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Folate production in lactic acid bacteria and determination of folate derivatives by HPLC

Laktik asit bakterilerinde folat üretimi ve HPLC ile folat türevlerinin tayini

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Abstract

Folat, insanların sentezleyemedikleri ve dışarıdan alınması gereken B grubu bir vitamindir. Bazı Laktik Asit Bakterileri folat üretebilse de, yoğurt starter kültürlerinin folat üretimi hakkında yeterli bilgi yoktur. Bu çalışmada yoğurt örneklerinden iki Lactobacillus ve iki Streptococcus cinsine ait dört bakteri izole edilmiş ve izolatlar API 50 CHL kiti ile 16S rDNA ile biyokimyasal olarak tanımlanmıştır. Bakterilerin hücre içi ve hücre dışı folat üretimi çeşitli ortam ve tampon uygulamalarında belirlenmiştir. En yüksek hücre içi folat üretimi Lactobacillus delbrueckii subsp. *bulgaricus* ZN951 *(126,3 µg/L) ve Streptococcus thermophilus* Z151 *(177,1 µg/L) suşlarında MRS/Elliker besiortamı ve potasyum fosfat tamponunda belirlenmiştir. L. delbrueckii subsp. bulgaricus* ZN541 *suşu, aynı ortam ve tamponda en yüksek hücre dışı folat üretimini (105,1 µg/L) gösterirken, S. thermophilus* Z151 *suşu, 86,4 µg/L folat üretimi sergilemiştir. Suşların folat türevleri ve pABA içerip içermediği HPLC analizi ile araştırılmış ve suşların hiçbirinde pABA tespit edilememiştir. Kültürler arasında en yüksek toplam folat içeriği 110.5 µg/L olarak L. delbrueckii subsp. bulgaricus ZN951 suşunda tespit edilmiştir. Bu suşun 5-MeTHF ve 5-FmTHF konsantrasyonlarına sahip olduğu bulunmuştur. Bu nedenle, bu bakterilerin kullanımı gıdalardaki folat içeriğini doğal olarak arttırmaya bir alternatif olabilir.*

Keywords: Hücre içi folat, hücre dışı folat, folat türevleri, laktik asit bakterisi

Öz

Folate is a B-group vitamin that humans cannot synthesize and must be taken externally. Although some types of Lactic Acid Bacteria can produce folate, there is insufficient information about the production of folate by yoghurt starter cultures. In this study, four bacteria belonging to two Lactobacillus, and two Streptococcus genera were isolated from yoghurt samples, and the isolates were biochemically identified by 16S rDNA with an API 50 CHL kit. Intracellular and extracellular folate production of the bacteria was designated in various media and buffer applications. The highest intracellular folate production was observed in Lactobacillus delbrueckii subsp. *bulgaricus* ZN951 *(126.3 µg/L) and Streptococcus. thermophilus* Z151 *(177.1 µg/L) strains in MRS/Elliker medium with potassium phosphate buffer. L. delbrueckii* subsp. *bulgaricus ZN541 strain showed the highest extracellular folate production (105.1µg/L) in the same medium and buffer, but S. thermophilus Z151 strain exhibited 86.4 µg/L folate productions. Whether the strains contained folate derivatives and pABA was investigated by HPLC analysis and pABA was not detected in any of the strains. Among cultures, the highest total folate content was detected as 110.5µg/L by L. delbrueckii subsp. bulgaricus ZN951 strain. This strain was found to have concentrations of 5-MeTHF and 5-FmTHF. Therefore, the use of these bacteria can be an alternative to naturally increase folate content in foods.*

Anahtar kelimeler: Intracellular folate, extracellular folate, folate derivates, Lactic Acid Bacteria

1 Introduction

Folate includes pteridine ring, para-aminobenzoic acid, and glutamic acid [1, 2]. Folate is very important for human life because it is included in the basic functions of cell metabolism, such as DNA synthesis, cell repair, replication, and methylation, as well as in the synthesis of nucleotides, some amino acids, and vitamins [3, 4]. A deficiency of folate, quite a crucial vitamin for human health, has been associated with a diverse of illnesses: Alzheimer's, coronary heart disease, osteoporosis, cancer, hearing loss, megaloblastic anaemia, and neural tube defects. Fortifying nourishments with synthetic folate or folic acid may be used to correct folate deficiency [5-7]. Folate can only be synthesized and produced by plants and microorganisms [8]. Since folate cannot be generated in the human body, it must be taken through foods [4].

The amount of folate that should be taken daily may vary in non-pregnant, pregnant, and lactating women. The recommended doses are respectively stated as 400(800/1000), 600(1000), and 500(1000) µg/d. [2, 9]. Even though folate status in fermented foods depends on the existence of Lactic Acid Bacteria (LAB) strains, it is relatively few belonging to the advised daily intake for an adult (400 µg/d). Therefore, to displace chemically synthesized folic acid, it is quite crucial to screen bacteria with high folate-generating capability that can be utilized as a folate enhancer [1].

It has been reported that many LABs, including Streptococcus, *Lactococcus, Lactobacillus*, and *Bifidobacteria*, can produce folate, and the folate production capacities of dissimilar strains can vary exceedingly [7, 10]. In addition, LAB strains that generate a great amount of folate and vital in the gastrointestinal system may be considered efficient probiotics to withstand folate deficiency [1, 11-13]. Tetrahydrofolate, 5-methyltetrahydrofolate (5-MTHF), and 5-formyltetrahydrofolate are synthesized by microbiological way. Between them, active folate (5-MTHF) is constant folate that may insert the circulation without metabolism and absorbed and used in the human [1, 14].

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The interest in functional foods has increased due to the increase in demand for people's consumption of quality food rich in nutrients and vitamins. Fermented dairy nourishments such as yoghurt and cheese are accepted as probiotic carrier food products in research and marketing sectors [15]. Milk contains nutrients suitable for the development of microorganisms, besides folate-binding proteins, which can enhance the stability of folate along fermentation. Yoghurt involves over levels of folate than raw milk, but yoghurt starter cultures generally generate low quantities of folate [7]. The studies to increase the folate production capabilities of yoghurt starter cultures are important. For this reason, the goal of this research was to isolate *Lactobacillus* and *Streptococcus* bacteria from traditionally fermented yoghurt samples obtained from four different provinces of Türkiye, biochemical and molecular identification of isolates, and to determine the effects of different media and different buffer on intracellular and extracellular folate production. Also, it was aimed to detect the presence of folate derivatives [tetrahydrofolate (THF), 5-Methyl Tetrahydrofolate (5-MTHF), 5-Formyl Tetrahydrofolate (5-FTHF, folinic acid)], and para-Aminobenzoate (pABA) by HPLC analysis.

2 Material and Method

2.1 Isolation

In this study yogurt samples obtained from Ankara-Yenimahalle (4 samples), Ankara-Çankaya (3 samples), Ankara-Sincan (4 samples), Ankara-Altındağ (2 samples),Çankırı-Orta (4 samples), Nevşehir- Gülşehir(2 samples), Çorum-Sungurlu (6 samples), Amasya-Merzifon (5 samples), Ankara-Keçiören (6 samples), Bolu-Göynük (3 samples), and Mersin-Silifke (6 samples) in Turkey. 0.1 g yoghurt samples from each were taken and suspended with 2 mL sterile phosphate-buffered saline buffer (PBS, 0.02% KCl, 0.144% Na_2HPO_4, 0.8% NaCl, 0.024% KH_2PO_4, pH 7.2). Samples diluted to 10^{-10} were inoculated on Man Ragosa-Sharp (MRS, Merck, Darmstadt, Germany) and Neutral Red Chalk Lactose (NRCLA, HiMedia, USA) solid media and incubated at 42°C for 16-18 hours. For better growth of the single colony selected samples, they were incubated again by inoculating on cysteine-MRS (MRSC, Merck, Darmstadt, Germany) and cysteine-Elliker (Merck, Darmstadt, Germany) broths. The morphologies of bacterial cultures were examined under a light microscope (Leica DM750), and Gram-positive bacilli and cocci were selected. Thirty three *Lactobacillus* spp. and thirty four *Streptococcus* spp. were isolated from 45 yogurt samples. Two *Lactobacillus* spp. and two *Streptococcus* spp. isolated from yogurt samples with the highest average starter culture count were selected randomly for the future studies. The remaining isolates (31 *Lactobacillus* and 32 *Streptococcus*) were used in another study (16).

2.2 Identification

Two *Lactobacillus* spp. and two *Streptococcus* spp. samples, which were isolated from yoghurt, were grown for 16-48 hours at 42°C in suitable media for identification (MRS and Elliker, respectively). API 50 CHL (Bio-Meriéux, France) test kit was used for the biochemical identification of the isolates. With the API kit, the enzymatic activities and sugar fermentations of the bacteria were determined, and their biochemical identifications were carried out qualitatively. Bacterial density (6×10^8 CFU/mL for *Lactobacillus;* 12×10^8 CFU/mL for *Streptococcus*) of microtubes containing dehydrated substrates was adjusted, then added, and incubated at 42°C for 24-48 hours. The colour change was detected in the metabolic end products of the bacteria. The change of purple colour to yellow was evaluated as positive, and if it remained the same, it was evaluated as negative. Evaluation of the API results of the isolates was made by comparing the API results of standard ATCC strains in the API WEB (NTSYSpc 2.0) program.

For molecular identification of isolates, DNA isolation of bacterial samples was performed using a Genomic DNA purification kit (Thermo Scientific, Catalog number: K0512). The purity and quantity of the isolated genomic DNA were designated in the ELISA (Microplate Readers, Epoch) instrument (OD260/280). Amplification of 16S rDNA was carried out using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) [17]. The genome sequencing of samples was performed using a genetic analyzer device Genetic Analyzer Applied Biosystems, 3130 model at the Gazi University Life Sciences Application and Research Center. The sequences results were matched with Genbank data (http://blast.ncbi.nlm.nih.gov/Blast.cgi) database.

2.3 Folate Production

Folate production of bacteria was carried out according to Sybesma et al [11, 12] and Aswathy et al's [18] methods with some modifications. MRS (for lactobacilli)/Elliker (for streptococci), Ten percent Skim milk (Oxoid, Ireland), and Folic Acid Casei Medium (FACM, HiMedia, USA) media and 0.1 M potassium phosphate, sodium acetate, and sodium phosphate as buffer were used to determine the intracellular and extracellular folate production ability of bacteria. Bacteria were inoculated into the media, grown at 42°C for 16 hours, and then centrifuged (12 000×g for 10 minutes). Pellet was used for intracellular production and the supernatant was used for extracellular production. It was washed with PBS buffer (pH 7.2) to remove the medium and other residues.

2.3.1 Intracellular folate production

The bacterial density in each buffer was adjusted to McFarland 5 (15×10^8 CFU/mL). Samples were sonicated for 5 minutes in an ultrasonication (VibraCell, USA) device set to 50 MHz frequency. In order to separate the folate-binding proteins from folate, the samples were kept at 100°C for 15 minutes. Then centrifugation was applied at 4000×g for 10 minutes at 4°C. The supernatant obtained after the second centrifugation process applied to the samples, which were re-incubated in a hot water bath for 5 minutes, was transferred to micro-wells by passing through a 0.45 µm filter. Intracellular folate production of cultures was determined by measuring at OD580 nm wavelength [11, 12, 18] Standard solutions of 10-100 µg/L were prepared from the folic acid stock, and the folic acid concentration in bacteria was measured by a spectrophotometer [19].

2.3.2 Extracellular folate production

Three different buffers were suspended in a 1:1 ratio with the supernatant samples. Folate-binding proteins were separated from folate by keeping the samples at 100°C for 15 minutes. After this process, centrifugation was applied at 4000×g for 10 minutes at 4°C. Two mL of supernatant was treated with 0.4 mL of human plasma, 0.1 M 2-mercaptoethanol, 0.5% sodium ascorbate solution, kept in an oven with shaking for 1 hour at 37°C. The supernatant obtained after the second centrifugation process applied to the samples, which were re-incubated in a hot water bath for 5 minutes, was transferred to micro-wells by

passing through a 0.45 µm filter. Extracellular folate production of cultures was designated at OD_{580} nm wavelength utilising the folic acid standard solutions [1, 11, 12, 19, 20].

2.4 HPLC Analysis

HPLC analysis was performed at Gazi University Life Sciences Application and Research Center to determine the amount of folate production and the folate derivatives. The study was carried out on a 1200 model HPLC instrument (Agilent Technologies) on a 250/4.6 ACE C18 100-5 type column. The presence and amount (µg/L) of folate derivatives Tetrahydrofolate (THF), 5-Methyl Tetrahydrofolate (5-MTHF), and 5-Formyl Tetrahydrofolate (5-FTHF) (folinic acid) and pABA (Sigma-Aldrich, St. Louis, Missouri, USA), which is involved in folate metabolism, were determined [11].

Lactobacillus bacteria were prepared for HPLC analysis in 0.1 M potassium phosphate buffer (pH 6.4) to investigate intracellular and extracellular folate contents after they were grown in MRS and *Streptococcus* bacteria in Elliker media at 42ºC for 16 hours [11, 21]. After the samples were kept at 90°C for 20 minutes, they were centrifuged (4000 g for 15 minutes). The supernatant was filtered through 0.45 µm filter and injected into the HPLC column [2, 22]. Mobile phases used for folate derivatives 39:1 ratio of 0.1 M potassium phosphate buffer (pH 6.4), acetonitrile (Sigma-Aldrich, St. Louis, Missouri, USA), and for pABA 39:1 ratio of 20 mM potassium phosphate buffer (pH 2.5) acetonitrile. The flow rate is 1 mL/min. Folate derivatives were observed at 280 nm and pABA at 210 nm with the help of a UV detector (ProStar 330 PDA, UV-VIS) at room temperature. Different concentrations of standard solutions (10, 25, 50, 100, and 200 µg/L) were prepared. The data were evaluated according to calibration curve (Figure 1) [23].

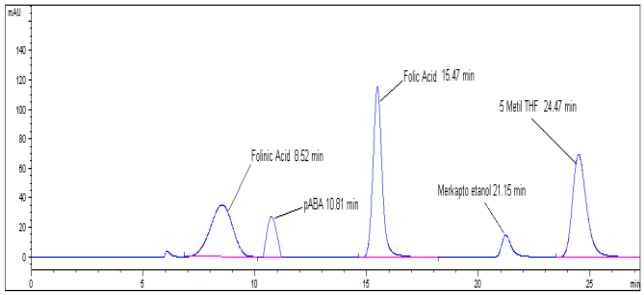

Figure 1. Chromatogram of folate derivatives and peak induction time of pABA

2.5 Statistical Analysis

SPSS Inc. Software (Ver. 22.0) was used for statistical analysis to detect whether there was a significant difference among various media and buffers used for intracellular and extracellular folate production. Tukey's test, which is one of the non-parametric tests that provide a one-way analysis of variance, was utilised to examine whether there was a significant difference in 3 dissimilar buffer of 3 different media ($p<0.05$).

3 Result and Discussion

3.1 Isolation and Identification

The average starter bacteria count in the yogurts from which *Lactobacillus* and *Streptococcus* were isolated from forty-five yogurt samples used in the study are shown in Table 1 as cfu/g. The study was carried out with bacteria isolated from Bolu-Göynük and Amasya-Merzifon region (*Lactobacillus* sp.) and Ankara-Keçiören and Mersin-Silifke region (*Streptococcus* sp.). As a result of biochemical identification using API 50 CHL test kit, *Lactobacillus* isolates were found to be *L. delbrueckii* subsp. *bulgaricus* (97.00-98.00%), and *Streptococcus* isolates were similar to *S. thermophilus* (95.00-97.00%).

Table 1. Total bacterial counts in yogurt samples

Yogurt Samples	Average *Lactobacillus* (kob/g)	Average *Streptococcus* (kob/g)
Ankara-Yenimahalle (n=4)	$8.4x10^6$±0.3	$8.5x10^6$ ±0.6
Ankara-Çankaya (n=3)	$6.3x10^5$ ±0.5	$1.5x10^5$ ±0.6
Ankara-Sincan (n=4)	$8.3x10^6$ ±0.5	$4.5x10^6$ ±0.6
Ankara-Altındağ (n=2)	$2.1x10^5$ ±0.5	$7.5x10^5$ ±0.6
Çankırı-Orta (n=4)	$5.4x10^6$ ±0.5	$8.3x10^6$ ±0.6
Nevşehir- Gülşehir (n=4)	$4.9x10^6$ ±0.5	$2.0x10^6$ ±0.1
Çorum-Sungurlu (n=6	$5.0.x10^7$ ±0.5	$9.2x10^7$ ±0.6
Amasya-Merzifon (n=5)	$9.0x10^7$±0.3	$21.5x10^8$±0.4
Ankara-Keçiören (n=6)	$20.0x10^8$ ±0.1	$9.6x10^7$ ±0.3
Bolu-Göynük (n=3)	$8.6x10^7$ ±0.2	$13.0x10^8$ ±0.6
Mersin-Silifke (n=6)	$10.8x10^8$ ±0.4	$8.4 x10^7$±0.1

ZN541, ZN951, Z151, and Z1052 isolates were randomly selected and used for further studies. After being biochemically identified, 16S rDNA molecular identifications of these isolates were carried out. The degree of purity (OD260/280 nm) was determined for *Lactobacillus* to be 1.92 and 1.96, 1.88 and 1.98 for *Streptococcus* (Table 2). The results of the sequence analysis of the DNAs of the samples were determined by scanning with the BLAST (Basic Local Alignment Search Tool) function in the NCBI (National Center for Biotechnology Information) GenBank. As a result of the molecular identification of all bacteria used in the study, it was determined that they were 99% similar to the model strains in the NCBI database, and the results are given in Table 2.

Table 2. API 50 CHL identification of bacteria, and determination of similarity rates and purity of 16S rDNA sequences according to NCBI Genbank results ($OD_{260/280}$ nm)

Code	Strain	EMBL/GenBank Number	Similarity (%)	$OD_{260/280}$ nm
ZN541	*L. delbrueckii* subsp. *bulgaricus*	JN675227.1	99	1.92
ZN951	*L. delbrueckii* subsp. *bulgaricus*	CP000156.1	99	1.96
Z151	*S. thermophilus*	GU195648	99	1.98
Z1052	*S. thermophilus*	HQ721249.1	99	1.88

3.2 Folate Production

While some of the folate produced by some *Lactobacillus* and *Streptococcus* bacteria, which contribute to the development of potential food technology with their capability to generate folate, accumulates in the cell, and some of it is released to the external environment [11, 22, 24]. Therefore, the folate production (intracellular and extracellular) abilities of the four bacteria as were investigated in this study. The high amount of intracellular folate production gives information about the metabolic activity of the bacteria, while the increase in the amount of extracellular folate gives an idea about the richness of the consumed foods in terms of folate content.

In our study, intracellular and extracellular folate production amounts (µg/L) were detected in three different media and buffers of four strains, and compared with control bacteria (*L. delbrueckii* ssp. *bulgaricus* ATCC 11842 and *S. thermophilus* ATCC 14425). The results are given in Table 3. In *L. delbrueckii* subsp. *bulgaricus* strains; it was determined that the highest intracellular folate production was in potassium phosphate buffer (126.3 µg/L) in MRS medium in ZN951 strain, and the lowest production was in sodium acetate buffer (9.7 µg/L) in

the skim milk medium. Considering the extracellular folate production, ZN541 strain showed the highest value in potassium phosphate buffer (105.1 µg/L) in MRS medium, while ZN951 strain showed the lowest value in sodium acetate buffer (3.9 µg/L) in the same medium. While in *S. thermophilus* strains, the highest intracellular and extracellular folate productions were determined in potassium phosphate buffer (177.1 µg/L, 86.4 µg/L) in Elliker medium in Z151 strain, respectively, the lowest folate production was in sodium phosphate buffer in FACM medium in Z1052 strain (20.4 µg/L) and in sodium acetate buffer (7.8 µg/L) on skim milk medium in strain Z151. Similar results were observed in the control group under all conditions. Sybesma et al. [11] determined intra/extracellular folate production amounts of 2 *S. thermophilus* cultures, which were isolated from Argentine yogurt, grown in an M17 medium. The first bacteria had an extracellular folate concentration of 6 µg/L, while the second bacteria had a concentration of 25 µg/L. Intracellular folate production was measured at 3 µg/L for the first bacteria and 83 µg/L for the second. Another study *S. thermophilus* CRL 808 exhibited the highest intracellular folate production (54.7 µg/L) in FACM medium. However, *S. thermophilus* CRL 415 demonstrated the highest level of extracellular folate production (76.6 µg/L) in the same medium [3]. The researchers investigated the folate production capabilities of twelve LAB strains. Notably, none of the tested bacteria, except for strains belonging to *Lactobacillus plantarum*, demonstrated any folate production. Interestingly, they even detected folate production in *L. plantarum* strains GSLP-7 (1.31 µg/mL) and SKT109 (0.51 µg/mL) despite these strains lacking the pabB gene [7]. A study involving five *S. thermophilus* strains (34v, 170v, 268v, 361v, and 341pc) grown in skim milk medium revealed that *S. thermophilus* 34v exhibited the highest folate production (208 ± 8 ng/mL) [25]. Albano et al. [26] investigated the extra and intracellular folate production in the MRS of 35-rod *Lactobacillus* strains, and the highest extracellular folate production was determined to be 72.99 ng/mL in *L. plantarum* VS513 and the highest intracellular folate production to be 14.51 ng/mL in *L. plantarum* VS513. The authors also researched extra/intracellular folate production of 12 cocci (*Lactococcus lactis* subsp. *lactis*, *S. thermophilus, Enterococcus faecium, E. lactis*). According to analysis results, they designated that among the 12 cocci, the maximum folate-generating strains were *E. faecium* VC223 (123.625 ng/mL) and *E. lactis* BT161 (384.22 ng/mL). Laino et al [26] examined the impact of diverse carbon sources on folate generation using the *Streptococcus macedonicus* CRL415 strain in their study. In the study using three different carbon sources, glucose, lactose, and sucrose, it was reported that the highest intracellular (9.1 µg/L) and extracellular (80 µg/L) folate production was observed in the glucose-containing environment. Our study identified strains *L. delbrueckii* subsp. *bulgaricus* ZN951 and *S. thermophilus* Z151 as having the highest capacities for both intracellular and extracellular folate production. When compared to other research, these strains exhibited production levels closer to those reported by Cucick et al. [25] using *L. plantarum*, rather than the highest values observed by Sybesma et al. [11], [7], [26]. Furthermore, our strains displayed higher folate production than the *Lactobacillus* and *Coccus* strains (except *E. lactis* BT161 at 384 ng/mL) investigated by Albano et al. [26]. Factors like fermentation time, temperature, precursor (pABA and glutamate) concentration, prebiotics, and even inhibitory elements can influence folate production by bacteria [27, 28]. It is thought that this difference between the folate productions of the bacteria may be owing to the isolation source, carbon source; the medium, buffer solution, and metabolic pathways of folates.

3.3 HPLC Analysis

Microbiological assays have been the conventional method for folate analysis [29]. However, these assays, despite their high sensitivity, are laborious and offer limited insight into the specific folate types within a sample. Moreover, the sample matrix can influence the accuracy of these assays. High-performance liquid chromatography (HPLC) emerges as a valuable alternative for folate measurement in various food products [30, 31]. Hence, the predominant forms of folate, namely tetrahydrofolate (THF), 5-methyltetrahydrofolate (5-MeTHF), and 5-formyltetrahydrofolate (5-FmTHF), were quantified utilizing the HPLC method in this study. While many studies directly quantify folate levels in yogurt using HPLC analysis [2, 7, 23, 32], this study uniquely employed HPLC analysis to determine the folate derivatives and quantities produced by the strains (ZN541, ZN951, Z151, Z1052) (Table 4). Analysis of *L. delbrueckii* subsp. *bulgaric*us strains ZN541 and ZN951 revealed the presence of several folate derivatives. These included folinic acid (intracellular: 20.0, 44.8 µg/L; extracellular: 56.8, 59.6 µg/L, respectively), THF (tetrahydrofolate) (intracellular: 0.31, 0.67 µg/L; extracellular: 0.61, 0.66 µg/L, respectively), and 5-methyl-THF (intracellular: 0.45, 2.23 µg/L; extracellular: 1.90, 2.53 µg/L, respectively). Analysis of *S. thermophilus* Z1052 revealed the presence of folinic acid (intracellular: 7.30 µg/L, extracellular: 12.64 µg/L) and 5-methyl-THF (intracellular: 0.13 µg/L, extracellular: 0.32 µg/L). Notably, no THF (tetrahydrofolate) derivatives were detected in this strain.

In addition, the presence of pABA could not be determined in the bacteria. The analysis of genome sequences using the KEGG database [33] suggests that members of the genus *Lactobacillus* and *Streptococc*us lack the ability to synthesize para-aminobenzoic acid (pABA) *de novo*. Specifically, the enzymes required for converting chorismate into pABA are absent in all sequenced *Lactobacillus* and *Streptococc*us species [34]. It is thought that our strains synthesize folate by either acquiring PABA from the external environment or synthesizing it through alternative pathways.

While research suggests that THF and MTHF are the main folate forms produced by some LAB during fermentation, our study identified folinic acid as the predominant folate form in the investigated strains [31]. High total folate production by bacteria is considered a desirable trait for starter cultures used in developing fermented foods with enriched nutritional value. In this study, we observed that the total folate content of the investigated strains was between 20.39-110.49 µg/L in *S. thermophilus* Z1052 and *L. delbrueckii* subsp. *bulgaricus* ZN951. Several studies have investigated folate production in various bacterial strains. Crittenden et al [35] determined folate production increased from 11.5 ng/g to 40-50 ng/g in *S. thermophilus*. Zahed et al [2] reported that 4 *Propionibacterium freudenreichii* ssp. *shermanii* and 4 *P. freudenreichii* ssp. *freudenreichii* strains folate production amounts varied between 21.2 and 31.9 µg/mL by HPLC analysis.

Table 3. Intracellular and extracellular folate production of *L. delbrueckii* subsp. *bulgaricus* ve *S. thermophilus* strains in different media

Bacteria Code	Medium	Potassium Phosphate Buffer µg/L (A)		Sodium Phosphate Buffer µg/L (B)		Sodium Acetate Buffer µg/L (C)	
		Intracellular	Extracellular	Intracellular	Extracellular	Intracellular	Extracellular
ZN541	MRS	104.9±1.5^	105.1±1.5+	93.6±1.6#	60.9±4.4+	31.3±0.6*	52.7±1.7*
	FACM	101.4±1.1^	62.0±4.9+	86.7±1.6#	59.3±1.0+	36.5±0.0*	51.1±0.3*
	Skimmilk	82.2±4.1^	70.8±4.2+	64.4±0.6#	32.6±1.7+	14.8±0.0*	14.9±0.1*
ZN951	MRS	126.3±3.7^	69.2±6.3Ω	96.1±4.1#	49.9±0.2#	28.9±0.0*	3.9±0.1Ω
	FACM	110.4±3.4^	57.7±1.3Ω	36.8±3.2#	27.2±1.6#	15.5±0.1*	18.7±0.1Ω
	Skimmilk	119.1±1.9^	86.9±0.5Ω	117.2±1.8#	8.9±0.1#	9.7±0.1*	6.2±1.3Ω
L. delbrueckii ssp. *bulgaricus* ATCC 11842	MRS	125.2±1.0	107.1±0.5	101.2±0.6	74.6±1.1	55.3±1.2	61.0±1.0
	FACM	117.8±0.1	71.0±4.9	81.9±1.0	63.9±1.1	42.2±1.4	26.9±1.7
	Skimmilk	98.2±1.5	80.1±2.3+	71.4±0.1	37.5±0.5	28.8±0.0	24.0±0.0
Z151	Elliker	177.1±3.9^	86.4±0.3^	55.8±0.9+	38.9±2.5#	25.1±0.7*	21.2±2.6*
	FACM	90.5±1.6^	68.9±1.3^	58.2±0.6+	22.8±2.6#	29.8±2.1*	23.7±2.9*
	Skimmilk	86.8±1.3^	50.5±3.2^	72.3±0.4+	13.6±1.5#	41.7±0.1*	7.8±0.1*
Z1052	Elliker	135.7±1.2^	75.4±0.7^	32.8±1.3#	28.4±2.2+	31.6±1.3*	12.1±1.7*
	FACM	91.1±1.8^	58.9±5.9^	20.4±0.9#	26.3±0.5+	26.5±1.6*	14.6±1.1*
	Skimmilk	95.1±5.2^	51.8±1.4^	56.4±1.8#	22.1±0.9+	39.5±0.6*	9.2±0.6*
S. thermophilus ATCC 14425	Elliker	142.1±1.2	79.1±1.2	40.1±1.0	32.1±1.3	29.1±0.1	25.0±1.0
	FACM	88.0±0.0	52.0±0.0	34.0±0.0	24.1±1.2	25.5±0.0	17.9±0.9
	Skimmilk	76.1±0.0	49.7±1.3	69.1±0.2	23.0±0.0	32.9±0.0	8.0±0.0

ΩB. +C. *A vs B. #A vs C. ^B vs C ($p<0.05$)

[a]Values are means ± standard deviations from experiments done in triplicate

Table 4. Folate derivatives and total folate amounts of bacteria by HPLC analysis

Strain	5-Formyl THF (Folinic Acid) (µg/L)		THF (µg/L)		5- Methyl THF (µg/L)		Total Folate (µg/L)
	Intracellular	Extracellular	Intracellular	Extracellular	Intracellular	Extracellular	
ZN541	20.0	56.8	0.31	0.61	0.45	1.90	80.07
ZN951	44.8	59.6	0.67	0.66	2.23	2.53	110.50
Z151	6.54	14.63	0.45	0	0.13	0	21.75
Z1052	7.30	12.64	0	0	0.13	0.32	20.39

Wu et al [36] analyzed the folic acid production of 3 LAB strains *(Lactobacilus casei, Lactobacilus acidophilus, L. plantarum*) isolated from fermented yogurt products by HPLC analysis. They observed variations in folic acid production between strains, with *L. plantarum* demonstrating the highest level at 63.23 µg/mL*. L. casei* and *L. acidophilu*s exhibited similar production levels, at 45.41 µg/mL and 42.78 µg/mL, respectively.

Different LAB strains produce different forms of folate [37]. Lin and Young [38] and Sanna et al [39] found that some *Bifidobacterium, Lactobacillus*, and *S. thermophilus* strains primarily synthesize 5-MTHF, THF, and 5-FTHF. Sybesma et al [11] reported *S. thermophilus* strains mainly producing 5,10-methenylTHF and 5-FTHF, while *L. lactis* strains favor 10-FTHF and 5,10-methenylTHF. Meucci et al [32] investigated the capability of *S. thermophilus* to produce folate along milk fermentation, and they found the 5-MeTHF and THF. Also, they declared that the generation of 5-MeTHF was higher than THF. In another study, the highest levels of 5-methyltetrahydrofolate and 5-formyltetrahydrofolate were found in the yoghurt made using *L. plantarum* 15HN [40]. Liu et al [1] investigated the presence of folate derivatives in their study and reported that *Lactobacilus sakei* LZ217 may produce 5-MTHF. In the study by Mahara et al [37], the twelve lactic acid bacteria (LAB) isolates, including the positive control WCFS1, contained a relatively low concentration of 5-MTHF. In the present study, folate derivatives of strains were examined by HPLC analysis, and the presence of FmTHF, MTHF (except *S. thermophilus* Z151-extracellular), and THF (except *S. thermophilus* Z1052- intra and extracellular and Z151-extracellular) was determined. The observed low levels of 5-MTHF might be due to oxidation or interconversion during analysis. These processes can happen naturally under the acidic conditions of the mobile phase used in HPLC separation, as reported by both Kariluoto et al [41] and Delchier et al. [42].

4 Conclusion

Folate is a crucial vitamin in the synthesis of nucleotides, amino acids, and vitamins and essential for healthy fetal development. Since the human system cannot synthesize folate on its own, this need must be obtained from external sources. The body wants a certain amount of folate during development and pregnancy, especially in children, as folate is essential for DNA synthesis and cell division, nervous system development, red blood cell formation and immune system function. Folate deficiency during pregnancy can lead to adverse events such as anaemia, miscarriage, and premature birth [40]. LAB synthesizes folate intracellularly and uses it for cellular growth. However, some are released into the growth medium, thus enhancing the folate concentration [43]. This extracellular folate can increase folate levels in fermented foods and can be an alternative natural resource of folate without adverse impacts on human health [3, 44]. This research, the intracellular and extracellular folate production of four bacteria

obtained from traditional yoghurts in various regions of Turkey was determined. The use of these bacteria as an alternative to naturally increase folate content in foods was investigated. *L. delbrueckii* subsp. *bulgaricus* ZN951 strain was found to have more 5-MeTHF and 5-FmTHF. Therefore, having a high total folate production ability, *L. delbrueckii* subsp. *bulgaricus* ZN951 (110.50 µg/L) strain is thought to be used as an auxiliary starter culture candidate in yoghurt production after other starter properties are determined. For this reason, enriching the food structure of this strain and using it in food production will both provide economic advantages and industrial and biotechnological benefits. This strain's high folate production ability can offer a promising alternative to synthetic folic acid fortification. Also, it could be harnessed for the development of natural folate supplements or functional foods, contributing to a healthier lifestyle.

5 Author contribution statements

Author 1 contributed to collecting data, searching for literature, performing the analysis, and writing the original draft.

Author 2 contributed to the creation of ideas, making the design, project administration, checking the spelling and checking the article in terms of content.

Author 3 contributed to writing the original draft, searching for literature, and performing statistical analyzes.

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7 Ethics committee approval and conflict of interest statement

This study does not require ethics committee approval".

There is no conflict of interest among authors.

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