International Journal of Advanced Research and Multidisciplinary Trends (IJARMT)
An International Open Access, Peer-Reviewed Refereed Journal
Impact Factor: 6.4 Website: https://ijarmt.com ISSN No.: 3048-9458

Dietary Botanicals and Rearing Environment Shape Mulberry Silkworm (Bombyx mori L., PM × CSR2) Pupae Oil: From Larval Physiology to Oil Functionality

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Abstract
This study tests whether upstream husbandry—specifically, botanical treatments during the fifth instar and rearing environment—can tune downstream quality of mulberry silkworm (Bombyx mori, PM × CSR2) pupae oil (SPO). We run a 4 × 2 factorial: four phytogenic treatments applied as standardised larval sprays versus controls, crossed with laboratory and field rearing. Outcomes span larval performance (growth, silk gland index, survival), chitin yield, and oil functionality: yield and proximate composition, fatty-acid profile (GC-FID), minor lipids (HPLC tocopherols/sterols), physicochemical and oxidative indices (iodine, peroxide, p-anisidine, TOTOX; OSI by Rancimat), and in-vitro bioactivities (DPPH/ABTS/FRAP/ORAC; antimicrobial zones and MIC/MBC against Staphylococcus aureus, Escherichia coli, Candida albicans). We add a 30-day micro-stability trial in two simple prototypes (salad dressing; O/W base cream) at ambient storage. We expect botanicals with juvenoid-like or antioxidant constituents to improve growth and silk metrics, modestly shift UFA: SFA and n-6:n-3, and strengthen antioxidant and anti-staphylococcal signals; field rearing may widen variability owing to temperature/humidity swings and feed consistency.
Keywords: Bombyx mori; dietary botanicals; rearing environment; pupae oil; antioxidant; antimicrobial; chitin; product stability.

Introduction

Edible insect oils are moving to become more than just a novelty in food and health applications. Silkworm pupae are also unique due to their mass production, which is a foreseeable by-product of silk reeling, and makes silkworm pupae oil (SPO) a lipid readily accessible to fit circular-bioeconomy objectives. A literature review of Bombyx mori reveals potential lipid compositions, antioxidant compounds and possible bioactivity. However, the reported oil yields, fatty-acid distributions and stability measures are varied. Much of this dispersion is due to upstream decisions (to pre-process (roasting versus gentle drying), to extract (solvent versus supercritical CO2), and to assay (conditions)). A sensible way to go to cleaner comparisons is to go further upstream to husbandry. Growth, the formation of silk glands and body composition depend on the larval diet and rearing conditions, insects are not oilseeds, and the lipid pools of insects vary with physiology and stress. Constant temperature and humidity are likely to increase the performance and swings of the field are likely to reduce performance. These variations probably spill over into the quality of oil through the alteration

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of the fatty-acid balance and the small lipids. Another lever is botanicals: multiple plant extracts have been shown to be juvenile hormone mimics or otherwise to alter larval physiology, and the growth and silk outputs of many have been reported to improve. When diet alters physiology it can also tune oil functionality. SPO generally contains a lot of oleic, linoleic and a-linolenic acids and tocopherols and sterols which influence oxidation and health-relevant characteristics. The UFA:SFA, n-6:n-3, and tocopherol might also change due to diet-induced changes in shifting Rancimat induction times and chemical indices. Environment can also have a relationship with diet: laboratory cohorts are under constant conditions and with a constant quality of leaves; field cohorts are subjected to thermal and humidity levels and unreliable food. In addition to oil, a co-product, chitin, is also useful, and the yield of chitin is sensitive to species, stage of life and process; so that improvements in botanicals and environment can increase the yield of chitin. The research question presented in this study is a related biological-technological one, namely, do simple, field-compatible botanicals and the rearing environment have a significant impact on larval physiology and downstream oil quality? Our factorial design and track (i) larval performance, silk gland index and chitin; (ii) oil composition and minor lipids; (iii) oxidative and physicochemical quality; (iv) in-vitro antioxidant and antimicrobial activities; and (v) 30-day ambient micro-stability in two basic prototypes of products.

Literature Review

1) Diet, physiology, and lipid pools

The lipids of the silkworm pupa are triacylglycerides that are rich in MUFA and PUFA, with oleic, linoleic, and a-linolenic acids prevailing. This trend is supported by systematic reviews but emphasizes the variability depending on the strain, diet, and method and follows on the need to make the rearing context clearer. In addition to composition tables, the entomological literature demonstrates that diet may alter lipid metabolism. Plant-based juvenile hormone-like additives in B. mori tend to elevate the weight of larvae and the production of silk, suggesting the existence of common metabolic drivers that may act to change lipid deposition. A second bridge is antioxidant intake: tocopherols and phenolics in leaves or sprays can be stored in pupal tissues and oils which can enhance radical-quenching assays and increase the induction times provided that these minors are retained during extraction.

2) Rearing environment: laboratory versus field

Sericulture is based on temperature and humidity control. Unstable conditions during late stages of instar development may decrease survival and productivity, and stable conditions may enhance these outcomes. Usually, field rearing introduces expanded diurnal ranges, humidity variations and variability in leaves, stressors that have the potential to redistribute energy, influence lipid metabolism and alter oil stability. Environment is hence a significant mediator of any dietary effect.

3) Reported composition and bioactivities of B. mori pupae and oils

In literature, SPO is rich in oleic acids and linoleic acids with a significant presence of a-linolenic, tocopherols and sterols. Unsaturated lipids and smaller bioactives are most commonly reported to have antioxidant activities (DPPH, ABTS, FRAP, ORAC) and antimicrobial effects are most commonly observed against Gram-positive bacteria, e.g.

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Staphylococcus aureus. Several constituents - fatty acids, tocopherols, peptides - are likely to play a role, and therefore mechanisms that preserve minor components might increase in-vitro performance.

4) Chitin as a co-product

Chitin and chitosan from insects are well explored. B. mori offers workable yields and established isolation routes. If botanicals or environment increase biomass or support robust cuticle formation, chitin output could improve, strengthening small-scale economics that valorise both oil and shell fractions.

5) Evidence gaps and rationale

Despite many reports, head-to-head tests linking botanicals and environment to oil quality under matched starting material and common analytics are rare. Reviews repeatedly flag method variability and missing metadata (strain, diet, environment). A factorial experiment that joins larval physiology, chitin, and oil functionality in one pipeline addresses this gap with village-level realism.

Methods

Design and scope

We evaluate whether selected botanicals and the rearing environment shape larval physiology (growth, silk gland index, survival), chitin yield and purity, and oil quality (composition, stability, antioxidant and antimicrobial activity). To isolate upstream effects, extraction is fixed to a single route (Soxhlet–acetone) across all samples (Zhou et al., 2022; Tassoni et al., 2022). Two simple product prototypes are prepared to test 30-day ambient micro-stability.

Experimental design and allocation

Randomised factorial design with five botanical levels (including a vehicle control) crossed with two environments (laboratory versus field). Each cell has n = 8 trays (independent rearing units), balanced across batches to limit day and leaf effects. Power targets medium main effects and interaction detection. Outcomes include larval growth rate, survival, silk gland index, chitin yield/purity, fatty-acid profile (GC-FID), tocopherols/sterols (HPLC), peroxide and p-anisidine values, Rancimat induction time, and in-vitro antioxidant (DPPH, ABTS, FRAP) and antimicrobial assays against representative Gram-positive and Gram-negative strains. Interaction terms test whether environment amplifies or attenuates botanical effects.

Table 1. Factorial structure and replication

Factor A: Botanical treatment	Factor B: Environment	Unit of replication	Replicates per cell
Vehicle control	Laboratory / Field	Tray (independent cohort)	8
Tectona grandis extract	Laboratory / Field	Tray	8
Lantana camara extract	Laboratory / Field	Tray	8
Santalum album extract	Laboratory / Field	Tray	8
Vitis vinifera extract	Laboratory / Field	Tray	8

Notes: Treatment order randomised within environment; analysts blinded for lab assays. (Rumpold & Schlüter, 2013; Zhou et al., 2022)

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Botanical application and dosing

Treatments are applied during the 5th instar as either a standardised leaf-dip or topical spray using food-grade carriers. Doses and schedules are pre-set from pilot tolerability, with a vehicle-only control to account for carrier effects. Basic phytochemical fingerprints ensure consistency between lots.

Table 2. Botanical application schedule and identity checks

Botanical	Application route	Dose & schedule	Identity/quality checks
Vehicle control	Leaf-dip	Matching schedule	Carrier-only blank; no actives
Tectona grandis	Leaf-dip	Day 1 & 3 of 5th instar	TLC/UV profile; retention indices
Lantana camara	Spray	Day 1, 2, 4	TLC/UV; marker peaks
Santalum album	Spray	Day 2 & 4	LC profiling of sesquiterpenes
Vitis vinifera	Leaf-dip	Day 1, 3, 5	LC for polyphenols (catechin/gallic ranges)

Notes: Doses expressed as mg extract per 100 g fresh leaf; vehicle and droplet size standardised. (Sericulture texts; method alignment to edible-insect diet manipulations in reviews)

Rearing, health checks, and sampling

Mulberry leaf **variety and maturity** are standardised; feed intervals fixed; stocking densities aligned with sericulture practice. **Laboratory** rooms hold tight temperature/humidity; **field** sheds are monitored (logger data). Cohorts are harvested at the same post-reeling age across arms.

Table 3. Rearing & sampling variables

Domain	Laboratory setting	Field setting	Recorded variables
Climate	25 ± 1 °C; 70 ± 5% RH	Ambient; logged T/RH	Hourly T/RH; dew point
Feeding	Fixed cultivar & schedule	Same cultivar; local logistics	Leaf moisture; Brix
Health	Routine screening	Routine screening	Mortality; disease flags
Sampling	Fixed harvest age	Same	Larval weight; SGI; survival (%)

*SGI: silk gland index. (FAO, 2013; sericulture practice guides)

Processing and single-route extraction

We **fix extraction** to **Soxhlet–acetone** to isolate upstream influences:

1. Depuration; 2) Controlled drying (e.g., 50 °C to constant mass); 3) Milling; 4) Soxhlet with fixed solvent:mass, cycle count, time, and solvent recovery; 5) Oil yield reported **% w/w (dry basis)**.

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Table 4. Processing & extraction SOP (Soxhlet–acetone)

Step	Parameter	Acceptance/record
Drying	Temp/time to constant mass	Δmass < 0.1% across 2 h
Milling	Screen size	±0.1 mm target
Extraction	Ratio, bath temp, cycles, duration	Fully recorded; recovery > 95%
Post-extraction	Nitrogen blanket; amber glass	Headspace minimised

*(AOCS/ISO framing for solvent recovery and oxidative handling)

Analytical pipeline

We quantify **chitin**, **oil composition**, **oxidation**, and **bioactivity** using validated methods.

Table 5. Analytical methods and outputs

Domain	Method	Output(s)	Key references
Chitin	Deproteination–decalcification; gravimetry; FTIR purity	Yield (% w/w), purity (%)	Insect chitin workflows
FA profile	GC-FID (FAMEs)	MUFA/PUFA/SFA; UFA:SFA; n-6:n-3	Standard GC practice; Nowak et al., 2016
Minor lipids	HPLC (tocopherols/sterols)	mg/100 g oil	Method papers for tocopherols
Oxidation	Iodine, PV, p-AV, TOTOX; **Rancimat OSI**	Indices; OSI (h @ fixed T/flow)	ISO 6886; AOCS Cd 12b-92
Antioxidant	DPPH, ABTS, FRAP, ORAC	IC_{50} or TE/Fe^{2+} eq.	Benzie & Strain, 1996; Re et al., 1999; Ou et al., 2001
Antimicrobial	Zones; MIC/MBC/MFC vs S. aureus, E. coli, C. albicans	mm; % v/v (or µg/mL)	CLSI M07; EUCAST

*(ISO 6886; AOCS Cd 12b-92; CLSI, 2024; EUCAST, 2024; Shahidi & Zhong, 2015)

Prototype stability micro-trial

We test real-world behaviour of SPO in **two simple prototypes** at 25 °C (plus a 40 °C accelerated subset) for **30 days**.

Table 6. Stability prototype designs and endpoints

Prototype	SPO level	Matrix	Endpoints (baseline, 7, 14, 30 d)
Vinaigrette	5%	Oil-in-vinegar with standard emulsifier	Phase separation; PV; pH; colour; basic sensory
O/W cream	5%	Cosmetic base with defined emulsifier	Phase separation; pH; colour; odour note; PV

*(Shahidi & Zhong, 2015—quality indices in formulations)

Quality assurance (QA/QC)

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We apply **calibration, precision, and control** checks across assays.

Table 7. QA/QC thresholds

Check	Target
Calibration linearity	$R^2 \geq 0.995$ (5-point)
Spike-recovery	85–115% (composition); 80–120% (bioassays)
Precision (CV)	≤10% composition; ≤15% bioassays
Rancimat control	OSI within control chart limits
MIC QC	Reference strain within CLSI/EUCAST ranges
Botanical ID	TLC/UV or LC fingerprints per batch

*(ISO/AOCS; CLSI/EUCAST)

Statistics

Primary models use **two-way ANOVA** (Botanical, Environment, interaction). **Tukey HSD** handles multiple comparisons; effects are reported as **partial η²**. **Repeated-measures ANOVA** is used for 30-day stability endpoints. Correlation networks (Pearson/Spearman) link **larval metrics ↔ composition ↔ bioactivity**.

Table 8. Statistical plan

Component	Detail
Primary model	Two-way ANOVA (+ interaction)
Post-hoc	Tukey HSD ($\alpha = 0.05$)
Effect size	Partial η^2
Assumptions	Shapiro–Wilk; Levene; transform if needed
Repeated measures	RM-ANOVA for PV, pH, colour over 30 days
Networks	Correlations and clustered heat maps

*(Shahidi & Zhong, 2015—oxidation endpoints; standard ANOVA practice)

Results and Analysis

We will report larval **growth**, **SGI**, **survival**, and **chitin** by treatment and environment with 95% CIs and interaction plots. Oil **yield**, **FA profile** (including **UFA:SFA** and **n-6:n-3**), and **minor lipids** (tocopherols/sterols) will be summarised in tables and visualised as heat maps. Oxidative metrics (iodine, PV, p-AV, TOTOX) will be paired with **Rancimat OSI** at a fixed temperature and airflow (conditions stated explicitly, as OSI is method-dependent: ISO 6886; AOCS Cd 12b-92). Antioxidant outputs (DPPH/ABTS/FRAP/ORAC) will be harmonised to IC_{50} or TE and interpreted by mechanism (electron transfer vs hydrogen-atom transfer). Antimicrobial outcomes (zones; MIC/MBC/MFC) will be benchmarked to CLSI/EUCAST read across.

Table 9. Planned reporting elements

Domain	Visuals / tables
Growth & SGI	Means ± 95% CI; interaction plots
Chitin	Yield and purity table; violin plots
FA profile	Heat map of species; ratio plots (UFA:SFA; n-6:n-3)
Minor lipids	Tocopherols/sterols table with CIs

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Oxidation	PV/p-AV/TOTOX table; OSI box/violin + interaction
Antioxidant	DPPH IC_{50}; ABTS/FRAP/ORAC equivalents; correlation panels
Antimicrobial	Zones; MIC/MBC/MFC table; QC notes
Stability	PV growth curves; pH and phase trend charts

Findings & Discussion

We expect botanicals to influence larval physiology through two routes: (i) endocrine-like cues (juvenoid-type) that increase growth and silk gland development; and (ii) antioxidant/phenolic inputs that can be routed into tissues and, after extraction, appear as stronger radical-quenching capacity in vitro. This is consistent with reports that plant metabolites can modulate B. mori performance and with broader observations that dietary antioxidants affect insect matrices (Tassoni et al., 2022; Zhou et al., 2022; Rumpold & Schlüter, 2013).

Environment ought to be a moderator. More predictable lipid profiles and longer OSI may be enabled by laboratory cohorts with steady temperature and humidity, and fewer stress signatures, which is more likely to detect fewer stress signatures, but only in endogenous antioxidants are spared. The village units are governed by field cohorts, which are less predictable, but which are the fact of botanical effects; they may weaken or strengthen, according to the fluctuations of heat and moisture. It is important to map the interaction (botanical x environment) hence: a treatment that increases the growth in the lab and not the field is less of use in MSMEs (FAO, 2013; sericulture practice).

On oil composition, UFA:SFA and n-6:n-3 changes are feasible in case of botanical changes in metabolic allocation, or stress-modulation changes desaturation patterns. The tocopherols/sterols minor lipid pool is particularly fussy; an increase in tocopherols is expected to be associated with decreased PV and increased OSI, which is in line with the anticipated chain-breaking protection during oxidation (Shahidi and Zhong, 2015). These differences are likely to find their way in antioxidant assays: DPPH/ABTS/FRAP (electron-transfer) and ORAC (HAT) can increase with retained tocopherols and phenolics (Benzie and Strain, 1996; Re et al., 1999; Ou et al., 2001). On antimicrobial tests, we anticipate stronger anti-staphylococcal signals than activity against E. coli, consistent with envelope susceptibility differences and prior edible-insect reports (Rumpold & Schlüter, 2013; Zhou et al., 2022). All antimicrobial readouts will be interpreted strictly against CLSI/EUCAST controls to avoid artefacts from emulsifiers.

The stability micro-trial connects analytics to practice. If an arm shows longer OSI and lower PV rise in the lab, it should also perform better in vinaigrette and O/W cream over 30 days—less phase separation, slower peroxide growth, and fewer off-notes. This translation step is crucial for MSMEs: it turns lab metrics into actionable product choices (e.g., “use botanical X under field conditions for better dressing stability”). Finally, by pairing larval metrics ↔ composition ↔ function, correlation networks should reveal mechanistic links (e.g., tocopherols ↔ OSI; SGI or growth ↔ FA ratios), helping to explain why a given botanical–environment pair works.

Limitations of the Study & Future Work

Batch-to-batch botanical variability is inevitable; we minimise it with fingerprints but cannot remove it entirely. The focus on a single hybrid and region limits external generalisability; repeating across strains/voltinity and geographies is a logical next step (Nowak et al., 2016). All bioactivity tests are in vitro; they indicate potential but not clinical efficacy. Future work should include scaled feeding trials, extended shelf-life in retail-like packaging, consumer acceptance studies for taste/odour, and in-vivo digestibility/allergenicity assessments (Rumpold & Schlüter, 2013; Shahidi & Zhong, 2015).

Implications

For farmers, low-cost botanicals are a practical lever to improve growth and quality under real field conditions. For processors, choosing SPO from better-performing botanical × environment arms can lift tocopherols and stability, reducing the need for fortification. For policy and extension, the results can feed into by-product valorisation guidelines and MSME training: SOPs for dosing, timing, extraction, and basic stability checks (FAO, 2013; Zhou et al., 2022).

Conclusion

Botanical inputs during the 5th instar and the choice of rearing environment can shape not only larval performance and **chitin** yield, but also the **composition and functionality** of the resulting silkworm pupae oil. A simple, standardised pipeline—consistent rearing records, one fixed extraction method, validated analytics, and a short stability test—delivers clear signals that MSMEs can use. In practice: select botanical–environment pairs that raise tocopherols and keep **UFA-rich** profiles stable, then confirm performance in basic food and cosmetic prototypes before scale-up.

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