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

2

3   Lesley Hoyles[1]*, Maria L. Jiménez-Pranteda[2]*, Julien Chilloux[1]*, Francois Brial[3], Antonis

4   Myridakis[1], Thomas Aranias[3], Christophe Magnan[4], Glenn R. Gibson[2], Jeremy D. Sanderson[5], Jeremy

5   K. Nicholson[1], Dominique Gauguier[1,3], Anne L. McCartney[2]† and Marc-Emmanuel Dumas[1]†

6

7   [1]Integrative Systems Medicine and Digestive Disease, Department of Surgery and Cancer, Faculty of

8   Medicine, Imperial College London, Exhibition Road, London SW7 2AZ, UK

9   [2]Food Microbial Sciences Unit, Department of Food and Nutritional Sciences, School of Chemistry,

10   Food and Pharmacy, Faculty of Life Sciences, The University of Reading, Whiteknights Campus,

11   Reading RG6 6UR, UK

12   [3]Sorbonne Universities, University Pierre & Marie Curie, University Paris Descartes, Sorbonne Paris

13   Cité, INSERM UMR_S 1138, Cordeliers Research Centre, Paris, France

14   [4]Sorbonne Paris Cité, Université Denis Diderot, Unité de Biologie Fonctionnelle et Adaptative, CNRS

15   UMR 8251, 75205 Paris, France

16   [5]Department of Gastroenterology, Guy's and St Thomas' NHS Foundation Trust and King's College

17   London, London, UK

18

19   *These authors made equal contributions to this work; shared first authorship.

20   †**Corresponding authors:** Anne L. McCartney, a.l.mccartney@reading.ac.uk; Marc-Emmanuel

21   Dumas, m.dumas@imperial.ac.uk

22

23   **Running title:** Reduction of TMAO by the human gut microbiota

24   **Abbreviations:** AUC, area under the curve; DMA, dimethylamine; FISH, fluorescence *in situ*

25   hybridization; FMO, flavin mono-oxygenase; MMA, monomethylamine; PC, phosphatidylcholine;

26   TMA, trimethylamine; TMAO, trimethylamine *N*-oxide; UPLC–MS/MS, ultra-performance liquid

27   chromatography–tandem mass spectrometry.

28   **Keywords:** co-metabolic axis; gut–liver axis; metabolomics; *Enterobacteriaceae*; lactic acid bacteria.

29   **ABSTRACT**

30   The dietary methylamines choline, carnitine and phosphatidylcholine are used by the gut microbiota

31   to produce a range of metabolites, including trimethylamine (TMA). However, little is known about

32   the use of trimethylamine *N*-oxide (TMAO) by this consortium of microbes. A feeding study using

33   deuterated TMAO in C57BL6/J mice demonstrated microbial conversion of TMAO to TMA, with

34   uptake of TMA into the bloodstream and its conversion to TMAO. Antibiotic-treated mice lacked

35   microbial activity necessary to convert TMAO to TMA, with deuterated TMAO being taken up

36   directly into the bloodstream. In batch-culture fermentation systems inoculated with human faeces,

37   growth of *Enterobacteriaceae* was stimulated in the presence of TMAO. Human-derived faecal and

38   caecal bacteria (n = 66 isolates) were screened on solid and liquid media for their ability to use

39   TMAO, with metabolites in spent media analysed by ^{1}H-NMR. As with the *in vitro* fermentation

40   experiments, TMAO stimulated the growth of *Enterobacteriaceae*; these bacteria produced most

41   TMA from TMAO. Caecal/small intestinal isolates of *Escherichia coli* produced more TMA from

42   TMAO than their faecal counterparts. Lactic acid bacteria produced increased amounts of lactate

43   when grown in the presence of TMAO, but did not produce large amounts of TMA. In summary,

44   TMA can be produced by the gut microbiota (predominantly *Enterobacteriaceae*) from TMAO. This

45   TMA is then taken up by the host and converted back to TMAO. That is, metabolic retroconversion

46   occurs. In addition, TMAO influences microbial metabolism depending on isolation source and taxon

47   of gut bacterium.

48

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

3

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

105   TMA from choline (*cutC*), glycine-betaine (*grdH*), L-carnitine (*cntA*) and γ-butyrobetaine (*cntA*)

106   (Borrel *et al.*, 2017). A search of the NCBI nucleotide database with the phrase 'TMAO reductase'

107   also suggests many other bacteria of human intestinal origin (namely, *Salmonella*, *Helicobacter*,

108   *Prevotella*, *Bacillus* and *Bacteroides* spp.) should be able to reduce TMAO to TMA. However, this

109   trait has not been examined *in vitro* for isolates of intestinal origin.

110   Consequently, we carried out an *in vivo* study in mice to confirm use of TMAO by the mouse

111   gut microbiota and to allow us to examine metabolic retroconversion of TMAO in a murine model.

112   We then used an *in vitro* fermentation system to highlight the effect of TMAO on the human faecal

113   microbiota. Finally, we screened a panel of human faecal and caecal/small intestinal isolates to

114   determine which members of the human gut microbiota were able to reduce TMAO to TMA, and

115   whether their metabolism was affected by being grown in the presence of TMAO.

116

117

118   **METHODS**

119   **Animal work**

120   Groups of six-week-old C57BL6/J mice (Janvier Labs, Courtaboeuf, France) were received

121   and acclimated in specific pathogen-free (SPF) maintenance conditions. They were fed with a

122   standard chow diet (R04-40, Safe, Augy, France) and were either given free access to tap water or

123   treated with the antibiotic cocktail (0.5 g/L vancomycin hydrochloride, 1 g/L neomycin trisulfate, 1

124   g/L metronidazole, 1 g/L ampicillin sodium; all antibiotics were purchased from Sigma-Aldrich) for

125   14 days. Mice were given in the morning by gavage either a solution of d_9-TMAO at 1×10^{-4} M

126   (Cambridge Isotope Laboratories Inc., DLM-4779-0, UK) or saline and euthanized 6 h later by

127   cervical dislocation. Blood samples were collected by tail tipping every 2 h in Microvette® CB 300

128   Lithium Heparin (Sarstedt, Marnay, France). Plasma was separated by centrifugation (10 min, 5000 ***g***,

129   4 °C) and stored at -80 °C until analysed by ultra-performance liquid chromatography–tandem mass

130   spectrometry (UPLC–MS/MS).

5

131   All procedures were authorized following review by the institutional ethics committee

132   (Sorbonne Universities) and carried out under national license conditions.

133

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

135   UPLC-MS/MS was employed for the determination of TMA, d_9-TMA, TMAO and d_9-

136   TMAO. Samples (10 μL) were spiked with 10 μL Internal Standard solution (d_9-choline and $^{13}C_3/^{15}N$-

137   TMA in water; 1 mg/L, Sigma-Aldrich). Ethyl 2-bromoacetate solution (45 μL) (15 g/L ethyl 2-

138   bromoacetate, 1 % NH_4OH in acetonitrile; ChromaSolv grade, Sigma-Aldrich) was added and

139   derivatization of TMAs (TMA, d_9-TMA and $^{13}C_3/^{15}N$-TMA) to their ethoxy-analogues was completed

140   after 30 min at room temperature. Protein/lipid precipitation solution (935 μL) (94 % acetonitrile/5

141   %water/1 % formic acid; ChromaSolv grade, Sigma-Aldrich) was added, samples were centrifuged

142   (20 min, 20,000 *g*, 4 °C) and were transferred to UPLC-autosampler vials. Sample injections (5 μL

143   loop) were performed with a Waters Acquity UPLC-Xevo TQ-S UPLC-MS/MS system equipped

144   with an Acquity BEH HILIC (2.1×100 mm, 1.7 μm) chromatographic column. An isocratic elution

145   was applied with 10 mM ammonium formate (Sigma-Aldrich) in 95:5 (v/v) acetronitrile:water for 7

146   min at 750 μL/min and 50 °C. Positive electrospray (ESI+) was used as ionization source and mass

147   spectrometer parameters were set as follows: capillary, cone and sources voltages at -700, -18 and

148   50 V, respectively, desolvation temperature at 600 °C, desolvation/cone/nebuliser gases were high

149   purity nitrogen (BOC) at 1000 L/h, 150 L/h and 7 bar, respectively. Collision gas was high-purity

150   argon (BOC). Mass spectrometer was operated in multiple reaction monitoring mode. The monitored

151   transitions were the following: for TMA, +146 → +118/59 *m/z* (23/27 V); for d_9-TMA, +155 →

152   +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 →

153   +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 →

154   +60/45 *m/z* (20/22 V). The system was controlled by MassLynx software, also used for data

155   acquisition and analysis.

156

157   **^{1}H-NMR spectroscopy and data analysis**

6

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**

7

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     $DF \times ACC \times 6{,}732.42 \times 50 \times DF_{sample}$

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

11

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

12

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

13

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

14

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

16

437 presence may contribute to cross-feeding associated with short-chain fatty acid production (Hoyles

438 and Wallace, 2010). It is difficult to determine relevance of correlations from mixed microbial

439 ecosystems in the absence of isotope labelling or pure culture work: i.e. correlation does not equate

440 with causation. As an example, three (*Clostridium* clusters I and II, bifidobacteria, coriobacteriia) of

441 the four groups of bacteria correlated with TMA production in our fermentation study did not produce

442 notable quantities of TMA from TMAO based on our pure culture work. Therefore, correlating

443 microbiota and metabolite data derived from complex systems will not give a true picture of which

444 members of microbiota contribute to specific metabolic processes, and work with pure cultures is

445 required to supplement functional studies to increase our understanding of poorly understood

446 microbially driven metabolic processes within the human gut.

447   In summary, we have demonstrated metabolic retroconversion – another example of host–

448 microbial co-metabolism – occurs in the mammalian system with respect to TMAO, whereby TMAO

449 is reduced by the gut microbiota to TMA and regenerated by host hepatic enzymes. We have also

450 demonstrated that growth and metabolism of members of the gut microbiota are affected by TMAO in

451 a source- and taxon-dependent manner, with the family *Enterobacteriaceae* making the greatest

452 contribution to production of TMA in the gut.

453

454

455 **ACKNOWLEDGEMENTS**

463

464

REFERENCES

al-Waiz, M., Ayesh, R., Mitchell, S.C., Idle, J.R. and Smith, R.L. (1987) Disclosure of the metabolic retroversion of trimethylamine *N*-oxide in humans: a pharmacogenetic approach. *Clin Pharmacol Ther* 42, 608–612.

Ando, H., Abe, H., Sugimoto, N. and Tobe, T. (2007) Maturation of functional type III secretion machinery by activation of anaerobic respiration in enterohaemorrhagic *Escherichia coli*. *Microbiology* 153, 464–473.

Barrett, E.L. and Kwan, H.S. (1985) Bacterial reduction of trimethylamine oxide. *Ann Rev Microbiol* 39, 131–149.

Benjamini, Y. and Hochberg, Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser B* 57, 289–300.

Bennett, B.J., de Aguiar Vallim, T.Q., Wang, Z., Shih, D.M., Meng, Y., Gregory, J., Allayee, H., Lee, R., Graham, M., Crooke, R., Edwards, P.A., Hazen, S.L. and Lusis, A.J. (2013) Trimethylamine-*N*-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. *Cell Metab* 17, 49–60.

Blaise, B.J., Shintu, L., Elena, B., Emsley, L., Dumas, M.-E. and Toulhoat, P. (2009) Statistical recoupling prior to significance testing in nuclear magnetic resonance based metabonomics. *Anal Chem* 81, 6242–6251.

Borrel, G., McCann, A., Deane, J., Neto, M.C., Lynch, D.B., Brugère, J.F. and O'Toole, P.W. (2017) Genomics and metagenomics of trimethylamine-utilizing *Archaea* in the human gut microbiome. *ISME J* 11, 2059–2074.

Bradbeer, C. (1965) The clostridial fermentations of choline and ethanolamine. 1. Preparation and properties of cell-free extracts. *J Biol Chem* 240, 4669–4674.

Cani, P.D., Amar, J., Iglesias, M.A., Poggi, M., Knauf, C., Bastelica, D., Neyrinck, A.M., Fava, F., Tuohy, K.M., Chabo, C., Waget, A., Delmée, E., Cousin, B., Sulpice, T., Chamontin, B., Ferrières, J., Tanti, J.F., Gibson, G.R., Casteilla, L., Delzenne, N.M., Alessi, M.C. and Burcelin, R. (2007) Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 56, 1761–1772.

Cani, P.D., Bibiloni, R., Knauf, C., Waget, A., Neyrinck, A.M., Delzenne, N.M. and Burcelin, R.

493   (2008) Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat

494   diet-induced obesity and diabetes in mice. *Diabetes* 57, 1470–1481.

495   Daims, H., Brühl, A., Amann, R., Schleifer, K.-H. and Wagner, M. (1999) The domain-specific probe

496   EUB338 is insufficient for the detection of all *Bacteria*: development and evaluation of a more

497   comprehensive probe set. *Syst Appl Microbiol* 22, 434–444.

498   de la Huerga, J. and Popper, H. (1951) Urinary excretion of choline metabolites following choline

499   administration in normals and patients with hepatobiliary diseases. *J Clin Invest* 30, 463–470.

500   de la Huerga, J., Gyorgy, P., Waldstein, S., Katz, R. and Popper, H. (1953) The effects of

501   antimicrobial agents upon choline degradation in the intestinal tract. *J Clin Invest* 32, 1117–1120.

502   de La Serre, C.B., Ellis, C.L., Lee, J., Hartman, A.L., Rutledge, J.C. and Raybould, H.E. (2010)

503   Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota

504   and gut inflammation. *Am J Physiol Gastrointest Liver Physiol* 299, G440–G448.

505   Dona, A.C., Jiménez, B., Schäfer, H., Humpfer, E., Spraul, M., Lewis, M.R., Pearce, J.T., Holmes, E.,

506   Lindon, J.C. and Nicholson, J.K. (2014) Precision high-throughput proton NMR spectroscopy of

507   human urine, serum, and plasma for large-scale metabolic phenotyping. *Anal Chem* 86, 9887–9894.

508   Dona, A.C., Kyriakides, M., Scott, F., Shephard, E.A., Varshavi, D., Veselkov, K. and Everett, J.R.

509   (2016) A guide to the identification of metabolites in NMR-based metabonomics/metabolomics

510   experiments. *Comput Struct Biotechnol J* 14, 135–153.

511   Dumas, M.-E., Barton, R.H., Toye, A., Cloarec, O., Blancher, C., Rothwell, A., Fearnside, J., Tatoud,

512   R., Blanc, V., Lindon, J.C., Mitchell, S.C., Holmes, E., McCarthy, M.I., Scott, J., Gauguier, D. and

513   Nicholson, J.K. (2006) Metabolic profiling reveals a contribution of gut microbiota to fatty liver

514   phenotype in insulin-resistant mice. *Proc Natl Acad Sci U S A* 103, 12511–12516.

515   Dumas, M.-E., Rothwell, A.R., Hoyles, L., Aranias, T., Chilloux, J., Calderari, S., Noll, E.M., Péan,

516   N., Boulangé, C.L., Blancher, C., Barton, R.H., Gu, Q., Fearnside, J.F., Deshayes, C., Hue, C.,

517   Scott, J., Nicholson, J.K. and Gauguier, D. (2017) Microbial-host co-metabolites are prodromal

518   markers predicting phenotypic heterogeneity in behavior, obesity, and impaired glucose tolerance.

519   *Cell Rep* 20, 136–148.

520   Franks, A.H., Harmsen, H.J.M., Raangs, G.C., Jansen, G.J., Schut, F. and Welling, G.W. (1998)

521   Variations of bacterial populations in human feces measured by fluorescent in situ hybridization

522   with group specific 16S rRNA-targeted oligonucleotide probes. *Appl Environ Microbiol* 64, 3336–

523   3345.

524   Gao, X., Liu, X., Xu, J., Xue, C., Xue, Y. and Wang, Y. (2014) Dietary trimethylamine *N*-oxide

525   exacerbates impaired glucose tolerance in mice fed a high fat diet. *J Biosci Bioeng* 118, 476–481.

526   Harmsen, H.J.M., Elfferich, P., Schut, F. and Welling, G.W. (1999) A 16S rRNA-targeted probe for

527   detection of lactobacilli and enterococci in faecal samples by fluorescent *in situ* hybridization.

528   *Microb Ecol Health Dis* 11, 3–12.

529   Harmsen, H.J.M., Raangs, G.C., He, T., Degener, J.E. and Welling, G.J. (2002) Extensive set of 16S

530   rRNA-based probes for detection of bacteria in human feces. *Appl Environ Microbiol* 68, 2982–

531   2990.

532   Harmsen, H.J.M., Wildeboer-Veloo, A.C.M., Grijpstra, J., Knol, J., Degener, J.E. and Welling, G.W.

533   (2000) Development of 16S rRNA-based probes for the *Coriobacterium* group and the *Atopobium*

534   cluster and their application for enumeration of *Coriobacteriaceae* in human feces from volunteers

535   of different ages. *Appl Environ Microbiol* 66, 4523–4527.

536   Hildebrandt, M.A., Hoffmann, C., Sherrill-Mix, S.A., Keilbaugh, S.A., Hamady, M., Chen, Y.Y.,

537   Knight, R., Ahima, R.S., Bushman, F. and Wu, G.D. (2009) High-fat diet determines the

538   composition of the murine gut microbiome independently of obesity. *Gastroenterology* 137, 1716–

539   1724.

540   Hold, G.L., Schwiertz, A., Aminov, R.I., Blaut, M. and Flint, H.J. (2003) Oligonucleotide probes that

541   detect qualitatively significant groups of butyrate-producing bacteria in human feces. *Appl Environ*

542   *Microbiol* 69, 4320–4324.

543   Hoyles, L. and McCartney, A.L. (2009) What do we mean when we refer to *Bacteroidetes*

544   populations in the human gastrointestinal microbiota? *FEMS Microbiol Lett* 299, 175–183.

545   Hoyles, L. and Wallace, R.J. (2010) Gastrointestinal tract: intestinal fatty acid metabolism and

546   implications for health. In *Handbook of Hydrocarbon and Lipid Microbiology*, vol. 4, part 7,

547   *Human-Animal-Plant Health and Physiology Consequences of Microbial Interactions with*

548   *Hydrocarbons and Lipids*, pp. 3120–3132. Edited by T. McGenity, K. N. Timmis, J. R. van der

549   Meer & V. de Lorenzo. New York: Springer.

550   Hoyles, L., Murphy, J., Neve, H., Heller, K. J., Turton, J. F., Mahony, J., Sanderson, J. D., Hudspith,

551   B, Gibson, G. R., McCartney, A. L. and van Sinderen, D. (2015) *Klebsiella pneumoniae* subsp.

552   *pneumoniae*–bacteriophage combination from the caecal effluent of a healthy woman. *PeerJ*

553   doi:10.7717/peerj.1061.

554   Jameson, E., Doxey, A.C., Airs, R., Purdy, K.J., Murrell, J.C. and Chen, Y. (2016) Metagenomic

555   data-mining reveals contrasting microbial populations responsible for trimethylamine formation in

556   human gut and marine ecosystems. *Microb Genom* 2, e000080.

557   Jebsen, J.W. and Riaz, M. (1977) Breakdown products of trimethylamine oxide in airdried stockfish.

558   Means of enhancing the formation of formaldehyde and dimethylamine. *Fish Dir Skr Ser Ernoering*

559   1, 145–153.

560   Kempf, V.A., Trebesius, K. and Autenrieth, I.B. (2000) Fluorescent in situ hybridization allows rapid

561   identification of microorganisms in blood cultures. *J Clin Microbiol* 38, 830–838.

562   Kloiber, O., Banjac, B. and Drewes, L.R. (1988) Protection against acute hyperammonemia: the role

563   of quaternary amines. *Toxicology* 49, 83–90.

564   Koeth, R.A., Wang, Z., Levison, B.S., Buffa, J.A., Org, E., Sheehy, B.T., Britt, E.B., Fu, X., Wu, Y.,

565   Li, L., Smith, J.D., Didonato, J.A., Chen, J., Li, H., Wu, G.D., Lewis, J.D., Warrier, M., Brown,

566   J.M., Krauss, R.M., Tang, W.H., Bushman, F.D., Lusis, A.J. and Hazen, S.L. (2013) Intestinal

567   microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med* 19,

568   576–585.

569   Langendijk, P.S., Schut, F., Jansen, G.J., Raangs, G.W., Kamphuis, G.R., Wilkinson, M.H.F. and

570   Welling, G.W. (1995) Quantitative fluorescent in situ hybridisation of *Bifidobacterium* spp. with

571   genus specific 16S rRNA targeted probes and its application in fecal samples. *Appl Environ*

572   *Microbiol* 61, 3069–3075.

573   Le Chatelier, E., Nielsen, T., Qin, J., Prifti, E., Hildebrand, F., Falony, G., Almeida, M., Arumugam,

574   M., Batto, J.M., Kennedy, S., Leonard, P., Li, J., Burgdorf, K., Grarup, N., Jørgensen, T.,

575   Brandslund, I., Nielsen, H.B., Juncker, A.S., Bertalan, M., Levenez, F., Pons, N., Rasmussen, S.,

576   Sunagawa, S., Tap, J., Tims, S., Zoetendal, E.G., Brunak, S., Clément, K., Doré, J., Kleerebezem,

577   M., Kristiansen, K., Renault, P., Sicheritz-Ponten, T., de Vos, W.M., Zucker, J.D., Raes, J., Hansen,

578   T.; MetaHIT consortium, Bork, P., Wang, J., Ehrlich, S.D. and Pedersen, O. (2013) Richness of

579   human gut microbiome correlates with metabolic markers. *Nature* 500, 541–546.

580   Lee, K.M., Park, Y., Bari, W., Yoon, M.Y., Go, J., Kim, S.C., Lee, H.I. and Yoon, S.S. (2012)

581   Activation of cholera toxin production by anaerobic respiration of trimethylamine *N*-oxide in *Vibrio*

582   *cholerae*. *J Biol Chem* 287, 39742–39752.

583   Lim, S.M., Jeong, J.J., Woo, K.H., Han, M.J. and Kim, D.H. (2016) *Lactobacillus sakei* OK67

584   ameliorates high-fat diet-induced blood glucose intolerance and obesity in mice by inhibiting gut

585   microbiota lipopolysaccharide production and inducing colon tight junction protein expression. *Nutr*

586   *Res* 36, 337–348.

587   Loy, A., Maixner, F., Wagner, M. and Horn, M. (2007) probeBase – an online resource for rRNA-

588   targeted oligonucleotide probes: new features 2007. *Nucleic Acids Res* 35, D800–D804.

589   Manz, W., Amann, R., Ludwig, W., Wagner, M. and Schleifer, K.-H. (1992) Phylogenetic

590   oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions.

591   *Syst Appl Microbiol* 15, 593–600.

592   Manz, W., Amann, R., Ludwig, W., Vancanneyt, M. and Schleifer, K.H. (1996) Application of a suite

593   of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum

594   cytophaga-flavobacter-bacteroides in the natural environment. *Microbiology* 142, 1097–1106.

595   Martín-Peláez, S., Gibson, G.R., Martín-Orúe, S.M., Klinder, A., Rastall, R.A., La Ragione, R.M.,

596   Woodward, M.J. and Costabile, A. (2008) *In vitro* fermentation of carbohydrates by porcine faecal

597   inocula and their influence on *Salmonella* Typhimurium growth in batch culture systems. *FEMS*

598   *Microbiol Ecol* 66, 608–619.

599   Miñana, M.D., Hermenegildo, C., Llsansola, M., Montoliu, C., Grisolía, S. and Felipo, V. (1996)

600   Carnitine and choline derivatives containing a trimethylamine group prevent ammonia toxicity in

601   mice and glutamate toxicity in primary cultures of neurons. *J Pharmacol Exp Ther* 279, 194–199.

602   Paiva, J.B., Penha Filho, R.A, Pereira, E.A, Lemos, M.V, Barrow, P.A., Lovell, M.A. and Berchieri,

603   A. Jr (2009) The contribution of genes required for anaerobic respiration to the virulence of

604   *Salmonella enterica* serovar Gallinarum for chickens. *Braz J Microbiol* 40, 994–1001.

605   Phetcharaburanin, J., Lees, H., Marchesi, J.R., Nicholson, J.K., Holmes, E., Seyfried, F. and Li, J.V.

606   (2016) Systemic characterization of an obese phenotype in the Zucker rat model defining metabolic

607   axes of energy metabolism and host–microbial interactions. *J Proteome Res* 15, 1897–1906.

608   Qin, N., Yang, F., Li, A., Prifti, E., Chen, Y., Shao, L., Guo, J., Le Chatelier, E., Yao, J., Wu, L.,

609   Zhou, J., Ni, S., Liu, L., Pons, N., Batto, J.M., Kennedy, S.P., Leonard, P., Yuan, C., Ding, W.,

610   Chen, Y., Hu, X., Zheng, B., Qian, G., Xu, W., Ehrlich, S.D., Zheng, S. and Li, L. (2014)

611   Alterations of the human gut microbiome in liver cirrhosis. *Nature* 513, 59–64.

612   Rebouche, C.J. and Chenard, C.A. (1991) Metabolic fate of dietary carnitine in human adults:

613   identification and quantification of urinary and fecal metabolites. *J Nutr* 121, 539–546.

614   Rebouche, C.J., Mack, D.L. and Edmonson, P.F. (1984) L-Carnitine dissimilation in the

615   gastrointestinal tract of the rat. *Biochemistry* 23, 6422–6426.

616   Roger, L.C., Costabile, A., Holland, D.T., Hoyles, L. and McCartney, A.L. (2010) Examination of

617   faecal *Bifidobacterium* populations in breast- and formula-fed infants during the first 18 months of

618   life. *Microbiology* 156, 3329–3341.

619   Seim, H., Schulze, J. and Strack, E. (1985) Catabolic pathways for high-dosed L(-)- or D(+)-carnitine

620   in germ-free rats? *Biol Chem Hoppe Seyler* 366, 1017–1021.

621   Spencer, M.D., Hamp, T.J., Reid, R.W., Fischer, L.M., Zeisel, S.H. and Fodor, A.A. (2011)

622   Association between composition of the human gastrointestinal microbiome and development of

623   fatty liver with choline deficiency. *Gastroenterology* 140, 976–986.

624   Stremmel, W., Schmidt, K.V., Schuhmann, V., Kratzer, F., Garbade, S.F., Langhans, C.D., Fricker, G.

625   and Okun J.G. (2017) Blood trimethylamine-*N*-oxide originates from microbiota mediated

626   breakdown of phosphatidylcholine and absorption from small intestine. *PLoS One* **12**, e0170742.

627   Taesuwan, S., Cho, C.E., Malysheva, O.V., Bender, E., King, J.H., Yan, J., Thalacker-Mercer, A.E.

628   and Caudill, M.A. (2017) The metabolic fate of isotopically labeled trimethylamine-*N*-oxide

629   (TMAO) in humans. *J Nutr Biochem* 45, 77–82.

630   Takagi, M. and Ishimoto, M. (1983) *Escherichia coli* mutants defective in trimethylamine *N*-oxide

631   reductase. *FEMS Microbiol Lett* 17, 247–250.

632   Tang, W.H., Wang, Z., Levison, B.S., Koeth, R.A., Britt, E.B., Fu, X., Wu, Y. and Hazen, S.L. (2013)

633   Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *N Engl J Med* 368,

634   1575–1584.

635   Thorasin, T., Hoyles, L. and McCartney, A.L. (2015) Dynamics and diversity of the '*Atopobium*

636   cluster' in the human faecal microbiota, and phenotypic characterization of '*Atopobium* cluster'

637   isolates. *Microbiology* 161, 565–579.

638   Trojanová, I., Vlková, E., Rada, V. and Marounek, M. (2006) Different utilization of glucose and

639   raffinose in *Bifidobacterium breve* and *Bifidobacterium animalis*. *Folia Microbiol (Praha)* 51, 320–

640   324.

641   van den Bogert, B., Boekhorst, J., Herrmann, R., Smid, E.J., Zoetendal, E.G. and Kleerebezem, M.

642   (2013) Comparative genomics analysis of *Streptococcus* isolates from the human small intestine

643   reveals their adaptation to a highly dynamic ecosystem. *PLoS One* 8, e83418.

644   van den Bogert, B., Meijerink, M., Zoetendal, E.G., Wells, J.M. and Kleerebezem, M. (2014)

645   Immunomodulatory properties of *Streptococcus* and *Veillonella* isolates from the human small

646   intestine microbiota. *PLoS One* 9, e114277.

647   Walker, A.W., Duncan, S.H., McWilliam Leitch, E.C., Child, M.W. and Flint, H.J. (2005) pH and

648   peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within

649   microbial communities from the human colon. *Appl Environ Microbiol* 71, 3692–3700.

650   Wang, Z., Klipfell, E., Bennett, B.J., Koeth, R., Levison, B.S., Dugar, B., Feldstein, A.E., Britt, E.B.,

651   Fu, X., Chung, Y.M., Wu, Y., Schauer, P., Smith, J.D., Allayee, H., Tang, W.H., DiDonato, J.A.,

652   Lusis, A.J. and Hazen, S.L. (2011) Gut flora metabolism of phosphatidylcholine promotes

653   cardiovascular disease. *Nature* 472, 57–63.

654   Wu, D., Cao, M., Peng, J., Li, N., Yi, S., Song, L., Wang, X., Zhang, M. and Zhao, J. (2017) The

655   effect of trimethylamine *N*-oxide on *Helicobacter pylori*-induced changes of immunoinflammatory

656   genes expression in gastric epithelial cells. *Int Immunopharmacol* 43, 172–178.

657 Wünsche, R. (1940) Uber die wirkung von darmbakterien auf trimethylammoniumbasen. *Ber Ges*

658   *Physiol Exp Pharm* 118, 303.

659 Yonejima, Y., Ushida, K. and Mori, Y. (2013) Effect of lactic acid bacteria on lipid metabolism and

660   fat synthesis in mice fed a high-fat diet. *Biosci Microbiota Food Health* 32, 51–58.

661 Zeisel, S.H. and Da Costa, K.A. (1986) Increase in human exposure to methylamine precursors of *N-*

662   nitrosamine after eating fish. *Cancer Res* 46, 6136–6138.

663 Zeisel, S.H., Wishnok, J.S. and Blusztajn, J.K. (1983) Formation of methylamines from ingested

664   choline and lecithin. *J Pharmacol Exp Ther* 225, 320–324.

665 Zhang, J., Cerny, M.A., Lawson, M., Mosadeghi, R. and Cashman, J.R. (2007) Functional activity of

666   the mouse flavin-containing monooxygenase forms 1, 3, and 5. *J Biochem Mol Toxicol* 21, 206–

667   215.

668 Zoetendal, E.G., Raes, J., van den Bogert, B., Arumugam, M., Booijink, C.C., Troost, F.J., Bork, P.,

669   Wels, M., de Vos, W.M. and Kleerebezem, M. (2012) The human small intestinal microbiota is

670   driven by rapid uptake and conversion of simple carbohydrates. *ISME J* 6, 1415–1426.

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).
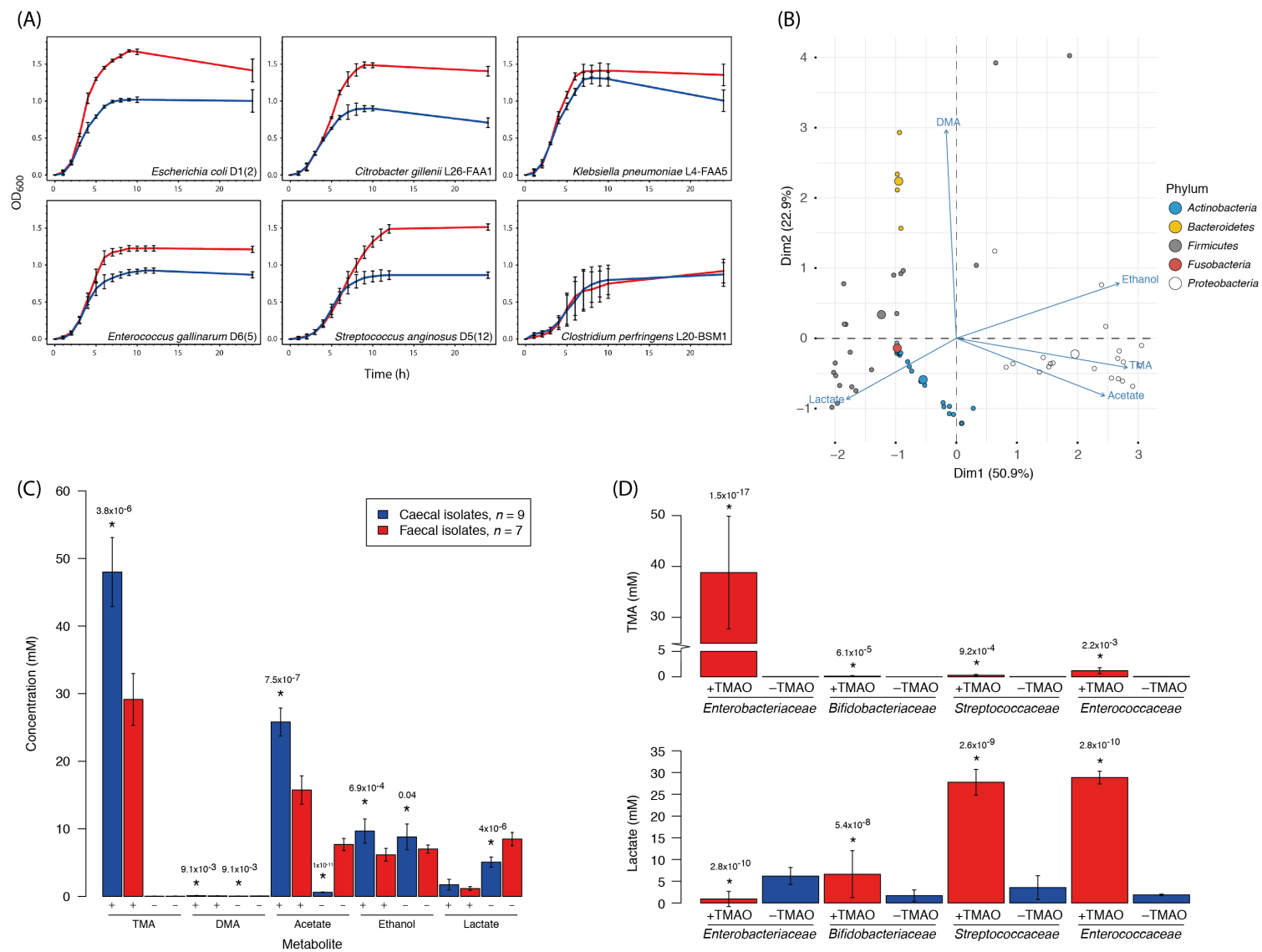
27

715 **Fig. 1**

716

717

Fig. 2

(A)

(B)

(C)

Fig. 3

(A)

(B)

(C)

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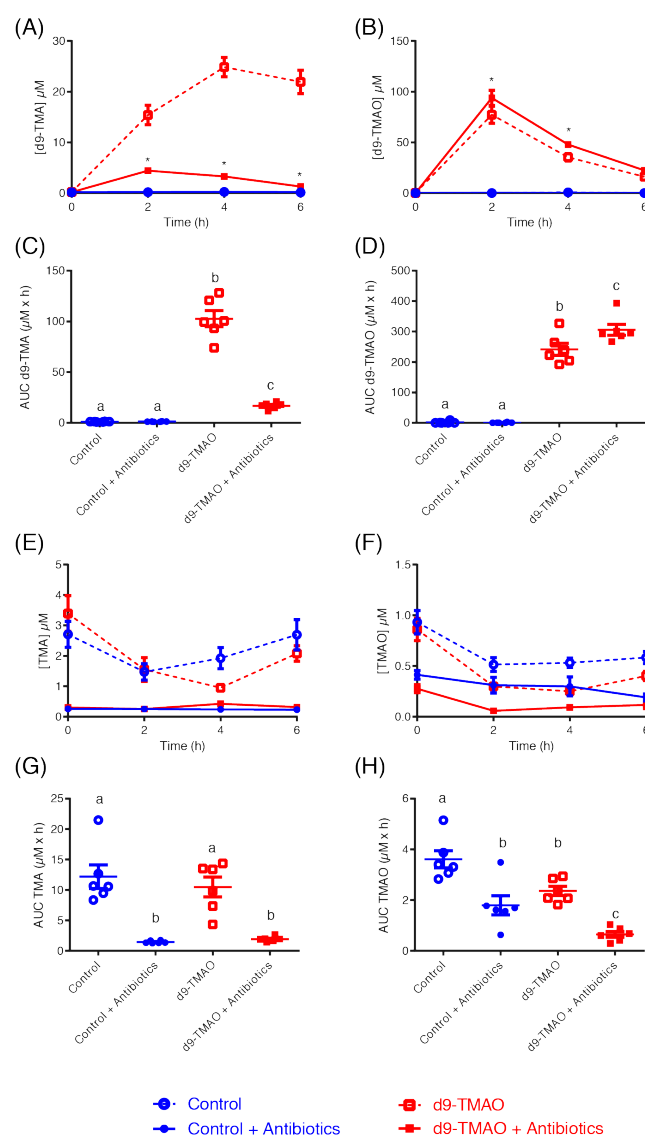
722 **Table 1.** Details for human-derived gut bacteria screened for their ability to reduce or utilize TMAO

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

31

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

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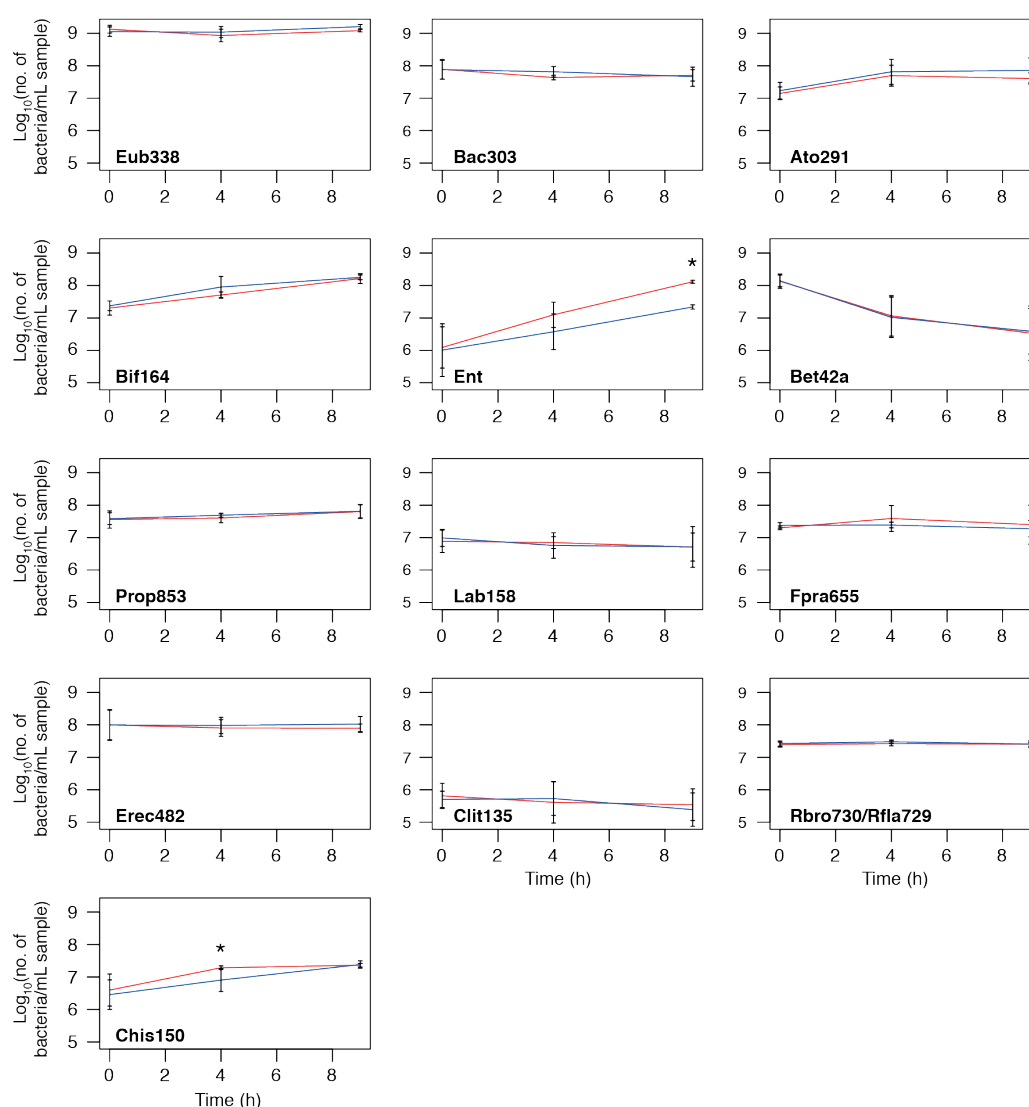
Supplementary Figure 1. Effect of TMAO on mixed faecal microbial population *in vitro*.

Enumeration of bacteria in fermentation vessels by FISH analysis. Red lines, TMAO-containing systems; blue lines, negative controls. Data are shown as mean + SD ($n = 3$). Eub338, total bacteria; Bac303, *Bacteroidales*; Ato291, '*Atopobium* cluster'; Bif164, *Bifidobacterium* spp.; Ent, *Enterobacteriaceae*; Bet42a, *Betaproteobacteria*; Prop853, *Veillonellaceae*; Lab158, lactic acid bacteria (*Lactobacillales*); Fpra655, *Faecalibacterium prausnitzii* and *Subdoligranulum* spp.; Erec482, *Lachnospiraceae*; Clit135, *Clostridium* cluster XI; Rbro730/Rfla729, *Ruminococcaceae*; Chis150, *Clostridium* clusters I and II. Detection limit of the method was 4.95 $\log_{10}$(number of bacteria/mL sample). *, Statistically significantly different (adjusted $P < 0.05$) from the control at the 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.

748

749

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

751

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).

754

755

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

757

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

758 *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

759 vessel without TMAO (negative control); E prefix, donor 3 vessel containing TMAO; F prefix, donor 3 vessel without TMAO (negative control).

760

40

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

762

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.