Characterization and antifungal properties against wood decaying fungi of hydrothermal liquefaction liquids from spent mushroom substrate and tomato residues

Aitor Barbero-López a,*, Yeray Manuel López-Gómez a, Jaime Carrasco b,c, Noora Jokinen d, Reijo Lappalainen d, Jarkko Akkanen e, Blas Mola-Yudego g, Antti Haapala a,f

a Department of Chemistry, University of Eastern Finland, P.O. Box 111, Joensuu 80101, Finland
b IRIAF – Agri-food and Forestry Regional Research and Development Centre, CIAF - Agroforestry Research Centre, Carretera Toledo-Cuenca km 174, Cuenca 16194, Spain
c Department of Biology, University of Oxford, South Parks Road, Oxford OX1 2JD, UK
d Department of Technical Physics, University of Eastern Finland, 70211 Kuopio, Finland
e Department of Environmental and Biological Sciences, University of Eastern Finland, P.O. Box 111, Joensuu 80101, Finland
f FSCN Research Centre, Mid Sweden University, SE-85170 Sundsvall, Sweden
g School of Forest Sciences, University of Eastern Finland, P.O. Box 111, Joensuu 80101, Finland

* Corresponding author.
E-mail address: aitorb@uef.fi (A. Barbero-López).

https://doi.org/10.1016/j.biombioe.2023.107035
Received 26 July 2023; Received in revised form 22 November 2023; Accepted 15 December 2023
Available online 24 December 2023

© 2023 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

ARTICLE INFO

Keywords:
Agricultural waste
Fungal inhibition
Liquefaction
Mushroom residues
Thermal treatment

ABSTRACT

This study aimed to investigate the potential of converting bio-based residues from industrial production of mushrooms and tomatoes into more valuable chemicals with antifungal properties using hydrothermal liquefaction (HTL). Liquid fractions were obtained from HTL of spent substrate of Agaricus bisporus (Lange) Imbach and Pleurotus ostreatus (jacq.) P. kumm., recomposted Agaricus bisporus spent substrate, and tomato residues. The quantitative 1H NMR spectroscopy analysis revealed that the HTL liquids of all residues contained antifungal constituents like phenols and organic acids. The HTL liquids at dilutions of 10% were able to inhibit the fungi by over 80%. Interestingly, the fungus P. ostreatus showed tolerance to these constituents as its growth was promoted at the lowest concentration of all the HTL liquids. The HTL liquids had lower ecotoxicity than the commercial wood preservative. These results suggest that the tested residues could be a promising source of preservative chemical constituents for the wood industry.

1. Introduction

European mushroom production amounts yearly to more than 1.107.000 tonnes, employing 50.000 people with an annual turnover of €1.470 M [1]. When mushroom production is completed, the substrate (lignocellulosic material such as wood and straw) remains “spent”, as it cannot be used for another mushroom growing cycle due to the depletion of nutrients and it is called “Spent Mushroom Substrate” (herein-after SMS), becoming a waste that needs to be managed according to regulations on waste, fertilisers and nitrates. Approximately 3.3 tonnes of SMS are produced per each tonne of mushrooms but the generation of up to 5 kg of SMS for each kg of mushrooms produced have been reported [2]. The world production of cultivated edible mushroom has been quantified in 34 Mtn in 2013, therefore over 100 Mtn of SMS are globally produced yearly [3]. SMS has multiple uses as substrate for primary decomposers (other cultivated mushrooms), as a source of compost through re-composting process, for animal feed and a source of industrial enzymes or to produce biomaterials or biofuels [4,5]. The most efficient use for SMS in terms of cost is to use it as biofuel, but it has low calorific value [6] what shows that other ways than direct combustion are needed for it.

Currently, fresh SMS is mostly used for agricultural land application since it is a good soil amendment and acts as a soil conditioner for soil mixtures and potting used in horticulture, in addition to be a fertiliser for vegetable production [4,7]. For these purposes, fresh SMS is usually passively re-composted with long composting times (6 months) and increased exposure to leachates. Due to the high content in water (60–70%), these uses are restricted to short distances due to both the difficulties to store SMS and the high transport costs [8]. Further, stored SMS is producing dangerous greenhouse gases such as hydrogen...
sulphide [8], due to partly anaerobic digestion within the composting process.

Tomato (Solanum lycopersicum L.) is an extensively cultivated horticul-
tural crop, holding significant importance in global agricultural practices. Europe is one of the main tomato producers worldwide with an annual production of 15 million tonnes, while Spain is the second European producer with an estimated annual production of 330,000 tonnes, harvested by around 1750 farmers (the number of farmers is an estimation, based on a surface average per farmer.) from 7270 Ha (FAOSTAT [9]). The residual biomass of tomato after the fruit is har-
vested is conformed by the stem biomass (about 70 %) and the leaf biomass (0.75 kg per plant), for a total >15 t/ha [10]. After the tomato campaign concludes, the plants are left to dry and then removed from the greenhouses. Subsequently, they are disposed within a conditioned space and burned. Currently in Spain this practice is authorized only for phytosanitary reasons, due to the tomato brown rugose fruit virus, toBRFV [11], transmitted by contact and very persistent. These green wastes for composting align with circular economy principles [12], although in several places there are no authorized composting plants to process the residues that has sufficient capacity to take all the plant remains. These discarded tomato-plant residues contain valuable ex-
tracts that have been studied as antimicrobials and antioxidants [13].

In the recent years new methods are gaining attention for valoriza-
tion of residues. One of these methods is hydrothermal liquefaction (HTL), which is gaining attention as a process for decomposing wet biomass at temperature between 250 °C and 450 °C and pressure between 100 and 350 bars [14]. As a result of HTL new compounds are generated —biochar, liquids and gas— which have very versatile properties depending on the feedstock and the parameters applied in the whole process. The processing of SMS via HTL can convert these residues into more valuable materials [15]. Spent mushroom substrate has also been treated using hydrothermal carbonization, a thermal process similar to HTL, resulting in charcoal with higher calorific value than the original SMS used [6], although the properties of the resulting liquids were not characterised. In addition, dried SMS has also been pyrolyzed to produce biochar with promising results [16], but the required feed-
stock drying before the process makes the process significantly expensive.

Development of bio-based antifungals is a relevant topic in wood industry. Several extractives have already been proven to be successful antifungals against wood-decaying fungi, such as Scots pine knotwood [17] and Rhizosphora spp. bark extractives [18], tannins [19] and monoterpenes [20]. Distillates from spruce and birch bark and hempo pyrolysis, another thermal process, had several antifungal constituents and showed strong antifungal activity against wood-decaying fungi in one of our previous experiments [21]. Pyrolysis has been also tested as a method for the extraction of wood tar from wood subjected to preser-
vative treatments, facilitating the recovery of the tar for subsequent utilization as a potential wood preservative [22]. Several constituents from pyrolysis distillates which provide them with antifungal properties are known to exist also in HTL liquids from lignocellulose biomass, such as phenolic compounds and organic acids [23].

This paper presents the chemical characteristics of HTL liquids from Pleurotus ostreatus and Agaricus bisporus substrate, recomposted Agaricus bisporus substrate and after-harvesting tomato plant residues. Also, their antifungal properties against wood-decaying fungi and acute ecotoxicity using photoluminescent bacteria are presented.

2. Materials and methods

2.1. HTL of the industrial and agricultural residues

The industrial and agricultural residues used for this experiment were fresh SMS from cultivation of A. bisporus and P. ostreatus (fresh SMS is collected from the growing facilities when the crop cycle is completed), recomposted A. bisporus SMS (fresh SMS passively re-
composted for 6 month) where provided by Sustratos de La Rioja S.L. (Pradejón, Spain). The tomato green waste used in this project has been provided by the company Hernandez Zamora, S.A. (Mazarrón Spain) and by Grupo Hortofruticola Paloma S.A. (Murcia, Spain). The remains have been collected from green house facilities located in Mazarrón (Spain) of the commercial variety Ramybelle. The dry residues were sent for biomass transformation to the University of Eastern Finland by the provider. Additionally, ready-to-use substrate commercially designed for cultivation of A. bisporus and P. ostreatus prior to inoculating the fungi (unused) provided by ASOCHAMP (Autol, Spain) were used as references in the Nuclear Magnetic Resonance (NMR) analysis of the HTL liquids.

To liquefy the residues, batches of 20 % dry SMS or tomato residues and 80 % water were prepared. Then the mixture was introduced in the HTL reactor, and 10 bars of nitrogen added to the atmosphere to prevent product incineration instead of thermal decomposition. The tempera-
ture in the HTL reactor was increased to 300 °C and kept at this tempera-
ture for 90 min while continuously stirring. The mixture was then cooled down in the reactor overnight. Product slurry was collected and filtered (Rundfilter Mm 640 d, Macherey-Nagel, Düren, Germany) to separate the resulting hydrochar and HTL liquid. The HTL liquid was then stored in fridge (5 °C) until used in the experiment.

2.2. NMR analysis of the HTL liquids

The HTL liquids were measured with a Bruker Avance III HD 600 MHz NMR spectrometer (Billerica, MA, USA). Each sample (50 μl) was filtered with a syringe filter (0.22 μm) and dissolved in deuterated methanol (475 μl) with trimethylsilyl propionic-2,2,3,3,-d4 acid sodium salt (TSP) (1.0 mM) as an internal reference standard. The analysis of 1H NMR spectra, and concentration calculation procedure of the compound groups were adapted from Ref. [24]. The spectra were processed and analyzed using Bruker TopSpin 4.1.4 software (Billerica, MA, USA). The concentration calculation of compound groups was carried out as follows: (1) Spectrum phase adjustment; (2) Baseline correction; (3) TSP peak integration and calibration to 9.000 (according to the number of chemically equivalent protons in TSP); (4) Integration over the compound group chemical shift areas, similar to Salami et al. [24] and Salami [25]; (5) Concentration calculation of the compound groups. The spectral peak area of each compound group was integrated, and corre-
sponding number of chemically equivalent protons were considered as shown in Equation (1).

\[
\text{Conc (mM)} = \frac{\text{Integrated area/ # of equivalent protons}}{\text{Sample volume (μl)/Solvent volume (μl)}} \times \text{TSP concentration (mM)} \tag{1}
\]

The choice for the number of chemically equivalent protons per compound group was based on previous research [25] on the same type of samples: Alcohols:2, Aldehydes:1, Alkenes:2, Aromatics:3,5, Hydro-
carbons:2,5, Ketones/acid (carbonyl compounds):2,5, Sugars:3, Phe-
nols:3,5 (Methoxyphenols:4,5).

2.3. Antifungal properties of the HTL liquids

To perform the antifungal test five wood-degrading fungi species were selected: the three brown rot fungi Gloeophyllum trabeum (strain BAM 115), Rhodonia (Poria) placenta (strain BAM 113) and Coniophora puteana (strain BAM 112) and the two white rot fungi Trametes versicolor (strain BAM 116) and Pleurotus ostreatus (strain BAM 96). All the strains were purchased from the Federal Institute for Materials Research and Testing (BAM, Berlin, Germany). Fresh colonies of the five fungi were grown in 4 % malt powder and 2 % agar culture media at 20 ± 2 °C and 65 ± 5 % relative humidity 10 days before setting the antifungal experi-
ment up.

The antifungal test was performed in petri dishes (0 90 mm). The
HTL liquids were dosed in the fungal growth media following the method introduced in Belt [26]. The growth media solutions contained 4 % malt powder and 2 % agar in MilliQ water. In addition, they contained HTL liquid in turn at 1 %, 3 %, 10 % or 100 % (w/w). This mixture was prepared for each HTL liquid. The solutions were autoclaved (120 °C, 15 min) and 15 mL were casted in each Petri dish under sterile conditions. The controls were prepared following the same procedure without HTL liquid, pouring 4 % malt powder and 2 % agar in MilliQ water. Afterwards, the Petri dish was sealed with parafilm and left in a fridge (8 °C) until inoculation.

Using a plug, one spherical piece of fungus (0.3 mm in radius, ca. 0.283 cm³) was inoculated under sterile conditions in each of the previously prepared Petri dish. The dishes were sealed with parafilm and incubated with no light at 22 ± 2 °C and 65 ± 5 % relative humidity in a growth chamber. The area of the mycelium was measured daily, starting from the 3rd day after inoculation, until the mycelium of one of the treatments (either controls or any of the HTL liquid containing media) reached the edge of the Petri dish (between 6 and 15 days depending on the fungal species). A total amount of six replicates were measured for all the fungi, but for C. puteana, for which eight replicates were measured due to its higher growth variability. Pictures of the petri dishes were taken the last measurement day. Fungal growth inhibition was measured by modifying equation (2) proposed by Chang et al. [27].

\[
\text{Inhibition} \, (\%) = \left( 1 - \frac{AT - IA}{AC - IA} \right) \times 100
\]  

(2)

Here, AT is the area of the experimental plate, AC is the area of the control plate, and IA is the surface area (mm²) of the inoculated plug.

The statistical analysis and figures were carried out using R v4.1.2 [28] and the statistical differences were tested using the Tamhane test of the ‘PMCMRplus’ package [29], in order to compare the inhibition of the different HTL liquids with respect to the growth in the control plate within the same fungus.

### 2.4. Acute ecotoxicity test of HTL liquids

The acute toxicity test of the HTL liquids was performed with light emitting bacteria, according to the kinetic ISO 21338:2010 [30] method that is capable of bypass the problems arising from the colour in the samples affecting the measured light emission. This acute ecotoxicity allows a fast ecotoxicity screening of the tested chemicals [31] but obviously does not represent the sensitivity of all species in the aquatic environment. A BioTox™ test kit (ISO 21338:2010) purchased from Aboatox Inc. (Masku, Finland) was used to measure the luminescence reduction caused by the HTL liquids in the *Alivibrio fischeri* photoluminescent bacteria. A cold 2 % NaCl solution was added to the bacterial solution, and after mixing, the solution incubated for 30 min at 4 °C and then for 30 min at 15 °C. Based on a preliminary test, 5 % solutions were prepared for *A. bisporus* SMS and recomposted *A. bisporus* SMS liquids, and 1 % (w/w) solution of *P. ostreatus* SMS and tomato residues, all in 2 % NaCl, were prepared. The pH of the test solutions was measured and adjusted using 0.1 M NaOH (ISO 21338:2010). Only 1 sample required adjustment, *P. ostreatus* SMS HTL liquid, and its pH was adjusted to 6.1. From each of these solutions two dilutions of 1:1 and 2:3 in 2 % NaCl were prepared, and from both, a series of two more 1:1 dilution in 2 % NaCl were prepared to have six test concentrations per sample.

Two replicates of 300 µL were prepared for the experiment from every dilution, and they were kept at 15 °C for 15 min. Then, using the luminometer (Berthold Sirius 1, Pforzheim, Germany) and Sirius Software (Berthold, Pforzheim, Germany), 300 µL of the previously prepared bacterial suspension was injected into the dilutions, and the luminescence of the bacterial suspension was measured right after the injection and exactly 30 min after the injection. As a control, 2 % NaCl solution with no other chemical was used. The photoluminescence reduction was measured by comparing the reduction in bioluminescence after 30 min in all dilutions and the control. The results were reported as effective chemical concentrations that reduced bioluminescence by 20 % (IC20) and 50 % (IC50).

Celcure C4 (Koppers Inc., Pittsburgh, USA), a commercial AB-class preservative, was used as a commercial reference for the acute ecotoxicity test. This preservative contains copper(II) carbonate (17 %), ethanolamine (<35 %), benzalkonium chloride (4.75 %), cyproconazol (0.096 %), sodium nitrite (<5 %), and polyethoxylated tallow amine (<5 %). A 1 % solution of the Cu-based preservative was prepared and tested following the same method as for HTL liquids, but its pH (9.8) was not adjusted as the solution is naturally basic and it precipitates when neutralized.

### 3. Results and discussion

#### 3.1. NMR analysis of the HTL liquids

The results from the NMR analysis of the HTL liquids showed that the HTL constituents differed only slightly when ready-to-use substrate and spent substrate were compared (Table 1). The concentrations of aldehydes were below detection limit in all samples, and the concentration of most of the constituents was similar in both unused and spent substrates of *P. ostreatus* and *A. bisporus*. The concentration of phenols decreased very slightly from unused to spent substrate of *P. ostreatus*, while it increased in the case of *A. bisporus*. The main differences in the mushroom substrates were found in *P. ostreatus* substrate prior to fungal inoculation, which had the highest concentration of alcohols, and in recomposted substrate of *A. bisporus*, which had the lowest. HTL liquids from tomato residues contained a much higher concentration of aromatic compounds, slightly higher concentration of sugars, alcohols and hydrocarbons and a much higher concentration of ketones/acid (carbonyl compounds) than the rest of the liquids. The observed differences among the liquids obtained from SMS and tomato residue liquids are caused by the singularities in the original feedstock matrix. While tomato plant included mostly stem and leaves of tomato plants left in the field after the tomato harvesting, the *P. ostreatus* and *A. bisporus* substrate were distinct from each other, for enhancing their respective growth and fruiting. Due to metabolic processes during mushroom cropping, the substrate employed lost between 35 and 50 % of the mass to confine the SMS which in essence consists of the initial biobased substrate (short-pasteurized straw-based substrate in the case of *Pleurotus* and composted material, straw and chicken manure based, in the case of *Agaricus*), extracellular enzymes secreted by the mycelium and the fungal biomass [32]. The changes caused in the substrate during the growth of *A. bisporus* and *P. ostreatus* might be caused by the different metabolic pathways used by each species, as well as other external factors such as the feedstock, microbes in the substrate, etc ...

The studied agricultural residues yield to several valuable liquid-phase constituents, agreeing with previous studies about wood-based SMS [15]. Several compounds found in the HTL liquids of all the tested SMS and tomato residues are known to inhibit wood-decaying fungi, including phenols [33,34] and organic acids, such as acetic acid, propionic acid [35–37], and citric acid [38].

Ketones and phenols identified in the HTL liquids have antimicrobial properties that protect plants against pathogens and herbivores [39]. Aldehydes, such as those found in olive fruit, are also able to inhibit several fungi [40]. Additionally, the concentration of sugars in all the samples was low, while in similar processes the cellulose converts to levoglucosan [41]. However, sugars were below the detection limit or at low concentrations in the liquids—due to them staying in the solid hydrochar or not being generated in the HTL process—what is positive as carbohydrates act as nutrients for fungi [36]. Due to it, the HTL liquids are a promising source of chemicals for antifungal formulations.

It is worth notice that the nature of the residue matrices under study varied from fresh a) *A. bisporus* SMS, based on a composted mixture of...
wheat straw and chicken manure [42] colonized by the mycelium biomass that can make up 6.8 % of the compost, of which 57 % has been reported dead [43]; b) *P. ostreatus* SMS, based on a wheat-straw pasteurized substrate [42], where the fungal biomass is also part of the by-product; 6-month recomposted material from *A. bisporus* SMS [8]; and naturally dried tomato plant residue (postharvest tomato plant) [44]. Those residues are difficult to manage by the productive industries and tend to be even incinerated like in the case of tomato plants [45].

### Table 1

Results of the $^1$H NMR analysis done to HTL liquids performed at 300 °C from: substrates prior to fungal inoculation (*P. ostreatus* unused and *A. bisporus* unused) as controls, Spent substrate of *Pleurotus ostreatus* (*P. Ostreatus* SMS) and *Agaricus bisporus* (*A. bisporus* SMS), recomposted *A. bisporus* SMS and post-harvesting tomato plant residues (tomato residues). Concentration of constituents below detection limit are presented in table as < LoD.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (mM)</th>
<th>Aldehydes</th>
<th>Aromatics</th>
<th>Phenols</th>
<th>Methoxyphenols</th>
<th>Alkenes</th>
<th>Sugars</th>
<th>Alcohols</th>
<th>Ketones/acids (Aliphatic) hydrocarbons</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. ostreatus</em> unused</td>
<td>&lt; LoD</td>
<td>3</td>
<td>8</td>
<td>3</td>
<td>&lt; LoD</td>
<td>1</td>
<td>78</td>
<td>82</td>
<td>37</td>
</tr>
<tr>
<td><em>P. ostreatus</em> SMS</td>
<td>&lt; LoD</td>
<td>4</td>
<td>7</td>
<td>1</td>
<td>&lt; LoD</td>
<td>51</td>
<td>75</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td><em>A. bisporus</em> unused</td>
<td>&lt; LoD</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>&lt; LoD</td>
<td>57</td>
<td>68</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td><em>A. bisporus</em> SMS</td>
<td>&lt; LoD</td>
<td>3</td>
<td>7</td>
<td>1</td>
<td>&lt; LoD</td>
<td>41</td>
<td>64</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Recomposted <em>A. bisporus</em> SMS</td>
<td>&lt; LoD</td>
<td>3</td>
<td>6</td>
<td>&lt; LoD</td>
<td>&lt; LoD</td>
<td>33</td>
<td>58</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Tomato residues</td>
<td>&lt; LoD</td>
<td>8</td>
<td>7</td>
<td>2</td>
<td>&lt; LoD</td>
<td>2</td>
<td>84</td>
<td>146</td>
<td>39</td>
</tr>
</tbody>
</table>

*SMS: Spent Mushroom Substrate (agricultural residue remaining after the crop cycle is completed).*

![Fig. 1. Mean inhibition values for the antifungal test performed with the HTL liquids at different concentrations with: Spent substrate of *A. bisporus* SMS, recomposted *A. bisporus* SMS, *Pleurotus ostreatus* SMS and post-harvesting tomato plant residues (tomato residues). Positive values indicate that the fungus was inhibited, while negative values indicate growth promotion of the fungi. Lines link the average values per concentration and species.](image-url)
Noteworthy the nature HTL from ready-to-use substrate designed for *A. bisporus* [42] barely differed from the one obtained from its SMS while ready-to-use substrate designed for *P. ostreatus* [46] showed higher differences (for instance the highest concentration of alcohols among the materials tested) when compared to its SMS. This observation suggests that the content of lignin in the SMS, that is degraded by the metabolic action of a primary decomposer such as *P. ostreatus* but not much by a secondary decomposer such as *A. bisporus*, may have an impact on the nature of the HTL obtained.

3.2. Antifungal properties of the HTL liquids

The antifungal test results show that all the HTL liquids were able to cause a complete inhibition of the wood-decaying fungi at their original concentrations (Fig. 1; Table A1). These liquids inhibited the fungi in most of the cases also after significant dilution. The mean results of the wood-decaying fungus *P. ostreatus* had high standard errors when the HTL liquids were tested at 1 %, but its growth was promoted instead of inhibited by the HTL liquids. The samples of *R. placenta* were not inhibited or showed very low inhibition by the tested liquids at 1 % concentration. The remaining wood-decaying fungi were slightly inhibited at 1 % concentration. At 3 % concentration of the liquids, *P. ostreatus* was inhibited by tomato residues and *P. ostreatus* SMS, did not differ from controls when exposed to 3 % recomposted *A. bisporus* SMS and caused a significant growth promotion when exposed to 3 % *A. bisporus* SMS HTL liquid. At a 5 % concentration the HTL liquids inhibited all the wood-decaying fungi. The growth of *G. trabeum* was inhibited about 40–50 % by all the liquids. The fungi *R. placenta* and *T. versicolor* were inhibited 65 % or above by all the liquids, while *P. ostreatus* was the most resistant fungal species with except when exposed to 5 % tomato residue HTL liquid, which inhibited the growth of *P. ostreatus* about 80 %. The fungus *C. puteana* was the most sensitive to the liquids and its growth was inhibited over 90 % by all the liquids already at 5 % concentration, reaching already 100 % inhibition when the *P. ostreatus* SMS was tested. At 10 % concentration, all the liquids caused a very strong growth inhibition of the fungi with values between 80 % and 100 %.

The inhibitory effects of HTL liquids primarily resulted from liquid concentration and strain sensitivity, while the mean antifungal activity of the liquids remained consistent across the feedstocks used to produce them (Fig. 2, Fig. 3). Only significant differences in the inhibition of HTL liquids were observed between *A. bisporus* SMS and tomato residue (p-value = 0.038, Tamhane’s T2 test). In fact, the HTL liquid of *A. bisporus* SMS was the least effective as an antifungal at the lowest concentrations (Fig. 3A), but had a similar antifungal activity than the rest of the HTL liquids already at 10 %.

Conversely, the differences in the antifungal activity were significant among the decay fungi exposed to the liquids (Figs. 2 and 3B), particularly at low concentrations. *Coniophora puteana* was the most sensitive strain to the liquids as its growth was inhibited over 30 % when the concentration of HTL liquids in the Petri dish was 1 % (p-value<0.001 for all comparisons based on the Tamhane’s T2 test). On the other extreme, the growth of *P. ostreatus* was promoted over 50 % when the concentration of liquids in Petri dish was 1 %, and at 3 % HTL liquid concentration had almost zero inhibition. However, at 10 % HTL liquid concentration the inhibition of these species was similar to the rest, although there were significant differences concerning *G. trabeum* (p-value<0.001 for all comparisons based on the Tamhane’s T2 test). In overall, *C. puteana* was the most sensitive species to the HTL liquids and *P. ostreatus* the least.

The chemical characterization showed that SMS HTL liquids contained a similar concentration of constituents, while postharvest tomato plant HTL liquid contained higher amount of carbonyl compounds. Despite those differences, the performance of all the HTL liquids as antifungals was similar no matter the feedstock. The studies on the antifungal activity of HTL liquids are scarce, but a previous study performed by Zhao et al. [47] found that cornstalk liquefaction liquids inhibited the plant pathogen *Fusarium oxysporum*, being those extracted at higher temperatures (270 °C) more effective against the fungus than those extracted at lower temperatures (190 °C). The same trend was found with pyrolysis distillates, where higher pyrolysis temperature leads to higher fungal inhibition caused by the liquids [48], possibly due to the higher total acid concentration. The properties of substrate used for mushroom cultivation can vary significantly depending on factors such as the type of agricultural by-products used, whether the substrate is composted or steam sterilized, and whether or not nutritional

---

**Fig. 2.** Inhibitions (%) caused by the different concentrations. Each column corresponds to a HTL liquid concentration, from 1 % to 10 %. Above: inhibitions for different fungi (*C. puteana*: PUT; *G. trabeum*: TRA; *P. ostreatus*: PLE; *R. placenta*: PLA; *T. versicolor*: VER). Below, inhibitions caused by different HTL liquids (*A. bisporus* SMS: *A. bis.;* *P. ostreata* SMS: *P. ost.*; Recomposted *A. Bisp*orus SMS: Rec.; Tomato residues: Tomato). Letters, when displayed, indicate statistical differences between fungi or HTL liquids (Tamhane’s T2 test). (HTL: Hydrothermal liquefaction; SMS: Spent Mushroom Substrate). Values are displayed in a boxplot, where the thick line corresponds to the median value, the box entails the 25th-75th percentile, whiskers include the 10th-90th percentile and points represent outliers.
faster harvesting of the fruiting bodies by the mushroom industry, while increasing the growth rate, i.e., to act as a biostimulant. This may yield to a strain selection, as HTL liquids at low concentration could be a useful tool to screen for fungi resistant to the antifungal constituents of the liquids, as toxicity in the testing with an IC50 value close to 170 mg/l and an IC50 value of about 195 mg/L (Table 2). Recomposed A. bisporus SMS HTL liquid showed to be the least toxic in the testing with IC20 and IC50 values over 2000 mg/l and 3500 mg/l respectively. The SMS HTL liquid of P. ostreatus showed an IC20 value close to 170 mg/l and an IC50 value close to 2400 mg/l, while the IC20 and IC50 shown by tomato residue HTL liquids were about 190 mg/l and 3200 mg/l respectively. The IC50 values shown by the liquids of A. bisporus SMS liquefaction were almost 750 mg/l for IC20 and about 1500 mg/l IC50.

These results are valid to compare the different HTL liquids between them and to the commercial Cu-based wood preservative, but further tests are also needed for understanding all the effects that they may have in the environment. The results showed that all the tested HTL liquids have lower ecotoxicity than the commercial Cu-Based wood preservative, which makes SMS a very promising source of wood preservative constituents. Within the studied HTL liquids, the highest ecotoxicities were found in P. ostreatus SMS when IC20 was measured and in A. bisporus SMS when IC50 was measured. In terms of acute ecotoxicity, recomposited A. bisporus SMS presented the best performance, as it was the HTL liquid requiring the highest concentrations to reach IC20 and IC50.

While previous papers highlighted that HTL can convert some of the constituents of the feedstock into compounds with higher toxicity, such as it happens with arsenic in microalgae HTL [51], the ecotoxicity of our samples varied depending on the feedstock but was not high when compared to the commercial wood preservative. The literature looking into the ecotoxicity of HTL liquids is scarce, but our research group found that distillates from bark and hemp pyrolysis, which has several similarities with HTL even if it is a different process, create liquids with also very variable ecotoxicity [52]. In that study, the values varied depending on the feedstock and some liquids had higher ecotoxicity than the commercial wood preservative. Additionally, Zhao et al. [53] found that liquids looking similar may still be very different due to the different processing temperatures and feedstock used. Therefore, the ecotoxicity of HTL liquids should be tested before using them for antifungal formulations. If the ecotoxicity of these liquids is high, they can also be treated to reduce their ecotoxicity, similarly to the study performed by Pham et al. [54], but possible change during the treatment in

### Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH (adjusted pH)</th>
<th>IC20 (mg/l)</th>
<th>IC50 (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. bisporus SMS HTL liquid</td>
<td>7.1</td>
<td>743.3</td>
<td>1520.1</td>
</tr>
<tr>
<td>Recomposed A. bisporus SMS HTL liquid</td>
<td>7.4</td>
<td>2056.5</td>
<td>3572.4</td>
</tr>
<tr>
<td>P. ostreatus SMS HTL liquid</td>
<td>5.3 (6.1)</td>
<td>173.1</td>
<td>2415.1</td>
</tr>
<tr>
<td>Tomato Residues HTL liquid</td>
<td>6.3</td>
<td>191.4</td>
<td>3231.1</td>
</tr>
<tr>
<td>Cu-based preservative</td>
<td>9.8</td>
<td>92.8</td>
<td>195.1</td>
</tr>
</tbody>
</table>
the antifungal properties of the liquids needs to be considered.

Despite the effectiveness found in the antifungal test and the lower ecotoxicity compared to the commercial Cu-based wood preservative, further tests are needed to confirm the possibility of using HTL liquids—or their constituents—from SMS in wood, as the effectiveness may differ depending on many factors. Antifungal tests exclude factors affecting the effectiveness of tested extracts such as their variability in distribution in wood [55], what may cause differences when applied in wood. Variations of effectiveness of treatments have also been found between different wood species [56], and different fungi may also be able to degrade the constituents of HTL liquids by using different degradative systems, proving the need for further tests with HTL liquids of SMS.

4. Conclusion

In conclusion, hydrothermal Liquefaction (HTL) is an effective technology for converting *P. ostreatus* and *A. bisporus* spent mushroom substrates, along with tomato plant residues, into valuable antifungal chemicals. The high fungal inhibition caused by all the HTL liquids and the lower ecotoxicity values than the commercial copper-based reference indicate the potential of the SMS as a source of antifungals in wood preservation. The HTL liquid fractions emerge as promising sources of antifungals against wood-decaying fungi, suggesting their potential inclusion in wood preservative formulations.

Funding

This work was supported by the Niemi foundation [grant number 2022007]; Academy of Finland project Ecodee [grant number 329884], the SNS project SYNERGIES and the European Union’s H2020 research and innovation programmes under the Marie Sklodowska-Curie DecisionES [grant number 101007950]; J.C. is the recipient of a Ramón y Cajal contract [RYC2021-032796-I], funded by MCIN/AEI/10.13039/19.22.1812/100100011033 (Ministerio de Ciencia e Innovacion, Spain) and the European Union “NextGenerationEU”/PRTR+.

CRediT authorship contribution statement

Aitor Barbero-López: Data curation, Investigation, Methodology, Software, Validation, Writing – original draft, Drafting – review & editing.

Jaime Carrasco: Conceptualization, Data curation, Investigation, Project administration, Resources, Writing – original draft, Writing – review & editing.

Noora Jokinen: Formal analysis, Investigation, Validation, Writing – original draft.

Reijo Lappalainen: Data curation, Formal analysis, Software, Validation, Investigation, Writing – original draft, Writing – original draft.

Blas Mola-Yudego: Data curation, Formal analysis, Software, Validation, Writing – original draft, Writing – original draft.

Antti Haapala: Funding acquisition, Invitation, Resources, Supervision, Writing – original draft, Writing – review & editing.

Jaime Opeza: Data curation, Methodology, Validation, Writing – original draft.

Aitor Barbero-Lopez: Data curation, Investigation, Methodology, Software, Validation, Writing – original draft, Drafting – review & editing.

Jaime Carrasco: Conceptualization, Data curation, Investigation, Project administration, Resources, Writing – original draft, Writing – review & editing.

Noora Jokinen: Formal analysis, Investigation, Validation, Writing – original draft.

Reijo Lappalainen: Data curation, Formal analysis, Software, Validation, Investigation, Writing – original draft, Writing – original draft.

Blas Mola-Yudego: Data curation, Formal analysis, Software, Validation, Writing – original draft, Writing – original draft.

Antti Haapala: Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to acknowledge the support provided by the UEF FORES doctoral school. We acknowledge the company Hernandez Zamora, S.A. and Grupo Hortofrutícola Paloma, S. A. for providing the tomato plant residues used in this work. We acknowledge Sustratos de La Rioja, S. L. and CTICH (Mushroom Technological Research Center of La Rioja) for providing the SMS employed in this research.

Appendices.

Table A.1

Mean results of the antifungal test performed with the HTL liquids from: Spent substrate of *Pleurotus ostreatus* (*P. ostreatus* SMS) and *Agaricus bisporus* (*A. bisporus* SMS), recomposted *A. bisporus* SMS and post-harvesting tomato plant residues (tomato residues). Positive values indicate that the fungus was inhibited, while negative values indicate growth promotion.

<table>
<thead>
<tr>
<th>HTL liquid</th>
<th>concentration (%)</th>
<th>G. trabeum</th>
<th>R. placenta</th>
<th>C. puteana</th>
<th>T. versicolor</th>
<th>P. ostreatus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato Residues</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>10.8 ± 1.1</td>
<td>9.3 ± 3.7</td>
<td>27.5 ± 15.3</td>
<td>14.7 ± 1.5</td>
<td>−55.5 ± 21.6</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>35.3 ± 3.1</td>
<td>44.5 ± 3.5</td>
<td>78.2 ± 2.2</td>
<td>41.0 ± 2.8</td>
<td>48.4 ± 13.0</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>44.7 ± 1.8</td>
<td>67.5 ± 3.1</td>
<td>96.9 ± 0.7</td>
<td>68.9 ± 4.5</td>
<td>79.4 ± 6.4</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>81.2 ± 1.3</td>
<td>99.7 ± 0.1</td>
<td>100.0 ± 0</td>
<td>96.5 ± 0.6</td>
<td>100.0 ± 0</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
</tr>
<tr>
<td><em>P. ostreatus</em> SMS</td>
<td></td>
<td>7.4 ± 1.8</td>
<td>−3.9 ± 3.4</td>
<td>39.2 ± 11.8</td>
<td>18.6 ± 3.7</td>
<td>−47.6 ± 20.0</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>34.8 ± 2.2</td>
<td>32.9 ± 2.7</td>
<td>79.5 ± 1.6</td>
<td>50.5 ± 3.2</td>
<td>26.3 ± 12.4</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>44.0 ± 1.0</td>
<td>76.2 ± 1.9</td>
<td>100.0 ± 0</td>
<td>75.6 ± 3.8</td>
<td>58.8 ± 8.2</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>89.6 ± 0.4</td>
<td>94.9 ± 0.5</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
</tr>
<tr>
<td><em>A. bisporus</em> SMS</td>
<td></td>
<td>11.0 ± 2.7</td>
<td>1.9 ± 3.3</td>
<td>23.0 ± 9.9</td>
<td>22.2 ± 6.2</td>
<td>−126.9 ± 24.3</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>30.1 ± 3.1</td>
<td>47.5 ± 3.1</td>
<td>65.3 ± 4.0</td>
<td>28.2 ± 4.3</td>
<td>−43.6 ± 14.0</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>48.7 ± 0.9</td>
<td>69.2 ± 0.7</td>
<td>94.9 ± 1.9</td>
<td>64.6 ± 2.9</td>
<td>28.5 ± 8.2</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>82.7 ± 0.7</td>
<td>97.6 ± 0.9</td>
<td>100.0 ± 0</td>
<td>91.7 ± 1.0</td>
<td>94.3 ± 1.2</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
</tr>
<tr>
<td>Recomposted <em>A. bisporus</em> SMS</td>
<td>6.3 ± 3.8</td>
<td>4.8 ± 2.5</td>
<td>46.5 ± 8.6</td>
<td>15.8 ± 2.7</td>
<td>−32.9 ± 12.9</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>23.9 ± 2.4</td>
<td>60.5 ± 1.0</td>
<td>73.7 ± 3.9</td>
<td>31.8 ± 2.7</td>
<td>−12.1 ± 16.2</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>51.5 ± 1.9</td>
<td>88.4 ± 2.2</td>
<td>92.4 ± 1.7</td>
<td>64.0 ± 1.7</td>
<td>−21.5 ± 14.4</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>94.5 ± 0.4</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>95.8 ± 0.3</td>
<td>89.9 ± 2.9</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
<td>100.0 ± 0</td>
</tr>
</tbody>
</table>

