Effect of probiotics on gut microbiome in patients with administration of surgical antibiotic prophylaxis: a randomized controlled study

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Authorship statement

All authors meet the ICMJE authorship criteria.

Competing interests

Kaku N and Yanagihara K received lecture fee from Biofermin Pharmaceutical Ltd (Kobe, Hyogo, Japan). Matsumoto N, Sasaki D, Tsuda K, Kosai K, Uno N, Morinaga Y, Tagami A, Adachi S, and Hasegawa H declare no potential conflict of interest.

Abstract

Surgical antibiotic prophylaxis (SAP) is recommended for the prevention of surgical site infections. However, there is a concern about adverse effects of SAP, such as antibiotic-associated diarrhea (AAD). To prevent AAD, administration of probiotics has been investigated. Although recent advances in next-generation sequencing makes it possible to analyze the gut microbiome, the effect of probiotics on the gut microbiome in the patients with SAP remains unknown. To test a hypothesis that SAP influences the gut microbiome and probiotics prevent the influence, a randomized controlled study was conducted with patients who underwent spinal surgery at Nagasaki University Hospital. After obtaining informed consent, the patients were automatically classified into the non-probiotics group and the probiotics group. In the probiotics group, the patients took 1 g of *Enterococcus faecium* 129 BIO 3B-R, 3 times a day on postoperative days (PODs) 1 to 5. The feces of all patients were sampled before administration of SAP and on PODs 5 and 10. We compared alpha and beta diversity and differential abundance analysis of the gut microbiome before and after SAP. During the study period, a total of 33 patients were evaluated, comprising 17 patients in the non-probiotics group and 16 in the probiotics group. There was no significant difference between the groups regarding patient characteristics. In alpha and beta diversity, there were no significant differences among all combinations. In differential abundance analysis at

operational taxonomic unit level, *Streptococcus gallolyticus* and *Roseburia* were significantly increased in the non-probiotics group and significantly decreased in the probiotics group.

Keywords

Prophylaxis; surgery; antibiotics; probiotics; microbiome

Introduction

Surgical site infections (SSIs) are among the most common healthcare-associated infections (HAIs), with an incidence rate at 31.0% of all HAIs among hospitalized patients [1]. In spinal surgery, the incidence rate of SSIs rates range from 0.72% for laminectomy with no risk factors to 8.7% for refusion in patients with risk factors ~~early SSI is a frequent complication, with incidence rates ranging from 2% to 10%~~.[2] Since several meta-analyses revealed that surgical antibiotic prophylaxis (SAP) can reduce the risk of SSIs [3–5], SAP has been recommended in guidelines of the Centers for Disease Control and Prevention (CDC) [6].

Despite the CDC recommendation, there is concern about the adverse effects of SAP, such as allergy, anaphylaxis, nausea, diarrhea, and emergence of antibiotic resistance. Among these adverse effects, antibiotic-associated diarrhea (AAD) ~~including *Clostridioides difficile* infection (CDI)~~ is important, because ~~owing to~~ because its frequency has risen ~~and severity~~.[7] ~~Because~~ Since AAD results from changes in the gut bacteria resulting from administration of antibiotics, probiotics, such as *Enterococcus faecium* 129 BIO 3B-R, *Enterococcus faecalis* BIO-4R, *Clostridium butyricum,* have been used as drugs to prevent the occurrence of AAD in Japan. In culture, post-antibiotic gut bacteria with addition of probiotics were more similar to pre-antibiotic gut bacteria than those without probiotics [8].

Nevertheless, it is difficult to assess the actual effect of probiotics on gut bacteria because there are many unculturable bacteria in stool samples [9].

Recently, with the development of new technologies like next-generation sequencing (NGS), it has become possible to detect and analyze gut bacteria, including unculturable bacteria (the gut microbiome). In the past decade, there have been many reports on the relationship between various diseases and changes in the gut microbiome. These studies have revealed that the gut microbiome has an important role in human health and disease [10]. However, there are few reports on the influence of antibiotics or the effect of probiotics on the gut microbiome. In particular, the influence of SAP on gut microbiota remains unknown. In this study, we investigated the effect of *Enterococcus faecium* 129 BIO 3B-R on the gut microbiome in the patients who received SAP using NGS.

Material and methods

Study design

We conducted a prospective randomized controlled study at Nagasaki University Hospital between July 2016 and October 2017. Forty adult patients who underwent spinal surgery in the Department of Orthopedic Surgery participated in this study. Patients were excluded if they had an allergy to probiotics preparations, a history of antibiotics administration within 1 month prior to hospitalization, administration history of a probiotics preparation within 1 month prior to hospitalization, inflammatory bowel diseases, or pregnancy. After obtaining informed consent, the patients were automatically classified into two groups using the permuted block method created by primary investigator: the non-probiotics group and the probiotics group. The patients classified into the probiotics group took 1 g of *Enterococcus faecium* 129 BIO 3B-R (Biofermin Pharamceutical Ltd., Kobe, Hyogo, Japan), 3 times a day on postoperative days (PODs) 1 to 5. All patients underwent surgery and were administered antimicrobial prophylaxis (SAP) on the decision of a physician. The feces of all patients were sampled before administration of SAP (pre-operatively) and on PODs 5 and 10. In the non-probiotics group, one patient was excluded for withdrawal of consent and another patient was excluded for a lack of samples. In the probiotics group, two patients were excluded for withdrawal of

consent and two patients were excluded for a lack of sample. Finally, we analyzed feces samples obtained from 33 patients, comprising 17 patients in the non-probiotics group and 16 patients in the probiotics group (Fig. 1). The primary outcome measure in this study was diversity difference between the non-probiotics and probiotics groups at PODs 5 and 10. The secondary outcome measures in this study was differences in bacterial species between the non-probiotics and probiotics groups at PODs 5 and 10.

Statistical analysis of patients' background

The backgrounds and clinical course of all included patients were investigated until discharge from the hospital. In a comparative study of the backgrounds of all included patients, we used IBM SPSS version 25 (IBM Japan, Tokyo, Japan) for all statistical analyses, which were unpaired, two-tailed, and tests of significance. The statistically significant alpha level was set at ≤ 0.05. Fisher's exact test was used to compare categorical variables. Continuous variables were expressed as the mean ± standard deviation (SD), and compared using the Student t-test.

PCR amplification and preparation for 16S rRNA gene sequencing (NGS)

Fecal samples were stored at −80°C until further analysis. DNA was extracted using a ZR Fecal DNA MiniPrep Kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions. The V1-V2 region of bacterial 16S rRNA genes was amplified using the following primers: forward (5'-AGAGTTTGATYMTGGCTCAG-3') with the Ion A adapter and sample-specific 13-base barcode sequences, and reverse (5'-TGCTGCCTCCCGTAGGAGT-3') with the Ion trP1 adapter sequence [11]. Emulsion PCR and enrichment were performed using an Ion PGM HiQ View OT2 Kit (Thermo Fisher Scientific, Waltham, MA, USA). The enriched samples were loaded onto an Ion 318 chip and sequencing was performed using the Ion Torrent Personal Genome Analyzer with an Ion PGM HiQ View Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Sequence analysis

The sequencing reads were analyzed using CLC Genomics Workbench version 11.0.1 and CLC Microbial Genomics Module version 3.6.11 (QIAGEN N. V., Venlo, Netherlands). After removing the primer sequences and trimming the read length to less than 240 bp, a total of 22,089,551 reads were obtained from 99 samples (range 53,550 to 793,981). Reads were categorized into operational taxonomic

units (OTUs) with 97% similarity and then assigned using Greengenes version 13.8. We removed OTUs with low abundance, combined abundance less than 10, or less than 0.01% of total reads.

The number of OTUs, Shannon index, and Simpson's index (alpha diversity) were calculated; the data were visualized as box and whisker plots. To compare alpha diversity, the data were analyzed using Tukey's honestly significant difference with IBM SPSS. The statistically significant alpha level was set as ≤0.05. Beta diversity was calculated and visualized with principal coordinate analysis (PCoA) plots using unweighted and weighted UniFrac distances. To compare beta diversity, the data were analyzed in PERMANOVA analysis using the CLC Microbial Genomics Module, and the statistically significant alpha level was set as ≤0.05 in false discovery rate (FDR) p value.

To reveal the change of abundance in each group, abundance pre-operatively and on PODs 5 and 10 in each group was compared using differential abundance analysis with the CLC Microbial Genomics Module, and statistical significance was set to alpha ≤0.05 in FDR p value. To reveal the effect of probiotics, we defined OTUs that were significantly increased in the probiotics group and decreased in the non-probiotics group as positive responders; OTUs that were significantly decreased in the probiotics group and increased in the non-probiotics group were defined as negative responders. To identify the species associated with positive and negative responders, their sequence data were analyzed using the

EzBioCloud 16S database [12]. When species showed pairwise similarity with OTUs, the scientific name of the higher taxonomic classification was used.

Ethics

This study followed the principles set forth in the Declaration of Helsinki and was approved by the ethics committee of Nagasaki University Hospital (approval number, 16042503). This trial was registered in UMIN-CTR (reference number, UMIN000021718; date of full registration, 02/05/2016). Written informed consent for participation and publication was obtained from all participants before the start of study.

Results

Comparison of patient characteristics

During the study period, a total of 33 patients were evaluated, 17 patients in the non-probiotics group and 16 in the probiotics group (Fig. 1). Patient characteristics are shown in Table 1. There were no significant differences between the non-probiotics and probiotics groups with respect to age, sex,

underlying diseases, and surgical site. Regarding SAP, all patients were administered cefazolin. The percentage of combination therapy with cefazolin and vancomycin was higher in the control group (n=4, 23.5%) than that in the probiotics group (n=2, 12.5%), but there was no significant difference. The mean days of cefazolin administration in the non-probiotics group and the probiotics group was 3.3 ± 1.0 and 3.0 ± 0.4, respectively. The patients were administered vancomycin once in the combination therapy. Postoperative complications occurred in the non-probiotics group only, where two patients had soft stools. Both patients were administered cefazolin and vancomycin and had soft stool at PODs 5.

Alpha and beta diversity

We identified a total of 629 OTUs. The average richness (observed OTUs) pre-operatively and on PODs 5 and 10 was 220.71, 226.00, and 225.71 in the non-probiotics group and 247.56, 253.25, and 226.06 in the probiotics group, respectively (Fig. 2A). The average Shannon index pre-operatively and on PODs 5 and 10 was 4.49, 4.33, and 4.40 in the non-probiotics group and 5.04, 4.88, and 5.00 in the probiotics group, respectively (Fig. 2B). The average Simpson's index pre-operatively and on PODs 5 and 10 was 0.87, 0.87, and 0.87 in the non-probiotics group and 0.92, 0.92, and 0.91 in the probiotics group, respectively (Fig. 2C). Regarding alpha diversity, there was no significant difference in all combinations.

The beta diversity was visualized with a PCoA plot using unweighted and weighted UniFrac distances (Fig. 3). There was no significant difference among all combinations in PERMANOVA analysis with unweighted and weighted UniFrac distances.

Changes of abundance in each group

In differential abundance analysis at OTU level, the number of OTUs in the non-probiotics group that significantly increased on PODs 5 and 10 in comparison with the pre-operative period was 62 and 36, respectively; in the probiotics group, the number of OTUs on PODs 5 and 10 was 77 and 62, respectively. In the non-probiotics group, the number of OTUs that significantly decreased at PODs 5 and 10 in comparison with the pre-operative period was 89 and 30, respectively; in the probiotics group, the number of OTUs on PODs 5 and 10 was 66 and 46, respectively. Among OTUs, there were four positive and three negative responders (Table 2). Based on their sequence data, we identified the taxonomic names of positive and negative responders (Table 2). *C. celatum* and *Eubacterium siraeum* (two OTUs) were identified as positive responders on both PODs 5 and 10. The genus *Enterobacter* was identified as a positive responder only on PODs 5. The genus *Roseburia* was identified as a negative responder at

PODs 5. *Streptococcus gallolyticus* subsp. *pasteurianus* and *S. gallolyticus* (subsp. *gallolyticus or pasteurianus*) were identified as negative responders at PODs 10.

Discussion

Our findings regarding alpha and beta diversity indicated that ~~administration of antibiotics~~ SAP did not change the composition of the gut microbiome. In this study, all participants were administered cefazolin as monotherapy or in combination with vancomycin. In a previous study on the risk of *Clostridioides difficile* infection (CDI)~~CDI~~, the adjusted hazard ratio for first- and second-generation cephalosporins in CDI was 2.4 [13]. Investigation of an outbreak of CDI revealed that the incidence of CDI was significantly associated with the administration of cefazolin, in multivariate analysis [14]. However, in the report, the authors described that the association between cefazolin use and CDI rates is a mathematical relationship driven by the consistently high use of cefazolin, because they could achieve sustained control of a CDI outbreak without a change in the use of antibiotics[14]. Additionally, in a randomized control trial of SAP with cefazolin, no patients experienced CDI [15]. Our ~~data~~ findings regarding alpha and beta diversity were the same as these previous studies. ~~suggested that SAP with~~

~~cefazolin did not influence the gut microbiome, and probiotics did not show efficacy for improving gut microbiota.~~ However, we found a potentially influential factor among our results. As shown in Fig. 3, the composition of the gut microbiome differed substantially among pre-operative samples from study participants. We planned this prospective study to include patients undergoing spinal surgery, as we expected that most of these patients would have fewer underlying diseases than those undergoing other types of surgery. Contrary to expectations, 45.5% of the patients participating in this study had underlying diseases. The composition of gut microbiota might depend on underlying diseases [10]. Thus, there is a possibility that the underlying diseases in our patients could account for the variation in the pre-operative composition of gut microbiota.

On the other hand, ~~I~~in the differential abundance analysis at OTU level, *S. gallolyticus* (two strains) and *Roseburia* were identified as negative responders, which were significantly decreased in the probiotics group and increased in the non-probiotics group. *S. gallolyticus* (previously *S. bovis*) has long been associated with colorectal cancer and infective endocarditis [16,17]. The strain identified as *Roseburia* was homologous to *R. cecicola* and *R. faecis*. These species have been isolated from murine cecal mucosa and human stool samples, respectively [18,19]; however, there are no reports on their pathogenicity and function in the human intestines. In our study, changes were observed in these species

at PODs 5 only. *C. celatum* and *Eubacterium siraeum* were identified as positive responders, which were significantly increased in the probiotics group and decreased in the non-probiotics group, at both PODs 5 and 10, and *Enterobacter* (KN150796, *E. soli,* or *E. asburiae*) was identified as a positive responder at PODs 5 only. *C. celatum* is a gram-positive rod that has been isolated from healthy adults [20]. Although there has been only one report of *C. celatum* infection in two patients [21]; its pathogenicity in the human intestine remains uncertain. There have been no reports on the pathogenicity and function of *E. siraeum* and *Enterobacter* in the human intestine. At this time, it is uncertain whether the effect of probiotics on negative responders (*Roseburia*) and positive responders (*C. celatum*, *E. siraeum,* and the genus *Enterobacter*) has any implications for patients administered SAP because the pathogenicity and function of these bacteria in the human intestine remains unknown. However, considering the pathogenicity of *S. gallolyticus* and the influence of SAP through PODs 10, the effect of probiotics shown in this study seems to be important.

The effect of probiotics has been studied for a long time. In a meta-analysis of the effect of probiotics on gastrointestinal diseases, a significant effect of probiotics has been observed in many gastrointestinal diseases, including AAD [22]. A previous report stated that administration of *Lactobacillus rhamnosus* GG (LGG) as a probiotic reduced the risk of AAD from 22.4% to 12.3% in patients treated with

antibiotics [23]; however, when children and adults were evaluated separately, this reduction was not observed in adults [23]. In a meta-analysis of the effect of probiotics on CDI prevention in adults and children, probiotics were effective in preventing CDI, with moderately strong evidence (risk ratio 0.40, $p < 0.001$) [24]. In the analysis, the risk ratio of CDI prevention with *Saccharomyces boulardii* and many combinations of various species showed a significant reduction in risk whereas *Saccharomyces cerevisiae*, LGG, *Lactobacillus acidophilus*, *L. plantarum*, *L. reuteri*, and *L. casei Shirota* did not have significant effects [24]. Based on these studies, the effect of probiotics on AAD, including CDI, seems to differ from species to species. In this study, we used *E. faecium* 129 BIO 3B-R. This bacterium has been used as a probiotic in Japan for patients who are administered antibiotics. Although enterococci, such as *E. faecium* and *E. faecalis,* are important nosocomial pathogens, they produce lactic acid and are used as probiotics. Several clinical studies have reported that enterococci probiotics have a significant effect in patients with AAD [25]. In this study, two patients (11.8%) had soft stools after surgery in the non-probiotics group whereas there no patients had soft stools in the probiotics group. Even though there was no significant difference between the groups, *E. faecium* might prevent the occurrence of AAD.

There were some limitations in this study. First, as discussed previously, the composition of gut microbiota differed substantially between participants pre-operatively. There is a possibility that this

condition influenced the results with respect to alpha and beta diversity. Further investigation with comparable preoperative conditions is needed to ascertain whether SAP indeed influences the gut microbiome. Second, there has been no confirmation of the pathogenicity and function of *Roseburia*, *C. celatum*, *E. siraeum,* and the genus *Enterobacter*. It is therefore difficult to assess the influence of SAP and the effect of probiotics in differential abundance analysis at the OTU level. Third, to date, there is no standard analytical method for the gut microbiome. We analyzed gut microbiota using commercial software and a widely recognized 16S rRNA gene database. However, there are many software programs and databases used for analysis of the gut microbiome, which might produce different results. Finally, two patients had soft stools, but no microbiological tests, including antigen testing for CDI, were performed for these stool samples.

Conclusions

Our findings showed that SAP did not influence the composition of the gut microbiome, but the relative abundance of *S. gallolyticus* was increased after SAP. In contrast, administration of probiotics significantly decreased the relative abundance of *S. gallolyticus*. Considering the pathogenicity of *S.*

gallolyticus, SAP had a negative influence on patients, and probiotics prevented adverse effects after surgery.

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Figure legends

Figure 1. Participant flow diagram

Participant flow diagram showing progression through phases of the randomized controlled trial.

PODs, postoperative days; NGS, next-generation sequencing.

Figure 2. Alpha diversity

Box and whisker plot of the alpha diversity indices for richness (observed OTUs; A), Shannon index (B), and Simpson's index (C). There was no significant difference between the groups with respect to richness, Shannon index, and Simpson's index.

PODs, postoperative days; OTU; operational taxonomic unit.

Figure 3. Beta diversity

Beta diversity was visualized using a principal coordinate analysis plot with unweighted (A and B) and weighted (C and D) UniFrac distances. There was no significant difference in all groups for the PERMANOVA test using unweighted and weighted UniFrac distances.

PCoA, principle coordinate analysis; PODs, postoperative days.

Tables

Table 1. Comparison of patients' characteristics

Characteristics	Non-probiotics group (n =17)	Probiotics group (n=16)	*P* value
Age, mean (SD)	66.0 (8.9)	66.6 (13.5)	0.888
Sex, female	7 (41.2)	8 (50.0)	0.732
Underlying diseases	13 (76.5)	13 (81.3)	1.000
Hypertension	8 (47.1)	8 (50.0)	1.000
Dyslipidemia	6 (35.3)	6 (37.5)	1.000
Diabetes mellitus	2 (11.8)	6 (37.5)	0.118
Cerebrovascular diseases	2 (11.8)	1 (6.3)	1.000
Rheumatic arthritis	2 (11.8)	0 (0.0)	0.485
Others	6 (35.3)	5 (31.3)	1.000
Site of operation			
Cervical spine	7 (41.2)	4 (25.0)	0.465
Thoracic spine	1 (5.9)	1 (6.3)	1.000
Lumbar spine	9 (52.9)	11 (68.8)	0.481
Antimicrobial prophylaxis			
Cefazolin	13 (76.5)	14 (87.5)	0.656
Cefazolin and vancomycin	4 (23.5)	2 (12.5)	0.656
Administration period, day, mean (SD)	3.3 (1.0)	3.0 (0.4)	0.270
Change of antimicrobial agents	2 (11.8)	0 (0.0)	0.485
Postoperative complication			
Soft stool	2 (11.8)	0 (0.0)	0.485

SD, standard deviation; Others, less than 10%.

Table 2. Positive and negative responder to probiotics

OTUs name (specific number)	Fold change in non-probiotics group	Fold change in probiotics group	Top hit taxonomy (similarity, %)
Positive responder at PODs 5			
*Clostridiales*_o (186984)	-89.7	70.8	*Clostridium celatum* (99.1)
*Enterobacteriaceae*_f (668514)	-29.0	49.0	*Enterobacter*_g (99.1)
*Ruminococcus*_g (187181)	-187.6	9628.2	*Eubacterium siraeum* (100)
*Ruminococcus*_g (291902)	-38.3	599.2	*Eubacterium siraeum* (99.1)
Positive responder at PODs 10			
*Clostridiales*_o (186984)	-163.7	-163.7	*Clostridium celatum* (99.1)
*Ruminococcus*_g (187181)	-152.5	-152.5	*Eubacterium siraeum* (100)
*Ruminococcus*_g (291902)	-16.5	-16.5	*Eubacterium siraeum* (99.1)
Negative responder at PODs 5			
*Ruminococcus*_g (547223)	34.2	-20.9	*Roseburia*_g (97.3)
Negative responder at PODs 10			
*Streptococcus*_g (290759)	913.4	-265.2	*Streptococcus gallolyticus* subsp. *pasteurianus* (100)
*Streptococcus*_g (328283)	1600.3	-151.7	*Streptococcus gallolyticus* (100)

Positive responders, OTUs that were significantly increased in the probiotics group and decreased in the non-probiotics group; negative responders, OTUs that were significantly decreased in the probiotics group and increased in the non-probiotics group; PODs, postoperative days; g, genus; s, species; o, order; f, family; NS, not significant.

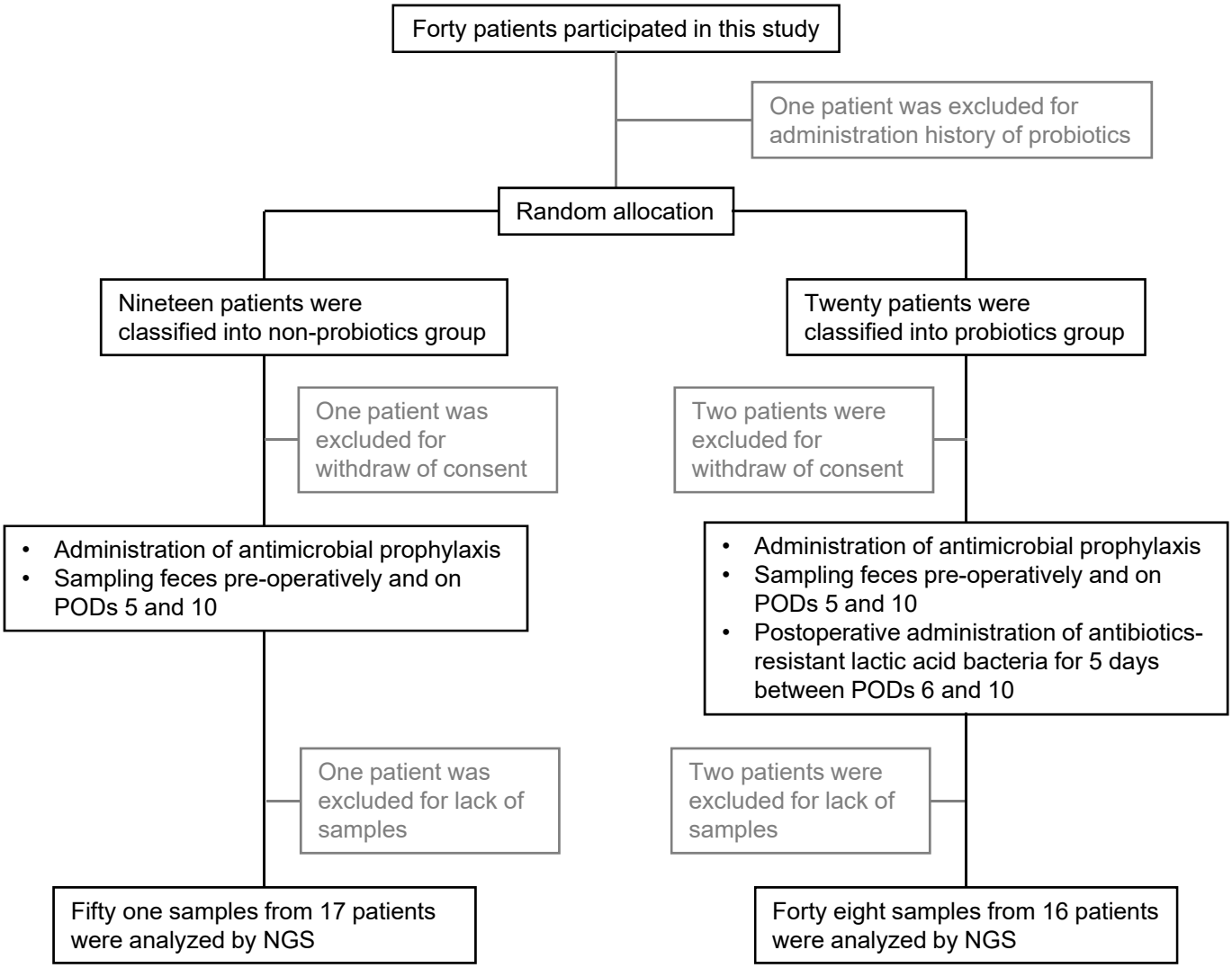

Figure 1. Participant flow diagram
Participant flow diagram showing progression through phases of the randomized controlled trial.
PODs, postoperative days; NGS, next-generation sequencing.

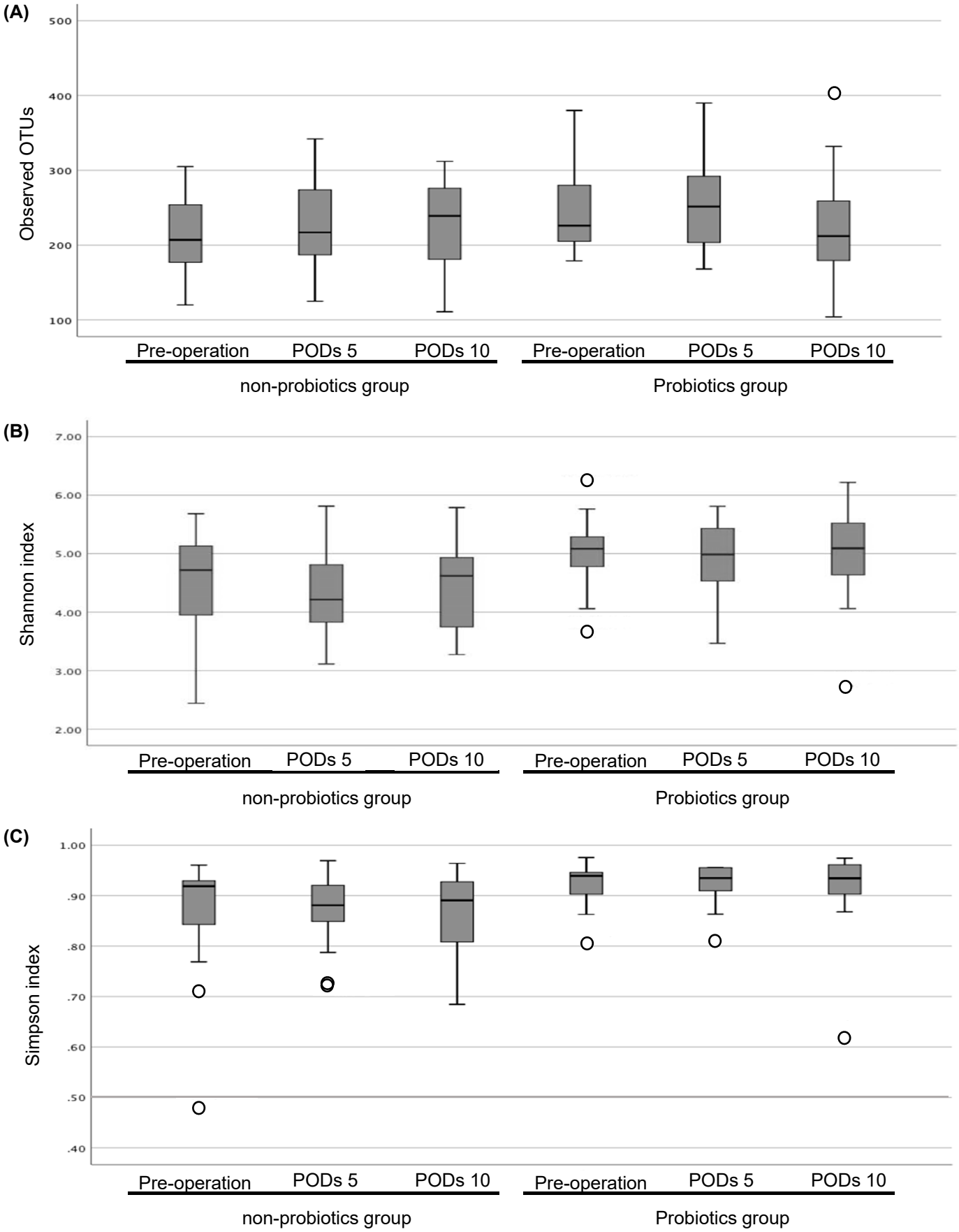

Figure 2. Alpha diversity

Box and whisker plot of the alpha diversity indices for richness (observed OTUs; A), Shannon index (B), and Simpson's index (C). There was no significant difference between the groups with respect to richness, Shannon index, and Simpson's index.

PODs, postoperative days; OTU; operational taxonomic unit.

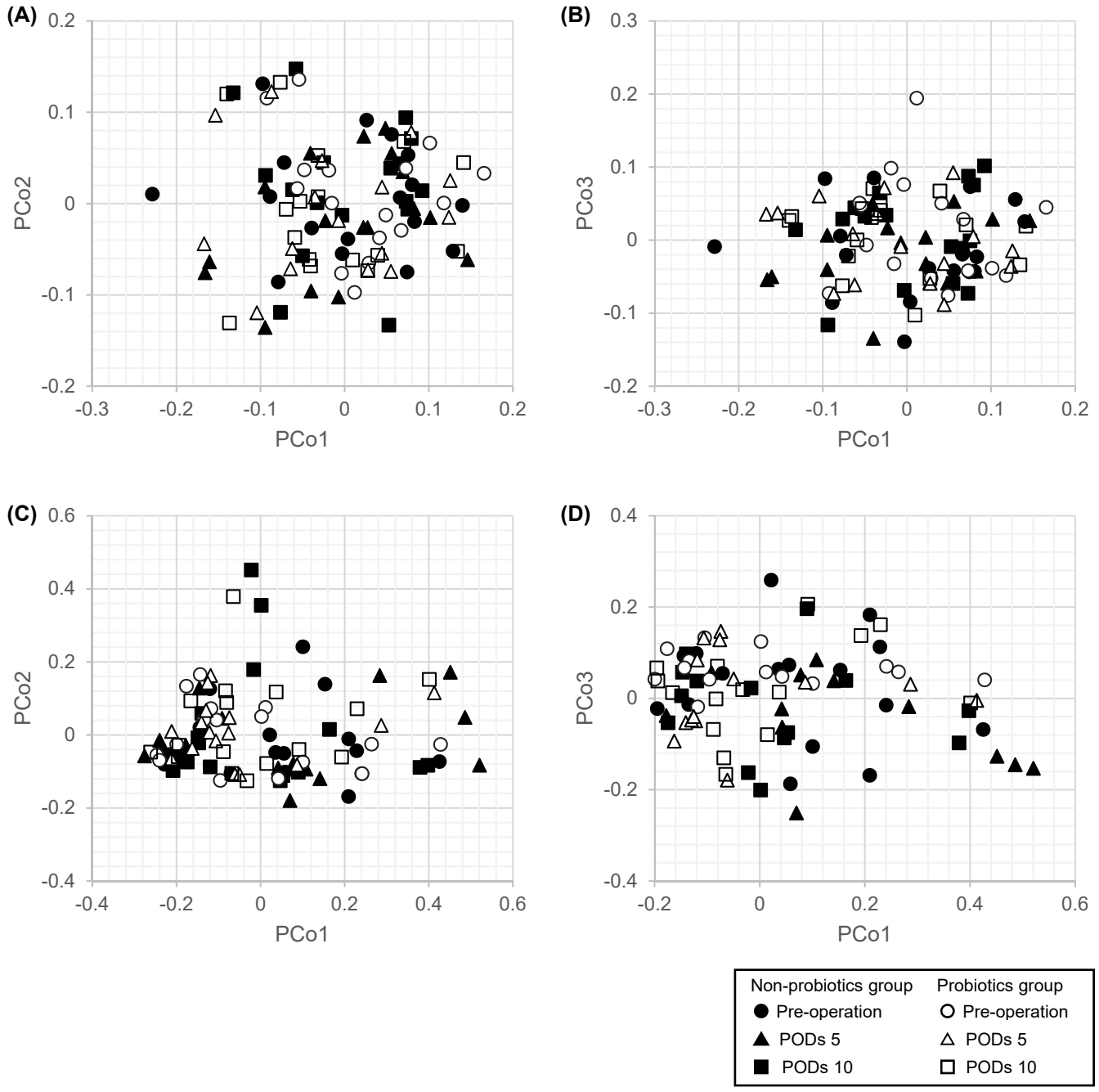

Figure 3. Beta diversity

Beta diversity was visualized using a principal coordinate analysis plot with unweighted (A and B) and weighted (C and D) UniFrac distances. There was no significant difference in all groups for the PERMANOVA test using unweighted and weighted UniFrac distances.

PCoA, principle coordinate analysis; PODs, postoperative days.