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The Manipulation of Folate Biosynthesis in Lactic Acid Bacteria with a Folate Analog and Enhancers

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ABSTRACT

Folate biosynthesis in Lacticaseibacillus rhamnosus R23 isolate of breast milk and Limosilactobacillus fermentum JK13 isolate of kefir granules was manipulated by stressing them with a folate analog (methotrexate) to increase extracellular folate production. Extracellular folate production was further enhanced in isolate R23 (as a folate-efficient probiotic) by adding various enhancer compounds (PABA, glutamate, combination PABA-glutamate, $CaCl_2$, and ascorbic acid) in a folate-free medium. Extracellular folate analysis was done using a microbiological assay that quantified all forms of folate in the samples. Both isolates could grow in folate-free medium containing methotrexate (2.5 mg/L); however, random mutant colonies of both had no increase in extracellular folate production. The resistance mechanism against methotrexate did not trigger excessive extracellular folate production but caused bacterial filamentation. Adding various enhancer compounds also did not significantly increase the extracellular folate production of isolate R23, probably due to the inadequate concentration of the compounds. This study's results indicate that stress exposure to methotrexate seems to be ineffective to increase the extracellular folate production of isolates R23 and JK13. The formation of bacterial filaments in response to stress exposure to methotrexate is possibly a new mechanism that has not been previously reported regarding the mechanism of methotrexate resistance by lactic acid bacteria. This study requires further investigation primarily evaluating intracellular folate concentrations and finding optimum concentrations of different folate biosynthesis enhancer compounds.

Keywords: *enhancer compound, extracellular folate production, folate analog, lactic acid bacteria, stress exposure*

INTRODUCTION

Folate is a water-soluble vitamin involved in amino acid synthesis, methylation cycles, and DNA synthesis (Lyon *et al.,* 2020; Shulpekova *et al.,* 2021). Folate deficiency will lead to various health problems such as anemia, homocysteinemia, cardiovascular disease, an increased risk of cancer, and neural tube defects in newborns (Imbard *et al*., 2013; Thaler, 2014; Warzyszynska and Kim, 2014; Zheng and Cantley, 2019; Lyon *et al.,* 2020). The daily recommended intakes of folate are 400 *µ*g per day for an average adult, 500 *µ*g for lactating women, and 600 *µ*g for pregnant women (FAO/WHO, 2001). Since the body cannot produce folate and must rely on external supplies, several ways can be done to increase folate levels in the body, *i.e.*, by consuming rich-folate foods, taking folic acid supplements, and fortifying folic acid in food products (Saini *et al.,* 2016; Ismail *et al.,* 2023; McNulty *et al.,* 2023). However, consuming rich-folate foods is insufficient to meet daily folate requirements since folate is highly water soluble, unstable, and easily oxidatively degraded during food processing and handling (Delchier *et al.,* 2016; Saini *et al.,* 2016). Meanwhile, consideration of the health risks of consuming synthetic folate (folic acid) is increasing due to its long-term health issues (Patel and Sobczyńska-Malefora, 2017).

Certain lactic acid bacteria (LAB) are known to have the ability to synthesize folate (Mosso *et al.,* 2018; Mahara *et al.,* 2021). The fermentation technique using folate-producing LAB has been widely applied to develop natural folate-rich fermented products, and selecting a suitable folate-producing LAB strain can increase folate levels in food (Laiño *et al.,* 2014). In addition, there is a growing interest in strain improvement to obtain folate-overproducing strains (Wegkamp, 2008; Zhang *et al.,* 2020); thus, their ability to produce folate does not have to be under certain conditions (affected by the available folate in the media), and further, they can be applied to various kinds of foods (Mahara *et al.,* 2021).

The selection of analog-resistant mutants is one of the strain improvements that can be used to obtain folate-overproducing mutants in a natural way

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(Wegkamp, 2008). Previous studies have successfully used this method for vitamin production by LAB (Wegkamp, 2008; del Valle *et al.,* 2014; Russo *et al.,* 2014; Zhang *et al.,* 2020). In this method, chemical compounds structurally analogous to folate or folate intermediates at sequentially different concentrations will inhibit the folate biosynthesis pathway. One of the antifolate compounds that can be used is methotrexate (MTX), an analog of dihydrofolate (DHF), which will compete with DHF for binding to dihydrofolate reductase (DHFR). The antifolate compounds can inhibit folate biosynthesis and, in turn, will significantly reduce bacterial growth. Sequential inhibition, however, can also lead to excessive folate synthesis, giving DHF more opportunities to compete with methotrexate for DHFR binding. As a result, that mechanism will further trigger the methotrexate resistance mechanisms, thus not affecting bacterial growth. By using this technique, *L. plantarum* WCFS1 has been reported to produce folate up to 52-fold in media without folate (Wegkamp, 2008). In addition to this, strategies to increase folate production include adding folate precursors (Mousavi *et al.,* 2013; Divya and Nampoothiri, 2015; Laiño *et al.,* 2019), folate inducers (Zhang *et al.,* 2020) or reducing agents (Divya and Nampoothiri, 2015).

This study aims to obtain the most effective manipulation strategy to increase extracellular folate production by manipulating them under stress with a folate analog, methotrexate, at sequentially different concentrations. The increased extracellular folate production in LAB isolates will also be done by the addition of certain compounds, such as folate precursors (PABA, glutamate, and PABA-glutamate), a folate inducer ($CaCl_2$), and a reducing agent (ascorbic acid).

MATERIALS AND METHOD

Materials

The LAB isolates used in this study were *Lacticaseibacillus rhamnosus* R23 isolated from breast milk (Genbank accession no. MF689061) and *Limosilactobacillus fermentum* JK13 isolated from kefir granules (Genbank accession no. ON005305.1), previously selected for their folate-producing abilities (Mahara *et al.,* 2021). These isolates belong to the SEAFAST Center, IPB University, Indonesia.

Microorganisms and inoculum preparation

All of the isolates were grown and kept in a mixture of de Man, Rogosa, and Sharpe broth (MRSB; CM0359, Oxoid Ltd., Basingstoke, United Kingdom) and glycerol (20%) (104094, Merck KGaA, Darmstadt, Germany) at −20 °C. The isolates were revived in MRSB and incubated at 37 °C for 24 h before use. After that, all isolates were washed by centrifuging them twice (refrigerated centrifuge LRF-B20; Labtron Equipment Ltd., Camberley, United Kingdom) at 10,000 ×*g* for 5 min at 4 °C and resuspended in 0.85% w/v NaCl (106404, KGaA, Darmstadt, Germany), followed by dilution to 5~6 log CFU/mL as the inoculums (Mahara *et al.,* 2021).

Increasing extracellular folate production

The increase in extracellular folate production in this study was done via several strategies, elaborating different mechanisms (Figure 1), *i.e.*, exposure to a folate analog (methotrexate) and adding different enhancer compounds, such as folate precursors (PABA, glutamate, and PABA-glutamate), a folate inducer ($CaCl_2$), and a reducing agent (ascorbic acid). As previously explained (in the Introduction section), exposure to methotrexate will inhibit the activity of the DHFR enzyme in the folate biosynthesis pathway, which is expected to trigger excessive folate synthesis; hence, it can lead to a mechanism of LAB resistance. Meanwhile, the addition of high concentrations of PABA and glutamate will inhibit/deactivate the activity of folylpolyglutamate synthase (FPGS) for polyglutamate tail extension, thereby increasing the synthesis of monoglutamate folate, which is readily excreted outside cells (Divya and Nampoothiri, 2015). Moreover, adding $CaCl_2$ to the medium can also induce folate biosynthesis and produce high folate production as the availability of Ca^{2+} ions might activate GTP cyclohydrolase (a metal-dependent enzyme), which catalyzes the first step of folate biosynthesis (Zhang *et al.,* 2020). The addition of reducing agents (antioxidants) such as ascorbic acid (Waters *et al.,* 1961) or sodium ascorbate (Gangadharan and Nampoothiri, 2011; Divya and Nampoothiri, 2015) was reported to prevent oxidative damage to folate, as folate is susceptible to oxidation due to exposure to heat, light, water, and an acidic pH.

Initially, the inoculum (2%) was grown in a folate-free medium broth, Folic Acid Casei Medium broth (FACM broth; M543, HiMedia Laboratories, Mumbai, India), at 37 °C for 24 h. The cultures were then streaked and cultured on FACM agar {FACM broth + 1.5% w/w Agar Bacteriological (LP0011, Oxoid LTD., Basingstoke, United Kingdom)} at concentrations of 0, 2.5, and 5.0 mg/L methotrexate (MTX, Sigma-Aldrich, St. Louis, MO, United States of America), sequentially, at 37 °C for 48 h. The colonies appearing on the FACM agar were randomly picked and cultured in FACM broth at 37 °C for 24 h to determine the folate productivity. The selected colonies were also subjected to cell morphology observation before, during, and after the exposure (grown on MRSA) under a microscope at 1000x magnification.

The analog-resistant mutants with the highest extracellular folate production were selected for

further enhancement of extracellular folate levels through the addition of various enhancer compounds. The inoculum (2%) of the selected LAB mutants was grown in FACM broth at 37 °C for 24 h. The isolate was then recultured in FACM broth supplemented with different compounds, *i.e.*, 100 µmol/L *p*-aminobenzoic acid (PABA; A9878, Sigma-Aldrich, United States of America), 100 µmol/L glutamate (G1251, Sigma-Aldrich, United States of America), PABA:glutamate with a ratio of 1:1 (100 µmol/L of each), 100 mg/L $CaCl_2$ (102382, Merck KGaA, Darmstadt, Germany), and 0.2% (w/v) ascorbate acid (100468, Merck KGaA, Darmstadt, Germany), based on the best concentrations from previous studies (Divya dan Nampoothiri, 2015; Zhang *et al.,* 2020) with modifications, and incubated at 37 °C for 24 h.

Folate extraction and analysis using the microbiological assay

The folate extraction was done by centrifugation (refrigerated centrifuge LRF-B20, Labtron Equipment Ltd., Camberley, United Kingdom) at 10,000 *xg* for 5 min, followed by supernatant filtration using a 0.2-µm nylon filter membrane (2.CF2202.0001, ANPEL Laboratory Technologies Inc., Shanghai, PRC). Folate was then quantified by a microbiological assay using the Vitafast® Folic Acid Test Kit (P1001, R-Biopharm AG, Germany) per the manufacturer instructions. Essentially, the procedure involves introducing the extracted folate into the wells of a microtiter plate that is coated with bacteria sensitive to folate (folate-consuming bacteria). Subsequently, the plate undergoes incubation in darkness at 37 °C for a period of 44-48 h. The growth of the bacteria sensitive to folate relies on the folate supply within the sample, and this growth is assessed by measuring turbidity at 610-630 nm through a microtiter plate reader (iMark, Bio-Rad Laboratories Inc., Hercules, CA, United States of America). The folate concentration is then determined by referencing a standard curve derived from various known folate concentrations, measured in a manner similar to the samples.

Data analysis

The statistical significance level for the data analysis was established at 95% (α<0.05) using SPSS 20.0. To compare the changes in extracellular folate synthesis, a one-way ANOVA was used.

RESULTS AND DISCUSSION

Exposure to methotrexate

The increase in folate production by LAB is done by exposure to methotrexate (a folate analog) at gradual concentrations. Exposure to methotrexate will trigger excessive folate production, eventually leading to methotrexate resistance. Only isolates with high folate productivity could survive growing on media containing high concentrations of methotrexate (Zhang *et al.,* 2020). In this study, several colonies growing on media containing methotrexate were taken randomly to measure their extracellular folate production (Figure 2). However, there was no increase in extracellular folate production after methotrexate exposure (Figure 3).

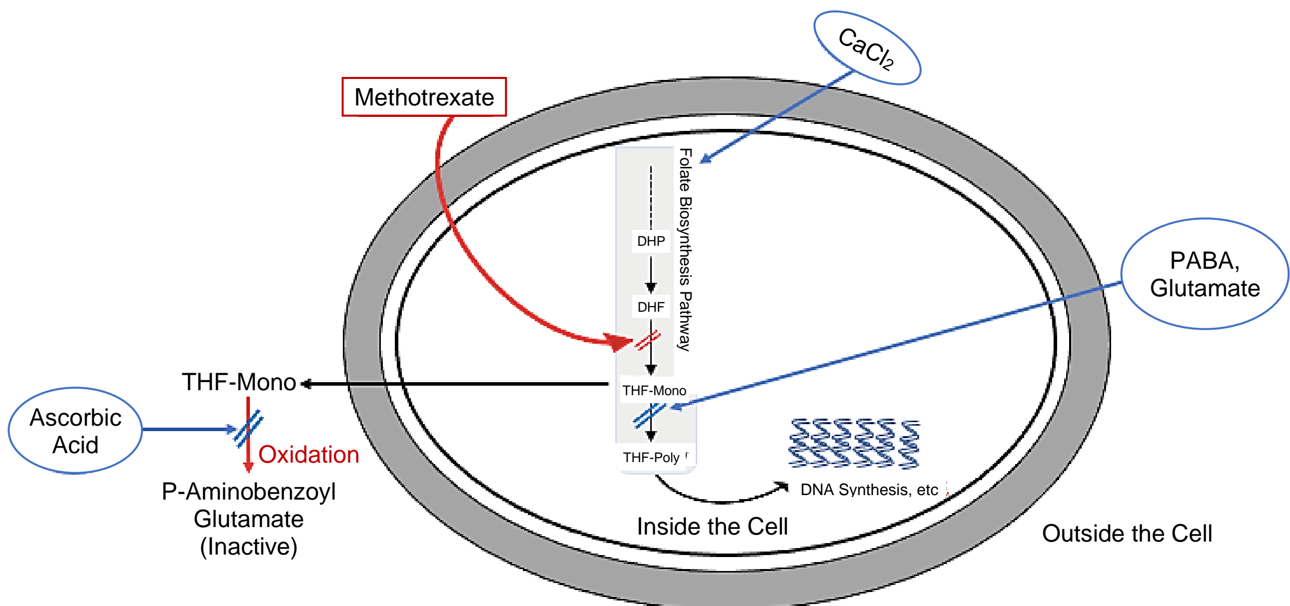

Figure 1. Illustration of strategies to increase extracellular folate production in this study

R23
MTX 0 mg/L

R23
MTX 2.5 mg/L

R23
MTX 5 mg/L

JK13
MTX 0 mg/L

JK13
MTX 2.5 mg/L

Figure 2. Growth of lactic acid bacteria colonies on folic acid casei medium (FACM) agar containing methotrexate (MTX). Colonies with black circles were taken and evaluated for folate production

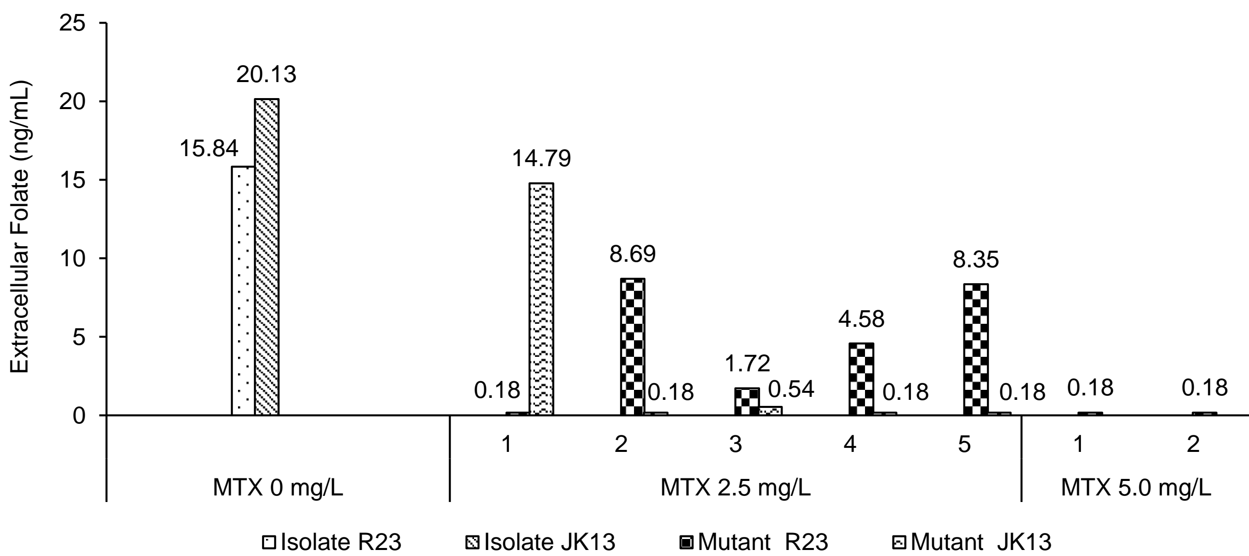

Figure 3. Extracellular folate production of methotrexate (MTX)-resistant mutant cells

Figure 2 shows that the number of colonies for two isolates, *L. rhamnosus* R23 and *L. fermentum* JK13, growing on media containing methotrexate was significantly reduced compared to those growing on media without methotrexate. This reduction may be attributed to the growth inhibition effect of methotrexate on most of the cell colonies. Stress treatment at a 5.0 mg/L concentration of methotrexate was not performed for the JK13 isolate (Figure 2) because its random mutant cell colonies experienced a drastic decrease in extracellular folate production after exposure to 2.5 mg/L methotrexate (Figure 3). Meanwhile, for the R23 isolate, although methotrexate exposure was performed up to a 5.0 mg/L concentration, the random mutant cell colonies remained drastically decreased.

Resistance to folate antagonists such as methotrexate is reported to occur via one or a combination of several mechanisms (Wegkamp, 2008), *e.g.*, (1) amplification of the gene encoding the DHFR enzyme (folA); (2) mutation to produce DHFR enzymes with decreased affinity for methotrexate; (3) decreased activity of FPGS enzyme and (4) increased activity of folate conjugase enzyme, both of which are aimed at reducing polyglutamate methotrexate synthesis (reducing methotrexate retention in cells); (5) increase in polyglutamate folate synthesis to maintain intracellular folate homeostasis; (6) decreased transport of methotrexate into cells through decreased expression of folate carriers or folate receptors; and (7) increased protein expression associated with multidrug resistance. For this reason, the mechanism of excessive folate production that triggers methotrexate resistance is not a common mechanism carried out by bacterial cells against the growth-inhibitory action of folate antagonists such as methotrexate (Wegkamp, 2008). No increase in folate production in the mutant cell colonies that grew on media containing methotrexate in this study can indicate that the mutant cells may have other mechanisms of countering methotrexate's growth inhibition.

Figure 4 shows that most of the cells exposed to methotrexate had continuously elongated cell growth and incomplete or delayed cell division; hence, they appeared to form long filaments. This finding aligns with the research results of Zhang *et al.* (2021), who reported that wild-type cells of *E. coli*, treated with a novel DHFR inhibitor compound (named CD15-3), exhibited a filamentous morphology. Cellular filamentation and morphological alterations have been reported to be indicators of stress responses triggered by the inhibition of DHFR in the folate pathway (Ahmad *et al.,* 1998; Justice *et al.,* 2008; Sangurdekar *et al.,* 2011; Zaritsky *et al.,* 2006; Zhang *et al.,* 2021). The present finding shows that MTX exposure similarly affected the cell morphology when observed qualitatively using a microscope. Further exploration and a better understanding of these mechanisms may require a quantitative study.

The change in cell morphology can occur as a microbial strategy for increasing defense against stress exposure, such as antibiotics (Yang *et al.,* 2016; Banerjee *et al.,* 2021). In such conditions, microbes will focus more on growth than cell division, thus increasing cell size and surface area (Ojkic *et al.,* 2022). This mechanism is reported to facilitate the development of cell resistance to toxic compounds and promote the emergence of beneficial mutations in response to environmental stress (Yang *et al.,* 2016).

Ojkic *et al.* (2022) explained the concept of antibiotic stress resistance via bacterial cell shape-shifting, in which bacteria modulate their cell morphology by changing their cell size, surface-to-volume ratio (S/V), or cell curvature, as a mode of resistance to antibiotic stress. Cell volume and surface area can increase (decreased S/V) when cells are exposed to toxic compounds that target DNA, RNA, ribosomes, or cell wall damage. In contrast, compounds that target membrane damage will bind directly to the membrane, thereby inducing a decrease in cell volume and surface area (increased S/V) to reduce the density of membrane-bound compounds.

Compounds targeting DNA damage will induce the SOS (“Save Our Ship”) response to inhibit cell division, resulting in elongated cells. Compounds targeting cell wall damage will inhibit peptidoglycan synthesis and affect septal cell wall synthesis, so cells grow elongated without dividing. The compounds that target ribosome damage will inhibit the translation process and increase the synthesis of new ribosomes to compensate for the inhibition. In this case, the increase in ribosome production will be allocated differently by bacteria depending on the availability of nutrients. In nutrient-rich media, more ribosomes will be allocated for division, resulting in smaller cells to encourage the entry of more nutrients. In contrast, in nutrient-poor media, more ribosomes will be allocated for growth, resulting in larger cells (to dilute the concentration of antibiotics in cells) (Ojkic *et al.,* 2022).

In this study, as an antifolate compound (which blocks folate biosynthesis), exposure to methotrexate stress also seems to induce an increase in cell size, resulting in the formation of bacterial filaments; hence, the cells can survive growing in media containing methotrexate. The mechanism of filamentation may have similarities with the mechanism of antibiotic stress resistance via bacterial cell shape-shifting. In both folate-producing isolates R23 and JK13, exposure to methotrexate may have induced an excessive increase in polyglutamate folate (intracellular) synthesis to compensate for inhibiting its biosynthesis (to maintain intracellular folate

homeostasis). Thus, the extracellular folate measured in this study decreased (as it had been allocated to intracellular folate production). Furthermore, since the medium used in this study is a folate-free (folate-poor) medium, the increase in intracellular folate production (which plays a role in DNA synthesis) will be focused more on cell growth than cell division. This will induce inhibition of cell division, resulting in cell growth that continues to elongate as an adaptation response to methotrexate stress exposure.

The increase in intracellular folate synthesis that triggers the modulation of bacterial cell morphology may be one of the mechanisms by which mutant cells of both R23 and JK13 isolates adapt against growth inhibition by methotrexate. However, it may work synergistically with other mechanisms that are not yet known. In this case, analyzing intracellular folate becomes important to study the possible mechanism of methotrexate resistance. This particular study focuses on extracellular folate production as it is more applicable and effective in increasing product folate levels (Mahara *et al.,* 2019).

The restoration of mutant cell morphology occurs in later generations (Figure 4C). Most of the mutant cells appeared to return to their wild-type cell shape after being grown on medium without methotrexate, despite the slightly visible filamentation. Wegkamp (2008) also reported that mutant cells of *L. plantarum* WCFS1 with a methotrexate-resistant folate overproducing phenotype turned back into wild-type cells and lost their mutant phenotype after 30 generations of growth in a medium without methotrexate. Therefore, phenotypic instability in the form of changes in the return of cells to their wild-type cell shape after several generations in this study may also be a common phenomenon in mutant cells due to the stress exposure of toxic compounds (Wegkamp, 2008). Banerjee *et al.* (2021) also explained that changes in cell shape and size would encourage faster growth, thus allowing bacteria to become more adaptive in surviving environmental stress, and subsequent changes in cell shape can return to their original shape after several generations.

Addition of various compounds to increase extracellular folate production

Although it had no increase in extracellular folate production from the previous stage, the isolate R23 was then chosen for the stage of increased extracellular folate production by the addition of various enhancer compounds due to its complete folate biosynthetic genes (Mahara *et al.,* 2023) and its potential as a probiotic with cholesterol-lowering activity (Maryati *et al.,* 2016) and preventing diarrhea (Nuraida *et al.,* 2012) as a value-added health benefit. However, as shown in Figure 5, addition of various enhancers in this study did not significantly increase extracellular folate production in isolate R23.

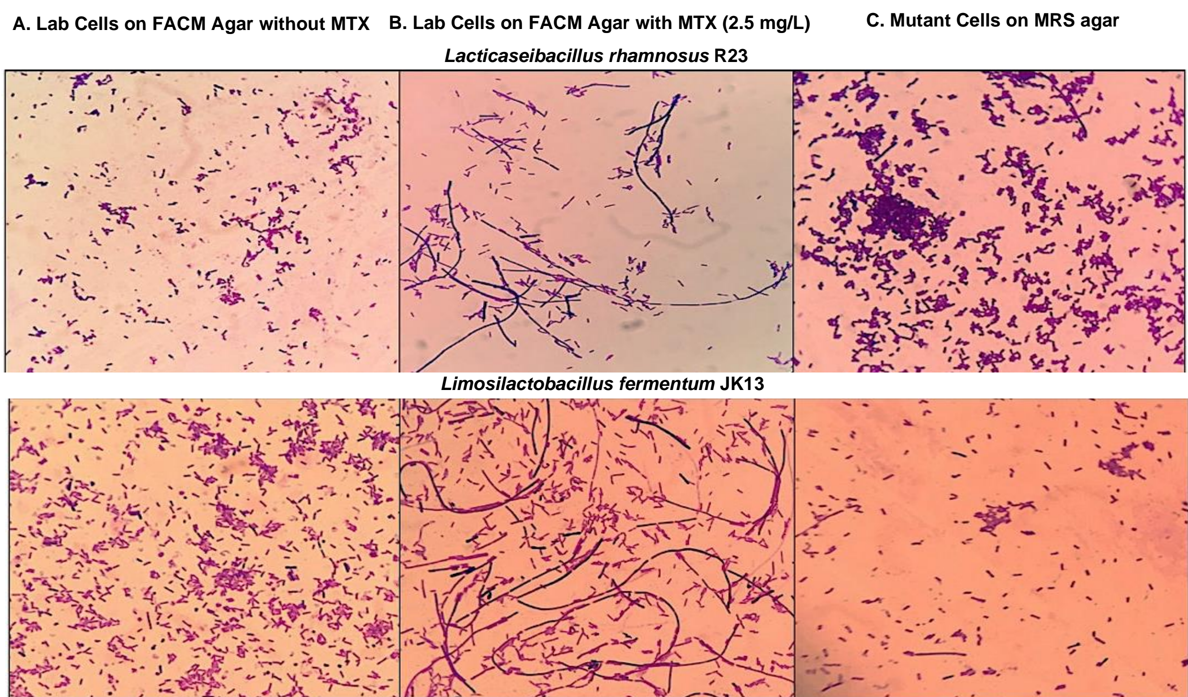

Figure 4. Differences in cell morphology of *Lacticaseibacillus rhamnosus* R23 and *Limosilactobacillus fermentum* JK13: A. Lactic acid bacteria (LAB) cells in folic acid casei medium (FACM) agar without methotrexate (MTX); B. LAB cells in FACM agar with MTX 2.5 mg/L (mutant cells); C. Mutant cells in MRS agar (MRSA; CM0361, Oxoid Ltd., Basingstoke, United Kingdom)

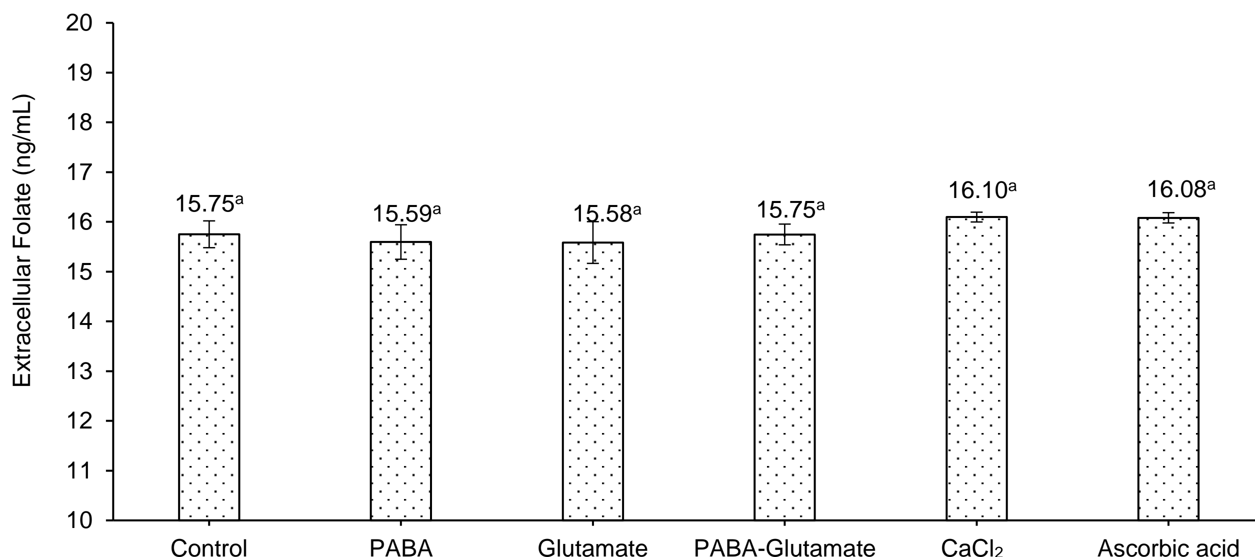

Figure 5. Effect of adding various enhancers on extracellular folate production by isolate R23

The production of extracellular folate remained unchanged, with or without addition of various enhancers. It only ranged from 15.59–16.08 ng/mL, not much different from the control treatment (without adding enhancers), which was 15.75 ng/mL.

Most LAB (especially *Lactobacilli*) cannot synthesize PABA precursors (Rossi *et al.,* 2011) and glutamate (Lapujade *et al.,* 1998), so the addition of these folate precursors is expected to increase extracellular folate production by LAB. However, there was no significant increase in extracellular folate production in this study, possibly due to the inadequate concentration of the added compounds to increase folate productivity.

Divya and Nampoothiri (2015) reported that the addition of 100 µmol/L PABA to skim milk medium significantly increased extracellular folate production in *Lactococcus lactis* CM28, while a higher concentration of PABA (125 µmol/L) had no significant effect. Laiño *et al.* (2019) also reported that adding 10 µmol/L PABA significantly increased folate production of *S. macedonicus* CRL415 but did not affect cell growth. However, when the PABA concentration was increased up to 1500 µmol/L, no further increase in folate production was obtained. Meanwhile, research by Mousavi *et al.* (2013) showed that the presence of PABA (1–150 µmol/L) did not significantly affect the folate yield and cell growth of *S. thermophilus* BAA-250, although the highest folate production was observed at 1 µmol/L PABA. On the other hand, the results of Hugenschmidt *et al.* (2011) showed that the addition of 10 mg/L PABA to SWP media significantly increased extracellular folate production more than 10 times in co-culture isolates of *L. plantarum* SM39 and *P. freudenreichii* DF13.

Likewise, with glutamate, adding glutamate at certain concentrations was also reported to increase extracellular folate production significantly. Divya and Nampoothiri (2015) reported an increase in extracellular folate production of up to around 15.63% in *Lactococcus lactis* in milk media supplemented with 75 µmol/L glutamate. However, Hugenschmidt *et al.* (2011) reported that the addition of glutamate (10 mg/L) combined with PABA (10 mg/L) in the SWP medium did not affect the level of folate production and the growth of co-culture isolates of *L. plantarum* SM39 and *P. freudenreichii* DF13. The results of these studies indicate that each bacterial strain may have different optimum concentrations of precursors. Therefore, in this study, the precursor addition of PABA, glutamate, or a combination of both may still require optimization of concentration to increase extracellular folate production significantly.

The addition of $CaCl_2$ has been reported to induce high folate productivity in LAB. High concentrations of extracellular calcium ions (Ca^{2+}) can disrupt intracellular calcium homeostasis, leading to an increase in free calcium concentration in the cytosol. This ultimately causes cell membrane injury and triggers the release of folate from the cell (Farber 1990; Zhang *et al.,* 2020). Moreover, the Ca^{2+} ion can activate GTP cyclohydrolase, a metal-dependent enzyme, catalyzing the first step of folate biosynthesis in bacteria. In addition, Ca^{2+} is considered a safe metal cation; thus, its use in food products can be a promising application to increase the folate productivity of LAB (Zhang *et al.* 2020). Zhang *et al.* (2020) reported that adding $CaCl_2$ (100 mg/mL) increased extracellular folate production by 18% in *L. plantarum* GSLP-7 V. However, adding a higher concentration of $CaCl_2$ had no significant effect.

On the other hand, folate is susceptible to oxidation by exposure to heat, light, air, and acidic pH, which causes loss of biological activity as it can

change folate to its inactive forms such as *p*-aminobenzoyl glutamate (Gangadharan and Nampoothiri 2011; Divya and Nampoothiri 2014). During growth, LAB will produce lactic acid, causing the pH of the media to be more acidic. The acidic pH of the media can cause oxidative damage to some unstable forms of extracellular folate when excreted into the medium, resulting in a measurable decrease in the production level (Padalino *et al.,* 2012). Hence, the addition of sodium ascorbate as an antioxidant can prevent oxidative damage of folate and restore the inactive form of folate to active folate, resulting in high folate measurements (Gangadharan and Nampoothiri 2011). Gangadharan and Nampoothiri (2011) reported that skimmed milk media added with sodium thioglycolate (0.2 g/100 mL) or sodium ascorbate (0.2 g/100 mL) could increase folate production up to 70 ng/mL in *L. lactis ssp. cremoris.* Divya and Nampoothiri (2015) also reported that adding 0.2% sodium ascorbate to skim milk media increased extracellular folate production to 61.02 ng/mL in *L. lactis ssp. cremoris.*

In this study, addition of 100 mg/L $CaCl_2$ and 0.2% ascorbic acid did not significantly increase extracellular folate production. Similar to folate precursors, adding $CaCl_2$ as a folate inducer and ascorbic acid as an antioxidant at different concentrations may have different effects on folate production for each bacterial strain. Therefore, these two compounds addition may also require optimization of concentration to increase extracellular folate production significantly.

The two folate-producing LAB isolates used in this study, *i.e.*, R23 and JK13, were reported to behave as folate-efficient bacteria, which will only produce folate as their growth needs (Mahara *et al.,* 2021). No increase in extracellular folate production after the methotrexate stress exposure and the addition of various enhancer compounds in this study may also relate to the characteristics of LAB. At this point, the available nutrients in FACM (including precursors) may have been sufficient for bacterial growth, so precursors and other enhancer compounds addition cannot increase folate production even more. Furthermore, the methotrexate stress exposure successfully applied to *L. plantarum* WCFS1 was also reported that this strain can produce excessive folate, thus resulting in a high increase in folate production (Wegkamp, 2008). Nevertheless, the results of this study indicate that the use of these two methods may not be appropriate for increasing extracellular folate production in folate-efficient strains.

CONCLUSION

Methotrexate exposure did not trigger excessive production of extracellular folate in the LAB isolates of R23 and JK13. The formation of bacterial filaments was found to be one of the bacterial adaptive responses against growth inhibition by methotrexate. The addition of various folate biosynthesis enhancer compounds requires further concentration optimization in order to provide a high increase in extracellular folate productivity. Further evaluation on the intracellular folate concentration produced after methotrexate exposure is also required.

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