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Artefactual source of 2-hydroxypyridine

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1 # Artefactual source of 2-hydroxypyridine

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Abstract

As part of the revisions of our original manuscript[1], we performed ^{13}C-labelling experiments with cultures of the microorganism most strongly correlated with 2-hydroxypyridine (2-HP), i.e. the archaeal species *Methanobrevibacter smithii*. Although unlabelled 2-HP was detected in the cultures, the measurements by gas chromatography-mass spectrometry (GC-MS) indicated that *M. smithii* was not the direct source of 2-HP as labelled 2-HP was not measured. Further experiments involving a labelled solvent (deuterated pyridine) and faecal samples from our original study alongside the use of additional analytical platforms and measurements on human blood plasma and mouse brain tissues demonstrate that 2-HP is an artefact of the measurements by GC-MS. It is produced in a sample-specific manner during the derivatisation process for GC-MS by a so far unknown chemical reaction. Our correlative links between archaea (*M. smithii*) and 2-HP remain but, based on these most recent results, cannot be directly mechanistically linked. Apart from this central limitation of our original study, we have so far not uncovered any reasons which would draw into question the validity of our *in vitro* and *in vivo* results linking 2-HP to the observed molecular, behavioural and pathological hallmarks of Parkinson's disease.

61 # Main

62 ## ^{13}C-labelling experiments with *Methanobrevibacter smithii*

63 To address the reviewers' comments of our original manuscript[1], which suggested further
64 experiments to clarify the archaeal source and metabolism of the 2-hydroxypyridine (2-HP)
65 measured in our faecal samples, we performed ^{13}C-labelling experiments with cultures of the
66 microorganism most strongly correlated with this metabolite, i.e. the archaeal species
67 *Methanobrevibacter smithii*, and corresponding measurements by gas chromatography-mass
68 spectrometry (GC-MS). We cultured *M. smithii* under four different conditions: in the presence
69 of either ^{13}C-2 acetate or $^{13}CO_2$, in the presence of both labelled carbon sources, as well as a
70 control condition without labelled carbon sources (Methods). Based on our present knowledge
71 of the metabolism of *M. smithii*, the uptake and metabolism of the ^{13}C-labelled carbon sources
72 was expected to lead to fully or partially labelled 2-HP. However, no labelled 2-HP was
73 detected in samples from these cultures, indicating that 2-HP was not a direct product of
74 metabolism by *M. smithii* (Fig 1a). In agreement with our previous results[1], unlabelled 2-HP
75 was nevertheless detected in the samples (Fig 1b).

76

77 ## Derivatisation for GC-MS

78 GC-MS measurements of complex polar metabolite mixtures require a 2-step derivatisation
79 process[2]. Derivatisation increases the volatility and decreases the polarity of the compounds,
80 enabling gas chromatographic separation and mass spectrometric measurements. First,
81 methoxyamine reacts with carbonyl groups forming oximes to suppress keto-enol tautomerism
82 and formation of multiple acetal or ketal structures. For this reaction, pyridine is used as a
83 solvent for methoxyamine. In a second step, N-trimethylsilyl-N-methyl trifluoroacetamide
84 (MSTFA) is used to displace active hydrogens from carboxylic, hydroxylic, phosphate, amine
85 and thiol groups by trimethylsilyl (TMS) groups.

86

87 It is known that small amounts of 2-HP are formed from pyridine during the derivatisation
88 procedure. We had been aware of this, and the levels that we had reported were always clearly
89 above this low background level as represented by our corresponding extraction blanks
90 (samples that did not contain the biological matrix and were processed in the same manner as
91 test samples; Fig 1c). In our original study, we had performed absolute quantification of 2-HP
92 via the standard addition approach[1]. Application of this method resulted in sample-specific
93 quantities well above the background[1]. Nevertheless, as the lack of labelled 2-HP in the
94 archaeal measurements had raised questions regarding the source of 2-HP in our results, we
95 decided to perform additional experiments to determine whether 2-HP was indeed sample-
96 derived, or a possible artefact of the derivatisation protocol.

97

98 GC-MS measurements using a labelled derivatisation solvent

99 As a way of distinguishing endogenous 2-HP from 2-HP formed during the derivatisation
100 procedure, we tested a modified derivatisation protocol in which we replaced the standard
101 pyridine with deuterated pyridine (Pyr-D5; Methods). Using this approach, 2-HP formed
102 during the derivatisation from pyridine-D5 would be detected as four times deuterated 2-HP
103 (2-HP-D4 1TMS; Fig 2a). Due to the mass shift of 4 Da, we would be able to distinguish
104 between the 2-HP 1TMS derivative (quantification ion m/z 152) and the 2-HP-D4 1TMS
105 derivative (quantification ion m/z 156) by mass spectrometry. First, to test the workflow, we
106 prepared calibration curves of 2-HP so that one batch of samples was derivatised using
107 unlabelled pyridine, and a second batch with Pyr-D5 (Fig 2b-c). The results showed that 2-HP-
108 D4 was present in reproducible amounts when Pyr-D5 was used as the solvent in the first step
109 of the derivatisation procedure (Fig 2d).

110

111 Since the key findings of our manuscript involved 2-HP measurements in human faecal
112 samples, we next measured such samples using the derivatisation with Pyr-D5. Faecal samples
113 as well as calibrants of 2-HP were derivatised and measured in selected ion monitoring mode,
114 which allows precise and accurate quantification of target molecules. Moreover, we selected

115 four faecal samples for standard addition experiments as performed in our original study[1]. As
116 expected, low levels of 2-HP-D4 were detected in reproducible amounts in all extraction blanks
117 (Fig 2e). In contrast, all faecal samples showed much higher levels of 2-HP-D4 (Fig 2e), yet
118 the levels of non-deuterated 2-HP remained below the limit of detection in all tested faecal
119 samples. In other words, all 2-HP seen in the results originated from the Pyr-D5 that was used
120 as the solvent, and sample-derived 2-HP was not detected. Intriguingly, the quantities of 2-HP-
121 D4 varied across the samples in a reproducible pattern, with some samples consistently
122 showing higher levels than others, in agreement with our original results[1].

123

124 2-HP measurements by liquid chromatography-mass spectrometry and 125 nuclear magnetic resonance spectroscopy

126 To verify the 2-HP results in faecal samples, we subsequently developed a liquid
127 chromatography-mass spectrometry (LC-MS)-based method to also measure 2-HP levels in the
128 faecal extracts using this complementary approach in which no derivatisation is needed during
129 sample preparation. An external calibration curve was used to accurately quantify 2-HP levels
130 ranging from 1 to 10 µM (Methods; Fig 3a). Using this method, we did not detect 2-HP in
131 faecal samples (Fig 3b). We additionally used nuclear magnetic resonance (NMR)
132 spectroscopy to further validate these results on independent faecal samples. Here, 2-HP was
133 not detectable in any of the analysed native faecal samples by NMR spectroscopy, further
134 underlining its absence in faecal samples (Fig 3c).

135

136 2-HP measurements in human blood plasma and mouse brain tissues

137 Due to our measurements of 2-HP in human blood plasma samples[1] as well as published reports
138 of 2-HP in human blood[3] and mouse brain tissues[4,5], we further measured 2-HP in plasma
139 samples and samples of mouse brain regions using the two approaches we established (GC-MS
140 with Pyr-D5 instead of pyridine, as well as the LC-MS platform). The results indicated no
141 endogenous 2-HP in plasma samples (only 2-HP-D4 with the GC-MS protocol, and levels

142 below the detection limit with LC-MS; Fig 4a-b). LC-MS measurements of mouse brain
143 samples also showed no 2-HP above the detection limit (Fig 4c), while measurements with the
144 GC-MS protocol resulted in a reproducible pattern whereby some brain regions exhibited
145 consistently higher levels of 2-HP-D4 than others (Fig 4d).

146

147 **Discussion**

148 In summary, none of the samples that we tested contained endogenous 2-HP. Instead, an
149 unknown chemical reaction involving pyridine leads to the formation of 2-HP during the
150 derivatisation procedure. Intriguingly, the quantities of 2-HP formed during the derivatisation
151 are sample-specific and this in a reproducible manner, indicating that its formation is dependent
152 on the features of the sample. In this context, our correlative links between archaea (*M. smithii*)
153 and 2-HP remain but, based on the present results, cannot be directly mechanistically linked,
154 as 2-HP is an artefact of the measurements by GC-MS. Apart from this central limitation of
155 our original study, we have so far not uncovered any reasons which would draw into question
156 the validity of our *in vitro* and *in vivo* results linking 2-HP to the observed molecular,
157 behavioural and pathological hallmarks of Parkinson's disease.

158 # Methods

159 ## Archaeal cultures

160 *Methanobrevibacter smithii* (DSM #861) was cultivated in closed serum bottles containing 50
161 mL minimal medium supplemented with 1 mM sodium sulphate, 2 mM cysteine, CO_2 and H_2
162 (in a ratio of 20% to 80%) and ampicillin to avoid bacterial contamination[6]. The cultures were
163 incubated at 37 °C. For monitoring growth, the turbidity at 600 nm was measured using an
164 Ultraspec 2100 pro Photometer (Amersham Biosciences). Cell numbers were determined using
165 a Thoma cell counting chamber. Cells were harvested by centrifugation at 2,455 x g for 20 min
166 and 4 °C. The cell pellets and the supernatants were separated. The cell pellets were
167 resuspended with 0.9% sodium chloride solution and centrifuged again (21,130 x g, 10 min,
168 4 °C).

169

170 For the labelling experiments, the following four culturing conditions were used:

171 1) With sodium acetate trihydrate (Carl Roth GmbH), and a gas phase consisting of a
172 mixture of labelled $^{13}CO_2$ (carbon-^{13}C dioxide, Sigma-Aldrich) and H_2 (Air Liquide) in
173 a ratio of 20% to 80%,
174 2) with labelled ^{13}C-2 acetate (sodium acetate-2-^{13}C, Sigma-Aldrich), and no labelling in
175 the gas phase (CO_2/H_2, 20%/80%, Air Liquide),
176 3) with both, ^{13}C-2 acetate (Sigma-Aldrich), and a gas mixture of labelled $^{13}CO_2$ (Sigma-
177 Aldrich) and H_2 (Air Liquide) in a ratio of 20% to 80%, and
178 4) control (no labelled substrates) with sodium acetate trihydrate (Carl Roth GmbH), and
179 CO_2/H_2 (20%/80%, Air Liquide).

180

181 For each of the four conditions, a 400 mL culture of *M. smithii* was split into 8 x 50 mL bottles
182 in the anaerobic tent. The eight cultures per condition were grown until mid-exponential phase.
183 For cultures with labelled CO_2, the gas mixture ($^{13}CO_2$ and H_2) was exchanged daily. If ^{13}C-2

184 sodium acetate was used, no other (non-labelled) sodium acetate was added. At the end of each
185 experiment, cell numbers were determined to harvest six replicates of approximately 1 x 10^{10}
186 cells per tube. The collected cells were washed with a 0.9% NaCl solution, centrifuged, and
187 frozen at -80 °C for storage and analysis.

188

189 ## Metabolite extractions

190 *Archaea*

191 Archaeal cells were ground using 500 µL of extraction fluid per 4 x 10^{10} cells. The extraction
192 fluid consisted of a methanolic solution (4:1, methanol/water mixture, v/v, -20 °C) including
193 three internal standards, namely U-13C5 ribitol (c = 10 µg/mL; Omicron Biochemicals),
194 pentanedioic-d6 acid (c = 10 µg/mL; C/D/N Isotopes Inc.) and tridecanoic-d25 acid (c =
195 20 µg/mL; C/D/N Isotopes Inc.). Cell walls were disrupted using 500 mg glass beads (100 µm)
196 and two 30 s cycles at 6,000 rpm (0 to 5 °C, Precellys24 homogeniser) with 30 s pause between
197 the two cycles. Then, 200 µL chloroform and 60 µL MilliQ water were added, and the
198 homogenate was incubated for 10 min at 4 °C and 2,000 rpm. Samples were subsequently
199 centrifuged at 21,000 x g for 5 min (4 °C). 150 µL water and 150 µL chloroform were then
200 added to 570 µL of the supernatant. After intensive mixing, samples were again centrifuged
201 and 250 µl of the upper phase of the biphasic system was transferred into a GC vial with a
202 micro insert. Samples were evaporated at -4 °C for 4 h, followed by an adaptation phase to
203 room temperature for 25 min (Labconco CentriVap) for subsequent derivatisation and GC-MS
204 analysis.

205

206 *Faecal samples*

207 To extract metabolites from stool samples, 500 µL of MilliQ water were added to 50 mg faecal
208 matter. Samples were subsequently homogenised using a Precellys24 homogeniser (Bertin
209 Technologies) using 600 mg ceramic beads (1.4 mm) and one 30 s cycle at 6,000 rpm at 0 to 5
210 °C. The homogenate was centrifuged at 21,000 g for 5 min at 4 °C. Polar metabolites were

211 extracted by transferring 100 µL of the supernatant into a 0.5 mL Eppendorf tube and adding
212 40 µL of internal standard mixture. The internal standard mixture consisted of U-^{13}C5 ribitol
213 (c = 150 µg/mL; Omicron Biochemicals), pentanedioic-d6 acid (c = 150 µg/mL; C/D/N
214 Isotopes Inc.) and caffeine trimethyl-^{13}C3 (c = 100 µg/mL; C/D/N Isotopes Inc.). The
215 metabolite extractions were followed by protein precipitation and a liquid-liquid extraction.
216 First, 80 µL of the particulate-free homogenate were added to 320 µL methanol. The mixture
217 was vortexed for 10 s, then incubated for 5 min at 2,000 rpm and 4 °C (Eppendorf
218 ThermoMixer Comfort), followed by a centrifugation at 21,000 x g for 5 min (4 °C). 350 µL
219 of the protein-free supernatant were then added to 280 µL chloroform and 180 µL MilliQ water.
220 The mixture was vortexed for 10 min at 2,000 rpm and 4 °C. After centrifugation at 21,000 x
221 g for 10 min (4 °C), two aliquots of 200 µL of upper phase containing polar metabolites were
222 transferred into separate GC vials with micro inserts and evaporated at -4 °C for 4 h, followed
223 by an adaptation phase to room temperature for 25 min (Labconco CentriVap). The two
224 aliquots were subsequently analysed by GC-MS using two independent derivatisation
225 workflows. In addition, one aliquot (50 µL) of the upper phase was filtered (PHENEX-RC
226 syringe filter; Phenomenex) and transferred into an amber LC vial with micro insert for
227 subsequent LC-MS analysis.

228

229 *Human plasma samples*

230 To extract metabolites from human plasma samples, 45 µL of MilliQ water-internal standard
231 mixture (4 °C) were added to 110 µL blood plasma. The internal standard mixture consisted of
232 U-13C5 ribitol (c = 150 µg/mL; Omicron Biochemicals), pentanedioic-d6 acid (c =
233 150 µg/mL; C/D/N Isotopes Inc.) and caffeine trimethyl-13C3 (c = 100 µg/mL; C/D/N Isotopes
234 Inc.). 120 µL of the mixture were then added to 480 µL methanol at 4 °C. To achieve complete
235 protein precipitation, samples were incubated for 15 min at 4 °C and 2,000 rpm (Eppendorf
236 ThermoMixer Comfort). After centrifugation at 21,000 x g for 5 min (4 °C), the extract was
237 split. One aliquot (350 µL) underwent a liquid-liquid extraction before GC-MS analysis as
238 described above. The other aliquot (50 µL) was filtered (PHENEX-RC syringe filter;

239 Phenomenex) and transferred into an amber 2 mL glass vial with micro insert for subsequent
240 LC-MS analysis.
241
242 *Mouse brain regions*
243 To extract metabolites from brain regions, samples were first homogenised. Frozen brain
244 samples were transferred into reinforced 2 mL-reaction tubes with screw caps. 600 mg ceramic
245 beads (1.4 mm) were then added to each sample. An equivalent of 4,000 µL extraction fluid
246 per 100 mg tissue was used to process brain samples between 10 to 30 mg, and 2,000 µL per
247 100 mg tissue for brain samples >30 mg tissue, respectively. A fixed volume of 400 µL was
248 used for samples below 10 mg. The extraction fluid consisted of a methanolic solution (4:1,
249 methanol/water mixture, v/v, -20 °C) including three internal standards, namely U-13C5 ribitol
250 (c = 10 µg/mL; Omicron Biochemicals), pentanedioic-d6 acid (c = 10 µg/mL; C/D/N Isotopes
251 Inc.) and caffeine trimethyl-13C3 (c = 0.8 µg/mL; C/D/N Isotopes Inc.). One 30 s cycle at
252 6,000 rpm (0 to 5 °C) was used to grind brain samples, followed by centrifugation at 21,000 x
253 g for 5 min (4 °C). One aliquot (300 µL) underwent a liquid-liquid extraction before GC-MS
254 analysis as described above. At the end of the protocol, 150 µL were dried in a 2 mL glass vial
255 with a micro insert. All brain samples were measured in splitless mode. The other aliquot (50
256 µL) was filtered (PHENEX-RC syringe filter; Phenomenex) and transferred into an amber 2
257 mL glass vial with a micro insert for subsequent LC-MS analysis.
258

259 GC-MS measurements

260 Metabolite derivatisation was performed using a multi-purpose sampler (Gerstel), with one of
261 the following approaches:

262 1) Derivatisation using pyridine (Pyridine anhydrous, 99.8%, Sigma-Aldrich)

263 Dried polar sample extracts were dissolved in 20 µL pyridine, containing 20 mg/mL of
264 methoxyamine hydrochloride (Sigma-Aldrich), and incubated under shaking for 120
265 min at 45 °C. After adding 20 µL N-methyl-N-trimethylsilyl-trifluoroacetamide

266 (MSTFA; Macherey-Nagel), samples were incubated for additional 30 min at 45 °C
267 under continuous shaking.

268 2) Derivatisation using pyridine-D5 (Pyridine D5 99.50%D, Eurisotop)

269 Dried polar sample extracts were dissolved in 20 µL pyridine-D5, containing 20 mg/mL
270 of methoxyamine hydrochloride (Sigma-Aldrich), and incubated under shaking for 120
271 min at 45 °C. After adding 20 µL N-methyl-N-trimethylsilyl-trifluoroacetamide
272 (MSTFA; Macherey-Nagel), samples were incubated for additional 30 min at 45 °C
273 under continuous shaking.

274

275 GC-MS analysis was performed using an Agilent 7890B GC – 5977A MS instrument (Agilent
276 Technologies). A sample volume of 1 µL was injected into a Split/Splitless inlet, operating in
277 split mode (10:1) at 270 °C. The gas chromatograph was equipped with a 5 m guard column +
278 30 m (I.D. 250 µm, film 0.25 µm) DB-35MS capillary column (Agilent J&W GC Column).
279 Helium was used as the carrier gas with a constant flow rate of 1.2 mL/min.

280

281 The GC oven temperature was held at 90 °C for 1 min and increased to 270 °C at 9 °C/min.
282 Then, the temperature was increased to 320 °C at 25 °C/min and held for 7 min. The total run
283 time was 30 min. The transfer line temperature was set constantly to 280 °C. The mass selective
284 detector (MSD) was operating under electron ionisation at 70 eV. The MS source was held at
285 230 °C and the quadrupole at 150 °C. For precise quantification of the selected target analytes
286 and mass isotopomer distribution, measurements were performed in selected ion monitoring
287 mode. Target fragment ions (m/z) and dwell times (ms) are listed in Table 1.

288

289 All GC-MS chromatograms were processed using MetaboliteDetector[7], v3.220190704.
290 MetaboliteDetector was used for mass spectrometric data post processing, quantification, mass
291 isotopomer distribution (MID) calculations, correction for natural isotope abundance and
292 determinations of fractional carbon contributions.

293

294 Compounds were annotated by retention time and mass spectrum using an in-house mass
295 spectral library. The following deconvolution settings were applied: Peak threshold: 2;
296 Minimum peak height: 2; Bins per scan: 10; Deconvolution width: 2 scans; No baseline
297 adjustment; Minimum 15 peaks per spectrum; No minimum required base peak intensity. The
298 internal standards (U-^{13}C5-ribitol and pentanedioic-d6 acid) were added at the same
299 concentration to every sample to correct for uncontrolled sample losses, and analyte
300 degradation during metabolite extraction and sensitivity drifts during measurements. The
301 dataset was normalized by using the response ratio of the integrated peak area of the analyte
302 and the integrated peak area of the internal standard (pentanedioic-d6 acid 2TMS, m/z 267).

303

304 LC-MS measurements

305 As an independent analytical platform for the detection of 2-HP, additional measurements were
306 performed using an Exion LC coupled to a 7500 Triple quad MS (SCIEX) equipped with an
307 Optiflow Pro Ion Source. The ion source was operated in electrospray ionisation mode.
308 Chromatography was performed using a Waters Acquity UPLC BEH C18 (130Å, 1.7 µm, 2.1
309 mm x 150 mm) column protected by a VanGuard pre-column (2.1 mm x 5 mm). The column
310 temperature was maintained at 40 °C. The autosampler was kept at 4 °C.

311

312 The mobile phases consisted of water + 0.1% formic acid (eluent A) and acetonitrile + 0.1%
313 formic acid (eluent B). The flow rate was set to 0.3 mL/min. The LC method consisted of 1
314 min isocratic delivery of 5% eluent B, a 11 min linear gradient to 95% eluent B and 6 min
315 isocratic delivery of 95% eluent B followed by a re-equilibration phase on starting conditions
316 at 5% eluent B for 7 min. The injection volume was 5 µL.

317

318 Target compounds were measured in multiple reaction monitoring mode. Specific transitions
319 of each target analyte are provided in Table 2. The source and gas parameters applied were as
320 follows: ion source gas 1 and 2 were maintained at 35 psi and 50 psi, respectively. The curtain
321 gas was at a pressure of 40 psi, CAD gas at 10 and source temperature was held at 550 °C.
322 Spray voltage was set to 2,000 V in positive ion mode.

323

324 Mass spectrometric data were acquired with SCIEX OS (Version 3.0.0) and analysed with
325 MultiQuant (Version 3.0.3). Target compounds were identified by retention time and ion ratio.
326 In addition, the identity of all targets was confirmed by MS/MS. The data was normalized by
327 using the response ratio of the integrated peak area of target compound and the integrated peak
328 area of the internal standard (caffeine trimethyl-^{13}C3).

329

330 **NMR measurements**

331 Faecal samples (40-60 mg) were suspended in 400 µL of ice-cold methanol and 200 µL of
332 MilliQ H_2O and transferred to Precellys tubes with 1.4 mm diameter zirconium oxide beads
333 (Bertin Technologies). Additional samples with different concentrations of 2-HP spiked into
334 the faecal samples were prepared with 10 µM as the lowest concentration. The resulting
335 suspension was homogenised two times for 20 s by Precellys24 tissue homogeniser (Bertin
336 Technologies) at 25 °C. The homogenised samples were then centrifuged at 8,700 x g for 30
337 min at 4 °C. The supernatants were transferred to new tubes for metabolomic analyses.
338 Supernatants were lyophilised at < 1 Torr, 850 rpm, 25 °C for 10 h in a vacuum-drying chamber
339 (Savant Speedvac SPD210 vacuum concentrator), with an attached cooling trap (Savant
340 RVT450 refrigerated vapor trap) and vacuum pump (VLP120; Thermo Scientific). Samples
341 were then re-dissolved in 500 µL of NMR buffer containing 0.08 M Na_2HPO_4, 5 mM 3-
342 trimethylsilyl propionic acid-2,2,3,3,-d_4 sodium salt (TSP) and 0.04 (w/v)% NaN_3 in D_2O,
343 adjusted to 7.4 pH with 8 M HCl and 5 M NaOH.

344

345 The prepared metabolite extracts were measured at 310 K using a 600 MHz Bruker Avance
346 Neo NMR spectrometer equipped with a TXI 600S3 probe head. The Carr–Purcell– Meiboom–
347 Gill (CPMG) pulse sequence was used to acquire 1D ^{1}H NMR spectra with a pre-saturation for
348 water suppression (cpmgpr1d, 128 scans, 73728 points in F1, 12019.230 Hz spectral width,
349 recycle delay 4 s).

350

351 The data were processed in Bruker Topspin version 4.0.2 using one-dimensional exponential
352 window multiplication of the FID, Fourier transformation, and phase correction. The NMR
353 data were then imported into Matlab2014b, TSP was used as the internal standard for chemical-
354 shift referencing (set to 0 ppm).

355

356 Signals for 2-HP were detectable by NMR down to the lowest concentration tested (10 μM).
357 To verify if 2-HP is detectable in other faecal samples, we re-evaluated a previously obtained
358 NMR data set[8], as well as more recent, so far unpublished data. 2-HP was not detected in any
359 of these independent faecal samples.

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386 # Tables

387

388 **Table 1. Target fragment ions and dwell times for gas chromatography-mass spectrometry**
389 **measurements.**

Derivative (m/z of selected fragments for quantification)	Fragment-Ions (m/z)	Dwell Time (ms / for each ion)
2-Hydroxypyridine(-D4) 1TMS (152, 156)	152.1 – 158.1	15
2-Hydroxypyridine(-D4) 1TMS	166.1 – 173.1	10
Internal Standard: Pentanedioic-d6 acid 2TMS (267)	206.1, 239.1, 267.1	50
Internal Standard: U-13C5 Ribitol 5TMS (220)	220.1, 310.1, 323,1	50

390

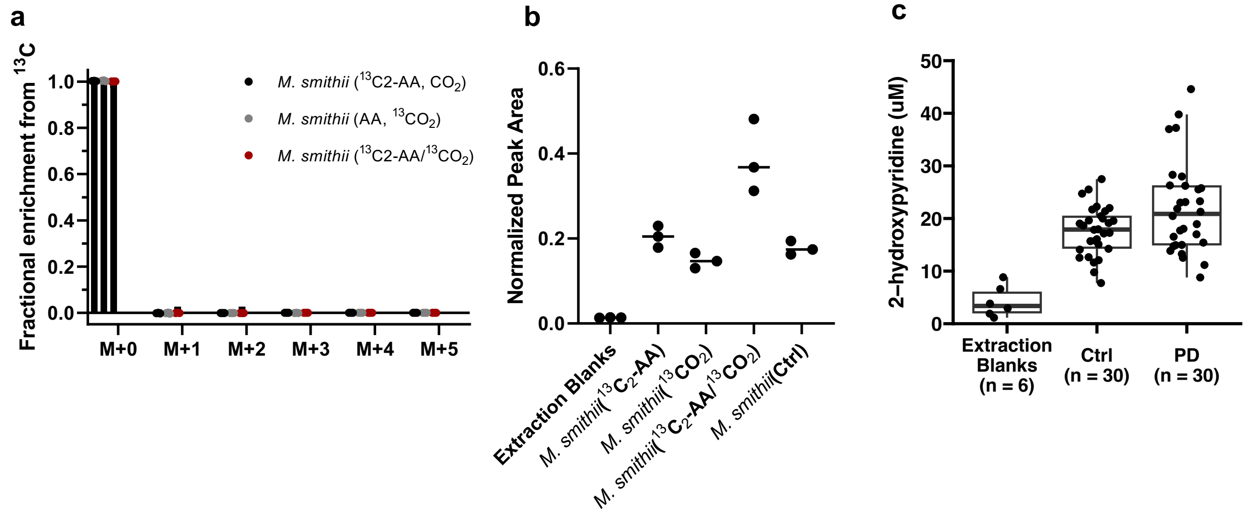
391 **Table 2. Mass transitions and compound dependent source parameters for liquid**
392 **chromatography-mass spectrometry measurements.**

Ionisation mode	Q1 (m/z, Da)	Q3 (m/z, Da)	ID	Dwell time (ms)	EP (Volts)	CE (Volts)	CXP (Volts)
POS.	198.1	140.02	IS_Caffeine_POS - 198.1-140.02	50	10	26	13
POS.	198.1	112.02	IS_Caffeine_POS - 198.1-112.02	50	10	30	6
POS.	96.016	78.01	2-Hydroxypyridine_POS - 96.01-78.01	50	10	18	10
POS.	96.016	50.99	2-Hydroxypyridine_POS - 96.01-50.99	50	10	27	15

393

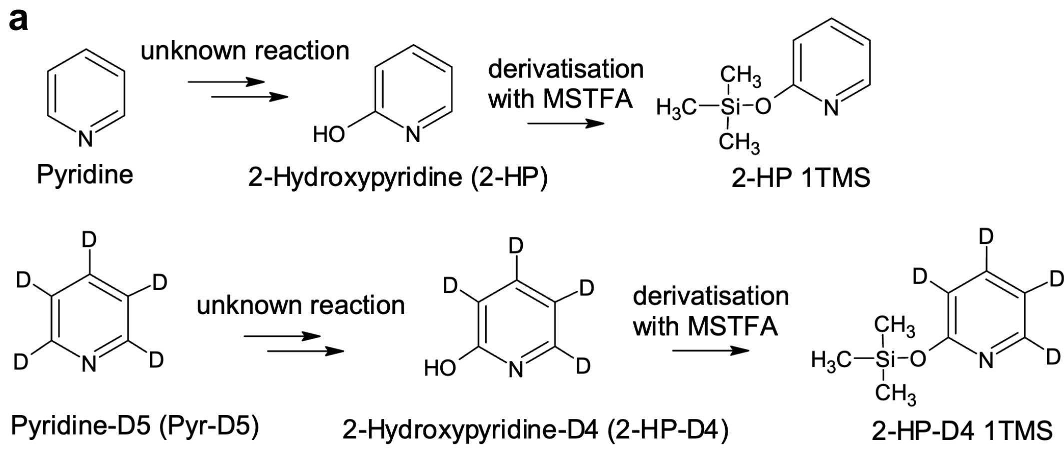
394 # Figures

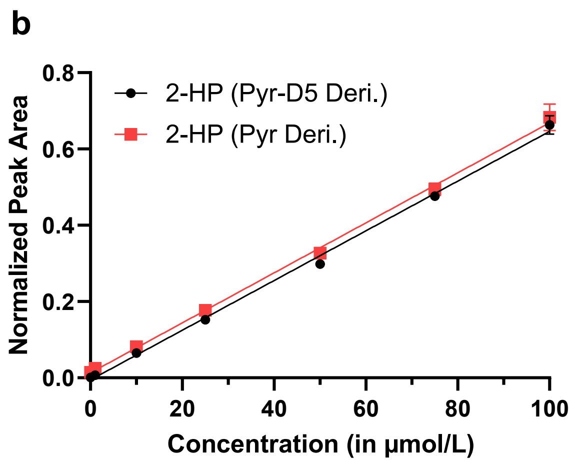
395

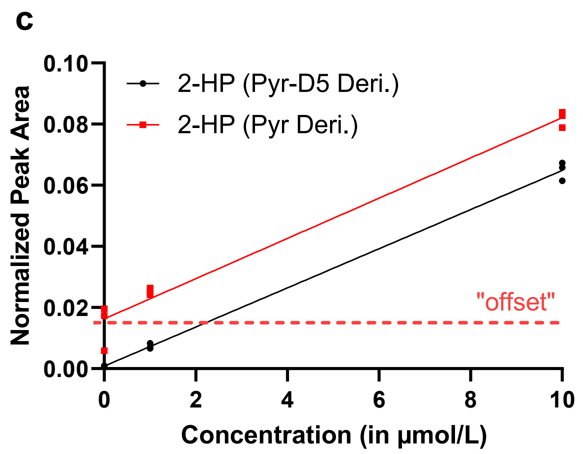


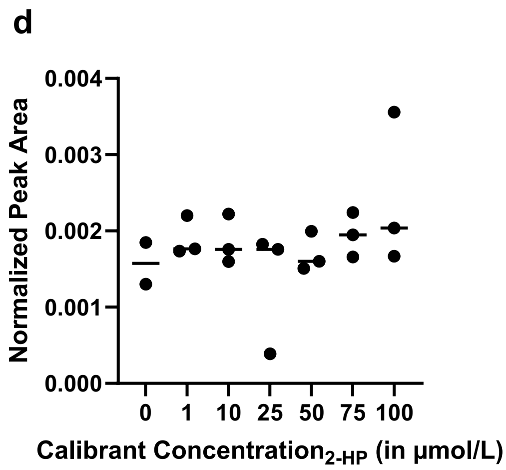
396

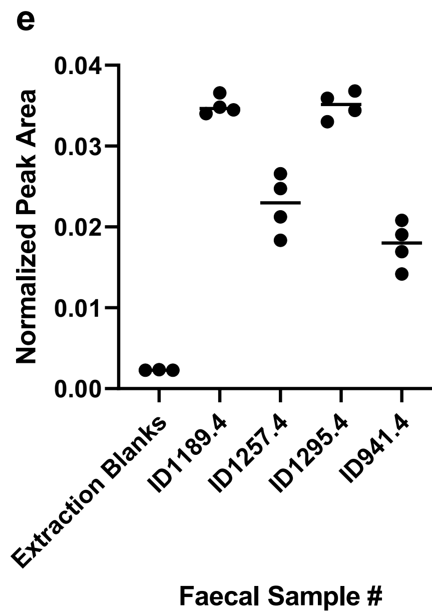
397 **Figure 1: Measurements of 2-hydroxypyridine (2-HP) in archaeal cultures and faecal samples. a.**
398 Fractional enrichment in 2-HP from cultures of *M. smithii* grown on ^{13}C2-acetic acid (^{13}C2-AA) and
399 CO_2, acetic acid (AA) and $^{13}CO_2$, and ^{13}C2-acetic acid and $^{13}CO_2$ as carbon source. **b.** Quantification of
400 unlabelled 2-HP in *M. smithii* cell extracts. **c.** Absolute quantification of 2-HP in human faecal samples
401 (Ctrl = control subject, PD = Parkinson's disease patient), also showing the background level detected
402 in extraction blanks. Box hinges: 1st and 3rd quartiles; line: median; whiskers: hinge to highest/lowest
403 values that is within 1.5*IQR of hinge.
404


a
unknown reaction
Pyridine
2-Hydroxypyridine (2-HP)
derivatisation with MSTFA
2-HP 1TMS
Pyridine-D5 (Pyr-D5)
2-Hydroxypyridine-D4 (2-HP-D4)
2-HP-D4 1TMS


b
2-HP (Pyr-D5 Deri.)
2-HP (Pyr Deri.)
Normalized Peak Area
Concentration (in µmol/L)

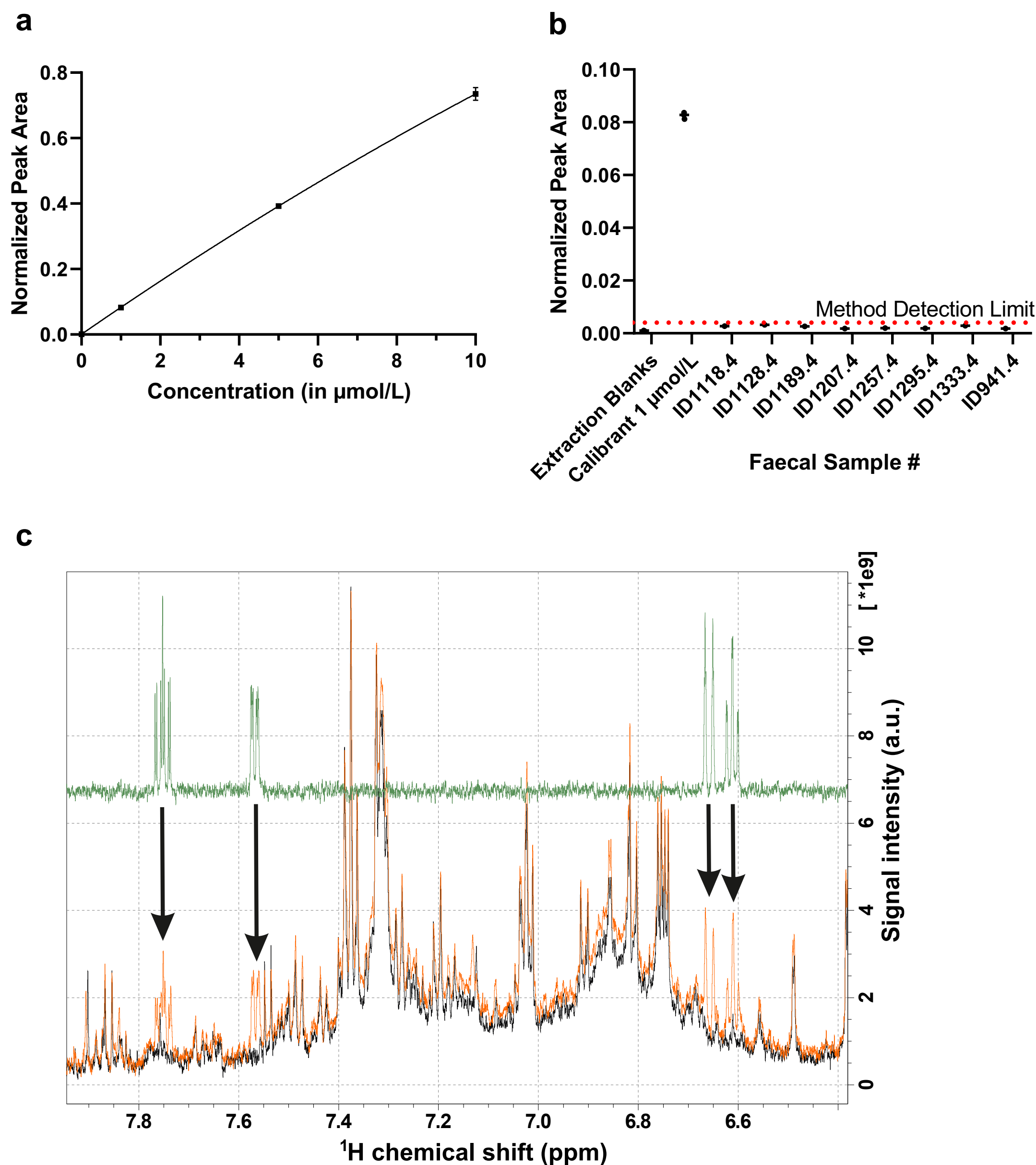

c
2-HP (Pyr-D5 Deri.)
2-HP (Pyr Deri.)
"offset"
Normalized Peak Area
Concentration (in µmol/L)


d
Normalized Peak Area
Calibrant Concentration2-HP (in µmol/L)


e
Normalized Peak Area
Extraction Blanks
ID1189.4
ID1257.4
ID1295.4
ID941.4
Faecal Sample #

406 **Figure 2: 2-Hydroxypyridine (2-HP) and derivatisation for gas chromatography-mass**
407 **spectrometry. a.** 2-HP formation during derivatisation with methoxyamine in pyridine (top) or
408 pyridine-D5 (bottom) followed by silylation with N-trimethylsilyl-N-methyl trifluoroacetamide,
409 MSTFA. **b.** External calibration curve of 2-hydroxypyridine. Pyridine or pyridine-D5 was used as
410 solvent during the first step of the derivatisation process. The calibrated range was from 1 to 100
411 µmol/L. Mean±SEM. **c.** The additional formation of 2-HP during the derivatisation ("offset") results
412 in a shift of the calibration curve. **d.** Reproducible levels of 2-HP-D4 were detected for each calibration
413 point (calibrant) using pyridine-D5 as solvent in the derivatisation process. **e.** 2-HP-D4 levels in selected
414 faecal samples used for the standard addition experiments with 2-HP.

415

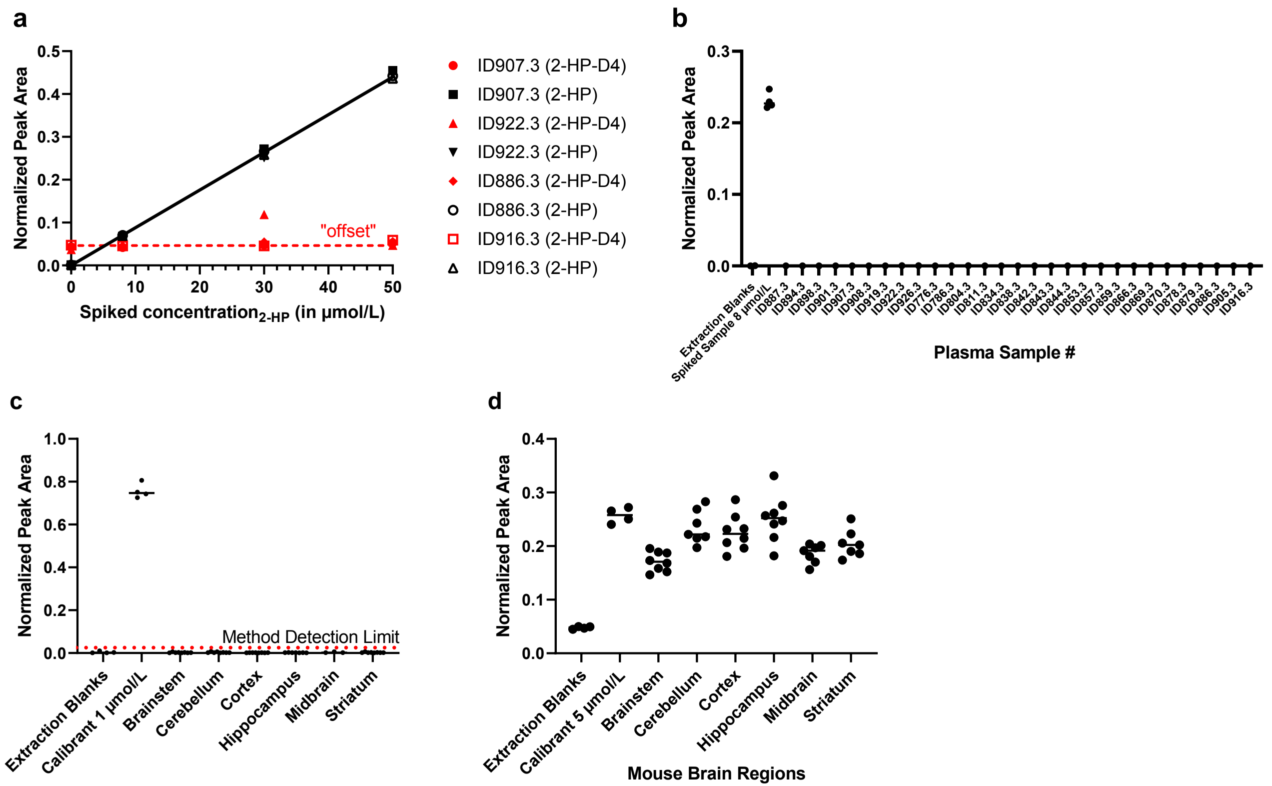


416

417 **Figure 3: Measurements of 2-hydroxypyridine (2-HP) by liquid chromatography-mass**
418 **spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy. a.** 2-HP calibration
419 curve, calibrated range: 1 to 10 µmol/L (LC-MS). Mean±SEM. **b.** 2-HP quantification in faecal samples
420 by LC-MS. **c.** NMR analysis of 2-HP. The overlay shows a representative faecal 1D ^{1}H NMR spectrum
421 (black) with the spectrum of the same stool sample spiked with 100 µM of 2-hydroxypyridine. The 1D
422 ^{1}H NMR spectrum of the 2-HP standard is shown in green and the ^{1}H resonances characteristic for 2-
423 HP are indicated by arrows.

424

425



426

427 **Figure 4: Measurements of 2-hydroxypyridine (2-HP) in human blood plasma samples and mouse**
428 **brain regions. a.** Spiked human blood plasma samples using pyridine-D5 as solvent for the first step
429 of the derivatisation for gas chromatography-mass spectrometry (GC-MS) measurement. **b.** Liquid
430 chromatography-mass spectrometry (LC-MS) measurements of 2-HP in human blood plasma. **c.** LC-
431 MS measurements of 2-HP in mouse brain regions. **d.** GC-MS measurements of pyridine-D5-derived
432 2-HP-D4 in mouse brain regions.

433

434 # References

435 1. Trezzi, J.-P. *et al.* An archaeal compound as a driver of Parkinson's disease pathogenesis.
436 Preprint at https://www.researchsquare.com/article/rs-1827631/v1 (2022).

437 2. Trezzi, J.-P. *et al.* Metabolic profiling of body fluids and multivariate data analysis.
438 *MethodsX* **4**, 95–103 (2017).

439 3. Chen, L. *et al.* Influence of the microbiome, diet and genetics on inter-individual variation
440 in the human plasma metabolome. *Nat. Med.* **28**, 2333–2343 (2022).

441 4. Jaeger, C. *et al.* The mouse brain metabolome. *Am. J. Pathol.* **185**, 1699–1712 (2015).

442 5. Ding, J. *et al.* A metabolome atlas of the aging mouse brain. *Nat. Commun.* **12**, 6021 (2021).

443 6. Ehlers, C., Veit, K., Gottschalk, G. & Schmitz, R. A. Functional organization of a single *nif*
444 cluster in the mesophilic archaeon *Methanosarcina mazei* strain Gö1. *Archaea* **1**, 143–150
445 (2002).

446 7. Hiller, K. *et al.* MetaboliteDetector: Comprehensive analysis tool for targeted and
447 nontargeted GC/MS based metabolome analysis. *Anal. Chem.* **81**, 3429–3439 (2009).

448 8. Kumpitsch, C. *et al.* Reduced B12 uptake and increased gastrointestinal formate are
449 associated with archaeome-mediated breath methane emission in humans. *Microbiome* **9**,
450 193 (2021).

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