



Isolation, Biochemical Characterization and Screening of Folate Producing Potential Starter Culture from Pigeon Pea and Brown Rice

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ABSTRACT

One essential B vitamin that is needed in numerous metabolic pathways is folate. Isolation and characterisation of lactic acid bacteria (LAB) from traditional fermented legumes and cereals was conducted in order to identify suitable strains capable of producing high folate as starter cultures. By adding methotrexate, a folate analogue, to the isolation medium, the folate-producing microbes were separated. The isolated bacteria underwent morphological, biochemical, and phenotypic characterization. They were also evaluated for folate production in a culture medium free of folate (Folic Acid Casei Medium (FACM)), and during milk fermentation. While the 3-aminophenol spectrophotometric approach was used to quantify the synthesis of folate, phenotypic characterisation was carried out using API 50 CHL. The isolates were compared with microbial strains procured from gene bank (USDA); *Lactobacillus delbruckii* sub. *bulgaricus* (B59387) and *Lactobacillus plantarum* (B1848). From the results obtained, 7 isolates that were capable of synthesising folate from the fermented raw materials were obtained and based on phenotypic characterization; they were identified as *L. plantarum*, *L. brevis*, *L. acidophilus*, *Lactococcus* and *Streptococcus* species. Four strains out of the nine microbial strains grew in FACM. Total folate content varied between 1.90 to 86.2 µg/ml among the strains. The strains significantly ($p < 0.05$) increased the folate concentration in fermented milk by triple of their initial values. Microorganisms isolated from fermented pigeon pea and rice produced low folate amount in comparison to the microbial strains procured from the gene bank. This study shows that microorganism isolated from fermented brown rice and pigeon pea can synthesize folate in growth media as well as fermented milk. Utilizing them can help prevent folate insufficiency through preventative methods or intervention research. Food manufacturers could leverage the study's findings to create fermented foods with enhanced folate content, providing consumers with a nutritious and healthy option.

Keywords: Folate, Lactic acid bacteria, Nutritional deficiency, Fermentation.

INTRODUCTION

A water-soluble vitamin that is vital to the B group family, folate (also known as folic acid or vitamin B9) has a recommended daily intake of 200–400 µg. It is essential for the synthesis of methylation, nucleic acids, and certain proteins and amino acids needed for human growth and replication (Jacob, 2000; Lucock, 2000). According to Rahman et al. (2015), folate plays a major part in the process of protein production, ribonucleic acid (DNA) and deoxyribonucleic acid (RNA) digestion, and the growth of new cells. Folate is also necessary for appropriate fetal growth and development in order to reduce the risk of neural tube abnormalities, which are birth disorders affecting the brain and/or spinal cord. To avoid nutritional deficiency, folate must be obtained externally because humans are unable to manufacture it (Kariluoto et al., 2014). Folate deficiency is a public health concern that significantly impacts expectant mothers and inexorably affects the fetus's development. By mandating the folic acid fortification of cereal products, a number of nations have worked to guarantee adequate folate intake and prevent the illnesses associated with folate insufficiency (Food fortification initiative, 2015). In most countries, food fortification programs are not always efficient since there aren't enough control systems, according to Youngblood et al. (2013). However, because of its possible negative consequences, folic acid has not been adopted by many nations as part of a National Fortification Program (Laino et al., 2013). Among other things, excessive folic acid absorption—the chemically synthesized version of folate—may conceal the signs of vitamin B12 insufficiency, which could lead to irreversible neuropathy development (FAO/WHO, 2005). Populations in danger, such as youngsters and pregnant mothers, may not be able to obtain enough folate through tablets and their regular meals due to the aforementioned circumstances. Given the risks

associated with folic acid enrichment, an increasing number of researchers are looking into enriching foods with naturally occurring forms of folate instead (Iyer et al., 2009; Leblanc et al., 2007). Conversely, natural forms of folate, including those present in food or synthesized by specific microbes, do not negatively impact an individual's health, even in cases where excessive consumption occurs (Laino et al., 2013). According to numerous researches, certain strains of lactic acid bacteria (LAB) used as starting cultures in fermentation process have the ability to produce, release, and/or enhance B-group vitamins (Levit et al., 2021; Ngene et al., 2019; Laino et al., 2013; Laino et al., 2012; Padalino et al., 2012; Iyer et al., 2010; Kariluoto et al., 2014; Crittenden et al., 2003; Lin & Young, 2000). In order to produce meals fortified with natural folate through fermentation, the use of lactic acid bacteria strains that produce folate so appears as an alternative (Laino et al., 2013).

MATERIAL AND METHODS

Procurement of Raw Material

Brown rice was purchased from the National Cereal Research Institute in Badeggi Bida, Niger State, and red pigeon pea (*Cajanus cajan* L.) was obtained from Ogige market in Nsukka LGA of Enugu state, Nigeria. After being cleaned, all the materials were placed in jute bags and kept at room temperature ($30\pm 2^{\circ}\text{C}$) until needed. The Agricultural Research Service (ARS) Culture Collection Center, USA, and the United States Department of Agriculture (USDA) provided *L. delbrueckii* sub. *bulgaricus* (B59387) and *L. plantarum* (B1848).

Preparation of Samples

Microorganisms and Growth Conditions

Along with the isolated microorganisms from fermented rice and pigeon pea, the lactic acid bacteria obtained from USDA, *Lactobacillus delbrueckii* sub. *bulgaricus* (B59387) and *Lactobacillus plantarum*, (B1848) were preserved at -20°C in a blend of De Man-Rogosa-Sharpe (MRS) broth (De Man Rogosa Sharpe, 1960) and glycerol (20%). The isolates were revived in MRSB and incubated at 37°C for 24 hours prior to use.

Fermentation of Pigeon Pea and Brown Rice

To enable fermentation, the brown rice and red pigeon pea samples were washed and immersed in distilled water in two separate containers for three days at room temperature. The fermented samples were brought to the laboratory for additional examination.

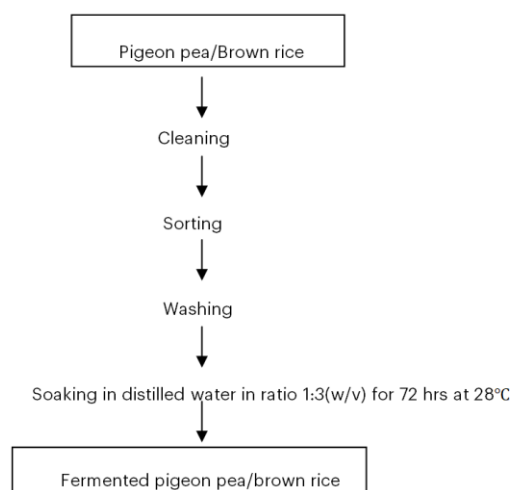


Figure 1: Flowchart for the fermentation of pigeon pea and brown rice

Isolation of Folate-Producing Microorganisms

Using the pour plate approach as outlined by Harrigan and McCance (1976), bacteria were isolated from the fermenting raw materials. Test tubes were cleaned, and then nine milliliters (9 ml) of distilled water were added. The test tubes were autoclaved for 15 minutes at 121°C and left to cool. To isolate lactic acid bacteria, deMan Rogosa and Sharpe (MRS) agar were used while malt extract agar was used for yeasts. One milliliter of the fermenting materials were diluted serially using distilled water, and thereafter it was pour plated. After cooling to around 40°C, the media was aseptically supplemented with 50 mg/ml of methotrexate, an analogue of folic acid. This is to filter the samples for bacteria that produce folate. For 48 hours, lactic acid bacteria and yeasts were incubated at 30± 5°C. Following incubation, the formation of different colonies was monitored on the plates. To acquire pure culture, these colonies were counted, randomly picked, and streaked.

Phenotypic Characterization of the Lab and Yeast Isolates

The examination of morphological and biochemical characteristics as reported by Harrigan and McCance (1976) and Kurtzman et al. (2011), respectively, was used to characterize the LAB and yeast isolates. Using the Genera of Lactic Acid Bacteria (Wood and Holzapel, 1995), the presumed identification of lactic acid bacteria isolates was carried out.

Biochemical Characterization

The production of gas, the capacity to grow at various temperatures, pH, and salt tolerance, as well as other fundamental biochemical characteristics was measured. After a 48-hour incubation period, gas generation was monitored by utilizing inverted Durham's tubes to look at the gas that formed from glucose in MRS broth. Visual evaluation was conducted after 24, 48, and 72 hours of incubation to assess the isolated bacteria's growth capacity at various temperatures (15, 37, and 45°C). In addition, growth was assessed in MRS agar at pH 4.5, 6.5, and 9.6. The pH was altered using HCl and NaOH, respectively. MRS broth with 2, 3, and 4% (w/v) sodium chloride (NaCl) was used to test salt tolerance, and the cultures were incubated for 72 hours at 37 degrees Celsius.

Folate Production Screening and Quantification in Culture Medium

The Ruengsitagoon and Hattanat (2012) method was used to screen the acquired isolates for folate synthesis. To put it succinctly, the isolates were cultured in their corresponding broths and incubated for 48 hours. The broth was then centrifuged for 20 minutes at 3000 g. When one milliliter of the supernatant was combined with one milliliter of 4 mol·L⁻¹ hydrochloric acid, one milliliter of sodium nitrate (1% w/v), one milliliter of sulfamic acid (1% w/v), and one milliliter of 3-amino phenol (1% w/v), an orange-yellow complex was produced, signifying the presence of folate. Using a UV visible spectrophotometer (Jenway, Essex, UK), the complexation's absorbance was measured at 460 nm. Using a standard curve created with the absorbance of folic acid at a given concentration, the amount of folate present in the samples was calculated. By introducing the isolates into Folic Acid Casei Medium, the isolates' capacity to produce folate was further assessed. As per Kodi et al. (2015), Folic Acid Casei Medium (FACM) is an assay medium that includes all the necessary nutrients and substances needed for the test organism's growth, with the exception of folic acid. Potential manufacturers of folate were the isolates that flourished in this chemically defined medium.

Folate production in fermented milk

Two percent (2%) (w/v) of selected folate-producing bacteria were added into reconstituted non-fat powdered milk, and the mixture was cultured for 24 hours at 37 and 42 °C. Samples were collected at various intervals (0, 6, 8 and 24 hours after inoculation) in order to assess folate synthesis and bacterial growth. A mixture of 500 µL protection buffer and 500 µL milk samples was used to determine the amount of folate. The resultant slurry was centrifuged at 10,000 g for 6 minutes after being heated at 100 °C for 5 minutes to precipitate proteins and liberate folate from milk-binding proteins. Before being utilized for the measurement of total folate, the supernatant was collected and kept at -70°C.

RESULTS & DISCUSSION

Isolation and biochemical characterization of folate producing microorganisms from the natural fermentation of pigeon pea and brown rice

The result for the isolation and biochemical characterization of starter culture from the natural fermentation is presented in Table 1 and Figure 2.

Pigeon pea and brown rice fermentation slurry produced microorganisms that synthesized folate. The development of microbes from the slurry in culture media supplemented with methotrexate (50 mg/ml), a folate analogue, indicates that these isolates are immune to the drug, this was discovered to be the reason of the isolates' ability to produce folate. This could be explained by the fact that these microbes used the folate analogue to produce necessary components including DNA, RNA, and mRNA, which allowed for their growth and replication (Okoroafor et al., 2019). To create a pure culture, the acquired isolates underwent further screening. Seven isolates in all were found from both brown rice and pigeon pea, according to the data.

Biochemical Characteristics

Based on cell shape, gas production from glucose, growth behavior at various temperatures, pH, and salt solutions, the isolates were classified to genus levels. Using the API 50 CHL database, the isolates were further classified to specie levels based on fermentation of carbohydrate. The morphological, physiological, and biochemical examinations revealed a variety of lactic acid bacteria (LAB), categorized according to the genera *Streptococcus*, *Lactobacillus*, and *Lactococcus*. The isolates had a rod- and cocci-shaped appearance, were non-spore-forming, non-motile, gram-positive, and showed negative catalase and oxidase reactions (Table 1). Past research has revealed that *Lactobacillus* species from fermented legumes and cereals are catalase negative and gram positive (Rao et al., 2015; Salvetti et al., 2012).

Since pH plays a major role in bacterial development, sustainability research in various pH values was conducted. Both acidic and alkaline environments were investigated for each strain. According to the results, the bacteria were able to survive in both extremely acidic and alkaline environments (Table 1). *Lactobacillus* isolated from fresh fruits and vegetables survived in pH ranges of 3.5 to 7.0, according to a prior work by Pundir et al. (2013). According to Mannan et al. (2017), pH tolerance is an essential criterion for LAB to proliferate and carry out their advantageous function in the digestive system. To determine how well *Lactobacillus* species tolerated sodium chloride (NaCl), a test was carried out. According to Hoque et al. (2010), NaCl is a hindering chemical that prevents some bacteria from growing. A NaCl tolerance test was performed at doses of 2%, 3%, and 4%. Growth at these concentrations was possible for each isolate.



Figure 2: Morphological characteristics of folate-producing microorganisms from pigeon pea



Figure 3: Morphological characteristics of folate-producing microorganisms from brown rice



Figure 4: Pure culture of bacteria isolates from fermented brown rice after streaking



Figure 5: Pure culture of bacteria isolates from fermented pigeon pea after streaking

Table 1: Physiological and biochemical characteristics of isolated strains

Characteristics	R1	R2	R3	R4	P1	P2	P3
Gram stain	+	+	+	+	+	+	+
Oxidase test	-	-	-	-	-	-	-
Catalase test	-	-	-	-	-	-	-
Indole test	-	-	-	-	-	-	-
Methyl red test	+	+	+	+	-	-	-
Motility test	-	-	-	-	-	-	-
Spore formation	-	-	-	-	-	-	-
Glucose fermentation	+	+	+	+	+	+	+
Lactose fermentation	+	+	+	+	+	+	+
Sorbitol	-	+	+	-	+	+	-
Production of CO ₂ with glucose	-	-	-	-	-	-	-
Growth at temp 15°C	+	+	+	-	+	+	-
Growth at temp 37°C	+	+	+	+	+	+	+
Growth at temp 45°C	-	-	-	+	-	-	-
Growth in a medium with NaCl (2%)	+	+	-	+	+	+	-
Growth in a medium with NaCl (3%)	+	-	+	+	+	+	-
Growth in a medium with NaCl (4%)	+	-	+	+	+	+	+
Growth at pH 4.5	+	+	-	+	+	+	-
Growth at pH 6.5	+	+	+	+	+	+	-
Cellular morphology	Rods	Rods	Cocci	Rods	Rods	Rods	Cocci
Probable organism	<i>L. casei</i>	<i>L. brevis</i>	<i>Lactococcus</i>	<i>L. acidophilus</i>	<i>L. plantarum</i>	<i>L. plantarum</i>	<i>Streptococcus</i>

Growth of Lactobacillus Strains in Folate Free Culture Medium (FACM)

Table 2 and Figure 4 and 5 show the growth of lactobacillus strain in FACM.

Natural folate can be synthesized by certain strains of LAB, according to research. Former researchers have reported that some LAB, including *L. acidophilus*, *L. plantarum*, *L. reuteri*, *Leuconostoc lactis*, *Propioni bacterium*, and *Bifidobacterium longum*, have the capacity to generate this vitamin from the B group in addition to some industrially important species, such as *Lactococcus lactis* and *S. thermophiles* (Crittenden et al., 2003; Gangadharan et al., 2010; LeBlanc et al., 2011; Lin and Young, 2000; Pompei et al., 2007).

But it's important to clarify that while some species can manufacture folate; this is a strain-dependent characteristic, meaning that finding strains that produce sufficient levels of this important vitamin requires careful strain selection.

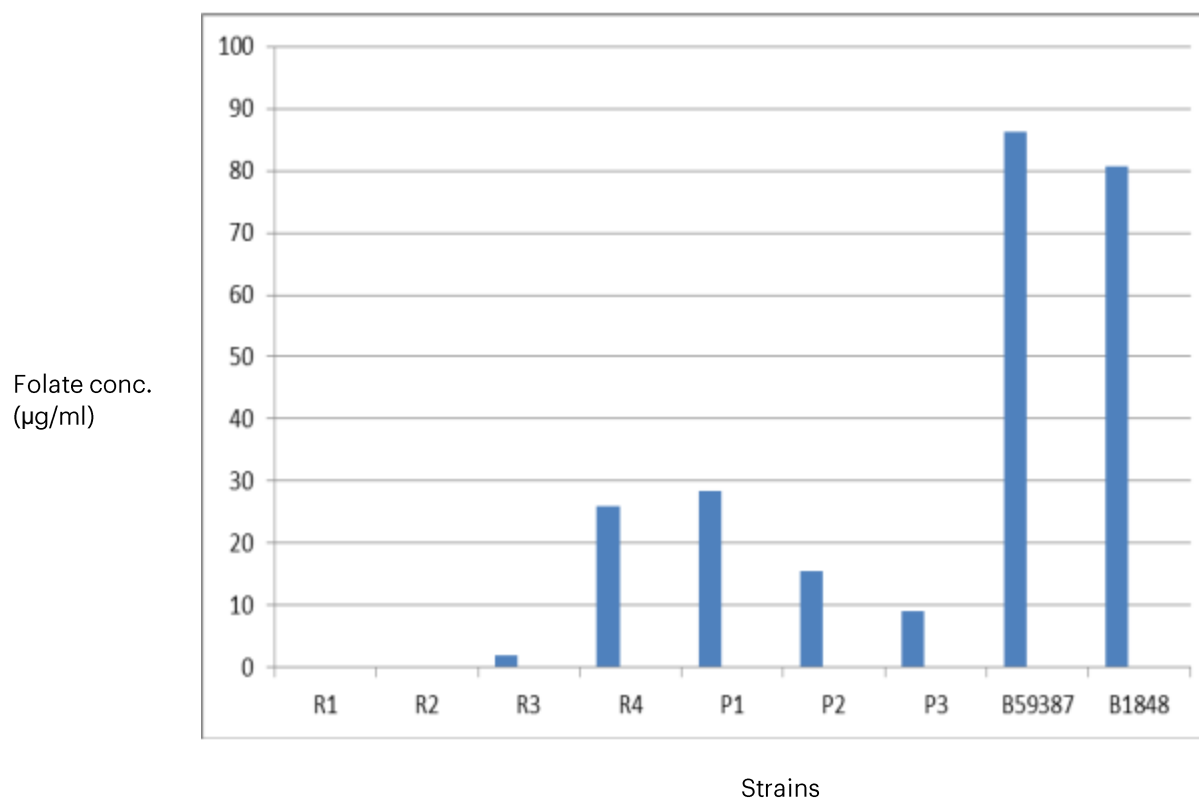
Only four of the seven strains obtained from fermented pigeon pea and brown rice were able to grow after seven subcultures in the folate-free culture medium. It was shown that strains R3 (*Lactococcus*), R4 (*Lactobacillus acidophilus*), P1 (*Lactobacillus plantarum*), P2 (*Lactobacillus plantarum*), P3 (*Streptococcus*), B59387 (*Lactobacillus*

delbrucki sub. *bulgaricus*), and B1848 (*Lactobacillus plantarum*) were capable of increasing the concentrations of folate in the culture media, but that effect was strain-dependent; that is, certain strains produced significant concentrations of the vitamin, while others produced hardly perceptible levels (Table 2 and Figure 4 and 5). No discernible growth was observed in the culture media for strains R1 (*Lactobacillus casei*) and R2 (*Lactobacillus brevis*). Though some strains of bacteria may not require folate for growth, Laino et al. (2012) stated that, growth in the absence of folate does not indicate that these microorganisms manufacture it. Nevertheless, it is a useful indicator and a useful tool for identifying possible LAB that synthesise folate. The range of total folate levels in the strains was 1.90 to 86.2 µg/ml. Isolate R3 (*Lactococcus*) produced the least amount of folate among the lactic acid bacteria, producing 1.90 µg/ml, while strain B59387 (*Lactobacillus delbrucki* sub. *bulgaricus*) produced the most, at 86.2 µg/ml (Table 2). The microbial strains obtained from the gene bank produced more folate than the bacteria acquired from the fermented raw materials. Despite the fact that it is usually challenging to equate data on entire folate content of LAB with current literatures as a result of variations in the procedure and in culture media used in various research, the entire folate concentrations obtained in this study are in the same range as previously reported folate concentrations (10.98 to 30.89 µg/l) produced by LAB isolated from fermented maize. (Okoroafor et al., 2019). From the results obtained, the maximum folate production in MRS medium among the isolated organisms from the fermented raw material was found to be *L. plantarum* P1. *L. fermentum* and *L. plantarum*, produced a folate concentration of 110 µg/ml and 120 µg/ml respectively in MRS medium in Burkina Faso's ben salaaga, an African grain gruel. (Greppi et al., 2017). In a different investigation, *Lactobacillus plantarum* and *Lactococcus* sp. isolated from Malaysian tapai ubi generated, in MRS media, 8.608µg/L and 10.71µg/L, respectively (Nor et al., 2008). Furthermore, strains of 36 LAB grown in a medium that is free of folate produced extremely high amounts of extracellular folate, whilst other strains produced very little and were almost negligible (Laino et al., 2012). According to various research (Laino et al., 2012; Greppi et al., 2017), strain dependence may be the cause of the variations in folate concentrations generated by bacteria in culture medium. Furthermore, the differences could be attributed to the techniques used in these investigations, since spectrophotometric and high-performance liquid chromatography (HPLC) methods typically yield more precise quantification of folate concentrations than do microbiological assay and ultra HPLC methods. This is because high-performance liquid chromatography and ultra-high-liquid chromatography aim for the particular folate generated rather than the raw vitamins detected using the spectrophotometric and microbiological assay methods (Kariluoto et al., 2004; Kariluoto et al., 2014). This is caused by differences in the sensitivity of the procedures.

Table 2: Growth of *Lactobacillus* strains in folate- free culture medium and folate production

Strains	Reaction	Folate (µg/ml)
R1	-	ND
R2	-	ND
R3	+	1.90
R4	+	25.89
P1	+	28.49
P2	+	15.45
P3	+	9.1
B59387	+	86.2
B1848	+	80.7

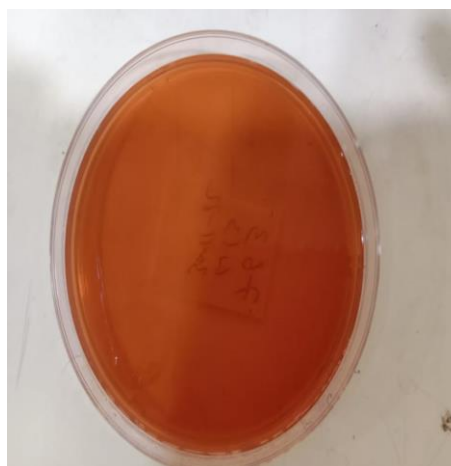
Presence (+) or absence (-) of growth in Folic Acid Casei Medium; ND indicates that no folate were detected
 CODES: R1-R4: folate-producing bacteria isolates from brown rice; P1-P3: folate-producing bacteria isolates from pigeon pea ; B59387: *L. delbrucki* sub. *bulgaricus*; B1848: *L. plantarum*



CODES: R1-R4: folate-producing bacteria isolates from brown rice; P1-P3: folate-producing bacteria isolates from pigeon pea ; B59387: *L. delbruki* sub. *bulgaricus*; B1848: *L. plantarum*
 Fig 5: Growth in folate-free culture medium (FACM) for 16 h @ 37° C



Figure 6: Growth in FACM



No growth seen in FACM

Production of Folate in Fermented Milk

Strain production of folate in fermented milk is shown in Figure 6 and 7.

Although milk is thought to be a nutritious food, it is not a recommended dietary source of folate (Laino et al., 2012). Milk only has 20-30 µg/L of folate, according to a research study by LeBlanc et al. (2007). However, a lot of dairy products, like yoghurt, are made by microbial fermentations, which may result in the production of folate. From the

results obtained, strains B59387, B1848, R4, and P1 were chosen based on their increased vitamin synthesis in FACM. The synthesis of folate of the four chosen lactic acid bacteria strains was assessed in fermented milk at various intervals after development in an incubator at 37 and 42°C. The findings revealed that, at 37°C, *L. plantarum* (B1848) increased the amount of folate significantly ($p < 0.05$) to approximately 30 percent following 24 hours of growth in an incubator whereas *L. delbrueckii* subsp. *bulgaricus* (B59387) increased the quantity of folate to approximately 25 percent following 24 hours of fermentation, in comparison with the beginning values. This indicates that the lactic acid bacteria were capable of synthesizing folate rather than consuming the vitamin. The results showed that the maximum folate concentration was achieved after 6 hours of fermentation, followed by 24 hours. *L. delbrueckii* subsp. *bulgaricus* (B59387) strain exhibited significantly, dissimilar behavior at 42°C incubation temperature compared to 37°C. Following a 6-hour fermentation period, B59387 raised the folate concentration of the milk to approximately 40% and then brought it back to the initial levels after a 24-hour period. This decrease may have occurred because the microorganisms responsible for producing folate began consuming it, as they required it for growth and metabolism (Gangadharan and Nampoothiri, 2011; Kundu and Deep, 2014). The results of Laino et al. (2012), who observed that the production of folate by a folate-producing starter culture peaked after 24 hours of fermentation, are consistent with the results gotten. For each of the strain, the same pattern was noted. Results showed that the presence of folate in milk had no effect on folate production by the strains. This is extremely important since certain strains have the ability to increase the quantity of folate in meals beyond what the bacteria require to grow. Higher amounts of folate in fermented milk have been linked to the production of folate by lactic acid bacteria, including *Lactobacillus delbrueckii* var. *bulgaricus* and *Lactococcus* sp. (Laino et al., 2012). The strains in the culture broth (FACM) produced significantly less folate than those in fermented milk, indicating that the presence of folate precursors or the makeup of the food matrix determine how much folate may be produced (Greppi et al., 2017). According to guidelines from the World Health Organization, adults should ingest 400 µg of folate per day, while children should consume 200 µg (FAO/WHO, 2002). Furthermore, it has been stated that if a meal provides at least 10% to 20% of the daily needed amount of folate, it is deemed to be a rich source of the nutrient (Ohio State University, 2005). In this study, milk products containing more than 80 µg/L of folate were produced by certain strains. As an alternative to using these strains separately, combining them could result in a product that has enough folate to meet the 10% Recommended Dietary Allowance for adults and the 20% Recommended Dietary Allowance for children. According to Okoroafor et al. (2019), the use of mixed starter cultures containing both yeast and lactic acid bacteria that produce folate has been shown to boost the amount of folate in fermented food more than the use of either bacterium or yeast as a single starter.

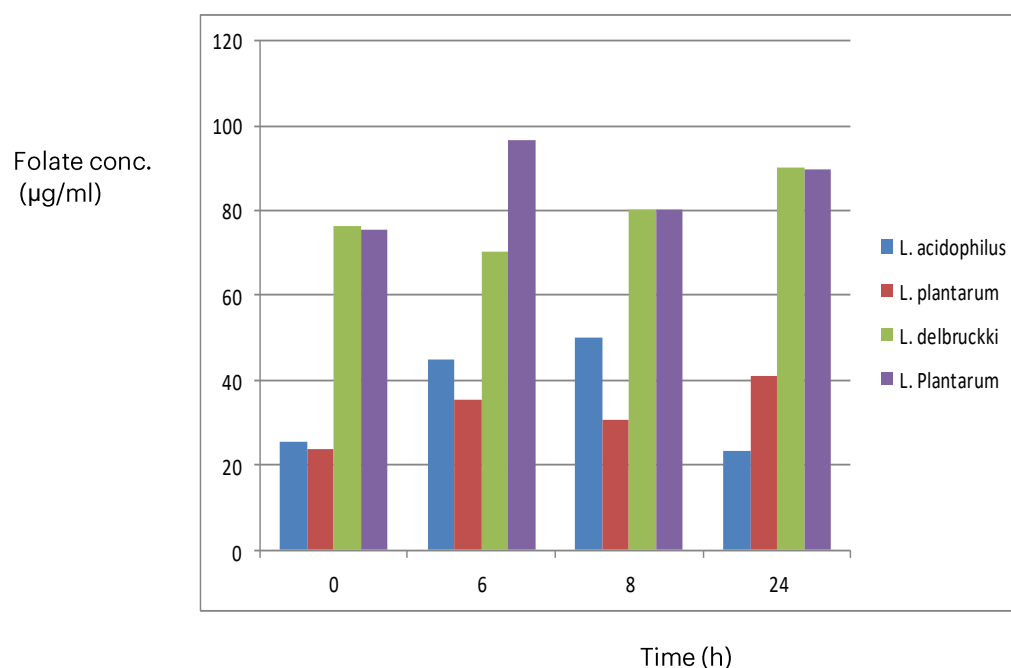


Figure 7: production of folate in fermented milk at 37°C. Values are means \pm standard deviation (SD) of duplicate determinations

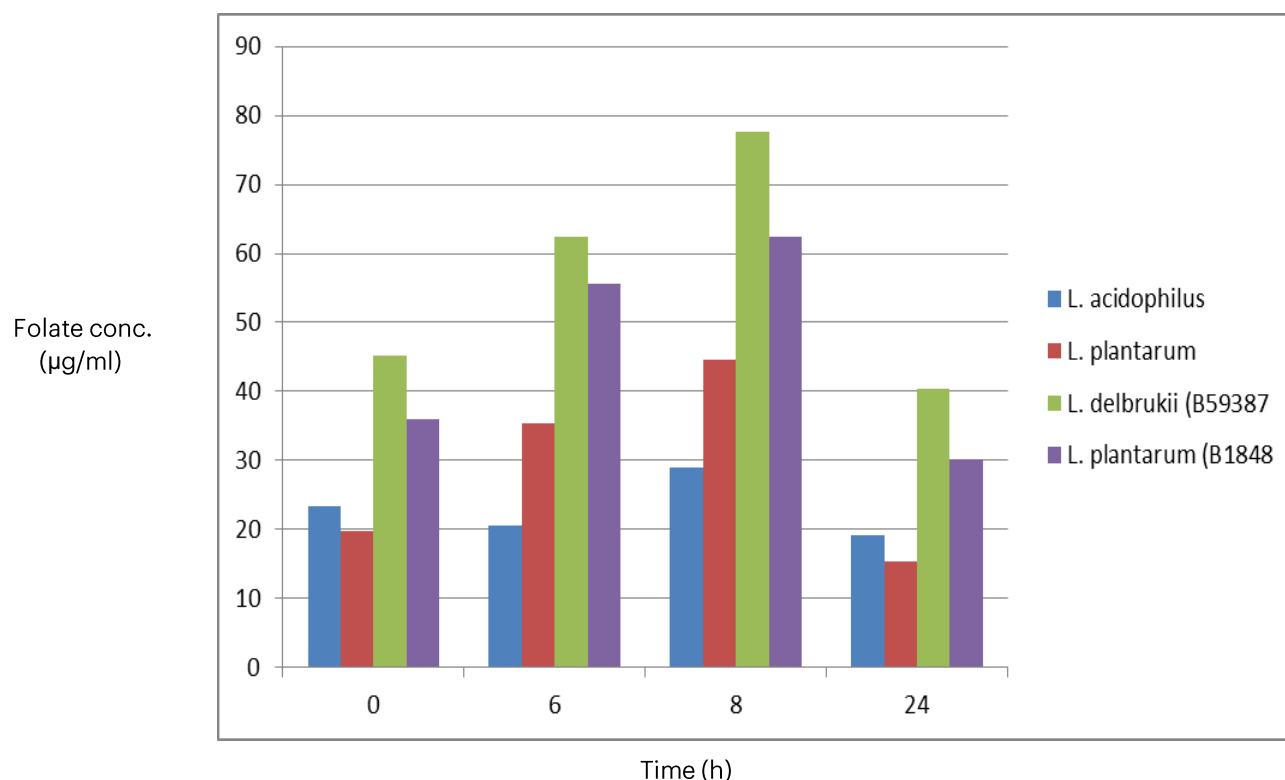


Figure 8: Production of folate in fermented milk at 42°C. Values are means \pm standard deviation (SD) of duplicate determinations

CONCLUSION

This work demonstrates the ability of microorganisms derived from fermented brown rice and pigeon pea to synthesize folate in both fermented milk and culture media. While the microbial strains obtained from the gene bank showed greater folate values, the strains obtained from the fermented raw materials could potentially have a higher folate concentration if folate precursors were added to the growth media and milk. Fermentation time influenced the folate-producing capacity of the strains. In low-income countries, the use of these strains in combination rather than singly as starter cultures for fermented food production could be a way to contribute to daily folate requirements and boost folate intake. Utilizing them can help prevent folate insufficiency through preventative methods or intervention research. Similarly, the chosen microbes can be bioengineered to enhance their folate-producing capabilities, hence increasing the amount of folate in fermented meals.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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