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Phenolic compounds extracted from spruce (*Picea abies*) by supercritical carbon dioxide as antimicrobial agents against gram-positive bacteria assessed by isothermal calorimetry

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Abstract
Antimicrobial agents are substances that, when present at low concentrations, can delay microbial growth. For many years, several powerful antimicrobial substances have been synthetized. However, because of their significant side effects, there is a growing interest nowadays to find natural alternatives to the synthetic one. The present study aims to recover extracts with antimicrobial activity from *Picea abies* residues using two different extraction technologies, respectively, Soxhlet and supercritical carbon dioxide. Their antimicrobial activity was tested on the growth of *Enterococcus faecalis* and *Streptococcus thermophilus.* Isothermal calorimetry was used as a technique to quantify the antimicrobial effect of the extracts. The heat flow curves, obtained during the microbial growth, were fitted by a modified Gompertz function obtaining the lag time (λ) and the maximum growth rate (μ_{max}) parameters. High-resolution mass spectrometry was used to identify the phenolic compounds responsible for the antimicrobial activity of the extracts. Regardless to the technology used, both extracts showed similar antimicrobial activity. For both microbial strains, the addition of the extract induced longer lag times (λ), while the maximum growth rate (μ_{max}) decreased. *S. thermophilus* showed a higher resistance compared to *E. faecalis* suggesting a different capacity to metabolize the substrate in the presence of *Picea abies* extracts. Catechin, dihydroquercetin, astringin and isorhapontin were the identified phenolic compounds responsible for such effect. In conclusion, the results of this study provided an exciting potential for the future in light of the shift away from artificial preservatives and the trend toward natural alternative compounds coming from sustainable sources.

Keywords *Picea abies* extracts · Supercritical fluid extraction · Solvent extraction · Antimicrobial activity · Soxhlet

Introduction

From ancient times, wood always played a central role in human life. Despite its use for applications like heat generation, building weapons or vehicles progressively declined during the course of human civilization, nowadays, wood still remains essential in a myriad of applications, such as buildings, furniture, papers, utensils, artworks, musical instruments and more. However, behind the use of wood, a great amount of wooden residues are generated. Often, such residues have very little economical value. Therefore, there is a growing interest to investigate new possible ways to recycle the residues into new materials.

One fascinating approach to exploit and recycle wood residues was based on the extraction of bioactive compounds [1, 2]. Barks and branches of many tree species were used from centuries as a source of flavors, fragrances or colorants. Recently, there has been a growing interest to study their potential antioxidant and antimicrobial properties [3–6]. As examples, wood extracts from chestnut and cherry were tested to control the microbial spoilage of wines [7], extracts from *Endopleura uchi* tree reported a high antimicrobial and cytotoxic activities [8], extracts from *Eucalyptus globulus* wood demonstrated high activity for inhibiting bacteria and yeast growth [9], and extracts from needles of *Abies alba* obtained by hydrodynamic cavitation showed an enhanced antioxidant activity [10].

Among the several tree species, the residues of Norway spruce (*Picea abies*) received great attention as an important

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source of antimicrobial compounds. This species was widely distributed in Europe. Norway spruce represented about 38% of trees in the European forests [11], and it was one of the dominant forest tree species in the Alps. Its industrial processing originated important quantities of residual materials. It was found that the bark of Norway spruce had a high content of extractives with a very good antioxidant activity from the crude polar fraction [12]. In addition, because of the high content of hemicellulose the production of oligomers with potential use in nutraceutical and pharmaceutical industries seemed to be a feasible option. In another study, it was demonstrated that Norway spruce bark extracts had a strong antimicrobial activity against human pathogens such as *Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [13]. Similarly, a significant antibacterial effect of *Picea abies* wood material against *Streptococcus pneumoniae* was also found [14].

Although the antimicrobial and antioxidant activity of *Picea abies* extracts was confirmed in several studies, what was not yet well understood is the effect of the extraction technology and the solvent used during the extraction on the resulting antimicrobial or antioxidant activity. For instance, the methanolic extract of *Thymus pectinatus* (*Lamiaceae*) did not show any antimicrobial activity. However, some antimicrobial activity was observed when the essential oil was obtained by Clevenger distillation [15]. Such differences could be linked to the solvent used for the extraction. Conversely, Salem reported that methanolic extract of *Picea abies* exhibited strong antibacterial and antioxidant activity [1].

Moreover, not only the solvent, but also the technology could affect the resulting functional properties of wood extracts. For instance, it was reported that pressurized methanol extraction from emblica (*Phyllanthus emblica* L.) plant had a superior antioxidant activity than supercritical fluid or conventional methanolic extraction [16]. However, this result was debated since the use of high temperatures was known to negatively affect the resulting antioxidant activity of the extract. Other works reported, instead, that supercritical fluid extraction, thanks to the mild temperatures and the addition of co-solvents, allowed obtaining essential oils with strong antimicrobial activity. For instance, extracts from Agarwood (*Aquilaria crassna*) exhibited antimicrobial activity against *S. aureus* and *C. albicans* [17]. Moreover, extracts from supercritical fluid obtained by cedarwood [18], *Pinus taeda* chips [19] and *Eucalyptus* wood industrial wastes exhibited all strong antioxidant activities [20]. Recently, the potential of supercritical fluid extracts obtained from *Picea abies* against the growth of *Escherichia coli* has also been shown [21]. However, no comparisons were carried out with other extracts obtained from an organic solvent extraction.

The studies published so far clearly evidenced the lack in knowledge and controversies regarding the effective antimicrobial activity of natural extracts obtained with different technologies such as those using organic polar solvents at high temperatures and those applying more eco-friendly approaches comprising the use of carbon dioxide as apolar solvent in supercritical state. The present manuscript aimed to fill this gap. In detail, the study focused on the antimicrobial effectiveness of *Picea abies* extracts obtained by two different technologies: Soxhlet extraction with ethanol and supercritical fluid extraction with carbon dioxide. The choice of these two types of extraction was based essentially on the polarity of the solvents. Ethanol–Soxhlet extraction generally led extracts composed of polar phenolic compounds. Instead, extracts obtained from carbon dioxide in its supercritical state provided essential oils rich in apolar components [22, 23]. These two extraction technologies will be applied on the same spruces of *Picea abies*, whose choice was mainly justified by its widespread use in building and manufacturing worldwide.

The antimicrobial activity of *Picea abies* extracts, obtained by supercritical fluid and ethanol–Soxhlet extraction, was then tested on two gram-positive microorganisms such as *Enterococcus faecalis* and *Streptococcus thermophilus*. These microbial strains were chosen as the first one is common in human body with high resistance to hot, salty and acidic environments, while the second one is present in several dairy products and, although nonpathogenic, its inactivation provides evidences on how a natural extract can act on a microorganism of a food-related matrix. The antimicrobial activity was monitored by isothermal calorimetry. HPLC-HRMS analysis of the extracts with and without their mixture with the microbial strains was also performed to identify the phenolic compounds responsible for the antimicrobial action.

Materials and methods

Spruce from *Picea abies*

Collection of Norway spruce (*Picea abies*) residues was carried out in the South Tyrol region (Italy). Upon arrival in the laboratory, the samples were ground to obtain a fine powder with 300–800 µm particle size (Mill-LM3100, Perten Instruments, Sweden). The final moisture content of the powder was equal to 7.8 ± 1.2, while the water activity was equal to 0.4.

Extraction from *Picea abies* by supercritical carbon dioxide

A high-pressure pilot plant (Superfluidi s.r.l., Padova, Italy) was used to perform the extraction with supercritical carbon dioxide from residues of *Picea abies*. The system comprised

an extractor (1 L volume of the vessel) and two gravimetric separators. Inside the extractor, a stainless steel vessel (800 mL volume), closed with porous stainless steel mesh filters on both ends, was loaded. A high-pressure diaphragm pump (Lewa LDC–M–9XXV1, Milano, Italy) was used to pump CO_2 inside the vessel. For the experiments, 80 ± 1 g of *Picea abies* powder was loaded in the high-pressure vessel. A first experimental plan was set up in order to define the conditions of pressure (from 10 to 30 MPa), temperature (from 35 to 50 °C) and time (10 to 180 min) to obtain the highest extraction yield. Ethanol as co-solvent with percentage equal to 10% (*w*/*w*) was added to increase the concentration of phenolic compounds in the final extracts. A low CO_2 flow rate of 2 L h^{-1} was selected in order to ensure higher contact times between the solvent and the sample. The highest yield, expressed as a ratio between the grams of extract and those of wood powder placed in the high-pressure vessel, was equal to 3.4 ± 0.5% (*w*/*w*) and was obtained at 45 °C, 20 MPa and 120 min.

Soxhlet extraction

Soxhlet extraction was performed with ethanol as solvent. About 150 mL of ethanol was recycled over 10 g of *Picea abies* powder in a Soxhlet apparatus for 6 h at the boiling temperature of the solvent. The yield was equal to 2.6 ± 0.7% (*w*/*w*).

Antimicrobial activity of extracts

Microbial growth conditions

Picea abies extracts were tested on two gram-positive microorganisms: *Enterococcus faecalis* (ATCC 29212) and *Streptococcus thermophilus* (ATCC 19258). The concentration of the microbial cultures ranged from 10^1 to 10^7 CFU mL^{-1}. The strains were stored in tryptone soy broth (TSB) at − 80 °C until needed. For experimental use, the stock cultures were maintained on tryptone soy agar (TSA) slants at 4 °C and transferred monthly.

Inhibition of microbial growth

The antimicrobial activity of *Picea abies* extracts was assessed on *E. faecalis* and *S. thermophilus* growth by isothermal calorimetry (Thermal Activity Monitor, Model 421 TAM III, TA Instruments). The methodology was the same applied in previous studies [21, 24, 25]. In these studies, the reliability of the methodology was assessed by the comparison with accepted standards endorsed by the National Committee for Clinical Laboratory Standards (NCCLS) such as plate count technique or disk diffusion test. To perform the experiment by isothermal calorimetry, a colony of each of the two strains was transferred to 10 mL of tryptic soy broth (TSB) and incubated at 37 and 40 °C for *E. faecalis* and *S. thermophilus*, respectively. The incubation time was set to 18 h to obtain fresh early-stationary-phase cells. The microbial suspensions with a final concentration of 10^8 CFU mL^{-1} (colony forming unit per mL) were serially diluted in TSB obtaining 10^5 CFU mL^{-1}. *Picea abies* extracts from supercritical fluid and Soxhlet extraction were also diluted in sterile TSB. Then, they were mixed with the microbial cultures reaching a final concentration equal to 1, 3 and 5 mg mL^{-1}. About 1 mL of the samples was transferred to 4-mL sterile stainless steel vials to start the analysis with isothermal calorimetry. The heat flow developed during the microbial growth was recorded every 10 s at 37 °C for *E. faecalis* and 40 °C for *S. thermophilus*. The experiments were performed in triplicate. From each calorimetric curve, the total heat (Q_{tot}), produced during the microbial growth, the maximum heat flow value (ϕ_{max}) and the time at which the maximum heat flow was recorded (t_p) were calculated and the results were expressed as mean values and standard deviations.

Thermodynamic parameters of microbial by isothermal calorimetry

The heat flow curves generated during the microbial growth were analyzed calculating the fractional reaction parameter α obtained from the integral of the heat flow curve. It was calculated using the following equation:

$$\alpha = \frac{q(t)}{Q_{tot}} \tag{1}$$

where $q(t)$ is the cumulative heat and Q_{tot} is the overall heat.

By plotting α calculated from Eq. (1) as a function of the time, a sigmoidal curve was obtained. The curve was fitted by an iterative least squares function using the following modified Gompertz function:

$$\alpha = \exp\left[-\exp\left(\mu_{max} \cdot e\right) \cdot (\lambda - t) + 1\right] \tag{2}$$

where λ is the lag time (h), μ_{max} is the maximum growth rate (h^{-1}) and e is the Euler's number.

Identification of phenolic compounds responsible for the antimicrobial activity of *Picea abies* extracts

The phenolic compounds of *Picea abies* extracts were identified by high-performance liquid chromatography–high-performance mass spectrometry (HPLC-HRMS). A Q Exactive Orbitrap HRMS instrument (Thermo Scientific, Milano, Italy) coupled to an UltiMate 300 UHPLC instrument was used. A flow rate equal to 0.2 mL min^{-1} with an Accucore RP-MS LC column (100 mm × 2.1 mm i.d., 2.6 µm) with a pre-column (Thermo Scientific, Milano, Italy) was used to

get the separation of the phenolic compounds. The following conditions were applied for full-MS analysis with the mass spectrometer operated in negative ionization mode: sheath gas equal to 20 (arbitrary units), aux gas equal to 5 (arbitrary units) and aux temperature of 250 °C. The spray voltage was set at ± 3.5 kV, the capillary temperature at 320 °C and RF S-lens at 65 °C. The mass ranges were selected from 100 to 1000 m z^{-1} with a full-MS set resolution of 70,000 at m z^{-1} 200, AGC target at 1×10^6 and max. injection time of 175 ms. The MS2 measurements of the selected ions were performed with a resolution of 17,500 and AGC target set at 5×10^5. The phenolic compounds were identified based on their retention time and UV absorbance at 280 nm. The retention times were compared with those of the external analytical standards. Correlation of chemical compounds' relative abundances and integration of the area under each peak were done using Compound Discoverer 2.1 software (Thermo Scientific, Milano, Italy).

To identify the phenolic compounds responsible for the inhibition activity, about 5 mg of supercritical carbon dioxide and Soxhlet extracts were added to 1 mL of TSB broth in the presence and absence of *E. faecalis* with a concentration of 10^6 CFU mL^{-1}. This microbial strain was chosen as it is the most sensitive to the addition of *Picea abies* extract. The samples were incubated at 37 °C for 24 h. A third sample was also prepared by adding 5 mg of supercritical carbon dioxide and Soxhlet extracts to 1 mL of TSB broth. The three samples were analyzed by HPLC-HRMS. For extraction of the phenolic compounds, 5 mL of MeOH/water (70:30) was added to 500 mg of the sample. The mixture was vortexed for 5 min, sonicated at room temperature for 15 min, and centrifuged at 10,000 rpm for 15 min. The supernatant was filtered through 0.2-µm syringe filters before analysis. Three independent extractions were performed for each sample. The analysis was performed in triplicate, and the results were expressed as mean values and standard deviations.

Statistical analysis

The analysis of variance (ANOVA) using IBM SPSS software was carried out to detect significant differences and main effects between the values of the overall heat (Q_{tot}), lag time (λ) and maximum growth rate (μ_{max}) obtained with different concentrations and type of *Picea abies* extract added to the microbial strains. The significant differences were analyzed by the Tukey test ($p < 0.01$). A comparison with previous published findings confirmed the goodness of the obtained results [21, 24, 25].

Results

Microbial growth by isothermal calorimetry

Figure 1A shows the isothermal calorimetric signals (thermal power vs. time) obtained at 37 °C during the microbial growth of *S. thermophilus*. The inoculum concentrations ranged from 10^1 to 10^7 CFU mL^{-1}. Figure 1B displays that the resulting cumulative heat (q) versus time (t) matched the modified Gompertz function:

$$q(t) = Q_{tot} \cdot \exp\left[-\exp\left(\mu_{max} \cdot e\right) \cdot (\lambda - t) + 1\right] \quad (3)$$

where Q_{tot} is the total heat produced during the whole growth process, calculated as the area swept by the heat flow curve; λ is the lag time, which is defined as the initial period between the starting of the experiments and the onset time of the exponential growth; finally, μ_{max} is the maximum growth rate, which is calculated as the highest slope observed along the heat flow curve. This corresponded to the maximum growth rate of the microbial strain. In addition, from the heat flow curve, it was also possible to measure the maximum heat flow value (ϕ_{max}) together with the time at which such value was recorded (t_p).

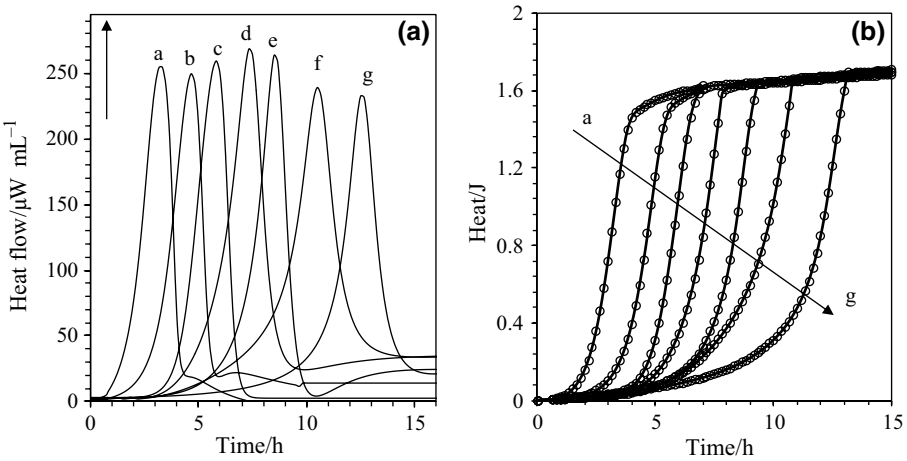

Fig. 1 **A** Heat flow traces of *S. thermophylus* at concentrations from (a) 10^7 to (g) 10^1 log(CFU mL^{-1}). **B** Heat versus time curves obtained by integrating the area delimited by the heat flow profile

These two parameters were also connected with the microbial growth and were used to identify the end of the exponential growth, i.e., when substrates or oxygen requirements became limiting. All the results are reported in Table 1.

Correlation analysis between isothermal calorimetry variables

Table 2 reports the Pearson correlation coefficients (r) between the variables reported in Table 1. This provides information about the relationships between variables. The strongest correlation was observed between the logarithm of the microbial load concentrations and either the lag time (λ) or the peak time (t_p), respectively, with $r=-0.995$, $n=7$, $p=0.01$ and $r=-0.990$, $n=7$, $p=0.01$. Higher microbial loads (from 10^1 to 10^7 CFU mL^{-1}) corresponded to shorter λ or t_p. In practice, within the observed range from 10^1 to 10^7 CFU mL^{-1} of *S. thermophilus*, the lag time significantly increased ($p<0.01$) from 2.3 ± 0.2 to 10.9 ± 0.6 h. Because of the substantial linearity of this correlation ($R^2=0.99$), a linear equation: $\lambda=-1.39\ C+11.11$ was used to determine the maximum inoculum concentration (C, expressed as log(CFU mL^{-1})) that could be detected. This resulted equal to 10^5 CFU mL^{-1} [24, 25]. Similar results were also obtained for the microbial growth of *E. faecalis*. Again, a linear trend was observed ($R^2=0.99$), with equation equal to $\lambda=-1.59\ C+19.57$. Other variables like the total heat (Q_{tot}), the maximum growth rate (μ_{max}, h^{-1}) and the maximum heat flow (ϕ_{max}, μW) values were nearly invariant to the inoculum concentration. For these reasons, the lag time was used as an index of microbial growth in all the next sections.

Table 2 Pearson correlation coefficients among initial microbial concentration and the kinetic and thermodynamic parameters

	Initial microbial concentration/ CFU mL^{-1}	μ_{max}/h^{-1}	λ/h	Q_{tot}/J	t_p/h
μ_{max}/h^{-1}	0.632	–			
λ/h	−0.995**	−0.625	–		
Q_{tot}/J	−0.439	−0.358	0.402	–	
t_p/h	−0.990**	−0.600	0.993**	0.497	–
ϕ_{max}/μW	0.488	0.237	−0.505	−0.740	−0.580

**Correlation is significant at 0.01 level (2-tailed)

Antimicrobial activity of *Picea abies* extracts

This section investigates the antimicrobial activity of wood extracts from *Picea abies*. Twelve experiments were designed in double to test the antimicrobial activity of ($n=12$) extracts obtained by Soxhlet extraction and by ($n=12$) supercritical fluid extraction. For each extraction technology, the lag time of the two microbial strains, respectively, *Streptococcus thermophilus* and *Enterococcus faecalis,* was tested by using three doses of the extracts. Figure 2A shows the average raw heat flow signals obtained exclusively for *S. thermophilus*, upon the addition of increasing concentrations of *Picea abies* extracts obtained by supercritical carbon dioxide extraction. Similarly, Fig. 2B shows the experiments obtained with extracts from Soxhlet extraction. In both cases, when the extract was present, the microbial growth was characterized by longer lag times (λ), shorter area (Q_{tot}) and lower maximum heat flow (ϕ_{max}) values. Also, the maximum growth rate (μ_{max}) decreased with higher

Table 1 Kinetic (μ_{max} and λ) and thermodynamic (Q_{tot}, t_p and ϕ_{max}) parameters obtained from the curves during the growth of *S. thermophilus* and *E. faecalis* ($n=3$)

Microorganism	Initial microbial concentration/ CFU mL^{-1}	$100*\mu_{max}$/h^{-1}	λ/h	Q_{tot}/J	t_p/h	ϕ_{max}/μW
S. thermophilus	10^7	6.7 ± 0.1^{ab}	2.3 ± 0.2^{g}	1.68 ± 0.31^{b}	4.0 ± 0.4^{e}	254 ± 12^{ab}
	10^6	6.3 ± 0.1^{bc}	3.5 ± 0.3^{f}	1.69 ± 0.24^{b}	4.7 ± 0.3^{de}	249 ± 19^{ab}
	10^5	6.5 ± 0.1^{ab}	4.8 ± 0.3^{e}	1.61 ± 0.43^{c}	5.8 ± 0.3^{d}	259 ± 11^{a}
	10^4	6.3 ± 0.1^{ab}	6.5 ± 0.4^{d}	1.60 ± 0.54^{c}	7.3 ± 0.3^{c}	268 ± 15^{ab}
	10^3	6.1 ± 0.1^{ab}	7.4 ± 0.3^{c}	1.62 ± 0.61^{b}	8.5 ± 0.4^{c}	263 ± 25^{ab}
	10^2	6.1 ± 0.1^{ab}	8.7 ± 0.4^{b}	1.61 ± 0.91^{a}	10.5 ± 0.4^{b}	239 ± 23^{ab}
	10^1	5.5 ± 0.1^{c}	10.9 ± 0.6^{a}	1.63 ± 0.72^{b}	12.3 ± 0.6^{a}	232 ± 28^{b}
E. faecalis	10^7	6.1 ± 0.1^{a}	3.1 ± 0.2^{f}	2.38 ± 0.31^{ab}	5.1 ± 0.2^{f}	239 ± 10^{a}
	10^6	6.3 ± 0.1^{a}	3.4 ± 0.2^{f}	2.38 ± 0.33^{ab}	5.3 ± 0.4^{f}	242 ± 12^{a}
	10^5	5.7 ± 0.2^{a}	4.5 ± 0.3^{e}	2.44 ± 0.22^{ab}	6.5 ± 0.3^{e}	245 ± 19^{a}
	10^4	5.5 ± 0.1^{b}	6.4 ± 0.3^{d}	2.76 ± 0.41^{b}	8.2 ± 0.2^{d}	229 ± 11^{a}
	10^3	5.1 ± 0.1^{b}	7.7 ± 0.4^{c}	2.29 ± 0.54^{ab}	9.7 ± 0.3^{c}	220 ± 15^{a}
	10^2	5.2 ± 0.1^{b}	10.7 ± 0.4^{b}	2.94 ± 0.62^{a}	11.5 ± 0.4^{b}	215 ± 25^{a}
	10^1	5.4 ± 0.1^{b}	12.5 ± 0.4^{a}	2.95 ± 0.89^{a}	13.2 ± 0.4^{a}	207 ± 23^{a}

Values are means of three replicates ± standard deviation. Values in the same column followed by the same superscript letters are not significantly different ($p<0.01$)

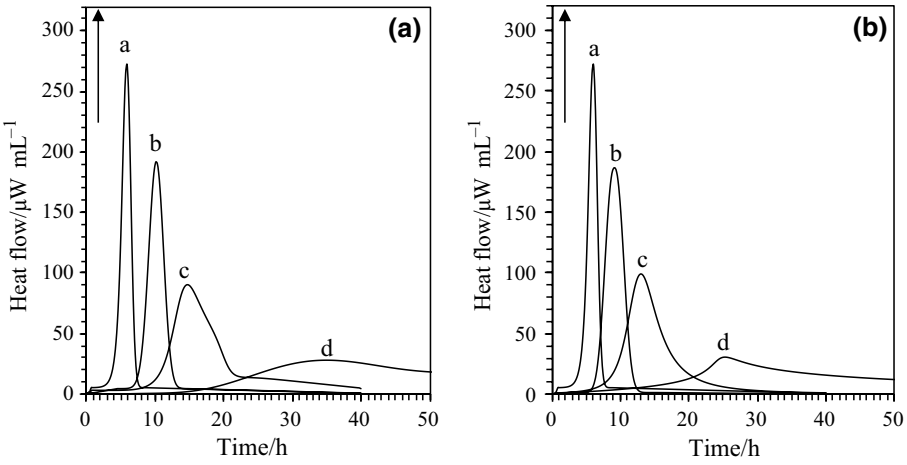

Fig. 2 Power time curves of (a) *S. thermophilus* growth (inoculum concentration equal to 10^5 CFU mL^{-1}) with (b) 1 (c) 3 and (d) 5 mg mL^{-1} of **A** SFE and **B** Soxhlet extract

dose of the extract. Despite all these changes, however, for the reasons explained in the previous section, the shifts on the lag time were selected as the primary index of the antimicrobial activity.

Three-way analysis of variance

With the results observed in Fig. 2, extended to both strains of *E. faecalis* and *S. thermophilus*, a three-way ANOVA was performed to examine the effect of: (1) extracts concentrations (1, 3 and 5 mg mL^{-1}), (2) type of extraction technology (supercritical carbon dioxide vs. Soxhlet) and (3) type of microorganism (*E. faecalis* vs. *S. thermophilus*). The dependent variable for this experimental design was the lag time, selected because of its strong negative correlation with the concentration of the microbial strains (see previous sections). As before, the isothermal calorimetric signal was recorded and fitted by a modified Gompertz function. The fitting parameters are reported in Table 3.

Considering the lag time as the dependent variable, the results of ANOVA in Table 4 show that the most important effect was given by the concentration of the extract, which accounted for more than 40% of the total variance (η^2). A further 16% of variance was also explained by the interaction between the extract concentration and the microbial strain. The presence of such interaction suggested that the *Enterococcus* strain was more sensitive to the addition of the *Picea abies* extract than the *Streptococcus* strain. In detail, at any concentration of the extract, the average lag time (in hours) observed for *Enterococcus* strain ($M = 16.9$, SD = 7.4)

Table 3 Kinetic (μ_{max} and λ) and thermodynamic (Q_{tot}, t_p and ϕ_{max}) parameters obtained from curves during the growth of *E. faecalis* and *S. thermophilus* in the presence of increasing extracts concentration (from 1 to 5 mg mL^{-1}) ($n = 3$)

Microorganism	Extraction technique	Extract concentration/mg mL^{-1}	$100*\mu_{max}$/h^{-1}	λ/h	Q_{tot}/J	t_p/h	ϕ_{max}/μW
S. thermophilus		–	6.7 ± 0.1	4.8 ± 0.3	2.2 ± 0.4	6 ± 0.3	259 ± 11
	Supercritical fluid	1	5.3 ± 0.1	7.9 ± 0.7	2.5 ± 0.2	10 ± 4	144 ± 2
		3	4.3 ± 0.2	8.9 ± 0.2	1.4 ± 0.4	15 ± 2	94 ± 8
		5	0.8 ± 0.2	12.4 ± 0.6	1.1 ± 0.3	35 ± 2	30 ± 16
	Soxhlet	1	5.1 ± 0.01	7.7 ± 2.0	2.1 ± 0.1	8 ± 1	189 ± 17
		3	3.6 ± 0.01	8.6 ± 0.5	1.8 ± 0.1	12 ± 3	105 ± 22
		5	2.8 ± 0.01	10.5 ± 0.8	1.7 ± 0.1	27 ± 1	24 ± 6
E. faecalis		–	6.0 ± 0.1	6.4 ± 0.3	2.8 ± 0.5	8 ± 1	229 ± 11
	Supercritical fluid	1	4.1 ± 0.1	11.2 ± 1.3	2.3 ± 0.3	12 ± 1	153 ± 14
		3	3.6 ± 0.1	18.2 ± 0.4	2.0 ± 0.1	24 ± 2	49 ± 1
		5	0.9 ± 0.4	27.7 ± 1.1	1.7 ± 0.5	37 ± 1	40 ± 13
	Soxhlet	1	4.0 ± 0.1	8.5 ± 0.1	2.4 ± 0.2	10 ± 1	149 ± 2
		3	1.5 ± 0.2	12.0 ± 0.9	2.1 ± 0.2	15 ± 2	42 ± 2
		5	0.5 ± 0.2	24.0 ± 0.9	1.6 ± 0.1	32 ± 2	32 ± 10

Values are means of three replicates ± standard deviation

Table 4 Results of ANOVA analysis performed on lag times (λ)

Source	SS	df	MS	F	p	η^2 (%)
A: *Stafylococcus* vs. *Enterococcus*	344.78	1	344.78	394.9	< 0.001	35
B: Supercritical fluid vs. Soxhlet	37.53	1	37.53	43.0	< 0.001	4
C: Extract concentration	403.16	2	201.58	230.9	< 0.001	41
AB:	17.00	1	17.00	19.5	< 0.001	2
AC:	156.75	2	78.37	89.8	< 0.001	16
BC:	3.64	2	1.82	2.1	< 0.167	0.4
ABC:	4.57	2	2.28	2.6	< 0.114	0.5
Error	10.48	12	0.87			1
Total	977.90	23				

Letters in the table indicated: *SS* sum of square, *df* degree of freedom, *MS* mean square value, *F* static value from ANOVA analysis, *p* probability, η^2 variance

was significantly higher than the one from *Streptococcus* ($M = 9.4$, SD = 1.9). This comparison led to a large value of the Student parameter t (22) = 3.46 ($p = 0.002$). This meant in practice that the extract of *Picea abies* showed a remarkably higher antimicrobial activity toward *Enterococcus* than *Streptococcus* strain. Such superior effect was even more evident at higher doses of the extract. Figure 3A shows that the effect of the dose used was maximized with *Enterococcus faecalis*. Such strong interaction between strain and dose was confirmed with the ANOVA results (Table 3), showing a $F(2,12) = 89.8$, $p < 0.001$.

The extraction technology also played a significant role ($F(1,12) = 43$, $p < 0.001$), although its effect was small ($\eta^2 = 4\%$). Such effect is displayed in Fig. 3B. In this plot, the extracts obtained by supercritical fluid extraction exerted a remarkable higher effect only when used with *Enterococcus* strain. Apparently, extracts from supercritical fluids had some compounds, which were more effective toward *Enterococcus* than *Streptococcus*. This hypothesis was confirmed by the significance of the interaction between the microbial strains and the extraction technology ($F(1,12) = 19.5$, $p < 0.001$, $\eta^2 = 2\%$).

Finally, Fig. 3C does not provide any evidence that the interaction between the extraction technology and the concentration was significant. For both extraction technologies, the lag time increased with the concentration of the extract used. The ANOVA confirmed the nonsignificant interaction. It was possible to conclude that, regardless to the technology used, both extracts succeeded similarly to inhibit the growth of microbial strains.

Antimicrobial compounds in *Picea abies* extracts

This section explores some of the main polyphenolic compounds responsible for the antimicrobial activity of *Picea abies* extracts. Among the two microbial strains, *E. faecalis* (10^6 CFU mL^{-1}) was chosen as test microorganism. This choice was based on the results reported in the previous section showing that *E. faecalis* was more sensitive to the addition of *Picea abies* extract than *S. thermophilus*. The

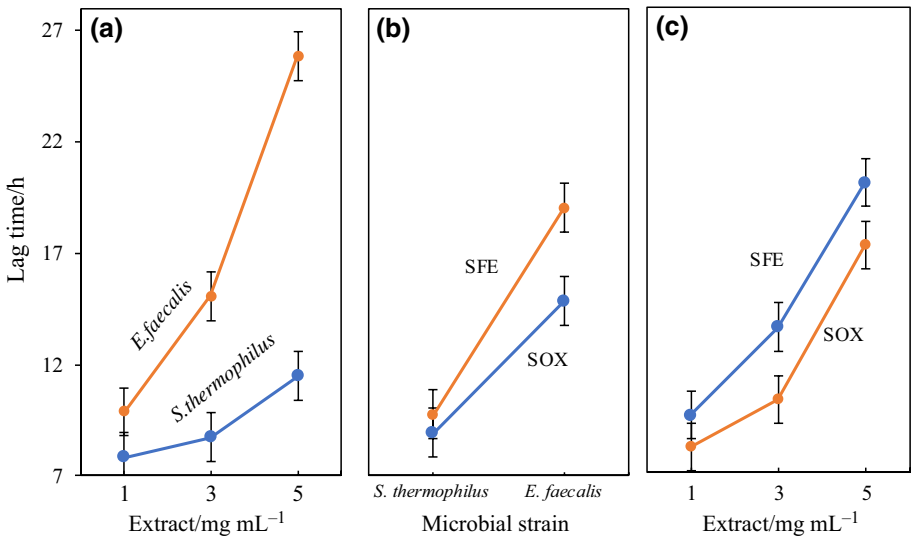

Fig. 3 **A** Effect of extract concentration on the lag time of *E. faecalis* (orange line) and *S. thermophilus* (blue line); **B** effect on the lag time of the two microbial strains to which *Picea abies* extracts from SFE (orange line) and Soxhlet (blue line) were added; **C** effect on the lag time of extract concentrations obtained by SFE (blue line) and Soxhlet (orange line) technology. (Color figure online)

Table 5 Antioxidant compounds detected by HPLC-HRMS in TSB inoculated with 10^6 CFU mL^{-1} of *E. faecalis* to which 5 mg mL^{-1} of *Picea abies* extracts were added

Compound	Formula	$[M–H]^-$ theoretical	$[M–H]^-$ measured	Δmi/Da	Δppm	RT in ESI/–	Supercritical fluid extraction			Soxhlet extraction		
							Without microbial strain $Area_1$ 10^6	With microbial strain $Area_1$ 10^6	Inhibition/%	Without microbial strain $Area_2$ 10^6	With microbial strain $Area_2$ 10^6	Inhibition/%
2-Methylbenzoic acid	$C_8H_8O_2$	135.0452	135.045	−0.0002	−1.1	10.7	61.8 ± 0.8	55 ± 1	11 ± 1.3	50.1 ± 0.5	48.3 ± 0.6	3.6 ± 0.5
Cinnamic acid	$C_9H_8O_2$	147.0451	147.0452	0.0001	0.7	14.9	2.03 ± 0.1	0.68 ± 0.06	66.5 ± 3.4	1.5 ± 0.4	1.1 ± 0.3	26.7 ± 3.2
Protocatechuic acid	$C_7H_6O_4$	153.0193	153.0192	−0.00001	−0.1	3.44	1.5 ± 0.1	0.5 ± 0.1	66.6 ± 3.1	1.1 ± 0.2	0.8 ± 0.01	27.3 ± 2.2
p-Coumaric acid	$C_9H_8O_3$	163.0401	163.0401	−0.00002	−0.1	11.7	1.6 ± 0.1	1.5 ± 0.04	6.3 ± 0.5	1.3 ± 0.4	1.2 ± 0.2	7.7 ± 1.1
Gallic acid	$C_7H_6O_5$	169.0142	169.0143	0.0001	0.6	1.96	4.1 ± 0.3	1.9 ± 0.6	53.7 ± 3.2	3.1 ± 0.5	2.5 ± 0.4	19.4 ± 1.2
Ferulic acid	$C_{10}H_{10}O_4$	193.0506	193.0507	0.0001	0.4	13.6	0.60 ± 0.13	0.51 ± 0.09	15 ± 2.3	0.55 ± 0.12	0.51 ± 0.01	7.3 ± 0.8
(+)-Catechin	$C_{15}H_{14}O_6$	289.0718	289.072	0.0002	0.7	6.7	1.6 ± 0.3	0.09 ± 0.05	94.4 ± 8.3	0.84 ± 0.01	0.33 ± 0.03	58.8 ± 4.1
Dihydroquercetin (Taxifolin)	$C_{15}H_{12}O_7$	303.0513	303.0513	0.0000	0.0	14.74	59 ± 2	19 ± 2	67.8 ± 3.5	42 ± 3	35 ± 3	16.7 ± 1.4
(+)-6-hydroxy-pinoresinol	$C_{20}H_{22}O_7$	373.1293	373.1293	0.0000	0.0	18.4	312 ± 23	265 ± 5	15.1 ± 1.8	201 ± 12	178 ± 6	11.4 ± 1.1
Astringin	$C_{20}H_{22}O_2$	405.1191	405.1196	0.0005	1.2	11.16	16 ± 1	1.25 ± 0.9	92.2 ± 5.4	10 ± 2	2.7 ± 0.7	73.1 ± 1.5
Isorhapontin	$C_{21}H_{24}O_9$	419.1348	419.1349	0.0001	0.2	18.4	432 ± 56	312 ± 23	27.8 ± 3.2	323 ± 15	260 ± 12	19.8 ± 2.1

Measurements were performed with and without the microbial strain after 24 h at 37 °C ($n = 3$). Results for supercritical fluid extract are indicated as A_1 and Soxhlet as A_2

microbial culture was left at 37 °C for 24 h with the addition of extracts obtained from supercritical fluid and Soxhlet extraction (5 mg mL^{-1}). Before and after incubation, the main phenolic compounds of the extracts were analyzed by HPLC-HRMS. All the results are reported in Table 4.

Before incubation, the most representative phenolic compounds (i.e., highest area under the peaks of the chromatogram) present in the extracts were: 2-methylbenzoic acid, gallic acid, catechin, dihydroquercetin, hydroxypinoresinol and isorhapontin. Such compounds were present in both extracts, either obtained by Soxhlet or obtained by supercritical carbon dioxide technology. A control experiment was performed by incubating the extracts in the medium at 37 °C for 24 h without the presence of the microorganism. The results did not show any significant change in these phenolic compounds.

After incubation, significant changes ($p < 0.05$) were observed on catechin, dihidroquercetin, astringin and isorhapontin. The area under the peaks of these phenolic compounds significantly decreased after the incubation of the microbial strain with *Picea abies* extracts. The consumption of these compounds was likely attributable to their antimicrobial effect. Both extracts showed a consistent inhibition effect as indicated by the values reported in Table 5. For some phenolic compounds (i.e., cinnamic acid, protocatechuic acid and gallic acid), the inhibition, calculated as decreased area under the peak, was higher for the extracts obtained by supercritical carbon dioxide.

Discussion

In this study, the antimicrobial activity of extracts from residues of *Picea abies* obtained by supercritical fluid and Soxhlet extraction was compared. Both extracts were able to inhibit similarly the microbial growth. For both microbial strains, when the extract was present, the growth was characterized by longer lag times (λ), lower heat developed during the growth (Q_{tot}) and consequently lower heat flow values (ϕ_{max}). Also, the maximum growth rate (μ_{max}) decreased with higher dose of the extracts. The microbial strain of *S. thermophilus* showed a higher resistance to *Picea abies* extracts compared to *E. faecalis.* This effect could be related to the capacity of *S. thermophilus* to form small flocks during the growth [26]. The microbial flocks reported higher resistance to the antimicrobial agent addition likely because, in this form, the colonies resulted are less exposed to the antimicrobial extract. Instead, *E. faecalis* developed short chains, which, apparently, left a larger number of cells in direct contact with the antimicrobial agent [27, 28]. The antimicrobial activity of *Picea abies* extracts against gram-positive and gram-negative bacteria and fungi has been already demonstrated in some studies [1, 29–31]. They showed the unambiguously antibacterial activity of *Picea abies* extracts obtained by hydrodistillation and solvent extraction and recognized some compounds such as quercetin, kaempferol and myricetin as responsible for the antimicrobial activity. A comprehensive study was also addressed assessing the antimicrobial effects of wood-associated polyphenols on food pathogens and spoilage organisms [32]. Polyphenolic compounds such as pinosylvin, pinosylvin monomethyl ether, astringin, piceatannol, isorhapontin, and isorhapontigenin reported high antimicrobial activity against gram-negative (*Salmonella*) and gram-positive bacteria (*Listeria monocytogenes*, *Staphylococcus epidermidis*, *Staphylococcus aureus*) and yeasts (*Candida tropicalis*, *Saccharomyces cerevisiae*). Similarly, in a recent review phenolics and extracted phenolic compounds from Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) were classified based on their antibacterial activity against several microorganisms [33]. It was reported that in Norway spruce (*P. abies*), the major flavonoids present were quercetin, dihidroquercetin and myricetin, which also reported a high antimicrobial activity against different bacteria.

In this study, the inhibition of *Picea abies* extracts was associated with some phenolic compounds, i.e., catechin, dihidroquercetin, astringin and isorhapontin, which were detected in lower concentration after the incubation with the microbial cultures. The majority of phenolic compounds in plants are synthesized via the phenylpropanoid pathway. They belong to a group of phenylalanine derivatives, which have a basic C6–C3 carbon skeleton, and are antimicrobial agents naturally synthesized in the wood to respond to microorganisms attacks [33]. This clearly explains their efficient antimicrobial action if properly extracted from wood. Although toxicity information on such phenolic compounds is still scarce, several studies showed their potential as antioxidant and antimicrobial substances. As an example, among these compounds, catechin, a pharmacologically active phenols, was found to inhibit intestinal tumor formation in mice [34], to retard the oxidation of low-density lipoprotein [35] and to enhance the antifungal effect of amphotericin B against *Candida albicans* [36]. Similar findings have been also published testing phenolic compounds such as astringin and isorhapontin as antioxidant and antimicrobial natural substances [33]. They all supported the results of the present study confirming the efficacy of extracts containing such compounds.

Conclusions

In this study, the antimicrobial activity of extracts from residues of *Picea abies* obtained by supercritical fluid and Soxhlet extraction was compared. Both extracts were able to inhibit similarly the microbial growth. For both microbial

strains, when the extract was present, the growth was characterized by longer lag times (λ), lower heat developed during the growth (Q_{tot}) and consequently lower heat flow values (ϕ_{max}). Also, the maximum growth rate values (μ_{max}) decreased with higher dose of the extract. Catechin, dihidroquercetin, astringin and isorhapontin were some of the detected phenolic compounds responsible for such antimicrobial activity. The results of the present study represented the first attempt to test extracts from *Picea abies* residues, obtained by a green extraction technology such as supercritical carbon dioxide, with the capability to inhibit microorganisms' growth.

On a more general perspective, the study provided an exciting potential for the future, especially in light of the shift away from artificial preservatives and the application of natural compounds coming from sustainable sources.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest in relation to this work.

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