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# *Impact of an upcycled Humulus lupulus extract on atopic skin microbiota*

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## 1. Introduction

Atopic Dermatitis (AD), also known as atopic eczema, is a chronic and inflammatory skin disease characterized by the combination of impaired skin barrier function, immune dysregulation, and alteration of the microbiome. Each of these can modulate genetic and environmental factors. The pathogenesis of AD is complex and multifactorial. Individuals with AD often have genetic defects in the *FLG* gene, which encodes the protein filaggrin, essential for maintaining skin barrier integrity. This barrier dysfunction allows the penetration of allergens and irritants, triggering an overactive Th2 immune response, which leads to the overproduction of inflammatory cytokines to drive chronic inflammation and worsen symptoms. Clinical presentation includes pruritus, xerosis, and eczematous lesions. Over the past few decades, the incidence of AD has been increasing, particularly in developed countries, becoming a health problem on a global scale. AD leads to high healthcare costs and considerable morbidity with compromised quality of life (QoL) of patients [1], [2], [3], [4], [5], [6].

Microbiota plays a crucial role in the inflammatory process in atopic AD by influencing skin barrier function and immune responses. AD patients' microbiota is often dominated by *Staphylococcus aureus* (*S. aureus*), a Gram-positive bacterium normally present in small numbers on healthy individuals' skin and nasal passages. However, in patients with AD, *S. aureus* is often found in much higher numbers, both on lesional and non-lesional disease severity, as the bacteria can produce a variety of virulence factors that can exacerbate inflammation and disrupt the skin barrier. Notably, *S. aureus* is capable of forming biofilms—structured bacterial communities that adhere to surfaces and resist immune responses—further disrupting the skin barrier and sustaining inflammation. The formation of *S. aureus* biofilms has been directly

associated with worsening AD symptoms. In addition to the direct effects of *S. aureus*, the overall composition of the skin microbiome plays a critical role in the pathogenesis of AD. The skin microbiome in AD patients is often characterized by reduced diversity and overgrowth of *S. aureus*, which can disrupt the balance of commensal microorganisms and contribute to disease development and progression. In AD, dysbiosis occurs, with *Staphylococcus aureus* (SA) dominating and forming biofilms that disrupt the skin barrier and promote inflammation. *Staphylococcus epidermidis* (SE) typically protects the skin by inhibiting SA, but in AD, it can either reduce SA biofilms or contribute to inflammation when overgrown. Restoring microbial balance is a promising strategy in AD management [5], [6].

*Humulus lupulus* (hops) is widely used in the brewing industry to impart bitterness to beer, counterbalancing malt sweetness. Beyond brewing, hops have a long history in traditional medicine and have recently gained interest for dermatological applications. Hops are rich in bioactive compounds such as humulone, lupulone, and xanthohumol, known for their anti-inflammatory, antioxidant, and antimicrobial properties [7], [8], [9]. These properties make hop extracts valuable candidates for therapeutic and cosmetic formulations targeting skin conditions. Notably, hops-rich trubs are a byproduct of craft beer production, making them an attractive, sustainable source for bioactive compound extraction.

This study aims to evaluate the antimicrobial potential of a hop extract, derived from brewing industry dry hopping trubs, against two key microorganisms implicated in atopic dermatitis (AD): *Staphylococcus aureus* and *Staphylococcus epidermidis*. The goal is to assess whether hop extracts can help modulate the skin microbiome and contribute to restoring microbial balance, thereby offering a novel, sustainable strategy for AD management.

## 2. Materials and Methods

The hop extract used in this study was obtained from the trub generated during the dry hopping stage in the production of an India Pale Ale (IPA) craft beer (Letra F), kindly provided by Cervejeira Letra (Vila Verde, Portugal). This beer was brewed using a combination of three hop varieties—Cascade, Mosaic, and Citra—sourced from Yakima Chief Hops (USA).

Upon receipt, the trub (solid dry hopping residue) was subjected to centrifugation at 17,168 × g using a Gyrozen centrifuge. The recovered solid fraction was then frozen for subsequent extraction procedures. Solid–liquid extraction was performed by mixing the thawed trub with 96% ethanol in a 1:1 (wet solid-to-solvent) ratio, followed by processing in a mixing extruder to enhance extraction efficiency.

After extraction, the liquid phase was separated from the solid residue. The liquid extract was subsequently characterized by high-performance liquid chromatography (HPLC) using a Waters 2695 system equipped with a Surf C18 Ti 100 Å 5 µm column (250 × 4 mm, ImChem). The mobile phases consisted of methanol:formic acid and ultrapure water.

Microbiological quality of the extracts was assessed according to the European Pharmacopeia 2.6.12 monograph by the pour-plate method. Using Petri dishes with 9 cm, 1 ml of the sample prepared with peptone water (1:10 dilution) was add to each dish and incorporated with liquefied agar medium suitable for the cultivation of bacteria (TSA), or liquefied agar medium suitable for the cultivation of fungi (SDA) at not more than 45 °C. For each medium, two Petri dishes

were prepared. The plates were incubated for five days at 30 °C to 35 °C for bacteria and 20 °C to 25 °C for fungi.

The minimum inhibitory concentration (MIC) of the extracts was determined against *Staphylococcus aureus* (ATCC 6538) and *Staphylococcus epidermidis* (ATCC 17870) using the microdilution broth method with minor modifications. Briefly, fresh bacterial cultures were prepared in Mueller-Hinton Broth (MHB) and adjusted to a turbidity equivalent to a 0.5 McFarland standard. The bacterial suspensions were subsequently diluted in MHB to achieve a final concentration of approximately $5 \times 10^5$ CFU/mL in each well of a 96-well microplate. The extract was dissolved in DMSO and prepared in sterile MHB to obtain a final concentration of 0.033 mg/μL. Six serial dilutions of the extract were prepared in MHB (1:2). Then, 100 μL of each bacterial suspension and 100 μL of the extract dilution were added to each well. Growth control wells containing only medium and bacterial inoculum, and negative control wells containing only medium and extract were included in each assay. A solvent control was also included, consisting of 5% DMSO in the culture medium without the addition of extract. The plates were incubated at 37 °C for 24 hours. MIC was defined as the lowest concentration of the extract that visibly inhibited bacterial growth, assessed by visual inspection. The MIC was determined analytically by measuring the absorbance of the bacterial growth in the microplate at 600 nm using a microplate reader. Analytically, MIC was defined as the lowest extract concentration that resulted in no significant increase in absorbance compared to the control wells, indicating the inhibition of bacterial growth.

For the determination of the minimum lethal concentration (MLC), 10 μL aliquots from each well were streaked onto Tryptic Soy Agar (TSA) plates and incubated at 37 °C for 24 hours. MLC was defined as the lowest concentration at which no bacterial growth was observed after incubation.

The impact on biofilm formation was assessed against *Staphylococcus aureus* (ATCC 6538) and *Staphylococcus epidermidis* (ATCC 17870) using the crystal violet (CV) staining method. Briefly, bacterial suspensions were prepared in Tryptic Soy Broth (TSB) and adjusted to a turbidity equivalent to a 0.5 McFarland standard, then seeded into 96-well microplates and incubated at 37 °C for 24 hours to allow biofilm formation. For the prevention assay, the extract was added simultaneously with the bacterial suspension in 96-well microplates (1/4 MIC, ½ MIC, MIC, 2x MIC, 5x MIC), followed by incubation at 37 °C for 24 hours to allow biofilm formation in the presence of the extract. For the disruption assay, biofilms were first allowed to form by incubating the bacterial suspension alone for 24 hours at 37 °C, after which fresh medium containing the extract was added (1/4 MIC, ½ MIC, MIC, 2x MIC, 5x MIC, 10x MIC), followed by an additional 24-hour incubation. Following incubation, the wells were gently washed two times with phosphate-buffered saline (PBS) to remove planktonic cells. The remaining biofilms were heat-fixed at 60°C for 1 hour, and stained with 0.1% (w/v) crystal violet solution for 15 minutes at room temperature. Excess stain was removed by rinsing the wells carefully with PBS. The bound crystal violet was solubilized with 30% (v/v) acetic acid. The absorbance was measured at 570 nm using a microplate reader to quantify the total biomass.

Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test, considering $p < 0.05$ as statistically significant.

## 3. Results

Solid–liquid extraction using 96% ethanol resulted in recovery yields ranging from 17% to 38%, with the highest yield corresponding to 127.56 grams of extract per kilogram of dry hopping trub. In terms of overall composition, the extracts contained approximately 3.19 g/L of proteins and a total phenolic content of around 23 mg/L.

HPLC analysis of the extracts revealed that the ethanolic extraction of Letra F dry hopping trub yielded a composition predominantly rich in key hop-derived compounds, namely (Ad)Humulone, Xanthohumol, Colupulone, and (Ad)Lupulone. These compounds were identified and quantified based on their characteristic retention times and UV absorption spectra. The results confirm that concentrated ethanolic extraction is a simple and effective method for isolating hop-derived bioactive compounds—particularly alpha and beta acids—from IPA beer production residues. Notably, the extract was especially rich in Xanthohumol (~31.5%) and Colupulone (~46.15%), highlighting the potential of this method to produce high-value extracts suitable for therapeutic or cosmetic applications, as it will be evaluated in this study for AD management.

The extract demonstrated microbiological quality, with no microbial growth detected, as shown in Figure 1.

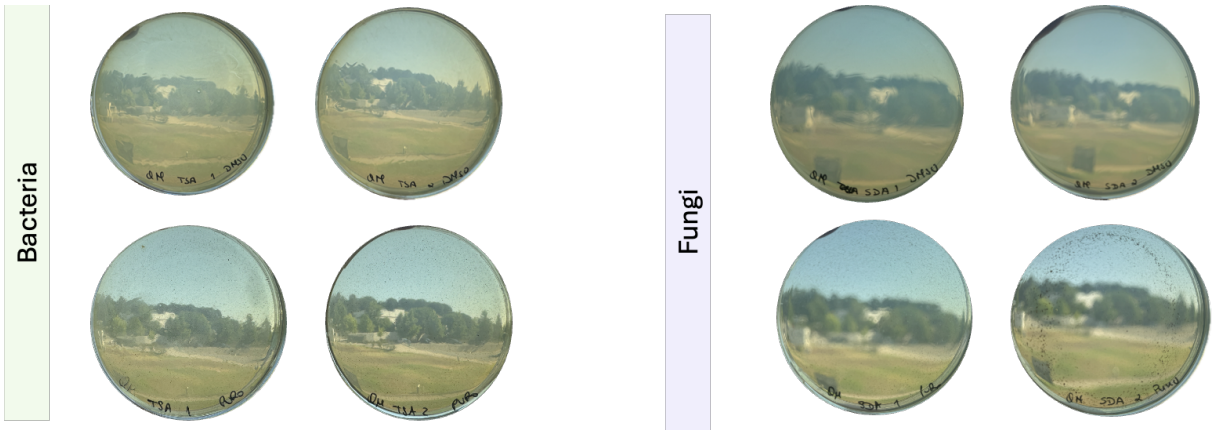


**Figure 1.** Microbiological quality results after 5 days. No bacterial or fungal growth was detected, and the landscape could be observed through the Petri dishes.

The results from visual MIC and MLC are compiled in Table 1 below. The extract demonstrated bacteriostatic activity, particularly in inhibiting *S. aureus´* growth.

**Table 1.** Visual MIC for both SA and SE was 0.129 (% w/v), while the MLC for SA was 0.515 (% w/v), and for SE was 1.030 (% w/v).

| | MIC (%w/v) | MLC (w/v) |
|---|---|---|
| *S.aureus* | 0.129 | 0.515 |
| *S. epidemridis* | 0.129 | 1.030 |

Analytically, for both microorganisms, the lowest extract concentration that resulted in no significant increase in absorbance compared to the control wells, indicating the inhibition of bacterial growth, starts at 0.002 (%w/v). The results from the analytical MIC are in Figure 2.

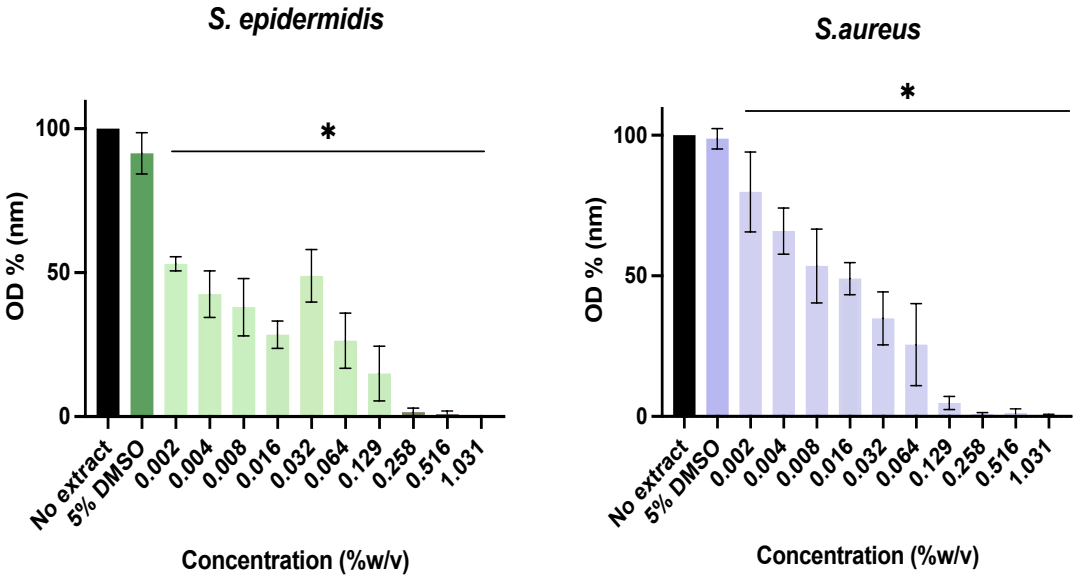


**Figure 2.** Analytical MIC of *S. aureus* and *S. epidermidis.* * Statistically significant result compared with control, as determined by one-way ANOVA with Dunnett's multiple comparisons test, with a p-value < 0.05.

When applied before biofilm formation, the extract showed a more significant effect on *S. aureus* biofilms at the MIC concentration, while a 5xMIC concentration was required for inhibiting the formation of biofilms of S*. epidermidis* and mixed biofilms of *S. aureus* and *S. epidermidis* (Figure 3).

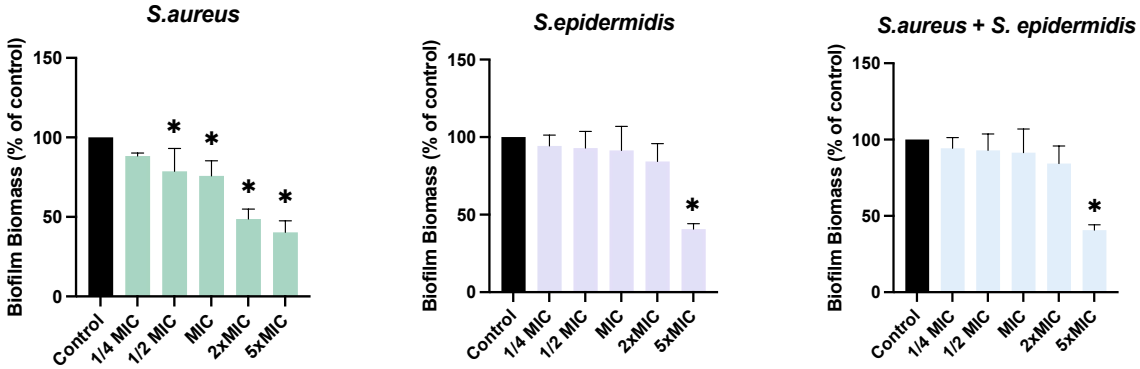


**Figure 3.** Extract applied before biofilm formation. These statistically significant results indicated that *S. aureus* is more susceptible to biofilm inhibition at lower concentrations. * Statistically significant result compared with control, as determined by one-way ANOVA with Dunnett's multiple comparisons test, with a p-value < 0.05.

When applied after biofilm formation, the extract also showed a more significant effect on the destruction of *S. aureus* biofilms and mixed biofilms of *S. aureus* and *S. epidermidis* at the

2xMIC concentration. In contrast, a 5xMIC concentration was required for the destruction of *S. epidermidis´* biofilms (Figure 4).

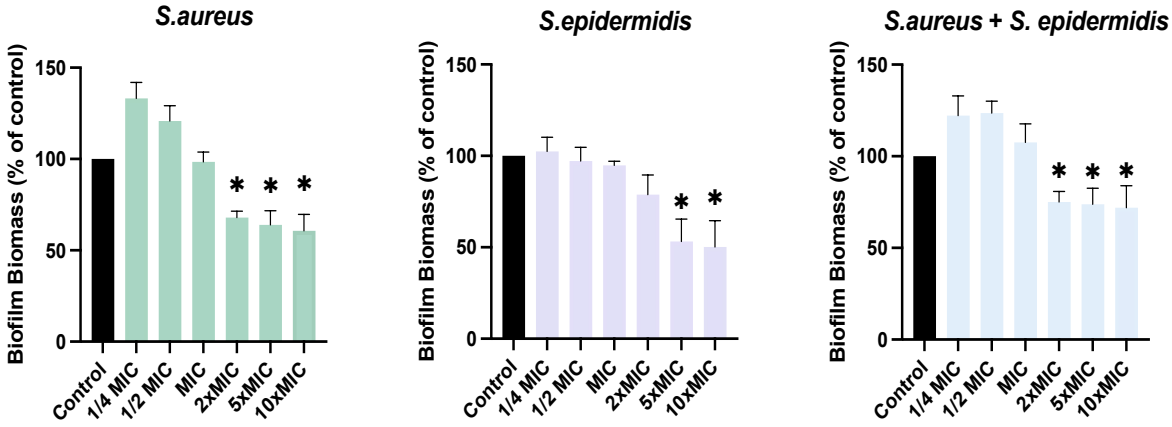


**Figure 4.** Extract applied after biofilm formation. These statistically significant results indicated that *S. aureus* and the combination of *S. aureus* and *S. epidermidis* are more susceptible to biofilm inhibition at lower concentrations. * Statistically significant result compared with control, as determined by one-way ANOVA with Dunnett's multiple comparisons test, with a p-value < 0.05.

## 4. Discussion

AD is a chronic inflammatory skin disease with complex pathogenesis, involving an interplay of genetic, immune, and environmental factors. One of the hallmark features of AD is skin barrier dysfunction, which predisposes affected individuals to increased colonization by pathogenic microorganisms such as *S. aureus*. *S. aureus* overgrowth in AD has been shown to exacerbate skin inflammation and disrupt the skin barrier by producing a range of virulence factors, including biofilm formation. As biofilm formation by *S. aureus* is linked to the severity of AD symptoms, disrupting this process presents a promising strategy for AD treatment. The role of the skin microbiota, particularly the balance between *S. aureus* and S. *epidermidis*, is crucial in the progression and modulation of AD. Dysbiosis, with an overgrowth of *S. aureus*, disrupts the normal microbiota and contributes to the pathogenesis of AD. Thus, strategies aimed at restoring the skin microbiome balance are essential for AD management [1], [4], [10].

The results of this study highlight the potential of hop extracts, derived from dry hopping trub—a byproduct of IPA beer production—as a promising strategy to modulate microbial imbalances associated with AD. The use of a 96% ethanolic solution proved effective, yielding up to 127.56 g extract per kg of trub and recovering key bioactive compounds, including high levels of co-lupulone (~46.15%) and xanthohumol (~31.5%). These compounds, identified through HPLC analysis, are well recognized for their antimicrobial and anti-inflammatory activities, making them particularly relevant in the context of AD [7], [11]. Importantly, the extract's composition aligns with the therapeutic goals in AD management. The condition is strongly associated with *S. aureus* overgrowth and reduced microbial diversity, often involving impaired *S. epidermidis* activity. The presence of compounds such as xanthohumol and humulone—which have been reported to inhibit *S. aureus* colonization and biofilm formation—suggests that the extract may

help reestablish microbial balance by selectively suppressing pathogenic strains while supporting commensals.

The microbiological quality of the hop extract was confirmed through a microbiological assay, demonstrating no microbial contamination or growth, ensuring its safety for further testing and supporting its potential application in cosmetic contexts.

The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of the extract were determined against *S. aureus* and *S. epidermidis.* The visual MIC for both bacteria was found to be 0.129% (w/v), indicating that the hop extract was effective in inhibiting bacterial growth at relatively low concentrations. The MLC for *S. aureus* and *S. epidermidis* were 0.515% and 1.030%, respectively, suggesting a bacteriostatic effect. These findings highlight the potential of the hop extract to effectively inhibit both *S. aureus* and *S. epidermidis*, which are implicated in the pathogenesis of AD. The extract demonstrated greater efficacy against *S. aureus*, consistent with the known ability of this bacterium to form biofilms and exacerbate AD symptoms.

The extract, applied before biofilm formation, significantly reduced biofilm formation by S*. aureus* at the MIC concentration, whereas a 5xMIC concentration was required to inhibit biofilm formation by *S. epidermidis* and the mixed biofilm of both bacteria. This differential effect suggests that *S. aureus* is more susceptible to the hop extract in terms of biofilm inhibition, which may be attributed to its more aggressive biofilm-forming ability. When applied after biofilm formation, the extract exhibited a similar trend, with *S. aureus* and the combined *S. aureus* + *S. epidermidis* biofilms being more readily inhibited at lower concentrations compared to *S. epidermidis* biofilms. This finding suggests that the hop extract may not only prevent the formation of bacterial biofilms but also disrupt pre-existing biofilms, a crucial consideration in AD management, where biofilm-associated bacteria are difficult to eradicate.

These results are promising and suggest that hop extracts may serve as a valuable adjunct in AD management, particularly by targeting microbial dysbiosis and biofilm formation. The antimicrobial properties of hop compounds, particularly their ability to inhibit *S. aureus* growth and biofilm formation, could help restore the microbial balance on the skin and mitigate the inflammatory response associated with AD.

## 5. Conclusion

In conclusion, the hop extract from beer production byproducts demonstrates significant antimicrobial (bacteriostatic) activity, particularly against *S. aureus*, a key player in AD pathogenesis. Its ability to inhibit biofilm formation offers additional therapeutic potential, supporting its role as a novel candidate for managing AD and related skin conditions. Further research is warranted to evaluate the extract's broader dermatological applications and its potential to restore microbial balance and improve skin health in atopic dermatitis.

## 6. Acknowledgments

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