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ORIGINAL CONTRIBUTIONS

Bariatric/Metabolic Surgery Induces Noticeable Changes of Microbiota and Their Secreting Extracellular Vesicle Composition in the Gut

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Abstract

Introduction Microbial ecology is reported to be an important regulator of energy homeostasis and glucose metabolism. Microbes secrete extracellular vesicles (EVs) during their proliferation and death to communicate with other cells. To investigate the roles of gut microbiota in glucose metabolism, we analyzed serial changes of gut microbe and microbial EV composition before and after bariatric/metabolic surgery (BMS).

Methods Twenty-eight Wistar rats were fed on high-fat diet (HFD) to induce obesity and diabetes. Five of them compared with 5 rats fed on regular chow diet (RCD). Among the remaining 23 rats, Roux-en-Y gastric bypass (RYGB) ($n = 10$), sleeve gastrectomy (SG) ($n = 10$), or sham operation ($n = 3$) was randomly performed. Gut microbiota and EVs from fecal samples were analyzed by 16S rDNA amplicon sequencing.

Results The present study showed that microbial diversity was decreased in HFD-fed rats versus RCD-fed rats. In addition, BMS reversed glucose intolerance and microbial richness which were induced by HFD. In terms of microbiota and microbial EV composition, both RYGB and SG enhance the composition of phyla Proteobacteria, Verrucomicrobia, and their secreting EVs, but decrease phylum Firmicutes and its EVs. We tried to demonstrate specific genera showed a significant compositional difference in obesity/diabetes-induced rats compared with normal rats and then restored similarly toward normal rats' level after BMS. At the genus level, *Lactococcus*, *Ruminococcus*, *Dorea* in Firmicutes(p), *Psychrobacter* in Proteobacteria(p), and *Akkermansia* in Verrucomicrobia(p) fit these conditions after BMS.

Conclusion We suggest that these genera are the candidates contributing to obesity and diabetes improvement mechanism after BMS.

Keywords Gut microbiota · Extracellular vesicle · Obesity · Diabetes · Bariatric/metabolic surgery

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Introduction

Globally, obesity and related metabolic diseases are spreading rapidly and becoming serious public health issues. Obesity is a major independent risk factor for the development of diabetes and over 90% of people with type 2 diabetes mellitus (T2DM) are overweight or obese [1]. Bariatric/metabolic surgery (BMS) has become a very important treatment option because it can improve or resolve T2DM by various mechanisms independent of weight loss [2], though we acknowledge that weight loss is one of the major treatment modalities [3]. The mechanisms of improvement in glucose metabolism after BMS remain incompletely understood. Reduced food ingestion, changes in food preferences, increased satiety, increased gastric emptying, changes of the gut hormone milieu, shifts in bile acid metabolism, and modulation of the gut microbiota are suggested as mechanisms.

In recent decades, the potential role of the gut microbiota in altering host health has drawn considerable attention. Emerging evidence suggests a link between gut microbiota and various diseases, including colorectal cancer, liver cirrhosis, arthritis, and atherosclerosis [4–7]. Microbial ecology is reported to be an important regulator of energy homeostasis and glucose metabolism [8–10]. Compositional alterations and reduced gut microbiota diversity have been hypothesized to be associated with obesity/diabetes, whereas gut microbiota changes after BMS have been observed to be related to metabolic improvements [11].

As gut microbiota are constrained to their gastrointestinal niche and normally cannot be absorbed systemically, how can they communicate with their host? Recent studies have determined that extracellular vesicles (EVs) play a key role in intercellular communication and can influence both neighboring and distant cells. EVs are formed as lipid-bilayered spheres ranging from 20 to 500 nm in diameter and are secreted by bacteria, archaea, and eukaryotes constitutively into the extracellular microenvironment [12]. EVs released from bacteria carry biologically important information, including bacterial DNA and RNA, endotoxins (lipopolysaccharides), and virulence proteins. Thus, EVs have been recognized as potent transporters of important materials to communicate between cells affecting various physiological and pathological phenomena such as cancers and autoimmune diseases [13]. Until now, the change of bacteria-derived EV composition after BMS has not been analyzed.

The purposes of this study were first to identify the differences in gut microbiota and EV composition between obese/diabetes-induced rats versus rats fed a normal regular chow diet and second to analyze the serial changes of gut microbiota and EV composition after BMS.

Material and Methods

Animals

Five-week-old male Wistar rats were purchased from Orient Bio Inc. (Sungnam, Korea). Twenty-eight rats were fed 60% high-fat diet (HFD) (Central Lab. Animal Inc., Seoul, Korea) and five rats were fed a regular chow diet (RCD) at the OO Medical Research Institute. All animal procedures were approved by the Institutional Animal Care and Use Committee of OO University OO Hospital (IACUC approval no. 15-0292).

Experimental Design

After 6 months of the HFD, randomly chosen five rats in the HFD group and five rats in the RCD group were examined for body weight and oral glucose tolerance test (OGTT), and stool samples were collected for metagenomic analysis. The

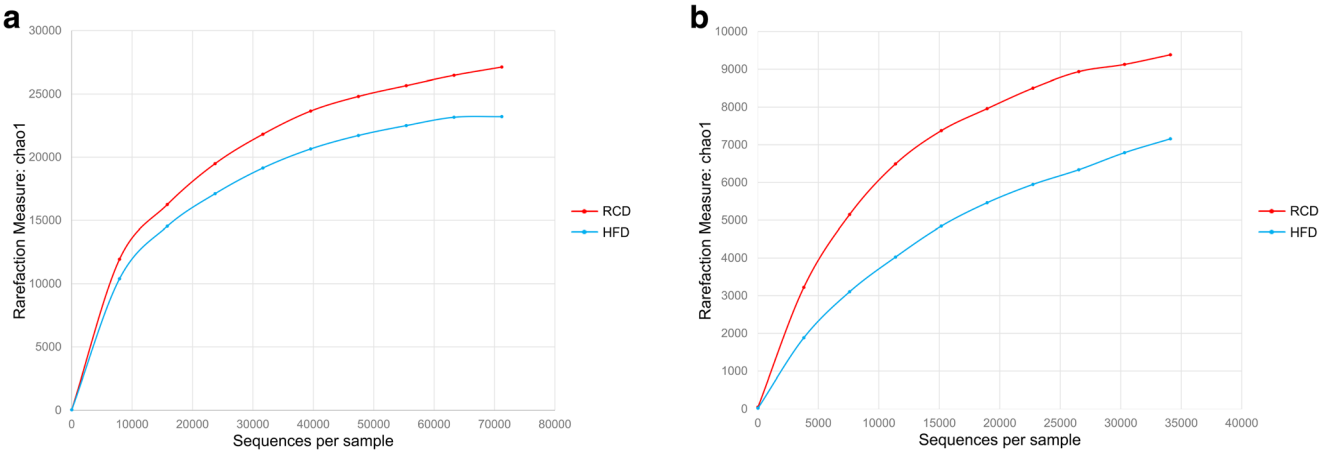
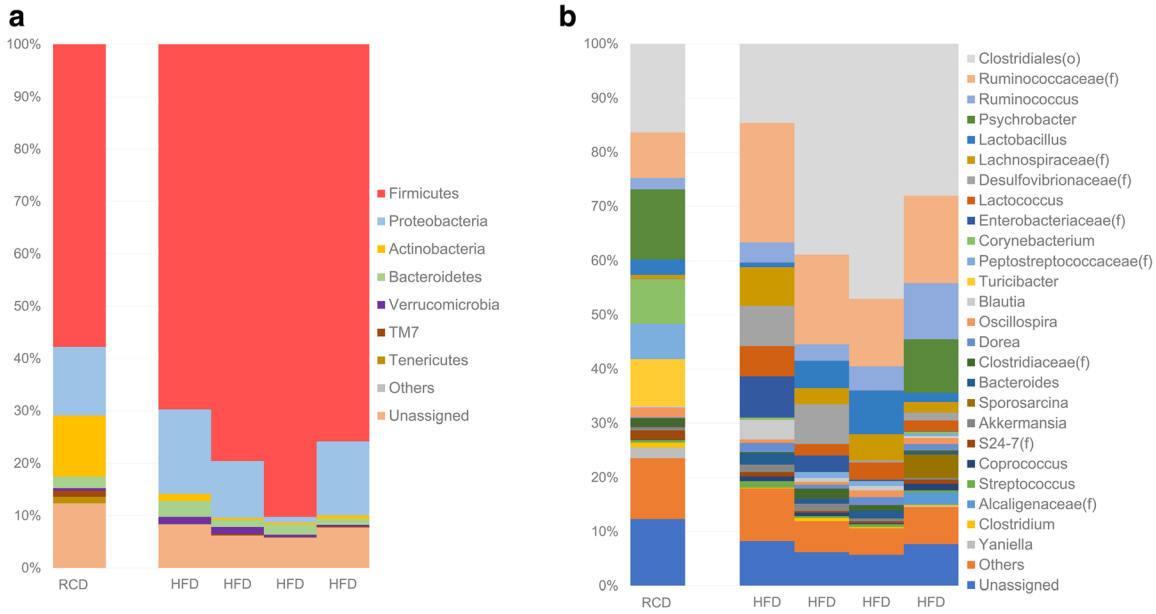


Fig. 1 α-Diversity between high-fat diet (HFD) group and regular chow diet (RCD) group. **a** Differences of bacteria. **b** Differences of bacteria-derived extracellular vesicles



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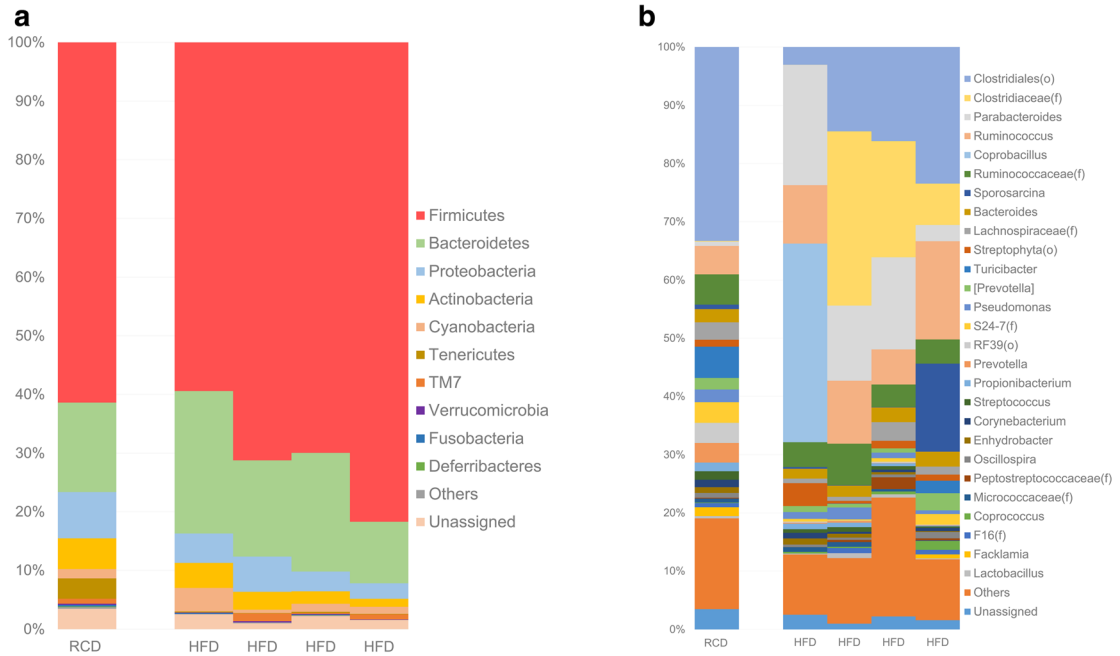
Phylum	RCD (%)	HFD (%)	Genus	RCD (%)	HFD (%)
Firmicutes	57.8	78.8	*Ruminococcus*	2.1	5.4
			Clostridiales(o)	16.3	32.2
			Ruminococcaceae(f)	8.4	16.8
			Dorea	0.2	1.3
			Lactococcus	0.01	3.2
Proteobacteria	13.1	10.6	*Psychrobacter*	12.9	2.5
			Neisseria	<0.01	<0.01
			Sutterella	0.01	0.2
			Enterobacteriaceae(f)	0.03	2.8
			Alcaligenaceae(f)	<0.01	0.6
Bacteroidetes	2.2	1.9	*Bacteroides*	0.2	1.2
			Parabacteroides	0.03	0.06
			Prevotella	0.3	0.01
			Rikenellaceae(f)	0.01	0.02
			S24-7(f)	1.7	0.6
Actinobacteria	11.6	0.7	*Corynebacterium*	8.3	0.2
			Yaniella	2.0	0.03
			Bifidobacterium	<0.01	<0.01
			Adlercreutzia	0.6	0.1
			Coriobacteriaceae(f)	0.09	0.02
Verrucomicrobia	0.5	0.9	*Akkermansia*	0.5	0.9

Fig. 2 Differences of bacterial distribution between high-fat diet (HFD) group and regular chow-diet (RCD) group. **a** Phylum level. **b** Genus level. **c** Changes of bacterial distribution after high-fat diet



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Phylum	RCD (%)	HFD (%)	Genus	RCD (%)	HFD (%)
Firmicutes	61.4	70.6	*Ruminococcus*	4.8	10.9
			Clostridiales(o)	33.3	14.3
			Ruminococcaceae(f)	5.2	4.9
			Dorea	0.04	0.2
			Lactococcus	0.04	0.3
			Weissella	0.3	0.4
Proteobacteria	7.9	4.2	*Psychrobacter*	0.1	0.03
			Neisseria	0.07	0.05
			Sutterella	<0.01	<0.01
			Enterobacteriaceae(f)	0.5	0.4
			Alcaligenaceae(f)	<0.01	<0.01
Bacteroidetes	15.2	17.9	*Bacteroides*	2.3	2.2
			Parabacteroides	0.7	13.1
			Prevotella	3.4	0.1
			Rikenellaceae(f)	0.08	0.1
			S24-7(f)	3.5	0.8
Actinobacteria	5.3	2.7	*Corynebacterium*	1.3	0.6
			Yaniella	0.2	0.08
			Bifidobacterium	0.07	0.1
			Adlercreutzia	0.08	0.01
			Coriobacteriaceae(f)	0.1	0.02
Verrucomicrobia	0.2	0.1	*Akkermansia*	0.2	0.1

Fig. 3 Differences of bacterium-derived extracellular vesicles distribution between high-fat diet (HFD) group and regular chow diet (RCD) group. **a** Phylum level. **b** Genus level. **c** Changes of bacterium-derived extracellular vesicle distribution after high-fat diet

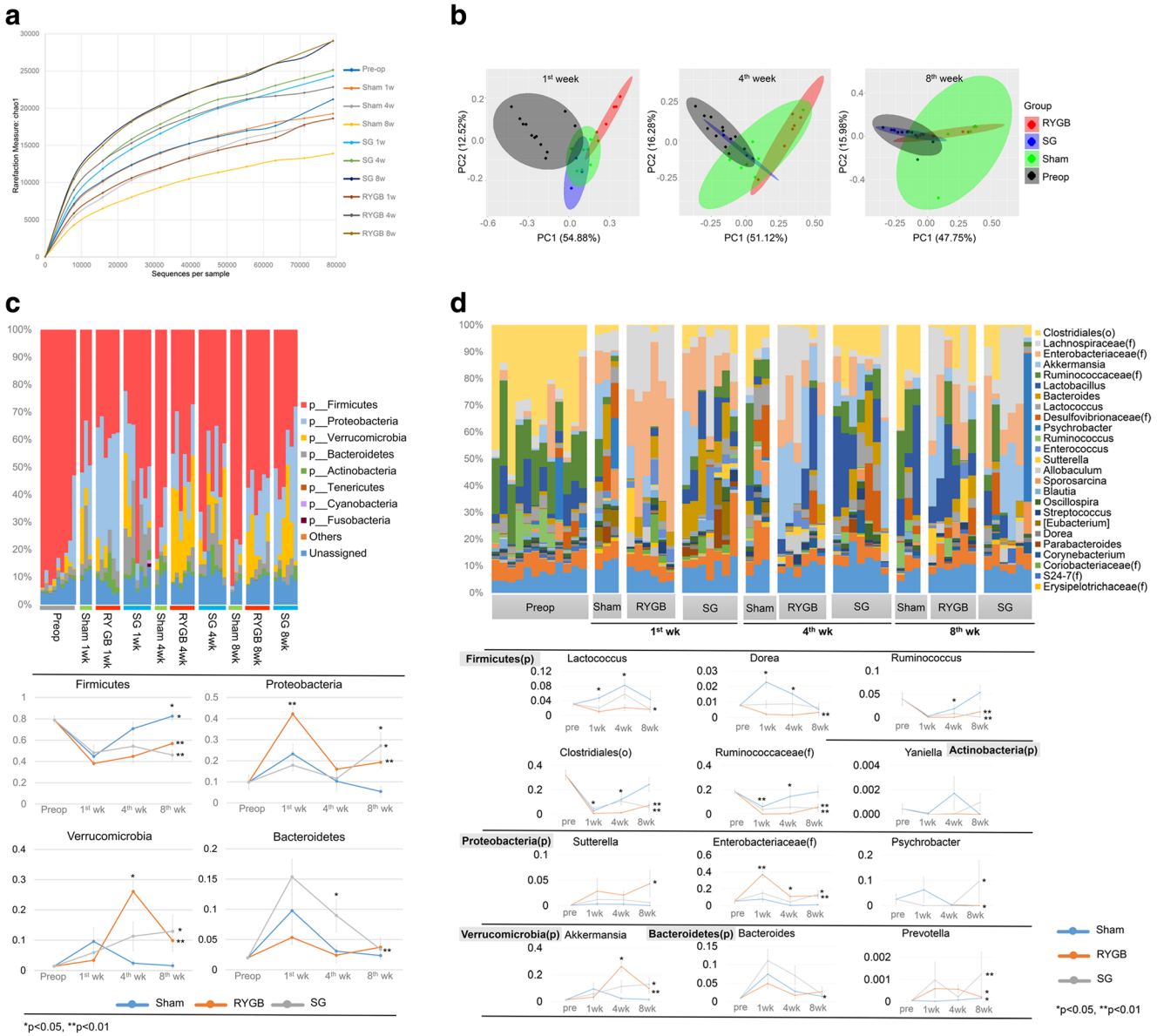


Fig. 4 Postoperative metagenomic data according to the operation types in bacteria. **a** Distinctly different α-diversity. **b** β-Diversity in genus level. **c** Serial changes of bacterial composition in phylum level. **d** Serial changes of bacterial composition in genus level. **Sham* sham operation; *SG* sleeve gastrectomy; *RYGB* Roux-en-Y gastric bypass

remaining 23 rats in the HFD group, which were confirmed to have induced glucose intolerance through OGTT, were randomly distributed to perform one of the following operations: Roux-en-Y gastric bypass (RYGB) ($n = 10$), sleeve gastrectomy (SG) ($n = 10$), and sham operation ($n = 3$). All rats maintained the same HFD for 8 weeks after operations. In all groups, postoperative measurements of body weight, random glucose, and food intake were carried out once a week for a total of 8 weeks. OGTT tests, using administration of 20% dextrose solution (2 g/kg) through oral gavage, were performed at the first and eighth week postoperatively. Fecal samples were collected for metagenomic analysis at the first, fourth, and eighth week postoperatively.

Surgical Techniques

After overnight fasting, operations were performed under general anesthesia with 2% isoflurane. For RYGB, after a laparotomy, the jejunum was divided at 40 cm from the Treitz ligament. Side-to-side jejunojejunostomy was performed at 15 cm of the Roux limb. The stomach was divided just below the gastroesophageal junction, and the distal part of the divided stomach (approximately 95%) was closed with running sutures. End-to-side gastrojejunostomy was performed. For SG, the vessels of the greater curvature were cauterized from the fundus to proximally 1 cm from the pylorus. SG was conducted, resecting about 80% of the stomach, including

the whole fundal portion. The resection line was closed with hand-sewn sutures. For sham operations, only manual exploration of the whole abdomen was performed. Water was given from the first postoperative day. On postoperative day 2, HFD and water were taken ad libitum.

Metagenomics

EV Isolation and DNA Extraction from Rat Fecal Samples

The fecal sample was filtered through a cell strainer after being diluted in 10 mL of PBS for 24 h. EVs in stool samples were isolated using centrifugation at 10,000×*g* for 10 min at 4 °C. After centrifugation, the pellet was comprised of bacteria and supernatant was comprised of EVs. Bacteria and foreign particles were thoroughly eliminated by sterilizing the supernatant through a 0.22-μm filter. To extract the DNA out of the bacteria and the bacterial EV membrane, bacteria and EVs were boiled for 40 min at 100 °C. To eliminate the remaining floating particles and waste, supernatant was collected after centrifugation at 13,000 rpm for 30 min at 4 °C. DNA was extracted using a DNA isolation kit (PowerSoil DNA Isolation Kit, MO BIO, USA). The standard protocol outlined in the kit was followed. Bacteria and EV-derived DNA in each sample were quantified using QIAxpert system (QIAGEN, Germany).

Analysis of Bacterial Composition in the Gut Microbiota

Raw pyrosequencing reads obtained from the sequencer were filtered according to the barcode and primer sequences using MiSeq (Illumina, USA). Taxonomic assignment was performed by the profiling program MDx-Pro ver.1 (MD Healthcare, Korea). This program selects high-quality sequencing reads after checking the read length (≥ 300 bp) and quality score (average Phred score ≥ 20). Operational Taxonomy Units (OTUs) were clustered using sequence clustering algorithm CD-HIT. Subsequently, taxonomy assignment was carried out using UCLUST and QIIME against the 16S rDNA sequence database in GreenGenes 8.15.13. Based on sequence similarities, all 16S rDNA sequences were assigned to the appropriate taxonomic levels. The bacterial composition at each level was plotted in the stacked bar. If case clusters could not be assigned at the genus level due to the lack of sequences or redundant sequences in the database, the taxon was assigned at the next highest level, which is indicated in parenthesis. Species richness (rarefaction curves) was estimated with the Chao1 estimator.

Statistical Analysis

Statistical analysis was performed with R version 3.4.3 software. Serial data of microbiome according to operations and difference between operations were analyzed with the Kruskal–Wallis test and the Mann–Whitney *U* test, as appropriate. Wilcoxon rank-sum tests were additionally performed for comparisons of relative abundances of microbiota between case and control groups. For bacterial and EV communities, PCA (principal component analysis) was also performed based on the abundance of each taxon. A p value < 0.05 after the Bonferroni correction was considered as statically significant.

Results

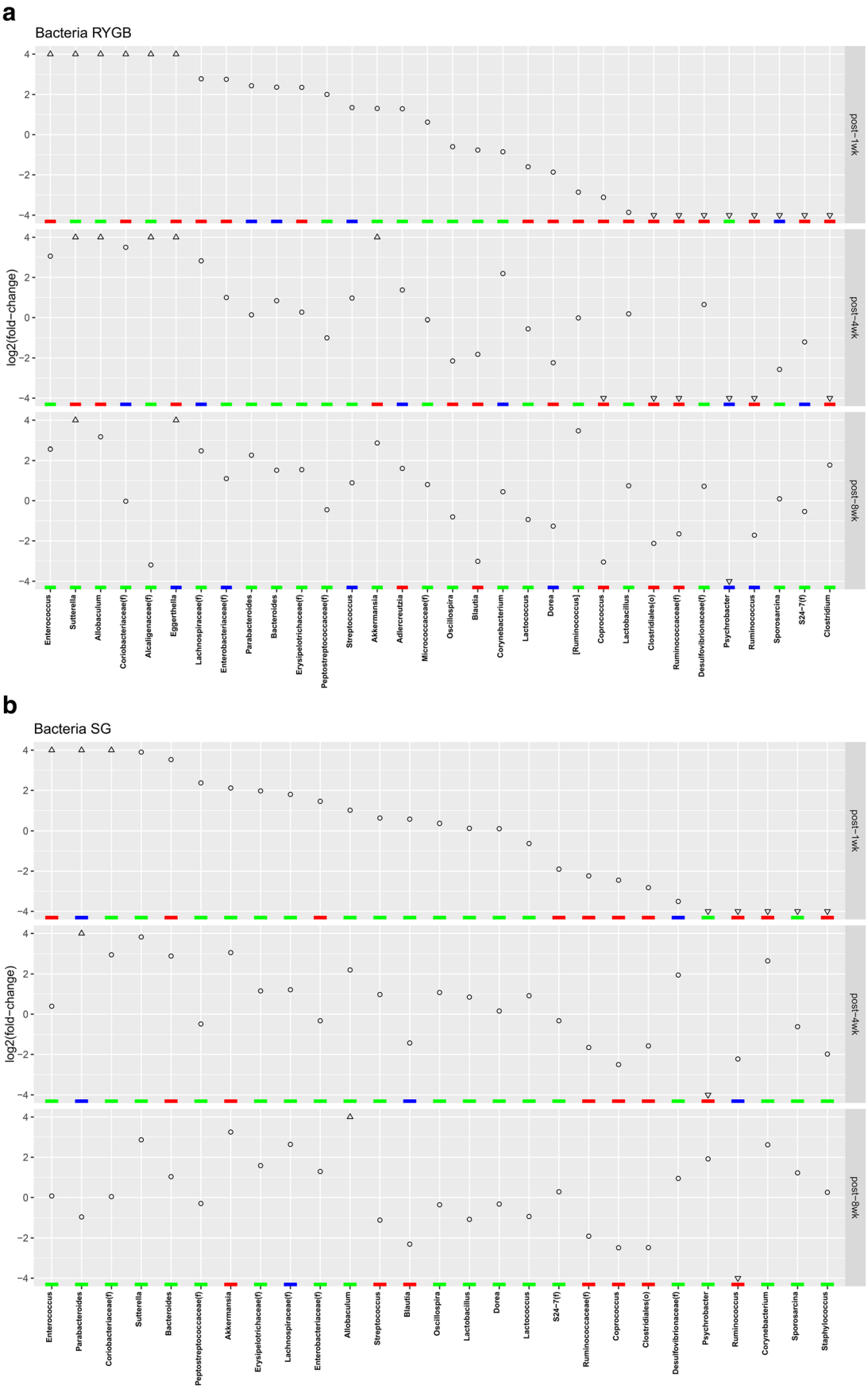
Comparison Between HFD Group and RCD Group

After 6 months, median values of body weight were 625.2 g (range, 569–673) in the RCD group and 796.8 g (range, 740–940) in the HFD group compared to initially 100–150 g. Through the OGTT, the glucose level confirmed the induction of glucose intolerance in the HFD group showing peak values of 187.8 and 164.6 mg/dL after 30 min and 154.4 and 72.6 mg/dL after 120 min in the HFD and RCD groups, respectively. Rarefaction curves based on Chao1, an estimator of species richness (α-diversity), showed considerable difference between the HFD and RCD groups. Species richness was lower in the HFD group than in the RCD group and this difference was more prominent in EVs than microbiota (Fig. 1).

The differences in composition of bacteria and bacteria-derived EVs in feces between HFD and RCD groups are shown in Figs. 2 and 3. For bacteria, the proportion of Firmicutes(p) was higher, while those of Actinobacteria(p), Proteobacteria(p), and Bacteroidetes(p) were lower in HFD group at the phylum level. At the genus level, the proportional differences of *Clostridiales(o)(g)*, *Ruminococcus(g)*, *Dorea(g)*, *Lactococcus(g)* in Firmicutes(p), *Psychrobacter(g)*, *Enterobacteriaceae(f)* in Proteobacteria(p), *Prevotella(g)*, *S24-7;g_(g)* in Bacteroidetes(p), *Corynebacterium(g)*, and *Yaniella(g)* in Actinobacteria(p) contributed to the compositional differences within significantly changed bacterial phyla (Fig. 2).

Meanwhile, the pattern of bacterium-derived EVs was somewhat different from that of bacteria. The difference of Firmicutes(p) EVs between the HFD and RCD groups was less than that of Firmicutes(p) bacteria. Overall, EVs of Bacteroidetes(p) were the second most common phylum in EVs following Firmicutes(p), and Bacteroidetes(p) proportion was higher in the HFD group. At the genus level, the genera, which showed significant changes in bacteria, also showed

Fig. 5 Changed individual bacteria in genus level following sham operation (sham), Roux-en-Y gastric bypass (RYGB), and sleeve gastrectomy (SG). Median fold changes in relative abundance of genus that changed between preop and first week, preop and fourth week, and preop and eighth week. **a** Roux-en-Y gastric bypass group. **b** Sleeve gastrectomy group. **c** Sham operation group. **d** Changes of bacterial distribution following sham operation, Roux-en-Y gastric bypass, and sleeve gastrectomy


a
Bacteria RYGB
log2(fold−change)
post−1wk
post−4wk
post−8wk
Enterococcus
Sutterella
Allobaculum
Coriobacteriaceae(f)
Alcaligenaceae(f)
Eggerthella
Lachnospiraceae(f)
Enterobacteriaceae(f)
Parabacteroides
Bacteroides
Erysipelotrichaceae(f)
Peptostreptococcaceae(f)
Streptococcus
Akkermansia
Adlercreutzia
Micrococcaceae(f)
Oscillospira
Blautia
Corynebacterium
Lactococcus
Dorea
[Ruminococcus]
Coprococcus
Lactobacillus
Clostridiales(o)
Ruminococcaceae(f)
Desulfovibrionaceae(f)
Psychrobacter
Ruminococcus
Sporosarcina
S24−7(f)
Clostridium
b
Bacteria SG
log2(fold−change)
post−1wk
post−4wk
post−8wk
Enterococcus
Parabacteroides
Coriobacteriaceae(f)
Sutterella
Bacteroides
Peptostreptococcaceae(f)
Akkermansia
Erysipelotrichaceae(f)
Lachnospiraceae(f)
Enterobacteriaceae(f)
Allobaculum
Streptococcus
Blautia
Oscillospira
Lactobacillus
Dorea
Lactococcus
S24−7(f)
Ruminococcaceae(f)
Coprococcus
Clostridiales(o)
Desulfovibrionaceae(f)
Psychrobacter
Ruminococcus
Corynebacterium
Sporosarcina
Staphylococcus

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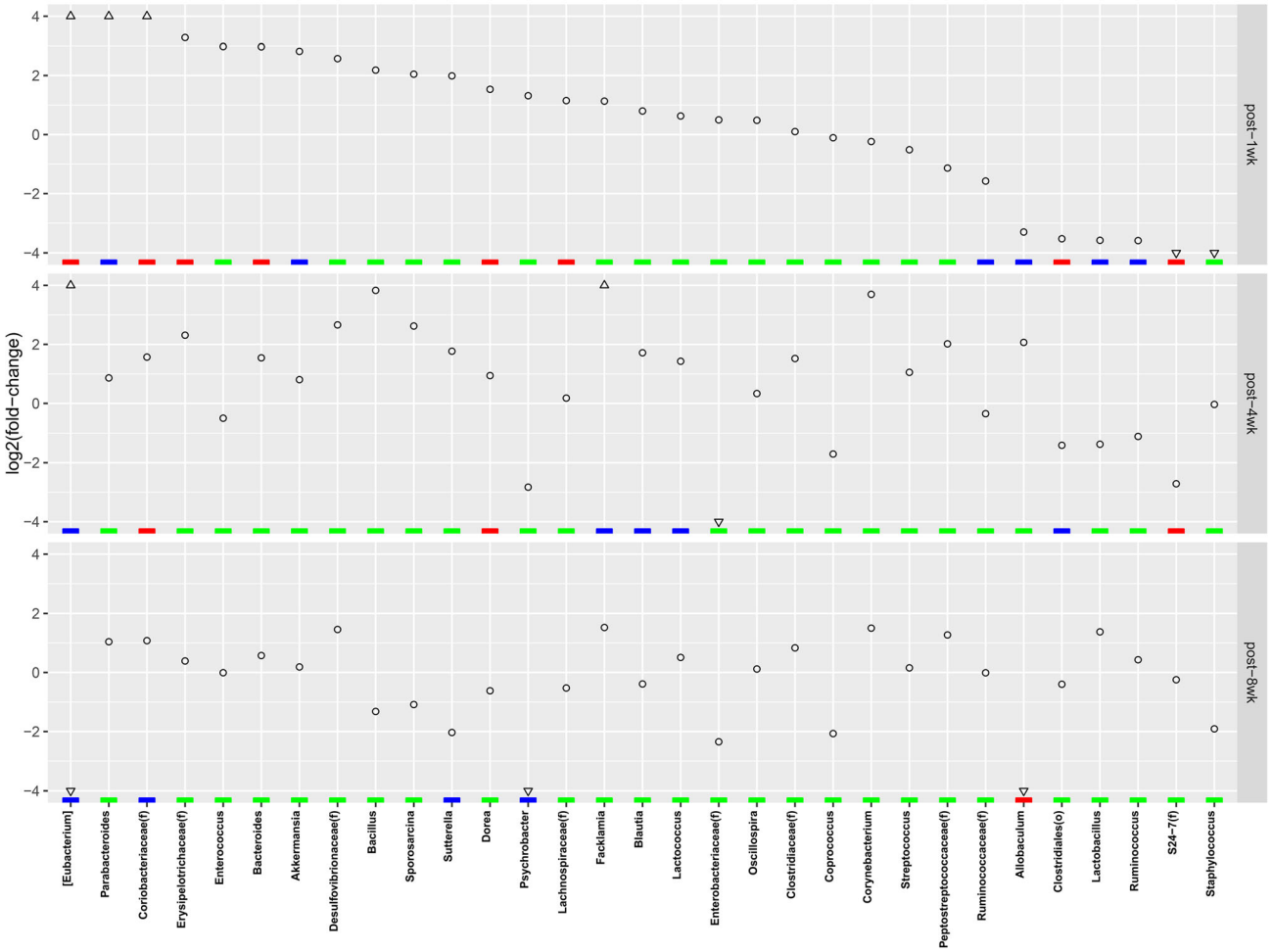


d

Phylum	Genus	Preop	RYGB			SG			Sham		
			1st week	4th week	8th week	1st week	4th week	8th week	1st week	4th week	8th week
Firmicutes	*Ruminococcus*	4.0	0.1	0.08	1.2	0.2	0.9	0.1	0.3	1.8	5.4
	Clostridiales(o)	32.2	0.7	1.3	7.4	4.5	10.8	5.8	2.8	12.1	24.4
	Ruminococcaceae(f)	18.6	0.2	0.6	5.9	3.9	5.9	4.9	6.2	14.7	18.4
	Dorea	0.8	0.2	0.2	0.3	0.9	0.9	0.6	2.3	1.5	0.5
	Lactococcus	3.1	1.0	2.1	1.6	2.0	5.8	1.6	4.7	8.2	4.4
Proteobacteria	*Psychrobacter*	2.5	0.01	0.03	<0.01	0.01	<0.01	9.5	6.3	0.4	<0.01
	Neisseria	<0.01	<0.01	<0.01	<0.01	0.2	<0.01	<0.01	<0.01	<0.01	<0.01
	Sutterella	0.1	2.9	2.1	4.3	1.2	1.1	0.6	0.3	0.3	0.02
	Enterobacteriaceae(f)	5.5	37.0	10.9	11.7	15.0	4.4	13.4	7.7	0.3	1.1
	Alcaligenaceae(f)	<0.01	1.4	0.3	<0.01	<0.01	0.1	0.3	<0.01	<0.01	<0.01
Bacteroidetes	*Bacteroides*	1.0	4.9	1.7	2.7	11.1	7.1	2.0	7.5	2.8	1.4
	Parabacteroides	0.1	0.3	0.1	0.3	3.5	1.0	0.03	2.1	0.1	0.1
	Prevotella	<0.01	0.06	0.1	0.02	0.1	0.02	0.1	<0.01	0.01	0.02
	Rikenellaceae(f)	0.03	0.04	0.2	0.08	0.3	0.2	0.2	0.1	0.03	0.04
	S24-7(f)	0.9	0.02	0.4	0.6	0.2	0.7	1.1	0.01	0.1	0.8
Actinobacteria	*Corynebacterium*	0.2	0.1	1.0	0.3	0.01	1.3	1.3	0.2	2.7	0.6
	Yaniella	0.05	<0.01	<0.01	<0.01	<0.01	<0.01	0.1	0.01	0.2	<0.01
	Bifidobacterium	<0.01	0.02	<0.01	<0.01	0.2	0.01	<0.01	0.01	<0.01	0.01
	Adlercreutzia	0.1	0.2	0.2	0.3	0.02	0.2	0.1	0.05	0.1	0.2
	Coriobacteriaceae(f)	0.02	2.8	0.2	0.02	1.7	0.1	0.02	0.5	0.05	0.04
Verrucomicrobia	*Akkermansia*	1.4	3.4	26.1	9.9	5.9	11.3	12.9	9.6	2.4	1.5

Fig. 5 (continued)

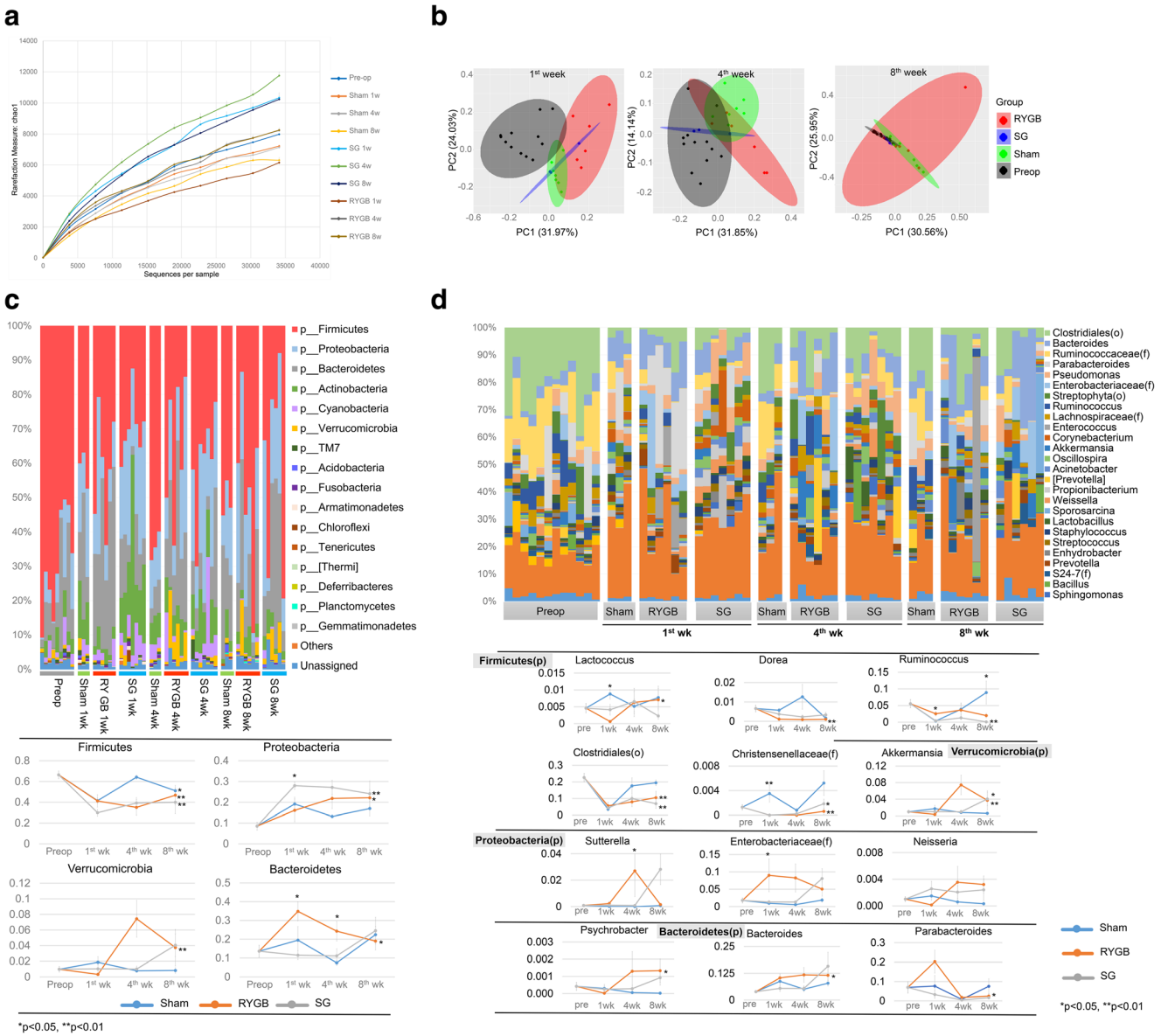


Fig. 6 Postoperative metagenomic data according to the operation types in extracellular vesicles. **a** Distinctly different α-diversity. **b** β-Diversity in genus level. **c** Serial changes of extracellular vesicle composition in phylum level. **d** Serial changes of extracellular vesicle composition in genus level. **Sham* sham operation; *SG* sleeve gastrectomy; *RYGB* Roux-en-Y gastric bypass

differences in EVs (Fig. 3). In particular, a decrease of *Akkermansia(g)* in Verrucomicrobia(p) was observed.

Changes According to Operative Type

Changes of Clinical Findings after Operations

Preoperative clinical findings of study subjects are described in the supplementary table. Before operations were performed, there were no significant differences among the groups in terms of weight ($p = 0.909$) and random glucose level ($p = 0.148$). The operative mortality rate was 30.4% (7 of 23; 4 after RYGB, 3 after SG). Body weights gradually decreased in both RYGB and SG groups and the gaps with sham operation tended to increase steadily for 8 weeks postoperatively, but no statistically significant difference was determined ($p = 0.08$) (Supplemental Figure S1A). The food intake was significantly reduced in all the three groups immediately after operations were performed compared to the preoperative average of 25 g/day, but intake of the RYGB and SG groups was less than the sham operation group in all observation periods. In the sham group, the food intake gradually increased to almost the same amount of preoperative intake at postoperative week 8. On the other hand, intake of RYGB

and SG was shown to plateau after 6 weeks postoperative and did not recover to the preoperative level (Figure S1B). The postoperative OGTT results of the RYGB and SG groups showed improved glucose intolerance. In the OGTT conducted in the first week, the fasting glucose levels were slightly lower and glucose clearance rates after oral glucose injection were shortened after RYGB and SG compared with the sham operation (Figure S1C). In the OGTT conducted during the eighth week, the glucose levels of both RYGB and SG groups at 120 min were normalized (Figure S1D).

Metagenomics for Serial Microbial Changes at the Phylum and Genus Levels

Diversity The α-diversity was different between all groups (Fig. 4a). Compared with the preoperative group, the diversity curves increased in both RYGB and SG groups except in the RYGB group 1 week postoperative. The sham group showed a decrease of diversity postoperative. The characteristic distributions of microbial communities (β-diversity) at the genus level after sham, RYGB, and SG operations were serially visualized using principal component analysis of the log-transformed relative abundances. At the first week, all three groups showed clearly different distribution patterns. However, the pattern of the sham operation group became similar to the baseline (preoperative) group, while those of RYGB and SG groups were clearly different from sham operation at the fourth and eighth week postoperative (Fig. 4b).

Compositional Changes Figure 4c shows the serial changes of microbial composition at the phylum level according to the operation types. The proportion of Firmicutes(p) decreased, and those of Proteobacteria(p) and Verrucomicrobia(p) increased after RYGB and SG. The preoperative proportion of Firmicutes(p) was 78.9%, but it decreased rapidly in all three groups at the first week after surgery. However, Firmicutes(p) recovered gradually to preoperative levels in the sham group, while it maintained a proportion of 56.8% and 46.1% in RYGB and SG groups, respectively, 8 weeks postoperative. Verrucomicrobia(p) steeply increased at the fourth week in the RYGB group and gradually increased up until the eighth week postoperative in the SG group, while it decreased to preoperative levels in the sham group. Proteobacteria(p) showed an increasing trend until the eighth week after RYGB and SG, and the proportion was decreased more than the preoperative value at the eighth week after the sham operation.

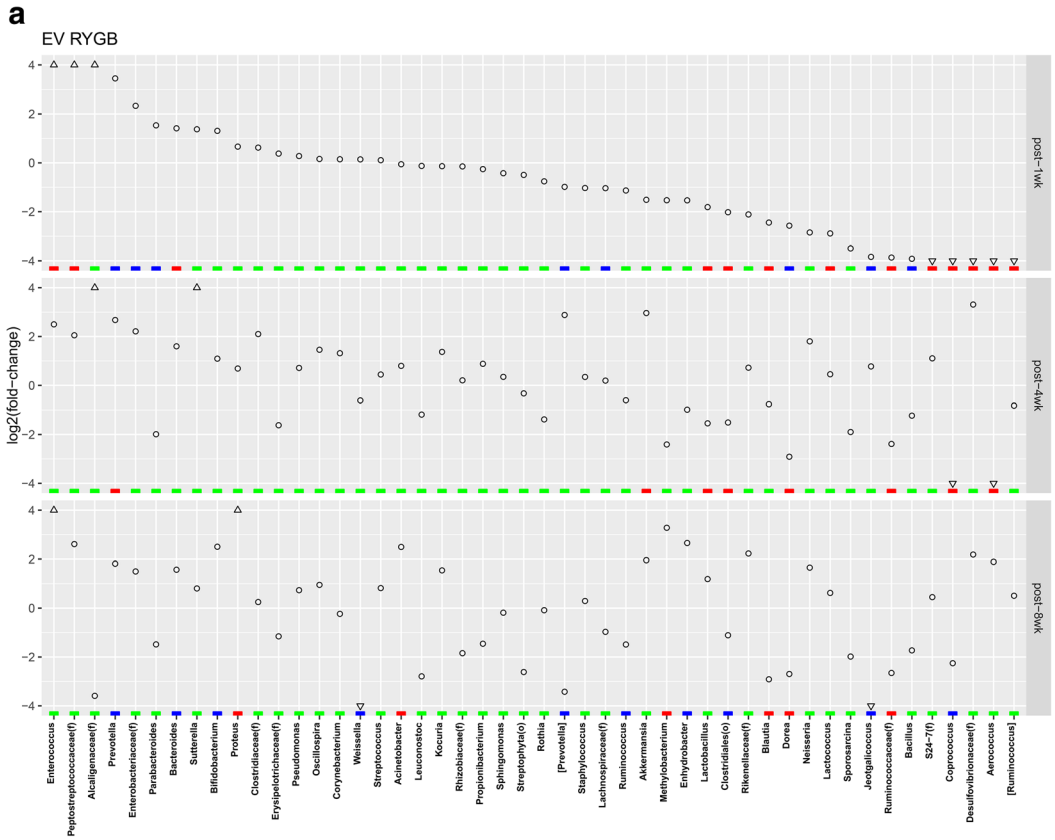
Figure 4d shows the serial changes of microbial composition at the genus level. Among the decreased Firmicutes(p) after surgery, *Lactococcus(g)*, *Dorea(g)*, *Ruminococcus(g)*, *Clostridiales(o)*, and *Ruminococcaceae(g)* were determined to be the decreased genus that caused the major changes. In Actinobacteria(p), *Yaniella(g)* only showed an increased proportion the eighth week after SG. *Sutterella(g)* and *Enterobacteriaceae(f)* were the major genus among the increased Proteobacteria(p) after surgery, but *Psychrobacter(g)* in Proteobacteria(p) increased only after SG. *Akkermansia(g)* was the most markedly increased genus in Verrucomicrobia(p). In Bacteroidetes(p), *Prevotella(g)* markedly increased after RYGB and SG, and *Bacteroides(g)* increased after SG and decreased after RYGB.

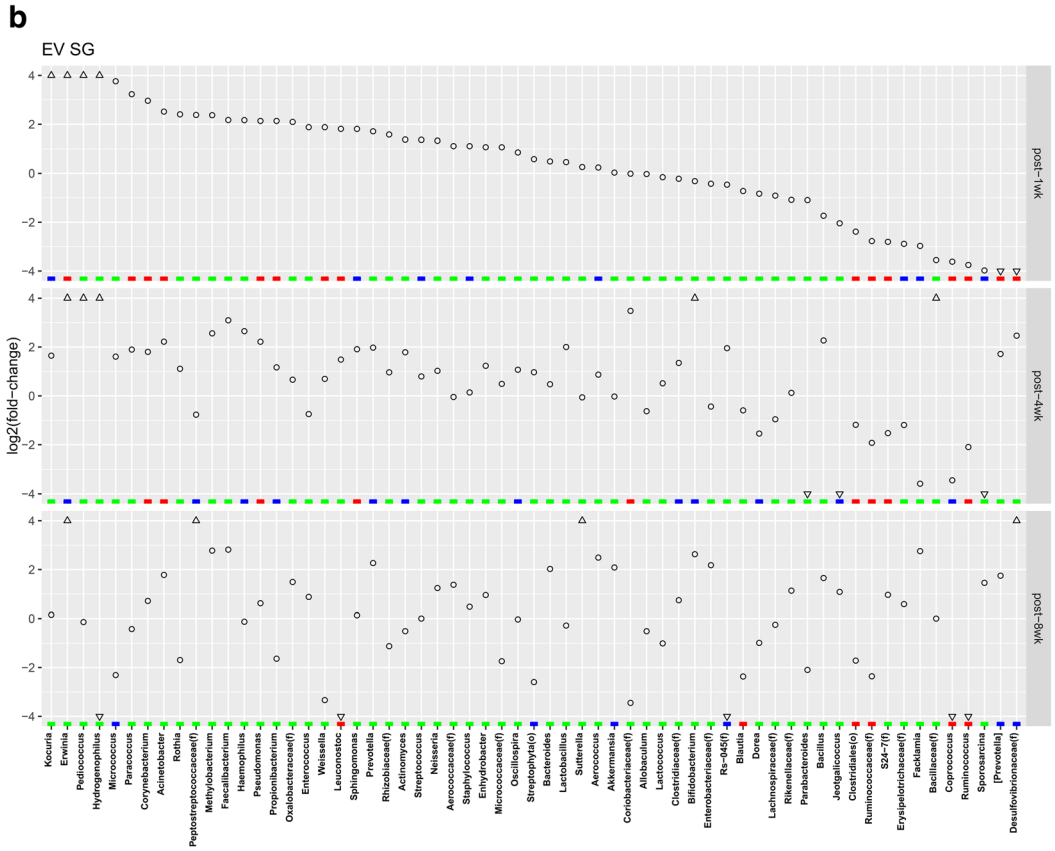
Figure 5 shows the changes in individual gut microbiota genera following RYGB (Fig. 5a), SG (Fig. 5b), and sham operation (Fig. 5c). Genera showing noticeable changes were listed in descending order of the median fold change between the baseline and first week, fourth week, and eighth week postoperative. These figures showed a series of genera that showed marked fold changes after operation regardless of their occupied proportion. The values − 1 and 1 mark when the microbes halved or doubled their relative abundance. After RYGB, *Eggerthella(g)* from Actinobacteria(p) significantly increased on the first week postoperatively and maintained the increased relative abundance throughout the 8-week observation. The fold change of *Sutterella(g)* from Proteobacteria(p) was also very large and lasted the total 8-week measure. The fold change of *Akkermansia(g)* from Verrucomicrobia(p) significantly increased more in the fourth week. *Ruminococcus(g)* and *Dorea(g)* from Firmicutes(p) showed significant decreased fold changes, and the changes remained until the eighth week. In the SG group, *Sutterella(g)* from Proteobacteria(p) and *Akkermansia(g)* from Verrucomicrobia(p) showed high fold changes on the first week, and those changes remained throughout 8 weeks. *Dorea(g)* did not demonstrate a significant fold change, but *Ruminococcus(g)* showed a more noticeable fold change. Genera that showed fold changes > 2 or occupied proportion > 0.1 percentage was tabulated in Fig. 5d.

Metagenomics for Serial Changes of Microbial EVs at the Phylum and Genus Levels

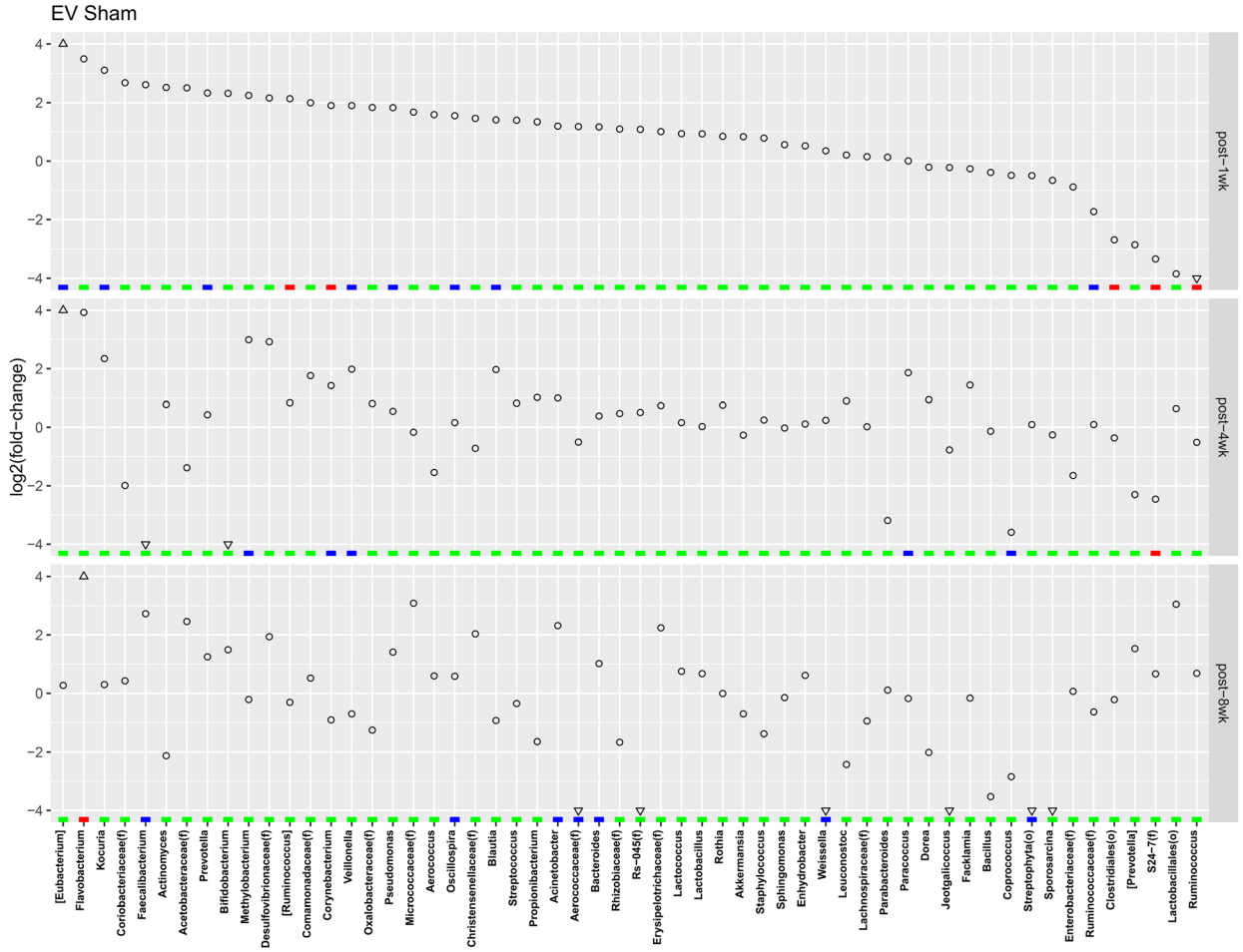
Diversity For the bacterium-derived EVs, microbial richness was shown to be different between groups (Fig. 6a). Compared with the preoperative results, the diversity curves increased in the SG group, whereas decreased in the sham group. Unexpectedly, richness did not increase after RYGB. Each group had a characteristic distribution at the genus level, shown in the β-diversity figures (Fig. 6b). At the first week, all three operational groups showed clearly distinct

Fig. 7 Changed individual extracellular vesicles (EVs) in genus level following sham operation (sham), Roux-en-Y gastric bypass (RYGB), and sleeve gastrectomy (SG). Median fold changes in relative abundance of genus that changed between preop and first week, preop and fourth week, and preop and eighth week. **a** Roux-en-Y gastric bypass group. **b** Sleeve gastrectomy group. **c** Sham operation group. **d** Changes of bacterium-derived extracellular vesicle distribution following sham operation, Roux-en-Y gastric bypass, and sleeve gastrectomy


a
EV RYGB
log2(fold−change)
post−1wk
post−4wk
post−8wk
Enterococcus
Peptostreptococcaceae(f)
Alcaligenaceae(f)
Prevotella
Enterobacteriaceae(f)
Parabacteroides
Bacteroides
Sutterella
Bifidobacterium
Proteus
Clostridiaceae(f)
Erysipelotrichaceae(f)
Pseudomonas
Oscillospira
Corynebacterium
Weissella
Streptococcus
Acinetobacter
Leuconostoc
Kocuria
Rhizobiaceae(f)
Propionibacterium
Sphingomonas
Streptophyta(o)
Rothia
[Prevotella]
Staphylococcus
Lachnospiraceae(f)
Ruminococcus
Akkermansia
Methylobacterium
Enhydrobacter
Lactobacillus
Clostridiales(o)
Rikenellaceae(f)
Blautia
Dorea
Neisseria
Lactococcus
Sporosarcina
Jeotgalicoccus
Ruminococcaceae(f)
Bacillus
S24−7(f)
Coprococcus
Desulfovibrionaceae(f)
Aerococcus
[Ruminococcus]


b
EV SG
log2(fold−change)
post−1wk
post−4wk
post−8wk
Kocuria
Erwinia
Pediococcus
Hydrogenophilus
Micrococcus
Paracoccus
Corynebacterium
Acinetobacter
Rothia
Peptostreptococcaceae(f)
Methylobacterium
Faecalibacterium
Haemophilus
Pseudomonas
Propionibacterium
Oxalobacteraceae(f)
Enterococcus
Weissella
Leuconostoc
Sphingomonas
Prevotella
Rhizobiaceae(f)
Actinomyces
Streptococcus
Neisseria
Aerococcaceae(f)
Staphylococcus
Enhydrobacter
Micrococcaceae(f)
Oscillospira
Streptophyta(o)
Bacteroides
Lactobacillus
Sutterella
Aerococcus
Akkermansia
Coriobacteriaceae(f)
Allobaculum
Lactococcus
Clostridiaceae(f)
Bifidobacterium
Enterobacteriaceae(f)
Rs−045(f)
Blautia
Dorea
Lachnospiraceae(f)
Rikenellaceae(f)
Parabacteroides
Bacillus
Jeotgalicoccus
Clostridiales(o)
Ruminococcaceae(f)
S24−7(f)
Erysipelotrichaceae(f)
Facklamia
Bacillaceae(f)
Coprococcus
Ruminococcus
Sporosarcina
[Prevotella]
Desulfovibrionaceae(f)

c



d

Phylum	Genus	Preop	RYGB			SG			Sham		
			1st week	4th week	8th week	1st week	4th week	8th week	1st week	4th week	8th week
Firmicutes	*Ruminococcus*	5.5	2.5	3.6	2.0	0.4	1.3	0.08	0.3	3.9	8.9
	Clostridiales(o)	22.6	5.6	8.0	10.5	4.3	10.0	6.9	3.5	17.6	19.5
	Ruminococcaceae(f)	14.5	1.0	2.8	2.3	2.1	3.8	2.8	4.4	15.4	9.3
	Dorea	0.7	0.1	0.1	0.1	0.4	0.2	0.3	0.6	1.3	0.2
	Lactococcus	0.5	0.1	0.6	0.7	0.4	0.7	0.2	0.9	0.5	0.8
	Christensenellaceae(f)	0.1	<0.01	<0.01	0.06	<0.01	0.02	0.2	0.4	0.1	0.5
Proteobacteria	*Psychrobacter*	0.04	<0.01	0.1	0.1	0.03	0.03	0.09	0.03	0.01	<0.01
	Neisseria	0.1	0.01	0.4	0.3	0.3	0.2	0.2	0.2	0.06	0.03
	Sutterella	0.1	0.2	2.7	0.2	0.1	0.08	2.8	0.01	<0.01	0.08
	Enterobacteriaceae(f)	1.8	9.0	8.3	5.1	1.3	1.3	8.1	1.0	0.6	1.9
	Alcaligenaceae(f)	<0.01	0.3	0.5	<0.01	<0.01	<0.01	0.2	0.02	0.04	<0.01
Bacteroidetes	*Bacteroides*	3.9	10.4	11.8	11.6	5.5	5.4	15.9	8.8	5.1	7.9
	Parabacteroides	7.0	20.3	1.8	2.5	3.3	0.4	1.6	7.7	0.8	7.5
	Prevotella	0.2	2.7	1.6	0.9	0.8	1.0	1.2	1.3	0.3	0.6
	Rikenellaceae(f)	0.2	0.04	0.3	0.9	0.09	0.2	0.4	0.2	<0.01	0.2
	S24-7(f)	1.0	0.04	2.2	1.4	0.2	0.4	2.0	0.1	0.2	1.7
Actinobacteria	*Corynebacterium*	0.6	1.0	2.2	0.8	7.0	3.1	1.5	3.4	2.4	0.5
	Yaniella	0.01	<0.01	<0.01	<0.01	<0.01	0.01	0.01	<0.01	0.07	<0.01
	Bifidobacterium	0.1	0.2	0.2	0.5	0.07	1.5	0.5	0.4	<0.01	0.2
	Adlercreutzia	0.1	<0.01	0.04	0.1	<0.01	0.04	0.2	0.02	0.2	0.04
	Coriobacteriaceae(f)	0.1	0.2	0.3	<0.01	0.08	0.9	0.01	0.5	0.02	0.1
Verrucomicrobia	*Akkermansia*	1.0	0.3	7.4	3.7	1.0	0.9	4.1	1.7	0.8	0.6

Fig. 7 (continued)

distribution patterns. However, the sham operation pattern became similar to the baseline (preoperative) group by the eighth week, while those of the RYGB and SG groups maintained clearly distinct patterns compared with the sham operation group after fourth and eighth weeks.

Compositional Changes EV compositional changes were generally similar to those of bacteria with some exceptions. The proportion of Firmicutes(p) decreased in all groups from the first week after surgery. The sham group increased gradually, while the RYGB group and SG group maintained at lower proportions in the eighth week. The proportion of Proteobacteria(p) increased from 8.5% before surgery to 22.1% for RYGB and 24.1% for SG after 8 weeks. Verrucomicrobia(p) also increased in both RYGB and SG groups. Bacteroidetes(p) showed a significant increase in the RYGB group, but not in the SG group (Fig. 6c).

Figure 6d shows the serial change of bacterium-derived EVs at the genus level. Among the decreased Firmicutes(p) taxa after surgery, similar genera, which showed changes in bacteria, caused the major changes. Of these, the change of *Christensenellaceae(f)* was not markedly observed in bacteria. *Sutterella(g)* and *Enterobacteriaceae(f)* in Proteobacteria(p) and *Akkermansia(g)* in Verrucomicrobia(p) showed common increase after surgery. Increases of *Neisseria(g)* and *Psychrobacter(g)* EVs in Proteobacteria(p) were observed after RYGB and SG, unlike bacterial changes. In Bacteroidetes(p), *Bacteroides(g)* was the major genus causing an increase in the RYGB group and a decrease in the SG group. *Parabacteroides(g)* was increased on the first week after RYGB, whereas after SG, it continuously decreased. The patterns of changes in bacterial EVs over time and between surgeries were not exactly the same as those of bacteria.

Genera showing noticeable changes following RYGB (Fig. 7a), SG (Fig. 7b), and sham operation (Fig. 7c) were listed in a descending order of median fold change. More variable and unpredictable changes were observed in EVs, and slight differences were observed compared with bacterial changes. *Enterococcus(g)* from Firmicutes(p), *Prevotella(g)*, and *Bacteroides(g)* from Bacteroidetes(p) showed significantly high fold changes after RYGB in the first week; however, those changes were only maintained on the total 8 weeks in *Prevotella(g)* and *Bacteroides(g)* with significance. *Coprococcus(g)*, *Dorea(g)*, and *Blautia(g)* from Firmicutes(p) showed significantly decreased fold changes at both first and eighth weeks (Fig. 7a). In the SG group, among the genera with increased fold changes, few showed continuous, maintained changes. Among the genera with reduced fold changes, *Ruminococcus(g)* and *Coprococcus(g)* showed significant, continuous, and distinct changes. Genera that showed fold changes > 2 with occupied proportion > 0.1% were tabulated in Fig. 7d.

Discussion

For over a decade, several studies have reported the differences in gut microbiota between obese and lean phenotypes, suggesting gut microbiota as a potential regulator of host metabolism [8, 9, 14], though the findings were inconsistent due to the limited number of samples and differing methodologies. BMS has emerged as an effective treatment for severe obesity and associated T2DM. In this animal study, we demonstrated obesity and glucose intolerance induced by HFD were associated with microbial dysbiosis, i.e., decreased diversity and compositional changes of gut microbiota. Furthermore, the resolution of obesity/diabetes and restoration of dysbiosis in part were observed after RYGB and SG even though HFD was continued. We could also find somewhat different results between the two procedures in terms of serial changes of microbial composition and diversity. Finally, we showed meaningful changes of bacteria-derived EVs which can act as intercellular communicators within our body.

According to recently published studies, microbial diversity was increased after BMS [15], which can be interpreted as a recovery from the unhealthy status of reduced diversity [14], though this conclusion is still under debate. After HFD, we could induce diabetes/obesity phenotype in concordance with decreased microbial diversity. With the significant weight reduction and glucose intolerance resolution observed after both RYGB and SG, microbial richness increased more and more over time from postoperative weeks 1 to 8. We also found microbial compositional dissimilarity after each operation. However, the distribution pattern of the sham operation group became similar to that of the preoperative baseline by weeks 4 and 8, while those of RYGB and SG groups was clearly different from the sham operation or baseline groups and each other as well. This means BMS influences microbial compositional distribution in a lasting way to maintain beneficial host metabolism effects. Furthermore, each operational procedure, RYGB and SG, affects compositional distribution differently according to the difference in anatomical modification. This finding is in line with similar results reported by Palleja et al. that gut microbial diversity increased within the first 3 months after RYGB and remained high until 1 year later [15].

Recent evidence shows there is strong evidence regarding the connection between BMS and microbial compositional change, but the suggested contributing microbiota varies from report to report, especially at the genus or species level [16]. In human and animal experiments, Firmicutes(p) is generally reported to decrease after BMS [17, 18] with shifts toward higher proportions of Proteobacteria(p), Bacteroidetes(p), Verrucomicrobia(p), and Fusobacteria(p) at the phylum level [15, 18, 19]. Our results also demonstrated phyla Proteobacteria, Verrucomicrobia, and Bacteroidetes were increased, but phylum Firmicutes was decreased after BMS.

At the genus level of gut microbial composition, we found approximately 400 kinds of genera present after BMS. Among them, we tried to determine which genera showed significant compositional difference in obesity/diabetes-induced rats compared with normal rats and were also restored similarly toward RCD levels after BMS, unlike sham-operated rats fed a HFD. Further, we only selected those bacteria that occupied a proportion greater than 0.1% or a fold change greater than 2 postoperative. Based on these criteria, *Akkermansia(g)* and *Prevotella(g)* were representatively increased genera that fit these conditions after both RYGB and SG. *Sutterella(g)* fits these conditions after RYGB but not SG, while *Psychrobacter(g) and Yaniella(g)* after SG, but not RYGB, also fit these conditions. Meanwhile, *Ruminococcus(g)*, *Lactococcus(g)*, *and Dorea(g)* were representatively decreased genera that fit these conditions after both RYGB and SG.

Although gut microbiome alteration is recognized as a potential contributor for regulating host metabolism after BMS, the mechanism of host-microbiota crosstalk is still under investigation [20]. Previous data indicate that microbiota-derived EVs are key players in insulin resistance, which provided important clues to understand the mechanism in the pathogenesis of T2DM [21]. Bacteria normally inhabit the gut, whereas EVs secreted from bacteria can enter systemic circulation by penetration of intestinal barriers thus can be distributed to target organs involved in insulin action. Given the fact that more active bacteria secrete more vesicles, it can be assumed that changes in vesicles rather than the simple amount of bacteria more accurately reflect the activity of bacteria and their effects on the human body. In this study, based on serial compositional changes of EVs at the phylum level after operations, we found that phylum Firmicutes EVs were decreased, but phyla Proteobacteria and Verrucomicrobia EVs were increased after BMS operation, like microbial compositional change. However, at the genus level, neither the composition of EVs of obesity/diabetes-induced rats nor the changes in EVs over time and between surgeries were exactly the same as those of bacteria, which might reflect the difference of microbial functional activity. The present study showed that EVs derived from genera *Akkermansia*, *Sutterella*, *Neisseria*, and *Psychrobacter* were representatively increased after both RYGB and SG. Conversely, EVs derived from genera *Ruminococcus*, *Parabacteroides*, and *Dorea* were increased after both RYGB and SG, and *Lactococcus* EVs decreased after only SG.

We also tried to determine which EVs derived from specific genera showed a meaningful compositional change in order to assign to be the candidates using the same selection criteria as bacteria. *Akkermansia(g)*, *Enterobacteriaceae(f)*, *Sutterella(g)*, *Neisseria(g)*, and *Psychrobacter(g)* were representatively increased genera that fit these conditions after both RYGB and SG. Meanwhile, *Ruminococcus(g)*, *Parabacteroides(g)*, and *Dorea(g)* were candidates shown to representatively decrease after both RYGB and SG, and *Lactococcus(g)* decreased after only SG representatively decreased genera fit these conditions.

There are several limitations to the present study. Metagenomics research analyzes all genes present in an environmental sample, yielding massive data sets. It is important to define not only what bacterial changes occur but also to determine the significance of those changes. Those that show consistent changes among microbiota and EVs need to be clarified for practical roles through functional validation studies such as microbial transplantation, and it will be necessary to ensure that repeated studies yield consistent results. If solid results are obtained through ongoing research, this may greatly affect the diagnosis and treatment paradigm of obesity-related diabetes.

In the present study, we investigated the effects of BMS on gut microbiota and microbe-derived EVs over time. Our results showed an increased diversity of gut microbiota and altered microbe and microbial EV composition with improved metabolic functions after RYGB and SG. Although we could not identify the precise role of gut microbiota and EVs, it is important that this study analyzed not only microbiota alterations but also the changes in microbial EVs as an actual cross talk player for the first time. In the future, further studies characterizing each specific change of bacteria and their secreted EVs after BMS will be needed.

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Compliance with Ethical Standards The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Ewha Womans University Mokdong Hospital (IACUC approval no. 15-0292).

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval The research followed all applicable institutional and/or national guidelines for the care and use of animals.

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