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Enteral feeding reduces metabolic activity of the intestinal microbiome in Crohn's disease: Observational study

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Abstract

Background

Enteral feeding will induce remission in as many as 80-90% of compliant patients with active Crohn's Disease (CD) but its method of action remains uncertain. This study was designed to examine its effects on the colonic microbiome.

Method

Healthy volunteers and patients with CD followed a regimen confined to enteral feeds alone for one or two weeks respectively. Chemicals excreted on breath or in faeces were characterised at the start and at the end of the feeding period by gas chromatography mass spectrometry (GC/MS).

Results

One week of feeding in healthy volunteers caused significant changes in stool colour and deterioration in breath odour, together with increased excretion of phenol and indoles on the breath. Feeding for two weeks in patients with CD produced significant improvements in symptoms and a decrease in the concentration of C-reactive protein. The faecal concentrations of microbial products including short chain fatty acids (SCFAs), and potentially toxic substances including 1-propanol, 1-butanol and the methyl and ethyl esters of SCFAs showed significant falls.

Conclusion

48   A significant change occurs in the production of microbial metabolites after enteral feeding in both healthy

49   volunteers and patients with CD. Many of those detected in CD are toxic and may feasibly lead to the

50   immunological attack on the gut microbiota, which is characteristic of IBD. The reduction in the production

51   of such metabolites after enteral feeding may be the reason for its effectiveness in CD.

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Introduction

Despite the increasing frequency of Crohn's disease, its treatment remains unsatisfactory. Many of the therapeutic agents used have unpleasant or even dangerous side effects and some are very expensive. The continuing perception of CD as a relapsing and remitting disorder emphasises the difficulty in maintaining long term control. A complete cure remains elusive.

Reports of a positive response to dietary manipulation in CD have emerged from several sources.[1-8] 2-4 weeks of total enteral feeding has been reported to reduce remission in 85-90% of compliant patients suffering active CD. [1-6.] Lack of understanding of the method of action of enteral feeds in CD has however, discouraged their use. [2-8]

Enteral feeds are nutritionally complete liquid mixtures of pre-digested foods presenting nitrogen as amino acids, oligopeptides or a single protein, carbohydrates as simple sugars, typically malto-dextrins, and fat as a single oil, (eg. Rapeseed oil), together with minerals and vitamins.

Suggestions as to the method of action of enteral feeding are many, but it is now known that bowel rest [7] and the reduction of potential food allergens [2-4] are incorrect. Enteral feeding is unlikely to have therapeutic benefit by producing immunosuppression as it is ineffective in the treatment of ulcerative colitis. [3] Reduction in inflammation can be detected before any improvement in nutritional state begins, [8] and the suggestion that dietary particles might be important was not supported by a controlled trial. [9]

The increasing evidence that inflammation in CD is provoked by an immune response targeted against the intestinal microbiome implies that manipulation of the metabolic activity of the microbiota might have a

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98 role in the treatment of this disease [10,12] We and others have recently demonstrated an association between

99 Crohn's disease and the profile volatile organic compounds obtained from breath and faecal headspace

100 samples [16,25]. These measurements are a useful indication of changes in the gut microbiome, being simple,

101 rapid and non-invasive. We have used this approach here in a study of the effects of enteral feeding. It has

102 been suggested that food intolerance, as distinct from food allergy, might reflect an interaction between

103 unabsorbed food residues and the intestinal microbiome.[13]   As the nutrients contained in enteral feeds are

104 absorbed high in the small intestine, they supply little in the way of energy substrates to micro-organisms

105 in the lower bowel. This might lead to changes in microbial metabolism which in turn could lead to a

106 reduction in inflammation. The present studies were designed to investigate this possibility.

107

108 **Methods**

109 **Study 1 Healthy volunteers**

110 Volunteers were recruited from students of either sex aged 18-65 years, at Cranfield University who were

111 in good health and eating a normal diet. A total of 12 subjects was recruited aged 23-32, of which 8 were

112 female. Subjects suffering conditions possibly requiring specific diets e.g. irritable bowel syndrome (IBS),

113 or coeliac disease were excluded. Other exclusions were pregnancy or lactation, a course of antibiotics in

114 the previous six weeks, bacterial products such as pro- or pre- biotics and any chronic medication other

115 than oral contraceptives.

116

117 Subjects were randomly allocated to take either E028 extra (Nutricia UK Liverpool), or Modulen-IBD

118 (Nestle Ltd, Croydon UK), for 7 days with all other foodstuffs excluded except water *ad libitum.*.

119 Nutritional requirements were calculated for each individual using Schofield's equation. [14]

120 After 7 days subjects returned to normal diets for 21 days before commencing the alternative enteral feed

121 for a further 7 days. The two feeds were administered 4 weeks apart in order that they were taken at the

5

122 same stage of the menstrual cycles of female volunteers. During enteral feeding, subjects were asked to

123 record how much feed they consumed and to complete symptom score sheets recording on a daily basis

124 stool frequency, consistency and colour and any changes in breath odour. Weights were recorded and

125 breath samples taken before the study and after each week of enteral feeding. This trial was an open,

126 randomised controlled study performed at Cranfield University and approved by the ethics committee of

127 Cranfield University and the NHS Cambridge local research ethics committee.

128 Volunteers were provided with a sheet depicting a range of faecal colours ranging from dark brown to

129 bright green (copies supplied to the editor) and asked to assess the stool colour, consistency and frequency.

130 They were asked to record daily changes in breath odour which was assessed subjectively on a scale from

131 1 (odourless) to 4 (extremely unpleasant).

132 Bio-VOC samplers were used according to manufacturer's recommendations to obtain a one-litre end-tidal

133 breath sample after breakfast on the first day of each feeding period. Samples were injected onto Thermal

134 desorption tubes containing 1:1 Tenax TA and Carbotrap adsorbents (Markes International, Llantrisant,

135 UK).

136 Study 2 Patients with Crohn's disease

137 Patients aged 18-65 years were recruited in the department of Gastroenterology, Addenbrooke's Hospital,

138 Cambridge. A total of 17 patients each provided a faecal sample before treatment with enteral feed

139 E028extra and again when they went into remission. At recruitment, all had symptoms of active disease.

140 The diagnosis of CD was made by standard diagnostic criteria and the severity of symptoms was assessed

141 using the Harvey and Bradshaw Index[15]. The concentration of C-reactive protein (CRP) in serum samples

142 obtained at each visit was determined by the Biochemistry Department of Addenbrooke's Hospital to

143 provide an objective measure of disease activity.

144

145 Any patients who had received antibiotics in the previous 6 weeks were excluded. Some were taking

146 medication including 5-aminosalicylic acid compounds and/or azathioprine which had been insufficient to

147 control their symptoms, but none had received previous dietary treatment. They were asked to continue

148 such medication during the period of feeding with elemental diet. Non-fasting morning samples of faeces

149 were obtained before starting two weeks treatment with E028 extra (Nutricia Liverpool UK) with amounts

150 again being calculated by Schofield's equation. A further faecal sample was obtained at the end of this

151 period. Samples were delivered to the hospital on the same day as passed with a maximum delay before

152 freezing of 4 hours. They were stored at -40°C until transferred to the laboratory for analysis.

153 Ethical permission for this study was granted by the Leeds West LREC (Ref: 07/Q1205/39).

154 **Laboratory analysis**

155 An internal standard solution comprising 50 ng deuterated (D8) toluene (Supelco Cat no 48,593) in

156 methanol was added to each tube according to the manufacturer's instructions (Markes International Ltd,

157 Llantrisant,UK). Head space samples were analysed by automated thermal desorption gas

158 chromatography/mass spectrometry. A Perkin Elmer system was used for analysis combining a TurboMass

159 MS 4.1 Autosystem XL GC and Automatic Thermal Desorption system (ATD 400 PerkinElmer, Wellesley

160 MA). The gas carrier was CP-grade helium (BOC gases Guildford UK) passed through a combined trap

161 for removal of hydrocarbons, oxygen and water vapour. A wall-coated Zebron ZB624 chromatographic

162 column was used with dimensions 60 x .04 x 0.25mm (internal diameter), the liquid phase comprising a

163 0.25 µm layer of 6% cyanopropylphenyl and 94% methylpolysiloxane.

164

165 Thermal desorption tubes were initially purged for 2 minutes to remove air and water vapour and then

166 desorbed for 5 minutes at 300°C. The automatic thermal desorption valve temperature was set at 180°C

167 and TD tubes were desorbed onto the secondary cold trap, which was initially maintained at 30°C. Once

168 desorption was complete, the secondary trap was heated to 320°C using the fastest available heating rate

169 and then maintained for 5 minutes. The effluent was transferred to the gas chromatograph through a transfer

170 line heated to 210°C. The gas chromatograph oven was maintained at 50°C for 4 minutes after injection

171 and then raised at a rate of 10°C/min to 220°C and then held for 9 minutes. Eluted products were transferred

172 to the mass spectrometer via a line heated to 240°C. Electron ionisation (70eV) was used. Full scan mode

173 was selected with mass-to-charge ratios from 33 to 350 m/z with a scan time of 0.3 second and 0.1 second

174 interscan delay to produce a total ion count (TIC) chromatogram.

175 **Study 2**

176 Samples were transferred to the laboratory packed in dry ice inside insulated containers and on arrival were

177 stored at -80°C until analysis.

178 Aliquots (5ml) of the defrosted samples were placed in gas sampling bags which were then sealed and filled

179 with hydrocarbon-free air and incubated for 10 minutes at body temperature. A portable air pump was then

180 used to draw 500ml of headspace through TD tubes packed with 50% Carbotrap and 50% Tenax. Full

181 details have been published elsewhere [16]

182

183 **Data and statistical analysis**

184   **Study 1**

185 Compound identification was achieved using Automated Mass Spectral Deconvolution and Identification

186 (AMDIS version 2.62) software and the National Institute of Standards and Technology mass spectral

187 library. Quantification was achieved by comparing the area of each compound peak with the peak area

188 associated with the known amount of d8 toluene.

189

190 Concentration data proved to be heavily right-skewed, therefore a non-parametric approach was adopted.

191 A McNemar test was used to determine whether the probability of a compound to be present before or after

192 the diet was significant. When present a Wilcoxon Rank Test was used to see if the compound was present

193 in different quantities. Raw TIC data (i.e. a matrix of time vs. ion abundance) were also subjected to

194 Principal Components Analysis (PCA)[27] using Matlab (version 6/5 Mathworks Inc USA incorporating

195 functions from the PLS Toolbox version 2.0 Eigenvector Research Inc USA).

196 **Study 2**

197 Compound identification and quantification were carried out as for study 1. In any given faecal headspace

198 sample, automated mass spectral deconvolution and identification (AMDIS) would identify between 100-

199 300 different compounds and it was therefore found necessary to select a subset of those we observed to

200 render statistical analysis tractable. Three approaches were followed to provide a list of what we have

201 termed 'candidate compounds'. The list comprised first compounds that appeared to be most abundant

202 from inspection of the results obtained using AMDIS; second compounds that appeared to discriminate

203 between patient groups by visual inspection of a subset of pre-treatment sample chromatograms and third

204 compounds selected on the basis of a search of the relevant literature. An initial generic list was made

205 including short-chain fatty acids (SCFAs) and their derivatives, phenolic compounds and indoles and

206 sulfides. This list was then refined according to publications dealing more explicitly with VOC profiles in

207 disease. A final list of compounds was obtained in this way.

208

209 **Results**

210 **Study 1**

211

212 Of the 12 volunteers recruited, two females withdrew before the feeding commenced. During the first

213 feeding period 2 withdrew after 2 days feeding, one (female having E028) because of persistent hunger and

214 the other (male having Modulen-IBD) because of insomnia attributed to an empty stomach. Eight subjects

215 completed the first phase. A further subject (male Modulen-IBD) withdrew after 4 days in the second phase

216 because of malaise and headaches.

217

9

218 Stool consistency and frequency showed no change. There was a consistent change in stool colour from

219 browns towards green on E028 extra (r=0.639, p<0.05 Spearman test), and a similar but less marked effect

220 was seen after Modulen-IBD (r=0.598, p<0.05). Faecal colour had returned to normal by the start of the

221 second feeding period.

222

223 All subjects showed deterioration in odour on E028 extra and 5 out of 6 on Modulen-IBD. One volunteer

224 did not record his breath changes on a daily basis. A Spearman test showed a significant difference between

225 the odour of the breaths of the volunteers before they started and the last day of the diet (E028 extra r=0.575

226 p<0.05, Modulen-IBD r=0.574 p<0.05). Subjects' breath odour had returned to normal at the start of the

227 second feeding period. Numerical results were presented as mean with upper and lower quartiles. The

228 frequency distributions for all compounds were found to be highly skewed with a proportion of nondetects;

229 therefore, a nonparametric statistical approach was adopted.

230

231 Over 140 compounds were seen in the breath analysis including aldehydes, ketones, saturated and non-

232 saturated hydrocarbons, organic acids, alkenes, alcohols and furans. The compounds also varied between

233 volunteers. As at least one third of compounds were known to be environmental contaminants, e.g. benzene,

234 toluene, xylene, we concentrated on two marker compounds known to be bacterial metabolites, phenol and

235 indole.

236

237 The mean alveolar gradient for indole on a normal diet was $0.034 \pm$ SD 0.029. There was little change

238 following Modulen-IBD $0.041 \pm$ SD 0.028 (NS). After E028 it rose to $0.149 \pm$ SD 0.099 (NS) The

239 differences between the values after diet did not differ significantly from those before, but the aveolar

240 gradient after E028 was significantly higher than that after Modulen-IBD (P<0.03).

241

242 The mean level of alveolar gradient for phenol on the breath on a normal diet was 0.024 ± SD 0.017. After

243 Modulen-IBD it rose to 0.055 ± SD 0.025 (NS). After E028 the levels were 0.229 ± SD 0.152 (p<0.05).

244 The increase after E028 was significantly greater to that after Modulen-IBD P=0.035   After 3 weeks of

245 normal eating, breath chemicals had in every case returned to levels indistinguishable from those present

246 at the start of the first period of enteral feeding.

247

248 **Results**

249 **Study 2:**

250

251 At the start of treatment all 17 patients had active disease as confirmed by a Harvey and Bradshaw index

252 of >6 and raised concentration of C-reactive protein (CRP) in the blood. 9 patients were receiving no

253 medication, 4 were taking 5ASA compounds, 2 were taking 5ASA with Azathioprine, 1 taking

254 Azathioprine alone and 1 taking Azathioprine and Prednisolone. Patients were asked to continue the same

255 medication throughout the study and this was not changed in any way, remission being achieved in all cases

256 by the addition of enteral feed. The mean Harvey & Bradshaw (H&B) before treatment was 6.88 ± SD

257 2.93 falling to 4 ± SD 5.50 after treatment, (p<0.05). The initial mean CRP was 36.0 ± SD 41.3mg/L

258 falling to 8.11 ± SD 3.59 after treatment (p<0.05).

259

260 The results of GC/MS faecal analysis are summarised in Table 1. Many compounds of known bacterial

261 origin were present in the initial sample. These included propanoic and butanoic acids, para-cresol, indole,

262 dimethyl disulphide and phenol. The concentrations of the SCFAs fell dramatically after enteral feeding.

263 No difference was discerned in the fall of concentrations of bacterial metabolites in those subjects receiving

264 enteral feeds alone, and those who continued their previous medication. Thus the results of all the patients

265 were analysed together.

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266

267 There were also however, a number of potentially toxic compounds present. These included the alcohols,

268 1-propanol and 1-butanol as well as the methyl and ethyl esters of propanoic acid and butanoic acid. After

269 treatment, the amounts of these compounds also fell significantly. The SCFA-esters disappeared virtually

270 completely and there was a significant fall in the concentration of 1-propanol and 1-butanol. However,

271 other chemicals including those derived by bacterial breakdown of amino acids, phenol and indole did not

272 change significantly (table 1).

273

274 **Discussion**

275

276 The present study demonstrates changes in chemicals of microbial origin in both healthy controls and in

277 patients with CD after administration of enteral feeds. Our first study confirms reports of stool colour

278 change during treatment with the development of breath odour. It is probable that this was the result of the

279 cessation of the normal microbial breakdown of biliverdin (green) to stercobilin (brown).

280

281 We also attempted to assess bacterial activity by determination on the breath of known bacterial metabolites

282 that might be absorbed into the blood stream from the colon. Many chemicals are present in breath and

283 urine and we detected 140. Their origins of many are poorly understood. We therefore concentrated on

284 changes in the excretion of two chemicals whose synthesis by the microbiota is well understood, namely

285 phenol and indole.[14,15]

286

287 Phenol and indole are produced by the microbial conversion of tyrosine and tryptophan respectively. Much

288 less is produced when carbohydrate fermentation is continuing in the colon. Conversely, when

289 carbohydrate was withdrawn from the diet, phenol production from endogenous protein sources such as

290 intestinal secretions and exfoliated cells was increased [17,18]

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291

In the present study, phenol and indole identified on the breath showed a significant increase in concentration after feeding with Modulen-IBD and an even greater increase after E028extra, which rapidly returned to base line on resumption of a normal diet.   This is consistent with a switch in colonic fermentation to a protein-based pattern, as an effect of ingesting carbohydrate in the form of maltodextrins - simple sugars that are absorbed high in the small intestine - rather than complex carbohydrates that may pass down to be fermented by the colonic flora. Indole is malodorous and may contribute to the unpleasant breath odour reported by our volunteers.

299

The effect of E028 on phenol and indole was greater than that of Modulen IBD. The may be related to the content of long chain triglyceride in the feeds which we and others have shown to be an important factor influencing their effectiveness. [2,28] The LCT content of Modulen IBD is greater than that of E028 extra.

303

The term 'enterometabolic disease' has been suggested for non-infective conditions arising from abnormal fermentation by the colonic microbiota [13]. Patients with IBS have a similar abnormal gut flora to that seen in CD.[11,20,23] and have a markedly increased excretion of a bacterial product, hydrogen. This was dramatically reduced, with highly significant reduction in symptoms, when patients were switched from a standard diet to an exclusion diet, suggesting that the diet reduced microbial activity [24]. Support for this concept was provided by the demonstration of reduced hydrogen excretion in patients with IBS , again with significant improvement in symptoms, when microbial activity was reduced by administration of antibiotics or by enteral feeding .[25]

Is it possible that CD like IBS may be an 'entero-metabolic disorder', [13] and that enteral feeding is effective because it reduces the metabolic activity of an abnormal colonic flora?

314

13

315 There is strong evidence that the host microflora provokes an immunological response in CD. Duchmann

316 and his colleagues showed that monocytes from the peripheral blood and the lamina propria were activated

317 when incubated with preparations of faecal bacteria from other subjects, but not by such preparations

318 derived from the faeces of the host. Monocyte activation occurred only when host faeces was incubated

319 with cells obtained from the lamina propria from sites of active CD. No activation was seen in monocytes

320 obtained from areas where no active CD was present, suggesting that monocytes in areas of active CD were

321 specifically targeted against the host microflora.[10]

322

323 This finding has been supported by later studies that demonstrated that the great majority of microorganisms

324 found in the faeces of patients with IBD were coated with immunoglobulin, including IgA, IgG and IgM,

325 whereas in normal subjects or those with IBS, less than 20% were so affected [12].

326 Furthermore, a significant reduction in the number of microorganisms coated with immunoglobulin was

327 seen after 2 weeks treatment with corticosteroids in UC, and a similar response occurred in CD after a two

328 week course of elemental feeding. This suggested that the immune response to the flora had been

329 significantly reduced, an interpretation supported by the finding that patients with CD and UC in long term

330 remission had similar numbers of coated bacteria to those seen in healthy controls.[12]

331 No specific pathogen has as yet been confirmed as being the cause of CD, but it has been demonstrated that

332 the faecal flora is abnormal with an overgrowth of facultative anaerobes and reduction in the numbers of

333 important beneficial species such as *Lactobacilli* and *Bifidobacter*.[11,20] Although previous studies of the

334 effects of enteral feeding on the composition of the bacterial flora in CD, had been inconclusive,[21,22]

335 a recent study of the entire gut mucosal microbiome in a child with CD before and after nutritional therapy

336 showed that the flora, initially markedly abnormal, returned after therapy, to a pattern very similar to that

337 found in a healthy control [26]. Likewise, it has also been shown that enteral nutrition in CD may reduce the

338 levels of certain bacteria within the *Firmicutes*. These bacteria are important producers of SCFAs and this

339 report is in keeping with our discovery of reduced SCFA production. [27]

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340

341 Unfortunately, it was not possible in the present study to perform complex studies of changes in the gut

342 microbiome, but changes in bacterial metabolites serve as valuable markers of its metabolic activity. SCFAs

343 have an important function in the colon especially butanoic acid which is a major source of nutrition for

344 colonocytes.[19] They are produced by the microbial fermentation of undigested complex carbohydrates

345 entering the caecum and the fall in faecal SCFA concentration found after enteral feeding in our patients

346 with CD was consistent with reduction in colonic fermentation.

347

348 Such a reduction in fermentation might be beneficial if it resulted in less production of toxic metabolites.

349 There were highly significant falls in the concentrations of number of chemicals including 1-propanol, p-

350 cresol, phenol, 1-butanol, dimethyl disulphide and fatty acid ethyl esters (Table 1). These are known to be

351 toxic chemicals which we have shown not to be present in the stools of healthy volunteers. [16]   It seems

352 possible that the production of such chemicals might be a factor initiating an immune attack on the

353 microflora. This could lead to coating of microflora with immunoglobulin – a suggestion which has been

354 supported by the significant reduction in bacterial coating seen after 2 weeks feeding with enteral feeds.[12,16]

355

356 Similar toxic chemicals also appear in UC, but in contrast to CD, **do not fall** after enteral feeding, but only

357 after successful treatment by immunosuppression with prednisolone[16.] Although evidence on the role of

358 diet in UC remains weak, this suggests that the microflora in UC differs from that in CD in that it derives

359 its nutritional requirements, not from food residues, but from other substances present in the large intestine

360 – possibly mucus or intestinal secretions.   It is therefore feasible, that the production of toxic chemicals

361 resulting from abnormal bacterial metabolism, may be an important factor in the initiation of an immune

362 attack on the microflora in inflammatory bowel disease.

363

364

15

Table 1 Changes in faecal chemicals before and after elemental feeding in patients with Crohn's disease

| Compound | VOC concentration (ng/l) Median (lower quartile, upper quartile) | | p-value |
	Pre-treatment	Post-treatment	
acetone	57 (38, 128)	80 (50, 104)	0.435
propanoic acid	169 (0, 328)	12 (0, 84)	0.031*
butanoic acid	1110 (316, 1596))	24 (0, 104)	0.001*
1-propanol	229 (41, 892)	36 (0, 233)	0.025*
propanoic acid, ethyl ester	19 (0, 117)	0 (0, 15)	0.008*
butanoic acid, methyl ester	19 (7, 121)	0 (0, 1)	0.013*
butanoic acid, ethyl ester	46 (4, 255)	0 (0, 15)	0.008*
p-cresol	518 (118, 1160)	480 (144, 1051)	0.687
indole	118 (54, 146)	20 (0, 128)	0.125
dimethyl disulphide	83 (34, 683)	39 (0, 140)	0.113
1-butanol	99 (57, 256)	58 (0, 199)	0.030*
butanoic acid, 3-methyl	147 (48, 504)	0 (0, 45)	0.015*
phenol	64 (16, 102)	24 (10, 177)	0.332

16

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473 **SUPPORTING INFORMATION**

474 Declaration of funding interests:

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478 STROBE STATEMENT

479 All items on the strobe checklist have been checked and confirmed to be included in this paper.