1 **Roux-Y Gastric Bypass and Sleeve Gastrectomy directly change gut microbiota** 2 **composition independent of operation type**

3 Running title: Microbiota changes in bariatric surgery patients

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24

25 **Competing Interests:** The authors declare no competing financial interests.

26 **Abstract**

27 **Background**

28 Bariatric surgery in patients with morbid obesity, either through gastric sleeve
29 gastrectomy or Roux-Y gastric bypass surgery, leads to sustainable weight loss,
30 improvement of metabolic disorders and changes in the intestinal microbiota. Yet, the
31 relationship between changes in gut microbiota, weight loss and the surgical
32 procedure remains incompletely understood.

33 **Subjects/Methods**

34 We determined temporal changes in microbiota composition in 45 obese patients
35 undergoing a crash diet followed by gastric sleeve gastrectomy ($n= 22$) or Roux-Y
36 gastric bypass ($n= 23$). Intestinal microbiota composition was determined before
37 intervention (baseline, S1), 2 weeks after a crash diet (S2), and 1 week (S3), 3 months
38 (S4) and 6 months (S5) after surgery.

39 **Results**

40 Relative to S1, the microbial diversity index declined at S2 and S3 ($p< 0.05$), and
41 gradually returned to baseline levels at S5. The crash diet was associated with an
42 increased abundance of Rikenellaceae and decreased abundances of
43 Ruminococcaceae and Streptococcaceae ($p< 0.05$). After surgery, at S3, the relative
44 abundance of Bifidobacteriaceae had decreased (compared to the moment directly
45 after the crash diet), whereas those of Streptococcaceae and Enterobacteriaceae had
46 increased ($p< 0.05$). Increased weight loss during the next 6 months was not
47 associated without major changes in microbiota composition. Significant differences
48 between both surgical procedures were not observed at any of the time points.

49 **Conclusions**

In conclusion, undergoing a crash diet and bariatric surgery were associated with an immediate but temporary decline in the microbial diversity, with immediate and permanent changes in microbiota composition, with no differences between patients undergoing gastric sleeve gastrectomy or Roux-Y gastric bypass surgery.

Introduction

Bariatric surgery is the only sustainable effective treatment for obesity¹. Surgical procedures such as the Roux-Y Gastric bypass (RYGB) and the sleeve gastrectomy (SG) facilitate a 50-70% decrease in excess body weight and fat mass¹. In addition, surgery leads to decreased caloric intake or malabsorption and to metabolic changes, such as an improved glucose metabolism, and is associated with a changed intestinal microbiota²⁻⁴. The role of altered host-microbial interactions in this process is incompletely understood¹. Studies on the composition of the distal gut microbiota in obesity and after RYGB in humans and rodents yielded long lasting changes in types and ratio of enteric bacteria^{3, 5-8}. Furthermore, transfer of the gut microbiota from RYGB-treated mice to non-operated, germ-free mice resulted in weight loss and decreased fat mass in the recipient animals⁵. These findings support a direct effect of the microbiota on weight and adiposity. Recently Liu *et al.* demonstrate using metagenomic shotgun sequencing that the abundance of glutamate-fermenting *Bacteriodes thetaiotaomicron* is decreased in obese Chinese individuals and glutamate levels are increased⁹. Weight loss by SG partially reversed metabolic and microbial alterations, including reduced abundance of *B. thetaiotaomicron* and increased serum glutamate⁹.

To further elucidate the results of the entire bariatric surgery procedure on the intestinal microbiota composition we investigated sequentially collected stool samples

75 from 45 morbid obese patients undergoing either RYGB or SG at five different time
76 points before and after surgery.

77

78 **Subjects and Methods**

79 *Ethics Statement*

80 The study protocol was in accordance with the regulations of the Ethics Committee of
81 Catharina Hospital Eindhoven.

82

83 *Study Design*

84 In this observational study patients with morbid obesity were recruited from the
85 Catharina Hospital Eindhoven out-patient obesity clinic between September 2014 and
86 November 2014. All 45 patients fulfilled the criteria for bariatric surgery and were
87 screened before surgery for eligibility by a team including a surgeon, dietician and a
88 psychologist. Two weeks before the planned surgery, patients were subjected to a
89 crash diet consisting of 500 calories a day for 2 weeks. Type of surgery was
90 determined based on clinical criteria and shared decision making between surgeon
91 and patient. Reasons to refrain from RYGB were medication dependence, increased
92 operation risk or super obesity. A reason to refrain from SG was gastroesophageal
93 reflux disease. During surgery, patients received 1 g cefazolin antibiotic prophylaxis
94 intravenously. After hospitalization, general practitioners managed adjustments of
95 insulin, oral diabetics and other medication in the home setting. Patients visited out-
96 patient clinic at 3, 6 and 12 months for evaluation and will remain in follow up for 5
97 years.

98

99 *Sample collection and DNA extraction.*

100 Stool samples (Sterilin specimen container, Thermo-Fisher) were gathered at the out-
 101 patient clinic or at patient homes. Samples were always stored in the freezer and
 102 collected at the homes of the patients using dry-ice and stored at the hospital at -80°C.
 103 Sample were collected at 5 different time points; before the start of the crash diet (S1),
 104 2 weeks after the crash diet (S2), and 1 week (S3), 3 months (S4) and 5 to 6 months
 105 after surgery (S5).

106 Total bacterial DNA from feces samples was isolated according to Godon *et al.* ¹⁰.
 107 When isolated DNA contained PCR inhibitors (20% of the samples random
 108 distributed over the time points), samples were submitted to an extra step of
 109 isopropanol precipitation and column purification with QiAamp stool mini kit
 110 (Qiagen). DNA was stored at -20°C prior to further analysis.

111

112 *16S rRNA gene sequencing strategy and analysis*

113 A 469 bp encompassing the V3 and V4 hypervariable regions of the 16S rRNA gene
 114 was amplified and sequenced using the Illumina MiSeq Reagent Kit v3 (600-cycle) on
 115 an Illumina MiSeq instrument according to Fadrosch *et al.* ¹¹. Negative controls, buffer
 116 controls were included in the DNA extraction, amplification and sequencing protocol
 117 to monitor for potential contamination. A total of 3 amplicon pools were sequenced,
 118 generating 8.9, 7.8 and 14.4 (mean of 10.3) million total reads. These 2x300 bp
 119 paired-end reads were pre-processed as follows. The first 12 bp of each paired-end
 120 containing the index sequences were extracted and afterwards concatenated to dual-

121 index barcodes of 24 bp specific for each read-pair and sample. Paired reads were
 122 merged, as an overlap of about 90 bp was expected, using FLASH (version 1.2.11) ¹².
 123 Subsequently, these merged reads were de-multiplexed using the
 124 split_libraries_fastq.py script from and analyzed by the QIIME microbial community
 125 analysis pipeline (version 1.9.1) ¹³. Quality filtering was also performed during this
 126 step, truncating reads with an average PHRED quality score of 20 or less. After
 127 removal of the barcodes, heterogeneity spacers, and primer sequences about 19.8
 128 million sequences were left with a mean length of 410 bp (median length of 405). The
 129 obtained sequences with a minimum of 97% similarity were assigned to operational
 130 taxonomic units (OTUs) using QIIME's open-reference OTU picking workflow
 131 (pick_open_reference_otus.py). This workflow was carried out using USEARCH
 132 (version 6.1.544) ¹⁴ for OTU picking, in addition to detection and removal of chimeric
 133 sequences. The obtained OTU sequences were aligned to the Greengenes 16S rRNA
 134 gene database (gg_13_8_otus), followed by removal of OTUs represented by less than
 135 0.005% of the total number of sequences. The generated OTU table and phylogenetic
 136 tree were used for assessing alpha- and beta-diversity using QIIME's
 137 core_diversity_analyses.py workflow with a rarefaction depth of 20001 sequences.
 138 The weighted UniFrac distance was used to calculate beta-diversity of the samples,
 139 while the shannon index was used for the alpha-diversity. For Principal Component
 140 Analysis (PCA) R 3.5.0 in an environment of RStudio 1.1.383 (RStudio Team,
 141 Boston, MA) ¹⁵ was employed, using zCompositions, clr transformation and ggplot
 142 R packages ¹⁶⁻¹⁸.

143

144 *Statistical analysis*

Microbiota changes between time points and operation types were investigated using ANCOM¹⁹ in R 3.3.3¹⁵. Changes in the clinical parameters (BMI, vitamin D, vitamin B6, cholesterol, bilirubin, glycated hemoglobin (HbA1c), iron, ferritin and folate) between baseline and 6 months after surgery was analyzed by applying t-test in Prism GraphPad (version 7.0). Associations between changes in total read counts at family level (at baseline versus 6 months after surgery) and changes in patient characteristics (at baseline versus 6 months after surgery) were investigated using a linear regression model. To eliminate possible confounding effects, age and sex were included as covariates. For these analyses, changes in total read counts were used as outcome, whereas changes in patient characteristics were used as predictor (model: $\text{change_in_read_counts} \sim \beta_1 \cdot \text{age} + \beta_2 \cdot \text{sex} + \beta_3 \cdot \text{change_in_patient_characteristic}$). For association analysis R 3.5.0 in an environment of RStudio 1.1.383 (RStudio Team, Boston, MA) was employed¹⁵. Results are presented using pheatmap package (<https://CRAN.R-project.org/package=pheatmap>). For statistical testing we used false discovery rates (FDRs) correct for multiple comparisons, and an FDR- adjusted p-value < 0.05 was considered as significant²⁰.

161

162 **Results**

163 *RYGB and SG resulted in significant decrease of BMI in all patients.*

164 In this study, 45 Caucasian Dutch patients were included with an average age of 43
165 years, 36 (84%) being female, 11 (24%) using proton pump inhibitors and 4 (9%)
166 having type 2 Diabetes Mellitus at baseline (Table 1). After a crash diet, 22 patients
167 underwent SG and 23 underwent RYGB. At baseline the mean BMI was 42.9 (+/-
168 6.56) and 43 (+/-4.13) for patients undergoing RYGB and SG, respectively. At 6

169 months after the procedure BMI declined to 30.81 (+/-5.35) and 31.52 (+/-3.86),
170 respectively, with no significant difference based on surgery type (Table 1).

171

172 *Crash diet reduces microbial alpha diversity, which is restored to baseline levels 6*
173 *months after surgery, irrespective of surgery type.*

174 In total 221 fecal samples were collected, with 4 samples missing from 4 unique times
175 points from 4 different patients. Using a pre-defined cut-off value of 20001 reads, 220
176 samples could be analyzed.

177 The initial crash diet had a stronger effect on total microbiota diversity as the Shannon
178 diversity index declined from 4.5 at baseline (S1) to 4.0 after the crash diet ($p < 0.05$)
179 (S2) and then gradually returned to 4.5 at 3 (S4) and 6 months (S5) after surgery
180 (Figure 1a). Differences in diversity are reflected by an initial decrease and
181 subsequent rise in numbers of distinct microbial OTUs. At baseline, 3 months and 6
182 months after surgery more than 500 OTUs were identified, whereas after the crash
183 diet and at 1 week after surgery this number was reduced to below 400 OTUs (Figure
184 1b).

185 In Principle Component Analysis (PCA) (Figure 1c), patients at baseline (S1) and
186 after crash diet (S2) are more similar to each other when compared to the times points
187 after the surgery (S3, S4, S5), which cluster together further apart.

188

189 *Distinct microbial changes appear directly after crash diet, but are replaced by*
190 *persistent distinct changes shortly after surgery.*

191 Significant changes in total relative abundance of specific families in the different
 192 time points were observed (Figure 2h). After the crash diet (S2) there was a
 193 significant reduction in relative abundance of 2 microbial families, Streptococcaceae
 194 and Ruminococcaceae (Figure 2a and d), and a significant increase in 1 family,
 195 Rikenellaceae (Figure 2e). Subsequent comparison of the microbial composition pre-
 196 surgery (S2) and 1-week post-surgery (S3) revealed a significant increase in the
 197 relative abundance of Streptococcaceae and Enterobacteriaceae families (Figure 2a
 198 and b) and a decrease in Bifidobacteriaceae, which persisted until 6 months post-
 199 surgery (S5) (Figure 2c). In these 6 months (at S5) microbiota complexity was
 200 restored (Figure 1a and b), which when compared with 1 week after surgery (S3)
 201 coincided with increased relative abundance of low abundance families
 202 Veillonellaceae and the Clostridiales order with no further family classification
 203 (Figure 2f and g).

204 When RYGB and SG surgeries were analyzed separated, no significant differences in
 205 microbiota composition based on beta diversity and relative abundance was observed
 206 at baseline (S1) (Figure 3a), 1 week (S3) (Figure 3b) or 6 months after surgery (S5)
 207 (Figure 3c) between patients that underwent either SG and RYGB (Figure 3d).

208

209 *Significant associations between microbiota changes and clinical markers*

210 Clinical parameters in patients were analyzed at baseline and 6 months after the
 211 surgery. Besides weight loss, serum levels of vitamin D, B6, cholesterol, bilirubin,
 212 HbA1c, iron, ferritin and folate improved 6 months after surgery when compared to
 213 baseline (FDR-adjusted, $p < 0.05$, Table 1).

214 These changes were associated to overall differences in microbial abundance in
 215 relation to the changes in clinical parameters at S5 versus S1, which are highlighted in
 216 Figure 4. Significant associations were only found in low abundance families.
 217 Decreased bilirubin level was associated with and Prevotellaceae, Bacteroidales and
 218 Peptococcaceae taxa; and increased iron level was associated with Pasteurellaceae. In
 219 addition, a decreased HbA1c was associated with Coriobacteriaceae and Clostridiales
 220 taxa The most pronounced measured effects in the dataset was a negative association
 221 between Prevotellaceae, Veillonellaceae, Streptococcaceae, Bifidobacteriaceae and
 222 Enterobacteriaceae taxa in relation to decreased serum cholesterol levels, whereas
 223 most pronounced positive associations were found between Lachnospiraceae and
 224 Coriobacteriaceae taxa in relation to decreased cholesterol levels (Figure 4). Yet,
 225 these associations were not statistically significant after FDR adjustment (Figure 4).

226

227 **Discussion**

228 This study is novel in the fact that it compares microbiota profiles before and very
 229 shortly after bariatric surgery with subsequent follow-up profiles. We describe the
 230 sequential impact of a crash diet followed by either RYGB or SG surgery, resulting in
 231 progressive weight loss and changes in the gut microbiota composition. Several other
 232 studies report sequential sampling of patients after bariatric surgery, but none of the
 233 studies define timing of the baseline sample in relation to a crash diet^{3, 9, 16, 21-23}, and,
 234 therefore, renders the relative impact of the different measures in the bariatric
 235 procedure difficult to dissect. In addition, this study is unique in that a very early
 236 postsurgical sampling time point is included. Apart from substantial weight loss and
 237 improvements in clinical parameters, as reported by others, bariatric surgery induces
 238 long-lasting changes in microbiota composition in most patients. The most apparent

239 immediate change in microbiota composition occurred after the crash diet, with a
240 concurrent reduction in alpha diversity, whereas surgery was associated with early
241 and sustained replacement of distinct bacterial taxa and restoration of the diversity.

242 Although significant microbial changes are identified in the gut microbiota of
243 bariatric surgery patients, at 6 months after surgery the total microbial diversity was
244 similar to microbial diversity measured at baseline. This sudden decline in alpha
245 diversity probably reflects a severe stress on the human microbiota by a crash diet,
246 with a significant change in catabolic state ²⁴. Persisting post-surgery microbiota
247 changes suggest adaptation to anatomic and physiologic changes, such as reduced
248 acid production, increased oxygen content, altered bile acid concentrations delivered
249 to the colon, induced by the surgery. Previously reported effects of bariatric surgery
250 on microbiota diversity have ranged from an increase in total diversity ^{25, 26} to absence
251 of change and even a decrease in alpha diversity ²⁷. We suspect that baseline sampling
252 in relation to crash diet may vary between studies, and might contribute to the
253 reported differences between studies.

254

255 Besides a stable bacterial alpha diversity after surgery, we observed profound
256 differences after each consecutive intervention on bacterial taxa composition.

257 The crash diet immediately resulted in an increase in the relative abundance of
258 Bifidobacteriaceae and decrease of Streptococcaceae, whereas the opposite effect was
259 observed after surgery; an increase in abundance of Streptococcaceae and decline in
260 Bifidobacteriaceae that persisted for at least 6 months. In addition to the observed
261 increased abundance of Veillonellaceae may reflect survival of oral microbiota into
262 the intestine. Observed persistent increase in Enterobacteriaceae after surgery,
263 confirms previous sustained changes reported in humans and animal models (rats),

264 associated with increased pH ^{7, 24, 28, 29}. Other main differences, exposure to
 265 undigested nutrients and biliopancreatic enzymes, may play important roles in the
 266 microbial composition, intestinal permeability and intestinal adaptation ³⁰. Since
 267 increased intestinal permeability is associated with inflammation and reductions in
 268 alpha diversity, which is also associated with obesity, it is questionable whether
 269 restoration of alpha diversity to baseline level may also reflect persistent
 270 inflammation in the post-surgery state at 6 months, which has been previous related to
 271 increase in Enterobacteriaceae ¹⁶. This corresponds to the observed higher alpha
 272 diversity of fecal samples from a healthy normal weight cohort compared to the
 273 although improved, yet lower diversity of postoperative patients ¹⁶.

274
 275 Although others observed microbiota changes only after RYGB ⁵, here we observed
 276 this in both surgery types. This suggests that despite the 2 procedures result in distinct
 277 anatomic differences, this did not seem to influence the post-surgery changes in
 278 relative abundance of Bifidobacteriaceae, Streptococcaceae and Enterobacteriaceae
 279 observed amongst both patient groups and which were similar for both types of
 280 surgery. Interestingly, unlike Liu *et al.* ⁹ and Ilhan *et al.* ³¹ both patients groups here
 281 after surgery develop comparable weight loss irrespective of surgery type, this may
 282 explain why we find similar changes in gut microbiota composition. Also baseline
 283 characteristics did not differ significantly. Moreover, we suggest that bariatric surgery
 284 in itself, unlike crash diet, results in an altered long-lasting composition of the
 285 microbiota.

286 Although a significant association with changed clinical parameters between baseline
 287 and 6 months after surgery was lacking, the relative abundance of Bifidobacteriaceae,
 288 Streptococcaceae and Enterobacteriaceae taxa changed significantly shortly after

289 surgery. This sudden adjustment further confirms that the altered postoperative
 290 microbiota more likely reflects surgery induced effects, rather than improved clinical
 291 parameters^{16, 23, 31}. We observed a significant association between increased serum
 292 bilirubin level and decreased relative abundance of Bacteroidales, Peptococcaceae and
 293 Prevotellaceae taxa in this dataset. The abundance of Bacteroidales in the gut
 294 microbiota could contribute to the increase in bilirubin level, since *Bacteroides*
 295 *fragilis*, which is part of Bacteroidales taxa, is one of the bacterial species described to
 296 be able to metabolize bilirubin in the gut^{32, 33}. In addition, a decreased HbA1c was
 297 found significantly associated with decreased Coriobacteriaceae and increased
 298 Clostridiales taxa. Nevertheless, the exact meaning of changes of these low
 299 abundance taxa is unknown.

300 This study failed to confirm the suggested relationship between increased abundance
 301 of Firmicutes and Bacteroidetes and obesity^{34, 35}, as the relative abundance of the
 302 family members of these phyla remained stable before and after surgery, despite
 303 significant weight loss. In addition, other studies described that *Faecalibacterium* (*F.*
 304 *prausnitzii*) was assumed to play a role in inflammation and glucose homeostasis in
 305 obesity with a reduced relative abundance after RYGB surgery^{3, 8, 36, 37}. In our study,
 306 a decreased abundance of the Ruminococcaceae family, to which *F. prausnitzii*
 307 belongs, was observed after the crash diet, yet this change did not sustain after
 308 surgery.

309

310 In conclusion, here we illustrate that temporal sampling of bariatric surgery patients
 311 with subsequent microbiota analysis can lead to increased insights into the relative
 312 contribution of interventions on stability and composition of the microbiota. We show
 313 that a crash diet invoked profound temporary changes in total microbiota diversity and

composition, yet surgery precluded early fixed changes of microbial composition and restoration of the microbial diversity that likely contribute to weight loss.

Acknowledgments

The authors declare that there are no conflicts of interest. Authors contribution: conceived and designed the experiments: ML, DA, MJMB, ACF, HLL. Performed the experiments: ML, DA, APAH, JG, MCV. Analyzed the data: FLP, MRCR, CMH, HWU, RMB. Wrote the paper: FLP, ML, CMH, HWU, MRCR, DA, RMB, APAH, MCV, JG, MJMB, ACF, RJLW, HLL.

Competing interests

The authors declare no competing financial interests.

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490 **Figures Legends**

491 **Figure 1.** Observed and estimated richness of gut microbiota at different time points
 492 during the bariatric surgery procedure. (A). Shannon diversity index estimated a
 493 decrease in bacterial richness at S2 and S3. (B). Rarefaction curves showed a
 494 reduction in bacterial richness at S2 and S3. (C). Principal component analysis (PCA)
 495 plot of similarity between the samples; each dot represents 1 sample, each color a
 496 different time point. S1. before surgery (red); S2. after 2 weeks of crash diet (orange);
 497 S3. 1 week after surgery (yellow); S4. 3 months after surgery (light blue); S5. 6
 498 months after surgery (dark blue).

499
 500 **Figure 2.** Relative abundance of bacterial families in the gut microbiota at the five
 501 time points analyzed. (A-G). Boxplots show the average relative abundance of 7
 502 families that significantly changed between 2 different time points. (A).
 503 Streptococcaceae. (B). Enterobacteriaceae. (C). Bifidobacteriaceae. (D).
 504 Ruminococcaceae. (E). Rikenellaceae. (F). Veillonellaceae. (G).
 505 O_Clostridiales_f_others. H. Relative abundance of all families identified at the
 506 different time points. Significant families are represented in the same color. Asterisk
 507 (in red) indicates significant fold change differences ($p < 0,05$) analyzed by ANCOM.

508
 509 **Figure 3.** (A-C). Principal coordinate analysis (PCoA) plots comparing beta diversity
 510 of Sleeve Gastrectomy (SG) versus Roux-Y Gastric bypass (RYGB) surgery at
 511 baseline (S1) (A) 1 week after surgery (S3) (B) and 6 months after surgery (S5) (C).
 512 SG is indicated in red, RYGB is indicated in blue (D). Relative abundance of bacterial
 513 families in the gut microbiota at the five time points analyzed in SG versus RYGB
 514 surgery.

515 **Figure 4.** Association between clinical parameters and family taxa calculated based
 516 on the difference between 6 months after surgery (S6) and baseline (S1). The
 517 significant association (false discovery rate (FDR) adjusted p-value<0.05) is indicated
 518 with a “x”. The red color indicates positive effect and the blue color negative effect.
 519 HbA1c, glycated hemoglobin; VitD, vitamin D; VitB6, vitamin B6.