

Characterizing Dominant Lactic Acid Bacterial Species in Traditionally Fermented Camel and Sheep Milk: Evaluating their Influence on Calcium Salt Bioavailability using the CaCO-2 Cell Line

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Background: Ninety-nine percent of calcium is found in the teeth and bones of the body, while 1% is found in the serum. Research has shown that bioavailability, rather than the amount of calcium ingested, is a major concern. Hence, the present study was initiated to investigate the effect of Lactobacillus on calcium bioavailability.

Methods: The 16S rRNA sequencing technique was used for the molecular identification of the dominant LAB isolates. The CaCO₂ cell line was grown on 24-well plates. Quantification of calcium transportation was carried out using ICPMS Agilent 7800.

Results: Molecular identification reported that the Fermented camel milk was dominated by Lactobacillus casei, while fermented sheep milk was dominated by Lactobacillus fermentum. The CaCO₂ cell line transportation of calcium carbonate from the apical to basolateral compartment was approximately 2.14%, whereas, in combination with vitamin D₃, the transportation of calcium carbonate was about 3.1%. When combined with Lactobacillus casei, it was 14.16%, and the combination of calcium carbonate, vitamin D₃, and Lactobacillus casei showed a 15.64% calcium influx. On the other hand, the calcium carbonate influx with Lactobacillus fermentum was about 11.7%, and along with Lactobacillus fermentum and vitamin D₃, the calcium influx was 12.48%. When studying the effects of Lactobacillus casei and Lactobacillus fermentum in combination with vitamin D₃, we found a calcium transport of about 29.58%.

Conclusion: Based on the result as reported the calcium carbonate has a very low bioavailability, The present study revolves around the fact that vitamin D₃ influency over the calcium bioavailability is 1%.

Keywords: Calcium, fermentation, camel milk, sheep milk, CaCO₂ cell line.

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1. INTRODUCTION

A process in maintaining Ca²⁺ homeostasis is intestinal calcium (Ca²⁺) absorption through the transcellular, metabolically driven transport of a passive, non-saturable conduit known as the paracellular pathway. These are the ways in which it occurs [1]. Due to their tremendous importance in the prevention of osteoporosis and other problems connected to Ca²⁺ metabolism, hormones, nutrients, and other variables that regulate these systems have been investigated for many years. The major influence of the hormonal metabolite of vitamin D, 1,25(OH)₂D₃, is on the transcellular pathway. This is achieved by its interaction with the classical nuclear vitamin D receptor (VDR), leading to the

activation of transcriptional genes. [2]. Some VDR polymorphisms, like the FOK I polymorphic site, appear to have an undetermined influence on intestinal Ca²⁺ absorption [3]. Ninety percent of total Ca²⁺ absorption occurs in the small intestine under healthy conditions, where Ca²⁺ ions are mostly absorbed [4].

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Marcus and Lengemann observed that 88% of Ca²⁺ absorption took place in the ileum, 4% in the jejunum, and 8% in the duodenum in a rat's small intestine [5]. Cramer reported that calcium absorption in the ileum, jejunum, and duodenum

of dogs was 80%, 16%, and 4%, respectively [6]. The ileum is more favorable for Ca^{2+} absorption than the other intestinal segments because Ca^{2+} spends more time there. Physiological Ca^{2+} requirements are also influential variables in the variability of intestinal Ca^{2+} absorption. When intake is low and the requirements become higher, the calcium absorption efficiency increases [7]. While intestinal Ca^{2+} absorption is boosted during growth, pregnancy, and lactation, it declines over time as individual's age. According to research, calcium is primarily involved in the development of teeth and bones, hormone secretion, muscle contraction, vasodilation, intercellular communication, and nerve transmission. Even slight changes in serum calcium levels can impact these bodily processes. The majority of bone development has been completed by the time a person is 25 to 30 years old. After the age of 30 years, bone density is sustained, and a loss occurs over time. Research suggests that bone density starts to decline around the age of 50. Therefore, maintaining bone mineral density and minimizing bone loss is essential to lower the risk of fractures and osteoporosis [8]. Previous research has consistently shown that numerous lactic acid bacteria possess health-enhancing abilities, particularly in their ability to combat various pathogens and diseases. These beneficial bacteria and microbes are commonly referred to as probiotics, as they contribute to promoting human health. The term "probiotics" was first coined by Nobel laureate Metchnikoff in 1907, and later, Lilly and Stillwell (1965) described probiotics as substances produced by one microorganism that stimulate the growth of another. For millennia, microbial cultures have been employed in the process of food fermentation. In the last century, numerous microbial cultures underwent evaluation for their potential in preventing and treating diseases, leading to the establishment of the term "probiotics." Presently, probiotics are widely available in diverse food products and nutritional supplements. The consumption of fermented milk, recognized as the earliest form of probiotic, has been known since ancient Vedic times. The publication of "The Prolongation of Life" by Elli Metchnikoff in 1908 [9] significantly boosted the interest and research in probiotics. Subsequently, other scientists have also offered their definitions of "probiotics" based on their expertise. In 1974, Parker defined probiotics as organisms and substances that contribute to the balance of intestinal microbial communities, while in 1989, Fuller characterized probiotics as live microbial feed supplements that beneficially influence the intestinal microbial equilibrium [10]. As per the World Health Organization (WHO), probiotics are live microorganisms that, when ingested in sufficient quantities, bestow health benefits upon the host. Scientific experts suggest that a minimum of 10^6 - 10^7 cfu/gm of probiotics in the product at the time of consumption can exert health-promoting effects. Research has demonstrated that consuming probiotics along with dairy food can help in buffering stomach acidity and enhancing bacterial survival in the intestine. Furthermore, dairy products containing probiotics offer essential nutrients like calcium [11]. Given the crucial significance of calcium, the bioavailability of ingested

calcium is a matter of great concern. Several studies have established the role of probiotics in enhancing calcium absorption. Consequently, the current investigation aims to identify a specific strain of lactic acid bacteria (LAB) that can effectively improve calcium bioavailability.

2. MATERIALS AND METHODS

The acquisition process involved obtaining specific items from different suppliers. From HiMedia Laboratories, the following items were procured: MRS HiVeg Agar (catalog number MV641), HiMedia Grams Stain-Kit (catalog number K001L), and Hugh Leifson Medium (catalog number M826). Additionally, the Montage PCR Clean up kit (provided by Millipore), ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase, DMEM (Dulbecco's Modified Eagle Medium) with 20% FBS (Fetal Bovine Serum), HEPES buffering agent, Trypsin, and Hanks balanced salt solution (HBSS) were sourced from Triyat Genomics, Nagpur, Maharashtra. Furthermore, as part of the process, gift samples of liquid paraffin and Vitamin D3 were received from Titan Bioscience Pvt Ltd.

2.1 MILK SAMPLES COLLECTION

Milk samples from camel and sheep were collected from various local regions within the Anand district of Gujarat, India. After collection, the samples were immediately stored in a deep freezer at a temperature of -20°C to preserve their integrity and ensure their suitability for subsequent analyses and investigations.

2.2 TRADITIONAL METHOD FOR FERMENTATION [12]

According to the FAO, Asian and African people traditionally fermented milk without the addition of any starter culture. In ancient Nepal, Bhutan, and India, they fermented milk by pouring the collected milk into an earthenware pot, covering it with lids, and placing it in a dark area. Fresh milk was added every day until the earthenware pot was filled, allowing fermentation to take place. In our investigation, we endeavored to replicate the conventional milk fermentation process with certain adjustments. Specifically, we positioned an earthenware container in a controlled environment at a temperature of 37°C for a duration of 24 hours. During this time, we carefully monitored its observable properties. Regrettably, when we conducted molecular identification through 16S rRNA sequencing, we discovered the proliferation of undesired impurities instead of the intended lactic acid bacteria (LAB) strains. After encountering difficulties in isolating the desired LAB strains using the earthen pots, we decided to alter our methodology. Instead, we collected milk samples in laboratory-grade bottles and allowed them to ferment in a light-protected environment at room temperature for a period of 8 hours. Subsequently, we analyzed the traits of the isolated samples and performed genotypic characterization through 16S rRNA sequencing to determine the prevailing isolates.

2.3 ISOLATION AND PHENOTYPIC CHARACTERIZATION

The medium selected for culturing lactic acid bacteria was lactobacillus MRS HIVEG agar (Himedia laboratories, MV641) medium. Serial dilutions of curd samples were prepared, and a loop full of the diluted samples was streaked onto sterile MRS agar petri plates. Quadrant streaking was performed under aseptic conditions. The petri plates were then incubated at 37°C for 24 to 48 hours. After incubation, colonies were restreaked on MRS agar petri plates to obtain well-isolated colonies. These isolated colonies were further restreaked on MRS agar slants, and sterile liquid paraffin was poured over them. The slants were then stored at 4°C for future use.

2.4 PHENOTYPIC CHARACTERIZATION [13-15]

All the isolates underwent phenotypic characterization, which involved an examination of their morphological and biochemical traits. The process encompassed the careful observation and analysis of the isolates' external features and behavior, alongside conducting diverse biochemical tests to assess their metabolic properties. These tests encompassed evaluating growth patterns, colony morphology, gram staining, catalase activity, carbohydrate fermentation, acid production, and other pertinent biochemical reactions. By scrutinizing these characteristics, we obtained valuable information regarding the phenotypic traits and behavior of the isolates, which facilitated their subsequent classification and identification.

2.4.1 MORPHOLOGICAL EXAMINATION OF CULTURE

The morphological assessments were carried out using Gram's staining, following the method established by Hans Christian Gram in 1884. To accomplish this, we employed the HiMedia Grams Stain-Kit (K001L). Gram staining is a differential staining technique used to distinguish bacteria into two main groups: Gram-positive and Gram-negative. The staining process involves applying a sequence of dyes, including crystal violet, iodine, ethanol or acetone, and a counterstain like safranin. By subjecting the bacterial cells to this staining process, we could observe them under a microscope and determine their cell wall characteristics. Gram-positive bacteria retain the crystal violet stain and exhibit a purple color, while Gram-negative bacteria do not retain the stain and display a pink or red hue. Gram's staining technique is a fundamental tool in microbiology for the initial identification and classification of bacteria based on their cell wall properties.

2.4.2 IDENTIFICATION OF THE PURE CULTURE

The identification process of the isolates involved conducting biochemical tests, including Hugh and Leifson's test, Endospore test, Catalase test, Motility test, and Sugar fermentation test.

2.4.3 ENDOSPORE TEST

Following strict aseptic protocols, a bacterial smear was prepared on a microscopic slide and subsequently heat-fixed. The primary stain, malachite green, was applied to the slide while positioned above a steaming water bath for a duration of five minutes. Afterward, the slide was removed from the water bath and carefully rinsed with water. Subsequently, the glass slide was immersed in the counter-

stain Safranin for 20 seconds, followed by a thorough washing. Once blot dried, these slides were observed under a light microscope for examination.

2.4.4 HUGH AND LEIFSON'S TEST

The primary objective of the Hugh and Leifson's test was to determine whether the isolates exhibited oxidative or fermentative characteristics based on their acid production under both aerobic and anaerobic conditions. To perform this test, we utilized Hugh and Leifson's Medium M826 (HiMedia). Before commencing the test, all glassware was autoclaved at 121°C for 15 minutes to prevent and eliminate contamination. To create the medium, a filtered and sterilized solution of 10% glucose was added to achieve a 1% concentration. Once prepared, the medium was allowed to cool and then inoculated by stabbing it with the test isolates. Subsequently, all the inoculated test tubes were placed in an incubator set at 37°C for 24 to 48 hours, maintaining both aerobic and anaerobic conditions. After the incubation period, all the test tubes were carefully observed and analyzed.

2.4.5 MOTILITY TEST

The motility of the bacteria was assessed through a motility test using Hugh and Leifson's medium, employing the stab inoculation technique to determine whether the bacteria exhibited motility or were non-motile.

2.4.6 CATALASE TEST

A catalase test was conducted to verify the presence of the enzyme catalase in the test isolates. This test involved placing a drop of 3% H₂O₂ on a glass slide, followed by mixing a loopful of isolates with a 3% H₂O₂ solution on the same slide. The slide was then observed for the production of bubbles.

2.4.7 SUGAR FERMENTATION TEST

Approximately 100 ml of the nutritional broth solution and one milliliter of phenol red were added to a conical flask. The medium was then subjected to autoclaving at 121°C for 15 minutes, followed by cooling to room temperature. In aseptic conditions, a syringe-filter sterilized solution of 1% glucose was prepared. Subsequently, 100 µl of the glucose solution and 5 ml of the broth were placed in each sterilized test tube. After 24 hours at room temperature, the test tubes were inspected for any signs of contamination. Following this, all the test tubes were inoculated with freshly produced bacterial culture and incubated at 37°C for 48 hours. If the bacteria were homofermentative, the medium would change from red to yellow due to acid production. On the other hand, if the bacteria were heterofermentative, the medium would turn from red to yellow, accompanied by gas production in a Durham tube.

2.5 MOLECULAR IDENTIFICATION OF ISOLATES (16 S RRNA SEQUENCING) [16-21]

Cell lysis and homogenization were carried out by suspending 1-3 colonies aseptically and combining them with 450 µl of "B Cube" lysis buffer in a 2 ml micro centrifuge tube. The cells were lysed by repeated pipetting, followed by the addition of 4 µl of RNase A and 250 µl of "B Cube" neutralization buffer. The contents were vortexed and incubated in a water bath at 65°C for 30 minutes to prevent excessive DNA shearing. To extract the DNA, the lysate was

centrifuged at 14,000 rpm for 20 minutes at 10°C, and the viscous supernatant was transferred to a fresh 2 ml micro centrifuge tube without disturbing the pellet. To further purify the DNA, 600 µl of "B Cube" binding buffer was added to the content, mixed thoroughly, and incubated at room temperature for 5 minutes. The mixture was then transferred to a spin column placed in a 2 ml collection tube and centrifuged at 14,000 rpm for 2 minutes to separate the DNA from the remaining impurities. The flow-through was discarded, and the washing process was repeated twice using "B Cube" washing buffers I and II. The final elution of DNA was achieved by adding 100 µl of "B Cube" Elution buffer to the middle of the spin column and incubating the tubes at room temperature for 5 minutes. After centrifugation at 6000 rpm for 1 minute, the eluted DNA was collected, and its concentration was determined by running aliquots on a 1% agarose gel. The DNA samples were stored at -20°C until further use. For sequencing, single-pass sequencing was performed on each DNA template using specific 16S rRNA universal primers. The resulting fluorescent-labeled fragments were purified from the unincorporated terminators through an ethanol precipitation protocol. Subsequently, the samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

2.5.1 PHYLOGENETIC ANALYSIS

The DNA databases available on NCBI were utilized for performing a basic phylogenetic local alignment search tool (blast) search to identify sequence similarities. Subsequently, from each blast result, five distinct strains or species were selected for further phylogenetic analysis. The analysis was carried out using molecular evolutionary genetics analysis software (mega version 6).

2.6 INFLUENCE OF ISOLATED LACTOBACILLUS BACTERIA FROM THE FERMENTED MILK OF CAMEL AND SHEEP ON CALCIUM TRANSPORT IN CACO2 CELL LINE [22-26]

The Caco-2 cell model has been extensively utilized and well-defined as a cell-based model for investigating the absorption, permeability, and efflux transport properties of potential drug candidates in the drug development process. These human colon adenocarcinoma Caco-2 cells have the ability to spontaneously differentiate into polarized cells with tightly connected monolayers, resembling the human intestinal epithelium in vitro. Moreover, research has demonstrated a strong correlation between the permeability of drugs through Caco-2 cell monolayers and their oral absorption in humans.

Permeability studies are performed by culturing Caco-2 cells for 21 days, which allows them to form a functional monolayer. Test compounds as listed in the table no.- 1 are added to the apical chamber along with an appropriate buffer volume. After a 60-minute incubation period, samples are collected from the basolateral chambers for further analysis.

2.6.1 ASSAY PROCEDURE

In this study, cells were collected when they reached approximately 80% coverage. After checking their viability,

the cells were centrifuged and then resuspended in 1 ml of DMEM medium. Subsequently, 60,000 cells were seeded into each well of a 24-well Millipore plate and cultured for 21 days, with media changed every other day. Before conducting experiments, each cell monolayer was washed twice with HBSS buffer and incubated for 30 minutes in a CO2 incubator with 500µl of HBSS buffer in each well. Then, the apical chamber plate was transferred to a 24-well transporter analysis plate, with 2.4mL of HBSS buffer in each basolateral well. After incubating the cells with HBSS buffer for 30 minutes, individual isolated Lactobacillus strains were added to the respective wells at a concentration of 1-2 x 10⁷ CFU/ml, along with 50µg/ml of Gentamycin. After a further 30 minutes of incubation with the bacteria, the cells were incubated for 6 hours. To study the permeability, 0.5ml of a test concentration (500µg/ml) of calcium samples was added to the apical chamber. The plate was secured on a shaker and incubated in an incubator at 37°C. After 60 minutes of treatment, samples were collected from the basolateral chambers. Quantification of calcium transportation was carried out using ICPMS Agilent 7800.

2.6.2 TEER VALUE MEASUREMENT USING MILLICELL SYSTEM

Set the mode switch to the "R" position and activate the POWER. Press the TEST button. If the Range switch is set to 2000Ω, the meter should display a value of 1000Ω+1Ω. If the Range switch is set to 20K, the meter should display 1KΩ. Connect the telephone-type plug into the electrode port on the meter. Submerge the electrode tip in a solution of 1X PBS or HBSS buffer. Now, switch the mode to "V" and turn on the power. The meter may show a reading of 0.1 or 0.2. Adjust the voltage potentiometer using a screwdriver until the meter reads 0.0. Switch the mode back to "R" and adjust the Ω potentiometer with a screwdriver until the reading reaches 0.0. Clean the electrode using an alcohol solution. Measure the resistance of two blanks (samples without cells). Finally, measure the cell resistance in the well where cells are present. $R_{cell} \text{ monolayer} = R_{sample} - R_{blank}$ Corrected Cell Monolayer = $R_{cell} \text{ monolayer} \times \text{area of the well}$ Note: Area of 24 well - 0.785cm².

2.6.3 SAMPLE

Table 1. Test Sample Details

S. No.	SAMPLES CODE	SAMPLE COMBINATION FOR CACO2 CELL LINE STUDY
1.	CC	Calcium carbonate
2.	CC Vit D3	Calcium carbonate + Vit. D3
3.	CC_TL1	Calcium carbonate + isolated TL1 strain

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4.	CC_TL1_Vit D3	Calcium carbonate + isolated TL1 strain + Vit. D3
5.	CC_TL2	Calcium carbonate + isolated TL2 strain
6.	CC_TL2_Vit D3	Calcium carbonate + isolated TL2 strain + Vit. D3
7.	CC_TL1_TL2_Vit D3	Calcium carbonate + isolated TL1 strain+ isolated TL2 strain + Vit. D3

*CC- Calcium carbonate, Vit. D3- vitamin D3, TL1-*Lactobacillus casei*, TL2- *Lactobacillus fermentum*

2.6.4 STANDARD SOLUTION PREPARATION

A volume of 50 µL of the sample was taken from the basolateral chamber of the well plate. This withdrawn sample was then subjected to digestion by adding 1 mL of Nitric acid (65%) in open vessels. The digestion process was carried out at room temperature for a duration of 20 minutes. As part of each digestion run, a blank sample was also included.

2.6.5 MEASUREMENT PARAMETERS

The measurements were performed using the Agilent Technologies 7800x ICP-MS Waldbronn, Germany and the measurement parameters set as listed in the table no.-2. The instrument was configured with specific parameters: 1550-W radiofrequency power, a spray chamber temperature of 13 °C (55.4 °F), a 10-mm sample depth, a carrier gas flow rate of 0.6 L/min, a dilution gas flow rate of 0.40 L/min, and a nebulizer pump speed of 0.10 reps. Argon gas was used as both the plasma-carrier gas and dilution gas. To mitigate polyatomic interferences, a helium flow rate of 0 mL/min was employed in the No Gas tuning mode, and 4.3 mL/min was used in the He tuning mode within the collision cell. Sample introduction was accomplished using the ASX-500 auto sampler from Cetac (Omaha, NB). For washing, a solution comprising 5% (v/v) nitric acid and 1% (v/v) hydrochloric acid was used, while an 8% (v/v) nitric acid solution was used for other purposes. The rinse duration for Solution 1 was set at 55 seconds with a nebulizer pump speed of 0.3 reps, and for Solution 2, the rinse time was set at 45 seconds with a nebulizer pump speed of 0.4 reps.

Table 2. Agilent 7800 Icp-Ms Measurement Parameters

S. No.	Element	Mode
1.	Ca	He
2.	Parameter	He mode
3.	Forward Power (W)	2000 W
4.	Nebulizer speed (rps)	0.1 rps

5.	Carrier gas (L/min)	0.6 L/min
6.	Kinetic Energy Discrimination (V)	4 V
7.	Cell Gas flow (mL/min, He)	4 mL/min, He

* Ca- Calcium, He - Helium

4. RESULTS

4.1 Phenotypic Characterization

As listed bellowed table no.- 3, The isolates were identified as Gram-positive, lacking endospores, not producing catalase enzyme, displaying no motility, and exhibiting heterofermentative characteristics with acid production.

Table 3. Phenotypic Characterization

S. No.	TEST	TL1	TL2
1.	Types of colony	Small	Small
2.	Gram staining	+Ve	+Ve
3.	Microscopic morphology	Roads shape	Roads shape
4.	Endospore test	-Ve	-Ve
5.	Hugh and leifson's test	F	F
6.	Motility test	-Ve	-Ve
7.	Catalase test	-Ve	-Ve
8.	Sugar Fermentation test	H	H

*TL1-*Lactobacillus Casei*, TL2- *Lactobacillus fermentum*, +ve- positive, -ve-negative, F- Fermentation, H-heterofermentative with acid production.

4.2 16s rRNA Gene Sequencing of Dominant Isolate of Fermented Camel Milk

For the CONTIG-TL1: sequencing done with reference listed in the table no.-4. AGCGGCGGACGGGTGAGTAACACGTGGGTAACCTG CCCATAAGACTGGGATAACTCCGGGAACCGGGGC TAATACCGGATAACATTTTGAACCGCATGGTTCGAA ATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGA CCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCT CACCAAGGCAACGATGCGTAGCCGACCTGAGAGGG TGATCGGCCACACTGGGACTGAGACACGGCCCAGA CTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA ATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAG

TGATGAAGGCTTTCGGGTCGTAAACTCTGTTGTTA
GGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACC
TTGACGGTACCTAACCAGAAAGCCACGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG
CGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCA
GGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCT
CAACCGTGGAGGGTTCATTGGAAACTGGGAGACTTG
AGTGCAGAAGAGGAAAGTGGAAATTCATGTGTAGC
GGTGAATGCGTAGAGATATGGAGGAACACCAGTG
GCGAAGGCGACTTCTGGTCTGTAACGACTGAG
GCGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAG
TGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAA
CGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCA
AGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCA
CAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC
GCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAA
ACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAG
TGACAGGTGGTGCATGGTTGTCTGTCAGCTCGTGTCTG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTTGATCTTAGTTGCCATCATTAAAGTTGGGCACTC
TAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTG
GGGATGACGTCAAATCATCATGCCCTTATGACCTG
GGCTACACACGTGCTACAATGGACGGTACAAAGAG
CTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAA
CCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCT
ACATGAAGCTGGAATCGCTAGTAATCGCGGATCAG
CATGCCGCGGTGAATACGTTCCCGGGCCTTGTACAC
ACCGCCCGTACACCACGAGAGTTTGTAACACCCG
AAGTCGGTGGGGTAACCTTTTGGAGCCAGCCGCCT
AAGG

Table 4. NCBI-BLAST- TL1-*Lactobacillus Casei*

S. N o.	Description	Max score	Total score	Query cover	E value	Ident	Accession
1.	<i>Lactobacillus casei</i> strain 090 16S ribosomal RNA gene, partial sequence	1971	1971	100%	0.0	99%	JN560917.1
2.	<i>Lactobacillus casei</i> strain A5 16S ribosomal RNA gene, partial	1965	1965	100%	0.0	99%	KU510332.1

	sequence						
3.	<i>Lactobacillus casei</i> strain 095 16S ribosomal RNA gene, partial sequence	1958	1958	100%	0.0	99%	JN560879.1
4.	<i>Lactobacillus casei</i> strain 02 16S ribosomal RNA gene, partial sequence	1954	1954	100%	0.0	99%	JN560892.1
5.	<i>Lactobacillus casei</i> strain SYB-H 16S ribosomal RNA gene, partial sequence	1940	1940	100%	0.0	99%	KY010410.1

*RNA- Ribonucleic acid.

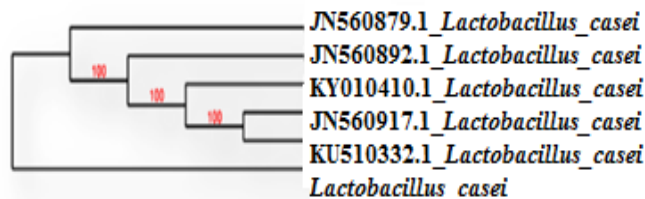


FIG. (1). Phylo Tree (Phylogenetic Analysis)

Table 5. NCBI-BLAST - TL2- *Lactobacillus fermentum*

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S. N o.	Descripti on	Ma x sco re	Tot al sco re	Que ry cov er	E val ue	Ide nt	Accessi on
1.	<i>Lactob acillus ferment um</i> strain L23 16s riboso mal RNA gene partial sequen ce	26 34	26 34	10 0 %	0. 0	9 9 %	KP317 693.1
2.	<i>Lactob acillus ferment um</i> strain L22 16s riboso mal RNA gene partial sequen ce	26 34	26 34	10 0 %	0. 0	9 9 %	KP317 692.1
3.	<i>Lactob acillus ferment um</i> strain L16 16s riboso mal RNA gene partial sequen ce	26 34	26 34	10 0 %	0. 0	9 9 %	KP317 687.1
4.	<i>Lactob acillus ferment um</i> strain L7 16s riboso	26 34	26 34	10 0 %	0. 0	9 9 %	KP317 680.1

	mal RNA gene partial sequen ce						
5.	<i>Lactob acillus ferment um</i> strain 2BF 16s riboso mal RNA gene partial sequen ce	26 34	26 34	10 0 %	0. 0	9 9 %	KY249 642.1

4.3 16s rRNA Gene Sequencing of Dominant Isolate of Fermented Sheep Milk

For the CONTIG-TL2: sequencing done with reference listed in the table no.-5.

ATTGATTGATGGTGCTTGCACCTGATTGATTTTGGT
CGCCAACGAGTGGCGGACGGGTGAGTAACACGTAG
GTAACCTGCCAGAAGCGGGGACAACATTTGGAA
ACAGATGCTAATACCGCATAACAACGTTGTTTCGCAT
GAACAACGCTTAAAAGATGGCTTCTCGCTATCACTT
CTGGATGGACCTGCGGTGCATTAGCTTGTGGTGGG
GTAACGGCCTACCAAGGCGATGATGCATAGCCGAG
TTGAGAGACTGATCGGCCACAATGGGACTGAGACA
CGGCCCATACCTACGGGAGGCAGCAGTAGGGAA
TCTTCACAATGGGCGCAAGCCTGATGGAGCAACA
CCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGC
TCTGTTGTTAAAGAAGAACACGTATGAGAGTAACT
GTTTCATACGTTGACGGTATTTAACCAGAAAGTCACG
GCTAAACTACGTGCCAGCAGCCGCGGTAATACGTA
GGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAA
GAGAGTGCAGGCGGTTTTCTAAGTCTGATGTGAAA
GCCTTCGGCTTAACCGGAGAAGTGCATCGGAAACT
GGATAACTTGAGTGCAGAAGAGGGTAGTGGAACCTC
CATGTGTAGCGGTGGAATGCGTAGATATATGGAAG
AACACCAGTGGCGAAGGCGGCTACCTGGTCTGCAA
CTGACGCTGAGACTCGAAAGCATGGGTAGCGAACA
GGATTAGATACCCTGGTAGTCCATGCCGTAAACGAT
GAGTGCTAGGTGTTGGAGGGTTTCGCCCTTCAGTG
CCGGAGCTAACGCATTAAGCACTCCGCCTGGGGAG
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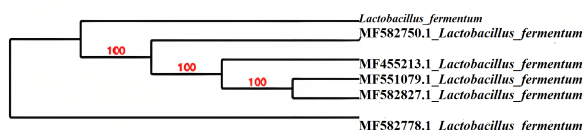


Fig. (2). Phylo Tree (Phylogenetic Analysis)

From the 16S rRNA sequencing we were found that the camel milk has dominancy of *Lactobacillus casei* (CONTIG TL1) and sheep milk has dominancy of *Lactobacillus fermentum* (CONTIG TL2).

4.3 Calcium Permeability

Table 6. Calcium Permeability

S. No.	Test sample	Calcium content at 0 min (Apical chamber) in µg	Calcium content at 120 min (Basal chamber) in µg	% transport
1.	Control	0.0	0.0	0.0
2.	Calcium carbonate	500 µg	10.7 µg	2.14%
3.	Calcium carbonate + Vit. D3(800IU)	500 µg	15.5 µg	3.1%
4.	Calcium carbonate + isolated TL1 strain	500 µg	70.8 µg	14.16%
5.	Calcium carbonate + isolated TL1 strain + Vit D3(800IU)	500 µg	78.2 µg	15.64%
6.	Calcium carbonate + isolated TL2 strain	500 µg	58.5 µg	11.7%
7.	Calcium carbonate + isolated	500 µg	62.4 µg	12.48

	TL2 strain + Vit D3(800IU)			
8.	Calcium carbonate + isolated TL1 strain + isolated TL2 strain + Vit D3(800IU)	500 µg	147.9 µg	29.58%

* CC- Calcium carbonate, Vit. D3- vitamin D3, TL1- *Lactobacillus casei*, TL2- *Lactobacillus fermentum*.

As listed in the table no.-6 the CaCO₂ cell line study results showed that the influence of vitamin D3 over the calcium carbonate influx is about 0.8%, while the well-optimized LAB strains *Lactobacillus casei* had increased in calcium transport of 11.06% and *Lactobacillus fermentum* had increased in calcium transport of 8.6%, while in the combination of both the strains with vitamin D3 had increased in calcium transport of 29.58%, which was the highest amongst all.

5. DISCUSSION

Calcium is a crucial nutrient, with the majority (approximately 99%) being stored in bones and teeth, while only about 1% is present in the serum. The focus on calcium's significance lies not so much in the quantity ingested but rather in its bioavailability. Numerous studies have demonstrated that Lactic Acid Bacteria (LAB) can enhance calcium absorption. Therefore, our current investigation aims to identify a specific LAB strain that promotes calcium bioavailability. To achieve this, we fermented camel and sheep milk using a modified traditional method. From the fermented milk, we isolated and characterized the lactic acid bacteria phenotypically and genotypically, using the 16S rRNA sequencing technique for molecular identification. Next, we cultivated a CaCO₂ cell line on 24 well plates to quantify calcium transportation. The quantification was carried out using ICPMS Agilent 7800. In the fermented camel milk, *Lactobacillus casei* was the dominant strain, while in fermented sheep milk, *Lactobacillus fermentum* dominated. The results of calcium carbonate transportation from the apical to basolateral compartment in the CaCO₂ cell line were as follows: 2.14% without vitamin D3, 3.1% with vitamin D3, 14.16% with *Lactobacillus casei*, and 15.64% with a combination of calcium carbonate, vitamin D3, and *Lactobacillus casei*. For *Lactobacillus fermentum*, the calcium carbonate influx was 11.7%, and with the addition of vitamin D3, it increased to 12.48%. When both *Lactobacillus casei* and *Lactobacillus fermentum* were present, along with vitamin D3, the calcium transport from the apical to basolateral chambers reached 29.58% in the well plates. Based on the findings, it is evident that the combination of calcium carbonate, both strains isolated from fermented camel and sheep milk, along with vitamin D3, demonstrated the

highest calcium transportation from the apical to the basal compartment of the well plate.

CONCLUSION

Among the LAB strains, *Lactobacillus casei* dominated the fermented camel milk, while *Lactobacillus fermentum* was the dominant isolate in fermented sheep milk. The CaCO₂ cell line study indicated that vitamin D3's influence on calcium carbonate influx was approximately 0.8%. However, well-optimized LAB strains, such as *Lactobacillus casei*, exhibited an 11.06% increase in calcium transport, and *Lactobacillus fermentum* showed an 8.6% increase. When both strains were combined with vitamin D3, the calcium transport increased to 29.58%, the highest among all combinations. The study highlights that calcium carbonate has relatively low bioavailability, despite its widespread use in various formulations such as tablets, powders, and syrups, either alone or in combination with vitamin D3. The research emphasizes that vitamin D3 contributes only 1% to calcium bioavailability. The real potential for enhancing calcium transport lies in leveraging the identified *Lactobacillus* strains, *Lactobacillus casei*, and *Lactobacillus fermentum*. In future studies, extending the duration of CaCO₂ cell line investigations will provide a more precise understanding of the maximum achievable calcium bioavailability within a specific timeframe.

LIST OF ABBREVIATIONS

LAB- Lactic acid bacteria, TL1- code for *Lactobacillus casei*, TL2- *Lactobacillus fermentum*, Caco-2 (Cancer coli-2) was established from a human colorectal adenocarcinoma by Jorgen Fogh at the Sloan-Kettering Cancer Research Institute (Fogh et al. 1977), CC- Calcium carbonate, Vit. D3- vitamin D3, RNA- Ribonucleic acid, +ve- positive, -ve-negative, F- Fermentation, H-hetrofermentative with acid production, Ca- Calcium, He - Helium

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CONFLICT OF INTEREST

No conflict of interest

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