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

Lesley Hoyles[1]*, Maria L. Jiménez-Pranteda[2]*, Julien Chilloux[1]*, Francois Brial[3], Antonis Myridakis[1], Thomas Aranias[3], Christophe Magnan[4], Glenn R. Gibson[2], Jeremy D. Sanderson[5], Jeremy K. Nicholson[1], Dominique Gauguier[1,3], Anne L. McCartney[2]† and Marc-Emmanuel Dumas[1]†

[1]Integrative Systems Medicine and Digestive Disease, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, Exhibition Road, London SW7 2AZ, UK

[2]Food Microbial Sciences Unit, Department of Food and Nutritional Sciences, School of Chemistry, Food and Pharmacy, Faculty of Life Sciences, The University of Reading, Whiteknights Campus, Reading RG6 6UR, UK

[3]Sorbonne Universities, University Pierre & Marie Curie, University Paris Descartes, Sorbonne Paris Cité, INSERM UMR_S 1138, Cordeliers Research Centre, Paris, France

[4]Sorbonne Paris Cité, Université Denis Diderot, Unité de Biologie Fonctionnelle et Adaptative, CNRS UMR 8251, 75205 Paris, France

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

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

†**Corresponding authors:** Anne L. McCartney; Marc-Emmanuel Dumas

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

Email addresses: L. Hoyles, lesley.hoyles11@imperial.ac.uk; M.L. Jiménez-Pranteda, jimepra@ugr.es; J. Chilloux, j.chilloux@imperial.ac.uk; F. Brial, francois.brial@crc.jussieu.fr; A. Myridakis, a.myridakis@imperial.ac.uk; T. Aranias, thomas.aranias@free.fr; C. Magnan, christophe.magnan@univ-paris-diderot.fr; G.R. Gibson, g.r.gibson@reading.ac.uk; J.D. Sanderson, jeremy.sanderson@kcl.ac.uk; J.K. Nicholson, j.nicholson@imperial.ac.uk; D. Gauguier, dominique.gauguier@upmc.fr; A.L. McCartney, a.l.mccartney@reading.ac.uk; M.-E. Dumas, m.dumas@imperial.ac.uk

ABSTRACT

BACKGROUND: 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.

RESULTS: 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. Microbial activity necessary to convert TMAO to TMA was suppressed in antibiotic-treated mice, 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. Clostridia (*sensu stricto*), bifidobacteria and coriobacteria were significantly correlated with TMA production in the mixed fermentation system but did not produce notable quantities of TMA from TMAO in pure culture.

CONCLUSIONS: Reduction of TMAO by the gut microbiota (predominantly *Enterobacteriaceae*) to TMA followed by host uptake of TMA into the bloodstream from the intestine and its conversion back to TMAO by host hepatic enzymes is an example of metabolic retroconversion. TMAO influences microbial metabolism depending on isolation source and taxon of gut bacterium. Correlation of metabolomic and abundance data from mixed microbiota fermentation systems did not give a true picture of which members of the gut microbiota were responsible for converting TMAO to TMA; only by supplementing the study with pure culture work and additional metabolomics was it possible to increase our understanding of TMAO bioconversions by the human gut microbiota.

BACKGROUND

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 [1–3]. TMA can be used by members of the order *Methanomassiliicoccales* (*Archaea*) present in the human gut to produce methane [4], or taken up by the host. Microbially produced TMA derived from PC is absorbed in the small intestine [5]. 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) [6]. 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 [7,8]. TMAO can also be detected in human skeletal muscle within 6 h of an oral dose of TMAO [8].

TMAO present in urine and plasma is considered a biomarker for non-alcoholic fatty liver disease (NAFLD), insulin resistance and cardiovascular disease (CVD) [9–12]. Feeding TMAO to high-fat-fed C57BL/6 mice exacerbates impaired glucose tolerance, though the effect on the gut microbiota is unknown [13]. Low plasma PC and high urinary methylamines (including TMAO) were observed in insulin-resistant mice on a high-fat diet, leading to the suggestion microbial methylamine metabolism directly influences choline bioavailability; however, the microbiota of the mice was not studied nor was labeled choline used to determine the metabolic fate of TMA/TMAO [9]. Choline bioavailability is thought to contribute to hepatic steatosis in patients [11]. Spencer *et al.* [11] manipulated dietary choline levels in 15 females for 2 weeks and monitored changes in the fecal microbiota during the intervention. They found the level of choline supplementation affected Gammaproteobacteria (inhibited by high levels of dietary choline, negatively correlated with changes in liver fat/spleen fat ratios) and Erysipelotrichi (positively correlated with changes in liver fat/spleen fat ratios), suggesting baseline levels of these taxa may predict the susceptibility of an individual to liver disease. However, the study was under-powered and requires replication with a much larger cohort to fully examine the link between choline bioavailability and liver disease. High levels of

circulating TMAO are associated with CVD [12], potentially via a platelet-mediated mechanism [14]. Circulating TMAO has been suggested to play a role in protection from hyperammonemia in mice, acting as an osmoprotectant, and from glutamate neurotoxicity in mice and primary cultures of neurons [15,16]. Recently, chronic exposure to TMAO was shown to attenuate diet-associated impaired glucose tolerance in mice, and reduce endoplasmic reticulum stress and adipogenesis in adipocytes [10]. The relevance of these findings to human health remains to be determined. There is a basal level of TMA and TMAO detected in human urine even in the absence of dietary supplementation [17], suggesting use of (microbial and/or host) cell-derived choline or PC in the intestinal tract by the gut microbiota.

While it is well established that choline [2,11,18], PC [2,12] and carnitine [19–22] are used by the human and rodent gut microbiotas to produce TMA, little is known about the reduction of TMAO (predominantly from fish) to TMA (or other compounds) by members of these consortia. TMAO is found at concentrations of 20–120 mg per 100 g fish fillet [23]. Ingestion of fish by humans leads to increased urinary excretion of dimethylamine (DMA) and TMA, from 5.6 to 24.1 and from 0.2 to 1.6 μmol/24 h/kg of body weight, respectively [24]. Individuals with trimethylaminuria (fish odour syndrome), in which TMA is not detoxified to TMAO by hepatic FMOs, have been shown to reduce 40–60 % of an oral dose of TMAO to TMA [25]. It was suggested the gut microbiota was responsible for reducing TMAO in these individuals, and in those individuals without trimethylaminuria the TMA was re-oxidized in the liver before being excreted in the urine. The process was termed '*metabolic retroversion*' "*(i.e., a cycle of reductive followed by oxidative reactions to regenerate TMAO)*" [25]. The reduction of TMAO to TMA is most commonly associated with the gut microbiota of marine fish, with the TMA generated by these bacteria contributing to the characteristic odour of rotting fish [26]. Members of the class *Gammaproteobacteria*, which includes a number of food spoilage organisms, are known to reduce TMAO to TMA quantitatively and are unable to reduce TMA further [26,27]. The conversion of TMAO to TMA by bacteria represents a unique form of anaerobic respiration, in which TMAO reductase acts as a terminal electron acceptor (from NADH or formate) for respiratory electron flow [27].

112 Mining of metagenomic data (*torA*) suggests *Proteobacteria* (particularly *Escherichia* and
113 *Klebsiella* spp.) are likely to contribute greatest to the production of TMA from TMAO in the human
114 gut via the TMAO reductase pathway, with *Actinobacteria* (*Eggerthellaceae*) becoming more
115 important under stress [28]. Other microbial genes are associated with production of TMA from
116 choline (*cutC*), glycine-betaine (*grdH*), L-carnitine (*cntA*) and γ-butyrobetaine (*cntA*) [4]. A search of
117 the NCBI nucleotide database with the phrase 'TMAO reductase' also suggests many other bacteria of
118 human intestinal origin (namely, *Salmonella*, *Helicobacter*, *Prevotella*, *Bacillus* and *Bacteroides* spp.)
119 should be able to reduce TMAO to TMA. However, this trait has not been examined *in vitro* for
120 isolates of intestinal origin.
121 Consequently, we carried out an *in vivo* study in mice to confirm use of TMAO by the mouse
122 gut microbiota and to allow us to examine metabolic retroconversion of TMAO in a murine model.
123 We then used an *in vitro* fermentation system to highlight the effect of TMAO on the human faecal
124 microbiota. Finally, we screened a panel of human faecal and caecal/small intestinal isolates to
125 determine which members of the human gut microbiota were able to reduce TMAO to TMA, and
126 whether their metabolism was affected by being grown in the presence of TMAO.
127
128
129 **METHODS**
130 **Animal work**
131 Groups of six-week-old C57BL6/J mice (Janvier Labs, Courtaboeuf, France) were received
132 and acclimated in specific pathogen-free (SPF) maintenance conditions. They were fed with a
133 standard chow diet (R04-40, Safe, Augy, France) and were either given free access to tap water or tap
134 water containing the antibiotic cocktail (0.5 g/L vancomycin hydrochloride, 1 g/L neomycin trisulfate,
135 1 g/L metronidazole, 1 g/L ampicillin sodium; all antibiotics were purchased from Sigma-Aldrich) for
136 14 days. Mice were given in the morning by gavage either a solution of d_9-TMAO at 1×10^{-4} M
137 (Cambridge Isotope Laboratories Inc., DLM-4779-0, UK) or saline and euthanized 6 h later by
138 cervical dislocation. Blood samples were collected by tail tipping every 2 h in Microvette® CB 300

139 Lithium Heparin (Sarstedt, Marnay, France). Plasma was separated by centrifugation (10 min, 5000 ***g***,
140 4 °C) and stored at -80 °C until analysed by ultra-performance liquid chromatography–tandem mass
141 spectrometry (UPLC–MS/MS).
142
143 **UPLC–MS/MS determination of plasma TMA, d_9-TMA, TMAO and d_9-TMAO**
144 UPLC–MS/MS was employed for the determination of TMA, d_9-TMA, TMAO and d_9-
145 TMAO. Samples (10 µL) were spiked with 10 µL Internal Standard solution (d_9-choline and $^{13}C_3/^{15}N$-
146 TMA in water; 1 mg/L, Sigma-Aldrich). Ethyl 2-bromoacetate solution (45 µL) (15 g/L ethyl 2-
147 bromoacetate, 1 % NH_4OH in acetonitrile; ChromaSolv grade, Sigma-Aldrich) was added and
148 derivatization of TMAs (TMA, d_9-TMA and $^{13}C_3/^{15}N$-TMA) to their ethoxy-analogues was completed
149 after 30 min at room temperature. Protein/lipid precipitation solution (935 µL) (94 % acetonitrile/5
150 %water/1 % formic acid; ChromaSolv grade, Sigma-Aldrich) was added, samples were centrifuged
151 (20 min, 20,000 ***g***, 4 °C) and were transferred to UPLC-autosampler vials. Sample injections (5 µL
152 loop) were performed with a Waters Acquity UPLC-Xevo TQ-S UPLC–MS/MS system equipped
153 with an Acquity BEH HILIC (2.1×100 mm, 1.7 µm) chromatographic column. An isocratic elution
154 was applied with 10 mM ammonium formate (Sigma-Aldrich) in 95:5 (v/v) acetronitrile:water for 7
155 min at 750 µL/min and 50 °C. Positive electrospray (ESI^+) was used as ionization source and mass
156 spectrometer parameters were set as follows: capillary, cone and sources voltages at -700, -18 and
157 50 V, respectively, desolvation temperature at 600 °C, desolvation/cone/nebuliser gases were high
158 purity nitrogen (BOC) at 1000 L/h, 150 L/h and 7 bar, respectively. Collision gas was high-purity
159 argon (BOC). Mass spectrometer was operated in multiple reaction monitoring mode. The monitored
160 transitions were the following: for TMA, +146 → +118/59 *m/z* (23/27 V); for d_9-TMA, +155 →
161 +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 →
162 +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 →
163 +60/45 *m/z* (20/22 V). The system was controlled by MassLynx software, also used for data
164 acquisition and analysis.
165

166 **^{1}H-NMR spectroscopy and data analysis**

167 Medium samples were randomized and centrifuged (5 min, 16000 ***g***). Aliquots (50 µL) of the
168 supernatants were diluted in 550 µL D_2O containing 1 mM trimethylsilyl-(2,2,3,3-2H_4)-1-propionate.
169 Samples were transferred to 5 mm NMR tubes and measured on a NMR spectrometer (Bruker)
170 operating at 600.22 MHz ^{1}H frequency as described [29]. ^{1}H-NMR spectra were pre-processed and
171 analysed as described [9] using the Statistical Recoupling of Variables-algorithm [30]. Structural
172 assignment was performed as described [31], using in-house and publicly available databases.
173

174 ***In vitro* fermentation systems**

175 Freshly voided faecal samples were obtained from three healthy human volunteers (one male
176 and two females; age range 29–31 years), none of whom had been prescribed antibiotics 6 months
177 prior to the study, eaten fish 3 days prior to sample collection or had any history of gastrointestinal
178 disease. The University of Reading's Research Ethics Committee (UREC) does not require that
179 specific ethical review and approval be given by UREC for the collection of faecal samples from
180 healthy human volunteers to inoculate *in vitro* fermentation systems. Samples were processed
181 immediately by diluting them 1 in 10 (w/w) in pre-reduced minimal broth and homogenizing them in
182 a filter bag in a stomacher (Stomacher 400 Lab System; Seward) for 2 min at 'high' speed. For each
183 donor, a batch culture vessel containing 175 mL of pre-reduced minimal broth was inoculated with
184 20 mL of the faecal homogenate and 5 mL of sterile H_2O containing 2 g TMAO dihydrate (Sigma-
185 Aldrich). Control vessels were inoculated with 20 mL of the faecal homogenate and 5 mL of sterile
186 H_2O. The final working volume of each batch culture vessel was 200 mL. The pH of each vessel (pH
187 6.5) was controlled automatically by the addition of 2 M HCl or 2 M NaOH. pH controllers were
188 supplied by Electrolab. The contents of each vessel were stirred constantly. An anaerobic
189 environment was maintained by constantly sparging the vessels with O_2-free N_2. The temperature of
190 each vessel was maintained at 37 °C by use of a circulating waterbath connected to each fermentation
191 vessel. The experiment was run for 9 h, with samples taken at 0, 1, 2, 3, 4, 5, 6 and 9 h.
192

193 **Fluorescence *in situ* hybridization (FISH) analysis**

194 Aliquots (2× 375 μL) of sample were fixed in ice-cold 4 % paraformaldehyde for 4 h, washed
195 in sterile phosphate-buffered saline and stored for FISH analysis as described [32]. **Supplementary**
196 **Table 1** gives details for probes used in this study. Probes were synthesized commercially (MWG-
197 Biotech) and labelled with the fluorescent dye cyanine 3 (Cy3; excitation λ, 514 nm; emission λ, 566
198 nm; fluorescence colour, orange–red). FISH was done as described [32].
199 Slides were viewed under a Nikon E400 Eclipse microscope. DAPI slides were visualized
200 with the aid of a DM400 filter; probe slides were visualized with the aid of a DM575 filter. Cells
201 (between 15 and 50 per field of view) were counted for 15 fields of view, and the numbers of bacteria
202 were determined by using the following equation:
203 $\mathrm{DF} \times ACC \times 6{,}732.42 \times 50 \times DF_{\mathrm{sample}}$
204 Where the DF (dilution factor) was calculated by taking into account the concentration of the
205 original sample (375 μL to 300 μL = 0.8×). *ACC* (average cell count) was determined by counting 15
206 fields of view and assumes that a normal distribution was observed for the counts. The figure
207 6,732.42 refers to the area of the well divided by the area of the field of view. DF_{sample} refers to the
208 dilution of sample used with a particular probe (e.g. 5× for t_0 Lab158 counts). The detection limit of
209 this method was 89,766 bacteria/mL of sample (= $\log_{10}$ 4.95).
210
211 **Screening of bacteria for ability to reduce TMAO**
212 Part of an in-house collection (University of Reading) of bacteria isolated from human
213 caecal/small intestinal and faecal samples (**Table 1**) was screened on minimal agar [g/L: glucose
214 (Fisher Scientific), 4; bacteriological peptone (Oxoid), 20; NaCl (Fisher Scientific), 5; neutral red
215 (Sigma-Aldrich), 0.03; agar technical no. 3 (Oxoid), 15; pH 7] with and without 1 % (w/v) TMAO
216 dihydrate (Sigma-Aldrich). The composition of the agar was based on [27], who used the medium
217 with crystal violet and bile salts to screen members of the *Enterobacteriaceae* for TMAO reductase
218 activity. Colonies of isolates able to ferment glucose were red, whereas those able to reduce TMAO
219 were white. Growth curves (OD_{600} measured hourly for 10–12 h, then at 24 h) were determined for
220 selected isolates grown anaerobically at 37 °C in anaerobic minimal broth [g/L: glucose, 4;

221 bacteriological peptone, 20; NaCl, 5; L-cysteine HCl (Sigma-Aldrich), 0.5; resazurin solution (0.25
222 mg/mL; Sigma-Aldrich), 4 mL; pH 7] with and without 1 % (w/v) TMAO dihydrate. Glucose was
223 substituted by raffinose (Sigma-Aldrich) [33] in the minimal broth when working with bifidobacteria
224 because of the poor growth of these bacteria on the glucose-based medium. pH and metabolite
225 profiles of culture medium were examined at the end of the growth experiment (i.e. at t_{24}).
226
227 **Statistical analyses**
228 Differences between metabolites produced by faecal and caecal/small intestinal isolates of
229 *Escherichia coli* and lactic acid bacteria in the presence and absence of TMAO were analysed using
230 Student's *t* test. FISH data from the *in vitro* fermentation systems were analysed using the
231 Kolmogorov–Smirnov test with statistical significance, after correction for multiple testing, taken at P
232 < 0.05. Because of the presence of ties in the metabolite data from *in vitro* fermentation systems, the
233 bootstrapped Kolmogorov–Smirnov test (10,000 replications) was used with these data. Data from *in*
234 *vitro* fermentation systems (FISH and NMR) were correlated using Spearman's rank correlation
235 (corrected for ties) with results corrected for multiple testing using the method of Benjamini and
236 Hochberg [34].
237
238
239 **RESULTS**
240 ***In vivo* confirmation of metabolic retroconversion**
241 First, to confirm *in vivo* metabolic retroconversion (i.e. microbial conversion of TMAO to
242 TMA, followed by host conversion of TMA to TMAO), we administered isotopically-labelled d_9-
243 TMAO or saline to mice that had or had not been treated with a broad-spectrum antibiotic cocktail for
244 14 days to suppress the gut microbiota [12]. Reduction of d_9-TMAO to d_9-TMA was quantified in
245 urine by UPLC–MS/MS up to 6 h after d_9-TMAO gavage, together with unlabelled TMA and TMAO
246 potentially produced from dietary sources (**Fig. 1**).
247 In the absence of antibiotics, d_9-TMAO was converted to d_9-TMA within 2 h of gavage. This
248 conversion was dramatically reduced, leading to a significantly lower concentration of d_9-TMA and a

249 higher concentration of d_9-TMAO, when the gut microbiota was suppressed by antibiotics (**Fig. 1A–**
250 **D**). We noted that in the absence of antibiotics all animals excreted approximately three times higher
251 levels of urinary unlabelled TMA than TMAO (**Fig. 1E–H**), corresponding to constitutively low
252 FMO3 activity in mice [35]. Levels of unlabelled TMAO/TMA were not significantly different from
253 one another in the control and d_9-TMAO-fed animals, while antibiotic treatment significantly reduced
254 the amount of both TMAO and TMA (**Fig. 1E**, **F**). Bioavailability of unlabelled TMAO/TMA, as
255 assessed by area under the curve (AUC), was significantly reduced by antibiotic treatment in both
256 saline and d_9-TMAO-gavaged animals, with almost no TMA detected in either experimental group
257 (**Fig. 1G**, **H**).
258
259 **Effect of TMAO on human gut bacteria within a mixed system**
260 After *in vivo* validation of the role of the gut microbiota in metabolic retroconversion, we
261 analysed the effect of TMAO on the faecal microbiota in an anaerobic batch-culture fermentation
262 system. Fermenter vessels filled with the glucose-containing medium supplemented or not with 1 %
263 (w/v) TMAO were inoculated with faecal slurries from three healthy donors and monitored for 9 h.
264 With the exception of enhanced growth of the *Enterobacteriaceae* (probe Ent), the presence of
265 TMAO in the medium had no statistically significant effect on the growth of bacteria within the
266 fermentation systems at 9 h (**Fig. 2A**, **Supplementary Figure 1**).
267 Huge variability, as measured by ^{1}H-NMR, was observed in the amount of TMA and DMA
268 produced by gut bacteria in the TMAO-containing fermentation systems, with the concentrations of
269 both metabolites increasing steadily from 0 to 9 h and differing significantly ($P < 0.05$) from the
270 control systems (**Fig. 2B**). The amount of TMAO in the systems was seen to decrease at 8 h.
271 Correlation of metabolite and FISH data demonstrated *Clostridium* clusters I and II
272 (Chis150), *Enterobacteriaceae* (Ent), bifidobacteria (Bif164) and coriobacteriia (Ato291) were
273 associated with TMA, acetate, ethanol and lactate (**Fig. 2C**). The *Betaproteobacteria* (Bet42a) were
274 anti-correlated with TMA, acetate, ethanol and lactate, which is unsurprising given this was the only
275 group of bacteria whose representation decreased in the fermentation systems over the course of the
276 experiment (**Supplementary Figure 1**, **Fig. 2C**). The *Enterobacteriaceae* and clostridia were

277 positively correlated with DMA production. The lactic acid bacteria (Lab158) were not significantly
278 correlated with any of the metabolites in the mixed culture.
279
280 **Growth of pure cultures of gut bacteria in the presence of TMAO**
281 Using an in-house collection of bacteria, we initially screened isolates on a modified version
282 of the agar of Takahi & Ishimoto [27] as a rapid means of screening bacteria for TMAO reductase
283 activity, and thereby their ability to reduce TMAO to TMA. While members of the
284 *Enterobacteriaceae* produced expected results (i.e. white colonies when grown in the presence of
285 TMAO and glucose, rather than red colonies on the glucose only control), we observed a number of
286 unexpected outcomes depending on the species under study. For example, colonies of lactic acid
287 bacteria were larger (almost twice their usual size) on TMAO-containing agar than on the glucose
288 control but remained red in colour, suggesting they had not reduced TMAO to TMA at detectable
289 levels but TMAO was influencing their growth. The clostridia examined produced mixed results on
290 the control and TMAO-containing media (i.e. white colonies on both plates or on the control plate
291 only, larger colonies on the TMAO-containing medium but without a colour change of the medium).
292 To determine whether these isolates were converting TMAO to TMA but a low level, we
293 examined the growth of all isolates in liquid culture and metabolites in the spent medium using NMR.
294 The growth of the *Enterobacteriaceae* was most greatly affected by the presence of TMAO in the
295 medium, with a faster, longer-lasting exponential phase than for the same isolates grown in the
296 control medium (**Fig. 3A**). pH of the spent medium (after 24 h) when *Enterobacteriaceae* were grown
297 in the presence of TMAO increased from a mean of 4.7 ± 0.3 (for the control) to 7.6 ± 0.3 (n = 20)
298 (the change in pH is what causes the colonies to appear white on TMAO-containing agar). The
299 growth of lactic acid bacteria, including *Enterococcus* and *Streptococcus* (**Fig. 3A**) spp., was
300 enhanced in the presence of TMAO, but not to the same extent as seen for the *Enterobacteriaceae*.
301 There was no significant difference (P = 0.27, t test) in the pH of the spent medium for these bacteria
302 after 24 h (mean 4.67 ± 0.9 compared with 4.33 ± 0.27 for the control). The growth of members of
303 *Clostridium* cluster I (e.g. *Clostridium perfringens*, **Fig. 3A**) was not enhanced in the presence of
304 TMAO, though some of these bacteria changed the colour of both the control and TMAO-containing

305 plates yellow during their growth. The pH of the spent liquid medium confirmed this observation to
306 be due to the alkalinity of the media in the control and TMAO-containing media after 24 h incubation:
307 e.g. *Clostridium sporogenes* D1(9) (pH 6.25 compared with 6.76 in the control medium), *Clostridium*
308 *paraputrificum* L16-FAA6 (pH 6.45 vs 5.38) and *Clostridium perfringens* L20-BSM1 (pH 5.56 vs
309 4.64).

310 ^{1}H-NMR analysis of spent medium from the TMAO-containing and control samples
311 demonstrated that, as expected, the *Enterobacteriaceae* produced the greatest amount of TMA from
312 TMAO (mean 38.79 ± 11.08 mM compared with 0.03 ± 0.01 mM, *n* = 20) (**Fig. 3B**; **Supplementary**
313 **Table 2**). Members of the families *Peptostreptococcaceae* (3.72 mM, *n* = 1), *Clostridiaceae* (Cluster
314 I) (2.62 ± 1.83 mM, *n* = 3), *Porphyromonadaceae* (1.42 mM, *n* = 1), *Bacteroidaceae* (1.40 ± 0.31
315 mM, *n* = 3), *Enterococcaceae* (1.19 ± 0.05 mM, *n* = 5), *Erysipelotrichaceae* (0.94 mM, *n* = 1;
316 [*Clostridium*] *ramosum*), *Staphylococcaceae* (0.34 mM, *n* = 1), *Streptococcaceae* (0.30 ± 0.16 mM, *n*
317 = 5), *Lactobacillaceae* (0.17 ± 0.07 mM, *n* = 2), *Pseudomonadaceae* (0.12 mM, *n* = 1) and
318 *Bifidobacteriaceae* (0.13 ± 0.1 mM, *n* = 17) produced low levels of TMA from TMAO (**Fig. 3B**;
319 **Supplementary Table 2**). There was great variability in the ability of the bifidobacteria to produce
320 TMA from TMAO, with several isolates and [*Clostridium*] *innocuum*, *Actinomyces odontolyticus*,
321 *Fusobacterium ulcerans* and *Actinomyces viscosus* not producing TMA from TMAO (**Fig. 3B**;
322 **Supplementary Table 2**).
323

324 **Differences in the metabolic capabilities of faecal and caecal *Escherichia coli* isolates**

325 Comparison of the amounts of TMA, and co-metabolites, produced by the faecal (*n* = 7) and
326 caecal (*n* = 9) isolates of *Escherichia coli* demonstrated significantly higher amounts of TMA were
327 produced by the caecal isolates compared with the faecal isolates in the TMAO-containing medium
328 (**Fig. 3C**). *Escherichia coli* of caecal origin produced more TMA than faecal isolates of the same
329 bacterium or other enterobacteria (*Hafnia*, *Citrobacter* and *Klebsiella* spp.) (**Supplementary Table**
330 **2**). The faecal isolates produced more acetate and lactate than the caecal isolates when grown in the
331 control medium. Taken together, these results demonstrate the different metabolic capabilities of
332 isolates of *Escherichia coli* recovered from different regions of the human gut.

333

334 **Lactic acid bacteria produce more lactate in the presence of TMAO**

335 Differences were also seen in the amount of lactic acid produced by lactic acid bacteria in the
336 presence and absence of TMAO (**Fig. 3B**, **D**). In raffinose-containing medium, bifidobacteria
337 produced increased of amounts of lactate when grown in the presence of TMAO (the bifidobacteria
338 grew poorly, if at all, in glucose-containing media). Unlike the bifidobacteria, the *Streptococcaceae*
339 and *Enterococcaceae* grew well in the glucose-containing medium and produced over 25 mM lactic
340 acid in the TMAO-containing samples compared with <5 mM in the control samples (**Fig. 3D**). To the
341 best of our knowledge, this is the first time TMAO has been shown to influence the metabolism of gut
342 bacteria – specifically lactic acid bacteria – without producing appreciable amounts of TMA.

343

344

345 **DISCUSSION**

346 TMAO is a circulating metabolite produced as a direct result of microbial degradation of
347 dietary methylamines in the intestinal tract, and can be readily detected along with its precursor TMA
348 in human urine, blood and skeletal muscle [8,12,18,22]. It worsens atherosclerosis in some mouse
349 models of CVD, and is positively correlated with CVD severity in humans. Beneficial effects
350 associated with TMAO include potential protection from hyperammonia and glutamate neurotoxicity,
351 alleviation of endoplasmic reticulum stress and improved glucose homeostasis by stimulating insulin
352 secretion by pancreatic cells [10,15,16].

353 Over many decades it has been established that choline, PC and carnitine are dietary
354 methylamines that contribute directly to microbiome-associated circulating levels of TMAO found in
355 humans and other animals – e.g. [2,12,18,19,21,22]. However, TMAO itself is a water-soluble
356 osmolyte found in high abundance in fish. Based on their observations that individuals with
357 trimethylaminuria could reduce an oral dose of TMAO to TMA could not detoxify it to TMAO, al-
358 Waiz *et al.* [25] suggested the gut microbiota could use TMAO as a substrate, and in those individuals
359 without trimethylaminuria the TMA was re-oxidized in the liver before being excreted in the urine.
360 This led these authors to propose the process of '*metabolic retroversion*'. Reference to the literature

361 associated with fish spoilage and recent mining of metagenomic data have predicted members of the
362 *Enterobacteriaceae* (particularly *Escherichia coli* and *Klebsiella pneumoniae*) have the potential to
363 convert TMAO to TMA in the intestinal tract, but this has not been tested *in vitro* or *in vivo* to date
364 [4,8,26–28]. Consequently, we instigated this study to demonstrate metabolic retroconversion of
365 TMAO, and to determine the effect of TMAO on the growth and metabolism of human-derived
366 intestinal bacteria in pure and mixed cultures.

367 Through *in vivo* administration of deuterated TMAO to mice via oral gavage in mice, we
368 unambiguously demonstrated that TMAO is converted to TMA, with this TMA detectable in plasma
369 within 2 h of administration. This conversion was highly dependent on the gut microbiota, as
370 conversion of TMAO to TMA was dramatically reduced when the microbiota was suppressed by
371 treatment of animals with broad-spectrum antibiotics. Even in the presence of antibiotics, there was
372 low-level conversion of TMAO to TMA, suggesting a subpopulation of the microbiota was resistant
373 to the antibiotics used in our experiment. However, administration of a broad-spectrum antibiotic
374 cocktail for 14 days has been demonstrated to be an effective means of suppressing the gut microbiota
375 in studies associated with gut microbial use of dietary methylamines [12].

376 Our *in vivo* experiment clearly demonstrates the gut microbiota converts TMAO to TMA, and
377 that this TMA is re-oxidized to TMAO, in line with the process of '*metabolic retroversion*' [25]. Gut-
378 associated microbial conversion of the majority of TMAO to TMA is at odds with recent findings [8],
379 in which it was suggested TMAO is taken up intact and not metabolised by the gut microbiota of
380 humans. The ratio of TMAO-to-TMA is around 10:1 in humans and 1:10 in mice, meaning TMAO
381 was *N*-oxidized before Taesuwan *et al.* [8] were able to observe it in circulating blood of their human
382 subjects. In a human system with high FMO3 activity, ^{17}O-labelled TMAO would need to be used in
383 any study evaluating O turnover to allow calculation of the true rate of retroconversion.

384 Based on work done in mice [10,36], metabolic retroconversion of TMAO may be protective,
385 and may even go some way to explaining why TMA and TMAO are detected at low levels in urine in
386 the absence of dietary methylamines [17]. Chronic exposure of high-fat-fed mice to TMAO reduced
387 diet-associated endoplasmic stress and adipogenesis, and improved glucose tolerance [10]. Exposure
388 of high-fat-fed mice to TMA reduced low-grade inflammation and insulin resistance via inhibition of

interleukin-1 receptor-associated kinase 4 (IRAK-4) [36]. Concentrations of the microbial signalling metabolite TMA used by Chilloux *et al.* [36] were comparable to those found in normal human plasma. Continual exposure of the human system to TMA/TMAO may bring similar benefits to host metabolic and inflammatory responses; these remain to be studied.

Having conducted our *in vivo* experiment in mice, we examined the ability of a range of human-derived gut bacteria to convert TMAO to TMA. In the mixed microbiota system and in pure cultures, the growth of the *Enterobacteriaceae* – the main TMA-producers – was quickly affected by the presence of TMAO. This is likely to happen in the human gut also. Consequently, we believe dietary TMAO undergoes metabolic retroconversion in mammals, with the TMA produced as a result of bacterial activity in the gut available to the host for conversion back to TMAO by FMOs in hepatocytes. It should be noted that [8] did not suppress or monitor the intestinal/faecal microbiota when they administered isotopically labeled TMAO to humans, nor did they measure d_9-TMA and d_9-TMAO in the portal vein, bypassing subsequent hepatic *N*-oxidation of d_9-TMA, so it is not possible to interpret their results in the context of presence/absence of microbial activity.

Of note is the finding that caecal isolates of enterobacteria produce more TMA from TMAO than faecal isolates of the same bacterium. The speed with which TMAO was reduced to TMA by the *Enterobacteriaceae* in the present study suggests bacterial conversion of TMAO to TMA takes place in the small intestine/proximal colon of humans and small intestine/caecum of mice. It is, therefore, unsurprising that caecal bacteria – representing the microbiota present at the intersection of the small and large intestine – are metabolically more active than their faecal counterparts with respect to TMAO metabolism. This finding is relevant to functional studies of the gut microbiota where gene annotations are based largely on faecal isolates, whose functionalities may be greatly different from those of bacteria in other regions of the human intestinal tract [37,38]. It has already been demonstrated that the microbiota of the small intestine is enriched for functions associated with rapid uptake and fermentation of simple carbohydrates compared with the faecal microbiota, and that streptococci isolated from this niche are functionally very different from the same bacteria isolated from different habitats [37,38]. It is, therefore, important we characterize the functions and genomes of bacteria isolated from all regions of the intestinal tract, not just those of faecal bacteria, to gain a

417 true picture of how microbial activity influences host health.

418 *Enterobacteriaceae* made the greatest contribution to the conversion of TMAO to TMA, both
419 in pure culture and in a mixed microbiota. These Gram-negative bacteria are a source of the virulence
420 factor lipopolysaccharide (LPS), which is associated with low-grade inflammation in high-fat-fed
421 mice and elevated plasma levels that define metabolic endotoxaemia [39,40]. High-fat feeding has
422 been shown to increase the representation of *Enterobacteriaceae* in the caecal microbiota of obesity-
423 prone Sprague–Dawley rats [41], though this mode of feeding is known to modulate the microbiota of
424 mice independent of obesity [42]. Non-LPS-associated virulence of *Enterobacteriaceae*, *Vibrio*
425 *cholerae* and *Helicobacter pylori* is increased when these bacteria are grown anaerobically or
426 microaerophilically in the presence of as little as 5–10 mM TMAO [43–46], and may be an additional
427 means by which the gut microbiota contributes to CVD and other diseases in which increased
428 representation of *Gammaproteobacteria* is observed. This warrants attention in future animal studies.

429 Lactic acid bacteria clearly grow better in the presence of TMAO. The relatively high
430 concentration (1 %) of TMAO used in this study may have contributed to this improved growth, as
431 TMAO is an osmolyte that stabilizes proteins. Future work will involve growing lactic acid bacteria in
432 a range of TMAO concentrations to determine how this compound affects their growth and gene
433 expression, and comparing faecal and caecal isolates. Similar to the *Enterobacteriaceae*, a large
434 number of these bacteria are facultative anaerobes able to grow over a range of conditions, and whose
435 representation is increased in obese, and cirrhotic patients [47,48]. *Streptococcus* and *Enterococcus*
436 spp. are commensal lactic acid bacteria of the gut microbiota known to modulate immune function;
437 but little is known about their metabolic activities in mixed microbial populations [49]. Understanding
438 how commensal lactic acid bacteria influence the host in dysbiosis in mixed microbial communities
439 may allow the development of approaches to modulate their activity and influence host health.

440 With respect to the lactic acid bacteria, it is important to note that our mixed culture work did
441 not highlight these as being relevant to TMAO metabolism. This is unsurprising given these bacteria
442 do not produce large quantities of TMA from TMAO. However, we have shown their metabolism is
443 affected by presence of TMAO in growth medium, and the increased lactate they produce in its
444 presence may contribute to cross-feeding associated with short-chain fatty acid production [50]. 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.

CONCLUSIONS

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.

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.

DECLARATIONS

Ethics approval: All animal procedures were authorized following review by the institutional ethics committee (Sorbonne Universities) and carried out under national license conditions. Ethical approval to collect caecal effluent from patients was obtained from St Thomas' Hospital Research Ethics Committee (06/Q0702/74) covering Guy's and St Thomas' Hospitals, and transferred by agreement to London Bridge Hospital. Patients provided written consent to provide samples.

Consent for publication: Not applicable.

Availability of data and material: The datasets used and/or analysed during the current study are available from the corresponding authors upon reasonable request.

Competing interests: The authors have no competing interests.

Funding: MLJP was sponsored by Fundación Alfonso Martin Escudero (Spain). Work was supported by a grant from the EU-FP7 (METACARDIS, HEALTH-F4-2012-305312) to DG and MED. LH is in receipt of an MRC Intermediate Research Fellowship in Data Science (grant number MR/L01632X/1, UK Med-Bio).

Authors' contributions: MLJP, LH and ALM performed the microbiological work, JC was responsible for NMR analysis and interpretation of the NMR data, TA and FB did the animal work, MED supervised the NMR work, ALM supervised the microbiological work, CM provided invaluable advice for the labelling study and DG supervised the animal work. All authors contributed to the writing of the manuscript.

Acknowledgements: Not applicable.

REFERENCES

[1] Wünsche R. Uber die wirkung von darmbakterien auf trimethylammoniumbasen. Ber Ges Physiol Exp Pharm. 1940;118:303.

[2] Zeisel SH, Wishnok JS, Blusztajn JK. Formation of methylamines from ingested choline and lecithin. J Pharmacol Exp Ther. 1983;225:320-24.

[3] Bradbeer C. The clostridial fermentations of choline and ethanolamine. 1. Preparation and properties of cell-free extracts. J Biol Chem. 1965;240:4669-74.

[4] Borrel G, McCann A, Deane J, Neto MC, Lynch DB, Brugère JF, et al. Genomics and metagenomics of trimethylamine-utilizing *Archaea* in the human gut microbiome. ISME J. 2017;11:2059-74.

[5] Stremmel W, Schmidt KV, Schuhmann V, Kratzer F, Garbade SF, Langhans CD, et al. Blood trimethylamine-*N*-oxide originates from microbiota mediated breakdown of phosphatidylcholine and absorption from small intestine. PLoS One. 2017;12:e0170742.

[6] Bennett BJ, de Aguiar Vallim TQ, Wang Z, Shih DM, Meng Y, Gregory J, et al. Trimethylamine-*N*-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. Cell Metab. 2013;17:49-60.

[7] de la Huerga J, Popper H. Urinary excretion of choline metabolites following choline administration in normals and patients with hepatobiliary diseases. J Clin Invest. 1951;30:463-70.

[8] Taesuwan S, Cho CE, Malysheva OV, Bender E, King JH, Yan J, et al. The metabolic fate of isotopically labeled trimethylamine-*N*-oxide (TMAO) in humans. J Nutr Biochem. 2017;45:77-82.

[9] Dumas ME, Barton RH, Toye A, Cloarec O, Blancher C, Rothwell A, et al. Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice. Proc Natl Acad Sci U S A. 2006;103:12511-6.

[10] Dumas ME, Rothwell AR, Hoyles L, Aranias T, Chilloux J, Calderari S, et al. Microbial-host co-metabolites are prodromal markers predicting phenotypic heterogeneity in behavior, obesity, and impaired glucose tolerance. Cell Rep. 2017;20:136-48.

[11] Spencer MD, Hamp TJ, Reid RW, Fischer LM, Zeisel SH, Fodor AA. Association between composition of the human gastrointestinal microbiome and development of fatty liver with choline deficiency. Gastroenterology. 2011;140:976-86.

[12] Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med. 2013;368:1575-84.

[13] Gao X, Liu X, Xu J, Xue C, Xue Y, Wang Y. Dietary trimethylamine *N*-oxide exacerbates impaired glucose tolerance in mice fed a high fat diet. J Biosci Bioeng. 2014;118:476-81.

[14] Zhu W, Wang Z, Tang WHW, Hazen SL. Gut microbe-generated trimethylamine *N*-oxide from dietary choline is prothrombotic in subjects. Circulation. 2017;135:1671-3.

[15] Kloiber O, Banjac B, Drewes LR. Protection against acute hyperammonemia: the role of quaternary amines. Toxicology. 1988;49:83-90.

[16] Miñana MD, Hermenegildo C, Llsansola M, Montoliu C, Grisolía S, Felipo V. Carnitine and choline derivatives containing a trimethylamine group prevent ammonia toxicity in mice and glutamate toxicity in primary cultures of neurons. J Pharmacol Exp Ther. 1996;279:194-9.

[17] de la Huerga J, Gyorgy P, Waldstein S, Katz R, Popper H. The effects of antimicrobial agents upon choline degradation in the intestinal tract. J Clin Invest. 1953;32:1117-20.

538 [18] Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of
539 phosphatidylcholine promotes cardiovascular disease. Nature. 2011;472:57-63.
540 [19] Rebouche CJ, Mack DL, Edmonson PF. L-Carnitine dissimilation in the gastrointestinal tract of
541 the rat. Biochemistry. 1984;23:6422-6.
542 [20] Rebouche CJ, Chenard CA. Metabolic fate of dietary carnitine in human adults: identification
543 and quantification of urinary and fecal metabolites. J Nutr. 1991;121:539-46.
544 [21] Seim H, Schulze J, Strack E. Catabolic pathways for high-dosed L(-)- or D(+)-carnitine in germ-
545 free rats? Biol Chem Hoppe Seyler. 1985;366:1017-21.
546 [22] Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota
547 metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med. 2013;19,
548 576-85.
549 [23] Jebsen JW, Riaz M. Breakdown products of trimethylamine oxide in airdried stockfish. Means
550 of enhancing the formation of formaldehyde and dimethylamine. Fish Dir Skr Ser Ernoering.
551 1977;1:145-53.
552 [24] Zeisel SH, Da Costa KA. Increase in human exposure to methylamine precursors of *N*-
553 nitrosamine after eating fish. Cancer Res. 1986;46:6136-8.
554 [25] al-Waiz M, Ayesh R, Mitchell SC, Idle JR, Smith RL. Disclosure of the metabolic retroversion
555 of trimethylamine *N*-oxide in humans: a pharmacogenetic approach. Clin Pharmacol Ther.
556 1987;42:608-12.
557 [26] Barrett EL, Kwan HS. Bacterial reduction of trimethylamine oxide. Ann Rev Microbiol.
558 1985;39:131-49.
559 [27] Takagi M, Ishimoto M. *Escherichia coli* mutants defective in trimethylamine *N*-oxide reductase.
560 FEMS Microbiol Lett. 1983;17:247-50.
561 [28] Jameson E, Doxey AC, Airs R, Purdy KJ, Murrell JC, Chen Y. Metagenomic data-mining
562 reveals contrasting microbial populations responsible for trimethylamine formation in human
563 gut and marine ecosystems. Microb Genom. 2016;2:e000080.
564 [29] Dona AC, Jiménez B, Schäfer H, Humpfer E, Spraul M, Lewis MR., et al. Precision high-
565 throughput proton NMR spectroscopy of human urine, serum, and plasma for large-scale
566 metabolic phenotyping. Anal Chem. 2014;86:9887-94.
567 [30] Blaise BJ, Shintu L, Elena B, Emsley L, Dumas ME, Toulhoat P. Statistical recoupling prior to
568 significance testing in nuclear magnetic resonance based metabonomics. Anal Chem.
569 2009;81:6242-51.
570 [31] Dona AC, Kyriakides M, Scott F, Shephard EA, Varshavi D, Veselkov K, et al. A guide to the
571 identification of metabolites in NMR-based metabonomics/metabolomics experiments. Comput
572 Struct Biotechnol J. 2016;14:135-53.

573 [32] Martín-Peláez S, Gibson GR, Martín-Orúe SM, Klinder A, Rastall RA, La Ragione RM, et al. *In*
574 *vitro* fermentation of carbohydrates by porcine faecal inocula and their influence on *Salmonella*
575 Typhimurium growth in batch culture systems. FEMS Microbiol Ecol. 2008;66:608-19.
576 [33] Trojanová I, Vlková E, Rada V, Marounek M. Different utilization of glucose and raffinose in
577 *Bifidobacterium breve* and *Bifidobacterium animalis*. Folia Microbiol (Praha). 2006;51:320-4.
578 [34] Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful
579 approach to multiple testing. J R Stat Soc Ser B. 1995;57:289-300.
580 [35] Zhang J, Cerny MA, Lawson M, Mosadeghi R, Cashman JR. Functional activity of the mouse
581 flavin-containing monooxygenase forms 1, 3, and 5. J Biochem Mol Toxicol. 2007;21:206-15.
582 [36] Chilloux J, Everard A, Zhang L, Blancher C, Fuchs J, Hoyles L, et al. Gut microbial
583 trimethylamine inhibits IRAK-4 and chronic inflammation in metabolic syndrome. bioRxiv.
584 2018;doi:10.1101/277434.
585 [37] Zoetendal EG, Raes J, van den Bogert B, Arumugam M, Booijink CC, Troost FJ, et al. The
586 human small intestinal microbiota is driven by rapid uptake and conversion of simple
587 carbohydrates. ISME J. 2012;6:1415-26.
588 [38] van den Bogert B, Boekhorst J, Herrmann R, Smid EJ, Zoetendal EG, Kleerebezem M.
589 Comparative genomics analysis of *Streptococcus* isolates from the human small intestine reveals
590 their adaptation to a highly dynamic ecosystem. PLoS One. 2013;8:e83418.
591 [39] Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia
592 initiates obesity and insulin resistance. Diabetes. 2007;56:1761-72.
593 [40] Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut
594 microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced
595 obesity and diabetes in mice. Diabetes. 2008;57:1470-81.
596 [41] de La Serre CB, Ellis CL, Lee J, Hartman AL, Rutledge JC, Raybould HE. Propensity to high-
597 fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut
598 inflammation. Am J Physiol Gastrointest Liver Physiol. 2010;299:G440-8.
599 [42] Hildebrandt MA, Hoffmann C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen YY, et al.
600 High-fat diet determines the composition of the murine gut microbiome independently of
601 obesity. Gastroenterology. 2009;137:1716-24.
602 [43] Ando H, Abe H, Sugimoto N, Tobe T. Maturation of functional type III secretion machinery by
603 activation of anaerobic respiration in enterohaemorrhagic *Escherichia coli*. Microbiology.
604 2007;153:464-73.
605 [44] Lee KM, Park Y, Bari W, Yoon MY, Go J, Kim SC, Lee HI, et al. Activation of cholera toxin
606 production by anaerobic respiration of trimethylamine *N*-oxide in *Vibrio cholerae*. J Biol Chem.
607 2012;287:39742-52.

608 [45] Paiva JB, Penha Filho RA, Pereira EA, Lemos, MV, Barrow PA, Lovell MA, et al. The
609 contribution of genes required for anaerobic respiration to the virulence of *Salmonella enterica*
610 serovar Gallinarum for chickens. Braz J Microbiol. 2009;40:994–1001.
611 [46] Wu D, Cao M, Peng J, Li N, Yi S, Song L, et al. The effect of trimethylamine *N*-oxide on
612 *Helicobacter pylori*-induced changes of immunoinflammatory genes expression in gastric
613 epithelial cells. Int Immunopharmacol. 2017;43:172-8.
614 [47] Qin N, Yang F, Li A, Prifti E, Chen Y, Shao L, et al. Alterations of the human gut microbiome
615 in liver cirrhosis. Nature. 2014;513:59-64.
616 [48] Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, et al. Richness of human gut
617 microbiome correlates with metabolic markers. Nature. 2013;500:541-6.
618 [49] van den Bogert B, Meijerink M, Zoetendal EG, Wells JM, Kleerebezem M. Immunomodulatory
619 properties of *Streptococcus* and *Veillonella* isolates from the human small intestine microbiota.
620 PLoS One. 2014;9:e114277.
621 [50] Hoyles L, Wallace RJ. Gastrointestinal tract: intestinal fatty acid metabolism and implications
622 for health. In *Handbook of Hydrocarbon and Lipid Microbiology*, vol. 4, part 7, *Human-Animal-*
623 *Plant Health and Physiology Consequences of Microbial Interactions with Hydrocarbons and*
624 *Lipids*. 2010, 3120-32. Edited by T. McGenity, K. N. Timmis, J. R. van der Meer & V. de
625 Lorenzo. New York: Springer.
626 [51] Roger LC, Costabile A, Holland DT, Hoyles L, McCartney AL. Examination of faecal
627 *Bifidobacterium* populations in breast- and formula-fed infants during the first 18 months of life.
628 Microbiology. 2010;156:3329-41.
629 [52] Hoyles L, Murphy J, Neve H, Heller KJ, Turton JF, Mahony J, et al. *Klebsiella pneumoniae*
630 subsp. *pneumoniae*–bacteriophage combination from the caecal effluent of a healthy woman.
631 PeerJ. 2015;doi:10.7717/peerj.1061.
632

633 **FIGURE LEGENDS**

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

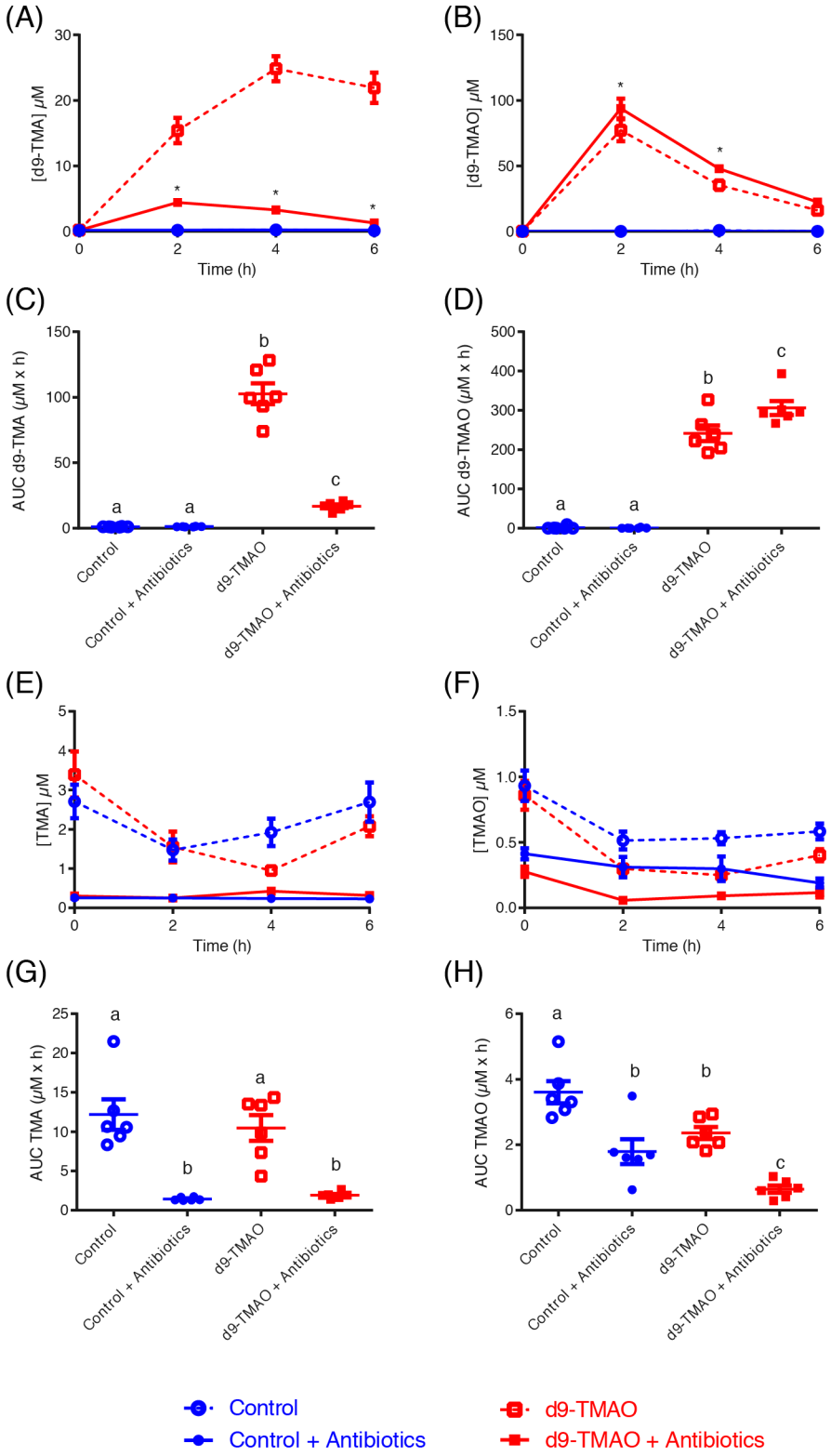
646

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

660

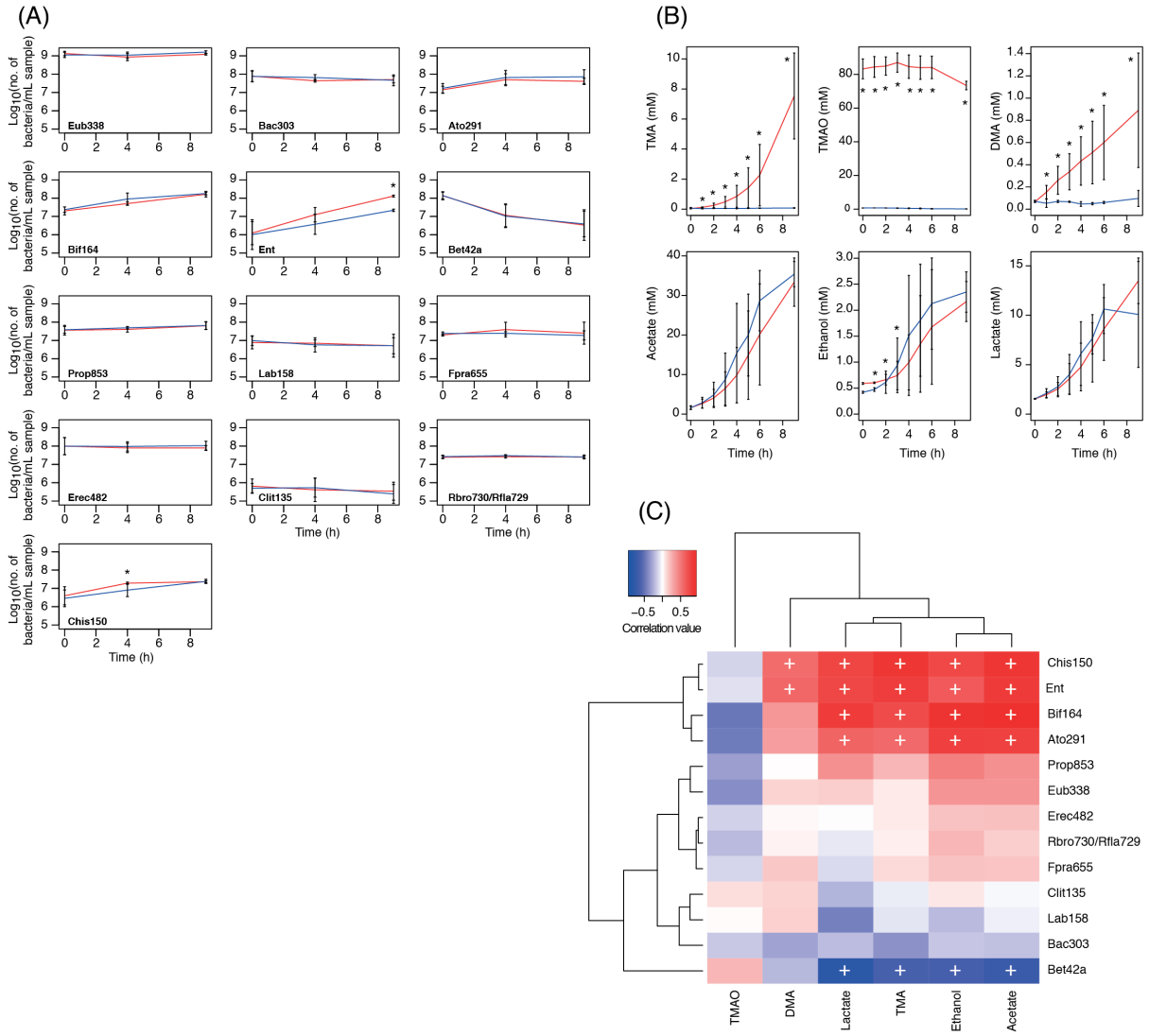
661 **Fig. 3.** Influence of TMAO on growth and metabolism of pure cultures of gut bacteria. (A)
662 Representative growth curves for isolates grown in the presence and absence of TMAO. Red lines,
663 TMAO-supplemented cultures; blue lines, negative controls. Data are shown as mean ± SD (n = 3).
664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

677 **Fig. 1**


(A)
[d9-TMA] μM
Time (h)
(B)
[d9-TMAO] μM
Time (h)
(C)
AUC d9-TMA (μM x h)
Control
Control + Antibiotics
d9-TMAO
d9-TMAO + Antibiotics
(D)
AUC d9-TMAO (μM x h)
(E)
[TMA] μM
Time (h)
(F)
[TMAO] μM
Time (h)
(G)
AUC TMA (μM x h)
(H)
AUC TMAO (μM x h)
Control
Control + Antibiotics
d9-TMAO
d9-TMAO + Antibiotics

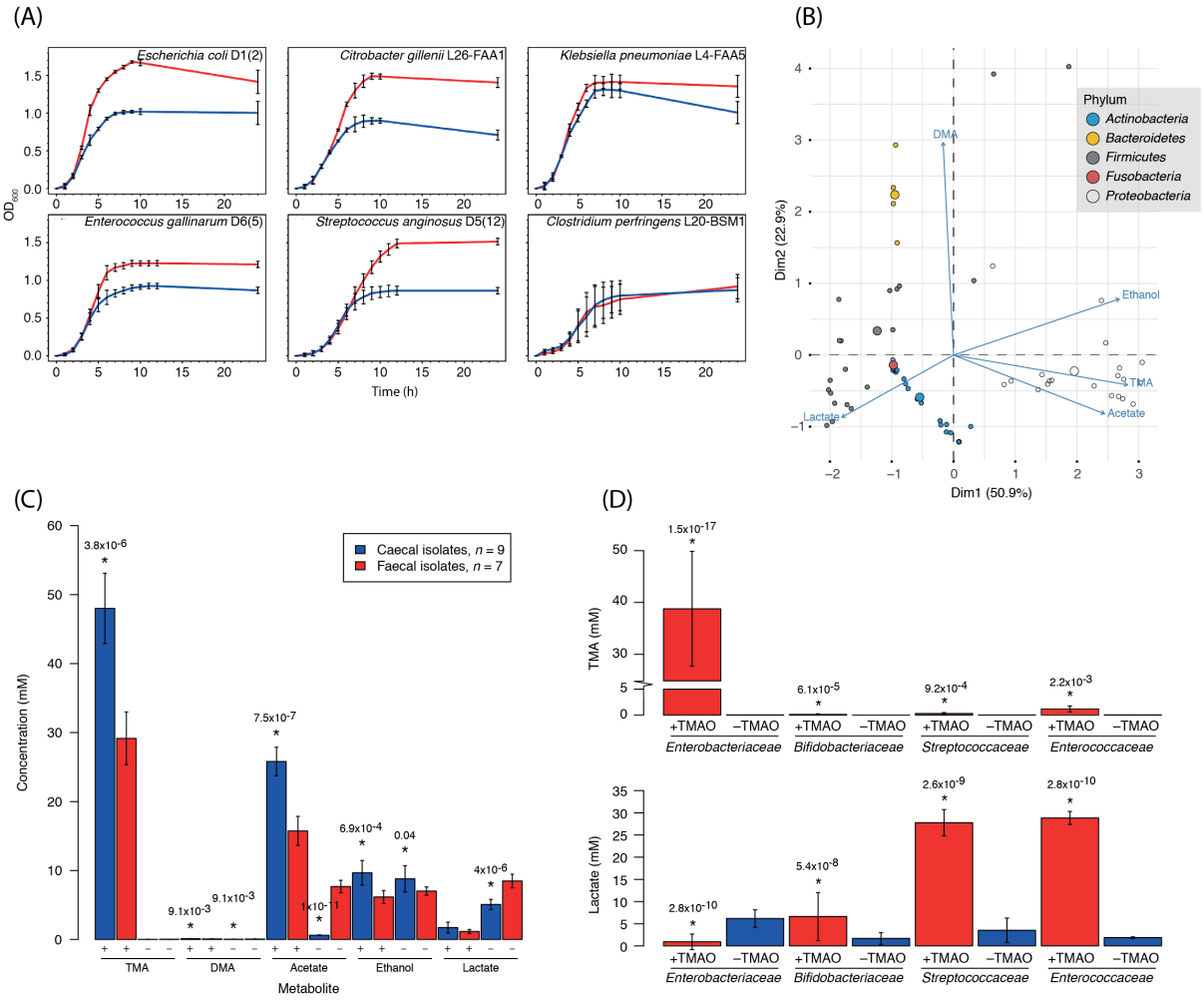
678

679 **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)
−0.5 0.5
Correlation value
Chis150
Ent
Bif164
Ato291
Prop853
Eub338
Erec482
Rbro730/Rfla729
Fpra655
Clit135
Lab158
Bac303
Bet42a
TMAO
DMA
Lactate
TMA
Ethanol
Acetate

680

Fig. 3


(A)
Escherichia coli D1(2)
Citrobacter gillenii L26-FAA1
Klebsiella pneumoniae L4-FAA5
Enterococcus gallinarum D6(5)
Streptococcus anginosus D5(12)
Clostridium perfringens L20-BSM1
OD600
Time (h)
(B)
Phylum
Actinobacteria
Bacteroidetes
Firmicutes
Fusobacteria
Proteobacteria
DMA
Ethanol
TMA
Acetate
Lactate
Dim1 (50.9%)
Dim2 (22.9%)
(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

683 **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	[51]	No
LCR11	*Bifidobacterium bidifum*	Infant faeces	[51]	No
L25-MRS2	*Bifidobacterium bifidum*	Human caecum	L. Hoyles, unpublished	No
LCR5	*Bifidobacterium breve*	Infant faeces	[51]	No
LCR8	*Bifidobacterium breve*	Infant faeces	[51]	No
LCR1	*Bifidobacterium dentium*	Infant faeces	[51]	No
DSM 20093^{T}	*Bifidobacterium gallicum*	Human intestine	DSMZ	No
LCR6	*Bifidobacterium longum*	Infant faeces	[51]	No
DSM 20088^{T}	*Bifidobacterium longum* subsp. *infantis*	Infant intestine	DSMZ	No
LCR2	*Bifidobacterium longum* subsp. *infantis*	Infant faeces	[51]	No
DSM 20219^{T}	*Bifidobacterium longum* subsp. *longum*	Human intestine	DSMZ	No
LCR3	*Bifidobacterium pseudocatenulatum*	Infant faeces	[51]	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	[52]	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	[51]	No

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

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