Folate compounds are among the water-soluble vitamins and are key nutrients required for cell division, DNA synthesis, and amino acid biosynthesis (1). Folate deficiency can result in neural tube defects (2) and megaloblastic anemia (3). Supplements and folic acid-fortified foods usually contain a synthetic monoglutamate form (PteGlu2). It has been well characterized yet. A chloramphenicol-resistant strain L. rhamnosus ATCC 7773 as well as the wild-type strain ATCC 7469 was usually used to assay folate in biological samples (6, 7). Particularly, the use of the chloramphenicol-resistant strain can readily determine folate in many samples using automated microtiter plate technology because it does not require sterilization or aseptic conditions (8, 9). However, the chloramphenicol-resistant strain has not been well characterized yet.

Food folate compounds are usually treated using folate conjugases from porcine kidney, chicken pancreas or rat serum (10, 11). Porcine kidney and rat serum conjugases produce PteGlu1 as an end-product, whereas chicken pancreas folate conjugase produces PteGlu2 (12, 13). Porcine kidney folate conjugase exhibits exopeptidase activity, whereas chicken pancreas and rat serum enzymes have endopeptidase activity (13). Of these commercially available folate conjugases, chicken pancreas has the highest specific activity (10, 11), so chicken pancreas preparation is recommended for use as a folate conjugase in microbiological folate assays as the AOAC official methods (10, 11). However, Gohl et al. (14) reported that different levels of growth of L. rhamnosus ATCC 7469 were stimulated by the addition of PteGlu1 and PteGlu2. If so, the folate content determined by treating food samples with chicken pancreas
folate conjugase would be incorrect.

In this study, we investigated whether two *L. rhamnosus* strains (ATCC 7469 and ATCC 27773) used for the folate microbiological assays showed similar or different growth responses to the addition of PteGlu₁ and PteGlu₂, and we compared the total folate contents of various foods determined using microbiological assays with PteGlu₁ or PteGlu₂ as the folate compound.

**Materials and Methods**

**Materials.** PteGlu₁ and PteGlu₂ were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Schircks Laboratories (Jona, Switzerland), respectively, and were used as folate standard compounds. Type II porcine kidney acetone powder (Sigma-Aldrich) and chicken pancreas powder (DIFCO, Franklin Lakes, NJ, USA) were used as folate conjugases. In addition, α-amylase (from *Aspergillus oryzae*), protease (Type XIV, from *Streptomyces griseus*), and the certified reference material BRC-485 (from mixed vegetables) were obtained from Sigma-Aldrich.

**Organisms and growth conditions.** The *L. rhamnosus* strains ATCC 27773 and ATCC 7469 were obtained from the American Type Culture Collection (Manassas, VA, USA). These were cultured at 37°C for 24 h in Lactobacilli Inoculum Broth (Nissui, Tokyo, Japan) and then cultured in Lactobacilli culture agar (Nissui). The agar cultures were stored at 4°C and then cultured in Lactobacilli Inoculum Broth at 37°C for 24 h before being used in a folate assay. For *L. rhamnosus* ATCC 27773, chloramphenicol (30 mg/L) was added to the Lactobacilli Inoculum Broth. Folate assay medium used was composed of dextrose D (+) 40 g/L, sodium acetate 40 g/L, charcoal-treated pancreatic digest of casein 10 g/L, dipotassium phosphate 1 g/L, monopotassium phosphate 1 g/L, l-asparagine 0.6 g/L, l-cysteine hydrochloride 0.5 g/L, DL-tryptophan 0.2 g/L, magnesium sulfate (anhydrous) 0.2 g/L, ferrous sulfate 20 mg/L, sodium chloride 20 mg/L, xanthine 20 mg/L, manganese sulfate 15 mg/L, adenine sulfate 20 mg/L, guanine hydrochloride 10 mg/L, reduced glutathione 5 mg/L, *p*-aminobenzoic acid 2 mg/L, riboflavin 1 mg/L, calcium pantothenate 0.8 mg/L, nicotinic acid 0.8 mg/L, pyridoxine hydrochloride 0.4 mg/L, and thiamine hydrochloride 0.4 mg/L. When using *L. rhamnosus* ATCC 27773, chloramphenicol (30 mg/L) was added to the medium.

**Preparation of folate conjugases.** The chicken pancreas (0.10 g) and porcine kidney (0.12 g) powders were dissolved in 20 mL of 0.1 mol/L sodium phosphate buffer, pH 7.8 and pH 6.1, respectively. Activated charcoal powder (2.0 g) was added to each solution, stirred for 1 h at 4°C, and centrifuged at 9000 × g for 10 min at 4°C to remove the endogenous folate compounds. The supernatant fractions were treated with a membrane filter (25AS020AS; ADVANTEC® Tokyo Roshi Kaisha Ltd., Tokyo, Japan) and used as the folate conjugases.

**Extraction of total folates from foods.** Folate compounds were extracted from various foods by the tri-enzyme method (11). Several grams of the food sample were homogenized with a mortar and pestle. With the chicken pancreas folate conjugase, aliquots (0.1–1.0 g) of the homogenate were extracted in 2 mL of 0.1 mol/L sodium phosphate buffer, pH 7.8. With the porcine kidney folate conjugase, 0.1 mol/L sodium phosphate buffer, pH 6.1, was used as the extraction buffer. Distilled water was added to the homogenates to obtain a final volume of 5 mL. Octanol (100 µL) was then added and the homogenates were autoclaved at 121°C for 15 min. After cooling to room temperature (25°C), 1 mL of 0.1 mol/L sodium phosphate buffer at either pH 6.1 (for porcine kidney folate conjugase) or pH 7.8 (for chicken pancreas folate conjugase) and 100 µL of protease solution (7.0 × 10⁻⁴ U) were added to each homogenate, and the homogenates were incubated at 37°C for 3 h. To stop the protease enzyme reaction, the homogenates were heated at 100°C for 3 min. After cooling with ice, they were treated with 100 µL of α-amylase solution (0.3 U) for 2 h at 37°C. Subsequently, 400 µL of either porcine kidney or chicken pancreas folate conjugase solution, prepared as described earlier, was added to the homogenates, which were then left for 16 h at 37°C. The treated homogenates were heated at 100°C for 3 min to stop the enzyme reactions and then cooled to room temperature (25°C). Each homogenate was diluted with distilled water to a final volume of 10 mL, filtered through filter paper (type 2, 90 mm, ADVANTEC®), and used as a food folate extract.

**Microbiological assay for total folates.** The folate assays were performed using 13 × 100 mm polypropylene tubes (Bio-Rad Laboratories, Hercules, CA, USA), into which were added the food extract (50 µL), 0.1 mol/L sodium phosphate buffer, pH 7.0, (200 µL), and *L. rhamnosus* folate assay medium (1 mL). The prepared assay mixture was diluted with distilled water to give a final volume of 2.0 mL and then autoclaved at 121°C for 5 min. After cooling to room temperature (25°C), the tube was inoculated aseptically with 50 µL of *L. rhamnosus* ATCC 27773 pre-cultured in Lactobacilli Inoculum Broth as described above, washed three times with 5 mL of saline buffer, and dissolved in saline buffer to achieve 92% light transmittance at 660 nm. After incubating the tube at 37°C for 22 h, its optical density at 660 nm was determined using a UV-2550 Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Each food sample was assayed for folate content in triplicates, and this was repeated at least thrice.

**Preparation of calibration lines.** PteGlu₁ (8.83 mg) and PteGlu₂ (3.42 mg) were weighed accurately and dissolved in 2.0 mL and 6.0 mL of 0.1 mol/L potassium phosphate buffer, pH 7.0, respectively, and they were then stored until use at −30°C. The solutions were diluted with distilled water to obtain a final concentration of 1 nmol/L. PteGlu₁ and PteGlu₂ concentrations were determined by measuring the absorbance at 346 nm (ε₃₄₆ = 7.200 M⁻¹ cm⁻¹) for folic acid (10). Various concentrations (0.0, 0.025, 0.05, 0.10, 0.15, and 0.20 nmol/L) of PteGlu₁ or PteGlu₂ were added in place of the food extract to folate assay mixture described earlier, and the folate assay tubes were treated under the same conditions as described. Based on the results from
preliminary experiments, *L. rhamnosus* ATCC 27773 was incubated at 37°C for 22 h, whereas *L. rhamnosus* ATCC 7469 was incubated for 15 h. The optical density of each tube was determined at 660 nm using the UV-2550 Spectrophotometer (Shimadzu). The calibration lines were made in triplicates.

**Statistical analysis.** The effects of various concentrations of PteGlu1 or PteGlu2 on the growth of the *L. rhamnosus* strains were evaluated by two-way ANOVA, with post hoc analysis using Sidak’s multiple comparison tests. The folate contents of the food samples were evaluated by one-way ANOVA, with post hoc analysis using Tukey’s multiple comparison tests. The analyses were performed with GraphPad Prism 3 for Windows version 2.01 (GraphPad Software Inc., La Jolla, CA, USA). The data are presented as the mean±SEM. Differences were considered statistically significant when *p*<0.05.

**Results**

Effects of various concentrations (0–0.20 nmol/L) of PteGlu1 or PteGlu2 on the growth of *L. rhamnosus* chloramphenicol-resistant strain ATCC 27773 and wild-type strain ATCC 7469 were studied (Fig. 1). Based on the results from preliminary experiments, the chloramphenicol-resistant strain was incubated at 37°C for 22 h, whereas the wild-type strain was incubated for 15 h. The addition of PteGlu1 and PteGlu2 stimulated the growth of the chloramphenicol-resistant strain in a dose-dependent manner (Fig. 1A); however, the degree of stimulated growth under the experimental conditions was significantly greater with PteGlu1 than with PteGlu2. The slopes of the calibration lines for PteGlu1 and PteGlu2 were shown *y*=2.073 *x* and *y*=1.606 *x*, respectively. With *L. rhamnosus* wild-type strain, there was no significant difference in the growth response to PteGlu1 and PteGlu2 (Fig. 1B), at which the slopes of the calibration lines differed (*y*=2.775 *x* and *y*=2.470 *x* for PteGlu1 and PteGlu2, respectively). These results suggest that PteGlu2 be used as the standard folate compound when folate is determined in the samples treated with chicken pancreas folate conjugase particularly for *L. rhamnosus* chloramphenicol-resistant strain bioassay because the end-product of this enzyme is PteGlu2.

Table 1 summarizes the total folate contents of various foods treated with chicken pancreas folate conjugase.

**Table 1. Folate contents of various food samples treated with chicken pancreas folate conjugase using PteGlu1 and PteGlu2 as standard folate compounds.**

| Folate conjugases: Standard compounds: | Chicken pancreas PteGlu1 | Chicken pancreas PteGlu2 | Porcine kidney PteGlu1 |
|----------------------------------------|--------------------------|--------------------------|-----------------------|
| Cow Liver                              | 741.1±14.4<sup>b</sup>   | 1038.6±20.2<sup>a</sup> | 996.1±43.4<sup>a</sup> |
| Broccoli                               | 147.3±9.0<sup>b</sup>    | 202.9±12.0<sup>a</sup>  | 220.4±2.3<sup>a</sup>  |
| Asparagus                               | 120.6±10.0<sup>b</sup>  | 216.0±9.8<sup>a</sup>   | 222.4±20.6<sup>a</sup> |
| Mung beans                              | 194.8±1.9<sup>b</sup>   | 584.9±58.3<sup>a</sup> | 432.3±18.7<sup>a</sup> |
| Whole wheat flour                      | 25.4±0.5<sup>b</sup>    | 50.2±2.5<sup>a</sup>   | 49.7±3.3<sup>a</sup>  |
| Mixed vegetables*                      | 220.0±8.5<sup>b</sup>   | 307.8±12.0<sup>a</sup>| 340.5±14.0<sup>a</sup> |

Folate compounds were extracted from various food samples by the tri-enzyme method. Pteroyl-poly-γ-glutamates found in the tested food homogenates were treated with porcine kidney folate conjugase or chicken pancreas folate conjugase to form PteGlu1 and PteGlu2, respectively. Folate content was determined using PteGlu1 or PteGlu2 as a standard compound in *Lactobacillus rhamnosus* ATCC 27773 bioassays. Food folate content was assayed in triplicates and repeated at least thrice. Different superscript letters indicate values that differ significantly (*p*<0.05).

PteGlu1, pteroyl-mono-γ-glutamate; PteGlu2, pteroyl-di-γ-glutamate.

<sup>*</sup>The certified reference material BCR-485 contains 315 μg of total folate per 100 g of dry weight.
determined by *L. rhamnosus* chloramphenicol-resistant strain bioassay using PteGlu1 and PteGlu2 as standard compounds. When PteGlu2 was used as the standard folate compound, the folate content values determined for all food samples tested were similar to those determined using a calibration line of PteGlu1 in the samples treated with porcine kidney folate conjugase because the end-product of the porcine enzyme is PteGlu1. However, the folate content values determined using a calibration line of PteGlu1 were significantly low in the food samples treated with chicken pancreas folate conjugase. In particular, a certified reference material BCR-485 is a lyophilized mixed vegetable preparation containing chopped tomatoes, frozen carrots, and sweetcorn, packaged into food-grade, heat-sealed, aluminum laminate sachets under an inert atmosphere, containing 31.5 μg of folate compounds per 100 g of dry weight; this was similar to the values determined using calibration lines of PteGlu1 and PteGlu2 in the samples treated with porcine kidney and chicken pancreas folate conjugases, respectively. These results indicate that when chicken pancreas folate conjugase is used in preparing the sample for *L. rhamnosus* chloramphenicol-resistant strain bioassay, PteGlu2 should be used as the standard folate compound.

**Discussion**

*L. rhamnosus* wild-type strain ATCC 7469 and chloramphenicol-resistant strain ATCC 27773 have been widely used to assay folate in biological samples (6, 7). From the comparison study of serum and red blood cell folate assay for population surveys, the chloramphenicol-resistant strain reportedly yielded a higher response to reduced folate forms such as 5-methyltetrahydrofolate and 5-formyltetrahydrofolate than to folic acid (6). Furthermore, the chloramphenicol-resistant strain (42–45 h) appears to require much longer incubation time to determine serum folate than the *L. rhamnosus* wild-type strain (16–20 h) (6). This indicates that growth rate is faster in *L. rhamnosus* wild-type strain than in the chloramphenicol-resistant strain. As shown in Fig. 1A and B, the chloramphenicol-resistant strain showed a higher response to PteGlu1 than to PteGlu2 as a folate calibrator compound relative to the wild-type strain. These observations clearly showed that the properties of *L. rhamnosus* wild-type strain and the chloramphenicol-resistant strain are different.

It was reported that the behavior of *L. rhamnosus* wild-type strain ATCC 7469 changes its character depending on the form of folate compounds present in the medium used for the final inoculation (5). We used a commercially available Lactobacilli Inoculum Broth (Nissui) for the inoculation. Although the inoculum broth used contains yeast extract (5.5 g/L) as a folate source, we do not have detailed information available on the forms of folate compounds present in the medium. Although the wild-type strain reportedly showed similar growth responses to PteGlu1 and PteGlu2 (5), there was slight difference in the growth response to these folate compounds in the wild-type strain (Fig. 1B). Such different behavior may be due to the difference in folate forms present in the inoculum culture.

Foods such as vegetables and fruits mainly contain PteGlu4 as folate compounds. Because it is difficult for both *L. rhamnosus* strains in the folate bioassay to use PteGlu4 for their growth, PteGlu4 must be treated with folate conjugase to convert them into PteGlu1 or PteGlu2. Food folate compounds are usually treated using folate conjugases from porcine kidney, chicken pancreas or rat serum (10, 11). The AOAC official methods state that chicken pancreas preparation is suitable for use as a folate conjugase in microbiological folate assays because this preparation has the highest enzyme activity (10, 11). Indeed, our preliminary experiments indicated that the specific activities of folate conjugases from commercially available chicken pancreas, rat serum, and porcine kidney were 8.2, 0.05, and 0.03 nmol/min/mg protein, respectively, in the enzymatic conversion of PteGlu2 to PteGlu1. However, rat serum has been widely used because it is readily available and has enough enzyme activity against appropriately diluted samples in food folate assay (12).

Because porcine kidney folate conjugase reportedly has optimum activity at pH 4.5–5.0 (13), pH of folate extracts is usually adjusted to pH 4.5–5.0 to treat with the folate conjugase after α-amylase and protease-treatments. However, we used a pH 6.1 buffer in the enzyme treatment, including porcine kidney folate conjugase, according to the folate bioassay method adopted in the Japanese Standard Tables of Food Composition (15). As shown in Table 1, the folate values determined for all food samples treated with chicken pancreas by *L. rhamnosus* ATCC 27773 microbiological assays using PteGlu2 as standard compounds were similar to those determined in the samples treated with porcine kidney folate conjugase. These results implied that porcine kidney conjugase has enough enzyme activity under the conditions used in the experiments. However, the treatment of porcine kidney folate conjugase should be done under the optimum acidic pH conditions because of its low specific activity.

Tamura and Stokstad described that no significant differences in food folate activity were found after treatment with either porcine kidney or chicken pancreas folate conjugase (16). However, as per the results presented here, to determine folate in the samples treated with chicken pancreas folate conjugase according to the AOAC official method, PteGlu2 (the end-product of this enzyme) should be used as the standard folate compound, especially for *L. rhamnosus* ATCC 27773 bioassay.

**Disclosure of state of COI**

The authors declare no competing financial interests.

**Author contributions**

K.K. and N.O. performed most experiments. S.E. supervised the experimental techniques for folate bioassay and discussed the results. K.K., T.B., Y.Y. and E.W. designed the experiments, interpreted the results, and
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RESERVED RESPONSES

All authors commented on the manuscript and approved the final version.

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