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Biotransformation of Bioactive Compounds in a Multi-Millet and Sprouted Fenugreek Formulation via Lactobacillus plantarum Fermentation

Jawahar Vel M V M[1] Dr.V. Sathiya[2]

[1]Ph.D. Research Scholar , Department of Nutrition and Dietetics,PSG College of Arts & Science.
[2]Assistant Professor, Department of Nutrition and Dietetics College of Arts & Science.

Abstract

Background: The synergistic fermentation of pseudocereals and legumes holds significant potential for developing functional foods with enhanced bioavailability of phytonutrients. Solid-substrate fermentation of grain and legume matrices with Lactobacillus plantarum has repeatedly been shown to increase free phenolic acid and flavonoid content relative to unfermented controls, largely through microbial esterase and glycosidase activity that liberates bound phenolics from the plant cell wall.

Methods: This study evaluates the functional enhancement of a standardized 522 g multi-millet and sprouted fenugreek powder mixture fermented with L. Plantarum. To assess the release of bound phenolic acids and intracellular components by fermentation with bacterial cultures targeted cell lysis via sonication, followed by comprehensive bioactive profiling using high-performance liquid chromatography (HPLC).

Results: Fermentation of the 522 g formulation reached peak acid production (1.52 pm 0.05% lactic acid) and a minimum ph of 3.72 pm 0.03 at 48 h. HPLC quantification revealed significant biotransformation across all targeted phenolics. Sonication-assisted cell lysis (F-L) yielded a +189.3% increase in Total Phenolic Content (412.30 pm 12.40 mg GAE100 g DW) and a +176.4% increase in Total Flavonoid Content (188.50 pm 6.80 mg QE100 g DW) compared to unfermented controls. Crucially, cell lysis released an additional 38.2% of total extractable phenolics over the non-lysed fermented group ($p < 0.001$).

Conclusion: Combining L. Plantarum fermentation with sonication-assisted cell lysis significantly enhances the yield and extractability of bound and intracellular bioactive compounds in multi-millet and sprouted fenugreek matrix formulations, providing a novel processing route for bioactive functional food development.

Keywords: Lactobacillus plantarum; millet; fenugreek; solid-state fermentation; phenolic acids; HPLC; functional food

1. Introduction

The rising demand for gluten-free and functional food products has driven research into the nutritional optimization of traditional grains and legumes. Millets are rich in dietary fiber, essential amino acids, and polyphenols, while sprouted fenugreek provides a robust profile of bioactive secondary metabolites,

including flavonoid glycosides, phenolic acids, and saponins. However, a substantial proportion of these phenolics exist in bound form, esterified to cell-wall polysaccharides or complexed with proteins, which limits their extractability and bioavailability in the raw matrix.Lactic acid bacteria (LAB) fermentation, particularly with Lactobacillus plantarum, is a well-established strategy for improving the phytochemical profile of cereal- and legume-based matrices. In sourdough-fermented quinoa, L. plantarum ATCC 8014 fermentation increased flavonoid and total phenolic content and radical-scavenging activity relative to unfermented flour (Chiș et al., 2020). Comparable enhancement has been reported across a range of substrates fermented with L. plantarum strains: solid-state fermentation of whole wheat flour markedly increased free-phenolic fractions and antioxidant capacity relative to unfermented flour, with total phenolic content rising by roughly 280–400% depending on the wheat genotype and fermentation regime.Pearl millet fermented with L. plantarum strains DHCU 70 and MCC 5231 for a probiotic beverage application showed total phenolic content rising from about 13.4 mg GAE100 g in the unfermented control to 42–48 mg GAE100 g post-fermentation, with total flavonoid content roughly doubling over the same period.A separate autoclavingfermentation-time study on pearl millet fermented with L. plantarum ATCC 8014 found that total phenolic and flavonoid content increased gradually over a 72-hour fermentation window, while total tannin content — an anti-nutritional factor — decreased, consistent with tannase activity in L. plantarum partially hydrolyzing condensed tannins. Notably, that study also observed a decline in phenolic content beyond roughly 60 hours of fermentation, attributed to further microbial catabolism of phenolic acids into non-phenolic derivatives, underscoring that the fermentation-time window itself needs to be empirically optimized rather than assumed.

Fenugreek responds similarly to bio-processing. Solid-state fungal fermentation of fenugreek seed cultivars produced significant increases in total phenolic and condensed tannin content by day 5, with HPLC analysis confirming the appearance of new bioactive compounds including vanillin, benzoic acid, and catechin that were not detected in the unfermented seed. Independently, simple soaking and germination (sprouting) of fenugreek seed has also been shown to raise total phenolic content and antioxidant activity relative to the raw seed, suggesting that the sprouting step already used in this formulation's fenugreek fraction should provide a phenolically enriched substrate before fermentation even begins.Taken together, the literature supports two testable expectations for the present matrix: first, that L. plantarum fermentation will measurably increase free phenolic acid, flavonoid, and total phenolic content relative to the unfermented multi-milletsprouted-fenugreek control; and second, that a meaningful fraction of that increase will only become quantifiable after disruption of bacterial and plant cell structures, since bound and intracellular phenolics are not captured by extraction alone. This study addresses the latter point directly by comparing sonication-assisted cell lysis against non-lysed extraction, a methodological contrast that is often assumed but rarely quantified in the millet–legume fermentation literature.

2. Materials and Methods

2.1 Formulation and Inoculation

A standardized 522 g powder mixture comprising multi-millet species—finger millet (Eleusine coracana), pearl millet (Pennisetum glaucum), and foxtail millet (Setaria italica) in equal proportions (130.5 g each, 75% ww total)—and sprouted fenugreek (Trigonella foenum-graecum, 130.5 g, 25% ww) was prepared under aseptic conditions. Fenugreek seeds were soaked in deionized water for 12 h at 28°C, germinated for 24 h at 28°C in dark conditions, cabinet-dried at 50°C for 12 h, and co-milled with raw millets to pass through a 60-mesh (250 µm) analytical sieve.

The matrix was inoculated with an active culture of Lactobacillus plantarum (ATCC 8014) at an inoculum density of 1.0 times 10^7 CFUg dry weight. Solid-state fermentation was performed in controlled environmental chambers at 37°C and 85% relative humidity for 48 h to drive enzymatic degradation and acidogenesis.

2.2 Experimental Design and Sampling

To isolate the independent contributions of fermentation and cell lysis to phytochemical yield, four experimental groups were evaluated:

Group	Description	Purpose
Unfermented control (UC)	522 g multi-millet + sprouted fenugreek matrix, no inoculation, held under identical time temperature conditions	Baseline phytochemical and microbiological profile
Fermented, non-lysed (F-NL)	Matrix inoculated with L. plantarum, fermented for 48 h, extracted without sonication	Isolates the fermentation effect on free (extractable) phenolics
Fermented, lysed (F-L)	Matrix inoculated with L. plantarum, fermented for 48 h, subjected to sonication-assisted cell lysis prior to extraction	Captures both fermentation-released and intracellularly boundentrapped phenolics
Sterile matrix control (SMC)	Matrix autoclaved (121^circC, 15 min), no live inoculum added, incubated identically	Controls for heatprocessing effects independent of L. plantarum metabolism

Each experimental group was evaluated in biological triplicate (n = 3), with each batch analyzed in analytical duplicate on HPLC (n = 6 data points per group). Sampling was performed at 0, 6, 12, 24, 36, and 48 h to evaluate kinetic progression.

2.3 Cell Lysis and Extraction

Cell lysis was executed using a high-intensity ultrasonic processor (VCX 750, Sonics & Materials Inc., USA) equipped with a 13 mm titanium probe operating at 20 kHz and 50% amplitude. Samples were sonicated for 10 min in pulse mode (5 s ON 5 s OFF) under continuous temperature monitoring (< 10^circC) in an ice-water bath. Bioactive compounds were extracted using acidified 80% aqueous methanol (0.1% vv HCl) at a 1:10 wv ratio for 2 h at room temperature under agitation. Homogenates were centrifuged at 10,000 times g for 15 min at 4°C, and supernatants were filtered through 0.22 mum PTFE syringe filters prior to chromatographic injection.

2.4 High-Performance Liquid Chromatography (HPLC) Analysis

Phytochemical quantification was performed on an Agilent 1260 Infinity II HPLC system equipped with

a Diode Array Detector (DAD) and a C18 reversed-phase column (250 mm times 4.6 mm, 5 mum particle size; Zorbax Eclipse Plus). The mobile phase consisted of 0.1% formic acid in ultrapure water (Solvent A) and 100% acetonitrile (Solvent B) delivered at a flow rate of 1.0 mLmin.

The linear gradient profile was: 0–5 min, 5–10% B; 5–25 min, 10–35% B; 25–35 min, 35–60% B; 35–40 min, 60–95% B; re-equilibration for 5 min. Standard calibration curves (1–100 mugmL) for gallic acid (R^2 = 0.9992), ferulic acid (R^2 = 0.9995), p-coumaric acid (R^2 = 0.9989), chlorogenic acid (R^2 = 0.9991), quercetin (R^2 = 0.9987), catechin (R^2 = 0.9994), and vanillic acid (R^2 = 0.9990) were constructed. Detection wavelengths were set to 280 nm (hydroxybenzoic acids, catechin) and 320 nm (hydroxycinnamic acids, quercetin).

2.5 Microbiological and Physicochemical Monitoring

Viable L. plantarum density was enumerated on de Man, Rogosa, and Sharpe (MRS) agar incubated at 37°C for 48 h. Slurry pH was measured with a calibrated digital pH probe, and titratable acidity (% lactic acid equivalent) was determined by titrating against 0.1 N NaOH using phenolphthalein indicator.

2.6 Statistical Analysis

Data are expressed as mean pm standard deviation. Normality and homoscedasticity were verified using Shapiro–Wilk and Levene's tests, respectively. Group means across UC, F-NL, F-L, and SMC were compared using one-way ANOVA followed by Tukey's HSD post-hoc test (alpha = 0.05). Kinetic dynamics were analyzed via two-way repeated-measures ANOVA. All statistical tests were conducted in SPSS Statistics version 28.0.

3. Results

3.1 Fermentation Kinetics

Fermentation of the 522 g multi-millet and sprouted fenugreek formulation demonstrated robust acidification and rapid microbial propagation over 48 h (Table 1).

Table 1: Fermentation kinetics of L. plantarum in multi-millet and sprouted fenugreek matrix

Time point (h)	pH	Titratable acidity (% lactic acid)	Viable L. plantarum count (log CFUg)
0	6.25 pm 0.05^a	0.18 pm 0.02^a	7.12 pm 0.10^a
6	5.80 pm 0.04^b	0.32 pm 0.03^b	7.85 pm 0.08^b
12	5.10 pm 0.05^c	0.58 pm 0.04^c	8.42 pm 0.11^c
24	4.25 pm 0.03^d	0.95 pm 0.05^d	9.15 pm 0.09^d
36	3.85 pm 0.04^e	1.35 pm 0.06^e	9.48 pm 0.12^e
48	3.72 pm 0.03^f	1.52 pm 0.05^f	9.20 pm 0.10^e

Values represent Mean pm SD (n = 3). Superscript letters within columns indicate statistically significant differences ($p < 0.05$).

The pH declined rapidly from an initial 6.25 pm 0.05 to 3.72 pm 0.03 at 48 h ($p < 0.001$), accompanied by a linear increase in titratable acidity to 1.52 pm 0.05% lactic acid equivalent. Viable cell counts peaked at 36 h (9.48 pm 0.12log CFUg), representing a 2.36log expansion before entering stationary phase at 48 h.

3.2 Phenolic and Flavonoid Profile

Solid-state fermentation combined with cell lysis produced substantial biotransformation of bound polyphenols (Table 2).

Table 2: Bioactive compound profile across experimental groups (mg100 g DW)

Compound	UC (mg100 g)	F-NL (mg100 g)	F-L (mg100 g)	% Change (F-L vs UC)
Gallic acid	12.45 pm 0.85^c	28.30 pm 1.20^b	38.60 pm 1.45^a	+209.9%
Ferulic acid	24.10 pm 1.15^c	62.40 pm 2.10^b	84.20 pm 2.80^a	+249.4%
p-Coumaric acid	15.20 pm 0.90^c	31.50 pm 1.50^b	42.80 pm 1.85^a	+181.6%
Chlorogenic acid	8.60 pm 0.40^c	16.20 pm 0.80^b	21.50 pm 1.10^a	+150.0%
Quercetin	6.80 pm 0.35^c	14.90 pm 0.70^b	22.30 pm 0.95^a	+227.9%
Catechin	10.15 pm 0.50^c	21.40 pm 1.05^b	29.80 pm 1.30^a	+193.6%
Vanillic acid	5.30 pm 0.25^c	12.10 pm 0.60^b	17.40 pm 0.80^a	+228.3%
Total phenolic content (TPC, mg GAE100 g)	142.50 pm 5.20^c	298.40 pm 9.10^b	412.30 pm 12.40^a	+189.3%
Total flavonoid content (TFC, mg QE100 g)	68.20 pm 3.10^c	135.60 pm 5.40^b	188.50 pm 6.80^a	+176.4%

Values represent Mean pm SD ($n = 6$). Distinct superscript letters within a row indicate significant differences by Tukey's HSD ($p < 0.05$). SMC values remained statistically identical to UC ($p > 0.05$) and are omitted for conciseness.

Ferulic acid exhibited the highest magnitude of absolute liberation, rising from 24.10 pm 1.15 mg100 g in UC to 84.20 pm 2.80 mg100 g in F-L (+249.4%). Gallic, quercetin, and vanillic acid concentrations all tripled post-lysis ($p < 0.001$). Overall TPC increased from 142.50 mg GAE100 g in unfermented matrix to 412.30 mg GAE100 g in the lysed fermented treatment.

3.3 Impact of Cell Lysis on Recovery

Sonication-assisted cell lysis significantly outperformed standard non-lysed solvent extraction across all quantified parameters ($p < 0.001$).

Table 3: Lysis recovery enhancement (F-L vs F-NL)

Parameter	Non-Lysed Yield (F-NL)	Lysed Yield (F-L)	Lysis Net Increase (%)	ANOVA (F-statistic, p-value)
TPC (mg GAE100 g)	298.40 pm 9.10	412.30 pm 12.40	+38.2%	F = 312.4, p < 0.001
TFC (mg QE100 g)	135.60 pm 5.40	188.50 pm 6.80	+39.0%	F = 224.8, p < 0.001
Ferulic acid (mg100 g)	62.40 pm 2.10	84.20 pm 2.80	+34.9%	F = 189.6, p < 0.001
Quercetin (mg100 g)	14.90 pm 0.70	22.30 pm 0.95	+49.7%	F = 156.2, p < 0.001

All comparative metrics demonstrate that conventional solvent extraction leaves nearly 40% of microbial-transformed phenolics unquantified within cell-wall structures and intracellular envelopes.

4. Discussion

The observed biotransformation confirms that solid-state fermentation of multi-millet and sprouted fenugreek matrices with L. plantarum produces marked gains in free phenolic acids and flavonoids. Total phenolic content nearly tripled post-fermentation (+189.3%), aligning with reported trends in single-substrate fermented pearl millet and whole wheat flour. Microbial esterases, tannases, and beta-glucosidases synthesized by L. plantarum cleave ester and glycosidic bonds binding phenolic acids to matrix arabinoxylans and protein complexes.

The high release of ferulic acid (84.20 mg100 g) is especially noteworthy; ferulic acid is the primary hydroxycinnamic acid esterified to cell-wall hemicellulose in finger and pearl millets. Sprouting fenugreek prior to fermentation initiates endogenous phytase and protease activity, partial-hydrolysis that primes the matrix for deeper microbial enzymatic cleavage.

The primary methodological finding lies in the stark contrast between F-L and F-NL groups. Standard extractions (F-NL) missed over 38% of total extractable phenolics and nearly 50% of accumulated quercetin. Sonication-induced cavitational collapse disrupts both residual botanical plant cell fragments and L. plantarum cellular envelopes. This confirms that a substantial portion of biotransformed phenolics either remains entrapped within the dense polysaccharide matrix or is internalized absorbed by microbial

biomass during active fermentation.

From a practical perspective, combining germinated legume-pseudocereal blending with L. plantarum solid-state fermentation and mechanical cell disruption offers a scalable pathway for high-density functional food ingredients. The elevated levels of free hydroxycinnamic acids and flavonols improve functional antioxidant capacity while simultaneously mitigating anti-nutritional factors.

5. Conclusion

The targeted fermentation of the 522 g multi-millet and sprouted fenugreek matrix using L. plantarum ATCC 8014 significantly altered its biochemical profile, yielding a +189.3% increase in total phenolic (412.30 mg GAE100 g) and a +176.4% increase in total flavonoids (188.50 mg QE100 g). Sonication-assisted cell lysis released an additional 38.2% of total phenolic content over standard non-lysed extraction ($p < 0.001$), demonstrating that cell wall disruption is essential for full recovery of bio transformed bioactive. This multi-millet sprouted fenugreek bioprocessing framework provides an effective model for producing nutrient-dense functional food formulations.

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