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EVALUATION OF ANTIMICROBIAL AND CYTOTOXIC ACTIVITIES OF COMMERCIAL LACTOBACILLUS ACIDOPHILUS AGAINST FOODBORNE PATHOGENS AND THE MCF-7 CELL LINE

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ABSTRACT

Background: *Lactobacillus acidophilus*, a key member of the lactic acid bacteria (LAB) group, contributes significantly to both human and animal health by enhancing the microbiome. This study explores the bioactive compounds produced by *L. acidophilus* isolated from the Synkromax probiotic, with a focus on their antimicrobial, antifungal, and anticancer properties. **Methodology:** The cell-free supernatant (CFS) of *L. acidophilus* was subjected to solvent extraction to isolate secondary metabolites, while peptides, including bacteriocins, were partially purified using ammonium sulfate precipitation. Metabolite profiling was conducted using Gas Chromatography-Mass Spectrometry (GC-MS/MS), and Thin Layer Chromatography (TLC) was used for qualitative analysis. The antimicrobial and antifungal activities of the extracts were evaluated using the well diffusion method against pathogens, including *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Aspergillus niger*, and *Aspergillus flavus*. The cytotoxic potential of the partially purified bacteriocin extract (PPBE) was assessed using the MTT assay on MCF-7 breast cancer cells. **Results and Discussion:** GC-MS/MS analysis identified a diverse range of bioactive secondary metabolites. Both CFS and partially purified peptides demonstrated significant antimicrobial and antifungal activity. The PPBE also exhibited strong cytotoxicity against MCF-7 cells, with an IC_{50} value of 72.3991 µg/mL, indicating promising anticancer potential. **Conclusion:** *Lactobacillus acidophilus* from Synkromax produces a variety of bioactive compounds with potent antimicrobial, antifungal, and anticancer activities. These findings support its potential application in therapeutic development and enhancing food safety.

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INTRODUCTION

Lactobacillus acidophilus is a type of probiotic bacteria that supports a healthy balance for our gut system through boosting the immune system and improving digestive health. It enhances the immune system, development, and feed efficiency in animals while reducing the need for antibiotics. In individuals, it supports immunity, digestive health, and may also benefit cardiovascular and mental health [1]. Across species, *L. acidophilus* plays a significant role in maximizing gut health and function. Additionally, by promoting the formation of antibodies and enhancing immune cell activity, these microorganisms strengthen the immune system and increase resistance to infections and illnesses [2]. Probiotics are an excellent substitute for antibiotics because they enhance the utilisation of nutrients by breaking down complex feed ingredients, which raises growth rates and feed conversion ratios. They also stabilise the gut flora and lessen stress. They also reduce the likelihood of antibiotic resistance. *L. acidophilus* is a symbiotic partner of the host in the digestive system [3]. It mainly coexists in the small intestine and cecum, where it helps to maintain a healthy microbial community [4]. *L. acidophilus* helps build a strong defense against a wide range of infections frequently found in animal farming conditions through this immunomodulatory function [5]. This is especially important in systems of intensive farming when animals are frequently subjected to stimuli that can impair their immune systems. Additionally, *L. acidophilus* is essential for enhancing nutritional absorption in the animal's digestive system [6]. This probiotic strain enhances growth rates and feed conversion ratios by producing enzymes that break down complex proteins and carbohydrates, thereby facilitating the absorption of essential nutrients. This is advantageous for the general health and well-being of the animal as well as for the profitability of livestock operations [7].

Moreover, adding *L. acidophilus* to animal feed provides an ecologically friendly and sustainable approach to livestock management [8]. Although there are many potential advantages to using *L. acidophilus* in animal feed, maintaining the probiotic's viability and stability throughout feed processing and storage poses difficulties. Considering the significance of L. acidophilus, optimizing its ability to support animal health necessitates attention to storage conditions, suitable formulation methods, and rigorous quality control measures. Commercial *Lactobacillus acidophilus* supplements have gained popularity in both human and animal nutrition due to the probiotic's advantages for immune system and digestive health [9]. These supplements offer benefits such as stronger immune system support, improved nutrient absorption, reduced reliance on antibiotics, and enhanced digestive health [10]. The need for a deeper understanding of strain-specific effects, formulation compatibility issues, stringent quality control procedures, and viability concerns is one of the challenges [11]. The sensitivity of *L. acidophilus* to environmental factors is still a consideration, even though these supplements utilize cutting-edge technologies to ensure stability during manufacturing and storage. To fully realize the potential benefits of commercially available *L. acidophilus* across various applications, different research studies with rigorous quality assurance are essential. These challenges include variability in product quality, the impact of formulation on efficacy, and cost implications [12]. In this study, we have assessed the *L. acidophilus* commercial spray-dried powder provided by SynkroMax Biotech Private Limited. Thirumazhisai, Chennai- 600124, Tamil Nadu, India, and preserved at the Centre of Bioscience and Nanoscience Research, Eachanari, Coimbatore- 641021, Tamil Nadu, India. The efficacy of the product was determined by examining the biological activity of cell-free supernatant (CFS) and partially purified bacteriocin extract (PPBE) against common foodborne pathogenic bacteria and fungi. The partially purified extract's cytotoxic effects were assessed on MCF-7 cancer cell lines. Furthermore, GC-MS/MS analysis was performed on the bacterial spent supernatant extracted with ethyl acetate overnight to profile the various beneficial metabolites secreted by *L. acidophilus*. By characterizing the functional profile of *L. acidophilus* from Synkromax, this study aimed to uncover its therapeutic relevance and provide insights that could enhance its application in food safety, human health, and veterinary medicine.

MATERIALS AND METHODS

Preparation of culture medium and streaking

MRS agar medium (Hi-Media) was used as a selective medium for preliminary screening, and 0.5 grams of L. acidophilus commercial spray-dried powder formulation, produced by SynkroMax Biotech Private Limited, was used in the study [13]. On the prepared sterile MRS agar, a loopful of the sample was streaked, and the plate was then incubated at 37 °C for 48 hours. The colony's shape on the cultured plate was inspected, and Gram staining was performed to confirm the culture's homogeneity.

Primary streak method for bacteriocin production screening

The individual colony was isolated, subcultured, and analyzed for its inhibition effect by the primary streak method [14]. The pathogens were lawn cultured on Mueller-Hinton agar medium, and the isolated L. acidophilus was streaked against a specific set of pathogens and incubated at 37°C for 24 h.

Inoculum preparation

To prepare the inoculum, a loopful of L. acidophilus culture was transferred into 100 mL of sterilized MRS broth. The mixture was then incubated for 48 hours at 36°C and 170 rpm in a rotary shaker. All analytical-grade chemicals used in the experiment were acquired from Hi-Media in India. CFS was prepared from the cultured broth by centrifuging it for 10 min at 6000 rpm in a cooling centrifuge. The supernatant was then separated into two portions; one portion was subjected to partial purification of bacteriocin using ammonium sulfate precipitation for additional characterization, while the CFS was sent for secondary metabolite analysis by Gas Chromatography-tandem mass spectrometry (GC-MS/MS) [14].

Extraction of Secondary Metabolites

The CFS was mixed with ethyl acetate in a separating funnel and left undisturbed overnight to allow thorough extraction of the bioactive compounds. After the extraction period, the ethyl acetate layer was carefully separated from the aqueous layer. The solvent was then evaporated to dryness, leaving behind a residue of the extracted compounds [15]. This residue was subsequently re-dissolved in a small volume of the same solvent in preparation for further analysis using GC-MS/MS (Eurofins EAG Materials Sciences LLC, Columbia) for secondary metabolite profiling.

Thin layer chromatography

TLC is a chromatographic method used to identify the different types of components in a mixture using a thin stationary phase supported by an inert solid backing. To trace the presence of bioactive components in CFS and PPBE. 10 µL of each sample was spotted on the bottom of the plate using a capillary tube. The mobile phase consists of a solvent mixture of methanol and acetic acid (9:1), which was used to dissolve both the crude and refined extracts. Then, the spotted TLC plates were positioned at an angle in a developing chamber with the mobile phase buffer system. The plates were taken out once the mobile phase reached its ¾ of its distance. The plates were then drizzled with ninhydrin (0.2 in 100 mL of acetone) and dried in an oven at 50 °C for 5 min [18]. Photography technique was used to visualize color development and detection. The distance moved by the solute and solvent was measured. The R_F value was calculated. The retention factor value was calculated for the separated spot.

$$R_F\ value = \frac{Solute\ movement\ from\ origin}{Solvent\ front}$$

Antimicrobial Activity

For antimicrobial activity assessment, the well diffusion method has been followed according to the CLSI guidelines. The antibacterial and antifungal potency of CFS and PPBE was tested against common foodborne pathogens, including *E. coli, S. aureus, K. pneumoniae, A. niger,* and *A. flavus*. 100µL of each bacterial culture was added to the respective Mueller-Hinton agar plates and spread using a sterile L-rod; likewise, fungal strains were added to the respective malt agar plates and spread uniformly using a sterile L-rod. Well were bored using a sterile cork borer to the centre of the test plates. Then the test samples were loaded into the allotted well, along with their positive and negative controls for checking antibacterial and antifungal activities against the test pathogenic microorganisms [16]. Each experiment was carried out in triplicate, and the average value has been recorded for the results. The experimental results have been confirmed as statistically significant using a t-test.

Cytotoxicity of the Purified Extract

The MCF-7 cell line cultures were grown in DMEM medium in a CO_2 incubator with controlled conditions, which included maintaining a temperature of 37°C, 80% humidity, and 5% CO_2. Following a 48-hour cultivation period, cells were examined under an inverted microscope to determine cellular viability, which was then analyzed using a microplate reader. Different concentrations of PPBE were used for the investigation. Every prepared sample was added along with the MCF-7 cell line and a negative control. Following 24 hours of incubation, cells were treated with trypsin and DMSO in turn. Then, after 20 microliters of MTT dye were added to the wells. Following an additional 24-hour incubation period, optical density readings were obtained using an ELISA reader (Robonik) at a wavelength of 570 nm [17]. The experimental results have been confirmed as statistically significant using a t-test.

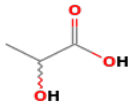
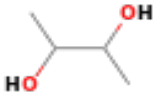
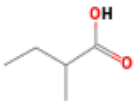
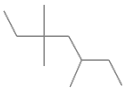
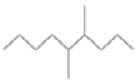
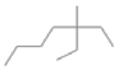
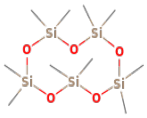
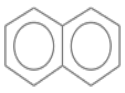
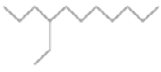
RESULTS AND DISCUSSION

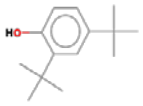
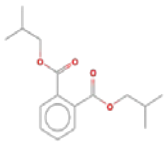
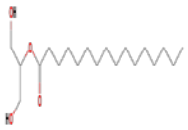
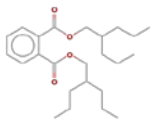
Screening of Secondary metabolites in CFS.

Gas Chromatography- tandem Mass Spectrometry was performed to analyze the secondary metabolites present in the overnight solvent-extracted sample derived from CFS. The

resulting chromatograms displayed distinct peaks, each representing significant compounds detected in the sample. Through MS/MS analysis, the molecular identities of these compounds were confirmed by examining their fragmentation patterns. This allowed for a precise characterization of the metabolites present, providing valuable insights into significant constituents of the sample and the potential bioactive compounds associated with bacteriocin production, as shown in Figure 1 and in Tables 1 and 2**.** A similar screening of secondary metabolites was reported in a study involving *Bacillus thuringiensis* culture supernatant [18,19]. This comprehensive analysis lays the groundwork for understanding the functional properties of these metabolites and their potential applications in various industries.

Table 1: List of compounds detected in chromatogram interpretation.

Peak	Retention Time	Compound Name	Match factor	Formula	2D-structure created using PubChem as a reference	Biological Activity
1	3.5680	Lactic acid	96.9	$C_3H_6O_3$		Antibacterial and Antifungal activity [20].
2	3.8969	2,3-Butanediol	94.1	$C_4H_{10}O_2$		Pesticidal, insecticidal, Antitumor, and anti-inflammatory [20].
3	5.0374	Butanoic acid,2-methyl	90.3	$C_5H_{10}O_2$		Antibacterial and Antioxidant [21].
4	7.9851	Heptane,3,3,5-trimethyl	85.8	$C_{10}H_{22}$		Antimicrobial properties [22].
5	8.8988	Nonane,4,5-dimethyl	94.7	$C_{11}H_{24}$		Antimicrobial, Antioxidant, Anticancer [23].
6	10.0726	3-Ethyl-3-methylheptane	89.9	$C_{10}H_{22}$		Antibacterial, Antifungal, Anti-inflammatory properties [22].
7	11.6072	Cyclopentadiene,decamethyl	83.2	$C_{10}H_{30}O_5Si_5$		Anti-oxidant, Anti- fungal [20].
8	12.3608	Naphthalene	99.3	$C_{10}H_8$		Anti-inflammatory, Antioxidant activities [23].
9	14.4771	Undecane,4-ethyl	88.6	$C_{13}H_{28}$		Antimicrobial [24].
10	16.2385	Dodecane,4,6-dimethyl	89.5	$C_{14}H_{30}$		Flavouring agent [25].
11	18.5279	Pentadecane	86.4	$C_{15}H_{32}$		Flavouring agent [26].

Peak	Retention Time	Compound Name	Match factor	Formula	2D-structure created using PubChem as a reference	Biological Activity
12	19.8823	2,6,10-Trimethyltridecane	93.8	$C_{16}H_{34}$		Antifungal, Antioxidant, Cancer preventive, Nematicide, Hypercholesterolemic, Lubricant [27].
13	19.8829	Hexadecane	92.0	$C_{16}H_{34}$		Cytotoxicity, Antimicrobial, Antioxidant, Antipyretic [28].
14	20.2142	2,4-Di-tert-butylphenol	92.7	$C_{14}H_{22}0$		Antimicrobial and Antitumor characteristics [29].
15	20.6014	Hexadecane,2,6,11,15-tetranethyl	98.3	$C_{20}H_{42}$		Cytotoxicity & Dyspepsia,[30].
16	22.9037	Heneicosane	92.3	$C_{21}H_{44}$		Anti-HIV, Antioxidant,Antimicrobial [31].
17	23.4205	Heptadecane	86.3	$C_{17}H_{36}$		Antimicrobial [32].
18	24.7621	1,2-Benzenedicarboxylic acid, bis-(2methylpropyl)este	96.1	$C_{16}H_{22}O_4$		Antibacterial and Antioxidant [33].
19	26.1622	Eicosane	84.7	$C_{20}H_{42}$		Antihyperglycemic, Antimicrobial, Cytotoxic & Antioxidant Properties [34].
20	31.3636	Hexadecanoic acid,2-hydroxy-1-(hydroxymethyl)ethyl ester	97.2	$C_{19}H_{38}O4$		Antimicrobial [35].
21	31.6781	Phthalic acid,di(2-propylprntyl)ester	97.9	$C_{24}H_{42}O_4$		Antimicrobial activity & Antioxidant [20].
22	33.0509	Octadecanoic acid,2-hydroxy-1-ethyl ester	92.5	$C_{21}H_{42}O_4$		Anti-tumourogenic, Antidiabetic, Anticancer, Haemolytic, Pesticide, Skin irritant [36].
23	33.3607	Tetracosane	84.1	$C_{24}H_{50}$		Cytotoxicity on malignant cells, Laxative, Antibacterialand Anti-inflammatory [37].

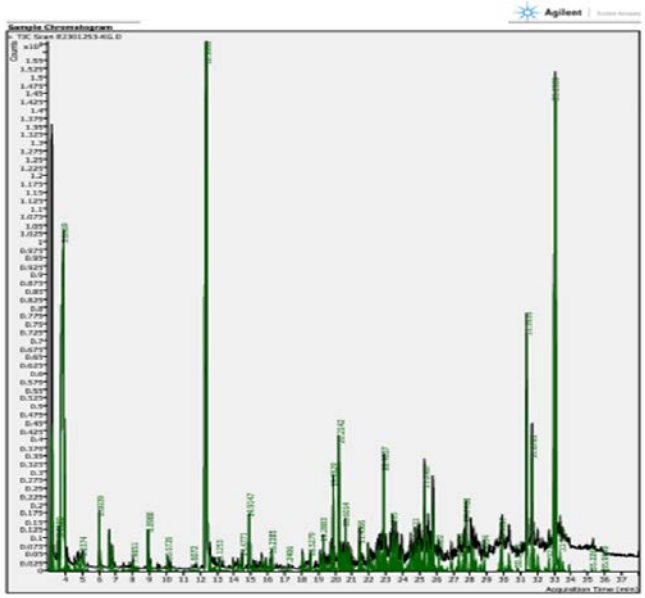


Figure 1: GC-MS/MS Chromatogram of CFS.

THIN LAYER CHROMATOGRAPHY

TLC was conducted to identify bacteriocins present in both the CFS and the PPBE. Upon visualization with ninhydrin, purple-colored spots were observed in both samples, as illustrated in Figure 2. The presence of these spots indicates the successful separation and detection of proteinaceous compounds, suggesting that bacteriocins were present in both crude and partially purified samples. The intensity and number of spots in the partially purified extract were more prominent, reflecting the enrichment of bacteriocin through the partial purification process. The Rf values for the predominant stained spots of CFS and PPBE have been determined to be 0.68 and 0.75, respectively. These findings confirm the successful partial purification of bacteriocin and highlight the effectiveness of TLC in providing a preliminary qualitative analysis. Further studies, such as an antimicrobial study, were conducted to investigate bacteriocin activity.

ANTIBACTERIAL ACTIVITY OF CFS AND PPBE

The antimicrobial activity of the CFS and the PPBE was evaluated against common foodborne pathogens, specifically Gram-negative and Gram-positive bacteria, as well as two pathogenic fungi, using the well diffusion method. As presented in Figure 3, both samples exhibited significant antibacterial and antifungal activity. According to the results, Table 3 illustrates the zone of clearance acquired from the results, including positive (Ampicillin) and negative (phosphate buffer saline) controls.

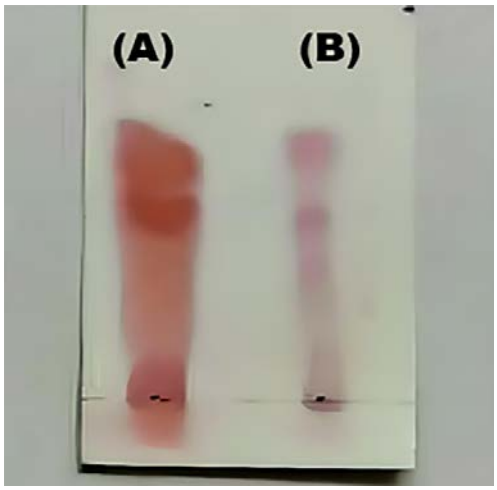


Figure 2: Thin layer chromatography for (A) CFS and (B) partially purified Bacteriocin extract using ninhydrin staining.

These findings suggest that the extracts contain bioactive compounds, likely including bacteriocins, which are effective in inhibiting a broad spectrum of bacterial and fungal pathogens. Notably, the partially purified extract demonstrated greater effectiveness, indicating that the purification process enriched the concentration of active antimicrobial agents. The outcome suggested that observed inhibition in test samples was not an artifact of the buffer or other application method.

In another recent study, bacteriocin was reported to show good inhibition against pathogens such as *Escherichia coli* and *Shigella flexneri* [38]. These results emphasize the effectiveness of the probiotic product against bacterial and fungal pathogens, highlighting its potential as a natural antimicrobial alternative. The enhanced activity of the partially purified extract suggests a promising formulation for various applications.

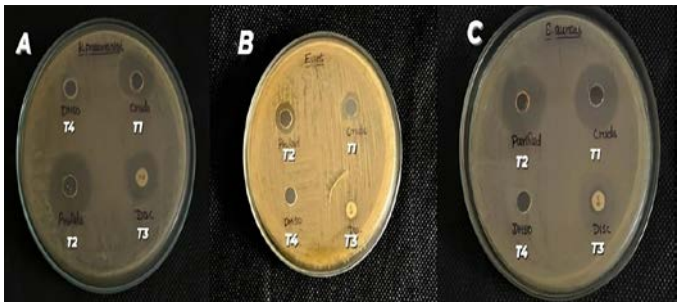


Figure 3: Antibacterial activity through the well diffusion method. Test microorganisms: (A) *K. pneumoniae*, (B) *E. coli*. (C) *S. aureus*. T1 - CFS, T2 - PPBE, T3 - Ampicillin standard disc (positive control), T4 - Phosphate buffer saline (negative control).

Table 3: Zone of inhibition (observed diameter in mm) against the microbes, *E. coli, K. pneumoniae,* and *S. aureus.*

Name of Organism	CFS (T1)	PPBE (T2)	Ampicillin (positive Control) (T3)	Phosphate buffer saline (negative control) (T4)
E. coli	11±0.1	12±0.5	10±0.2	Nil
S. aureus	19±0.2	21±0.2	10±0.2	Nil
K.pneumoniae	16±0.1	20±0.4	15±0.3	Nil

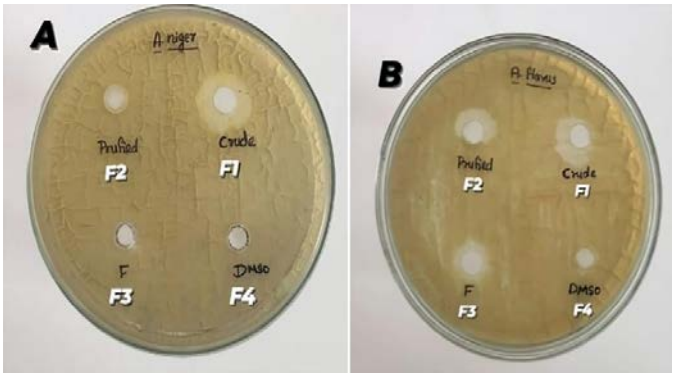


Figure 4: Antifungal activity through well diffusion method Test Fungus (A) *Aspergillus niger,* (B) *Aspergillus flavus* F1- CFS, F2- PPBE, F3- Positive control Disc, F4- Phosphate buffer saline.

Table 4: Zone of inhibition (diameter) against *A. niger* and *A. flavus*.

Name of Organism	CFS F1 (mm)	Purified F2 (mm)	Fluconazole F3 (mm)	Phosphate buffer saline F4(mm)
A.niger	15±0.2	Nil	Nil	Nil
A.flavus	13±0.4	11±0.19	10±0.24	Nil

CYTOTOXICITY OF THE PPBE

The PPBE exhibited strong cytotoxicity against MCF-7 cells, with an IC_{50} value of 72.3991 µg/mL, indicating promising anticancer potential. Figure 5 presents the dose-response curve for MCF 7 cells treated with PPBE. Table 5 provides a summary of the data obtained for samples ranging from 25 µg/mL to 100 µg/mL. Figure 6 depicts the microscopic image of MCF-7 cells treated with PPBE at concentrations ranging from 25 µg/mL to 100 µg/mL. Concentration-dependent trends in lowering cell viability with increasing concentrations. These results are consistent with previous studies that have identified probiotics and their metabolites as potential anti-cancer agents [39].

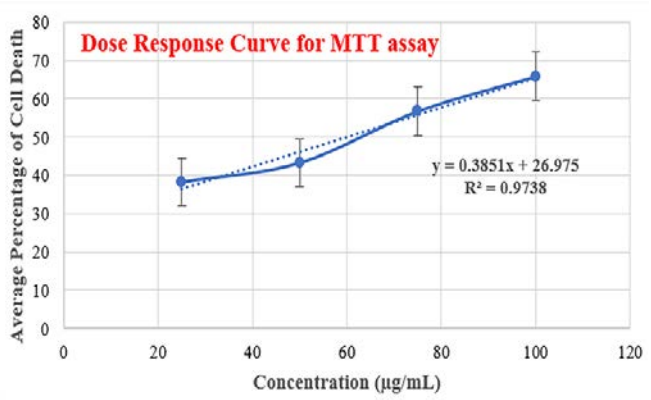


Figure 5: Dose response curve for MCF 7 cells treated with PPBE

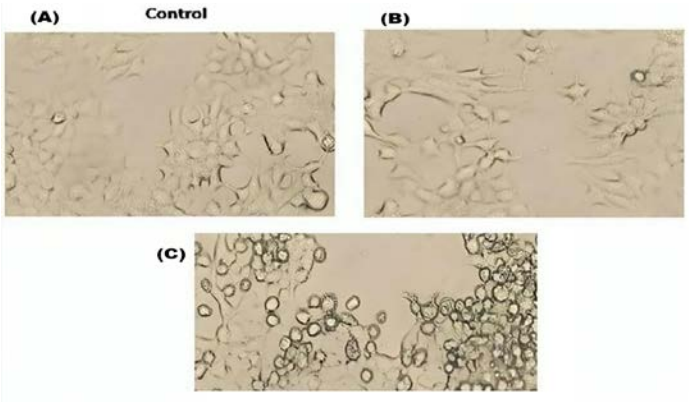


Figure 6: Microscopic image of MCF 7 cells treated with PPBE in the range of (25µg-100 µg/mL). Control without extract (B), treated with 25 µg/mL of PPBE sample (C), treated with 100 µg/mL of PPBE sample.

Table 5: Effect of the PPBE obtained from *L. acidophilus* on MCF-7 Cell viability

Concentration (µg/mL)	Average percentage of cell death (Mean average percentage calculated in triplicate)
25	38.55 ± 0.33%
50	43.50 ± 0.19%
75	56.50 ± 0.28%
100	65.75 ± 0.24%

CONCLUSION

The study results provide promising evidence of the effectiveness of SynkroMax Biotech's *Lactobacillus acidophilus* product. The cell-free supernatant (CFS) extract, partially purified bacteriocin extract (PPBE), and other metabolites produced by *L. acidophilus* during the stationary phase are rich in bioactive compounds, exhibiting better antimicrobial, probiotic, and therapeutic potential. These substances, including primary and secondary metabolites,

contribute to the bacterium's ability to inhibit pathogenic microorganisms, promote gut health, and improve immune function. The antimicrobial activity of bacteriocins, combined with the acidification caused by lactic acid production, creates an effective defense mechanism against foodborne pathogens and spoilage organisms, making these metabolites valuable in food preservation. Moreover, the probiotic benefits of *L. acidophilus* are supported by its production of health-promoting metabolites that improve digestive health and enhance the gut microbiome. Even though *L. acidophilus* is a widely used probiotic and generally considered safe for most people. There are some limitations and potential risks to be aware of, such as people with weakened immune systems who have undergone chemotherapy and organ transplant recipients.

Additionally, it may be at risk of allergic reactions to inactive ingredients in supplements, such as dairy-based media and capsules. Furthermore, its effects can be strain-specific and dose-dependent. However, further in vivo validation studies are needed in the future. In conclusion, the bioactive compounds produced by this microbe offer numerous applications in food, health, and pharmaceutical industries, supporting the development of natural preservatives, probiotics, and novel antimicrobial agents.

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AUTHOR'S CONTRIBUTION
Venkatesa Prabhu Sundramurthy carried out the original draft writing, visualization, and investigation. Sasidharan Satheesh Kumar and Ramachandra Ragunathan contributed to the investigation, review, validation, and editing. Madhusree was involved in editing, formal analysis, data curation, and conceptualization. Lochana Devi P. contributed to the formal study. All authors have contributed significantly to the work and approved the final manuscript.

CONFLICT OF INTEREST
Proper consent was obtained from SynkroMax Biotech Private Limited for validating the efficacy of its probiotic product. The authors declare no conflicts of interest related to this research.

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None

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