

Fig. 1 - Dry mass remaining during 200 days in SSF in (A) *Celtis tala* and (B) *Ligustrum lucidum* litter inoculated with *Marasmiellus candidus* (circles), *Leratiomyces ceres* (squares), and *Marasmius haematocephalus* (triangles). Data are the mean of three replicates, one standard error of the mean is represented.

Fig. 2 - Characterization of the water soluble fraction during 200 days in SSF with *Celtis tala* (dotted lines) or *Ligustrum lucidum* litter (solid lines) and inoculated with *Marasmiellus candidus* (circles), *Leratiomyces ceres* (squares) or *Marasmius haematocephalus* (triangles): (A) pH (B) Glucose (C) Ammonium-N (D) Proteins (E) Phenolic compounds. Data are the mean of three replicates, one standard error of the mean is represented.

Fig. 3 - Analysis of enzyme activities related to lignocellulose system during 200 days in SSF with *Celtis tala* (dotted lines) or *Ligustrum lucidum* litter (solid lines) and inoculated with *Marasmiellus candidus* (circles), *Leratiomyces ceres* (squares) or *Marasmius haematocephalus* (triangles): (A) Endoglucanase (B) β-glucosidase (C) Endoxylanase (D) Mn Peroxidase (E) Laccase. Data are the mean of three replicates, one standard error of the mean is represented.

Fig. 4 - ^{13}C CPMAS NMR spectra of initial litter samples (C.t and L.l) and after 200 days in SSF with the selected basidiomycetous fungi (C.t-M.c, L.l-M.c, C.t-L.c, L.l-L.c, C.t-M.h and L.l-M.h): C.t, *Celtis tala*; L.l, *Ligustrum lucidum*; M.c, *Marasmiellus candidus*; L.c, *Leratiomyces ceres*; M.h, *Marasmius haematocephalus*. Spectra chemical shift range of litter was characterized by the following dominant peaks: alkyl C (0–45 ppm), O-alkyl C

(45–110 ppm), methoxyl C (50–60 ppm), aromatic C (110–140 ppm), phenolic C (140–160 ppm) and carboxyl C (160–190 ppm).

Fig. 5 - Coinertia analysis using PCA-PCA of the chemical parameters (analytical determinations of the WSF and NMR results of the SF) and the lignocellulolytic enzymes in the WSF produced by the selected basidiomycetous fungi after 200 days in SSF. The analysis represents the individual treatments (from 1 to 12; n = 2) corresponding to the substrate (C.t, *Celtis tala;* L.l, *Ligustrum lucidum*) inoculated with the fungal species (M.c, *Marasmiellus candidus*; L.c, *Leratiomyces ceres*; M.h, *Marasmius haematocephalus*). (A) Inertia projection of the chemical and enzymatic parameters (B) Biplot showing the covariance optimization by the coinertia (C) The eigenvalues showing the total variance and the eigenvalues considered in the analysis (D) The Y and X loadings (canonical weights) for PC1 (chemical parameters: Alk.C.Carb.C., Alkyl C/Carboxyl C; Rem. mass, Remaining mass; O. Alk. C, O. Alkyl C; Phen, Phenolic compounds; Prot, Preteins; pH) and PC2 (enzymatic activities: Endo, Endoglucanase; Endoxy, Endoxylanase; b.glu, β glucosidase; MnP, Mn-Peroxidase; Lac, Lacasse).