2016•2017
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Paxillus involutus alleviates forest litter toxicity on bacterial growth

Promotor :
Prof. dr. Francois RINEAU

Promotor :
dr. MICHIEL OP DE BEECK

Copromotor :
Prof. dr. ANDERS TUNLID

Thomas Van Dijck

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

2016•2017
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Paxillus involutus alleviates forest litter toxicity on bacterial growth

Promotor :
Prof. dr. Francois RINEAU

Promotor :
dr. MICHIEL OP DE BEECK

Copromotor :
Prof. dr. ANDERS TUNLID

Thomas Van Dijck

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

universiteit hasselt | Maastricht University

ABSTRACT

Background: It has been observed that when ectomycorrhizal fungi (EMF) are excluded from soils, forest litter degradation increases significantly. Furthermore, several fungal secondary metabolites are known for their antibacterial effects. Therefore, it is hypothesized that forest litter extracts processed by EMF inhibit bacterial growth.

Material & methods: *Paxillus involutus* – a model EMF – was grown for 7 days on liquid forest litter extracts. FTIR-spectroscopy was used to investigate fungal-induced changes in the litter composition. Extracts were fractionated by 3 different methods: phenolic compounds were separated via solid-phase extraction; low-molecular weight compounds (3 kDa cut-off) via ultrafiltration; apolar compounds via double ethyl acetate extraction. Bacterial growth inhibition of the different fractions was tested for by ^{3}H-leucine incorporation.

Results: *P. involutus* processing of the litter extracts resulted in a decrease in aromatic C=C stretches and an increase in carboxylic C=O stretches. The addition of unprocessed extract to bacterial slurries reduced bacterial growth rates by 80%. If the extract had been processed by *P. involutus* bacterial growth was only reduced by 20%. This effect was reflected in both <3 kDa and >3 kDa fractions, with no significant difference between both fractions. Both phenolic and apolar compounds inhibited bacterial growth but no difference due to EMF processing was found. However, the aqueous fractions did strongly reduce bacterial growth and reflected the observed difference between unprocessed and processed litter extracts. Salts, sugars, metals, and carboxylic acids were found not to affect bacterial growth at the investigated concentrations.

Discussion & conclusions: When growing on litter extracts, *P. involutus* has not been found to induce bacterial toxicity. On the contrary, litter extracts themselves exert antibacterial effects which are partially neutralized after *P. involutus* processing. This effect is exerted by unknown water-soluble molecules, and further research is required to identify these antibacterial compounds in forest litter.

SAMENVATTING

Achtergrond: Het is vastgesteld dat de degradatie van de strooisellaag in bossen significant toeneemt indien ectomycorrhizale fungi (EMF) worden verwijderd uit de bodem. Ook weet men dat sommige van deze schimmels secondaire metabolieten produceren die een antibacterieel effect uitoefenen. Gebaseerd op deze 2 bevindingen is er gehypothetiseerd dat wanneer extracten van de strooisellaag bewerkt zijn door EMF, deze de bacteriële groei inhiberen.

Materiaal & methoden: *Paxillus involutus* – een model-EMF – werd voor 7 dagen gegroeid op een vloeibaar extract gemaakt van de strooisellaag van een bos. FTIR-spectroscopie werd gebruikt om na te gaan hoe de chemische samenstelling van het extract veranderde tijdens deze periode. Drie technieken werden toegepast om de extracten te fractioneren: fenolische componenten werden geïsoleerd door vastefase-extractie; moleculen kleiner dan 3 kDa door ultrafiltratie; apolaire moleculen door een dubbele ethylacetaat extractie. Van elke fractie werd de inhiberende capaciteit op de bacteriële groei onderzocht via de incorporatie van ^{3}H-leucine.

Resultaten: Nadat *P. involutus* op het extract gegroeid had, waren er minder aromatische C=O verbindingen en meer carboxylische C=O verbindingen aanwezig. Wanneer onbewerkt extract werd toegevoegd aan bacteriën, werd de groei ervan gereduceerd met 80%. Als *P. involutus* hier eerst op gegroeid had, was de reductie slechts 20%. Dit effect werd teruggevonden in zowel de fractie die moleculen <3 kDa bevatte als deze die moleculen >3 Kda bevatte. Zowel de fenolische fractie als de fractie met de apolaire componenten zorgde voor een inhibitie van de bacteriële groei, maar geen verschil werd opgemerkt tussen onbewerkte en door *P. involutus* bewerkte extracten. De fractie die de polaire moleculen bevatte reduceerde de bacteriële groei ook en reflecteerde het opgemerkte verschil tussen onbewerkt en bewerkt extract. Zouten, suikers, metalen en carboxylzuren hadden geen invloed op de bacteriële groei bij de onderzochte concentraties.

Discussie & conclusies: Er zijn geen aanwijzingen gevonden dat *P. involutus* extracten van de strooisellaag toxisch maakt voor bacteriën. In tegendeel, deze extracten blijken zelf toxisch te zijn voor bacteriën en *P. involutus* verlicht dit gedeeltelijk. Wateroplosbare moleculen blijken verantwoordelijk te zijn voor deze toxiciteit, maar verder onderzoek is nodig om deze antibiotica te identificeren.

TABLE OF CONTENTS

ABSTRACT 1

SAMENVATTING 3

TABLE OF CONTENTS 5

ACKNOWLEDGMENTS 7

LIST OF KEY ABBREVIATIONS 9

INTRODUCTION 11

- Global climate change – a modern threat 11
- Soil C-sequestration – an ecological process 11
- Interactions between EMF and soil bacteria 12
- Goals and objectives of this study 12

MATERIAL & METHODS 13

- Soil and litter sampling 13
- Litter extracts 13
- Fungal strain and culture conditions 13
- Litter processing by P. involutus 14
- Characterization of litter processing 14
- Molecular size-fractionation 14
- Ethyl acetate extraction 15
- Fractionation of phenolics 15
- Screening for antibacterial effect 15
- Statistical analyses 16

RESULTS AND DISCUSSION 17

- Bacterial growth rates are pH-sensitive 17
- P. involutus alleviates bacterial toxicity of forest litter extracts 17
- *P. involutus* modifies FHE during 7 days of growth 21
- Water-soluble components are responsible for antibacterial effect 22

CONCLUSION & SYNTHESIS 27

REFERENCES 29

ACKNOWLEDGMENTS

First and foremost, I would like to thank my parents for providing the necessary support, both mentally and financially, during the course of my education. It has been a long road, and without this, it would not have come to making this thesis.

Secondly, I would like to thank prof. dr. François Rineau. During my Junior Practical Training in my first year as a Master` student, he brought back the interest in performing research for me. Performing science is all about gaining in-depth knowledge of a specific topic of interest. The fun in this process, however, lies for me in the possibility to be able to deviate from the originally proposed research objectives upon an interesting observation. One can only find answers upon asking questions, and questions are only asked when one is being interested. Also, I would like to thank him for introducing me to the research group of prof. dr. Anders Tunlid while I was searching for a thesis topic.

I thank prof. dr. Anders Tunlid for taking me on as a Master`s student during the last year. It was a nice experience to see such a large group of scientists working on the same project and being closely involved in each other's research. The weekly group meetings and lunch seminars proved the value of communicating with your fellow peers to me.

Next, I would like to thank my supervisor dr. Michiel Op De Beeck. His guidance throughout this project was invaluable. I would like to think that I have adapted his punctuality and critical opinion towards performing research. Furthermore, supervising a student in the lab is not only about helping them to obtain the necessary skills. One should be capable to allow one to adopt his own methods and at his own pace. For that, I would also like to thank him.

Lastly, I would like to thank the Erasmus programme for providing me with a scholarship to perform my Master thesis abroad.

^{3}H-LEU	L-[4,5-3H]-Leucine
DRIFTS	Diffuse reflectance infrared Fourier transform spectroscopy
EMF	Ectomycorrhizal fungi
ETOAC	Ethyl acetate
FCE	Forest cold extract
FHE	Forest hot extract
HMWC	High molecular weight compounds
LMWC	Low molecular weight compounds
TCA	Trichloroacetic acid
TN	Total nitrogen
TOC	Total organic carbon

· Global climate change – a modern threat

Global climate change is a modern acknowledged problem, resulting from an increase in the average global temperature. Over the last 30 years, the global surface temperature increased with 0.2˚C per decade and it is predicted that global warming of more than 1˚C will lead to dramatic effects on agriculture, human health, water resources and sea level (1).

Global warming is caused by the increased accumulation of greenhouse gases in the atmosphere and this increase has been attributed to human activities, particularly the burning of fossils fuels. One of those greenhouse gases that is stated to be a major contributor to the `greenhouse effect` is atmospheric carbon dioxide (CO_2).

· Soil C-sequestration – an ecological process

Soil carbon (C)-sequestration is the ecological process in which atmospheric CO_2 is taken up by photosynthesizing higher plants and transferred to the soil. This can be via the roots in the form of exudates, but also as living or dead plant biomass. This process has led to the creation of major carbon sinks.

Of all the ecotypes, boreal forest biomes are of major importance. They cover approximately 11% of the terrestrial surface and contain 16% of the total sequestered C in soils worldwide (2). These forests are thus characterized by a thick litter layer. In this litter layer, ectomycorrhizal fungi appear to be dominant. These microbial organisms form mutualistic symbioses with the roots of their host plants. They extend the root system by forming extensive mycelial networks in the surrounding soil. This allows them to scavenge the soil for mineral nutrients which they exchange with their hosts for energy-rich carbohydrates.

EMF comprise only a part of the microbial organisms found in soils. Also saprotrophic organisms, which includes both fungi and bacteria, are found. They are dependent on the dead organic matter in the soil for their energy source. The respiration of all these organisms results in a flux of CO_2 back into the atmosphere, and this contributes much higher to the atmospheric CO_2 content than any human activity (3).

· Interactions between EMF and soil bacteria

In 1971 and 1975, Gadgil & Gadgil observed that when EMF were excluded from the soil, the degradation of the litter layer increased significantly (4, 5). Since then, many hypotheses have been investigated to account for this observed effect (**Figure 1**). Physical parameters such as temperature and soil moisture content, but also biological interactions between the microbial organisms have been investigated (6). To date, no single parameter has been found to be able to account for this observation solely, raising the possibility that this effect depends on multiple factors. Interestingly, it has been observed that in coniferous forest soils, fungi are found to quantitatively dominate bacteria in the decomposing litter layer while the importance of bacteria increases with soil depth (7). This can indicate that fungi exert specific effects on soil bacteria, providing a valid hypothesis for explaining the Gadgil effect. Further evidence comes from the observation that some EMF species are found to reduce the bacterial activity in soils (8).

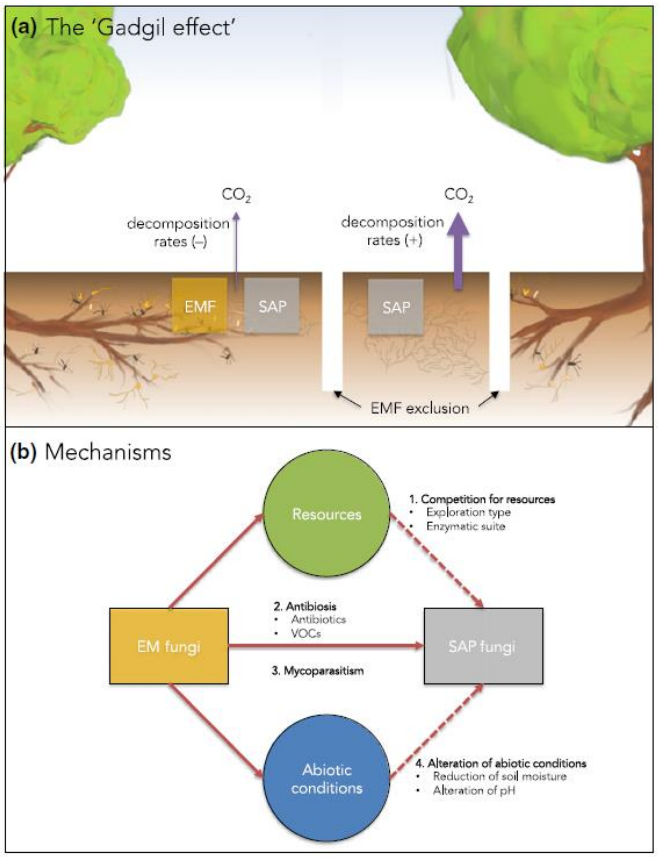

Figure 1 Schematic review of the Gadgil effect (a), and possible mechanisms that induce this effect (b). Adapted from (4).

· Goals and objectives of this study

It is hypothesized that EMF induce bacterial toxicity when degrading forest litter. To investigate this, *Paxillus involutus*, a model EMF, will first be grown on liquid forest litter extracts. The degradation of this extracts will be characterized via diffuse reflectance infrared Fourier transform spectroscopy. Bacterial growth rates will be tested for *P. involutus*-induced toxicity by adding fractions of unprocessed and processed litter extracts to them and comparing the observed results. Should *P. involutus* be found to induce bacterial toxicity, the nature of this toxicity will be further characterized.

· Soil and litter sampling

Soil samples were taken from both the litter layer (November 2015) and the underlying mineral layer (February 2017; SUPPORTING INFORMATION, table S1) of a pure spruce forest site in southern Sweden (56˚ 42` 2.63" N, 13˚ 6` 57.57" E, Halmstad, Skåne lan, Sweden). Samples were homogenized by sieving them (2 mm mesh size) and stored fresh in plastic bags at 4˚C until further usage.

· Litter extracts

Two different litter extracts were made to use in the experiments. 120 g of litter was suspended in 600 mL of distilled water and either boiled for 1 h (Forest Hot Extract, FHE) or magnetically stirred for 24 h at room temperature (Forest Cold Extract, FCE). Solids were removed by filtration through a cheese mesh and subsequent centrifugation (30 min, 10000 *g*). To obtain homogeneous extracts, the supernatants were sequentially filtered through glass microfibre filters of three different pore sizes (Whatman GF/D 2.6 µm, GF/A 1.6 µm, GF/F 0.7 µm; GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). Extracts were sterilized via syringe filtration (Filtropur S 0.22 µm; Sarstedt, Nümbrecht, Germany) and stored at 4˚C until further analyses.

· Fungal strain and culture conditions

Paxillus involutus (Batsch) Fr. (strain ATCC 200175; Manassas, VA, USA) (Basidiomycota, Boletales), a model EMF, was chosen to use in the experiments. Cultures were maintained aseptically in the dark at 21˚C on 1% (w/v) agar plates containing modified Fries` medium (9) (33 mM glucose, 3.7 mM NH_4Cl, 74 µM $FeCl_3 \cdot 6H_2O$, 0.4 mM $MgSO_4 \cdot 7H_2O$, 5 µM $CuSO_4 \cdot 5H_2O$, 20 µM $ZnSO_4 \cdot 7H_2O$, 50 µM $MnSO_4 \cdot H_2O$, 0.2 µM $(NH_4)_6Mo_7O_4 \cdot 4H_2O$, 0.3 mM NaCl, 1.3 mM KCl, 0.2 mM H_3BO_3, 0.2 mM KH_2PO_4, 0.2 mM $CaCl_2 \cdot 2H_2O$, 55 µM myo-inositol, 0.1 µM biotin, 0.6 µM pyridoxine, 0.3 µM riboflavin, 0.8 µM nicotinamide, 0.7 µM p-aminobenzoic acid, 0.3 µM thiamine·HCl, 0.4 µM Ca-pantothenate; pH adjusted to 4.8).

· Litter processing by *P. involutus*

A fungal inoculum was obtained by taking a single 5- by 5-mm plug of actively growing mycelium from the margins of a 2-week-old *P. involutus* culture. This inoculum was transferred to a Petri dish, filled with a single layer of 4 mm diameter glass beads and 10 mL of liquid Fries` medium (see above; without agar). After 9 days of incubation, the medium was removed and replaced with 10 mL of liquid $NH_4$-free Fries` medium to induce N-deprivation in the mycelium (10). After 24 h, the medium was again removed and replaced with 10 mL of either one of the two litter extracts: FHE or FCE. To prevent that remaining glucose in the medium would interfere in the subsequent experiments, it was added as a solid 1 M glucose patch (0.5 cm diameter, 0.5 cm height; 1% (w/v) agar) on top of the mycelium. After 7 days of fungal growth, the mycelium was discarded and the extracts were collected and stored at -20˚C until further analyses. At the same time, aliquots (10 mL) of unprocessed litter extract were also taken from the bulk solution and stored at -20˚C to serve as controls.

· Characterization of litter processing

The chemical compositions of unprocessed and processed litter extracts were compared via diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS). To prepare the samples for analyses, each sample (10 mL) was freeze-dried and ground to a fine powder in an agate mortar. Next, 5 mg of sample was diluted with 295 mg of KBr and this mixture was again ground. Samples were stored at 60˚C until being measured. The infrared spectra were recorded under low vacuum using a Vertex 80v spectrometer (Bruker, Billerica, MA, USA) and each spectrum was the average of 512 consecutive scans at a resolution of 4 cm^{-1}. Spectral peaks were assigned to specific chemical bonds according to (11).

· Molecular size-fractionation

Low molecular weight compounds (LMWC) were separated (3 kDa cut-off) from high molecular weight compounds (HMWC) for both the unprocessed and processed litter extracts via ultrafiltration (3K Omega Macrosep advance centrifugal devices; Pall corporation, Port Washington, NY, USA). After centrifugation (1 h, 5000 *g*) of the samples (10 mL), both fractions were resuspended in Milli-Q water to 10 mL and stored at -20˚C until further analyses.

· Ethyl acetate extraction

Unprocessed and processed litter extracts were extracted with ethyl acetate (EtOAc) to separate apolar from polar compounds. Each sample (10 mL) was divided into 2 subsamples of 5 mL. Each subsample was protonated through the addition of 1 mL 1 M HCl and subsequently mixed with 5 mL 99.8% EtOAc. After centrifugation (40 min, 3500 rpm), the EtOAc fractions were carefully taken off with glass Pasteur pipettes as to not disturb the formed interlayer between the EtOAc fraction and the aqueous fraction. Water fractions were extracted a second time with fresh EtOAc (without the protonation step). EtOAc fractions from the same sample were pooled together, evaporated with N_2 gas and resuspended in Milli-Q water to 10 mL. The water fractions were carefully collected by pipetting throughout the interlayer to the bottom of the glass vials, freeze-dried and resuspended in 10 mL Milli-Q water. All fractions were stored at -20˚C until further analyses.

· Fractionation of phenolics

Phenolic compounds were isolated from the unprocessed and processed litter extracts through an optimized solid-phase extraction using Oasis HLB cartridges (Waters corporation, Milford, MA, USA). Cartridges were prepared for analysis by rinsing them with 2 mL 99.8% methanol and acidifying them with 4 mL 0.01 M HCl. Before analyses, 1050 µL -samples were taken from the litter extracts and acidified with 15 µL 1 M HCl. For each subsample, two cartridges were each loaded sequentially with 500 µL sample and 500 µL 0.01 M HCl. Next, the bound phenolic compounds were eluted from the cartridges with 500 µL methanol solution (50% (v/v) 99.8% methanol in 0.02 M NaOH). These fractions were collected in 15 µL 1 M HCl to neutralize the pH. The subsamples of each fraction were then pooled, freeze-dried, resuspended with 1 mL Milli-Q water and stored at 20˚C until further analyses.

· Screening for antibacterial effects

Unprocessed and processed litter extracts, and their respective fractions, were tested for antibacterial effects by the ^{3}H-labelled leucine (^{3}H-Leu) incorporation method (12, 13). This method measured protein synthesis as a proxy for bacterial growth. Bacterial slurries were obtained by shaking 3 g mineral soil or litter in 30 mL distilled water on a Multi Reax Vortex (Heidolph instruments, Schwabach, Germany) for 3 min at maximum intensity. Particles and fungal contaminants were excluded by centrifugation (10 min, 1000 *g*) and filtration of the

supernatant (MF-Millipore 1.2µm; Tullagreen, Carrigtwohill, County Cork, Ireland). The resulting bacterial suspensions from the same soil layer were pooled and 1.35 mL subsamples were used to measure bacterial growth. Toxicity tests were performed by adding 150 µl of the test solute to each bacterial subsample together with 20 µL ^{3}H- Leu solution (2 µL ^{3}H-labelled Leu (37 MBq mL^{-1} and 5.74 TBq mmol^{-1}; PerkinElmer, Boston, MA, USA) and 18 µL non-labelled Leu, resulting in 275 nM Leu in each bacterial suspension). Samples were briefly vortexed and incubated for 2 h in the dark at 21˚C. Bacterial growth was terminated by the addition of 75 µl 100% trichloroacetic acid (TCA), and the radioactive pellets were sequentially washed with 1.5 mL 5% TCA and 1.5 mL 80% ethanol. After the termination step and each washing step, samples were briefly vortexed, centrifuged (8 min, 13000 *g*) and the supernatant was carefully aspirated as to not disturb the radioactive pellets. Pellets were then resolubilized in 0.2 mL 1 M NaOH for 1 h at 90˚C. After the samples were cooled down to room temperature, 1 mL Ultima gold LSC scintillation cocktail (PerkinElmer, Boston, MA, USA) was added, and the amount of incorporated radioactive leucine was detected using a Tri-Carb 2910TR liquid scintillation analyser (PerkinElmer, Boston, MA, USA).

· Statistical analyses

MATLAB R2015b (MathWorks, Natick, MA, USA) was used for processing the spectra obtained via DRIFTS analysis. Spectra were pre-processed by performing an alternating least squares (ALS) baseline correction (λ=66000, p=0.001) of the spectral region between 950-1820 cm^{-1}. Data were then interpreted by multivariate curve resolution (MCR), followed by cluster analysis (14). R version 3.3.3 (R foundation for statistical computing, Vienna, Austria) was used for the remaining statistical analyses. Bacterial growth responses due to the addition of processed or unprocessed litter extracts were compared for statistically significant differences. Initial growth rate values were expressed as percentages related to the control samples by dividing the values with the average of the control samples and multiplying by 100. If the assumptions of normality and homoscedasticity were fulfilled, a one-way or two-way analysis of variance (one-way ANOVA) was performed followed by the Tukey`s range test. If the assumption of normality was met but the data appeared to be heteroscedastic, the Welch`s ANOVA test was used in combination with the Games-Howell post-hoc test. In case both assumptions could not be met, a Kruskal-Wallis test was used followed by a Pairwise Wilcoxon rank sum test.

· Bacterial growth rates are pH-sensitive

One of the most crucial factors affecting bacterial growth rates is the pH of the environment bacteria grow in (15, 16). Both bacterial slurries extracted from the mineral soil layer and litter layer had similar pH values, i.e. pH = 4.95 ± 0.07 and pH = 5.06 ± 0.06 respectively (pH ± S.D.). However, these values differed significantly ($p < 0.01$) from the pH of the litter extracts (FHE) (**TABLE 1**). Furthermore, *P. involutus* reduced the pH of the litter extracts even more ($p < 0.05$) after 7 days of growth. A comparison between the effect of unprocessed or *P. involutus*-processed FHE on bacterial growth rates can therefore be subjected to pH-induced differences. This depends on the sensitivity of the bacteria to changes in pH, and therefore bacterial growth rates across a pH gradient were investigated.

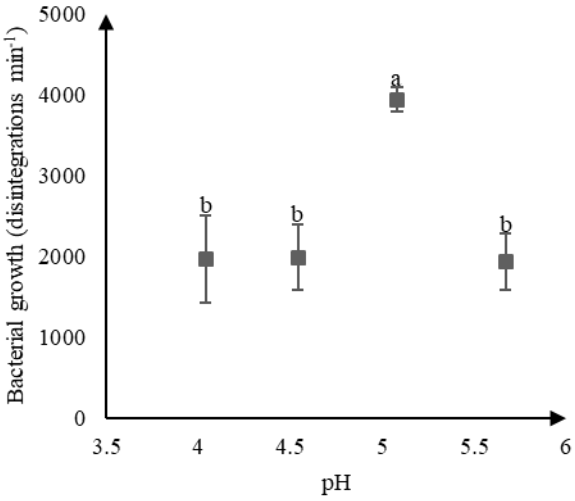

FIGURE 1 Bacterial growth rates across a pH gradient of bacteria extracted from the mineral soil layer (pH = 5.08). Bacterial growth rates are expressed as disintegrations of ^{3}H-Leu x min^{-1}. Error bars represent ± S.D. (n = 3). Statistically significant differences are annotated via different letters (Tukey`s range test ($p < 0.01$)).

Bacteria extracted from the mineral soil layer (pH = 4.95 ± 0.07; table 1) showed a significant decrease ($p < 0.01$) in growth rate when the pH was reduced to pH ≤ 4.54 or increased to pH = 5.67, down to approximately half the normal growth rate (**FIGURE 1**). Therefore, all solutions that were tested for antibacterial properties in the subsequent experiments were first adjusted to a pH of 5.

· *P. involutus* alleviates bacterial toxicity of forest litter extracts

To investigate whether *P. involutus* secretes antibacterial metabolites during litter degradation, soil bacteria were exposed to a dilution series of both processed and unprocessed FHE and bacterial growth rates were compared. Both bacteria extracted from the mineral soil layer and litter layer were tested to account for community-specific effects.

TABLE 1 pH values, total organic carbon (TOC) and total nitrogen (TN) content of the different soil layers and Forest Hot Extracts (FHEs), before (reference) and after 7 days of *P. involutus*-growth (processed). Values are expressed as means (n = 3 – 7) ± S.D.

Soil layer	**pH (H_2O)**	**TOC (%)**	**TN (%)**
Mineral layer	4.95 ± 0.07	3.60 ± 0.19*	0.14 ± 0.01*
Litter layer	5.06 ± 0.06	47.62 ± 0.73*	2.02 ± 0.05*
FHE batch	**pH (H_2O)**	**TOC (mgL^{-1})**	**TN (mgL^{-1})**
Batch 1, reference	$3.66 \pm 0.01^{a`}$	$1679.63 \pm 76.17^{a`}$	97.48 ± 3.49^{a}
Batch 1, processed	$3.21 \pm 0.14^{b`}$	$1365.63 \pm 83.62^{bc`}$	42.19 ± 7.76^{b}
Batch 2, reference	$3.74 \pm 0.01^{c`}$	$1461.00 \pm 18.36^{b`}$	92.10 ± 1.66^{a}
Batch 2, processed	$3.11 \pm 0.18^{b`}$	$1135.13 \pm 24.13^{c`}$	31.81 ± 2.50^{b}
Batch 3, reference	$3.66 \pm 0.01^{a`}$	$2109.75 \pm 157.27^{d`}$	125.60 ± 5.42^{c}
Batch 3, processed	$3.11 \pm 0.12^{b`}$	$1445.50 \pm 135.20^{abc`}$	60.28 ± 6.70^{d}

* Values within a column that are statistically different ($p < 0.01$, one-way ANOVA; n = 3)

a-d Values within a column that are statistically different ($p < 0.01$, Tukey`s range test; n = 3 – 4)

a`-d` Values within a column that are statistically different ($p < 0.05$, Games-Howell Test; n = 3 – 7)

Bacterial growth rates were found to be inhibited by litter extracts, and the intensity of this inhibition increased significantly (Wilcoxon, $p < 0.05$) as the amount of added extract increased (**FIGURE 2**). At the highest concentration of processed FHE, bacterial growth rates were reduced by 20% (**FIGURE 2A** for soil bacteria, **FIGURE 2B** for litter bacteria). However, when unprocessed FHE was added at the highest concentration, bacterial growth rates were reduced by 80%.

One possible explanation for the observed toxicity of FHE on soil bacteria can be found in the preparation of FHE, in which forest litter is boiled for 1 h to extract the organic matter. It has previously been shown that forest litter proved to be toxic for microbial organisms after being autoclaved (17), which is most likely due to an increase in aromaticity because of the heating process.

To investigate this hypothesis, new litter extracts were prepared in which the organic matter was not extracted by heating the samples but instead by stirring them at room temperature (FCE). After *P. involutus* had grown on it for 7 days, both unprocessed and processed FCE were tested for inhibitory effects on bacterial growth. Similar observations were obtained from the experiments with FCE (**FIGURE 2C, D**) as from those with FHE, indicating that litter extracts themselves are toxic for bacteria and the toxicity is not introduced by boiling the litter. Because both *P. involutus* was shown to grow better on FHE and FHE extracted more organic C than FCE (data not shown), FHE was used in the following experiments.

After *P. involutus* had grown on the litter extracts, the toxic effects of these extracts on bacteria seemed to be partially alleviated. However, it is possible that this observed reduction was only the result of an uptake of naturally occurring Leu from the medium by *P. involutus* as amino-acids were possibly co-extracted while making the litter extracts. Then the ratio of unlabelled Leu to ^{3}H-Leu would be much higher in unprocessed litter extracts, resulting in a technical artefact. Therefore, an experiment was set up in which bacterial growth rates were compared between unprocessed and processed FHE for 2 different concentrations of radiolabeled leucine. Because both bacteria extracted from the mineral soil layer and litter layer previously gave comparable results, only bacteria from the mineral soil layer were tested in the following experiments.

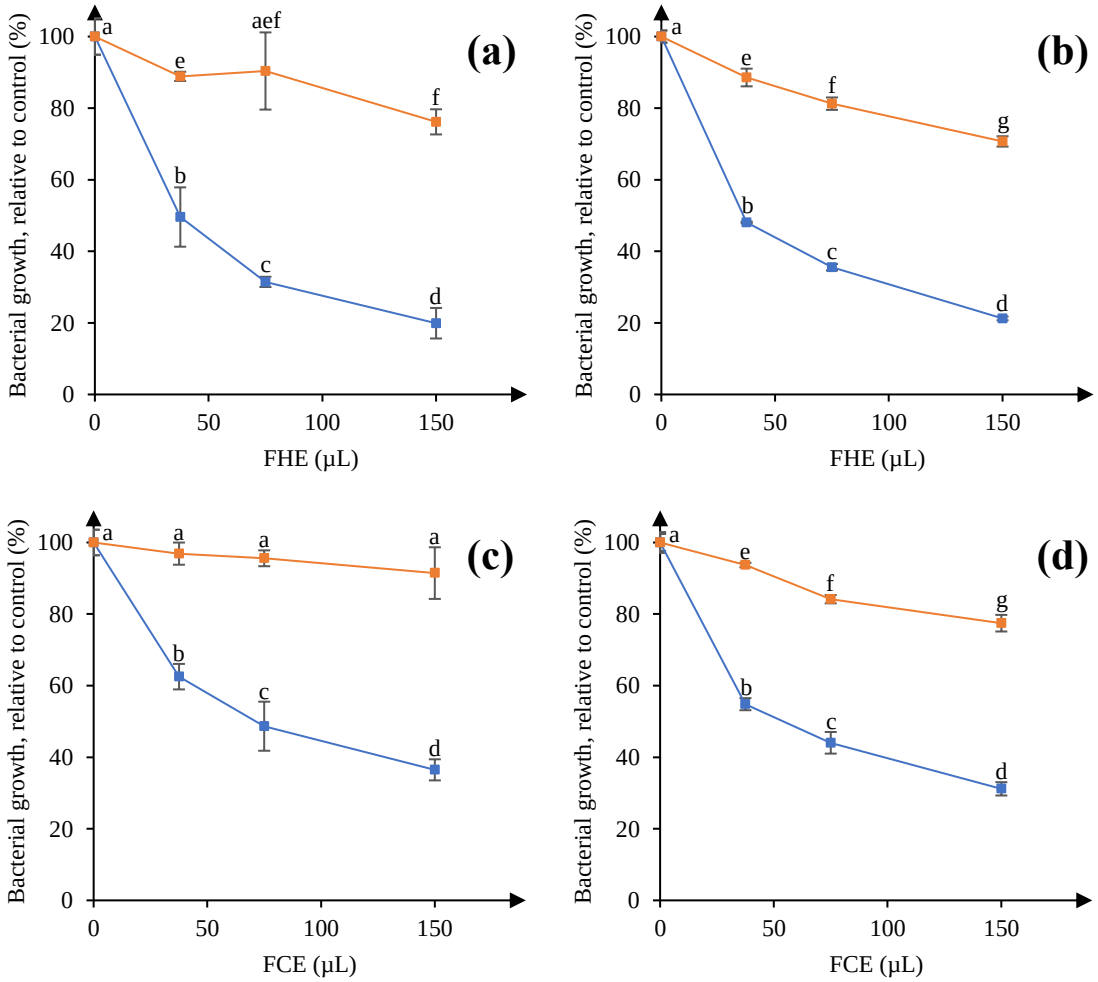

FIGURE 2 Bacterial growth rates after the addition of a dilution series of unprocessed (blue line) and *P. involutus*-processed (orange line) litter extracts. (a) The effect of FHE on bacterial growth extracted from the mineral soil layer; (b) the effect of FHE on bacteria from the litter layer; (c) the effect of FCE on bacteria from the mineral layer: (d) the effect of FCE on bacteria from the litter layer. Growth rates are expressed as relative values, compared to control samples. Data points represent averages of 4 replicates (n = 4). Error bars represent ± S.D.. Statistically significant differences in each treatment are indicated with different letters (Wilcoxon rank sum test ($p < 0.05$)).

Increasing the amount of radiolabeled leucine to 4x the normal concentration gave no significant difference in bacterial growth rates, both for unprocessed FHE and processed FHE (ANOVA, $p = 0.09$ and $p = 0.15$, respectively; **Figure 3**). These results reject the possibility that the observed effect was due to a technical error and confirms that *P. involutus* alleviates the induced bacterial toxicity by FHE.

When comparing the results in **Figure 2A** with **Figure 3**, different outcomes in growth responses are observed. The preparation of litter extracts involved the suspension of a certain amount of litter sample in water. Litter samples, however, are quite heterogeneous and different batches of FHE were found to vary strongly in the amount of extracted organic C and N (**Table 1**). The observed effect of FHE on bacterial growth rates is therefore not necessarily representative for the total litter layer. To investigate this, 3 different batches of FHE were tested for their effect on bacterial growth rates. Although bacterial growth rates were statistically differently affected by the different batches of FHE (ANOVA, $p < 0.01$; **Figure 4**), each batch reduced bacterial growth strongly and *P. involutus*-processing always alleviated this toxicity. However, to represent the effect of FHE on bacterial growth more accurately, bacterial growth rates were in

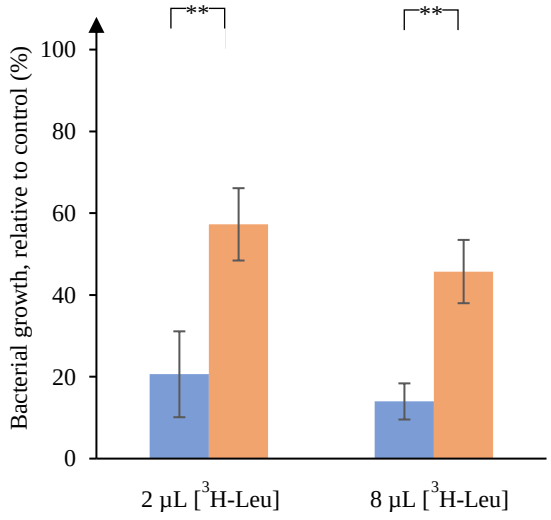

Figure 3 The effect of increasing the amount of radiolabeled leucine [^{3}H-Leu] on bacterial growth rates after the addition of unprocessed (blue bars) and *P. involutus*-processed (orange bars) FHE. No significant difference was found in bacterial growth rates after increasing the amount of ^{3}H-Leu to 4x the normal concentration ($p = 0.09$ for unprocessed FHE, $p = 0.15$ for processed FHE). Growth rates are expressed as relative values, compared to control samples. Bars represent averages of four replicates (n = 4). Error bars represent ± S.D.. ** $p < 0.01$ (ANOVA).

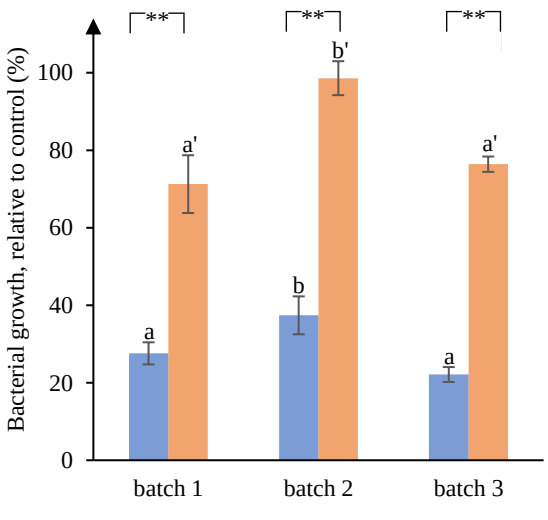

Figure 4 A comparison of bacterial growth rates after the addition of unprocessed (blue bars) and *P. involutus*-processed (orange bars) FHE for different batches of FHE. Growth rates are expressed as relative values, compared to control samples. Bars represent averages of four replicates (n = 4). Error bars represent ± S.D.. ** $p < 0.01$ (ANOVA). Statistically significant differences between different batches are indicated with different letters (Tukey`s range test ($p < 0.01$)).

the following experiments investigated for each FHE batch and represented as the average value thereof (**Figure 6A**).

The finding that *P. involutus* alleviates FHE toxicity on soil bacteria can be explained by either one of two mechanisms; either *P. involutus* indeed reduces or modifies the antibacterial compounds of FHE during litter degradation, or *P. involutus* secretes compounds that promote bacterial growth. The latter would suggest that the litter toxicity remains, but that the unaffected bacterial strains at the same time grow faster. To further characterize the toxic effect of FHE, we decided to 1) identify the changes in the chemical composition of FHE that occur during *P. involutus*-processing and 2) to investigate the chemical nature of these toxic molecules.

· *P. involutus* modifies FHE during 7 days of growth

To characterize *P. involutus*-induced changes in FHE, the chemical compositions of unprocessed and processed FHE were compared via DRIFTS. It was found that *P. involutus* strongly modified the chemical composition of FHE after 7 days of growth (**Figure 5**), mostly by altering the present carboxylates and aromatic structures. Both the spectral bands denoted to carboxylate C=O stretching (1720 cm^{-1}) and to C-O stretching of phenolic OH groups (1265 cm^{-1}) showed an increased intensity, while those of carboxylate C-O stretching and/or aromatic C=C stretching (1426 cm^{-1} and 1600-1650 cm^{-1}) were strongly reduced. This indicates that *P. involutus* oxidized the litter extract during degradation, on observation that also confirmed findings made in other studies (18, 19).

However, another explanation for the increase in the absorption band at 1720 cm^{-1} was given in a previously conducted study which found that this could be the result of acidification of the sample (20). Although *P. involutus*-processing of FHE resulted in a decrease of the pH, this explanation was disregarded because the pH values of both our unprocessed and processed FHE were acidic (pH < 3.74, **Table 1**).

Also, no changes in the polysaccharide fingerprint region (1030 – 1080 cm^{-1}) were observed after *P. involutus*-processing of the extracts, indicating that adding glucose in a patch on top of the mycelium instead of in the medium itself successfully excluded glucose from the medium. The observed changes in spectral bands can therefore be said to be the result of modifications of the litter extracts, without bias induced by the glucose itself.

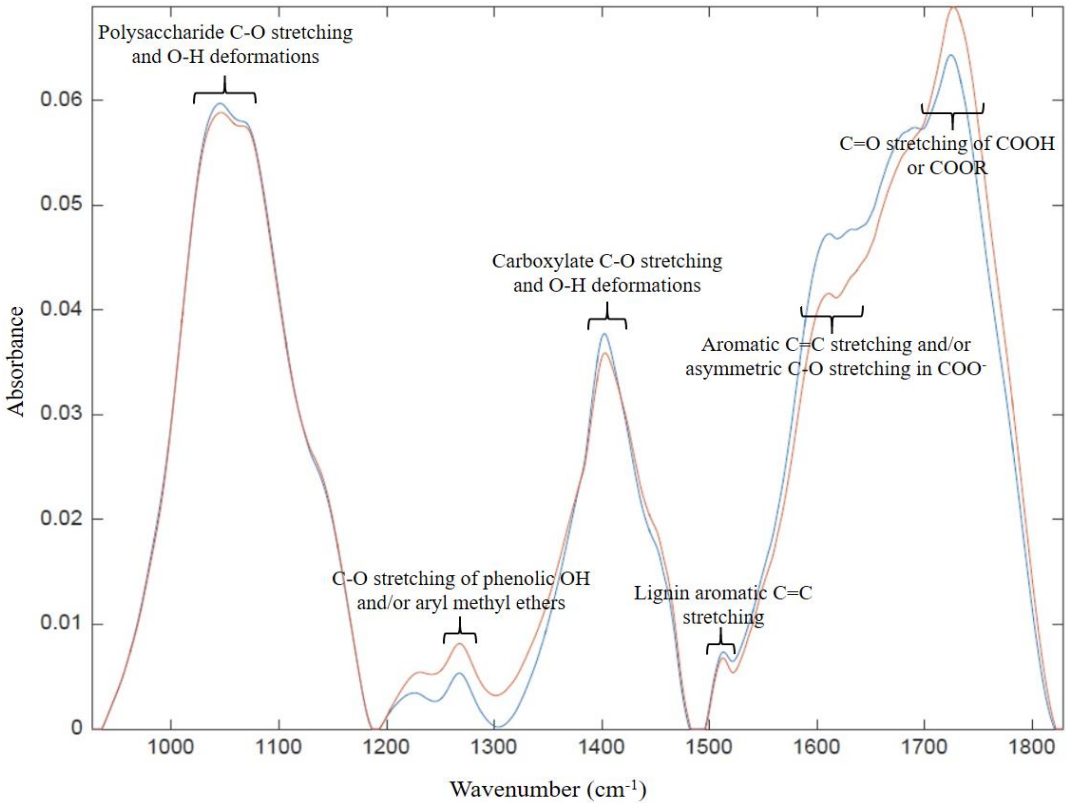

Figure 5 Chemical composition of Forest Hot Extract (FHE) before (blue line) and after (orange line) 7 days of incubation with *P. involutus*, as determined by DRIFTS analysis. Spectral peaks are assigned to their most likely origin according to (3). All spectra have been normalized to the same total area over the wavenumber region displayed (n = 3, unprocessed; n = 6, processed).

· Water-soluble components are responsible for antibacterial effect

To further characterize the molecules that were responsible for the observed forest litter toxicity, litter extracts were fractionated via 3 different methods and each fraction was tested for their effect on bacterial growth rates.

First, FHE was fractionated into LMWCs and HMWCs (3 kDa cut-off) to determine the size of the toxic compounds (**Figure 6b**). For each fraction, the effect on bacterial growth rates was similar to that of the total extract, i.e. a significantly stronger reduction in bacterial growth by the addition of unprocessed FHE when compared with processed FHE (Kruskal-Wallis, $p < 0.01$). This indicates that the toxic molecules cannot be separated based on their size. However, the separation of LMWCs from FHE did not appear to be successfully completed. Large retention volumes were still observed after the centrifugation step, most probably due to the saturation of the filter membranes. It is possible that small components were still present in the

HMWC fraction. Because the separation proved to be inconclusive, these results could not account for the observed effects.

Next, phenolic compounds were extracted from FHE because they are known to be capable of exhibiting toxicity to bacteria (21). The alleviation by *P. involutus* of phenolic-induced toxicity on bacteria could then be the result of the oxidation of these compounds during the incubation period (**Figure 5**). Phenolic compounds were indeed found to reduce bacterial growth rates (**Figure 6C**), but no difference was found between phenolic compounds from unprocessed or processed FHE (ANOVA, $p = 0.31$). However, the other fraction obtained during this procedure, which mostly contained small ionic molecules, also inhibited bacterial growth rates and differed between unprocessed and processed FHE (ANOVA, $p < 0.01$).

At last, the compounds in FHE were fractionated according to their polarity. While the apolar fraction did inhibit bacterial growth rates, the amount of reduction was not as high as observed originally and no significant difference was found between unprocessed and processed FHE (Kruskal-Wallis, $p = 0.30$). However, the polar fraction also reduced bacterial growth rates and was found to be statistically different for unprocessed and processed FHE (Kruskal-Wallis, $p < 0.01$; **Figure 6D**). Therefore, it can be concluded that the antibacterial effect of FHE is exerted by polar molecules.

The results of the fractionation experiments indicate that small, water-soluble molecules are responsible for the observed difference in bacterial toxicity between unprocessed and processed FHE.

As mentioned before, the observed difference in bacteria growth rates between unprocessed and processed FHE can be explained by two possible mechanisms: either *P. involutus* reduces the toxicity of FHE, or it secretes bacterial growth-promoting compounds. In the latter mechanism, the toxicity would not be alleviated but the growth of the unaffected bacterial strains would be increased. To test for possible growth-promoting effects, bacterial growth rates were measured after the addition of a dilution series of glucose (**Table 2**).

During the timescale of the incubation periode (2 h), bacterial growth rates were found not to be affected by the addition of glucose (ANOVA, $p = 0.18$), not even at the highest concentration which corresponded with an amount of 5 g glucose L^{-1}. This strongly indicates that the

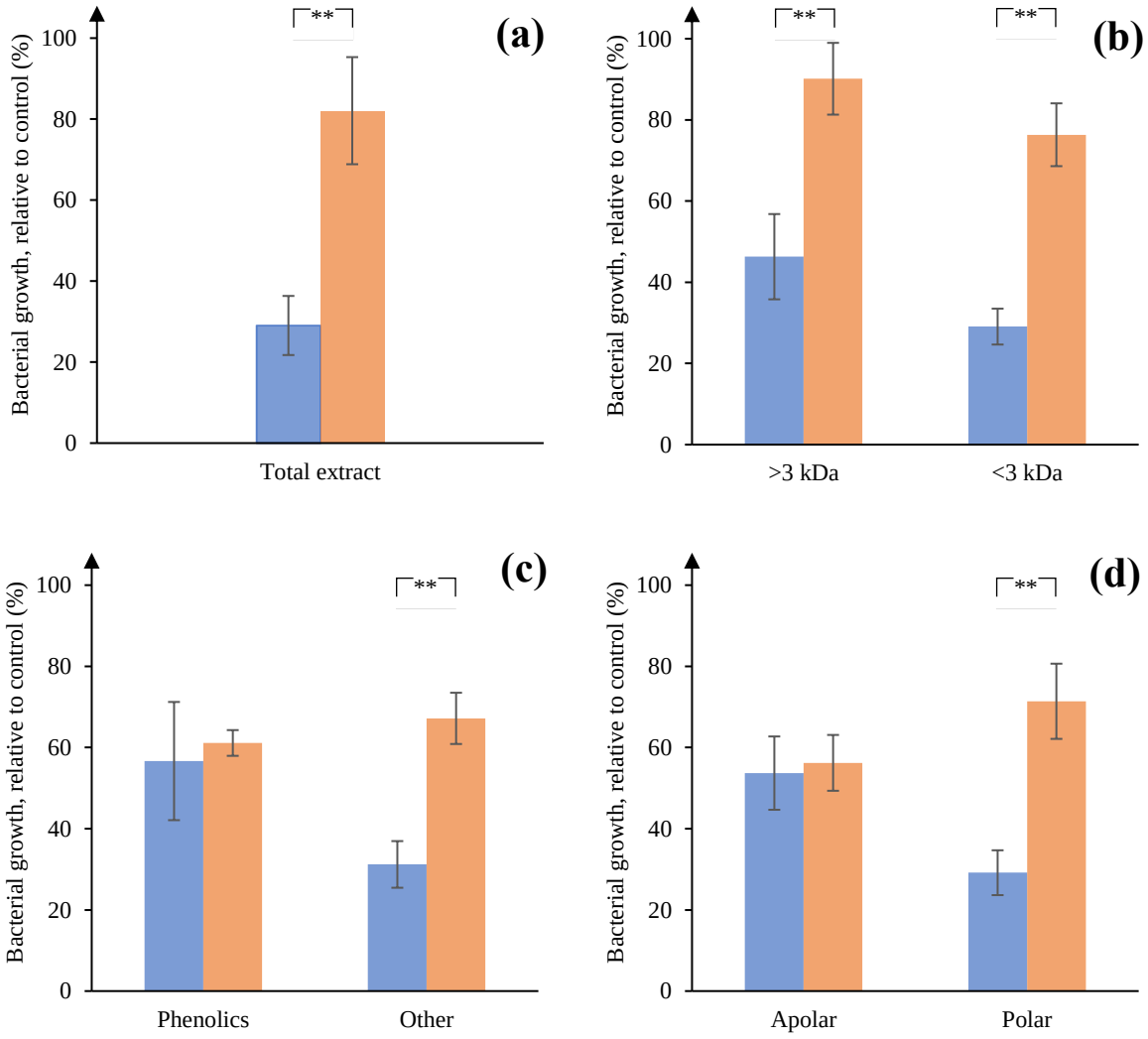

FIGURE 6 Bacterial growth rates after the addition of unprocessed (blue line) and *P. involutus*-processed (orange line) litter extracts and different fractions thereof. (a) The effect of the total fraction; (b) the effect of LMWC (<3 kDa) and HMWC (>3 kDa) as fractionated by ultrafiltration; (c) the effect of phenolics and the remaining part of FHE as fractionated by solid-phase extraction; (d) the effect of polar and apolar compounds as fractionated by ethyl acetate extraction. Growth rates are expressed as relative values, compared to control samples. Bars represent averages of twelve replicates (n = 12; 4 for each FHE batch). Error bars represent ± S.D.. ** $p < 0.01$ (ANOVA or Kruskal-Wallis).

alleviated FHE toxicity by *P. involutus* is not due to the secretion of growth-promoting compounds, but results from the neutralization of the toxic compounds present in FHE.

Next, the possibility of metal-induced toxicity was investigated. The litter that was used in the experiments contained significant amounts of aluminium and iron (data not shown) and both metals are known to exert antibacterial properties at certain concentrations (22, 23). However, the amount of aluminium and iron found in FHE varied significantly between the different batches, ranging both from 1 mgL^{-1} to 10 mgL^{-1}. Therefore, bacterial growth was investigated across a dilution series of each metal (**TABLE 2**), ranging from 10 mgL^{-1} to 0.1 mgL^{-1}. Even at

the highest concentrations, bacterial growth rates were not significantly affected by either one of the metals (ANOVA, $p = 0.26$), suggesting that these are not responsible for the observed toxicity of FHE.

The following possible explanation that was investigated was the presence of small carboxylic acids in FHE. Carboxylic acids are involved in many processes operating in soils and are known to be capable of inducing toxicity in a wide range of microbial organisms (24, 25). Should these compounds prove to be toxic, the alleviation of *P. involutus* could be explained by induced modifications to these molecules due to degradation or uptake (**FIGURE 5**). To test this hypothesis, two different carboxylic acids were tested, i.e. oxalic acid and citric acid. Both were added to the bacteria in a dilution series with similar concentration of carbon compared to the glucose experiment. Also, no statistical differences were found in bacterial growth rates after the addition of citric acid (ANOVA, $p = 0.23$), but oxalic acid reduced bacterial growth rates significantly at the highest concentration, i.e. 2 g carbon L^{-1}. However, this concentration was an overestimation of the actual concentration that is likely to be found in soils. Thus, these compounds were also concluded not to be responsible for the observed toxicity of FHE.

TABLE 2 The influence of diluted series of iron, aluminum, glucose, citric acid and oxalic acid on bacterial growth rates. Values are expressed as the average growth percentage (n = 3) ± S.D., relative to the control value.

	[Metal] (mgL^{-1})			
Metal	**0.1**	**0.5**	**5**	**10**
Aluminum	104 ± 4	102 ± 5	96 ± 3	92 ± 5
Iron	92 ± 3	95 ± 2	90 ± 4	101 ± 2
	[C] gL^{-1}			
Carbon source	**0.002**	**0.02**	**0.2**	**2**
Glucose	95 ± 2	111 ± 10	111 ± 16	96 ± 6
Citric acid	100 ± 12	96 ± 3	92 ± 10	87 ± 11
Oxalic acid	113 ± 6	115 ± 10	103 ± 8	70 ± 4*

* Values within a row that are statistically different from the control value ($p < 0.01$, ANOVA; n = 3)

Litter extracts made from forest litter reduce bacterial growth rates on by 80%. This effect is both observed for bacteria extracted from the mineral soil layer and from the litter layer itself. By fractionating these litter extracts, it was found that water-soluble molecules are responsible for this induced bacterial toxicity. Metals and carboxylic acids were found not to be the cause of this effect and the molecules responsible for the observed effects remain unknown. Further studies on the causative agents could be promising as their identification could result in the finding of a novel antibiotic. Size-exclusion chromatography followed by gas chromatography-mass spectrometry would be a viable method to explore further the characteristics of the molecules of interest.

Paxillus involutus is not found to induce antibacterial effects during the degradation of these litter extracts. In contrary, *P. involutus*-processing of litter extracts results in an alleviation of the induced bacterial toxicity to a merely 20% of the normal bacterial growth rates. This process is due to altering the toxic molecules, either by taken them up or modifying them. *P. involutus* has been found to oxidize aromatic and carboxyl compounds in the litter extracts, but due to the unknow character of the toxic molecules, it remains unknown if this was also the case for the toxic compounds. By investigating this further, these findings can contribute to gaining a better understanding of the complex interactions that EMF exert on soil bacteria. Once the toxic molecules are identified, possible *P. involutus*-induced modifications can be investigated by FTIR.

Two possible candidate groups of antibacterial compounds that can be found in soils are proposed. The first group consist of glycopeptides and their derivated aminosugars. Various members of this expanding group which are known to be of microbial origin exert antibacterial effects towards Gram-positive bacteria. Other candidates can possibly be found in the class of small polyketides. In soils, these secondary metabolites are produced in high quantities by microbial organisms and form a rich source of bioactive molecules.

REFERENCES

1. Hansen J, Sato M, Ruedy R, Lo K, Lea DW, Medina-Elizade M. Global temperature change. Proc Natl Acad Sci U S A. 2006;103(39):14288-93.
2. Knight A. Systematic reviews of animal experiments demonstrate poor human clinical and toxicological utility. ATLA-Altern Lab Anim. 2007;35(6):641-59.
3. Read DJ, Leake JR, Perez-Moreno J. Mycorrhizal fungi as drivers of ecosystem processes in heathland and boreal forest biomes. Can J Bot-Rev Can Bot. 2004;82(8):1243-63.
4. Gadgil PD, Gadgil RL. Suppression of litter decomposition by mycorrhizal roots of Pinus radiata: New Zealand Forest Service; 1975.
5. Gadgil RL, Gadgil PD. MYCORRHIZA AND LITTER DECOMPOSITION. Nature. 1971;233(5315):133-+.
6. Fernandez CW, Kennedy PG. Revisiting the 'Gadgil effect': do interguild fungal interactions control carbon cycling in forest soils? New Phytol. 2016;209(4):1382-94.
7. Baath E, Anderson TH. Comparison of soil fungal/bacterial ratios in a pH gradient using physiological and PLFA-based techniques. Soil Biol Biochem. 2003;35(7):955-63.
8. Olsson PA, Chalet M, Baath E, Finlay RD, Soderstrom B. Ectomycorrhizal mycelia reduce bacterial activity in a sandy soil. FEMS Microbiol Ecol. 1996;21(2):77-86.
9. Fries N. BASIDIOSPORE GERMINATION IN SOME MYCORRHIZA-FORMING HYMENOMYCETES. Transactions of the British Mycological Society. 1978;70(JUN):319-24.
10. Shah F, Rineau F, Canback B, Johansson T, Tunlid A. The molecular components of the extracellular protein-degradation pathways of the ectomycorrhizal fungus Paxillus involutus. New Phytol. 2013;200(3):875-87.
11. Artz RRE, Chapman SJ, Robertson AHJ, Potts JM, Laggoun-Defarge F, Gogo S, et al. FTIR spectroscopy can be used as a screening tool for organic matter quality in regenerating cutover peatlands. Soil Biol Biochem. 2008;40(2):515-27.
12. Baath E. MEASUREMENT OF PROTEIN-SYNTHESIS BY SOIL BACTERIAL ASSEMBLAGES WITH THE LEUCINE INCORPORATION TECHNIQUE. Biol Fertil Soils. 1994;17(2):147-53.
13. Baath E, Pettersson M, Soderberg KH. Adaptation of a rapid and economical microcentrifugation method to measure thymidine and leucine incorporation by soil bacteria. Soil Biol Biochem. 2001;33(11):1571-4.
14. Felten J, Hall H, Jaumot J, Tauler R, de Juan A, Gorzsas A. Vibrational spectroscopic image analysis of biological material using multivariate curve resolution-alternating least squares (MCR-ALS). Nat Protoc. 2015;10(2):217-40.
15. Wu YC, Zeng J, Zhu QH, Zhang ZF, Lin XG. pH is the primary determinant of the bacterial community structure in agricultural soils impacted by polycyclic aromatic hydrocarbon pollution. Sci Rep. 2017;7:7.
16. Baath E, Arnebrant K. GROWTH-RATE AND RESPONSE OF BACTERIAL COMMUNITIES TO PH IN LIMED AND ASH TREATED FOREST SOILS. Soil Biol Biochem. 1994;26(8):995-1001.
17. Laiho O. Paxillus involutus as a mycorrhizal symbiont of forest trees1970.
18. Rineau F, Roth D, Shah F, Smits M, Johansson T, Canback B, et al. The ectomycorrhizal fungus Paxillus involutus converts organic matter in plant litter using a trimmed brown-rot mechanism involving Fenton chemistry. Environ Microbiol. 2012;14(6):1477-87.
19. Rineau F, Shah F, Smits MM, Persson P, Johansson T, Carleer R, et al. Carbon availability triggers the decomposition of plant litter and assimilation of nitrogen by an ectomycorrhizal fungus. Isme J. 2013;7(10):2010-22.
20. Gondar D, Lopez R, Fiol S, Antelo JM, Arce F. Characterization and acid-base properties of fulvic and humic acids isolated from two horizons of an ombrotrophic peat bog. Geoderma. 2005;126(3-4):367-74.
21. Deanross D, Rahimi M. TOXICITY OF PHENOLIC-COMPOUNDS TO SEDIMENT BACTERIA. Bull Environ Contam Toxicol. 1995;55(2):245-50.
22. Wood M. A MECHANISM OF ALUMINUM TOXICITY TO SOIL BACTERIA AND POSSIBLE ECOLOGICAL IMPLICATIONS. Plant Soil. 1995;171(1):63-9.
23. Sorokina EV, Yudina TP, Bubnov IA, Danilov VS. Assessment of iron toxicity using a luminescent bacterial test with an Escherichia coli recombinant strain. Microbiology. 2013;82(4):439-44.
24. Rozycki H. PRODUCTION OF ORGANIC-ACIDS BY BACTERIA ISOLATED FROM SOIL, RHIZOSPHERE AND MYCORRHIZOSPHERE OF PINE (PINUS-SYLVESTRIS L). Acta Microbiologica Polonica. 1985;34(3-4):301-8.
25. Lehner A, Meimoun P, Errakhi R, Madiona K, Barakate M, Bouteau F. Toxic and signalling effects of oxalic acid: Oxalic acid—Natural born killer or natural born protector? Plant Signaling & Behavior. 2008;3(9):746-8.

Auteursrechtelijke overeenkomst

Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:
Paxillus involutus alleviates forest litter toxicity on bacterial growth

Richting: **master in de biomedische wetenschappen-milieu en gezondheid**
Jaar: **2017**

in alle mogelijke mediaformaten, - bestaande en in de toekomst te ontwikkelen - , aan de Universiteit Hasselt.

Niet tegenstaand deze toekenning van het auteursrecht aan de Universiteit Hasselt behoud ik als auteur het recht om de eindverhandeling, - in zijn geheel of gedeeltelijk -, vrij te reproduceren, (her)publiceren of distribueren zonder de toelating te moeten verkrijgen van de Universiteit Hasselt.

Ik bevestig dat de eindverhandeling mijn origineel werk is, en dat ik het recht heb om de rechten te verlenen die in deze overeenkomst worden beschreven. Ik verklaar tevens dat de eindverhandeling, naar mijn weten, het auteursrecht van anderen niet overtreedt.

Ik verklaar tevens dat ik voor het materiaal in de eindverhandeling dat beschermd wordt door het auteursrecht, de nodige toelatingen heb verkregen zodat ik deze ook aan de Universiteit Hasselt kan overdragen en dat dit duidelijk in de tekst en inhoud van de eindverhandeling werd genotificeerd.

Universiteit Hasselt zal mij als auteur(s) van de eindverhandeling identificeren en zal geen wijzigingen aanbrengen aan de eindverhandeling, uitgezonderd deze toegelaten door deze overeenkomst.

Voor akkoord,

Van Dijck, Thomas

Datum: **12/06/2017**