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**MK Aashifaa**
Department of Agricultural Microbiology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

**Dr. Z John Kennedy**
Centre for Post-Harvest Technology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

**Dr. D Balachandar**
Department of Agricultural Microbiology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

**Correspondence**
**Dr. Z John Kennedy**
Centre for Post-Harvest Technology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

# Characterization of folate producing potency of lactic acid bacteria as starter culture for dairy industry

**MK Aashifaa, Dr. Z John Kennedy and Dr. D Balachandar**

**Abstract**
Folate or Vitamin $B_9$ can't be synthesized by humans and obtained exogenously to prevent folate deficiency and related ailments. Lactic acid bacteria (LAB) are capable of producing folate and exploiting this folate producing potency in fermentation is a novel approach to increase concentrations of folate in foods. Nearly 49 isolates were made from different sources and based on biochemical and molecular techniques 16 isolates were characterized as Lactic acid bacteria (LAB). These 16 LAB isolates were screened for folate grown in folate-free culture medium (FACM) and evaluated for folate production by microbiological assay. Among the isolates the highest folate producer was LAB 33 (76.078µg/L) followed by LAB 28 (69.804), LAB 29 (68.558), LAB 45 (64.725) and LAB 07 (48.451) was the least folate producer. Also potency of the LAB isolates to convert milk into curd could be used as starter cultures in dairy industries to produce novel bio-folate enriched dairy foods which could be able to increase the folate levels without the need for chemical fortifications.

**Keywords:** Lactic acid bacteria (LAB), folate, folic acid casei medium (FACM), autotrophs, microbiological assay

## Introduction
Folates or Vitamin B9 belong to water-soluble B-group of vitamins and is one of the essential components of human diet as it is involved in many metabolic pathways. It plays major role in biosynthesis of DNA and RNA (Shane 2001) [20] and involved in synthesis of many vitamins and aminoacids (Hanson *et al*., 2001) [11]. It also improves level of haemoglobin and reduces the risk of strokes in brain. The anti-oxidant properties of folate protect the human genomes from free radicles (Duthie *et al*., 2002) [8]. Although folate plays an important role in human life, it can't be synthesized by humans. In-order to prevent folate deficiency and related ailments its obligate to depend on exogenous supply from plants and microorganisms which can partially synthesize this vitamin (Gangadharan *et al*., 2010; LeBlanc *et al*., 2010) [10, 15].
Deficiency in folate levels leads to many health disorders such as breast, pancreatic, colorectal cancer, osteoporosis, megaloblastic anemia, heart diseases and also increases the risk of birth defects like neural tube defects (NTD) (Boushey *et al*., 1995; Rossi *et al*., 2011; Safi *et al*., 2012) [7, 18, 19]. The daily recommended intake of folate according to World Health Organisation (WHO) is 400 mcg/day for adults and 600 mcg/day in-case of pregnant women (FAO/WHO 2002) [9]. It is reported that intake of folic acid during preconception can help to reduce 62-70% of NTDs. To offset nutritional deficiencies some countries has adopted vitamins supplementation and fortification programmes using folic acid which is chemical form of folate. But high in-take of this chemical form may mask early clinical manifestations of vitamin B12 deficiency, alteration in the activity of hepatic dihydrofolate reductase enzyme and thereby it will promote cancer (Bailey *et al*., 2009; Baggott *et al*., 2012) [5, 6]. So the natural folates would be a more potential and secure alternative than supplementation with folic acid (Lamers *et al*., 2006) [14]. As these form of folates such as 5-methyltetrahydrofolate, which are normally found in foods and also produced by microorganisms are less likely to cause un-desirable side-effects.
So many researchers were in thrust for a novel approach to increase concentrations of naturally occurring folate in foods. Several studies show that certain strains of lactic acid bacteria (GRAS) organism are able to produce folate (Aryana, 2003; Lin and Young, 2000) [2, 16]. In the present study lactic acid bacteria from different sources was isolated and characterized by biochemical and molecular techniques and screened for their folate production.

## 2. Materials and methods
All the media used for the study were procured from M/s. Himedia, India and all the chemicals

used for experiments were of Analytical Reagent (AR) grade chemicals obtained from M/s. Sigma Chemicals, India. The reference strain *Lactobacillus rhamnosus* MTCC 1408 used for microbiological assay was bought from Microbial type culture collection centre and GeneBank (MTCC), Chandigarh, India.

**2.1. Isolation of bacterial strains**

Various sources such as milk, fermented foods like kambu koozh, idly batter, curd, butter milk and butter and plant sources like banana, tomato, leafy vegetable (*Sesbania grandiflora*) were used for isolation of lactic acid bacteria. The samples were serially diluted to $10^{-2}$ and $10^{-3}$ using sterile peptone water and spread and pour plated on MRS agar plates, incubated at 37˚C for 48 hrs. The dilutions of samples from dairy source was spread plated on *Streptococococus thermophillus* isolation agar and incubated at 37˚C for 2 days. After incubation the colonies formed on agar plates were streaked repeatedly to get purified colonies and the morphological characters were recorded (Table 1).

**2.2. Screening for lactic acid bacteria by biochemical studies**

The isolates were primarily screened according to their colony morphology, catalase, oxidase and gram reaction (Aneja, 2007) [1]. The gram positive, oxidase negative and catalase negative isolates were selected for further studies. The other biochemical test such as phenol red carbohydrate fermentation test was performed. To find homo-fermentative or hetero-fermentative nature of isolates production of gas from glucose test was performed. (Aswathy *et al*., 2008) [3].

**2.3. Characterization of isolates using molecular techniques**

The genomic DNA of 17 selected isolates was extracted using CTAB method and amplified using 16S rRNA universal primer i.e., FD1(5'AGA GTT TGA TCC TGG CTC AG 3') and RP2 (5'ACG GCT ACC TTG TTA CCA CTT 3') and was also amplified using lactic acid bacterial specific primer i.e., forward primer Lac1 (5'-AGC AGT AGG GAA TCT TCC A-3') and reverse primer Lac2 (5'- CAT GTG TAG CGG TGR AAT -3') (Walter *et al*., 2001) [22]. Agarose gel electrophoresis was performed to check the quality of DNA and also to separate the PCR amplified products and the banding pattern was analyzed and documented using InGenius (Syngene, UK) documentation and analysis system.

**2.4. Estimation of lactic acid production**

Fresh culture was inoculated in broth and incubated at 37˚C for 24 hours. After incubation 10ml of broth culture was centrifuged at 8000 rpm for 15 min at 4˚C and supernatant was used for quantification of organic acid produced by the isolates. Titremetrical estimation was performed against 0.1N NaOH using phenolphthalein as indicator. In accordance to the standards of A.O.A.C., 1ml of 0.1N NaOH is equivalent to 90.08mg of lactic acid and the amount of lactic acid produced by the isolates were calculated accordingly (Gowri Sukumar *et al*., 2010) [21].

**2.5. Screening for folate producing isolates**

The selected isolates were screened for its folate producing ability by growing them in folate free FACM medium (folic acid casei medium). Overnight cultures of LAB grown in MRS broth was centrifuged and the cell pellets were washed 3 times with 0.85% saline solution and re-suspended in this solution at the original culture volume then 2% of culture suspension was inoculated in fresh FACM and incubated for 18 hours at 37˚C. Washing and re-suspension step was repeated (to deplete the folate residues) for 7 times. The cultures showing good growth (by increased turbidity) were used for further studies (Laino *et al*., 2012) [13].

**2.6. Extraction of folate from folate autotrophs**

A sample (500µl) of fresh LAB grown in FACM (seven times sub-cultured) was taken and 500µl of protecting buffer (0.1 mol/L phosphate buffer, pH 6.8, containing 1% (m/v) ascorbic acid to prevent vitamin oxidation and degradation) was added and mixed well, followed by immediate centrifugation for 10 min at 10000 rpm. The supernatant was collected and considered as extracellular folate then the cell pellet was again re-suspended in 500µl of protecting buffer which is considered as intracellular folate sample. Both samples were then boiled (100 °C) for 5 min then centrifuged at 14000 rpm for 10 minutes. After centrifugation supernatant was collected and stored at –20 °C until used for folate determination (Laino *et al*., 2012) [13].

**2.7. 16S rRNA gene sequencing and phylogeny of LAB isolates**

**7** LAB isolates showing good growth in FACM medium were further characterized using 16S rRNA gene sequencing. Similarity search of 16S rRNA gene sequence was performed against GenBank database.

**2.8. Milk coagulation test**

The selected folate producing isolates was inoculated in sterilized milk and incubated at room temperature to examine its curdling property. Observation for coagulation of milk was recorded at 12, 24 and 36 hours. The LAB isolates which has ability to convert milk into curd (coagulate milk) was used for further studies.

**2.9. Folate determination**

Folate was determined by modified microbiological assay by using folate auxotrophic organism *Lactobacilllus rhamnosus* MTCC 1408 as indicator organism (Horne and Patterson, 1988) [12]. The reference culture was grown in MRS broth and fresh culture was washed thrice with 0.85% saline solution. 2-4% of cell suspension was inoculated in FACM containing 0.3µg of folate/litre and incubated at 37˚C for 24 hours and second subculture was used for folate determination.

The frozen samples of folate extracted from selected LAB isolates were thawed and processed in reduced light conditions and diluted 40 folds with protection buffer (0.1 mol/L phosphate buffer, pH 6.8, containing 1% (m/v) ascorbic acid) and 100µl of each sample was placed in 1 well of 96 well microplate. The folate auxotroph *Lactobacilllus rhamnosus* MTCC 1408 (reference strain) 4% was inoculated in 10 ml of 2×FACM containing chloramphenicol (20µg/ml- in order to decrease other microbial contamination) and 100µl of this inoculated media was added to each well and mixed well. Un-inoculated samples were taken as control. The folate concentration of each sample was estimated in triplicate. Instead of samples chemical folic acid was prepared in different concentrations i.e., 0.2, 0.4, 0.6, 0.8 and 1ng/ml diluted in protection buffer and 100µl of it was placed in each well along the reference strain. Then the microtitre plate was covered with sterile plate covers and incubated at 37˚C for 48 hours. The optical density (OD) was measured at 580nm using a microtitre plate reader after incubation period. The

standard curve was realized using the values obtained from the different concentrations of chemical folic acid and the final folate concentrations of samples was obtained by multiplying the values obtained from the standard curve with dilution factor, expressed in micrograms per litre (Laino *et al*., 2012) [13]. Three best folate producing strains were selected and used for further studies.

## 3. Results

### 3.1. Isolation and characterisation of lactic acid bacteria

In total 49 isolates were isolated among them 17 isolates were tentatively identified as LAB as they show the characters similar to that of lactic acid bacteria i.e., gram positive, catalase negative and oxidase negative. The biochemical characterization results are as given in the Table 1.

All 17 DNA samples of isolates got amplified by universal primer but only 16 DNA samples of the isolates got amplified by specific primer of lactic acid bacteria (Fig.1) which indicates that 16 isolates were lactic acid bacteria.

All the 16 isolates were found to produce varying concentrations of lactic acid and was estimated by titrating against 0.1N NaOH and calculated according to the A.O.A.C. standards (Table 2). Isolate LAB 04 produced maximum lactic acid i.e., 1.540 gm/10 ml of supernatant compared to other isolates.

**Table 1:** Morphological and biochemical characterization of lactic acid bacteria

| S. No. | Isolate | Colony morphology | Gram reaction | Catalase test | Oxidase test | Carbohydrate fermentation test | | | | | | Production of gas from glucose (heterofermentative or homofermentative) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Glucose | Lactose | Fructose | Sucrose | Maltose | Galactose | |
| 1. | LAB 02 | Circular, flat, punctiform, entire, smooth and slimy white colonies. | + | - | - | + | - | + | +* | +* | - | + |
| 2. | LAB 03 | Circular, raised, small, entire, smooth and creamy white colonies. | + | - | - | + | + | + | + | + | - | - |
| 3. | LAB 04 | Circular, raised, punctiform, entire, smooth, slimy white colonies. | + | - | - | + | - | + | +* | +* | + | + |
| 4. | LAB 06 | Circular, raised, punctiform, entire, glistering and slimy white colonies. | + | - | - | + | + | + | + | + | + | - |
| 5. | LAB 07 | Circular, flat, small, entire, glistering and slimy cream coloured colonies. | + | - | - | +* | + | + | + | +* | - | + |
| 6. | LAB 08 | Circular, raised, small, entire, glistering, slimy white colonies. | + | - | - | +* | +* | + | + | +* | + | + |
| 7. | LAB 09 | Circular, flat, small, entire, glistering and slimy white colonies. | + | - | - | +* | + | + | - | +* | - | + |
| 8. | LAB 28 | Circular, raised, smooth, white coloured colonies. | + | - | - | + | + | + | + | + | + | - |
| 9. | LAB 29 | Circular, raised, smooth, white coloured colonies. | + | - | - | + | + | + | + | + | + | - |
| 10. | LAB 31 | Circular, flat, medium sized, entire, dull and light brown colour colonies. | + | - | - | + | + | + | +* | + | + | + |
| 11. | LAB 33 | Circular, convex, punctiform, entire, smooth creamy white coloured colonies. | + | - | - | - | + | - | - | - | - | - |
| 12. | LAB 35 | Circular, flat, medium sized, entire, glistering, light brown colour colonies. | + | - | - | + | + | + | + | + | + | + |
| 13. | LAB 36 | Circular, flat, small, entire, glistering, creamy white coloured colonies. | + | - | - | + | + | + | +* | + | - | + |
| 14. | LAB 40 | Circular, raised, punctiform, entire, glistering and slimy white coloured colonies. | + | - | - | + | + | + | + | + | + | - |
| 15. | LAB 41 | Circular, raised, punctiform, entire, smooth, white coloured colonies. | + | - | - | + | + | + | + | + | - | + |
| 16. | LAB 45 | Circular, raised, smooth, white coloured colonies. | + | - | - | + | + | + | + | + | + | - |
| 17. | LAB 49 | Circular, raised, small, entire, glistering, slimy white colonies. | + | - | - | + | + | + | + | + | - | - |

LAB: Lactic acid bacteria
+ : positive -: negative
+* - Gas production

**Table 2:** Quantitative estimation of lactic acid production

| Strain | Lactic acid (gm/10 ml of 24 hrs supernatant) |
|---|---|
| LAB 02 | 0.882±0.006 |
| LAB 04 | 1.540±0.003 |
| LAB 06 | 1.432±0.025 |
| LAB 07 | 0.963±0.006 |

| | |
|---|---|
| LAB 08 | 1.071±0.012 |
| LAB 09 | 0.846±0.006 |
| LAB 28 | 1.504±0.007 |
| LAB 29 | 1.504±0.011 |
| LAB 31 | 0.945±0.003 |
| LAB 33 | 0.783±0.013 |
| LAB 35 | 0.927±0.006 |
| LAB 36 | 0.918±0.016 |
| LAB 40 | 1.459±0.006 |
| LAB 41 | 0.810± 0.013 |
| LAB 45 | 1.531± 0.007 |
| LAB 49 | 1.531±0.018 |

± represents standard deviation

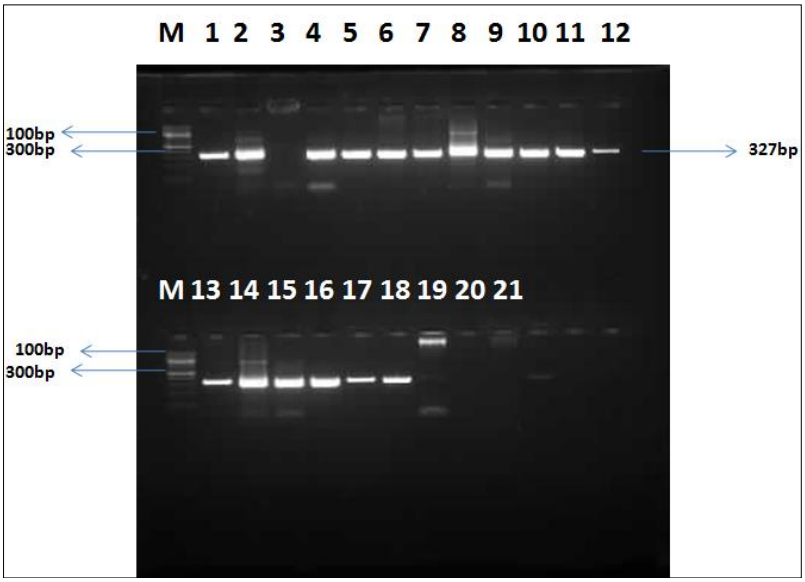


**Fig 1:** Specific primer amplification of DNA of LAB isolates.
Lane M: 100bp DNA ladder Lane 1: MTCC1408, Lane 2: LAB02, Lane 3: LAB 03, Lane 4: LAB04, Lane 5: LAB06, Lane 6: LAB07, Lane 7: LAB08, Lane 8: LAB09, Lane 9: LAB28, Lane 10: LAB29, Lane 11: LAB31, Lane 12: LAB33, Lane 13: LAB35, Lane 14: LAB36, Lane 15: LAB40, Lane 16: LAB41, Lane 17: LAB45, Lane 18: LAB49, Lane 19, 20, 21: Negative control

### 3.2. Screening for folate producing isolates and folate extraction

LAB isolates were screened for their folate producing ability by growing them repeatedly in FACM (folate free medium). Folate was extracted from the 7 isolates which showed good growth (maximum turbidity) and stored at -20˚C for further studies.

### 3.3. 16S rRNA gene sequencing and phylogeny of folate producing LAB isolates

The nearly full length of 16SrRNA gene of selected folate producing LAB was amplified using FD1 and RP2 primers and sequenced. The molecular identification of 7 isolates was given in Table no.3.

**Table 3:** Molecular identification of Lactic acid bacterial isolates

| Isolate | Homologous microorganism | Accession no. of homologous microorganism | Identity % |
|---|---|---|---|
| LAB 07 | *Lactobacillus fermentum* | CP021964.1 | 99.19% |
| LAB 09 | *Lactobacillus fermentum* | JF414110.1 | 99.80% |
| LAB 28 | *Lactobacillus plantarum* | KM485570.1 | 99.32% |
| LAB 29 | *Lactobacillus plantarum* | CP031140.1 | 99.38% |
| LAB 33 | *Streptococcus thermophillus* | CP029252.1 | 99.24% |
| LAB 36 | *Lactobacillus fermentum* | JX272057.1 | 99.46% |
| LAB 45 | *Lactobacillus plantarum* | EU419599.1. | 99.39% |

### 3.4. Milk coagulation test

The observation for coagulation of milk showed that 5 out 7 LAB isolates has milk coagulation property. LAB 33 coagulated milk within 12 hours of incubation and other isolates LAB 07, LAB 28, LAB 29, LAB 45 coagulated milk after 36 hours of incubation.

### 3.5. Determination of folate

The folate produced by isolates was determined by microbiological assay. The standard curve was realized using the values obtained from the different concentrations of chemical folic acid. The extra and intracellular folate concentrations of samples was obtained by multiplying the

values obtained from the standard curve with dilution factor, from which the total folate was estimated and expressed in micrograms per litre. Among 5 isolates the highest folate producer was LAB 33 (76.078µg/L) followed by LAB 28 (69.804), LAB 29 (68.558), LAB 45 (64.725) and LAB 07 (48.451) was the least folate producer (Table 4).

**Table 4:** Determination of folate produced by isolates in folate free culture medium

| S. No. | Isolate | Folate concentration (µg/L) | | |
|---|---|---|---|---|
| | | Extracellular folate | Intracellular folate | Total folate |
| 1. | LAB 07 | 15.51 | 32.941 | 48.451 |
| 2. | LAB 28 | 35.294 | 34.51 | 69.804 |
| 3. | LAB 29 | 35.451 | 33.137 | 68.588 |
| 4. | LAB 33 | 36.333 | 39.745 | 76.078 |
| 5. | LAB 45 | 29.961 | 34.764 | 64.725 |

## 4. Discussion

In the present study lactic acid bacteria was isolated from dairy, plant sources and from fermented foods. The biochemical characters such as gram positive, catalase negative, ferment carbohydrates was authenticated as in Bergey's manual of Determinative Bacteriology (Edition no.7). Walter *et al*., (2000) [20] used Denaturing gradient gel electrophoresis (DGGE) of DNA fragments generated by PCR with 16S ribosomal DNA targeted group specific primers with GC-clamp to detect lactic acid bacteria of different genera like *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella*. But in this study we used the same specific primer without GC-clamp, 16 out of 17 isolates got amplified which was analysed by agarose gel electrophoresis. Laino *et al*., (2012) [13] isolated *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* from artisanal Argentinean yogurts evaluated for intracellular and extracellular folate production by growing them in folate-free culture medium (FACM). They found that strains *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 863 and *Streptococcus thermophilus* CRL 415 and CRL 803 produced highest extracellular folate levels i.e., 22.3 to 135 µg/L in FACM which was determined by microbiological assay. Similarly in our study the folate autotrophs was screened by growing them in folate free medium and folate was extracted and estimated by microbiological assay. The results showed that among 5 isolates the highest extracellular folate producer was LAB29 (47.161µg/L) and the highest intracellular folate producer was isolate LAB 28 (44.405 µg/L) and LAB 33 (76.078µg/L) was highest total folate producer. In contradictory to microbiological assay Bhagya *et al*., (2018) [5] screened for extracellular folate production and folate concentration of 64 lactic acid bacteria and was determined by using HPLC. In our study folate producing lactic acid bacteria genera such as *Lactobacillus*, *Streptococcus* was studied wereas Pompei *et al*., (2006) [17] investigated the folate producing ability of 76 *Bifidobacterium* strains by culturing them in the folate free semi synthetic medium SM7 and found that most of the *Bifidobacterium* strains needed folate for their growth and concluded that concluded that 6 strains out of 17 produced higher concentrations (41 to 82ng/ml) of vitamin which was analysed by microbiological assay.

## 5. Conclusion

The results shows that lactic acid bacterial strains isolated in our study are folate autotrophs and could be used as starter cultures in dairy industries to produce novel bioenriched foods. The use of folate producing strains as starter cultures in fermented foods could be able to increase the folate levels without the need for chemical fortifications and genetic engineering techniques. The natural folates produced by these strains are most effective and economically viable alternative tool to overcome folate deficiencies.

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