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# Natural Bioactive System for Enhancing the probiotic Survival and Functional Efficiency

**B. Raghava Tej[1], Durga Prameela Gaddam[1]*, Kanneppagari. Akhila[2]**
1&2 *Department of Biological and Chemical Sciences, Mohan Babu University, Tirupati-517102
Corresponding Author: durgaprameelag@gmail.com

**ABSTRACT**
*Probiotics play a crucial part in balancing gut health and improving digestion and protection from different pathogens, however probiotic survival under stress conditions is a major limitation. In the present study, Neera (non-fermented drink) samples were collected from palm tree and cultured on MRS agar using spread plate method. These isolates were purified and identified by using gram staining, Biochemical test and 16S rRNA sequencing. The isolate was analyzed as a Gram-positive rod-shaped bacterium. Biochemical characterization showed catalase-negative and carbohydrate fermentation test positive reactions.16S rRNA sequencing identified the isolate as Bacillus paramycoides. Stress tolerance of the isolates was examined under Bile salts, acidic, UV and oxidative stress conditions to enhance probiotic survival and functional properties, milk derived exosomes were isolated and used to develop an exosome mediated probiotic system. The functional properties of the developed system were examined in Danio rerio as a in vivo model. Danio rerio were exposed to antibiotic treatment followed by probiotic and exosome mediated probiotic feeding for 21 days. After experimental period, digestive enzyme activities were analysed. Exosome mediated probiotics showed improved survival under bile salt, UV, oxidative stress while acid tolerance is limited, this study suggests that exosome-mediated delivery improved probiotic survivability under selected stress conditions.*
***Keywords:*** *Probiotics, Exosome mediated delivery, stress tolerance, Gut microbiome, In vivo studies, Milk Derived Exosomes*

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## INTRODUCTION

Microorganisms are essential for maintaining ecosystem stability across diverse systems like environmental and host associated ecosystems. In the human body gut microbiome contributes digestion, immunity, nutrient absorption and protection against harmful microbes and these microorganisms is called probiotics.it is very supportive for immunosuppressed individuals like cancer patients, chronic illness, transplantation patients etc. Probiotics are beneficial bacteria that promote health benefits to the host when consumed in adequate amounts. Mainly probiotics are used in clinical and nutritional applications to restore microbes, gut health and protection from infectious diseases. Effectiveness of probiotics can be evaluated by their ability to survive in any condition within the host environment. One of the major challenge of probiotics is reduction of survivability in stressful conditions inside host. Bacteria are exposed to different stress triggers such as acid, bile salts, UV exposure and oxidative stress. These factors may reduce the survivability of probiotics and efficiency [1-6].

Among these factors oxidative stress is major contributor to microbial damage. Oxidative stress occurs due to accumulation reactive oxygen species in body. Acidic gastric conditions and oxidative stress can reduce probiotic survival in the gastrointestinal tract. This leads to imbalance of beneficial microbes in gut microbiome and reduce survival rate in immune compromised patients [10-14].

This limitation highlights gap in probiotic strategies. Many strategies focus on increasing probiotic dosage; however, maintaining microbial viability remains a challenge this method is not useful for microbial survival in stressful conditions. Recent advancements in gut microbiome research have introduced the concept of natural bioactive support systems to enhance microbial stability. natural bioactive systems defined as products that obtained from natural sources like milk example of these natural bioactive systems are milk derived exosomes. These exosomes act as protectant for probiotics under stressful conditions. these vesicles are nanoscale structures contain lipids, proteins and signalling molecules that interact with microbial cells and influence the behaviour. Based on this concept, the present study focuses on developing novel solution to improve probiotic survivability in adverse conditions this study explores where

probiotics are combined with natural bioactive system to enhance their stability.to evaluate this concept a in vivo model is used to stimulate stress conditions and microbial response [15-18]. This study is particularly applicable for immunocompromised conditions, where maintain adequate microbes is essential but challenging. By improving stability and efficiency, it may be possible to enhance their effectiveness.

## MATERIAL AND METHODS

### Isolation of microorganisms from Neera:

Neera sample were collected from palm trees in Tirupati, Andhra Pradesh, and transported to laboratory within 2hrs in cold conditions and microbial isolation was performed immediately to main viability and palm Neera were isolated on De Man, Rogosa, and Sharpe agar plates and incubated at $37^0$C for 24 hours.

### Purification and subculturing of isolates:

Bacterial colonies were subcultures repeatedly using streak plate method to obtain pure cultures. Subculturing was performed up to four times to maintain purity. The purified isolates were maintained in 4c for further studies

### Identification and characterization of isolates:

The Bacterial isolates were put through morphological and biochemical analysis. Gram staining was performing to identify bacteria is gram positive or negative. Biochemical tests such as catalase test and carbohydrate fermentation test were executed. For molecular identification 16s rRNA sequencing the bacterial isolate was identified by amplifying the 16S rRNA gene using universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR mixture consisted of:

- Distilled water: 18µL
- 10× $MgCl_2$ buffer: 2.67µL
- dNTP mix: 2µL
- Taq DNA polymerase: 0.33µL
- Primer mix: 1µL
- Template DNA: 1µL

PCR cycling conditions were:

- Initial denaturation: 94°C for 5 min
- 35 cycles of:
- Denaturation at 94°C for 30 s
- Annealing at 56°C for 30 s
- Extension at 72°C for 1 min 10 s
- Final extension at 72°C for 7 min

The amplified products were verified by 1.5% agarose gel electrophoresis and sequenced using the BigDye Terminator v3.1 kit. Sequence analysis was performed with MEGA 7 software, and BLAST results identified the isolate as *Bacillus paramycoides.*

The probiotic potential was examined by different tolerance tests using of microbiological culture media studies by colony count, staining and biochemical tests.

Table 1. Morphological and Biochemical Characteristics of *Bacillus paramycoides*

| Test | Result |
|---|---|
| Gram Staining | Gram positive |
| shape | Rod |
| Catalase test | Negative |
| Microbe | *Bacillus paramycoides* |

**Stress tolerance studies:**

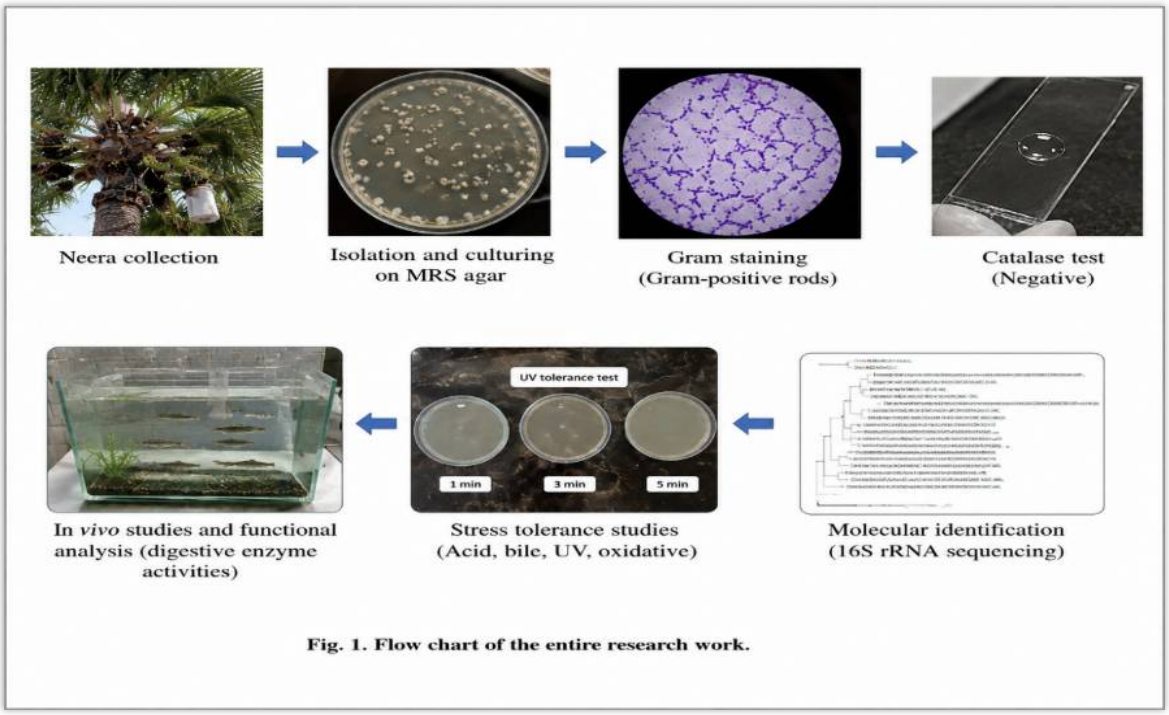



**Fig. 1. Flow chart of the entire research work.**

**Acid tolerance test**

The isolates were cultured into MRS broth adjusted different Power of hyderogen(pH) 2 and 3 and incubate 37C determine survival. Exosome mediated probiotic also tested under similar conditions for comparison [6].

**Bile tolerance test**

Bile salt tolerance was evaluated by adding of bile salts to MRS agar at different concentrations 0.3% and 2% to Petri plates and inoculated the probiotic culture to the plates. Exosome mediated probiotic also tested under similar conditions for comparison [8].

**UV tolerance**

Bacterial cultures were isolated on De Man, Rogosa, and Sharpe agar plates and exposed to UV light for different time durations 1min, 3min, 5min and. Exosome mediated probiotic also tested under similar conditions for comparison [19].

**Oxidative stress tolerance:**

Oxidative stress was induced using hydrogen peroxide. The probiotic culture was exposed to oxidative conditions and Exosome mediated probiotic culture also tested under similar conditions for comparison based on bubbles and colour intensity [20].

**Exosome mediated probiotics:**

Exosomes are tiny vesicles range between 30-200nm which is rich in protein, lipids and miRNAs it acts as delivery system. Exosomes were obtained from Bovine milk samples using differential centrifugation. The isolated exosomes were purified and collected. Natural sources like Fresh milk (50 mL) used for exosome isolation. The sample was initially centrifuged at 4000 rpm for 15 minutes to remove debris and fat globules. Following centrifugation, a thick cream layer formed on the surface, which was carefully removed and discarded to obtain defatted milk. The defatted milk was further centrifugation at 8000 rpm for 15 minutes to remove residual particulate matter. The pellet formed at the bottom was discarded, and the supernatant was collected for further processing. To precipitate exosome mixture, the pH was adjusted by the gradual addition of acetic acid (drop-wise) with gentle mixing until the pH reached approximately 4.6. This resulted in the formation of visible white clumps. The mixture was incubated for a short duration to allow complete precipitation and then centrifuged at 10,000 rpm for 15 minutes. The resulting pellet was discarded, and the supernatant containing exosome-enriched fractions was collected. The obtained preparation was stored at 4°C overnight and used for additionally experimental analysis [21].

Transmission electron microscopy revealed spherical vesicle-like structures in the nanometer range. The range of around 100-200 nm confirmed that the particles were within the expected size range of extracellular vesicles."

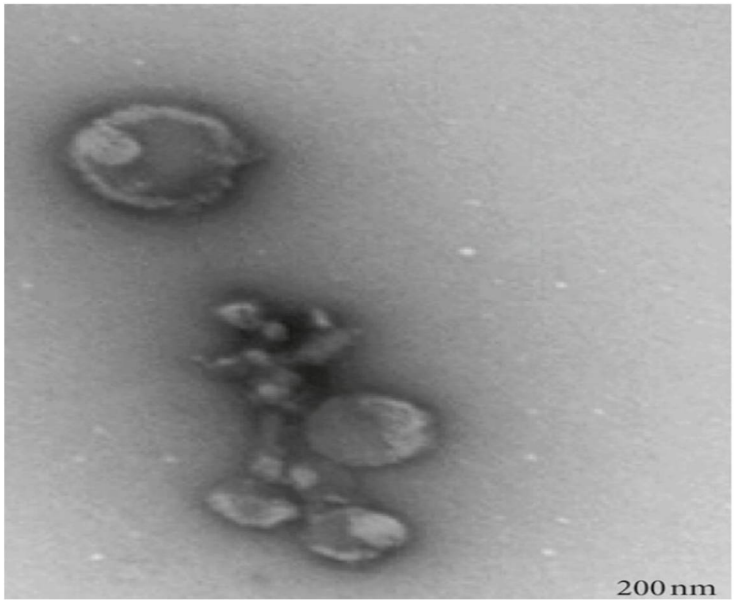



**Fig 2: TEM image of exosomes**

***In vivo* studies (Danio rerio):**

Healthy zebrafish were selected and acclimatized under laboratory conditions. The Danio rerio was partitioned into control and treatment groups. the experiment was consisting 21days feeding schedule, initially, Danio rerio were fed Tetracycline-containing feed (2mg/g feed) to reduce the existing gut microbiota using water immersion method. This was followed by feeding of probiotic and exosome mediated probiotic in alternating cycles through fish food.

Adult zebrafish (Danio rerio) aged 4–6 months were housed in glass aquaria (2 ft × 2 ft × 2 ft) at 27 ± 1°C with a 14 h light/10 h dark cycle. Fish were acclimatized for 7 days prior to the experiment. Water conditions were controlled with a pH of 7.0–7.5 and dissolved oxygen above 6 mg/L. Stocking density was 6 fish per aquarium. For a duration of 21 days. Feeding occurred twice daily, and 30–40% of the aquarium water was replaced every 3 days. All zebrafish experiments were performed according to institutional guidelines.

Fish were carefully handled using a soft net to minimize stress and avoid injury. After acclimatization, fish were dissected under controlled conditions, and at the end of the experiment, fish were dissected to collect the gastrointestinal tract for digestive enzyme analysis [22].

**Preparation of Gut Homogenate**

The dissected gut tissues were collected and homogenized using sterile normal saline (0.9% NaCl) to obtain a uniform suspension. The homogenate was used immediately for enzymatic assays.

**Enzyme activities of gut samples**

**Amylase Activity Assay**

Amylase activity was analyzed using the starch-iodine method. The gut homogenate was incubated with starch solution for a specific period. After incubation, iodine solution was added, and the color intensity was observed. A reduction in blue-black coloration indicated the hydrolysis of starch and the presence of amylase activity [23].

**Protease Activity Assay**

Protease activity was assessed using casein extracted from milk as a substrate. The gut homogenate was added to the casein solution and incubated under suitable conditions. The digestion of casein was indicated by a reduction in turbidity or the formation of a clearer solution, confirming protease activity [24].

**Lipase Activity Assay**

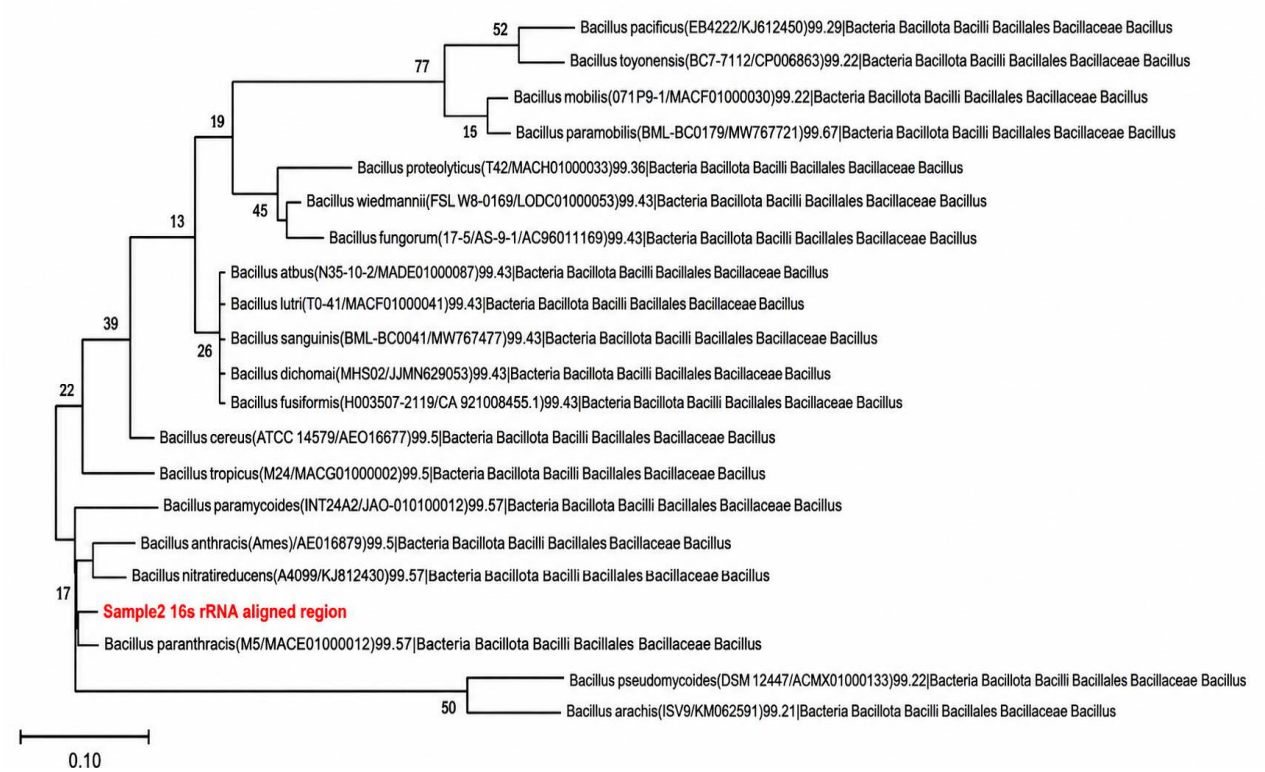
Lipase activity was evaluated using ghee as a lipid substrate. Since lipids are not soluble in water, Tween 20 was added as an emulsifying agent to facilitate interaction between the enzyme and substrate. The gut homogenate was added to the mixture and incubated [25].

**RESULTS**

Microorganisms were isolated from Neera samples using and spread and streak plate techniques. Distinct colonies were observed on MRS agar after incubation. Repeated subculturing resulted in pure cultures.

Morphological characterization of the isolates gives that the bacterial cells were rod-shaped and an appeared purple in Gram staining, it indicates Gram-positive bacteria.

Biochemical characterization showed that the isolates were catalase negative, which is a characteristic feature of probiotic bacteria. Molecular characterization through 16s rRNA sequencing, and the obtained sequences showed similarity with *Bacillus paramycoides* species.




**Tolerance tests:**

**Bile tolerance test:**

Bile salt tolerance was examined through MRS agar added with different concentrations of bile salts (0.3% and 2%).

However, the exosome-mediated probiotic culture showed enhanced growth and colony formation at 2% bile concentration compared to normal probiotic culture, indicating enhanced resistance under extreme bile stress.

The isolate was able to survive under bile salt conditions. Exosome mediated probiotics showed improved survival compared to normal probiotic. Mainly in 2% bile conditions exosome mediated probiotic grown well in MRS bile agar.




**Fig 3: comparison between normal probiotic and ex-probiotic in bile salts**

**Acid tolerance test:**

Acid tolerance of the isolated probiotic strain was examined using MRS broth adjusted to pH 2 and pH 3. Importantly, both normal probiotic culture and no significant improvement in survival was observed under acidic conditions, indicating the isolate was sensitive to low PH as evidenced by the absence (or very minimal presence) of turbidity and colony formation.

These results indicate that the isolated microorganism lacks strong acid tolerance and is sensitive to low pH environments.

Both probiotic cultures exhibited poor survival under high acidic conditions. Exosome mediated probiotic did not show good tolerance

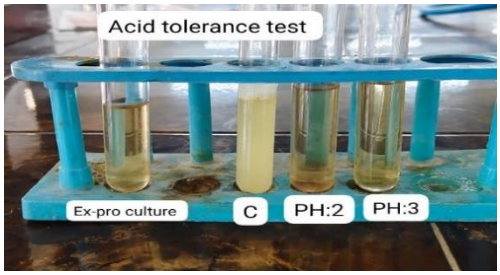



**Fig 4: Acid tolerance**

**UV tolerance**

UV tolerance was assessed by examined the cultures to ultraviolet radiation for 1 minute, 3 minutes, and 5 minutes.

At 5 minutes exposure, reduced but visible colony formation was observed, indicating survival under prolonged UV stress. The exosome-mediated probiotic culture exhibited higher colony survival at 5 minutes exposure compared to normal cultures, indicating enhanced UV resistance.

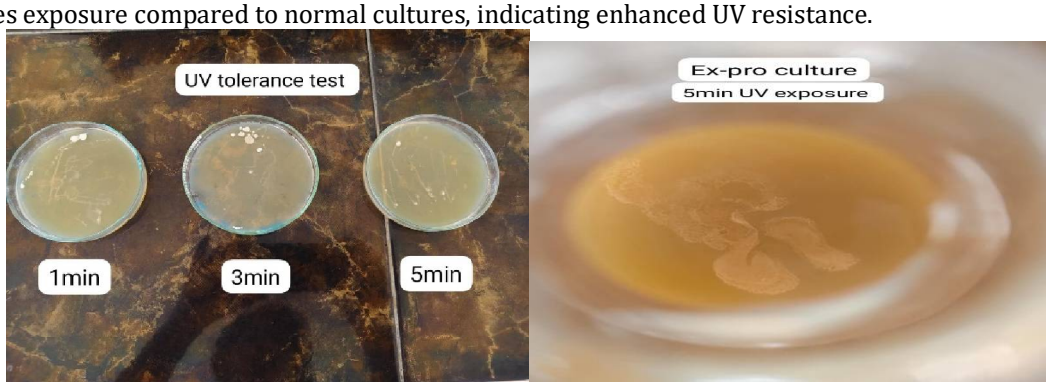



Fig 5: comparison between normal probiotic and ex-probiotic efficiency in UV exposure

Exosome mediated probiotic exhibited great survival to UV exposure compares with untreated probiotics. This study concluded a protective role of exosomes against UV stress.

**Oxidative stress tolerance**

Oxidative stress tolerance was evaluated using hydrogen peroxide ($H_2O_2$).

The exosome-mediated probiotic culture showed higher turbidity compared to $H_2O_2$-treated samples, indicating improved resistance to oxidative stress.

Exosome mediated probiotic cultures showed improved survival under oxidative stress. These studies suggested exosomes maintains the protective role against hydrogen peroxide.

**Amylase activity**

Amylase activity was assessed using the iodine-starch method. The exosome-mediated probiotic group showed higher digestive enzyme activity than the control group. Amylase activity was detected in 50% (3/6) of samples in the exosome-treated group versus 33.3% (2/6) in the control group, indicating enhanced starch hydrolysis following exosome-mediated probiotic supplementation.

| Group | Amylase-positive samples |
|---|---|
| Control | 33.3% (2/6) |
| Exosome group | 50.0% (3/6) |

Table 2: Amylase activity in gut samples in Danio rerio

**Protease Activity**

Protease activity was evaluated using casein as a substrate. Protease activity was detected in 66.7% (4/6) of the exosome-treated samples, whereas only 33.3% (2/6) of the control samples showed proteolytic activity, suggesting improved protein digestion in zebrafish supplemented with exosome-mediated probiotics.

Table 2: Protease activity in gut samples in Danio rerio

| Group | Protease-positive samples |
|---|---|
| Control | 33.3% (2/6) |
| Exosome group | 66.7% (4/6) |

**Lipase Activity**

ipase activity was determined using ghee emulsion and confirmed by phenolphthalein indicator. Lipase activity was observed in 66.7% (4/6) of the exosome-treated samples, compared with 16.7% (1/6) in the control group, indicating enhanced lipid digestion in the exosome-mediated probiotic group.

**Table 2: Lipase activity in gut samples in Danio rerio**

| Group | Lipase-positive samples |
|---|---|
| Control | 16.7% (1/6) |
| Exosome group | 66.7% (4/6) |

**DISCUSSION**

The study shows, probiotic was successfully isolated from Neera and examined for their morphology, Biochemical and molecular identification. The isolate was evaluated as gram-positive, rod-shaped bacteria with catalase negative, molecular identification shows *Bacillus paramycoides* spp.

Stress tolerance is an important factor for determining probiotic potential. In this study, the isolate was expose to multiple stress conditions like acid bile UV and oxidative stress tolerance. The results of the stress tolerance isolates exhibit good tolerance to bile salts, UV radiation and oxidative stress, only isolate show limited tolerance to acidic conditions. Exosome mediated probiotics improved survival of probiotics in the form of microbial colonies. Exosome mediated probiotic showed high colonies compare to normal probiotic, except in acidic conditions [27, 28].

Danio rerio (Zebra fish) as an in vivo model for determining the functional efficacy of exosome mediated probiotic system [29].

Overall, the findings of this study demonstrate that milk-derived exosomes can improve probiotic survival under bile, UV and oxidative stress conditions while also enhancing digestive enzyme activities in *Danio rerio*. Although acid tolerance was not significantly improved, the results indicate that exosome-mediated probiotic delivery has potential as a natural bioactive strategy for improving probiotic stability and functional performance. Further studies involving quantitative analysis and advanced exosome characterization are required to better understand the mechanisms responsible for these beneficial effects.

**CONCLUSION**

In the current study, *Bacillus paramycoides* was successfully isolated from Neera and identified using morphological, biochemical, and 16S rRNA sequencing. Milk-derived exosomes were isolated and utilized as a natural bioactive component to improve the survival and functional efficiency of the probiotic bacterial isolate from neera. The results demonstrated that exosome-mediated bacteria showed better survival under bile salt, ultraviolet radiation, and oxidative stress conditions, whereas only limited tolerance was observed under acidic conditions compared to other tolerance assays.

Transmission electron microscopy confirmed the presence of nanosized exosomes, and in vivo studies using *Danio rerio* showed improved digestive enzyme activities, including amylase, protease, and lipase, in the exosome-treated group. The results indicate that milk-derived exosomes may contribute significantly to the survival and functional efficiency of *Bacillus paramycoides* under stressful conditions.

Although the study produced promising results, further investigations involving quantitative analysis, advanced molecular characterization, and direct gut microbiome studies are necessary to better understand the underlying mechanisms and explore the applications of this exosome-mediated probiotic system in food and biomedical research.

**CONFLICT OF INTEREST**

The authors confirm that no conflict of interest exists.

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