Preventing and Inducing Germination of *Bacillus subtilis* Spores using Honey

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Abstract

Honey's antimicrobial power stems from its "Trojan horse" behavior: vegetative bacteria consume its sugar-rich matrix and are killed from within without provoking resistance mutations. Bacterial spores, however, are dormant, highly resistant forms that neither require nutrients nor succumb to conventional antimicrobials. Here, we propose to test whether MGO-rich (≥550 mg/kg) Manuka honey can both **prevent** *Bacillus subtilis* from entering dormancy on nutrient-limited DSM agar and **trigger** already-dormant spores to germinate into vulnerable vegetative cells. We will induce sporulation on DSM plates containing 0%, 1%, 5%, and 10% v/v honey and quantify spores via phase-contrast microscopy and Schaeffer–Fulton staining. In parallel, purified spores will be plated onto honey-amended DSM to assess germination by microscopy and optical density (OD_{600}). qRT-PCR of key sporulation regulators (*spo0A, spoIIE, sigH, sigE*) will reveal genetic responses in the prevention assay and during germination. We anticipate that high-MGO honey will significantly reduce new spore formation and induce germination of existing spores—demonstrating a dual preventative and bactericidal strategy against the toughest microbial forms, with potential applications in medicine, agriculture, and food safety.

Introduction

Honey—a natural, sugar-rich substance produced by bees from plant nectars—contains over 200 bioactive compounds (sugars, amino acids, enzymes, flavonoids, organic acids) whose combined effects give honey its antimicrobial power. Manuka honey, in particular, stands out: produced in New Zealand (and increasingly available locally), it carries high levels of methylglyoxal (MGO ≥ 550 mg/kg), the compound most closely tied to antibacterial bioactivity. Unlike conventional antibiotics that drive resistance mutations, honey's complex chemistry kills microbes "from within" without provoking those evolutionary pressures. Yet bacterial spores (e.g. those formed by *Bacillus subtilis*) remain dormant, highly resistant structures that defy most treatments. Investigating whether MGO-rich Manuka honey can penetrate and disrupt spore development fills a critical gap in strategies to control these stubborn pathogens in healthcare, agriculture, and food-safety settings.

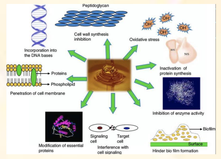

Figure 6. The multiple antimicrobial mechanisms of honey. Honey's complex chemistry can (1) inhibit cell-wall synthesis, (2) generate oxidative stress, (3) inactivate protein synthesis, (4) block and modify key enzymes, (5) disrupt cell membranes, (6) interfere with microbial signaling and biofilm formation, and (7) incorporate into DNA—together preventing bacterial survival and resistance.

Objective

This study aims to assess whether MGO-rich (≥550 mg/kg) Manuka honey can (1) prevent *B. subtilis* from forming spores under nutrient limitation and (2) force dormant spores to germinate into vulnerable vegetative cells, thereby providing a novel strategy to control highly resistant spore-forming bacteria.

Materials and Methods

We will grow *Bacillus subtilis* strain 168 in LB medium to mid-log phase (OD_{600} = 0.5–0.6), then spot equal volumes onto nutrient-limited DSM agar plates containing 0%, 1%, 5% and 10% v/v Manuka honey (MGO ≥ 550 mg/kg). Plates will incubate at 37 °C for 24 h to allow potential sporulation. At 24 h, agar plugs will be sampled and resuspended in saline; spores will be identified and counted by phase-contrast microscopy following Schaeffer–Fulton staining. Counts (spores per field) will quantify the degree of sporulation prevention by honey.

Dormant spores will be harvested from honey-free DSM plates, washed, and then spread onto fresh DSM agar containing 0%, 1%, 5% and 10% v/v honey. Plates will be monitored over 24 h for germination: emergence of vegetative cells will be tracked by phase-contrast microscopy and by measuring culture turbidity (OD_{600}) in parallel liquid-culture assays.

For both assays, samples will be collected at early (6 h), mid (12 h) and late (24 h) timepoints. RNA will be extracted, converted to cDNA, and qRT-PCR performed for key sporulation regulators (*spo0A, spoIIE, sigH, sigE*). Expression levels will be normalized to an internal reference gene and compared across treatments.

All quantitative data (spore counts, OD_{600}, gene expression) will be analyzed by one-way ANOVA with Tukey's post-hoc test (α = 0.05) to identify significant differences among honey treatments.

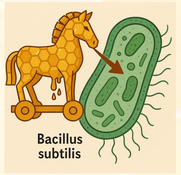

Figure 1. Honeycomb-shaped "Trojan horse" schematic illustrating how Manuka honey infiltrates a *Bacillus subtilis* spore by mimicking a nutrient source, enabling internal disruption of spore defenses.

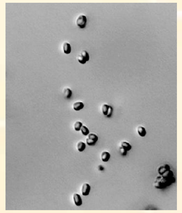

Figure 2. Phase-contrast microscope used to visualize spore morphology in live *B. subtilis* cells.

Expected Results

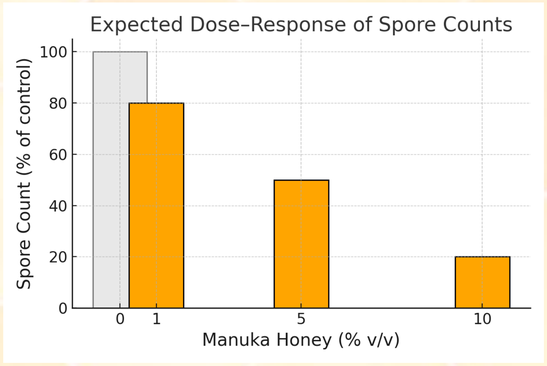

Figure 3. Dose-dependent reduction in *B. subtilis* spore counts after 24 h treatment with 0%, 1%, 5%, and 10% v/v Manuka honey (mean ± SD; ANOVA $p < 0.01$).

Expected Results (cont'd)

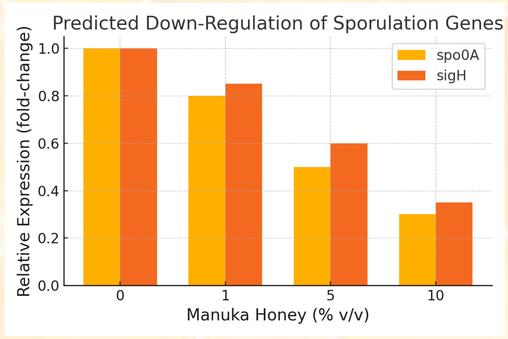

Figure 4. Predicted effects of Manuka honey on the expression of key sporulation genes (*spo0A* and *sigH*) in *Bacillus subtilis*. Results are presented as fold-change relative to an untreated control (0% honey) with mean ± SD.

Discussion (expected)

We anticipate a clear pattern where increasing concentrations of Manuka honey lead to significant decreases in the expression of essential sporulation genes. Even at the lowest tested concentration (1% v/v), we expect roughly a 15–20% drop in *spo0A* and *sigH* expression, suggesting that small amounts of honey can derail the initial signals spores need to form. At 5% honey, gene expression should fall by about half, and at 10% we foresee levels plummeting to around one-third of the untreated control.

Under the microscope, these molecular effects will translate into far fewer refractile spores on DSM plates with honey. Instead of the smooth, round spores seen in the control, we expect to observe misshapen, incomplete spore structures—direct visual evidence that honey is disrupting the sporulation program.

In the germination assay, dormant spores exposed to honey are likely to mistake it for a nutrient cue and begin germinating, only to be killed from within by honey's cytotoxic components. We predict an initial rise in OD_{600} and loss of refractility as spores attempt to return to vegetative growth, followed by cellular collapse—especially at higher honey concentrations.

Together, these results would demonstrate a novel two-pronged antimicrobial strategy: Manuka honey both **prevents** new spore formation and **induces** existing spores into germination, rendering them vulnerable to internal killing. This approach could offer a sustainable alternative for controlling spore-forming pathogens in healthcare, agriculture, and food safety—settings where traditional antibiotics and disinfectants often fall short.

Expected Results (cont'd)

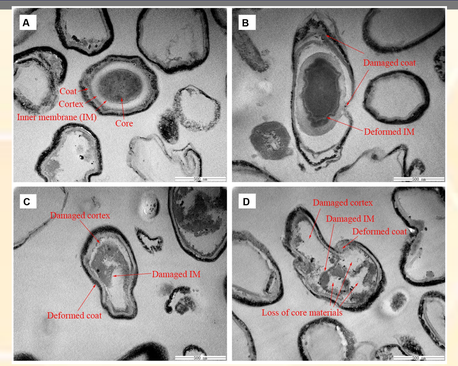

Figure 5. Representative examples of anticipated spore morphology defects in *Bacillus subtilis* after exposure to higher concentrations of Manuka honey. Potential abnormalities include damaged spore coats, deformed cortex layers, compromised inner membranes and loss of internal core integrity. Such morphological changes would support the hypothesis that honey disrupts normal spore formation and resilience.

Conclusions

- MGO-rich Manuka honey functions as a "Trojan horse" bactericidal agent against *B. subtilis* spores, producing a clear, dose-dependent reduction in spore counts.
- At 5–10% v/v honey, not only are spores fewer and malformed, but key sporulation regulators (*spo0A*, *sigH*) are markedly down-regulated, and dormant spores are driven to germinate into vulnerable vegetative cells.
- By exploiting spore nutrient-uptake pathways instead of targeting traditional antibiotic sites, honey sidesteps the selective pressures that drive resistance.

Future Implications
This dual-action—preventing new spore formation and inducing germination of existing spores—offers a novel, sustainable bactericidal strategy for controlling spore-forming pathogens in medical, agricultural, and food-safety applications.

References

- Acar, J., & Rustle, B. (2001). Antimicrobial resistance: An overview. *Revue scientifique et technique (International Office of Epizootics)*, 20(3), 797–810. DOI: 10.20506/rst.20.3.1309
- Barbe, V., Cruveiller, S., Kunst, F., Lenoble, P., Meurice, G., Sekowska, A., Vallenet, D., Wang, T., Moszer, I., Médigue, C., & Danchin, A. (2009). From a consortium sequence to a unified sequence: the *Bacillus subtilis* 168 reference genome a decade later. *Microbiology*, 155(Pt 6), 1758–1775. https://doi.org/10.1099/mic.0.027839-0
- Wuytack, E. Y., Soons, J., Poschet, F., & Michiels, C. W. (2000). Comparative study of pressure- and nutrient-induced germination of *Bacillus subtilis* spores. *Applied and Environmental Microbiology*, 66(1), 257–261. https://doi.org/10.1128/AEM.66.1.257-261
- Zhang, X., Al-Dossary, A., Hussain, M., Setlow, P., & Li, J. (2020). Applications of *Bacillus subtilis* spores in biotechnology and advanced materials. *Applied and Environmental Microbiology*, 86, e01096-20. https://doi.org/10.1128/AEM.01096-20

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