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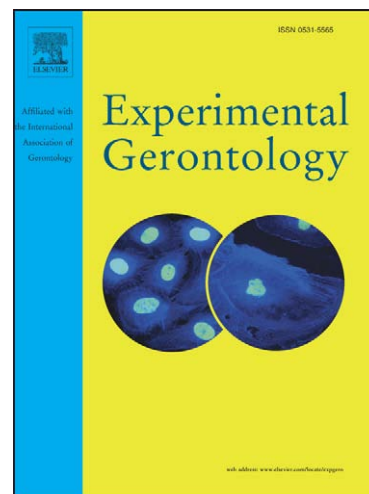
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1 **Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in**
2 **fecal population sizes and diversity of *Bacteroides*, bifidobacteria and**
3 **clostridium cluster IV in institutionalized elderly.**

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41 18 **running title : Bacterial diversity in elderly.**

42
43 19 Keywords: microbiota, PCR-DGGE, qPCR, *Bacteroides*, bifidobacteria, *Clostridium*,
44 feces, ageing

45
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48 20
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50
51 22 **Abbreviations:**

52
53 23 APS ammonium-persulfate

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55 24 TEMED N,N,N',N'-tetramethylethylenediamine

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58 25 GIT gastro-intestinal tract

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26 **ABSTRACT**

1
2 27 Aims: This study aimed at determining ageing-related shifts in diversity and
3
4 28 composition of key members of the fecal microbiota by comparing institutionalized
5
6
7 29 elderly (n=17, 78-94 yrs) and young volunteers (n=17, 18-31 yrs).

8
9 30 Methods and Results: A combination of molecular methods was used to characterize
10
11
12 31 the diversity and relative abundance of total gastro-intestinal flora, along with relevant
13
14 32 subsets within the genera *Bacteroides*, *bifidobacteria* and *Clostridium* cluster IV. The
15
16 33 institutionalized elderly harbored significantly higher numbers of *Bacteroides* cells
17
18
19 34 than control (28.5 % \pm 8.6%; 21.4 % \pm 7.7%; p=0.016) but contained less
20
21
22 35 *bifidobacteria* (1.3 % \pm 0.9, 2.7 % \pm 3.2%, p=0.026) and *Clostridium* cluster IV (26.9%
23
24 36 \pm 11.7%, 36.36% \pm 11.26%, p=0.036). The elderly also displayed less total *Bacteria*
25
26
27 37 diversity and less diversity with the *Clostridium* cluster IV (p<0.016) and *Bacteroides*.
28
29 38 Conclusion: Despite high individual variations, our analyses indicate the composition
30
31
32 39 of microbiota in the elderly comprises a less diverse subset of young healthy
33
34 40 microbiota.

35
36 41 Significance and Impact of the Study: A better understanding of the individual
37
38
39 42 composition of the human microbiota and the effects of ageing might result in the
40
41 43 development of specifically targeted supplementation for elderly citizens in order to
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44 44 support healthy ageing.

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46 INTRODUCTION

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5 48 It is estimated that between 60-80 % of the microbial diversity resident within the
6
7 49 human gastro-intestinal tract (GI) has yet to be cultivated (Suau *et al.* 1999).
8
9
10 50 However, the use of culture-independent molecular methods has enabled
11
12 51 researchers to identify important characteristics of this community. We now know that
13
14 52 the composition of GI microbiota is highly distinct between individuals (Dethlefsen *et*
15
16 53 *al.* 2006). Despite this variability, some general features are apparent. There appear
17
18 54 to be 'core species' present in a majority of humans which are quite resilient to
19
20 55 external influences (Dore 2007). In addition to this group, there are also 'passengers'
21
22 56 or transients, sometimes in great numbers, sometimes below detection limit (Favier
23
24 57 *et al.* 2003). However, the majority of sequences seem to be unique to some
25
26 58 individual and not one single phylotype may be present in all humans (Turnbaugh *et*
27
28 59 *al.* 2009). A number of factors have been identified which influence community
29
30 60 composition. The type of bacteria introduced to the environment is determined by
31
32 61 those associated with food sources (Duncan *et al.* 2007; Pryde *et al.* 2002). The
33
34 62 nature and composition of non-digestible carbohydrates in a diet can further stimulate
35
36 63 different types of bacteria (Kolida and Gibson 2007), whilst hereditary dispositions
37
38 64 and personal gut environmental factors may account for unique personal
39
40 65 characteristics (Eckburg and Relman 2007).
41
42 66 Correlations between the GI microbiota and diseases such as allergenicity (Shreiner
43
44 67 *et al.* 2008), inflammatory bowel diseases (Sokol *et al.* 2006) and individual
45
46 68 dispositions such as obesity (Zhang *et al.* 2009) have been discussed.
47
48 69 The colon harbors butyrate-producing species of several genera such as *Clostridium*,
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51 70 *Eubacterium* and *Fusobacterium* (Pryde *et al.* 2002). Among strains that produce
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71 high levels of butyrate *in vitro* are those related to *Faecalibacterium prausnitzii*, an
72 ubiquitous member of *Clostridium* cluster IV (Pryde et al. 2002). *Bacteroidetes*
73 species have been reported to show high variations between individuals, although
74 *Bacteroides thetaiotaomicron* is found in all human beings (Layton et al. 2006). Both
75 *Bacteroidetes* and *Clostridium* cluster IV are known to be involved in beneficial
76 functions, including nutrient absorption, production of short chain fatty acids (SCFAs)
77 and epithelial cell maturation and maintenance (Woodmansey 2007).
78 Another important subgroup of the human GI microbiota is the bifidobacteria.
79 Stimulation of these bacteria has previously been shown after prebiotic intervention
80 with inulin and fructo-oligosaccharides (Kolida and Gibson 2007). Furthermore, they
81 have been shown to be involved in prevention of atopic disease (Ouwehand *et al.*
82 2007), obesity and insulin resistance via enhanced barrier function of the gut
83 epithelium (Cani *et al.* 2007).
84 With ageing, a decrease in beneficial organisms such as *Lactobacilli* and
85 bifidobacteria, amongst other anaerobes, and an increase in the number of
86 facultative anaerobes (Guigoz *et al.* 2008) have been reported. Population cross-
87 sectional studies with relevant numbers of healthy elderly also show age-related
88 changes in GI microbiota. These include a consistent global increase in
89 nonpathogenic Gram-negative bacteria (mainly *Enterobacteria*), as well as country-
90 specific changes in bifidobacteria (Guigoz *et al.* 2008). This, along with a general
91 reduction in species diversity within most bacterial groups, changes to diet and
92 altered digestive physiologies such as intestinal transit time, may result in increased
93 putrefaction in the colon and a greater susceptibility to disease. The aged gut is
94 characterized by increased proteolytic activity, decreased amylolytic activity and
95 reduced levels of SCFA (Woodmansey 2007). Ageing is associated with reduced

96 levels of prostaglandins such as PGE₂ and PGF₂α as shown in specimens of
97 stomach and duodenum biopsies (Tiihonen *et al.* 2008). Subclinical intestinal
98 inflammation in elderly populations has been detected and is believed to contribute to
99 impaired immune functions, the underlying cause of mortality beyond 75-80 years of
100 age (Guigoz *et al.* 2008).

101 Analyses of individual dispositions associated with changes of the microbiota should
102 consider quantitative and qualitative aspects of gut community structure. Analysis of
103 stool samples can target changes in colonic microbiota, since feces are
104 representative of inter-individual differences (Turnbaugh *et al.* 2007).

105 The aim of this work was to investigate shifts in GI microbiota associated with aging,
106 by comparing institutionalized elderly with young healthy volunteers. To analyze
107 changes in total bacterial community composition, along with specific compositional
108 changes within the *Bacteroidetes*, bifidobacteria and *Clostridia* cluster IV, we used
109 the polymerase chain reaction (PCR) based community fingerprinting method
110 Denaturing Gradient Gel Electrophoresis. The resolution of this method allows for the
111 characterization of the dominant members of a targeted microbial community.
112 Further, we used quantitative-PCR (q-PCR) to determine the relative load of
113 *Bacteroidetes*, bifidobacteria and *Clostridia* cluster IV groups within our samples.
114 Thus, use of these methods in combination allowed the characterization of both
115 diversity and relative abundance of our targeted organisms.

117 MATERIAL AND METHODS

118 Probands

119 Seventeen institutionalized elderly aged 86 ± 8 years, BMI 21.75 ± 5.08, from a
120 geriatric department in Vienna and 17 students from Vienna joined the study.

121 Probandns were interviewed following a questionnaire assessing: age; gender; body
122 length and weight; individual health status, including chronic or acute diseases and
123 blood lipid levels; and life-style aspects, such as physical activity and dietary habits.
124 Five percent of geriatric patients suffered from manifest diabetes mellitus type 2. Ten
125 patients were bed-ridden and seven mobile. Causes for loss of mobility were
126 Parkinson's disease, dementia and osteoporosis. Nursing staff reported the
127 application of NSAIDs (non-steroidal anti-inflammatory drugs) on demand.
128 Seventeen young healthy volunteers were aged 24 ± 2.5 years, BMI 22.68 ± 3.41
129 and their dietary habits were typical for Central Europe.
130 Study populations were gender balanced, with 55% females in the group of elderly
131 and 50% in the young group. Only non-pregnant probands with no diagnosed gastro-
132 intestinal disease and no antibiotic or chemotherapeutic treatment three months prior
133 to sampling were included in the study. All probands agreed to participate in the
134 study and gave their informed consent.

135

136 **Sampling and DNA extraction from Stools and Type Strains**

137 From each proband, three stool samples were taken within the course of a week and
138 immediately stored at -70°C . Portions of the three samples from each patient were
139 pooled, a 200 mg aliquot was treated twice for 45 sec in a bead-beater (Mini-
140 Beadbeater-8) and DNA extracted with the QIAamp® DNA Stool Mini Kit (QIAGEN)
141 following the manufacturer's protocol and then immediately stored at -20°C .

142 Type strains known to be associated with GI microbiota were grown, and DNA
143 extracted, for use as part of the markers in DGGE analysis (see below). Type strains
144 *L. casei* DSM 20011^T, *L. delbrueckii* subsp. *lactis* DSM 20072^T, *Bacteroides fragilis*
145 DSM 2151^T, *Bacteroides thetaiotaomicron* DSM 2079^T, *Bifidobacterium longum*

146 DSM20219^T, *B.longum* DSM 20211, *B.pseudolongum* DSM 20099 and
147 *B.thermophilus* DSM 20210 were anaerobically cultivated on blood agar, *E.coli* IMBH
148 252/07 and clones were aerobically cultivated on LB-agar (liquid broth medium). The
149 biomass was resuspended in sterile phosphate buffered saline (1x PBS from 10x
150 PBS Roti®stock, ROTH). Tenfold dilutions from these suspensions in sterile 1x PBS
151 were plated in duplicate on blood agar or LB-agar, colony forming units (CFU/ml)
152 were counted and DNA extracted from serial dilutions. DNA was extracted from
153 clones using the Wizard® Plus.SV Minpreps DNA Purification System (Promega).
154 The DNA of all Gram-negative bacteria was extracted with the DNA Mini Kit
155 (QIAGEN), for Gram- positive bacteria, the FastDNA Spin Kit for Soil (MP-
156 Biomedicals) was used following the instructions of the manufacturer.

157

158 **Polymerase Chain Reaction (PCR)**

159 PCR was used to amplify 16S ribosomal RNA gene sequences from type strains and
160 *Bacteria* in stool samples for use in DGGE analysis and manufacture of clone
161 libraries (see below). All reactions were carried out using a ready-to-use mastermix
162 (Promega) with 1.5mM MgCl₂. Bovine serum albumin (10mg/ml, Fermentas) was
163 added to a final concentration of 400µg/ml, primer concentration in the reaction
164 volume was 0.5µM. Amplifications were carried out in a Robocycler (Stratagene).

165

166 **Clone libraries**

167 Clone libraries were constructed from stool samples to identify dominant members of
168 the *Clostridium* cluster IV and the *Bacteroidetes*. Selected clones were then used,
169 along with the cultured type strains, to generate appropriate reference markers for
170 DGGE analysis. Amplifications were carried out using primer pair 27f Edwards et al.

171 1989) and sg-Clep-R (Matsuki *et al.* 2004), which are specific for members of the
172 *Clostridium* cluster IV, and 32f and 708r (Bernhard and Field 2000) which are
173 specific for members of the *Bacteroides*. Amplified products were cloned into a p-
174 GEM Easy Vector (Promega) following the instructions of the manufacturer. Clone
175 libraries were screened as previously described (Schabereiter-Gurtner *et al.* 2001).
176 Clone inserts were sequenced by 'DNA confidence' (Vienna). Nucleotide sequences
177 were corrected for vector and primer sequences in CodonCode Aligner
178 (www.codoncode.com) and taxonomically identified by comparison to previously
179 published sequences using the online tools of the ribosomal database project
180 (<http://rdp.cme.msu.edu/>).

181

182 DGGE

183 PCR amplifications of 16S rRNA gene fragments from total DNA extracted from stool
184 samples were carried out using the specific primer sets outlined in Table 2.

185 Separate DGGE gels were run to analyse samples for patterns in a) total *Bacteria*, b)
186 bifidobacteria, c) *Clostridium* cluster IV and d) *Bacteroides*. DGGE gels were
187 prepared as described previously (Muyzer and Smalla 1998) with a linear gradient of
188 25-65% for *Bacteria* (general), 30-65% for bifidobacteria, 20-50% for *Bacteroides* and
189 30-50% for *Clostridium* cluster IV, using a gradient mixer (Hoefer SG 30) and a
190 peristaltic pump. We generated reference markers appropriate for each set of DGGE
191 analyses (i.e. a different reference marker was used for each of a,b,c and d above) to
192 enable meaningful comparisons across multiple gels and to provide putative
193 identification of some bands. These reference markers contained fragments of 16S
194 rRNA genes from cultured bacteria and clones generated from fecal material (as
195 described above). Each marker was loaded in triplicate on each gel to allow gel-to-

196 gel comparison. The reference marker for DGGE fingerprinting of general *Bacteria*
197 consisted of *E.coli* IMBH 252/07, *Enterococcus faecium* DSM 20477^T, *Bacteroides*
198 *thetaiotaomicron* DSM 2079^T, *Bifidobacterium longum* DSM 20219^T, *Clostridium*
199 *perfringens* (laboratory isolate), clone BT11 (98.8% similarity with *Bacteroides*
200 *uniformis* JCM 5828^T), clone BT17 (96.0% similarity with *Bacteroides vulgatus*^T and
201 bacterium LY88 previously identified in human feces (Eckburg *et al.* 2005)) and clone
202 CL16 (98.4% similarity with an uncultured bacterium from human feces (Turnbaugh
203 *et al.* 2006) and 96.7% similarity with *Faecalibacterium prausnitzii*^T). The reference
204 marker for bifidobacteria specific DGGE analysis consisted of *Enterococcus faecium*
205 DSM 20477^T, *E.coli* IMBH 252/07, *Bifidobacterium longum* DSM 20219^T, *B. longum*
206 DSM 20211, *B. thermophilus* DSM 20210^T and *B. pseudolongum* DSM 20099^T. The
207 reference marker for *Bacteroides* specific DGGE analysis was composed of
208 *Bacteroides thetaiotaomicron* DSM 2079^T, fecal clones Bt 17 and Bt11 and
209 *Enterococcus faecium* DSM 20477^T. The reference marker for *Clostridium* cluster IV
210 DGGE analysis was constructed from 7 clones all representing previously uncultured
211 species from cluster IV: Similarities with type strains from this cluster were 81.8%,
212 96.6%, 96.7% and 97.8% similarity with *Faecalibacterium prausnitzii*^T, 92.1% and
213 92.9% similarity with *Eubacterium desmolans*^T and 95.9% similarity with
214 *Subdoligranulum variabile*^T.

216 **TaqMan RTQ-PCR**

217 The TaqMan-assay was carried out in a Rotorgene 3000 (Corbett Life Science) in
218 duplicate in a volume of 10 µl containing 5 µl TaqMan SensiMix DNA Kit (Quantace),
219 1 µl of each primer and probe (final concentrations table 1) and 2 µl of the 100-fold
220 dilution of the template. All probes were labeled with 6-FAM at the 5' end and carried

221 a BHQ-1 quencher at the 3' end. TaqMan Probe (Clep-P) for *Clostridium* cluster IV
1
2 222 was designed with CLC DNA Workbench (www.clcbio.com). Analysis with
3
4
5 223 ProbeMatch (rdp.9.58) indicated that Clep-P binds to all members of *Clostridium*
6
7 224 cluster IV. The PCR program for bifidobacteria, *Bacteroides* and universal bacteria
8
9 225 consisted of denaturing at 95°C for 3min and 45 cycles of 95°/60°C for 15/ 45 sec.
10
11
12 226 The amplification program for clostridium cluster IV was: denaturation at 95° for 5 min
13
14 227 and 45 cycles at 95°/ 55°C for 30/ 45 sec.
15
16
17 228 DNA of *Bacteroides thetaiotaomicron*^T and *Bifidobacterium longum*^T, clone CL16 and
18
19 229 one fecal sample were used to construct standard curves for comparison of PCR
20
21
22 230 reaction efficiencies among different experiments and enumeration of all bacterial
23
24 231 groups. Reaction efficiency (E) was estimated using the slope of the standard curve
25
26 232 and the formula $E=10^{(-1/\text{slope})} - 1$ as described elsewhere (Penders *et al.* 2005).
27
28
29 233 Quantification was done using standard curves obtained from known concentrations
30
31
32 234 of organisms containing the respective amplicons for each set of primers. The
33
34 235 percentage of bacterial group rRNA gene copies in relation to total rRNA gene copies
35
36 236 (relative abundance) was calculated for each individual, and the mean was
37
38
39 237 determined for each subject group. Relative quantification (% of bacteria) was
40
41 238 performed using Rotor-Gene 3000 calculation software (Corbett operator manual)
42
43
44 239 and Excel. Cross reactivity with non-target strains was tested using the Probe-Match
45
46 240 tool at the RDP website and using the strains mentioned above.
47

48 241
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51 242 **Statistical analysis.**
52
53 243 Food frequency data were analyzed based on Chi-square approximation as
54
55
56 244 implemented in SPSS. P values <0.05 were considered significant. Band comparison
57
58 245 tables were analyzed with principal component analysis (PCA) using the default
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246 settings in 'R-software environment for statistical computing' (www.r-project.org) until
1
2 247 100% variance was explained. Transformed data were plot in a bi-plot as a function
3
4
5 248 of the first two principal components. Clustering was applied to DGGE fingerprinting
6
7 249 data as implemented in the GelComparII environment (www.applied-maths.com).
8
9
10 250 Clustering was performed based on Dice coefficient as well as based on Pearson
11
12 251 correlation. Those methods are supplementary to each other, clustering after Dice
13
14 252 takes band positions into account, whereas Pearson correlation based clustering
15
16 253 analyzes the densitometric curves of each fingerprint. UPGMA dendrograms were
17
18
19 254 generated and Jackknife analysis was performed using average similarities and 100
20
21
22 255 resamplings. Jackknife analysis is a leave-one-out method that tests the reliability of
23
24 256 the clustering similar to bootstrap analysis. Shannon and Simpson's diversity index
25
26 257 were calculated on binary band information (presence-absence) with the default
27
28
29 258 settings implemented in the 'vegan' package in 'R'. Shannon index is defined as $H = -$
30
31
32 259 $\sum p_i \ln p_i$, where p_i is the proportional abundance of species i . For Simpson's index D ,
33
34 260 this is $1 - \sum (p_i)^2$, where p_i is the relative frequency of the i -th species. Prior to
35
36
37 261 application of Student's t-test for diversity indices and qPCR data, equality of
38
39 262 variances of the three datasets was tested using the F-test as implemented in
40
41
42 263 Microsoft Excel.

265 RESULTS

51 267 Dietary aspects

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54 268 Analysis of the participant's dietary habits indicated similar consumption patterns of
55
56 269 fruits, vegetables and milk products in both groups. Young volunteers stated
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58
59 270 significantly less frequent (Chi^2 Test; $p < 0.04$) consumption of meat than elderly
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271 probands and regular consumption of whole grain products several times a week.

272 The institutionalized elderly of this study did not consume any whole grain products

273 at all but received supplements with soluble fiber (Benfiber®, Novartis).

274

275 **TaqMan-quantification**

276 TaqMan assays were set up quantifying bacterial sub-populations as percentage of

277 the total bacterial DNA. We detected no cross-reactivity of group-specific primers and

278 probes with non-target strains. Test-retest variations were between 2.7% and 5.2%,

279 values after relative quantification varied by less than 4%. The sensitivity was

280 corresponding to a 100 000 fold dilution of DNA from feces, that is 20 copies of 16S

281 rRNA gene per reaction.

282 The elderly harbored only $69\% \pm 21.6\%$ of the total bacterial load in their faeces

283 compared to control (figure 1). *Bacteroides* were found to represent a larger

284 percentage than bifidobacteria in all samples (figure 1) and the institutionalized

285 elderly harbored significantly more *Bacteroides* than young volunteers ($p=0.016$).

286 Although relative levels of bifidobacteria were highly variable among samples, the

287 differences between young and elderly probands were statistically significant

288 ($p=0.026$). Furthermore, elderly citizens had significantly less members of *Clostridium*

289 cluster IV (figure 1) in their fecal microbiota than young volunteers ($p=0.036$).

290

291 **DGGE bandpattern analysis**

292 DGGE fingerprinting with primer pair 341GC-518, which amplified the total microbial

293 community, showed high inter-individual variations (figure 2). The mean numbers of

294 bands per individual were 16.6 ± 3 bands for institutionalized elderly and 20 ± 3

295 bands in control. Two bands, highlighted in figure 2, occurred significantly less

296 frequently in elderly probands. Shannon and Simpson indices of diversity were
297 significantly lower for elderly than control (figure 3). The highly diverse dataset was
298 subjected to principal component analysis (PCA). Extraction of underlying
299 components within the dataset indicated grouping of samples according to their
300 variance (figure 4) along the first two principal components. Cluster analysis could
301 separate the fingerprints of young and elderly (figure 5). Jackknife analysis
302 suggested that the dominant bacteria of the elderly microbiota are a subpopulation of
303 the microbiota of young individuals: Predictability of groupings was 100% for young
304 and only 13.33% for elderly.

305 Bifidobacteria

306 An average of thirteen bands were observed in individual DGGE bandpatterns
307 obtained with the primer pair specific for bifidobacteria. Two bands with the same
308 melting behaviour as the type strains *B.longum* DSM 20219^T and *B.pseudolongum*
309 DSM 20099^T in our marker were abundant in the majority of probands. PCA was
310 performed and the first two PC's explained 23.64% of variance (figure 4). Although
311 cluster analysis showed high similarity of young and elderly bandpatterns, jackknife
312 testing demonstrated greater similarity of bandpattern for control than for elderly
313 (expressed in predictability of groupings: 91.67% for control and 35.72% for elderly).
314 PCA supported these results and could not separate young and elderly according to
315 variances in the dominant bifidobacteria of their microbiota.

316 *Bacteroides*

317 DGGE fingerprints obtained with the *Bacteroides* specific primers contained an
318 average of 7.6 ± 2.5 bands for the elderly, whilst young individuals averaged 9.5 ± 3

bands. Diversity indices (figure 3) showed a tendency to be lower for elderly than for control. PCA results suggest a tendency for less *Bacteroides* diversity with ageing (figure 4). Elderly subjects grouped along the first principal component. Clustering of Pearson correlations could separate young and elderly individuals according to DGGE fingerprinting. Jackknife analysis using average similarities could predict groupings of young bandpattern with 76.92% reliability and 75% for elderly.

Clostridium cluster IV

DGGE fingerprints obtained with the *Clostridium* cluster IV specific primers yielded an average of 10 ± 3.5 bands per elderly individual and 13 ± 3 bands per young individual. Diversity indices (figure 2) were significantly ($p=0.02$) lower for aged citizens than for the young. One band that occurred more frequently in the young than in the elderly had the same melting characteristics as a band in our marker. This band was generated from a clone from our library related to the genus *Faecalibacterium*. This clone had the highest similarity (99.2%) to an uncultured bacterium EF403698 and also displayed 96.6% similarity to *Faecalibacterium prausnitzii*^T. Three more bands occurred more frequently in the young than in elderly. These bands were identified from our clone libraries as relating to the genera *Ruminococcus* (clone had 95.9% similarity to *Ruminococcus bromii*^T, 99% similarity to uncultured AJ408987 from human colon) and *Subdoligranulum* (clones had 94.7%/ 97.5% similarity to *Subdoligranulum variabile*^T, and 97.9%/ 98% similarity to uncultured bacterium DQ793301).

PCA (figure 4) indicated separation of young and elderly according to their *Clostridium* cluster IV DGGE fingerprints along PC1.

345 Clustering and Jackknife analysis did not result in distinct clustering of elderly and
1
2 346 young individuals. *Clostridium* cluster IV representatives in the elderly microbiota are
3
4
5 347 most likely to be a subset of the species present in young individuals: Only in 35.71%
6
7 348 of resamplings elderly bandpattern grouped with each other, whereas Jackknife
8
9 349 value for young individuals was 100%.

350

351 DISCUSSION

352 Metagenomic analysis of the human GI microbiota is presently the subject of large
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19 353 research consortia and has already substantiated the concepts of a 'core
20
21 354 microbiome' and inter-individual variations (Turnbaugh *et al.* 2007). However, the
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23
24 355 collection of data on 'reference microbiota' is far from complete and thus no definition
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26 356 of a healthy microbiota is available yet. Important information comes from analyses
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28
29 357 addressing the abundance and diversity of specific bacterial populations with
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31 358 relevance to disease, diet or probiotic intervention. We used a combined molecular
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34 359 approach to compare patterns in several target GI microbial groups between the
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36 360 institutionalized elderly and young healthy volunteers. The community fingerprinting
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39 361 method PCR-DGGE was used to compare the diversity present in total *Bacteria* and
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41 362 also specifically within the *Clostridium* cluster IV, bifidobacteria and *Bacteroides*,
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44 363 whilst q-PCR was used to quantify the relative population abundance of these same
45
46 364 bacterial groups in all samples.

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48 365 Our results demonstrate some significant shifts in patterns in GI microbiota between
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50
51 366 our study groups. Faeces from the institutionalized elderly had less total Bacterial
52
53 367 abundance and lower total Bacterial diversity than that from the young subjects,
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56 368 However, samples from the elderly displayed an increase in the relative abundance
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58 369 of *Bacteroides*, although this group tended to display less diversity than *Bacteroides*

370 in the young. The relative abundance of the bifidobacteria and *Clostridium* cluster IV
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2 371 were significantly higher in the young, and the *Clostridium* cluster IV also displayed
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5 372 greater diversity in the young. Furthermore, cluster analysis revealed that for all
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7 373 microbial groups analysed, the members of the GI microbiota in the elderly could be
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10 374 considered a subset of that present in the young.

11
12 375 The results of our bifidobacteria analysis are in agreement with previous studies
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14 376 which have identified a reduction and loss of diversity of bifidobacteria associated
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16
17 377 with aging (Woodmansey et al. 2007, Hopkins et al. (2002)) or hospitalization of aged
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19 378 citizens (Bartosch et al. 2004). Our study population of elderly was supplemented
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21
22 379 with soluble fiber. This prebiotic intervention alone was apparently not able to
23
24 380 antagonize ageing-related changes in the bifidobacteria. In this respect Ouwehand et
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26
27 381 al. (2008) recently reported that supplementation with lactitol and a probiotic
28
29 382 *Lactobacillus* led to an increase in bifidobacteria in the microbiota of elderly.
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31
32 383 Bartosch et al (2004) also reported a marked reduction in the abundance of
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34 384 *Faecalibacterium prausnitzii*, a member of the *Clostridium* cluster IV which decreased
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36 385 in our aged group. However several studies (Hopkins et al. 2002, Woodmansey et al.
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39 386 2007, Bartosch et al. 2004) have reported a decrease in the relative abundance of
40
41 387 the *Bacteroides*, whereas our aged study population displayed a relative increase in
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43
44 388 abundance of this group. Like us, all the previous studies reported a decrease in
45
46 389 *Bacteroides* diversity. Increased levels of *Bacteroides* have been found in individuals
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48
49 390 with infectious colitis (Sokol et al. 2006). Discrepancies in changes of the *Bacteroides*
50
51 391 abundance might be due to country-specific differences in this bacterial subgroup as
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53 392 indicated by Mueller et al. (2006). Mueller et al. (2006) also observed gender effects
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56 393 within *Bacteroides*, with levels being generally higher in males than in females. Our
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58 394 study populations were gender balanced with volunteers being 55% females in the
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395 group of elderly and 50% in the young. The results presented here do not support
396 gender differences in *Bacteroides* abundance. Reductions in amylolytic activity
397 observed in a healthy elderly population have been correlated with the occurrence
398 and diversity of *Bacteroides* (Woodmansey 2007).

399
400 Reduced numbers of *Bacteria* in the fecal content of elderly reflect the physiological
401 alterations associated with ageing. These include prolonged colonic transit time and
402 reduced dietary energy requirement and food uptake (Morley et al. 2007). Further
403 reductions in the relative abundance of important sub-populations such as
404 *Clostridium* cluster IV and bifidobacteria might result in reduced formation of SCFAs,
405 altered epithelial cell maturation and maintenance, and altered barrier function of the
406 gut epithelium in elderly probands. Those changes in the GI microbiota have
407 previously been linked to impaired immune functions prevalent in individuals of
408 advanced age and may result in a greater susceptibility to disease.

409
410 Improved analytical concepts for the characterization of the microbiota of consumers
411 and patients might become important as a rationale for individualized probiotic
412 intervention. Probiotic supplementation is a promising concept in restoring impaired
413 functions or enhancing specific desirable functions of the microbiota. Encouraging
414 effects of probiotic supplementation have been reported for aspects such as direction
415 of host immunity, pathogen defense, maintenance of integrity of the gut epithelium,
416 alleviation of lactose intolerance symptoms and immune effects, such as in atopic
417 disease. For further insight into the relationships between phylogenetic information
418 and metabolic activities, sequence information in addition to 16S rRNA based
419 fingerprinting will be mandatory.

420

421 **CONCLUSION**

422 Studies comparing elderly and young volunteer microbiota with PCR-DGGE
 423 fingerprinting and qPCR are still rare. We found that ageing is associated with less
 424 overall bacteria and significantly decreased *Clostridium* cluster IV and bifidobacteria
 425 and an increase of *Bacteroides*. Diversity of dominant bacteria, *Bacteroides* and
 426 *Clostridia* cluster IV were reduced. Those changes in the GI microbiota are
 427 suggested to be cause and effect of impaired immune functions in individuals of
 428 advanced age and may result in a greater susceptibility to disease.

429

430 **Tables:**

431

432 **Table 1:** Primers and probes used for quantification of fecal bacteria using TaqMan
 433 assays targeting 16S rRNA coding regions.

Target organism	Primer or probe	Sequence (5' - 3')	Fragment Size (bp)	Conc. [nM]	Reference
bifidobacteria	Fwd primer	GCG TGC TTA ACA CAT GCA AGT C	125	300	
	Rev primer	CAC CCG TTT CCA GGA GCT ATT		300	
	Probe	(FAM)- TCA CGC ATT ACT CAC CCG TTC GCC -(BHQ-1)		150	(Penders <i>et al.</i> 2005)
<i>Bacteroides</i>	AllBac296f	GAG AGG AAG GTC CCC CAC	106	300	
	AllBac412r	CGC TAC TTG GCT GGT TCA G		300	
	AllBac375Bhq	(FAM)-CCA TTG ACC AAT ATT CCT CAC TGC TGC CT-(BHQ-1)		100	(Layton <i>et al.</i> 2006)
Bacteria (general)	BAC-338-F	ACT CCT ACG GGA GGC AG	468	1000	
	BAC-805-R	GAC TAC CAG GGT ATC TAA TCC		1000	

	BAC-516-P	(FAM)-TGC CAG CAG CCG CGG TAA TAC-(BHQ-1)	200	(Yu 2005)
1				
2				
3				
4	<i>Clostridium</i>			
5	sg-Clep-F	GCA CAA GCA GTG GAG T	239	400
6	cluster IV			
7				
8				(Matsuki <i>et al.</i>
9				
10	sg-Clep-R	CTT CCT CCG TTT TGT CAA	400	2004)
11				
12				
13				
14		(FAM)-AGG GTT GCG CTC GTT-		
15	Clep-P ^a	(BHQ-1)	200	This study
16				
17				

434 ^a position of target site (numbering corresponding to *E. coli* 16S rRNA gene) 1082 to
 435 1107.

436

437 **Table 2:** Primers applied for PCR-DGGE fingerprinting of 16S rRNA coding regions.

Target organism	Primer pairs	Sequence (5'-3')	Ann.temp (°C)	Reference
bacteria	27f	GTGCTGCAGAGAGTTTGA	57	(Edwards <i>et al.</i> 1989)
		TCCTGGCTCAG		
	985r	GTAAGGTTCTTCGCGTT	57	(Heuer <i>et al.</i> 1999)
	341f-GC	CCT ACG GGA GGC AGC	55	(Muyzer <i>et al.</i> 1993)
		AG		
	518r	ATT ACC GCG GCT GCT	55	(Neefs <i>et al.</i> 1991)
		GG		
<i>Bacteroides</i>	32f			(Bernhard and Field 2000)
		AACGCTAGCTACAGGCTT	56	
	708r			(Bernhard and Field 2000)
		CAATCGGAGTTCTTCGTG	56	

1	bifidobacteria g-BifidF			(Matsuki <i>et al.</i>
2		CTCCTGGAAACGGGTGG	58	2002)
3				
4		g-BifidR	GGTGTTCCTCCCGATATC	(Matsuki <i>et al.</i>
5				
6				
7		TACA	58	2002)
8				
9				
10	<i>Clostridia</i>	sg-Clep-		
11				
12	cluster IV	F-GC	see table 1	55
13				
14		sg-Clep-		
15				
16				
17		R		
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438

439

440 Figures

441

442 **Figure 1:** Proportions of *Bacteroides*, *bifidobacteria* and *Clostridium* cluster IV of the
 443 total bacterial load in stool samples of institutionalized elderly and young healthy
 444 volunteers. Total amount of bacteria is depicted relative to the mean counts for
 445 healthy young young.

446

447 **Figure 2:** PCR-DGGE bandpattern of 16S rRNA coding regions of dominant bacteria
 448 amplified with primer pair 341GC-518. Bands that were observed more frequently in
 449 young than in elderly are indicated with arrows. Organisms and sequences listed
 450 were used for the construction of the reference lanes.

451 A, G, I, J, young; B, C, E, F, elderly; D, H, reference lanes

452

453 **Figure 3:** Diversity indices derived from DGGE fingerprinting of 16S rRNA coding
 454 regions.

455 Y, young; E, elderly; Cl.IV, *Clostridium* cluster IV; B, *Bacteroides*, Bif, bifidobacteria;

456 *, $p < 0.05$.

457 **Figure 4:** PCA of DGGE fingerprints of 16S rRNA coding regions of dominant

458 bacteria in fecal samples.

459 Y, young; O, elderly

460

461 **Figure 5:** UPMGA dendrogram showing clustering (Dice) based on the similarities of

462 DGGE fingerprints of dominant bacteria obtained with primer pair 341GC-518.

463 , elderly; , young

464

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469

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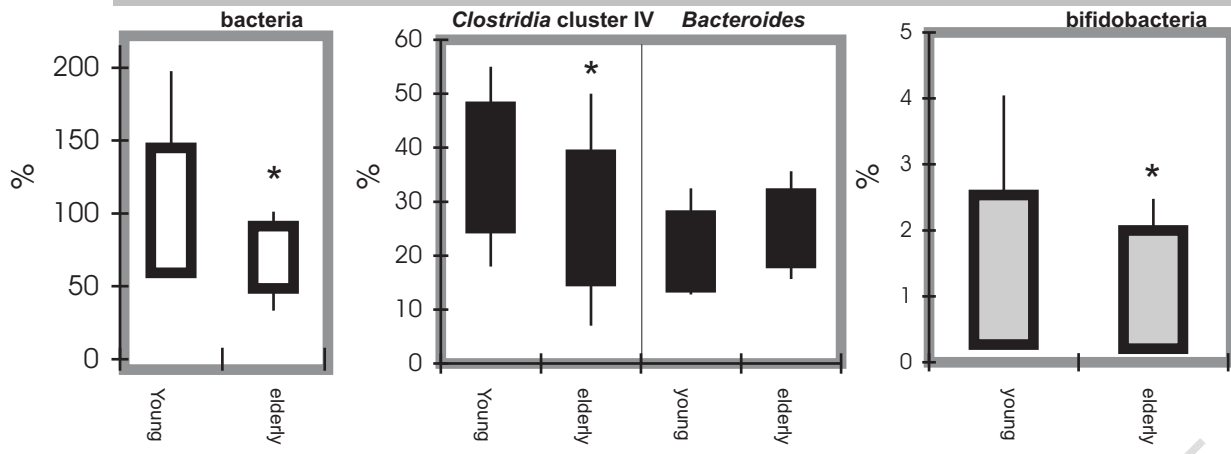
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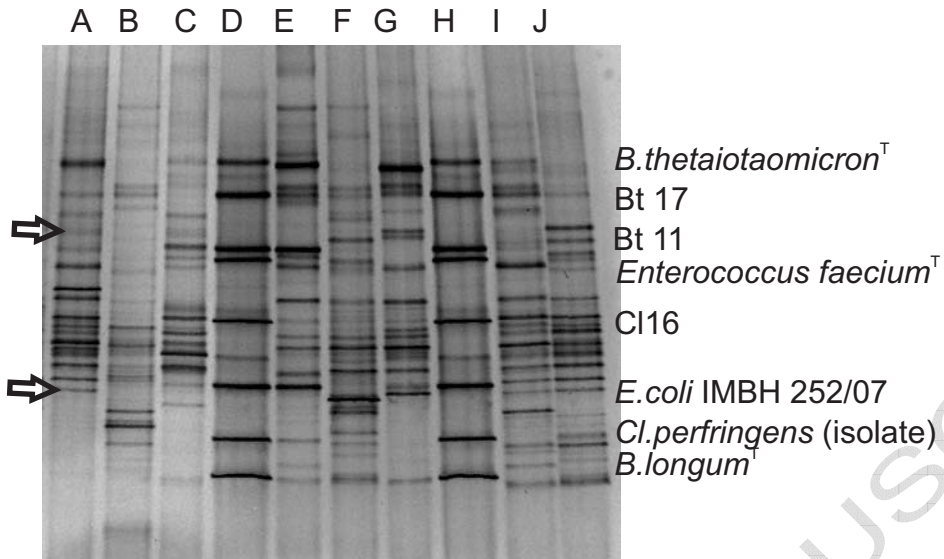
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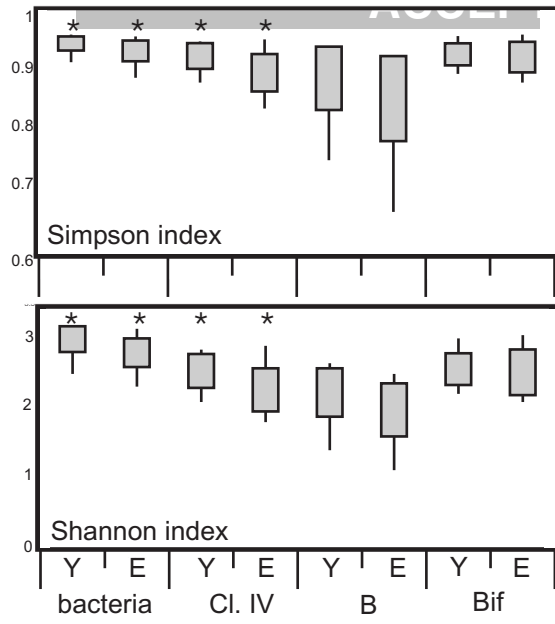
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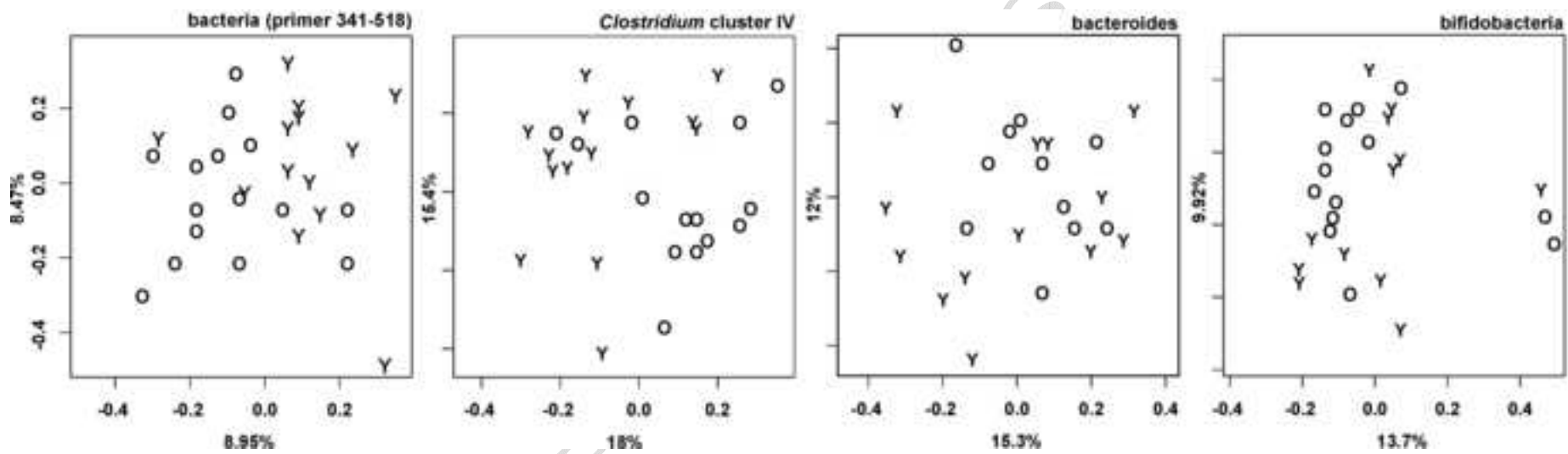


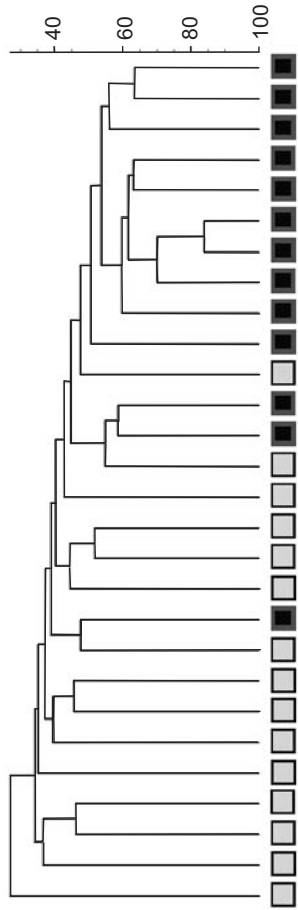
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