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SCREENING FOR OXALATE AND URIC ACID DEGRADING LACTIC ACID BACTERIA FROM PROBIOTIC FOOD ITEMS USING CLASSICAL MICROBIOLOGICAL METHODS.

Microbiology

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ABSTRACT

Calcium oxalate and uric acid are the major components involved in hyperoxaluria and hyperuricemia respectively.This study was aimed at discovering LAB's (Lactic acid bacteria) which can degrade uric acid and calcium oxalate to alleviate these conditions. 7 isolates were procured from commonly consumed fermented food items in the Indian subcontinent and commercially available probiotic supplements and cultured in anaerobic conditions using MRS Agar. Biochemical tests were conducted by referring to the 'Bergey's manual for classical identification' and were identified to the genus level. 2 strains showed significant uric acid degradation, D3A and Y2 both showed 79% and 29% reduction respectively showing viability in alleviating hyperuricemia. None of the isolates showed any significant calcium oxalate degradation. A survey was conducted to test/analyse the health benefits of probiotic consumption in a population. After running statistical analysis and tests, it was concluded that probiotic foods and supplements do have a positive impact on the general wellbeing of an individual.

KEYWORDS

LAB's , probiotics, Hyperoxaluria, hyperuricemia, Henry Caraway method, calcium oxalate, uric acid, Lactobacillus.

INTRODUCTION:

Calcium oxalate is a primary cause of kidney stones in humans whereas uric acid leads to Gout arthritis. Sources of uric acid involve purine rich diets like red meat, alcohol, high fructose diets, etc [10] . These purines get converted to uric acid by xanthine oxidation pathway. Calcium oxalate accounts for approximately 70%-80% of the makeup of kidney stones [2]. Some derivatives of oxalate are also generated endogenously in the liver as a byproduct of the metabolism of glyoxylate, ascorbic acid, and glycine. Numerous medical diseases, including hyperoxaluria (high concentration of oxalate), renal failure, and calcium oxalate urolithiasis, can be brought on by high oxalate levels in the body[3].

The human body does not contain the necessary oxalate molecule metabolising enzymes, so the body deals with this potentially hazardous substance in three ways:

- Elimination is by absorbing these compounds in the direction of the urinary system and excreting them in urine.
- Combining the poisonous oxalate chemical in the gut with calcium to create insoluble calcium oxalate, which is then eliminated in stool.
- Microbial breakdown by gastrointestinal tract bacteria, *Oxalobacter formigenes*, which uses calcium oxalate as its sole energy source. Oxalate absorption and urine excretion rates are significantly influenced by the levels of oxalate and calcium in the body. Excessive amounts of oxalate in the human body can result in a variety of medical diseases, including hyperoxaluria, renal failure and calcium oxalate urolithiasis. [4]

Human purine metabolism results in uric acid, a byproduct that cannot be further digested since uricase enzyme is absent [10]. Hyperuricemia (increased uric acid levels) may be brought on by either an increase in the buildup of uric acid or a decrease in its elimination, or potentially both. It is characterised by gout, urolithiasis, and urate nephropathy as symptoms, due to urate crystal production in the bones, joints, and kidneys and has emerged as a major international health concern [10]. On the other hand, people accumulate uric acid by the body's natural synthesis of uric acid, which results from purine metabolism in the intestines, liver, kidneys and muscle tissues in addition to consumption of purine rich foods. Two thirds of the uric acid that humans produce is eliminated by the kidneys, while the remaining is eliminated through the intestines [10].

The World Health Organization (WHO) defines probiotics as "live microorganisms, which, when administered in adequate amounts, confer a health benefit to the host." Since most of the microorganisms utilized in probiotic therapy are non-pathogenic and of human origin, they are typically selected [9]. These probiotic treatment plans are quite effective at restoring the natural flora and have minimal to no negative effects, which strengthens innate immunity. Most probiotic organisms have gastrointestinal origins and are used to treat a variety of conditions, including candidiasis, rheumatoid arthritis and lactose intolerance [9].Taking medications like Lumasiran, thiazide diuretics, and vitamin B-6, phosphates, and citrates are some of the current methods used to treat kidney stones [3]. But these techniques have moderate to severe adverse effects including sulfonamide allergy, hyperglycemia (elevated blood sugar content), hyperuricemia, and mild dermatitis. In the five years following therapy, hyperoxaluria recurs frequently, despite the substantial risk of side effects associated with these medications. Gout is usually treated with a combination of over-the-counter NSAIDs when you're experiencing symptoms and prescription medication to help lower your uric acid levels. Colchicine (a prescription that prevents gout attacks) or corticosteroids are the default medications [10]. Corrective actions such as consuming copious amounts of fluids and altering one's diet can help avoid this problem, but they are very erratic and have negligible impact on primary hyperoxaluria and hyperuricemia, which is caused by genetic inheritance and gender [3][10]. This study aims to close the gap between these two domains by introducing oxalate and/or uric acid-degrading bacteria via probiotics, hence improving the effectiveness and accessibility of hyperoxaluria and hyperuricemia treatment.

MATERIALS AND METHODS:

Sample collection, isolation, and preservation.

Commercially available and homemade samples of curd, Dosa batter, Idli batter and Yakult were procured.These samples were subsequently stored under refrigeration conditions.In the process of isolation, we took two grams out of each collected sample, and it was mixed with five milligrams of sterile saline to bring the samples in suspension form. Now serial dilution of each sample was done using sterile saline up to 10^{-3}. All three dilutions of all the samples were plated on the MRS (De Man, Rogosa, and Sharpe agar by HiMediaTM formulation) [5][8] using the spread plate technique by taking 0.1 ml of the suspension on a plate. The MRS plates were incubated at 37°C for 48 hours and as lactobacillus species need anaerobic conditions to grow the plates were kept in anaerobic candle jars. Initially 21 isolates were preserved on the MRS slants and stored in refrigerator conditions and after every 20 days, the isolates are reisolated onto a new MRS slant to maintain the viability of the isolates. [4]

Staining And Identification Of LAB's Using Biochemical Test:

Gram staining was carried out for all 21 isolates to select the isolates which correspond to the group of Lactic acid bacteria (LAB's). Along with staining procedures and colony characteristics, catalase test was performed. To perform this test, 15% hydrogen peroxide was taken on a cavity slide and the microbial colony to test was suspended in the cavity slide. If immediate and instantaneous bubble formation or effervescence is seen, it confirms a positive catalase test. The test organism was not older than 24 hr.

Calcium Oxalate Degradation Assay:

7 strains from the previous tests were identified as LAB's and were tested for oxalate degradation activity. For this assay, HiMedia Formulated MRS broth was prepared with 0.9 mg/dl of amorphous calcium oxalate. All 7 strains were inoculated in the MRS + oxalate broth (MRS-ox) and were incubated at 37°C for 48 hours. MRS broth (-oxalate +bacterial culture) was used as blank for each isolate. Controls were prepared using just MRS broth[7]

Uric Acid Degradation Assay:

Uric acid estimation was done by the Henry Caraway/ Phosphotungstate method. 0.5 mg/dl uric acid crystals were added in 100 ml MRS broth. MRS broth without uric acid was used as a blank. The MRS broth with isolates were first centrifuged at 1000 rpm for 5 minutes before using the suspension for the assay. A kit based assay by NSN was used for the assay. Amount of tungstate was estimated colorimetrically at 660 nm by using kit based phosphoric acid and sodium tungstate reagents under alkaline condition at pH 10.2, which forms a blue colour complex tungstate. The intensity of the blue colour complex is directly proportional to the amount of uric acid present in the sample.[1]

RESULTS:

7 pure isolates were obtained from the samples. Isolates labelled based on the initial of their food source.

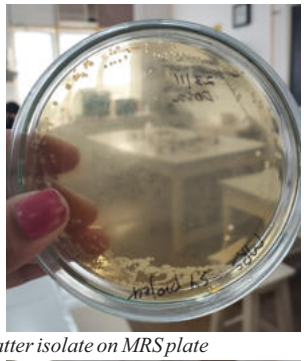


Fig 1.A: *Dosa batter isolate on MRS plate*

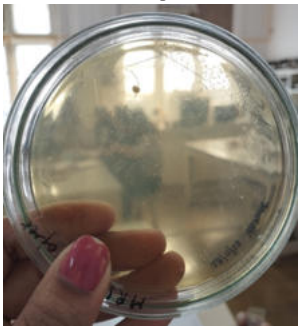
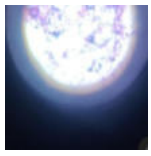
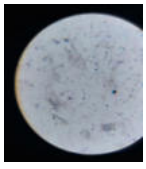
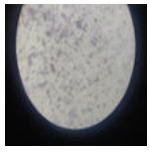
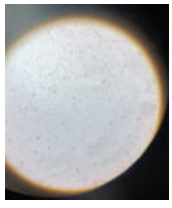
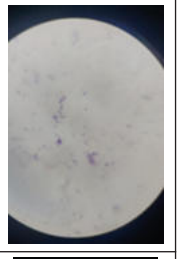
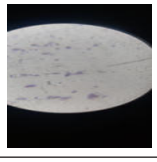
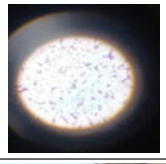


Fig 1.B: Yakult isolates on MRS agar plate

Fig 1.C: *Curd isolates on MRS agar plate.*

All seven isolates were found to be belonging to the genus lactobacillus and this conclusion was found after performing the gram's staining and the results of gram's staining for all the seven isolates are as tabulated below:

Isolate Label	GRAM CHARACTER	MORPHOLOGY	GRAM STAINING IMAGE
IDLI-1	Gram positive	Cocci	
CURD-2	Gram positive	Cocci	
YAKULT-1	Gram positive	Bacillus	
YAKULT-2	Gram positive	Bacillus	
D-3A	Gram positive	Cocci	
YOGURT-1	Gram positive	Coccobacillus	
YOGURT-2	Gram positive	Cocci	

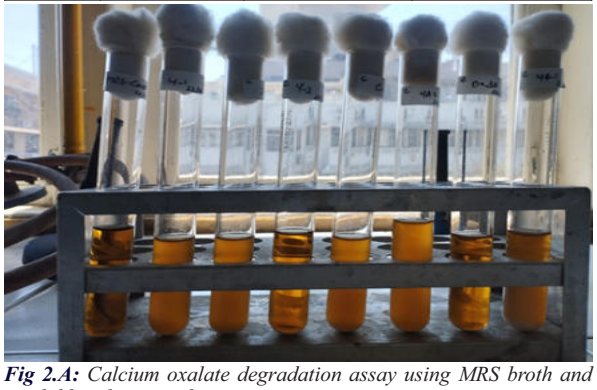


Fig 2.A: *Calcium oxalate degradation assay using MRS broth and insoluble calcium oxalate.*

The colonies obtained from the microbial isolates of D-3B, D4, and D-3A were cream coloured, circular in shape, elevation seen was convex, moist, with smooth edges, whereas for Yakult isolate the colonies were light yellow in colour, circular in shape, elevation seen was convex and colonies were moist with smooth edges. All the isolates gave Catalase a negative test, as no effervescence/bubbles can be seen which is characteristic of LAB's [9].

Calcium Oxalate Degradation Assay:

All seven isolates were found to be non-oxalate degrading. The turbidity was due to the insoluble calcium oxalate and the bacterial growth was analysed and detected to be negative using spectrophotometric methods using a blank.

URIC Acid Degradation Test.

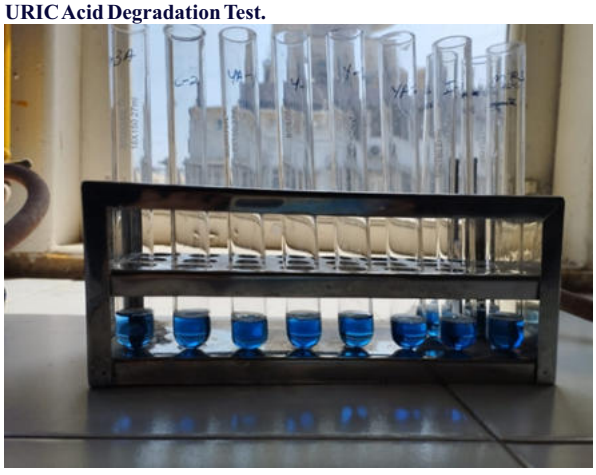


Fig 2.B: *Uric acid degradation assay by Henry Caraway's method.*

Isolates	Absorbance at 660 nm	Uric acid content (mg/dL)	Uric acid utilisation (%)
Standard 5mg/dL uric acid	0.39	5.00	-
MRS+uric acid	0.24	3.08	Initial uric acid content
Yakult-1	0.26	5.42	increase
Yakult-2	0.11	2.29	29%
Idli-1	0.33	6.88	increase
Dosa-3A	0.03	0.63	79%
Yoghurt-1	0.28	5.03	increase
Curd-2	0.23	4.79	increase
Yoghurt-2	0.31	6.46	increase

Two of the isolates, Dosa-3A and Yakult-2, showed uric acid utilisation which was 79% and 29% respectively.

Survey And Statistical Analysis:

A survey was conducted among 151 individuals from diverse socio-economic backgrounds, different age groups, colleges, and social media platforms to assess the potential benefits of probiotic consumption. The study ensured that external incentives did not influence responses, maintaining unbiased data collection.

Two key research questions were analysed using descriptive statistics and a z-test:

1. Do people consume probiotics?
2. Did people have to consult a doctor regarding digestive issues?

Responses were grouped and assessed using descriptive analysis (Fig 3.B). Graphical representation of the survey responses is provided in Figure 3.A. Descriptive statistical measures, including mean, median, and skewness, indicated that the dataset followed a normal distribution. Given that the sample size was greater than 30 (N > 30), a parametric z-test was performed to assess the hypothesis [6].

Hypothesis Testing

The hypothesis tested was:

- Null Hypothesis (H_0): Eating probiotics is linked to improved stomach health, potentially reducing digestive issues and improving mood compared to non-users.

With a 95% confidence interval, the significance (alpha) value was set at 0.05. The p-value obtained for the one-tailed and two-tailed tests was 0.5 and 1, respectively, both exceeding the significance threshold. Therefore, the null hypothesis was accepted, indicating no statistically significant difference between probiotic users and non-users regarding digestive health and mood improvement.

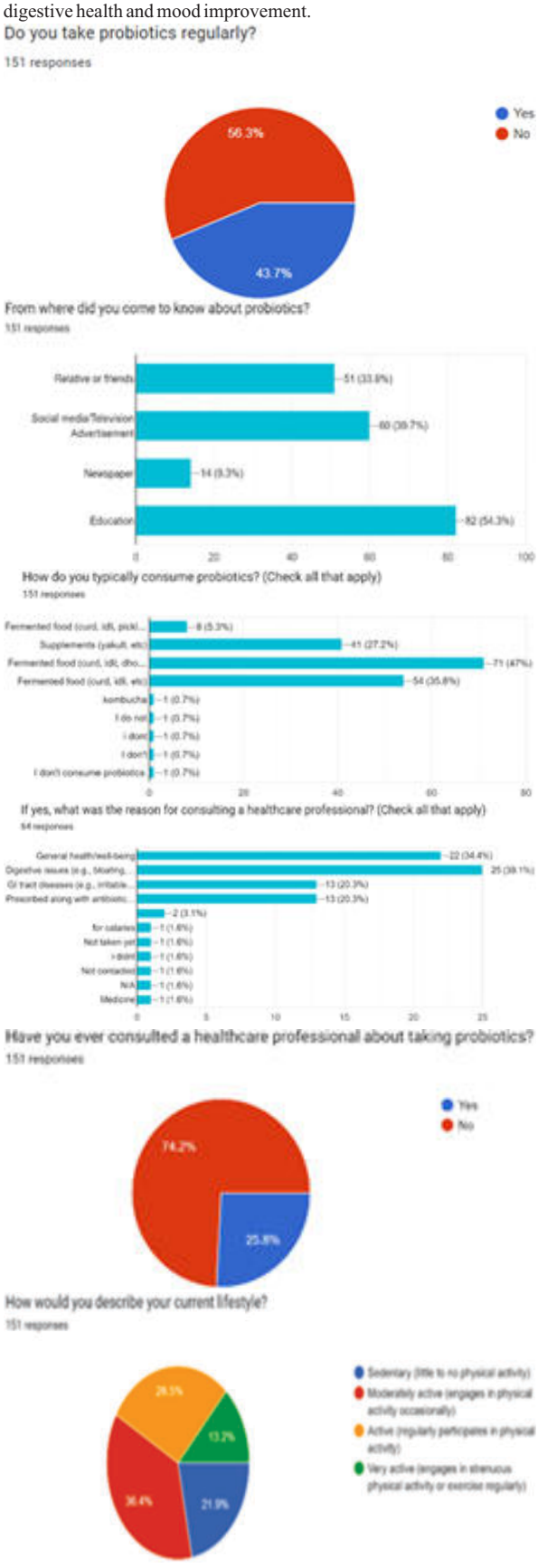


Fig 3.A: *Graphical representation of the results from the survey*

	Q1: Frequency of consumption	Q2: Frequency of consultance
YES	66	39
NO	85	112

	Q1		Q2	
Mean	75.5	Mean	75.5	
Standard Error	9.5	Standard Error	36.5	
Median	75.5	Median	75.5	
Mode	85	Mode	112	
Standard Deviation	13.43502884	Standard Deviat	51.61879503	
Sample Variance	180.5	Sample Variance	2664.5	
Kurtosis	#N/A	Kurtosis	#N/A	
Skewness	#N/A	Skewness	#N/A	
Range	19	Range	73	
Minimum	66	Minimum	39	
Maximum	85	Maximum	112	
Sum	151	Sum	151	
Count	2	Count	2	
Largest(1)	85	Largest(1)	112	
Smallest(1)	66	Smallest(1)	39	
Confidence Level(95%)	120.708945	Confidence Leve	463.7764729	

66	z-Test: Two Sample for Means		
67			
68		Q1	Q2
69	Mean	75.5	75.5
70	Known Variance	180.5	2664.5
71	Observations	2	2
72	Hypothesized Mean Difference	0	
73	z	0	
74	P(Z<=z) one-tail	0.5	
75	z Critical one-tail	1.644853627	
76	P(Z<=z) two-tail	1	
77	z Critical two-tail	1.959963985	

Fig 3.B: *Descriptive analysis of the Q1 (left) and Q2 (right) to analyse the null hypothesis*.

DISCUSSION:

The findings of this study highlight the potential of specific lactic acid bacteria (LAB) isolates in uric acid degradation while revealing limitations in calcium oxalate degradation. Among the seven LAB isolates screened from probiotic food sources, two strains, D3A and Y2, demonstrated significant uric acid degradation, with reduction rates of 79% and 29%, respectively. These results suggest the feasibility of utilizing these strains as probiotic candidates for alleviating hyperuricemia, a condition associated with elevated uric acid levels that can lead to gout and kidney complications [(2)(12).] The ability of LAB to degrade uric acid aligns with previous studies that have reported microbial interventions as an alternative approach to managing hyperuricemia through gut microbiota modulation.

In contrast, none of the isolates exhibited calcium oxalate degradation under the tested conditions. This outcome may be attributed to multiple factors, including the concentration of calcium oxalate in the assay medium, the absence of specific oxalate-degrading enzymes in the tested strains, and potential growth limitations in anaerobic environments. Previous studies have identified *Oxalobacter formigenes* as a primary oxalate-degrading bacterium, with some Lactobacillus species also showing mild oxalate degradation capabilities [(7).] However, the isolates in this study did not demonstrate such activity, suggesting the need for further optimization in experimental conditions. On the contrary many isolates showed increase in Uric acid content. This is highly unlikely since there haven't been studies suggesting any LAB's that synthesise uric acid. The increase in absorbance reading might be due to 2 possibly factors:

1. Interference of other metabolites that result in release of free tungstate which is assessed by the assay.
2. Cell lysis causes purine residues to collect in the broth suspension. If there's contamination by a foreign microbe which produces xanthine oxidase enzyme, this can lead to production of uric acid.

Future studies could explore alternative methodologies, such as reducing the standard calcium oxalate concentration from 50 mM to 20 mM, employing agar overlay methods to ensure anaerobic growth, and utilizing soluble oxalate sources like potassium or sodium oxalate. The application of flame photometry for more precise oxalate quantification may also enhance detection sensitivity [(7)].

The identification of D3A and Y2 as promising uric acid-degrading strains necessitates further characterization at the species level. Classical biochemical methods or molecular identification techniques, such as VITEK or 16S rRNA sequencing, could provide insights into their genetic profiles and metabolic pathways involved in uric acid degradation. Additionally, probiotic suitability tests, including pH tolerance, bile salt resistance, and gastrointestinal survival, are essential to determine their efficacy in real-world applications. Since probiotics must withstand harsh stomach conditions and bile exposure before reaching the intestines, these characteristics are crucial for commercial viability [(3)].

Another critical aspect requiring investigation is the potential antimicrobial activity of these isolates against other gut microbiota. While probiotic bacteria confer health benefits, they may also exert inhibitory effects on other beneficial gut microbes, altering gut homeostasis [(3)(4)]. Further research should assess whether D3A and Y2 interact positively or negatively with native gut flora, ensuring their safe application in probiotic formulations. Additionally, pathogenicity assessments must be conducted to confirm their safety for human consumption.

Beyond laboratory analysis, the study also incorporated a survey evaluating public perceptions and health benefits associated with probiotic consumption. The statistical analysis confirmed the hypothesis that probiotic intake is linked to improved digestive health and overall well-being. The findings suggest that awareness regarding probiotics primarily stems from educational and academic sources, with traditional fermented foods being the most common dietary sources [(6).] This highlights an opportunity for targeted awareness programs and the potential for probiotics to be incorporated into public health initiatives as preventive measures against gastrointestinal and metabolic disorders.

Moving forward, research should focus on developing probiotic formulations incorporating uric acid-degrading LAB strains while ensuring their stability, efficacy, and safety. The impact of these strains on clinical conditions such as gout and kidney diseases should also be assessed through in vivo studies or controlled clinical trials. Additionally, further investigation into genetic modifications or adaptive evolutionary strategies to enhance the oxalate-degrading capabilities of LAB could open new avenues for probiotic-based treatments for hyperoxaluria [(7)].

In conclusion, this study identified two promising LAB strains capable of uric acid degradation, presenting a potential probiotic-based strategy for hyperuricemia management. However, the inability of isolates to degrade calcium oxalate indicates the need for further research into optimized methodologies and alternative microbial candidates. The integration of probiotics into preventive healthcare strategies holds promise, but comprehensive safety, efficacy, and regulatory evaluations must precede their commercialization.

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