Metabolic retroconversion of trimethylamine *N*-oxide and the gut microbiota

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Running title: Reduction of TMAO by the human gut microbiota

Abbreviations: AUC, area under the curve; DMA, dimethylamine; FISH, fluorescence *in situ* hybridization; FMO, flavin mono-oxygenase; MMA, monomethylamine; PC, phosphatidylcholine; TMA, trimethylamine; TMAO, trimethylamine *N*-oxide; UPLC–MS/MS, ultra-performance liquid chromatography–tandem mass spectrometry.

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ABSTRACT

The dietary methylamines choline, carnitine and phosphatidylcholine are used by the gut microbiota to produce a range of metabolites, including trimethylamine (TMA). However, little is known about the use of trimethylamine *N*-oxide (TMAO) by this consortium of microbes. A feeding study using deuterated TMAO in C57BL6/J mice demonstrated microbial conversion of TMAO to TMA, with uptake of TMA into the bloodstream and its conversion to TMAO. Antibiotic-treated mice lacked microbial activity necessary to convert TMAO to TMA, with deuterated TMAO being taken up directly into the bloodstream. In batch-culture fermentation systems inoculated with human faeces, growth of *Enterobacteriaceae* was stimulated in the presence of TMAO. Human-derived faecal and caecal bacteria (n = 66 isolates) were screened on solid and liquid media for their ability to use TMAO, with metabolites in spent media analysed by ^{1}H-NMR. As with the *in vitro* fermentation experiments, TMAO stimulated the growth of *Enterobacteriaceae*; these bacteria produced most TMA from TMAO. Caecal/small intestinal isolates of *Escherichia coli* produced more TMA from TMAO than their faecal counterparts. Lactic acid bacteria produced increased amounts of lactate when grown in the presence of TMAO, but did not produce large amounts of TMA. In summary, TMA can be produced by the gut microbiota (predominantly *Enterobacteriaceae*) from TMAO. This TMA is then taken up by the host and converted back to TMAO. That is, metabolic retroconversion occurs. In addition, TMAO influences microbial metabolism depending on isolation source and taxon of gut bacterium.

INTRODUCTION

Dietary methylamines such as choline, trimethylamine *N*-oxide (TMAO), phosphatidylcholine (PC) and carnitine are present in a number of foodstuffs, including meat, fish, nuts and eggs. It has long been known that gut bacteria are able to use choline in a fermentation-like process, with trimethylamine (TMA), ethanol, acetate and ATP among the known main end-products (Wünsche, 1940; Bradbeer, 1965; Zeisel *et al.*, 1983). TMA can be used by members of the order *Methanomassiliicoccales* (*Archaea*) present in the human gut to produce methane (Borrel *et al.*, 2017), or taken up by the host. Microbially produced TMA derived from PC is absorbed in the small intestine (Stremmel *et al.*, 2017). TMA diffuses into the bloodstream from the intestine via the hepatic vein to hepatocytes, where it is converted to trimethylamine *N*-oxide (TMAO) by hepatic flavin-containing mono-oxygenases (FMOs; Bennett *et al.*, 2013). The bulk of TMAO, and lesser amounts of TMA, derived from dietary methylamines can be detected in urine within 6 h of ingestion, and both compounds are excreted in urine but not faeces (de la Huerga and Popper, 1951; Taesuwan *et al.*, 2017). TMAO can also be detected in human skeletal muscle within 6 h of an oral dose of TMAO (Taesuwan *et al.*, 2017).

TMAO present in urine and plasma is considered a biomarker for non-alcoholic fatty liver disease (NAFLD), insulin resistance and cardiovascular disease (Dumas *et al.*, 2006; Spencer *et al.*, 2011; Tang *et al.*, 2013; Dumas *et al.*, 2017). Feeding TMAO to high-fat-fed C57BL/6 mice exacerbates impaired glucose tolerance, though the effect on the gut microbiota is unknown (Gao *et al.*, 2014). Low plasma PC and high urinary methylamines (including TMAO) were observed in insulin-resistant mice on a high-fat diet, suggesting microbial methylamine metabolism directly influences choline bioavailability (Dumas *et al.*, 2006), and choline bioavailability is known to contribute to hepatic steatosis in patients (Spencer *et al.*, 2011). High levels of circulating TMAO are associated with cardiovascular disease (Tang *et al.*, 2013). However, circulating TMAO has been suggested to play a role in protection from hyperammonemia, acting as an osmoprotectant, and from glutamate neurotoxicity (Kloiber *et al.*, 1988; Miñana *et al.*, 1996). Recently, chronic exposure to TMAO has been shown to attenuate diet-associated impaired glucose tolerance in mice, and reduce endoplasmic reticulum stress and adipogenesis in adipocytes (Dumas *et al.*, 2017). There is a basal

77 level of TMA and TMAO detected in human urine even in the absence of dietary supplementation (de
78 la Huerga *et al.*, 1953), suggesting use of (microbial and/or host) cell-derived choline or PC in the
79 intestinal tract by the gut microbiota.

80 While it is well established that choline (Zeisel *et al.*, 1983; Wang *et al.*, 2011; Spencer *et al.*,
81 2011), PC (Zeisel *et al.*, 1983; Tang *et al.*, 2013) and carnitine (Rebouche *et al.*, 1984; Seim *et al.*,
82 1985; Rebouche and Chenard, 1991; Koeth *et al.*, 2013) are used by the human and rodent gut
83 microbiotas to produce TMA, little is known about the reduction of TMAO (predominantly from fish)
84 to TMA (or other compounds) by members of these consortia. TMAO is found at concentrations of
85 20–120 mg per 100 g fish fillet (Jebsen and Riaz, 1977). Ingestion of fish by humans leads to
86 increased urinary excretion of dimethylamine (DMA) and TMA, from 5.6 to 24.1 and from 0.2 to 1.6
87 μmol/24 h/kg of body weight, respectively (Zeisel and Da Costa, 1986). Individuals with
88 trimethylaminuria (fish odour syndrome), in which TMA is not detoxified to TMAO by hepatic
89 FMOs, have been shown to reduce 40–60 % of an oral dose of TMAO to TMA (al-Waiz *et al.*, 1987).
90 It was suggested the gut microbiota was responsible for reducing TMAO in these individuals, and in
91 those individuals without trimethylaminuria the TMA was re-oxidized in the liver before being
92 excreted in the urine. The process was termed '*metabolic retroversion*' "*(i.e., a cycle of reductive*
93 *followed by oxidative reactions to regenerate TMAO)*" (al-Waiz *et al.*, 1987). The reduction of
94 TMAO to TMA is most commonly associated with the gut microbiota of marine fish, with the TMA
95 generated by these bacteria contributing to the characteristic odour of rotting fish (Barrett and Kwan,
96 1985). Members of the class *Gammaproteobacteria*, which includes a number of food spoilage
97 organisms, are known to reduce TMAO to TMA quantitatively and are unable to reduce TMA further
98 (Takagi and Ishimoto, 1983; Barrett and Kwan, 1985). The conversion of TMAO to TMA by bacteria
99 represents a unique form of anaerobic respiration, in which TMAO reductase acts as a terminal
100 electron acceptor (from NADH or formate) for respiratory electron flow (Takagi and Ishimoto, 1983).

101 Mining of metagenomic data (*torA*) suggests *Proteobacteria* (particularly *Escherichia* and
102 *Klebsiella* spp.) are likely to contribute greatest to the production of TMA from TMAO in the human
103 gut via the TMAO reductase pathway, with *Actinobacteria* (*Eggerthellaceae*) becoming more
104 important under stress (Jameson *et al.*, 2016). Other microbial genes are associated with production of

TMA from choline (*cutC*), glycine-betaine (*grdH*), L-carnitine (*cntA*) and γ-butyrobetaine (*cntA*) (Borrel *et al.*, 2017). A search of the NCBI nucleotide database with the phrase 'TMAO reductase' also suggests many other bacteria of human intestinal origin (namely, *Salmonella*, *Helicobacter*, *Prevotella*, *Bacillus* and *Bacteroides* spp.) should be able to reduce TMAO to TMA. However, this trait has not been examined *in vitro* for isolates of intestinal origin.

Consequently, we carried out an *in vivo* study in mice to confirm use of TMAO by the mouse gut microbiota and to allow us to examine metabolic retroconversion of TMAO in a murine model. We then used an *in vitro* fermentation system to highlight the effect of TMAO on the human faecal microbiota. Finally, we screened a panel of human faecal and caecal/small intestinal isolates to determine which members of the human gut microbiota were able to reduce TMAO to TMA, and whether their metabolism was affected by being grown in the presence of TMAO.

METHODS

Animal work

Groups of six-week-old C57BL6/J mice (Janvier Labs, Courtaboeuf, France) were received and acclimated in specific pathogen-free (SPF) maintenance conditions. They were fed with a standard chow diet (R04-40, Safe, Augy, France) and were either given free access to tap water or treated with the antibiotic cocktail (0.5 g/L vancomycin hydrochloride, 1 g/L neomycin trisulfate, 1 g/L metronidazole, 1 g/L ampicillin sodium; all antibiotics were purchased from Sigma-Aldrich) for 14 days. Mice were given in the morning by gavage either a solution of d_9-TMAO at 1×10^{-4} M (Cambridge Isotope Laboratories Inc., DLM-4779-0, UK) or saline and euthanized 6 h later by cervical dislocation. Blood samples were collected by tail tipping every 2 h in Microvette® CB 300 Lithium Heparin (Sarstedt, Marnay, France). Plasma was separated by centrifugation (10 min, 5000 ***g***, 4 °C) and stored at -80 °C until analysed by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS).

All procedures were authorized following review by the institutional ethics committee (Sorbonne Universities) and carried out under national license conditions.

UPLC–MS/MS determination of plasma TMA, d_9-TMA, TMAO and d_9-TMAO

UPLC-MS/MS was employed for the determination of TMA, d_9-TMA, TMAO and d_9-TMAO. Samples (10 µL) were spiked with 10 µL Internal Standard solution (d_9-choline and $^{13}C_3/^{15}N$-TMA in water; 1 mg/L, Sigma-Aldrich). Ethyl 2-bromoacetate solution (45 µL) (15 g/L ethyl 2-bromoacetate, 1 % NH_4OH in acetonitrile; ChromaSolv grade, Sigma-Aldrich) was added and derivatization of TMAs (TMA, d_9-TMA and $^{13}C_3/^{15}N$-TMA) to their ethoxy-analogues was completed after 30 min at room temperature. Protein/lipid precipitation solution (935 µL) (94 % acetonitrile/5 %water/1 % formic acid; ChromaSolv grade, Sigma-Aldrich) was added, samples were centrifuged (20 min, 20,000 ***g***, 4 °C) and were transferred to UPLC-autosampler vials. Sample injections (5 µL loop) were performed with a Waters Acquity UPLC-Xevo TQ-S UPLC-MS/MS system equipped with an Acquity BEH HILIC (2.1×100 mm, 1.7 µm) chromatographic column. An isocratic elution was applied with 10 mM ammonium formate (Sigma-Aldrich) in 95:5 (v/v) acetronitrile:water for 7 min at 750 µL/min and 50 °C. Positive electrospray (ESI+) was used as ionization source and mass spectrometer parameters were set as follows: capillary, cone and sources voltages at -700, -18 and 50 V, respectively, desolvation temperature at 600 °C, desolvation/cone/nebuliser gases were high purity nitrogen (BOC) at 1000 L/h, 150 L/h and 7 bar, respectively. Collision gas was high-purity argon (BOC). Mass spectrometer was operated in multiple reaction monitoring mode. The monitored transitions were the following: for TMA, +146 → +118/59 *m/z* (23/27 V); for d_9-TMA, +155 → +127/68 *m/z* (21/23 V); for $^{13}C_3/^{15}N$-TMA, +150 → +63/122 *m/z* (27/22 V); for TMAO, +76 → +59/58 *m/z* (12/13 V); for d_9-TMAO, +85 → +68/66 *m/z* (18/20 V); and for d_9-choline, +108 → +60/45 *m/z* (20/22 V). The system was controlled by MassLynx software, also used for data acquisition and analysis.

^{1}H-NMR spectroscopy and data analysis

158 Medium samples were randomized and centrifuged (5 min, 16000 ***g***). Aliquots (50 µL) of the
159 supernatants were diluted in 550 µL D_2O containing 1 mM trimethylsilyl-(2,2,3,3-$2H_4$)-1-propionate.
160 Samples were transferred to 5 mm NMR tubes and measured on a NMR spectrometer (Bruker)
161 operating at 600.22 MHz ^{1}H frequency as described previously (Dona *et al.*, 2014). ^{1}H-NMR spectra
162 were pre-processed and analysed as described previously (Dumas *et al.*, 2006) using the Statistical
163 Recoupling of Variables-algorithm (Blaise *et al.*, 2009). Structural assignment was performed as
164 described in Dona *et al.* (2016), using in-house and publicly available databases.
165
166 ***In vitro* fermentation systems**
167 Freshly voided faecal samples were obtained from three healthy human volunteers (one male
168 and two females; age range 29–31 years), none of whom had been prescribed antibiotics 6 months
169 prior to the study, eaten fish 3 days prior to sample collection or had any history of gastrointestinal
170 disease. The University of Reading's Research Ethics Committee (UREC) does not require that
171 specific ethical review and approval be given by UREC for the collection of faecal samples from
172 healthy human volunteers to inoculate *in vitro* fermentation systems. Samples were processed
173 immediately by diluting them 1 in 10 (w/w) in pre-reduced minimal broth and homogenizing them in
174 a filter bag in a stomacher (Stomacher 400 Lab System; Seward) for 2 min at 'high' speed. For each
175 donor, a batch culture vessel containing 175 mL of pre-reduced minimal broth was inoculated with
176 20 mL of the faecal homogenate and 5 mL of sterile H_2O containing 2 g TMAO dihydrate (Sigma-
177 Aldrich). Control vessels were inoculated with 20 mL of the faecal homogenate and 5 mL of sterile
178 H_2O. The final working volume of each batch culture vessel was 200 mL. The pH of each vessel (pH
179 6.5) was controlled automatically by the addition of 2 M HCl or 2 M NaOH. pH controllers were
180 supplied by Electrolab. The contents of each vessel were stirred constantly. An anaerobic
181 environment was maintained by constantly sparging the vessels with O_2-free N_2. The temperature of
182 each vessel was maintained at 37 °C by use of a circulating waterbath connected to each fermentation
183 vessel. The experiment was run for 9 h, with samples taken at 0, 1, 2, 3, 4, 5, 6 and 9 h.
184
185 **Fluorescence *in situ* hybridization (FISH) analysis**

186 Aliquots (2× 375 μL) of sample were fixed in ice-cold 4 % paraformaldehyde for 4 h, washed
187 in sterile phosphate-buffered saline and stored for FISH analysis as described by Martín-Peláez *et al.*
188 (2008). **Supplementary Table 1** gives details for probes used in this study. Probes were synthesized
189 commercially (MWG-Biotech) and labelled with the fluorescent dye cyanine 3 (Cy3; excitation λ,
190 514 nm; emission λ, 566 nm; fluorescence colour, orange–red). FISH was done as described by
191 Martín-Peláez *et al.* (2008).
192 Slides were viewed under a Nikon E400 Eclipse microscope. DAPI slides were visualized
193 with the aid of a DM400 filter; probe slides were visualized with the aid of a DM575 filter. Cells
194 (between 15 and 50 per field of view) were counted for 15 fields of view, and the numbers of bacteria
195 were determined by using the following equation:
196 $\mathrm{DF} \times ACC \times 6{,}732.42 \times 50 \times DF_{\mathrm{sample}}$
197 Where the DF (dilution factor) was calculated by taking into account the concentration of the
198 original sample (375 μL to 300 μL = 0.8×). *ACC* (average cell count) was determined by counting 15
199 fields of view and assumes that a normal distribution was observed for the counts. The figure
200 6,732.42 refers to the area of the well divided by the area of the field of view. DF_{sample} refers to the
201 dilution of sample used with a particular probe (e.g. 5× for t_0 Lab158 counts). The detection limit of
202 this method was 89,766 bacteria/mL of sample (= $\log_{10}$ 4.95).
203
204 **Screening of bacteria for ability to reduce TMAO**
205 Part of an in-house collection (University of Reading) of bacteria isolated from human
206 caecal/small intestinal and faecal samples (Table 1) was screened on minimal agar [g/L: glucose
207 (Fisher Scientific), 4; bacteriological peptone (Oxoid), 20; NaCl (Fisher Scientific), 5; neutral red
208 (Sigma-Aldrich), 0.03; agar technical no. 3 (Oxoid), 15; pH 7] with and without 1 % (w/v) TMAO
209 dihydrate (Sigma-Aldrich). The composition of the agar was based on that of Takagi and Ishimoto
210 (1983), who used the medium with crystal violet and bile salts to screen members of the
211 *Enterobacteriaceae* for TMAO reductase activity. Colonies of isolates able to ferment glucose were
212 red, whereas those able to reduce TMAO were white. Growth curves (OD_{600} measured hourly for 10–

213 12 h, then at 24 h) were determined for selected isolates grown anaerobically at 37 °C in anaerobic
214 minimal broth [g/L: glucose, 4; bacteriological peptone, 20; NaCl, 5; L-cysteine HCl (Sigma-Aldrich),
215 0.5; resazurin solution (0.25 mg/mL; Sigma-Aldrich), 4 mL; pH 7] with and without 1 % (w/v)
216 TMAO dihydrate. Glucose was substituted by raffinose (Sigma-Aldrich) (Trojanová *et al.*, 2006) in
217 the minimal broth when working with bifidobacteria because of the poor growth of these bacteria on
218 the glucose-based medium. pH and metabolite profiles of culture medium were examined at the end
219 of the growth experiment (i.e. at t_{24}).
220
221 **Statistical analyses**
222 Differences between metabolites produced by faecal and caecal/small intestinal isolates of
223 *Escherichia coli* and lactic acid bacteria in the presence and absence of TMAO were analysed using
224 Student's *t* test. FISH data from the *in vitro* fermentation systems were analysed using the
225 Kolmogorov–Smirnov test with statistical significance, after correction for multiple testing, taken at P
226 < 0.05. Because of the presence of ties in the metabolite data from *in vitro* fermentation systems, the
227 bootstrapped Kolmogorov–Smirnov test (10,000 replications) was used with these data. Data from *in*
228 *vitro* fermentation systems (FISH and NMR) were correlated using Spearman's rank correlation
229 (corrected for ties) with results corrected for multiple testing using the method of Benjamini and
230 Hochberg (1995).
231
232
233 **RESULTS**
234 ***In vivo* confirmation of metabolic retroconversion**
235 First, to confirm *in vivo* metabolic retroconversion (i.e. microbial conversion of TMAO to
236 TMA, followed by host conversion of TMA to TMAO), we administered isotopically-labelled d_9-
237 TMAO or saline to mice that had or had not been treated with a broad-spectrum antibiotic cocktail for
238 14 days to suppress the gut microbiota (Tang *et al.*, 2013). Reduction of d_9-TMAO to d_9-TMA was
239 quantified in urine by UPLC–MS/MS up to 6 h after d_9-TMAO gavage, together with unlabelled
240 TMA and TMAO potentially produced from dietary sources (**Fig. 1**).

241 In the absence of antibiotics, d_9-TMAO was converted to d_9-TMA within 2 h of gavage. This
242 conversion was dramatically reduced, leading to a significantly lower concentration of d_9-TMA and a
243 higher concentration of d_9-TMAO, when the gut microbiota was suppressed by antibiotics (**Fig. 1A–**
244 **D**). We noted that in the absence of antibiotics all animals excreted approximately three times higher
245 levels of urinary unlabelled TMA than TMAO (**Fig. 1E–H**), corresponding to constitutively low
246 FMO3 activity in mice (Zhang *et al.*, 2007). Levels of unlabelled TMAO/TMA were not significantly
247 different from one another in the control and d_9-TMAO-fed animals, while antibiotic treatment
248 significantly reduced the amount of both TMAO and TMA (**Fig. 1E, F**). Bioavailability of unlabelled
249 TMAO/TMA, as assessed by area under the curve (AUC), was significantly reduced by antibiotic
250 treatment in both saline and d_9-TMAO-gavaged animals, with almost no TMA detected in either
251 experimental group (**Fig. 1G**, **H**).
252
253 **Effect of TMAO on human gut bacteria within a mixed system**
254 After *in vivo* validation of the role of the gut microbiota in metabolic retroconversion, we
255 analysed the effect of TMAO on the faecal microbiota in an anaerobic batch-culture fermentation
256 system. Fermenter vessels filled with the glucose-containing medium supplemented or not with 1 %
257 (w/v) TMAO were inoculated with faecal slurries from three healthy donors and monitored for 9 h.
258 With the exception of enhanced growth of the *Enterobacteriaceae* (probe Ent), the presence of
259 TMAO in the medium had no statistically significant effect on the growth of bacteria within the
260 fermentation systems at 9 h (**Fig. 2A**, **Supplementary Figure 1**).
261 Huge variability, as measured by ^{1}H-NMR, was observed in the amount of TMA and DMA
262 produced by gut bacteria in the TMAO-containing fermentation systems, with the concentrations of
263 both metabolites increasing steadily from 0 to 9 h and differing significantly ($P < 0.05$) from the
264 control systems (**Fig. 2B**). The amount of TMAO in the systems was seen to decrease at 8 h.
265 Correlation of metabolite and FISH data demonstrated *Clostridium* clusters I and II
266 (Chis150), *Enterobacteriaceae* (Ent), bifidobacteria (Bif164) and coriobacteriia (Ato291) were
267 associated with TMA, acetate, ethanol and lactate (**Fig. 2C**). The *Betaproteobacteria* (Bet42a) were
268 anti-correlated with TMA, acetate, ethanol and lactate, which is unsurprising given this was the only

269 group of bacteria whose representation decreased in the fermentation systems over the course of the
270 experiment (**Supplementary Figure 1**, **Fig. 2C**). The *Enterobacteriaceae* and clostridia were
271 positively correlated with DMA production. The lactic acid bacteria (Lab158) were not significantly
272 correlated with any of the metabolites in the mixed culture.
273
274 **Growth of pure cultures of gut bacteria in the presence of TMAO**
275 Using an in-house collection of bacteria, we initially screened isolates on a modified version
276 of the agar of Takagi and Ishimoto (1983) as a rapid means of screening bacteria for TMAO reductase
277 activity, and thereby their ability to reduce TMAO to TMA. While members of the
278 *Enterobacteriaceae* produced expected results (i.e. white colonies when grown in the presence of
279 TMAO and glucose, rather than red colonies on the glucose only control), we observed a number of
280 unexpected outcomes depending on the species under study. For example, colonies of lactic acid
281 bacteria were larger (almost twice their usual size) on TMAO-containing agar than on the glucose
282 control but remained red in colour, suggesting they had not reduced TMAO to TMA at detectable
283 levels but TMAO was influencing their growth. The clostridia examined produced mixed results on
284 the control and TMAO-containing media (i.e. white colonies on both plates or on the control plate
285 only, larger colonies on the TMAO-containing medium but without a colour change of the medium).
286 To determine whether these isolates were converting TMAO to TMA but a low level, we
287 examined the growth of all isolates in liquid culture and metabolites in the spent medium using NMR.
288 The growth of the *Enterobacteriaceae* was most greatly affected by the presence of TMAO in the
289 medium, with a faster, longer-lasting exponential phase than for the same isolates grown in the
290 control medium (**Fig. 3A**). pH of the spent medium (after 24 h) when *Enterobacteriaceae* were grown
291 in the presence of TMAO increased from a mean of 4.7 ± 0.3 (for the control) to 7.6 ± 0.3 (n = 20)
292 (the change in pH is what causes the colonies to appear white on TMAO-containing agar). The
293 growth of lactic acid bacteria, including *Enterococcus* and *Streptococcus* (**Fig. 3A**) spp., was
294 enhanced in the presence of TMAO, but not to the same extent as seen for the *Enterobacteriaceae*.
295 There was no significant difference (P = 0.27, t test) in the pH of the spent medium for these bacteria
296 after 24 h (mean 4.67 ± 0.9 compared with 4.33 ± 0.27 for the control). The growth of members of

297 *Clostridium* cluster I (e.g. *Clostridium perfringens*, **Fig. 3A**) was not enhanced in the presence of
298 TMAO, though some of these bacteria changed the colour of both the control and TMAO-containing
299 plates yellow during their growth. The pH of the spent liquid medium confirmed this observation to
300 be due to the alkalinity of the media in the control and TMAO-containing media after 24 h incubation:
301 e.g. *Clostridium sporogenes* D1(9) (pH 6.25 compared with 6.76 in the control medium), *Clostridium*
302 *paraputrificum* L16-FAA6 (pH 6.45 vs 5.38) and *Clostridium perfringens* L20-BSM1 (pH 5.56 vs
303 4.64).
304 ^{1}H-NMR analysis of spent medium from the TMAO-containing and control samples
305 demonstrated that, as expected, the *Enterobacteriaceae* produced the greatest amount of TMA from
306 TMAO (mean 38.79 ± 11.08 mM compared with 0.03 ± 0.01 mM, *n* = 20) (**Fig. 3B**; **Supplementary**
307 **Table 2**). Members of the families *Peptostreptococcaceae* (3.72 mM, *n* = 1), *Clostridiaceae* (Cluster
308 I) (2.62 ± 1.83 mM, *n* = 3), *Porphyromonadaceae* (1.42 mM, *n* = 1), *Bacteroidaceae* (1.40 ± 0.31
309 mM, *n* = 3), *Enterococcaceae* (1.19 ± 0.05 mM, *n* = 5), *Erysipelotrichaceae* (0.94 mM, *n* = 1;
310 [*Clostridium*] *ramosum*), *Staphylococcaceae* (0.34 mM, *n* = 1), *Streptococcaceae* (0.30 ± 0.16 mM, *n*
311 = 5), *Lactobacillaceae* (0.17 ± 0.07 mM, *n* = 2), *Pseudomonadaceae* (0.12 mM, *n* = 1) and
312 *Bifidobacteriaceae* (0.13 ± 0.1 mM, *n* = 17) produced low levels of TMA from TMAO (**Fig. 3B**;
313 **Supplementary Table 2**). There was great variability in the ability of the bifidobacteria to produce
314 TMA from TMAO, with several isolates and [*Clostridium*] *innocuum*, *Actinomyces odontolyticus*,
315 *Fusobacterium ulcerans* and *Actinomyces viscosus* not producing TMA from TMAO (**Fig. 3B**;
316 **Supplementary Table 2**).
317
318 **Differences in the metabolic capabilities of faecal and caecal *Escherichia coli* isolates**
319 Comparison of the amounts of TMA, and co-metabolites, produced by the faecal (*n* = 7) and
320 caecal (*n* = 9) isolates of *Escherichia coli* demonstrated significantly higher amounts of TMA were
321 produced by the caecal isolates compared with the faecal isolates in the TMAO-containing medium
322 (**Fig. 3C**). *Escherichia coli* of caecal origin produced more TMA than faecal isolates of the same
323 bacterium or other enterobacteria (*Hafnia*, *Citrobacter* and *Klebsiella* spp.) (**Supplementary Table**
324 **2**). The faecal isolates produced more acetate and lactate than the caecal isolates when grown in the

325 control medium. Taken together, these results demonstrate the different metabolic capabilities of
326 isolates of *Escherichia coli* recovered from different regions of the human gut.
327
328 **Lactic acid bacteria produce more lactate in the presence of TMAO**
329 Differences were also seen in the amount of lactic acid produced by lactic acid bacteria in the
330 presence and absence of TMAO (**Fig. 3B, D**). In raffinose-containing medium, bifidobacteria
331 produced increased of amounts of lactate when grown in the presence of TMAO (the bifidobacteria
332 grew poorly, if at all, in glucose-containing media). Unlike the bifidobacteria, the *Streptococcaceae*
333 and *Enterococcaceae* grew well in the glucose-containing medium and produced over 25 mM lactic
334 acid in the TMAO-containing samples compared with < 5 mM in the control samples (**Fig. 3D**). To
335 the best of our knowledge, this is the first time TMAO has been shown to influence the metabolism of
336 gut bacteria – specifically lactic acid bacteria – without producing appreciable amounts of TMA.
337
338
339 **DISCUSSION**
340 TMAO is a circulating metabolite produced as a direct result of microbial degradation of
341 dietary methylamines in the intestinal tract, and can be readily detected along with its precursor TMA
342 in human urine, blood and skeletal muscle (Wang *et al.*, 2011; Koeth et al., 2013; Tang *et al.*, 2013;
343 Taesuwan *et al.*, 2017). It worsens atherosclerosis in some mouse models of cardiovascular disease,
344 and is positively correlated with cardiovascular disease severity in humans. Beneficial effects
345 associated with TMAO include potential protection from hyperammonia and glutamate neurotoxicity,
346 alleviation of endoplasmic reticulum stress and improved glucose homeostasis by stimulating insulin
347 secretion by pancreatic cells (Kloiber *et al.*, 1988; Miñana *et al.*, 1996; Dumas *et al.*, 2017).
348 Over many decades it has been established that choline, PC and carnitine are dietary
349 methylamines that contribute directly to microbiome-associated circulating levels of TMAO found in
350 humans and other animals (e.g. Zeisel *et al.*, 1983; Rebouche *et al.*, 1984; Seim *et al.*, 1985; Wang *et*
351 *al.*, 2011; Koeth et al., 2013; Tang *et al.*, 2013). However, TMAO itself is a water-soluble osmolyte
352 found in high abundance in fish. Based on their observations that individuals with trimethylaminuria

353 could reduce an oral dose of TMAO to TMA could not detoxify it to TMAO, al-Waiz *et al.* (1987)
354 suggested the gut microbiota could use TMAO as a substrate, and in those individuals without
355 trimethylaminuria the TMA was re-oxidized in the liver before being excreted in the urine. This led
356 these authors to propose the process of '*metabolic retroversion*'. Reference to the literature associated
357 with fish spoilage and recent mining of metagenomic data have predicted members of the
358 *Enterobacteriaceae* (particularly *Escherichia coli* and *Klebsiella pneumoniae*) have the potential to
359 convert TMAO to TMA in the intestinal tract, but this has not been tested *in vitro* or *in vivo* to date
360 (Takagi and Ishimoto, 1983; Barrett and Kwan, 1985; Jameson *et al.*, 2016; Borrel *et al.*, 2017;
361 Taesuwan *et al.*, 2017). Consequently, we instigated this study to demonstrate metabolic
362 retroconversion of TMAO, and to determine the effect of TMAO on the growth and metabolism of
363 human-derived intestinal bacteria in pure and mixed cultures.
364 Through *in vivo* administration of deuterated TMAO to mice via oral gavage in mice, we
365 unambiguously demonstrated that TMAO is converted to TMA, with this TMA detectable in plasma
366 within 2 h of administration. This conversion was highly dependent on the gut microbiota, as
367 conversion of TMAO to TMA was dramatically reduced when the microbiota was suppressed by
368 treatment of animals with broad-spectrum antibiotics. Even in the presence of antibiotics, there was
369 low-level conversion of TMAO to TMA, suggesting a subpopulation of the microbiota was resistant
370 to the antibiotics used in our experiment. However, administration of a broad-spectrum antibiotic
371 cocktail for 14 days has been demonstrated to be an effective means of suppressing the gut microbiota
372 in studies associated with gut microbial use of dietary methylamines (Tang *et al.*, 2013).
373 Our *in vivo* experiment clearly demonstrates the gut microbiota converts TMAO to TMA, and
374 that this TMA is re-oxidized to TMAO, in line with the process of '*metabolic retroversion*' defined
375 by al-Waiz *et al.* (1987). Gut-associated microbial conversion of the majority of TMAO to TMA is at
376 odds with the findings of Taesuwan *et al.* (2017), who suggested TMAO is taken up intact and not
377 metabolised by the gut microbiota of humans. The ratio of TMAO-to-TMA is around 10:1 in humans
378 and 1:10 in mice, meaning TMAO was *N*-oxidized before Taesuwan *et al.* (2017) were able to
379 observe it in circulating blood of their human subjects. In a human system with high FMO3 activity,
380 O^{17}-labelled TMAO would need to be used in any study evaluating O turnover to allow calculation of

381 the true rate of retroconversion.

382 Having conducted our *in vivo* experiment in mice, we examined the ability of a range of
383 human-derived gut bacteria to convert TMAO to TMA. In the mixed microbiota system and in pure
384 cultures, the growth of the *Enterobacteriaceae* – the main TMA-producers – was quickly affected by
385 the presence of TMAO. This is likely to happen in the human gut also. Consequently, we believe
386 dietary TMAO undergoes metabolic retroconversion in mammals, with the TMA produced as a result
387 of bacterial activity in the gut available to the host for conversion back to TMAO by FMOs in
388 hepatocytes. It should be noted that Taesuwan *et al.* (2017) did not suppress or monitor the
389 intestinal/faecal microbiota when they administered isotopically labeled TMAO to humans, nor did
390 they measure d_9-TMA and d_9-TMAO in the portal vein, bypassing subsequent hepatic *N*-oxidation of
391 d_9-TMA, so it is not possible to interpret their results in the context of presence/absence of microbial
392 activity.

393 Of note is the finding that caecal isolates of enterobacteria produce more TMA from TMAO
394 than faecal isolates of the same bacterium. The speed with which TMAO was reduced to TMA by the
395 *Enterobacteriaceae* in the present study suggests bacterial conversion of TMAO to TMA takes place
396 in the small intestine/proximal colon of humans and small intestine/caecum of mice. It is, therefore,
397 unsurprising that caecal bacteria – representing the microbiota present at the intersection of the small
398 and large intestine – are metabolically more active than their faecal counterparts with respect to
399 TMAO metabolism. This finding is relevant to functional studies of the gut microbiota where gene
400 annotations are based largely on faecal isolates, whose functionalities may be greatly different from
401 those of bacteria in other regions of the human intestinal tract. It has already been demonstrated that
402 the microbiota of the small intestine is enriched for functions associated with rapid uptake and
403 fermentation of simple carbohydrates compared with the faecal microbiota, and that streptococci
404 isolated from this niche are functionally very different from the same bacteria isolated from different
405 habitats (Zoetendal *et al.*, 2012; van den Bogert *et al.*, 2013). It is, therefore, important we
406 characterize the functions and genomes of bacteria isolated from all regions of the intestinal tract, not
407 just those of faecal bacteria, to gain a true picture of how microbial activity influences host health.

408 *Enterobacteriaceae* made the greatest contribution to the conversion of TMAO to TMA, both

409 in pure culture and in a mixed microbiota. These Gram-negative bacteria are a source of the virulence
410 factor lipopolysaccharide (LPS), which is associated with low-grade inflammation in high-fat-fed
411 mice and elevated plasma levels that define metabolic endotoxaemia (Cani *et al.*, 2007, 2008). High-
412 fat feeding has been shown to increase the representation of *Enterobacteriaceae* in the caecal
413 microbiota of obesity-prone Sprague–Dawley rats (de La Serre *et al.*, 2010), though this mode of
414 feeding is known to modulate the microbiota of mice independent of obesity (Hildebrandt *et al.*,
415 2009). Non-LPS-associated virulence of *Enterobacteriaceae*, *Vibrio cholerae* and *Helicobacter pylori*
416 is increased when these bacteria are grown anaerobically or microaerophilically in the presence of as
417 little as 5–10 mM TMAO (Ando *et al.*, 2007; Lee *et al.*, 2012; Paiva *et al.*, 2009; Wu *et al.*, 2017),
418 and may be an additional means by which the gut microbiota contributes to cardiovascular and other
419 diseases in which increased representation of *Gammaproteobacteria* is observed. This warrants
420 attention in future animal studies.

421 Lactic acid bacteria clearly grow better in the presence of TMAO. The relatively high
422 concentration (1 %) of TMAO used in this study may have contributed to this improved growth, as
423 TMAO is an osmolyte that stabilizes proteins. Future work will involve growing lactic acid bacteria in
424 a range of TMAO concentrations to determine how this compound affects their growth and gene
425 expression, and comparing faecal and caecal isolates. Similar to the *Enterobacteriaceae*, a large
426 number of these bacteria are facultative anaerobes able to grow over a range of conditions, and whose
427 representation is increased in obese, and cirrhotic patients (Le Chatelier *et al.*, 2013; Qin *et al.*, 2014).
428 *Streptococcus* and *Enterococcus* spp. are commensal lactic acid bacteria of the gut microbiota known
429 to modulate immune function; but little is known about their metabolic activities in mixed microbial
430 populations (van den Bogert *et al.*, 2014). Understanding how commensal lactic acid bacteria
431 influence the host in dysbiosis in mixed microbial communities may allow the development of
432 approaches to modulate their activity and influence host health.

433 With respect to the lactic acid bacteria, it is important to note that our mixed culture work did
434 not highlight these as being relevant to TMAO metabolism. This is unsurprising given these bacteria
435 do not produce large quantities of TMA from TMAO. However, we have shown their metabolism is
436 affected by presence of TMAO in growth medium, and the increased lactate they produce in its

presence may contribute to cross-feeding associated with short-chain fatty acid production (Hoyles and Wallace, 2010). It is difficult to determine relevance of correlations from mixed microbial ecosystems in the absence of isotope labelling or pure culture work: i.e. correlation does not equate with causation. As an example, three (*Clostridium* clusters I and II, bifidobacteria, coriobacteriia) of the four groups of bacteria correlated with TMA production in our fermentation study did not produce notable quantities of TMA from TMAO based on our pure culture work. Therefore, correlating microbiota and metabolite data derived from complex systems will not give a true picture of which members of microbiota contribute to specific metabolic processes, and work with pure cultures is required to supplement functional studies to increase our understanding of poorly understood microbially driven metabolic processes within the human gut.

In summary, we have demonstrated metabolic retroconversion – another example of host–microbial co-metabolism – occurs in the mammalian system with respect to TMAO, whereby TMAO is reduced by the gut microbiota to TMA and regenerated by host hepatic enzymes. We have also demonstrated that growth and metabolism of members of the gut microbiota are affected by TMAO in a source- and taxon-dependent manner, with the family *Enterobacteriaceae* making the greatest contribution to production of TMA in the gut.

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671 **FIGURE LEGENDS**

672 **Fig. 1.** *In vivo* confirmation of metabolic retroconversion of TMAO. Reduction of d_9-TMAO to d_9-
673 TMA was quantified by UPLC–MS/MS up to 6 h after d_9-TMAO gavage and antibiotic treatment,
674 together with unlabelled TMA and TMAO levels. Plasma quantification of post-gavage (A) d_9-TMA
675 and (B) d_9-TMAO. *, Significantly ($P < 0.05$; *t* test and corrected for multiple comparison using the
676 Holm–Sidak method) different from the respective groups not treated with antibiotics. (C) d_9-TMA
677 bioavailability (AUC). (D) d_9-TMAO bioavailability (AUC). Plasma quantification of post-gavage
678 unlabelled/endogenous (E) TMA and (F) TMAO. *, Significant between d_9 and d_9 antibiotic
679 treatment; $, significant between TMAO and TMAO antibiotic treatment. (G) Unlabelled/endogenous
680 TMA bioavailability (AUC). (H) Unlabelled/endogenous TMAO bioavailability (AUC). Data ($n = 6$
681 per group) are shown as mean ± SEM. (A, B, E and F). Differences between the bioavailabilities (C,
682 D, G and H) were assessed using one-way analysis of variance (ANOVA), followed by Holm–Sidak
683 post hoc tests. Data with different superscript letters are significantly different ($P < 0.05$).

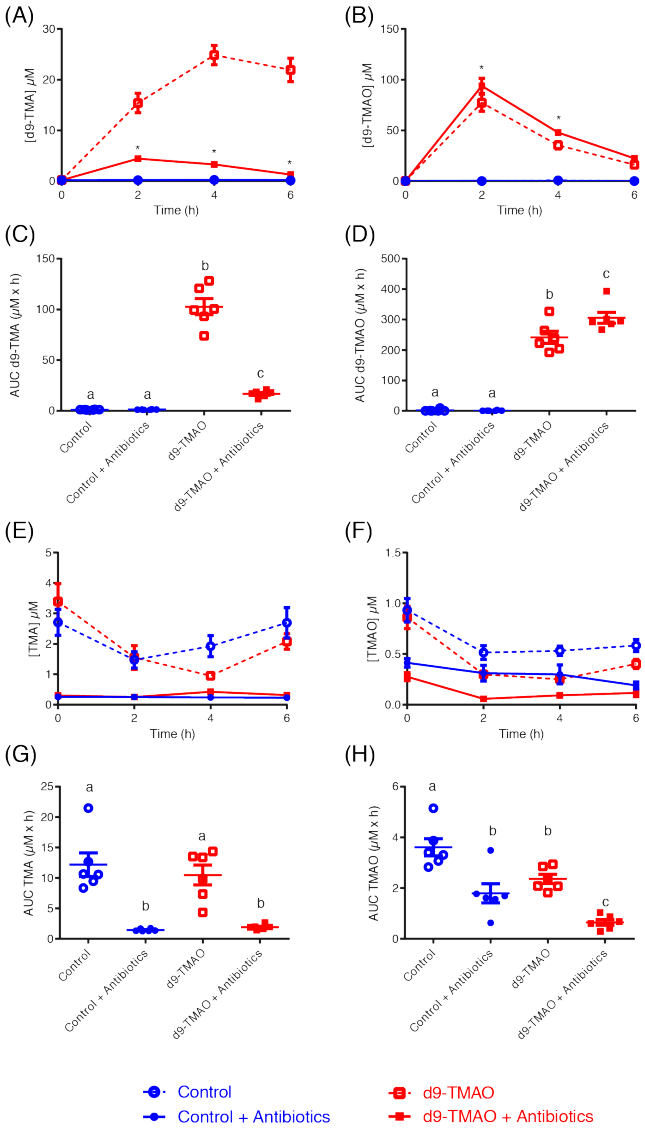
684

685 **Fig. 2.** Effect of TMAO on mixed faecal microbial population *in vitro*. (A) Enumeration of selected
686 bacteria in fermentation vessels by FISH analysis. Red lines, TMAO-containing systems; blue lines,
687 negative controls. Data are shown as mean + SD ($n = 3$). Eub338, total bacteria; Ent,
688 *Enterobacteriaceae*; Bif164, *Bifidobacterium* spp.; Lab158, lactic acid bacteria. *, Statistically
689 significantly different (adjusted $P < 0.05$) from the control at the same time point. Full data are shown
690 in **Supplementary Figure 1**. (B) ^{1}H-NMR data for batch culture samples. Data are shown mean ± SD
691 ($n = 3$). Red lines, TMAO-containing systems; blue lines, negative controls. *, Statistically
692 significantly different ($P < 0.05$) from the negative control at the same time point. (C) Bidirectional
693 clustering of correlation matrix of FISH data and data for the six metabolites found in highest
694 amounts in the NMR spectra from the batch-culture samples. +, Adjusted *P* value (Benjamini–
695 Hochberg) statistically significant ($P < 0.05$). FISH and metabolite data and a table of correlations and
696 adjusted *P* values (Benjamini–Hochberg) for the batch-culture samples are available in
697 **Supplementary Tables 3–5**.

698

699 **Fig. 3.** Influence of TMAO on growth and metabolism of pure cultures of gut bacteria. (A)
700 Representative growth curves for isolates grown in the presence and absence of TMAO. Red lines,
701 TMAO-supplemented cultures; blue lines, negative controls. Data are shown as mean ± SD (n = 3).
702 (B) Biplot showing production of various metabolites when isolates were grown in the presence of
703 TMAO. Summary of data from **Supplementary Table 2**. The larger a circle, the more of the
704 metabolite produced by an isolate. (C) Differences in metabolites produced when caecal and faecal
705 isolates of *Escherichia coli* were grown in the presence (+) and absence (–) of 1 % TMAO. Adjusted
706 (Benjamini–Hochberg) P values indicate the caecal isolates were significantly different from the
707 faecal isolates for a particular metabolite. (D) Lactate production by lactic acid bacteria was increased
708 in the presence of TMAO. *Enterobacteriaceae*, n = 20; *Bifidobacteriaceae*, n = 17; *Streptococcaceae*,
709 n = 7; *Enterococcaceae*, n = 5. Members of the *Enterococcaceae* and *Streptococcaceae* are
710 homofermenters (produce only lactic acid from glucose fermentation), whereas the
711 *Bifidobacteriaceae* are heterofermenters (produce ethanol, CO_2 and lactic acid from glucose
712 fermentation), though it should be noted the bifidobacteria included in this study were grown on
713 raffinose-containing media. Red, TMAO-containing medium; blue, negative control. *, Statistically
714 significantly different from its negative control (adjusted P value < 0.05).

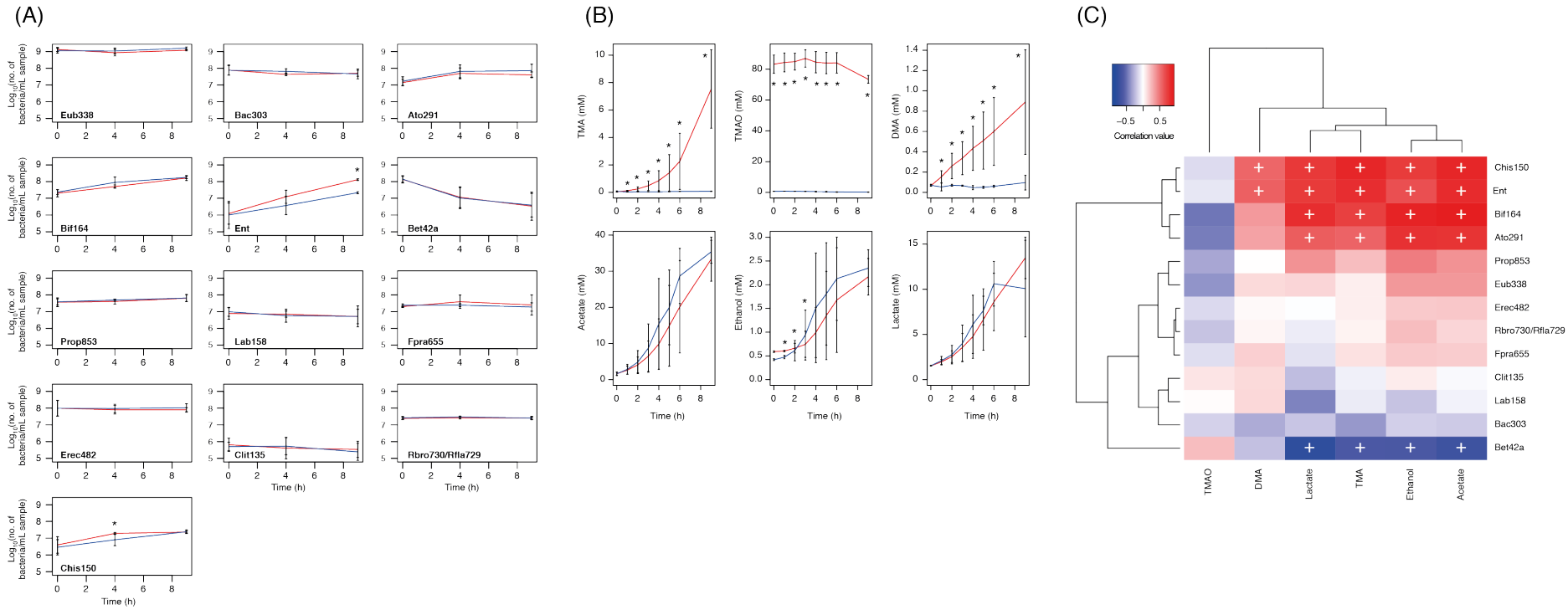
715 **Fig. 1**



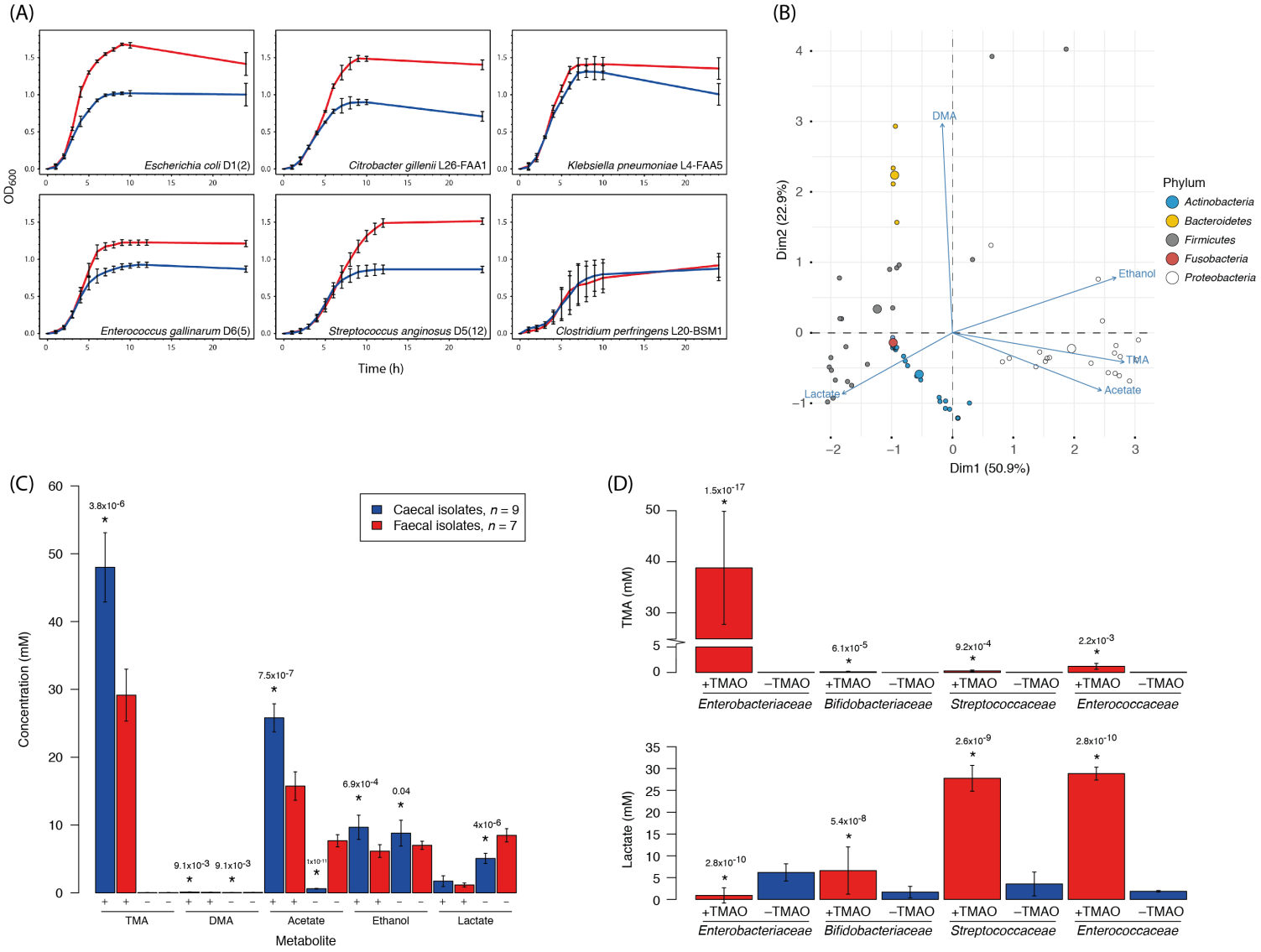
716

717

Fig. 2


(A)
Log10(no. of bacteria/mL sample)
Eub338
Bac303
Ato291
Bif164
Ent
Bet42a
Prop853
Lab158
Fpra655
Erec482
Clit135
Rbro730/Rfla729
Chis150
Time (h)
(B)
TMA (mM)
TMAO (mM)
DMA (mM)
Acetate (mM)
Ethanol (mM)
Lactate (mM)
Time (h)
(C)
Correlation value
−0.5
0.5
Chis150
Ent
Bif164
Ato291
Prop853
Eub338
Erec482
Rbro730/Rfla729
Fpra655
Clit135
Lab158
Bac303
Bet42a
TMAO
DMA
Lactate
TMA
Ethanol
Acetate

720 **Fig. 3**


(A)
OD600
Escherichia coli D1(2)
Citrobacter gillenii L26-FAA1
Klebsiella pneumoniae L4-FAA5
Enterococcus gallinarum D6(5)
Streptococcus anginosus D5(12)
Clostridium perfringens L20-BSM1
Time (h)
(B)
Dim2 (22.9%)
Dim1 (50.9%)
DMA
Ethanol
TMA
Acetate
Lactate
Phylum
Actinobacteria
Bacteroidetes
Firmicutes
Fusobacteria
Proteobacteria
(C)
Caecal isolates, n = 9
Faecal isolates, n = 7
Concentration (mM)
3.8x10-6
9.1x10-3
9.1x10-3
7.5x10-7
1x10-11
6.9x10-4
0.04
4x10-6
TMA
DMA
Acetate
Ethanol
Lactate
Metabolite
(D)
TMA (mM)
1.5x10-17
6.1x10-5
9.2x10-4
2.2x10-3
Lactate (mM)
2.8x10-10
5.4x10-8
2.6x10-9
2.8x10-10
+TMAO
−TMAO
Enterobacteriaceae
Bifidobacteriaceae
Streptococcaceae
Enterococcaceae

721

722 **Table 1.** Details for human-derived gut bacteria screened for their ability to reduce or utilize TMAO

Isolate*	Identified as	Source	Reference	Facultative anaerobe?
D2(9)	[*Clostridium*] *innocuum*	Human faeces	M.L. Jiménez-Pranteda, unpublished	No
L16-FAA1	[*Clostridium*] *ramosum*	Human caecum	L. Hoyles, unpublished	No
L12-BSM1	*Actinomyces odontolyticus*	Human caecum	L. Hoyles, unpublished	No
L6-BSM10	*Actinomyces viscosus*	Human caecum	L. Hoyles, unpublished	No
L6-FAA7	*Bacteroides fragilis*	Human caecum	L. Hoyles, unpublished	No
D1(4)	*Bacteroides vulgatus*	Human faeces	M.L. Jiménez-Pranteda, unpublished	No
L9-FAA7	*Bacteroides vulgatus*	Human caecum	L. Hoyles, unpublished	No
DSM 20083^{T}	*Bifidobacterium adolescentis*	Human intestine	DSMZ†	No
LCR26	*Bifidobacterium animalis* subsp. *lactis*	Infant faeces	Roger *et al.* (2010)	No
LCR11	*Bifidobacterium bidifum*	Infant faeces	Roger *et al.* (2010)	No
L25-MRS2	*Bifidobacterium bifidum*	Human caecum	L. Hoyles, unpublished	No
LCR5	*Bifidobacterium breve*	Infant faeces	Roger *et al.* (2010)	No
LCR8	*Bifidobacterium breve*	Infant faeces	Roger *et al.* (2010)	No
LCR1	*Bifidobacterium dentium*	Infant faeces	Roger *et al.* (2010)	No
DSM 20093^{T}	*Bifidobacterium gallicum*	Human intestine	DSMZ	No
LCR6	*Bifidobacterium longum*	Infant faeces	Roger *et al.* (2010)	No
DSM 20088^{T}	*Bifidobacterium longum* subsp. *infantis*	Infant intestine	DSMZ	No
LCR2	*Bifidobacterium longum* subsp. *infantis*	Infant faeces	Roger *et al.* (2010)	No
DSM 20219^{T}	*Bifidobacterium longum* subsp. *longum*	Human intestine	DSMZ	No
LCR3	*Bifidobacterium pseudocatenulatum*	Infant faeces	Roger *et al.* (2010)	No
L19-MRS1	*Bifidobacteroum longum* subsp. *longum*	Human caecum	L. Hoyles, unpublished	No
L25-MRS8	*Bifidobacteroum longum* subsp. *longum*	Human caecum	L. Hoyles, unpublished	No
L26-MRS4	*Bifidobactrium animalis* subsp. *lactis*	Human caecum	L. Hoyles, unpublished	No
L26-FAA1	*Citrobacter gillenii*	Human caecum	L. Hoyles, unpublished	Yes
L8-FAA3	*Citrobacter koseri*	Human caecum	L. Hoyles, unpublished	Yes
L16-FAA6	*Clostridium paraputrificum*	Human caecum	L. Hoyles, unpublished	No
L20-BSM1	*Clostridium perfringens*	Human caecum	L. Hoyles, unpublished	No
D1(9)	*Clostridium sporogenes*	Human faeces	M.L. Jiménez-Pranteda, unpublished	No
D2(14)	*Enterococcus faecalis*	Human faeces	M.L. Jiménez-Pranteda, unpublished	Yes
D3(1)	*Enterococcus faecalis*	Human faeces	M.L. Jiménez-Pranteda, unpublished	Yes
D5(2)	*Enterococcus faecalis*	Human faeces	M.L. Jiménez-Pranteda, unpublished	Yes
D6(1)	*Enterococcus faecium*	Human faeces	M.L. Jiménez-Pranteda, unpublished	Yes
D6(5)	*Enterococcus gallinarum*	Human faeces	M.L. Jiménez-Pranteda, unpublished	Yes
D1(2)	*Escherichia coli*	Human faeces	M.L. Jiménez-Pranteda, unpublished	Yes
D2(1)	*Escherichia coli*	Human faeces	M.L. Jiménez-Pranteda, unpublished	Yes
D2(2)	*Escherichia coli*	Human faeces	M.L. Jiménez-Pranteda, unpublished	Yes
D2(8)	*Escherichia coli*	Human faeces	M.L. Jiménez-Pranteda, unpublished	Yes
D3(8)	*Escherichia coli*	Human faeces	M.L. Jiménez-Pranteda, unpublished	Yes
D4(15)	*Escherichia coli*	Human faeces	M.L. Jiménez-Pranteda, unpublished	Yes
D5(1)	*Escherichia coli*	Human faeces	M.L. Jiménez-Pranteda, unpublished	Yes
L1-FAA5	*Escherichia coli*	Human caecum	L. Hoyles, unpublished	Yes
L13-FAA2	*Escherichia coli*	Human caecum	L. Hoyles, unpublished	Yes
L16-FAA5	*Escherichia coli*	Human caecum	L. Hoyles, unpublished	Yes
L19-FAA2	*Escherichia coli*	Human caecum	L. Hoyles, unpublished	Yes
L20-FAA3	*Escherichia coli*	Human caecum	L. Hoyles, unpublished	Yes
L24-FAA5	*Escherichia coli*	Human caecum	L. Hoyles, unpublished	Yes
L5-FAA2	*Escherichia coli*	Human caecum	L. Hoyles, unpublished	Yes
L6-FAA1	*Escherichia coli*	Human caecum	L. Hoyles, unpublished	Yes
L9-MRS1	*Escherichia coli*	Human caecum	L. Hoyles, unpublished	Yes
L9-FAA5	*Fusobacterium ulcerans*	Human caecum	L. Hoyles, unpublished	Yes
L15-FAA9	*Hafnia paralvei*	Human caecum	L. Hoyles, unpublished	No
L4-FAA5	*Klebsiella pneumoniae* subsp. *pneumoniae*	Human caecum	Hoyles *et al.* (2015)	Yes
L26-MRS5	*Lactobacillus fermentum*	Human caecum	L. Hoyles, unpublished	No

L26-FAA6	*Lactobacillus rhamnosus*	Human caecum	L. Hoyles, unpublished	No
L13-FAA10	*Parabacteroides johnsonii*	Human caecum	L. Hoyles, unpublished	No
D4(1)	*Paraclostridium bifermentans*	Human faeces	M.L. Jiménez-Pranteda, unpublished	No
L1-FAA6	*Pseudomonas aeruginosa*	Human caecum	L. Hoyles, unpublished	Yes
D2(4)	*Staphyloccoccus hominis*	Human faeces	M.L. Jiménez-Pranteda, unpublished	Yes
D1(5)	*Streptococcus anginosus*	Human faeces	M.L. Jiménez-Pranteda, unpublished	Yes
D5(12)	*Streptococcus anginosus*	Human faeces	M.L. Jiménez-Pranteda, unpublished	Yes
L25-MRS1	*Streptococcus gallolyticus*	Human caecum	L. Hoyles, unpublished	Yes
L4-MRS5	*Streptococcus oralis*	Human caecum	L. Hoyles, unpublished	Yes
L4-MRS1	*Streptococcus sanguinis*	Human caecum	L. Hoyles, unpublished	Yes
D4(3)	*Streptococcus* sp.	Human faeces	M.L. Jiménez-Pranteda, unpublished	Yes
L26-MRS7	*Streptococcus vestibularis*	Human caecum	L. Hoyles, unpublished	Yes
LCR4	Unknown *Bifidobacterium*	Infant faeces	Roger *et al.* (2010)	No

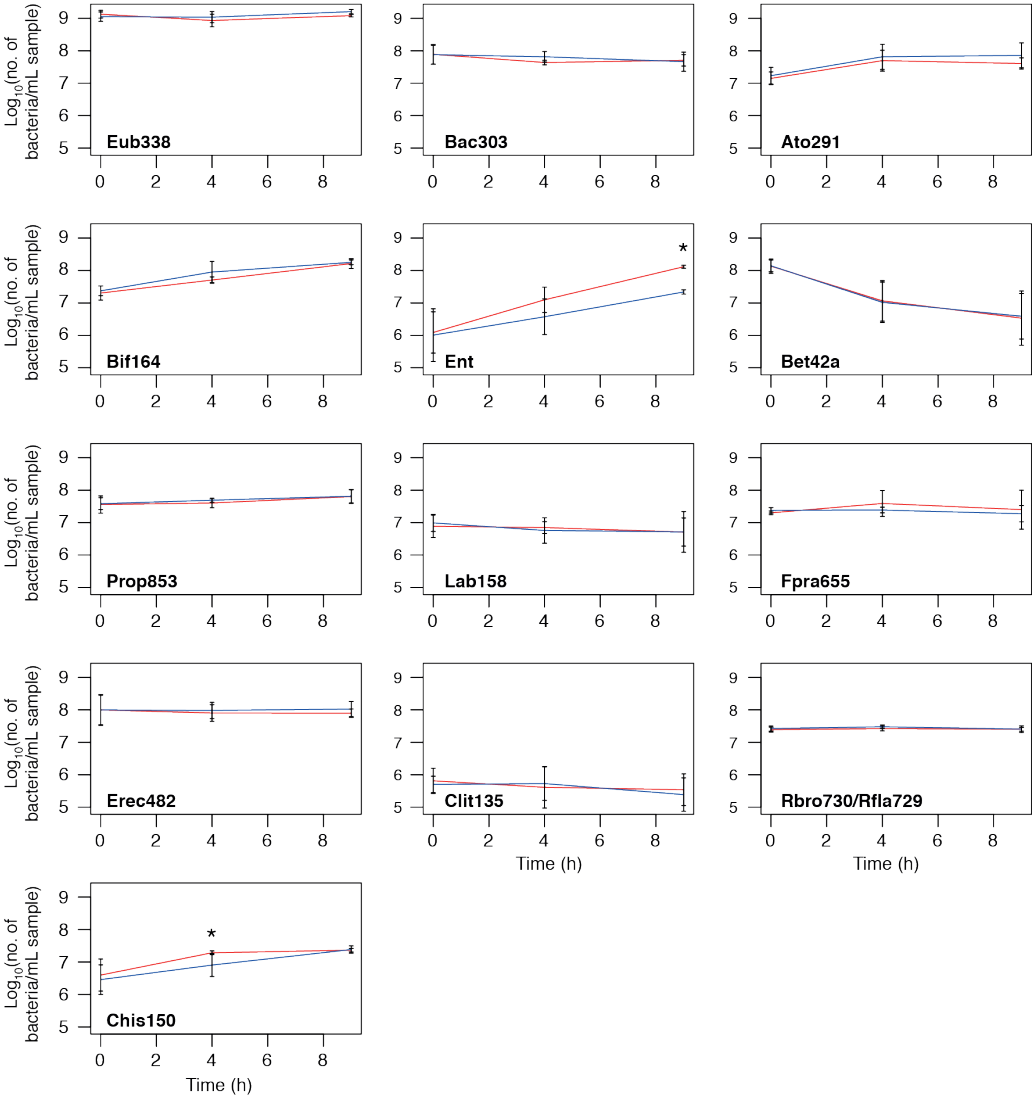
723

724 *L*n*- prefix, different numbers indicate isolates recovered from different individuals.

725 †DSMZ, Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures.

726

727 **SUPPLEMENTARY MATERIAL**



728

729 **Supplementary Figure 1.** Effect of TMAO on mixed faecal microbial population *in vitro*.

730 Enumeration of bacteria in fermentation vessels by FISH analysis. Red lines, TMAO-containing

731 systems; blue lines, negative controls. Data are shown as mean + SD (n = 3). Eub338, total bacteria;

732 Bac303, *Bacteroidales*; Ato291, '*Atopobium* cluster'; Bif164, *Bifidobacterium* spp.; Ent,

733 *Enterobacteriaceae*; Bet42a, *Betaproteobacteria*; Prop853, *Veillonellaceae*; Lab158, lactic acid

734 bacteria (*Lactobacillales*); Fpra655, *Faecalibacterium prausnitzii* and *Subdoligranulum* spp.;

735 Erec482, *Lachnospiraceae*; Clit135, *Clostridium* cluster XI; Rbro730/Rfla729, *Ruminococcaceae*;

736 Chis150, *Clostridium* clusters I and II. Detection limit of the method was 4.95 $\log_{10}$(number of

737 bacteria/mL sample). *, Statistically significantly different (adjusted $P < 0.05$) from the control at the

738 same time point.

739 **Supplementary Table 1.** Oligonucleotide probes used in this study

Probe name	Detects	Probe accession no.*	Hybridization pre-treatment†	Formamide (%) in hybridization buffer	Temperature (°C)		Reference(s)
					Hybridization	Washing	
Ato291	*Cryptobacterium curtum*, *Gordonibacter pamelaeae*, *Paraeggerthella hongkongensis*, all *Eggerthella*, *Collinsella*, *Olsenella* and *Atopobium* species; '*Enorma*' and *Senegalemassilia* spp. (most members of the class *Coriobacteriia*; phylum *Actinobacteria*)	pB-00943	Lysozyme	0	50	50	Harmsen *et al.* (2000); Thorasin *et al.* (2015)
Bac303	Most members of the genus *Bacteroides*, some *Parabacteroides* and *Prevotella* species, *Paraprevotella*, *Xylanibacter*, *Barnesiella* species and *Odoribacter splanchnicus* (order *Bacteroidales*; phylum *Bacteroidetes*)	pB-00031	None	0	46	48	Manz *et al.* (1996); Hoyles and McCartney (2009)
Bet42a	*Betaproteobacteria* (class *Betaproteobacteria*; phylum *Proteobacteria*)	pB-00034	None	35	46	48	Manz *et al.* (1992)
Bif164	Most *Bifidobacterium* species and *Parascardovia denticolens* (family *Bifidobacteriaceae*; phylum *Actinobacteria*)	pB-00037	Lysozyme	0	50	50	Langendijk et al. (1995)
Chis150	Most members of *Clostridium* cluster I, all members of *Clostridium* cluster II (family *Clostridiaceae*; phylum *Firmicutes*)	pB-00962	None	0	50	50	Franks *et al.* (1998)
Clit135	Nine members of [*Clostridium*] cluster XI, including [*Clostridium*] *difficile* (family *Peptostreptococcaceae*; phylum *Firmicutes*)	pB-00961	None	0	50	50	Franks *et al.* (1998)
Ent	*Enterobacteriaceae* except *Proteus* spp. (order *Gammaproteobacteria*; phylum *Proteobacteria*)	pB-00351	None	30	46	48	Kempf *et al.* (2000)
Erec482	Most members of *Clostridium* cluster XIVa (family *Lachnospiraceae*; phylum *Firmicutes*)	pB-00963	None	0	50	50	Franks *et al.* (1998)
Eub338/ Eub338II/ Eub338III‡	*Bacteria*	pB-00159/ pB-00160/ pB-00161	None	35	46	48	Daims *et al.* (1999)
Fpra655	*Faecalibacterium prausnitzii* and *Subdoligranulum* spp. (*Clostridium* cluster IV; family *Ruminococcaceae*; phylum *Firmicutes*)	pB-00734	None	0	58	58	Hold *et al.* (2003)
Lab158	All *Oenococcus*, *Vagococcus*, *Melissococcus*, *Tetragenococcus*, *Enterococcus*, *Catellicoccus*, *Paralactobacillus*, *Pediococcus* and *Lactococcus* species, most *Lactobacillus*, *Weissella* and *Leuconostoc* species (order *Lactobacillales*; phylum *Firmicutes*)	pB-03928	Lysozyme	0	50	50	Harmsen *et al.* (1999)
Prop853	Most members of *Clostridium* cluster IX (family *Veillonellaceae*; phylum *Firmicutes*)	pB-03930	None	0	50	50	Walker *et al.* (2005)
Rbro730/ Rfla729‡	*Anaerotruncus colihominis*, *Ruminococcus bromii*, *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Desulfotomaculum alcoholivorax* (Clostridium cluster IV; family *Ruminococcaceae*; phylum *Firmicutes*)	pB-00558/ pB-00557	Lysozyme	20	50	50	Harmsen *et al.* (2002)

740 *According to probeBase (Loy *et al.*, 2007).

741 †As described by Martín-Peláez *et al.* (2008).

742 ‡These probes were used together in equimolar concentrations (each at 50 ng/μL).

743

744 **Supplementary Table 2.** NMR-based analysis of metabolites in growth medium of pure cultures after 24-h incubation (anaerobic) in the presence and absence of
745 TMAO
746

Species and isolate*	Family, phylum	TMA (mM)		DMA (mM)		Acetate (mM)		Ethanol		Lactate	
		+TMAO	-TMAO	+TMAO	-TMAO	+TMAO	-TMAO	+TMAO	-TMAO	+TMAO	-TMAO
***Escherichia coli* L6-FAA1**	*Enterobacteriaceae*, *Proteobacteria*	53.94	0.03	0.03	0.05	28.17	0.70	7.64	8.20	0.19	5.10
***Escherichia coli* L24-FAA5**	*Enterobacteriaceae*, *Proteobacteria*	53.94	0.04	0.07	0.05	27.67	0.56	7.14	4.00	2.40	6.00
***Escherichia coli* L9-MRS1**	*Enterobacteriaceae*, *Proteobacteria*	52.94	0.02	0.08	0.04	27.17	0.60	9.14	10.20	0.00	4.70
***Escherichia coli* L19-FAA2**	*Enterobacteriaceae*, *Proteobacteria*	51.94	0.03	0.03	0.05	24.17	0.60	7.64	9.80	0.02	4.80
***Hafnia paralvei* L15-FAA9**	*Enterobacteriaceae*, *Proteobacteria*	45.44	0.03	0.08	0.07	23.97	5.20	6.44	14.60	0.80	2.50
***Escherichia coli* L13-FAA2**	*Enterobacteriaceae*, *Proteobacteria*	44.94	0.03	0.00	0.04	23.47	0.60	8.64	10.00	0.00	4.50
***Escherichia coli* L16-FAA5**	*Enterobacteriaceae*, *Proteobacteria*	44.94	0.03	0.06	0.05	23.17	0.60	9.64	9.30	0.06	4.80
***Escherichia coli* L20-FAA3**	*Enterobacteriaceae*, *Proteobacteria*	43.94	0.04	0.07	0.07	24.17	0.70	12.64	9.00	0.15	6.50
***Escherichia coli* L1-FAA5**	*Enterobacteriaceae*, *Proteobacteria*	42.94	0.03	0.03	0.05	23.67	0.60	10.64	9.00	0.03	5.20
***Citrobacter koseri* L8-FAA3**	*Enterobacteriaceae*, *Proteobacteria*	42.24	0.02	0.35	0.04	21.17	0.74	10.64	11.00	5.30	5.00
***Escherichia coli* L5-FAA2**	*Enterobacteriaceae*, *Proteobacteria*	41.94	0.02	0.07	0.03	23.17	0.60	10.64	9.70	0.32	4.10
***Citrobacter gillenii* L26-FAA1**	*Enterobacteriaceae*, *Proteobacteria*	37.94	0.03	0.13	0.06	18.77	0.60	12.64	11.00	4.60	5.40
***Escherichia coli* D3(8)**	*Enterobacteriaceae*, *Proteobacteria*	32.64	0.04	0.03	0.08	16.77	6.80	6.14	6.90	0.00	8.70
***Escherichia coli* D2(8)**	*Enterobacteriaceae*, *Proteobacteria*	31.84	0.03	0.02	0.06	16.47	8.20	6.74	7.00	0.00	8.50
***Escherichia coli* D2(2)**	*Enterobacteriaceae*, *Proteobacteria*	31.54	0.03	0.01	0.06	16.27	8.00	6.34	6.80	0.00	9.50
***Escherichia coli* D1(2)**	*Enterobacteriaceae*, *Proteobacteria*	30.34	0.03	0.00	0.07	16.17	8.60	5.44	8.00	0.00	9.40
***Escherichia coli* D4(15)**	*Enterobacteriaceae*, *Proteobacteria*	29.94	0.03	0.03	0.06	14.67	6.75	6.64	6.40	0.20	7.30
***Escherichia coli* D2(1)**	*Enterobacteriaceae*, *Proteobacteria*	24.44	0.05	0.00	0.08	11.67	8.70	4.14	7.60	0.00	9.00
***Escherichia coli* D5(1)**	*Enterobacteriaceae*, *Proteobacteria*	22.94	0.04	0.00	0.05	12.37	6.70	5.14	6.40	0.00	7.00
***Klebsiella pneumoniae* subsp. *pneumoniae* L4-FAA5**	*Enterobacteriaceae*, *Proteobacteria*	14.94	0.02	0.41	0.05	10.17	0.52	7.14	12.00	4.40	5.90
Clostridium sporogenes D1(9)	*Clostridiaceae* (Cluster I), *Firmicutes*	4.64	0.12	1.03	0.07	17.37	16.90	16.24	13.50	0.10	0.90
[*Clostridium*] *bifermentans* D4(1)	*Peptostreptococcaceae*, *Firmicutes*	3.72	0.00	1.08	0.00	12.15	0.00	8.81	0.00	0.03	0.00
Clostridium perfringens L20-BSM1	*Clostridiaceae* (Cluster I), *Firmicutes*	2.19	0.03	0.23	0.06	1.97	0.81	10.64	5.30	4.80	5.90
Enterococcus gallinarum D6(5)	*Enterococcaceae*, *Firmicutes*	1.74	0.04	0.30	0.15	0.00	0.77	1.24	0.83	29.80	2.00
Bacteroides vulgatus L9-FAA7	*Bacteroidaceae*, *Bacteroidetes*	1.70	0.00	0.88	0.00	2.65	0.00	0.00	0.00	0.00	0.00
Enterococcus faecalis D5(2)	*Enterococcaceae*, *Firmicutes*	1.64	0.05	0.45	0.13	0.00	0.67	0.82	1.04	27.80	1.60
Parabacteroides johnsonii L13-FAA10	*Porphyromonadaceae*, *Bacteroidetes*	1.42	0.00	0.65	0.00	1.51	0.00	0.00	0.00	0.24	0.00
Bacteroides fragilis L6-FAA7	*Bacteroidaceae*, *Bacteroidetes*	1.42	0.00	0.51	0.00	2.67	0.00	0.00	0.00	0.56	0.00
Enterococcus faecalis D3(1)	*Enterococcaceae*, *Firmicutes*	1.24	0.06	0.29	0.16	0.00	0.74	1.24	1.02	28.60	2.00

Bacteroides vulgatus D1(4)	*Bacteroidaceae*, *Bacteroidetes*	1.08	0.02	0.71	0.01	1.66	2.82	0.06	0.00	0.12	0.33
Clostridium paraputrificum L16-FAA6	*Clostridiaceae* (Cluster I), *Firmicutes*	1.04	0.03	0.33	0.07	1.37	0.63	1.04	2.50	2.00	3.60
Enterococcus faecalis D2(14)	*Enterococcaceae*, *Firmicutes*	1.00	0.05	0.17	0.14	0.00	0.69	1.34	1.40	27.20	1.80
[*Clostridium*] *ramosum* L16-FAA1	*Erysipelotrichaceae*, *Firmicutes*	0.94	0.07	0.33	0.09	1.47	0.60	1.34	1.80	4.30	5.20
Streptococcus sanguinis L4-MRS1	*Streptococcaceae*, *Firmicutes*	0.49	0.03	0.02	0.06	0.00	0.60	0.34	0.33	22.90	9.20
Streptococcus anginosus D5(12)	*Streptococcaceae*, *Firmicutes*	0.48	0.02	0.16	0.12	0.00	0.60	0.09	0.46	29.30	2.00
Streptococcus anginosus D1(5)	*Streptococcaceae*, *Firmicutes*	0.44	0.03	0.11	0.14	0.00	0.64	0.06	0.38	29.10	2.20
Bifidobacterium breve LCR5	*Bifidobacteriaceae*, *Actinobacteria*	0.38	0.03	0.04	0.00	32.04	26.51	2.00	0.89	12.77	0.68
Staphylococcus hominis D2(4)	*Staphylococcaceae*, *Firmicutes*	0.34	0.03	0.33	0.08	1.47	2.30	0.08	0.32	2.50	4.00
Enterococcus faecium D6(1)	*Enterococcaceae*, *Firmicutes*	0.31	0.06	0.13	0.14	0.00	0.70	0.30	0.37	30.80	1.95
Bifidobacterium infantis DSM 20088T	*Bifidobacteriaceae*, *Actinobacteria*	0.27	0.02	0.04	0.00	27.56	29.81	0.34	0.21	12.01	0.94
Streptococcus vestibularis L26-MRS7	*Streptococcaceae*, *Firmicutes*	0.25	0.02	0.00	0.05	0.00	0.40	0.28	0.65	29.40	2.20
Bifidobacterium bifidum LCR11	*Bifidobacteriaceae*, *Actinobacteria*	0.24	0.02	0.02	0.01	33.14	33.91	0.50	0.49	14.13	0.91
Lactobacillus fermentum L26-MRS5	*Lactobacillaceae*, *Firmicutes*	0.22	0.02	0.16	0.03	0.47	1.50	0.19	0.80	0.70	2.70
Bifidobacterium longum LCR6	*Bifidobacteriaceae*, *Actinobacteria*	0.22	0.02	0.02	0.01	23.86	23.77	0.39	0.06	10.47	0.77
Bifidobacterium longum DSM 20219^{T}	*Bifidobacteriaceae*, *Actinobacteria*	0.20	0.02	0.02	0.00	27.92	23.19	0.58	0.28	13.51	0.83
Bifidobacterium longum subsp. *infantis* LCR2	*Bifidobacteriaceae*, *Actinobacteria*	0.18	0.03	0.02	0.01	25.11	0.00	0.38	0.02	11.65	0.74
Streptococcus oralis L4-MRS5	*Streptococcaceae*, *Firmicutes*	0.16	0.02	0.00	0.05	0.00	0.60	1.39	0.80	24.60	5.10
Streptococcus sp. D4(3)	*Streptococcaceae*, *Firmicutes*	0.16	0.03	0.06	0.13	0.00	0.56	0.25	0.40	27.90	1.90
Streptococcus gallolyticus L25-MRS1	*Streptococcaceae*, *Firmicutes*	0.14	0.04	0.00	0.06	0.00	0.60	0.09	0.35	31.20	2.20
Bifidobacterium pseudocatenulatum LCR3	*Bifidobacteriaceae*, *Actinobacteria*	0.14	0.03	0.01	0.02	28.26	19.68	0.78	0.27	12.66	1.25
Pseudomonas aeruginosa L1-FAA6	*Pseudomonadaceae*, *Proteobacteria*	0.12	0.02	0.04	0.00	0.09	0.00	0.03	0.00	0.00	0.00
Lactobacillus rhamnosus L26-FAA6	*Lactobacillaceae*, *Firmicutes*	0.12	0.03	0.02	0.00	0.16	0.00	0.11	0.00	12.49	0.00
Bifidobacterium animalis subsp. *lactis* L26-MRS4	*Bifidobacteriaceae*, *Actinobacteria*	0.10	0.02	0.01	0.00	6.76	6.32	0.10	0.01	3.12	2.73
Bifidobacterium longum subsp. *longum* L19-MRS1	*Bifidobacteriaceae*, *Actinobacteria*	0.09	0.02	0.01	0.01	14.86	12.64	0.22	0.13	6.79	3.06
Unknown *Bifidobacterium* LCR4	*Bifidobacteriaceae*, *Actinobacteria*	0.07	0.02	0.01	0.00	8.51	6.84	0.06	0.00	4.10	3.75
Bifidobacterium breve LCR8	*Bifidobacteriaceae*, *Actinobacteria*	0.05	0.02	0.01	0.00	13.11	6.33	0.14	0.00	6.07	3.49
Bifidobacterium longum subsp. *longum* L25-MRS8	*Bifidobacteriaceae*, *Actinobacteria*	0.05	0.02	0.02	0.01	5.87	9.40	0.09	0.05	2.63	4.68
Bifidobacterium bifidum L25-MRS2	*Bifidobacteriaceae*, *Actinobacteria*	0.05	0.02	0.01	0.01	1.32	1.72	0.06	0.07	0.34	0.90
Bifidobacterium dentium LCR1	*Bifidobacteriaceae*, *Actinobacteria*	0.04	0.02	0.02	0.00	2.18	1.75	0.04	0.01	0.88	1.14
[*Clostridium*] *innocuum* D2(9)	*Erysipelotrichaceae*, *Firmicutes*	0.03	0.03	0.01	0.00	0.26	0.00	0.04	0.00	0.10	0.00
Bifidobacterium adolescentis DSM 20083^{T}	*Bifidobacteriaceae*, *Actinobacteria*	0.02	0.02	0.01	0.00	1.85	2.31	0.02	0.01	0.92	1.57
Bifidobacterium animalis subsp. *lactis* LCR26	*Bifidobacteriaceae*, *Actinobacteria*	0.02	0.02	0.01	0.00	1.26	1.75	0.01	0.06	0.39	0.79
Actinomyces odontolyticus L12-BSM1	*Actinomycetaceae*, *Actinobacteria*	0.02	0.02	0.01	0.00	0.82	0.00	0.00	0.00	0.02	0.00
Fusobacterium ulcerans L9-FAA5	*Fusobacteriaceae*, *Fusobacteria*	0.02	0.02	0.02	0.00	0.14	1.46	0.07	0.00	0.00	0.10

Bifidobacterium gallicum DSM 20093^{T}	*Bifidobacteriaceae*, *Actinobacteria*	0.01	0.01	0.01	0.00	0.31	0.33	0.02	0.00	0.06	0.43
Actinomyces viscosus L6-BSM10	*Actinomycetaceae*, *Actinobacteria*	0.00	0.00	0.00	0.00	0.04	0.22	0.03	0.00	0.00	0.06

747 *Species and isolates shown in bold produce large quantities of TMA from TMAO.

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750 **Supplementary Table 3.** FISH data for the batch culture samples, with data presented as $\log_{10}$(number of bacteria/mL sample)

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System, time (h)*	EUB338	Bac303	Ato291	Bif164	Ent	Bet42a	Prop853	Lab158	Fpra655	Erec482	Clit135	Rbro/Rfla	Chis150
A0	9.08	7.78	6.96	7.17	5.65	8.22	7.68	6.70	7.36	8.52	6.21	7.44	7.05
A4	8.89	7.70	7.40	7.64	7.36	7.32	7.44	7.04	7.41	8.20	5.65	7.46	7.34
A9	9.13	7.90	7.48	8.04	8.10	6.10	7.62	6.92	7.24	7.98	5.25	7.39	7.30
B0	8.97	7.81	6.95	7.26	5.65	8.22	7.69	6.93	7.45	8.54	5.99	7.49	6.84
B4	8.99	7.80	7.43	7.57	7.01	7.37	7.62	7.20	7.43	8.26	5.65	7.42	7.08
B9	9.25	7.92	7.63	8.17	7.40	6.18	7.63	6.98	7.42	8.11	4.95	7.40	7.27
C0	9.03	7.65	7.14	7.19	5.80	8.29	7.74	6.67	7.25	7.70	5.43	7.30	6.07
C4	8.76	7.56	7.64	7.81	6.64	6.35	7.74	6.81	7.31	7.73	4.95	7.34	7.22
C9	9.05	7.57	7.54	8.33	8.17	5.99	7.76	6.21	6.89	7.75	5.25	7.36	7.41
D0	8.97	7.63	7.30	7.31	5.43	8.27	7.69	6.76	7.29	7.67	5.56	7.44	5.95
D4	8.89	7.66	7.80	8.12	5.95	6.30	7.71	6.47	7.29	7.77	5.25	7.53	6.50
D9	9.12	7.73	7.65	8.26	7.33	6.18	7.77	5.99	6.98	7.76	5.25	7.31	7.49
E0	9.26	8.23	7.35	7.55	6.82	7.88	7.25	7.29	7.29	7.77	5.80	7.43	6.68
E4	9.15	7.65	8.04	7.66	7.28	7.51	7.63	6.69	8.05	7.78	6.23	7.47	7.29
E9	9.07	7.63	7.80	8.26	8.07	7.49	8.03	7.00	8.06	7.97	6.10	7.47	7.38
F0	9.22	8.20	7.45	7.54	6.94	7.94	7.37	7.28	7.39	7.78	5.56	7.34	6.58
F4	9.23	7.98	8.21	8.16	6.76	7.39	7.73	6.60	7.45	7.92	6.28	7.48	7.14
F9	9.24	7.34	8.30	8.32	7.28	7.40	8.04	7.16	7.42	8.21	5.95	7.51	7.39

752 *A prefix, donor 1 vessel containing TMAO; B prefix, donor 1 vessel without TMAO (negative control); C prefix, donor 2 vessel containing TMAO; D prefix, donor 2
753 vessel without TMAO (negative control); E prefix, donor 3 vessel containing TMAO; F prefix, donor 3 vessel without TMAO (negative control).

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Supplementary Table 4. NMR data for the batch culture samples, with concentrations of metabolites given in mM

System, time (h)	TMA	TMAO	DMA	Ethanol	Acetate	Lactate
A0	0.03	89.45	0.05	0.44	1.63	1.44
A4	0.77	92.3	0.45	0.59	6.68	3.69
A9	5.46	71.4	0.86	1.76	26.51	13.61
B0	0.03	0.58	0.04	0.31	1.51	1.42
B4	0.03	0.56	0.03	0.92	9.79	4.53
B9	0.06	0.21	0.05	2.14	33.5	12
C0	0.02	80	0.04	0.4	0.89	1.44
C4	0.16	81.1	0.19	0.55	4.61	2.96
C9	6.33	76.83	0.4	2.07	33.7	15.51
D0	0.03	0.62	0.05	0.31	0.97	1.5
D4	0.03	0.55	0.03	0.69	6.41	3.74
D9	0.06	0.2	0.04	2.01	32.4	13.94
E0	0.03	79.6	0.05	0.41	1.76	1.4
E4	1.61	80	0.61	1.52	17.5	7.38
E9	10.81	70.8	1.4	2.5	38.5	10.7
F0	0.03	0.58	0.05	0.27	1.77	1.37
F4	0.05	0.23	0.05	2.78	29.5	9.75
F9	0.08	0.05	0.16	2.76	38.3	3.95

*A prefix, donor 1 vessel containing TMAO; B prefix, donor 1 vessel without TMAO (negative control); C prefix, donor 2 vessel containing TMAO; D prefix, donor 2 vessel without TMAO (negative control); E prefix, donor 3 vessel containing TMAO; F prefix, donor 3 vessel without TMAO (negative control).

761 **Supplementary Table 5.** Spearman correlation (corrected for ties) and adjusted *P* values (Benjamini–Hochberg) for FISH and metabolite data for batch culture samples

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Probe	Spearman correlation values*					
	Acetate	**DMA**	**Ethanol**	**Lactate**	**TMA**	**TMAO**
Eub338	0.37	0.14	0.38	0.15	0.06	-0.38
Bac303	-0.18	-0.30	-0.17	-0.21	-0.36	-0.16
Ato291	0.78	0.33	0.79	0.60	0.57	-0.46
Bif164	0.93	0.37	0.87	0.81	0.72	-0.48
Ent	0.82	0.59	0.65	0.75	0.81	-0.09
Bet42a	-0.69	-0.22	-0.62	-0.78	-0.62	0.24
Prop853	0.40	0.01	0.47	0.40	0.24	-0.32
Lab158	-0.05	0.14	-0.21	-0.43	-0.08	0.02
Fpra655	0.18	0.17	0.19	-0.10	0.12	-0.12
Erec482	0.20	0.03	0.20	-0.01	0.06	-0.13
Clit135	-0.03	0.13	0.07	-0.24	-0.06	0.10
Rbro730/Rfla729	0.15	0.04	0.24	-0.07	0.05	-0.22
Chis150	0.85	0.56	0.74	0.76	0.85	-0.13
Probe	**Adjusted *P* values**					
	Acetate	**DMA**	**Ethanol**	**Lactate**	**TMA**	**TMAO**
Eub338	2.51×10^{-1}	7.05×10^{-1}	2.51×10^{-1}	7.05×10^{-1}	8.05×10^{-1}	2.51×10^{-1}
Bac303	5.23×10^{-1}	5.23×10^{-1}	5.23×10^{-1}	5.23×10^{-1}	5.23×10^{-1}	5.23×10^{-1}
Ato291	3.88×10^{-4}	1.84×10^{-1}	3.88×10^{-4}	1.67×10^{-2}	1.99×10^{-2}	6.89×10^{-2}
Bif164	1.13×10^{-7}	1.33×10^{-1}	8.41×10^{-6}	8.01×10^{-5}	1.04×10^{-3}	5.55×10^{-2}
Ent	1.63×10^{-4}	1.26×10^{-2}	5.32×10^{-3}	6.66×10^{-4}	1.63×10^{-4}	7.15×10^{-1}
Bet42a	5.06×10^{-3}	3.71×10^{-1}	9.87×10^{-3}	7.82×10^{-4}	9.87×10^{-3}	3.71×10^{-1}
Prop853	2.09×10^{-1}	9.64×10^{-1}	2.09×10^{-1}	2.09×10^{-1}	4.15×10^{-1}	3.02×10^{-1}
Lab158	9.35×10^{-1}	9.35×10^{-1}	9.35×10^{-1}	4.38×10^{-1}	9.35×10^{-1}	9.35×10^{-1}
Fpra655	6.83×10^{-1}	6.83×10^{-1}	6.83×10^{-1}	6.83×10^{-1}	6.83×10^{-1}	6.83×10^{-1}
Erec482	9.71×10^{-1}	9.71×10^{-1}	9.71×10^{-1}	9.71×10^{-1}	9.71×10^{-1}	9.71×10^{-1}
Clit135	9.09×10^{-1}	9.09×10^{-1}	9.09×10^{-1}	9.09×10^{-1}	9.09×10^{-1}	9.09×10^{-1}
Rbro730/Rfla729	8.65×10^{-1}	8.65×10^{-1}	8.65×10^{-1}	8.65×10^{-1}	8.65×10^{-1}	8.65×10^{-1}
Chis150	2.80×10^{-5}	2.00×10^{-2}	6.10×10^{-4}	5.37×10^{-4}	2.80×10^{-5}	6.21×10^{-1}

763 *Green cells show statistically significant results.