Abstract: Saprotrophic fungi, key players in global carbon cycling, have been identified as methane (CH$_4$) sources not yet accounted for in the global CH$_4$ budget. This study, for the first time, explores the influence of oxygen (O$_2$) and temperature on CH$_4$ production by two fungi, *Lactiporus sulphureus* and *Pleurotus sapidus*. To explore the relationship between these parameters and fungal CH$_4$ formation, we examined CH$_4$ formation under varying O$_2$ levels (0 to 98%) and temperatures (17, 27, and 40 $^\circ$C) during fungal growth on pine wood, beech wood, and grass under sterile conditions. Our findings show that fungal CH$_4$ formation strongly depends on O$_2$ levels. Methane formation was highest when O$_2$ levels exceeded 5%, whilst no CH$_4$ formation was observed after complete O$_2$ consumption. Reintroducing O$_2$ immediately resumed fungal CH$_4$ production. Methane formation normalized to O$_2$ consumption (CH$_4$$_{\text{norm}}$) showed a different pattern. *L. sulphureus* showed higher CH$_4$$_{\text{norm}}$ rates with higher O$_2$ levels, whereas *P. sapidus* showed elevated rates between 0 and 5%. Temperature also significantly influenced CH$_4$ and CH$_4$$_{\text{norm}}$ rates, with the highest production at 27 $^\circ$C, and comparatively lower rates at 17 and 40 $^\circ$C. These findings demonstrate the importance of O$_2$ levels and temperature in fungal CH$_4$ emissions, which are essential for refining CH$_4$ source predictions.

Keywords: aerobic methane formation; fungi; oxygen-dependency; temperature-dependency; wood decay

1. Introduction

Methane (CH$_4$) is a potent climate gas, with a greenhouse gas (GHG) potential approximately 100 times greater than carbon dioxide (CO$_2$) over a 10-year period [1]. A significant portion of global CH$_4$ emissions originates from biotic sources, exceeding contributions from abiotic sources like fossil fuel and biomass burning as well as geogenic processes [2,3].

Contrary to the earlier assumption that biotic CH$_4$ production occurs exclusively under anaerobic conditions by methanogenic archaea in certain environments, such as wetlands, landfills, and rice paddies, and in the digestion system of termites and ruminants, recent research has revealed that biotic CH$_4$ can also be produced in the presence of oxygen (O$_2$). The first evidence of aerobic CH$_4$ formation by plants was presented by Keppler et al. [4], and subsequent research expanded this finding to a range of eukaryotic CH$_4$ and prokaryotic sources, including mosses and lichens [5], marine algae [6,7], terrestrial and marine cyanobacteria [8], plant cell cultures [9,10], non-methanogenic archaea [10], animals [11,12], human cell cultures, and humans [10,13–16], as well as fungi [10,17,18].

For some years, the mechanisms behind CH$_4$ formation in these organisms remained elusive. However, a recent breakthrough came with the discovery by Ernst et al. [10] of a universal non-enzymatic CH$_4$ formation mechanism potentially occurring in all organisms that produce reactive oxygen species (ROS). This mechanism, based on Fenton chemistry,
involves the reaction of ROS with free iron (II) ions and methylated precursor compounds within cells, encompassing all three domains of life.

Despite these advancements, little is known about the detailed mechanism and the physical and chemical factors that drive CH$_4$ formation in many newly discovered sources and organisms. This is especially true for (saprotrophic) fungi that play an essential role in decomposing organic matter, such as wood lignocellulose, thus playing a crucial role in global carbon recycling [19]. The challenge lies in determining fungal biomass and correlating it with CH$_4$ emissions. This is further complicated by the species and medium dependency of these emissions (e.g., [18]), as well as the potential for yet unknown CH$_4$ formation pathways, which complicates our understanding of the global impact of fungal CH$_4$ emissions.

Before the studies by Lenhart et al. [17] and Schroll et al. [18], it was assumed that fungi initiated the decomposition process by breaking down macromolecules, like those in wood, thereby providing the precursor compounds for CH$_4$ production through methanogenic archaea in anoxic microsites (e.g., [20–23]). Another CH$_4$-producing pathway involving facultative anaerobic fungi and a halomethane-dependent pathway was identified by Huang et al. [24], where CH$_4$ formation correlated with the formation of chloromethane (CH$_3$Cl), as previously reported by McNally et al. [25]. These authors further highlighted that the function of the enzymes involved in the halomethane-dependent CH$_4$ formation pathway is independent of O$_2$ and thus might also be involved in the observed fungal CH$_4$ production by Lenhart et al. [17] and Schroll et al. [18].

A common consensus is that fungal CH$_4$ emissions are strongly dependent on the fungal species and the wood substrates [17,18,26–28]. However, the influence of key parameters like O$_2$ availability and temperature on fungal CH$_4$ formation has not been investigated, even though these factors strongly influence the physiological activity and growth of fungi. Studies have shown that the activity and growth of xylotrophic fungi depend on prevailing O$_2$ mixing ratios [29–31]. For instance, xylotrophic fungi, which include the two investigated fungi in this study, can consume all available O$_2$ in their woody habitat and still grow under anoxic conditions [31]. On the other hand, studies have indicated that below a concentration of 0.2% O$_2$, fungal growth is completely inhibited [29,30], while the decay of wood debris by saprotrophic fungi decreased with decreasing O$_2$ and increasing CO$_2$ mixing ratios and vice versa [29,32]. This suggests that prevailing O$_2$ concentrations significantly influence fungal activity and metabolism, potentially controlling fungal CH$_4$ emissions.

Temperature is another critical driver of fungal metabolism. Numerous studies have investigated the relationship between wood decay and temperature [27,28,33–35], finding that increased temperatures usually lead to higher CH$_4$ emissions due to wood decay. This observation is likely linked to the role of saprotrophic fungi as significant producers of extracellular enzymes needed for wood decomposition, which is predicted to increase due to higher temperatures [33]. Fungal growth even quadrupled with a 10 °C increase in temperature across a tropical elevation gradient [34], indicating a substantial impact of temperature on the amount of prevailing fungal biomass and, consequently, on CH$_4$ emissions from xylotrophic fungi.

In our study, we investigated the effects of different O$_2$ levels and temperatures on fungal CH$_4$ production by two saprotrophic fungi, *Pleurotus sapidus* and *Laetiporus sulphureus*. Both fungal species were grown on various substrates, including beech wood, pine wood, and grass, and incubated under sterile conditions. Oxygen consumption rates were measured under different temperatures and patterns of CH$_4$ production, and CH$_4$ production normalized to O$_2$ consumption (CH$_4$$_{\text{norm}}$) was examined.
2. Results

In order to evaluate the dependency of fungal CH₄ formation on prevailing O₂ concentrations starting at ambient levels (20.9% O₂, see Section 2.1) and at elevated levels (starting at >90% O₂, see Section 2.2) as well as different temperature (17 to 40 °C, Section 2.3), two different saprotrophic fungal species were incubated with different growth media (beech, pine, grass). For methodological details, we refer to Section 4, Materials and Methods. Please note that CH₄ formation and O₂ consumption rates were based on a per flask basis and not related to fungal dried biomass because it was not possible to determine it after each measurement step. Thus, in all conducted incubation experiments, CH₄ production rates were normalized to the O₂ consumption rates (CH₄,norm) to directly link fungal CH₄ production to its metabolic activity (inferred from O₂ consumption; Table 1). In addition, changes in CO₂ concentrations in the flask were also measured and their formation rates were estimated, and these are also indicators of the metabolic activity of the fungi. As the focus of the manuscript is on the role of O₂, the accompanying CO₂ data are shown in the Supplement (Text S1 and Figures S1–S3). Please also note that all presented CH₄ formations and CH₄,norm rates were corrected by subtracting the observed CH₄ rates in the medium controls.

2.1. Dependence of Fungal CH₄ Production on Ambient O₂ Concentrations

All incubation experiments in which the two fungal species P. sapidus or L. sulphureus were grown on different substrates at various O₂ levels showed measurable CH₄ formation rates compared with the respective substrate control (Table 1). Formation rates of CH₄ for controls were smaller compared with fungal incubations, accounting for 0.30 ± 0.05, 0.91 ± 0.09, and 3.26 ± 0.53 nmol h⁻¹ for pine wood incubated at 17 °C, 27 °C, and 40 °C, respectively, as well as 0.01 ± 0.001 and 0.31 ± 0.01 nmol h⁻¹ for grass incubated at 17 °C and 40 °C, respectively. Calculated CH₄ formation and O₂ consumption rates for experiments with the two fungal species were in the range of 0 to 5.34 ± 0.64 nmol h⁻¹ and 0.06 ± 0.01 to −0.88 ± 0.06 mmol h⁻¹, respectively.

No measurable CH₄ formation was observed when O₂ levels were below ~0.5%. As soon as O₂ was reintroduced, following an O₂-induced dilution of CH₄ levels in the vials, this led to an immediate increase in CH₄ formation, indicating a rapid response to the availability of O₂ for fungal metabolism. This is exemplarily shown in Figure 1 (arrows indicate the addition of O₂) for L. sulphureus (Figure 1A) and P. sapidus (Figure 1B) grown on beech wood.

For both fungi, the amount of CH₄ gradually increased within the flasks when O₂ was present. Notably, CH₄ formation rates substantially decreased in both fungi when O₂ mixing ratios fell, eventually ceasing completely, indicating no further CH₄ formation. However, upon reintroduction of O₂ to the flasks (Figure 1, as indicated by arrows, e.g., at an incubation time of 370 h for L. sulphureus grown on beech wood), the amount of O₂ initially increased (from 0.05 ± 0.03 to 16.8 ± 0.7 nmol) while that of CH₄ decreased (from 240.0 ± 7.8 to 190.3 ± 7 nmol) a result of the dilution effect from the supplemented gas volume. Subsequently, CH₄ formation resumed immediately leading to a consequent increase in CH₄ (~60 nmol). This pattern was repeatedly observed during the incubation of the two fungi grown on beech wood (Figure 1). It is important to note that during the incubation of P. sapidus grown on beech wood (Figure 1B), the CH₄ yield in the flask gradually decreased with successive O₂ additions from 167 nmol at the start of the incubation to 94.7 ± 6.0 nmol at the end of the incubation. This reduction was attributed to the dilution effect of the O₂ additions, which surpassed the fungal CH₄ formation rate. Nonetheless, a distinct increase in CH₄ was noted following each O₂ addition. Controls containing pine wood and grass (excluding beech due to unavailability, although Lenhart et al. [17] showed that beech controls show negligible CO₂ emissions) exhibited much lower but still measurable CH₄ as well as CO₂ emission rates regardless of prevailing O₂ levels (Figures S1–S3). A more detailed description and a discussion of these data can be found in the Supplement (Text S1).
Table 1. Overview of incubation experiments: fungal species, growth medium, and various temperatures (17 to 40 °C). The term “O₂ range” refers to the categories of different O₂ levels that prevailed within the incubation flasks during incubations. “N” represents the number of observations used to determine the rates of CH₄ formation, O₂ consumption, and the CH₄ formation to O₂ consumption ratio (CH₄ norm). These rates are presented as the arithmetic mean accompanied by the standard deviation. Please note that all rates are on a per flask basis because it was not possible to determine the dry weight of fungal biomass after each measurement step.

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Medium</th>
<th>Temperature [°C]</th>
<th>O₂ Range [%]</th>
<th>N</th>
<th>CH₄ Formation Rate [nmol h⁻¹]</th>
<th>O₂ Consumption Rate [mmol h⁻¹]</th>
<th>CH₄ norm [10⁻⁶]</th>
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<td>27</td>
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<td>beech</td>
<td>27</td>
<td>14 to 94</td>
<td>4</td>
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<td>0.04 ± 0.07</td>
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<td>pine</td>
<td>27</td>
<td>14 to 94</td>
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<td>0.40 ± 0.05</td>
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<td>grass</td>
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<td>grass</td>
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<td>5 to 20.9</td>
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Beyond the dependency of fungal CH₄ formation on the presence of O₂, we further found that the prevailing mixing ratios of O₂ in the incubation flasks influenced the CH₄ formation rates by *P. sapidus* and *L. sulphureus*. This is shown in Figure 2, where CH₄ formation as well as CH₄ norm rates and O₂ consumption rates (as negative values) are illustrated. While in all experiments, O₂ consumption rates were generally higher when higher O₂ mixing ratios prevailed (Table 1), this observation was more obvious for CH₄ production and CH₄ norm rates.
Figure 1. Amount of CH₄ and the mixing ratio of O₂ in the incubation flasks of (A) L. sulphureus and (B) P. sapidus grown on beech wood. Arrows indicate the points of O₂ addition to the individual flasks during incubation. Data points represent the arithmetic mean and standard deviation (n = 4).

Figure 2. CH₄ production and O₂ consumption, along with CH₄_norm rates at different O₂ levels of L. sulphureus (left panel) and P. sapidus (right panel) grown on beech wood. The bars represent the arithmetic mean and standard deviation of observations during replicate experiments (n = 4, N = 3 to 12, see Table 1).
Generally, we observed differences between CH$_4$ production rates and CH$_4_{\text{norm}}$ rates of _P. sapidus_ and _L. sulphureus_. While CH$_4$ production rates were similar when both fungi were grown on beech wood, _L. sulphureus_ showed higher rates when grown on pine wood ($p = 0.005$). Similarly, we found that CH$_4_{\text{norm}}$ rates were generally higher for _L. sulphureus_ compared with _P. sapidus_ ($p < 0.001$ for beech wood and $p = 0.003$ for pine wood; Figure 2). For _P. sapidus_ (except for when grown on grass at 17 °C), higher CH$_4_{\text{norm}}$ rates were observed when O$_2$ levels ranged between 0 and 5% compared with higher levels between 5% and ambient (pine wood, $p = 0.057$; grass at 17 °C, $p = 0.063$; grass at 40 °C, $p < 0.001$; Table 1). For _L. sulphureus_, we observed an opposite trend, where higher CH$_4_{\text{norm}}$ rates prevailed in a range between 5 and 21% for beech wood ($p = 0.002$) and pine wood at 27 °C (Figure 2, Table 1).

In all incubations, CO$_2$ mixing ratios demonstrated an opposite trend to O$_2$ levels, serving as a clear indicator of the fungi’s metabolic activities. Please note that CO$_2$ measurements were conducted less frequently than those for CH$_4$ and O$_2$ due to logistical reasons. During some experiments where O$_2$ was added, CO$_2$ concentrations exceeded ~21%, a level expected when all O$_2$ was consumed and converted to CO$_2$ (Supplement Text S1 and Figures S1–S3).

### 2.2. Fungal CH$_4$ Production Starting at Elevated O$_2$ Mixing Ratios of Near 100%

In another approach investigating the O$_2$ dependency of fungal CH$_4$ formation, both _L. sulphureus_ and _P. sapidus_ were grown on beech wood at a temperature of 27 °C and exposed to a starting O$_2$ mixing ratio of approximately 95% by flushing the flasks with pure O$_2$ (Figure 3). At the beginning of the experiment, the amount of CH$_4$ in the incubation flasks was $27.3 \pm 5.2$ nmol for _L. sulphureus_ and $16.9 \pm 1.1$ nmol for _P. sapidus_ (reflecting prevailing CH$_4$ mixing ratios of around 0.2 and 0.4 ppmv in the flasks, respectively). These values increased to $161.3 \pm 23.7$ nmol (2.2 ± 0.3 ppmv) and $61.6 \pm 7.1$ nmol (0.8 ± 0.1 ppmv) over 1055 h and 70 h, respectively, whilst O$_2$ levels decreased to $16.6 \pm 5.3\%$ and $13.9 \pm 3.5\%$. This corresponds to a CH$_4$ formation rate of $0.6 \pm 0.1$ nmol h$^{-1}$ for _P. sapidus_ and a lower rate of $0.09 \pm 0.02$ nmol h$^{-1}$ for _L. sulphureus_ ($p = 0.002$). Interestingly, the O$_2$ consumption rate varied substantially between the two fungal species. _P. sapidus_ had a much higher rate accounting for $-0.88 \pm 0.06$ mmol h$^{-1}$, in contrast to _L. sulphureus_, which reached a considerably lower O$_2$ consumption rate of $-0.06 \pm 0.001$ mmol h$^{-1}$ ($p = 0.001$). This disparity in O$_2$ consumption directly influenced the CH$_4_{\text{norm}}$ rates, which were substantially higher for _L. sulphureus_ compared with _P. sapidus_, accounting for $1.56 \pm 0.25$ ($\times 10^{-6}$) and $0.70 \pm 0.12$ ($\times 10^{-6}$), respectively ($p = 0.02$).

When comparing these CH$_4_{\text{norm}}$ rates to the experiments starting at ambient O$_2$ levels of 21%, we found that CH$_4_{\text{norm}}$ rates were in a similar range for _P. sapidus_ ($0.95 \pm 0.76$ ($\times 10^{-6}$)) and lower compared to _L. sulphureus_ with $3.76 \pm 1.28$ ($\times 10^{-6}$).

### 2.3. Temperature Dependency of Fungal CH$_4$ Formation, O$_2$ Consumption, and CH$_4_{\text{norm}}$ Rates

_P. sapidus_ and _L. sulphureus_, grown on pine wood and grass (only _P. sapidus_), were chosen to investigate whether CH$_4$ formation and O$_2$ consumption, as well as CH$_4_{\text{norm}}$ rates, differed based on different incubation temperatures of 17 °C, 27 °C, and 40 °C. Similarly to what is described in Sections 2.1 and 2.2, the above-mentioned rates of CH$_4$ formation, O$_2$ consumption, and CH$_4_{\text{norm}}$ showed a clear influence of prevailing O$_2$ mixing ratios. This influence, along with that of different temperatures, is additionally presented in this section.
Figure 3. Amount of CH\textsubscript{4} and the mixing ratio of O\textsubscript{2} in the incubation flasks of (A) \textit{L. sulphureus} and (B) \textit{P. sapidus} grown on beech wood at 27 °C with incubation starting at 98% and 95% O\textsubscript{2}, respectively. Data points represent the arithmetic mean and standard deviation of replicate experiments (\(n = 4\)).

Figure 4A,B show the trend of the amount of CH\textsubscript{4} and O\textsubscript{2} levels of the incubation experiments of \textit{L. sulphureus} grown on pine wood at three different temperatures (17, 27, 40 °C), while Figure 4C,F show the course of these parameters of \textit{P. sapidus} grown on pine wood (17 °C and 40 °C) and grass (17 °C and 27 °C), respectively, along with the corresponding controls for pine wood and grass. Please note that for \textit{P. sapidus} grown on pine wood and grass, only two temperature steps were available due to logistical reasons. All three incubation experiments clearly showed that CH\textsubscript{4} production rates were highest at a temperature of 27 °C (\(p = 0.02\) for \textit{L. sulphureus} and \(p = 0.001\) for \textit{P. sapidus} grown on grass) or at 40 °C for \textit{P. sapidus} grown on pine wood compared with the rates at a temperature of 17 °C (\(p = 0.002\); Figure 5, Table 1). Similar observations were made for O\textsubscript{2} consumption rates, which were highest at a temperature of 27 °C compared with the other temperature steps. Moreover, calculated CH\textsubscript{4}_\text{norm} rates at each O\textsubscript{2} level (see Table 1 and Figure 5) echoed this pattern. However, we observed a more complex distribution of CH\textsubscript{4}_\text{norm} rates depending on the fungal species when we considered the different O\textsubscript{2} levels within each incubation experiment (\textit{L. sulphureus} grown on pine and beech wood and \textit{P. sapidus} grown on grass, pine, and beech wood; Figures 2, 4 and 5). For incubations with \textit{L. sulphureus} grown on pine wood at 27 °C, CH\textsubscript{4}_\text{norm} rates were higher for O\textsubscript{2} mixing ratios between 5 and 21% (\(13.7 \pm 0.97 \times 10^{-6}\)) compared with rates below 5% O\textsubscript{2} (\(10.4 \times 10^{-6}\)). However, for \textit{P. sapidus} grown on pine wood, CH\textsubscript{4}_\text{norm} rates were higher when O\textsubscript{2} mixing ratios were <5% compared with O\textsubscript{2} levels between 5 °C and 21 °C, with rates accounting for \(4.18 \pm 1.26 \times 10^{-6}\) vs. \(2.08 \pm 1.23 \times 10^{-6}\) and \(4.94 \pm 0.25 \times 10^{-6}\) vs. \(3.97 \pm 0.38 \times 10^{-6}\) for 17 °C and 27 °C, respectively. The same observation was made for \textit{P. sapidus} grown on grass at a temperature of 40 °C, with values of \(11.6 \pm 0.17 \times 10^{-6}\) vs. \(3.41 \pm 1.73 \times 10^{-6}\) for O\textsubscript{2} mixing ratios below 5% and above 5%, respectively. Contrastingly, at a temperature of 17 °C, \textit{P. sapidus} grown on grass exhibited higher CH\textsubscript{4}_\text{norm} rates when O\textsubscript{2} levels ranged between 5 and 21% (\(0.86 \pm 0.62 \times 10^{-6}\)) as opposed to O\textsubscript{2} mixing ratios.
below 5% (0.19 ± 0.05 (×10⁻⁶)). Pine wood and grass controls showed a comparatively small increase in CH₄ levels over time depending on the temperature, with CH₄ formation rates ranging from 0.30 ± 0.05 to 3.36 ± 0.53 nmol h⁻¹ for pine wood and 0.01 ± 0.001 to 0.31 ± 0.01 nmol h⁻¹ for grass (Table 1, Text S2, and Figure S4).

Figure 4. Changes in CH₄ amounts and O₂ levels in the flasks during incubation of (A,B) *L. sulphureus* grown on pine wood at 17, 27, and 40 °C, (C,D) *P. sapidus* grown on pine wood at 17 and 27 °C, and (E,F) *P. sapidus* grown on grass at 17 and 40 °C. Black arrows indicate the points of O₂ addition to the individual flasks containing fungi, while pink arrows indicate O₂ removal by flushing of the incubation flask with helium. Data points represent the arithmetic mean and standard deviation of replicate experiments (n = 3 to 4).
Figure 5. CH₄ production and O₂ consumption, along with CH₄₃.norm rates at varying O₂ levels (0 to 21%) and temperatures (17, 27, 40 °C), of (A) L. sulphureus grown on pine wood and (B) P. sapidus grown on pine wood and (C) grass. Bars represent the arithmetic mean and standard deviation of observations during replicate experiments (n = 3 to 4, N = 2 to 6, see Table 1).
3. Discussion

3.1. Dependence of Fungal CH₄ Formation on O₂ Levels

Despite existing studies on the growth, wood decay, and eco-physiological adaptations of xylotrophic fungi, the impacts of O₂ levels on CH₄ production rates and CH₄norm rates have not been explored to date [36]. Our results clearly demonstrate that fungal metabolism due to the availability of O₂ is a crucial factor driving fungal CH₄ production. When O₂ was completely consumed by the fungi (meaning below ~0.5%, as indicated by the sensitivity of the deployed O₂ sensors), CH₄ formation ceased in our experiments (Figures 1 and 4). This finding contradicts earlier beliefs that linked CH₄ formation in wood debris to anoxic microsites and the activity of methanogenic archaea (Figures 1 and 4) [20–23,37]. If methanogenic archaea would have been responsible for the observed CH₄ formation in our experiments, we would have expected a strong increase in CH₄ levels once O₂ was depleted as anaerobic conditions are a prerequisite for methanogenic CH₄ formation. Instead, we observed the opposite, as no CH₄ was produced by the fungi once O₂ was depleted. This finding clearly indicates that fungal CH₄ formation is dependent on the occurrence of O₂. However, our fungal incubations were performed under sterile conditions and excluding the activity of bacteria and archaea (see Lenhart et al. [17]). Our observation is further in line with previous findings of Schroll et al. [18] that showed that δ¹³C-CH₄ values of fungal CH₄ covers a wide range from ~42 to ~70 ‰, which is not exclusively indicative of methanogenic CH₄ but overlaps with many other CH₄ sources, such as thermogenic degradation of organic matter and other eukaryotes, such as algae and cyanobacteria [8,38].

Intriguingly, upon reintroduction of O₂ to the incubation flasks, inducing aerobic metabolism, O₂ consumption, and CO₂ production, fungal CH₄ formation promptly resumed (e.g., Figure 1B). Thus, our study distinctly establishes for the first time that aerobic CH₄ formation by the investigated fungal species, P. sapidus and L. sulphureus, and likely saprotrophic fungi in general, occurs exclusively for metabolically active cells in the presence of O₂. This observation is in agreement with previous results by Ernst et al. [10] showing that bacteria, such as Bacillus subtilis, produce CH₄ when they are active and O₂ is present. Conversely, CH₄ production by this organism halts when either the cells are in a dormant state or O₂ is absent. Thus, the pattern of CH₄ formation, dependent on O₂ levels and the metabolic activity of B. subtilis, appears to be a clear analogy to the two fungal species investigated in this study. Therefore, it seems likely that fungal CH₄ was produced via a mechanism similar to that described by Ernst et al. [10] via the generation of methyl radicals by oxidative demethylation of a methylated nitrogen, sulfur, or oxygen compound in the presence of ROS and iron (II) (Figure 6).

Further exploring this dependency, we discovered that the level of O₂ present in the incubation flasks significantly influenced CH₄ formation by P. sapidus and L. sulphureus. In all experiments, O₂ consumption rates were generally higher when higher O₂ mixing ratios were present. This observation aligns with prior studies indicating that O₂ levels below 0.2% strongly inhibit fungal growth [29] and O₂ levels below 10% diminish wood decay activities of saprotrophic fungi [32]. Contrarily, Mukhin and Diyarova [31] found that xylotrophic basidiomycetes could completely consume O₂ in their environment and withstand high CO₂ levels, even up to 100%. These fungi are, therefore, facultative anaerobes that produce CO₂ in O₂-deprived environments. Our observations support this, as we noticed a significant increase in CO₂ levels, even when O₂ was entirely consumed in the incubations containing medium and fungi (Figures S1–S3). It is noteworthy, however, that CO₂ mixing ratios rose more rapidly when O₂ was available to the fungi.

Our study further found that O₂ consumption, CH₄ production, and CH₄norm rates differed substantially between the two fungal species, P. sapidus and L. sulphureus. The differences were most prominent in the O₂ consumption rates, where P. sapidus exhibited much higher rates compared with L. sulphureus under similar incubation conditions. This could be due to differences in biomass, however, this parameter could not be determined within the scope of this study. Another reason could be differences in their metabolic
activity, as they are different types of white rot and brown rot fungi, respectively. And, finally, the growth substrates might have impacted the observed rates. Several studies have found that fungal CH₄ production, also in relation to CO₂ emissions as an indicator of metabolic activity, is dependent on the growth substrate and substrate quality [17,18,27]. In our experiments, the most likely explanation for the observed differences is the different enzyme sets of L. sulphureus, as a brown rot fungus, and P. sapidus, as a white rot fungus.

Figure 6. Overview of potential mechanisms for fungal CH₄ formation, adapted after Ernst et al. [10] (upper part) and Huang et al. [24] (lower part). The upper part illustrates the mechanisms proposed for CH₄ formation driven by reactive oxygen species (ROS) in living systems, which involves the production of methyl radicals via Fenton chemistry from methylated compounds. The lower part depicts the mechanism proposed for halomethane-mediated CH₄ formation. This process begins with a methanogenic substrate and progresses through its conversion to methylated compounds via multiple enzymatic steps (blue boxes, MCT = methyl chloride transferase, DH = dehalogenase), leading to the generation of halomethanes (X⁻ = Cl⁻, I⁻, Br⁻) and, ultimately, methyl radicals.

Generally, there were notable differences in CH₄ production rates and CH₄-norm rates between these two fungal species. In line with previous studies by Lenhart et al. [17] and Schroll et al. [18], CH₄ production rates were typically up to 2.5 times higher in incubations containing L. sulphureus (grown on pine) compared with those with P. sapidus grown on pine (Table 1) and similar in magnitude when both fungi were grown on beech wood. Please note that for experiments where the fungi were grown on beech wood, no wood controls could be obtained. Nevertheless, previous studies by Lenhart et al. [17] clearly showed that incubation studies with sterilized beech wood controls, analogous to this study, did not increase CH₄ levels. Moreover, this observation was further validated by incubation studies in this study (see Figure 1), where no CH₄ increase was observed when O₂ was completely consumed, thus indicating that neither the fungal species nor the beech wood released CH₄ during this experiment. However, we now demonstrate for the first time that CH₄-norm rates normalized to O₂ consumption also exhibited a similar trend, with L. sulphureus showing up to 2.5 times higher values than P. sapidus (Figures 2 and 3).

Moreover, we observed distinct differences between P. sapidus and L. sulphureus concerning CH₄-norm rates under varying O₂ levels. For P. sapidus, CH₄-norm rates were often higher when O₂ levels ranged from 0 to 5%, compared with higher levels between 5% and ambient mixing ratios (Figure 5B,C; P. sapidus grown on pine and grass). Conversely, for L. sulphureus, higher CH₄-norm rates were observed in a range between 5 and 20%. The
underlying reason for this disparity remains unclear, but a potential explanation might lie in the differences in their metabolic pathways. *P. sapidus*, a white rot fungus, predominantly uses oxidative enzymes to decompose wood compounds, such as lignin, cellulose, and hemicellulose. In contrast, *L. sulphureus*, a brown rot fungus, relies on non-enzymatic oxidative systems to primarily depolymerize cellulose and, to a lesser extent, lignin, by generating ROS. Brown rot fungi deploy a mechanism dependent on Fenton-type reactions with ROS for wood decomposition [39–41]. However, the specific O₂ requirements for both mechanisms are not well-understood. While the O₂ requirement for brown rot fungi is relatively known due to the direct production of ROS, it is more complex for white rot fungi, where O₂ is utilized as a substrate for the enzymes associated with wood decay [40,42,43]. This aspect requires further evaluation and could be linked to the observed differences in CH₄ production and CH₄,norm rates between *P. sapidus* and *L. sulphureus*.

In general, the growth and wood decay of these fungi at different O₂ levels indicate that metabolic activity is closely connected to fungal CH₄ formation, given that no CH₄ is produced by either fungus when O₂ was absent. While the exact mechanism of CH₄ formation by these fungi remains elusive, initial evidence from Lenhart et al. [17], which identified methionine as a precursor of fungal CH₄, suggests that the universal CH₄ formation mechanism proposed by Ernst et al. [10] involving Fenton chemistry with methylated compounds and ROS likely represents a significant contributor to the observed formation of CH₄ (Figure 6, upper part). Nonetheless, the potential involvement of other mechanisms, such as the halomethane-dependent pathway reported by Huang et al. [24], represents another possibility (Figure 6, lower part). This study proposes a halomethane-mediated CH₄ formation mechanism in fungi, where CH₄ formation starts with a methanogenic substrate and progresses through its conversion via methylated substrates and biomass. This process leads to the formation of methylated compounds, which are then ultimately converted to halomethane and methyl radicals through multiple enzymatic steps. Although demonstrated under anaerobic conditions, the activity of the relevant enzymes for this mechanism also persists under aerobic conditions, presenting another possible CH₄ formation mechanism that warrants future investigation, particularly under aerobic conditions.

### 3.2. Influence of Temperature on Fungal CH₄ Formation Dynamics

To date, there is a noticeable gap in in the understanding of the influence of the temperature on fungal CH₄ formation. Previous studies have primarily focused on the growth or decomposition rates of wood via basidiomycetes, which are also critical factors for fungal CH₄ emissions. Previous studies have consistently shown that both fungal growth [34] and wood decomposition rates [27,28,33] increase with rising temperature. However, it should be noted that the fungal biomass and changes during incubation experiments could not be determined during this study, and thus CH₄ formation rates per unit biomass of fungi could not be calculated. Instead, we used consumption of O₂ and CO₂ production rates in relation to CH₄ formation as an indicator for fungal metabolic activity (see Supplement Text S1 and Figures S1–S3). We observed that the highest CH₄ formation and CH₄,norm rates occurred at 27 °C for both of the studied fungi. This peak in activity was likely attributed to the temperature being close to the optimal metabolism [44,45]. At temperatures both lower (17 °C) and higher (40 °C) than 27 °C, a decrease in the CH₄,norm rates was noted, suggesting a decline in metabolic activity, as further indicated by lower O₂ consumption and CO₂ production rates.

It is important to acknowledge that higher fungal biomass resulting from elevated growth rates most likely leads to increased CH₄ formation. This is due to CH₄ being produced by the fungus itself, as reported by Ernst et al. [10]. This study found that two fungal species produced CH₄ and that elevated levels of ROS, which can increase in organisms as a stress response, even amplified the observed CH₄ formation. This aligns with findings from Lenhart et al. [17] and Schroll et al. [18], which showed fungal CH₄ formation independent of the presence of methanogenic archaea. Therefore, it appears that
CH₄ formation may be a function of not only the fungal biomass and experienced stress (ROS) levels but also the metabolic activity of the fungal species.

At 17 °C, P. sapidus grown on grass exhibited smaller CH₄ production rates compared with when it was grown on pine wood, despite similar O₂ consumption rates across different O₂ regimes. Consequently, CH₄ norm rates were much higher for P. sapidus grown on pine wood. This indicates a substrate-specific component regulating CH₄ formation rates, supporting previous studies that highlighted the strong effect of fungal substrate on CH₄ formation and decomposition rates [18,27]. Additionally, CH₄ production rates for P. sapidus grown on grass and pine wood increased at elevated temperatures of 40 °C and 27 °C, respectively, likely due to higher metabolic activity, as indicated by increased O₂ consumption rates, particularly when O₂ mixing ratios exceeded 5%. This observation could also be influenced by increased stress levels, especially for P. sapidus grown on grass at 40 °C, in a similar way as the previously discussed higher CH₄ formation in fungi due to increased ROS [10].

A similar pattern was noted for L. sulphureus grown on pine wood at 17 °C, 27 °C, and 40 °C. The highest CH₄ production rates were found at 27 °C, which is presumably closest to the optimal fungal growth temperature, whereas the lowest CH₄ formation rates at 17 °C coincided with the lowest O₂ consumption rates, indicating reduced metabolic activity at this temperature compared to 27 °C and 40 °C. However, contrastingly, CH₄ norm rates were in a similar range for both 17 °C and 40 °C, suggesting that while metabolic activity (inferred from O₂ consumption) was higher at 40 °C, the ratio of CH₄ formation to O₂ consumption (CH₄ norm) remained in the same range of around 3.5 (10⁻⁶). Mukhortova et al. [28] also observed that CH₄ and CO₂ levels clearly increased with temperature, ranging from 5 °C to 25 °C, during the decomposition of woody debris in a northern boreal forest, indicating an aerobic mechanism for CH₄ formation as reported by [10,17,18], which opposes the notion of an exclusive anaerobic origin of CH₄ through methanogenic archaea in anoxic microsites of these woody debris.

At various temperatures, we found that without O₂, no or substantially less CH₄ was emitted during our incubation experiments. Notably, CH₄ formation observed in the controls was also temperature-dependent, suggesting an additional abiotic mechanism, as previously suggested by Lenhart et al. and Schroll et al. [17,18]. For the results of the control experiments and explanations of abiotic CH₄ formation, we refer to the Supplement Text S2 and Figure S4. Regarding the lack of knowledge about abiotic formation of CH₄ and CO₂ from the studied substrates, substantially more research is required to fully comprehend this phenomenon.

4. Materials and Methods

4.1. Selected Fungi

In this study, we selected P. sapidus (Pleurotaceae, DSMZ 8266) and L. sulphureus (Polyporaceae, DSMZ 1014) due to their documented CH₄ emission properties, as reported in previous studies by Lenhart et al. and Schroll et al. [17,18]. These organisms were specifically chosen not only for their CH₄ emission capabilities but also for their distinct ecological and physiological traits. P. sapidus, a white rot fungus, and L. sulphureus, a brown rot fungus, are both notable for their ease of cultivation and management in laboratory settings, making them ideal species for our experimental analysis.

4.2. Incubation Experiments

We investigated the O₂ and temperature dependency of fungal CH₄ emissions, focusing on the two fungal species P. sapidus and L. sulphureus. P. sapidus was cultivated on a variety of media, including beech, pine, and a grass mixture, whereas L. sulphureus was grown on beech wood and pine wood. The cultivation process involved using autoclaved wood chips from these trees and the grass mixture, sterilized at 121 °C and 2 bar pressure for 20 min (Figure 7).
Experimental setup

Fungal CH₄ emissions

O₂ dependency

Temperature dependency

Table 1. Experimental setup for investigating the O₂ and temperature dependency of fungal CH₄ formation. Fungal CH₄ formation was examined through concentration measurements in incubation flasks containing fungi *L. sulphureus* or *P. sapidus* grown on beech wood, pine wood, or grass under varying O₂ levels (left column) and incubation temperatures of 17 °C, 27 °C, or 40 °C (right column). “n” represents the number of replicate experiments.

<table>
<thead>
<tr>
<th>Fungi</th>
<th><em>L. sulphureus</em></th>
<th><em>P. sapidus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>beech wood</td>
<td>pine wood</td>
</tr>
<tr>
<td>O₂ levels [%]</td>
<td>0 to 8.6</td>
<td>0 to 26.0</td>
</tr>
<tr>
<td>Temperature [°C]</td>
<td>16.6 to 97.5</td>
<td>14.0 to 94.5</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 2. Controls experiment for investigating the O₂ dependency of fungal CH₄ emissions, especially in cases where O₂ was depleted or fell below certain concentrations.

<table>
<thead>
<tr>
<th>Fungi</th>
<th><em>L. sulphureus</em></th>
<th><em>P. sapidus</em></th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>pine wood</td>
<td>pine wood</td>
<td>grass</td>
</tr>
<tr>
<td>Temperature [°C]</td>
<td>17</td>
<td>27</td>
<td>40</td>
</tr>
<tr>
<td>O₂ levels [%]</td>
<td>11.1 to 20.9</td>
<td>0 to 20.9</td>
<td>20.9</td>
</tr>
<tr>
<td>n</td>
<td>3</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

These sterilized media (~350 g) were then placed into 2.7 L glass flasks (Weck, Bonn, Germany) and inoculated with pure fungal submerged cultures (50 mL), maintaining sterile conditions as described by Lenhart et al. [17]. In parallel, controls of the medium were prepared in a similar way, except for the inoculation with fungal cultures. To facilitate gas exchange, each flask was sealed with a rubber band and a glass lid, which incorporated a hole plugged with a cotton stopper. Before the beginning of the incubation period, the flasks were aerated under sterile conditions to establish the initial atmospheric ratios of CH₄, O₂, and CO₂. Subsequently, the cotton stoppers were replaced with sterile silicone stoppers (Saint-Gobain Performance Plastics, Courbevoie, France) to limit uncontrolled gas exchange while allowing for precise gas sampling.

In order to investigate the temperature dependency of fungal CH₄ emissions, the fungi were placed in a climate chamber set at three different temperatures: 17 °C, 27 °C, and 40 °C. The duration of incubation varied, extending up to ~1100 h (ca. 46 days).

Gas samplings were performed as follows. First, the pressure within the glass flasks was measured using an EX- portable pressure-measuring instrument (GHM Messtechnik GmbH, Regenstauf, Germany) with a precision of ±1%. Then, a plastic syringe (Plastikpak, NJ, USA) was used to extract 10 mL of gas for CH₄ measurements and 6 mL for CO₂. Measurement of CH₄ and CO₂ mixing ratios was performed according to the procedures detailed in Section 4.3, with atmospheric air replacing the extracted gas. Alongside gas sampling, we continuously monitored O₂ concentrations within the flasks using calibrated O₂ spots (Section 4.3).

Furthermore, to maintain specific O₂ mixing ratios and to test the hypothesis of the O₂ dependency of fungal CH₄ emissions, especially in cases where O₂ was depleted or fell below certain concentrations, we regulated the O₂ levels in the flasks by adding pure O₂ until the desired concentration (usually to atmospheric O₂ levels), which was immediately controlled using the calibrated O₂ spots. For the pine wood control incubated at 40 °C, CH₄ formation was, in addition to ambient O₂ mixing ratios, investigated without the presence of O₂ by exchanging the whole headspace volume with pure helium.
4.3. Measurement of \( \text{CH}_4 \), \( \text{CO}_2 \), and \( \text{O}_2 \) Concentrations

Mixing ratios of \( \text{CH}_4 \) in this study were measured using a gas chromatograph coupled with a flame ionization detector (GC-FID; Shimadzu 14b, Kyoto, Japan). To facilitate this, sample gas was injected into the GC-FID through a six-port valve (Valco Instruments, Houston, TX, USA), which was linked to a chemical trap filled with Drierite®. This setup was crucial for drying the gas before entering the analytical system via a sample loop with a volume of 2 mL that was employed using a 20 mL plastic syringe (Plastipak BD, Franklin Lakes, NJ, USA). The GC itself was equipped with a stainless-steel column (3.175 mm in inner diameter) packed with a 60–80-mesh molecular sieve 5A (Supelco, St. Louis, MO, USA), effectively separating \( \text{CH}_4 \) from other gas components in the samples. The oven temperature was maintained constantly at 125 \( ^\circ \text{C} \). To quantify \( \text{CH}_4 \) within the samples, daily measurements (ranging from 3 to 4) of two reference standards with \( \text{CH}_4 \) mixing ratios of 2.192 and 9.655 ppmv were conducted.

For measuring \( \text{CO}_2 \) mixing ratios, a GC coupled with a barrier discharge ionization detector (BID; Shimadzu, Kyoto, Japan) was employed. Here, 50 \( \mu \text{L} \) of sample gas was injected into the GC-BID via an autosampler AOC-20-i (Shimadzu, Kyoto, Japan) using a split injection method (5:1). This GC was equipped with a ShinCarbon ST packed column (80/100 mesh; length: 2 m; diameter: 0.53 mm; Shimadzu, Kyoto, Japan). The quantification of \( \text{CO}_2 \) was achieved by measuring various reference standards (400 ppm, 0.5%, 10%, and 40% by volume each conducted in triplicate). As part of quality control, one reference standard was measured after every 6 to 9 single measurements.

Oxygen concentrations were determined using non-invasive optical sensors (\( \text{O}_2 \) spots; PST3 sensor type) and a Fibox 4 portable measuring instrument (both from PreSens Precision Sensing GmbH, Regensburg, Germany). These \( \text{O}_2 \) spots were installed in the glass flasks before the start of the incubation experiments and calibrated using a two-point calibration with ambient air (20.9% \( \text{O}_2 \)) and helium (0% \( \text{O}_2 \)). The precision of the \( \text{O}_2 \) spots is 0.4% at 20.9% \( \text{O}_2 \) and 0.05% at 0.2% \( \text{O}_2 \).

4.4. Calculations and Statistical Methods

In this study, all \( \text{CH}_4 \) emission rates were carefully normalized against the respective \( \text{O}_2 \) consumption rates to ensure the investigation of \( \text{CH}_4 \) formation based on the fungal metabolism. The \( \text{CH}_4 \) formation rates, based on \( \text{O}_2 \) levels, were categorized into three distinct groups: 0% \( \text{O}_2 \), 0–5% \( \text{O}_2 \), as well as >5 to 21% \( \text{O}_2 \) (for experiments with fungi grown on pine wood and grass) and >5 to 98% (for experiments with fungi grown on beech wood). This categorization was pivotal, as it was observed that below an \( \text{O}_2 \) mixing ratio of 5%, there was a noticeable reduction in \( \text{CH}_4 \) production.

To systematically categorize the normalized \( \text{CH}_4 \) rates within these defined \( \text{O}_2 \) levels for each flask, sections corresponding to the respective \( \text{O}_2 \) mixing ratios were manually defined. Within these sections, \( \text{CH}_4 \) emission and \( \text{O}_2 \) consumption rates were calculated using linear regression analysis for all incubation experiments. Importantly, respective \( \text{CH}_4 \) formation rates of the controls were subtracted from the calculated rates in the control of the respective substrate.

For each incubation experiment, arithmetic means and standard deviations were calculated to discern differences between the treatments. Furthermore, t-tests were conducted to provide statistical backing for the observations made. However, it is important to note that this study adhered to the recommendations of the American Statistical Association. Consequently, \( p \)-values and other statistical parameters were not solely relied upon as the criteria for drawing conclusions [46]. Thus, the term “statistically significant” was consciously avoided in the interpretation of the results.

5. Conclusions

This study, to our knowledge, is the first to investigate how different \( \text{O}_2 \) mixing ratios and temperatures control \( \text{CH}_4 \) emissions in two saprotrophic fungi, \textit{L. sulphureus} and \textit{P. sapidus}. We observed that \( \text{CH}_4 \) formation rates are highly dependent on the prevailing
O₂ mixing ratios. Notably, in all of our incubation experiments, we observed that fungal CH₄ formation rates diminished when O₂ mixing ratios fell below approximately 0.5%. Conversely, CH₄ formation increased immediately after O₂ was reintroduced or when O₂ levels remained above this threshold, unambiguously highlighting the role of aerobic metabolism for fungal CH₄ formation. Furthermore, we found that CH₄ NORM rates varied based on the fungal species and their substrates, including beech wood, pine wood, and grass. These findings suggest that the investigated fungal species, and possibly also further fungal species, produce CH₄, and that aerobic metabolism controlled by O₂ levels is a critical factor in this process. This further challenges the previous assumption that in the fungal realm, CH₄ is only formed under anoxic conditions and that saprotrophic fungi simply provide methanogenic archaea with precursor compounds via the decomposition of woody components. Additionally, our study shows that temperature has a substantial effect on fungal CH₄ formation. We observed lower CH₄ formation and CH₄ NORM rates at temperatures of 17 °C and 40 °C, while the highest rates of CH₄ formation occurred at 27 °C, indicating that this temperature is closer to the optimum metabolic activity for the investigated fungi.

Thus, our research clearly demonstrates that both the availability of O₂ and temperature are key in controlling fungal CH₄ emissions. From an environmental perspective, temperature increases due to climate change could significantly increase fungal CH₄ emissions through enhanced fungal growth and biomass. Therefore, understanding CH₄ formation and its controlling factors, especially temperature and O₂ levels, is crucial for assessing the role of fungal CH₄ emissions regarding global CH₄ fluxes. Additionally, exploring these fungal CH₄ dynamics in various environments, such as forests, soils, and aquatic systems, ref. [47] under both aerobic and anaerobic conditions is necessary to better understand their impact on carbon and greenhouse gas dynamics.

**Supplementary Materials:** The following supporting information can be downloaded at: [https://www.mdpi.com/article/10.3390/methane3020015/s1](https://www.mdpi.com/article/10.3390/methane3020015/s1). Text S1: Oxygen and temperature dependency of CO₂ mixing ratios during fungal incubations; Figure S1: Changes in CH₄ amounts (A,D) as well as O₂ (B,E) and CO₂ levels (C,F), respectively, in the flasks during incubation of *P. sapidus* grown on pine wood at 17 and 27 °C. Black arrows indicate the points of O₂ addition to the individual flasks containing fungi. Data points represent the arithmetic mean and standard deviation of replicate experiments (n = 3 to 4). Figure S2: Changes in CH₄ amounts (A,D) as well as O₂ (B,E) and CO₂ levels (C,F), respectively, in the flasks during incubation of *P. sapidus* grown on grass at 17 and 40 °C. Black arrows indicate the points of O₂ addition to the individual flasks containing fungi. Data points represent the arithmetic mean and standard deviation of replicate experiments (n = 3 to 4); Figure S3: Changes in CH₄ amounts (A,D,G) as well as O₂ (B,E,H) and CO₂ levels (C,F,I), respectively, in the flasks during incubation of *L. sulphureus* grown on pine wood at 17, 27, and 40 °C. Black arrows indicate the points of O₂ addition to the individual flasks containing fungi, while pink arrows indicate O₂ removal by flushing of the incubation flask with helium. Data points represent the arithmetic mean and standard deviation of replicate experiments (n = 3 to 4); Figure S4: Changes in CH₄ amounts as well as O₂ and CO₂ levels in the flasks during control incubation of pine wood at 17, 27, and 40 °C and grass at 17 and 40 °C. The pink arrow indicates O₂ removal by flushing of the incubation flask with helium. Data points represent the arithmetic mean and standard deviation of replicate experiments (n = 3 to 4). Refs. [48–56] have been cited in the Supplementary Material.

**Author Contributions:** M.S.: conceptualization, methodology, formal analysis, investigation, writing—original draft, writing—review and editing, visualization. K.L.: conceptualization, methodology, resources, writing—review and editing, supervision, funding acquisition. T.B.: formal analysis, investigation. P.H.: formal analysis, investigation. A.R.: formal analysis, investigation. S.S.: formal analysis, investigation. F.K.: conceptualization, methodology, resources, writing—review and editing, supervision, project administration, funding acquisition. All authors have read and agreed to the published version of the manuscript.

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