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ISOLATION OF PROBIOTIC BACTERIA FROM NON-DAIRY PRODUCTS : A NEW APPROACH IN FUNCTIONAL FOODS FOR LACTOSE INTOLERANCE

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Abstract:
Individuals with lactose intolerance often seek dairy-free alternatives for pro-biotic consumption. This study aimed to isolate pro-biotic bacteria from non-dairy sources suitable for lactose-intolerant and vegan individuals. Non-dairy foods which include idly and dosa batter were screened for potential pro-biotic strains using selective media and molecular identification techniques. Bacterial isolates were evaluated for basic tests againt pro-biotic characteristics such as acid and bile tolerance. A total of 25 samples were isolated from the selected food samples. From which potential pro-biotic strains were isolated and identified, including Lactobacillus plantarum, Lactobacillus fermentum and Bacillus mojavensis known for their beneficial effects on gut health. Further studies are warranted to assess the efficacy of these non-dairy derived pro-biotics in alleviating symptoms of lactose intolerance and improving overall gut microbiota composition in human trials. This research highlights the potential of non-dairy sources as viable alternatives for pro-biotic delivery, catering specifically to lactose-intolerant individuals.

Keywords: Probiotic bacteria, Lactose intolerance, Vegan, Non diary products

Introduction

Probiotic bacteria are beneficial microorganisms that promote a healthy balance in the gut microbiome, aiding digestion and supporting immune function. Probiotics are isolated from many consumable materials but the isolates with high performance, long-term persistence and viability are considered as good probiotics in market[1]. Probiotics need to match the criteria of nutraceuticals. Probiotics have been proven in many beneficial functions like maintaining gut balance, proficient in inhibiting pathogenic microorganisms by colonizing and adherence to epithelial layers, secretion of antimicrobial compounds, immunomodulation[2]. Probiotics are commonly found in fermented foods like yogurt, kefir, and kimchi, as well as in dietary supplements. The main difference between probiotic bacteria in dairy and non-dairy products lies in their strains and sources. Dairy products typically contain strains such as *Lactobacillus acidophilus* and *Bifidobacterium*, which are well-studied for their health benefits[3]. Non-dairy probiotics, found in foods like sauerkraut or tempeh, may include different strains such as *Lactobacillus plantarum* or *Saccharomyces boulardii*, offering diverse health advantages depending on the strain and the food source[4]. Both dairy and non-dairy probiotics contribute to overall gut health, but individual preferences and dietary restrictions often dictate which form is chosen for supplementation or consumption. Since vegans abstain from

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consuming animal products, they may have a different dietary composition that can impact their gut microbiota. Probiotics help maintain a balanced gut environment by promoting the growth of beneficial bacteria and suppressing harmful ones[5]. This can enhance digestion and nutrient absorption from plant-based foods, which often contain high amounts of fiber and complex carbohydrates that can be challenging to digest without adequate microbial support[6].

Materials and methods

In accordance to the objective of the study pro-biotic bacteria are to be isolated from non diary sources. Thus idly and dosa batter were selected as the sources for obtaining these bacteria.

Idly batter preparation for isolation of bacteria

Urad dal (Vigna mungo) or black gram and rice sooji were purchased from local market were taken in the ratio of 1:2 respectively and soaked overnight. It was grinded into fine paste and allowed to ferment for 8 hours at normal room temperature.

Preparation of dosa batter

Urad dal (Vigna mungo) or black gram, fenugreek seeds and rice were purchased from local market. Black gram and rice were taken in the ratio of 1:2 including 1 teaspoon of fenugreek seeds . All the contents were soaked for 5 -8 hours. All the material was grinded into fine paste and allowed to ferment for 5 hours at room temperature. Change in odor, taste and texture is an indication of fermentation[7].

Isolation of pure colonies:

For isolation of pro-biotic bacteria from selected sources 6.0g of nutrient agar powder (Sisco research laboratories) was weighed and suspended into 200 ml water taken in a conical flask. The medium is autoclaved at 121^0C for 15 minutes. 0.1 g of each batter sample was taken and suspended in a test tube.

Serial dilution was performed till 10^{-10} dilution. From each sample 10^{-3}, 10^{-4} and10^{-5}dilutions were used for plating. 100µL from each sample is taken and plated on nutrient agar media. Plates are labeled properly and kept for incubation at 37^0C for 24 hours. Desired colonies are isolated and plated. The same protocol is continued till pure colonies are isolated. Pure colonies are subjected for streaking and incubation to obtain individual colony plates.

Colony characterization and morphology identification:

The bacterial colonies are observed for morphological variation and all the charterers are noted. Grams staining was performed for the initial characterization based on which further identification can be made. Among the 25 samples isolated initially 15 different bacteria were found based on colony morphology and staining[8]. All the 15 samples were then subjected for basic screening for biochemical and pro-biotic tests for bacteria which includes acid tolerance and bile tolerance assay[9]. Among the 15 cultures isolated only 3 were showing positive for pro-biotic nature which were further subjected for molecular identification based on 16srDNA sequencing[10].

Biochemical characterization of Bacteria:

For the complete biochemical annotation of selected bacterial strains they are subjected for basic biochemical tests like Motility, Catalase, Glucose fermentation, IMViC and Urease test in accordance with Bergey's manual[11].

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Basic tests for identification of Pro biotic bacteria:
Bile tolerance assay and acid tolerance test were performed as a pre requisites for the identification of pro-biotc bacteria. All the 15 isolates were subjected fr these 2 basic tests among which only 3 bacterial isolates were positive indicating their efficacy as pro-biotic in nature. All the three were further subjected for molecular identification based on 16sRNA sequencing.

Molecular Identification of Bacteria
The procedure for molecular identification of bacteria using 16S rRNA gene sequencing involves several key steps. Initially, bacterial DNA is extracted from all the 3 selected bacterial pure cultures using standard techniques of phenol chloroform method[12]. The 16S rRNA gene is then amplified using polymerase chain reaction (PCR) with primers specific to conserved regions flanking the variable regions of the gene. Primers used are specified below. The PCR product, which contains the amplified 16S rRNA gene segment, is purified and sequenced using Sanger sequencing . Next, the obtained sequences are compared to NCBI[13] database suing BLASTN[14] approach to identify closely related sequences. Based on the BLAST result the identity of the organism was conformed at the species level.

Results and Discussion
The study included isolation of 25 bacterial strains from 2 different sources (idly batter and dosa batter) after serial dilution and plating technique. All the 25 isolates were subjected for Grams staining and morphological characterization after which only 15 were found to be different and other were repeated cultures. Further, all 15 cultures were subjected for biochemical tests and basic pro biotic tests like acid and bile tolerance which confirmed only 3 isolates to be pro biotic in nature. These 3 were further subjected for DNA extraction for molecular identification. All the results are showcased below.

Physiological characterization
All the 15 strains were inoculated and incubated at different temperatures 30°C, 37°C and 45°C . At 30°C, 37°C all the strains grew well and within 24 hours. Growth was observed at 45°C after 48 hours. After physiological characterization all the isolates were tested for biochemical characterization.

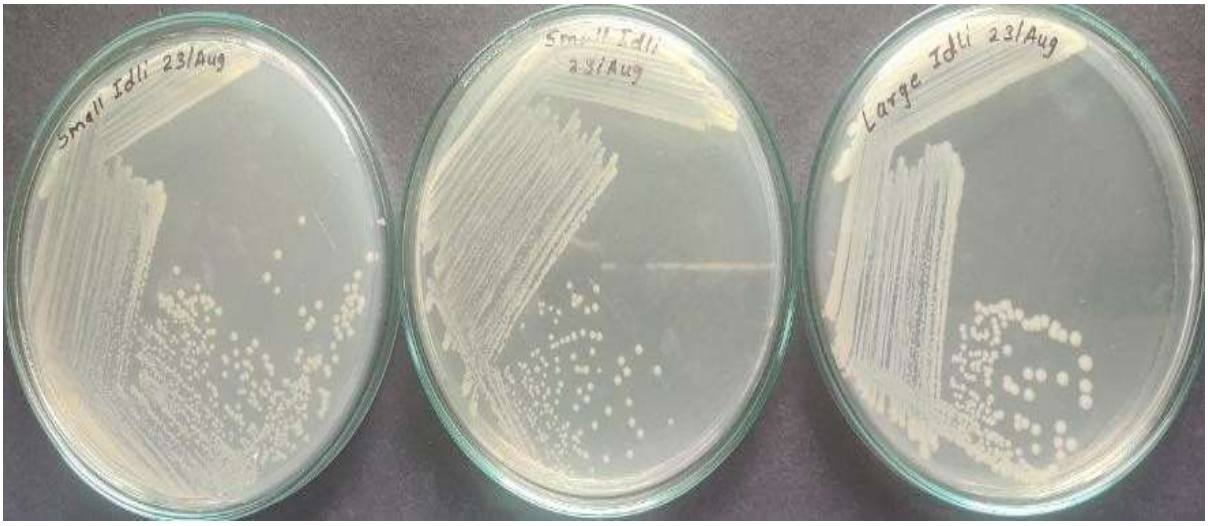

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Fig. 1. Isolated bacterial strains from fermented samples positive for pro biotic nature.

The above figure shows the 3 selected bacterial strains on MRS media

Table: 1 Physiological characteristics of 15 bacterial isolates

Isolates	Gram's staining	Shape	Margin	Size	Color
IS	Positive	Circular	Entire	Small	Creamish white
IL	Positive	Circular	Entire	Large	Creamish white
DF	Negative	Irregular	Undulate	Large	Pale white
DO	Positive	Circular	Entire	Small	Yellowish orange
DF1	Negative	Irregular	Undulate	Large	White
1	Negative	Polyp	Undulate	Large	White
2	Negative	Circular	Dry scaly	small	White
3	Positive	Circular	Entire	Small	White
4	Positive	Irregular	Entire	Small	Orange
5	Positive	Polyp	Undulate	Small	Pale white
6	Negative	Irregular	Dry scaly	Small	Pale white
7	Positive	Circular	Wavery	Large	White
8	Positive	Circular	Plain	Small	Creamish white
9	Negative	Polyp shape	Undulate	Small	Creamish white
TY	Negative	Circular	Plain	Small	Yellow

Table 2: Biochemical characterization of 15 strains isolated

Biochemical Tests	**IS**	**IL**	**DF**	**DO**	**DP**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**TY**
Gram's stain	+	+	+	-	-	-	-	+	-	-	+	-	-	-	+
Starch agar plate	-	-	-	-	-	+	+	+	-	-	-	-	-	-	+
Catalase Test	-	-	+	+	_	+	+	+	+	+	+	+	+	+	+
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+

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Fructose	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+
Indole test	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Methyl-Red test	-	+	+	+	-	-	+	-	-	+	+	+	-	-	+
Voges Proskauer's test	-	-	+	Variable	+	-	-	-	-	-	-	-	-	-	+
Citrate utilization Test	-	-	+	-	-	-	-	+	-	+	+	+	+	+	+
Gas from glucose	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Urea hydrolysis	-	-	W +	-	+	-	-	+	+	-	-	-	-	-	-

Acid and Bile tolerance test for Pro biotic bacterial identification:
Among all the 15 samples tested for acid and bile tolerance only 3 of them were identified to be positive which are shown below.

Figure 2, 3, 4: Graphs showing the degree of acid tolerance by isolates

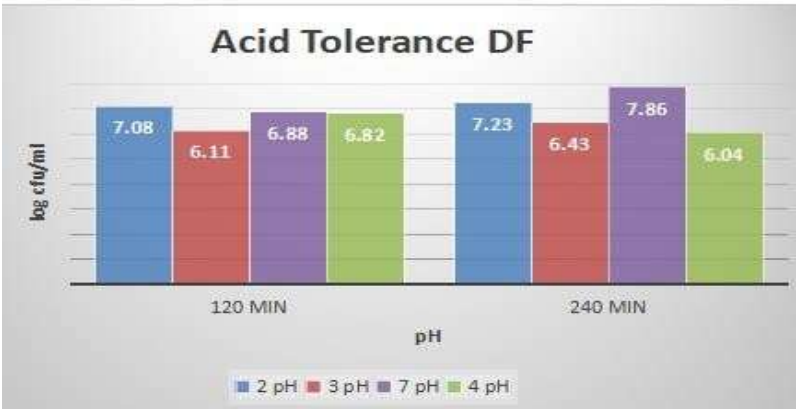

Fig 2: Acid tolerance of Culture 1 (DF)

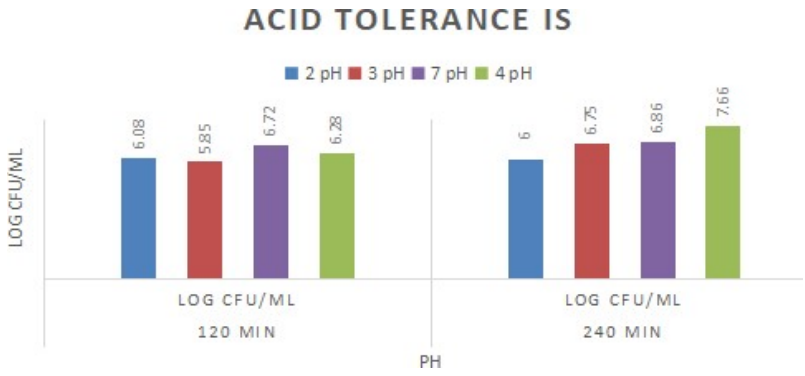

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Fig 3: Acid tolerance of Culture 2 (IS)

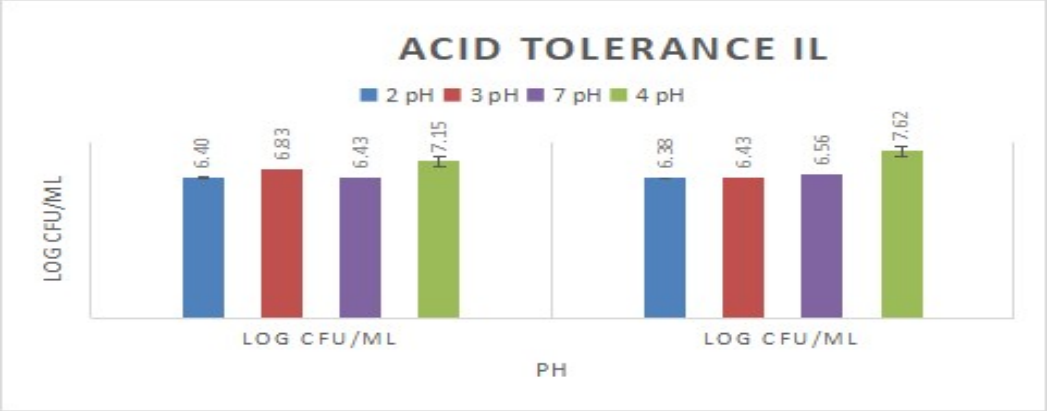

Fig 4: Acid tolerance of Culture 3 (IL)

All the above 3 plots of Fig 2,3 and 4 shows the degree of acid tolerance exhibited by the isolated cultures for a pH range of 2, 3, 4 and 7. Growth was measured by testing the turbidity spectrophotometrically.

Table 3 : Bile Tolerance potential of 3 isolates with time

	OD of Culture 1 (DF)	OD of Culture 2 (IS)	OD of Culture 3 (IL)
Control	0.06	0.062	0.061
0 hr	0.0159	0.155	0.156
1 hr	0.215	0.262	0.214
2 hr	0.274	0.278	0.275
3 hr	0.388	0.396	0.394

The above table shows varied turbidity of the cultures upon exposing them to bile salts at for varied duration. All the 3 were positive for bile tolerance showing high tolerance rate.

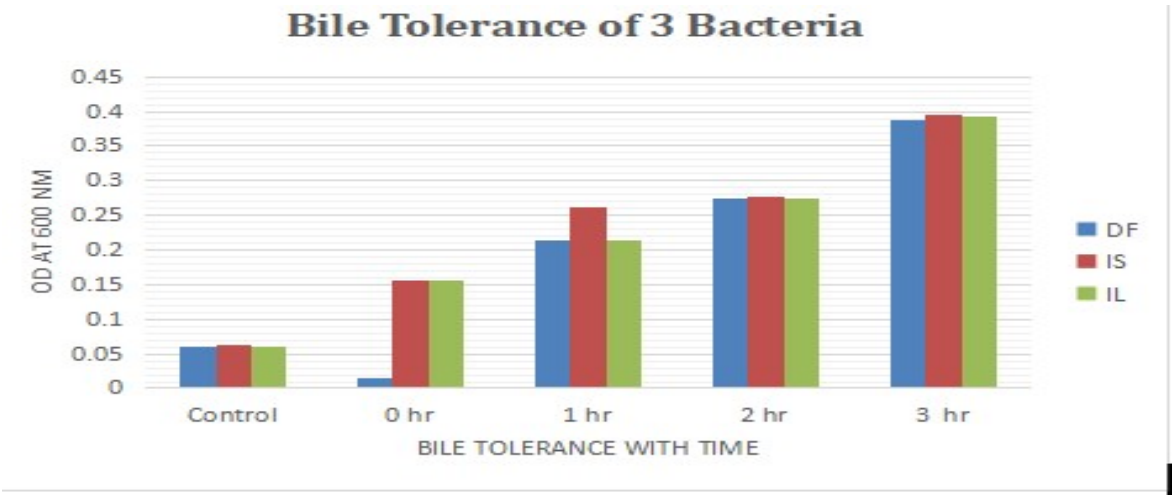

Fig 5: Bile tolerance of all the three cultures

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The above graph shows the bile tolerance ability of test cultures which is measured after every hour till 3 hrs. Comparatively high degree of tolerance was shown by Culture 2 (IS) where as the other 2 cultures exhibited nearly same tolerance.

Molecular Identification of 3 bacterial isolates:
Sequencing results obtained for the 3 bacteria were as follows:

Culture 1:
>DF
GCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTA
ACACGTGGGTAACCTGCCTGTAAGACT
GGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGTTTGAACCGCATGGTTCA
AACATAAAAGGTGGCTTCGGCTACCAC
TTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAA
CGATGCGTAGCCGACCTGAGAGGGTGA
TCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGA
ATCTTCCGCAATGGACGAAAGTCTGACG
GAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAA
GAACAAGTACCGTTCGAATAGGGCGGT
ACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAA
TACGTAGGTGGCAAGCGTTGTCCGGAA
TTATTGGGCGTAAAGGGCTCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCCCCCGGC
TCAACCGGGGAGGGTCATTGGAAACTG
GGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTA
GAGATGTGGAGGATCACCAGTGGCGAAG
GCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGAT
TAGATACCCTGGTAGTTCACGCCGTAA
ACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAA
GCACTCCGCCTGGGGAGTACGGTCGC
AAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT
TAATTCGAAGCAACGCGAAGAACCTTAC
CAGGTCTTGACATCTTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGT
GACAGGTGGTGCATGGTTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATTTTAGTTGC
CAGCATTCAGTTGGGCACTCTAAGGT
GACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTA
TGACCTGGGCTACACACGTGCTACAAT
GGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCA

Culture 2
>IS
ACCCTTATTATCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAA
ACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAC
ACGTGCTACAATGGATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTT
AAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCT
AGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCC

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CGTCACACCATGAGAGTTTGTAACACCCAAAGTCGGTGGGGTAACCTTTTAGGAACCA
GCCGCCTAAGGTGGGACAGATGATTAGGGTGAAGTCGTAACAAGGTAGCCGTAGGAG
AACCTGCGGCTGGATCACCTCCTTTCTAAGGAATATTACGGAAACCTACACATTCTTCG
A
AACTTTGTTTAGTTTTGAGAGATTTAACTCTCAAAACTTGTTCTTTGAAAACTAGATAAT
ATCAAATATATTTTTTCATAATGAAACCGAGAACACCGCGTTTTTTGAGTTTTTTATTGA
AGTTTAATTATCGCTAAACTCATTAATCGCATTTACCGTTAGGTAAATGAGGTTAAGTT
AACAAGGGCGCATGGTGAATGCCTTGGCACTAGGAGCCGATGAAGGACGGGACTAAC
ACCGATATGCTTCGGGGAGCTGTACGTAAGCTATGATCCGGAGA
TTTCCGAATGGGGTAACCCAGCAGTTTTAATCAACTGTTACCACTAGATGAATTCATAG
TCTAGTTGGAGGTAAACGCTGTGAACTGAAACATCTCATTAGCAGCAGGAATATAAAG
AAATTTCGATTCCCTAAGTAGCGGCGAGCGAACGGGGAACAGCCCAAACCAAAGTGCT
TGCACTTTGGGGTTGTAGGACTGAACATTTGAGTTACCAAAGAACTTGATAGTCGAAG
GATTTGG

Culture 3:
>IL
CACCTGATTGATTTGGTCGCAACGAGTGGCGGACGGGTGAGTAACACGTAGGTAACCT
GCCCAGAAGCGG
GGACAACATTTGGAAACAGATGCTAATACCGCATAACAACGTTGTTCGCATGAACAAC
GCTTAAAAGATG
GCTTCTCGCTATCACTTTGGATGGACCTGCGGTGCATTAGCTTGTTGGTGGGGTAACGG
CCTACCAAGGC
GATGATGCATAGCCGATTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCA
TACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGCCTGATGGAGCAACACCGCGT
GAGTGAAGAAGGG
TTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACACGTATGAGAGTAACTGTTCTACG
TTGACGGTATT
TAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTATCCGGA
TTTATTGGGCGTAAAGAGAGTGCAGGCGGTTTTTAAGTCTGATGTGAAAGCCTTCGGCT
TAACCGGAGAA
GTGCATCGGAAACTGGATAACTTAGTGCNGAA

Based on the BLASTN results for all the above sequences the organisms were identified as *Bacillus mojavensis, Lactiplantibacillus plantarum* (previously known as *Lactobacillus plantarum*) and *Lactobacillus fermentum strain LB22.*

Conclusion
Based on the finding of the study the current work concludes the presence of pro-biotic bacteria in non dairy sources which can be of high use for vegan and lactose intolerant individuals. The bacteria which were isolated and found to be pro-biotic were identified to be *Lactobacillus plantarum, Lactobacillus fermentum and Bacillus mojavensis*. All the three were subjected for both biochemical tests and molecular identification based on 16s RNA sequencing which could successfully identify the bacteria at strain level. Identified bacteria are known for their potential health benefits and pro-

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biotic properties. These findings underscore the diverse sources of pro-biotic strains beyond traditional dairy products, offering new avenues for enhancing the nutritional and functional properties of fermented foods. Further research into the viability and efficacy of these bacteria in non-dairy matrices could contribute to expanding the range of probiotic-rich foods available to consumers seeking dietary alternatives.

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