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Research Article

Characterization of Dairy and Non-Dairy Probiotic Isolates and their Molecular Identification

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Abstract
This study evaluated the fitness and potential capacity of probiotic strains from both dairy and non-dairy sources through a comparative analysis. Probiotic isolates were characterized through microbiological, biochemical methods and identified by molecular analyses, following 16S rRNA sequence analysis. The bacterial strains (14) isolated from fermented dairy products (cow curds, buffalos curd, homemade cheese, packed curds) and non-dairy items (sauerkraut, pickles, vinegar) were Gram positive, rod-shaped, non-motile and non-haemolytic in nature. Tests for acid, bile tolerance, and stainless-steel plate adhesion test for biofilm formation activity was carried out.
Results indicated that both dairy and non-dairy probiotics maintained high viability. About 74 to 87% probiotic isolates tolerated acidic environments (pH 2.0) and the 0.3 % bile salts (w/v) was tolerated by 47 to 90% isolates. Among all the isolates, strains P1, V1, CC1 and MCz showed higher adherence and curdling ability. Statistical analysis revealed no significant differences between dairy and non-dairy probiotics in terms of acid tolerance (p=0.765), bile tolerance (p=0.953).
These findings highlight the potential of non-dairy probiotics as viable alternatives to dairy probiotics, particularly for individuals following vegan diets or people with lactose intolerance, or dairy allergies. Non-dairy probiotics are equally effective in promoting gut health and preventing infections, making them suitable for incorporation into probiotic formulations and health products to meet diverse consumer needs.

Keywords: Probiotics, 16S rRNA, Dairy products, Non-dairy products, *Bacillus tequilensis* KCT 13622, *B. velezensis* CR-502

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Introduction

Probiotics are widely acknowledged for their potential health benefits and applications in food industry. Probiotics are live microorganisms that confer health advantages on the host when ingested in sufficient quantities (Ali et al., 2021). According to Anggriani and Taofiqurohman (2012), *Bacillus spp*. functions as probiotic bacteria that can rapidly break down feed and reduce the presence of pathogenic bacteria in the intestine; the bacterial activity in the gut can quickly change when microbes enter via feed and water. Similarly, Febryana (2017) noted that *Bacillus* sp. can secrete enzymes such as protease, lipase, and amylase. Research on *Bacillus* spp. has been extensive due to its

ability to adhere to surfaces and produce bacteriocins (anti-microbial peptides) and immunostimulants (Barbosa et al., 2005). This strain has been demonstrated to serve as a commercial probiotic product that enhances shrimp production and has anti-microbial properties (Decamp and Moriarty, 2006). Additionally, *Bacillus spp*. has the advantage of having spores that can be stored for long periods (Hong et al., 2005).

These beneficial organisms enhance gastrointestinal health, strengthen immunity, and prevent various gastrointestinal diseases. Probiotics are commonly found in fermented foods and supplements, with major sources including dairy products like yogurt and kefir, as well as non-dairy options such as fermented vegetables and soy-based products (Alkalbani et al., 2022). Strains of dairy probiotics, such as *Lactobacillus* and *Bifidobacterium* species, have been extensively researched and are well-known for their health benefits. These species found in dairy products improve lactose digestion, enhance immune response, and inhibit pathogenic bacteria (Ansari et al., 2021). These beneficial bacteria thrive best during the fermentation process in dairy products, ensuring their viability and effectiveness. However, the increasing incidence of lactose intolerance and the rise of veganism have driven demand for non-dairy probiotic sources (Barzegar et al., 2021). Non-dairy probiotics, derived from fermented vegetables, fruits, grains, and more, offer an alternative for those who do not consume dairy products.

Non-dairy probiotics have their benefits and challenges compared to dairy counterparts. These strains must adapt to different fermentation substrates and environmental conditions, which can influence their survival and probiotic properties. Non-dairy sources are often rich in dietary fibers and antioxidants, which react differently with bacteria compared to components found in dairy (Cosme et al., 2022). Non-dairy probiotic organisms can enhance the diversity of gut microbiota and offer additional nutritional advantages (Dahiya & Nigam, 2022).

Understanding the comparative merits of dairy versus non-dairy probiotics is crucial for their health implications and industrial applications. Furthermore, a comparative analysis may identify which specific probiotic strains are more suitable for certain health conditions, leading to targeted probiotic therapies (Hill et al., 2014).

Historically, research on probiotics began in the mid of 20th century, focusing primarily on beneficial bacteria from dairy products. The term "probiotics" became clearer when Garcia-Gonzalez et al. (2022) defined it as “any microorganism-derived compound that stimulates another organism's growth”. This definition expanded the scope of probiotics beyond just dairy sources. Advances in microbiology and molecular biology in the 1970s and 1980s enabled scientists to identify different strains more accurately. By using selective culture media and anaerobic conditions, strains like *Lactobacillus acidophilus* and *Bifidobacterium bifidum* were found to have significant positive effects on human health, including improved gastrointestinal function and immune system reinforcement (Gomes et al., 2021).

Probiotics promote health by occupying space to prevent harmful bacteria from establishing themselves, producing substances that enhance immune function, Bibi and generating compounds that inhibit pathogenic microorganisms (Kanwal et al., 2021). Scientists have also explored non-dairy sources like pickles or sauerkraut as potential sources of probiotics, offering alternatives for those with lactose intolerance or following vegan diets (Kariyawasam et al., 2020). Non-dairy probiotics have been shown to offer health benefits comparable to their dairy counterparts, expanding the field of research into new probiotic strains and their potential applications in healthcare and industry. Non-dairy probiotics come with dietary fibers and polyphenols, offering additional antioxidative benefits. These probiotics can improve metabolic markers of health and protect the immune system, similar to dairy-based probiotics (Terzić-Vidojević et al., 2020).

In view of above the present research has been carried out to study the characteristic features of dairy and non-dairy probiotic isolates and molecular characterization of potential strains.

Materials and Methods

Probiotic bacterial isolates from dairy and non-dairy products

Initially a total of 58 samples were collected from various dairy and non-dairy sources, including homemade cow curd, buffalo curd, home-made cheese, market cheese, vinegar, mango pickles, sauerkraut, and pineapple juice. Using these samples the bacteria were cultured on MRS agar plates. Fourteen bacterial isolates were selected for further studies, due to their unique characteristics including better growth, stronger lactic acid production or higher potential as probiotics (Kushwaha and Kumar 2024). The preserved cultures from previous study were used for the present research.

Characterization of probiotic properties

Haemolysis test

The test for β-haemolysis was carried out by culturing bacteria on sheep blood agar plates. The plates were incubated at 37 °C for 24 to 48 hours. Following incubation, the haemolysis was observed and interpreted as described earlier (Hargrove and Alford, 1978).

Motility test

The motility was tested following hanging drop method for the evaluation of motility in bacteria. A clean slide with depression on a side was taken, loopful of fresh bacterial culture grown in liquid broth was place in the center of a clean coverslip, the petroleum jelly was applied on the corners of coverslip. The depression slide was placed on coverslip in inverted position making a hanging drop of bacterial culture. The hanging drop was examined under bright light microscope at x40 magnification for bacterial motility (Aneja KR, 2023).

IMViC test

The **IMViC tests** were conducted by inoculating the respective media with the bacterial culture and incubating them at 35–37°C for 24–48 hours. For the **Indole test**, tryptone broth was used, and Kovac's reagent was added after incubation; a red layer indicated a positive result. In the **Methyl Red test**, Methyl Red indicator was added to MR-VP broth, and a red color confirmed acid production. For the **Voges-Proskauer test**, Barritt's A and B reagents were added to MR-VP broth, and a red color after standing indicated acetoin production. In the **Citrate test**, Simmon's citrate agar was streaked, and growth with a blue color signified citrate utilization. (Powers and Latt,1977; Hayet et al., 2021; MacWilliams, 2009).

Curdling test

For the milk curdling test, 2% (v/v) inoculum of fresh overnight-grown bacterial culture was combined with 10 ml of 10% (w/v) sterile skim milk suspension and incubated for 48 hours at 37°C (De et al., 2017).

Acid and bile tolerance

To assess acid tolerance, the isolated strains were exposed to simulated gastric juice adjusted to pH 2.0 for 3 hours. After exposure, viability was evaluated by plating the samples on MRS agar and counting the colony forming units (CFUs). For bile tolerance, the isolates were cultured in MRS broth amended with 0.3% bile salts and incubated at 37 °C for 4 hours. Growth was quantified spectrophotometrically at 600 nm to determine tolerance levels. Survival rates were calculated by comparing CFU counts before and after exposure to these stress conditions (Kumar et al., 2012).

Bacterial adhesion to stainless-steel plate

The steel plates sterilized by passing overnight duration in acetone, were submerged in a Tween 20 detergent solution and stirred for 45 minutes at 50 °C. Each stainless-steel plate was repeatedly cleaned with distilled water, air dried, and finally autoclaved. 500 µl of the overnight-grown bacterial culture was placed in a sterile test tube containing 4500 µl of sterile MRS broth. The sterile stainless-steel plate was then placed inside the tube, and it was incubated for 24 hours at 37 ºC. After incubation the plates were removed aseptically and transferred in a tube containing 10 ml of 1% sterile peptone water for 5 minutes for washing. Repeat washing was done by transferring the steel plate to a fresh-tube containing 6 ml of 1% sterile peptone water. The tubes were vortexed for 3 minutes to detach bacterial cells from the steel plate surface. The live cells adhered with stainless-steel plate were counted by spreading 0.1 ml peptone water on MRS agar medium. The positive control was made by mixing 500 ml culture and 4500 MRS broth, which was incubated overnight at 37 ºC. The c.f.u. in positive control was counted by spreading 0.1 ml of positive control on MRS agar. The inoculated Petri-dishes were incubated at 37 ºC for 24 h (Rim El-Jeni et al., 2016).

Statistical analysis

Experimental data from the Acid tolerance test and adherence test were analyzed using one-way analysis of variance (ANOVA) conducted with IBM SPSS Statistics (version 21, IBM, Armonk, NY, USA). Significance was determined at $p < 0.05$ using Duncan's test (Jose et al., 2015).

Bacterial DNA isolation

Genomic DNA was extracted using a modified CTAB method described previously by Doyle et al. (1986), briefly: a single colony was inoculated in 5 mL of nutrient broth and incubated overnight at 37 °C. The culture was centrifuged at 8,000 rpm for 5 minutes to pellet the cells. The supernatant was discarded, and the pellet was resuspended in 500 µL of CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 0.1 M Tris-HCl pH 8.0, and 20 mM EDTA). The suspension was incubated at 65°C for 30 minutes. Following this, an equal volume of chloroform alcohol (24:1) was added, and the mixture was centrifuged at 12,000 rpm for 10 minutes. The upper aqueous phase was transferred to a new tube, and 0.6 volumes of isopropanol was added to precipitate the DNA. The mixture was centrifuged at 12,000 rpm for 10 minutes, and the pellet was washed with 70% ethanol, air-dried, and dissolved in 50 µL of TE buffer made up of 10 mM Tris-HCl, 1 mM EDTA (Doyle et al., 1986). The gel electrophoresis was performed as per standard protocol described by Sambrook and Russell (2001).

16S rRNA Gene amplification and Sequencing

The 16S rRNA gene was amplified using specific primers: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The optimization of annealing temperature was carried out in 96-well gradient PCR machine (Techne, TC-512, UK), and 55 °C was selected as annealing temperature. The PCR reaction was carried out in a total volume of 50 µL, which included 25 µL of PCR Master Mix (2X), 2 µL of each primer (10 µM), 1 µL of template DNA, and 20 µL of nuclease-free water. The PCR conditions included an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 5 minutes. The PCR products were checked on a 1% agarose gel as described earlier and then purified using a PCR purification kit (Himedia® labs) according to the manufacturer's instructions. Sequencing of the purified PCR products was performed using Sanger sequencing technology (Peattie, 1979) at Biokart Pvt. Ltd. (Bangaluru).

Phylogenetic analysis of 16S rRNA gene sequence of the isolates

To identify the probiotic strains, the resulting sequences obtained were analyzed by performing similarity search against quality-controlled databases of 16S rRNA sequence data on Eztaxon server (https://www.ezbiocloud.net, Chalita et al., 2024**)**. The determination of Phylogenetic neighbours was done using multiple sequence alignment programme in

ClustalW (Thmpson et al.,1994). The phylogenetic relations of similarity showing sequences was determined using MEGA 11 software (Tamura et al., 2021). The phylogenetic tree was constructed on MEGA 11 using neighbour-joining (NJ) method and significance of junctions was established using bootstrap (1,000 replicates) method (Felsenstein, 1985).

Results and Discussion

Characterization of probiotic isolates

None of the bacterial isolates showed motility on SIM media, as well as no haemolysis was reported on blood agar medium. The isolates, S2, S3, MC2, OJ4, BC1 and BC2 were MR positive while, others were negative. Voges-Proskauer test was positive for S1, S2, S3, MC2, OJ4, and HMC3 isolates. Indole was produced by BC1, BC2 and HMC3, while none of the isolates utilized citrate. The minimum time taken for curdling was 5.33 hours by the isolate V1, while other bacteria look longer, the isolate CC2 took longest time for curdling. The results are presented in Figure 1 and Table 1.

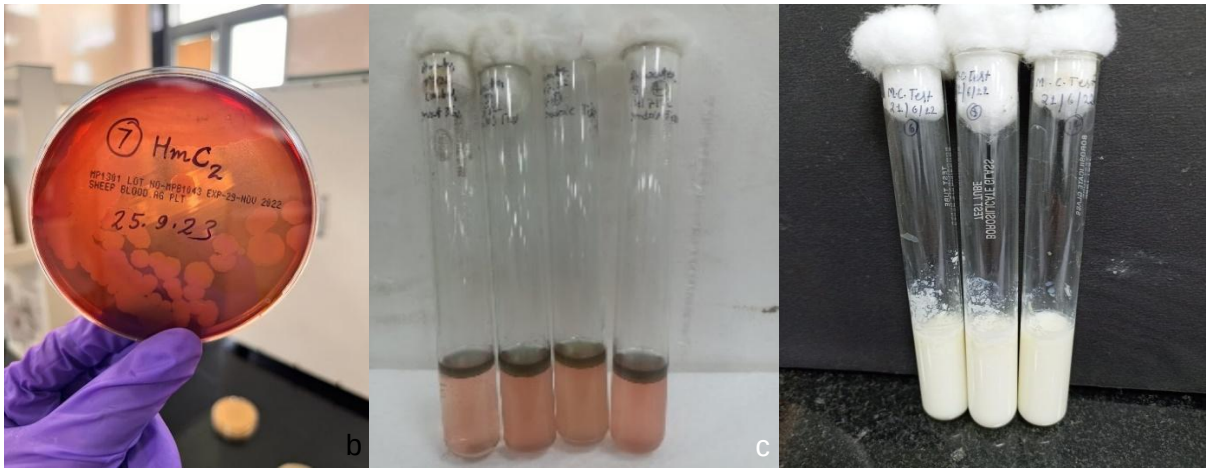

Figure 1: Biochemical tests of the probiotic isolates: a. haemolysis; b. indole test; c. curdling test of milk with probiotic isolates

Table1: Biochemical tests results

Sr. No.	Isolate ID	haemolysis test	Motility test	MR -test	VP Test	indole test	Citrate Utilization test	Mean Duration for curdling in hrs (S.D.)
1	S1	---	---	---	+++	---	---	22.43 (±0.51)
2	S2	---	---	+++	+++	---	---	31.67 (±6.51)
3	S3	---	---	+++	+++	---	---	33.67 (±3.51)
4	MC1	---	---	---	---	---	---	23.00 (±3.61)
5	MC2	---	---	+++	+++	---	---	26.00 (±5.29)
6	OJ4	---	---	+++	+++	---	---	16.33 (±2.52)
7	CC1	---	---	---	---	---	---	13.00 (±1.00)
8	CC2	---	---	---	---	---	---	34.33 (±4.04)
9	BC1	---	---	+++	---	+++	---	23.33 (±2.31)
10	BC2	---	---	+++	---	+++	---	15.00 (±1.73)
11	V1	---	---	---	---	---	---	05.33 (±1.53)
12	HMC3	---	---	---	+++	+++	---	23.33 (±3.06)
13	P1	---	---	---	---	---	---	13.33 (±1.53)
14	MCz	---	---	---	---	---	---	14.33 (±1.53)

Similar to our result, Khushboo et al., (2023) have also reported that none of the probiotic isolates were haemolytic. Others have also reported that there is no haemolysis by probiotic bacteria (Kong et al., 2020). Like our fundings, the results of MR-VP tests and indole production tests have been reported mixed type by other researchers also (Jauharah et al., 2020). The research report of Khushboo et al. (2023) also shows that none of the probiotic strains utilize citrate. The maximum curdling time was reported 16 hrs at 45 ºC by Adikari et al. (2021). Ali et al. (2021) reported that the minimum

curdling time was 6 hrs. Negi et al. (2018) reported that the curling time was about 24 hrs.

Stainless steel adhesion test

Stainless steel exhibits a particular behaviour that is typified by strong adherence in data related to physicochemical conditions (pH, ionic strength). In vitro, all of the new strains were able to adhere to the stainless-steel plates at varying rates. High Adhesion Percentage: Indicates more bacteria adhered compared to the total number available in the positive control. Low Adhesion Percentage: Indicates fewer bacteria adhered relative to the total number in the positive control. Strain MC2, OJ4, CC2 and HMC3 exhibited stainless steel adhesion about 20-40% while other strains S1, S2, S3, MC1, BC2, and BC2 shows about 60-65%. Strains P1, V1, MCz and CC1 has exhibited more than 70%. The results are presented in Table 2.

Mishra and Prasad (2005) reported the adhesion ranged between 52.8 to 66%. While, other researchers have reported that bacterial adhesion ranged between 33.17 and 36.30 % (Mulaw et al., 2019).

Table2: Bacterial adhesion to stainless steel plate

S.No.	Isolate ID	Bacterial growth		Percentage of cells Adhered to stainless steel
		Number of c.f.u. in positive control **(±SD)**	Number of c.f.u. adhered on steel plate **(±SD)**	
1	S1	153.33 (±15.28)	98.00 (±2.00)	63.91
2	S2	151.00(±9.54)	99.00(±3.00)	65.56
3	S3	152.33(±4.93)	94.67(±1.53)	62.15
4	MC1	136.67(±7.57)	91.00(±1.53)	66.58
5	MC2	40.00(±10.00)	16.33(±1.53)	40.83
6	OJ4	99.33(±2.52)	19.67(±6.51)	19.80
7	CC1	150.00(±5.00)	105.00(±12.12)	70.00
8	CC2	50.00(±4.58)	13.00(±2.00)	26.00
9	BC1	40.00(±4.00)	24.00(±3.79)	60.00
10	BC2	52.33(±5.51)	25.67(±2.52)	49.05
11	V1	102.00(±4.00)	72.00(±5.00)	70.59
12	HMC3	99.33(±5.86)	40.67(±2.65)	40.94
13	P1	151.33(±5.59)	109.33(±11.68)	72.25
14	MCz	130.00(±7.94)	99.33(±2.08)	76.41

Acid and bile tolerance test

The data presented in the Table 3, reflect that the acid and bile tolerance of various probiotic isolates. The P1 isolate stands out with the highest acid tolerance at 87%, demonstrating a strong ability to survive harsh gastric conditions. This resilience is crucial for effective probiotic action, as it ensures that the strain can reach the intestines in a viable state. Furthermore, CC1 and MCz also show impressive acid tolerance rates at 84% and 86%, respectively.

Table 3: Acid and bile tolerance

Sr. No.	Isolate code	Mean Acid Tolerance (±SD)	Mean Bile Tolerance (±SD)
1	BC1	82 (±2.65)	47(±2.65)
2	BC2	80(±4.00)	46(±2.00)
3	MC1	78(±11.79)	81(±4.36)
4	MC2	74(±4.58)	60(±5.00)
5	CC1	84(±7.81)	87(±4.00)
6	CC2	80(±7.81)	60(±4.00)
7	HMC3	76(±5.29)	57(±2.65)
8	V1	83(±11.79)	88(±1.73)
9	P1	87(±3.00)	90(±5.00)
10	S3	77(±9.54)	63(±3.00)
11	S4	81(±6.56)	50(±8.66)
12	S5	80(±9.17)	49(±7.81)
13	OJ4	76(±6.56)	58(±2.65)
14	MCz	86(±4.00)	88(±6.00)

For the bile salt tolerance, P1 leads with a 90% survival rate, indicating that it can thrive in the presence of bile salts. This is equally important for probiotic efficacy, as bile tolerance contributes to a strain's ability to survive and function in the intestinal tract. Other notable strains include MCz (88% survival) and V1 (88% survival), both indicating good bile tolerance.

Considering both acid and bile tolerance, the most potent probiotic organism from our finding appears to be P1. With an acid tolerance of 87% and the highest bile

tolerance of 90%, P1 demonstrates a strong ability to endure the gastrointestinal environment, making it a highly suitable candidate for probiotic applications. Other commendable strains such as MCz and CC1 also show promising tolerance profiles, but V1 emerges as the overall most resilient isolate based on these criteria.
The statistical analysis of the acid and bile tolerance data was performed using analysis of variance ANOVA. The result has been presented in Tabe 4.

Table 4: Statistical comparison of acid and bile tolerance of bacterial isolates from dairy and nondairy products using ANOVA

Property	**Dairy Mean**	**Non-Dairy Mean**	**p-value**
Acid Tolerance (%)	80.00	80.66	0.765
Bile Tolerance (%)	65.75	66.33	0.953

The p values for acid and bile tolerance have been calculated as 0.765 and 0.953, respectively. P values calculated are less than typical value 0.05. Therefore, we can conclude that there is no significant difference in acid and bile tolerance of the isolates from dairy and non-dairy products.
Kim et al. (2018) have been reported that acid tolerance survivability percentage ranged from 74.73 to 98.75 while, bile salt survivability percentage was observed between 30.99 and 97.94. Similar reports were presented by other researchers also (Torshizi et al., 2008; Padmavathi et al., 2018; Sieladie et al., 2011).

Selection of probiotic isolates for molecular characterization based on acid bile tolerance and bacterial adhesion to stainless steel tests

On the basis of the results from acid & bile tolerance tests and stainless-steel adhesion test, four isolates were selected for molecular identification. Two of which were dairy isolates, CC1 and MCZ, which demonstrated robust survival rates under acidic conditions and high bile tolerance, indicating their potential as effective probiotics. Additionally, two non-dairy isolates, V1 and P1, also exhibited promising probiotic characteristics.

Molecular identification of isolates

The DNA isolation and gel electrophoresis procedures yielded significant insights into the identity of the bacterial isolates, V1, P1, MCz, and CC1. The 16S rRNA sequencing analysis identified V1 as *Bacillus tequilensis*, showing a high similarity of **99.63%** to the top-hit strain KCTC 13622. In contrast, P1 was identified as *Bacillus velezensis*, exhibiting an impressive similarity of **99.92%** to strain CR-502. Both MCZ and CC1 were identified as *Bacillus tequilensis*, where MCZ shared **99.92%** similarity and CC1 **99.85%** similarity to KCTC 13622. The phylogenetic tree prepared using MEGA 11 is shown in Figure 2.
The sequence data was submitted on genbank at National Centre for Biotechnology Information (NCBI). The accession numbers of the submitted sequences are given in Table 5.

Table 5: NCBI accession number of the sequences submitted.

S. No.	Sample	Identified bacterial species	Accession Number provided by NCBI
1	CC1	*Bacillus tequilensis* KCT 13622	PQ325255
2	MCz	*Bacillus tequilensis* KCT 13622	PQ325256
3	P1	*Bacillus velezensis* CR-502	PQ325257
4	V1	*Bacillus tequilensis* KCT 13622	PQ325258

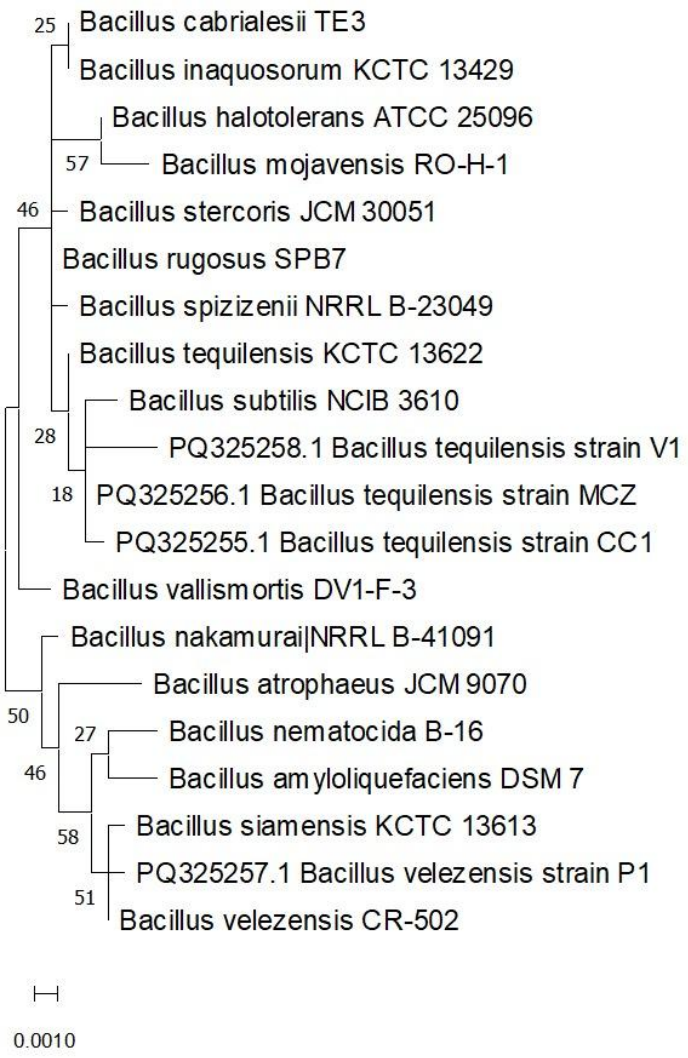

Figure 2: The phylogenetic Tree of Probiotic Isolates

The probiotic bacteria *Bacillus tequilensis* has been reportedly isolated form various sources, viz., Tunisian fermented goat milk, the gut of Masheer fish (*Tor Puititora*), Korean artisanal fermented food products (kimchi and gochujang) (Abid et al., 2019; Choi et al., 2021; Bibi et al., 2024). Similarly *Bacillus velezensis* has also been isolated from various fermented dairy and non-dairy products, viz., Tibetan sheep, fermented rice beer of Assam, fermented sauce, kimchi, cheese, and vinegar (Borah et al., 2019; Sultana et al., 2021; Cheng et al., 2024).

Conclusion:

The present research has been carried out for the characterization and identification of bacterial isolates from dairy and non-dairy products for their potential application as probiotic strains. The findings suggest the isolated bacteria have good probiotic characteristics as discussed above. The statistical analysis, ANOVA revealed there is no significant difference between dairy and non-dairy probiotic isolates. Through molecular identification the bacterial isolates were identified as *Bacillus tequilensis* and *Bacillus velezensis*. These bacteria have already been identified as good probiotic species. *Bacillus tequilensis* KCT 13622 has been consistently reported in CC1 (Homemade Cow Curds), MCz (Market Cheese) and V1 (Isolated from Vinegar), indicating its prevalent role in the fermentation processes of these dairy products and vinegar. This species is known for its ability to enhance flavor, contribute to food preservation, and offer potential health benefits due to its probiotic properties. Selecting effective probiotic sources, such as market cheese, vinegar, and mango pickles, can be particularly beneficial for enhancing food safety and promoting health.

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Manuscript was prepared by Triyugi Narain Kushwaha and finalized by Shailendra Kumar.

Competing Interests The authors declare no competing interests.

Conflict of Interest None

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