Brief Report

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Hot-water extraction, ethanol precipitation, and silkworm bioassays detect innate immune-stimulating activity in coffee extracts

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SUMMARY: A workflow combining hot-water extraction and ethanol precipitation can provide macromolecule-enriched fractions, but rapid *in vivo* evaluation of their innate immune-stimulating activity is not well established. We used coffee as a test matrix and evaluated the resulting fractions using a silkworm muscle-contraction assay and a *Pseudomonas aeruginosa* infection assay. Ethanol-precipitated fractions were prepared from three coffee origins, with or without lactic acid bacterial fermentation, under light- or dark-roast conditions. All sample groups showed measurable muscle-contraction activity. Specific activity ranged from 0.74 to 9.1 U/mg and was higher after light roasting in 11 of 12 matched conditions. Selected fractions increased survival at approximately 24 h after *P. aeruginosa* challenge, most clearly among Colombia EX samples; protection varied with bean origin, fermentation condition, roast, and dose. Non-fermented fractions were also active, indicating that fermentation was not uniformly required. These results support the feasibility of combining hot-water extraction, ethanol precipitation, and silkworm testing to detect bioactivity in macromolecule-enriched fractions. The method may be useful for prioritizing samples for chemical characterization.

Keywords: *Bombyx mori*, *Pseudomonas aeruginosa*, prophylaxis, polysaccharide

1. Introduction

Hot-water extracts can contain diverse water-soluble macromolecules, but practical workflows for enriching these materials and evaluating their *in vivo* biological activity remain limited. Coffee was selected as a test matrix because brewed coffee contains substantial soluble dietary fiber, including galactomannan- and arabinogalactan-related structures, whose abundance and fine structure depend on bean type, roasting, and brewing conditions (*1-3*). Ethanol precipitation has also been used to recover a coffee polysaccharide fraction that stimulated macrophage and intestinal immune readouts (*4*). These findings support the use of coffee to evaluate a workflow for biological screening of macromolecule-enriched fractions.

Silkworm bioassays can provide an intact-host component of such a screening workflow. In the silkworm *Bombyx mori*, the cytokine paralytic peptide is produced as an inactive precursor and is activated after tissue damage or recognition of microbial cell-wall components (*5-8*). Activated paralytic peptide induces cellular and humoral immune responses, and polysaccharide-dependent activation can be quantified using the silkworm muscle-contraction assay (*7-10*).

Silkworm infection models also provide functional readouts of host protection and have been used to study immune priming, lactic acid bacteria, bacterial virulence, and anti-infective agents (*11-20*). In particular, Miyashita *et al.* demonstrated that Gram-negative peptidoglycan or ingested bacterial stimuli can induce resistance to subsequent *P. aeruginosa* infection (*11,12*). Because lactic acid bacteria have been reported to activate innate immunity in silkworms (*13*), fermented and non-fermented coffee beans were included to examine whether fermentation was associated with the activity of the recovered fractions. We therefore examined whether a workflow combining hot-water extraction, ethanol precipitation, and silkworm testing could detect innate immune-stimulating activity, using coffee as a proof-of-concept matrix.

2. Materials and Methods

2.1. Coffee preparation, fermentation, and hot-water extraction

Green coffee beans from Guatemala SHB, Vietnam G1,

and Colombia EX were examined. Where indicated, beans were incubated with *Lactococcus lactis* 11/19-B1, *Weissella hellenica* 0916-4-2, or *Enterococcus faecalis* YM0831. Non-inoculated beans were included as comparators. Beans were roasted under light- and dark-roast conditions, ground, and extracted with hot water. Bean origin, fermentation condition, and roast were treated as sample descriptors rather than independent causal factors.

2.2. Ethanol precipitation

Two volumes (100 mL) of absolute ethanol were added to 50 mL of coffee extract. After incubation on ice for 1 h, precipitates were recovered by centrifugation (8,000 rpm, 10 min), washed with ethanol, dried, and redissolved in 5 mL of physiological saline. This operational fraction was expected to enrich macromolecules but was not chemically homogeneous.

2.3. Silkworm rearing

Silkworm eggs (*Bombyx mori*, strain KINSYU × SHOWA) were obtained from Ehime-Sanshu (Ehime, Japan). The larvae were reared at 27°C on an artificial diet (Silkmate 2S). On the first day of the fifth instar, each larva was provided with approximately 1 g of the diet, and larvae on the second day of the fifth instar were used for the experiments.

2.4. Silkworm muscle-contraction assay

Two-fold serial dilutions of each fraction were injected into isolated silkworm muscle specimens according to the established assay (*9*). Contraction was calculated as C = (initial length − post-injection length)/initial length. One unit was defined as the dose producing C = 0.15, and specific activity was expressed as U/mg of recovered material.

2.5. Silkworm *P. aeruginosa* infection assay

The silkworm infection assay was performed based on the methods described by Miyashita *et al.* (*11,12*). Silkworm larvae received ethanol-precipitated fractions at 0.5 or 5.0 mg/mL before being challenged with 50 µL of a 10^6-fold dilution of an overnight culture of *P. aeruginosa* PAO1. Physiological saline alone was administered before PAO1 challenge as the vehicle-treated, infected control. Survival was recorded approximately 24 h after infection. Each condition contained four larvae (n = 4). Survival results are presented descriptively as percentages without inferential statistical analysis.

3. Results and Discussion

3.1. Muscle-contraction activity

All sample groups yielded measurable activity, with specific activities ranging from 0.74 to 9.1 U/mg (Figure 1). Light-roast fractions were more active than their corresponding dark-roast fractions in 11 of 12 matched conditions; the exception was Vietnam G1 fermented with *E. faecalis* YM0831. The highest activity was observed for light-roasted Guatemala SHB fermented

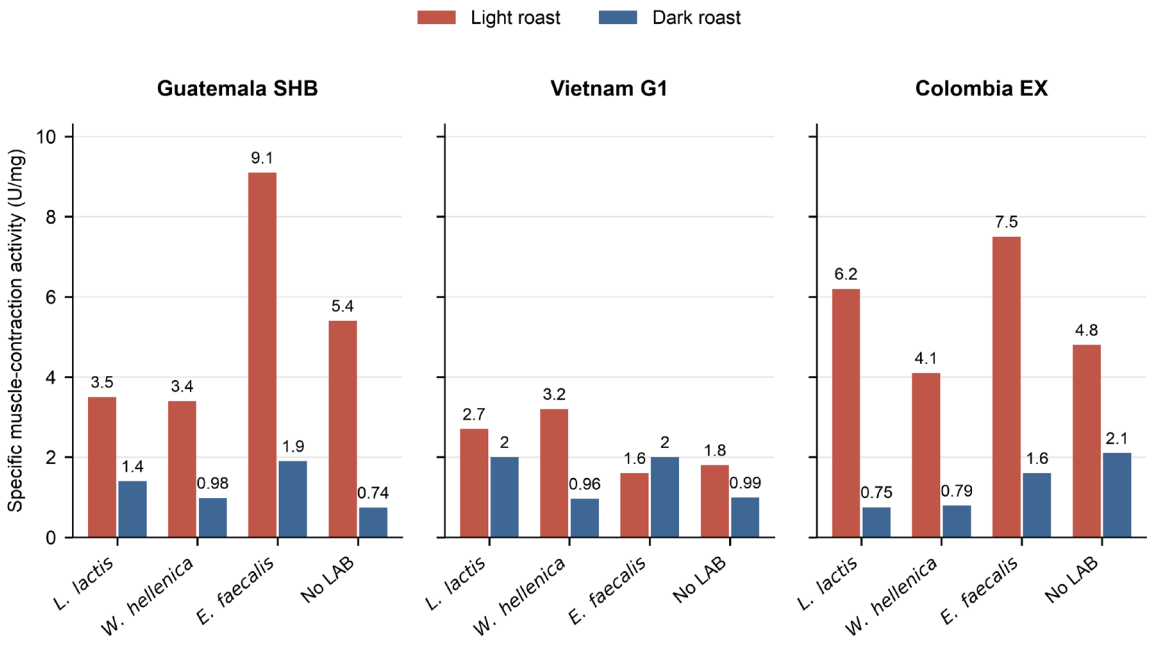


Figure 1. Specific silkworm muscle-contraction activity of ethanol-precipitated coffee fractions. Panels show Guatemala SHB, Vietnam G1, and Colombia EX fractions under the indicated fermentation and roast conditions. Numerical values above bars are specific activities in U/mg of recovered material. No LAB indicates the non-fermented comparator.

with *E. faecalis* YM0831 (9.1 U/mg), followed by light-roasted Colombia EX fermented with *E. faecalis* YM0831 (7.5 U/mg) and *L. lactis* 11/19-B1 (6.2 U/mg). Non-fermented controls were also active. Thus, ethanol-precipitable coffee constituents can stimulate the paralytic peptide-linked assay, consistent with previous work on immunostimulatory polysaccharides and silkworm cytokine activation (*6-10*), but fermentation was not uniformly required.

3.2. Prophylactic activity against *P. aeruginosa*

Survival in the saline-pretreated, PAO1-infected control group was 0/4 (0%). In the silkworm *P. aeruginosa* infection assay, survival at 0.5 mg/mL was limited for Guatemala SHB and Vietnam G1 fractions, whereas several Colombia EX fractions produced 50-100% survival (Figure 2). At 5.0 mg/mL, protective activity was broader: most Vietnam G1 and Colombia EX conditions produced 50-100% survival, while Guatemala SHB remained comparatively weak. Both fermented and non-fermented preparations were active in some conditions. These results do not establish fermentation as the sole determinant of survival. Muscle-contraction activity did not consistently predict survival. For example, light-roasted Guatemala SHB fermented with *E. faecalis* YM0831 had the highest specific activity (9.1 U/mg), but survival was 0% at both tested concentrations. The two assays measure different outcomes, and activity in the muscle-contraction assay does not necessarily translate into protection against infection.

3.3. Utility and limitations of the workflow

The principal outcome is methodological. When applied to coffee as a test matrix, the combined workflow produced ethanol-precipitated fractions that retained activity in two complementary silkworm assays. The muscle-contraction assay supplied a rapid quantitative readout, whereas the infection assay measured survival after bacterial challenge. Silkworm models are inexpensive, accommodate direct administration, and have supported infection biology and drug-discovery studies across bacterial and fungal pathogens (*14-20*). These features make the paired assays potentially useful for prioritizing ethanol-precipitated fractions from hot-water extracts before detailed purification.

The study has limitations. The muscle-contraction results were evaluated descriptively without inferential statistical analysis. The infection experiment used four larvae per group. Direct inhibition of *P. aeruginosa* by the coffee fractions was not excluded; improved survival therefore cannot be attributed specifically to host immune stimulation. Endotoxin and microbial carryover

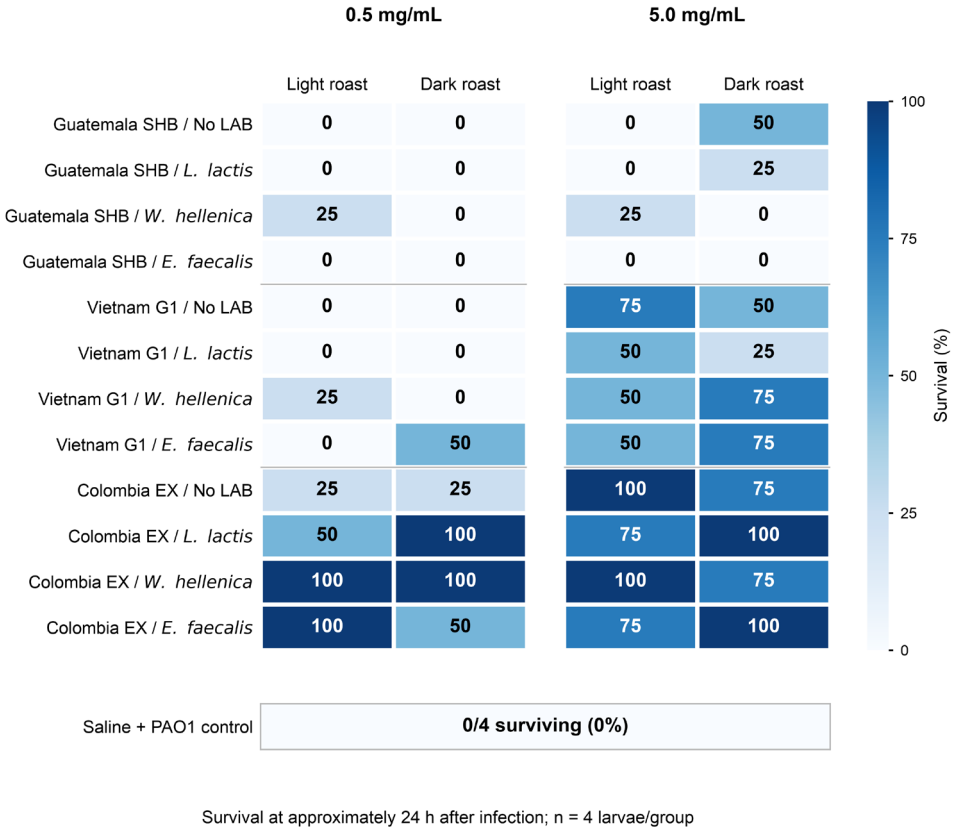


Figure 2. Prophylactic activity of ethanol-precipitated coffee fractions in the silkworm *Pseudomonas aeruginosa* infection model. Heatmaps show survival percentages at approximately 24 h after infection following pretreatment with 0.5 or 5.0 mg/mL fractions. Each cell represents four larvae. The saline-pretreated, PAO1-infected control is shown separately and had 0/4 surviving larvae (0%). No LAB indicates the non-fermented comparator.

were not quantified, and ethanol precipitation may recover polysaccharides, glycoproteins, aggregates, and other complexes. Generalization to tea, broth, or other beverages requires direct testing. Future studies should include individual-level data, measure extraction yield, include endotoxin controls, and chemically fractionate only samples with reproducible activity.

In conclusion, a workflow combining hot-water extraction, ethanol precipitation, and silkworm bioassays detected muscle-contraction activity and increased survival after bacterial challenge in selected groups treated with ethanol-precipitated fractions prepared from a hot-water extract. Coffee served as a proof-of-concept matrix; application to other hot-water extracts requires direct evaluation.

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