Microbial Metabolic Capacity for Intestinal Folate Production and Modulation of Host Folate Receptors

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Microbial metabolites, including B complex vitamins contribute to diverse aspects of human health. Folate, or vitamin B9, refers to a broad category of biomolecules that include pterin, para-aminobenzoic acid (pABA), and glutamate subunits. Folates are required for DNA synthesis and epigenetic regulation. In addition to dietary nutrients, the gut microbiota has been recognized as a source of B complex vitamins, including folate. This study evaluated the predicted folate synthesis capabilities in the genomes of human commensal microbes identified in the Human Microbiome Project and folate production by representative strains of six human intestinal bacterial phyla. Bacterial folate synthesis genes were ubiquitous across 512 gastrointestinal reference genomes with 13% of the genomes containing all genes required for complete de novo folate synthesis. An additional 39% of the genomes had the genetic capacity to synthesize folates in the presence of pABA, an upstream intermediate that can be obtained through diet or from other intestinal microbes. Bacterial folate synthesis was assessed during exponential and stationary phase growth through the evaluation of expression of select folate synthesis genes, quantification of total folate production, and analysis of folate polyglutamylation. Increased expression of key folate synthesis genes was apparent in exponential phase, and increased folate polyglutamylation occurred during late stationary phase. Of the folate producers, we focused on the commensal Lactobacillus reuteri to examine host–microbe interactions in relation to folate and examined folate receptors in the physiologically relevant human enteroid model. RNAseq data revealed segment-specific folate receptor distribution. Treatment of human colonoid monolayers with conditioned media (CM) from wild-type L. reuteri did not influence the expression of key folate transporters proton-coupled folate transporter (PCFT) or reduced folate carrier (RFC). However, CM from L. reuteri containing a site-specific inactivation of the folC gene, which prevents the bacteria from synthesizing a polyglutamate tail on folate,
INTRODUCTION

The human gut microbiota harbors a complex and dynamic microbial community that is dominated by Firmicutes and Bacteroidetes, with a lesser proportion of Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia (D’Argenio and Salvatore, 2015). Collectively the intestinal microbiota plays a pivotal role in nutrient digestion and production of host-modulating metabolites (O’Connor, 2013; Berding and Donovan, 2016; Maruvada et al., 2017). The fermentation and metabolic conversion of dietary components by intestinal bacteria results in diverse metabolites such as SCFAs, long chain fatty acids, lactic acid, and biogenic amines (Cummings and Macfarlane, 1997; Kimura et al., 2002; Hayakawa et al., 2004; Izquierdo Canas et al., 2009; Tabanelli et al., 2012; Thomas et al., 2012; Frei et al., 2013; Fersl et al., 2014; Hollister et al., 2014; Kibe et al., 2014; Luk et al., 2018; Shimizu et al., 2018). These metabolites may have an impact on the intestinal milieu and mammalian health status. Vitamins are another such end-product (Birnbaum et al., 1967; Friend et al., 1983; Cummings and Macfarlane, 1997; Hugenholtz and Smid, 2002; Crittenden et al., 2003; Streit and Entcheva, 2003; Burgess et al., 2006; Jayashree et al., 2010; LeBlanc et al., 2011, 2013b). Vitamins cannot be synthesized by mammals and therefore must be obtained through intestinal absorption from exogenous sources, including dietary sources and the gut microbiota (Rossi et al., 2011). This point is clearly demonstrated by the fact that germ-free animals lacking a microbiota require supplementation of vitamin K and certain B vitamins, while their conventionalized complete microbiota counterparts do not require these vitamins (Rossi et al., 2011). Bacterial genera common in the distal intestine, including Bacteroides, Bifidobacterium, and Enterococcus, are known to synthesize vitamins (Morowitz et al., 2011). Previous studies have identified individual species capable of producing vitamins (Birnbaum et al., 1967; Friend et al., 1983; Cummings and Macfarlane, 1997; Hugenholtz and Smid, 2002; Crittenden et al., 2003; Streit and Entcheva, 2003; Burgess et al., 2006; Jayashree et al., 2010; LeBlanc et al., 2011, 2013b). Additionally, several studies have performed in-depth analyses of vitamin production by microbes from the human intestine (Magnusdottir et al., 2015; Rowland et al., 2018; Das et al., 2019) These studies have highlighted the need for an evaluation of the folate synthesis capabilities of a broad cohort of bacterial strains, in conjunction with a detailed evaluation of the folates synthesized, as well as the specific effects of those folates on the human intestine.

Microbes produce B complex vitamins, including B1, B6, B12, and vitamin B9. Folate, or vitamin B9, is a broad category of biomolecules characterized by containing a complex of pterin, pABA, and glutamate subunits (Blakley and Benkovic, 1984). Folate biosynthesis requires a complex pathway involving 16 enzymatic processes (KEGG, 2017; Kanehisa and Goto, 2000). First, chorismate is produced from the glycolysis product, phosphoenolpyruvate which combines with pABA. GTP is converted to dihydropterin pyrophosphate prior to combining pABA and dihydropterin pyrophosphate intermediates. Finally, glutamylation and reduction yield THF, the simplest form of bioavailable folate (Scott, 1999; Rossi et al., 2011). The majority of naturally occurring folates are THF, 5-methyl THF, and 5-formyl THF, which are polyglutamylated. Bacteria synthesize mono- and poly-glutamylated folate (also known as 5,10-methenyl-THF or FPG), folate forms that are easily processed and absorbed by mammalian cells (Kim T. H. et al., 2004; Thomas et al., 2016). Microbe-generated vitamins, including folate, are primarily absorbed in the colon, while dietary vitamins are largely absorbed in the small intestine (Ichihashi et al., 1992; Saïd and Mohammed, 2006). Several bacterial strains within the human microbiome synthesize and secrete THF which can be absorbed by the intestinal epithelium (Camilo et al., 1996; Asrar and O’Connor, 2005; Maynard et al., 2018). Previous studies have evaluated potential probiotic strains such as Bifidobacterium and Lactobacillus as a source of folate compounds (LeBlanc et al., 2011, 2013b). However, few studies have examined the capacity of the gut microbiome to generate folate. Moreover, a paucity of data have been published defining microbe-mediated folate modifications, such as polyglutamylation.

On the host side, dietary and microbe-produced folate uptake occurs through four proteins: folate receptor alpha (FOLR1), folate receptor beta (FOLR2), PCFT, or RFC (Zhao and Goldman, 2007; Visentin et al., 2014). FOLR1 and FOLR2 are localized to tissue outside the intestine and neither receptor is believed to contribute significantly to intestinal folate absorption (Hague et al., 1995; Barber et al., 1999; Kim, 1999, 2006; Kamen and Smith, 2004; Kim D. H. et al., 2004; Blom et al., 2006; Pitkin, 2007; Vollset et al., 2013; Wikowo et al., 2013; Zhao and Goldman, 2013; Visentin et al., 2014; Shen et al., 2015; Balashova et al., 2018). In contrast PCFT and RFC folate transporters are postulated to play a significant role in intestinal folate transport (Visentin et al., 2014). To the best of our knowledge, no study has examined folate transporter distribution along the length of the human intestine or identified folate transport/assimilation mechanisms

Abbreviations: CHCA, α-cyano-4-hydroxycinnamic acid; CM, conditioned media; DTT, dithiothreitol; folC2, folylpolyglutamate 2 gene; FPG, folylpolyglutamates; GCPII, glutamate carboxypeptidase II; GTP, guanosine triphosphate; pABA, para-aminobenzoic acid; PCFT, proton-coupled folate transporter; RFC, reduced folate carrier; SAM, S-adenosylmethionine; SCFAs, short chain fatty acids; THF, tetrahydrofolate.

Keywords: B vitamin, enteroids, folate transporters, folylpolyglutamate, Lactobacillus reuteri, Lactobacilli, microbiome
in different regions of the gastrointestinal tract. Existing studies examining folate receptor expression and function have relied on cancer-derived cell lines. However, intestinal cancer cells exhibit a hallmark upregulation of FOLR1, which is not normally present in the healthy colon and this upregulation may affect other folate transporter responses. Another major gap in knowledge is the lack of studies describing folates produced by microbes and mammalian folate transporters. A more accurate model of the intestine is needed to identify folate-related functions in response to microbial folate compounds.

Folate is essential for several metabolic processes including one-carbon transfer, methylation metabolism through the synthesis of SAM, and the synthesis of thymidylate, purines, and several amino acids (Baggott and Tamura, 2015; Brosnan et al., 2015). Once absorbed, folate also participates in nucleotide synthesis and replication, repair, and methylation of DNA. Given the important role of folates in cell maintenance, deficiency in folate has been linked to a large spectrum of health disorders including coronary heart disease, osteoporosis, Alzheimer’s disease, and colorectal cancer (LeBlanc et al., 2011, 2013a). Bacterial folate has been put forward as a potential nutrition disease, and colorectal cancer (LeBlanc et al., 2011, 2013a). However, this effect has been hindered by the lack of characterization of folate-secreting intestinal bacterial strains.

In this study, the predicted folate synthesis capabilities of representative members of the human intestinal microbiome were evaluated to determine the patterns and prevalence of bacterial folate metabolism genes. Bacterial folate production was examined in a phylogenetically diverse set of intestinal microbes representing six key phyla of the human intestinal microbiome. Additionally, a more in-depth characterization of folate glutamylation by two key commensal microbes was examined by mass spectrometry. To connect microbe-derived folate metabolism with uptake by the host, we assessed folate-associated processes in human intestinal enteroids by RNaseq and identified the potential for microbial folate compounds and FPGs to alter the expression of folate transporters in human enterocytes.

**MATERIALS AND METHODS**

**Bacterial Strains and Culture Conditions**

All bacterial strains and growth conditions used in this study are described in Supplementary Table S1. Bifunctional dihydrofolate synthase/folylpolyglutamyl synthase type 2 (folC2) and bifunctional dihydrofolate synthase/folylpolyglutamyl synthase (folC) genes (GenBank NZ_ACGX02000001—007, HMPREF0536_11260, and HMPREF0536_10555, respectively) were inactivated in *Lactobacillus reuteri* ATCC PTA 6475 (*L. reuteri* 6475) by site-specific integration of plasmid pORI28 into the *L. reuteri* 6475 chromosome as described previously (Walter et al., 2005; Thomas et al., 2016). Dideoxy DNA sequencing was used to confirm site-specific insertional mutagenesis. *L. reuteri* 6475, *L. reuteri* 6475 folC2:pORI28 (referred to as folC2), and *L. reuteri* 6475 folC:pORI28 (referred to as folC) were cultured in MRS medium. Insertion mutants were cultured in the presence of 10 µg/mL erythromycin. *Bifidobacterium longum* subspecies *infantis* ATCC 15697 was also cultured in MRS medium. *Prevotella copri* CB7 DSM 18205 and *Clostridium sporogenes* DSM 795, ATCC 3584 were grown in brain–heart-infusion (BHI) broth supplemented with 2% yeast extract and 0.2% cysteine. *Akkermansia muciniphila* ATCC BAA-835 was cultured in BHI supplemented with 0.4% pig stomach mucin (Sigma Aldrich). *Escherichia coli* Nissle 1917 was cultured in Luria-Bertani (LB) broth. All cultures were grown in an Anaerope Systems AS-580 anaerobic chamber supplied with a mixture of 10% CO2, 5% H2, and 85% N2. Following initial growth, cultures were subcultured into the appropriate fully defined medium. Bacterial media recipes and reagents for the fully defined media are labeled as LDM4 and CDMM and compiled in Supplementary Tables S2, S3, respectively.

For growth curves, cultures of *L. reuteri* 6475, *L. reuteri* 6475:folC2, and *L. reuteri* 6475:folC grown at exponential phase in MRS were used to inoculate the fully-defined medium LDM4 (OD_{600nm} adjusted to 0.1) and incubated anaerobically at 37°C. Growth was examined by optical density (OD_{600nm}) at time 0, 1, 3, 6, 9, 12, 24, and 48 h. Optical densities were examined at OD_{600nm} on a Smartspec Plus Spectrophotometer (Bio-Rad Laboratories Inc.) and colony forming unit (CFU) counts were performed at 6, 24, and 48 h on MRS or MRS-ERM agar plates to examine growth.

**Generation of Bacterial Supernatants**

To generate bacterial Conditioned media (CM) for cell treatment, *L. reuteri* 6475, *L. reuteri* 6475:folC2, and *L. reuteri* 6475:folC were grown at exponential phase in MRS and used to inoculate LDM4 (OD_{600nm} adjusted to 0.1). Cultures were grown anaerobically at 37°C for 24 h (stationary phase). Following incubation, cells were pelleted by centrifugation (5,000 × g for 5 min) and the supernatant was treated with DTT (Sigma 10708984001) to release protein-bound folates, then filtered using Amicon Ultra-15 centrifugal filter units using the ultracel-3 membrane (Millipore UFC900324, Millipore, Bedford, MA, United States). The filtrate (<3 kDa) was lyophilized and resuspended in enteroid media to generate 25% CM in the appropriate cell culture medium. All CM was pH-adjusted to pH = 7.4.

**Folate Quantification**

Bacterial cultures were collected at exponential or stationary phases (strain-specific media and incubation duration in Supplementary Table S1). Cultures were centrifuged at 3,000 × g for 15 min at 4°C. Bacterial culture supernatants were reserved for folate quantification. The cell pellet was resuspended in STE buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM Ethylenediaminetetraacetic acid (EDTA), pH 8.0) and centrifuged at 7,500 × g for 5 min at 4°C, and supernatants were removed. Bacterial cell pellets were resuspended in 100 µl of “Fol diluent,” included with the ARCHITECT Folate Assay (Abbott Laboratories, Abbott Park, IL, United States). One hundred microliters of culture supernatants was diluted in 100 µl of “Fol diluent.” The concentration of folate present in each sample was determined using the ARCHITECT Folate assay,
a competitive immunoassay using direct chemiluminescence. Samples were treated with 0.1 M DTT for 5 min to release folate conjugates from binding proteins in the sample. Folate quantification was determined through a competitive binding assay utilizing an acridinium–folate-bound biotin–folate-binding protein. Binding was detected by the ARCHITECT i System using avidin–conjugated paramagnetic solid phase particles. The inter- and intra-assay variation of the assay was <10% and recovery of added folate was >90%.

MALDI TOF MS Analysis of Bacterial Folates
Bacterial cultures were collected at exponential or stationary phases (strain-specific media and incubation duration in Supplementary Table S1). The cultures were centrifuged at 4,500 × g for 10 min at 4°C, and bacterial supernatants were removed. Bacterial cell pellets were washed with water and centrifuged at 6,000 × g for 10 min at room temperature. Folates were extracted from cell pellets by adding extraction buffer (0.1% TFA, 50% acetonitrile in water), vortexing, and centrifuging at 16,000 × g for 10 min at room temperature. The folate-containing supernatants were spotted on CHCA matrix (ProteoChem P9100, Hurricane, UT, United States) onto a MALDI target (SimuTOF Systems, Marlborough, MA, United States). A standard consisting of eight peptides ranging from 8 to 28 amino acids was spotted onto the MALDI target as calibrant. Spectra were acquired on a SimuTOF Combo 200 Mass Spectrometer (Virgin Instruments, Marlborough, MA, United States) in reflector mode and annotated with MoverZ software (Proteometrics, LLC, New York, NY, United States).

Human Gastrointestinal Microbial Genome Analysis
Distribution of the folate synthesis genes in the gastrointestinal reference genomes of the Human Microbiome Project (HMP) was determined using the Integrated Microbial Genomes and Metagenomes (IMG/M) (Markowitz et al., 2012) available through the Joint Genomes Institute (JGI). Version 5.0 (updated August of 2018) was accessed through https://img.jgi.doe.gov/ on November 14, 2018. The Profile and Alignment Tool (in JGI) was used to determine identities of folate biosynthesis genes present in the 512 gastrointestinal bacterial reference genomes of the HMP. Genes encoding enzymes involved in folate synthesis were designated by the enzyme commission (E.C.) number. The E.C. number for each enzyme involved in folate synthesis was queried against each genome using the Profile and Alignment Tool in JGI. Folate metabolic capacity was established on the basis of genes present in each genome.

Evaluation of Bacterial RNA by qPCR
Bacterial cells were fixed by adding an equal volume of cold methanol to exponential or stationary phase bacterial cultures. Fixed cells were pelleted at 16,000 × g for 1 min. RNA was isolated from bacterial cell pellets using the QuickRNA kit (Zymo Research, Irvine, CA, United States) according to the manufacturer's instructions with modifications. Briefly, bacterial pellets were resuspended in 100 µl STE buffer (100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA), and transferred to tubes containing 0.1 mm glass beads. Samples were disrupted on a Fast Prep bead homogenizer (MP Biologicals) for 20 s at 4.0 m/s. Following disruption, Zymo Quick RNA lysis buffer was added to each glass bead containing tube, and the samples were disrupted on the Fast Prep homogenizer for a second time. Cell debris and beads were removed by centrifugation at 10,000 × g for 1 min, and the supernatant was processed for RNA according to the manufacturer's protocol. Genomic DNA removal was performed using the Ambion Turbo DNA-free kit following manufacturer instructions (ThermoFisher, Waltham, MA, United States). Quantitation and quality control (A260/A280 and 260/230 ratios) were performed with a Nanodrop OneC (ThermoFisher Scientific, Waltham, MA, United States) Spectrophotometer. Synthesis of cDNA from RNA was completed by reverse transcription using the SensiFast cDNA synthesis kit (Bioline, London, United Kingdom). For quantitative real-time PCR (qRT-PCR) analysis of bacterial folate genes, bacterial cDNA was examined using FAST SYBR green and primers (Supplementary Table S4) on a Stratagene 3005q qPCR System (ThermoFisher, Waltham, MA, United States). For relative quantitation, the ΔΔCT method was used and microbial mRNA quantities were normalized to amounts of 16S rRNA.

Treatment of Human Colonoid Monolayers With Bacterial Folates
Differentiated human colonoid monolayers were generated as previously described (Zou et al., 2017; Rajan et al., 2018). The colonoid lines AsCl09 and C103 were established from colonic tissue sampled via endoscopic-guided biopsy procedure from the ascending colon (Baylor College of Medicine, IRB # H-35094). Three-dimensional colonoids were cultured in complete medium with growth factors (CMGF+) as previously described (Sato et al., 2011; Saxena et al., 2016). Briefly, CMGF+ consisted of advanced Dulbecco’s modified Eagle medium (DMEM)/F-12 medium (Invitrogen) supplemented with 1× Glutamax (Invitrogen), 10 mM HEPES buffer (Invitrogen), and 100 U/mL penicillin–streptomycin (Invitrogen), with 50% (v/v) Wnt3A-conditioned medium, 20% (v/v) R-spondin conditioned medium, 10% (v/v) Noggin-conditioned medium, 1× N-2 supplement (Invitrogen), 1× B-27 supplement (Invitrogen), 1 mM N-acetylcysteine (Sigma–Aldrich), 50 ng/mL mouse epidermal growth factor (EGF) (Invitrogen), 10 nM Leu-Gastrin I (Sigma–Aldrich), 500 nM A-83-01 (Tocris Bioscience), 10 nM SB202190 (Sigma–Aldrich), and 10 mM nicotinamide (Sigma–Aldrich, St. Louis, MO, United States). Colonoids were passaged in phenol red-free, growth factor-reduced Matrigel (Corning). Colonoids at passage 6 were seeded into flat 96-well plates as described previously (VanDussen et al., 2015; Ettayebi et al., 2016) to generate monolayers. Briefly, 96-well plates were pre-treated with Matrigel diluted in 1× phosphate-buffered saline (PBS) (1:40) and incubated at 37°C. The Matrigel was removed from 3D colonoids and the colonoids were washed with an ice-cold solution of 0.5 mM EDTA in 1× PBS and dissociated with 0.05% trypsin/0.5 mM EDTA at 37°C for 4 min. Following
the incubation, the trypsin was inactivated with advanced DMEM/F12, 1 x Glutamax, 1 x HEPES + 10% fetal bovine serum. The cell solution was pipetted vigorously and filtered via a 40 µm nylon cell strainer (Falcon, Cat. No. 352340). The resulting single cells were centrifuged at 400 x g for 5 min, resuspended with CMGF+ and 10 µM Y-27632 Rock inhibitor, and plated into Matrigel-coated wells. After 48 h, the medium was changed to differentiation medium, which contains the same components as CMGF+ without Wnt3A conditioned medium, R-spondin conditioned medium, SB202190, and nicotinamide and only 5% (v/v) Noggin conditioned medium, with the addition of 10 µM Y-27632 Rock inhibitor. Differentiation medium with Y-27632 was changed daily for 4–5 days to differentiate cells.

For bacterial supernatant treatment, the differentiation media on colonoid monolayers on 96-well plates was changed to DMEM without folate supplemented with 1 x HEPES, 1 x Glutamax, and 1 x pyruvate. Colonoids were treated for 16 h with either media alone, 25% CM from uninoculated LDM4, wild-type L. reuteri media alone, 25% CM from uninoculated LDM4, wild-type L. reuteri + 10 µM Y-27632 Rock inhibitor. Differentiation medium with Y-27632 was changed daily for 4–5 days to differentiate cells.

Mammalian RNA Extraction and RNAseq
To assess the transcriptional profile of human intestinal enteroids, sterile monolayers were prepared on transwells and differentiated as previously described (Zou et al., 2017). RNA was extracted via TRIZOL according to the manufacturer’s instructions and rRNA integrity was checked on a 2100 Bioanalyzer (Agilent, Santa Clara, CA, United States) from two independent biological replicates per segment. Using mRNA enriched samples by Novogene, paired-end Illumina sequencing was performed following the standard work-flow procedure. Raw sequence reads were mapped to human genome hg19 using STAR software to generate the number of fragments per kilobase per million mapped reads (FPKM) for each gene (n = 2 biological replicates).

Evaluation of Mammalian RNA by qPCR
Colonoid RNA was extracted via TRIZOL according to the manufacturer’s instructions and genomic DNA removal was performed using the Ambion Turbo DNA-free kit (ThermoFisher, Waltham, MA, United States). Quantitation and quality control (A260/A280 and 260/230 ratios) were performed with a Nanodrop OneC (ThermoFisher Scientific, Waltham, MA, United States) Spectrophotometer. cDNA was synthesized from RNA by reverse transcription using the SensiFast cDNA synthesis kit (Bioline, London, United Kingdom). Expression of key folate-related genes was analyzed using qRT-PCR. The reaction was performed with colonoid cDNA and FAST SYBR green master mix (Applied Biosystems, Foster City, CA, United States) per manufacturer instructions. The following amplification conditions were used: after an initial denaturation at 95°C for 20 s, 40 amplification cycles were performed at 95°C for 3 s and 60°C for 30 s on a Stratagene 3005 qPCR System (ThermoFisher, Waltham, MA, United States). Primers used in this study are listed in Supplementary Table S4. The ΔΔCt method was used for relative quantification and mRNA levels were normalized to human 18S.

Statistical Analyses
Statistical analyses were performed using GraphPad Prism version 198 5.04 (GraphPad Software, San Diego, CA, United States). All data were normally distributed and examined using non-parametric tests. All data were analyzed by one-way ANOVA with a Bonferroni post hoc test. Statistical test results are presented as mean ± standard deviation (SD). P-values are indicated by asterisks as follows: *p < 0.05.

RESULTS
Folate-Generating Capacity Is Ubiquitous Throughout the Human Intestinal Microbiome
The folate metabolic capacity of 512 well-defined, phylogenetically diverse reference bacterial genomes from the human gastrointestinal tract was evaluated. Bacterial genomes were binned as potential contributors of folate biosynthesis if the genome contained at least one set of genes representing folate biosynthetic metabolic pathway. Pathways include genes required for synthesis of (1) chorismate from phosphoenolpyruvate (seven enzymatic functions), (2) pABA from chorismate (two enzymatic functions), (3) dihydropterin pyrophosphate from GTP (four enzymatic functions), and (4) THF from pABA and dihydropterin pyrophosphate intermediates (four enzymatic functions) (Figure 1A). A summary of the relative capacity of these gut microbes to synthesize specific folate intermediates is illustrated in a stacked graph stratified by phylum (Figure 1B).

Chorismate acts as an intermediate for diverse metabolic pathways including folate and amino acid biosynthesis and the genes required to synthesize chorismate from the glycolysis product, phosphoenolpyruvate, were found in 256 genomes (Figure 1B). These genomes represented all key phyla except Bacteroidetes. We found 134 genomes encode the capacity to convert chorismate to pABA including microbes from the phyla Proteobacteria, Firmicutes, Actinobacteria, and Fusobacteria. A total of 368 genomes, 72% of the reference genomes, representing six key intestinal phyla encode the capacity of synthesizing dihydropterin pyrophosphate from GTP (Pterin Synthesis). Genes involved in the synthesis of THF from pABA and dihydropterin pyrophosphate (folate synthesis) were found in 291 (57%) of the bacterial genomes representing six key phyla of the human intestinal microbiome, Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Proteobacteria, and Verrucomicrobia (Figure 1B). A total of 392 (77%) bacterial
Engevik et al. Bacterial Folate Affects Human Receptors

FIGURE 1 | Distribution of folate synthesis genes in the gastrointestinal human microbiome. (A) Schematic of de novo folate synthesis divided into four modules: (1) chorismate synthesis, (2) pABA synthesis, (3) pterin synthesis, and (4) folate synthesis. Each protein is listed by name and enzyme commission (E.C.) number. (B) Distribution of genomic folate synthesis capabilities within the Human Microbiome Project’s Gastrointestinal Reference Genomes stratified by phylum.

genomes contain genes capable of synthesizing THF if pABA is present as a precursor (Figure 1B). We also found that 267 genomes had the capacity for both pterin and folate synthesis. Finally, we found that 68 genomes were capable of using chorismate to synthesize folates completely de novo (Figure 1B). The phyla Firmicutes and Proteobacteria exhibited the highest number of genomes capable of de novo folate synthesis. This dataset indicates that the majority of microbes must obtain precursors in the form of dietary nutrients or microbial cross-feeding in order to produce luminal folate in the intestine.

Functional Characterization of Folate Production by Intestinal Microbes

The biochemical functionality of bacterial folate biosynthesis genes identified in situ was verified in a cohort of 10 bacterial strains representing six key phyla of the human intestinal microbiome. These microbes included three strains which are currently available as probiotics (L. reuteri ATCC PTA 6475, Enterococcus faecalis Symbioflor DSM 16431, and E. coli Nissle 1917) (Gronbach et al., 2010; Mu et al., 2018; Baccouri et al., 2019). Figure 2 depicts the genetic capacity of 10 bacterial
strains to synthesize folate de novo. Circle shading corresponds to genetic capacity; with completely shaded circles representing complete gene sets for the indicated pathways and empty circle indicating no genes present (Figure 2). From this representative cohort, only two strains (B. longum subsp. infantis ATCC 15697 and E. coli Nissle 1917) representing two phyla (Actinobacteria and Proteobacteria) contain complete pathways for generating folate de novo. In the phylum Firmicutes, three of four strains (C. sporogenes DSM 795, E. faecalis Symbioflor DSM 16431, and L. reuteri ATCC PTA 6475) are completely devoid of genes necessary to synthesize pABA and hence their ability to synthesize folate depends on the presence of pABA in their environmental milieu. This is consistent with previous work demonstrating the requirement of pABA for L. reuteri folate production (Spinel et al., 2014; Thomas et al., 2016).

To verify our in situ findings, we characterized folate production by one strain representing each of the six major intestinal phyla: B. longum subsp. infantis, C. sporogenes, L. reuteri, E. coli Nissle, A. muciniphila, and P. copri. We cultured each strain to exponential and stationary phase growth since bacterial metabolism differs significantly between growth phases (Rolfe et al., 2012; Robador et al., 2018; Supplementary Table S1). We collected supernatants and cell pellets and analyzed folate concentrations by competitive immunoassay. The intermediate pABA was included in all defined medias (LDM4 and CDMM). We found that in exponential growth phase, B. longum subsp. infantis (123.3 ± 11.2 ng/ml), L. reuteri (62.6 ± 1.8 ng/ml), and A. muciniphila (64.4 ± 0.15 ng/ml) produced net amounts of folate, while C. sporogenes, E. coli Nissle, and P. copri were folate consumers (Table 1). However, in stationary phase the majority of bacteria were net folate producers, with B. longum infantis generating the highest concentration (261.1 ± 0.15 ng/ml). Based on net production or consumption of folate, representative intestinal microbes can be divided into three functional groups: folate consumers, folate producers, and conditional producer/consumer. Regardless of growth phase, P. copri DSM 18205 is a net folate consumer and B. longum subsp. infantis ATCC 15697 is a net folate producer. E. coli Nissle 1917, on the other hand, is a conditional producer, consuming folate in exponential phase and producing folate during stationary phase (Table 1). This in vitro data indicate that several intestinal species are capable of generating folate.

**Different Folylpolyglutamate Profiles Generated by Intestinal Microbes**

We examined two members of the gut microbiota, E. coli and L. reuteri, to assess expression of folate genes and the ability...
to polyglutamatylate folate. Consistent with folate production (Table 1), qPCR analysis of key folate genes indicated increased expression in the exponential phase for E. coli Nissle 1917 (Figure 3A). Genes such as folK, the final enzyme required for pterin synthesis, were upregulated in E. coli Nissle 1917 exponential phase compared to stationary phase. Similar to E. coli Nissle 1917, folK was also upregulated in L. reuteri ATCC 6475 during exponential phase. Additionally folP, the enzyme which combines the pterin and pABA intermediates was significantly upregulated in exponential phase compared to stationary phase.

TABLE 1 | Total folate production quantified by chemiluminescence immunoassay.

| Strain information | Exponential phase | Stationary phase |
|--------------------|------------------|-----------------|
| Phylum Species Strain Designation (exponential, stationary) Media folate (ng/mL) Supernatant and cell pellet folate (ng/mL) | | |
| Actinobacteria Bifidobacterium longum infantis ATCC 15697 Producer, producer 133.7 ± 11.7 123.3 ± 11.2 | 261.0 ± 0.15 |
| Firmicutes Lactobacillus reuteri ATCC 6475 Producer, producer 133.7 ± 11.7 62.6 ± 1.8 | 155.3 ± 4.0 |
| Firmicutes Clostridium sporogenes ATCC 5858 Consumer, producer 133.7 ± 11.7 −88.0 ± 1.5 | 1.2 ± 0.1 |
| Proteobacteria Escherichia coli Nissle 1917 Consumer, producer 100.0 ± 4.1 −59.6 ± 1.3 | 49 ± 2.7 |
| Verrucomicrobia Akkermansia muciniphila BAA-835 Producer, producer 106.8 ± 1.1 64.4 ± 0.15 | 12.73 ± 1.3 |
| Bacteroidetes Prevotella copri DSM18205 Consumer, consumer 133.7 ± 11.7 −38.7 ± 9.7 | −44.9 ± 1.9 |

All data are represented as average ± SD (n = 3 biological replicates). Total bacterial folate (ng/mL) was determined by subtracting the amount of folate in the uninoculated media from the sum of the average folate concentration in the bacterial supernatant and the average folate concentration in the cell pellet. The colors indicate production of folate (green) or consumption of folate (blue).

Examine folate-associated processes (Foulke-Abel et al., 2016; Saxena et al., 2016; Zou et al., 2017). Moreover, human enteroids grown as 2D monolayers represent an optimal tool for examining host-microbe interactions. Using RNaSeq, we identified several folate-related genes in human enteroids derived from duodenum, jejunum, ileum, and colon (Figures 4A–E). High expression was observed in genes associated with nucleotide synthesis, methylation, one-carbon cycle, and folate transporters (Figures 4B–E). The most highly expressed genes included methylenetetrahydrofolate reductase (MTHFR), folypolyglutamate synthase (FPGS), 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR), PCFT (SLC46A1) and methylenetetrahydrofolate dehydrogenase (NADP + Dependent) 2 (MTHFD2), methylenetetrahydrofolate cyclohydrolase, and folate hydrolysis (FOLH) (Figure 4A). These genes represent unique host pathways involved in folate homeostasis. Interestingly, of these highly expressed genes, robust expression was observed in the small intestine (duodenum, jejunum, and ileum) and to a slightly lesser degree in the colon. FOLH is responsible for converting FPG to monoglutamate which can then be absorbed by mammalian cells and FOLH1 expression was high throughout all intestinal segments, including the colon (35.7 ± 12.5 FPKM). Since the colon is the primary site of microbe-produced folate absorption (Pompei et al., 2007a; Aufreiter et al., 2009; Lakoff et al., 2014), we chose to focus our attention on the colon.

We modeled host-microbe interactions by examining folate receptor/transporter gene expression in response to folate generated by L. reuteri 6475. We also included two L. reuteri 6475 mutants which harbored insertions in either the folC gene (6475:folC) or the folC2 gene (6475:folC2). Our previous study showed that 6475:folC lacks the ability to produce a polyglutamate tail on 5,10-methenyl THF and that 6475:folC2 does not produce any 5,10-methenyl THF (Thomas et al., 2016). We confirmed that these mutants exhibit normal growth in LDM4 by optical density (OD600nm) and CFU over 48 h (Supplementary Figures S1A,B). The CFU counts of all L. reuteri strains (WT, 6475:folC, or 6475:folC2) after 24 h of growth mirror the concentrations of L. reuteri observed in patients (10^8) (Million et al., 2013) and concentrations that are well tolerated and safe in humans (Mu et al., 2018). As a result, 24 h was selected for further analysis.

Bacterial Folate-Mediated Modulation of Human Folate Transporter Gene Expression

The novel human enteroid model has transformed the study of intestinal epithelial cells. Enteroids derived from intestinal stem cells represent non-transformed, multicellular epithelial cultures that can express all the native differentiated intestinal cell types (Sato et al., 2009, 2011; Clevers, 2016; Saxena et al., 2016; Zou et al., 2017). Enteroids appear to recapitulate human intestinal physiology, making them an ideal model to...
FIGURE 3 | Folate synthesis during exponential and stationary growth phase by qPCR and MALDI-TOF mass spectrometry. Expression of folate synthesis genes at exponential and stationary phase growth in (A) Escherichia coli Nissle 1917 and (B) Lactobacillus reuteri ATCC PTA 6475 as determined by qPCR. aroC is involved in chorismate synthesis; pabB in pABA synthesis; folP, folC, and folA in pterin and folate synthesis. Expression is normalized to 16S rRNA. One-way ANOVA with Bonferroni post hoc test. *p < 0.05 (n = 3 biological replicates). Differential glutamylation profiles of folates determined by MALDI-TOF mass spectrometry for (C) E. coli Nissle 1917 and (D) L. reuteri ATCC PTA 6475. L. reuteri produces both the 5,10-methenyl THF as well as the 5,10-ethenyl THF form with +14 glutamate (GLU) residues. Blue boxes highlight the peaks which represents MTHF + nGLU.

By mass spectrometry, we quantified the folate produced by our L. reuteri strains after 24 h growth (Supplementary Figure S1C). Consistent with our previous work, we demonstrate that the 6475:folC mutant is capable of producing glutamylated folate (170 ± 25.6 ng/ml), while the 6475:folC2 mutant is unable to produce folate above the LDM4 media control (16.7 ± 1.2 ng/ml) (Supplementary Figures S1D,E).

Using wild-type L. reuteri 6475 and insertion mutants 6475:folC and 6475:folC2 as a model for microbe folate production, the relative abilities of microbial-derived folates to modulate human folate receptor gene expression could be examined. Two human colonoid lines were used for all analyses: Asc109 and C103. Colonoid monolayers were treated with DMEM containing no folate in the presence of 25% LDM4.
or 25% L. reuteri (WT, 6475:folC, or 6475:folC2) LDM4 CM overnight (16 h). After treatment, the colonoid monolayers maintained their intestinal architecture as observed by light microscopy (data not shown). To confirm the viability of the enteroid monolayers following treatment, we assessed viability by trypan blue staining (Figures 5A,E) and metabolic activity by resazurin assay (Figures 5B,F). Consistent with the microscopic images, we observed no changes in viability or metabolic activity in colonoids treated with either L. reuteri strain. Analysis of mRNA levels of the PCFT (Figures 5C,G) and reduced folate transporter (RFC) (Figures 5D,H) by qPCR revealed unique patterns in response to our bacterial supernatant. Application of bacterial supernatants had no effect on the levels of the PCFT in either colonoid line (Figures 5C,G). However, the application of bacterial supernatants containing a non-glutamylated folate (L. reuteri 6475:folC-mutant strain) to human colonoids yielded enhanced expression of the folate transporter RFC (Figures 5D,H). This effect was not observed with the L. reuteri 6475:folC2-mutant strain which is unable to synthesize folate, pointing to the potential role of non-glutamylated folate in modulating RFC levels. In addition to receptor levels, other folate-related genes were queried (Supplementary Figure S2). No changes were observed in the GCPII (folh1), which is required to deconjugate polyglutamylated folates, with any of the treatment conditions (Supplementary Figures S2A,B). Likewise, no significant changes were observed in any MTHFD genes (Supplementary Figures S2C–H) or serine hydroxymethyltransferase 2 (SMTH2) (data not shown). Together these data demonstrated that bacteria in the human gastrointestinal microbiome produce bioavailable folates and that the glutamylation status of microbial folate can influence folate transporter expression in the human intestine (Figure 6).

**DISCUSSION**

Our study has demonstrated that folate biosynthesis is a major biochemical feature of the human intestinal microbiome. Four basic metabolic modules in folate biosynthesis have been defined, and described in terms of prevalence of these separate modules in the reference set of 512 microbial genomes attributed to the human gastrointestinal tract. These modules include chorismate, pABA, pterin, and folate synthesis. Our data demonstrate that these genes are distributed among the six phyla of the human microbiome (Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria, and Verrucomicrobia). However, only strains from Proteobacteria and to a lesser degree Firmicutes, Actinobacteria, and Verrucomicrobia (68 strains in total) are able to completely generate folate de novo. This study provides insight into the genetics of the
FIGURE 5 | Expression of folate transporters in human colonoids treated with L. reuteri conditioned media. Human colon monolayers (lines Asc109 and C103) were incubated with 25% uninoculated bacterial medium (LDM4) or 25% L. reuteri ATCC 6475 conditioned media for 16 h. In addition to WT L. reuteri, the 6475:folC mutant, which can produce THF, but can’t synthesize a polyglutamate tail, and the 6475:folC2 mutant, which can’t synthesize any THF, were included in the analysis. Viability assessment of colonoids following 16 h incubation as determined by (A,E) trypan blue staining and (B,F) resazurin assay. Expression of proton-coupled folate transporter, PCFT (C,G) and reduced folate carrier, RFC (D,H) determined by qPCR normalized to 18S. One-way ANOVA with Bonferroni post hoc test. *p < 0.05 (n = 2 biological replicates, three technical replicates).

human intestinal microbiome and suggests a cooperative role of microbial communities to generate B complex vitamins in the human host. Our studies revealed different glutamylation profiles for commensal gut microbes that may affect folate bioavailability in the intestine. We specifically showed that L. reuteri and E. coli Nissle generate moderate amounts of folate (49–77 ng/µl) and differ in the extent of polyglutamylation. Finally, our data showed that in the absence of bacterial THFs, folate transporter expression in human colonic enterocytes is significantly increased. This work has advanced the scientific understanding of bacterial folate metabolism and expanded the potential role for the microbiome in treating vitamin deficiencies.

Folate deficiency contributes to serious health problems and can result from insufficient dietary intake, poor absorption of ingested folate, and altered folate metabolism due to genetic defects or drug interactions (Rajagopalan et al., 2002; Hiraoka and Kagawa, 2017). In the absence of a genetic inability to absorb folate, folate deficiency can be treated through dietary changes, increasing consumption of dark green vegetables, beans, liver, and folate-enriched foods. Long-term correction of low folate through the diet has yielded mixed results (Menon et al., 2005; Kemperman et al., 2006; Nair and Iyengar, 2009; Kaehler et al., 2012). Folate deficiency can also be treated by supplementation with folic acid, a synthetic, fully oxidized, mono-glutamylated form of folate. In contrast to the synthetic folic acid, naturally occurring dietary and microbe-produced folates are polyglutamylated, fully reduced THFs with methyl, methylene, or formyl groups. THF is the form of folate that can be processed through the one-carbon cycle, making this form a more desirable format for absorption. As a result, we speculate that the delivery of microbial folates may be amenable for the treatment of low folate. Microbe-derived folate is particularly attractive for patients with inactivating single-nucleotide polymorphisms (SNPs) in dihydrofolate reductase (DHFR), which is required to convert folic acid to THF. These patients are unable to efficiently absorb synthetic folates (Askari and Krajinovic, 2010; Hiraoka and Kagawa, 2017) and thus need alternative sources of THFs. By delineating the mechanisms regulating the production and secretion of folate by the microbiome, manufacturers, physicians, and researchers can make informed decisions regarding probiotic recommendations and nutritional counseling based on human microbiome science.

We found that 13.3% of 512 bacterial reference genomes from the human intestinal microbiome contain the genes spanning all four metabolic modules involved in de novo synthesis of THF. This sizeable fraction of intestinal microbes containing complete sets of folate biosynthetic genes suggests that the microbiome serves as an important folate-generating “organ.” The remaining
Engevik et al. Bacterial Folate Affects Human Receptors

FIGURE 6 | Model of microbial folate production and microbe:host interactions. Polyglutamylated tetrahydrofolates are synthesized by the human intestinal microbiome. Human gamma-glutamylhydrolase, GGH, and gamma-glutamyltransferase, GGT, remove glutamate moieties making monoglutamylated folates available for uptake by other luminal bacteria or human colonocytes. Folate can activate folate receptors (Frα or Frβ) or be taken up into enterocytes by folate transporters (RFC or PCFT).

genomes (>86%) require folate or folate intermediates from other bacteria of the human microbiome or the human diet. Therefore, when proposing a potential probiotic source of folate, either complete capacity to generate folates or effective combinations of folate producing strains (combining a pABA-producing strain with a pABA-dependent strain) should be considered. Our data are consistent with previous genomic studies that predicted that 26% of Actinobacteria, 79% of Fusobacteria, 71% of Proteobacteria, and 15% of Firmicutes in the human intestinal microbiome are fully capable of synthesizing folate de novo (Magnusdottir et al., 2015). Magnusdottir et al. (2015) suggested that the human microbiome was potentially capable of synthesizing 37% of the daily recommended intake of folate for non-pregnant, non-breastfeeding adults. This recommendation highlights the potential importance of the gut microbiome as a source of folate production. We have identified strains including B. ovatus ATCC 8483 and A. muciniphila ATCC BAA 835 that contain the complete complement of 16 enzyme functions needed to synthesize folates. These strains have not historically been considered probiotics and as a result remain uncharacterized and may have undesired effects. Therefore, it is advantageous to utilize a well-characterized probiotic strains for folate delivery. The human breast milk-derived strain L. reuteri ATCC PTA 6475 has been used in clinical trials (NCT02422082, NCT00774163, NCT03360253) and is known to be safe to animals and humans (Adams and Marteau, 1995; McCabe et al., 2013; Britton et al., 2014; Fatheree et al., 2017; Galofre et al., 2018; McCabe and Parameswaran, 2018; Schepper et al., 2019). The genome of L. reuteri ATCC PTA 6475 lacks genes needed to synthesize chorismate or pABA, and depends on folate precursors. As a result, we propose addition of L. reuteri and pABA to harness the full folate producing potential of L. reuteri.

Knowledge of folate-related genes in the intestine has been limited by the lack of clinically relevant systems. The majority of existing work has been performed in rodent models and cancer-derived cell lines. Work using PCFT-deficient mice has identified that the presence of PCFT is a requirement for the transport of folates across the apical brush-border membrane of the proximal small intestine (Wang et al., 2013; Visentin et al., 2014). In immortalized rat small intestinal epithelial IEC-6 cells, PCFT was also found to facilitate entry of folate at low pH, while RFC enabled folate absorption at neutral pH (Rajgopal et al., 2001). Both these studies focused on the small intestine and did not examine the colon, which is the primary site for microbe-produced folate absorption (Pompei et al., 2007a; Aufreiter et al., 2009; Lakoff et al., 2014). Although PCFT is present in the colonic mucosa, the extent of colonic folate absorption in vivo is not known. Rodents are the experimental tool of choice for the majority of intestinal absorption studies and these types of studies have yielded tremendous insight into intestinal homeostasis. Nevertheless, significant differences...
between mice and humans may affect our knowledge of folate transport (Mestas and Hughes, 2004). Lakoff et al. (2014) verified that humans can absorb folates through the colon using a pH-sensitive capsule for targeted delivery. However, this study did not evaluate the composition of the human microbiome in their subjects or the cumulative effects of microbial metabolites on folate absorption as it used a single defined monoglutamylated folate. Our work is the first to identify folate-related processes in epithelial cells within different intestinal regions based on human intestinal enteroids. Human intestinal enteroids yield a model of intestinal epithelium exhibiting a similar cellular composition and function as the intact epithelium of the human intestine (Sato et al., 2011; Sato and Clevers, 2013; Foulke-Abel et al., 2014, 2016; Middendorp et al., 2014; Saxena et al., 2016; Zachos et al., 2016). Additionally, enteroids exhibit region-specific attributes of the human gastrointestinal epithelium (Middendorp et al., 2014; Saxena et al., 2016) and are non-transformed cells derived from intestinal stem cells. Enteroids have been used to examine host–microbe and host–viral interactions (Finkbeiner et al., 2012; Engvik et al., 2013; Yin et al., 2015; Saxena et al., 2016, 2017; Zou et al., 2017; Blutt et al., 2018; Rajan et al., 2018; Yin and Zhou, 2018). To the best of our knowledge, we are the first to identify folate genes in enteroids from diverse regions of the intestine. This work suggests that although the colon expresses lower levels of the major genes involved in nucleotide synthesis, methylation, one-carbon cycle, and folate transporters compared to the small intestine, it still expresses high levels of several genes including MTHFR, DHFR, MTRR, FPGS, PCFT, and RFC. Dietary FPGs are hydrolyzed to folylmonoglutamate derivatives prior to absorption by the intestinal epithelial cells. Of particular interest, the colonoids express high levels of folate hydrolase (FOLH) and γ-glutamyl hydrolase (GGH), enzymes found of the brush border and in the lysosome of the intestinal epithelial cells (Chave et al., 2003). These enzymes participate in the dietary FOLH. Additionally, only the colon expressed folate hydrolase 2 (FOLH1B), another hydrolase known to convert FPGs to folylmonoglutamates. These findings indicate that folate absorption by the human colon may significantly contribute to net folate absorption.

This study extends our knowledge of intestinal folate metabolism by using human enteroids to examine microbial folate–host interactions. Our work indicates that bacterial supernatants containing non-polyglutamylated THFs (L. reuteri 6475:folC-mutant strain) results in upregulation of the RFC transporter. Both PCFT and RFC are highly specific for the monoglutamate form of folate (Visentin et al., 2014). We speculate that in our enteroid model, the GCPII (folh1) cleaves the glutamate moieties to provide entrance of the L. reuteri folate polyglutamates into the cell. Interesting, we did not observe any changes in folh1 expression in our colonoids following bacterial supernatant treatment. Our data indicate that WT L. reuteri, producing a glutamylated THF and L. reuteri 6475:folC2, which produces no folate, had no effect on RFC levels in human colonoids. However, we do demonstrate that L. reuteri 6475:folC, which makes a non-glutamylated folate upregulates RFC. We reason that WT L. reuteri’s glutamylated folate requires extensive deconjugation by Folh1 before absorption occurs.
enhance human health by enhancing cellular functions and intestinal homeostasis.

DATA AVAILABILITY STATEMENT
The raw data supporting the conclusions of this manuscript will be made available by the authors upon request.

ETHICS STATEMENT
This study (protocol H-35094) was carried out in accordance with the policies of, and approved by, the Baylor College of Medicine Institutional Review Board with written informed consent from all subjects.

AUTHOR CONTRIBUTIONS
MAE, CM, and JV conceived and designed the research, and drafted the manuscript. MAE, CM, DR, KE, SD, and SC performed the experiments. MAE, CM, DR, KE, and JS analyzed the data. MAE, CM, and DR prepared the figures. MAE, CM, DR, KE, SD, SC, MKE, MK, and JV edited and revised the manuscript. JV approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02305/full#supplementary-material

FIGURE S1 | Lactobacillus reuteri strains (wild-type, 6475:folC, and 6475:folC2) exhibit normal growth, but varying degrees of folate production. (A) Diagram of the genes required to convert 7,8-dihydropteroate to 5,10-methenyl-THF (tetrahydrofolate). Key genes include foxC (purple) and foxC2 (light blue). (B) Growth of WT L. reuteri and two L. reuteri mutants, 6475:folC which can produce folate, but cannot add a glutamate tail, and 6475:folC2, which cannot produce folate, in LDM4 over 48 h. Samples were collected at 1, 3, 6, 12, 16, 24, and 48 h. (C) Colony forming units (CFUs) of L. reuteri strains at time 6, 24, and 48 h post-inoculation in LDM4. (D) Folate quantification in un inoculated LDM4 or WT L. reuteri, 6475:folC and 6475:folC2 LDM4 conditioned media after 24 h incubation by competitive immunoassay. (E) Glutamation profile of folates produced by L. reuteri 6475:folC determined by MALDI-TOF mass spectrometry. The diagram depicts no clear glutamate peaks for 6475:folC folate, indicative of no polyglutamate residues. *p < 0.05 (n = 3 biological replicates, 3 technical replicates).

FIGURE S2 | Expression of downstream folate targets in human colonoids treated with L. reuteri conditioned media. Human colon monolayers (lines Asc109 and C103) were incubated with 25% uninoculated bacterial medium (LDM4) or 25% L. reuteri ATCC 6475 conditioned media for 16 h. In addition to WT L. reuteri, the 6475:folC mutant, which can produce THF, but can’t synthesize a polyglutamate tail, and the 6475:folC2 mutant, which can’t synthesize any THF, were included in the analysis. Examination of mRNA levels (A-E) folh1, required for removing folate glutamates and (B-H) methylenetetrahydrofolate dehydrogenase, required for intracellular folate processing, by qPCR revealed no changes between groups. All qPCR data were normalized to 18S One-way ANOVA with Bonferroni post hoc test (n = 2 biological replicates, 3 technical replicates).

TABLE S1 | Bacterial strains, growth media, and exponential and stationary phase time points. LDM4 supplement: 0.1 mg/L vitamin K and 5 mg/L hemin.

TABLE S2 | Lactobacillus defined medium 4 (LDM4) recipe.

TABLE S3 | Chemically defined minimal media (CDMM) recipe.

TABLE S4 | Bacterial folate synthesis qRT-PCR primers.

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Engevik et al.
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