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Development of folate enriched yoghurt incorporating *Lacticaseibacillus paracasei*#

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Abstract

The aim of the study was to develop folate-enriched yoghurt incorporating lactic acid bacterial isolate obtained from natural sources. Ten lactic acid bacterial isolates obtained from natural sources, available in the Department of Dairy Science, College of Veterinary and Animal Sciences, Mannuthy, were screened to assess their potential for folate production. Optimisation studies were designed at two varying temperatures (37°C and 42°C) and three fermentation intervals (4h, 8h and 12h). In the study isolate "L1" was identified as a superior folate producer (17.67±1.78 µg/L) as enhanced folate production was achieved when grown at a temperature of 42°C for a period of 4 h which is considered as ideal fermentation condition for yoghurt preparation. Lactobacillus rhamnosus (NCDC 18), the standard strain yielded 11.38±0.91 µg/L. Molecular characterisation revealed that the isolate "L1" is Lacticaseibacillus paracasei. Functional yoghurt (treatment T) was formulated incorporating L. paracasei as an adjunct culture along with yoghurt cultures. Control 1 (C1) was prepared using standard yoghurt cultures, while control 2 (C2) was supplemented with L. rhamnosus as an adjunct culture in addition to the standard yoghurt cultures. A significantly higher ($p<0.05$) folate content was observed in functional yoghurt sample (T), with a value of 105.72±15.48 µg/L, when compared to control. In the sensory evaluation of yoghurt samples, a significantly higher overall score was observed for sample T. This suggests that the addition of L. paracasei can be a viable approach for developing folate-enriched yoghurt with acceptable sensory quality.

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Notably, folate production ability of isolate "L6" (224±36.93 µg/L over a 12h period at 37°C) can also be exploited for increasing folate level in other fermented dairy products, by way of natural synthesis.

Keywords: *folate, yoghurt, Lacticaseibacillus paracaei*

Folate, also known as vitamin B9, is an essential water-soluble vitamin crucial for numerous biological processes in the human body, including DNA synthesis, cell division and amino acid metabolism. Insufficient folate intake can lead to various health issues such as megaloblastic anaemia, birth defects during pregnancy and an increased risk of cardiovascular diseases. Consequently, addressing folate deficiency has become a global public health concern. Dairy products, particularly yoghurt, are widely consumed by people of all age groups and demographics, making them an ideal target for fortification to enhance essential nutrient intake. The incorporation of bioavailable folate into yoghurt offers an innovative approach to combat folate deficiency and improve public health.

Milk is not widely considered as a significant dietary source of folate (Vanitha *et al.* 2018). Nevertheless, numerous dairy products undergo microbial fermentations during processing, providing an opportunity for folate synthesis. In this context, it has been previously noted that milk fermented with certain specific lactic acid bacteria (LAB) exhibit a three-fold increase in folate concentrations when compared to non-fermented milk (Wouters *et al.* 2002). The daily recommended intake of folate for an adult varies between 200 and 400 µg. For women of child-bearing age and for pregnant women, dietary intake of 400 µg per day is recommended (Rekha *et al.* 2018). In the present study an attempt was made to develop folate enriched yoghurt using folate producing lactic acid bacterial isolate, available in the Department culture collection, isolated from natural sources. This study aims to introduce an innovative strategy for fortifying yoghurt with folate by utilising the potential of natural LAB isolates. The goal is to offer a readily adoptable approach for the food industry, contributing to improvements in human health. The findings of this study are anticipated to provide valuable insights for researchers, food scientists and policymakers in their endeavours to combat nutrient deficiencies and enhance the nutritional value of functional foods. Hence in this study the concept of increasing folate levels in dairy products through natural synthesis by the bacterial cultures rather than by fortification is explored. The study also seeks to introduce an innovative strategy for fortifying yoghurt with folate, exploiting the potential of natural lactic acid bacterial (LAB) isolates in order to provide a readily adoptable approach for the food industry.

Materials and methods

Ten lactic acid bacterial cultures used for this study were obtained from culture collections of Department of Dairy Science, College of Veterinary and Animal Sciences, Mannuthy. Kerala Veterinary and Animal sciences University. Standard LAB strain, *Lactobacillus rhamnosus* (NCDC 18) having folate production ability was procured from National collection of Dairy cultures, NDRI, Karnal, which served as control. Test strains (L1, L2, L3, L4, L5, L6, L7, L8, L9, L10) were analysed for their purity by streaking on MRS agar and incubating at 37°C for 24 hours. Their colony characteristics were also studied.

Preparation of standard curve

Standard folic acid solution (0.1 ng/mL concentration) was prepared. The standard solution was taken in test tubes at levels of 0.6, 1.2, 2.5 mL in triplicates. To all tubes equal volume of 2X basal medium (Folic acid casei medium- FACM) was added to bring the volume in each to 5 mL. A blank was also run having no standard folic acid. The contents of the tubes were shaken with a vortex mixer and the absorbance was read at 580 nm by spectrophotometer (Perkin Elmer, U.S). Calibration curve was drawn by plotting concentrations against optical density. Following linear regression equation was generated to estimate folate concentration of samples. The method suggested by Iyer and Tomar (2009) was being followed for this purpose.

Y= a + bx, a = 2.9411, b = 4443.3, x = optical

density, Y = folate concentration(ng/ml)

Preparation of Sample and Estimation of folate

The LAB isolates were cultivated in MRS broth. A sample (500 µL) of LAB was taken and 500 µL of protecting buffer (0.1 mol/L phosphate buffer, pH 6.8, containing 1.5% (m/v) ascorbic acid to prevent vitamin oxidation and degradation) was added and mixed, followed by immediate centrifugation for five min. at 5000g. The supernatant was collected and then boiled (100°C) for 5 min, centrifuged for six min. at 10000g, and the supernatant were stored at -70 °C until used for folate determinations (Iyer and Tomar, 2009).

The determination of folate was done by spectrophotometric method, as described by Horne and Patterson (1988). The supernatant was inoculated at 2 per cent concentration in folic acid casei medium (FACM), supplemented with 2 mg/L of p-aminobenzoic acid (pABA). By taking reading at 580 nm, the turbidity in the medium was assessed. To obtain the final folate concentrations, the values obtained was used in linear regression equation and expressed as µg/L.

Study of impact of fermentation condition

Optimisation studies for folate production were conducted at different temperatures (37 and 42 °C) and various fermentation period (4h, 8h, and 12h) for all LAB isolates, with a comparison made against the standard strain, *L. rhamnosus* (NCDC 18). The isolate that produces maximum folate content at 42°C during 4h of fermentation was selected to prepare folate-enriched yoghurt, since it is the most suitable conditions for yoghurt preparation.

Identification of LAB strains

Biochemical and molecular tests were conducted to define the isolate that produced maximum folate. Pure culture colonies of the selected isolate, also underwent characterization using morphology, cultural and biochemical characteristics. Subsequently, PCR was employed for confirmatory identification of isolate L1 at species level (Patil *et al.* 2010).

Method of yoghurt preparation

Yoghurt was prepared following the methodology proposed by Tarakci and Kucukoner (2003), utilising whole milk that underwent pasteurisation at 90°C for five minutes, followed by rapid cooling to 42°C. The control batch was aseptically inoculated with 3 per cent yoghurt culture. Experimental batch was inoculated with a combination of 1.5 per cent yoghurt culture and 1.5 per cent of the selected culture known for its maximum folate production. Subsequently, the incubated at 42°C for 4h and later stored under refrigeration. The cultures used in different treatment yoghurt samples are mentioned in table 1.

Quality evaluation of yoghurt

The analysis involved assessing the folate content in both the treatment and control samples of yoghurt, along with an additional examination of folate levels in the milk. Furthermore, the folate-enriched yoghurt and control samples undergone sensory evaluation for a comprehensive understanding of their sensory attributes. Sensory evaluation of yoghurt samples was carried out by 6 member panellists including teaching staff (four) and post-graduation students (two) using a nine-point hedonic scale as per Larmond (1977). Score card was also prepared. Different sensory parameters such as appearance and

Table 1. Different treatments of yoghurt prepared for analysis

Treatments	Details of starter lactic acid bacteria added
Control 1 (C1)	Yoghurt cultures (*S. thermophilus*, *L. bulgaricus*)
Control 2 (C2)	Yoghurt cultures + *L. rhamnosus* (NCDC 18)
Treatment (T)	Yoghurt cultures + identified best folate producer LAB isolate

colour, body and texture, flavour and overall scores were analysed.

Result and discussion

Effect of different temperature and fermentation period on folate production by LAB isolates

Ten lactic acid bacterial isolates (L1, L2, L3, L4, L5, L6, L7, L8, L9, L10) were examined for their colony characteristics. The colonies were appeared white, smooth and round in shape. Preliminary screening for folate production potential revealed that all ten isolates exhibited the ability to produce folate. Optimisation study revealed that isolate L6 exhibited highest folate production of 13.97±1.66 µg/L at 37°C within 4 hours. However no significant difference observed in folate yield among all tested strains and also between test strain and standard. The highest folate production by isolate L7 occurred at 37°C over an 8-hour duration, yielding a concentration of 31.74±3.74 µg/L, and again, no significant difference was observed in folate yield among the tested strains. Isolate L6 continued to demonstrate optimal folate production, reaching a peak of 224.21±36.93 µg/L within a 12-hour period at 37°C. However, significant difference in folate yield was not observed among the tested strains.

Isolate L1 exhibited its peak folate production, reaching 17.67±1.78 µg/L within a 4-hour time frame at 42°C, with a significant difference in folate yield across all tested strains. The optimal folate production for L1 occurred at 42°C within 8 h, resulting in a concentration of 42.04±5.41 µg/L, with a significant difference in folate yield among all tested strains. Isolate L6 demonstrated optimal folate production at 42°C within a 12-hour time frame, yielding 215.99±16.19 µg/L, with a significant difference in folate yield among the tested strains. The optimisation studies demonstrated that isolate L6 achieved the highest overall folate production, reaching 224±36.93 µg/L over a 12-hour period at 37°C. In contrast, isolate L1 exhibited peak folate production at 42°C within 4 h, yielding a value of 17.67±1.78 µg/L. Taking into account of conditions acceptable for yoghurt preparation (42°C for 4 h), isolate L1 was chosen for the production of folate-enriched yoghurt. Optimisation of fermentation processes trials had performed by researchers such as Crittenden *et al.* (2003)*;* Sybesma *et al. (*2003)*;* Laino *et al. (*2012)*;* Laino *et al. (*2013) and Mosso *et al.(* 2020). Their studies aimed to explore the most favourable conditions for the growth of LAB isolates and folate production.

Table.2 Mean Folate Production (µg/L) by LAB strains at different temperature and different fermentation time

	37°C			42°C		
	4h	8h	12h	4h	8h	12h
L1	10.57±0.85^{n}	29.74±1.93^{n}	154.01±43.38^{n}	17.67±1.78^{a}	42.04±5.41^{a}	122.98±10.86^{b}
L2	10.34±0.37^{n}	22.93±3.23^{n}	170.37±24.43^{n}	10.42±0.68^{bc}	34.78±4.58^{ab}	130.38±13.37^{b}
L3	10.71±1.25^{n}	24.49±2.88^{n}	131.64±15.35^{n}	7.82±0.47^{c}	30.26±2.27^{abc}	212.96±32.54^{a}
L4	10.57±1.49^{n}	22.19±4.92^{n}	168.52±36.44^{n}	12.79±0.63^{b}	27.67±3.48^{bc}	142.52±21.58^{ab}
L5	10.93±0.67^{n}	28.41±2.75^{n}	133.72±28.84^{n}	10.64±1.01^{bc}	34.56±3.03^{ab}	199.26±48.67^{ab}
L6	13.97±1.66^{n}	27.00±4.47^{n}	224.21±36.93^{n}	9.90±0.68^{bc}	31.89±4.66^{ab}	215.99±16.19^{a}
L7	11.16±1.46^{n}	31.74±3.74^{n}	202.14±21.21^{n}	10.27±0.68^{bc}	19.45±2.25^{c}	158.45±31.74^{ab}
L8	11.53±1.49^{n}	30.85±3.48^{n}	180.00±43.49^{n}	10.93±1.59^{bc}	26.04±5.50^{bc}	192.67±24.84^{ab}
L9	10.12±0.79^{n}	28.63±2.27^{n}	185.33±13.31^{n}	10.42±0.64^{bc}	34.34±2.30^{ab}	137.79±15.37^{ab}
L10	13.23±0.63^{n}	31.00±2.19^{n}	215.62±26.88^{n}	10.86±0.60^{bc}	27.45±2.62^{bc}	118.83±16.85^{b}
S	10.56±1.17^{n}	24.86±2.18^{n}	140.75±22.00^{n}	11.38±0.91^{b}	30.04±2.70^{abc}	136.24±17.11^{ab}

Means with different superscript in same column differ significantly ($p<0.05$)

Non-significant as n

Current study provided valuable insights into the fermentation condition preferred by LAB for better folate production.

Identification of LAB strains

Characterisation using morphology, cultural, and biochemical characteristics of pure culture colonies of selected L1 isolate confirmed that the isolate belongs to genus Lactobacillus. The PCR products, with a size of 657 bp (Fig.1), were further sequenced, leading to the identification of isolate L1 as *Lacticaseibacillus paracasei.* PCR based methods for detection have been widely in use and are recommended by OIE (*Chaitanya et al.* 2021). According to Morgan *et al*. (2009), in comparison to conventional methods, the 16S rRNA-based technique offers a significantly higher percentage of reliability.

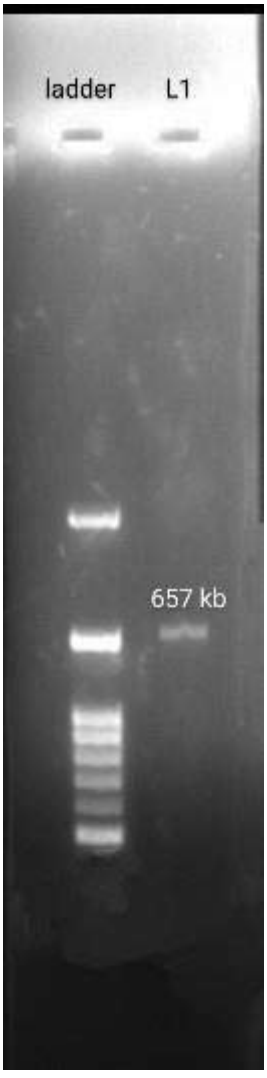

Fig. 1. PCR profile of LAB isolate (L1)

Preparation of functional yoghurt and quality evaluation

The *L. paracasei culture* (isolate L1) was introduced as an adjunct culture in yoghurt to enhance its functionality. Folate levels in yoghurt samples (control C1, C2, and treatment T) and milk were assessed through spectrophotometric method (Table 3), revealing folate contents of 70.17±5.05, 89.73±9.00, and 105.72±15.48 µg/L for C1, C2, and T, respectively. The mean folate content in milk was 32.18±3.16 µg/L. Significantly higher folate content ($p \leq 0.05$) was observed in treatment T compared to C1, and a similar trend noted between T and C2. Kneifel *et al.* (1989) reported that milk is not a rich source of dietary folate, but fermented dairy products such as yoghurt prepared using starter bacteria are regarded as important dietary sources of folate. The World Health Organization (WHO) recommends a daily intake of 400 micrograms (µg) of folate for adults and 200 micrograms per day for children (FAO/WHO, 2002). The folate content in current research meets the above-mentioned recommendation. The sensory evaluation of yoghurt yielded overall mean scores for samples C1, C2, and T, with scores of 17.59 ± 0.29, 17.30 ± 0.90, and 19.20 ± 0.41, respectively. Notably, treatment (T) achieved a higher score, indicating greater acceptability by the panellists in terms of appearance, colour, body, texture and flavour. Non-parametric test analysis revealed a p-value of ≤0.05, suggesting no significant difference in overall mean scores among the different yoghurt

Table 3. Folate content in milk and yoghurt samples

Treatments	Folate content (µg/L)
Control 1 (C1)	70.17±5.05
Control 2 (C2)	89.73±9.00
Treatment (T)	105.72±15.48
Milk	32.18±3.16

Means with different superscript in same column differ significantly ($p<0.05$)

Table 4. Sensory scores of yoghurt samples

Yoghurt samples	Sensory scores			
	Appearance and colour	Body and texture	Flavour	Overall sensory score
C1	4.17 ± 0.17^{n}	4.08 ± 0.16^{n}	9.33 ± 0.08^{n}	17.59 ± 0.29^{b}
C2	4.25 ± 0.28^{n}	4.08 ± 0.34^{n}	8.97 ± 0.17^{n}	17.30 ± 0.90^{b}
T	4.86 ± 0.09^{n}	4.79 ± 0.10^{n}	9.55 ± 0.15^{n}	19.20 ± 0.41^{a}

Means with different superscript in same column differ significantly ($p<0.05$)

Non-significant as n

samples. Additionally, no significant variations were observed in the scores for appearance and colour, as well as body and texture.

Conclusion

Out of the ten LAB isolates evaluated for folate production potential, isolate L6 showed highest folate production of 224.21±36.93 µg/L, when grown at 37°C for 12h. Isolate L1 exhibited superior folate production (17.67±1.78 µg/L) when grown at 42°C for 4h. which was more than the yield from the standard culture, *L. rhamnosus* (NCDC 18) which was recorded as 11.38±0.91 µg/L. Taking into account of conditions acceptable for yoghurt preparation (42°C for 4 h), isolate L1 was selected as most suitable isolate for further studies. Biochemical and molecular characterization confirmed that the L1 isolate is *L. paracasei.* The folate production exhibited by yoghurt incorporated with *L. paracasei* was comparable with that of yoghurt incorporated with standard strain, *L. rhamnosus* (NCDC 18), indicating that the former strain is capable of generating levels of folate equivalent to that produced by the established reference strain. The folate content of 105.72±15.48 µg/L was observed in the functional yoghurt sample, as determined by spectrophotometric assay. On examining the sensory scores of yoghurt samples, functional yoghurt (T) received better overall scores. Folate production ability of isolate L6 can be exploited for increasing folate level in fermented dairy products, by the way of natural synthesis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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