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1 **Gut microbiota of type 1 diabetes patients with good glycaemic control and high**
2 **physical-fitness is similar to matched non-diabetic controls: an observational study**

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16

17 **What's new?**

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

23

24 **Abstract**

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

31 **Methods:** Ten male type 1 diabetes patients (T1D) and ten matched controls (CON) were
32 recruited; groups were matched for age, BMI, VO_{2max} , exercise habits. Stool samples were
33 analysed using next generation sequencing of the 16S rRNA gene to obtain bacterial profiles
34 from each individual. Phylogenetic investigation of communities by reconstruction of
35 unobserved states (PICRUSt) was implemented to predict functional content of the bacterial
36 OTUs.

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

43 **Conclusions:** We show that both gut microbiota and resulting functional bacterial profiles
44 from patients with longstanding T1D in good glycaemic control and high physical-fitness
45 levels are comparable to matched non-T1D controls.

46 **Introduction**

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

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

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

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

76 This study seeks to explore gut microbiota in T1D patients with good glycaemic control and
77 high levels of physical-fitness and matched non-T1D controls. While the gut microbiota
78 potentially contributes to the T1D onset, we aimed to determine if long-term active sufferers
79 are able to develop a gut microbiome comparable to healthy controls or if important
80 differences persist long after onset.

81 **Materials and Methods**

82 **Participant recruitment and preliminary testing**

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

91 T1D patient eligibility criteria consisted of being aged between 18-35 years, a duration of
92 diabetes > 5 years, and an HbA_{1c} < 8.0% (64 mmol/mol). In addition, patients were required
93 to be absent of diabetes-related complications, other than mild-background retinopathy, not
94 receiving any medication other than insulin (assessed against recent medical notes), and
95 regularly and consistently undertaking exercise (participating in aerobic based exercise for a
96 minimum of 30 minutes at a time, at least three times per week). Ten male T1D patients were
97 recruited (aged 27±2 years, BMI 23.5±0.7 kg.m², VO₂peak 51.3±2.2 ml/kg/min, duration of
98 diabetes 12±2 years, HbA_{1c} 7.1±0.4% [54.5±2.1 mmol/mol]). Patients were treated with a
99 basal-bolus regimen composed of long-acting insulins glargine (n = 8) or detemir (n = 2), and
100 rapid-acting insulin aspart. Eligibility criteria for non-diabetic control participants consisted
101 of being between 18-35 years, regularly and consistently undertaking exercise. Ten non-
102 diabetic control participants (CON) were recruited (aged 27±2 years, BMI 22.4±0.8 kg/m²,
103 VO₂max 50.9±1.2 ml/kg/min). T1D and CON groups were matched for age, fitness and BMI
104 (P>0.05). Both groups were habitually consuming a predominantly carbohydrate rich diet

105 (>60% carbohydrate) assessed via 24 hour recall. Patient demographics are summarised in
106 Table 1.

107

108 **16S rRNA gene bacterial profiling**

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

123

124 **Statistical analysis**

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

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

133 **Results**

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

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

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

161 Discussion

162 Alterations in the gut microbiota, whether causative or as a result of T1D, may have
163 important implications for the health of patients. The aim of the present study was to explore
164 gut microbiota in T1D patients with good glycaemic control and high levels of physical-
165 fitness against matched non-T1D controls. We show for the first time that intensively
166 managed T1D patients with optimal glycaemic control and good physical-fitness display
167 comparable gut microbiota profiles to matched non-T1D individuals.

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

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

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

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

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

209 ranging HbA_{1c}, with some patients in the T1D groups exhibiting HbA_{1c} outside the range
210 considered excellent for glycaemic control. Existing comparable data is limited, with studies
211 to date focusing on differences in the gut microbiota in patients at the time of diagnosis (i.e.
212 childhood) [9,10]. While the gut microbiota may serve as an environmental trigger in the
213 onset of T1D in patients where genetic elements alone cannot account for the pathogenesis,
214 an important finding of this study is that active T1D adults have a gut microbiota reflective of
215 non-T1D adults. Further work should sample greater numbers of patients temporally and seek
216 to include sedentary sufferers and those with poorer glycaemic control. Future work should
217 also consider T1D patients with other pathologies, such as retinopathy or cardiovascular
218 disease. Considering the lack of available data pertaining to the influence of exercise on gut
219 microbiota, profiling patients across a range of glycaemic control and physical-activity levels
220 is warranted to ascertain whether alterations in gut microbiota are influenced by exercise,
221 glycaemic control, or both, and if intervention or therapeutic manipulation of the gut
222 microbiota could confer improvements to well-being. The potential influence of differences
223 in HLA genotype between those with T1D and control participants should also be considered
224 in future studies.

225 In summary, this study confirmed existing data relating to the dominant bacterial organisms
226 in the healthy active adult gut microbiota. Importantly, we show that both gut microbiota and
227 resulting functional bacterial profiles from patients with longstanding T1D in good glycaemic
228 control and high physical-fitness levels are comparable to matched non-T1D controls.

229 **COMPETING INTERESTS**

230 None to declare.

231

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237

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314

Table 1 – Individual participant characteristics

Group	Patient 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. Patients 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 PICRUSSt analysis from type 1 diabetes and matched controls. Each function represented as a % of the total community. Patients 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 T1D patients 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.