

In vitro Probiotic Efficacy and Molecular Identification of *Decalepis hamiltonii* Wight & Arn (Swallow root) Pickle

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Abstract

Fermentation, harnessing beneficial gut bacteria, plays a crucial role in enhancing the flavour and nutritional value of food. This research aimed to create Swallow Root pickle using traditional ingredients such as curd, chili powder, lemon, and raw mustard. The study assessed microbial safety, sensory qualities, nutritional composition, shelf-life, in vitro probiotic effectiveness, and molecular identification. Results indicated microbial growth below safety limits, with a high sensory appeal (rated 8.7). Proximate composition analysis revealed moisture content (65%), carbohydrates (28.46g), protein (2.8g), fat (0.8g), iron (0.34mg), calcium (6.654mg), ash (2.54%), energy (133.23kcal), and crude fiber (0.54g). Fermentation notably increased B vitamin levels. Over 30 days, shelf-life analysis showed a gradual rise in yeast, with negligible mold and *E. coli* presence. Probiotic efficacy tests demonstrated substantial pH (89%) and bile salts (97%) tolerance, bacterial adhesion (37%), and antimicrobial activity. Antibiotic susceptibility profiling revealed resistance to oxacillin, chloramphenicol, and cefoxitin, with sensitivity to tetracycline and vancomycin. Molecular identification via 16S rRNA sequencing confirmed *Bacillus subtilis* as the probiotic strain. Swallow Root pickle presents itself as a nutritious dietary option, promoting enhanced gut health.

Key words: *Decalepis hamiltonii* Wight & Arn, Pickle, *In vitro* probiotic efficacy, Molecular identification, Fermentation

The term 'probiotic' is derived from the Greek phrase "for life". Over the years, this has been given many different definitions. "Active substances that are essential for a healthy development of life" was a term coined for probiotics in 1953 by the German scientist Werner Kollath [13]. *Decalepis hamiltonii* Wight & Arn (*D. hamiltonii*) is an endemic and endangered species that is found in Peninsular India and the South Indian region and very rarely found in the Western Ghats region. *D. hamiltonii* belongs to the family Asclepiadaceae [35]. It is widely used in traditional medicine as a bacteriostatic, blood purifier, and appetizer as well as in many Ayurvedic treatments. According to reports, the plant contains pharmacological actions that make it an antioxidant. Lupeol, 4-O-methyl-resorcyraldehyde, a- and b- amyrins, coumarin, kaempferol, quercetin, rutin, and sterols are all abundant in the root of *D. hamiltonii* Wight & Arn [36]. It is commonly known as swallow root and also known by various names in different regions like makali beru, Magali kizhangu, or vagani beru [24]. Many probiotic bacteria have been identified from a variety of unusual sources, including fruits, vegetables, and traditional fermented meals [40]. Food items should have probiotic colonies with an average of 8–9 log CFU g⁻¹ according to daily recommendations [39] and 6–7 CFU g⁻¹ should be present before consumption for the colon to receive them. [25] In vitro, digestion investigations are commonly employed to determine structural modifications gastrointestinal conditions, and digestibility [17]. Several *Bacillus* strains can be found in the

environment, including the human gut, soil, air, and fermented food sources [34].

Pickles are beneficial to human health because they include vitamins, minerals, carbohydrates, and specific pigments including lycopene, β-carotene, anthocyanin, and flavonoids [18] [37]. The capacity of probiotic bacteria to survive in the GI tract, auto-aggregate, and be hydrophobic is connected to stability and probiotic adherence. With their antibacterial and antiadhesion properties against pathogen strains, probiotics modify gastrointestinal diseases. These compounds include bacteriocin, short-chain fatty acids, and organic acids [21]. The 16S rRNA gene serves as a valuable means to differentiate bacteria at the genus level, akin to a genetic identifier. However, in cases where closely related species exhibit nearly identical sequences, its effectiveness diminishes. This gene, spanning a considerable length, contains both consistent and variable segments, enabling meaningful comparisons. The sequencing of this gene for numerous bacteria aids in enhancing our understanding of them [9]. All bacteria have the 16S rRNA gene, which makes it possible to quantify connections between them [43-44]. The main objective of the study is to Formulate a traditional fermented pickle incorporating *D. hamiltonii*, conduct comprehensive analysis encompassing physical, sensory, microbial, and proximate composition, determine both shelf life and in vitro probiotic efficacy, and execute bacterial isolation and identification utilizing molecular identification techniques.

MATERIALS AND METHODS

This study entitled “*In vitro* probiotic efficacy and molecular identification of *Decalepis hamiltonii* Wight & Arn (Swallow Root) pickle” has been approved by the Institutional Human Ethics Committee (IHEC) with protocol no – SDNBVC/HSC/IHEC/2023/12 conducted on November 20th, 2023, by the Department of Home Science, S.D.N.B. Vaishnav College for Women, Chromepet, Chennai-44. Swallow roots were purchased from a vegetable vendor. Other ingredients are brought from the local grocery shop. The ingredients purchased to make *Decalepis hamiltonii* Wight & Arn pickle were swallow root, dry chili powder, salt, lemon, mustard, and turmeric powder. Homemade curd was inoculated and prepared using standard fermentation techniques. The SIDDHA CENTRAL RESEARCH INSTITUTE certified the raw material, *Decalepis hamiltonii* Wight & Arn root, with the code D03012403H.

Preparation of *Decalepis Hamiltonii* Wight & Arn Pickle

The root has been soaked for 7-8 hours to remove the dirt from the skin of the root. Following the pre-preparation process, the pickle, prepared by removing the peel and center stem, involves grinding raw ingredients into a paste, then adding lemon as needed. After two days, curd is incorporated into the mixture.

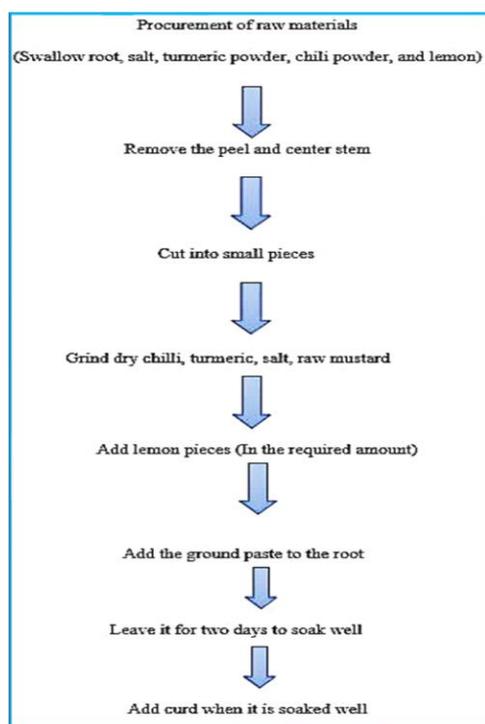


Fig 1 Flowchart of formulation of *Decalepis hamiltonii* Wight & Arn pickle

The pickle underwent a thorough examination, encompassing its physical characteristics, microbial content, sensory attributes, proximate composition, and shelf-life.

The physical analysis included assessing the pickle's pH and titratable acidity, as well as determining the levels of *E. coli* [20] and Total Yeast and Mold Count. The sensory evaluation utilized a 9-point hedonic scale [42], while proximate analysis covered various nutritional aspects such as energy, protein, fat, ash, moisture, crude fiber, carbohydrates, minerals, and vitamin B complex. The shelf-life was determined by monitoring microbial counts like *E. coli*, yeast, and mold count on days 0th, 1st, 5th, and 30th days [2].

In vitro probiotic efficacy and molecular identification of the isolate

The study employed various methodologies to investigate the effectiveness and characterization of the probiotic bacteria. Initially, the probiotic isolate was isolated and identified by diluting samples and growing them on specialized agar. Subsequently, its resilience to harsh conditions such as low pH and bile salts, susceptibility to antibiotics, anti-microbial activity, adhesion capabilities, and morphological characteristics were assessed. Concurrently, DNA extraction, amplification of a specific DNA region, and subsequent analysis were conducted to determine its genetic composition. This combined approach provided insights into both the functional attributes and genetic makeup of the probiotic [22].

Cost analysis

Cost analysis of the formulated pickle was also done to check their affordability for common people. The production cost of the pickle was calculated based on raw material cost and processing cost [19].

$$\text{Cost production per litre cost} = \frac{\text{Cost A} + \text{Cost B}}{Q}$$

Where;

cost A – the cost of raw materials

cost B – the cost of processing and Q - Quantity of pickle (g)

Statistical analysis

The product values have been statistically analyzed using SPSS software. The findings of each parameter collected from the pickle were given as mean ± standard deviation.

RESULTS AND DISCUSSION

Physical characteristics of the pickle

pH

The pickle's pH of 3.09±0.09 (Table 1) falls within the range of 2.9 to 3.9 as observed in other studies [1]. The slight acidity ensures safety and the absence of germs, with refrigeration increasing acidity due to LAB fermentation [28] [7a].

Table 1 Physical characteristics of the pickle

Parameters	Physical characteristics
pH	3.09±0.09
Titratable acidity	0.92±0.03

Titratable acidity

"Titratable acidity" denotes acidity measured in acetic acid units, controlling pH to support preferred bacterial growth while inhibiting competitors [41]. The pickle exhibited an acidity of 0.92±0.03 (Table 1), while the garlic pickle ranged from 0.70 to 2.66% [8b].

Table 2 Microbial characteristics of the pickle

Analysis	Microbial growth
<i>E. coli</i> count	Nil growth
Yeast count	3x10 ⁴ CfU/MI
Mold count	Nil growth

Microbial analysis of the pickle

The (Table 2) indicates the microbial characteristics of the pickle. In the context of food safety, the presence of food-borne pathogens poses a significant risk, necessitating careful attention to food handling practices to prevent potential illness,

as highlighted by [6]. While *Escherichia coli* is typically harmless, its proliferation to high levels can cause discomfort, particularly among vulnerable populations like young individuals. The absence of *E. coli* in the examined pickle sample is a reassuring outcome attributed to rigorous hygienic measures, as reported by [33]. In the complex realm of food fermentation and preservation, yeasts play a crucial role, as emphasized by [4] However, their dual capacity as facilitators of fermentation and potential contributors to spoilage requires careful monitoring. In this study, the yeast count in the sampled product, at 3×10^4 colony-forming units per milliliter (cfu/ml), remained within safe limits, as confirmed by [3].

Table 3 Sensory evaluation of the pickle

Sensory attributes	Average score
Appearance	8.7 ± 0.47
Taste	8.77 ± 0.41
Flavour	8.62 ± 0.48
Texture	8.65 ± 0.48
Colour	8.9 ± 0.30
Overall acceptability	8.77 ± 0.41

Sensory evaluation of the pickle

Data in (Table 3) indicates the sensory evaluation of the pickle. Sensory assessment has significantly advanced food product development, with standards for evaluating sensory impacts greatly enhanced [30]. The Nine-Point Hedonic Scale evaluated parameters such as overall acceptability, taste, texture, appearance, colour, and flavour. The sensory evaluation of the pickle showcased meticulous attention to detail across various dimensions. With its impressive uniformity of colour, suggesting freshness and quality, the pickle scored a notable 8.7 ± 0.47 in appearance. Taste-wise, its creamy curd base intertwined with subtle bitterness and tangy notes yielded a refined flavour profile, earning it an average score of 8.77 ± 0.41 . The appealing flavour, scoring 8.62 ± 0.48 , evoked positive associations and memories, enhancing overall satisfaction. Texture analysis unveiled a perfect chewiness, with a mean value of 8.65 ± 0.48 , significantly impacting consumer satisfaction. The consistent colour, enriched by spices and chili powder, scored an average of 8.9 ± 0.30 , aligning with consumer expectations. With a high acceptability rating of 8.77 ± 0.41 , the pickle exemplified a harmonious balance of sensory attributes, ensuring widespread appeal and desirability. The meticulous sensory assessment highlighted the pickle's attention to detail, promising a delightful culinary experience.

Proximate composition of the pickle

The proximate composition of the sample was examined. The nutrients such as energy, protein, fat, carbohydrates, moisture, crude fiber, ash, iron, calcium, Vitamin B complex, and mineral were analyzed. The in-depth examination of the pickle's composition uncovers intricate details regarding its nutritional content and culinary qualities. With a moderate energy content of 133.84 kcal/100g, it serves as a lighter option compared to conventional beetroot pickles. However, its nutritional richness extends beyond calorie count, boasting ample antioxidants, digestion-friendly microbes, and hydrating properties due to its high-water content, potentially offering significant health advantages. Upon comprehensive analysis of its macronutrient composition, significant insights emerge. The protein content, measured at $2.8 \text{ gm} \pm 0.21$, reflects a refined fermentation process that enriches both its savoury essence and nutritional density. Notably, [38], discovered that beetroot pickles contain 2.39g/100g of protein, indicating variability

among pickle varieties. Likewise, the fat content, quantified at $0.8 \text{ gm} \pm 0.12$, underscores deliberate attention to flavour and texture. [5], discovered a 5.30g fat content in oil-based cucumber pickles, signifying variations among pickle types and accentuating subtle distinctions in fat composition with nutritional implications. As ash increased, the mineral content also rises [31], as indicated by the ash content noted at $2.54\% \pm 0.02$. This signifies the infusion of crucial minerals during fermentation, enriching both nutritional value and flavour complexity. Additionally, the moisture content, documented at $65\% \pm 0.02$, suggests a meticulous blending of ingredients and preparation methods, potentially enhanced by the inclusion of curd for heightened texture and flavour enhancement [1]. The pickle's crude fiber content, outlined in Fig 2, measured at $0.54 \text{ gm} \pm 0.43$. [10] reported significantly higher fiber content, ranging from 4.73 to 34.73g in fenugreek seed pickle. Additionally, the pickle's carbohydrate content, as indicated in Fig 2, was 28.46g ± 1.10 . In contrast, [29] found a lower carbohydrate content of 11g in moringa leaves pickle. The observed rise could be attributed to the pickle's composition.

Minerals

Minerals like calcium and iron play crucial roles in fermentation, serving as essential nutrients and catalysts for enzymes. They regulate pH levels, support microbial growth, and influence the flavour and texture of fermented products [1] In the analyzed sample, calcium was measured at $6.65 \text{ mg} \pm 4.235$, while iron content was $0.34 \text{ mg} \pm 7.934$. Typically, carrot pickles contain around 0.11mg/ml of calcium and 0.18 $\mu\text{g}/\text{ml}$ of iron [1]. The increased calcium content may be attributed to the addition of 250g of root to 300 milliliters of curd in the pickle formulation.

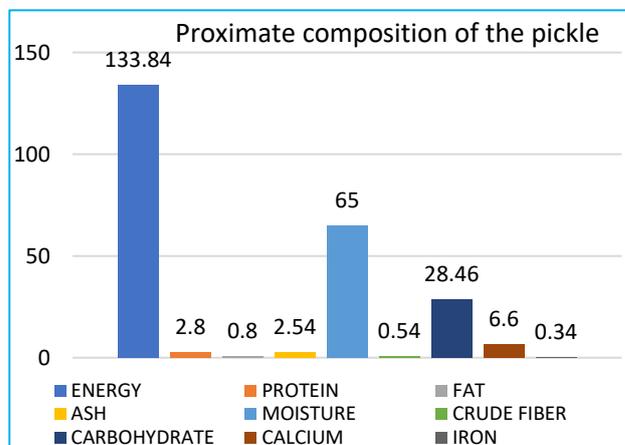


Fig 2 Proximate composition of the pickle

Table 4 Vitamin B composition of the pickle

Types of vitamin B	Vitamin value (μg)
Vitamin B1	2.81 ± 0.10
Vitamin B2	1.30 ± 0.92
Vitamin B3	5.22 ± 0.10
Vitamin B5	0.95 ± 0.12
Vitamin B6	1.36 ± 0.68
Vitamin B7	0.42 ± 0.09

Vitamin B composition of the pickle

Data in (Table 4) shows the vitamin B complex composition. The assessment of vitamin B complex levels in the formulated pickle unveils significant enhancements compared to garlic pickles across various vitamins. Notably, thiamine (vitamin B1) content displayed a marked increase,

measuring $2.81\mu\text{g} \pm 0.10$ in the formulated pickle, contrasting with the 0 to $0.055\mu\text{g}$ range observed in garlic pickles [7a]. Similarly, riboflavin (vitamin B2) exhibited elevated levels in the formulated pickle, registering at $1.30 \pm 0.92\mu\text{g}$, while ranging from 0.013 to $0.032\mu\text{g}$ in garlic pickles [8b]. The niacin (vitamin B3) content also saw a substantial rise in the formulated pickle, measuring $5.22\mu\text{g} \pm 0.10$, compared to $0.932\mu\text{g}$ reported in beetroot pickles [22]. Furthermore, pantothenic acid (vitamin B5) content was notably increased, measured at $0.95\mu\text{g} \pm 0.12$, highlighting a significant improvement compared to the $0.2\mu\text{g}$ value reported by USDA (2002). The pyridoxine (vitamin B6) content in the formulated pickle exhibited a noteworthy elevation, measuring $1.36\mu\text{g} \pm 0.68$, compared to $0.5\mu\text{g}$ in carrot pickles [1]. Moreover, the biotin (vitamin B7) content in the formulated pickle was measured at $0.42\mu\text{g} \pm 0.09$.

These findings not only underscore the enriched nutritional profile of the formulated pickle but also emphasize its potential as a valuable dietary component, offering essential B vitamins crucial for energy metabolism, cellular function, and overall health [16]. Additionally, it's worth noting that vitamin B levels, particularly those of thiamine and niacin, have been demonstrated to increase during fermentation processes. This

suggests that the fermentation of foods not only enhances their flavour and preservation but also leads to an enrichment of essential nutrients, providing added health benefits to consumers [14].

Shelf-life study of the pickle

Data in (Table 5) indicates the shelf life of the pickle. During the 30-day shelf-life analysis of the pickle, yeast count gradually increased, while mold and *E. coli* counts remained undetectable. Monitoring microbial growth from day 0 revealed no initial presence of *E. coli*, with a yeast count of 4×10^4 cfu/gm. Observations on day 1 showed minimal mold and no *E. coli* growth, although yeast count began to rise. By the 15th day, *E. coli* and mold counts remained stable, while yeast count steadily increased. On the 30th day, *E. coli* and mold counts were still controlled, but yeast count notably increased. Continuous monitoring and potential preservation adjustments are vital for ensuring product quality and safety throughout its shelf life. Yeasts, categorized as eukaryotic fungi, play a crucial role in food preservation. The absence of coliforms and molds reflects effective hygiene during storage. After the 30th day, yeast growth increased gradually within safe limits, indicating the pickle's suitability for consumption.

Table 5 Shelf-life study of the pickle

Intervals	<i>E. coli</i> x10 ⁴ cfu/gm	Yeast x 10 ⁴ cfu/gm	Mold x 10 ⁴ cfu/gm
0 th day	Nil	4	Nil
1 st day	Nil	5	Nil
15 th day	Nil	6	Nil
30 th day	Nil	12	Nil



0th day



15th day



30th day

Plate 1 Shelf-life study of the pickle

Table 6 Biochemical characterization of the isolate

Biological characterization	Results
KOH test	Negative
Catalase test	Positive
Spore forming	Positive
Gram staining	Positive

In vitro probiotic activity and molecular identification of the isolate

Biochemical characterization of the isolate

Data in (Table 6) presents the biochemical characterization results of the isolate, with one selected as more probiotic efficient. Gram staining confirmed all isolates as gram-positive, exhibiting blue-purple coloration with long rod-shaped bacilli. This aligns with previous studies on commercial and traditional pickles [12]. Catalase activity was positive, consistent with findings from commercial pickle analysis. However, the isolate tested negative for the KOH test but positive for spore formation.

In vitro probiotic activity

a) Tolerance to pH of the isolate

Data depicted in (Table 7) shows the acid tolerance patterns of probiotic isolate at different pH values after 3 and

6h exposure. The survival of probiotic LAB in the pickle was assessed at varying pH levels, ranging from 2.0 to 4.0, to simulate different stomach acidities [15]. The selected isolate underwent an acid tolerance test at pH 2 and pH 3 for 3 to 6 hours, demonstrating continued growth at all levels for up to six hours. Survival rates were recorded at 89.02% for 3 hours, 93.63% for 6 hours at pH 2, 90.45% for 3 hours, and 94.53% for 6 hours at pH 3.

Table 7 Tolerance to pH

pH 2		pH 3	
3 hours	6 hours	3 hours	6 hours
89.02	93.63	90.45	94.53



Plate 2 Tolerance to pH

b) *Tolerance to bile salts of the isolate*

Bile salts disrupt lipids and fatty acids in bacterial cell membranes, compromising survival rates [26]. In this study, a physiological concentration of human bile in the duodenum (0.3%) was simulated [32]. Table 8 illustrates that the isolates maintained over 90% survival at 0.3% bile salt, with one isolate reaching 97.34% survival after 24 hours of incubation. The study highlights the substance's ability to withstand bile salts 97.3% of the time and cope with varying pH levels 94.5% of the time, underscoring its practical potential for gastrointestinal applications.

Table 8 Bile salts

Parameters	Bile salt value
Bile salts	24 hours 97.34

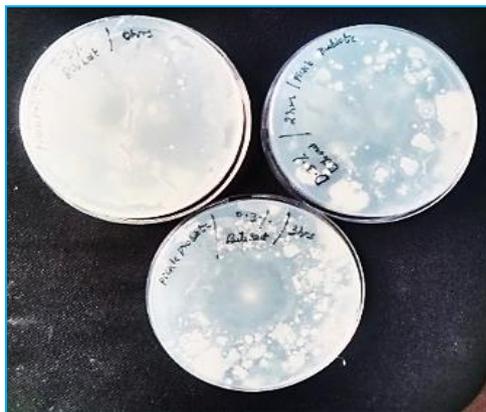


Plate 3 Tolerance to bile salts

c) *Bacterial adhesion to stainless steel plate of the isolate*

The study found *Bacillus* isolates adhering to stainless steel plates at a rate of 37.89%. This suggests effective colonization of gut mucosa, highlighting their potential for promoting gut health. The enhanced adhesion rate underscores their suitability for therapeutic applications and warrants further

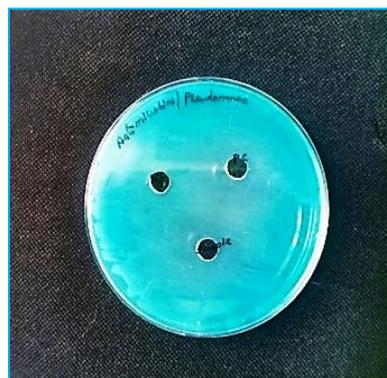


Plate 5 Antimicrobial activity

Table 10 antibiotic activity

Antibiotics	Activity
Oxacillin	Resistant
Tetracycline	Sensitive
Vancomycin	Sensitive
Chloramphenicol	Resistant
Cefoxitin	Resistant

a) *Antibiotic activity*

The assessment of antibiotic activity in the selected isolates revealed sensitivity to tetracycline and vancomycin, indicative of their susceptibility to these antibiotics.

investigation. The *in vitro* adherence rates to stainless steel plates for the four screened probiotic LAB isolates ranged from 32.75% to 36.30% [23]. These findings emphasize the importance of probiotic research and development in enhancing gut health [11].

Table 9 Antimicrobial activity

Bacteria	Antimicrobial activity (ZOI in mm)
<i>Streptococcus aureus</i>	11
<i>E. coli</i>	7
<i>Pseudomonas aeruginosa</i>	3



Plate 4 bacterial adhesion to stainless steel plate

d) *Antimicrobial activity of the isolate*

The selected strains were assessed for antimicrobial activity against *Streptococcus aureus*, *E. coli*, and *Pseudomonas aeruginosa*. Notably, *Streptococcus aureus* exhibited a substantial 11mm inhibition zone, while *E. coli* and *Pseudomonas aeruginosa* had zones of 7mm and 3mm, respectively. These results highlight the isolate's strong antimicrobial efficacy and potential applications in microbial control and health management (Table 9). The findings suggest that acidic fermented foods serve as a rich reservoir of *Bacillus* isolates exhibiting antibacterial properties against common foodborne pathogens like *E. coli* and *Streptococcus* [27].

Conversely, the probiotic isolates demonstrated resistance against oxacillin, chloramphenicol, and cefoxitin, suggesting a diminished response to these specific antimicrobial agents (Table 10).

Molecular identification of the isolate

Isolate 1, identified for its high probiotic potential, underwent gene sequencing to ascertain its species. Analysis of the 16S rRNA gene sequences revealed significant similarity to various bacterial species in the database. (Fig 5) illustrates the distribution of individual species, presenting percentages derived from sequence alignment. *Bacillus species* included

77% *Bacillus anthracis*, 70% *Bacillus paramycooides*, 79% *Bacillus cereus*, 89% *Bacillus zanthoxyli*, and 96% *Bacillus velezensis* and *Bacillus siamensis*. The dataset comprised 276 positions, and BLAST analysis showed 100% similarity to *Bacillus subtilis* strains in the Gene Bank. This comprehensive assessment sheds light on the bacterial isolates' composition, genetic identity, taxonomic classification, and evolutionary relationships within the *Bacillus* genus. Molecular sequences of the 16S rDNA gene, named *Bacillus subtilis* strain, have been submitted to NCBI GenBank under accession number PP264525.

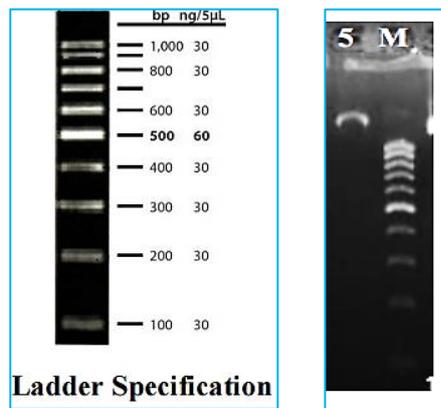


Fig 4 Gel picture of 16S rRNA PCR for bacterial isolate

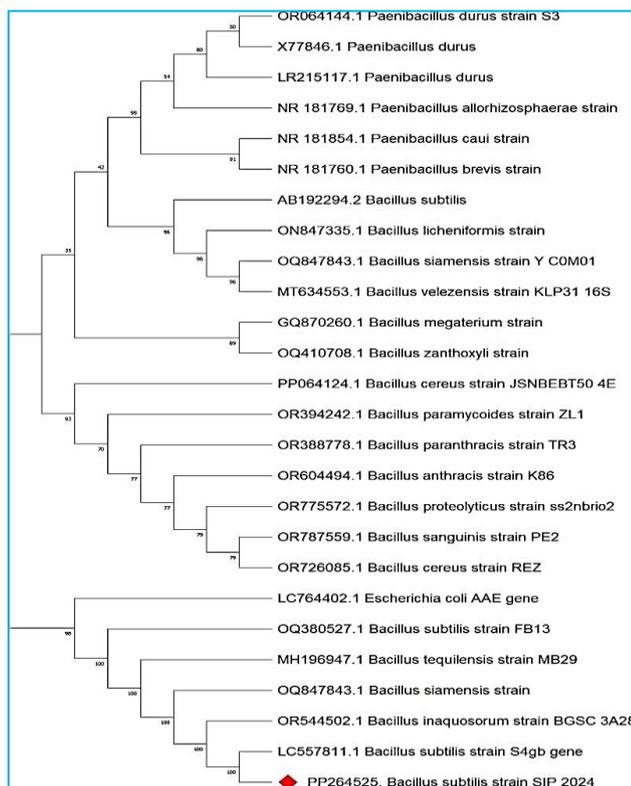


Fig 5 Phylogenetic tree for probiotic bacteria

Cost analysis

The cost analysis of the formulated pickle, based on a 100g serving size shown in (Table 11), revealed a production cost of Rs. 61.4/-. In comparison, the market price for the pickle was Rs. 70/-, resulting in a marginal difference of Rs. 8.6/-. This disparity between production cost and retail price reflects various factors including ingredient costs, labour, and desired profit margins. Such pricing considerations aim to ensure competitiveness in the market while maintaining profitability and consumer affordability. By evaluating the cost-effectiveness of the formulation, businesses can make informed decisions regarding pricing strategies, striking a balance between remaining competitive in the market and achieving financial objectives. Overall, the nominal difference between production cost and retail price suggests that the formulated pickle is competitively priced, providing value to consumers while also meeting profitability targets.

Table 11 cost analysis of the pickle

Materials requirement	Cost (Rs)
Total raw materials (Cost A)	484/-
Total processing cost (Cost B)	130/-
Total cost	614/-
Total cost (per 100g)	61.4/-
Cost of the standard pickle	70/-

CONCLUSION

The research on *Decalepis hamiltonii* Wight & Arn pickle presents a thorough examination of its physical, microbial, and nutritional properties. Through meticulous analysis, favourable physical attributes such as optimal pH levels and titratable acidity were uncovered, indicating sound quality. Additionally, microbial safety measures revealed the absence of *E. coli* growth and yeast levels within acceptable thresholds. Furthermore, the developed pickle displayed commendable sensory attributes, garnering positive feedback across various parameters, and exhibited substantial nutritional content, highlighting its potential as a flavorful and nutritious dietary option. Notably, observed antimicrobial and antibiotic properties underscore the potential health benefits of *Decalepis hamiltonii* Wight & Arn. Moreover, the isolation of *Bacillus* strains from the pickle suggests its potential effectiveness as a probiotic, offering promising contributions to gut health and overall well-being. This thorough examination underscores the favourable outlook for *Decalepis hamiltonii* Wight & Arn pickle, presenting it as an appealing choice for health-conscious consumers. The thorough analysis of *Decalepis hamiltonii* Wight & Arn pickle underscores its favorable physical, microbial, and nutritional properties, alongside promising health benefits. These attributes position the pickle as an appealing choice for health-conscious consumers, offering a combination of good taste, safety, nutritional value, and potential probiotic benefits.

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