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1 **Gut microbiota of type 1 diabetes patients with good glycaemic control and high**
2 **physical-fitness is similar to people without diabetes: an observational study**

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16

17 **What's new?**

- 18 • This study is the first to explore the gut microbiota in people with type 1 diabetes
19 (T1D), but otherwise have good glycaemic control and high physical-fitness
- 20 • The gut microbiota from the people with T1D and good glycaemic control and high
21 physical-fitness was comparable to matched non-diabetic healthy controls

22

23 **Abstract**

24 **Aim:** Type 1 diabetes (T1D) is the product of a complex interplay between genetic
25 susceptibility and exposure to environmental factors. Existing bacterial profiling studies
26 focus on people who are most at risk at the time of diagnosis; there is limited data on the gut
27 microbiota of people with long standing T1D. This study compared gut microbiota of people
28 with T1D and good glycaemic control and high levels of physical-fitness with matched non-
29 diabetic controls.

30 **Methods:** Ten males with T1D and ten matched controls without diabetes (CON) were
31 recruited; groups were matched for gender, age, BMI, VO_{2max} , exercise habits. Stool samples
32 were analysed using next generation sequencing of the 16S rRNA gene to obtain bacterial
33 profiles from each individual. Phylogenetic investigation of communities by reconstruction of
34 unobserved states (PICRUSt) was implemented to predict functional content of the bacterial
35 OTUs.

36 **Results:** *Faecalibacterium* sp., *Roseburia* sp., and *Bacteroides* sp. were typically the most
37 abundant members of the community in both T1D and CON and were present in every
38 sample in the cohort. Each bacterial profile was relatively individual and no significant
39 difference was reported between the bacterial profiles or the Shannon diversity indices of
40 T1D compared with CON. The functional profiles were more conserved and the T1D group
41 were comparable to that of the CON group.

42 **Conclusions:** We show that both gut microbiota and resulting functional bacterial profiles
43 from people with longstanding T1D in good glycaemic control and high physical-fitness
44 levels are comparable to matched people without diabetes.

45 **Introduction**

46 Type 1 diabetes (T1D) is the product of a complex interplay between genetic susceptibility
47 and exposure to environmental factors [1]. Environmental exposure has long been implicated
48 in the pathogenesis of the disease and now, with decades of evidence mapping an increased
49 rate of incidence, it is clear that disease progression occurs at a rate at which genetic change
50 alone cannot be solely accountable [2].

51 Previous research has shown that the gut microbiota, which is the collection of
52 microorganisms colonizing the gut, has important roles in the disease [3–5]. Germ-free (GF)
53 mice models of T1D may acquire the disease at higher rates, but this has been challenged
54 with no significant differences between GF and colonized mice [6]. In the same study a
55 Gram-positive organism was isolated which reduced the incidence of the disease.
56 Administering ‘probiotic’ (live microorganisms which confer health benefits) to mouse
57 models further demonstrated the potential of intervention targeting the gut microbiota to
58 reduce disease incidence [6]. Antibiotic administration earlier in life may also predispose
59 patients to T1D through modulation of the gut microbiota, where certain antibiotic
60 combinations were recently found to increase diabetes risk [7], although in mice the
61 incidence was reduced with vancomycin from birth to weaning [8].

62 Research in children has shown that the gut microbiota in Finish people with T1D had greater
63 Bacteroidetes relative to Firmicutes and reduced overall diversity [9]. More recently in a
64 Spanish cohort, people with T1D had increased abundance of *Clostridium*, *Bacteroides* and
65 *Veillonella* and reduced abundance of *Bifidobacterium* and *Lactobacillus* compared to
66 controls [10]. Interestingly the latter two organisms are regarded as beneficial and have been
67 used extensively as probiotic candidates. Overall these findings indicate that interactions
68 between the intestinal microbiota and the innate immune system are critical for disease
69 development [9,11]. However, T1D has a wide spectrum of severity and these studies tend to

70 focus on people at who are most at risk at the time of diagnosis. Thus an important
71 knowledge gap remains in the literature regarding the status of people in adulthood with
72 longstanding diabetes. Moreover, there is limited data examining such individuals who are
73 intensively managed, demonstrating good glycaemic control and high levels of physical
74 fitness.

75 This study seeks to explore gut microbiota in people with T1D and good glycaemic control
76 and high levels of physical-fitness, matched to people without diabetes. While the gut
77 microbiota potentially contributes to the T1D onset, we aimed to determine if long-term
78 active sufferers are able to develop a gut microbiome comparable to healthy controls or if
79 important differences persist long after onset.

80 **Materials and Methods**

81 **Participant recruitment and preliminary testing**

82 Fully informed written consent was obtained from all persons following the study's approval
83 from National Health Service NRES Committee - Tyne and Wear South. Participants
84 attended the Newcastle National Institute for Health Research Clinical Research Facility to
85 establish peak cardio-respiratory parameters during the completion of an incremental-
86 maximal treadmill running protocol as previously described [12]. Participants provided stool
87 material on tissue paper that was deposited in a sterile falcon tube and stored at -80 °C until
88 processing. Tissue paper was sterilised under UV and a negative control sample of toilet
89 paper was also carried out.

90 T1D eligibility criteria consisted of being aged between 18-35 years, a duration of diabetes >
91 5 years, and an HbA_{1c} < 8.0% (64 mmol/mol). In addition, people with T1D were required to
92 be absent of diabetes-related complications, other than mild-background retinopathy, not
93 receiving any medication other than insulin (assessed against recent medical notes), and
94 regularly and consistently undertaking exercise (participating in aerobic based exercise for a
95 minimum of 30 minutes at a time, at least three times per week). Ten male people with T1D
96 were recruited (aged 27±2 years, BMI 23.5±0.7 kg.m², VO₂peak 51.3±2.2 ml/kg/min,
97 duration of diabetes 12±2 years, HbA_{1c} 7.1±0.4% [54.5±2.1 mmol/mol]). Patients were
98 treated with a basal-bolus regimen composed of long-acting insulins glargine (n = 8) or
99 detemir (n = 2), and rapid-acting insulin aspart. Eligibility criteria for non-diabetic control
100 participants consisted of being between 18-35 years, regularly and consistently undertaking
101 exercise. Ten male people without diabetes (CON) were recruited (aged 27±2 years, BMI
102 22.4±0.8 kg/m², VO₂max 50.9±1.2 ml/kg/min). T1D and CON groups were matched for age,
103 fitness and BMI (P>0.05). Both groups were habitually consuming a predominantly

104 carbohydrate rich diet (>60% carbohydrate) assessed via 24 hour recall. Study demographics
105 are summarised in Table 1.

106

107 **16S rRNA gene bacterial profiling**

108 Participants were provided 3 sections of toilet paper from the same roll that had all undergone
109 UV sterilisation. Following excrement the participants used the toilet paper once, the soiled
110 tissue was then collected in sterile universal tubes. Nucleic acid extraction of stool was
111 carried out on a section of the soiled toilet paper using the PowerLyzer™ PowerSoil® DNA
112 Isolation Kit (MoBio, CA, USA) in accordance with the manufacturer's instructions.
113 Bacterial profiling utilised the 16S rRNA gene targeting variable region 4 and was carried out
114 by NU-OMICS (Northumbria University) based on the Schloss wet-lab MiSeq SOP and
115 resulting. raw fastq data were processed using Mothur (version 1.31.2) as described
116 previously [13]. Briefly, combined reads were trimmed to 275 reads with 0 ambiguous bases.
117 Chimeric sequences were detected by Chimera.uchime and removed from downstream
118 analysis. Alignment was generated via the Silva v4 database [14] and Chloroplast,
119 Mitochondria, unknown, Archaea, and Eukaryota lineages were removed from the analysis. In
120 total, 5,165,964 reads were generated from the 20 samples. Sequences were deposited in MG-
121 RAST under the accession numbers 4603090.3 - 4603109.3.

122

123 **Statistical analysis**

124 Data was normalised by subsampling and rarefying all samples to 104,142 reads. The data
125 was automatically transformed and analysed by principal coordinate analysis (PCA) using
126 SIMCA 13.0 (Umetrics, Stockholm, Sweden) [15]. The community structure between the
127 T1D and CON groups were analysed by Parsimony and weighted UniFrac analysis [16].
128 Significant operational taxonomic unit (OTUs) were classified by the metastats function in

129 Mothur using 1000 permutations with multiple hypothesis testing correction [17].
130 Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt)
131 was implemented to predict functional content of the bacterial OTUs [18].

132 **Results**

133 The number of reads used in the subsampling (104,142) facilitated robust coverage of the gut
134 microbiota of each individual in the cohort. No significant difference was found between the
135 T1D and control groups using Parsimony ($P = 0.309$) and weighted UniFrac ($P = 0.107$)
136 *Faecalibacterium* sp., *Roseburia* sp., and *Bacteroides* sp. were typically the most abundant
137 members of the community in both T1D and CON and were present in every sample in the
138 cohort (Figure 1). Levels of *Bacteroides* sp. tended to be higher in CON ($P = 0.06$) and
139 *Bifidobacterium* sp. tended to be higher in T1D ($P = 0.08$), but neither was significant.

140 The bacterial profiles of T1D were comparable to the CON group with no distinct clusters
141 based on the bacterial profiles (Figure 2A). To account for potential false negatives resulting
142 from some people with T1D, where HbA_{1c} was outside the range for truly excellent control,
143 further ordination analysis was conducted by stratifying T1D by HbA_{1c} by $>$ or $<$ 53
144 mmol/mol. PCA analysis with this classification showed no distinct clustering based on the
145 overall bacterial community, with resulting PLS-DA predictive (Q) scores of -0.106 in >53
146 mmol/mol and 0.022 in <53 , where scores of >0.5 represent significant differences and
147 predictively between the groups (Supplementary Figure 1). Only 17 OTUs from a total of
148 3,062 were found to be significantly different between the groups (Table 2). *Actinomyces* sp.
149 (OTU00428) was the most significant OTU ($P = 0.008$) in the T1D group and this was most
150 associated with the T1D group in the PLS-DA loadings plot (Figure 2B). However, this OTU
151 was detected in all but 2 participants (both from CON) and only comprised of 62 reads
152 from a total of 2,082,840 (0.003%), where 49 reads were from people with T1D and 13 reads
153 were from CON. No significant difference ($P = 0.344$) was found in the Shannon Diversity
154 (H') between each group. The average T1D H' was 3.37 (range 2.16 – 3.92), whereas the
155 CON H' was 3.13 (range 2.62 – 4.49).

156 PICRUSt was implemented to predict functional content of the bacterial OTUs. This showed
157 that despite the relatively large variation in of the bacterial community between individuals,
158 the functional profiles were much more comparable (Figure 3). Functional profiles from the
159 T1D group were comparable to that of the CON group.

160 Discussion

161 Alterations in the gut microbiota, whether causative or as a result of T1D, may have
162 important implications for the health of people with T1D. The aim of the present study was to
163 explore gut microbiota in people with T1D but good glycaemic control and high levels of
164 physical-fitness, matched to people without diabetes. We show for the first time that
165 intensively managed T1D suffers with optimal glycaemic control and good physical-fitness
166 display comparable gut microbiota profiles to matched non-T1D individuals.

167 The gut microbiota profiles were highly individual across the whole cohort, but there is
168 general conformity between the most dominant members of the community.
169 *Faecalibacterium* sp., *Roseburia* sp., and *Bacteroides* sp. were found to be the most abundant
170 in the cohort and generally represented a substantial proportion of the gut microbiota in each
171 person. These have been previously shown to be prevalent in a healthy adult gut microbiota
172 [19]. The most significant OTUs driving the separation of the T1D and control gut
173 communities were generally low in abundance and reflected only a small proportion of the
174 overall reads. For example the *Actinomyces* sp. (OTU00428), which was the most significant
175 OTU in the T1D group, only comprised of 62 reads (49 reads from T1D group) from a
176 total of 2,082,840 (0.003%). Thus OTUs with such universally low relative abundance are
177 unlikely to be contributing to disease pathophysiology and implying causality to disease
178 should be avoided. While the cohort employed in this study is small, 10 T1D suffers are
179 comparable to that of previously published studies and should not influence the lack of
180 clinically important OTUs discriminating people with T1D and controls [10]. Previous
181 studies have also inferred associations at diagnosis of increasing *Bacteroides* and reduced
182 *Bifidobacterium* in T1D [9,10]. While these organisms were relatively abundant overall we
183 see opposing trends, with lower *Bacteroides* and increased *Bifidobacterium* in T1D; although

184 these differences are noteworthy they were not significant, but further work in a larger cohort
185 is necessary to confirm these observations.

186 The Shannon diversity was comparable between T1D and controls with no significant
187 difference found between the groups. Interestingly, previous studies suggest that children
188 with T1D undergo dysbiosis of the gut microbiota, resulting in reduced diversity compared to
189 people without diabetes [9,20]. The diversity reported in this study is comparable to that of a
190 non-T1D adult population, but a lack of published aged-matched controls prevents any
191 comparison with T1D adults. Nonetheless, the observation that active adults with T1D have a
192 similar diversity to adults without T1D is important.

193 Previous studies have suggested an increase of butyrate-producing and mucin-degrading
194 bacteria in controls, whereas bacteria that produce short chain fatty acids (SCFAs) other than
195 butyrate were higher in disease cases [21]. Thus synthetic pathways may represent a key
196 etiological trigger in the onset of T1D. Functional analysis of the bacterial community in this
197 dataset demonstrated comparability between the bacterial pathways of the OTUs found in
198 people with T1D and matched controls. Despite large variation at the OTU level, the function
199 profiles showed much greater comparability, as has been previously reported [22].
200 Noteworthy is that these functional pathways represent only those of the bacterial community
201 based on the classification OTUs and thus do not account for differential gene expression
202 between the two groups.

203 Given the individual nature of the gut microbiota within each group of the cohort, it is
204 perhaps not surprising that the ordination analysis of the bacterial profiles showed no distinct
205 separation of people with T1D and matched controls. Thus, in adulthood the gut microbiota is
206 not significantly altered in active persons as a result of being diagnosed with T1D. Notably
207 this finding was not influenced when the T1D group was further stratified to account for

208 ranging HbA_{1c}. Existing comparable data is limited, with studies to date focusing on
209 differences in the gut microbiota in patients at the time of diagnosis (i.e. childhood) [9,10].
210 While the gut microbiota may serve as an environmental trigger in the onset of T1D in
211 patients where genetic elements alone cannot account for the pathogenesis, an important
212 finding of this study is that active T1D adults have a gut microbiota reflective of non-T1D
213 adults. Further work should sample greater numbers of people temporally and seek to include
214 sedentary sufferers and those with poorer glycaemic control. Future work should also
215 consider T1D patients with other pathologies, such as retinopathy or cardiovascular disease.
216 Considering the lack of available data pertaining to the influence of exercise on gut
217 microbiota, profiling patients across a range of glycaemic control and physical-activity levels
218 is warranted to ascertain whether alterations in gut microbiota are influenced by exercise,
219 glycaemic control, or both, and if intervention or therapeutic manipulation of the gut
220 microbiota could confer improvements to well-being. The potential influence of differences
221 in HLA genotype between those with and without T1D should also be considered in future
222 studies.

223 In summary, this study confirmed existing data relating to the dominant bacterial organisms
224 in the healthy active adult gut microbiota. Importantly, we show that both gut microbiota and
225 resulting functional bacterial profiles from people with longstanding T1D in good glycaemic
226 control and high physical-fitness levels are comparable to matched people without diabetes.

227 **COMPETING INTERESTS**

228 None to declare.

229

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233 data; in the writing of the manuscript; or in the decision to submit the manuscript for

234 publication.

235

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312

Table 1 – Individual participant characteristics

Group	Subject ID	Age (years)	BMI	VO _{2peak} (ml/kg/min)	Fasting Blood Glucose (mMol/L)	Diabetes Duration (years)	HbA _{1c} (mmol/mol)
Control	C1	25	22.1	50	4.20		
	C2	23	21.4	51	4.32		
	C3	31	21.7	56	4.33		
	C4	30	20.1	52	3.87		
	C5	28	26.9	48	3.46		
	C6	26	21.4	55	4.02		
	C7	26	23.7	50	3.29		
	C8	30	25.4	51	4.22		
	C9	25	21.8	45	4.28		
	C10	26	20.4	49	4.22		
T1D	T1	29	22.8	57	5.44	5	54
	T2	24	25.9	48	5.75	11	42
	T3	19	22.5	64	5.01	12	49
	T4	34	22.4	50	3.90	5	60
	T5	21	22.5	56	8.43	12	55
	T6	33	27.1	52	7.32	19	58
	T7	29	26.9	41	6.45	5	58
	T8	25	22.8	51	6.31	24	43
	T9	24	22.4	45	3.45	13	50
	T10	31	22.5	46	3.22	19	61

VO_{2peak}: peak oxygen uptake; BMI: Body mass index. Between group comparisons assessed with independent samples t-test.

Table 2 – OTUs which differ significantly between T1D and matched controls

Group	<i>P</i> value	OTU	Phylum	Class	Order	Family	Genus
CON	0.003	Otu00082	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>unclassified</i>
CON	0.017	Otu01214	Firmicutes	Bacilli	Bacillales	Bacillaceae_1	<i>Anoxybacillus</i>
CON	0.019	Otu00865	Proteobacteria	Alphaproteobacteria	Rhizobiales	Aurantimonadaceae	<i>Aurantimonas</i>
CON	0.021	Otu00820	Deinococcus-Thermus	Deinococci	Deinococcales	Deinococcaceae	<i>Deinococcus</i>
CON	0.026	Otu00625	Firmicutes	Clostridia	Clostridiales	Clostridiaceae_1	<i>Clostridium_sensu_stricto</i>
CON	0.027	Otu00217	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Coproccoccus</i>
CON	0.027	Otu00230	Proteobacteria	Betaproteobacteria	Burkholderiales	unclassified	<i>unclassified</i>
CON	0.032	Otu00807	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	<i>Schlegelella</i>
CON	0.033	Otu01323	Proteobacteria	Betaproteobacteria	Burkholderiales	unclassified	<i>unclassified</i>
CON	0.036	Otu01060	Actinobacteria	Actinobacteria	Coriobacteriales	Coriobacteriaceae	<i>unclassified</i>
CON	0.039	Otu00363	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	<i>Zoogloea</i>
CON	0.041	Otu00384	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	<i>unclassified</i>
T1D	0.008	Otu00428	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	<i>Actinomyces</i>
T1D	0.03	Otu00020	Actinobacteria	Actinobacteria	Coriobacteriales	Coriobacteriaceae	<i>Collinsella</i>
T1D	0.03	Otu00021	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>unclassified</i>
T1D	0.047	Otu00023	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>unclassified</i>
T1D	0.047	Otu00025	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	<i>Dialister</i>

Figure Legends

Figure 1 – Bar Chart of OTUs from type 1 (T1) diabetes and matched controls. Each OTU represented as a % of the total community. Samples ordered by *Faecalibacterium* abundance.

Figure 2 – SIMCA analysis of type 1 (T1) diabetes samples and matched control. A) PCA score scatter plot. $R^2X[1] = 0.124$, $R^2X[2] = 0.0998$. B) Loadings Plot showing taxa associated with each group. Green (Y) represents each OTU detected, where only the significantly different OTUs between cases and control are labelled. Blue (X) shows different classification of the model, where OTUs associated with control samples are shown on the upper right and OTUs associated with cases are shown on the lower left.

Figure 3 – Bar Chart of PICRUSt analysis from type 1 diabetes and matched controls. Each function represented as a % of the total community. Samples ordered in accordance with Figure 1.

Supplementary Figure Legends

Supplementary Figure 1 – PCA analysis of type 1 diabetes (T) samples and matched controls (C), with the T1D group split to account for differing glycaemic control. T1D samples split by $HbA_{1c} > 53$ mmol/mol (orange) and $HbA_{1c} < 53$ mmol/mol with PLS-DA scores of -0.106 and 0.022, respectively.