



Brief Report

Boiled Water Extract of Wild-Derived *Flammulina velutipes* Inhibits Biofilm Formation by *Staphylococcus aureus* Without Bactericidal Effects

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Abstract

Biofilm formation by *Staphylococcus aureus* presents a large challenge in clinical settings, as it confers resistance to antibiotics. Therefore, anti-biofilm agents that do not exert bactericidal effects are sought after. Extracts of cultivated *Flammulina velutipes* have been shown to inhibit biofilm formation by *Streptococcus mutans* and to exhibit antibacterial activity against *S. aureus*. However, the effects on biofilm formation by *S. aureus* have not been investigated. Therefore, the effects of a boiled water extract (BWE) from wild-derived *F. velutipes* on *S. aureus* biofilm production and growth were investigated. *Staphylococcus aureus* and *Cutibacterium acnes* were treated with BWE and biofilm formation was assessed using crystal violet staining. The BWE substantially reduced biofilm formation by *S. aureus* without inhibiting bacterial growth. This study is the first to demonstrate that wild-derived *F. velutipes* extracts inhibit biofilm formation in *S. aureus* without exerting bactericidal effects. Biofilm formation of *C. acnes* was also inhibited by BWE treatment.

Keywords: wild-derived *Flammulina velutipes*; *Staphylococcus aureus*; anti-biofilm agent; non-bactericidal activity

1. Introduction

Biofilms are complex structures formed by bacteria and consist of polysaccharides, proteins, and extracellular DNA [1]. Biofilm formation enables bacteria to resist external stresses, including antimicrobial agents [2]. Many bacteria form biofilms in natural environments [3]. In particular, biofilm formation by pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus mutans* on medical devices and within the human body represents a health concern, as it reduces the effectiveness of antibiotics [4–9]. Excessive use of antibiotics may induce the emergence of antimicrobial-resistant bacteria and can also harm beneficial commensal bacteria and the human body itself [10–15]. Thus, agents that specifically inhibit biofilm formation without exerting bactericidal effects are needed [10–15].

Staphylococcus aureus is an opportunistic pathogen commonly present on human skin [16]. It causes nosocomial infections by adhering to medical devices, such as artificial joints and catheters, and resists antibiotic action through biofilm formation [17]. The emergence of drug-resistant *S. aureus* is a major issue, necessitating the urgent development of new control methods [18].

Edible mushrooms have long been known to exhibit various medicinal activities and have therefore been used as sources for exploring active compounds [19–21]. Many



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edible mushrooms are produced through commercial cultivation. As the concentration of active ingredients in mushrooms can vary considerably depending on cultivation methods and habitat, rare wild-type mushrooms may exert different effects to widely distributed, cultivated varieties [22].

In the present study, the biological activities of extracts derived from a wild-derived and a commonly cultivated cultivar of *Flammulina velutipes* were compared. Whereas commercially cultivated *F. velutipes* (enoki mushroom) is generally represented by white cultivars, the strain examined here (Yaseishu-enoki) is a brown cultivar originally collected from a natural mountain habitat and subsequently domesticated for commercial production. Extracts from white *F. velutipes* inhibit biofilm formation by *Streptococcus mutans* and exhibit antibacterial activity against *Staphylococcus aureus* [22,23]. However, the anti-biofilm effects of wild-derived *F. velutipes* against *S. aureus* have not been explored. Therefore, the effects of a boiled water extract (BWE) prepared from wild-derived *F. velutipes* on biofilm formation and growth of *S. aureus* were investigated. To our knowledge, this is the first report describing the anti-biofilm activity of a wild-derived *F. velutipes* cultivar against *S. aureus*.

2. Materials and Methods

2.1. Strains and Culture Conditions

All mushrooms (wild-derived *F. velutipes*; Yaseishu-enoki, white *F. velutipes*; Shiro-enoki, cultivated *F. velutipes*; Amashiyaki-enoki, *Pleurotus ostreatus*, *Pleurotus eryngii* var. *tuoliensis*) were purchased from Shinko Industrial (Nagano, Japan). *Staphylococcus aureus* strain NBRC 100910 was purchased from the Biological Resource Center, NITE (NBRC, Kisarazu, Japan). *Staphylococcus aureus* was cultured on Luria–Bertani (LB) agar (Nacalai Tesque, Kyoto, Japan) plates or in LB broth (Nacalai) under aerobic conditions at 37 °C. The *Cutibacterium acnes* JCM6245 strain was purchased from the RIKEN Bio Resource Research Center (BRC, Tsukuba, Japan). *Cutibacterium acnes* was cultured in Gifu anaerobic medium broth (GAM, Nissui, Tokyo, Japan) under anaerobic conditions (10% CO₂, 0.1% O₂) at 37 °C.

2.2. Preparation of Boiled Water Extract of Wild-Derived *F. velutipes*

Wild-derived *F. velutipes* (15 g) was soaked in 500 mL of Milli-Q water and boiled for 1 h. After boiling, the solution was filtered through No. 5B filter paper (Kiriyaama Glass Works, Tokyo, Japan) and concentrated using evaporation. The obtained extract was dissolved and diluted in Milli-Q water to achieve the desired concentration.

2.3. Inhibition of Biofilm Formation

The pre-cultured *S. aureus* broth was diluted five-fold in LB medium with 1% glucose to an optical density [OD₆₀₀] of 0.2–0.3. Since glucose promotes biofilm formation in *S. aureus* [24], LB medium supplemented with glucose was used in this study. Samples included: Blank (LB medium with 1% glucose only), Control (Milli-Q water), Amp (ampicillin, final concentration: 20 µg/mL), and mushroom extracts. Briefly, 200 µL of each sample solution was added to three wells of a flat-bottom, 96-well plate (Violamo, AS ONE, Osaka, Japan) and incubated anaerobically at 37 °C for 3 d.

2.4. Crystal Violet Staining

After incubation, the supernatant was removed, and 200 µL of phosphate-buffered saline (1 mM sodium phosphate in distilled water, pH = 6.8) was added and removed. This washing step was repeated twice. Next, 200 µL of MeOH was added and left for 15 min to fix the biofilm. After removing MeOH, the plate was air-dried in an incubator at 65 °C. Subsequently, 150 µL of 0.1% crystal violet (Nacalai) solution was added, followed by

staining for 20 min. After staining, the crystal violet solution was removed, and the wells were washed twice with phosphate-buffered saline. After washing, 200 μ L of 99.5% ethanol was added, and the dye was eluted by pipetting the entire volume five times. Next, 100 μ L of the supernatant was transferred to a new 96-well plate, and the absorbance at 595 nm was measured using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Antibacterial Activity

Staphylococcus aureus was pre-cultured in LB medium supplemented with glucose for 24 h. The crude extract or active fractions obtained were dissolved in Milli-Q water. Aliquots of these fractions were added to *S. aureus* culture broth ($OD_{600} = 0.2$ – 0.25) at a final concentration of 10%. For the negative control, Milli-Q water was added, and ampicillin (20 μ g/mL) was used as the positive control. All samples were incubated anaerobically at 37 °C for 24 h. After incubation, the turbidity of the culture broths was adjusted to an OD_{600} of 0.1 in LB medium, and the bacterial suspension was diluted 10,000-fold. Next, 50 μ L of the diluted suspension was spread onto three LB agar plates for each sample and incubated at 37 °C for 24 h to allow colony formation. The resulting colonies were enumerated to assess the growth of *S. aureus*.

2.6. Statistical Analysis

All experimental results are expressed as the mean $\pm$ standard deviation of three replicates. Differences were analyzed using Dunnett's test to compare experimental samples (triplicates) or the Tukey–Kramer test for duplicate samples using Microsoft Office Excel and R software (version 4.0.2; R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. *Flammulina velutipes* BWE Inhibited Biofilm Formation of *S. aureus*

The BWE of *F. velutipes* fruiting bodies was mixed with the *S. aureus* culture medium to evaluate its effect on biofilm formation. Water was used as a negative control, and Amp was used as a positive control. Quantification of the formed biofilms using crystal violet staining showed that BWE treatment substantially reduced the amount of biofilm that adhered to the wells in a concentration-dependent manner (Figure 1A,B). These results indicate that BWE exhibited anti-biofilm activity against *S. aureus*. Furthermore, the BWE of wild-derived *F. velutipes* showed stronger anti-biofilm activity than that of other *F. velutipes* strains (white and Amashiyaki) and other mushrooms (*P. ostreatus* and *P. eryngii* var. *tuoliensis*) (Figure 2).

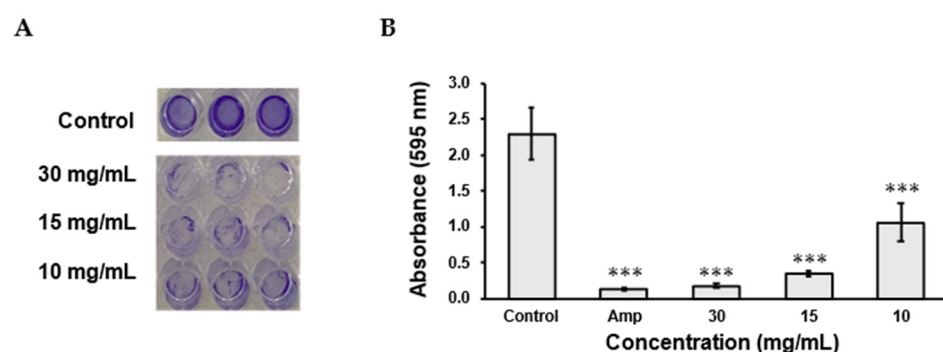


Figure 1. Inhibition of biofilm formation in *Staphylococcus aureus* by boiled water extract (BWE) of *Flammulina velutipes*. (A) Quantification of biofilm formation by *S. aureus* in the presence of water (negative control, (upper panel)) and BWE (bottom panel) using crystal violet staining. The amount of biofilm adhered to the wells decreased substantially with BWE treatment. (B) The effect of BWE on biofilm formation was concentration dependent. Data are presented as the mean $\pm$ standard deviation of three replicates. Statistical significance was determined using Dunnett's test (***: $p < 0.001$).

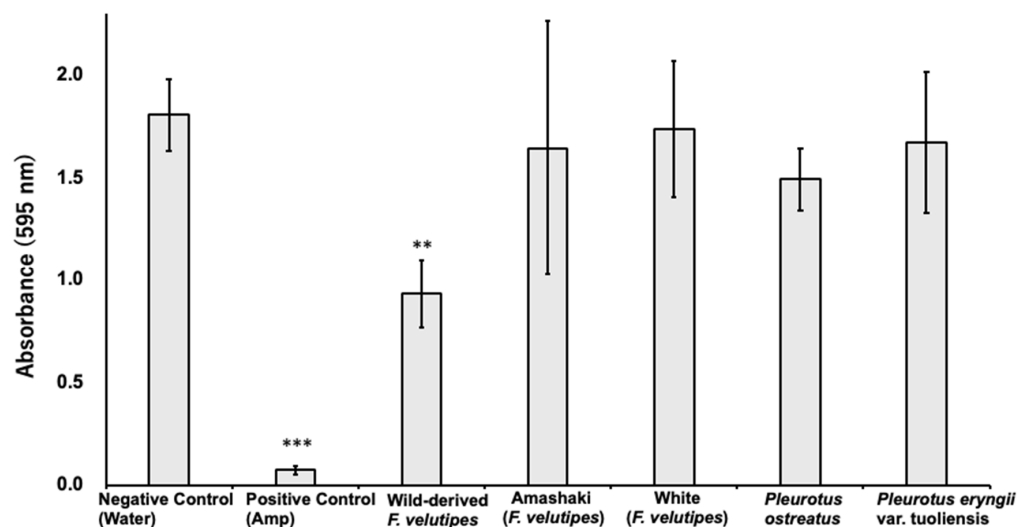


Figure 2. Comparative anti-biofilm activities of extracts from different *Flammulina velutipes* strains and other mushrooms against *Staphylococcus aureus*. The BWE (10 mg/mL) of wild-derived *F. velutipes* exhibited stronger anti-biofilm activity than that of extracts from other *F. velutipes* strains and other mushroom species. Biofilm formation was quantified using the crystal violet staining method. Data are presented as the mean $\pm$ standard deviation of three replicates. Statistical significance was determined using Dunnett's test; ***: $p < 0.001$; **: $p < 0.01$; Amp: ampicillin.

3.2. *Flammulina velutipes* BWE Did Not Inhibit *S. aureus* Growth

The cell concentration (colony-forming units) of *S. aureus* cultures after BWE treatment was measured to verify whether the anti-biofilm activity was attributable to bactericidal activity. The results confirmed that BWE treatment did not reduce the cell concentration of *S. aureus* (Figure 3). The cell concentration of BWE-treated samples increased, likely because *S. aureus* used components of the BWE, such as amino acids and sugars.

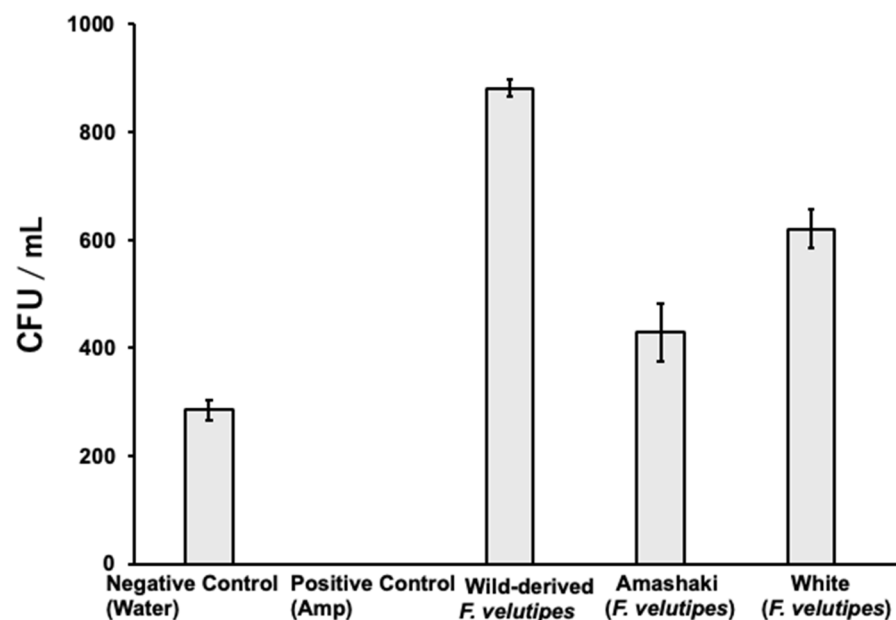


Figure 3. Effect of boiled water extract (BWE) of *Flammulina velutipes* on the growth of *Staphylococcus aureus*. The cell concentration (colony-forming units: CFU) of *S. aureus* was determined after treatment with BWE to evaluate its growth-inhibitory effects. BWE (2.5 mg/mL) treatment did not reduce the cell concentration of *S. aureus*, indicating that the observed anti-biofilm activity was not due to bactericidal effects. Data are presented as the mean $\pm$ standard deviation of three replicates.

3.3. BWE Inhibited Biofilm Formation of *C. acnes*

To examine the specificity of BWE activity, an anti-biofilm assay was conducted on *C. acnes*. BWE inhibited biofilm formation of *C. acnes* (Figure 4). These findings suggest that the anti-biofilm activity of BWE is not limited to *S. aureus* but extends to other clinically relevant, biofilm-forming bacteria. This broad-spectrum inhibitory effect indicates that BWE may target a conserved mechanism involved in biofilm development across multiple species.

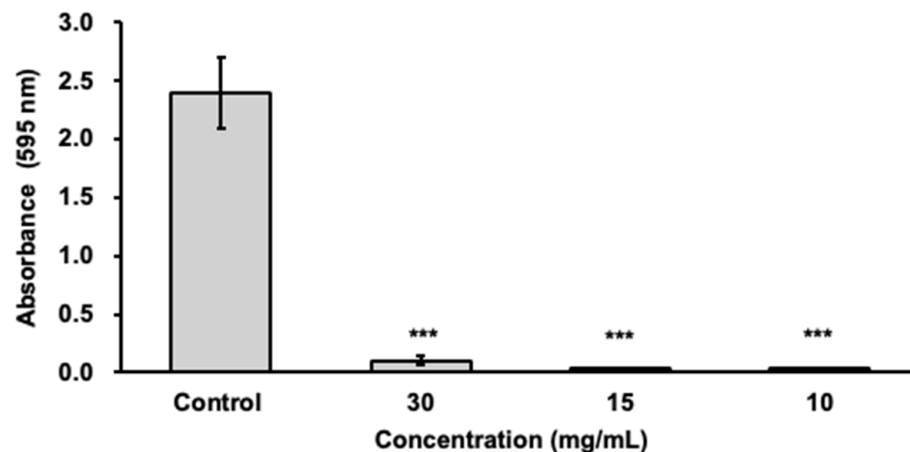


Figure 4. Inhibition of biofilm formation in *Cutibacterium acnes* by boiled water extract (BWE). The biofilm formation of *Cutibacterium acnes* was substantially inhibited by BWE. Data are presented as the mean $\pm$ standard deviation of three replicates. Statistical significance was determined using Dunnett's test (***: $p < 0.001$).

4. Discussion

Flammulina velutipes (white variant) extracts have been reported to exhibit various bioactivities [22]. Specifically, MeOH extracts inhibit biofilm formation by *S. mutans*, and water extracts show growth-inhibitory activity against *S. aureus* [22,23]. To the best of our knowledge, the present study is the first to report an extract that inhibits biofilm formation by *S. aureus* without exhibiting antibacterial activity. This unique finding suggests that the active components in the BWE of wild-derived *F. velutipes* (brown variant) function via a novel mechanism that does not rely on bactericidal effects.

Shah et al. [22] reported that water extracts of cultivated *F. velutipes* (white variant) exhibited antibacterial activity against *S. aureus* and suggested that phenolic and flavonoid compounds were responsible for this effect. The BWE of the wild-derived *F. velutipes* cultivar (brown variant) examined in the present study showed no antibacterial activity. Because phenolic and flavonoid compounds are generally considered relatively heat-stable, they are expected to be extracted even when hot water is used as the solvent. Therefore, the discrepancy between the findings of Shah et al. and those of the present study is more likely attributable to strain-dependent differences in chemical composition than to differences in extraction procedures. Shah et al. also suggested that the composition of bioactive compounds in mushrooms varied depending on genetic background and growth conditions. Consistent with this notion, the present study indicated substantial differences in biological activity among *F. velutipes* cultivars. The strain examined here was originally isolated from a wild population in a mountainous environment and was subsequently domesticated for commercial cultivation. Thus, the distinctive anti-biofilm activity observed in this study may reflect characteristics associated with its wild-derived genetic background. Taken together, these findings suggest that the anti-biofilm activity reported here may be specific to the wild-derived brown cultivar of *F. velutipes* used in this study.

Although the detailed structure of the active component was not identified in this study, nuclear magnetic resonance (NMR) spectroscopy suggested that it was a sugar alcohol (Figures S1–S3). Certain sugar alcohols have been reported to exhibit anti-biofilm activity [25–28]. Of these, the most well-known sugar alcohol is xylitol, which exhibits anti-biofilm activity by targeting genes important for polysaccharide-mediated adherence [25,28,29]. As xylitol cannot be metabolized by bacteria, it also exhibits antibacterial activity [30,31]. In contrast to xylitol, the BWE of wild-derived (brown variant) *F. velutipes* did not inhibit the growth of *S. aureus*, suggesting that its anti-biofilm activity is mediated through a mechanism distinct from those of sugar alcohols with known antibacterial properties. BWE inhibited biofilm formation in both *S. aureus* and *C. acnes*, despite the fact that these species use different mechanisms of biofilm formation. This observation suggests that BWE may target a common stage of biofilm development such as initial surface attachment or alternatively disrupt biofilm formation through a detergent-like effect. Further studies are needed to identify the active compound(s) responsible for this activity and to elucidate the underlying mechanism.

Biofilm control in medical devices relies primarily on chemical disinfectants and physical cleaning. However, biofilms are highly tolerant to disinfectants, often requiring high concentrations of antimicrobial agents for effective control. In addition, cleaning procedures may involve detergents that can adversely affect human health and the environment. The wild-derived (brown variant) *F. velutipes* used in this study is a commercially available edible mushroom, suggesting a favorable safety profile and low toxicity. Because anti-biofilm activity is observed only with BWE treatment, BWE is expected to have a low environmental impact. Therefore, wild-derived (brown variant) *F. velutipes* and BWE are considered useful materials that could contribute to the development of new strategies for controlling biofilm production in *S. aureus*.

5. Conclusions

This study demonstrated for the first time that BWE from wild-derived (brown variant) *F. velutipes* inhibited biofilm formation by *S. aureus* without affecting bacterial growth. BWE also inhibited biofilm formation by *C. acnes*, suggesting a broader anti-biofilm potential. These findings indicate that BWE may serve as a natural anti-biofilm agent capable of reducing biofilm-associated antimicrobial tolerance while exerting less selective pressure for the development of antimicrobial resistance than conventional antibacterial agents. Given that *F. velutipes* is an edible and commercially available mushroom, BWE may have potential applications in the prevention and control of biofilm-related infections, either alone or in combination with existing antimicrobial therapies. Further studies are required to identify the active compound(s) responsible for this activity and to elucidate the underlying mechanism of action.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/amh71030025/s1>, Supplementary Methods; Figure S1: Proton nuclear magnetic resonance (^1H NMR; 400 MHz; deuterated methanol, MeOD) spectra of fraction 2; Figure S2: Magnified proton nuclear magnetic resonance (^1H NMR; 400 MHz; deuterated methanol, MeOD) spectra shown in Figure S1; Figure S3: ^{13}C nuclear magnetic resonance (NMR; 100 MHz; deuterated methanol, MeOD) spectra of fraction 2.

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