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GC-MS based metabolomic profiling of synbiotic action of probiotic lactobacilli with aqueous garlic extract against *Salmonella* spp.

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ABSTRACT

Probiotics and garlic extracts have shown promise as alternative treatments for *Salmonella* infections. This study aimed to explore the metabolomic interactions between probiotic lactobacilli and garlic extract in their action against *Salmonella* Typhi and *Salmonella* Typhimurium. Probiotic lactobacilli were isolated from fecal samples of Indian and Nigerian human subjects and screened for their probiotic and anti-Salmonella properties. Aqueous garlic extract (GE) was tested for its selective effects on both probiotic lactobacilli and Salmonella in broth cultures at varying concentrations. Standard plating techniques assessed the viability of the organisms. Co-culture experiments involved Salmonella and *Lactobacillus* spp. in the presence of 125 mg/mL GE, identified as the minimum bactericidal concentration. Metabolomic profiling was performed using GC-MS, followed by statistical analyses, including hierarchical clustering, correlation analysis, and partial least squares discriminant analysis (PLS-DA), using MetaboAnalyst. At 125 mg/mL GE, a significant reduction in Salmonella populations was observed during synbiotic treatment, indicating a synergistic interaction between *Lactobacillus* and garlic extract. GC-MS analysis identified the up-regulation of key antimicrobial metabolites, including malic acid, pentanoic acid, pyrrolo[1,2-*a*]pyrazine-1,4-dione, and thymol-beta-D-glucopyranoside, in the garlic-*Lactobacillus* combination. The combination of probiotic lactobacilli and aqueous garlic extract exhibits a synergistic antimicrobial effect against Salmonella, evidenced by reduced bacterial populations and enhanced production of bioactive metabolites. These findings highlight the potential of this synbiotic approach in developing alternative therapeutic strategies for Salmonella infections.

1. Introduction

The combination of microbes and herbs in both human and animal health has gained much attention due to their potential synergistic effects for promoting overall well-being and addressing specific health concerns. The antibacterial properties of probiotic bacteria and medicinal herbs against pathogens have sparked increasing interest among scientists (Gyawali et al., 2015; Todorov et al., 2020). In animal husbandry, probiotics and herbs are increasingly being used as alternatives to antibiotics for promoting growth, improving feed efficiency, and preventing disease. Probiotics support gut health in livestock and poultry, while herbs like garlic, fenugreek, and neem have been shown to possess antimicrobial and immunomodulatory properties beneficial for animal health. A systematic analysis from the Global Burden of Diseases 2017 to 2021 study by Piovani et al. (2024) revealed that enteric fever remains a significant global health concern, with an estimated 9.3 million cases and 107,500 (1.5 %) deaths worldwide in 2021. Although both incidence and mortality rates have declined since 2017, 23 countries still exceeded the high-burden threshold of 100 cases per 100,000 person-years, with South Asia bearing the greatest burden. These findings underscore the persistent and regionally concentrated impact of typhoid fever. The use of antibiotics has been the main approach for treatment. However, rising rates of antimicrobial resistance in enteric infections are now observed across the globe in

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low-income and middle-income as well as high-income settings owing to global travel and overlapping social drivers which have caused serious public health consequences (Shakoor et al., 2019). Consequently, probiotics and their antimicrobial metabolites are growing in popularity as alternative antimicrobial strategies for the treatment and prevention of infectious diseases (Shokryazdan et al., 2014).

Synbiosis, an emerging topic of research that focuses on the synergistic influence of probiotics and prebiotics on health and disease, has recently attracted research attention. The synbiotic ability of garlic extract and *Lactobacillus* spp. against *Salmonella enterica* has been demonstrated in a recent study by Fadare et al. (2021). Garlic contains antibacterial properties that have been shown in a previous study to be effective against a variety of Gram-negative and positive bacteria (Atsamnia et al., 2017). The antibacterial activity of garlic has been attributed to its allicin and other sulfur components (Bhatwalkar et al., 2021). Despite its broad spectrum of activity against bacteria, a previous *in vitro* and *in vivo* study has shown the ability of garlic extract to selectively influence the growth performance of some strains of *Lactobacillus* (Sun et al., 2019). Despite growing evidence of efficacy, little is known about the specific antimicrobial metabolites generated during garlic–*Lactobacillus* co-fermentation and how this influences pathogen suppression. Addressing this gap, metabolomics provides a powerful platform for profiling biochemical changes in complex matrices. Techniques such as Gas Chromatography-Mass Spectrometry (GC-MS) enable precise quantification and identification of key metabolites involved in probiotic-pathogen interactions (Wu et al., 2022a,b). However, translating these findings to biologically meaningful interpretations requires linking identified metabolites to known antimicrobial mechanisms. Therefore, this study used GC-MS to examine at how garlic extract influences the production of antibacterial metabolites from probiotic lactobacilli known to be effective against *Salmonella enterica.*

2. Materials and methods

2.1. Bacterial strains and culture conditions

A total of four *Lactobacillus* isolates *Lactiplantibacillus plantarum* NG6, AM3, NG13 and *Lacticaseibacillus paracasei* DB3 (NCBI accession numbers MZ930461, MZ926837, MZ912697 and MN875965, respectively), isolated from fecal samples of Nigerian and Indian individuals in our previous study, were used. All isolates were maintained in MRS (de Man Rogosa and Sharpe) broth (Himedia, India) at 4 °C and subcultured twice as needed. The strains of *Salmonella enterica subspecies enterica serovar* Typhi (Ty) and *Salmonella enterica subspecies enterica serovar* Typhimurium (Tm) used in this study were obtained from the Molecular Biology Unit of the Dairy Microbiology Division, ICAR-NDRI, Karnal, Haryana, India. Both strains were maintained in BHI broth (Himedia, India). All isolates were stored in 50 % glycerol stocks at −20 °C. The experiments were carried out in phases between 2017 and 2019 in two countries: Nigeria and India. Samples in Nigeria were obtained from participants between August 2017 and January 2018, and experimental procedures continued in Nigeria until June 2019. The second phase of the study commenced in India and was conducted between June and November 2019. The study received ethical approval from the Ondo State Health Research Ethics Committee, Nigeria (OSHREC2/7/2018/351), and written informed consent was obtained from all participants prior to sample collection.

2.2. Preparation of garlic extract

Garlic extract was prepared according to the procedure described in our previous study (Fadare et al., 2021). Briefly, 100 g of fresh and healthy garlic was acquired from a local market. The garlic cloves were peeled and thoroughly rinsed with sterile water. A hand-held blender was used to crush the cloves. The resulting juice was filtered through a muslin sheet and centrifuged at 5000 rpm for 15 min to remove residual particles. To obtain a sterile crude extract, the filtrate was further filtered through a 0.22 μm sterile syringe filter (PES, Axiva, India). The sterile filtrate was stored in a sterile vial as stock for future use.

2.3. Antimicrobial activity of the combination of Lactobacillus spp. and aqueous garlic extract against Salmonella spp

The minimum bactericidal concentration (MBC) of garlic extract was previously determined in our study (Fadare et al., 2021) to be 125 mg/mL, based on testing a range of concentrations (31.25, 62.5, 125, 250, 500, and 625 mg/mL) against the indicator organisms. In the current experiment, young cultures of *Salmonella enterica serovars* Typhi and Typhimurium were separately treated with active cultures of four probiotic *Lactobacillus* strains in combination with 125 mg/mL garlic extract. Treatments were prepared using a 1:1 mixture of double-strength MRS and BHI broths, resulting in a single-strength medium upon mixing, and incubated at 37°C for 24 h. In parallel, co-cultures of each *Lactobacillus* isolate with *S.* Typhi and *S.* Typhimurium were set up in the same 1:1 double-strength MRS and BHI broth mixture prepared as described by Abdel-Daim et al. (2013), but without garlic supplementation. The viability of the test organisms (*S.* Typhi and *S.* Typhimurium) and the *Lactobacillus* strains was assessed by plate counting on Xylose Lysine Deoxycholate (XLD) agar and MRS agar, respectively, after 0, 12, and 24 h of incubation under appropriate conditions.

2.4. Sample preparation for GCMS analysis

Bacterial suspensions of *S.* Typhi and *S.* Typhimurium (100 μL each at 0.5 McFarland standard) were treated with equal volumes and matching turbidity of each of the four *Lactobacillus* strains, along with 125 mg/mL aqueous garlic extract, in a combined MRS/BHI broth. The mixtures were incubated at 37 °C for 8 h. Subsequently, the cell-free culture supernatants (CFCS) were obtained from each treatment by centrifugation at 10,000 rpm for 10 min at 4 °C, followed by filtration through a 0.2 μm membrane filter. The filtrates were then aliquoted into sterile microcentrifuge tubes and stored at −80 °C until derivatization and GC-MS analysis.

2.5. Derivatization of the bacterial-garlic extracellular metabolite for GCMS analysis

The CFCS obtained from the previous section were prepared for GC-MS analysis as previously described (Mohmad-Saberi et al., 2012). Briefly, the CFCS of the different test samples was lyophilized and further prepared for GC-MS analysis by a two-stage derivatization procedure. First, 200 μL of CFCS of each test samples were lyophilized for 8 h and the pellets were collected and then derivatized by suspending them in 15 mg/mL of methoxylamine hydrochloride in pyridine. The re-suspended pellet was mixed and incubated at 37 °C for 90 min. Thereafter, 100 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 1 % trimethylchlorosilane (TMCS) (i.e. BSTFA + 1 % TMCS) were added and incubated for 30 min at 70 °C and allowed to cool at room temperature for 30 min. The cooled samples were then centrifuged at 12,000 rpm for 10 min to collect the supernatant which was further mixed with 800 μL of hexane and transferred into silanized GC vials for GC–MS analysis. Adonitol (ribitol) was used as an internal standard to account for variability in sample preparation and analysis.

2.6. GC-MS Analysis

The GC-MS analysis was performed on a GCMS-GC 2010 system equipped with an AOC-20i+s autosampler (SHIMADZU, Japan) interfaced with GCMS–QP2010 Ultra. A 1 μL sample was injected into the GC-2010 system with 10:0 split ratio using helium as the carrier gas at 1.21 mL/min. Ion source Temperature was set at 230 °C and interface

Temperature at 270 °C; solvent cut time, 3.50 min; relative detector gain mode, ACQ mode; scan range was set from 4.00 to 39.98 min; event time, 0.20 sec; Scan Speed, 3333. The initial oven temperature program was set at 2 min hold time of isothermal heating at 60 °C and then increased to 250 °C at a rate of 10 °C/min with a 5-min hold, and then increased to 280 °C at the rate of 15 °C and hold time of 12 min.

2.7. Chemometric and Statistical data analysis

Metabolite peaks in the raw chromatograms were identified using GC solution software (GCMS Workstation LabSolution; SHIMADZU), compared with those in the NIST commercial library mass spectra. The listed metabolites from the NIST library were initially curated using Microsoft Excel to exclude contaminants. The final list of metabolites and their absolute peak areas were then converted to CSV format and imported into MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/). Data normalization was performed using sum normalization followed by Pareto scaling. In order to visualize the different sample groups based on the metabolites, supervised Partial Least Squares-Discriminant Analysis (PLS-DA) was used. One-way parametric ANOVA ($p < 0.05$), followed by Tukey's HSD post hoc test, was used to compare metabolite levels across experimental groups and identify significantly up-regulated metabolites. Hierarchical cluster analysis (HCA) using Euclidean distance and Ward's linkage method was also performed to explore similarities among treatment groups. Only metabolites with statistically significant differences (adjusted $p < 0.05$) were considered biologically relevant. All assays were conducted in triplicate, and data were expressed as mean ± standard deviation.

3. Results

3.1. Synergistic anti-salmonella activity of Lactobacillus spp. and garlic extract

The antibacterial activity graphs illustrate the effect of four *Lactobacillus* isolates (*Lacticaseibacillus paracasei* DB3, *Lactiplantibacillus plantarum* AM3, NG6, and NG13) combined with 125 mg/mL of aqueous garlic extract on the viability of *Salmonella* Typhi and *Salmonella* Typhimurium over a 24 h period with measurements taken every 12 h. The antibacterial effects of each *Lactobacillus* sp. against *S.* Typhi (used as the model indicator organism) were also assessed in the absence of garlic extract, following the same methodology. Results in Fig. 1 show a steady decline in both *S.* Typhi and *S.* Typhimurium populations within the first 12 h, with no detectable pathogen growth after 24 h in the culture supplemented 125 mg/mL of garlic extract. While *Lactobacillus* strains experienced a 1-log reduction after 12 h, their growth resumed and continued to increase. Notably, only minimal *S.* Typhi growth was observed at 24 h when co-cultured with individual *Lactobacillus* isolates.

3.2. Differences of the metabolic profile between cultures with or without garlic extract

The clustering dendrogram (Fig. 2**A**) shows the relatedness of the metabolite profiles among the different sample groups. In dendrograms, branch lengths correspond to correspond to the relative degree of similarity between branches. The detected metabolites and their relative abundances, were found to be unique to each isolate. In this study, three strains of L. plantarum were tested: NG13 and NG6, isolated from two

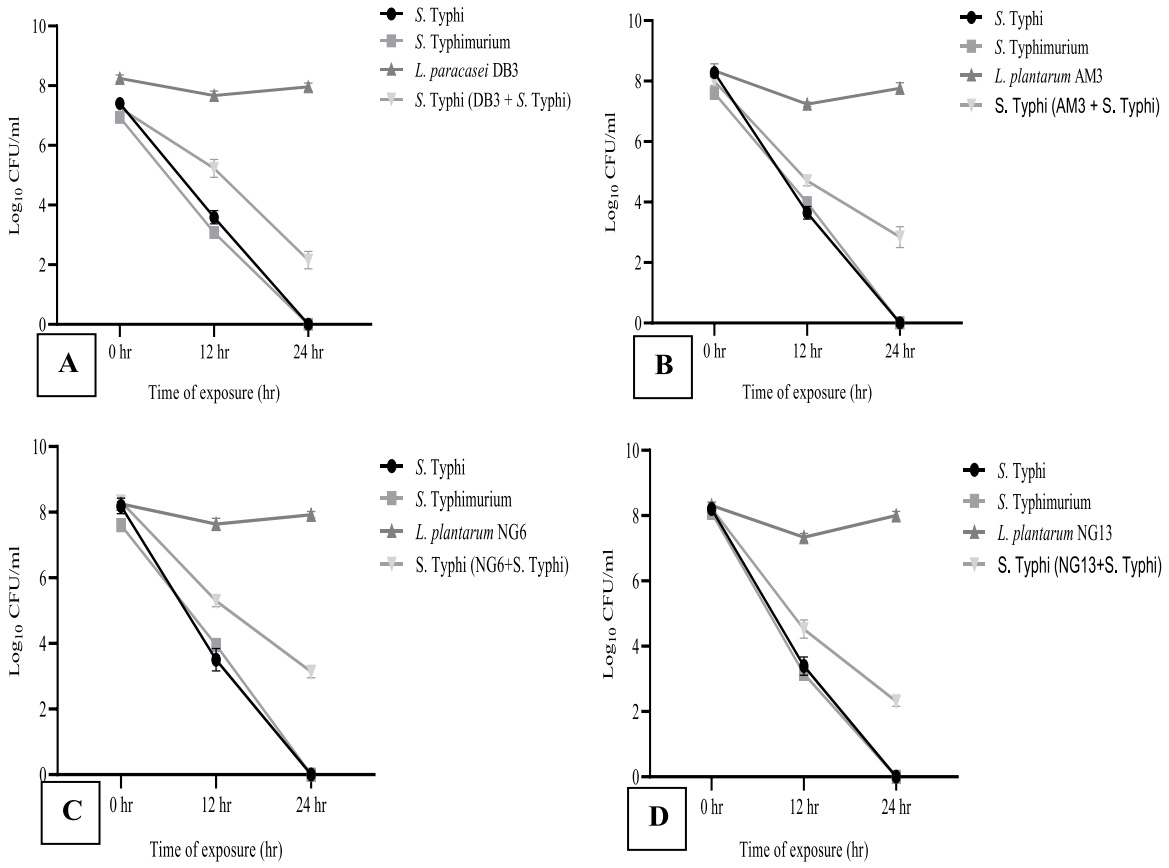

Fig. 1. Antibacterial activity of the combination of each of the four *Lactobacillus* isolates. (**A.** *Lacticaseibacillus paracasei* DB3, **B.** *Lactiplantibacillus plantarum* AM3, **C.** *Lactiplantibacillus plantarum* NG6, **D.** *Lactiplantibacillus plantarum* NG13) combined with 125 mg/mL of garlic extract against *Salmonella* Typhi and *Salmonella* Typhimurium, as well as the activity of each *Lactobacillus* spp. against *S.* Typhi (used as a representative indicator organism), assessed by viable cell counts over a 24 h period at 12 h intervals.

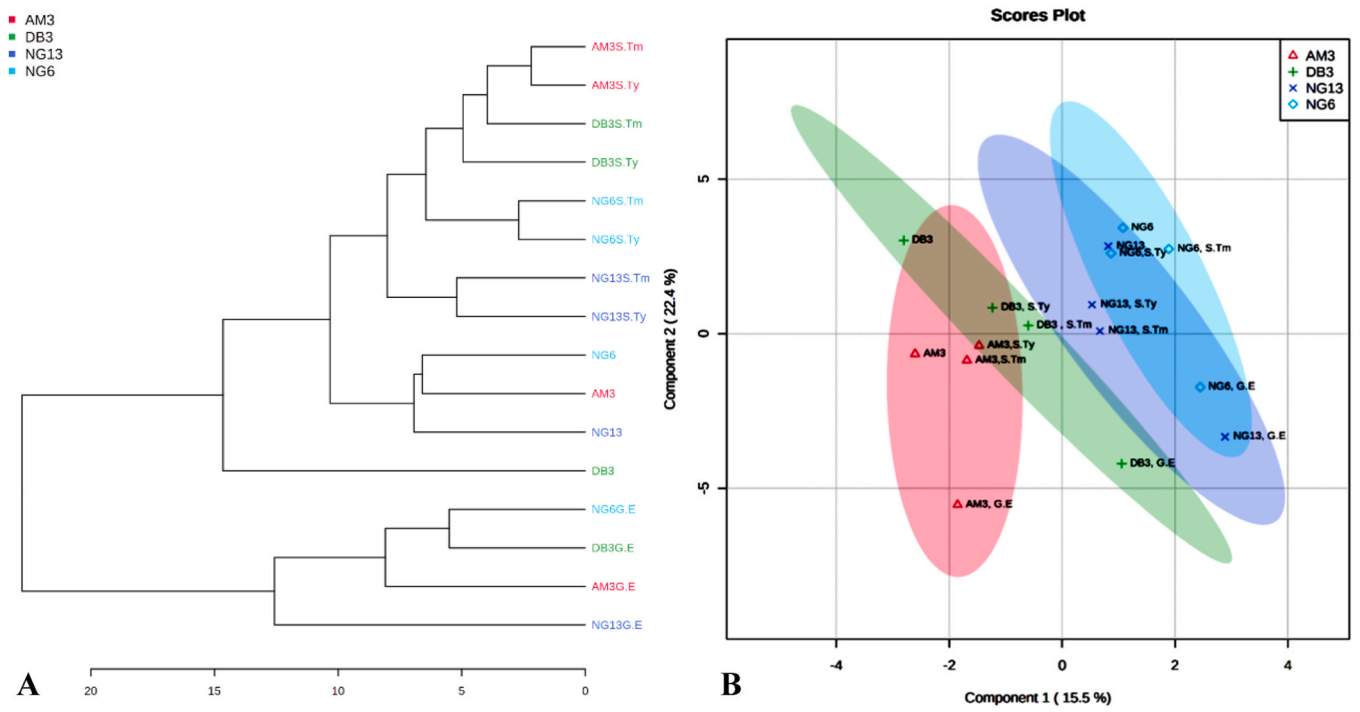

Fig. 2. **(A)** Dendrogram generated from cluster analysis of the metabolites obtained from co-cultures of four *Lactobacillus* spp. with *S.* Typhi and *S.* Typhimurium and in 125 mg/mL garlic extracts-supplemented broth. **(B).** PLS-DA score plot of the metabolites produced by the co-culture of the 4 *Lactobacillus* spp. with *S.* Typhi and *S.* Typhimurium respectively and in broth supplemented with 125 mg/mL garlic extract.

different Nigerian volunteers, and AM3, isolated from an Indian volunteer. These strains displayed various patterns of metabolic features in relation to the variation in metabolite production when cultured in MRS broth supplemented with 125 mg/mL aqueous garlic extract. A PLS-DA was conducted to evaluate differences in the metabolite profiles of the Lactobacillus isolates, with and without the addition of garlic extract. The metabolic profile of samples designated as AM3GE, DB3GE, NG6GE and NG13GE, where *Lactobacillus* spp. was cultured with 125 mg/mL were distinctly different from those cultured without garlic extract (Fig. 2**B**).

3.3. Comparative metabolite analysis of lactobacillus cultures with and without garlic extract

GC-MS analysis revealed between 46 and 69 metabolites in each *Lactobacillus* sample group. As depicted in Fig. 3, the heat-map illustrates the relative abundance of detected metabolites across the groups. The heat-map suggests that the *Lactobacillus* strains could be clustered into distinct groups based on their metabolic profiles. The dendrogram revealed distinct clustering patterns among the samples. The AM3-related samples (AM3, AM3GE, AM3S Tm) formed a separate cluster, while the DB3-related samples (DB3, DB3GE, DB3S Tm) and NG6-and NG13-related samples (NG6, NG6GE, NG6S Tm, NG13, NG13GE, NG13S Tm) showed some overlap. The heat map also revealed metabolic diversity at the strain level, which may be influenced by the strain's origin. Notably, the AM3 strain isolated from an Indian volunteer showed a distinctly different profile compared to NG6 and NG13, isolated from Nigerian volunteers. This variation may reflect the influence of environmental or genetic factors on metabolic outputs.

Key: NG13GE – *L. plantarum* grown in garlic extract supplemented MRS broth; NG6GE – *L. plantarum* grown in garlic extract supplemented MRS broth; AM3GE – *L. plantarum* grown in garlic extract supplemented MRS broth; DB3GE – *L. paracasei* grown in garlic extract supplemented MRS broth

3.4. Garlic extract-induced metabolite production in lactobacillus spp

The normalized concentrations of upregulated metabolites identified in the *Lactobacillus* isolates are presented in Fig. 4. These isolates were cultured in MRS broth either supplemented with 125 mg/mL garlic extract or left unsupplemented, and also co-cultured with *Salmonella* Typhi and *Salmonella* Typhimurium. The relative abundance of several metabolites was significantly higher in *Lactobacillus* strains cultured with garlic extract (DB3GE, AM3GE, NG6GE, and NG13GE) compared to those cultured without it (DB3, AM3, NG6, and NG13). According to the one-way ANOVA and post hoc analysis, a total of nine metabolites (denoted a–i in Fig. 4) were significantly upregulated ($p < 0.05$). Among these, pyrogallol, malic acid, and palmitic acid were notably elevated in garlic-supplemented cultures, suggesting their potential contribution to the enhanced antimicrobial activity observed in the synbiotic treatments. Table 1 summarizes the key metabolites and their documented antibacterial mechanisms.

4. Discussion

Although an acidic pH (5−6) has been shown to enhance the stability of allicin in garlic (Wang et al., 2015), possibly explaining the increased activity from *Lactobacillus*-induced acidity, several metabolites may also contribute to the observed effect. Therefore, the GC-MS based metabolomic profiling in the current study was an attempt to provide insight into the metabolic changes influenced by probiotic *Lactobacillus* isolates and aqueous garlic extract and their possible role in *Salmonella* spp. inhibition. To explore these metabolic changes, PLS-DA was conducted to identify differences among treatment groups. This supervised multivariate statistical method enhances group separation by identifying key metabolites that contribute to these differences (Kalivodová et al., 2014). In this study, Principal Component 1 (PC1) accounted for 15.5 % of the variance, while Principal Component 2 (PC2) accounted for 12.2 %, together capturing a moderate portion of the metabolic variance. The distinct clustering patterns indicated that the combination of probiotic *Lactobacillus* isolates and garlic extract led to unique metabolic

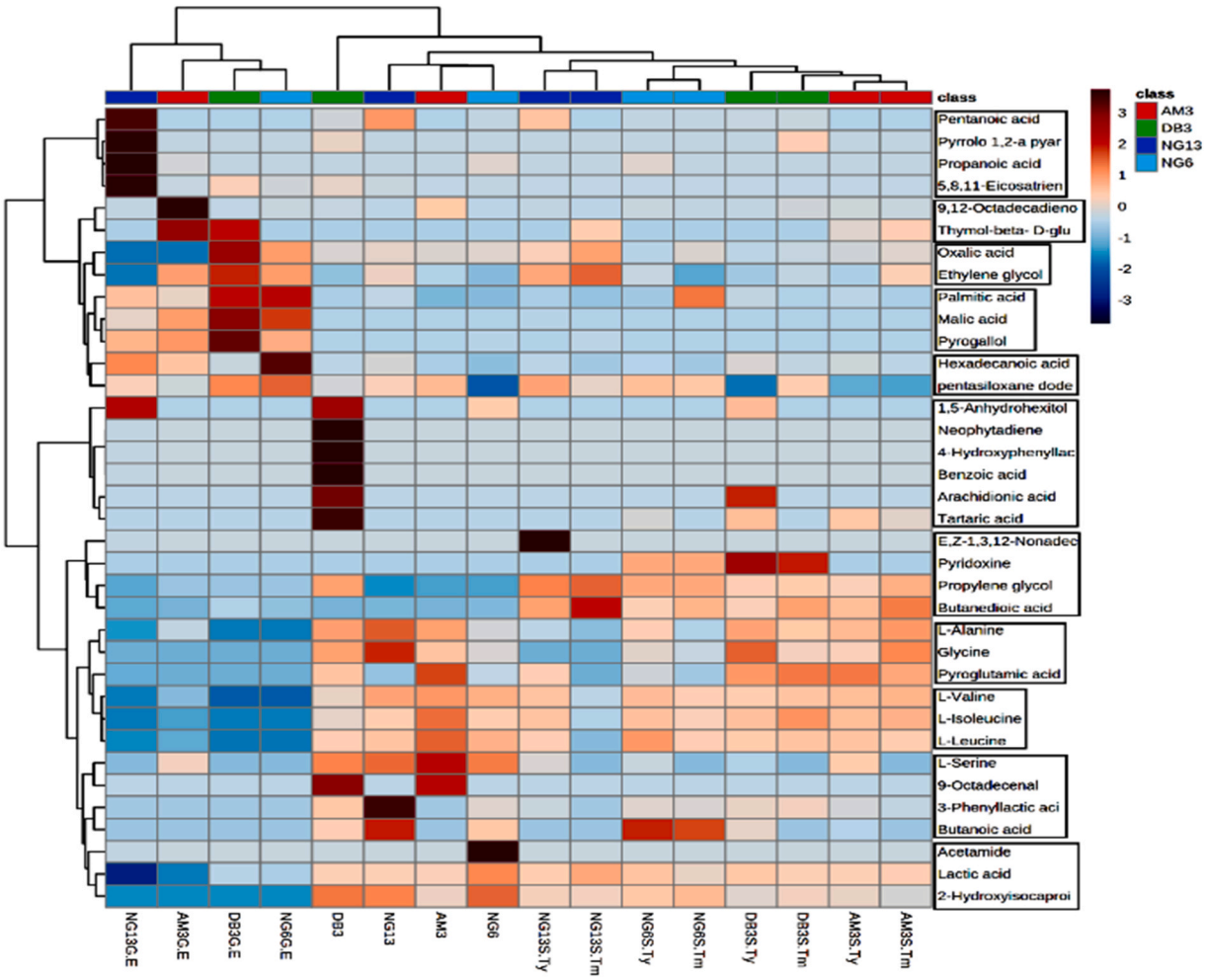

Fig. 3. Heat map and hierarchal clustering of metabolic profiles of fermented garlic extract by fecal isolates including *L. paracaesi* and *L. plantarum* (3 different strains) and also co-culture of the *Lactobacillus* isolates with *S.* Typhi and *S.* Typhimurium respectively.

changes, which may help inhibit Salmonella.

The clear separation of the AM3, DB3, and NG groups confirms that synbiotic and probiotic treatments result in different metabolic responses, likely through mechanisms involving SCFA-stimulated production, amino acid metabolism, and organic acid biosynthesis. The bacterial viability assay demonstrated the impact of these metabolic changes on bacterial survival, showing a notable decrease in the number of viable *Salmonella* Typhi and *Salmonella* Typhimurium cells over a 24 h period. When considered alongside the PLS-DA and heatmap findings, the observed bacterial inhibition can be linked to specific metabolic changes in the groups treated with probiotics. The distinct separation of NG6 and NG13 in the PLS-DA plot aligns with their stronger antibacterial effects seen in the viability assay. To validate these findings statistically, One-way ANOVA and post-hoc analyses were conducted to identify metabolites that were significantly upregulated in probiotic-fermented garlic extract. ANOVA is widely applied in metabolomic studies for assessing statistical differences in metabolomic studies and has been used in previous studies to distinguish metabolite abundance in probiotic interventions (Trivedi et al., 2016). The results from the ANOVA indicated that probiotic fermentation significantly modified the metabolomic profile of garlic extract ($p < 0.05$), which is is consistent with clustering patterns observed in the PLS-DA and heatmap analyses.

The metabolites that were significantly upregulated also correlate with the antibacterial activity graphs, supporting the idea that probiotic metabolism enhances the production of antimicrobial compounds derived from garlic. The ANOVA results further indicated that the metabolic changes observed in PLS-DA and the heatmap were not simply random fluctuations; they reflect significant biochemical changes brought about by the probiotic fermentation of garlic extract. These results are consistent with earlier research showing that probiotic metabolism influences gut microbiota composition and boosts the production of antimicrobial metabolites (Zhang et al., 2011). The antibacterial activity graphs provide strong phenotypic evidence that the probiotic *Lactobacillus* isolates, when combined with aqueous garlic extract, significantly reduce *Salmonella* viability, indicating a promising synbiotic approach for combating these enteric pathogens. Bacteria are known to produce a wide range of volatile secondary metabolites (Lemfack et al., 2017), which can exhibit antibacterial, antifungal, antiviral, and antioxidant properties, vital in the face of growing drug-resistant microbial infections (Abdel-Nasser et al., 2024). These metabolites are thought to serve as infochemicals that mediate inter- and intra-specific interactions (Schulz-Bohm et al., 2016).

In this study, the combination of *Lactobacillus* strains and garlic extract led to a progressive decline in the populations of *S.* Typhi and *S.* Typhimurium, with no detectable growth after 24 h in treated cultures. Our earlier study showed that the time required to inhibit these pathogens was reduced when the probiotics were combined with garlic extract compared to their use only. Similar findings have been reported elsewhere, where the combination of *Lactobacillus* spp. and *Curcuma longa* rhizome extract was more effective against *Cutibacterium acnes* than either agent alone in an agar well diffusion assay (Kim et al., 2020). Various antibacterial mechanisms of *Lactobacillus* spp. have been

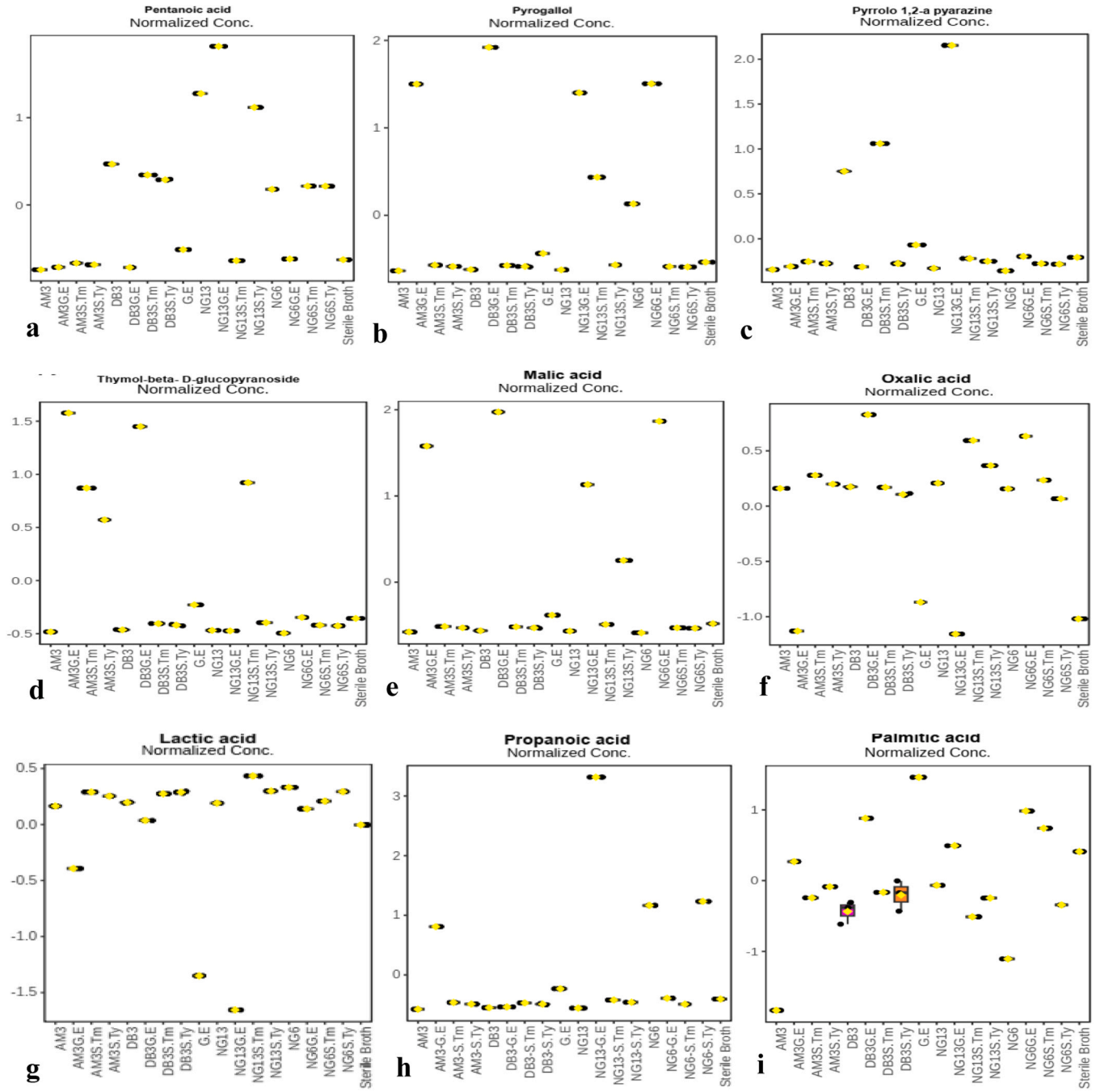

Fig. 4. One-way parametric ANOVA and post hoc Tukey's test showing normalized concentrations of significantly upregulated metabolites ($p < 0.05$) in *Lactobacillus* isolates cultured in MRS broth supplemented with 125 mg/mL garlic extract. The plot highlights nine metabolites (labeled a–i) that were consistently elevated across garlic-treated *Lactobacillus* strains (DB3GE, AM3GE, NG6GE, NG13GE) compared to their untreated counterparts. Results are presented as mean values, normalized and scaled using sum normalization and Pareto scaling.

identified, including nutrient competition and the production of inhibitory substances like bacteriocins, lactic acid, acetic acid, formic acid, and other organic acids that lower the pH (Nair et al., 2017, Chen et al., 2019). In this study, garlic extract at 125 mg/mL exhibited notable bactericidal activity against *Salmonella enterica serovars* Typhi and Typhimurium, while only transiently reducing *Lactobacillus* populations by approximately 1-log at 12 h post-treatment, followed by a recovery in viable counts at 24 h. This observation suggests a selective antimicrobial effect indicating that pathogenic bacteria are more susceptible to garlic's bioactive constituents than commensal probiotics. The differential response may be attributed to inherent structural and functional differences between Gram-negative enteropathogens and Gram-positive *Lactobacillus* spp., including variations in membrane permeability, efflux pump activity, detoxification enzymes, and stress adaptation mechanisms (Leus et al., 2023). Moreover, garlic is rich in inulin-type fructan carbohydrates known for their prebiotic activity, which may provide a competitive growth advantage to *Lactobacillus* under selective pressure (Lu et al., 2021). These fructooligosaccharides have been documented to enhance the colonization and resilience of beneficial gut microbes, further contributing to the observed recovery pattern. Although the exact mechanisms underlying this selective recovery remain unclear, the current findings support the potential application of garlic-based interventions in modulating gut microbiota by selectively inhibiting pathogens while promoting probiotic viability.

Table 1
Key metabolites and their antibacterial mechanism.

Metabolites	Source	Proposed antibacterial mechanism	Reference(s)
Propanoic acid (Propionic acid)	*L. plantarum* NG13 + garlic	SCFA; causes intracellular acidification, disrupts proton motive force, inhibits Salmonella	Jacobson et al., 2018; Ricke, 2003; Lamas et al., 2019.
Pentanoic acid	*L. plantarum* NG13 + garlic	SCFA with antimicrobial activity against Gram-negative and Gram-positive bacteria	Kovanda et al., 2019
Hexadecanoic acid (Palmitic acid)	Garlic-fermented cultures	Inserts into membranes; increases fluidity and leakage of ions/ metabolites	Casillas-Vargas et al., 2021; Shaaban et al., 2021
Pyrrolo[1,2-*a*] pyrazine−1,4-dione	*L. plantarum* NG13 + garlic	Disrupts cell functions; active against MDR Staphylococcus aureus	Kiran et al., 2018
Thymol-beta-D-glucopyranoside	*L. plantarum* AM3, *L. paracasei* DB3 + garlic	Disrupts bacterial cell membranes; derived from thymol, known for strong antibacterial activity	Gómez-García et al., 2020; Escobar et al., 2020
Ethylene glycol	*L. paracasei* DB3 + garlic	Alcohol with antimicrobial properties; disrupts metabolism and cell integrity	Krasowski, 2020; Salusjärvi et al., 2019
Malic acid	*L. paracasei* DB3 + garlic	Lowers pH; chelates metal ions; inhibits microbial enzymes	Raybaudi-Massilia et al., 2008; Mani-López et al., 2011
Pyrogallol	*L. plantarum* DB3 + garlic	Polyphenol that inhibits *Salmonella* quorum sensing and efflux pump genes	Jiménez et al., 2013; Birhanu et al., 2021; Bar et al., 2022

All metabolites significantly upregulated ($p < 0.05$)

Furthermore, strain-specific unique metabolic profiles were detected in the L. plantarum strains obtained from Nigerian and Indian volunteers which highlight the potential influence of geographical and individual factors on the metabolic differences at the strain level. These findings suggest that the specific metabolites produced by *Lactobacillus* spp. may vary across different populations, potentially contributing to variations in health outcomes. Many of the metabolites produced in high abundance in 125 mg/mL garlic extract supplemented MRS broth are known to have antimicrobial activities. A study by Ghosh et al. (2020) demonstrated that strains of *Lactobacillus* isolated from different geographic locations exhibit distinct metabolic profiles, which may contribute to variations in their probiotic properties. This supports the observation in our study where *L. plantarum* strains from Nigerian and Indian volunteers displayed unique metabolic patterns. The addition of garlic extract appears to have a significant impact on the metabolic profiles of the strains. The GE-treated samples (AM3GE, DB3GE, NG6GE, and NG13GE) exhibited distinct patterns compared to the control samples. Zhao et al. (2022) investigated how the metabolic profiles of *L. plantarum* varied based on the substrate provided during fermentation. Their findings corroborate our observation of strain-specific metabolic patterns when cultured with garlic extract. *L. plantarum* isolate NG13 exhibited a unique ability to produce high levels of pentanoic acid when cultured with garlic extract, suggesting a potential synergistic interaction. Pentanoic acid, a SCFA with known antimicrobial properties, has been shown to inhibit various pathogens, including *Clostridium perfringens*, *E. coli*, and *Salmonella* Typhimurium (Kovanda et al., 2019). While a significant amounts of pentanoic acid was detected in *L. plantarum* NG13 grown in the presence of garlic extract, its presence was notably reduced in monocultures and co-cultures with *Salmonella* Typhi. This suggests that garlic extract plays a crucial role in enhancing pentanoic acid production in this strain. Such variability in SCFA production among *Lactobacillus* strains, even when exposed to the same prebiotic, has been well-documented. For instance, Saulnier et al. (2009) demonstrated that different *Lactobacillus* strains responded differently to identical prebiotic substrates, resulting in varying levels and types of SCFAs. The marked increase in pentanoic acid production by *L. plantarum* NG13 when cultured with garlic extract underscores the strain-specific metabolic pathways activated by this substrate.

L. plantarum NG13 exhibited unique metabolic profiles when cultured with garlic extract, producing higher levels of pyrrolo[1,2-*a*] pyrazine-1,4-dione, propanoic acid, hexadecanoic acid, and 1,5-anhydrohexitol compared to the other strains. These metabolites have known antimicrobial properties, including activity against multiple drug-resistant *Staphyloccous aureus* (Kiran et al., 2018). Garlic extract appears to play a role in stimulating the production of pyrrolo[1,2-*a*] pyrazine-1,4-dione in NG13, as it was not detected in the strain when cultured alone. However, the production of this metabolite varied among the *Lactobacillus* strains. These findings highlight the potential of *Lactobacillus* strains and garlic extract combinations for combating bacterial infections. Propanoic acid, also known as propionic acid is one of the compounds that have been deemed promising for treating illnesses brought on by bacterial strains that are multi-drug resistant (Ricke, 2003). Propionic acid's effectiveness against enteric pathogens *in vivo* was shown to result in colonization resistance and growth inhibition of *Salmonella* Typhimurium (Jacobson et al., 2018). Lamas et al. (2019) reported that propionic acid exhibited minimum inhibitory and bactericidal concentrations of 3750 mg/L (50.6 mM) against various *Salmonella enterica* strains *in vitro*. Although such concentrations exceed the typical levels produced by microbial metabolism, the detection of propionic acid in the NG13 garlic-fermented supernatant (NG13G.E) in this study suggests a potential antimicrobial contribution. This may be supported by the presence of prebiotic carbohydrates like inulin-type fructans in garlic, which can enhance the metabolic activity of *Lactobacillus* isolates, possibly leading to increased production of short-chain fatty acids including propionate. However, the abundance of propionic acid detected in our samples was not at the MIC level reported by Lamas et al., and thus its antimicrobial role here is likely synergistic or supportive rather than directly inhibitory at that threshold. *Lactobacillus* spp. has been shown to ferment these prebiotics, resulting in the production of SCFAs like propionic acid (Dalile et al., 2019). Its ability to create a hostile environment both outside and inside microbial cells enhance its effectiveness in inhibiting bacterial growth and survival. Hexadecanoic acid, commonly known as palmitic acid, is the most frequently encountered saturated fatty acid in animals, plants, and microbes (Carta et al., 2017). Prior research has not identified hexadecanoic acid as one of the by-products of fermentation of aqueous garlic crude extract. However, hexadecanoic acid compound has been identified in a number of plants including moringa leaf (Devi et al., 2020), and alcoholic extract of clove (Shaaban et al., 2021). In the current study, GC-MS analysis of garlic-CFSC of the four *Lactobacillus* spp. showed higher abundance level of hexadecanoic acid compared to the culture of the same isolates without garlic, which suggest that garlic extract may constitute substrate required for the production of this metabolite. The antibacterial effects of hexadecanoic acid methyl ester against *S. aureus* W35, *P. aeruginosa* D31, *K. pneumoniae* DF30, and *K. pneumoniae* B45 have been documented (Shaaban et al., 2021). These compounds are known to insert into bacterial membranes, increasing permeability and causing leakage of vital ions and metabolites (Casillas-Vargas et al., 2021). This mechanism may synergize with acidic stress to compromise Salmonella cell integrity.

Cultures of *L. plantarum* AM3 and *L. paracasei* DB3 in 125 mg/mL garlic-supplemented MRS broth detected higher levels of thymol-beta-D-

glucopyranoside than *L. plantarum* strains NG13 and NG6. AM3 and DB3, isolated from the Indian population, appear more efficient at metabolizing garlic to produce high level of thymol-beta-D-glucopyranoside compared to the Nigerian isolates, possibly due to host-specific adaptations. Long-term garlic consumption in India may also have influenced the metabolic pathways of these strains. While no direct data compares garlic consumption between India and Nigeria, Saif et al. (2020), in their report ranks India among the highest in per capita garlic consumption. Pure thymol is originally from essential oils of plants such as *Thymus vaulgaris* and other species as well as *Oliveria decumbens* (Escobar et al., 2020). Thymol has been demonstrated to be effective against pure cultures of enteric pathogens such as *Salmonella*, *Campylobacter* and *E. coli* with minimum inhibitory doses ranging between 1.00 and 1.55 μmol/mL (Gómez-García et al., 2020). The mechanism of thymol's bactericidal activity has been proposed to be associated with its ability to insert into the target bacteria's cell wall, consequently leading to disintegration of the cell wall integrity (Gómez-García et al., 2020). Ethylene glycol, a toxic alcohol with known antimicrobial properties (Krasowski, 2020), was also detected in higher levels in *L. paracasei* DB3 compared to other isolates when cultured with garlic extract. While the production of ethylene glycol by lactic acid bacteria is less well-studied, biotechnological approaches using genetically modified microorganisms have been explored for its sustainable production (Salusjärvi et al., 2019). The detection of ethylene glycol in our study suggests that *Lactobacillus* strains may possess the metabolic capacity to produce this compound, potentially contributing to their antimicrobial effects. Malic acid, a dicarboxylic acid with known antimicrobial properties (Raybaudi-Massilia et al., 2008), was exclusively detected in the *Lactobacillus* strains cultured with garlic extract. *Lactobacillus paracasei* exhibited the highest malic acid production, suggesting a synbiotic effect between the strain and garlic. The detection of malic acid suggests that mild acidification and potential metal ion chelation could play a supporting role in antimicrobial activity, though its individual effect may be modest (Mani-López et al., 2011). Garlic supplementation in this study appeared to be a key factor in stimulating malic acid production in *Lactobacillus* strains, highlighting its potential role in enhancing the antimicrobial activity of these bacteria. Another potential antibacterial compound identified in the current study was pyrogallol, a hydroxylated polyphenol found in various fruits and vegetables, and commonly used in pharmaceuticals for therapeutic purposes (Sarikaya, 2014). Earlier, Birhanu et al. (2021) had demonstrated that pyrogallol exhibited antibacterial activity against *Salmonella* Typhimurium, inhibiting bacterial invasion and intracellular survival by down-regulating genes related to quorum sensing, pathogenicity and efflux pump mechanisms. In previous study, *L. plantarum* has been shown to produce pyrogallol by hydrolyzing hydrolyzable tannins to release gallic acid, which is then decarboxylated to form pyrogallol (Jiménez et al., 2013). This suggests that *L. plantarum* may contribute to pyrogallol production through metabolic processes involving tannins. Notably, tannins and other phytochemicals have been detected in aqueous garlic extract in earlier study (Bar et al., 2022), indicating that the presence of these compounds could facilitate pyrogallol production in the fermented garlic cultures observed in the current research. This highlights the potential for synergistic antimicrobial effects between garlic extract and *Lactobacillus*-derived metabolites.

5. Limitations of the study

This study employed simplified *in vitro* broth co-culture systems, which, while useful for preliminary mechanistic insights, do not replicate the complexity of the gastrointestinal environment. Important physiological factors such as gut pH, host immunity, epithelial interactions, anaerobic conditions, and the presence of a diverse native microbiota were not simulated, potentially limiting the relevance of the observed effects to *in vivo* conditions. Additionally, the co-culture medium composed of a 1:1 ratio of MRS (optimal for probiotic growth) and BHI (suitable for Salmonella growth) may have created suboptimal conditions for both organisms. This could introduce bias in microbial viability or metabolite expression, affecting the interpretation of competitive interactions. Furthermore, while PLS-DA was employed to discern group separation based on metabolite profiles, it is important to acknowledge that the cumulative variance explained by the first two components (PC1 and PC2) was 27.7 %. This relatively low proportion of total variance suggests that the model may not fully capture all biologically meaningful variability within the dataset. Consequently, some critical metabolic features contributing to group differences may reside in higher-order components not visualized in the scores plot. Future work should address these limitations by increasing the diversity and number of probiotic strains tested, validating findings in animal models or dynamic gut simulators, and employing targeted metabolomic approaches to quantify specific antimicrobial metabolites identified in this study.

While the GC-MS analysis successfully identified a range of metabolites produced by probiotic *Lactobacillus* isolates cultured with garlic extract, one technical limitation must be acknowledged. The scanning in our protocol commenced at 4.00 min, a standard practice to avoid solvent interference; however, this approach may have inadvertently excluded early-eluting volatile compounds, such as low molecular weight compounds which may also contribute to the reported antimicrobial activity. Acknowledging this limitation in future analyses and optimizing the solvent cut time may enhance the detection of these potentially significant metabolites.

6. Conclusions

The present study highlights the metabolomic interplay of the synergistic antibacterial effects of human fecal *Lactobacillus* spp. with aqueous garlic extract against *Salmonella* Typhi and *Salmonella* Typhimurium. The results revealed a significant reduction in pathogen counts when *Lactobacillus* spp. were co-cultured with aqueous garlic extract, indicating a promising synbiotic approach to combating enteric pathogens. Metabolomic profiling identified several metabolites with known antimicrobial properties, including valeric acid, propanoic acid, pyrrolo [1,2-*a*] pyrazine-1,4-dione, and hexadecanoic acid, with notable strain-specific differences in metabolite production. The study also found that *L. plantarum* strains isolated from different geographical regions exhibited varied garlic metabolism, likely due to host-specific adaptations. Additionally, the ANOVA-based metabolite analysis confirms that probiotic fermentation of garlic extract leads to the upregulation of bioactive metabolites with antimicrobial properties. The significant increase in SCFAs, organic acids, and other fermentation-derived metabolites aligns with the PLS-DA clustering and heatmap findings, explaining the strong *Salmonella* suppression observed in the antibacterial activity graph. These results reinforce the potential of synbiotic interventions as an effective antimicrobial strategy, highlighting the need for further targeted metabolomics and *in vivo* studies to fully characterize the functional role of these metabolites in pathogen inhibition.

Ethical clearance

The research received approval from the Ondo State Health Research Ethics Committee (OSHREC2/7/2018/351), Nigeria (Study allocated number: NHREC/18/08/2016; Protocol Number: OSHREC 2/7/2018/351).

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CRediT authorship contribution statement

Fadare Olalekan Shadrach: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Enabulele Onaiwu Idahosa:** Validation, Supervision, Conceptualization. **Vibhor Singh:** Writing – review & editing, Software, Data curation. **Diwas Pradhan:** Writing – review & editing, Supervision, Data curation, Conceptualization.

Declaration of Competing Interest

The authors have nothing to declare.

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Authors' Contribution

O.S. Fadare, O.I. Enabulele and D. Pradhan Conceptualized and designed the study. O.S. Fadare collected the fecal samples. O.S. Fadare carried out the Microbiological and GCMS analyses. O.S. Fadare, D. Pradhan and V. Singh performed the statistical analyses. O.S. Fadare drafted the manuscript. O.S. Fadare and D. Pradhan reviewed and edited the manuscript. All authors contributed equally based on their area of expertise. All authors have read and approved the final version of the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.microb.2025.100494.

Data availability

Data will be made available on request.

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