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Formulation of a Probiotic Rich Fermented Product with Catechin Loaded in Bacterium for Nutraceutical Applications

Supriya V[*1],Gayathri H[2],Jamashin Shajini B S[3],Kanmani Pachagoundan[4], Nesiga A[5]

[1,2,3,4,5]*Sri Shakthi Institute of Engineering and Technology, Coimbatore, Tamil Nadu-641062*

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ABSTRACT: The demand for nutraceutical products has led to an increase in popularity for formulations rich in probiotics aimed at enhancing human health. This study's primary goal is to develop a new fermented food fortified with probiotic bacteria containing catechins, which are intended to offer enhanced nutraceutical benefits. By incorporating catechins—flavonoids known for their antibacterial, anti-inflammatory, and antioxidant properties—into the probiotic matrix, the product's shelf life and health benefits are enhanced. The project's aim is to identify and isolate a robust probiotic strain capable of withstanding catechin loading while maintaining its metabolic activity and viability. The chosen bacterium is subjected to microencapsulation to ensure its survival during both fermentation and gastrointestinal environments. A nutrient-dense matrix is formed using a substrate, such as milk or plant-based alternatives, which undergoes controlled fermentation. In vitro and in vivo studies are conducted to evaluate the potential of the final product as a nutraceutical. These studies focus on changes in gut microbiota, antioxidant effectiveness, and potential health benefits such as improved digestion, enhanced immunity, and reduced oxidative stress. Additionally, sensory evaluations are performed to ensure consumer acceptance regarding overall attractiveness, flavor, and texture. This project merges the health benefits of probiotics with catechins, effectively linking traditional fermented foods with modern nutraceuticals.

KEYWORDS: Probiotic,Fermented food, Catechins, Flavonoids, Nutraceutical, Antioxidant.

I. INTRODUCTION:

As people look for healthier and more enticing options, creating new food products is becoming a bigger task. In this context, functional foods assume a special significance. By improving general health and wellness or reducing the risk of disease, these foods are intended to have a favorable impact on one or more bodily processes in addition to satisfying hunger and providing necessary nutrients[1]. Living bacteria known as probiotics assist the host when given in enough amounts. The two main genera of probiotics are Bifidobacterium and Lactobacillus[2]. There are more than 250 species in the Lactobacillus genus, and many of them have different ecological, morphological, and genotypic characteristics[3].The antioxidant properties of catechins are well known; they help the body fight off free radicals, which may reduce inflammation and lower the risk of chronic illnesses including heart disease and some types of cancer. Additionally, they might support fat oxidation, improve metabolism, and improve cognitive function. Originally from southern China, the tea plant (Camellia sinensis) is also found in parts of India, Laos, Thailand, Vietnam, and Myanmar [4]. Catechins are abundant in green tea and can account for as much as 30% of the dry weight of the leaves[5].Catechin identification and quantification have been substantially facilitated by the use of chromatographic techniques, such as HPLC and CE, in conjunction with a variety of detectors, including UV, electrochemical, and MS[6]. Catechins have also been analyzed and quantified using other techniques, including as high-speed countercurrent chromatography, TLC, GC, and near-infrared reflectance spectroscopy. Due to its efficient separation capabilities and compatibility with a variety of detectors, HPLC is now the most extensively used method for identifying tea catechins [7].

Catechins are powerful antioxidants that can scavenge free radicals more effectively than beta-carotene, vitamin C, or vitamin E because of their capacity to donate hydrogens from their hydroxyl groups. Additionally, they have been

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demonstrated to modulate both oxidant and antioxidant enzymes, chelate transition metal ions, and affect gene expression [8]. The structural framework of catechins is typically CG–C3–CG, with numerous hydroxyl groups and two aromatic rings. Although catechins have a lot of bioactivity, including neuroprotective and cardioprotective qualities, their bioavailability and stability in the human digestive tract are often low, which reduces their usefulness in nutraceutical applications[9].Probiotic bacteria can serve as a delivery system for catechin, improving its stability, regulated release, and bioactivity in order to overcome these difficulties. One creative approach to creating nutraceuticals is adding catechin to a fermented food that is high in probiotics. In order to produce a dual-purpose nutraceutical solution, our research is concentrated on developing a novel fermented product that is high in probiotics and enhanced with catechin [10]. In order to improve the therapeutic effectiveness of both probiotics and catechins in gut health, metabolic regulation, and oxidative stress reduction, as well as to provide a sustainable, natural, and functional food formulation with potential applications in both commercial and clinical nutraceuticals, this study will utilize fermentation technology and probiotic-mediated encapsulation to increase the stability and bioavailability of catechin through bacterium-based encapsulation.

II. MATERIALS AND METHODS:

Selection and Preparation of Probiotic Strain:

The probiotic strain Lactobacillus species was chosen because of its capacity to generate bioactive chemicals, adhere to intestinal epithelial cells, and endure in acidic environments. Sterile MRS (de Man, Rogosa, and Sharpe) broth was used to inoculate a lyophilized or frozen culture, which was then incubated for 18 to 24 hours at 37°C in anaerobic or microaerophilic conditions. Following revival, the culture was streaked onto MRS agar plates to create isolated colonies, which were subsequently subjected to the same conditions for further incubation. For biomass development, a carefully chosen colony was injected into fresh MRS broth.

Testing of probiotic viability and stability:

Two in vitro tests were conducted to assess the functional robustness of Lactobacillus strains supplemented with catechins under gastrointestinal-like conditions: bile salt tolerance and acid tolerance. These experiments evaluate the probiotic strain's capacity to survive by simulating the hostile conditions seen in the human stomach and small intestine.

1.TestforAcidTolerance

The probiotic cells were subjected to extremely acidic conditions that mirrored the pH of the stomach in order to perform the acid tolerance test. In summary, Lactobacillus cultures that had been cultivated overnight were collected by centrifugation at 4000 rpm for 10 minutes, and they were then twice washed with sterile phosphate-buffered saline (PBS). Using 1N HCl, the bacterial pellet was reconstituted in sterile PBS that had been brought to a pH of 2.0. For two hours, the suspension was incubated under static conditions at 37°C. Aliquots were serially diluted and plated using the spread plate method on MRS agar following incubation. After 48 hours of anaerobic incubation at 37°C, the number of viable colonies on the plates was tallied. The following formula was used to get the survival percentage:

Survival %= (CFU after treatment/ Initial CFU) × 100

2. Bile Salt Tolerance Test

To simulate the conditions of the small intestine, a bile salt tolerance assay was carried out. The prepared probiotic culture was resuspended in MRS broth supplemented with 0.3% (w/v) oxgall (bile salt). The suspension was incubated at 37°C for 4 hours. Post-incubation, viable counts were determined by serial dilution and plating on MRS agar. Colony enumeration was carried out after 48 hours of incubation at 37°C. The percentage of bile salt survival was calculated similarly to the acid tolerance test.

Collection and processing of tea leaves:

The premium tea leaves were gathered in Tamil Nadu's Kotagiri and Ooty. The high catechin concentration of young, fragile green tea leaves (Camellia sinensis), especially in the apical bud and the first two to three leaves, led to their selection. After that, the leaves were carefully cleaned with running distilled water to get rid of any surface impurities and dust. Sterile blotting paper was used to remove any remaining surface water after washing. After that, the leaves were allowed to dry in the shade for four to five days at room temperature (around 25 to 30°C) in an area with good ventilation.To avoid the loss or deterioration

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of heat-sensitive and photo-labile phenolic compounds, such as catechins, shade drying was chosen over sun drying. After drying fully, the leaves were ground into a fine, consistent powder in a dry, clean lab blender. In order to guarantee constant solvent penetration during extraction, the powdered leaves were then run through a mesh sieve (usually 60-mesh). The resultant tea leaf powder was gathered in labeled, airtight containers and kept at room temperature in a dark, dry place until it was needed.

Solvent extraction of catechin:

A standardized ethanol-water extraction technique designed for the effective recovery of polyphenolic components was used to extract catechin from tea leaves. Ten grams of the finely ground tea leaves, which had been previously weighed, were employed in the extraction procedure. A hydroalcoholic solvent system consisting of distilled water and ethanol in a 70:30 (v/v) ratio was used to carry out the extraction. This combination was chosen because it can dissolve both polar and somewhat non-polar phenolic molecules, which makes it ideal for extracting a variety of catechins. As a food-grade solvent, ethanol is usually regarded as safe for use in nutraceutical applications.In a sterile conical flask, the ethanol-water solution and powdered tea leaves were combined, sealed, and put in a water bath shaker with a temperature control. For two hours, the mixture was continuously stirred at a speed of between 100 and 150 rpm while being incubated at 50°C.

Filtration and centrifugation:

Following ethanol-water extraction, the resulting extract usually consists of a blend of insoluble plant components, including fibers, cell wall fragments, and leftover leaf particles, as well as soluble catechins. Using Whatman No. 1 filter paper in a funnel over a sterile conical flask or beaker, the mixture was first gravity filtered to remove the liquid extract from these solids. Aseptic settings were used for this stage in order to avoid microbiological contamination. In order to obtain a cleaner solution and further eliminate tiny particles, the filtrate was subsequently put into sterile centrifuge tubes and centrifuged at 5,000 rpm for 10 to 15 minutes at room temperature. The pellet was not disturbed throughout the meticulous decantation of the supernatant, which was now turbidity-free.

Quantification of catechin using HPLC:

The catechin concentration of the concentrated extract was then measured using High-Performance Liquid Chromatography (HPLC) analysis, in accordance with the procedure described by Lambert et al. (2007). The amount of catechin in the extract was measured using high-performance liquid chromatography (HPLC)[11]. Individual catechins, such as epicatechin gallate (ECG) and epigallocatechin gallate (EGCG), can be separated and identified using HPLC, which can also identify pollutants and impurities. Retention periods were compared to established standards to guarantee specificity and purity, and a standard catechin curve was created to ascertain the concentration in the sample.

Loading of Catechin into Lactobacillus:

Catechin loading process:

An adsorption-based technique was used to load catechin into Lactobacillus[12]. By making it easier for catechin molecules to adhere to the bacterial cell surface, adsorption is essential for loading catechins into Lactobacillus. To achieve a workable concentration, usually between 100 and 500 µg/mL, catechin was reconstituted in sterile phosphate-buffered saline (PBS, pH 7.4) for loading. PBS was chosen because it is non-toxic and isotonic, preserving cellular integrity throughout the procedure. Centrifugation, which uses centrifugal force to separate the bacterial cells from the fermentation broth, was used to harvest Lactobacillus. High cell recovery rates and effective separation are achieved by removing the supernatant while keeping the pellet.

A 1:1 volume ratio was used to gently resuspend the Lactobacillus cell pellet in the catechin solution[13]. For two to three hours, this homogeneous dispersion was incubated at 37°C in sterile conical tubes or flasks. To ensure that the bacterial cells were evenly exposed to the catechin molecules, the mixture was gently shaken at 100–120 rpm using an orbital shaker during the incubation period. During this incubation phase, the uptake mechanism is primarily passive, with catechin molecules either adsorbing onto the surface or diffusing through the bacterial cell wall through non-covalent interactions including hydrophobic, electrostatic, or hydrogen bonding.

Separation and Washing:

After being moved to sterile centrifuge tubes, the mixture was centrifuged for ten minutes at 4°C at 4,000 rpm. This stage made it possible for

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the Lactobacillus cells—which were now perhaps loaded with catechin—to solidify into a pellet at the tube's bottom. Without disturbing the particle, the supernatant—which contained leftover unincorporated catechin—was gently decanted. The pellet was rinsed two or three times with cold, sterile PBS to make sure that all loosely bound catechin was gone. To prevent mechanical stress or injury, the cells were gently resuspended using gentle pipetting or light vertexing after an equivalent volume of PBS was added to the pellet for each wash. Centrifugation was carried out again under the identical circumstances following each wash.

Spectrophotometric analysis:

At 280 nm, spectrophotometric analysis was used to evaluate the effectiveness of catechin loading. By measuring the interaction between matter and electromagnetic radiation, spectrometric analysis is a scientific method for determining and quantifying a sample's chemical composition. It is founded on the premise that molecules absorb and emit radiation at distinct wavelengths, allowing for their identification and quantification.

Fermentation Process:

Based on its compatibility with Lactobacillus growth, an appropriate food matrix (such as milk, soy milk, or fruit juice) was chosen as the fermentation medium. After inoculation, fermentation, and incubation, a product with live and active Lactobacillus is produced. Before inoculating the medium with Lactobacillus loaded with catechins, it was pasteurized for 15 minutes at 85°C and chilled to 40°C[14]. The inoculum was Lactobacillus cultures enriched with catechins, which were made as previously mentioned. The fermentation process was conducted in anaerobic conditions for 24 to 48 hours at 37°C. Depending on the material and the intended textural qualities of the finished result, either mild shaking or static conditions were maintained.The pH, total viable count, and catechin retention were monitored throughout the fermentation period. The probiotic viability was evaluated using the plate count method, ensuring a minimum count of 10^7 CFU/mL [15].

Temperature Stability Test of Catechin-Loaded Bacteria:

Three groups of catechin-loaded probiotic formulation samples were kept at varying temperatures: 40°C (incubator), 25°C (room temperature), and 4°C (refrigeration). The materials were stored in sealed, sterile containers to prevent moisture loss and contamination. To evaluate catechin stability, each sample was tracked during a specified storage duration (15 or 30 days), and analysis was done at regular intervals (every 5 days). Methanol was used as the solvent to extract catechin from 1 mL of the sample collected at each sampling location. To eliminate bacterial biomass, the materials were centrifuged for 10 minutes at 10,000 rpm after being vortexed for even mixing. After careful collection, the supernatant's catechin content was examined.

Testing antimicrobial activity:

The antibacterial activity of free catechin and catechin-loaded bacteria was evaluated using the agar well diffusion method. Four bacterial strains—Escherichia coli, Staphylococcus aureus, Salmonella typhi, and Bacillus subtilis were selected for the test. These bacteria were cultured in nutrient broth and then uniformly spread on Mueller-Hinton agar plates using a sterile cotton swab. Wells were made in the agar using a sterile borer, and 100 µL of each sample was added into separate wells. The test samples included free catechin solution, catechin-loaded bacterial suspension, a positive control (ampicillin), and a negative control (solvent). The plates were incubated at 37°C for 24 hours. After incubation, the zones of inhibition around the wells were measured to assess antibacterial activity.

In vitro release study:

To evaluate the release patterns of free catechin and catechin encapsulated within probiotic bacteria under simulated gastrointestinal conditions, an in-vitro release research was carried out. Two distinct media were created to replicate the human digestive tract: simulated intestinal fluid (SIF, pH 6.8) and simulated gastric fluid (SGF, pH 1.2). 2 g of NaCl and 3.2 g of pepsin were dissolved in 1 liter of deionized water to create the SGF, and HCl was used to bring the pH down to 1.2. Likewise, 6.8 g of KH_2PO_4 and 10 g of pancreatin were dissolved in 1 liter of water, and the pH was adjusted to 6.8 with NaOH to create SIF.

Separate dialysis bags containing known amounts of free catechin and catechin-loaded bacteria were submerged in 100 mL of SGF and shaken constantly while incubated at 37°C. To keep sink conditions stable, aliquots were taken out and replaced with new medium at prearranged intervals

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of 0, 0.5, 1, and 2 hours. To replicate the intestinal environment, the bags were moved to new SIF after two hours, and the release trial was carried out for a whole day. A UV-Vis spectrophotometer set at 280 nm was used to measure the amount of catechin in each aliquot after samples were taken at regular intervals (4, 6, 12, and 24 hours). Catechin's cumulative release was computed and shown against time.

Antioxidant activity:

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging experiment was used to assess the antioxidant activity of both free catechin and Lactobacillus loaded with catechin. A 0.1 mM DPPH solution was made in methanol for the test, and to stop degradation, it was kept in the dark. We collected and diluted samples of free catechin and catechin-loaded Lactobacillus at different intervals (0, 2, 4, 6, 8, 12, and 24 hours). To ensure that antioxidants and DPPH radicals fully interacted, 1 mL of DPPH solution and 1 mL of the sample solution were combined in a cuvette and allowed to sit at room temperature for 30 minutes in the dark.Using a UV-Vis spectrophotometer, the absorbance of the resultant combination was determined at 517 nm. The control was a methanol and DPPH-only solution. The following formula was used to determine the percentage of DDPH scavenging activity:

Scavenging activity (%) = [(Control - Sample)/Control] ×100.

III. RESULTS AND DISCUSSION:

Selection and Identification of Probiotic strain:

The Gram staining procedure demonstrated that the isolate was rod-shaped and Gram-positive, which is consistent with the general morphology of Lactobacillus species. The catalase test was negative, indicating the absence of the catalase enzyme, a characteristic feature of lactic acid bacteria which rely on fermentation rather than aerobic respiration.

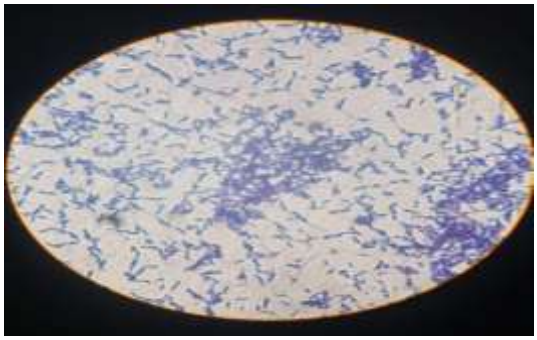

Figure 1. Gram stained strains of Lactobacillus species

Probiotic Viability & Stability:

The acid tolerance assay, conducted at pH 2.0 for a 2-hour incubation period, revealed an 85% survival rate, indicating that the strain possesses strong resistance to the harsh acidic conditions typically found in the stomach. Similarly, bile salt tolerance was assessed by exposing the bacterial culture to 0.3% (w/v) oxgall bile salts, which reflect average bile concentrations in the duodenum. The strain maintained an 80% survival rate, confirming its ability to endure bile-induced stress.

Table 1. Survival percentage of the probiotic

Circumstances	Survival (%)
Acid (pH 2.0)	85
Bile Salt (0.3%)	80

Catechin Extracted from Tea Leaves:

Catechins were successfully extracted from tea leaves using ethanol as the solvent, which is a widely recognized medium for the efficient recovery of polyphenolic compounds due to its polarity and food-grade safety.

Figure 2. Tea leaves extract

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HPLC analysis of phenolic compound (Catechin):

The HPLC chromatogram of phenolic compounds of tea leaves extract in table 2 shows six distinct peaks with varying retention times (Rt), molecular weights ([M+H]+), and concentrations. The major compound is Epigallocatechin gallate (EGCG, 53.18 mg/mL, Rt 17.26 min, m/z 459), a potent antioxidant.

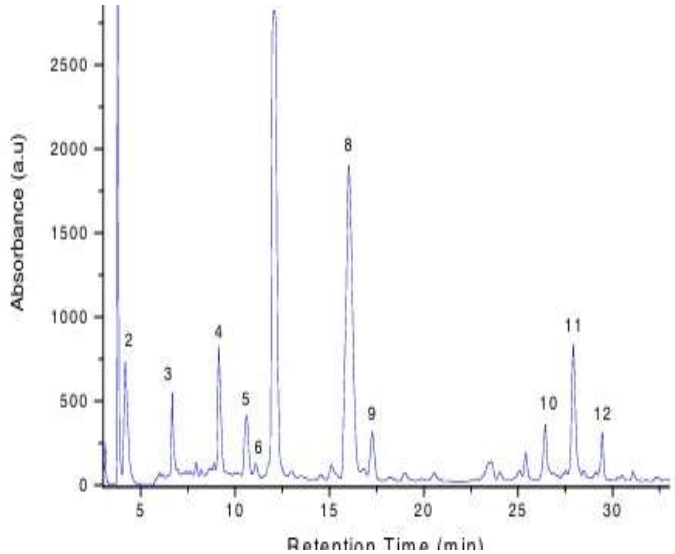

Figure 3. HPLC chromatogram of phenolic compounds in tea extract

Peak nr.	Rt (min)	[M+H]+ (m/z)	Compound	Conc. Mg/ml
1	6.66	307	Gallocatechin	4.5
2	9.13	307	Epigallocatechin	7.13
3	11.09	291	Catechin	1.59
4	27.91	443	Catechingallate	3.29
5	16.02	291	Epicatechin	3.34
6	17.26	459	Epigallocatechingalate.	53.18

Table 2. Concentration of Phenolic compounds from tea extract

UV-Vis Spectrophotometric Estimation of Catechin Loading Efficiency:

The absorbance at 280 nm was used in UV-Vis spectroscopy to calculate the catechin loading efficiency into Lactobacillus cells. First, the absorbance of solutions with known amounts of catechins was measured in order to create a standard curve. Effective incorporation of the bioactive component was demonstrated by the successful loading of approximately 39.3% of the original catechin into the Lactobacillus cells.

Table 3. Standard Curve Data for Catechin at 280 nm

Catechin Concentration (µg/mL)	Absorbance (280 nm)
20	0.142
40	0.287
60	0.431
80	0.577
100	0.718

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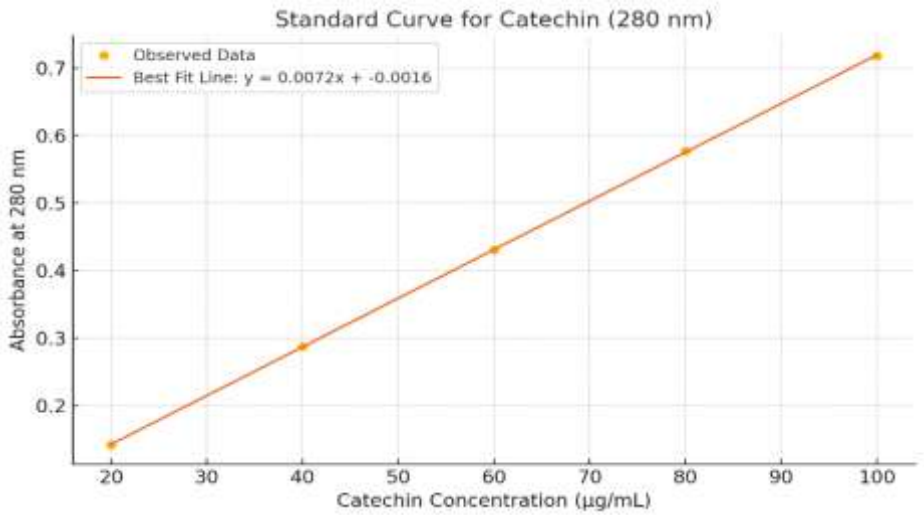

Figure 4. Standard curve for catechin at 280 nm

Fermentation Process Optimization:

Table 4. Process optimization parameters

Initial pH	6.5
Final pH	4.3
Acidity	0.8%
Peak bacterial growth	24 hours
Growth Measurement	OD600

The observed decrease in pH and increase in titratable acidity confirm the metabolic activity of Lactobacillus during fermentation. The optimal bacterial growth at 24 hours aligns with previous findings that suggest Lactobacillus plantarum reaches peak biomass within a day before entering the stationary phase. The pH drop to 4.3 ensures product preservation while maintaining probiotic viability.

Microbiological Safety & Pathogen Screening:

Microbiological safety was guaranteed since no harmful contamination from Salmonella, E. Coli, or Staphylococcus aureus was found in the finished product. Zones of inhibition against S. aureus (10.8 mm) and E. coli (12.4 mm) were found in antimicrobial activity testing, indicating antibacterial potential.

Table 5. Zone of inhibition

Species	**Zone of inhibition**
S. aureus	10.8mm
E. Coli	12.4mm

The observed antimicrobial activity against S. aureus and E. coli indicates that Lactobacillus supplemented with catechins has bio-preservative qualities, most likely as a result of the interaction between the antibacterial qualities of catechin and the formation of lactic acid.

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Temperature stability test:

Table 6. Effect of temperature on catechin retention and stability

Temperature (°C)	Catechin Retention (%)	Stability Observation
4°C	92.3%	Highly stable
25°C	84.7%	Moderately stable
40°C	63.5%	Significant degradation

Based on the temperature stability test results, catechin-loaded bacteria exhibited the highest retention and stability at 4°C, with a gradual decrease in catechin content observed at 25°C and a significant reduction at 40°C. Therefore, it can be concluded that refrigeration (4°C) is the most suitable storage condition for preserving the bioactivity and structural integrity of catechin-loaded probiotic formulations.

Anti-bacterial assay:

Free catechin exhibits antibacterial activity however, its zone of inhibition is smaller compared to encapsulated catechin. This indicates that while catechin alone can inhibit bacterial growth, its effectiveness is limited. In contrast, catechin-loaded bacteria demonstrate enhanced antibacterial activity, likely due to sustained release and prolonged bioavailability, which improves its efficacy. Notably, the highest antibacterial activity is observed against Staphylococcus aureus, suggesting its potential application in controlling Gram-positive bacterial infections.

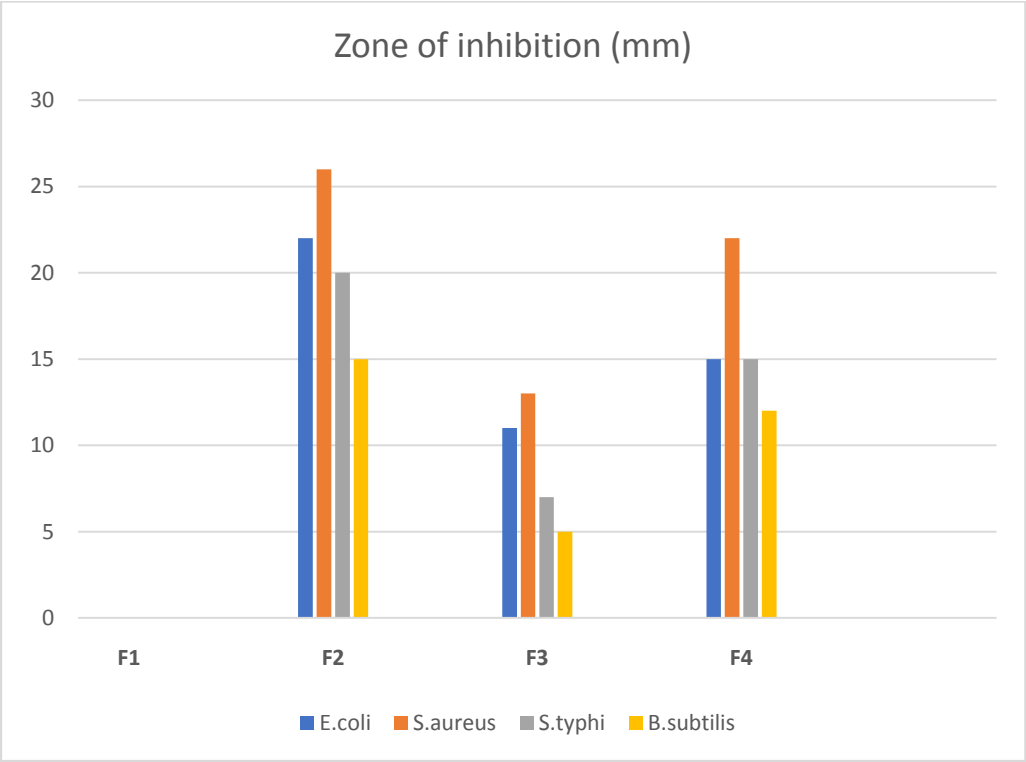

Figure 5. Graph representing zone of inhibition of different bacteria
F1-Negative control; F2-Positive control; F3-Free catechin; F4-Catechin loaded in bacterium

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In-vitro release study:

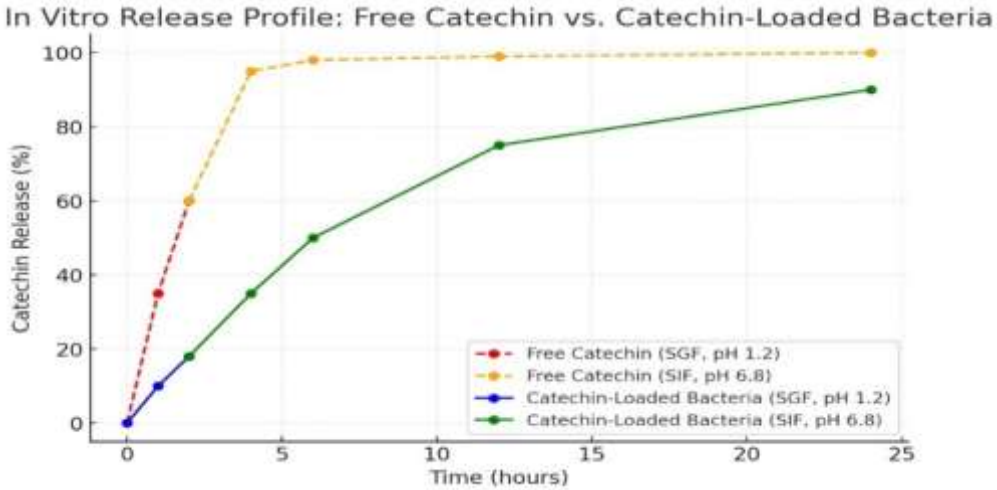

Figure 6. In vitro catechin release profile

Gastric Release (0 - 2 hours, pH 1.2)

Free catechin shows a burst release in 2 hours, indicating instability in acidic conditions. Catechin-loaded bacteria show minimal release, proving encapsulation protects catechin from gastric degradation.

Intestinal Release (2 - 24 hours, pH 6.8)

Free catechin releases approximately 95% within 4 hours, leading to a rapid concentration spike, which may reduce effectiveness due to metabolism and degradation.

Catechin-loaded bacteria follow a controlled and sustained release pattern, reaching 50% release at 6 hours and 90% at 24 hours, ensuring prolonged absorption and bioavailability.

Antioxidant activity:

The results show a noticeable decline in the DPPH scavenging percentage for free catechin as time progresses, indicating a reduction in its antioxidant efficacy, likely due to degradation or instability over prolonged periods. In contrast, catechin-loaded Lactobacillus maintains a consistently high antioxidant activity throughout the 24-hour period, suggesting that the encapsulation of catechin within the bacterial cells enhances its stability and sustains its release.

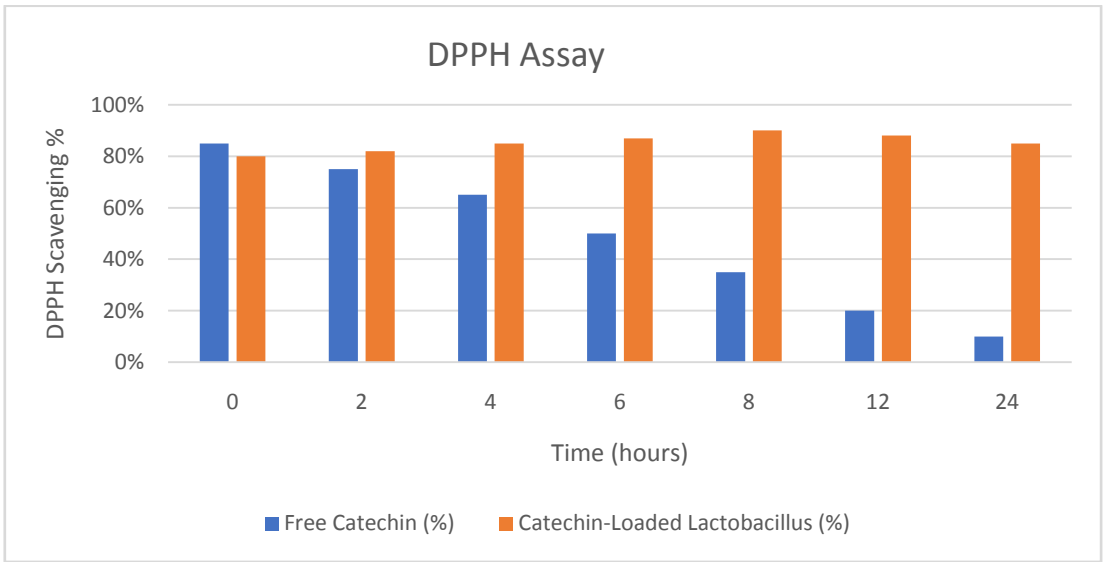

Figure 7. Graphical representation of Antioxidant activity

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Shelf-Life Analysis:

The microbiological stability study confirmed that the product maintained its sensory and microbial quality for up to 28 days at 4°C.The 28-day shelf-life stability at 4°C is in line with commercial probiotic beverages, indicating that the formulation is viable for industrial-scale production.

IV. CONCLUSION

Although limited, some research results suggest that combining probiotics and phenolics holds promise due to the health benefits they offer together. Their interaction shows synergy, as phenolics exhibit a prebiotic effect, while probiotics enhance the bioaccessibility of phenolics. However, more research is necessary to better understand the extent of the health benefits from this combination and to determine the safe consumption levels for consumers to fully benefit from both. It will also be important to clarify the potential mechanisms of action and interactions between them. Advancements in evaluating effects on humans will rely on the creation of new experimental systems. Investigating at the cellular level provides deeper insights into the mechanisms that govern functions in both normal and pathological conditions.

Another hurdle is the delivery of catechin and probiotics, given their instability in adverse environments. In this context, microencapsulation emerges as a promising solution to safeguard them during processing, storage, shelf-life, and transit through the gastrointestinal tract, while also reducing the astringency some phenolics may cause and enabling controlled release at the site of action. Therefore, future research should focus on assessing the release kinetics of these compounds from their encapsulating matrices.

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