Bioscience of Microbiota, Food and Health Vol. 40 (3), 135–143, 2021

Full Paper

Analysis of ileostomy stool samples reveals dysbiosis in patients with high-output stomas

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Received September 14, 2020; Accepted February 4, 2021; Published online in J-STAGE February 17, 2021

Construction of a diverting stoma can significantly reduce the onset of severe anastomotic leakage in patients with rectal cancer. High-output stoma is one of the most important potential surgical complications after anal function-preserving surgery with ileostomy. Culture-independent techniques have revealed the interaction of the complex intestinal bacterial ecology with various diseases. Our objective was to evaluate the differences in patient characteristics and gut microbiota distribution features in patients with high-output stomas. The cases of 24 consecutive patients who underwent curative resection for rectal cancer at our hospital between November 2016 and June 2018 were reviewed, and the patients were categorized into high-output and low-output groups. Their microbiota were analyzed using next-generation sequencing of ileostomy stool samples collected on postoperative day 7. There was a significant difference in the percentage of Bacteroidetes between the high-output and low-output groups (14.8% vs 0.5%; p=0.01). The percentage of *Clostridium butyricum* was increased in the low-output group (p=0.01). After the exclusion of those treated with the probiotic Miya-BM, whose principal component is *C. butyricum*, analyses revealed no significant differences between the high-output and low-output groups. This pilot study provides the first evidence correlating gut microbiota with the pathogenesis of high- output stoma compared with low-output stoma.

Key words: dysbiosis, high output, ileostomy, rectal cancer

INTRODUCTION

Construction of a diverting stoma is a useful means of significantly reducing the onset of severe anastomotic leakage (AL) that requires additional surgery in patients undergoing low anterior resection (LAR) or intersphincteric resection (ISR) for rectal cancer [1]. Although one of the most important potential surgical complications after anal function-preserving surgery is AL, which can result in morbidity and/or mortality, stoma-related complications such as stoma infection, obstructive complications, and electrolyte imbalance can also lead to severe problems [2]. High-output ileostomy may result from partial or intermittent bowel obstruction.

High-output stoma is characterized by an increased loss of fluids and sodium through fecal drainage, which may lead to hyponatremia, dehydration, and hyperaldosteronism [3]. Studies have reported that the majority of patients with a high-output stoma presented with only moderate decreases in glomerular filtration rate that were not clinically significant; however, 30% of the patients were found to have renal failure secondary to dehydration, requiring readmission [3, 4]. Patients with a daily stomal loss of <1,200 mL can usually maintain sodium balance by adding extra salt to the limit of palatability at the table and when cooking. When the stomal loss is in the range of 1,200–2,000 mL, or sometimes more, patients are more likely to have issues related to dehydration due to water and sodium loss through sweat, especially in hot weather [5].

Culture-independent techniques for bacterial identification based on 16S ribosomal RNA (rRNA) sequences have revolutionized the understanding of the complex intestinal bacterial ecology associated with various diseases [6, 7].

Despite advances in lower rectal surgery to preserve the sphincter and prevent AL using ileostomy, the consequences of stoma-related dehydration may be underestimated. The pathogenesis underlying high-output stoma is unclear. Therefore, the objective of the current study was to evaluate the differences in patient characteristics and the gut microbiota distribution in patients with high-output stomas.

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doi: 10.12938/bmfh.2020-062

MATERIALS AND METHODS

The data of 24 consecutive patients who underwent curative resection for rectal cancer with ileostomy by a laparoscopic approach at Juntendo University Hospital between November 2016 and June 2018 were reviewed in this retrospective study. The inclusion criteria were stage I–IV cancer, lateral pelvic lymph node dissection, and previous treatment with neoadjuvant pelvic chemoradiotherapy and chemotherapy. Patients who underwent emergency surgery, those with synchronous cancers, and those who underwent abdominoperineal resection were excluded from the current study.

Most of the total and tumor-specific mesorectal excisions were performed by the same team of staff colorectal surgeons. An ileostomy was created in the lower right abdominal wall with 30–40 cm of the terminal ileum. Ileostomy stool samples were collected on postoperative day 7. Rectal anastomosis was performed using the double stapling technique in patients undergoing LAR. Reconstruction comprised hand-sewn coloanal anastomosis in patients undergoing ISR. In all patients, cancer staging was based on the eighth edition of the TNM classification of malignant tumors. The indications for loop ileostomy were the following: anastomosis <5 cm from the anal verge, obstruction, neoadjuvant therapy, intraoperative technical issues, and severe diabetes mellitus. Preoperative neoadjuvant chemotherapy (such as FOLFOX or CapeOX) or chemoradiotherapy (such as S-1 with 45-Gy radiation) were administered in patients with clinical Stage II–III rectal cancer according to the TNM classification.

No chemical preparations, such as those including kanamycin and metronidazole, were administered preoperatively [8, 9]. All patients were intravenously administered 3 g/day cefmetazole, a cephamycin antibiotic. The use of probiotics, such as Miya-BM, which contains *Clostridium butyricum* M588, was at the surgeon's discretion.

AL was defined by the following clinical criteria: pelvic abscess, fecal discharge from the wound and drain, septicemia, and peritonitis, with or without radiologically confirmed leakage [10]. Grading of AL was performed as described by Rahbari *et al.* [11]. Surgical site infections were defined using the U.S. Centers for Disease Control definitions [12]. Postoperative ileus was defined as the inability to tolerate food in the presence of abdominal distension, absence of bowel sounds, and the need to delay enteral feeding [13]. Grading was based on the Clavien–Dindo classification [14]. High output was defined as an ostomy output ≥1,500 mL, as described in previous studies [15, 16].

DNA extraction from stool samples and 16S rRNA sequencing

Ileal stool samples from stoma pouches were suspended in 900 µL buffer containing 4 M guanidium thiocyanate, 100 mM Tris-HCl (pH 9.0), and 40 mM ethylenediaminetetraacetic acid (pH 8.0) and centrifuged at 20,800 *g* for 5 min. The supernatants were discarded, and 600 µL TE buffer (10 mmol/L Tris-HCl pH 8.0, 1 mmol/L ethylenediaminetetraacetic acid pH 8.0) was used to wash the pellets twice. Glass beads (diameter, 0.15–0.21 mm) were then added, and the samples were homogenized at 7,000 rpm for 20 sec using a MagNA Lyser instrument (Roche, Penzberg, Germany). Next, 2 µL lysozyme (10 mg/mL; Wako, Osaka, Japan) was added, the samples were incubated for 1 hr at 37°C. Subsequently, 100 µL of 10% sodium dodecyl sulfate (Kanto Chemical, Tokyo, Japan) and 600 µL of the phenol/chloroform/isoamyl alcohol solution (Nippon Gene, Tokyo, Japan) were added, and the samples were processed at 7,000 rpm for 20 sec in the MagNA Lyser. The samples were then centrifuged at 20,800 *g* for 5 min, and 600 µL of the supernatants were transferred to 1.5-mL tubes, followed by the addition of 600 µL isopropanol and 60 µL of 3 M sodium acetate (Nippon Gene). The samples were centrifuged at 20,800 *g* for 5 min, and the supernatants were removed by decanting. The DNA pellets were washed with 70% ethanol, dried with a centrifugal evaporator (Eyela, Tokyo, Japan), and dissolved by incubation in 200 µL TE buffer and 2 µL RNase (1 mg/mL; Nippon Gene) for 1 hr. The purity of the samples was determined with a High Pure PCR Template Preparation Kit (Roche, Basel, Switzerland). The DNA concentrations were quantified using a QuantiFluor One dsDNA system (Promega, Madison, WI, USA).

The variable V3–4 regions of the 16S rRNA gene were amplified by polymerase chain reaction using a Takara Ex Taq® Hot Start Version kit (Takara, Otsu, Japan) and the following primers: 341F, 50-AATGATACGGCGACCACCGAGATCTACAC (adaptor sequence) + barcode (eight bases) + ACACTCTTTCCCTACACGACGCTCTTCCGATCT (sequence primer) + CCTACGGGNGGCWGCAG-30, and 805R, 50-CAAGCAGAAGACGGCATACGAGAT (adaptor sequence) + barcode (eight bases) + GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT (sequence primer) + GACTACHVGGGTATCTAATCC-30 . The amplicons generated from the samples were purified using SPRIselect (Beckman Coulter, Brea, CA, USA). The DNA concentrations of purified amplicons were quantified using the QuantiFluor One dsDNA system, and equal DNA amounts were pooled.

The pooled samples were sequenced using MiSeq Reagent Kit v3 (600-cycle; Illumina, San Diego, CA, USA) on a MiSeq system according to the manufacturer's instructions. Sequence data were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (version 1.9.1).

Determination of α-diversity and β-diversity

The number of observed species and Chao1 and Shannon phylogenetic diversity indices were calculated using the phyloseq package for R and statistically analyzed using Wilcoxon's test. β- diversity was estimated using the UniFrac metric to calculate the distances between the samples and visualized by principal coordinate analysis. The graphs were generated using the QIIME pipeline (version 1.9.1).

Statistical analysis

The JMP version 13 (SAS Institute, Cary, NC, USA) statistical software was used for all statistical analyses. Categorical variables were compared using the χ^2 or Fisher's exact test, as appropriate. Continuous variables are presented as medians and were compared using the Mann-Whitney *U* test or analysis of variance. All data are presented as the median ± standard error of the mean (SEM). P values <0.05 were considered to indicate statistical significance.

Ethics approval and consent to participate

This study was performed in accordance with the ethical standards of the Committee on Human Experimentation of the study institution (Institutional Review Board No.16-124).

doi: 10.12938/bmfh.2020-062

Table 1. Characteristics and outcomes of patients

	High-output (n=12)	Low-output (n=12)	p-value
Sex (Male:Female)	9:3	8:4	0.65
Age (years)	61.5 (37–81)	65 (43–82)	0.75
BMI (kg/m^2)	23.2 (19.6–30.1)	23.0 (16.4–28.7)	0.45
Neoadjuvant chemotherapy	2	6	0.08
Neoadjuvant chemoradiotherapy	1	0	0.31
Procedure (LAR:ISR)	11:1	10:2	0.82
Lateral lymph node dissection	4	4	1
Operating time (min)	577 (339–657)	438.5 (230–769)	0.33
Blood loss (g)	50 (15–500)	45 (5–195)	0.6
During the use of antibiotic (day)	3 (2–13)	3 (2–6)	0.36
Antidiarrheals	10	9	0.62
Probiotics (Miya-BM)	3	5	0.39
Stage (I:II:III:IV)	4:5:2:1	5:2:5:0	0.3
Complication			
Anastomotic leakage (Grade A:B:C)	0:5:0	1:0:0	0.06
Ileus	1	0	0.31
Postoperative hospital stay (day)	16 (13–52)	12 (10–25)	0.004

This table shows the characteristics of the patients divided into two groups, high output and low output.

RESULTS

The study patients were categorized into the high-output (n=12) and low-output (n=12) groups. There were no significant differences in sex, age, body mass index, preoperative therapy, surgical procedure, operation time, blood loss, duration of antibiotic use, Miya-BM use, or postoperative complications, such as AL and postoperative ileus, between the two groups (Table 1).

Comparison of the α-diversity between the high-output and low-output groups using several indices, including the observed species, PD whole index, and Chao1 index, revealed that there were no statistically significant differences in α-diversity between the two groups (Fig. 1A). The overall community structure of the gut microbiota in both groups was evaluated using β-diversity indices calculated by the weighted UniFrac distance (Fig. 1B, C). The patients in the high-output group exhibited the highest UniFrac distances by Bonferroni test for multiple comparisons, indicating that the microbiota composition of the high-output group was significantly different from that of the low-output group.

The differences in the gut microbial structure were taxonomically evaluated at the phylum level (Fig. 2A). The major phyla of bacteria were Bacteroidetes (14.8% and 0.5% in the high-output and low-output groups, respectively; p=0.01), Firmicutes (64.9% and 70.3% in the high-output and low-output groups, respectively; p=0.63), Proteobacteria (15.5% and 25.9% in the high-output and low-output groups, respectively; p=0.95), Fusobacterium (2.5% and 0.1% in the high-output and low-output groups, respectively; p=0.73), and Actinobacteria (2.0% and 1.8% in the high-output and low-output groups, respectively; p=0.68).

There was no significant difference at the genus level. *Bacteroides* accounted for 11.6% of the bacteria in the high-output group, whereas it accounted for 0.1% of the bacteria in the low-output group (p=0.08; Fig. 2B). The taxonomic changes in the microbial communities in the high-output group of patients were evaluated at the species level as well. As shown in Fig. 2C, the low-output group showed a higher percentage of *C. butyricum* (p=0.01) and a lower percentage of *Parabacteroides gordonii* (p=0.07). We could not accurately distinguish between *C. butyricum* and Miya-BM. However, *C. butyricum* is thought to be the main component of the probiotic drug Miya-BM. Therefore, a secondary analysis was performed by categorizing patients not treated with Miya-BM into those with high output (n=9) and those with low output (n=7). There were no significant between-group differences in sex, age, body mass index, preoperative therapy, surgical procedure, operation time, blood loss, duration of antibiotic use, disease stage, or postoperative complications, such as AL and postoperative ileus (Table 2). Comparison of α- and β-diversity using the indices indicated above revealed that there were no statistically significant differences between the high-output and low-output patients with ileostomy who were not treated with Miya-BM (Fig. 3A–C).

In the subset of patients not treated with Mya-BM, the differences in the gut microbial structure between the high-output and low-output groups were taxonomically evaluated at the phylum level (Fig. 4A). The major phyla of bacteria were Bacteroidetes (16.8% and 0.1% in the high-output and low-output groups, respectively; p=0.03), Firmicutes (66% and 61.9% in the high-output and low-output groups, respectively; p=0.63), Proteobacteria (14.3% and 32.9% in the high-output and low-output groups, respectively; p=0.95), and Actinobacteria (2.4% and 2.6% in the high-output and low-output groups, respectively; p=0.87). Furthermore, as shown in Fig. 4B, there were no significant differences in the taxonomic changes of the microbial communities between the high-output and low-output patients not treated with Miya-BM. Although not statistically significant, the percentage of *Corynebacterium* at the genus level was lower in the low-output group than in the high-output group among the patients not treated with Miya-BM (p=0.09). Furthermore, the percentage of *Corynebacterium durum* at the species level was lower in the low-output group (p=0.09; Fig. 4B, C).

DISCUSSION

The current study represents the first assessment of the

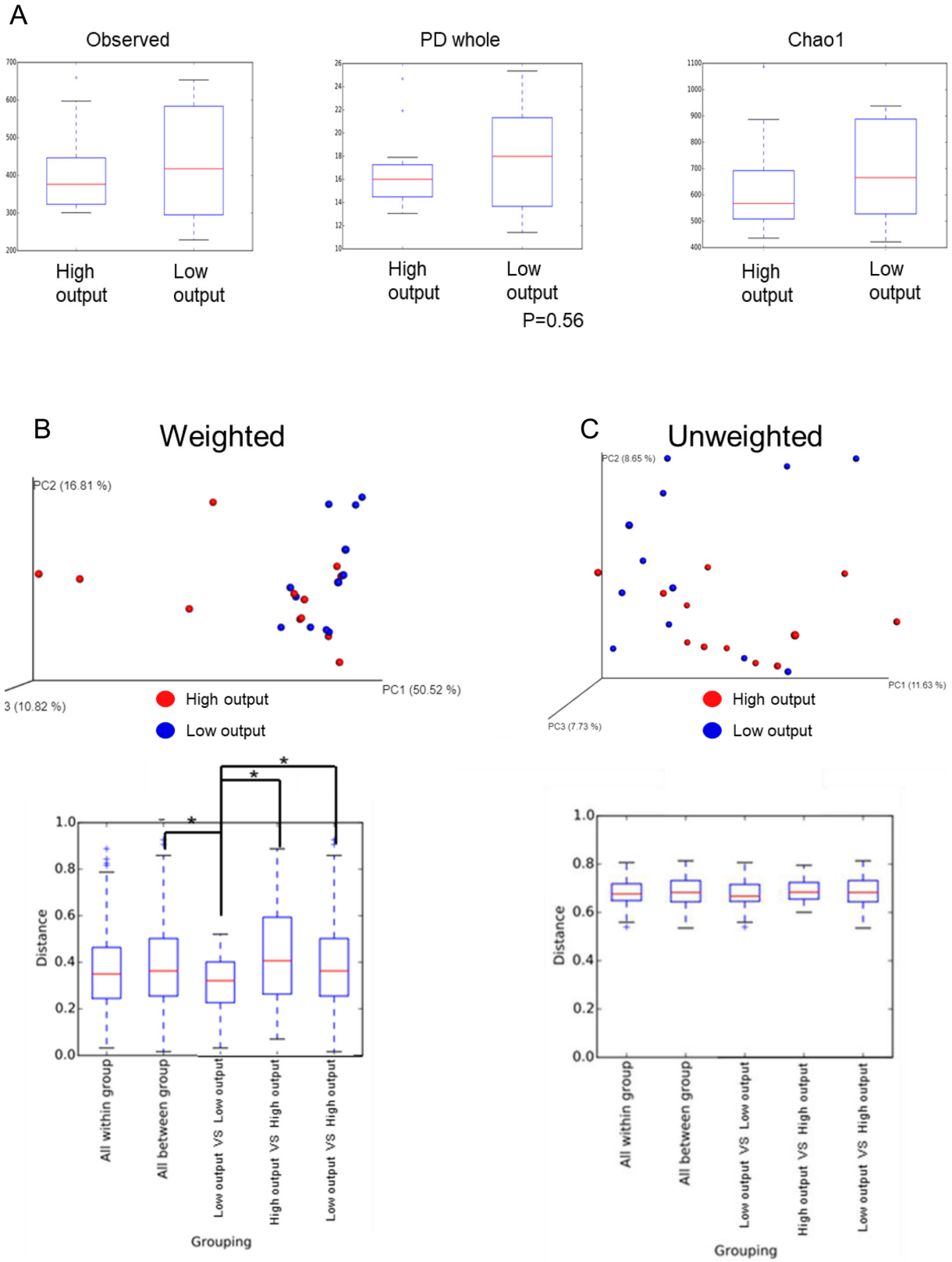

Fig. 1. The microbial communities of the high-output and low-output groups of patients with ileostomy. (A) α-diversity indices and (B) weighted and (C) unweighted principal coordinate analysis (pCoA) of β-diversity measures for all samples. Values represent means ± standard error of the mean (SEM).

*$p<0.05$.

doi: 10.12938/bmfh.2020-062

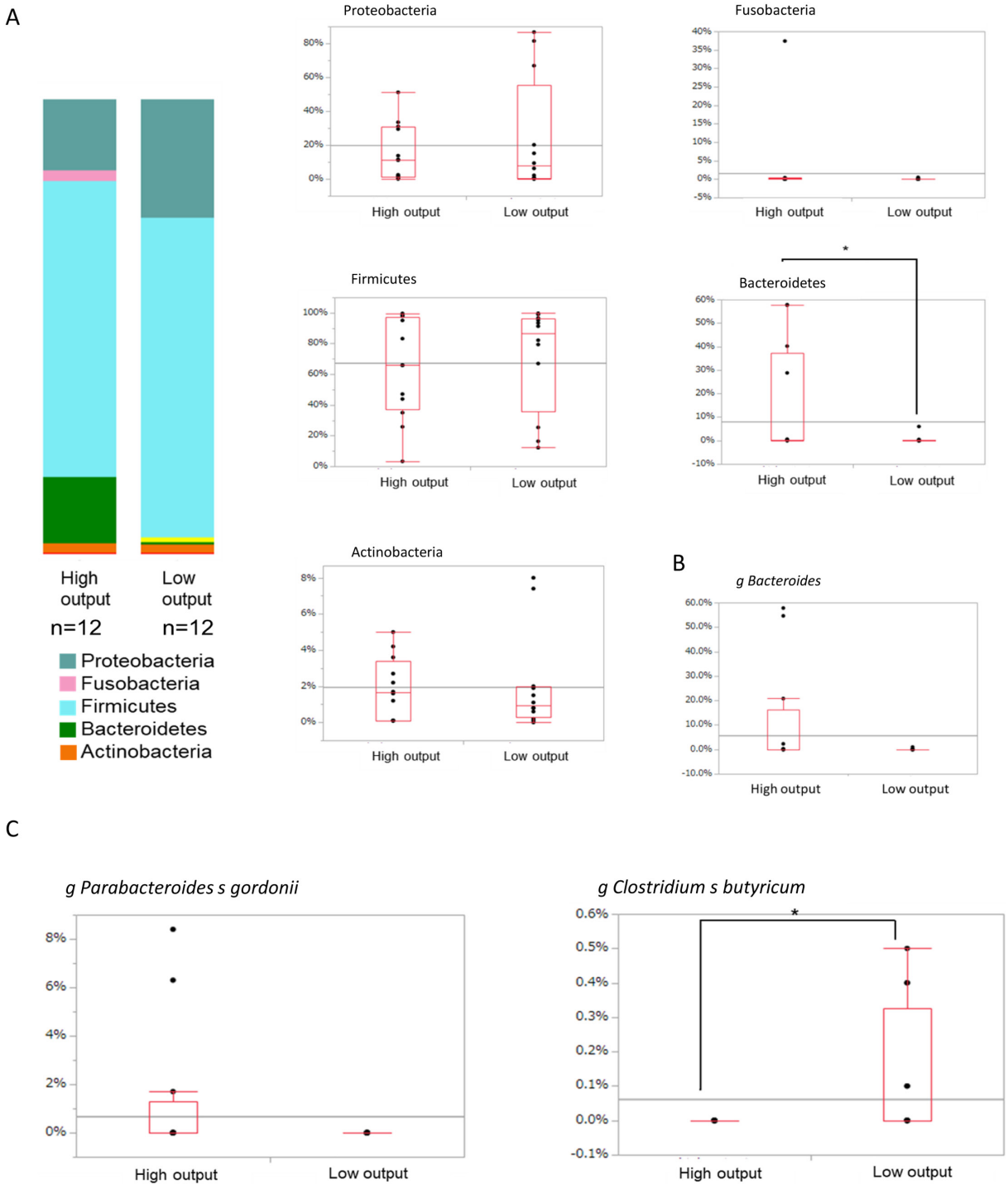

Fig. 2. Comparative analyses of the taxonomic composition of the microbial community at the phylum (A), genus (B), and species (C) levels. Representative bar charts with significant differences between the groups are presented. Values represent means ± SEM.
*p<0.05.

bacterial composition in patients with high-output stomas using next-generation sequencing. Excessive output from the stoma and related electrolyte abnormalities are precursors of dehydration and renal dysfunction [17]. In clinical practice, testing for *Clostridioides difficile* is important in detecting the underlying cause of high output due to the increasing number of diarrhea cases associated with *C. difficile* during hospitalization, which affects 3–10 patients per 1,000 hospitalizations [18]. In general, long-term antibiotic use has been widely documented to disturb the gut microbiota and to be associated with *C. difficile* infection (CDI) [18]. However, the current study did not show a correlation between high-output stomas and CDI.

Previous studies have shown that the relative abundance of microbes is a function of location along the cephalocaudal axis

Table 2. Characteristics and outcomes of patients with ileostomy after rectal surgery with high and low output among those not treated with Miya-BM

	High-output (n=9)	Low-output (n=7)	p-value
Sex (Male:Female)	6:03	5:02	0.84
Age (years)	60 (37–81)	66 (43–77)	0.67
BMI (kg/m^2)	22.3 (19.6–26.2)	24.4 (19.6–28.7)	0.56
Neoadjuvant chemotherapy	1	4	0.05
Neoadjuvant chemoradiotherapy	0	1	0.36
Procedure (LAR:ISR)	8:1	5:2	0.37
Lateral lymph node dissection	2	3	0.38
Operating time (min)	582 (339–657)	545 (270–769)	0.96
Blood loss (g)	30 (15–500)	75 (5–195)	0.4
During the use of antibiotic (day)	3 (2–13)	3 (2–6)	0.51
Antidiarrheals	7	4	0.38
Stage (I:II:III:IV)	3:3:2:1	2:2:3:0	0.71
Complication			
Anastomotic leakage (Grade A:B:C)	0:4:0	1:0:0	0.2
Ileus	1	0	0.36
Postoperative hospital stay (day)	17 (13–52)	12 (11–25)	0.09

This table shows only patients who were not taking Miya-BM.

of the distal gut. Bacteroidetes and Firmicutes were isolated from the terminal ileum [19, 20]. In the current study, we found decreased β-diversity in low-output patients and a very low percentage of Bacteroidetes in the low-output group, despite Bacteroidetes being the major intestinal commensal bacteria. Another point of consideration is that cefmetazole administered in all patients was ineffective against some *Bacteroides* species. *Bacteroides* have a stronger ability to induce the production of immunoglobulin A from the lymphocytes in Peyer's patches, providing protection against inflammation [21]. The reason why Bacteroidetes was very low in the low-output group warrants future investigation. Studies have previously demonstrated that Miya-BM is effective for the prevention and reduction of antibiotic-associated diarrhea [22]. Miya-BM has also been used in antibiotic-associated diarrhea during *Helicobacter pylori* eradication therapy [23]. There has been an increase in studies demonstrating interaction between the intestinal microbiota and a wide variety of conditions, including obesity, cardiometabolic diseases, inflammatory bowel disease (IBD), CDI, irritable bowel syndrome, insulin resistance, multiple sclerosis, and idiopathic thrombocytopenic purpura. Well-designed randomized controlled trials are needed to establish the efficacy of fecal microbiota transplantation for other diseases as well. Future studies should analyze the composition of the small intestinal and fecal microbiota before and after fecal microbiota transplantation [24]. The results of the current study suggest that probiotics might be a candidate approach that might be utilized to control issues associated with high-output stomas following ileostomy. Whether the flora was pathological could not be determined in either group. There was a loss of Bacteroides in the terminal ileum of the low-output patients, whereas *C. butyricum* was absent in the intestinal fluid of high-output patients. Furthermore, the analysis of the cohort after the exclusion of patients treated with Miya-BM indicated additional important differences associated with output. This result is in agreement with previous studies that have reported that the differences in the composition and diversity of the gut microbiota are related to specific food products, dietary habits, geographical origin, and antibiotics [25, 26].

The current study has several limitations, including the small number of patients, the lack of investigation of the influence of diet, and the lack of investigation of the impact of potential biases, including the uneven distribution of sex, previous antibiotic use, dietary habits, and geographical origin between the groups. The findings of the current study merit further investigation in well-designed, large, confirmatory studies, but our research has the potential to be a meaningful pilot study. Aside from conclusions about the use of probiotics such as Miya-BM, definitive conclusions cannot be drawn without increasing the number of cases. The amount of defecation from an ileostomy is usually large, and it was found that the intestinal microbiota of a high-output stoma was similar to the normal intestinal microbiota. Low-output stomas have low levels of Bacteroidetes. The relationship between low-output stomas and low levels of Bacteroidetes was confirmed, but the etiology of low- and high-output stomas is still unknown and merits future investigation.

In conclusion, this pilot study provides the first evidence of a correlation between the gut microbiota and the pathogenesis of high-output stoma compared with that of low-output stoma.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

The authors thank Yasutoshi Kuroki, Seiya Higashi, Kentaro Oka, and Motomichi Takahashi, Miyarisan Pharmaceutical Co. Ltd., for kindly providing technical assistance and performing the statistical analysis.

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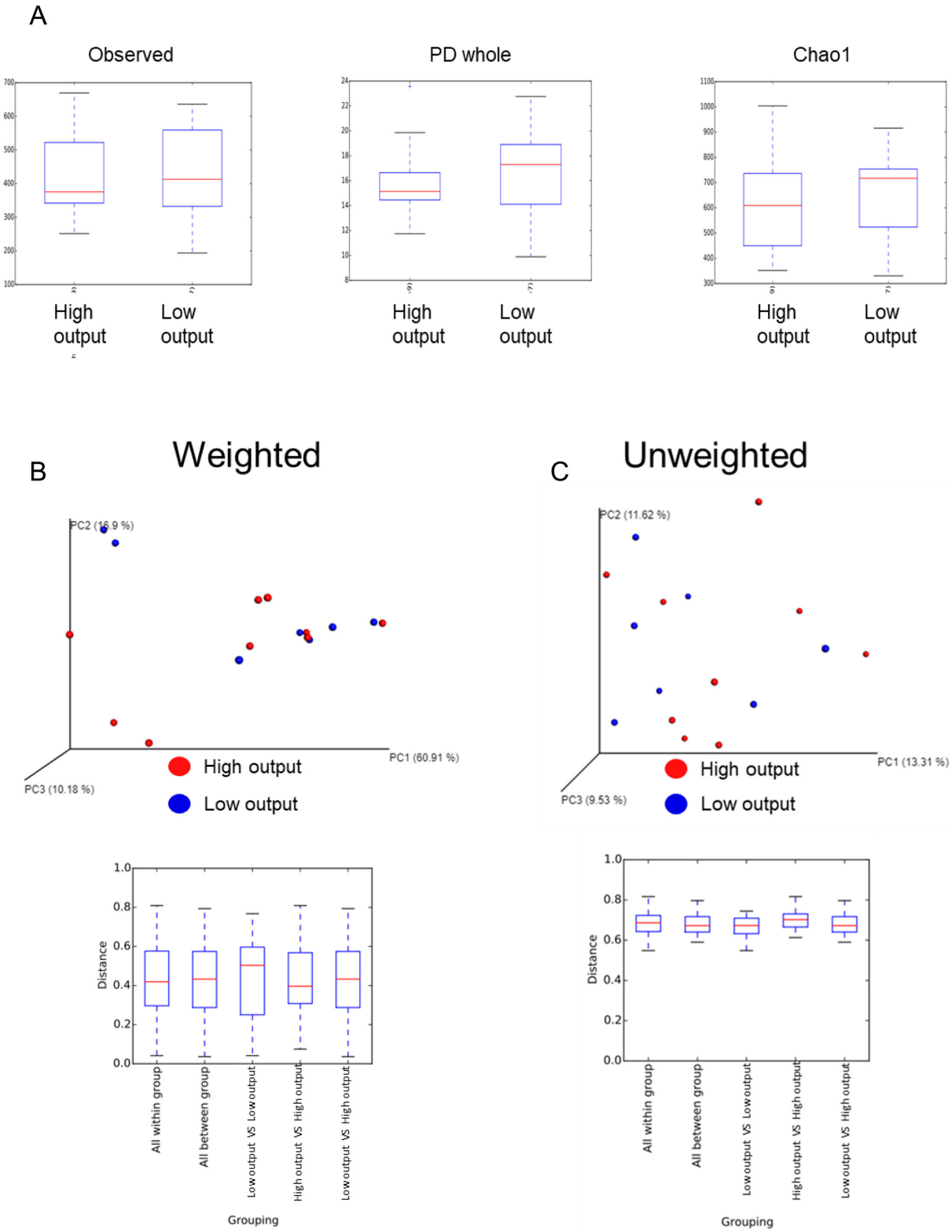

Fig. 3. The microbial communities of the high-output and low-output groups of patients with ileostomy not treated with Miya-BM. (A) α-diversity indices and (B) weighted and (C) unweighted pCoA of β-diversity measures for all samples. Values represent means ± SEM.

doi: 10.12938/bmfh.2020-062

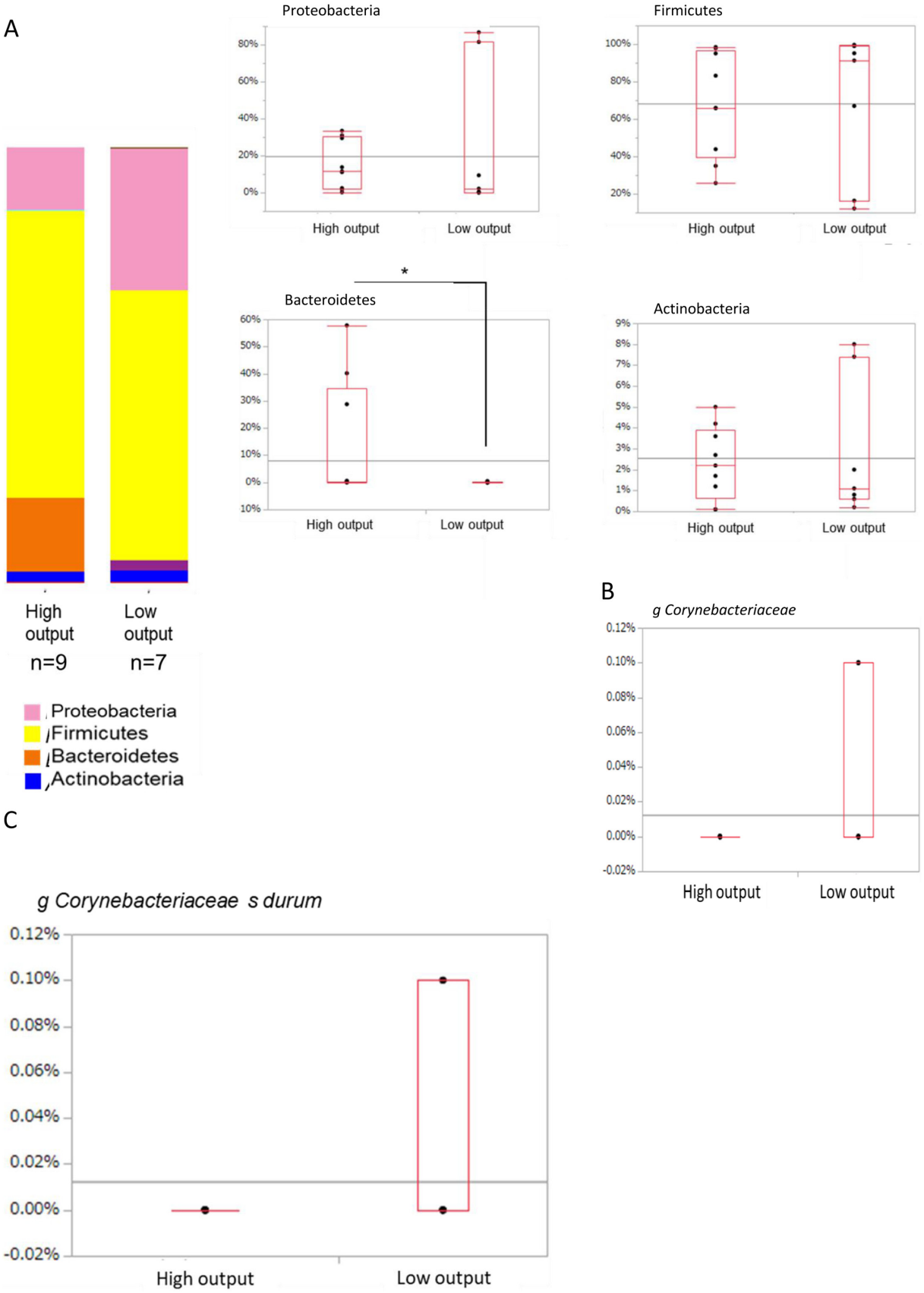

Fig. 4. Comparative analyses of the taxonomic composition of the microbial community at the phylum (A), genus (B), and species (C) levels in patients with ileostomy not treated with Miya-BM. Representative bar charts with significant differences between the groups are presented. Values represent means ± SEM.

*$p<0.05$.

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