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Postbiotic Potential of Enterococcus faecium Cell-Free Supernatant: Dual Antimicrobial Efficacy Against Bacterial and Fungal Pathogens with GC-MS/LC-MS-Q-TOF Metabolite Characterization

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Abstract

Rising antibiotic resistance and interest in natural, gut-friendly ingredients have brought postbiotics into focus as a safer alternative to live probiotics. In present study we looked at the CFS from *E. faecium* and tested what it could do against bacteria and fungi, then dug into what compounds were actually driving that activity. The results were strong. At 10% concentration, the CFS knocked down *S. aureus* growth by 96.22% and *S. typhi* by 95.08%, and the inhibition climbed steadily as we moved from 1% up to 10%. Against *C. albicans*, it suppressed growth by 91.2% at the 9–10% range, not far off fluconazole's 94.7%. To figure out what was behind this, we ran a methanolic extract through GC-MS and LC-MS-Q-TOF. Lactic, palmitic and stearic acids were detected by GC-MS as organic and fatty acids. Enterocin 900, spermidine, phosphatidylglycerol, riboflavin, and biotin were all detected by LC-MS-Q-TOF, which displayed a broader spectrum. Numerous of these have been demonstrated to have their own antibacterial, anti-inflammatory, or gut-barrier qualities, indicating that several compounds are cooperating rather than a single standout molecule carrying out the majority of the work. In summary, *E. faecium* CFS is an interesting postbiotic candidate for functional food and antimicrobial applications due to its potent dual antibacterial activity and gut-relevant metabolite profile.

Introduction

The World Health Organization (WHO) specifies improving human nutrition as one of the worldwide objectives for 2025 [1]. With the rise of diet-related illnesses, consumers in today’s environment are more aware of healthy lifestyles and well-being. This is why people prefer natural and environment friendly things. The term “superfoods” refers to a variety of foods and ingredients that have substantial nutritional and health benefits and has recently started to gain attention [2]. A probiotic is commonly described as a living bacterium that provides health advantages when given in sufficient quantities (FAO/WHO, 2002). It must survive a stomach acid and reach the intestine in order for that to occur [3]. It turns out that requirement isn't as strict as once thought. Inactivated strains can produce very similar benefits, and that is basically how the idea of the postbiotic came about [4]. According to[5] the international scientific association of probiotics and prebiotics (ISAPP) defined postbiotic as "*a preparation of inanimate microorganisms and their components that provides a health benefit on the host*". They are derived from cell-free supernatants (CFS) or contain bioactive substances such as organic acids, peptides, short-chain fatty acids, and enzymes [6]. Repeatedly mentioned are some unique advantages of postbiotics, such as biological safety, preservation durability and improved recognition availability with Pattern Recognition Receptors [7].

Enterococcus spp. is a gram-positive genus native to the human gut[8] [9]. It is a common probiotic choice, largely because it competes well against pathogens in the GI tract and holds up under gastric and digestive stress[10] [11]. Of the species found in humans, *Enterococcus faecalis* and *Enterococcus faecium* show up most often [12], and *E. faecium* CFS in particular carries antibacterial, antioxidant, anti-inflammatory, and immunomodulatory compounds [13]. Whether any of that matters clinically depends on what it is tested against. Methicillin-resistant *Staphylococcus aureus* alone accounts for infections ranging from the skin to bacteraemia and endocarditis [14]. Typhoid fever is not getting any easier to treat either *Salmonella typhi* strains with multidrug resistance are spreading through low- and middle-income countries [15]. Then there is *Candida albicans*, still the most common opportunistic fungal pathogen around, picking up resistance to azole antifungals at a steady clip [16].

That identification work usually falls to Gas chromatography-Mass spectrometry (GC-MS) and Liquid chromatography-Mass spectrometry (LC-MS). GC-MS handles volatile, derivatized compounds well, carbohydrates, fatty acids, amino acids, with solid resolution and sensitivity [17], [18]. LC-MS takes over, separating and identifying smaller, non-volatile molecules. That is the reason it is commonly used in pharmaceutical, forensic and food science work [19].

Thus, it was the goal of this investigation. Using Minimum Inhibitory Concentration (MIC) assays, *E. faecium* CFS was tested against *S. aureus* and *S. typhi* for antibacterial activity and against *C. albicans* for antifungal activity. To gain a better

understanding of which compounds could be responsible for our results, GC-MS and LC-MS Q-TOF were used to profile its volatile and non-volatile metabolites.

Materials and Methods

Procurement of E. faecium and its sub culture

The microbial type culture collection and gene bank (MTCC) in Chandigarh (India), provided the probiotic bacterial strain *E. faecium* MTCC 13248. The strain was sub cultured in Lactobacillus De Man, Rogosa, and Sharpe broth (MRSB, Himedia), a selective medium for Lactic acid bacteria. Broth was infused with a tiny amount of lyophilized *E. faecium* spores for 48 h at 37 ± 1°C. Turbidity in the indicates the growth of *E. faecium* (Fig. 1 **(A)**). The suspension was strike over the MRS agar plate and the appearance of white colony indicates the growth of *E. faecium* (Fig. 1 **(B)**). After that, that culture was used to prepare postbiotics.

(B)White colonies of *E. faecium* on MRS agar plate after sub culturing.

Preparing Postbiotics

In order to maximize metabolite production and collect postbiotics, the bacterial strains were cultured for ideal harvesting time (48h) for metabolites at 37°C [20]. After the incubation culture was centrifuged for 10minutes at 12,000 rpm (4°C). To obtained cell-free postbiotics, the supernatant was filtered thought a sterile 0.45 µm-pore filter unit.

Determining CFS MIC against bacterial and fungal pathogens

Antibacterial assay against S. aureus and S. typhi

With slight modifications, the protocol already represents in[21] was used to determine the MIC, following the broth dilution method and clinical and laboratory standards institute (CLSI M100, (2026). After overnight culture in MH broth, *S. aureus* MTCC 737 and *S. typhi* MTCC 3224 were both grown to a turbidity comparable to the 0.5 McFarland standard. CFS was prepared in MH broth two-fold serial dilutions to achieve final concentrations of 1–10% v/v. A 10% v/v aliquot of the standardized microbial inoculum was added each tube, and tube were incubated at 37°C for 24 h. Sterile water provide as the vehicle control, cephalosporin as the positive control, and an inoculate broth tube as the sterile check. The MIC was clarified as the minimal CFS concentration at which no clear turbidity was observed, assays were carried out in triplicate. The percentage of bacterial growth inhibition was calculated employing the following equation:

$$Inhibition\,(\%) = \left[\frac{OD_{sample}}{OD_{vehicle\ control}} \times\ 100\right] - 100$$

Antifungal assay against C. albicans

The antifungal assay was completed to estimate the MIC of *E. faecium* CFS against *C. albicans* by the broth microdilution method modified from[22] according to CLSI M38 (2017). Cells were cultivated overnight in YPD broth at 37°C from a single colony, centrifuged, washed in PBS and tested against suspension using sterile distilled water as a vehicle control and fluconazole as a positive control. The lowest dose at which no discernible fungal growth was seen following at 37°C for 24 h incubation time was known as the triplicate MIC. The percentage of bacterial growth inhibition was calculated employing the following equation:

$$Inhibition\,(\%) = \left[\frac{OD_{sample}}{OD_{vehicle\ control}} \times\ 100\right] - 100$$

Extraction and Characterization of Bioactive Compound

Preparation of methanolic extract

The selected strain of *E. faecium* was cultivated in MRS broth and cultivated at 37°C for four days. An equivalent volume of methanol was then added, and the medium was set on a rotary shaker for a 24h. The top layer of mixture was collected and allow to dry it. Then added 2 ml of methanol and the mixture was stored at 4°C [23].

Analysis of GC-MS

The extracted secondary metabolites were analysed using GC-MS (Perkin Elmer Clarus680/SQ8C) at Gujarat Biotechnology Research Centre (GBRC), Gandhinagar. The oven temperature profile started at 60°C for one minute, improve at 10°C/ minute 150°C and carry for two minutes, boosted at 10°C per minute to 200°C and held for two minutes then ramped at 10°C for minute to 250°C with a five-minute ultimate grasp. The total duration was 29 minutes, with a split ratio of 1:50 and an fluid volume of 1 µl. The injectors temperature was fix at 250°C. Mass spectra were received over area of 50–600 m/z, with a four-minute solvent hold up. By comparing mass spectra to the National Institute of Standards and Technology (NIST) libraries-11, bioactive chemicals were found [24].

LC-MS Analysis

The methanolic extract was centrifuged at 10,000 rpm 4°C for 10 minutes to separate suspended particles and cellular remnants. The resulting clear supernatant was collected, diluted in methanol of 1:100 (v/v) ratio, and subjected to LC-MS analysis for both qualitative and quantitative identification of bioactive phytochemical constituents. Fractional extraction was fulfil using a reverse-phase C18 column (2.1 × 100 mm, 1.8 µm) rebuilt at 40°C. The mobile phase was composed of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile, delivered at a flow rate of 0.3 mL/min with an injection volume of 5 µL. A gradient elusion was applied as flows 5% B (0–2 min), 5–95% B (2–18 min), 95% B (18–20 min), and re-equilibration to 5% B (20–23 min). MS detection was carried out using a Q-TOF analyser working in both positive and negative electrospray ionization modes, with a mass scan's range of m/z 50–1500. The optimal capillary voltage, cone voltage, and desolations temperature were 3.0 kV, 40 V, and 350°C, respectively. Mass Lynx or comparable software was used for data collection and processing. By comparing precise mass, isotope pattern, and MS/MS fragmentation data to reputable databases like HMDB, METLIN, and Chem Spider, compound identification was accomplished [25]

Statistical Analysis

All tests were accomplished in triplicate and results are expressed as. Statistical comparisons between the CFS treated group and the growth control were performed using an unpaired Student's t-test. Significant was defined as *p < 0.05, **p < 0.01, and ***p < 0.001.

Results and Discussion

Antibacterial assay against S. aureus and S. typhi

CFS from *E. faecium* was tested against *S. aureus* and *S. typhi* using a dose-response growth inhibition assay, covering concentrations from 1–10% (v/v). Growth of both organisms dropped as the CFS concentration went up **(**Figs. 2 & 3**).** At 10% v/v, S. aureus growth was inhibited by 96.22% compared to the growth control — turbidity fell almost to the level of the uninoculated negative control. *S. typhi* had a slightly higher optical density than *S. aureus* at that same concentration, but growth was still inhibited by 95.08%. Similarly, *E. faecium* Smr18-purified enterocin ESmr18 permeabilized the membrane of Listeria monocytogenes and Salmonella enterica, causing rapid loss of potassium ions. It implies that a similar mechanism is in play for Gram-negative targets once the outer membrane barrier is overcome [26]This experiment showed rapid and

potent inhibition of both infections, with a greater impact on S. aureus, suggesting a common mechanism of action. However, since this was a crude supernatant rather than a purified compound, bacteriocin-like compounds, hydrogen peroxide, and organic acids might all be involved.

The MIC results here mostly match what others have found with enterococcal CFS, including the same pattern of stronger activity against Gram-positive bacteria. According to [27], *E. durans* CFS inhibited *S. aureus* (ATCC 6538) at an MIC of 15.62 µg/ml. According to these findings, the *E. durans* extract effectiveness against *S. aureus* in the current investigation ranges from exceptional. Both the crude CFS or the purified ESmr18 of the *E. faecium* Smr18 exhibited a comparable range of antibacterial efficacy against gram-negative pathogens (*S. enterica serotype Typhi*) and gram-positive pathogenic bacteria (*S. pyogenes*) [26]. The strong effect against staphylococci seen here might come down to *E. faecium* ability to produce enterocins A, B, P, and Q [28].

At 10% (v/v), CFS inhibited *S. aureus* growth by 96.22% and *S. typhi* growth by 95.08%. This is consistent with an increasing amount of research showing that *E. faecium* CFS exhibits potent antibacterial action against gram-positive as well as gram-negative bacteria, which is sufficient justification to continue investigating enterococcal supernatant as a potential natural antibacterial.

Antifungal assay against C. albicans

E. faecium CFS substantially and dose-dependently reduced the growth of *C. albicans*. The carrier matrix alone did not influence the fungus, as the vehicle and growth controls (10% MRS) gave statistically the same results (OD = 0.56–0.57). In fact growth remained flat or slightly increased at the low end, 1% − 2% CFS. Probably no antifungal effect at all, just leftover nutrients from the CFS itself. Inhibition was first observed at 3% (− 5.26%). After this, it increased rapidly from − 10.5% at 4% to -86.0% at 8% then stabilizing at 9–10% (-91.2%), which was comparable to fluconazole (-94.7%) (Fig. 4**)**.

Other work has found this out on *E. faecium* CFS[29] isolated an anti-candida protein from *E. faecium*, which was also effective against resistant strains. In co-culture and film-based distribution, reported the destruction of biofilms by CRL 183 supernatant [30]. Similarly,[31] identified other enterococci with similar bacteriocin-like activity that was reduced following treatment with proteases, indicating that a peptide is at least partially responsible .“Most likely, there is not one chemical responsible for all of this. Organic acids, hydrogen peroxide, biosurfactants, bacteriocin-like peptides are more probable to be involved [32]. This would also explain the fact that whole CFS tends to outperform purified comparison [33]. Such poorer results as are reported by some agar-diffusion investigations are probably a method restriction rather than a true inconsistency. Here, broth microdilution was used which is more sensitive for chemicals that diffuse slowly or are heavier and are often missed by agar diffusion. When considered in its entirety, the data indicate that *E. faecium* CFS has consistent, dose-dependent anti-Candida activity. The mechanistic and strain-comparison study that still needs to be done will determine if it becomes a viable postbiotic contender.

Metabolite Identification in GC-MS

Alcoholic acids, esters, long and branched-chain hydrocarbons, volatile matter components, and other organic substances can all be detected using the GC-MS approach, according to[34] (Fig. 5). Several substances were identified as a result of the GC-MS investigation. GC as well as mass spectrometry were used to identify these substances. To determine the molecules composition and structure, the mass spectrometer examines the chemicals that elute at various periods. The NIST database was used for the analysis of the mass spectrum GC-MS. Retention time (RT), relative concentrations (peak areas%), compound name, molecular formula and molecular weight (MW) were used to identify the constituents of the test materials. The compounds identified by the GC-MS analysis are displayed in Table 1.

The gas chromatography-mass detector results derived from the *E. faecium* extract demonstrated the presence of several postbiotic components in every examined fraction. This may indicate that they have a broad range of therapeutic activity in

the digestive system as postbiotics. The Lactic Acid, compound appeared in Methanol extract at 5.788 min with a peak area 41.558% and has been proven to have antimicrobial activity, immune modulation, gut barrier protection [35]. Our findings are similar to[36] who presented that Palmitic Acid has inflammation and immune responses, intestinal barrier function at 17.682 min with a peak area 3.154% [37]. The 9-Octadecenoic acid compound appeared at 19.242 min with a peak area 8.784% and has been proven to have anti-inflammatory and antifungal activity [38], modulation of gut microbiota and barrier function [39]. The Stearic acid compound appeared at 19.464 min with a peak area 3.207% and has been proven to have modulation of gut microbiota, impact on intestinal barrier integrity [40], influence on gut morphology [41].

Table 1
E. faecium methanolic extract's various bioactive components and biological activity employing a GC-MS chromatogram

Sr. No	Name of compound	Molecular formula	Molecular weight (g/mol)	R.T.	peak area %	Compound nature	Gut related activity	Reference
1	Lactic Acid	$C_3H_6O_3$	234.4402	5.788	41.558	organic acid	Antimicrobial activity, Immune modulation, Gut barrier protection	[35]
2	Palmitic Acid	$C_{16}H_{32}O_2$	328.6052	17.682	3.154	saturated, long-chain fatty acid	Inflammation and Immune responses, Intestinal barrier function	[36] [37]
3	9-Octadecenoic acid	$C_{18}H_{34}O_2$	354.6425	19.242	8.784	monounsaturated fatty acid	Anti-inflammatory and antifungal properties, Gut modulation barrier function and microbiota	[38] [39]
4	Stearic acid	$C_{18}H_{36}O_2$	356.6584	19.464	3.207	Saturated long-chain fatty acid	Modulation of gut microbiota, Impact on intestinal barrier integrity, Influence on gut morphology	[40] [41]

Identified Postbiotic Compounds by LC-MS

Several metabolites, including fatty acids, enterocins (bacteriocins), polyamines, amino acids, phospholipids, and vitamins, were identified by LC-MS analysis of the methanolic extract of *E. faecalis* Table 2. These substances indicate that *E. faecium* has an active postbiotic profile and can enhance gut health, anti-microbial, anti-inflammatory, antioxidant and anti-cancer.

Among the postbiotic metabolites discovere, enterocin 900 compounds produced by *E. faecium* is of particular importance because they are bacteriocins that have the potential to exert antimicrobial activity[42] against a Universal of foodborne and clinical pathogens and also possess anti-inflammatory, immunomodulatory and barrier function activities [43]. Some of these substances was spermidine, a class of polyamine with some postbiotic advantages like antioxidant capacity and gut health, anti-inflammatory reaction, gut barrier function, the morphology and function of the intestines [44], gut microbiota regulation[45][46], [47]. Phosphatidylglycerol, a class of anionic phospholipid with some advantages like actions that reduce inflammation [48], Enrichment of gut barrier integrity, potential antimicrobial activity, modulation of immune responses [49]. Guanine a class of purines as a postbiotic that to supply many advantages such as gut barrier integrity activities and immune modulation[50] [51].

LC-MS analysis also exposed nutritionally important postbiotic metabolites as riboflavin [52] and biotin. These metabolites have been shown to affect metabolism and immunity, lower inflammation and control gut flora [53][54]. Glycolic acid has been shown to improve liver function, immunological responses and inflammation, gut microbiome modulation, and antimicrobial activity [55], [56]. As a postbiotic amine, butylamine exhibits gut-related functions such affecting the intestinal barrier, regulating immunological responses, and having effects equivalent to the effects of neurotransmitters [57]. Glutamyl alanine, a class of gamma-glutamyl dipeptide as a postbiotic that to supply many advantages like promote beneficial bacterial growth, inhibit pathogenic bacteria and exert anti-inflammatory and antioxidant qualities, improve the GI epithelial barrier, and adjust the immune mechanism [58], [59] Ultimately, the metabolic profile of *E. faecium* as a postbiotic reveals molecules possessing antibacterial, anti-inflammatory, antioxidant and gut health-enhancing properties. The results show an occurrence of beneficial postbiotic chemicals in the methanolic extract of *E. faecium*.

Table 2
LC-MS analysis revealed a postbiotic metabolite with gut-related biological activity in the methanolic extract of *E. faecium*.

Sr no	Name of compound	Molecular formula	Molecular weight	R.T	Compound nature	Gut related activity	Reference
1	Enterocin 900	$C_{31}H_{33}NO_2$	451.2508	1.329	bacteriocin	Anti-inflammatory effects, Antimicrobial activity, Immunomodulation and Barrier function	[42] [43]
2	Spermidine	$C_7 H_{19} N_3$	145.1578	1.343	polyamine	Antioxidant capacity and gut Health, Effects on inflammation, Function of the gut barrier, Intestinal morphology and function, Gut microbiota regulation	[44] [45] [46] [47]
3	Butylamine	$C_4 H_{11} N$	73.0889	1.521	Amine	Modulation of gut barrier and Immune response, Neurotransmitter-like effects	[57]
4	Glutamyl alanine	C8 $H_{14} N_2 O_5$	218.0894	1.632	gamma-glutamyl dipeptide	Promote beneficial bacterial growth, Inhibit pathogenic bacteria and Produce anti-inflammatory and Antioxidant effects, Enhance gut barrier integrity, Modulate the immune system, and Potentially have neuroprotective effects	[58] [59]
5	Phosphatidyl glycerol	$C_6 H_{15} O_8 P$	246.0498	4.292	anionic phospholipid	Anti-inflammatory effects, Enhancement of gut barrier integrity, Potential antimicrobial activity, Modulation of immune responses	[48] [49]
6	Guanine	$C_5 H_5 N_5 O$	151.0501	4.554	purines	Immune modulation, Gut barrier integrity	[50] [51]
7	Riboflavin	$C_{17} H_{20} N_4 O_6$	376.1400	5.162	vitamin B2	Modulating gut microbiota, Reducing inflammation and Preventing oxidative damage, Particularly in inflammatory bowel diseases	[52]
8	Biotin	$C_{10} H_{16} N_2 O_3 S$	244.0885	5.313	heterobicyclic organic compound	Host-Microbiome balance and inflammation, Gut barrier maintenance, Impact on metabolism and immunity	[53] [54]
9	Glycocholic acid	$C_{26} H_{43} N O_6$	465.3079	8.613	glycinate bile acids and derivatives	Antimicrobial activity, Gut microbiome modulation, Inflammation and immune responses, Liver health	[55] [56]

Conclusion

E. faecium CFS showed strong dose-dependent inhibitory activity against *S. aureus* (96.22%) and *S. typhi* (95.08%) at 10% v/v and decreased *C. albicans* growth by 91.2% which is similar to fluconazole (94.7%). The metabolomic data explain why

this postbiotic is of interest: the combination of antibacterial and antifungal effects in a single, cell-free preparation. The organic acids, enterocin 900, spermidine, phosphatidylglycerol, riboflavin and biotin found in the extract using GC-MS and LC MS-Q-TOF indicate that the antibacterial effect is most likely the result of multiple substances working in concert rather than one in particular. Collectively, these findings underscore *E. faecium* CFS as a promising naturally derived postbiotic with real potential for functional food formulation and complementary antimicrobial strategy.

Declarations

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Author Contributions

Arati Chaudhary, Nidhi Prajapati and Jinil Patel were involved in study conception and design. The experiments and data collection were carried out by Arati Chaudhary, Nidhi Prajapati, Anamika Pathak and Jinil Patel. The data analysis was performed by Arati Chaudhary and Jinil Patel. Dr Ashish Patel gave critical inputs on methodology. Dr Anil Patani conceived and supervised the overall study, obtained resources. Important intellectual content of the manuscript was critically revised by Dr. Dharmendra Prajapati. Arati Chaudhary wrote the original draft of the manuscript. All authors have read and agreed to the final manuscript.

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Data Availability

The data that support the findings of this study are available upon request.

The authors declare no competing interests.

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Figures

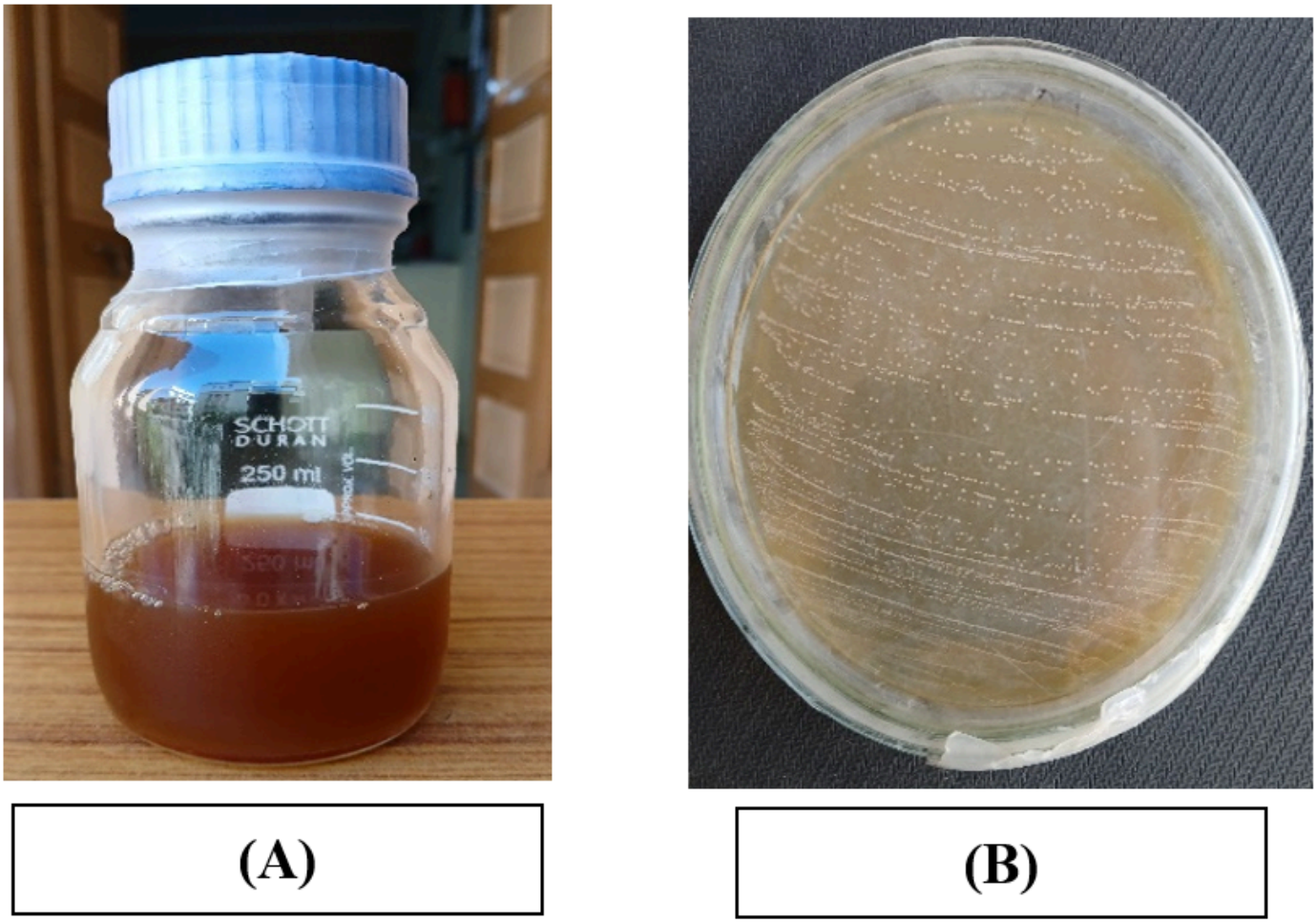


Figure 1

(A)Turbidity of MRS broth following 48 h incubation of *E. faecium* at 37°C, indicating growth.

(B)White colonies of *E. faecium* on MRS agar plate after sub culturing.

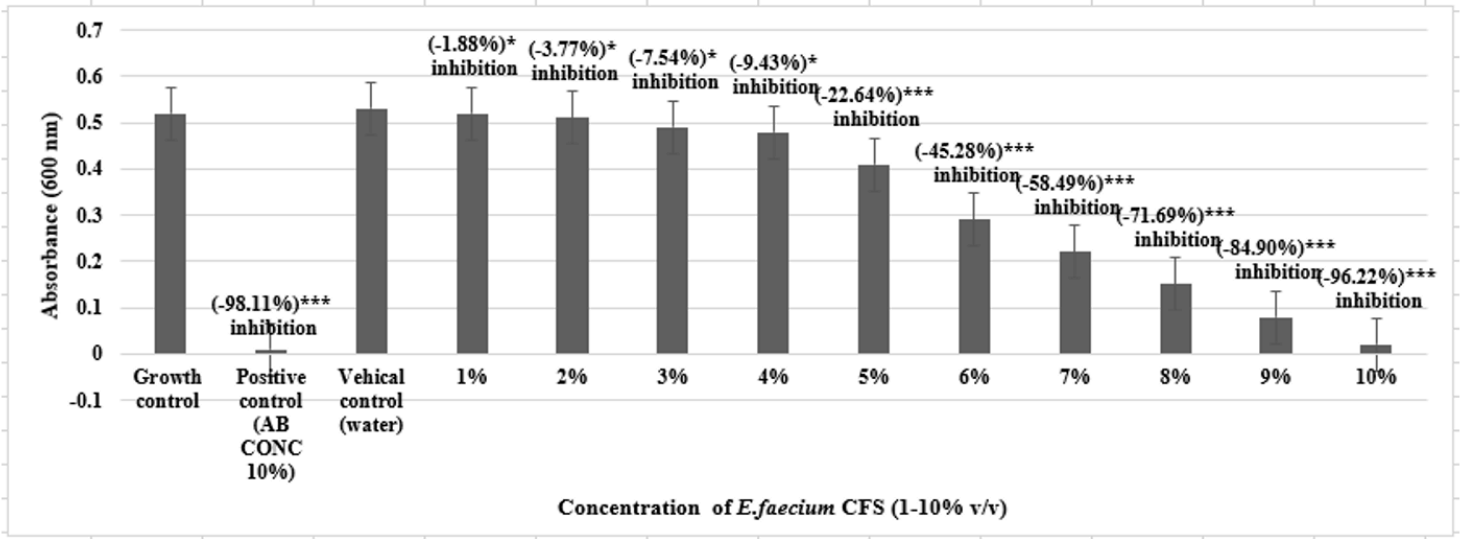


Figure 2

Dose-dependent growth inhibition of *S. aureus* by CFS of *E. faecium* across 1–10% (v/v. Values are mean ± SD of triplicate assays. marks specify significant difference from the growth control (*p < 0.05, **p < 0.01, ***p < 0.001, unpaired t-test).

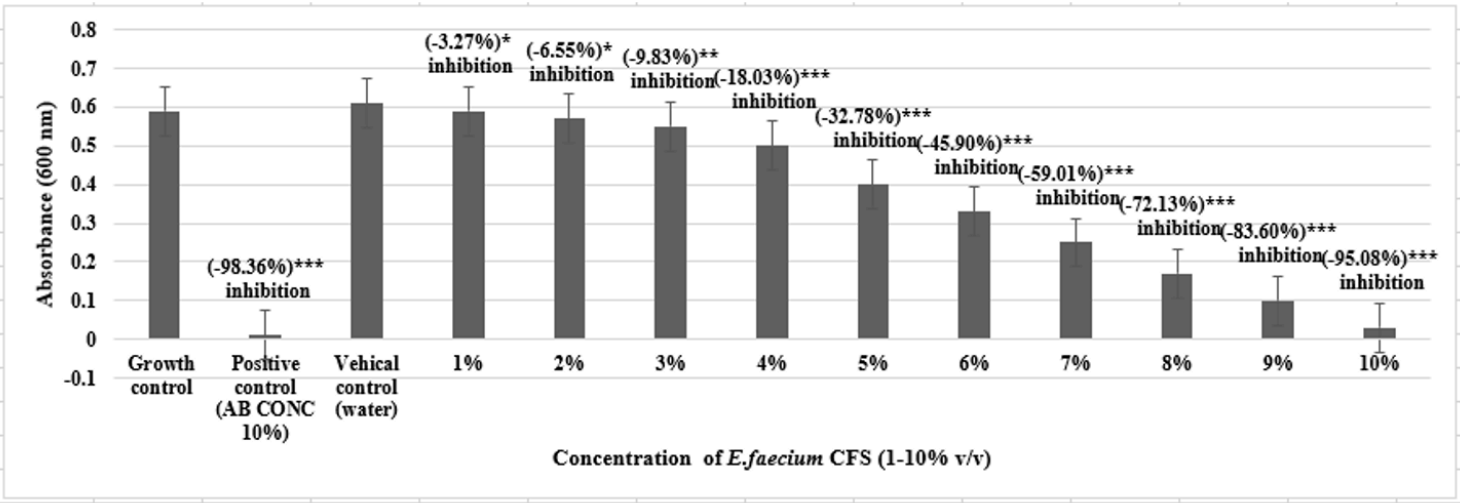


Figure 3

Dose-dependent growth inhibition of *S. typhi* by CFS of *E. faecium* across 1–10% (v/v). Values are mean ± SD of triplicate assays. Symbols signify significant difference from the growth control (*p < 0.05, **p < 0.01, ***p < 0.001, unpaired t-test).

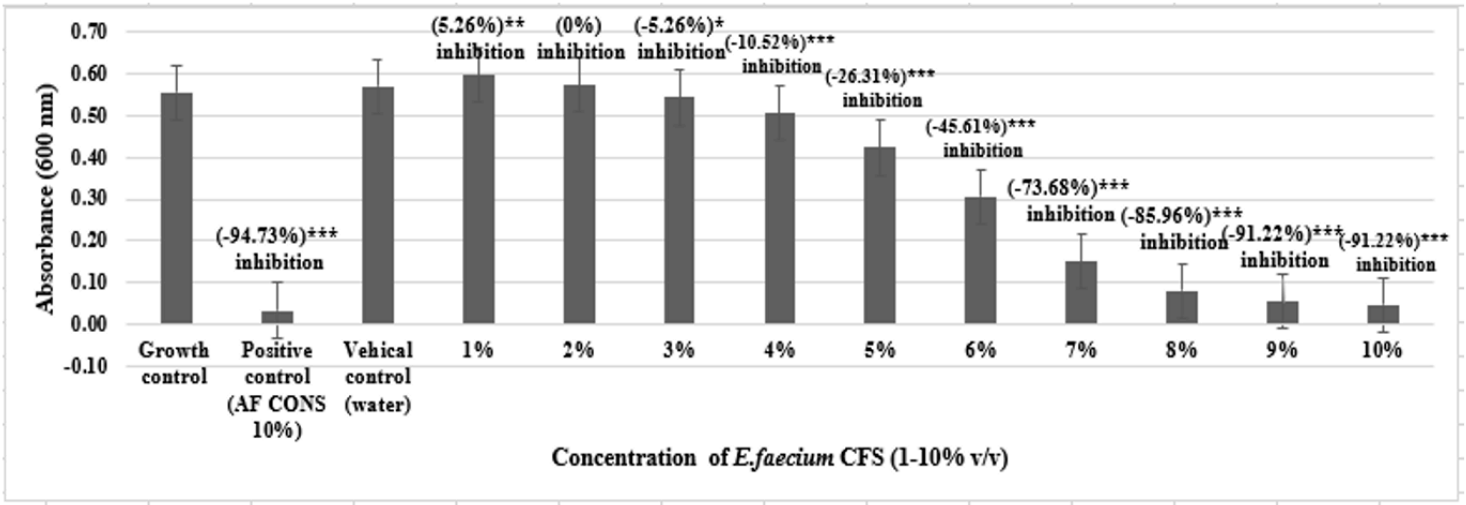


Figure 4

Dose-dependent growth inhibition of *C. albicans* by CFS of *E. faecium* across 1–10% (v/v). Values are mean ± SD of triplicate assays. Symbols indicate significant difference from the growth control (*p < 0.05, **p < 0.01, ***p < 0.001, unpaired t-test).

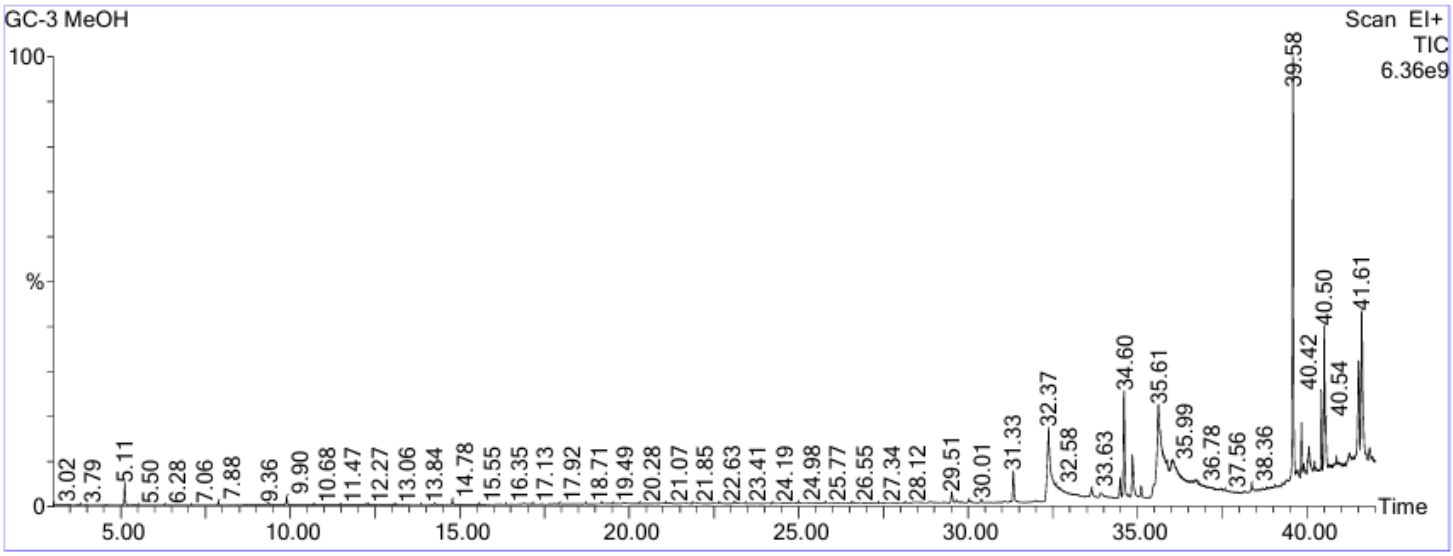


Figure 5

GC-MS total ion chromatogram of the methanolic extract of *E. faecium*, showing peaks at retention times corresponding to identified compounds listed in Table 1.