RESEARCH PAPER

Exploration of Antimicrobial Drug Targets from Human Breast Milk Lactobacillus for the Control of Vaginal Infections

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Abstract
Probiotics are live microorganisms that, when given in the right doses, can improve the health of the person who takes them. In this work, Lactobacillus species were isolated from the breast milk of a 26-year-old woman and assessed for their antibacterial efficacy against certain vaginal infections. The isolates underwent testing at varying concentrations (50 µL, 40 µL, and 30 µL). Motility tests, Gram staining, and routine biochemical assays were used to make a first guess about what Lactobacillus was. The agar disc diffusion method was used to test the isolated strain's ability to kill vaginal infections. Antibiotic susceptibility testing was also performed on the pathogenic isolates. The findings indicated that the Lactobacillus strain derived from breast milk shown considerable antibacterial efficacy against the examined vaginal pathogens, particularly demonstrating the most pronounced inhibitory effect on Klebsiella pneumoniae. We used Gas Chromatography–Mass Spectrometry (GC–MS) to do more research and find the bioactive components in the extract. The investigation showed that the active fraction had bioactive chemicals that could be new. The main goal of this study was to find out if Lactobacillus from breast milk may help prevent cervical and vaginal infections by testing its ability to kill bacteria and finding the bioactive substances that do so. These discoveries may expand existing research paradigms and facilitate the advancement of natural, human-derived medicinal molecules with reduced adverse effects relative to traditional chemical pharmaceuticals.
Keywords: Probiotic, Vaginal pathogens, Lactobacillus, LMW compounds, GC-MS, Antimicrobial compounds

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INTRODUCTION

Probiotics are live microorganisms that provide health advantages to the host when given in the right proportions [1–3]. A perfect probiotic strain should come from humans, not be harmful to humans, and not include genes or toxins that may be passed on that make it resistant to antibiotics. It should also be able to endure physiological stressors like acidic pH, bile salts, and digestive enzymes, fight off pathogens, change how the host's immune system works, and stay alive and stable during processing and storage [4,5]. Comprehensive research, encompassing systematic reviews, has elucidated the advantageous effects of probiotics in the prevention and management of gastrointestinal disorders, including infectious diarrhea, antibiotic-associated diarrhea, irritable bowel syndrome (IBS), Clostridioides difficile–associated diarrhea, and ulcerative colitis [6–11]. Researchers have also looked into how probiotics might help with allergy problems such atopic dermatitis and allergic rhinitis [12,13]. Lactobacillus and Bifidobacterium are the most popular types of probiotics that people buy [14]. Probiotics are very important for keeping the urogenital system healthy, in addition to keeping the gut healthy. Lactobacillus species are the most common type of bacteria in a healthy vagina. They guard against infections by making organic acids, hydrogen peroxide, and bacteriocins, and by competing with pathogens for places to attach to epithelial cells [24]. When this balance of microbes is upset, women are more likely to get reproductive tract infections (RTIs), which are especially common during pregnancy. RTIs, such as candidiasis, bacterial vaginosis (BV), and trichomoniasis, are more common in the third trimester because of hormonal changes. They can also cause problems with the pregnancy [17,18]. The increasing prevalence of antibiotic resistance in vaginal infections underscores the necessity for alternate, organically sourced treatment strategies. Recent research has identified human breast milk as a source of a diverse microbial community that has a role in the development of the baby gut microbiota and immunological

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maturation [22,23]. It acts as a way to pass on helpful bacteria from the mother to the baby during breastfeeding, such as Lactobacillus species [25]. These probiotic strains produced from humans may exhibit distinctive antibacterial characteristics and bioactive substances with prospective medicinal significance. Due to Lactobacillus's protective role in the female urogenital tract and the growing interest in natural bioactive compounds, Lactobacillus generated from breast milk is a promising option for the development of new antimicrobial medicines. The current study seeks to isolate and characterize Lactobacillus from human breast milk, assess its antimicrobial efficacy against certain vaginal infections, and identify the bioactive components responsible for this action by Gas Chromatography–Mass Spectrometry (GC–MS). This study aims to examine breast milk-derived probiotic strains as prospective natural medicinal agents for the prevention and treatment of vaginal infections.

MATERIALS AND METHODS

Sample Collection and Isolation of Lactobacillus spp.

A healthy 26-year-old lady who was breastfeeding sent us breast milk in a sterile container, and we processed it right away. We made serial dilutions of the sample in sterile saline and then dispersed the right amounts of those dilutions onto De Man, Rogosa, and Sharpe (MRS) agar plates. Plates were kept at 37°C for 24 hours in an aerobic environment. Subcultured distinct colonies to yield pure isolates, which were stored on 0.8% MRS agar supplemented with 50% glycerol at −20°C. Initial identification of putative Lactobacillus isolates was conducted using colony morphology, Gram staining, and conventional biochemical traits. The final identification was done using the rules in Bergey's Manual of Determinative Bacteriology and Bergey's Manual of Systematic Bacteriology.

Morphological and Biochemical Characterization

Morphological analysis was conducted utilizing Gram staining as delineated by Hans Christian Gram (1884). We used a microscope to capture the form, organization, and Gram response of the cells. Biochemical characterization encompassed catalase, oxidase, urease, indole, methyl red (MR), Voges–Proskauer (VP), citrate utilization, Triple Sugar Iron (TSI), carbohydrate fermentation, and motility assessments via the soft agar stab method. The identification was based on a combination of the morphological and biochemical profiles.

Test Microorganisms and Culture Conditions

We tested the isolated Lactobacillus strain's ability to kill germs against the following clinical pathogens: • Klebsiella pneumoniae • Staphylococcus aureus, • Enterococcus faecalis All strains were acquired from Magnum Diagnostic Centre (Trichy, India). The cultures were kept on Brain Heart Infusion (BHI) agar slants at 4°C and subcultured before the experiments.

Antibacterial Activity Assay

The disc diffusion method on MHA plates was used to test the test microorganisms' resistance to antibiotics, using normal microbiological methods. Fresh bacterial solutions (0.5 McFarland standard) were evenly spread on agar surfaces with a swab. We used the following antibiotic discs: ampicillin, ofloxacin, gentamicin, amphotericin B, vancomycin, and nitrofurantoin. We evaluated the inhibition zones in millimeters after 18 to 24 hours of incubation at 37°C. Results were analyzed based on conventional susceptibility criteria.

Antibiotic Susceptibility Testing

The disc diffusion method on MHA plates was used to test the test microorganisms' resistance to antibiotics, using normal microbiological methods. Fresh bacterial solutions (0.5 McFarland standard) were evenly spread on agar surfaces with a swab. We used the following antibiotic discs: ampicillin, ofloxacin, gentamicin, amphotericin B, vancomycin, and nitrofurantoin. We evaluated the inhibition zones in millimeters after 18 to 24 hours of incubation at 37°C. Results were analyzed based on conventional susceptibility criteria.

Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

Gas chromatography–mass spectrometry (GC–MS) was used to look at the extracellular metabolites of the isolated Lactobacillus strain (BMSD). We used methanol and chloroform to extract cell-free supernatants separately. An Agilent GC system with an HP-5MS non-polar capillary column (30 m × 0.18 mm) and an Agilent mass selective detector was used to inject 1 µL of each extract. It was adjusted to 250°C for the injector. The oven was first set to 50°C for 1 minute, then raised to 160°C at a rate of 25°C per minute (held for 1 minute), and then elevated to 230°C at a rate of 10°C per minute (held for 4.6 minutes). Helium was employed as a carrier gas at a pressure of 5 kPa. The temperature of the interface stayed at 250°C. Ionization was done by hitting the electrons with 70 eV in positive mode. We were able to identify the compound by comparing the mass spectra we got with reference spectra from the NIST library database.

RESULTS AND DISCUSSION

Isolation and Phenotypic Characterization of BMSD

A presumptive Lactobacillus strain, designated BMSD, was successfully isolated from human breast milk using MRS agar. Colonies were smooth, circular, convex, and creamy white after 24–48 h incubation at 37°C. Repeated subculturing confirmed purity and stability of the isolate. Microscopic examination revealed Gram-positive, rod-shaped bacilli arranged singly and in short chains, consistent with the general morphological characteristics of the genus Lactobacillus [26]. The absence of catalase activity further supports its classification within lactic acid bacteria (LAB), which are typically catalase-negative due to the lack of cytochrome systems [27]. These findings align with previous reports describing the isolation of Lactobacillus spp. from human breast milk as part of the natural maternal microbiota [28].

Biochemical Characterization

The biochemical profile of BMSD demonstrated positive fermentation of glucose, lactose, sucrose, and Triple Sugar Iron (TSI) agar, while showing negative reactions for catalase, oxidase, urease, citrate utilization, indole, methyl red (MR), and Voges–Proskauer (VP) tests. Carbohydrate fermentation patterns are key taxonomic features used in the identification of Lactobacillus species [29]. The negative catalase and oxidase reactions are characteristic of LAB and distinguish them from other Gram-positive bacilli [30]. Similar biochemical profiles have been reported for probiotic Lactobacillus strains isolated from breast milk and fermented foods [31].

Antibiotic Susceptibility of Pathogenic Strains

The antibiotic susceptibility patterns of Klebsiella pneumoniae, Staphylococcus aureus, and Enterococcus faecalis were evaluated using the disc diffusion method, revealing that K. pneumoniae showed the highest sensitivity to ofloxacin, E. faecalis exhibited greater sensitivity to nitrofurantoin compared to vancomycin, and S. aureus demonstrated maximum sensitivity to gentamicin; these results highlight the variable intrinsic and acquired resistance mechanisms among clinical pathogens [32] and underscore the importance of targeted antimicrobial therapy, particularly in the context of increasing multidrug resistance in Gram-negative bacteria such as K. pneumoniae [33-36].

Antimicrobial Activity of BMSD

The antimicrobial activity of BMSD cell-free supernatant was assessed at 30 µL, 40 µL, and 50 µL concentrations, demonstrating a clear concentration-dependent increase in inhibition zone diameter against all tested pathogens. At 50 µL, maximum inhibition zones were observed against Klebsiella pneumoniae (38 mm), followed by Staphylococcus aureus (35 mm) and Enterococcus faecalis (34 mm). The comparatively higher inhibition against K. pneumoniae indicates strong antagonistic potential against Gram-negative bacteria. Lactic acid bacteria (LAB) inhibit pathogens through multiple mechanisms, including organic acid production, bacteriocin secretion, hydrogen peroxide generation, and competitive exclusion [37-40]. The substantial inhibition zones observed suggest that BMSD produces potent extracellular antimicrobial metabolites, consistent with previous reports on breast milk–derived Lactobacillus strains exhibiting broad-spectrum antimicrobial activity [41].

GC–MS Analysis of Extracellular Metabolites

GC–MS analysis of the culture supernatant identified lactic acid as the predominant compound (62.47% peak area in methanolic extract). Additional fatty acids such as palmitic acid, stearic acid, oleic acid, dodecanoic acid, and tetradecanoic acid were also detected. Lactic acid is a primary metabolic product of Lactobacillus spp. and plays a critical role in pathogen inhibition by:Lowering environmental pH. Disrupting membrane permeability,Interfering with intracellular metabolic processes [42-43]. Fatty acids such as oleic and palmitic acid have also been reported to possess antimicrobial properties through membrane destabilization mechanisms [44-51]. The predominance of lactic acid strongly correlates with the significant antimicrobial activity observed in agar well diffusion assays, suggesting that organic acid production is a major inhibitory mechanism of BMSD

Fig 1 : Pure culture of BMSD

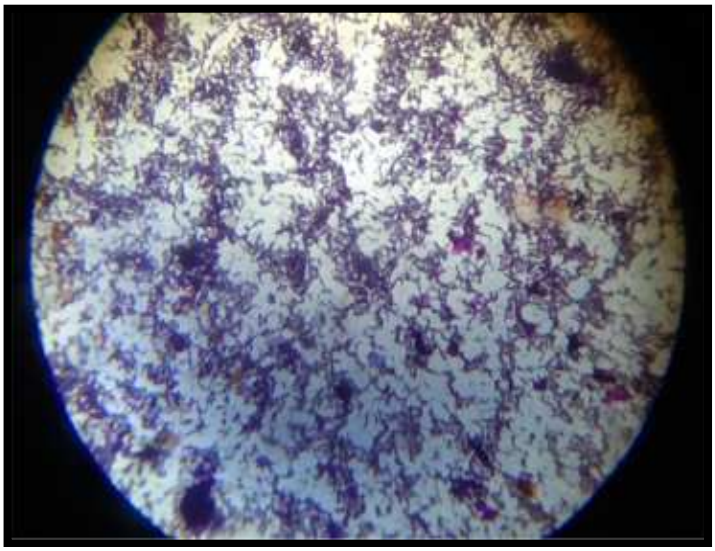

Fig 2 : Gram staining of BMSD

Table:1 Biochemical characterization of BMSD

BIOCHEMICAL TEST	**SAMPLE**
	BMSD
Triple sugar iron agar	+
Urease test	-
Citrate utilization test	-
Catalase test	-
Oxidase test	-
Indole test	-
Methyl red test	-
Voges Prauskauer test	-
Sucrose	+
Glucose	+
Lactose	+

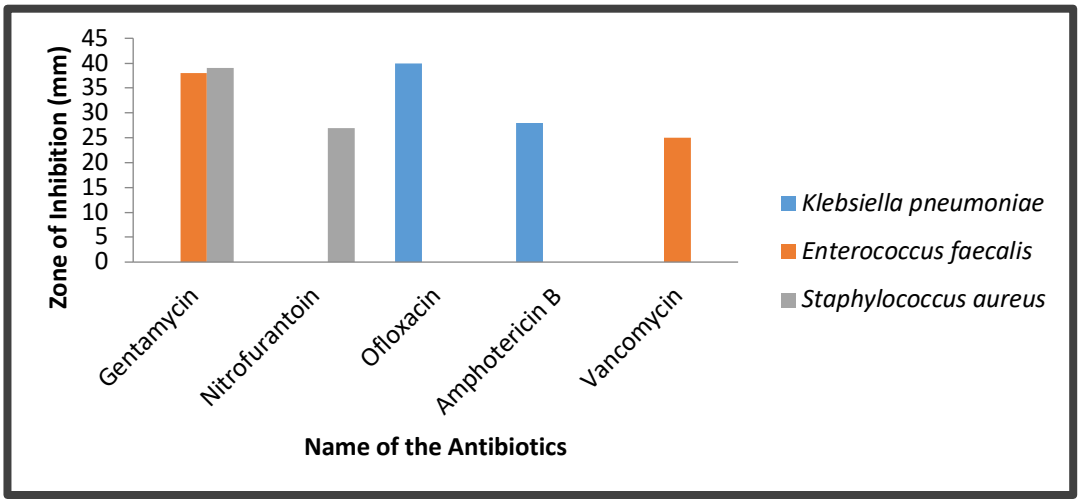

Fig: 3. Comparative zone of inhibition (mm) of different antibiotics against Klebsiella pneumoniae, Enterococcus faecalis, and Staphylococcus aureu

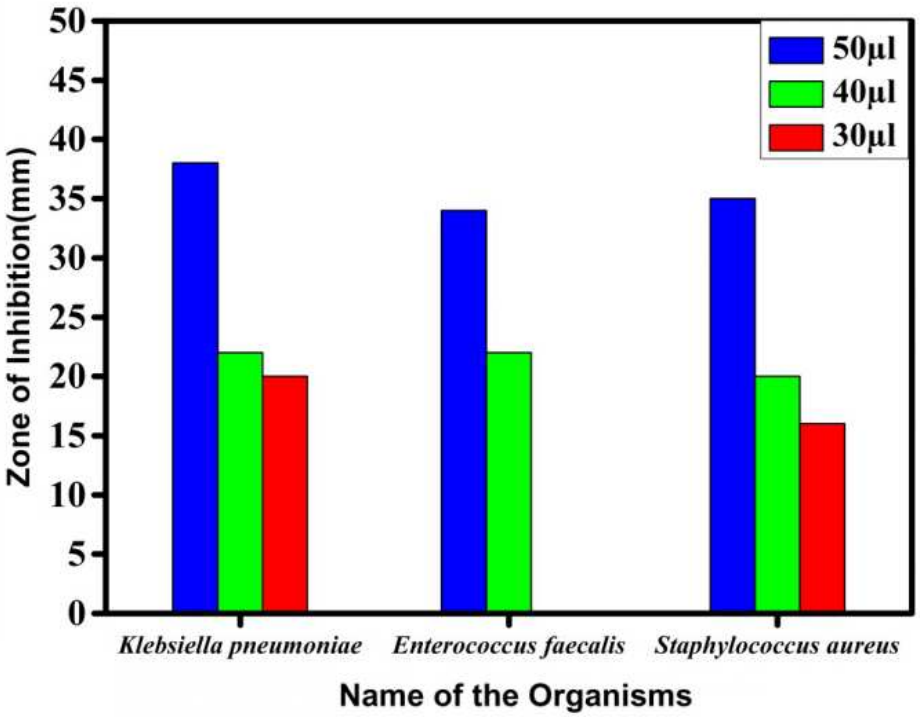

Fig: 4 Dose-dependent zone of inhibition (mm) against tested bacterial strains at concentrations of 30, 40, and 50 µl.

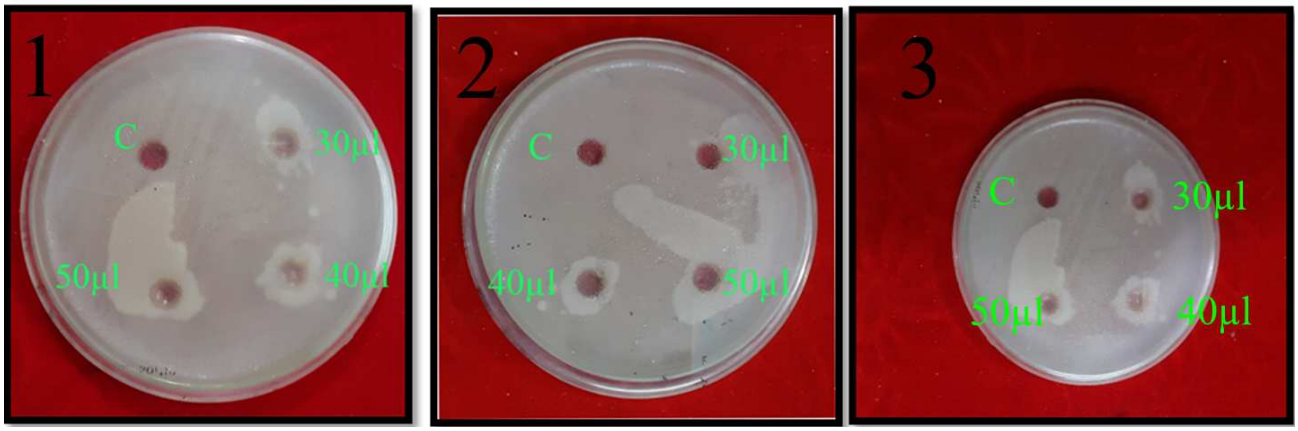

Fig 5 : Maximum zone of inhibition shown by BMSD-1 against 1. Klebsiella pneumoniae, 2. Enterococcus faecalis, 3. Staphylococcus aureus

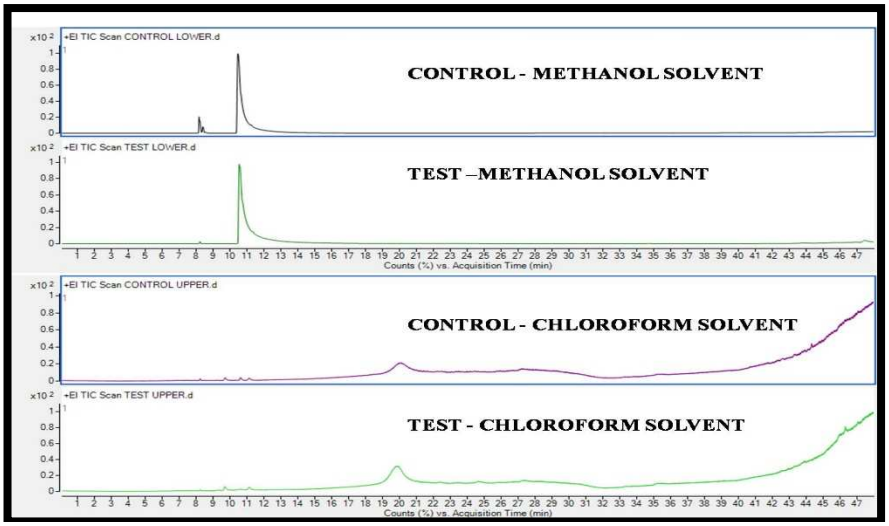

Fig. 6. GC–MS study of extracellular metabolites identified

Table 2. GC–MS analysis of extracellular metabolites detected in methanolic and chloroform extracts of BMSD culture supernatant

Peak No.	Retention Time (min)	Compound Identified	Molecular Formula	Molecular Weight (g/mol)	Peak Area (%)	Extract
1	11.84	Lactic acid	$C_3H_6O_3$	90.08	62.47	Methanol
2	13.21	Propanoic acid derivative	$C_3H_6O_2$	74.08	8.36	Methanol
3	21.47	Hexadecanoic acid (Palmitic acid)	$C_{16}H_{32}O_2$	256.42	9.84	Chloroform
4	22.93	Octadecanoic acid (Stearic acid)	$C_{18}H_{36}O_2$	284.48	6.52	Chloroform
5	24.76	9-Octadecenoic acid (Oleic acid)	$C_{18}H_{34}O_2$	282.46	7.19	Chloroform
6	27.18	Dodecanoic acid	$C_{12}H_{24}O_2$	200.32	3.21	Chloroform
7	29.64	Tetradecanoic acid	$C_{14}H_{28}O_2$	228.37	2.41	Chloroform

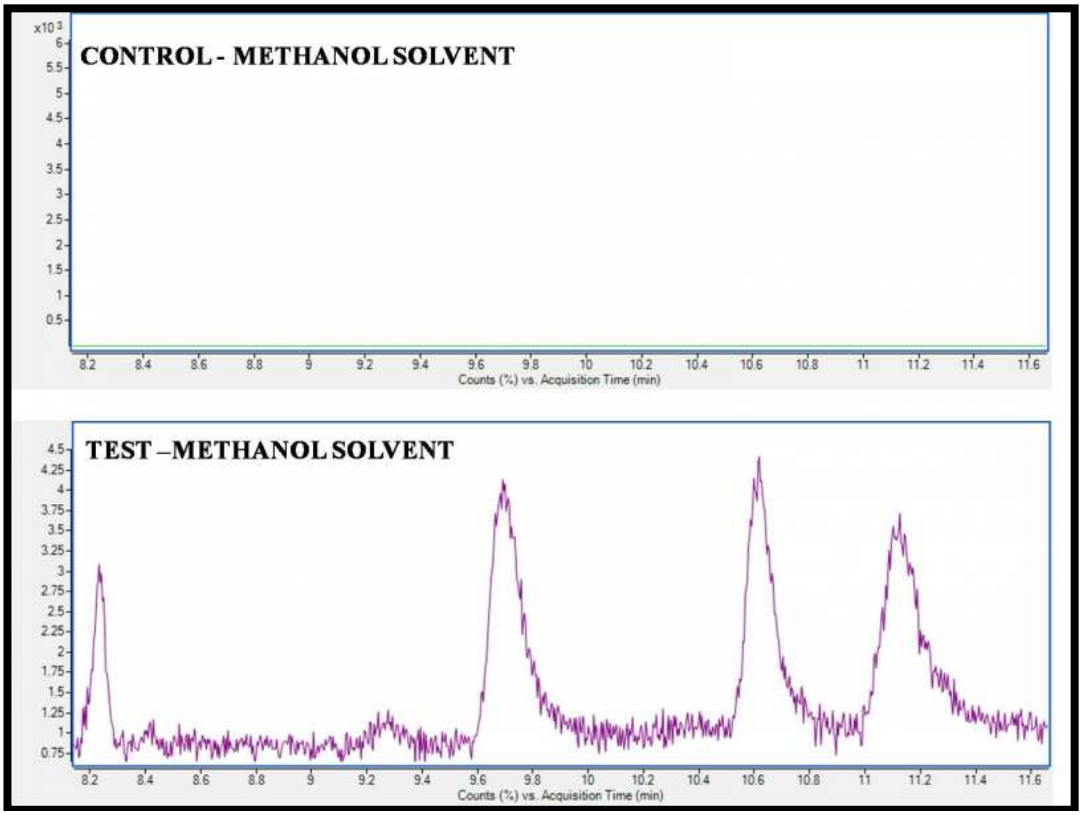

Fig. 7. GC–MS study of extracellular metabolites identified

Table 3: Prediction of Target for Lactic Acid: Probability scores for lactic acid - higher probability with indicated protein class is assumed as bioactive and to have this protein as target.

Target	Common name	Uniprot ID	ChEMBL ID	Target Class	Probability*
Tyrosine-protein kinase LCK	LCK	P06239	CHEMBL258	Kinase	0
Tyrosine-protein kinase FYN	FYN	P06241	CHEMBL1841	Kinase	0
Matrix metalloproteinase 9	MMP9	P14780	CHEMBL321	Protease	0
Matrix metalloproteinase 2	MMP2	P08253	CHEMBL333	Protease	0
HMG-CoA reductase	HMGCR	P04035	CHEMBL402	Oxidoreductase	0
Histone deacetylase 3	HDAC3	O15379	CHEMBL1829	Eraser	0
Estrogen receptor beta	ESR2	Q92731	CHEMBL242	Nuclear receptor	0
Egl nine homolog 1	EGLN1	Q9GZT9	CHEMBL5697	Oxidoreductase	0
Aldose reductase	AKR1B1	P15121	CHEMBL1900	Enzyme	0.085
Adenylosuccinate synthetase 2	ADSS	P30520	CHEMBL4875	Enzyme	0
DNA Adenine Methylase	DAM	P0AEE9	CHEMBL1075075	Enzyme	0.11

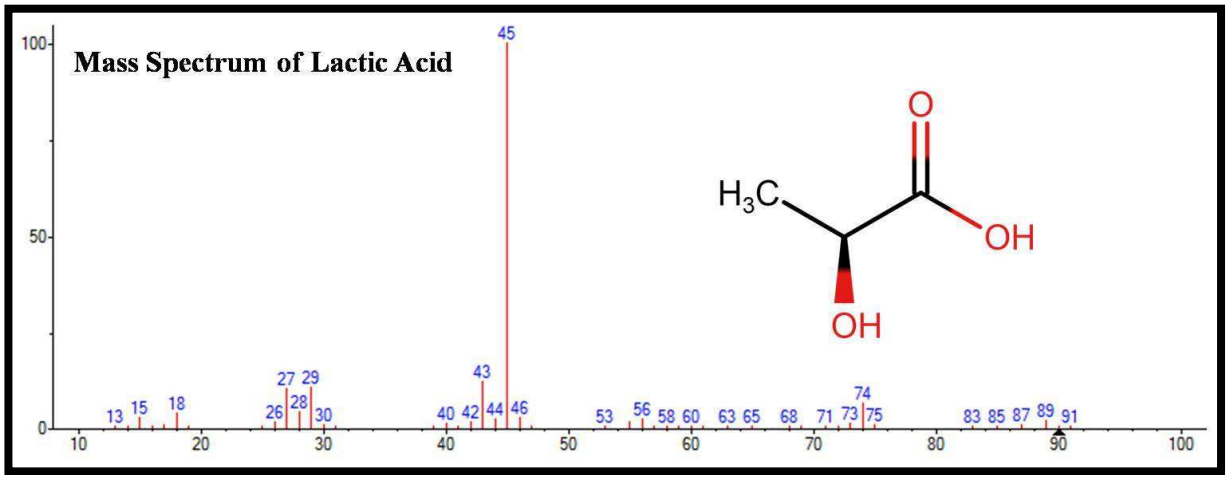

Fig. 8. GC–MS study of extracellular metabolites identified

CONCLUSION

The breast milk–derived strain BMSD exhibited typical morphological and biochemical characteristics of Lactobacillus spp. and demonstrated strong, dose-dependent antimicrobial activity against clinically important pathogens. GC–MS analysis confirmed lactic acid as the major extracellular metabolite, supporting its antimicrobial mechanism. These findings reinforce the probiotic and therapeutic potential of human milk–derived Lactobacillus strains as alternative or adjunct antimicrobial agents, particularly in the era of rising antibiotic resistance. Further molecular identification (16S rRNA sequencing), purification of bacteriocins, and in vivo validation studies are warranted to confirm clinical applicability.

Authors' contributions

ST and **JB** conceived the research, assisted in its design, execution, and analysis, as well as data interpretation and manuscript writing. **AT** helped with the implementation of the experiment as well as the manuscript revision. The manuscript was written by both contributors, who also provided feedback. The final manuscript has been read by all writers and authorized.

Data availability

Data will be made available on request.

Consent to publish

Not applicable.

Competing interests

The authors declare no competing interests.

Ethical approval and consent to participate

Not applicable.

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