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ORIGINAL RESEARCH PAPER

FOLATE PRODUCTION AND ITS DISTRIBUTION DURING GROWTH OF LACTIC ACID BACTERIA ISOLATED FROM FERMENTED FOOD AND BREAST MILK

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Abstract

Production of folate by lactic acid bacteria (LAB) isolated from tempe, fermented mustard, kefir, tapai, and breast milk was analyzed after 24 h of growth in folate-free media. The LAB isolates with the highest folate production were further investigated for folate distribution in intracellular and extracellular cells in a folate-free medium for 48 h. The two main forms of folates, *i.e.*, tetrahydrofolate (THF) and 5-methyl-tetrahydrofolate (5-MTHF), as well as folic acid, were detected and quantified using the HPLC-DAD method with each folate standard as calibration curves. Two folate peaks were found in the spent medium of folate-producing isolates; one of them was identified as 5-MTHF, while the other peak was identified as other folates. *Lacticaseibacillus rhamnosus* R23 from breast milk and *Limosilactobacillus fermentum* JK13 from kefir granules were the selected folate-producing isolates, with the production of total folates at 98.37 μg/ml and 85.67 μg/ml, respectively, after 24 h of incubation. The two isolates showed nearly similar patterns of intra- and extracellular folate distribution; that is, extracellular folate excreted into the media has a much higher proportion than intracellular folate. Therefore, the two LAB isolates can be utilized for extracellular folate production.

Keywords: distribution, folate, HPLC, lactic acid bacteria, production

Introduction

Folate is an essential vitamin that plays an important role in cellular metabolism, with the recommended daily folate requirement (RDA) of 400 μg for the average

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adult and 600 µg for pregnant women (FAO/WHO, 2001). Despite being found in various food sources (Saini *et al.*, 2016), natural folate from daily food intake still falls short of meeting daily folate requirements; hence, folate deficiency issues persist, especially in the group of pregnant women (Gernand *et al.*, 2016; Rogers *et al.*, 2018). The safety of using synthetic folate to alleviate folate insufficiency has been doubted, as many studies have reported its potential long-term side effects (Wright *et al.*, 2007; Laiño *et al.*, 2014; Patel and Sobczyńska-Malefora, 2017). Alternative sources of folate that are more efficient and cost-effective are required since the manufacture of synthetic folate entails a multi-step chemical process leading to immense energy and cost uses and harmful pollution impacts (Lu *et al.*, 2021).

Folate is also naturally found in many fermented foods, which contain various types of microorganisms, such as lactic acid bacteria (LAB) (Dana *et al.*, 2010; Laiño *et al.*, 2012; Mosso *et al.*, 2018; Mahara *et al.*, 2021). Certain strains of lactic acid bacteria have been reported to have the ability to produce folate (Laiño *et al.*, 2012; Laiño *et al.*, 2014; Greppi *et al.*, 2017) and are also capable of synthesizing various forms of folate, with the dominant forms produced being THF (tetrahydrofolate), 5-MTHF (5-methyltetrahydrofolate), and 5-FTHF (5-formyltetrahydrofolate) (Lin and Young, 2000; Sanna *et al.*, 2005; Mahara *et al.*, 2019). The THF and MTHF forms are the two primary forms of folate involved as cofactors in one-carbon metabolism (Lin and Young, 2000; Saini *et al.*, 2016) and have better bioavailability than synthetic folate (Patel and Sobczyńska-Malefora, 2017). For this reason, folate-rich fermented foods can be a better natural source of folate than chemical supplements in terms of effectiveness and efficiency.

Determination of folate levels in food is usually carried out by the microbiological assay, which is the AOAC standard method of folate analysis (Iyer and Tomar, 2009). This method is based on a quantitative relationship between sample folate levels and turbidimetry of indicator bacterial growth (a folate consumer). However, this method cannot analyze a single form of folate and has significant differences in the growth response to different forms of folate (Patring *et al.*, 2005; Iyer and Tomar, 2009). Therefore, the HPLC (High-Performance Liquid Chromatography) method is becoming more frequently used in the analysis of folate in foods as it can separate and quantify single forms of folate (Patring *et al.*, 2005; Iyer and Tomar, 2013; Öncü-Kaya, 2017).

The ability of LAB to synthesize folate and the forms of folate synthesized are highly dependent on the bacterial strain (Laiño *et al.*, 2014; Greppi *et al.*, 2017; Mahara *et al.*, 2021). In this study, folate production from LAB isolated from various sources (tempe, fermented mustard, kefir, tapai, and breast milk) was carried out in folate-free test media (FACM) using the HPLC-DAD (diode array detector) method. The distribution of intracellular and extracellular folate during growth and the time course for maximum production of folate were evaluated. This study aimed to obtain folate-producing LAB of fermented food and breast milk origin and to evaluate the distribution of intracellular and extracellular folate during growth.

Materials and methods

Microorganisms and growth conditions

All LAB isolates used in this study (Table 1) belonged to the culture collection of the SEAFAST Center, IPB University, Bogor, Indonesia, and were previously isolated from fermented foods (tempe, fermented mustard, kefir granules, cassava tape, and sticky rice tape) and breast milk. *Lactiplantibacillus plantarum* WCFS1 was also included as a reference strain for folate production (Greppi *et al.*, 2017). All strains were grown in de Man-Rogosa-Sharpe (MRS) broth culture medium (CM0359, Oxoid Ltd., Basingstoke, UK) at 37°C for 24 h.

Extracellular folate production in folate-free medium

After grown in the above-mentioned condition, all LAB were washed twice and resuspended in saline solution (0.85% w/v NaCl), followed by dilution to 5∼6 log CFU/ml as the inoculums (Mahara *et al.*, 2021). The inoculum (2%) was then grown in folate-free culture medium (Folic Acid Casei Medium, FACM; M543, HiMedia Laboratories, Mumbai, India) at 37°C for 24 h and subcultured twice in the same medium and growth conditions. The extracellular folate content was then extracted and analyzed.

Folate production kinetics in folate-free medium

The strains selected as the best producers of extracellular folate from the previous stage were inoculated in FACM and incubated at 37°C for 48 h. Samples were aseptically withdrawn at 6, 12, 18, 24, 36, and 48 h, and immediately extracted for extracellular and intracellular folate to determine the concentrations.

Extracellular and intracellular folate extraction

Briefly, samples were centrifuged at 10,000 ×g for 5 min at 4°C to obtain free-cell supernatants and cell biomass for analyses of extracellular and intracellular folate, respectively. The supernatant was then filtered with a 0.2 μm nylon filter membrane and analyzed further as extracellular folate (Mahara *et al.*, 2021). Meanwhile, the intracellular folate extraction from the cell biomass was performed according to Greppi *et al.* (2017), with some modifications. After centrifugation, the cell pellet was resuspended in 1 ml of ascorbic acid (0.5% w/v) to prevent folate oxidation during the extraction process. The suspension was added with 0.5 g of 0.5-mm-diameter zirconia/silica beads (BioSpec Products, 11079105z). The cell was then lysed using TissueLyser II Retsch (Qiagen, IKA, Wilmington, NC) at 30 Hz for 3 min, followed by centrifugation at 10,000 ×g for 5 min at 4°C. The supernatant was then filtered with a 0.2 μm nylon filter membrane and analyzed for intracellular folate. All samples were analyzed without the deconjugation reaction of folate polyglutamate and not purified further for the rapid determination of folates. All samples were stored at –20°C prior to folate quantification.

Folate quantification

Extracellular and intracellular folate were analyzed using an Agilent 1260 Infinity HPLC System with a Diode Array Detector (DAD; Agilent Technologies, USA) and an Eclipse XDB-C18 chromatography column (15 cm × 4.6 mm, 5 m) at 282 nm.

The mobile phase used was adapted from Kodi *et al.* (2015), which consisted of water (HPLC grade) containing glacial acetic acid (0.66%) and methanol (pure HPLC grade) in the 70:30 ratio of water:methanol under a flow rate of 0.8 ml/min. The retention times (Rt) of individual folate standards (Sigma, St. Louis, MO), i.e., 5-MTHF (Rt 2.1 min), THF (Rt 2.3 min), and folic acid (3.8–4.1), as well as their calibration curves, were used for peak identification and folate quantification. In this work, the individual folate and its retention time were accurately determined by spiking the standards into the supernatant (1:1). Meanwhile, the concentrations of other folates were calculated using the standard curve of 5-MTHF. Total folates were calculated as the total of THF, 5-MTHF, folic acid, and other folates.

Statistical analysis

All statistical analysis was performed using SPSS 20.0 with a 95% level of statistical significance ($\alpha<0.05$). A one-way ANOVA was used to analyze the differences in folate production in different LAB isolates, both intracellular and extracellular. The Duncan test was used for a post hoc test if a significant difference was found.

Results and discussion

Folate production by different LAB isolates in folate-free media

Folate production by various LAB isolates from fermented food and breast milk in folate-free media was analyzed using the HPLC-DAD method. Chromatograms of folate samples produced by folate producers and non-producers on folate-free media are presented in Figure 1. All peaks on the chromatogram were well separated, and other peaks that appeared were not adjacent to the detected form of folate, thus not interfering with quantification. The folate peaks detected in a folate-free medium grown by the R23 isolate as a folate producer (Mahara *et al.*, 2021) were 5-MTHF and other folates, whereas no peaks were detected for 5-MTHF, THF, folic acid, and other folates in the JK6 isolate as a non-producer of folate (Mahara *et al.*, 2021) (Figure 1). Other folates may include all forms other than 5-MTHF, THF, and folic acid, used as standards in this study.

Folate production of eleven folate-producing LAB isolates from fermented food and breast milk varied widely, ranging from 33.12–98.37 μg/ml. In contrast, two non-folate-producing LAB isolates (JK6 and BG8) from kefir granules (as a comparison) did not have detectable folate production (Table 1). *Lacticaseibacillus rhamnosus* R23 from breast milk and *Limosilactobacillus fermentum* JK13 from kefir granules were the two highest folate-producing isolates, with total folate concentrations of 98.37 μg/ml and 85.67 μg/ml, respectively. The folate levels produced by both isolates were 186–213% higher than those by the reference strain (WCFS1) used in this study, with a production rate of 46.11 μg/ml, and were also 136–156% higher than those by the isolate of *L. plantarum* reported by Wu *et al.* (2017), with folate production of 63.23 μg/ml in yogurt products. However, the folate-producing yeast strains were reported to have higher folate production, producing folate at 107.80 μg/ml by yeast isolates from kefir granules (Patring *et al.*, 2006) and at 145.00 μg/ml

by commercial yeast strains (Hjortmo *et al.*, 2005). Nonetheless, isolates R23 and JK13 in this study could be categorized as high-folate-producing LAB strains.

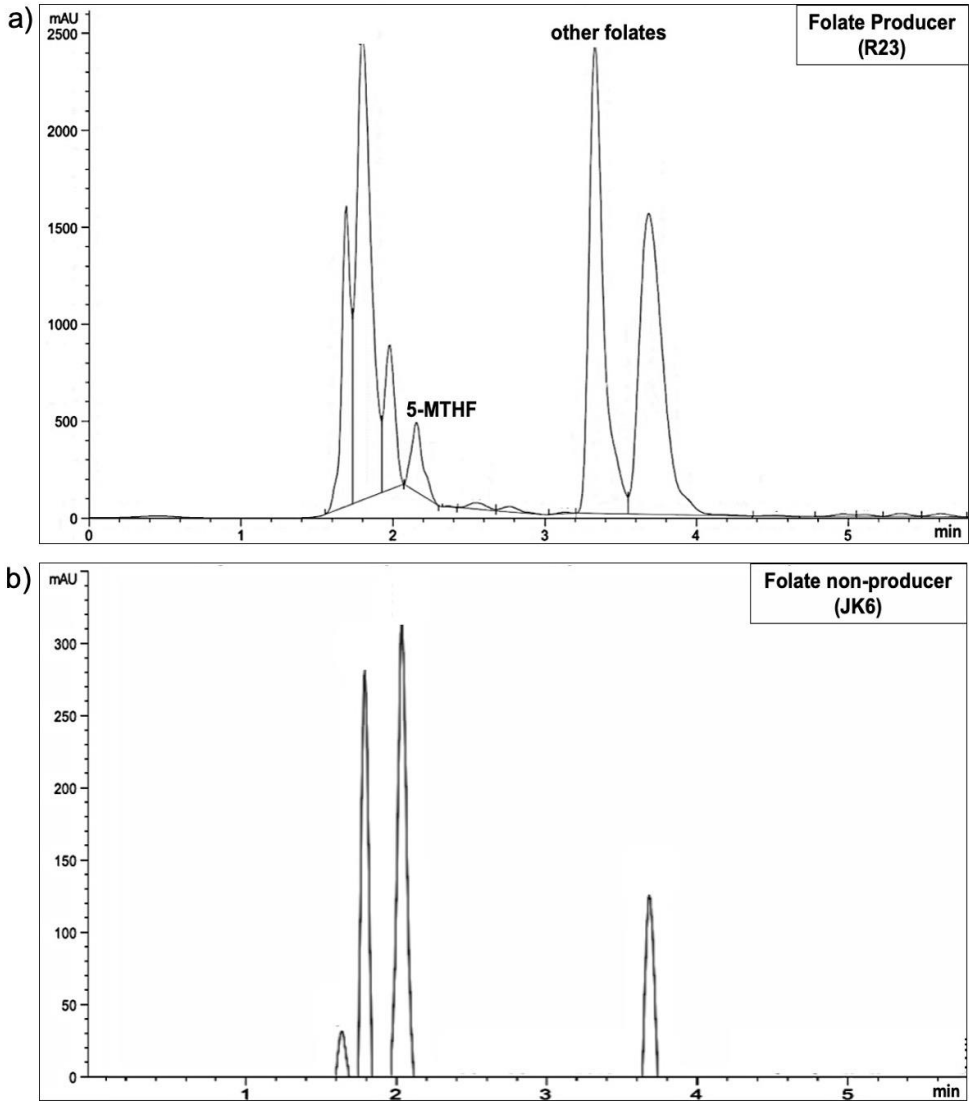

Figure 1. Chromatograms of folate as detected by HPLC-DAD (282 nm) in the spent medium of (a) folate-producing lactic acid bacteria (*Lacticaseibacillus rhamnosus* R23) and (b) non-folate-producing lactic acid bacteria (*Lactobacillus kefiri* JK6).

Table 1. Folate concentration (μg/ml) found in folate-free medium produced by different lactic acid bacteria isolated from fermented food and breast milk.

Isolate	Source	5-MTHF[b]	Other folates[c]	Total folate
Lactiplantibacillus plantarum WCFS1[a]	Human saliva	2.45	43.66	46.11
Lactiplantibacillus plantarum 4C261	Fermented mustard	3.40	47.11	50.51
Lactiplantibacillus plantarum R12	Breast milk	1.78	47.85	49.63
Lacticaseibacillus rhamnosus R23	Breast milk	9.28	89.09	98.37
Lacticaseibacillus rhamnosus R15	Breast milk	1.68	57.04	58.73
Lacticaseibacillus rhamnosus BD2	Kefir granules	2.64	59.21	61.86
Limosilactobacillus fermentum JK13	Kefir granules	8.78	76.89	85.67
Limosilactobacillus fermentum JK16	Kefir granules	1.85	47.70	49.55
Limosilactobacillus fermentum BK27	Sticky rice tapai	6.06	46.60	52.66
Limosilactobacillus fermentum BG7	Kefir granules	1.70	41.88	43.58
Leuconostoc mesenteroides S2SR08	Tempe	3.06	30.06	33.12
Pediococcus acidilactici NG64	Cassava tapai	6.86	53.65	60.51
Lactobacillus kefiri JK6	Kefir granules	ND	ND	ND
Lactobacillus kefiri BG8	Kefir granules	ND	ND	ND

[a] = a reference strain; [b]5-MTHF = 5-methyltetrahydrofolate; ND = not detected;
[c] = Other folates are folate other than 5-MTHF, THF and folic acid used as standard in this present study

The form of folate synthesized by LAB can vary between species and strains. According to Lin and Young (2000) and Sanna *et al.* (2005), several strains of *Bifidobacterium*, *Lactobacillus*, and *S. thermophilus* synthesized 5-MTHF, THF, and 5-FTHF as the main forms of folate. However, Sybesma *et al.* (2003) reported that *S. thermophilus* strains produced folate mainly in the forms of 5,10-methenylTHF and 5-FTHF, whereas *Lactococcus lactis* strains primarily produced 10-FTHF and 5,10-methenylTHF. In this study, the twelve LAB isolates (including the positive control WCFS1) had a relatively low concentration of 5-MTHF, which was only in the range of 1.68–9.28 μg/ml, and other forms of folate were detected at much higher concentrations, ranging from 30.06–89.09 μg/ml (Table 1). Given the significant amounts of other folates detected, these LAB isolates might have another major type of folate. However, the ratio of 5-MTHF to other folates does not appear to differ much among isolates. In this case, the folate in the spent medium analyzed

may not directly reflect the actual folate excretion due to the labile property of folate. It may have undergone folate oxidation or interconversion, which occurs spontaneously during the analysis process in the acidic mobile phase during HPLC separation (Kariluoto *et al.*, 2010; Delchier *et al.*, 2016).

Folate production and its distribution in selected isolates in folate-free media during growth

As the highest folate-producing isolates, *Lacticaseibacillus rhamnosus* R23 and *Limosilactobacillus fermentum* JK13 were used further to study folate production and its distribution during growth in folate-free media (Figure 2 and Figure 3).

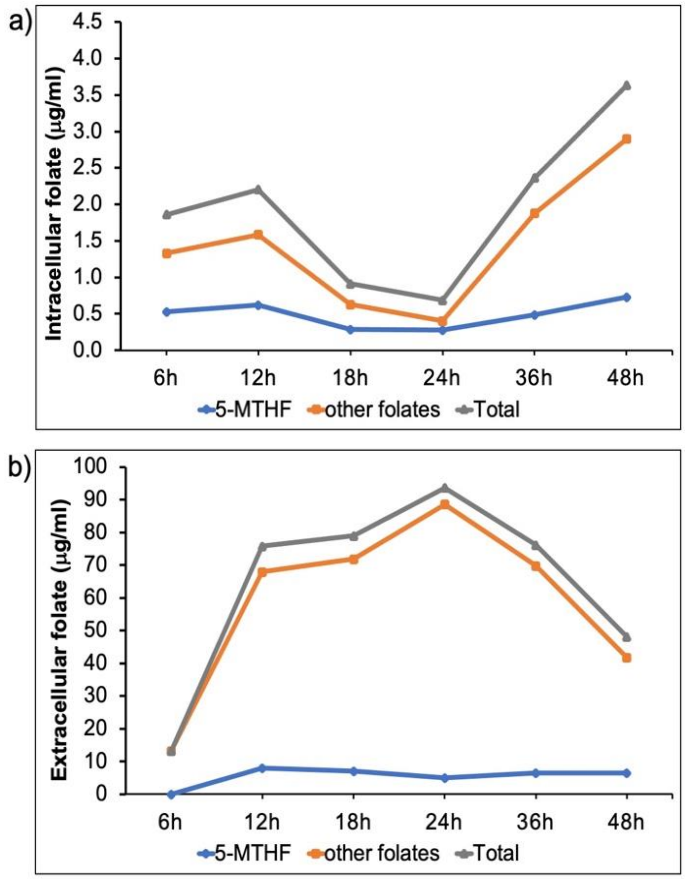

Figure 2. Production and distribution of (a) intracellular folate and (b) extracellular folate in *Lacticaseibacillus rhamnosus* R23 during incubation in a folate-free medium.

This aimed to determine the patterns of folate accumulation and excretion into the media. During growth, the two isolates, R23 and JK13, showed nearly identical patterns of total folate production, both intracellular and extracellular (Figure 2 and Figure 3). Total intracellular folate production of both isolates increased until the

12th hour of incubation, with the highest level for JK13. Subsequently, the production drastically decreased until the 18th hour for JK13 and the 24th hour for R23. However, after that, there was a significant increase in concentration up to 48 hours for both isolates. Meanwhile, total extracellular folate production of both isolates significantly increased in the first 12 hours of incubation, continued to rise until the 24th hour, and then declined until the 48th hour.

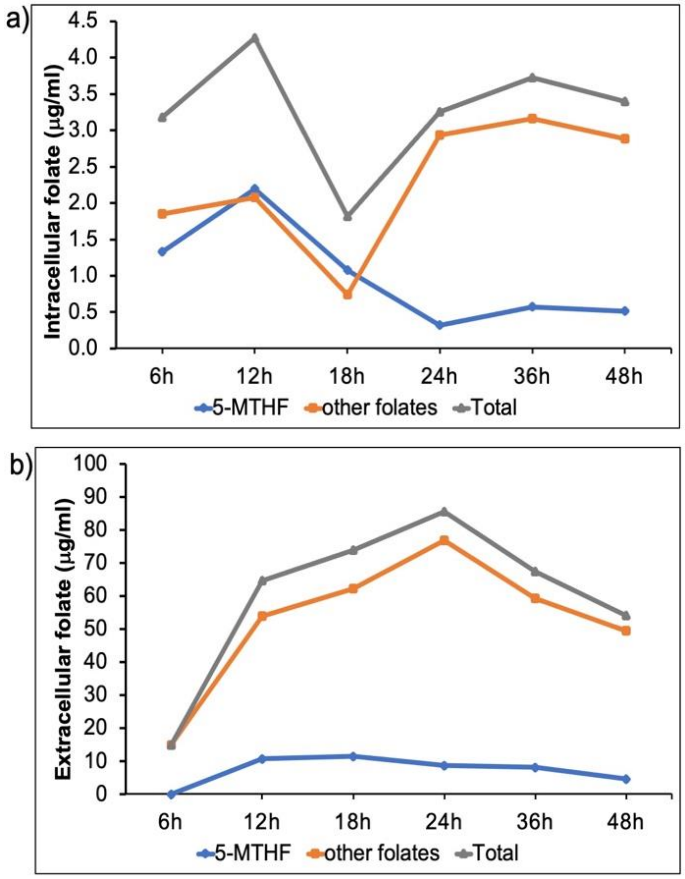

Figure 3. Production and distribution of (a) intracellular folate and (b) extracellular folate in *Limosilactobacillus fermentum* JK13 during incubation in a folate-free medium.

The decrease in intracellular folate levels between 12 and 24 h of incubation in both isolates was probably due to the increased folate utilization for cell growth and division (Divya and Nampoothiri, 2015; Mahara *et al.*, 2021). However, this decrease went along with the rise in extracellular folate concentrations. Previous studies have shown that folate accumulation and excretion are highly heterogeneous and bacterial strain-dependent (Kariluoto *et al.*, 2010; Greppi *et al.*, 2017). Greppi *et al.* (2017) reported that several LAB strains had the same proportion of intracellular and extracellular folate production. The folate levels accumulated in the

cells increased along with the increased concentration of folate excreted into the medium. In this case, the rate between folate synthesis and excretion may be at the same level; hence, the cells will immediately synthesize the excreted folate.

On the contrary, Kariluoto *et al.* (2010) reported that the folate synthesized and excreted into the medium had opposite proportions in certain microorganisms. Microorganisms with high levels of intracellular folate production were reported to have low levels of folate release into their culture media. Meanwhile, other bacteria with low intracellular folate concentrations excreted good folate levels into their culture media. The biosynthesis of folate in cells in monoglutamate form will be easily excreted into the medium. This may occur in microorganisms that release folate into the medium and do not accumulate it in cells, as in this study. In the following biosynthetic stage, folate-monoglutamate will gain more glutamate residues to form folate-polyglutamate, which has high cell retention (Sybesma *et al.*, 2003; Wegkamp *et al.*, 2007). This may occur in microorganisms with higher intracellular folate levels than folate excreted into the medium.

In this study, isolates R23 and JK13 had intracellular folate production with maximum concentrations of only 4–5% of the total folate produced (intra- and extracellular). The low proportion of folate accumulated by the two isolates is in line with several other studies. Some *Bifidobacterium* strains were reported to synthesize intracellular folate for only 9–38% of the total folate produced when grown in a folate-free semisynthetic medium (SM7) for 48 h (Pompei *et al.*, 2007). Meanwhile, the strain *L. amylovorus* CRL887 accumulated folate for around 15–71% of the total folate produced when grown in FACM from 0 to 24 h (Laiño *et al.*, 2014). Furthermore, the strain *S. macedonicus* CRL415 could only accumulate folate at a maximum level of around 21% after 10 h of incubation and excrete folate at a maximum concentration of approximately 79% after 8–12 h of incubation (Laiño *et al.*, 2019). This may also be related to their nutritional requirements, which may vary between bacteria (Pompei *et al.*, 2007; Mahara *et al.*, 2021).

During the 24–48 hour incubation period, there was an increase in the intracellular folate concentration in both isolates R23 and JK13, although it was only around 1–3 µg/ml. In contrast, the extracellular folate concentration decreased by 30–40 µg/ml. Mahara *et al.* (2021) reported that during growth, LAB would produce lactic acid at increasing concentrations along with the lengthening of the incubation time, thus lowering the pH value of the culture medium. During the 24–48 h of incubation, the available nutrients for bacteria may also have been used up; hence, they may no longer grow. For this reason, the trend that occurs tends to be caused by the acidic conditions of the medium's pH.

Sybesma *et al.* (2003) reported the effect of pH on intra- and extracellular folate distribution. They found that the low pH of the culture medium would increase folate excretion, while the high pH condition would boost intracellular folate synthesis. The effect of high pH on increased intracellular folate production could be because folate biosynthetic enzymes have optimum activity at a pH between 7.3 and 9.5; hence, high external pH conditions will create more alkaline cytosolic conditions in cells, which in turn increase the activity of folate biosynthetic enzymes.

Meanwhile, the increased extracellular folate caused by the low pH may be due to the activity of folate carrier proteins that function optimally at a low pH (Shane and Stokstad, 1975; Henderson *et al.*, 1977; Kumar *et al.*, 1987). Lactic acid bacteria have a folate transport system in the form of a binding protein that acts as an external receptor for folate (anionic) and a transmembrane folate carrier (Kumar *et al.*, 1987). This protein facilitates the movement of folate across the membrane through the mechanism of co-transport of cations (requiring cations for optimal binding of folate) (Henderson *et al.*, 1977; Kumar *et al.*, 1987). This carrier protein also has a high affinity for folate with short glutamate chains (monoglutamate) (Shane and Stokstad, 1975). A low pH of the medium will enhance the function of the folate carrier protein in transporting monoglutamate folate into cells or excreting it outside cells. In this study, the increase in intracellular folate that occurred after 24 h of incubation may be due to the activity of folate carrier proteins, which tend to take folate from the environment due to the absence of folate synthesis in the cells. Folate can be taken only as a monoglutamate; hence, the folate excreted in monoglutamate form will be easily transported back into the cells.

Aside from bacterial resorption, the decrease in extracellular folate levels could also be caused by folate oxidation due to the low pH of the medium. Extracellular folate is unstable; thus, the acidic pH of the medium may cause folate destruction. Paine-Wilson and Chen (1979) found that pH had a major influence on folate stability, in which increasing folate breakdown was found along with decreasing pH. Therefore, prolonging the incubation time to 48 h in this study may lower the medium's pH to a level that can trigger the destruction of some labile forms of folate when excreted into the medium (Padalino *et al.*, 2012).

Extracellular folate excretion by LAB can be utilized directly to increase folate concentrations in fermented products (Mahara *et al.*, 2019), while intracellular folate production can be utilized after cell lysis in the digestive tract (LeBlanc *et al.*, 2015). Therefore, both intracellular and extracellular folate must be available in sufficient amounts to be utilized properly. As a non-probiotic folate producer (Yusuf *et al.*, 2020; Mahara *et al.*, 2021), the isolate JK13 can provide both intracellular and extracellular folate because the cell will undergo lysis in the digestive tract. Meanwhile, the isolate R23, a probiotic folate producer (Mahara *et al.*, 2021), can be optimized for the utilization of extracellular folate since it can survive in the digestive tract and continue to produce extracellular folate after colonization in the colon.

Conclusions

The proper selection of bacterial strains and incubation time can result in high levels of folate production. In this study, two LAB isolates had the highest folate production with a nearly identical pattern of intra- and extracellular folate distribution, both of which had a much lower proportion of intracellular folate than the folate excreted into the medium. Both LAB isolates can be utilized as extracellular folate producers. Although the dominant form of folate (other folates, which are other than THF and folic acid) produced in this study is unknown, two high-folate-producing isolates in

this study were shown to produce 5-MTHF, which is a form of folate that can be directly absorbed and used in the body when consumed, thereby providing added value regarding the bioavailability of the folate synthesized by the two LAB.

Acknowledgments

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