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Running head:

Bariatric surgery alters gut microbiota.

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Abbreviation: dipeptidyl peptidase-4 (DPP 4)

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MO; TAL: study concept and design;

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MO; PDC; TAL: analysis and interpretation of data;

MO; TAL: drafting of the manuscript.

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Abstract

Roux-en-Y gastric bypass (RYGB) surgery might modify the gut microbiota composition differently in the three distinct anatomical sections of small intestine compared to sham surgery. We showed that RYGB induced changes in the microbiota of the alimentary limb and the common channel resembling those seen after prebiotics treatment or weight loss by dieting. These changes may be associated with altered production of intestinal hormones known to control energy balance. Postsurgical modulation of gut microbiota may significantly contribute to the beneficial metabolic effects of RYGB surgery.

1. Introduction

Recent studies examined the association between changes in intestinal microbial diversity in obese rodents and humans; some bacterial groups were associated with changes in the nutritional status. Obesity was associated with higher Firmicutes and lower *Bifidobacterium* spp, *Bacteroides*-related bacteria and *Lactobacillus* spp in comparison with the lean counterparts [1-4]. Interestingly, weight loss achieved by dieting was able to reverse those changes [5]. Furthermore, nutrients with prebiotic properties induced qualitative changes in the composition of the gastrointestinal microbiota and peptide release (e.g., glucagon-like peptide-1 (GLP-1)) similar to those seen after dieting. In diet-induced obese and type 2 diabetic (T2DM) mice the release of gut peptides induced by treatment with prebiotics improved glucose and lipid metabolism as well as systemic inflammation [6].

Roux-en-Y gastric bypass (RYGB) is currently, the most effective strategy for long term weight loss maintenance. RYGB significantly reduces body weight, improves T2DM and changes the postprandial enteric endocrine responses.

Gut microbiota analysis of fecal samples from humans and rats after RYGB suggested that the reduction of Firmicutes and Bacteroidetes may partly explain the weight loss and beneficial effects on metabolism and inflammation associated with the RYGB surgery [7-9]. Liou [10] et al. confirmed these findings and also showed that cecal transplants from mice after RYGB to unoperated germ free mice decreased body weight and adiposity compared to recipients of microbiota from sham-operated mice.

There are currently no data on the impact of gut microbiota on the hormonal and metabolic changes associated with RYGB. In most of the human and rodent studies investigating the ecology and activity of intestinal microbiota, fecal or cecal samples have been used. However, these may not be representative of the microbiome in RYGB where the intestine is surgically manipulated into three discrete section which may each contribute to distinct metabolic signals compared to feces that represents a amalgamate of the microbiome from the intestine as a whole. Therefore, we assessed the bacterial composition in the different anatomically corresponding intestinal segments after RYGB or sham surgery.

2. Materials and Methods

2.1 Subjects and Housing

Sixteen male Wistar rats (Harlan Laboratories Inc., Blackthorn, UK; Elevage Janvier, Le-Genest-St. Isle, France) were individually housed under a 12 h /12 h light-dark cycle at a room temperature of 21 ± 2 ºC. Water and standard chow were available ad libitum. All experiments were approved by the Veterinary Office of the Canton Zurich, Switzerland.

All rats were given one week of acclimatization before being randomized to RYGB (n= 8) or sham-operation (n= 8). After surgery, rats received Ensure (chocolate Ensure Plus, Abbott Nutrition, Baar, Switzerland) for 3 days before access to normal chow was reinstalled. Body weight was measured weekly. A food restricted sham-operated group of rats (n= 7), whose postoperative weight matched the weight of bypass-treated animals, was also studied (data not shown; see also ref [11]); data were comparable to the sham-operated *ad libitum* fed controls except for one parameter (see below).

2.2 Surgery

Rats were allocated to either RYGB (N= 8, body weight, mean ± SEM, 445 ± 5 g) or sham-operated (sham, N= 8; 435 ± 5 g) surgery groups. Anesthesia was induced in a chamber filled with 5% isoflurane in oxygen (1 L/min). After an adequate depth of anesthesia was achieved, rats were shaved from sternum to pelvis followed by disinfection with Betadine scrub (Mundi Pharma, Basel, Switzerland). Rats were then placed in a supine position on a heating pad and positioned in a nose cone to maintain anesthesia (2%–4% isoflurane in oxygen, 0.5 L/min) for the duration of the surgery. All surgeries were conducted as previously described [11-13] and the small intestinal segments after RYGB (biliopancreatic limb; alimentary limb; common channel) are depicted in Figure 1. Briefly, a midline incision of approximately 4 cm starting just below the xiphoid process was performed. For the RYGB procedure, the small bowel was transected approximately 20 cm distal to the pylorus of the stomach, creating a proximal and distal end of small bowel. The proximal end, being still continuous with the remaining portion of the stomach, constituted the biliopancreatic limb and was anastomosed to the ileum approximately 25–30 cm from the cecum, creating the common channel. For formation of the gastric pouch, the stomach was transected

approximately 5 mm below the gastroesophageal junction, creating a gastric pouch of a size of no more than 2%–3% of original stomach size. The Roux-en-Y reconstruction was completed by connecting the distal end of the small bowel to the gastric pouch, leading to formation of the alimentary limb. One single RYGB procedure lasted approximately 70 minutes. For sham operations, an anterior gastrostomy and a jejunostomy with subsequent closures were performed. One single sham procedure lasted approximately 30 minutes. The abdominal wall and the skin were closed in layers after both operations.

2.3 *DNA isolation*

The content of intestinal tracts (N= 6-8 per group) collected immediately after euthanasia was stored at −80°C (biliopancreatic limb, alimentary limb, common channel, caecum and colon after RYGB and in the anatomically corresponding segments after sham surgery).

Metagenomic DNA was extracted from the intestinal content using a QIAamp-DNA stool minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.4 *qPCR: primers and conditions*

The primers and probes used to detect Total bacteria, *Bifidobacterium* spp, *Lactobacillus* spp and *Bacteroides-Prevotella* spp. were based on 16S rRNA gene sequences and as described by Vincent et al. [14, 15]. Detection was achieved with a STEP one PLUS instrument and software (Applied Biosystems, Foster City, CA) using MESA FAST quantitative PCR MasterMix Plus for SYBR Assay (Eurogentec, Verviers, Belgium). Each assay was performed in duplicate in the same run. The cycle threshold of each sample was then compared to a standard curve (performed in triplicate) made by diluting

genomic DNA (five-fold serial dilution) (BCCM/LMG, Ghent, Belgium). The statistical analysis was done on logarithmic values.

2.5 DDP IV activity

Snap frozen intestinal tissue samples (200 mg) were homogenized in 1 mL cold homogenization buffer (10 mM Tris-HCl, pH 8.2), and analyzed immediately for enzyme activity. The serum from blood samples was separated by centrifugation and stored at - 20°C until analysis of enzyme activity.

Determination of intestinal and serum dipeptidyl peptidase-IV (DPP IV) activities, which degrades gut peptides like GLP-1, was performed as described by Kreisel et al. [16]. DPP IV activities were determined by measuring the release of 4-nitroaniline from an assay mixture containing 0.1 mol Tris-HCl (pH 8.0), 2mmol Gly-Pro p-nitroanilide (Sigma-Aldrich, Saint Louis MO, USA) as the substrate and enzyme in a total volume of 0.20 mL. After 30 minutes of incubation at 37°C, the reaction was stopped by the addition of 0.4 mL of 2 M sodium acetate buffer (pH 4.5). Human recombinant DPP IV (Sigma-Aldrich, Saint Louis MO, USA) was used as standard. The absorbance at 405 nm was measured by use of a Lab Systems Multiskan RC 96-well plate reader (Thermo Fisher Scientific, Waltham MA, USA). All reactions were performed in duplicate.

Protein concentrations in homogenates were determined according to the method of Pierce [17]. Enzyme activities in homogenates were expressed as international units per gram of protein, and in serum as international units per liter of serum. One unit corresponds to the hydrolysis of 1 mmol of substrate per minute under the assay conditions.

2.6 Statistical Analyses

Results are presented as mean ± S.E.M. Statistical significance of difference between groups was assessed by Mann Whitney non parametric t-test (Graph-Pad Prism Software, San Diego, CA, USA; www.graphpad.com).

3   Results and Discussion

Average presurgical body weight of rats was 430 ± 4 g. Seven days after surgery, sham-operated controls weighed significantly more compared with gastric bypass rats (sham: 370 ± 9 g vs. bypass: 450 ± 6 g, P < 0.001). Body weight changes for both groups are shown in Fig. 2.

Total bacteria content was significantly increased in the alimentary limb and common channel after RYGB compared to sham rats. In the caecum after RYGB the changes in the microbial ecology were similar to that seen after prebiotic treatment [4], with *Bifidobacterium* spp and *Lactobacillus* spp significantly lower. RYGB also increased *Bifidobacterium* spp and *Bacteroides-Prevotella* spp in the common channel, the alimentary limb and in the colon (Fig. 3). After RYGB surgery, DPP IV activity was decreased by 27% in the alimentary limb (p = 0.01) and by 32% in serum (p = 0.002) (Fig. 4).

Most changes in microbial composition seen in the RYGB rats were body weight-independent. In fact, sham-operated body weight-matched rats did not show significant modification of gut microbiota compared to *ad libitum* fed sham-operated controls, with the exception for the increase in *Bacteroides Prevotella* spp. in the alimentary limb and the common channel of the body-weight matched rats (data not shown). Although changes in gut microbiota seen after RYGB resembled those obtained after weight loss in obese rodents [5], our data indicate that RYGB may have specific effects on intestinal microbiota.

The major finding of the study is that the most substantial shifts in the composition of the microbiota were observed in the alimentary limb and the common channel. Interestingly, we recently found that the total gene expression of the gut hormones preproglucagon, peptide YY and cholecystokinin was increased in the alimentary limb and common channel of RYGB rats but not in sham-operated rats that were body weight-matched to the RYGB rats [18].

Our findings suggest that the bypass of the proximal intestine may contribute to the changes of the gut microbiota observed after RYGB. The exclusion of the proximal intestine from contact with ingested nutrients has been demonstrated to play a major role in the beneficial effects of RYGB surgery [19]. Although a putative mechanism promoting such beneficial effects of the surgery remains to be elucidated, our data suggest a possible role for changes in the microbial composition of the proximal small bowel.

In the present study, a decreased activity of intestinal and serum DDP IV activity was detected. A direct link between the decreased DPP IV activity and changes in gut microbiota composition was not investigated; however, we have previously shown that prebiotic-induced changes in gut microbiota and increased GLP-1 levels were associated with reduced DPP IV activity [20]. The gut microbiota changes that we observed in this study resemble in part those seen after treatment with prebiotics, thus we think that similar mechanisms may have prevailed under the conditions of our study.

The modest decrease in serum DPP IV activity in response to RYGB compared to pharmacological approaches is consistent with other studies in humans [21]. It could be secondary to the experimental conditions; it is e.g. known that the diabetic status may influence the level of DPP4 activity [22]. In our RYGB model, however, the difference of

glucose tolerance between sham-operated and RYGB is not supposed to differ as much as in some models of obesity and type 2 diabetes frequently used to test pharmacological approaches. Hence, we expected the difference of DPP-4 activity in our study to be smaller.

Further, because DPP-4 is mainly a tissue enzyme, changes in serum DPP-4 activity may not completely reflect the total decrease in activity of the enzyme and the consequent decreased inactivation of incretins. In the present study, DPP-4 activity in the alimentary limb was also decreased and might explain the robust increase in circulating incretin levels usually detected in our RYGB rat model [1]. In other words, reduced intestinal and serum DDP IV activity may contribute to enhanced bioavailability of endogenous GLP-1 and be consistent with the modulation of jejunal microbiota altering the production or breakdown of gastrointestinal hormones known to control energy balance.

4 Conclusions

In conclusion, these data are critical because they accurately represent how RYGB surgery may affect microbial communities in different intestinal segments after surgery. Because most changes in gut microbiota were independent from weight loss, we conclude that other mechanisms than weight loss per se seem to be responsible for the alteration of the intestinal microbial population after RYGB surgery.

Postsurgical modulations of the gastrointestinal microbial community, e.g. the bypass of the intestinal foregut, may influence gut peptide synthesis, release and breakdown; hence, these changes may significantly contribute to the beneficial metabolic effects of RYGB surgery independent of the RYGB-induced body weight loss.

The impact of RYGB on the microbiota of the different intestinal tracts remains to be confirmed in other experimental models, e.g. in a high fat diet or a genetic model of obesity, and in large-scale studies including body weight-matched controls and using pyrosequencing, metagenomic analysis and metabolic profiling. These tools will enable further insight into the composition of the intestinal microbiota and its functional evolution after bariatric surgery. Elucidating the mechanisms by which gut microbiota interact with the host will provide a new basis for putative pharmacological or dietary intervention for obesity and its related comorbidities.

Aknowledgments

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Figure captions

Figure 1:

Schematic illustration of the surgical diagram of our animal model of RYGB

A: Normal gut anatomy   **B:** Gut anatomy after RYGB surgery:

1: biliopancreatic limb/duodenum

2: alimentary limb/proximal jejunum

3: common channel/distal jejunum

4: caecum

Figure 2:

Body weight change for the gastric bypass (-●-) (n = 8) and sham-operated rats ad libitum fed (-○-) (n = 8). Data are expressed as mean ± SD.
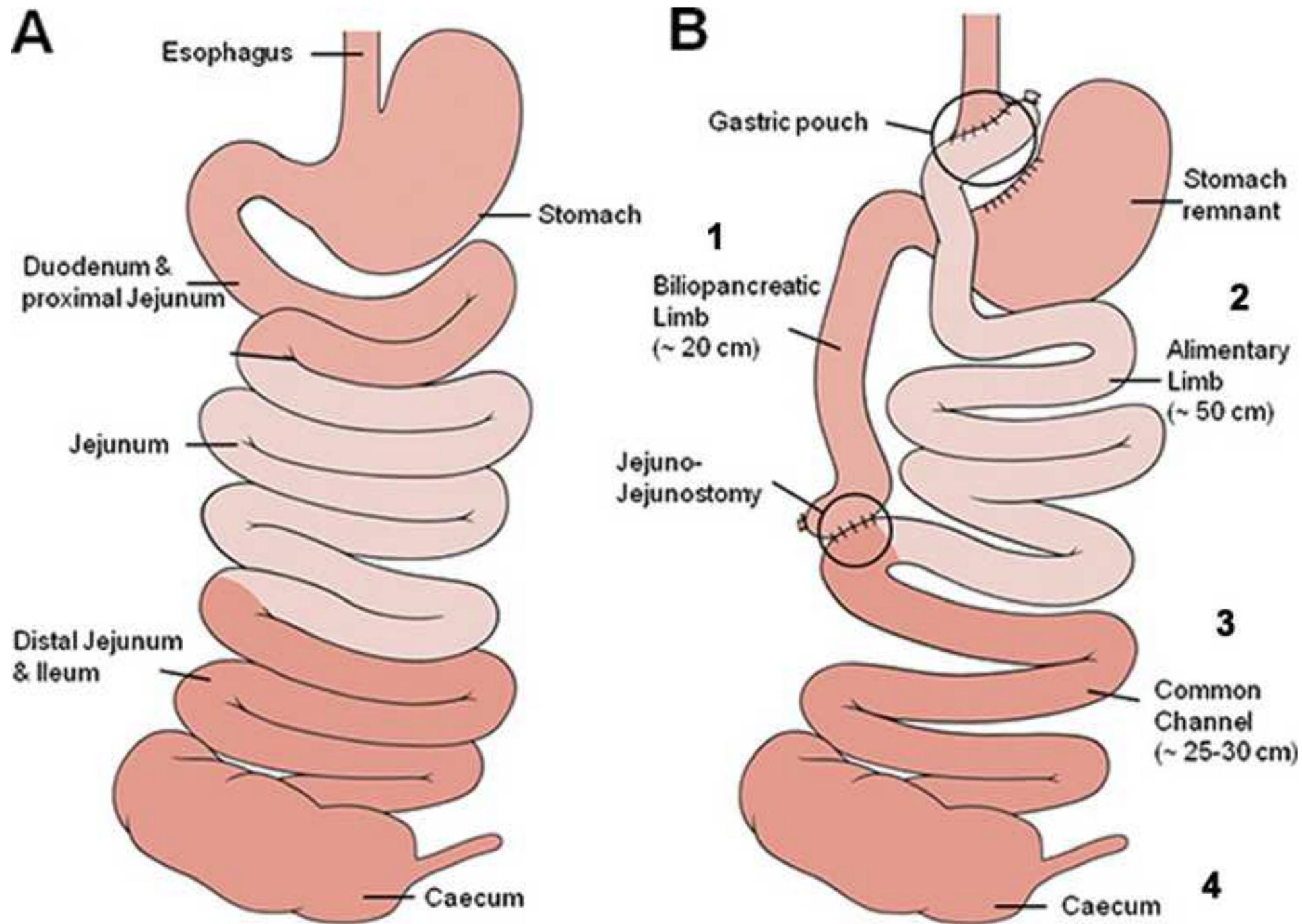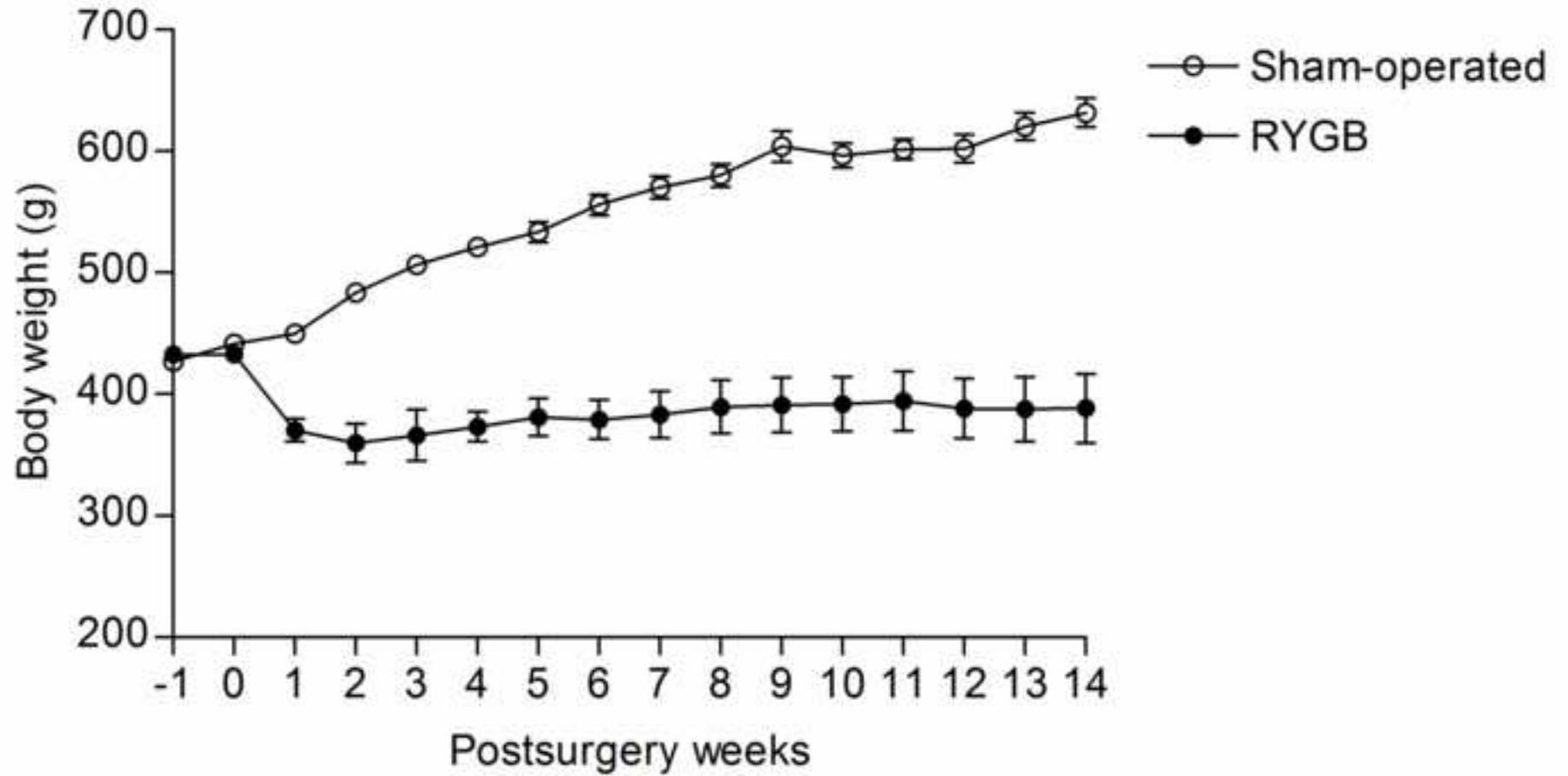
Figure 3:

RYGB-associated changes in gut microbiota.

Results are given as the Log_{10} of bacteria/intestinal content (g). Data are expressed as mean ± S.E.M * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to sham-operated rats.

Figure 4:

RYGB-associated changes in DPP 4 activity. Data are expressed as mean ± S.E.M * $p < 0.05$; ** $p < 0.01$ compared to sham-operated rats.

A

Esophagus

Stomach

Duodenum & proximal Jejunum

Jejunum

Distal Jejunum & Ileum

Caecum

B

Gastric pouch

Stomach remnant

1

Biliopancreatic Limb (~ 20 cm)

2

Alimentary Limb (~ 50 cm)

Jejuno-Jejunostomy

3

Common Channel (~ 25-30 cm)

4

Caecum