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Fenugreek sprout extract as a natural probiotic enhancer and antimicrobial agent for sustainable reproductive health management

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Abstract

Reproductive pathologies such as mastitis and uterine infections remain major challenges in dairy production. with limited treatment success, they are difficult to treat, often recur, and lead to economic losses due to reduced fertility, antibiotic residues, withdrawal periods, and antimicrobial resistance. Probiotics, particularly lactic acid bacteria (LAB), are increasingly explored as sustainable alternatives; however, their effectiveness depends on growth, adhesion, and biofilm formation within host environments. This study evaluated the prebiotic and antimicrobial potential of germinated fenugreek seed extract (GSF), rich in polyphenols (170.3 ± 10.7 mg GAE/g) and flavonoids (79.8 ± 9.3 mg QE/g), on two LAB strains: *Streptococcus thermophilus (S. thermophilus)* and *Enterococcus durans* (*E. durans)*. At optimal concentrations (0.8–0.9 mg/mL), GSF significantly enhanced LAB planktonic growth by 25%, autoaggregation (71.9% vs. 65.1% in *E. durans*), and biofilm formation (30% in *S. thermophilus*). GSF also modulated membrane permeability, with protein release increasing up to 85.4% in *E. durans* compared to 4.9% in *S. thermophilus*, supporting quorum-sensing activity. Importantly, the combination of LAB cell-free supernatants with GSF extract exhibited synergistic inhibitory effects, with inhibition zones against *Escherichia coli* reaching 2.35 cm and *Staphylococcus aureus* 1.20 cm. These findings highlight the dual role of fenugreek sprout extracts as probiotic enhancers and antimicrobial agents, supporting their potential use as natural, antibiotic-free interventions to improve reproductive health and productivity in livestock.

Introduction

Lactic acid bacteria (LAB) are Gram-positive, non-sporulating, catalase-negative microorganisms that convert carbohydrates into lactic acid as the primary fermentation product (Holzapfel & Wood 1995). They are widely distributed in diverse ecological niches, including milk and dairy products, meat, mucosal surfaces, and fermented plant foods, where they contribute to food preservation and flavor development (Axelsson et al. 2024; Orhan & Pekacar 2019). Beyond their technological importance, LAB possess well-documented probiotic properties in both humans and animals, including antioxidant, anti-inflammatory, immunomodulatory, and antimicrobial effects derived from the production of organic acids, exopolysaccharides, and bacteriocins (Fontana et al. 2015; Li et al. 2014; Vieco-Saiz et al. 2019). Their ability to colonize the gastrointestinal tract and form biofilms enhances persistence and pathogen exclusion, though adhesion and viability remain key determinants of probiotic efficacy (Morikawa 2006; Van Halbeek 1994).

In veterinary medicine, reproductive pathologies such as mastitis, metritis, and endometritis remain major constraints to livestock health and productivity. Mastitis, primarily caused by *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli (E. coli)*, is one of the most prevalent and costly diseases in dairy cattle, reducing milk yield and quality while impairing animal welfare (Dalanezi et al. 2020; Sheldon & Dobson 2004; Williams et al. 2008). Similarly, uterine infections delay conception and compromise fertility, leading to substantial economic losses (Williams et al. 2008). Current treatments rely heavily on antibiotics, yet chronic infections often respond poorly, and the widespread use of antimicrobials raises serious concerns regarding residues, withdrawal periods, treatment costs, and post-therapy infertility (Várhidi et al. 2024). Moreover, the escalation of antimicrobial resistance (AMR) has emerged as a critical public health and food safety issue (WHO 2025). These challenges highlight the need for sustainable, antibiotic-free alternatives to promote reproductive health in livestock.

Probiotic microorganisms, including *Streptococcus thermophilus (S. thermophilus)* and *Enterococcus durans (E. durans)*, have emerged as promising candidates for preventing and mitigating reproductive tract infections (Adnane et al. 2024). heir beneficial effects derive from competitive exclusion of pathogens, secretion of antimicrobial metabolites, biofilm formation on mucosal surfaces, and modulation of host immune responses (Ashaolu et al. 2025; Khalid et al. 2025; Khera et al. 2025). However, the success of probiotic interventions depends on their ability to withstand hostile environments, adhere to epithelial cells, and form stable biofilms, characteristics that can be enhanced through exposure to natural bioactive compounds.

Plant-derived phytochemicals, particularly polyphenols and flavonoids, have been shown to modulate probiotic activity while concurrently suppressing pathogenic growth (Boubakeur 2016; Boubakeur et al. 2018; Milutinović et al. 2021; Pacheco-Ordaz et al. 2018; Sharma et al. 2024). Among these, fenugreek (*Trigonella foenum-graecum*) is recognized for its rich composition of bioactive molecules with antioxidant, antimicrobial, and immunomodulatory potential (Arya et al. 2023; Singh et al. 2022). Germination of fenugreek seeds enhances the concentration and bioavailability of these compounds, including polyphenols, flavonoids, and saponins, yielding sprouts with superior biological activity (Andrianne 2008; Chaubey et al. 2018). This transformation, also applied

in gemmotherapy or phytoembryotherapy, exploits the embryonic tissues of plants, which are abundant in nucleic acids, vitamins, trace minerals, and secondary metabolites such as rutin, isoquercitrin, astragalin, and chlorogenic acid (Andrianne 2008).

The present study investigates the potential of fenugreek sprout extracts as natural modulators of probiotic performance. Specifically, it evaluates their effects on the growth, aggregation, and biofilm formation of *S. thermophilus* CNRZ447 and *E. durans* NCBI 53345, as well as the combined antimicrobial activity of these LAB cell-free supernatants with fenugreek extracts against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922. By bridging plant phytochemistry with probiotic biotechnology, this research aims to establish fenugreek sprouts as a dual-function enhancer, strengthening probiotic efficacy while reducing pathogen load, thereby contributing to sustainable reproductive health management in livestock.

Materials and methods

LAB Strains and inoculum preparation

Two lactic acid bacteria (LAB) strains were used: *S. thermophilus* CNRZ447 and *E. durans* NCBI 53345. *S. thermophilus CNRZ 447* was obtained from the INRAE cultute collectin (Rennes, France), wherease *E. durans* was locally isolated from the traditional fermented product "Hamoum". Both strains had been previously characterized for pH and bile tolerance, thermal resistance, antibiotic susceptibility, antagonistic activity, adhesion capacity, and exopolysaccharide (EPS) production (Boubakeur et al. 2021). For inoculum preparation, each strain was cultured in M17 broth and adjusted to an optical density (OD) of 0.08 using the MacFarland 0.5 scale (Andrews & Testing 2008). Bacterial density was verified spectrophotometrically, and optimal absorbance wavelengths were determined by spectral scanning to ensure accuracy in cell concentration.

Fenugreek seeds disinfection and germination

The disinfection and germination of fenugreek seeds were performed following published protocols (Ojha et al. 2018; Zhu et al. 2024). Seeds were first immersed in hydrogen peroxide (H_2O_2) for 10 min with constant agitation, then rinsed three times with sterile distilled water. A secondary disinfection was performed with 75% ethanol for 2 min, followed by triple rinsing. For germination, disinfected seeds were transferred into perforated glass jars containing distilled water. After 4 h of soaking, water was discarded, and jars were inverted to promote drainage and aeration. Seeds were moistened daily with sterile water and incubated at 25°C under aseptic conditions. Germination rate was monitored using 20 seeds on moistened cotton in sterile 90 mm Petri dishes; germinated seeds were counted every 12 h for 4 days. Sprouts were then shade-dried, ground to 100 µm particle size, and stored for extraction.

Extract preparation and phenolic analysis

Two solvents were used to extract bioactive compounds from sprouted fenugreek seeds: distilled water and a glycerin-based solution. The glycerinic maceration was carried out directly in a mixture of water, glycerin, and alcohol, a combination that provides complementary physicochemical properties to improve extraction efficiency. This ternary solvent system promotes the recovery of a broader molecular spectrum and enhances the transfer of bioactive compounds from the fresh buds. Furthermore, the buds were stabilized immediately after harvesting without freezing, preserving their intrinsic biological activity and energetic potential (Andrianne 2008). After optimization, glycerin solution was prepared by mixing 20 mL glycerin, 10 mL methanol (99%), and 70 mL distilled water, resulting in a final volume of 100 mL. Extraction was conducted according to previously established protocol (Dixit et al. 2005). For each extraction, 5 g of powdered sprouted seeds were macerated in 50 mL of the respective solvent (1:10). The aqueous extract was macerated for 7 days at 4°C, whereas the glycerinic extract was incubated for 21 days under the same conditions. After maceration, the extracts were filtered, centrifuged and stored at 4°C. Microfiltration was performed prior to each use to ensure sterility and purity.

Total polyphenol (TP) were determined using the Folin-Ciocalteu method assay (Sachin 2023). Briefly, 100 µL of diluted extract was mixed with 500 µL of 1:10 Folin-Ciocalteu reagent and 1 mL of distilled water. After 1 min of incubation, 1 mL of 20% sodium carbonate solution was added. The mixture was vortexed and incubated for 8 min, followed by centrifugation at 6000 rpm for 10

min (Sachin 2023). Absorbance was measured at 730 nm using a spectrophotometer. Results were expressed as milligrams of gallic acid equivalents per gram of crude extract (mg GAE/g).

Total flavonoid were quantified by the aluminum chloride ($AlCl_3$) colorimetric method (Brighente et al. 2008). Equal volumes (2 mL) of extract and 2% $AlCl_3$ solution were mixed and incubated at room temperature for 1 h. Absorbance was measured at 415 nm. The concentration of flavonoids was calculated using a quercetin standard curve and expressed as milligrams of quercetin equivalents per gram of crude extract (mg QE/g). The extract with the highest concentrations of polyphenols and flavonoids was further subjected to qualitative profiling using high-performance liquid chromatography-mass spectrometry (HPLC-MS).

HPLC-MS/MS analysis

To characterize conjugated and free phenolic acids, two complementary extraction methods were employed (Fischer et al. 2011):

Method 1: Sugar-conjugated phenolics

To analyze phenolic acids bound to sugars, 100 µL of extract was mixed with 900 µL of an extraction solution composed of water, methanol, and formic acid in a volume ratio of 79:20:1 (v/v/v) (Fischer et al. 2011). The mixture was vortexed for 30 seconds, then sonicated for 10 minutes at 45°C to enhance homogenization and extraction efficiency.

Method 2: Free phenolics

To identify free phenolic acids released by hydrolysis, 100 µL of extract was vortexed with 200 µL of 2 M hydrochloric acid (HCl) for 30 seconds. The solution was hydrolyzed using sonication at 90°C for 40 minutes. After cooling, 700 µL of the same extraction solvent (water/methanol/formic acid, 79:20:1) was added (Fischer et al. 2011).

Supernatants were injected into the LC-MS/MS system, allowing detection of both glycosylated and aglycone phenolic species.

Effect of fenugreek sprout extract on LAB bacterial growth

To evaluate the prebiotic potential, *S. thermophilus* and *E. durans* were cultured in M17 broth containing fenugreek sprout extract at 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 mg/mL. Cultures were incubated aerobically at 42°C for 18 h. Growth was monitored by measuring OD at 558 nm, which was converted to colony-forming units (CFU/mL) using the correlation: 70% absorbance ≈ 1.5 × 10^8 CFU/mL (Lambin & German 1961). The concentration eliciting maximal growth without inhibition was considered optimal.

Biofilm formation assay

The effect of extract concentration on biofilm formation was assessed in 96-well microtiter plates as previously described (Boubakeur et al. 2022; Khadem et al. 2020). Overnight cultures (10^8 CFU/mL) were mixed with extract concentrations (0.5-1.0 mg/mL) and incubated at 42°C for 24 h under static conditions. Biofilms were washed with PBS (pH 7.2), stained with 0.1% crystal violet (30 min), rinsed, and air-dried. Bound dye was solubilized in 30% citric acid, and absorbance was measured at 570 nm to estimate biofilm biomass.

Membrane permeability was evaluated by quantifying extracellular proteins (Hashemi et al. 2018). Cultures grown with optimal extract concentrations (1.0 mg/mL for *S. thermophilus*, 0.8 mg/mL for *E. durans*) were centrifuged, and supernatant absorbance at 280 nm was compared to controls. The relative increase in extracellular proteins was calculated using the following equation:

$$\varvec{P}\ (\varvec{\%}) = \left[\raisebox{1ex}{\$}\left(\varvec{A}\varvec{} - \varvec{A}\right)\ \$\Big/\raisebox{-1ex}{\$}\varvec{A}\$\right] \times\ 100$$

where A is the absorbance at 280 nm of the supernatant from cultures treated with extract, and A_o is the absorbance of the control (without extract).

Auto and coaggregation assays

Autoaggregation and coaggregation abilities were measured following Trunk et al. (2018) Bacterial suspensions (OD_{600} = 1.0) were incubated with or without fenugreek extract at 37°C for 4 h. After sedimentation, absorbance of the upper phase was recorded to calculate aggregation (%). Coaggregation was determined by mixing equal volumes of both LAB strains and quantifying the decrease in OD over time.

Antibacterial activity of cell-free supernatants (CFS) and extract combinations

Antimicrobial activity of CFS derived from LAB cultures (with or without fenugreek extract) was evaluated using the agar-well diffusion method (Ma et al. 2022). Each strain was cultured in MRS broth with or without GSF (1 mg/mL) for 24 h at 37°C. Supernatants were harvested by centrifugation (7000 × g, 30 min, 4°C) and filtration through 0.25 µm membranes. To determine the contribution of reactive oxygen species, aliquots were treated with catalase (1 mg/mL, 30 min, 25°C). The antibacterial activity of the resulting supernatants was assessed using agar diffusion tests against *Staphylococcus aureus (S. aureus)* and *Escherichia coli* (*E. coli)*, with the inhibition zones measured to quantify activity. Supernatants obtained from probiotic culture without fenugreek seed extract served as controls.

Results and discussion

Phenolic content and chemical composition

Phenolic compounds were extracted from germinated fenugreek sprouts using two solvents: distilled water and a glycerin-based solution. The total phenolic and flavonoid contents of the resulting extracts are presented in Fig. 1, while the detailed chemical composition identified through HPLC-MS/MS analysis is shown in Table 1 and Fig. 2.

The extraction solvent had a statistically significant effect on the concentration of phenolic compounds ($p = 3.77 \times 10^{-5}$, post hoc test). The aqueous extract yielded the highest values, with 170.33 ± 10.67 mg GAE/g dry extract of total phenolics and 79.82 ± 9.27 mg QE/g dry extract of total flavonoids. These results demonstrate the strong solvent dependency of phenolic recovery and agree with previous reports.

For instance, Lohvina et al. (2022) reported total phenolic contents of 120.3 ± 6.1 and 102.6 ± 5.1 mg GAE/g dry extract in two fenugreek varieties extracted with 70% ethanol. Similarly, Hameed et al. (2019) compared water, methanol, and mixed solvents, finding that 70% methanol yielded the highest phenolic (281.8 ± 10.6 mg GAE/100 g) and flavonoid (174.4 ± 4.8 mg QE/100 g) levels.

Extraction efficiency is influenced by factors such as solvent polarity, target compound solubility, particle size, and extraction conditions (Lohvina et al. 2022). The elevated polyphenol and flavonoid concentrations observed in the present study can also be attributed to seed germination, which activates hydrolytic enzymes and enhances the bioavailability of bound phenolics. Supporting this, Ojha et al. (2018) demonstrated that soaking and germination of fenugreek seeds significantly increased phenolic and ascorbic acid content while reducing tannins, thereby boosting antioxidant capacity.

To further characterize the bioactive profile, the aqueous extract was analyzed by HPLC-MS/MS. Using standard compound libraries, 15 out of 30 reference phenolics were identified (Table 1; Fig. 2). The extract contained a diverse array of bioactives, including flavonoids such as naringin (flavanone glycoside), luteolin (flavone), isorhamnetin and kaempferol (flavonols); phenolic acid derivatives like propyl gallate, ethyl gallate; phenolic acids, mainly gallic acid, protocatechuic acid, 2,5-dihydroxybenzoic acid, salicylic acid, *p*-coumaric acid, sinapic acid, and trans-ferulic acid; and other secondary metabolites such as indole-3-acetic acid and abscisic acid

This profile corroborates earlier reports by Benayad et al. (2014) and Khole et al. (2014), who identified luteolin, gallic acid, p-coumaric acid, and kaempferol as dominant constituents in germinated fenugreek. The abundance of hydroxycinnamic and flavonol derivatives underscores the strong antioxidant and antimicrobial potential of the germinated extract, which likely contributes to its probiotic-enhancing activity observed in subsequent assays.

Table 1
HPLC-MS/MS profile of the aqueous extract of germinated fenugreek seeds

Compound	RT (min)	Response	Concentration (ng/mL)
Gallic Acid	1.669	1346	31.03
Protocatechuic Acid	1.814	4303	200.86
2,5-Dihydroxybenzoic Acid	2.025	1716	1028.50
Salicylic Acid	3.691	913	101.76
p-Coumaric Acid	3.978	455	160.57
Naringin	3.988	439	68.46
Sinapic Acid	4.026	118	236.56
Ethyl Gallate	4.027	15	0.21
Trans-Ferulic Acid	4.045	55	86.84
Indole-3-Acetic Acid	4.122	49	2.95
Propyl Gallate	4.222	81	0.47
Luteolin	4.313	695	7.10
Abscisic Acid	4.339	214	55.37
Isorhamnetin	4.362	568	1.35
Kaempferol	4.397	45	6.58

Effect of GSF Extract on Planktonic and Sessile Growth of S. thermophilus and E. durans

The effect of GSF on both planktonic and sessile (biofilm) growth of *S. thermophilus* CNRZ 447 and *E. durans* NCBI is illustrated in Fig. 3. Distinct concentration-dependent responses were observed for each strain, revealing a differential physiological adaptation to the extract. For *S. thermophilus*, a slight reduction in planktonic growth was observed at low extract concentrations (C_1-C_3; 0.5–0.7 mg/mL), where cell densities decreased from 8.40 Log CFU/mL in the control to 8.15–8.20 Log CFU/mL. This reduction coincided with a modest, dose-dependent increase in biofilm formation, with the Log N of biofilm-associated cells ranging from 8.10 to 8.28, compared with 8.16 in the control. These findings suggest that under mild phytochemical stress, *S. thermophilus* may favor a sessile mode of growth as a protective adaptation, consistent with the biofilm activation mechanism (Bassi et al. 2017).

At higher extract concentrations (C_4 and above; ≥0.8 mg/mL), an inverse trend was noted. Planktonic growth increased significantly, with Log N values ranging from 8.72 to 9.03, exceeding the control (8.40), whereas biofilm biomass declined slightly (Log N = 7.90–8.01 vs. 8.16 in control). This inverse correlation between planktonic proliferation and biofilm formation may reflect a concentration-dependent inhibition of adhesion or quorum-sensing pathways by certain fenugreek metabolites, leading to reduced surface colonization but enhanced cell division in suspension.

Conversely, *E. durans* exhibited a steady, concentration-dependent increase in planktonic growth across all extract levels, with Log N values ranging from 8.75 to 10.04, compared with 8.74 for the control. In contrast, biofilm-associated cell counts showed a slight yet consistent reduction (Log N = 7.88–8.01 vs. 7.73 in control). These results imply that the extract may interfere with the sessile transition of *E. durans*, potentially by modulating cell-surface proteins or disrupting quorum-sensing signals required for adhesion and biofilm maturation.

Taken together, these observations highlight that germinated fenugreek seed extract exerts strain-specific and concentration-dependent effects on LAB physiology. *S. thermophilus* tends to shift toward biofilm formation under low phytochemical stress but reverts to planktonic proliferation at higher concentrations, whereas *E. durans* maintains robust planktonic growth with limited biofilm development. Such differential behaviors likely arise from intrinsic differences in cell wall composition, exopolysaccharide synthesis, and regulatory networks governing stress adaptation and adhesion dynamics.

Effect of GSF on Bacterial Aggregation: Auto and Coaggregation

The aqueous extract of GSF markedly enhanced the aggregation behavior of both *E. durans* NCBI 53345 and *S. thermophilus* CNRZ 447. As shown in Fig. 4 (a, b), the extract stimulated autoaggregation in both strains after four hours of sedimentation.

For *E. durans*, the autoaggregation rate increased from 65.08 ± 0.02% in the control to 71.88 ± 0.88% in the presence of the extract, representing a statistically significant enhancement ($p < 0.05$). *S. thermophilus* also showed a moderate improvement, from 67.5 ± 3.30% in the untreated control to 68.13 ± 0.83% with the extract. These findings confirm that the germinated fenugreek extract exerts a measurable influence on bacterial cell–cell interaction and surface adhesion properties.

Coaggregation between the two lactic acid strains was comparatively low but exhibited a clear upward trend in the presence of the extract. The maximum coaggregation value observed after four hours reached 15.27 ± 0.04%, compared to 12.98 ± 0.31% for the control. Such enhancement, although moderate, indicates that certain components of the extract may facilitate interspecies adhesion, a key step in the establishment of mixed-species biofilms. Aggregation represents a critical early phase in biofilm development and contributes to colonization and ecological stability in complex microbial systems (Alotaibi 2021; Trunk et al. 2018). Both *S. thermophilus* and *E. durans* are naturally chain-forming bacteria with inherent autoaggregative properties (Tuncer & Tuncer 2014). The observed increase in aggregation following exposure to germinated fenugreek extract may be attributed to its rich polyphenolic composition.

Polyphenols, particularly gallic acid, are known to modulate bacterial surface charge and hydrophobicity, thereby enhancing aggregation and adhesion potential. Supporting this hypothesis, phenolic-rich extracts such as those from *Thymus fontanesii* have been reported to improve lactic acid bacteria aggregation behavior (Boubakeur 2016; Boubakeur et al. 2022). Likewise, (Kos et al. 2003) demonstrated that gallic acid significantly increased autoaggregation among probiotic strains. Collectively, these findings suggest that germinated fenugreek sprout extract promotes both auto and coaggregation in LAB through phenolic-mediated modulation of cell surface properties. This enhanced aggregative capacity may ultimately influence biofilm formation dynamics and contribute to improved colonization efficiency and probiotic stability.

Effect of GSF Extract on Membrane Permeability

A significantly higher protein permeability was observed in *E. durans* (85.42 ± 2.95%) compared with *S. thermophilus* (4.86 ± 1.00%), indicating a species-dependent membrane response to the extract (Table 2). Notably, no extracellular DNA was detected in either strain (ND), suggesting that the extract did not compromise membrane integrity to the extent of causing cell lysis or DNA leakage.

This pattern implies that the fenugreek sprout extract selectively modulates bacterial membrane permeability, possibly enhancing surface activity without structural disruption. Such controlled permeability may underlie the elevated autoaggregation and biofilm formation observed in *E. durans* (Fig. 4).

Table 2
Effect of fenugreek sprout seed extract on membrane permeability of lactic acid strains

Lactic Strain	Protein Permeability (%)	DNA Permeability
E. durans	85.42 ± 2.95	ND
S. thermophilus	4.86 ± 1.00	ND
(*ND: Non-detectable*)		

Kamaraju et al (2011) demonstrated that increased membrane permeability can facilitate the release of quorum-sensing autoinducers such as AI-1 (N-(3-oxodecanoyl)-L-homoserine lactone), which are crucial for intercellular communication and biofilm coordination (Kamaraju et al. 2011). The present findings are consistent with this mechanism, particularly for *E. durans*, where elevated protein release could enhance cell–cell signaling and cooperative behaviors within biofilm communities.

While the precise molecular effects of polyphenols on bacterial membranes remain incompletely characterized, several studies have linked phenolic interactions to modulation of lipid bilayer fluidity and selective permeability (Kamaraju et al. 2011; Saha et al. 2018). In probiotic systems, mild physical or enzymatic treatments, such as sonication or lysozyme exposure, have been shown to

increase membrane permeability, improve hydrophobicity, and strengthen biofilm formation (Giordano & Mauriello 2023; Khadem et al. 2020). The results of this study suggest that natural polyphenol-rich extracts, such as germinated fenugreek sprout extract, may achieve comparable membrane conditioning effects through a non-destructive, plant-based mechanism, thereby supporting probiotic stability and functionality.

Combined antibacterial activity of CFS and GSF

Antimicrobial assays using CFS from both LAB strains revealed differential inhibition patterns against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 (Table 3).

Table 3
Zone of inhibition (cm) of CFS and GSF combinations against *Staphylococcus aureus* and *Escherichia coli*

Test Condition	*Staphylococcus aureus*	*Escherichia coli*
S. thermophilus CFS	1.25 ± 0.07	2.15 ± 0.07
Catalase-treated *S. thermophilus* CFS	0.95 ± 0.07	1.85 ± 0.07
S. thermophilus CFS + GSF extract	1.20 ± 0.00	2.35 ± 0.21
Treated *S. thermophilus* CFS + GSF extract	1.00 ± 0.14	1.95 ± 0.07
E. durans CFS	1.15 ± 0.07	1.20 ± 0.00
Catalase-treated *E. durans* CFS	0.90 ± 0.10	1.05 ± 0.07
E. durans CFS + GSF extract	1.05 ± 0.07	1.50 ± 0.07
Treated *E. durans* CFS + GSF extract	1.18 ± 0.07	1.25 ± 0.07

The untreated *S. thermophilus* CFS exhibited the strongest inhibitory activity, particularly against *E. coli* (2.15 cm), compared to *S. aureus* (1.25 cm). Catalase treatment led to a marked reduction in inhibition, indicating that H_2O_2 or other reactive oxygen species (ROS) contributed significantly to the antibacterial activity. When combined with GSF extract, the inhibitory effect increased, especially against *E. coli* (2.35 cm), suggesting a synergistic interaction between bacterial metabolites and plant-derived phytochemicals.

For *E. durans*, overall antimicrobial activity was milder, with inhibition zones of 1.15 cm (*S. aureus*) and 1.20 cm (*E. coli*). Catalase treatment again reduced activity, reinforcing the role of peroxide-mediated inhibition. When combined with GSF extract, inhibition improved for both pathogens, though the magnitude of enhancement was less pronounced than that observed with *S. thermophilus*. Interestingly, the catalase-treated CFS + GSF combination of *E. durans* produced a slightly larger inhibition zone against *S. aureus* (1.18 cm) compared to the untreated mixture (1.05 cm), indicating that catalase treatment may alter the balance of active metabolites, potentially enhancing the activity of non-peroxide compounds when interacting with phytochemicals.

Overall, the presence of the fenugreek sprout extract consistently enhanced the antimicrobial effect of both bacterial supernatants, whereas catalase treatment diminished it, confirming the contribution of ROS and H_2O_2 to the observed inhibition. These results align with previous studies demonstrating that lactic acid bacteria produce a variety of antimicrobial agents, such as organic acids, bacteriocins, and peroxides, that collectively inhibit pathogenic bacteria (Arrioja-Bretón et al. 2020). The inhibition zones obtained here (1.20–2.35 cm) are consistent with those reported by Arrioja-Bretón et al. (2020) for *E. coli* (1.21–2.02 cm) and *S. aureus* (1.12–2.28 cm). Similarly, Masumuzzaman et al. (2021) observed strong antibacterial activity from *S. thermophilus* SMQ 301, particularly against *S. aureus*, although *E. coli* was generally more sensitive, a pattern mirrored in the present findings. This resistance pattern was similarly observed in the current study.

Reports on the antimicrobial activity of fenugreek extracts alone have been variable. Shaheed et al. (2018) observed inhibition zones of 0.9–1.05 cm against *E. coli* using different concentrations (50 and 100 mg/mL). In contrast, Babaei et al. (2018) found no inhibition with solvent extracts in agar diffusion assays, although the hydroalcoholic extract showed minimum inhibitory concentration (MIC) values of 100 mg/mL in broth microdilution tests. Such variability likely stems from differences in extraction method, solvent polarity, and bacterial susceptibility. Randhir et al. (2004) demonstrated that germination enhances antioxidant

and antimicrobial properties of seeds, which supports the current observation that germinated fenugreek extract exhibits stronger bioactivity and synergizes effectively with bacterial metabolites. However, other studies report opposite trends, Youssef & Sabra (2021) found that seed extracts were more active than germinated forms, while Kumar et al. (2023) attributed differential bacterial sensitivity to structural differences in the cell wall and surface charge, particularly in response to fenugreek-based nickel oxide (NiO) nanoparticles.

The relatively small inhibition zones obtained in this study may reflect high inoculum density, specific CFS composition, or strain-dependent antimicrobial potency. Nonetheless, even moderate inhibition levels are biologically relevant, as they indicate synergistic enhancement between probiotic secretions and plant phytochemicals. While direct *in vitro* evidence on LAB-fenugreek combinations is limited, *in vivo* studies have demonstrated that dietary supplementation of probiotics with fenugreek improves animal growth performance and reproductive parameters. Abdel-Wareth et al. (2021) reported that combined administration of probiotics and fenugreek in white rabbits improved weight gain, feed efficiency, and reproductive hormone levels, supporting the synergistic potential observed in the present work.

Conclusion

This study demonstrates that GSF, enriched with polyphenols and flavonoids, functions as a potent prebiotic enhancer for LAB strains *S. thermophilus* and *E. durans*. At optimal concentrations (0.8–0.9 mg/mL), the extract significantly enhanced bacterial growth, adhesion, and biofilm formation. Importantly, the extract also exhibited synergistic antibacterial activity against key reproduction tract pathogens, *S. aureus* and *E. coli*, highlighting its dual functionality as both a probiotic stimulant and antimicrobial agent. These findings underscore the promising potential of plant-derived germinated seed extracts to strengthen probiotic-based interventions in veterinary medicine. Such approaches offer a sustainable, natural alternative to conventional antibiotics for managing infectious diseases in livestock. Future investigations should focus on in vivo validation to confirm safety and efficacy, as well as on optimizing delivery systems for practical field applications.

Declarations

Research involving human participants and/or animals

Not applicable

Competing Interests

The authors declare that there are no conflicts of interest associated with this study.

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Author's contributions

Badra Boubakeur: Conceptualization, Resource, Methodology, Validation, Formal analysis, Writing - Original Draft. **Hafidha Khadem**: Conceptualization, Methodology, Reviewing and Editing. **Mounir Adnane**: Writing - Original Draft, Reviewing and Editing.

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Data Availability

The data that support the findings are available upon request from the corresponding author

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Figures

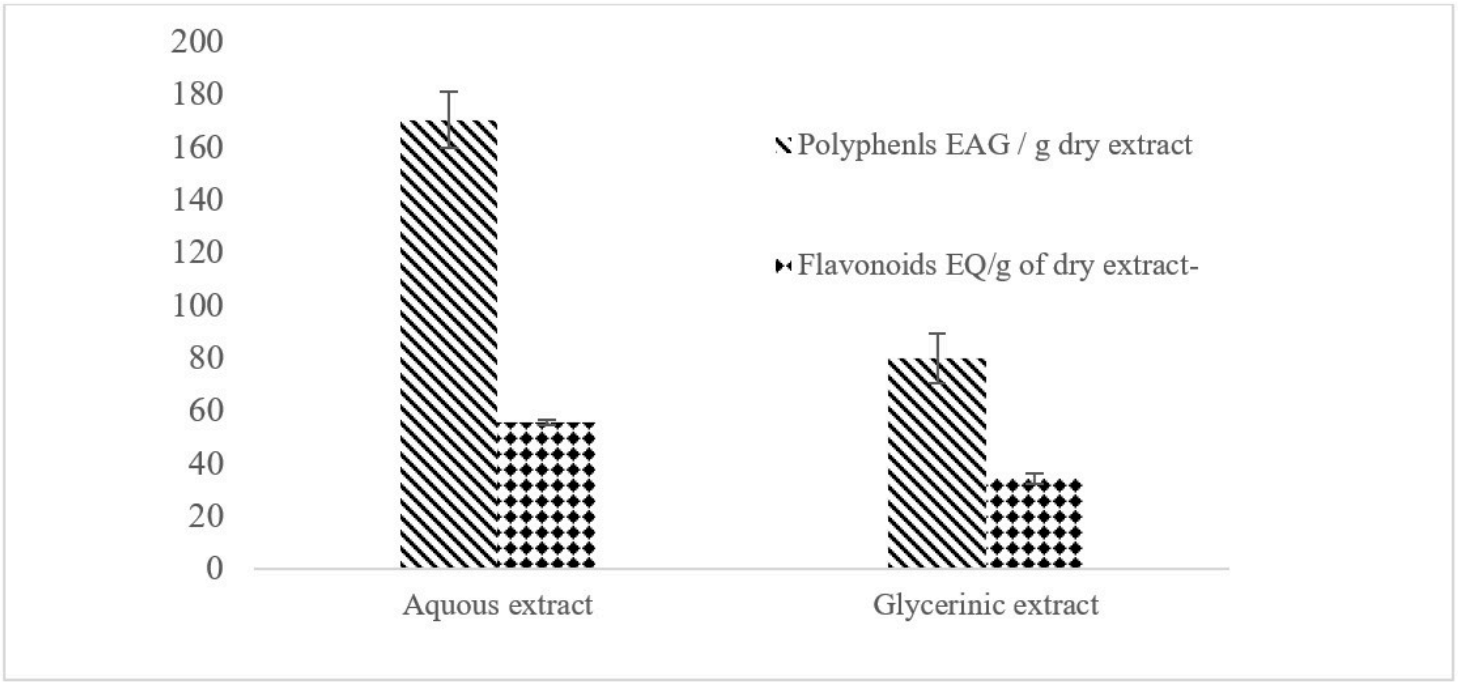


Figure 1

Total phenolic and flavonoid content of fenugreek sprout extracts obtained using two different solvents. The aqueous extract yielded a significantly higher concentration of both total phenolics (mg GAE/g dry extract) and total flavonoids (mg QE/g dry extract) compared to the glycerin-based extract. Data are presented as mean ± SD

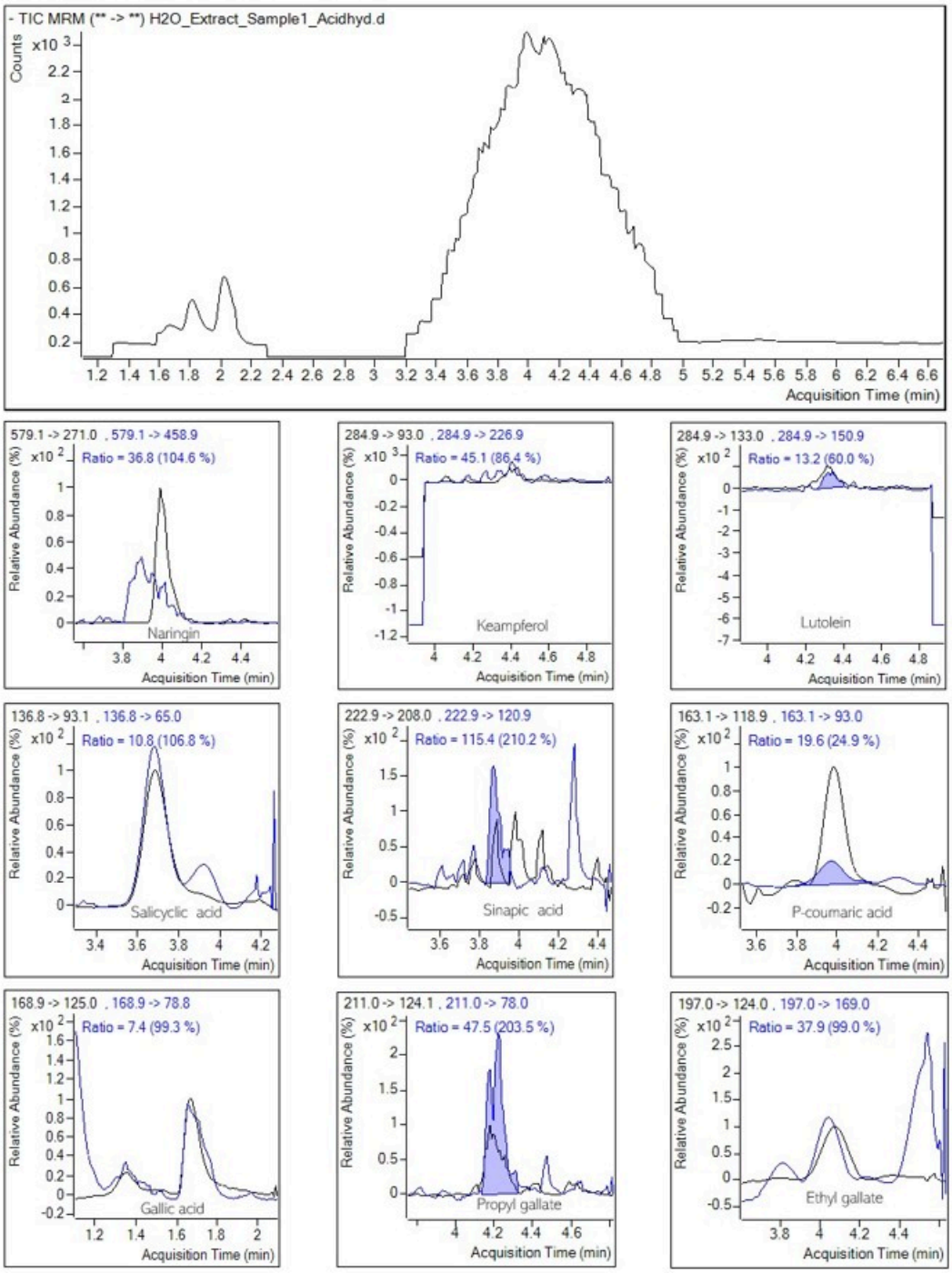


Figure 2

HPLC-MS/MS chromatogram of the aqueous germinated fenugreek seed extract. Key identified phenolic compounds are labeled, corresponding to the data in Table 1. The profile is dominated by phenolic acids such as 2,5-dihydroxybenzoic acid and sinapic acid, alongside flavonoids like naringin and luteolin

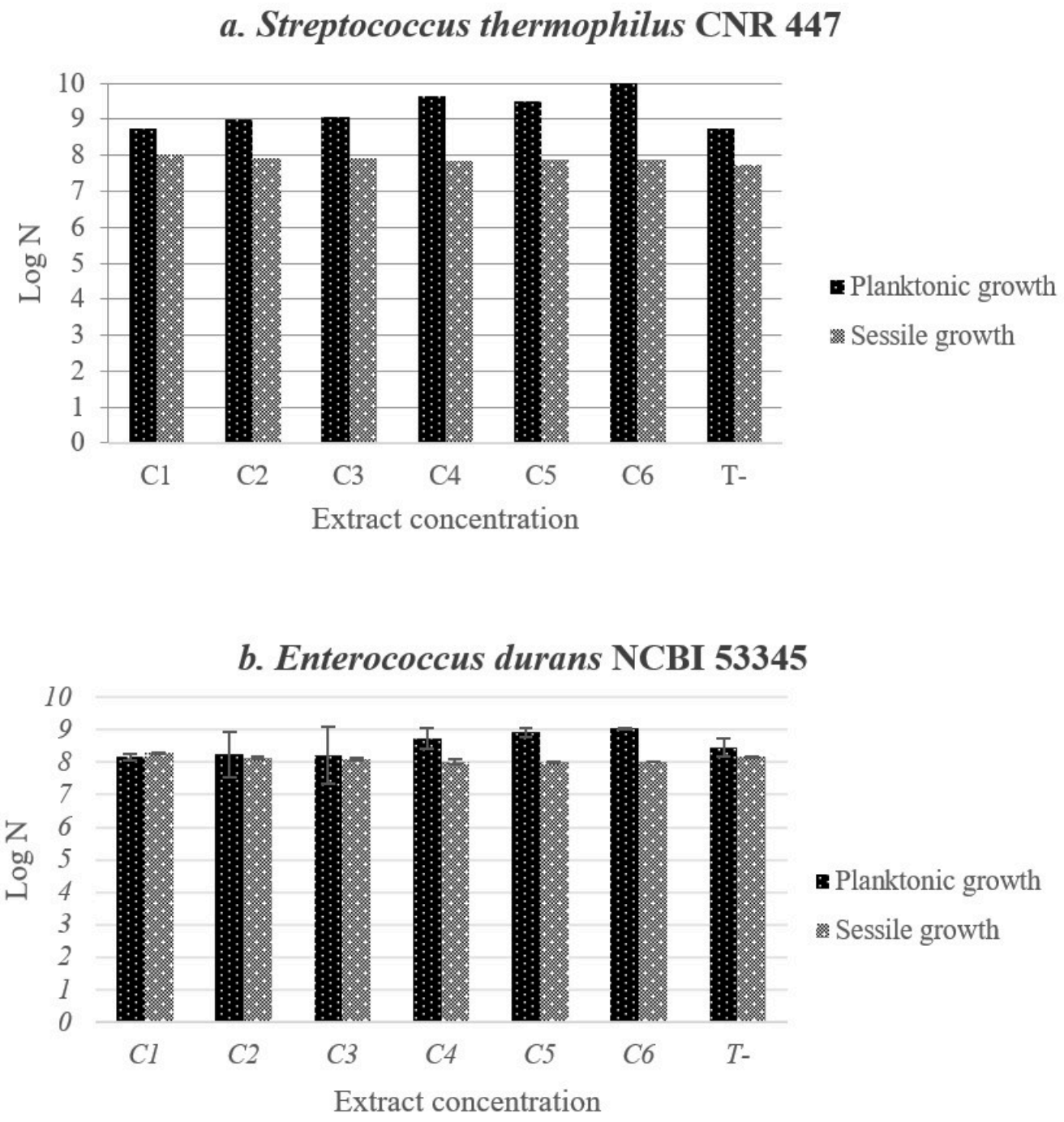
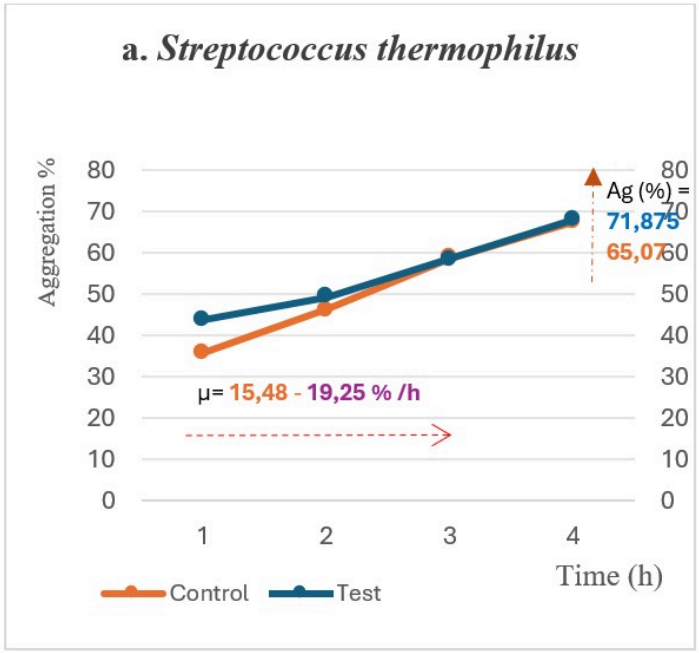


Figure 3

Differential response of bacterial growth modes to germinated fenugreek seed (GSF) extract. Planktonic and biofilm-associated growth (Log CFU/mL) of *Streptococcus thermophilus* (*S. thermophilus*) CNRZ 447 and *Enterococcus durans* (*E. durans)* NCBI 53345 under increasing concentrations of GSF extract (C1-C6 mg/mL). *S. thermophilus* shows a shift from sessile to planktonic dominance at higher concentrations, whereas *E. durans* exhibits enhanced planktonic growth with reduced biofilm across the concentration range. Error bars represent the standard deviation of three independent replicates. T-: Control culture without extract



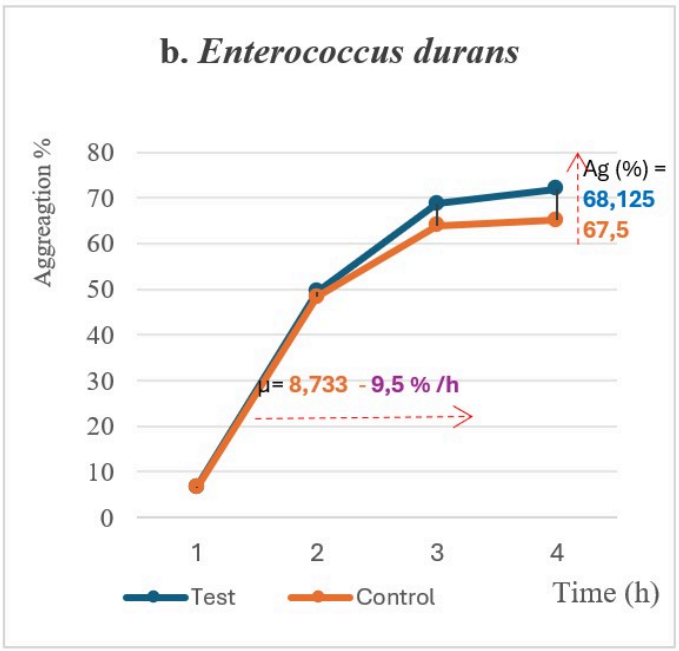


Figure 4

Effect of germinated fenugreek sprout (GSF) extract on bacterial aggregation. Autoaggregation of (a) *S. thermophilus* CNRZ 447 and (b) *E. durans* NCBI 53345 was measured after 4 hours of sedimentation. Values represent mean ± SD