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ORIGINAL ARTICLE

Alternative pathway for dopamine production by acetogenic gut bacteria that *O*-Demethylate 3-Methoxytyramine, a metabolite of catechol *O*-Methyltransferase

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Abstract

Aims: The gut microbiota modulates dopamine levels in vivo, but the bacteria and biochemical processes responsible remain incompletely characterized. A potential precursor of bacterial dopamine production is 3-methoxytyramine (3MT); 3MT is produced when dopamine is *O*-methylated by host catechol *O*-methyltransferase (COMT), thereby attenuating dopamine levels. This study aimed to identify whether gut bacteria are capable of reverting 3MT to dopamine.

Methods and Results: Human faecal bacterial communities *O*-demethylated 3MT and yielded dopamine. Gut bacteria that mediate this transformation were identified as acetogens *Eubacterium limosum* and *Blautia producta*. Upon exposing these acetogens to propyl iodide, a known inhibitor of cobalamin-dependent *O*-demethylases, 3MT *O*-demethylation was inhibited. Culturing *E. limosum* and *B. producta* with 3MT afforded increased acetate levels as compared with vehicle controls.

Conclusions: Gut bacterial acetogens *E. limosum* and *B. producta* synthesized dopamine from 3MT. This *O*-demethylation of 3MT was likely performed by cobalamin-dependent *O*-demethylases implicated in reductive acetogenesis.

Significance and Impact of the Study: This is the first report that gut bacteria can synthesize dopamine by *O*-demethylation of 3MT. Owing to 3MT being the product of host COMT attenuating dopamine levels, gut bacteria that reverse this transformation—converting 3MT to dopamine—may act as a counterbalance for dopamine regulation by COMT.

KEYWORDS

biotransformation, cobalamin-dependent *O*-demethylase, intestinal microbiology, metabolic processes, metabolism

INTRODUCTION

Hormone levels are tuned in the body through ways that we are only beginning to understand. There is accumulating evidence that a key piece of this metabolism puzzle is the gut microbiota, which encodes for millions of diverse biochemical reactions that remain largely uncharacterized. Robust differences in the levels of several hormones have been detected in rodents based on whether the intestine is colonized with bacteria or entirely devoid of these microorganisms (i.e., germ-free) (Luczynski et al., 2016). Additionally, variations in the collections of bacteria that compose the gut microbiota are correlated with changes in circulating levels of hormones, including serotonin and oestrogens (Fuhrman et al., 2014; Reigstad et al., 2015; Yano et al., 2015). The sources of such hormone changes remain incompletely defined, although there is evidence that the gut microbiota can impact hormone levels by two mechanisms: bacterial regulation of hormone production by the host (Xue et al., 2018) and synthesis of these endocrine molecules by gut bacteria (Cryan & Dinan, 2012; Galland, 2014). Here, we present a previously unexamined route for gut bacterial dopamine synthesis, which the gut microbiota may use to dictate levels of dopamine in vivo.

Dopamine, although most widely known for its neurotransmitter role, is a potent hormone that is present in the bloodstream at varying levels across people (Barbeau et al., 1961). One route by which dopamine enters the bloodstream is following its absorption from the intestine (Goldstein et al., 1999), a body site harbouring nearly half of peripheral dopamine (i.e., dopamine outside the brain) (Eisenhofer et al., 1997). There is evidence that the gut microbiota is a significant variable in control of peripheral dopamine levels: colonizing germ-free mice with a murine gut microbiota resulted in over a two-fold increase in dopamine levels in the colonic lumen (Asano et al., 2012). Although peripheral dopamine has significant and diverse biological effects that include modulating blood pressure, insulin levels, metabolic disease, and gastrointestinal (GI) motility, the precise bacteria and their biochemical pathways that modulate dopamine levels remain poorly understood (Martin et al., 1994; Nira, 2020; Rubí & Maechler, 2010; Sonne et al., 2021).

One route by which gut bacteria can contribute to dopamine production is through biotransformation of small molecules that are endogenous to the host. Gut bacteria *Enterococcus faecalis* and *Enterococcus faecium* produce dopamine by decarboxylating levodopa (L-dopa), an anti-Parkinsonian drug, via a tyrosine decarboxylase (TyrDC) (Figure 1) (Maini Rekdal et al., 2019; van Kessel et al., 2019). L-dopa may be produced from tyrosine, as faecal metagenomes encode for tyrosinase homologues, which are enzymes that convert tyrosine to L-dopa (Valles-Colomer et al., 2019); however, the biochemical activities of these homologues in gut bacteria remain to be functionally characterized. There is evidence that intestinal dopamine may also derive from gut bacterial hydrolysis of dopamine glucuronides (Asano et al., 2012), which are metabolites formed in a variety of tissues, including the liver and intestine (Itäaho et al., 2009). Several gut bacteria have also been reported to synthesize dopamine from undefined culture media (Wang et al., 2021), but the molecular precursors and the biochemical pathways that they traverse to be converted to dopamine remain incompletely characterized.

Here, we examine whether gut bacteria have the metabolic potential to elevate intestinal dopamine levels by counteracting host mechanisms for dopamine regulation. Peripheral dopamine levels are regulated by catechol *O*-methyltransferase (COMT) (Axelrod & Tomchick, 1958). This enzyme, which is highly expressed in the liver and GI tract (Uhlén et al., 2015), methylates dopamine to the aryl methyl ether 3-methoxytyramine (3MT) (Figure 1). There are several examples of gut bacteria *O*-demethylating aryl methyl ethers (Bess et al., 2019; Clavel, Borrmann, et al., 2006; Misoph et al., 1996; Sharak Genthner & Bryant, 1987), although gut bacterial *O*-demethylation of 3MT has remained unexamined. Studies in rats suggest that the gut microbiota can counteract COMT's regulation of dopamine levels in vivo. In rats intraperitoneally dosed with ^{14}C-labelled 3-*O*-methyldopa (the metabolite afforded by COMT *O*-methylation of L-dopa),

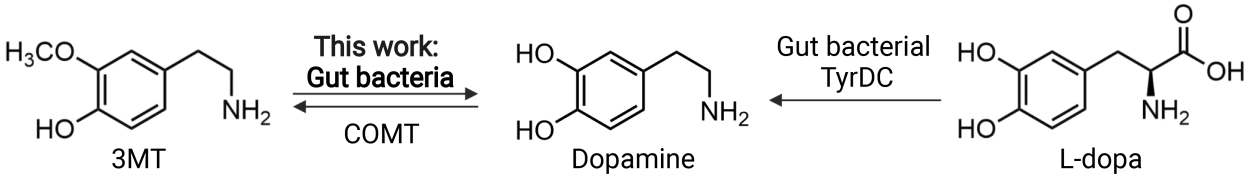

FIGURE 1 Dopamine can be *O*-methylated to 3-methoxytyramine (3MT) by catechol *O*-methyltransferase (COMT); this biotransformation attenuates the dopaminergic properties of dopamine. In this work, we identify that gut bacteria have the metabolic potential to counteract COMT's metabolism of dopamine by *O*-demethylating 3MT. Gut bacteria have previously been characterized to synthesize dopamine by decarboxylating levodopa (L-dopa) using tyrosine decarboxylase (TyrDC). Figure created with BioRender.com (agreement number RX2311G12H).

demethylation of 3-*O*-methyldopa only occurred once this compound was excreted in bile to the GI tract; no demethylation occurred in rats with fistula that prevented biliary excretion (Rivera-Calimlim, 1974). These findings indicate that 3-*O*-methyldopa, which is structurally analogous to 3MT, is *O*-demethylated in vivo, but only upon entering the GI tract. Whether bacteria or human enzymes are responsible for this demethylation remains undefined.

The GI lumen is a putative reservoir of 3MT, owing to 3MT being naturally present in the faecal excreta of rats (Jianguo et al., 2019; Ma et al., 2021), although the 3MT level in the human intestinal lumen remains to be determined. Within the gut microbiome, 3MT is in the midst of gut bacteria that are capable of *O*-demethylating a diverse array of molecules that also harbour the aryl methyl ether motif (Bess et al., 2019; Clavel, Henderson, et al., 2006; Misoph et al., 1996; Sharak Genthner & Bryant, 1987). Such *O*-demethylation is commonly carried out by acetogens (Bache & Pfennig, 1981; Frazer & Young, 1985; Misoph et al., 1996). These bacteria use cobalamin-dependent enzymes to funnel the methyl group of aryl methyl ethers into production of acetate through a process called reductive acetogenesis (Frazer & Young, 1986; Naidu & Ragsdale, 2001). Several gut bacterial acetogens have been functionally characterized to demethylate dietary aryl methyl ethers (Burapan et al., 2017; Cocaign et al., 1991; Possemiers et al., 2008; Wang et al., 2000) and encode genetic homologues for aryl methyl ether-dependent reductive acetogenesis (Kelly et al., 2016; Naidu & Ragsdale, 2001; Schilhabel et al., 2009). These gut bacteria include *Eubacterium limosum* (Sharak Genthner & Bryant, 1987) and *Blautia producta* (Misoph et al., 1996), which are members of the dominant gut microbiota (Clavel, Borrmann, et al., 2006). Here, we report the ability of *E. limosum* and *B. producta* to produce dopamine via the *O*-demethylation of 3MT. Furthermore, our findings provide supporting evidence for the involvement of cobalamin-dependent *O*-demethylases in the reversion of 3MT to dopamine.

MATERIALS AND METHODS

Bacteria, media and chemicals

Blautia producta (DSM 2950 and DSM 3507) and *Eubacterium limosum* DSM 20543 were obtained from DSMZ and were cultured in Brain Heart Infusion (BHI, Difco) supplemented with 0.05% w/v L-cysteine HCl (BHI + C). Bacteria were cultured in anoxic conditions (2%–5% H_2, 20% CO_2, with the balance being N_2) in a Coy Anaerobic Chamber. Experiments assessing the growth inhibition of conversion of 3MT to dopamine by propyl iodide and the production of acetate were performed in an acetogenic media (defined below). Faecal samples were cultured in Gifu Anaerobic Broth (GAM, Fisher Scientific). Media was equilibrated in anoxic conditions overnight to remove oxygen.

Chemicals were sourced as follows: 3-methoxytyramine HCl (3MT, 99%+, Acros Organics, 172851000), dopamine HCl (Alfa Aesar, AAA1113606), isoproterenol HCl (Cayman Chemical Company, 15592), methanol (HPLC Grade, Avantor/J.T. Baker), acetonitrile (LiChrosolv, Millipore Sigma), propyl iodide (99%, Sigma-Aldrich, 171883), and hexanes (HPLC Grade, Fisher Chemical).

Media and components for media were sourced as follows: brain heart infusion (BD Difco), brain heart infusion Agar (BD Difco, DF0418-17-7), Gifu anaerobic broth (GAM, Fisher Scientific, M1801), vitamin supplement, 100X (MD-VS, ATCC), D-glucose anhydrous (VWR Life Science, Biotechnology Grade), phosphate-buffered saline (10X) pH 7.4 (Thermo Fisher Scientific, 70011044), sodium bicarbonate (Sigma-Aldrich, S6014), L-cysteine HCl (Fisher Scientific, BP376) and casamino acids (Fisher Scientific DF0288-15-6).

Acetogenic media was prepared to contain the following: 5 mM glucose, 0.2% casamino acids, 0.2% sodium bicarbonate, 1X ATCC vitamins, 22 mM PBS, and 0.05% L-cysteine HCl in basal media. Basal media was prepared to contain the following: 1X Pfennig and Lippert trace elements, 116 nM sodium selenite, 136 nM sodium tungstate, 230 mM ammonium chloride, 2 mM calcium chloride, 2 mM magnesium sulphate, and 34 mM sodium chloride in double-deionized water. 333X Pfenning and Lippert trace elements were prepared to contain the following: 17 mM ethylenediaminetetraacetic acid, 8.4 mM iron (II) sulphate, 348 μM zinc sulphate, 152 μM manganese chloride, 4.9 mM boric acid, 841 μM cobalt (II) chloride, 58 μM copper (II) chloride, 77 μM nickel chloride, 82 μM sodium molybdate in double-deionized water adjusted to pH 4.0.

Ex vivo incubation of faecal samples

Faecal samples were obtained from seven healthy humans. All subjects consented to participate in the study, which was approved by the University of California, Irvine Institutional Review Board. Faecal samples were introduced into an anaerobic chamber, and each was resuspended in pre-reduced phosphate buffer saline (PBS), pH 7.4 at a final concentration of 0.1 g ml^{-1}. Suspensions were vortexed for 5 min and then allowed to settle for 5 min. The faecal slurries were diluted 1:100 into Gifu anaerobic media (GAM) supplemented with 3MT (500 μM in sterile double-deionized water) or vehicle control. The cultures were anaerobically cultured at 37°C for 72 h.

Quantification of 3MT and dopamine was conducted using the LC–MS/MS assay described below.

Incubations of bacteria with 3MT in vitro

Incubations with gut bacteria were carried out in brain heart infusion supplemented with 0.05% w/v L-cysteine HCl. Seed cultures were grown overnight in triplicate in anoxic conditions (2%–5% H_2, 20% CO_2, with the balance being N_2) at 37°C. Seed cultures were normalized to an OD_{600} of 0.5 in fresh media, then diluted 1:100 into media that was subsequently supplemented with 3MT (500 μM in sterile double-deionized water) or an equal amount of its vehicle. Cultures were incubated at 37°C for 72 h in triplicate with sterile controls. After 72 h, samples were removed from the anaerobic chamber and centrifuged at 2000 RPM for 20 min at 4°C. Supernatant (50 μl) was diluted five-fold in 0.2% L-ascorbic acid in water and immediately frozen at −20°C until analysed using LC–MS/MS.

Light-reversible inhibition of 3MT *O*-demethylation by propyl iodide

Incubations with *E. limosum* DSM 20543 and *B. producta* DSM 3507 were carried out in acetogenic media. Seed cultures were grown overnight in triplicate in brain heart infusion supplemented with 0.05% w/v L-cysteine HCl and in anoxic conditions (2%–5% H_2, 20% CO_2, with the balance being N_2) at 37°C. Seed cultures were normalized to an OD_{600} of 0.5 in fresh acetogenic media, then diluted 1:100 into acetogenic media that was subsequently supplemented with 3MT (500 μM in double-deionized water) and either propyl iodide (20 μM in ethanol) or an equivalent volume of vehicle (ethanol). Two sets of each media type were prepared in triplicate with sterile controls. Both sets of incubations containing 3MT and propyl iodide and one set of incubations containing 3MT and propyl iodide's vehicle were wrapped in aluminium foil to block out light; the fourth culture containing 3MT and propyl iodide's vehicle was exposed to light. Growth was tracked by measuring OD_{600}, and 100 μl aliquots of culture were sampled periodically over the course of incubation to assess metabolism of 3MT to dopamine. Aliquots were centrifuged at 2000 RPM for 10 min at 4°C, and 50 μl of supernatant was diluted five-fold in 0.2% w/v L-ascorbic $acid_{(aq.)}$. Samples were then immediately frozen at −20°C to preserve for analysis using LC–MS/MS. After reaching mid- to late-logarithmic phase of growth, one set of cultures containing 3MT and propyl iodide and another set with 3MT but without propyl iodide were exposed to light using a 60 Watt incandescent light bulb. Light exposure was maintained for the duration of the incubation.

Quantification of 3MT and dopamine by LC–MS/MS

Concentrations of 3MT and dopamine from incubations of gut bacteria with 3MT were assessed by electrospray ionization (ESI) triple-quadrupole liquid chromatography–mass spectrometry (LC–MS/MS, Acquity UPLC and Quattro Premier XE; Waters Micromass) in positive-ion mode with single reaction monitoring. Solvent A was 0.2% acetic acid and 1.8% acetonitrile in water, while solvent B was 0.2% acetic acid in acetonitrile. The concentration of solvent B was 10–90% for 0–2 min, 90–10% for 2–3 min, and 10% for 3–4 min. The column temperature was 50°C. A C18 column (Acquity UPLC BEH; 1.7 μM; 2.1 mm × 50 mm; Waters part number 186002350) was used. The injection volume was 10 μl. Retention times were as follows: 3MT, 0.79 min; dopamine, 0.76 min; isoproterenol, 0.83 min. The capillary voltage was 4.00 kV and the cone voltage was 10 V, with a source temperature of 125°C. The desolvation gas flow was 800 $L\,h^{-1}$ and the desolvation temperature was 400°C. The $[M–H]^+$ *m/z* values of parent/daughter ions were as follows: 3MT, 167.86/151.03; dopamine, 153.85/137.03; isoproterenol, 212.10/193.90.

Stock solutions of 3MT and dopamine were prepared at 50 mM in 0.2% L-ascorbic $acid_{(aq.)}$. These were used to generate a standard containing 3MT and dopamine (each standard at 1 mM in either brain heart infusion media (for screening bacteria for metabolism) or in acetogenic media (for assessing the inhibition of metabolism by propyl iodide)). These 1 mM standard solutions were serially diluted two-fold in their respective sterile media to 244 nM. Each standard (50 μl) was then diluted two-fold into isoproterenol (50 μM in 0.2% L-ascorbic $acid_{(aq.)}$). Standards were further diluted ten-fold in methanol, chilled at −80°C for 15 min, and centrifuged at 10,000 × **g**, 4°C for 10 min to precipitate proteins. Supernatant (50 μl) was diluted ten-fold in 10% acetonitrile in water. The calibration curve ranges used for quantification were 4.9 nM to 5 μM for 3MT and 20 nM to 5 μM for dopamine, with a limit of quantification set at a signal-to-noise ratio of 10:1. Calibration curves were generated by measuring peak areas and performing linear regression against known concentrations.

Supernatant (50 μl) from incubations was diluted five-fold in 0.2% L-ascorbic acid and immediately frozen at −20°C to preserve samples for LC–MS/MS. After thawing, preserved supernatants (50 μl) from incubations with 3MT were diluted two-fold into isoproterenol (50 μM in

0.2% L-ascorbic acid$_{(aq.)}$). Next, samples were diluted ten-fold in methanol, chilled at −80°C for 15 min, and centrifuged at 10,000 × ***g***, 4°C for 10 min to precipitate proteins. Supernatant (50 µl) was diluted ten-fold in 10% acetonitrile in water, and injected into the LC–MS/MS instrument. Dopamine and 3MT concentrations were quantified using calibration curves. Representative chromatograms are displayed in Figures S1–S3.

Quantification of acetate, propionate, and butyrate by GC–MS

Short-chain fatty acid concentrations (acetate, propionate and butyrate) were quantified by a GC–MS method adapted from Kage et al. (2004). All concentrations for short-chain fatty acids are reported relative to the sterile media used to construct the calibration curve.

Acetate, propionate, and butyrate from incubations of bacterial isolates were derivatized with pentafluorobenzyl bromide, and their concentrations were assessed by positive-ion electron ionization (EI+) single-quadrupole gas chromatography–mass spectrometry (TRACE™ GC ULTRA and ISQ; Thermo-Fisher Scientific). Samples were run on a TG-SQC column (15 m × 0.25 mm × 0.25 µm). Oven temperature was initially set at 40°C with a 20°C min^{-1} ramp to 290°C. Retention times of the short-chain fatty acid derivatives were as follows: pentafluorobenzyl acetate, 6.734 min; pentafluorobenzyl propionate, 7.425 min; pentafluorobenzyl butyrate, 8.054 min; ethyl phenylacetate (internal standard), 8.024 min. The $[M–H]^+$ m/z values for the pentafluorobenzyl fragment ($m/z = 181.05$) of each derivative were used to quantify each acid derivative. Ethyl phenylacetate was quantified using the benzyl fragment peak ($m/z = 91.09$). Signal for each derivative was calculated as a ratio to the ethyl phenylacetate internal standard signal (i.e., response for quantitation).

Stock solutions of sodium acetate, sodium propionate, and sodium butyrate were prepared at 1 M in double-deionized water. These were used to generate a standard containing acetate, propionate, and butyrate at 200 mM in double-deionized water, which was subsequently serially diluted, two-fold, to create standards ranging from 50 mM to 49 µM in double-deionized water. To 50 µl of each standard were added 50 µl of sterile media, 100 µl of a solution containing both ethyl phenylacetate (5 mM) and pentafluorophenol (5 mM) in sodium phosphate$_{(aq)}$ (500 mM, pH 6.8), followed by 200 µl of pentafluorobenzyl bromide (500 mM in acetone). Standards were vortexed to mix and incubated at 60°C for 1.5 h with shaking at 1000 RPM. Standards were then centrifuged at 10,000 × ***g*** for 1 min. To each standard, 1000 µl of hexanes were added, vortexed for 15 s to extract, and centrifuged at 10,000 × ***g*** for 1 min. Then, 100 µl of extract was diluted ten-fold in hexanes and transferred to GC–MS vials for analysis. The calibration curve ranges used for quantification were 98 µM to 12.5 mM for acetate, propionate, and butyrate, with a limit of quantification set at a signal-to-noise ratio of 10:1. Calibration curves were generated by measuring peak areas and performing linear regression against known concentrations. Pentafluorophenol did not end up being used in the quantification process.

To 50 µl of whole-cell culture from samples were added 50 µl of double-deionized water, 100 µl of a solution containing both ethyl phenylacetate (5 mM) and pentafluorophenol (5 mM) in sodium phosphate$_{(aq)}$ (500 mM, pH 6.8), followed by 200 µl of pentafluorobenzyl bromide (500 mM in acetone). Samples were vortexed to mix and incubated at 60°C for 1.5 h with shaking at 1000 RPM. Samples were then centrifuged at 10,000 × ***g*** for 1 min. To each sample, 1000 µl of hexanes were added, vortexed for 15 s to extract, and centrifuged at 10,000 × ***g*** for 1 min. Then, 100 µl of extract was diluted 10-fold in hexanes and transferred to GC–MS vials for analysis. Representative chromatograms are displayed in Figure S4.

Assessment of growth advantage via 3MT supplementation

Seed cultures of gut bacteria were grown overnight in triplicate in brain heart infusion supplemented with 0.05% w/v L-cysteine HCl in anoxic conditions (2%–5% H_2, 20% CO_2, with the balance being N_2) at 37°C. Seed cultures were normalized to an OD_{600} of 0.5 in acetogenic media, then diluted 1:100 into acetogenic media supplemented with 3MT (1 mM or 0.1 mM in sterile double-deionized water) or equal amounts of its vehicle. Cultures were incubated at 37°C for 38 h in triplicate with sterile controls. Growth was monitored by OD_{600} in 15-min intervals with 30 s of shaking at 500 RPM prior to each spectrophotometric reading.

RESULTS

3MT is reverted to dopamine by communities of faecal bacteria

In order to assess whether gut bacteria are capable of *O*-demethylating 3MT, faecal samples collected from healthy human donors ($n = 7$) were anaerobically incubated with 3MT (500 µM) or vehicle in nutrient-rich media for 72 h. In cultures of faecal samples from four of seven donors, 3MT was degraded to dopamine (Figure 2). These data indicate that gut bacteria can revert 3MT to the

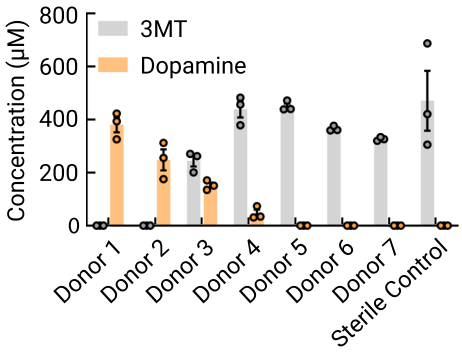

FIGURE 2 Faecal samples from healthy human donors ($n = 7$) were cultured with 3MT (500 μM) or vehicle. Dopamine and 3MT were quantified using LC–MS/MS. No dopamine was detectable in vehicle controls for any donor. ($n = 3$ technical replicates; bars are mean ± SEM). Figure created with BioRender.com (agreement number US2311BDRL).

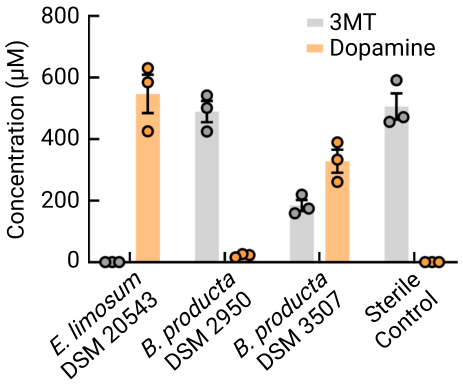

FIGURE 3 *E. limosum* and *B. producta* were incubated with 3MT (500 μM) for 72 h, at which point *O*-demethylation was quantified by LC–MS/MS. ($n = 3$ biological replicates; bars are mean ± SEM). Figure created with BioRender.com (agreement number YH239QJKA5).

active hormone, dopamine, but that this metabolic capacity may vary across people. Incubations of faecal samples from two donors (Donors 1 and 2) resulted in complete consumption of 3MT, whereas only 51% of 3MT was consumed by Donor 3 and 12% by Donor 4.

In these cultures, dopamine production was similarly detected at varying levels. Dopamine was not produced in any vehicle control from any donor, suggesting that the observed production of dopamine was a result of 3MT *O*-demethylation. In cultures of Donors 1 and 2, a substoichiometric quantity of dopamine formed, despite 3MT being completely consumed; the amount of dopamine produced relative to 3MT consumed was 76% and 50%, respectively. The unaccounted for metabolic fate of 3MT may be due to dopamine degradation, which can be carried out by some gut bacteria (Maini Rekdal et al., 2020). Taken together, our data show that faecal bacterial communities are capable of reverting 3MT to dopamine, although the occurrence of this metabolic capacity as well as the extent of metabolism likely varies across individuals.

Gut bacterial acetogens *E. limosum* and *B. producta* produce dopamine by *O*-demethylating 3MT

Seeking to identify members of the gut microbiota that may be responsible for the *O*-demethylation of 3MT, we focused on two prominent acetogenic gut bacteria, *Eubacterium limosum* and *Blautia producta*, that are known to *O*-demethylate dietary aryl methyl ethers (Burapan et al., 2017; Possemiers et al., 2008; Sharak Genthner & Bryant, 1987). Additionally, two strains of *B. producta* were screened to assess possible strain-level variation in 3MT metabolism. *E. limosum* DSM 20543 and *B. producta* DSM 2950 and DSM 3507 were anaerobically cultured with 3MT (500 μM) or vehicle in nutrient-rich media. Following incubation, concentrations of 3MT and dopamine were assessed using LC–MS/MS (Figure 3). *E. limosum* DSM 20543 quantitatively converted 3MT to dopamine. Both strains of *B. producta* converted 3MT to dopamine, although to a lesser extent than did *E. limosum*.

Conversion of 3MT to dopamine is likely performed by a cobalamin-dependent *O*-demethylase

Having identified prominent members of the gut microbiota that are capable of degrading 3MT to dopamine, we endeavoured to identify the cellular machinery responsible for this transformation. Literature precedent indicates that *O*-demethylation of aryl methyl ethers by acetogens, including *E. limosum* and *B. producta*, is carried out by cobalamin-dependent *O*-demethylase (Berman & Frazer, 1992; Burapan et al., 2017). These enzymes can be inhibited by propyl iodide, and inhibition is reversed by photolysis (Brot & Weissbach, 1965). To determine whether 3MT *O*-demethylation is performed by cobalamin-dependent enzymes, we tested whether this biotransformation could be inhibited by propyl iodide and, subsequently, rescued by light.

E. limosum DSM 20543 and *B. producta* DSM 3507 were each cultured in acetogenic media with 3MT (500 μM) as well as propyl iodide (20 μM) or vehicle; *B. producta* DSM 2950 was not used in these studies due to this strain's decreased efficiency in converting 3MT to dopamine. As propyl iodide inhibition of cobalamin-dependent *O*-demethylases is sensitive to light, bacteria were initially cultured in the dark and subsequently

exposed to light. Growth was monitored by OD_{600}, and *O*-demethylation was periodically assessed using LC–MS/MS. Neither propyl iodide nor light significantly impacted growth of *E. limosum* DSM 20543 (Figure 4a) or *B. producta* DSM 3507 (Figure 4b). In the absence of light, *O*-demethylation of 3MT by *E. limosum* DSM 20543 was completely inhibited by propyl iodide (Figure 4c). For *B. producta* DSM 3507, 83% of 3MT *O*-demethylation was inhibited at 15 h relative to cultures not exposed to propyl iodide (Figure 4d). Upon exposing cultures with propyl iodide to light, *E. limosum* DSM 20543 regained the ability to *O*-demethylate 3MT, affording a 2.4-fold increase (at 24 h) in dopamine concentration relative to cultures that were maintained in the dark; for *B. producta*, a 1.8-fold increase in dopamine concentration was observed upon exposure of cultures to light (at 17 h). The light-reversible inhibitory effects of propyl iodide suggest that cobalamin-dependent *O*-demethylases in both *E. limosum* DSM 20543 and *B. producta* DSM 3507 are responsible for the *O*-demethylation of 3MT to form dopamine.

3MT drives increases in acetate production by *E. limosum* and *B. producta*

Owing to the reported abilities of bacterial acetogens to use aryl methyl ethers as a carbon source for acetate synthesis (Kelly et al., 2016; Naidu & Ragsdale, 2001; Schilhabel et al., 2009), we sought to determine whether

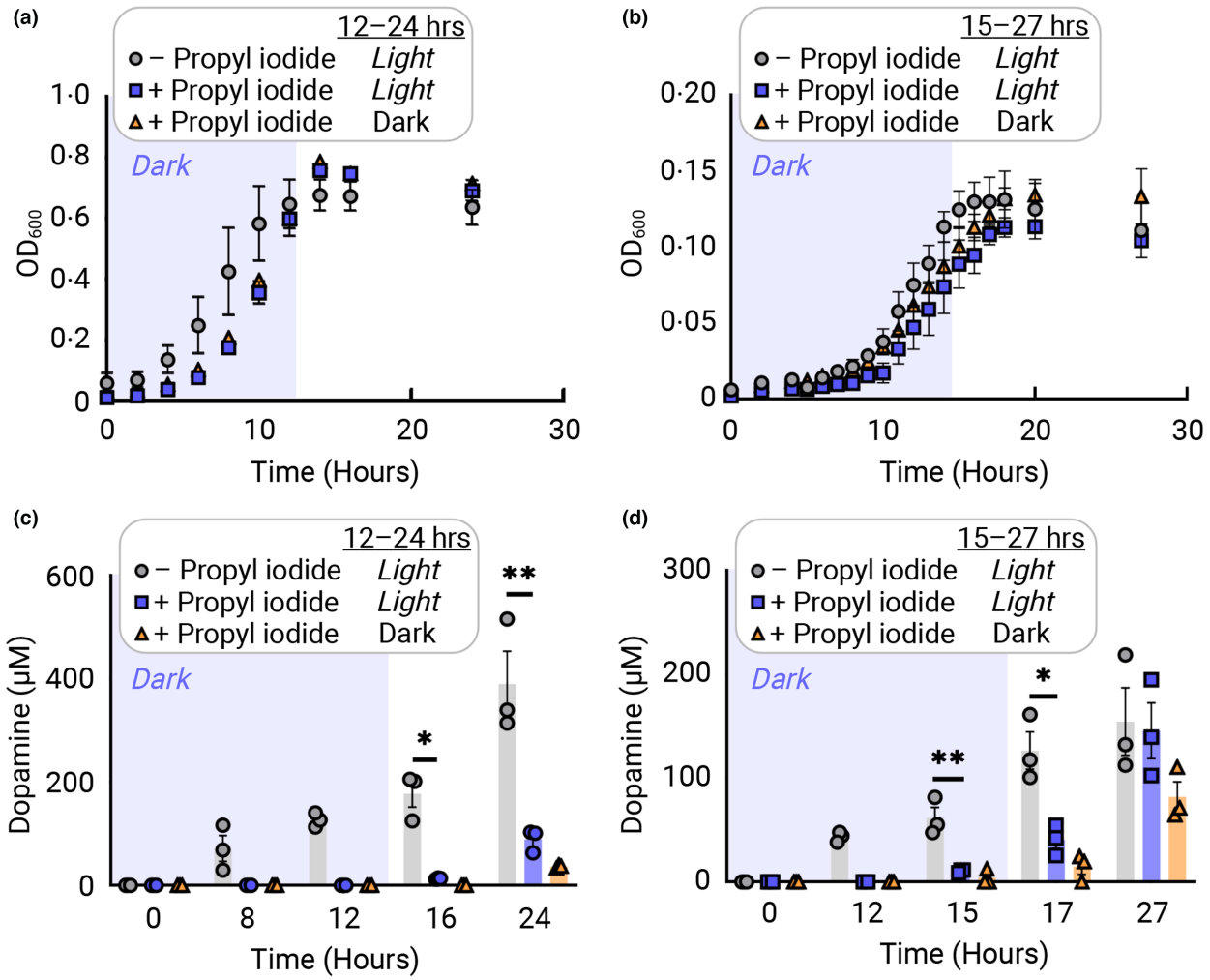

FIGURE 4 (a) *E. limosum* DSM 20543 and (b) *B. producta* DSM 3507 were each anaerobically cultured with 3MT (500 μM) as a function of propyl iodide (20 μM) and light exposure. Growth was measured using the optical density of cultures at 600 nm (OD_{600}). Cultures were maintained in the dark from 0–12 h (*E. limosum* DSM 20543) or 0–15 h (*B. producta* DSM 3507). Next, cultures without propyl iodide and half of the cultures with propyl iodide were exposed to light. Cultures of (c) *E. limosum* DSM 20543 and (d) *B. producta* DSM 3507 were periodically sampled, and dopamine production was measured by LC–MS/MS. ($n = 3$ biological replicates; values/bars are mean ± SEM; significance was determined by 1-way ANOVA; $^*P \leq 0.05$, $^{**}P \leq 0.01$). Figure created with BioRender.com (agreement number HU2311UO31).

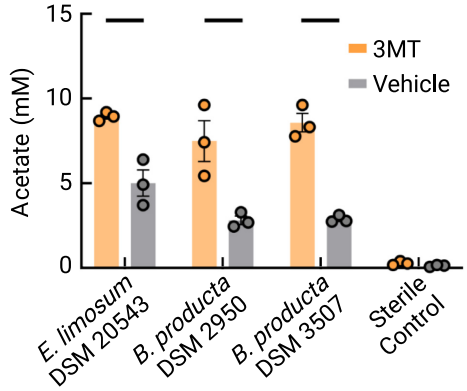

FIGURE 5 *E. limosum* DSM 20543 as well as the *B. producta* strains DSM 2950 and DSM 3507 were each cultured in acetogenic media with 3MT (2 mM) or its vehicle. Acetate levels were quantified using GC–MS ($n = 3$ biological replicates; bars are mean ± SEM; significance was determined by unpaired *t*-test; $^{**}P \leq 0.006$; $^{***}P = 0.0002$). Figures created with BioRender.com (agreement number HR2311YSN5).

the demethylation of 3MT to dopamine could drive acetate production. *E. limosum* DSM 20543 as well as *B. producta* strains DSM 2950 and DSM 3507 were each cultured in the presence of 3MT (2 mM) or vehicle and in a medium designed to promote acetogenesis (Groher & Weuster-Botz, 2016). Concentrations of short-chain fatty acids acetate, propionate, and butyrate were quantified through a GC–MS method adapted from Kage et al. (2004). For all strains tested, acetate levels were significantly higher in cultures incubated with 3MT as compared with vehicle controls (Figure 5). Measures of propionate (Figure S5A) and butyrate (Figure S5B) did not differ between treated and vehicle groups, which suggests that the acetate produced is not coming from fermentation, where increases in each of these short-chain fatty acids would be expected. It has been reported that *E. limosum* and *B. producta*, as well as other acetogens, are sometimes capable of deriving a growth advantage from certain aryl methyl ethers (Bache & Pfennig, 1981). A preliminary assessment to determine if *B. producta* could derive a growth advantage from 3MT was conducted; however, no growth advantage was detected (Figure S6).

DISCUSSION

Our data suggest the existence of a novel way that affords gut bacteria the potential to regulate dopamine levels. This gut bacterial metabolism entails formation of dopamine by *O*-demethylation of 3MT, which is a metabolite endogenously produced by COMT to attenuate dopamine levels (Männistö & Kaakkola, 1999). Conversion of 3MT to dopamine was observed in incubations with faecal bacteria from four of seven different human donors. This variability is likely due to differences in gut bacterial species as well as strains of species that exist across people. Towards elucidating a source of this variability, we sought to identify specific gut bacteria that can mediate 3MT *O*-demethylation. Because other aryl methyl ethers are *O*-demethylated by gut bacterial acetogens *E. limosum* and *B. producta* (Burapan et al., 2017; Hur & Rafii, 2000; Possemiers et al., 2008), we tested the abilities of these organisms to *O*-demethylate 3MT. While both of these bacterial species were capable of converting 3MT to dopamine, the extent of conversion differed between both of the two species tested as well as the two strains of *B. producta* examined. *E. limosum* DSM 20543 completely metabolized 3MT, while the two *B. producta* strains only partially metabolized 3MT. Furthermore, of the two strains of *B. producta* tested, DSM 3507 metabolized significantly more 3MT than did DSM 2950. Determining whether these differences in extent of 3MT metabolism are biologically significant or artefacts of in vitro culturing requires characterization of both the enzymes responsible for this process as well as the occurrence of this biochemical process in vivo.

Discovering the biochemical origin of the 3MT *O*-demethylation pathway will enable assessment of this pathway's contribution to dopamine levels in GI tracts harbouring a complex gut microbiota. To begin this process, we set out to determine the class of enzyme responsible for *O*-demethylating 3MT. Prior studies in other organisms determined that cobalamin-dependent *O*-demethylases *O*-demethylate aryl methyl ethers (Chen et al., 2016) and that this process is inhibited by propyl iodide in a light-reversible fashion (Ghambeer et al., 1971). Cobalamin-dependent *O*-demethylases operate by transferring the methyl abstracted from the aryl methyl ether to the cobalt centre of the cobalamin cofactor. Then, cobalamin relays the methyl to a methyl acceptor, which in this system is tetrahydrofolate (Matthews et al., 2008). When cobalamin-dependent *O*-demethylases are exposed to propyl iodide, the result is a cobalt–propyl bond where the propyl group is not transferred to tetrahydrofolate, impairing further *O*-demethylation (Brot & Weissbach, 1965). Although the cobalt–propyl bond cannot be biochemically cleaved, this bond is photolysed, which reverses inhibition of *O*-demethylation (Brot & Weissbach, 1965).

Owing to *E. limosum* and *B. producta* encoding several putative cobalamin-dependent *O*-demethylases (Chen et al., 2016; Kelly et al., 2016; Naidu & Ragsdale, 2001; Schilhabel et al., 2009), we used propyl iodide as a diagnostic for whether 3MT is *O*-demethylated by members of this enzyme class. Our studies exposing *E. limosum* DSM 20543

as well as *B. producta* DSM 3507 to propyl iodide, in the absence of light, demonstrated that 3MT *O*-demethylation was inhibited by propyl iodide. Importantly, propyl iodide had no significant impact on bacterial growth, indicating that the observed metabolic inhibition was not due to a growth defect but likely due to inhibition of a cobalamin-dependent *O*-demethylase. Moreover, exposing cultures to light partially reversed inhibition, further diagnostic of a cobalamin-dependent *O*-demethylase mediating the conversion of 3MT to dopamine. Identifying the specific gene and enzyme responsible for this biotransformation of 3MT is the subject of ongoing work in our laboratory and will be critical to determining the contribution of 3MT *O*-demethylation to inter-individual differences in dopamine metabolism in the GI tract.

Bacterial acetogens that *O*-demethylate aryl methyl ethers using cobalamin-dependent *O*-demethylase have been reported to funnel both the methyl group of this substrate and CO_2 into the synthesis of acetate through a process called reductive acetogenesis (Frazer & Young, 1986; Naidu & Ragsdale, 2001). Owing to *E. limosum* and *B. producta* each being capable of reductive acetogenesis (Chang et al., 1999; Geerligs et al., 1987), we suspected that these bacteria may use 3MT to produce acetate. Indeed, 3MT drove significant increases in the acetate produced by *E. limosum* DSM 20543 and both *B. producta* strains when each was cultured using acetogenic media that contained 3MT and glucose as the sole carbon sources. Concentrations of propionate and butyrate, other short-chain fatty acids that are abundantly produced by gut bacteria (Høverstad & Midtvedt, 1986; Miller & Wolin, 1996), were not impacted by the presence of 3MT. These data suggest that the unique increase in acetate was not due to 3MT inducing a generalized increase in short-chain fatty acid synthesis but, instead, was a direct consequence of 3MT *O*-demethylation. Curiously, we observed a supra-stoichiometric increase in acetate concentration relative to the amount of 3MT supplied. This could mean that 3MT induces an alternative pathway for reductive acetogenesis, perhaps the canonical route in which CO_2 and H_2 are coupled to produce acetate (Barker & Kamen, 1945; Ljungdahl et al., 1965; Wood, 1952).

Taken together, the reported role of cobalamin-dependent enzymes in producing acetate from aryl methyl ethers combined with our findings that 3MT enhances acetate production provides further support for 3MT *O*-demethylation being performed by a cobalamin-dependent *O*-demethylase. These findings also provide motivation for investigating secondary metabolites that are exposed to and metabolized by the gut microbiota that may serve as undiscovered regulators of gut bacterial central metabolism.

It remains necessary to examine the in vivo relevance of 3MT *O*-demethylation to the regulation of dopamine levels. The biogeography of 3MT-to-dopamine metabolism is likely critical to whether this process contributes to variation in blood dopamine levels. Whether dopamine produced in the GI tract by 3MT *O*-demethylation may reenter the systemic circulation requires further study in vivo. Regardless, dopamine produced from 3MT *O*-demethylation may have impacts within the GI tract, where dopamine regulates gut motility (Haskel & Hanani, 1994).

Next steps towards establishing the potential biological relevance of 3MT demethylation include measuring 3MT concentrations in intestinal digesta of mice as well as in human faecal samples to determine the concentrations at which the gut microbiota may be exposed to 3MT. Additionally, isotope tracing studies using *O*-[$^{13}CH_3$]3MT dosed to both germ-free mice and mice mono-colonized with 3MT-demethylating bacteria (e.g., *E. limosum* or *B. producta*) will enable determination of whether 3MT demethylation to dopamine occurs by gut bacteria in vivo. Use of *O*-[$^{13}CH_3$]3MT will enable assessment of the methyl group's metabolic fate upon demethylation and whether the methyl is funnelled into acetate.

As gut bacterial species in addition to *E. limosum* and *B. producta* have been reported to metabolize various aryl methyl ethers, the scope of 3MT-demethylating gut bacteria may extend beyond the species investigated here. Ongoing work to identify the genetic origin of 3MT demethylation is expected to enable a more comprehensive view of the potential scope of 3MT demethylation by the gut microbiota.

In conclusion, our data demonstrate that the gut microbiota, both bacterial isolates and faecal bacterial communities, are capable of *O*-demethylating dopamine metabolites in vitro. Here, we identified two prominent members of the gut microbiota, *E. limosum* and *B. producta*, that can revert 3MT to dopamine. Additional studies provided supporting evidence that the degradation of 3MT to dopamine is carried out by cobalamin-dependent *O*-demethylase in these species. Taken together, our findings point to a new pathway by which the gut microbiota might impact dopamine metabolism in the GI tract. If operative in vivo, gut bacterial 3MT *O*-demethylation to form dopamine would counteract a process by which the host attenuates levels of dopamine through its *O*-methylation to 3MT. The balance between host methylation of dopamine to form 3MT and bacterial *O*-demethylation of 3MT to form dopamine may be significant to the regulation of dopamine levels outside the brain. Such alterations to peripheral dopamine metabolism could have profound impacts on host health. Consequently, these in vitro findings motivate further studies to assess the occurrence of

3MT-to-dopamine metabolism in vivo and the potential impacts of this metabolic process on the host.

ASSOCIATED CONTENT

The following files are available free of charge: supplemental figures (Figures S1–S6) (.pdf).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

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