

1 **Parkinson's disease-associated alterations of the gut microbiome can**
2 **invoke disease-relevant metabolic changes**

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4 Federico Baldini^{1*}, Johannes Hertel^{2,3*}, Estelle Sandt⁴, Cyrille C. Thinnès^{1,2}, Lorieza
5 Neuberger-Castillo⁴, Lukas Pavelka^{1,5}, Fay Betsou⁴, Rejko Krüger^{1,5,6}, Ines Thiele^{1,2,7} # on
6 behalf of the NCER-PD Consortium

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8 * These authors contributed equally.

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10 ¹ Luxembourg Centre for Systems Biomedicine, University of Luxembourg, Campus Belval,
11 Esch-sur-Alzette, Luxembourg.

12 ² School of Medicine, National University of Ireland, Galway, Ireland.

13 ³ Department of Psychiatry and Psychotherapy, University Medicine Greifswald, Greifswald,
14 Germany.

15 ⁴ Integrated BioBank of Luxembourg, 1, Rue Louis Rech, 3555 Dudelange, Luxembourg

16 ⁵ Parkinson Research Clinic, Centre Hospitalier de Luxembourg (CHL), Luxembourg

17 ⁶ Transversal Translational Medicine, Luxembourg Institute of Health (LIH), Luxembourg

18 ⁷ Discipline of Microbiology, School of Natural Sciences, National University of Ireland,
19 Galway, Ireland.

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21 # email: ines.thiele@nuigalway.ie

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25 **ABSTRACT**

26

27 Parkinson's disease (PD) is a systemic disease clinically defined by the degeneration of
28 dopaminergic neurons in the brain. While alterations in the gut microbiome composition have
29 been reported in PD, their functional consequences remain unclear. Herein, we first analysed
30 the gut microbiome of patients and healthy controls by 16S rRNA gene sequencing of stool
31 samples from the Luxembourg Parkinson's study (n=147 typical PD cases, n=162 controls).
32 All individuals underwent detailed clinical assessment, including neurological examinations
33 and neuropsychological tests followed by self-reporting questionnaires. Second, we predicted
34 the potential secretion for 129 microbial metabolites through personalised metabolic modelling
35 using the microbiome data and genome-scale metabolic reconstructions of human gut
36 microbes. Our key results include: 1. eight genera and nine species changed significantly in
37 their relative abundances between PD patients and healthy controls. 2. PD-associated microbial
38 patterns statistically depended on sex, age, BMI, and constipation. The relative abundances of
39 *Bilophila* and *Paraprevotella* were significantly associated with the Hoehn and Yahr staging
40 after controlling for the disease duration. In contrast, dopaminergic medication had no
41 detectable effect on the PD microbiome composition. 3. Personalised metabolic modelling of
42 the gut microbiomes revealed PD-associated metabolic patterns in secretion potential of nine
43 microbial metabolites in PD, including increased methionine and cysteinylglycine. The
44 microbial pantothenic acid production potential was linked to the presence of specific non-
45 motor symptoms and attributed to individual bacteria, such as *Akkermansia muciniphila* and
46 *Bilophila wardsworthia*. Our results suggest that PD-associated alterations of gut microbiome
47 could translate into functional differences affecting host metabolism and disease phenotype.

48

49

50 INTRODUCTION

51 Parkinson's Disease (PD) is a complex multifactorial disease, with both genetic and
52 environmental factors contributing to the evolution and progression of the disease (Kalia et al.
53 2015). While several studies have elucidated the role of genetic factors in the pathogenesis of
54 the disease (Kitada et al. 1998; Bonifati et al. 2003; Paisan-Ruiz et al. 2004; Di Fonzo et al.
55 2009), the role and the contribution of various environmental and lifestyle factors are still not
56 completely understood (Gatto et al. 2010). Importantly, about 60% of the PD patients suffer
57 from constipation (Fasano et al. 2015), which can start up to 20 years before the diagnosis and
58 is one of the prodromal syndromes (Savica et al. 2009; Cersosimo et al. 2013).

59 The human being is considered to be a superorganism recognising a complex interplay
60 between the host and microbes (Sleator 2010). For instance, the human gut microbiome has
61 been shown to complement the host with essential functions (trophic, metabolic, protective)
62 and to influence the host's central nervous system (CNS) via the gut-brain axis through the
63 modulation of neural pathways and GABAergic and serotonergic signalling systems
64 (Carabotti et al. 2015).

65 Recent studies have reported an altered gut composition in PD (Hasegawa et al. 2015;
66 Keshavarzian et al. 2015; Scheperjans et al. 2015; Bedarf et al. 2017; Hill-Burns et al. 2017;
67 Hopfner et al. 2017; Petrov et al. 2017; Heintz-Buschart et al. 2018; Barichella et al. 2019).
68 One of these studies has been conducted using samples from recently diagnosed, drug-naive
69 patients (Bedarf et al. 2017). These studies have demonstrated that PD patients have an altered
70 microbiome composition, compared to age-matched controls. However, the functional
71 implications of the altered microbiome remain to be elucidated, e.g., using animal models
72 (Sampson et al. 2016). A complementary approach is computational modelling, or constraint-
73 based reconstruction and analyses (COBRA) (Orth et al. 2010), of microbiome-level
74 metabolism. In this approach, metabolic reconstructions for hundreds of gut microbes

75 (Magnusdottir et al. 2017) are combined based on microbiome data (Baldini et al. 2018;
76 Heirendt et al. 2019)). Flux balance analysis (FBA) (Orth et al. 2010) is then used to compute,
77 e.g., possible metabolite uptake or secretion flux rates of each microbiome model (microbiome
78 metabolic profile) (Heinken et al. 2019) or to study of microbial metabolic interactions (cross-
79 feedings) (Klitgord and Segre 2010; Heinken and Thiele 2015). This approach has been applied
80 to various microbiome data sets to gain functional insights (Thiele et al. 2018; Heinken et al.
81 2019; Hertel et al. in revision), including for PD where we propose that microbial sulphur
82 metabolism could contribute to changes in the blood metabolome of PD patients (Hertel et al.
83 in revision).

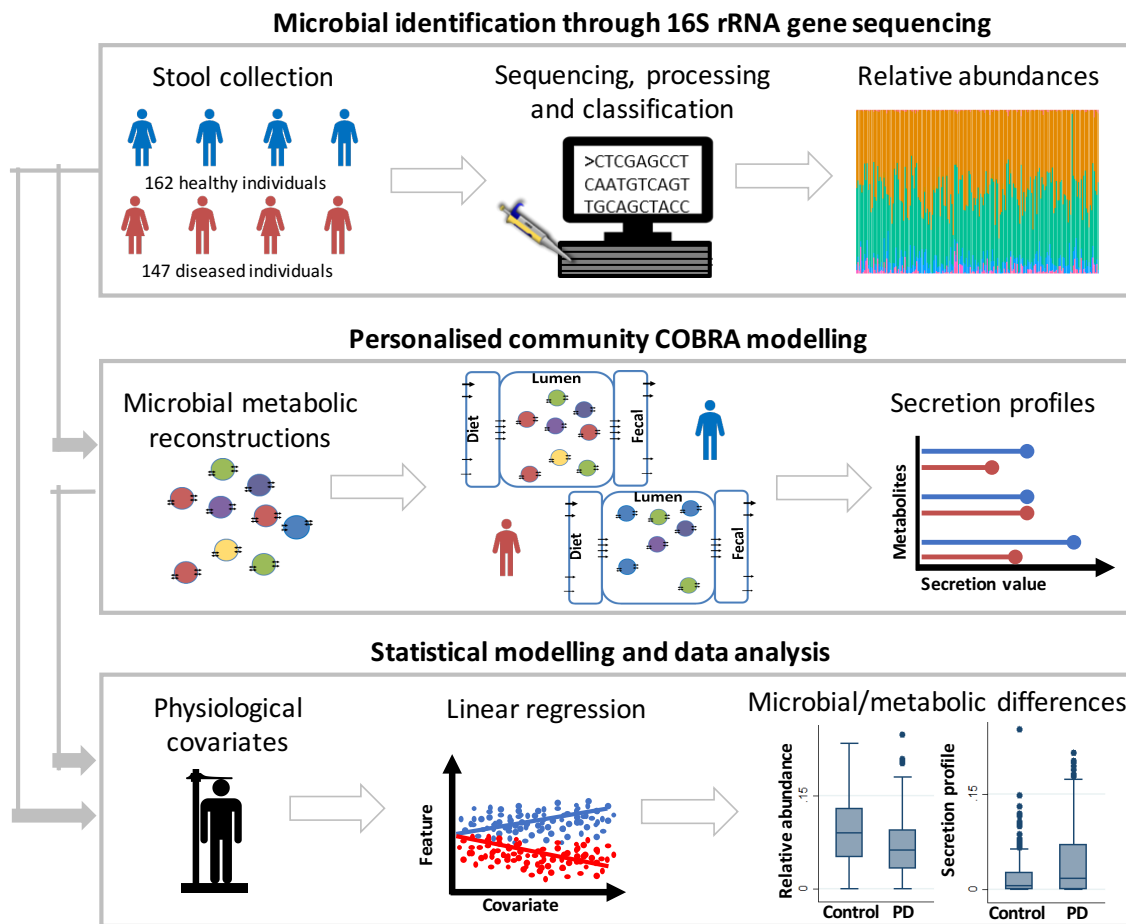
84 In the present study, we aim at investigating microbial changes associated with PD
85 while focusing on possible covariates influencing microbial composition and at proposing
86 functional, i.e., metabolic, consequences arising from the microbiome changes. First, we
87 analysed the faecal microbial composition of PD patients and controls from the Luxembourg
88 Parkinson's study (Hipp et al. 2018) (Figure 1). Second, based on the observed significant
89 differences in the composition of microbial communities between PD patients and controls, we
90 created and interrogated personalised computational models representing the metabolism of
91 each individual's microbial community. We demonstrate that the combined microbial
92 composition and functional metabolite analysis provides novel hypotheses on microbial
93 changes associated with PD and disease severity, enabling future mechanism-based
94 experiments.

95

96 **RESULTS**

97 The Luxembourg Parkinson's Disease study includes patients with typical PD and
98 atypical parkinsonism, as well as matched healthy control subjects from Luxembourg and its
99 neighbouring regions from a broad age-range (Hipp et al. 2018). For the present study, we

100 focused on typical PD patients and healthy controls over the age of 50 (Table 1, Methods).
101 Stool samples were analysed for 147 PD patients and 162 controls using 16S rRNA gene
102 sequences (Methods: Analysis of the microbial composition with 16S rRNA gene sequencing).
103



104

105 **Figure 1: Overview of the study approach and the key methods used.** Relative abundances
106 were derived from 16S rRNA gene sequences (Methods: Analysis of the microbial composition
107 with 16S rRNA gene sequencing) and used as input for the personalised community modelling
108 to simulate metabolites secretion profiles. Relative abundances and secretion profiles were
109 statistically analysed to identify microbial or metabolic differences between PD patients and
110 controls.

111

112

Variable	PD	Control	Missing values, in %		Genera influenced by PD-covariate interaction effects (FDR<0.05)	Genera associated with trait (up/down, FDR<0.05)	NMPCs associated with trait (up/down, FDR<0.05)
			PD	Control			
Cases vs. Controls	147	162	0%	0%	--	<i>Anaerotruncus</i> , <i>Christensenella</i> , <i>Lactobacillus</i> , <i>Streptococcus</i> , <i>Akkermansia</i> , <i>Bilophila</i> , <i>Turicibacter</i>	D-alanine, Oxalate, D-Mannitol, Cysteinylglycine, L-Methionine, L-alanine, D-Ribose, 4Hydroxybenzoic acid, Uracil,
Sex (female subjects)	31.5 %	35.8%	0%	0%	<i>Paraprevotella</i>	--	--
Age at basic assessment ^a	69.3 ± 8.6	63.3 ± 8.3	0%	0%	<i>Anaerotruncus</i> , <i>Roseburia</i>	--	Phosphate, Glycine
Body mass index ^a	27.3 ± 4.5	27.9 ± 4.8	0.7%	0%	<i>Paraprevotella</i>	<i>Victivallis</i>	--
Sniff score ^a	7.1 ± 3.4	12.7 ± 2.1	0%	0%	--	--	--
Metabolic diabetes	4.1 %	3.1%	0%	0%	--	--	--
Non-motor symptoms questionnaire score ^a	9.3 ± 5.1	3.9 ± 3.9	9.5%	3.7%	--	---	Pantothenate
Constipated	36.7%	6.2 %	0%	0%	<i>Bifidobacterium</i>	<i>Bifidobacterium</i>	Xanthine, D-Alanine, Pantothenate, L-Lactate, D-Ribose
PD disease duration	5.9 ± 5.7	--	6.1%	--	--	<i>Lactobacillus</i>	--
UPDRS-part I	10.0 ± 5.9	4.5 ± 4.4	3.4%	3.1%	--	--	--
UPDRS-part II	11.8 ± 8.1	1.3 ± 2.8	1.4%	2.4%	--	--	--
UPDRS-part III	34.6± 16.1	2.3± 2.9	1.4%	0%	--	<i>Peptococcus</i> , <i>Flavonifractor</i> , <i>Paraprevotella</i>	--
UPDRS-part IV	1.7 ± 3.2	--	1.4%	--	--	--	--
Hoehn and Yahr	2.2 ± 0.6	--	0%	--	--	<i>Bilophila</i> , <i>Paraprevotella</i>	--
L-DOPA intake	66.7%	0%	0%	0%	--	--	--
Dopamine agonist intake	56.5%	0%	0%	0%	--	--	--
MAO-B COMT inhibitors intake	41.5%	0%	0%	0%	--	--	--

113 **Table 1: Descriptive statistics of the analyses sample from the Luxembourg Parkinson's**

114 **Disease study and overview over associations.** A red label means increased in PD, blue
115 decreased in PD, while -- “nothing to report”. PD disease duration refers to time since diagnosis
116 at the date of stool sampling. UPDRS=Unified Parkinson Rating Scale, L-DOPA=levodopa,
117 MAO-B=monoaminoxidase B, COMT=Catecholamine-Methyl-Transferase, NMPC=Net
118 maximal production capability.

119

120 **Species and genus level changes in PD microbiomes**

121 We investigated disease-associated microbial changes at the species level. We found
122 that the mean species diversity (i.e., the alpha-diversity) did not significantly differ between
123 PD cases and controls (b=-0.04351, 95%-CI:(-.107;0.177), p=0.177), in agreement with earlier
124 studies (Scheperjans et al. 2015; Bedarf et al. 2017) (Hopfner et al. 2017), but in disagreement

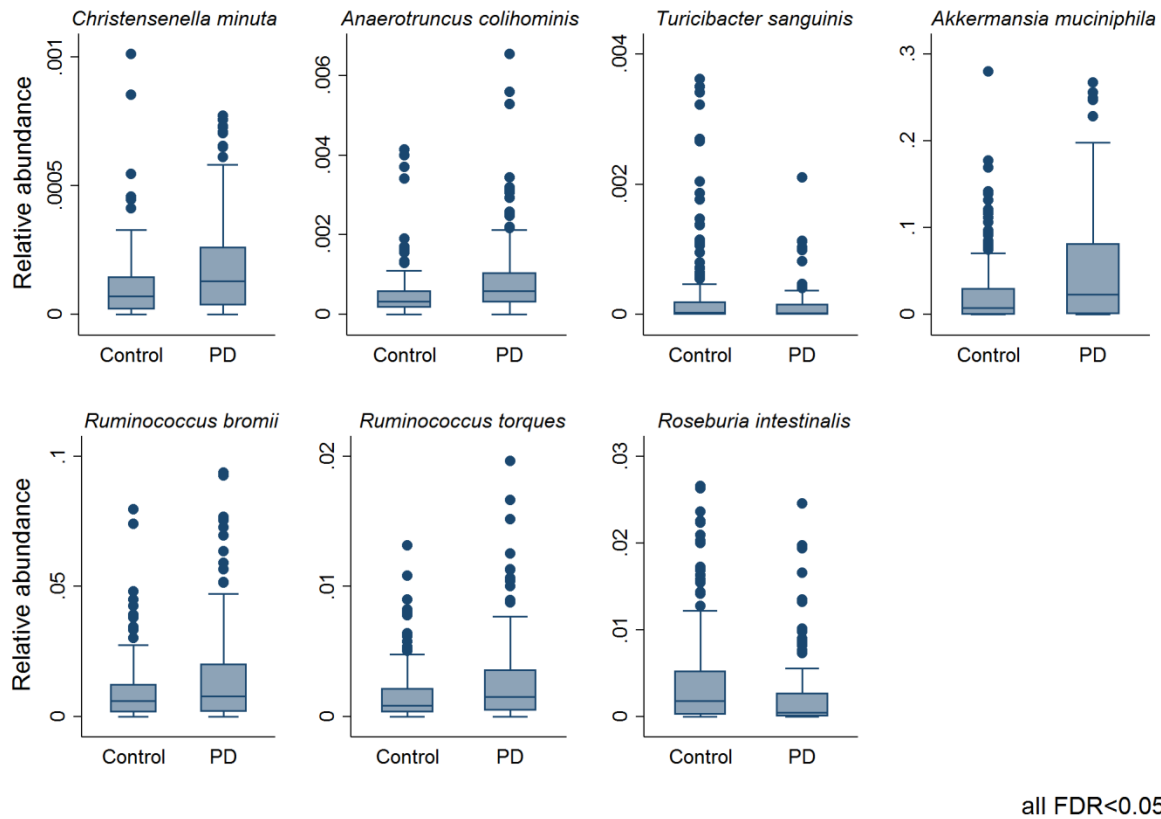
125 with two other studies (Keshavarzian et al. 2015; Heintz-Buschart et al. 2018). However, seven
126 species were significantly altered in PD (FDR<0.05, Figure 2). Note that when comparing
127 results between different taxonomic levels, changes observed for *Ruminococcus* and *Roseburia*
128 species were not significant on the genus level but only on the species level, highlighting the
129 importance of species-level resolution. The highest effect size was associated with
130 *Akkermansia muciniphila* (Odds ratio (OR)=1.80, 95%-CI=(1.29, 2.51), p=6.02e-04,
131 FDR<0.05; Supplementary Table 1) in agreement with the previously reported higher
132 abundance of *A. muciniphila* in PD patients (Bedarf et al. 2017; Heintz-Buschart et al. 2018)).
133 Subsequently, we examined possible differences at the genus level by performing
134 semiparametric fractional regressions while adjusting for age, sex, the body mass index (BMI),
135 batch, and total read counts. We identified eight genera to be significantly increased in PD
136 (FDR<0.05; Figure 3A, Table 1), with *Lactobacillus* showing the highest effect size (Odds
137 ratio (OR)=5.75, 95%-CI=(2.29, 14.45), p=1.96e-04, FDR<0.05; Supplementary Table 2). In
138 contrast, the genera *Turicibacter* decreased significantly in PD cases (FDR<0.05). To
139 summarise, significant changes could be observed on the species and genus level.

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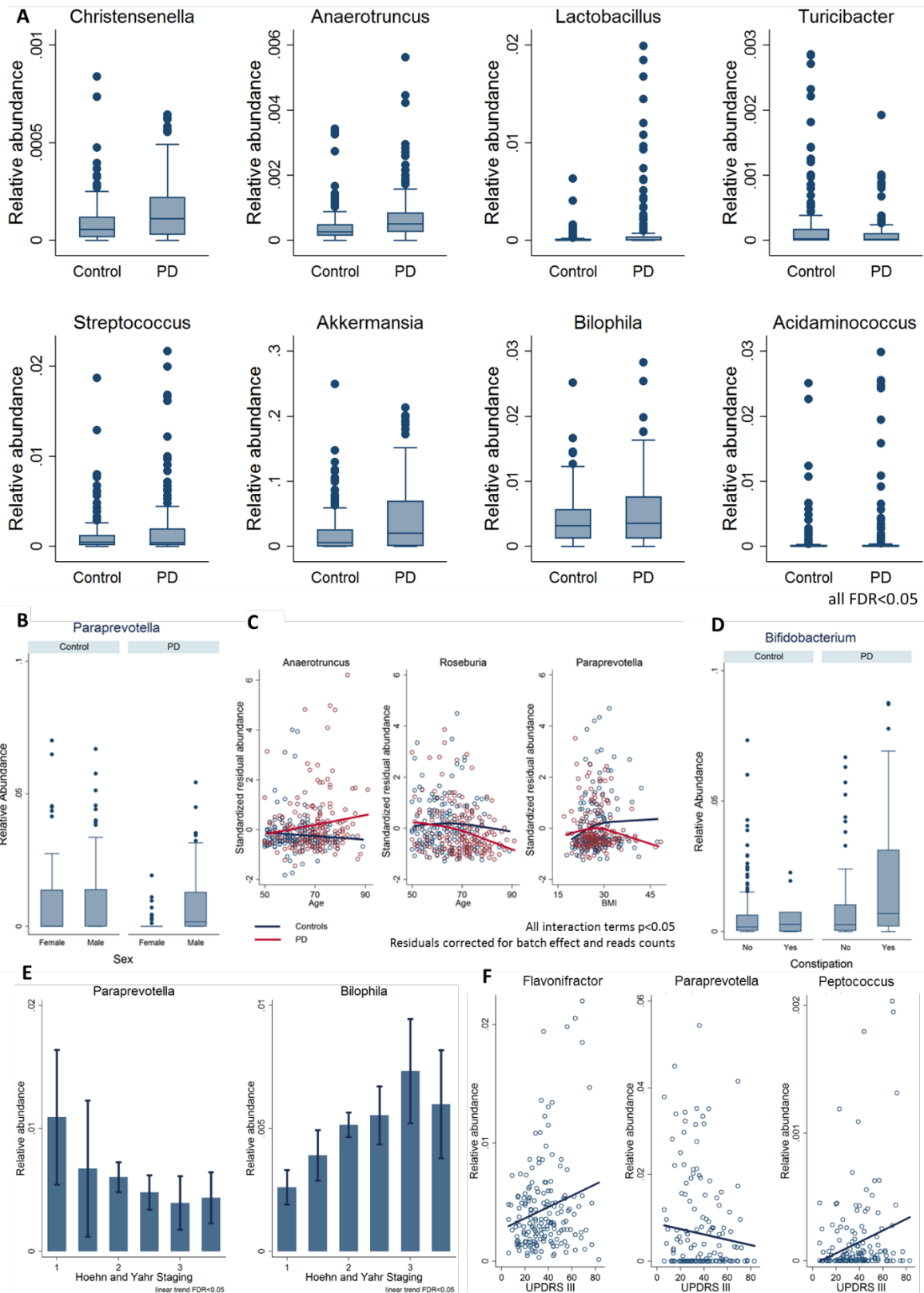
141 **PD modifies the effects of basic covariates on the microbiome**

142 Furthermore, we investigated whether the genus level alterations in PD were affected
143 by basic confounding factors. This interaction analyses uncovered rich effect modifications,
144 revealing that microbiome changes in PD have to be considered in the context of age, BMI,
145 and gender. Our analyses demonstrate that the effects of PD are not homogeneous among
146 important sub-groups of patients. For example, *Paraprevotella* was exclusively reduced in
147 female patients but not in female controls (Figure 3B), highlighting gender-dependent
148 alterations of microbial communities in PD. In addition, the effects of BMI and age were
149 modified in PD cases. The PD cases had increased *Anaerotruncus* abundance with age, while

150 non-linear, overall decreasing abundances of *Roseburia* and *Paraprevotella* were observed
151 with age and BMI, respectively (Figure 3C). Taken together, these analyses suggest that
152 microbial abundances are shifted in PD cases and that also the effects of important covariates
153 were altered in PD, reflecting the systemic and complex nature of PD.
154



155
156 **Figure 2: Boxplots of seven significantly changed species in PD versus controls**
157 (FDR<0.05). Significance levels were determined using multivariable semi-parametrical
158 fractional regressions with the group variable (PD vs. control) as predictor of interest, including
159 age, gender, BMI, and technical variables (i.e., total read-counts and batch effect) as covariates.
160 FDR=false discovery rate.



161

162 **Figure 3: Genus alterations in PDs due to interactions with basic covariates. Relative**

163 abundance is given on a logarithmic scale. **A.** Boxplots of the seven significant species

164 (**FDR<0.05**). **B.** Female PD patients have a reduced abundance of *Paraprevotella* (**FDR<0.05**).

165 C. Genus abundance age and BMI dependencies of *Anaerotruncus*, *Roseburia*, and
166 *Paraprevotella* (global test on all interaction terms, FDR<0.05). For graphical assessment of
167 the interaction terms the z-transformed residual abundances are displayed after correction for
168 technical covariates (batch and read counts). D. The genus relative abundance of
169 *Bifidobacterium* was increased in patients reporting to be constipated (FDR<0.05). E. Genus
170 association with disease staging showed a decrease of relative abundance of *Paraprevotella*
171 and an increase of *Bilophila* genus over increasing Hoehn and Yahr scale values (FDR<0.05).
172 F. An increased score in motor symptoms (UPDRS III) was associated with an increased trend
173 in abundances of *Flavonifractor* and *Peptococcus* and a decreased trend in *Paraprevotella*
174 abundance (FDR<0.05). UPDRS=Unified Parkinson Rating Scale, BMI=body mass index,
175 FDR=false discovery rate.

176

177 **Microbial abundances, medication intake, and constipation in PD**

178 The Luxembourg Parkinson's study enrolls patients of all stages of PD. Therefore, the
179 patients have considerable inter-individual variance in PD-related features, such as constipation
180 and intake of medication (Table 1). We analysed whether these features had an impact the
181 microbiome composition in PD. In our data, we could not find any evidence for an effect of the
182 three medication types on the microbiome, i.e., levodopa, COMT inhibitors, or MAO-B
183 inhibitors, when correcting for multiple testing (Supplementary Table 2). In contrast,
184 constipation, a prevalent non-motor symptom in PD patients (Lesser 2002), was associated
185 with an increased abundance of *Bifidobacterium*, with a clear effect in constipated PD cases
186 (Figure 3D). However, since there were only ten constipated controls (Table 1), these results
187 must be confirmed in larger cohorts.

188

189

190 **Genus association with the disease severity**

191 We next investigated whether the stage of the disease, i.e., defined by Hoehn and Yahr
192 staging, NMS, and UPDRS (Unified Parkinson Rating Scale) scores, and its subscales, was
193 associated with altered genus abundance. For the Hoehn and Yahr staging, *Paraprevotella*
194 showed a negative association and *Bilophila* showed a positive association, both of which were
195 significant after multiple testing (Figure 3E). For the UPDRS III subscale score (i.e., motor
196 symptoms, Table 1), three genera, being *Peptococcus*, *Flavonifractor*, and *Paraprevotella*,
197 survived correction for multiple testing (Figure 3F). In contrast, the other UPDRS subscales
198 and the NMS were not significantly associated with microbial changes, after correction for
199 multiple testing. Note that these analyses were performed while adjusting for disease duration.
200 When analysing the association pattern of disease duration, we found *Lactobacillus* positively
201 correlated with the disease duration (FDR<0.05, Supplementary Figure S1). In conclusion, our
202 data suggest that the microbial composition may be utilised as a correlate of disease severity.

203

204 **Metabolic modelling reveals distinct metabolic secretion capabilities of PD microbiomes**

205 To obtain insight into the possible functional consequence of observed microbiome
206 changes in PD, we used metabolic modelling (cf. Methods). Briefly, we mapped each of the
207 309 microbiome samples on the generic microbial community model consisting of 819 gut
208 microbial reconstructions (Magnusdottir et al. 2017; Heinken et al. 2019) (cf. Supplementary
209 Material) to derived personalised microbiome models (Baldini et al. 2018). We then computed
210 a net maximal production capability (NMPC) for 129 different metabolites that could be
211 secreted by each microbial community model (cf. Methods), providing thereby a
212 characterisation of the differential microbial metabolic capabilities in PDs and controls. The
213 secretion of nine metabolites had differential NMPCs in PD (Figure 4A, all FDR<0.05) as
214 determined by multivariable regressions adjusting for age, sex, BMI, and technical covariates.

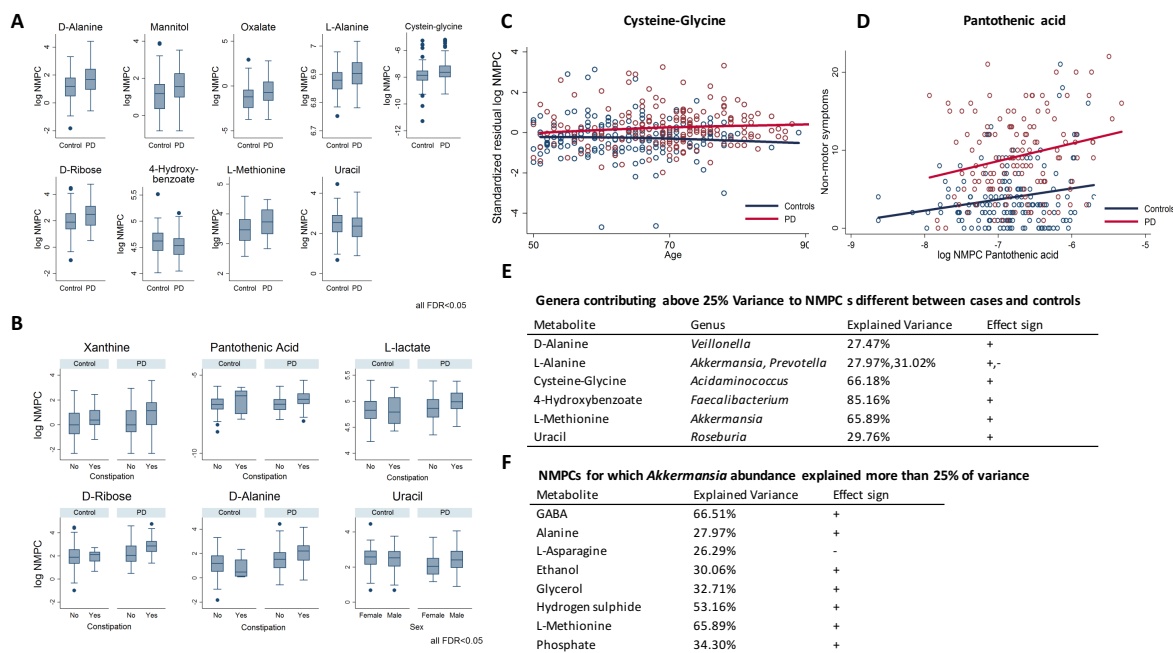
215 Moreover, although less dominant in comparison to the abundance data, PD-covariate
216 interactions were also prevalent, with the uracil secretion potential showing a sex-specific
217 effect and cysteine-glycine showing a BMI-dependent PD-effect (Figure 4B, 4C). In
218 subsequent analyses, we tested for associations of the NMPCs with constipation, medication,
219 disease duration, Hoehn-Yahr staging, NMS, and UPDRS III scores, complementing thereby
220 the analyses on the abundance level. Notably, we found xanthine, D-alanine, L-lactic acid, D-
221 ribose, and pantothenic acid positively associated with constipation (Figure 4B), while no
222 NMPC was associated with medication or with disease duration. However, the pantothenic acid
223 secretion potential was positively associated with higher NMS scores, interestingly both in PD
224 and in controls (Figure 4D), while no NMPC survived correction for multiple testing regarding
225 associations with the UPDRS III score and Hoehn-Yahr staging. To conclude, these results
226 suggest that the altered microbial composition in PD could result in broad changes in metabolic
227 capabilities, which manifested themselves additionally in non-motor symptoms and
228 constipation.

229

230 **PD specific secretion profiles were altered due to changed community structure and** 231 **species abundances**

232 Next, we analysed which microbes contributed to the differential secretion profiles by
233 correlating the NMPCs to the abundance data (Figure 4E/F, Supplementary Table 3). Six
234 metabolite NMPCs had strong contribution or were even dominated by single genera (Figure
235 4D), while for the other four NMPCs no single dominant genus could be identified. We then
236 computed the contribution value of each genus to the production of each secreted metabolite
237 (NMPC). From the aforementioned genera, which were associated on genus or species level
238 with PD, only *Akkermansia*, *Acidaminococcus*, and *Roseburia* had substantial metabolic
239 contributions (over 25%). *Acidaminococcus* was responsible for 64% of the variance in

240 cysteine-glycine production and *Roseburia* for 30% of the variance in uracil production
 241 potential. *Akkermansia* impacted the secretion profiles the most and substantially contributed
 242 to the metabolism of nine metabolites (Figure 4F), including the neurotransmitter gamma-
 243 aminobutyric acid (GABA) and two sulphur species, being hydrogen sulphide and methionine.
 244 GABA was also significantly altered between PD and controls on a nominal level missing FDR
 245 corrected significance narrowly ($b=0.18$, 95%-CI:(0.06;0.30), $p=0.003$, FDR=0.0501). These
 246 analyses demonstrate the added value of metabolic modelling to investigate altered metabolic
 247 functions from the whole microbial composition.
 248



249 **Figure 4: Result of analysing secretion profiles of microbial communities.** **A.** Box plots for
 250 NMPCs differential between cases and controls with FDR<0.05. **B.** NMPCs with sex-specific
 251 PD signature or constipation effects (all FDR<0.05). **C.** Differential age trajectory between
 252 cases and controls for cysteine-glycine ($p<0.05$). **D.** Association of pantothenic acid with non-
 253 motor symptoms. **E.** Genera contributing more than 25% to NMPCs different between cases
 254 and controls. **F.** *Akkermansia* contribution to community production of 12 metabolites
 255 expressed as a percentage of total production for each compound. Metabolites highlighted in
 256

257 red were significantly increased in PD (FDR<0.05). NMPC=net maximal production capacity,
258 GABA=gamma-aminobutyrate, H2S=hydrogen sulphide, FDR=false discovery rate. Effect
259 sign “-“: negative correlation. Effect sign “+“: positive correlation.

260

261 **DISCUSSION**

262 In this study, we aimed to elucidate compositional and functional changes in the faecal
263 microbiome of PD patients. Therefore, we analysed 16S rRNA data from a cohort of typical
264 PD patients (n=147) and controls (n=162), and performed personalised microbial
265 computational modelling. We identified i) eight genera and nine species that changed
266 significantly in their relative abundances between PD patients and healthy controls. ii) PD-
267 associated microbial patterns that were dependent on sex, age, BMI, constipation, and iii) in
268 PD patients altered secretion potentials, particularly in sulphur metabolism, using metabolic
269 modelling of microbial communities. Overall, our work demonstrated compositional and
270 functional differences in the gut microbial communities of Parkinson’s disease patients
271 providing novel experimentally testable hypothesis related to PD pathogenesis.

272 The microbial compositional analyses of our cohort identified significantly different
273 microbial abundance distributions between PD patients and healthy controls (Table 1). Up to
274 date, 13 studies have described altered colonic microbial compositions associated with PD and
275 an overall picture starts to arise (Figure 5). For instance, the microbial the families of
276 Verrucomicrobiaceae and Lactobacillaceae have been consistently found to have an increased
277 abundance in PD (Figure 5). In accordance, our study also reports increased abundance in PD
278 of Akkermansia, Christensenella, and Lactobacillus. Similarly, Bifidobacteria has also been
279 repeatedly associated with PD (Figure 5) but in our study, we could show that the
280 Bifidobacteria association dependent on constipation (Figure 3) highlighting the need for
281 incorporating disease-specific phenotypes as covariates into the statistical design.

		Bedarf, 2017	Barichella, 2019	Hasegawa, 2015	Heintz-Buschart, 2018	Hill-Burns, 2017	Hopfner, 2017	Keshavarzian, 2015	Li, 2017	Lin, 2018	Petrov, 2017	Qian, 2018	Scheperjans, 2015	Unger, 2016	Current study
Study size	PD cases	31*	193	52	76	197	29	38	14	75	89	45	72	34	147
	Controls	28	191	36	78	130	29	34	24	45	66	45	72	34	162
Method	Shotgun	x													
	16S rRNA (region)		V3-V4		V4	x	V1-V2	V4	V3-V4;V4-V5	x	V3-V4	V3-V4	V1-V3		V3-V4
	Quantitative PCR			x										x	
Family	Bacteroidaceae			b											
	Bifidobacteriaceae										c				
	Christensenellaceae										d				d
	Clostridiaceae			e							f	g			h
	Comamonadaceae											i			
	Coriobacteriaceae														
	Enterobacteriaceae														
	Enterococcaceae														
	Erysipelotrichaceae												j		
	Lachnospiraceae														
	Lactobacillaceae			k								k			k
	Pasteurellaceae														
	Prevotellaceae														
	Ruminococcaceae											l			m
	Sphingomonadaceae												n		
	Streptococcaceae														o
Verrucomicrobiaceae		p			p									p	

282

283 **Figure 5: Reported microbial changes at the family level associated with PD in different**

284 **studies.** Only those bacterial families are shown, for which significant associations with

285 species or genera have been reported in at least two studies comparing stool samples from

286 patients and controls. Red - increased in PD, Blue - decreased in PD. a: *Actinomycetales*, b:

287 *Bacteroides fragilis*, c: *Bifidobacterium*, d: *Christensenella*, e: *Clostridium coccooides/ leptum*,

288 f: *Faecalibacterium*, *Dorea*, g: *Clostridium IV/XVIII*, *Butyricoccus*, *Anaerotruncus*, h:

289 *Anaerotruncus*, i: *Aquabacterium*, j: *Holdemania*, k: *Lactobacillus*, l: *Oscillospira*, m:

290 *Ruminococcus romii*, *Ruminococcus torques*, n: *Sphingomonas*, o: *Streptococcus*, p:

291 *Akkermansia*. * Drug-naive, de novo PD patients only. Based on (Barichella et al. 2019).

292

293 At the same time, inconsistencies between the studies remain and they may be due to

294 differences in study design, inclusion criteria, faecal sampling, RNA extraction protocols, and

295 metagenomic and statistical methods. For instance, we used a relatively large, PD cohort while

296 Bedarf and colleagues (Bedarf et al. 2017) studied a small cohort of drug-naïve, male PD
297 patients and male controls (Figure 5). Three studies included individuals of Chinese descent
298 (Li et al. 2017; Lin et al. 2018; Qian et al. 2018) while the other studies focused on Caucasian
299 individuals. It has been shown that microbial composition is associated with ethnic
300 background, geography, and dietary habits (Turnbaugh et al. 2008; De Filippo et al. 2010;
301 Yatsunenko et al. 2012), which may explain some of the discrepancies. The differences
302 between the studies hence highlight the importance of performing meta-analysis to identify
303 global microbial signatures, as it has been done for, e.g., colorectal cancer (Wirbel et al. 2019).
304 Such meta-analysis may also permit to investigate subgroups of PD, as the number of cases
305 and controls would be substantially increased and thus provide higher statistical power. For
306 instance, we observed various effect modulators that were not reported before in humans (Table
307 1), such as *Paraprevotella* abundance reduction being specific to women. This result is
308 apparently in contradiction with findings from Bedarf and colleagues (Bedarf et al. 2017) who
309 reported decreased levels of Prevotellaceae in a cohort of only male PD patients. However,
310 once again, differences might be explained by different inclusion criteria, methodologies, and
311 related possible sex-specific effects. Interestingly, a recent study reported a higher abundance
312 of *Paraprevotella* in male mice compared to female mice (Huang et al. 2018). Despite the lack
313 of extensive studies on gender-specific differences in microbiome composition, we suggest that
314 machine learning procedures on microbiome data should be performed in a sex-stratified
315 manner. Larger cohorts, e.g., through meta-analysis of published cohorts would allow the
316 identification of generalizable microbial differences in PD patients and also, specific microbial
317 changes associated with certain traits and physiological characteristics, as suggested by our
318 data.

319 We could not detect an effect of the dopaminergic, PD specific medication on the
320 microbiome composition, after correction for multiple testing. Also the fact that key findings

321 from the study of Bedarf and colleagues were reproduced in other cohorts of PD patients under
322 medication, including ours, support that notion. Nonetheless, in previous studies, Dorea and
323 Phascolarctobacterium genera have been negatively associated with levodopa equivalent doses
324 (Qian et al. 2018) and members of the family of *Bacillaceae* have been correlated with
325 levodopa treatment (Heintz-Buschart et al. 2018). Consequently, it cannot be excluded that
326 medication is associated with microbial changes, albeit the association may be weaker than the
327 effects of other covariates. As PD drugs are often taken in combinations, it would require a
328 larger sample size than used in our study to permit the investigation of all possible drug
329 combinations. The lack of clear association is somewhat expected as levodopa is absorbed in
330 the upper part of the small intestine (Streubel et al. 2006) and thus small intestinal rather than
331 large intestinal microbes may play a more prominent role in levodopa bioavailability.
332 Consistently, a recent study showed that bacterial tyrosine decarboxylases restrict the
333 bioavailability of levodopa (van Kessel et al. 2019). Interestingly, 193/818 reconstructed
334 microbes (Magnusdottir et al. 2017), commonly found in the human gut, carry genes encoding
335 for proteins that convert levodopa into dopamine (Noronha et al. 2019). Levodopa is always
336 given with decarboxylase inhibitors, such as carbidopa or benserazide, targeting the human
337 decarboxylases, but it cannot be excluded that they also act on the microbial counterpart.
338 However, Van Kessel et al. have shown that carbidopa as well as benserazide is only a weak
339 inhibitor of the microbial tyrosine decarboxylase (van Kessel et al. 2019).

340 We identified a positive association of *Bilophila* abundance with the Hoehn and Yahr
341 staging, which captures motor impairment and disability independent of disease duration.
342 Indeed, the abundance of *Bilophila* was not associate with disease duration, indicating mainly
343 dependency on the progression of symptoms. This finding is consistent with experimental mice
344 studies demonstrating the pro-inflammatory effect of *Bilophila* overgrowth (Devkota et al.
345 2012; Natividad et al. 2018). Notably, the Hoehn and Yahr staging was also positively

346 associated on a nominal level with the predicted pyruvate secretion profile (Supplementary File
347 4), which was accordingly significantly increased in PD patients on a nominal level alongside
348 with L- and D-alanine. *Bilophila* has the rare capability to use taurine, an inhibitory
349 neurotransmitter with neuroprotective effects (Saransaari and Oja 2007; Wu et al. 2009), as an
350 energy source (Laue and Cook 2000). This pathway is initiated by the taurine: pyruvate
351 aminotransferase (Laue and Cook 2000), converting pyruvate and taurine into L-alanine and
352 sulfoacetaldehyde. The only microbe of the 818 species in our AGORA collection encoding
353 the corresponding gene was *Bilophila*, which was significantly increased (FDR<0.05) and
354 hence, the corresponding reaction (VMH ID: TAURPYRAT) was increased in abundance in
355 PD microbiomes as well. In a previous study (Hertel et al. in revision), we have shown that
356 blood taurine conjugated bile acids were positively associated with motor symptoms. *Bilophila*
357 may be a marker of disease progression in PD, and it could modulate human sulphur
358 metabolism through its taurine degradation capabilities. Alterations in sulphur metabolism
359 have been already described when using computational modelling of microbiomes from a
360 cohort of early diagnosed and levodopa naive PD patients (Bedarf et al. 2017; Hertel et al. in
361 revision) as well as an increased concentration of methionine and derived metabolites in blood
362 samples (Hertel et al. in revision). Furthermore, we and others have reported alterations in bile
363 acids and taurine-conjugated bile acids in PD patients (Graham et al. 2018; Hertel et al. in
364 revision). Our present study suggests again a key role of *Bilophila* in host-microbiome sulphur
365 co-metabolism, which may link with bile acid metabolism.

366 Interestingly, an increased abundance of *B. wadsworthia* has been linked to constipation
367 (Vandeputte et al. 2017). *B. wadsworthia* is the only microbe in the AGORA collection capable
368 of the metabolic reaction converting pyruvate and taurine to L-alanine and sulphoacetaldehyde
369 (VMH ID: TAURPYRAT). Therefore, an increased production of L-alanine might be due to
370 the increased *B. wadsworthia* abundance. This resulting higher production rate of L-alanine

371 could then lead to an increased conversion into D-alanine via the alanine racemase (VMH ID:
372 ALAR), which was present in 808/818 gut microbes in the AGORA collection. Accordingly,
373 D-alanine was one of the three metabolite secretion profiles increased in constipated PD
374 patients (Figure 4E). This hypothesis of *B. wadsworthia* playing a role in constipation of PD
375 patients would need to be experimentally validated, especially since we could not find
376 statistically significant changes in the association between the abundance of *B. wadsworthia*
377 and constipated individuals. In contrast, we found an increase in *Bifidobacteria* abundance in
378 constipated individuals and particularly in constipated PD patients. This result disagreed with
379 an earlier study on individuals with chronic constipation, which reported a decrease in
380 *Bifidobacteria* abundance (Khalif et al. 2005). Overall, the available data suggest that complex
381 alterations in microbial composition are associated with constipation but may differ between
382 diseases.

383 The mucin degrading microbe, *A. muciniphila*, represents about 1-4% of the faecal
384 microbiome in humans (Naito et al. 2018). Numerous diseases have been associated with a
385 decrease in *A. muciniphila* abundance (Schneeberger et al. 2015; Grander et al. 2018), while
386 an increase has been consistently reported in PD patients (Figure 5). The *A. muciniphila*
387 abundance had the largest contribution to the significantly altered metabolite secretion profiles
388 (Figure 4E), including the neurotransmitter gamma-aminobutyric acid (GABA). While its
389 predicted secretion potential was only nominally increased in PD patients the present study,
390 higher GABA secretions rates have also been predicted based on microbiome data from early
391 stage levodopa naive PD patients (Hertel et al. in revision). Importantly, GABA receptors have
392 been found in the enteric nervous system, gut muscle, gut epithelial layers, and endocrine-like
393 cells (Hyland and Cryan 2010) and its gut receptors are thought to be related to gastric motility
394 (peristalsis), gastric emptying, and acid secretion (Hyland and Cryan 2010). Experiments with
395 the GABA_B agonist baclofen have shown that GABA_B receptors can reduce gastric mobility

396 in the colon of rabbits (via cholinergic modulation) (Tonini et al. 1989). Interestingly, *A.*
397 *muciniphila* has been shown to be positively associated with gastrointestinal transit time
398 (Gobert et al. 2016; Vandeputte et al. 2016). GABA could reach the CNS via blood stream as
399 a lipophilic compound, being able to pass the blood brain barrier. Additionally, microbial
400 GABA could affect the brain-gut axis by contributing the human GABA pools, especially as it
401 has been shown that the microbiome can affect GABA receptor density in the CNS via the
402 vagus nerve (Bravo et al. 2011). To establish whether and which role *A. muciniphila* and GABA
403 may play a role in prodromal PD, further experimental studies will be required.

404 In order to move beyond mere cataloguing of microbial changes associated with
405 diseases, pathway-based tools (Abubucker et al. 2012) have been developed, in which
406 microbial sequences (or reads) are mapped, e.g., onto KEGG ontologies present in the KEGG
407 database (Kanehisa et al. 2017). Using such tools, Bedarf et al reported decreased glucuronate
408 degradation and an increase in tryptophan degradation and formate conversion (Bedarf et al.
409 2017). Similarly, Heinz-Buschart et al. reported 26 KEGG pathways to be altered in PD
410 microbiomes (Heintz-Buschart et al. 2018). In our study, we complemented the compositional
411 analysis with computational modelling to gain insight into potential functional, i.e., metabolic,
412 consequences of changed microbe abundances in PD. The advantage of our approach is that
413 the functional assignments may be more comprehensive than more canonical methods, such as
414 KEGG ontologies because (1) the underlying genome-scale metabolic reconstructions have
415 been assembled based on refined genome annotations and have been manually curated to
416 ensure that the reaction and gene content is consistent with current knowledge about the
417 microbe's physiology, and (2) each of these reconstructions, alone or in combinations, are
418 amenable to metabolic modelling and thus functional and metabolic consequences of a changed
419 environment (e.g., nutrients or other microbes in the models) can be computed. These

420 simulations are thus allowing to predict functional consequences and not only pathway or
421 reaction enrichment, as typically done.

422

423 **Strength and limitation**

424 Here, we present microbiome analyses in a large population-based, monocentric case-
425 control study on PD from a defined area (Figure 5). Capitalising on the overall clinical spectrum
426 of PD of the LuxPark cohort, which reflects a representative sample of PD patients of different
427 disease stages from a defined geographical area, we demonstrated that microbial composition
428 is not only altered in PD but also that the observed associations of PD with changes in the
429 composition of the microbiome should be interpreted in the context of age, sex, BMI, and
430 constipation. This information is of importance for clinical translation, highlighting the need
431 for both, (i) a personalised and (ii) a holistic approach, to understand the role of microbial
432 communities in PD pathogenesis. In a second step targeting the potential functional changes
433 related to PD-associated microbiomes, we performed metabolic modelling based on the
434 AGORA collection (Magnusdottir et al. 2017) of genome-scale metabolic reconstructions,
435 allowing for the predictions of metabolite secretion profiles. Thus, our analyses facilitated a
436 detailed investigation of the altered metabolism of PD-related microbial communities in the
437 gut, pointing towards a role of the known pro-inflammatory species *B. wadsworthia* interacting
438 with the host on sulphur metabolism. Hence, metabolic modelling provides a valuable tool for
439 deciphering the metabolic activity of microbial communities in PD.

440 However, despite the partial confirmation of previous results by our study (Table 5),
441 several limitations should be kept in mind. First, certain covariates were not investigated, such
442 as diet, exercise, and smoking. Whether these covariates alter the PD-specific signature is yet
443 to be analysed. Although our study belongs to the three largest studies performed yet on PD,
444 our sample size was still too small to deliver insights on combinations of drugs. Furthermore,

445 16S RNA sequencing, as applied in our study, is not allowing analyses on the strain level and
446 may lead to misclassifications (Janda and Abbott 2007), and follow-up studies based on
447 shotgun sequencing are needed to further corroborate our results. However, our results are
448 notably well aligned with a previous shotgun sequencing study (Bedarf et al. 2017), which
449 would further support a role of 16S RNA sequencing as a cost-efficient screening method.
450 Being cross-sectional in nature, causal inference is not possible. Consequently, although
451 metabolic modelling has been numerous times been shown to correctly predict attributes of
452 living systems (Oberhardt et al. 2009; Aurich and Thiele 2016; Nielsen 2017), our hypothesis
453 on the role of *B. wadsworthia* in PD interlinking sulphur metabolism with disease severity
454 requires experimental validation. To conclude, by combining metabolic modelling with
455 comprehensive statistical analyses, we identified a promising research target in PD and refined
456 the understanding of PD-related microbial changes.

457

458 **METHODS**

459 **Description of the Luxembourg Parkinson's study**

460 For this study, data and biospecimen of the LuxPark cohort were utilised (Hipp et al.
461 2018). The Luxembourg Parkinson's study includes a variegated group of patients with typical
462 PD and atypical parkinsonism, and controls from Luxembourg and its neighbouring regions
463 (Hipp et al. 2018). Controls were partly sampled among relatives of patients. The
464 corresponding information on the family relation between controls and cases was not available.
465 Cancer diagnosis with ongoing treatment, pregnancy, and secondary parkinsonism (drug-
466 induced parkinsonism and parkinsonism in the frame of normotensive hydrocephalus) were
467 exclusion criteria for enrolling in the patient or healthy control group. For 454 individuals
468 (controls: n=248, PD: n=206) from the LuxPark cohort, stool samples were available and used
469 for 16S RNA gene sequencing data (see below). Within LuxPark, controls were selected among

470 spouses of chosen patients and volunteers and individuals from other independent
471 Luxembourgish studies (Crichton and Alkerwi 2014; Ruiz-Castell et al. 2016). As we aimed to
472 target specifically typical PD (IPD), we excluded all individuals with age below 50 (controls:
473 n=47, PD: n=9) and all individuals with an unclear status of PD diagnosis or an atypical PD
474 diagnosis (PD: n=47). PD patients were defined as typical PD, according to the inclusion
475 criteria by the United Kingdom Parkinson's Disease Society Brain Bank Clinical Diagnostic
476 Criteria (Hughes et al. 1992). Furthermore, we excluded control patients with a United
477 Parkinson's Disease Rating Scale (UPDRS) III score above ten, except for one control where
478 the high UPDRS III score was caused by an arm injury. Furthermore, we excluded control
479 persons who took dopaminergic medications (n=5), and individuals who reported to have taken
480 antibiotics in the last six months (controls: n=20, PD: n=13). Note that excluded observations
481 behave sub-additive, because of overlap between the exclusion criteria (i.e. individuals below
482 age 50 and taking antibiotics). Finally, 309 individuals (controls: n=162, cases: n=147) were
483 included in the statistical analyses.

484 All study participants gave written informed consents, and the study was performed in
485 accordance with the Declaration of Helsinki. The LuxPark study (Hipp et al. 2018) was
486 approved by the National Ethics Board (CNER Ref: 201407/13) and Data Protection
487 Committee (CNPD Ref: 446/2017).

488

489 **Measurements and neuropsychiatric testing**

490 All patients and healthy controls were assessed by a neurologist, neuropsychologist or
491 trained study nurse during the comprehensive battery of clinical assessment. Olfaction testing
492 was conducted using the Sniffin' Sticks 16-item version (SS) within the LuxPark cohort (Hipp
493 et al. 2018). Antibiotics usage was defined as intake of antibiotic within the previous six months
494 to stool collection. For assessing PD-related motor and non-motor symptoms, the UPDRS

495 rating scales I-IV were used (Goetz et al. 2008). The severity of the disease was reflected by
496 the Hoehn and Yahr staging (Hoehn and Yahr 1967). Non-motor symptoms were measured via
497 the NMS questionnaire (Romenets et al. 2012). The use of medication was recorded, and PD-
498 specific medication was classified into three classes, 1) levodopa, 2) dopamine receptor
499 agonist, and 3) MAO-B/COMT inhibitors.

500

501 **Collection and processing of stool samples**

502 All samples were processed following standard operating procedures (Lehmann et al.
503 2012; Mathay et al. 2015): stool samples were collected at home by patients using the
504 OMNIgene.GUT stool tubes (DNA Genotek) and sent to the Integrated Biobank Luxembourg
505 (IBBL) where one aliquot of 1 ml was used for DNA extraction. For the DNA extraction, a
506 modified Chemagic DNA blood protocol was used with the MSM I instrument (PerkinElmer),
507 the Chemagic Blood kit special 4 ml (Ref. CMG-1074) with a lysis buffer for faecal samples,
508 and MSM I software. Samples were lysed using the SEB lysis buffer (included in the kit) and
509 vortexed to obtain a homogenous suspension that was incubated for 10min at 70°C, then 5min
510 at 95°C. Lysates (1.5mL) were centrifuged for five minutes at 10,000 g at RT. Supernatants
511 were transferred to a 24XL deep-well plate. Plates were processed using the MSM I automated
512 protocol.

513

514 **Analysis of the microbial composition with 16S rRNA gene sequencing**

515 The V3-V4 regions of the 16S rRNA were sequenced at IBBL using an Illumina
516 Platform (Illumina MiSeq) using 2x300bp paired-end reads (Hipp et al. 2018). The gene-
517 specific primers targeted the V3 - V4 regions of the 16S rRNA gene. These primers were
518 designed with Illumina overhang adapters and used to amplify templates from genomic DNA.
519 Amplicons were generated, cleaned, indexed, and sequenced according to the Illumina-

520 demonstrated 16S Metagenomic Sequencing Library Preparation Protocol with certain
521 modifications. In brief, an initial PCR reaction contained at least 12.5 ng of DNA. A subsequent
522 limited-cycle amplification step was performed to add multiplexing indices and Illumina
523 sequencing adapters. Libraries were normalised, pooled, and sequenced on the Illumina MiSeq
524 system using 2x300 bp paired-end reads.

525 The demultiplexed samples were processed merging forward and reverse reads and
526 quality filtered using the dedicated pipeline "Merging and Filtering tool (MeFit)" (Parikh et al.
527 2016) with default parameters. To obtain a reliable microbial identification, identification to
528 both genus and species taxonomic level was obtained using the SPINGO (SPecies level
529 Identification of metaGenOmic amplicons) classifier (Allard et al. 2015) with default
530 parameters. Relative abundances were computed, for each sample, using an R (R Foundation
531 for Statistical Computing, Vienna, Austria) (Ihaka and Gentleman 1996) custom script. Briefly,
532 for each sample, the counts of each genera/species were retrieved, and then the sum of the
533 counts of all the genera/species was used to normalise to a total value of 1 each genera/species
534 count.

535

536 **Personalised constraint-based modelling of microbial communities**

537 AGORA consists of a set of 819 strains of microbes commonly found in the human gut
538 (Magnusdottir et al. 2017; Noronha et al. 2019). To match species taxonomic resolution, we
539 combined strain models of the same species in one species model ('panSpeciesModel.m') using
540 the function 'createPanModels.m' of the microbiome modelling toolbox (Baldini et al. 2018).
541 Briefly, reactions of multiple strains are combined into one pan-reconstruction. The pan-
542 biomass reaction is built from the average of all strain-specific biomass reactions. Microbial
543 abundances were mapped onto a set of 646 species performing an automatic name matching
544 between SPINGO species taxonomic assignment and panSpecies names. A threshold for

545 assessing the bacterial presence of a relative abundance value of 0.0001 was used to reduce the
546 time of computations while limiting the order of magnitude simulations results of
547 stoichiometric coefficients to ten. A total of 259 species overlapped between our set of species
548 models and SPINGO species assignment when considering species identified at least in 10 %
549 of samples (Supplementary Material). The retrieved microbial abundance information for each
550 sample was integrated into a community modelling setup obtaining personalised microbiome
551 models using the automated module of the microbiome modelling toolbox (Baldini et al. 2018)
552 called mgPipe within the COBRA toolbox (Heirendt et al. 2019) (commit:
553 b097185b641fc783fa6fea4900bdd303643a6a7e). Briefly, the metabolic models of the
554 community members are connected by a common compartment, where each model can
555 secrete/uptake metabolites. An average European diet was set as input for each microbiome
556 model (Noronha et al. 2019). A community objective function was formulated based on the
557 sum of each microbial model objective function and constrained to a lower bound of 0.4 per
558 day and upper bound of one per day. A set of exchange reactions connects the shared
559 compartment to the environment enabling to predict metabolite uptake and secretion flux rates
560 (metabolic profiles/NMPCs) consistent with the applied constraints. The personalisation of
561 each microbiome model was achieved by adjusting stoichiometric coefficients in the
562 community biomass reactions to each sample's relative microbial abundance and removing
563 species undetected from the community models.

564 Relative reactions abundances were calculated by summing the number of species
565 having the reaction in a microbiome model and scaling the sum by the respective species
566 relative abundance. Community metabolic profiles of these microbial communities were
567 assessed using flux variability analysis on the exchange reactions (Gudmundsson and Thiele
568 2010). AGORA microbial metabolic reconstructions used for the construction of the
569 community models were downloaded from the VMH (www.vmh.life, (Noronha et al. 2019)).

570 All computations were performed in MATLAB version 2018a (Mathworks, Inc.), using the
571 IBM CPLEX (IBM, Inc.) solver through the Tomlab (Tomlab, Inc.) interface.

572

573 **Analyses of relative abundances**

574 For descriptive statistics, metric variables were described by means and standard
575 deviations, while nominal variables were described by proportions. Missing values were not
576 imputed, and the pattern of missing values was not assessable via the ADA platform (Hipp et
577 al. 2018). The read counts for each metagenomic feature (e.g., genera and species) were divided
578 by total read counts such that relative abundances were retrieved. Relative abundances were
579 checked for outliers. Observations with more than four standard deviations from the mean were
580 excluded from analyses. Only genera and species detected in more than 50% of all samples
581 were included in the analyses, resulting in 62 genera and 127 species.

582

583 The metagenomic data was analysed using fractional regressions as developed by
584 (Papke and Wooldridge 1996). Fractional regressions, first applied to econometric problems,
585 are semiparametric methods designed to model fractional data without the need of specifying
586 the distribution of the response variable. Fractional regressions are further inherently robust
587 against heteroscedasticity and can be parametrised in odds ratios, delivering convenient
588 interpretations of the regression coefficients. All statistical models included technical
589 covariates, batch, total read counts, and unclassified read counts (reads for which a taxonomic
590 assignment was not possible independently from any threshold of confidence estimate value
591 used). The read count variables were included into the statistical model, as it has been shown
592 that normalisation by division can introduce bias if certain statistical assumptions implied by
593 the application of division are not fulfilled (Hertel et al. 2018). In the case of metagenomic
594 data, the effect of read counts would be removed by division if the observations would be

595 sampled from a multinomial distribution. However, this is not a given as species and genera
596 correlate amongst each other, violating the assumptions needed to construct multinomial
597 distributions. In consequence, read count normalisation by division is prone to introduce a bias
598 into metagenomic data; a potential bias, we corrected for by including the read counts as
599 covariates into the model.

600 Before fitting the final statistical models, we explored the associations of basic
601 covariates (age, sex, and BMI) with metagenomic features using fractional regressions as
602 described above to avoid misspecifications of the statistical models. Since the data showed a
603 high range in age and BMI, we checked for potential non-linear associations by including these
604 variables into the models as restricted cubic splines (Harrell 2001) using three knots defined
605 by the 5%-percentile, the median, and the 95%-percentile. As in the case for age, we found
606 species with indications of non-linear age-associations with $p < 0.01$, age was modelled in all
607 analyses via restricted cubic splines.

608 All p-values are reported two-tailed. Statistical analyses were performed in STATA
609 14/MP (College Station, Texas, USA). Summary statistics of the performed analyses are given
610 in the Supplementary files ‘Supplementary Tables’ 1-4.

611

612 **Differences between PD and controls in microbial composition and the influence of** 613 **covariates**

614 To analyse difference between genus abundances between PD and controls, fractional
615 regressions were carried out with the relative abundance of the genus as the response variable,
616 while including technical covariates, age (restricted cubic splines), sex, and BMI into the
617 statistical modelling. The predictor of interest was the study group indicator variable. We
618 corrected for multiple testing using the Benjamini-Hochberg procedure (Benjamini 2010) by
619 setting the false discovery rate (FDR) to 0.05. Consequently, we corrected for 62 tests when

620 reporting genera results. These analyses were repeated analogously for the taxonomic level of
621 species, while correcting for multiple testing via the FDR.

622 Next, we explored the possibility of statistical interactions between basic covariates
623 (age, sex, and BMI) and the group indicator. For these analyses, we once again modelled age
624 and BMI via restricted cubic splines allowing for non-linear interaction terms. We only tested
625 two-way interaction terms. All interaction terms were introduced simultaneously into the
626 statistical model and tested on significance via a Wald test (Harrell 2001), correcting for
627 multiple testing via the FDR. For the globally significant test, the single interaction terms were
628 investigated to explore which covariate-group interaction contributed to the overall
629 significance. For interpretation, the interaction terms were visually inspected by plotting the
630 predictions conditional on technical covariates. These analyses were then rerun with species
631 abundances as response variable instead of genus abundances.

632 We assessed the influence of constipation on the microbial composition. We introduced
633 the binary predictor constipation (yes/no) as additional predictor into the model and the
634 corresponding group-constipation interaction term. Both terms were tested simultaneously on
635 zero with a Wald test. The analyses were once again adjusted for technical covariates, age
636 (restricted cubic splines), sex, and BMI, and we corrected for multiple testing via the FDR.

637

638 **Analyses of within PD phenotypes in relation to microbial composition**

639 We investigated the association pattern of medication and clinical features regarding
640 the microbial composition. These analyses were only performed on the IPD cases, while
641 controls were excluded from the analyses. First, we analysed the disease duration as measured
642 in years between the date of the stool sampling and the year of the diagnosis. The analyses were
643 conducted as before via fractional regressions with the genus abundances as the response
644 variable, while adjusting for technical covariates, age (restricted cubic splines), sex, and BMI.

645 Then, we assessed in separate analyses the UPDRS III score as an indicator for motor
646 symptoms, the non-motor symptoms as measured by the NMS, the Hoehn-Yahr staging of the
647 disease as a global measure of disease progression, and the sniff-score. All these analyses were
648 performed adjusted for technical covariates, age (restricted cubic splines), sex, BMI, and
649 disease duration. Each of these series of regression represents 62 test, which was accounted for
650 using the FDR. The impact of medication was analysed by examining three classes of
651 medication, a) levodopa, b) mono-amino oxidase/catechol-O-methyltransferase inhibitors, and
652 c) dopamine receptor agonists. We generated three corresponding binary phenotypes (intake/no
653 intake) and added these three variables simultaneously to the statistical model determining the
654 significance of this add-on via a Wald test. We then tested each medication-class in separate
655 analyses, strictly correcting for multiple testing via the FDR (186 tests in total). The analyses
656 were performed adjusted for technical covariates, age (restricted cubic splines), sex, BMI, and
657 disease duration.

658

659 **Statistical analyses of fluxes**

660 The NMPCs were log transformed such that the skewness of the distribution was
661 minimised (Box and Cox 1964)). This type of transformation was applied because of the very
662 differently skewed distributions of the single NMPCs. Then, outliers were excluded using the
663 4-SD outlier rule as before. Only fluxes with more than 50% non-zero values were retained in
664 analyses. Furthermore, NMPCs with distributions not suitable for statistical analyses (e.g.,
665 distributions with a high number of observations with exact the same numerical value) were
666 excluded resulting in 129 NMPCs included into analyses.

667 The NMPCs were analysed with mixed linear regressions including the batch as random
668 effects. Including the batch variable as a random effect has a higher statistical power in
669 comparison to the fixed effect approach, but relies on more restrictive assumptions. We tested

670 the corresponding random effect assumption by Hausman specification tests and found no
671 indications of violations of the Hausman specification test. Note that this possibility to account
672 for batch effects via random effects is not available with fractional regressions where batch
673 effects were corrected via fixed effects.

674 We performed the same analyses as with the metagenomic data, with the sole exception
675 of replacing the fractional regression model with the linear mixed model. In all other aspects,
676 the analyses followed the same scheme.

677

678 **Analyses of species contribution to fluxes**

679 To investigate the contribution of species and genera, we calculated for all included
680 genera and all analysed fluxes the pairwise correlation and the corresponding variance
681 contribution (the squared correlation). We classified every correlation above 0.5 (equal to 25%
682 of variance contribution) as a strong correlation in accordance with classical classifications of
683 effect size (Cohen 1988).

684

685 **Material availability**

686 All 16S rRNA sequences can be requested from I.T. (ines.thiele@nuigalway.ie). The
687 mgPipe pipeline is available within the COBRA toolbox
688 (<https://github.com/opencobra/cobratoolbox>), and the custom scripts with related
689 documentation are available at the GitHub repository:
690 https://github.com/ThieleLab/CodeBase/ND_collect.

691

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699

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