Impact of synbiotics treatment on bacteremia induced during neoadjuvant chemotherapy for esophageal cancer: A randomised controlled trial

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Abstract

Background & Aims: To elucidate the impact of synbiotics on bacterial translocation and subsequent bacteremia during neoadjuvant chemotherapy for esophageal cancer.

Methods: Patients requiring neoadjuvant chemotherapy for esophageal cancer were randomized to receive synbiotics (synbiotics group) or no synbiotics (control group) during chemotherapy.

Blood and fecal samples were taken before and after every chemotherapy cycle, and 1 day before surgery. Mesenteric lymph nodes (MLNs) were harvested at laparotomy (MLN-1) and after resection of the tumor (MLN-2). Bacteria in each sample were detected. Fecal microbiota and organic acid concentrations were also determined. The primary endpoint was the detection of bacteria in the blood samples, as well as the incidence of side effects during chemotherapy. The secondary endpoint was the detection rate of bacteria in the MLN samples collected during surgery.

Results: The study recruited a total of 42 patients (22 in the control group, 20 in the synbiotics group). Bacteria were detected in 16 of 101 blood samples in the control group, whereas those were detected only 2 of 100 blood samples in the synbiotics group ($p<0.001$) during neoadjuvant chemotherapy. Additionally, bacteria were detected in 12 of 34 MLN samples in the control group, whereas no bacteria were detected in 38 MLN samples in the synbiotics group ($p<0.001$). Suppression of bacterial translocation was at least partly associated with an increased fecal acetic acid concentration as well as a lowered fecal pH by synbiotics. The incidence rate of grade 3 gastrointestinal toxicity during chemotherapy was lower in the synbiotics group compared to the control group (8/22 vs. 1/20, $p = 0.022$).

Conclusions: Neoadjuvant chemotherapy for esophageal cancer may induce bacterial translocation and subsequent bacteremia, which can be prevented by synbiotics

administration.

Trial registration: The University Hospital Medical Information Network (http://www.umin.ac.jp; registration number ID 000007651)

Introduction

Previous studies demonstrated that bacterial translocation, in which bacteria are detected in the mesenteric lymph nodes (MLNs), is induced by highly invasive gastrointestinal surgery such as major hepatectomy with extrahepatic bile duct resection,[1] esophagectomy,[2, 3] and pancreatoduodenectomy.[4] In these studies, the presence of live bacteria was detected using bacterium-specific 16S and 23S ribosomal RNA-targeted reverse transcriptase–quantitative polymerase chain reaction (RT-qPCR), which is highly sensitive compared to the conventional culture method or PCR method that targets bacterial DNA.[5, 6, 7] Moreover, genetically identical bacteria detected in the MLNs were also detected in the blood one day after surgery, indicating that the bacteria translocated to the MLNs also move into the blood stream.[2] Notably, the presence of bacteria in the MLNs is significantly associated with the incidence of postoperative infectious complications in both hepatectomies[8] and esophagectomies.[3]

Synbiotics is a combination of prebiotics and probiotics. Preoperative administration of synbiotics improves the intestinal microenvironment (indicated by the fecal concentration of organic acids) and decreases the incidence of postoperative infectious complications in hepatectomy with extrahepatic bile duct resection.[9] Preoperative administration of synbiotics also decreases the incidence of bacterial translocation through improvement of intestinal microenvironment, in patients undergoing esophagectomy[3] and pancreatoduodenectomy.[4]

Neoadjuvant chemotherapy is generally performed as a standard treatment for esophageal cancer.[10, 11] Combination therapy using cisplatin and 5-fluorouracil is one of the most common regimens used in patients with advanced esophageal cancer. This regimen, however, strongly affects the gastrointestinal mucosa and frequently induces nausea, vomiting, stomatitis, and diarrhea.[12] Therefore, we hypothesized that neoadjuvant

chemotherapy using cisplatin and 5-fluorouracil may induce bacterial translocation during treatment. However, this has not been demonstrated in patients with esophageal cancer. Based on the observations in our previous studies, we hypothesized that preoperative use of synbiotics contributes to prevention of bacterial translocation and unfavorable events that occur during neoadjuvant chemotherapy.

The aim of this study was to clarify whether neoadjuvant chemotherapy for esophageal cancer induces bacterial translocation and to investigate whether synbiotics treatment can prevent bacterial translocation during chemotherapy and subsequent esophagectomy by performing a randomized controlled study.

Materials & Methods

Patient involvement

Patients were not involved in the design and conduct of this research. Once the trial has been published, participants will be informed details of the results in a study newsletter suitable for a non-specialist audience.

Randomisation and masking

All patients with esophageal cancer scheduled to undergo neoadjuvant chemotherapy at Nagoya University Hospital were eligible to participate in this randomized controlled trial. Written informed consent for participation was obtained from each patient before enrolment. The study was approved by the Human Research Review Committee of Nagoya University Hospital (approved number 2011-1337-2) and registered in the University Hospital Medical Information Network (http://www.umin.ac.jp; registration number ID 000007651). Patients who had been routinely ingesting foods or beverages containing probiotics or prebiotics were

excluded. Patients were randomized to the group with synbiotics treatment (synbiotics group) or no synbiotics treatment (control group). Using a computerized random number table, randomization was performed at least 1 week before starting neoadjuvant chemotherapy. This study was neither single- nor double-blinded, and no placebo was used in the control group. The primary endpoint was the detection of bacteria in the blood samples collected during neoadjuvant chemotherapy, as well as the incidence of side effects occurring during chemotherapy. The secondary endpoint was the detection of bacteria in the MLN samples collected during surgery~~before and after the surgical intervention~~.

Protocol for synbiotics treatment and perioperative feeding

Patients in the synbiotics group received a synbiotics formula that was previously found to be effective in preventing postoperative infectious complications after major hepatectomy with extrahepatic bile duct resection for biliary cancer.[9, 13] The following agents were administered orally or via a feeding tube daily from 7 days before starting neoadjuvant chemotherapy to 1 day before surgery: one 80-mL bottle of Yakult 400 (Yakult Honsha, Tokyo, Japan), which contained at least 4×10^{10} living *Lacticaseibacillus paracasei* strain Shirota (YIT9029) previously called *Lactobacillus casei* strain Shirota; one 100-mL bottle of MILMIL-S (Yakult Honsha), which contained at least 1×10^{10} living *Bifidobacterium breve* strain Yakult; and 15 g of Oligomate S-HP (Yakult Honsha) containing at least 4.95g of galacto-oligosaccharides. The composition of each supplement was as follows: Yakult 400 (80-ml bottle); energy, 62 kcal; protein, 1.0 g; lipids, 0.1 g; carbohydrates, 14.4 g; and sodium, 0-0.1 g. MILMIL-S (100-mL bottle); energy, 49 kcal; protein, 3.2 g; lipids, 0.1 g; carbohydrates, 13.7 g; and sodium, 0.1 g; galacto-oligosaccharides, 1.0 g. Oligomate S-HP (15 g); energy, 40.35 kcal; protein, 0 g; lipids, 0 g; carbohydrates, 11.25 g; and sodium, 0 g; galacto-oligosaccharides,

1.0 g. The synbiotics supplements were provided from the primary investigator to the patients with free of const. Yakult Honsha did not donate the synbiotics. Patients in the control group consumed an ordinary diet without synbiotics.

Neoadjuvant chemotherapy

Chemotherapy consisted of two cycles of intravenous administration of cisplatin (80 mg/m^2) on days 1 and 22, and 5-fluorouracil (800 mg/m^2) on days 1 to 5 and 22 to 26. In some patients, radiotherapy consisting of 40 Gy radiation to the primary tumor was combined with chemotherapy. In such cases, chemotherapy consisted of two cycles of intravenous administration of cisplatin (70 mg/m^2) on days 1 and 22, and 5-fluorouracil (700 mg/m^2) on days 1 to 4 and 22 to 25. The surgery was scheduled within 4 to 5 weeks after completion of chemotherapy. Radiological tumor assessments were conducted using computed tomography every 8 weeks in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1.[14] Adverse events during chemotherapy were assessed using the National Cancer Institute's CTCAE v5.0.

Sample collection

Blood samples (1 mL) were collected into a test tube containing 2 mL RNAprotect™ Bacterial Reagent (Qiagen, Hilden, Germany) before starting the first chemotherapy (pre-1st), on day 7 after starting the first cycle (post-1st), before starting the second cycle (pre-2nd), on day 7 after starting the second cycle (post-2nd), and 1 day before surgery (pre-op). MLNs were harvested at two different ~~laparotomy moments~~time point during the operation. First, we performed laparotomy and the first sample (MLN-1) was harvested before ~~surgical intervention~~esophageal resection. The second sample (MLN-2) was ~~sampled~~harvested after

the resection of esophageal cancer. ~~Then the abdomen was closed, and the esophagus was removed through thoracotomy. The second sample (MLN-2) was harvested at the second laparotomy before upper abdominal nodes dissection and gastrolysis. Both samples were~~Each sample was harvested from the jejunal mesentery using a fresh forceps and scalpel. A patient who was diagnosed as unresectable at laparotomy did not undergo MLN sampling. The MLN samples were collected into a test tube containing 1 mL RNAprotect™ Bacterial Reagent and were held at room temperature for 5 min before storage at −80°C. Feces were sampled at the same timing as blood sampling. The fecal samples were placed directly into two tubes (~1.0 g/tube) by patients; one tube contained 2 mL RNAlater (an RNA stabilization solution; Ambion, Austin, TX), and the other was empty. The samples with RNAlater were placed in a refrigerator at 4°C (for the analysis of fecal microbiota), and the others were placed in a freezer at −80°C (for the analysis of fecal organic acid concentrations and fecal pH) within 30 min of excretion. Samples were transported to the Yakult Central Institute at −20°C for feces and at −80°C for blood and MLN samples for the analysis. The patient's identity, clinical information, and study group (synbiotics or control) were unknown to the technician performing the analysis.

Gut microbiota analysis

After the tube containing the fecal sample was weighed, a 9-fold volume of RNAlater was added to prepare a fecal suspension. An aliquot (40 µL) of the resulting fecal homogenate was diluted into 1 mL sterile PBS, and the mixture was then centrifuged at 5,000 ×*g* for 10 min. The supernatant was discarded, and the pellet was stored at −80°C until extraction of RNA. RNA was isolated using a modification of the acid guanidinium thiocyanate-phenol-chloroform extraction method. The nucleic acid fraction was suspended in 1 mL nuclease-free

water (Ambion). To quantify the bacteria present in the fecal samples, total RNA fractions from feces were extracted according to the method described above. The microbiota composition was analyzed using the YIF-SCAN® system.[5, 6, 15] Three serial dilutions of the extracted RNA sample were used for bacterial rRNA-targeted RT-qPCR, and the threshold cycle values in the linear range of the assay were applied to the standard curve to obtain the corresponding bacterial cell count in each nucleic acid sample. These data then were used to calculate bacterial counts per sample. The specificity of the RT-qPCR assay using group-, genus-, or species-specific primers was determined as described previously.[5, 6, 15]

Detection of bacteria in the blood and MLN samples

Microorganisms in both blood and MLN samples were detected with YIF-SCAN®,[5, 6, 15] as described elsewhere. RT-qPCR was performed to detect representative bacteria associated with postoperative infectious complications and that were considered pathogenic based on previous studies.[9, 13, 16] The examined bacteria included obligate anaerobes (*Clostridium coccoides* group, *Clostridium leptum* subgroup, *Bacteroides fragiles* group, *Atopobium* cluster, *Prevotella*, and *Clostridium perfringens*), facultative anaerobes (*Enterobacteriaceae*, *Enterococcus*, *Streptococcus*, and *Staphylococcus*), and aerobes (*Pseudomonas*). The sequence homology of the rRNA genes among bacteria isolated from the samples was determined using 16S and 23S rRNA gene fragments. The RT-qPCR products that were amplified using several primer sets were purified using Amicon® Ultra-0.5 Centrifugal Filter Devices (Merck Millipore Corporation, Cork, Ireland). The sequences were automatically analyzed on an Applied Biosystems 3500XL genetic analyzer (Thermo Fisher Scientific, Waltham, MA). The resulting rRNA gene sequences were analyzed using the BLAST program of the DNA Data Bank of Japan (https://blast.ddbj.nig.ac.jp/) to assign each strain to

a particular species. The minimum detectable number of all the target bacteria by RT-qPCR was 1 bacterial cell per 1 mL blood or 5 bacterial cells per 1 g MLN samples.

Measurements of fecal organic acid concentrations and pH

A portion of the homogenized stool was isolated, weighed, mixed with a 4× volume of 0.15 M perchloric acid, and reacted at 48°C for 12 hours. Next, the mixture was centrifuged at 4°C at 20,000 ×*g* for 10 min, and the supernatant was filtered with a 0.45-μm membrane filter (Millipore, Tokyo, Japan) and sterilized. The concentration of organic acids in this sample was measured using a Waters high performance liquid chromatography system (Waters 432 Conductive Detector; Waters, Milford, MA) and a Shodex Rspack KC-811 column (Showa Denko, Tokyo, Japan). The concentrations of organic acids were calculated with the use of external standards and expressed as μmol/g of wet feces. The lower limits for the fecal organic acid concentrations using this procedure were 0.075 μmol/g for succinic acid, 0.2 μmol/g for lactic acid, 0.05 μmol/g for formic acid, 0.4 μmol/g for acetic acid, 0.5 μmol/g for propionic acid, 0.55 μmol/g for butyric acid, 0.8 μmol/g for isovaleric acid and 0.65 μmol/g for valeric acid. The stool pH was measured by directly inserting the glass electrode of a D-51 pH meter (Horiba Seisakusho, Tokyo, Japan) into a sample of homogenized stool.

Data collection

Demographic data including age, sex, body mass index, clinical stage, and depth of tumor invasion were recorded. Indexes for nutritional status and cancer prognosis such as prognostic nutritional index (PNI)[17, 18] and neutrophil-to-lymphocyte count (NLR)[19, 20] were calculated using the blood sample data of serum albumin, lymphocyte count, and neutrophil count. PNI was assessed using the following equation as described previously[21]: PNI = 10 × serum

albumin [g/dL] + 0.005 × total lymphocyte count in the peripheral blood [/mm^3].

Statistical analyses

The data analyses were performed using SPSS software version 23.0 J and R version 3.5.0. Continuous data were expressed as medians (range) or average (standard deviation). The non-parametric Wilcoxon rank-sum test or Student's t test was used to analyze continuous data between the two groups. The χ^2 test or Fischer's exact test was used for the analysis of categorical variables. Correlation coefficients were calculated in the R 'corrr' package with Spearman's rank correlation test, and the correlation matrix was visualized in the R 'corrplot' package. When the bacterial cell count and fecal organic acid concentration were below the lower detection limit, a half value of lower detection limit were applied for the statistical analysis. Two-sided p values were calculated and presented. A p value of <0.05 was considered statistically significant.

Results

A total of 42 patients (22 patients in the control group and 20 patients in the synbiotics group) who were diagnosed with resectable esophageal cancer from November 2012 to March 2016 were included in the analysis (Figure 1). In the synbiotics group, all patients could take synbiotics supplements without any side effects. In the control group, three patients terminated chemotherapy due to severe side effects. In addition, one patient in the control group was excluded because the tumor progressed during chemotherapy, and was not eligible for surgery. Finally, 19 patients in the control group underwent surgery. However, one patient in the control group was further excluded because the tumor was highly advanced and was not resectable at thoracotomy. In contrast to the control group, all patients tolerated and

completed two cycles of chemotherapy in the synbiotics group. The tumor of one patient progressed during chemotherapy and was not eligible for surgery. Therefore, MLN samples for the detection of bacteria were finally analyzed in 17 patients in the control group and 19 patients in the synbiotics group.

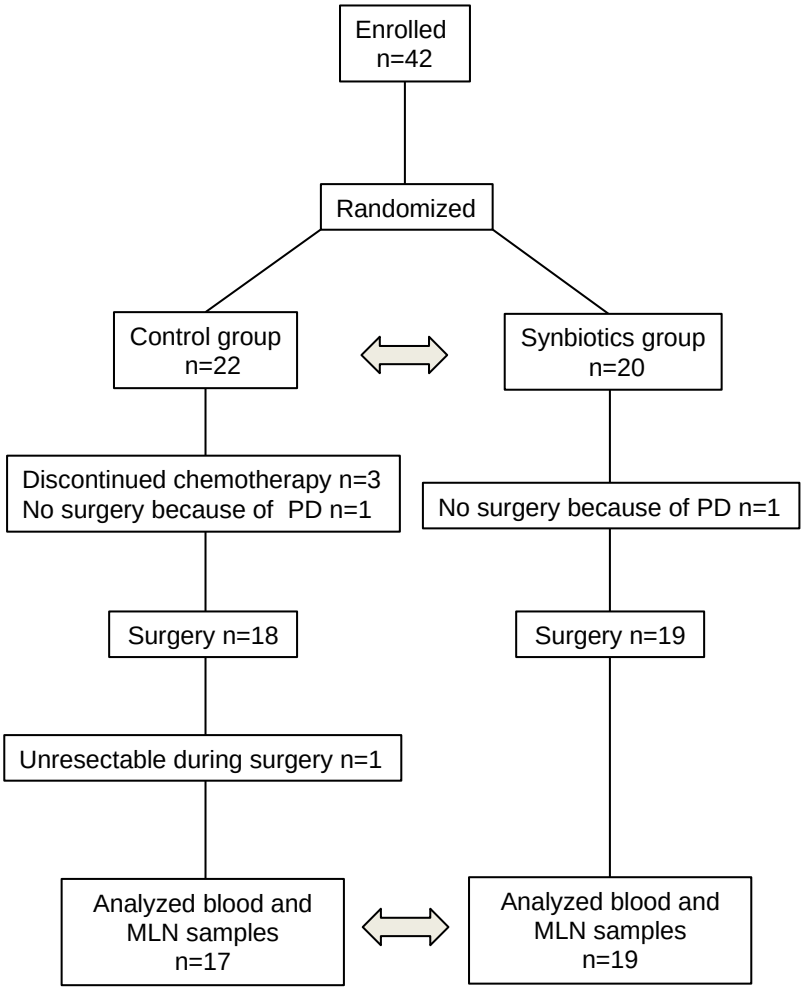


Figure 1.

Flow chart of the study patients.

PD, progressive disease; MLN, mesenteric lymph node.

Background characteristics of study patients

No significant differences were observed between the control and synbiotics groups in terms of the age, gender, clinical stage of esophageal cancer, depth of tumor invasion, proportion of patients who underwent preoperative chemoradiotherapy, or previous gastrectomy. The PNI and NLR before chemotherapy were also not significantly different between the two groups (Table 1).

Table 1. Background characteristics of study patients (before neoadjuvant therapy)

	Control (n=22)	Synbiotics (n=20)	*P*
Age [years]	67 (44-77)	63 (48-77)	0.193
Gender (M/F)	21/1	16/4	0.174
Clinical stage (UICC ver7)			0.965
Ⅰ	4	4	
Ⅱ	5	6	
Ⅲ	11	9	
Ⅳ	2	1	
Depth of invasion			0.552
T1	3	1	
T2	3	5	
T3	16	14	
Selected neoadjuvant therapy			0.491
Chemotherapy	15	16	
Chemoradiotherapy	7	4	
Previous gastrectomy	2	2	1.00
Body mass index [kg/m^2]	21.4 (15.0-26.9)	22.3 (16.6-25.7)	0.435
Serum albumin [g/dL]	4.0 (3.0-4.6)	4.0 (3.3-4.9)	0.560
Lymphocyte counts [×10^2/μL]	16 (8-28)	13 (9-30)	0.139
Neutrophil counts [×10^2/μL]	37.5 (17-97)	33.5 (17-53)	0.257
PNI	47.5 (34.5-56.0)	47.5 (38.0-57.5)	0.990
NLR	2.33 (0.94-8.88)	2.36 (1.07-4.70)	0.743

Note, continuous variables are described as median (range)

UICC ver7, Union for International Cancer Control version 7; PNI, Prognostic nutritional index; NLR, Neutrophil-to-lymphocyte ratio.

Detection of bacteria in the blood and MLN samples

First, we performed a quantitative analysis for the bacteria detected in blood and MLN at the

level of bacterial group, family, and genus using YIF-SCAN®. Bacteria were detected in 16 of all 101 blood samples in the control group, whereas those were detected only 2 of all 100 blood samples in the synbiotics group ($p<0.001$) during neoadjuvant chemotherapy (Figure 2A). Additionally, bacteria were detected in 12 of 34 MLN samples in the control group, whereas no bacteria were detected in 38 MLN samples in the synbiotics group ($p<0.001$) (Figure 2A). The detection rate was significantly higher (5/19, 26%) in the samples collected before the second cycle of chemotherapy in the control group than in the synbiotics group (0/20, 0%) (Figure 2B). In contrast, the detection of bacteria in the blood was observed in only one patient after the first cycle and in another one patient after the second cycle of chemotherapy in the synbiotics group (Figure 2B, Figure 3). We found a significant difference in the detection rate of bacteria in the blood samples collected before the second cycle of chemotherapy between the two groups (5/19 vs. 0/20, $p = 0.020$). With respect to the MLN samples in the control group, the detection rate of bacteria in the samples harvested at laparotomy (MLN-1) was 29% (5/17), whereas for samples harvested after resection of the tumor (MLN-2), the rate was 41% (7/17) (Figure 2B). During chemotherapy, *Enterobacteriaceae* and *Enterococcus*, which are pathogenic intestinal bacteria, and obligate anaerobes, which are predominant intestinal bacteria, were detected in the blood samples in the control group, indicating that the bacteria in the blood samples had originated from intestinal microorganisms (i.e., a consequence of bacterial translocation) (Figure 3). In contrast, we detected no *Enterobacteriaceae*, *Enterococcus*, or other obligate anaerobes in the blood samples of the synbiotics group. *Enterobacteriaceae*, *Enterococcus*, *Streptococcus*, and obligate anaerobes were frequently detected in the MLN samples in the control group.

Next, the bacterial species were estimated by sequence homology analysis of the amplified product of RT-PCR. As a result, among 7 patients with positive bacteria in the

MLN-2 samples in the control group, 5 patients were found to have the same bacterial species based on the sequence homology of the PCR products between the MLN and the blood samples. These results indicated that the bacteria detected in the blood sample during chemotherapy were mainly originated from the gut (Figure 4, Supplementary figure 1).

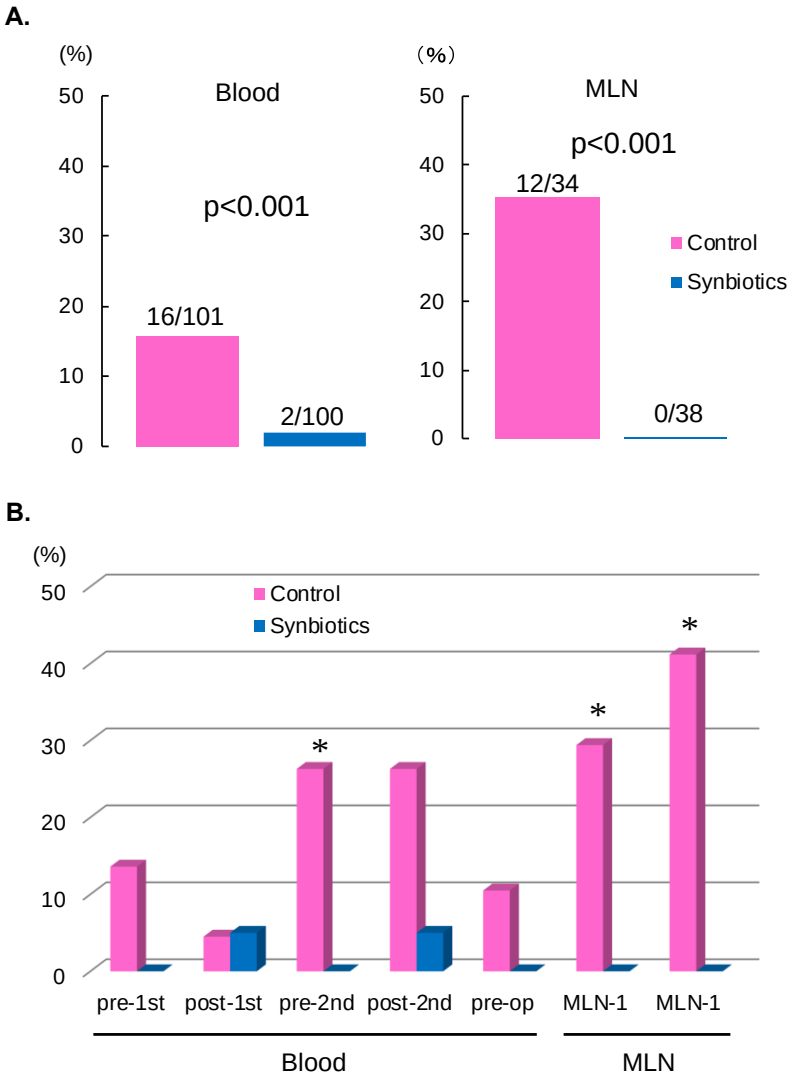


Figure 2.

The detection rate of bacteria in total blood and MLN samples (A) and that in each time point (B). Blood samples were collected before starting the first chemotherapy (pre-1st), on day 7 after starting the first cycle (post-1st), before starting the second cycle (pre-2nd), on day

7 after starting the second cycle (post-2nd), and 1 day before surgery (pre-op). MLN samples harvested during surgery at laparotomy (MLN-1) and after tumor resection (MLN-2).

*p < 0.05 vs. synbiotics group.

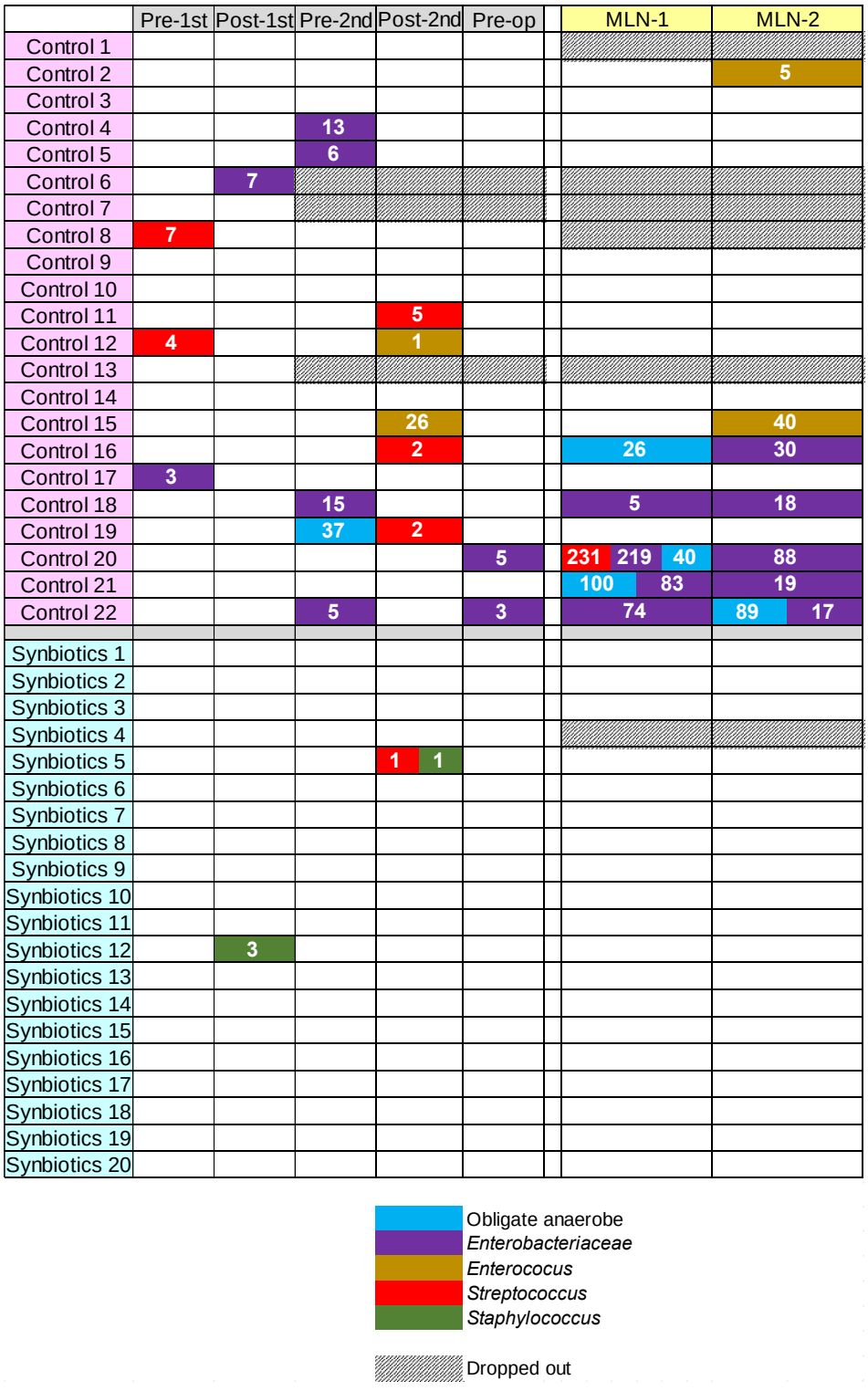


Figure 3.

The type of bacteria detected in each blood and MLN sample. The number of detected bacteria is expressed as cells/mL blood or cells/g MLN.

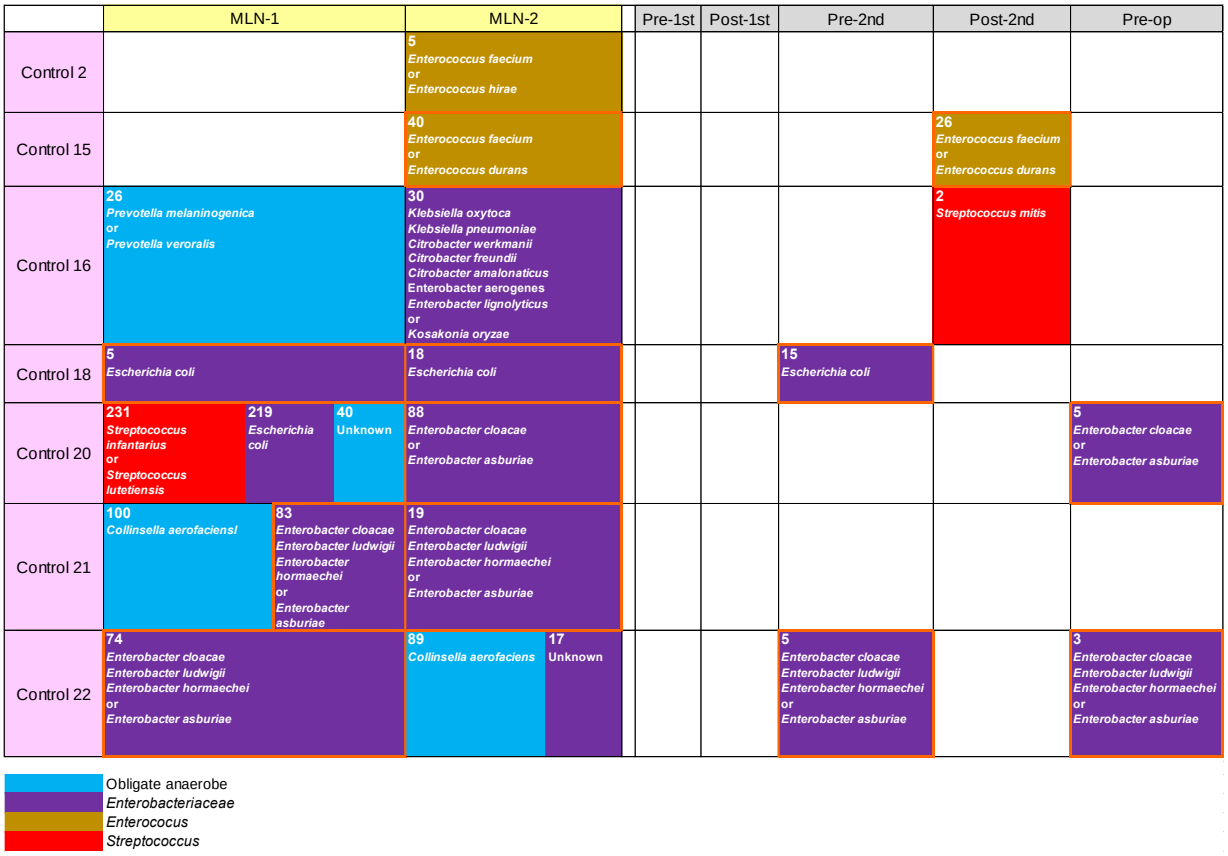


Figure 4.

Relevance of bacterial species isolated from MLN and blood. The numbers of detected bacteria are expressed as cells/mL blood or cells/g MLN. Orange border: the bacteria with homologous sequence were detected in both MLN and blood samples.

Response rate and side effects during chemotherapy

Although no patient had a complete response to neoadjuvant chemotherapy in either group, a partial response was achieved in 12 patients (55%) in the control group and 12 patients (60%) in the synbiotics group. In the control group, three patients discontinued chemotherapy after the first course of treatment; one due to liver failure with hyperammonemia, one due to severe renal dysfunction, and the other due to bleeding from the tumor. In the synbiotics group, no patient discontinued due to side effects. One patient in the control group did not undergo surgery because of emergence of pulmonary metastasis. One patient in the synbiotics group did not undergo surgery because of tumor progression to the aorta.

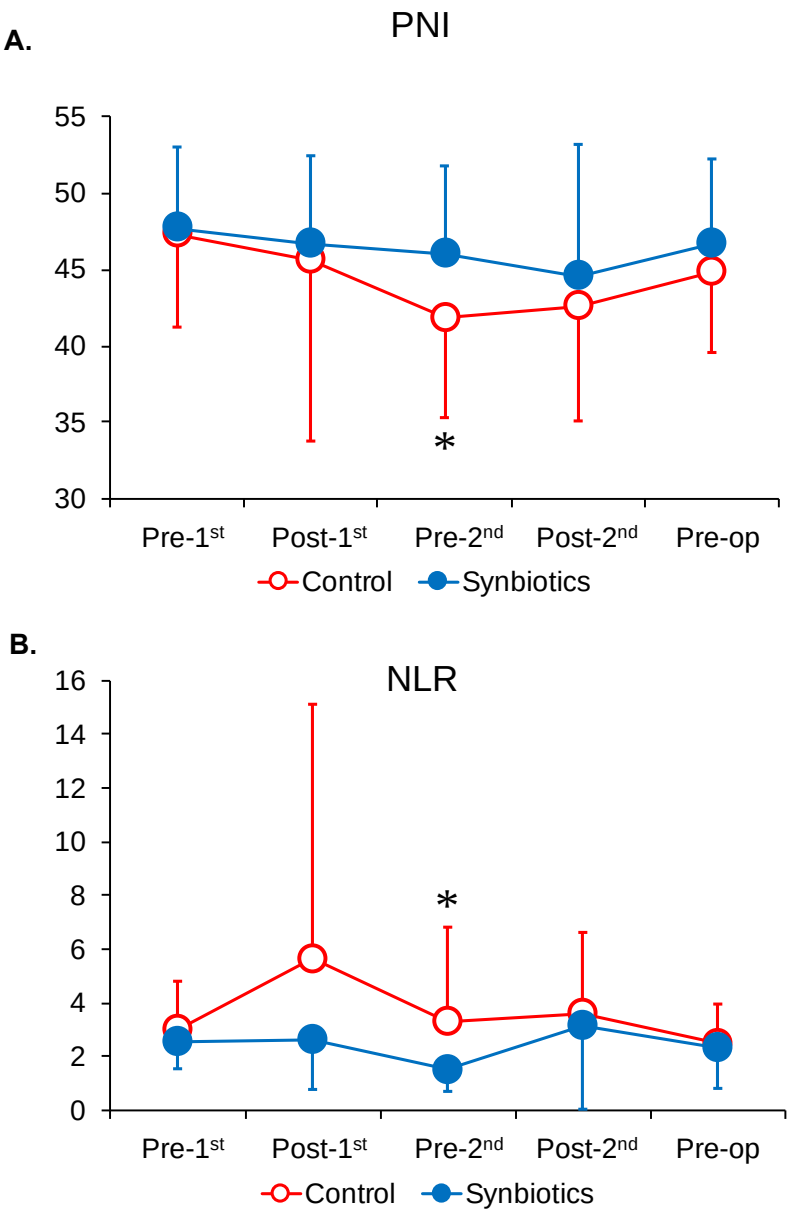
The incidence of side effects including malaise and hyperbilirubinemia during chemotherapy was significantly higher in the control group compared to the synbiotics group (Table 2). In terms of grade 3 or higher side effects, the incidence of gastrointestinal toxicity was significantly higher in the control group compared to the synbiotics group ($p = 0.022$).

Table 2. Side effects during neoadjuvant chemotherapy

	All grades, n (%)			≥Grade3, n (%)		
	Control (n=22)	**Synbiotics (n=20)**	***p***	**Control (n=22)**	**Synbiotics (n=20)**	***p***
Any blood toxicity	22 (100)	20 (100)	-	7 (32)	7 (35)	1.000
Leukopenia	18 (82)	16 (80)	1.000	0	2 (10)	0.221
Neutropenia	17 (77)	15 (75)	1.000	5 (23)	6 (30)	0.730
Platelets	17 (77)	14 (70)	0.730	0	1 (5)	0.476
Hemoglobin	21 (96)	20 (100)	0.489	2 (9)	1 (5)	1.000
Any gastrointestinal toxicity	22 (96)	19 (95)	1.000	8 (36)	1 (5)	0.022
Anorexia	16 (73)	10 (50)	0.204	7 (32)	1 (5)	0.047
Nausea	14 (64)	8 (40)	0.216	2 (9)	0	0.489
Vomiting	0	1 (5)	0.476	0	0	-
Diarrhea	7 (32)	4 (20)	0.491	1 (5)	0	1.000
Constipation	8 (36)	6 (30)	0.750	0	0	-
Malaise	11 (50)	3 (15)	0.023	3 (14)	0	0.233
Stomatitis	9 (41)	12 (60)	0.354	0	1 (5)	0.476
Liver function						
ALT	15 (68)	8 (40)	0.120	1 (5)	0	1.000
AST	8 (36)	3 (15)	0.166	1 (5)	0	1.000
Bilirubin	6 (27)	0	0.022	0	0	-
Renal function						
Creatinine	7 (32)	7 (35)	1.000	0	0	-
Hyponatremia	21 (96)	15 (75)	0.087	0	2 (10)	0.221
Sensory neuropathy	2 (9)	2 (10)	1.000	0	0	-
Infection	5 (23)	1 (5)	0.187	3 (14)	1(5)	0.608
Fever	6 (27)	1 (5)	0.096	0	0	-

Trends in nutritional status during chemotherapy

The PNI tended to deteriorate during neoadjuvant chemotherapy in both groups. The levels of PNI decreased after completion of the first and second cycles of chemotherapy in the control group. However, the deterioration of PNI was attenuated in the synbiotics group, and we observed a significant difference in the levels of PNI between the two groups before the second cycle of chemotherapy ($p = 0.037$) (Figure 5A). The levels of NLR increased after starting chemotherapy in the control group, whereas the elevation in NLR was attenuated in the synbiotics group (Figure 5B). We also found a statistically significant difference in the levels of NLR before the second cycle of chemotherapy between the two groups ($p = 0.024$).



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347 **Figure 5.**

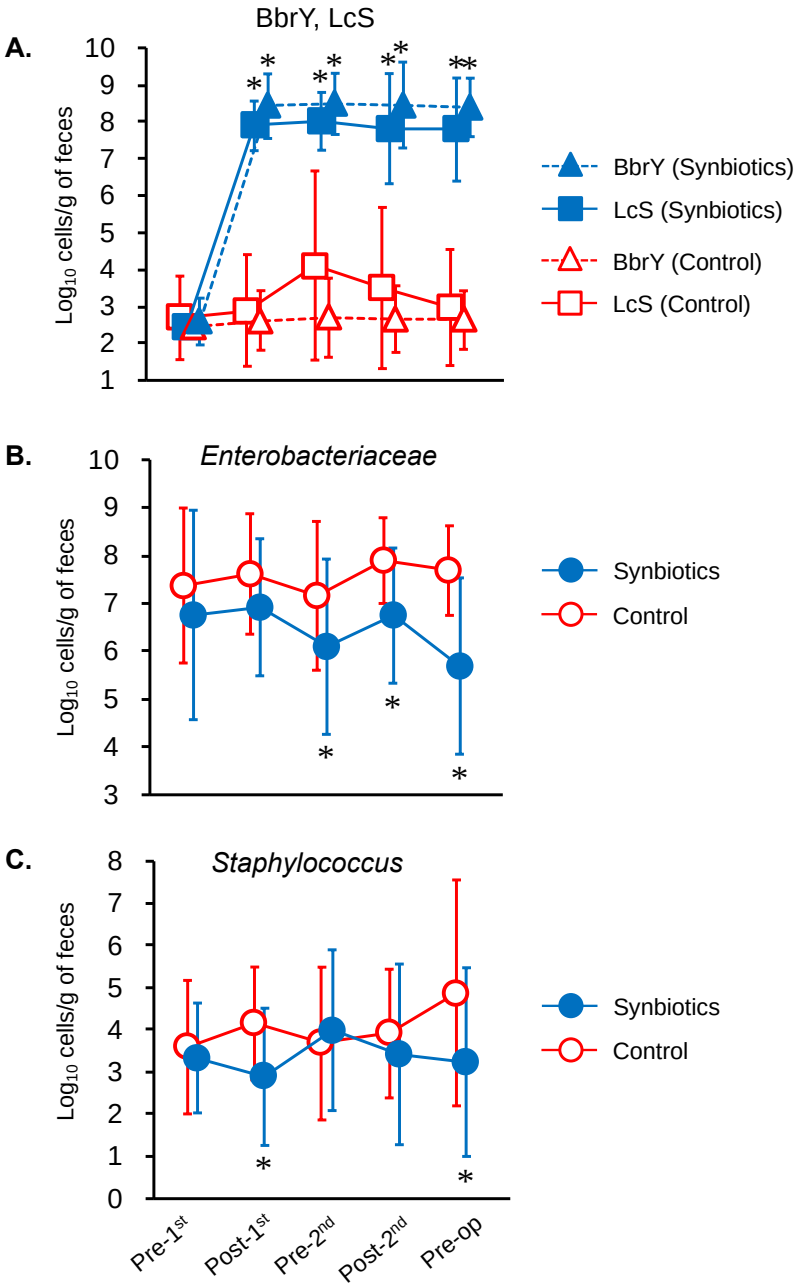
348 **Trend in prognostic nutritional index (PNI) (A) and neutrophil-to-lymphocyte ratio**

349 **(NLR) (B) during neoadjuvant chemotherapy.**

350 *$p < 0.05$ vs. synbiotics group.

Changes in the fecal microbiota

In the synbiotics group, a high number (~10^8 cells/g feces) of *Bifidobacterium breve* strain Yakult and *Lacticaseibacillus paracasei* strain Shirota, which were administered as probiotics, were detected in the feces throughout the whole course of chemotherapy in all patients (Figure 6A). However, these bacteria were scarcely detected in the control group. Overall, in feces, the number of pathogenic bacteria such as *Enterobacteriaceae* (Figure 6B) and *Staphylococcus* (Figure 6C) was lower in the synbiotics group compared to the control group while undergoing chemotherapy. The number of other bacteria in feces are described in Supplementary table 1.



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361 **Figure 6.**

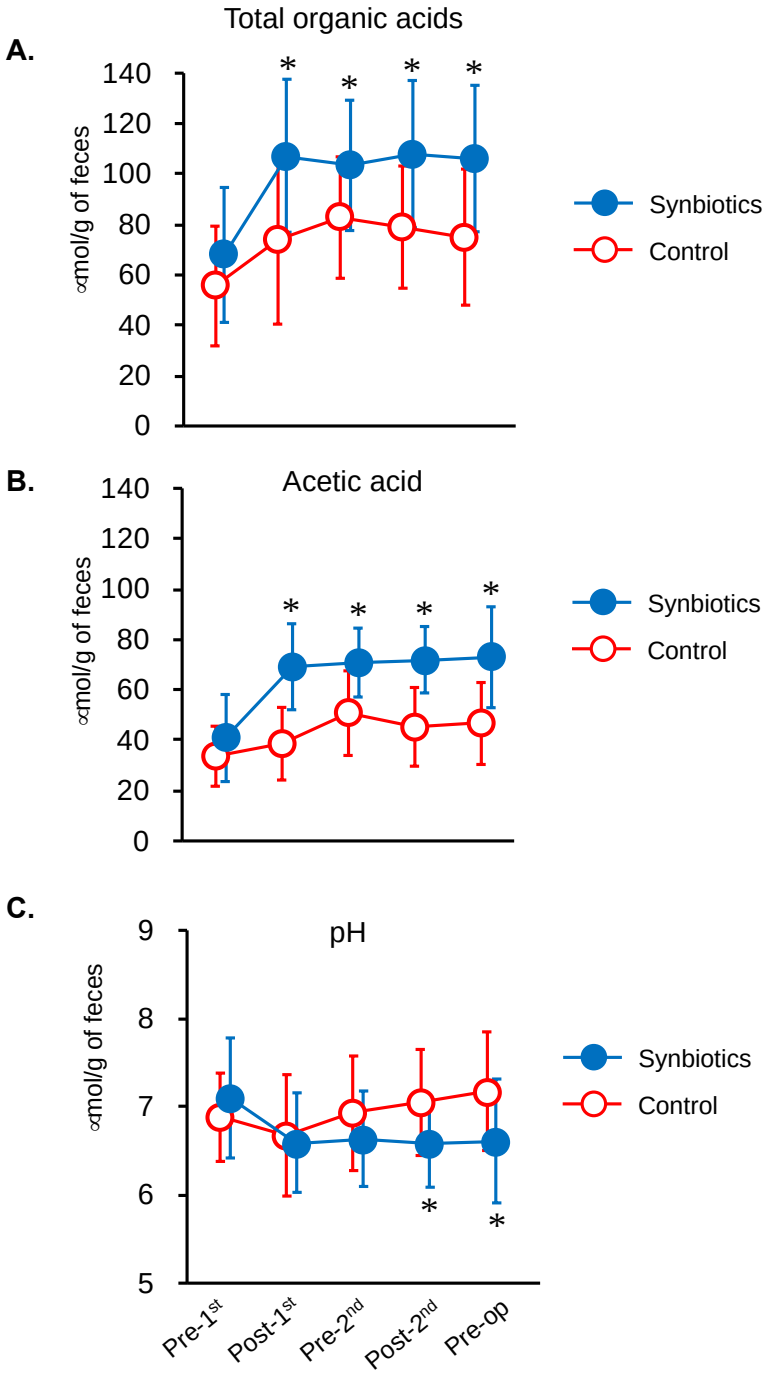
362 **Effect of synbiotics treatment on fecal microbiota.** BbrY, *Bifidobacterium breve* strain

363 Yakult; LcS, *Lacticaseibacillus paracasei* strain Shirota.

364 *$p < 0.05$ vs. control group.

Changes in fecal organic acid concentrations and pH

The fecal concentrations of total organic acids were significantly higher in the synbiotics group compared to the control group at all time points during chemotherapy (Figure 7A). In particular, the concentrations of acetic acid, which is the most predominant organic acid in the feces and is important for preserving intestinal mucosa integrity, were maintained at a high level in the synbiotics group compared to the control group (Figure 7B). Although the fecal pH gradually increased during chemotherapy in the control group, it was maintained at a lower level in the synbiotics group (Figure 7C). The concentrations of other organic acids are described in Supplementary table 2.



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375 **Figure 7.**

376 **Effect of synbiotics treatment on fecal organic acid concentrations and pH.**

377 *$p < 0.05$ vs. control group.

Relationships between fecal microbiota and organic acid concentrations and pH

Fecal concentrations of *Bifidobacterium breve* strain Yakult and *Lacticaseibacillus paracasei* strain Shirota showed a strong positive correlation with the fecal concentration of acetic acid, whereas they showed a strong negative correlation with the fecal pH (Figure 8). Other correlations are shown in Supplementary figure 2.

Interestingly, the fecal pH was significantly higher ($p = 0.025$) in patients whose blood was positive for bacteria after the second chemotherapy ($n = 6$) compared with those whose blood was negative for bacteria during chemotherapy ($n = 33$) (Supplementary table 3). In addition, the fecal concentration of acetic acid was significantly lower ($p = 0.026$) and pH was significantly higher ($p = 0.039$) in patients whose MLN-2 was positive for bacteria ($n = 7$) compared with those whose MLN-2 was negative for bacteria ($n = 30$) (Supplementary table 4).

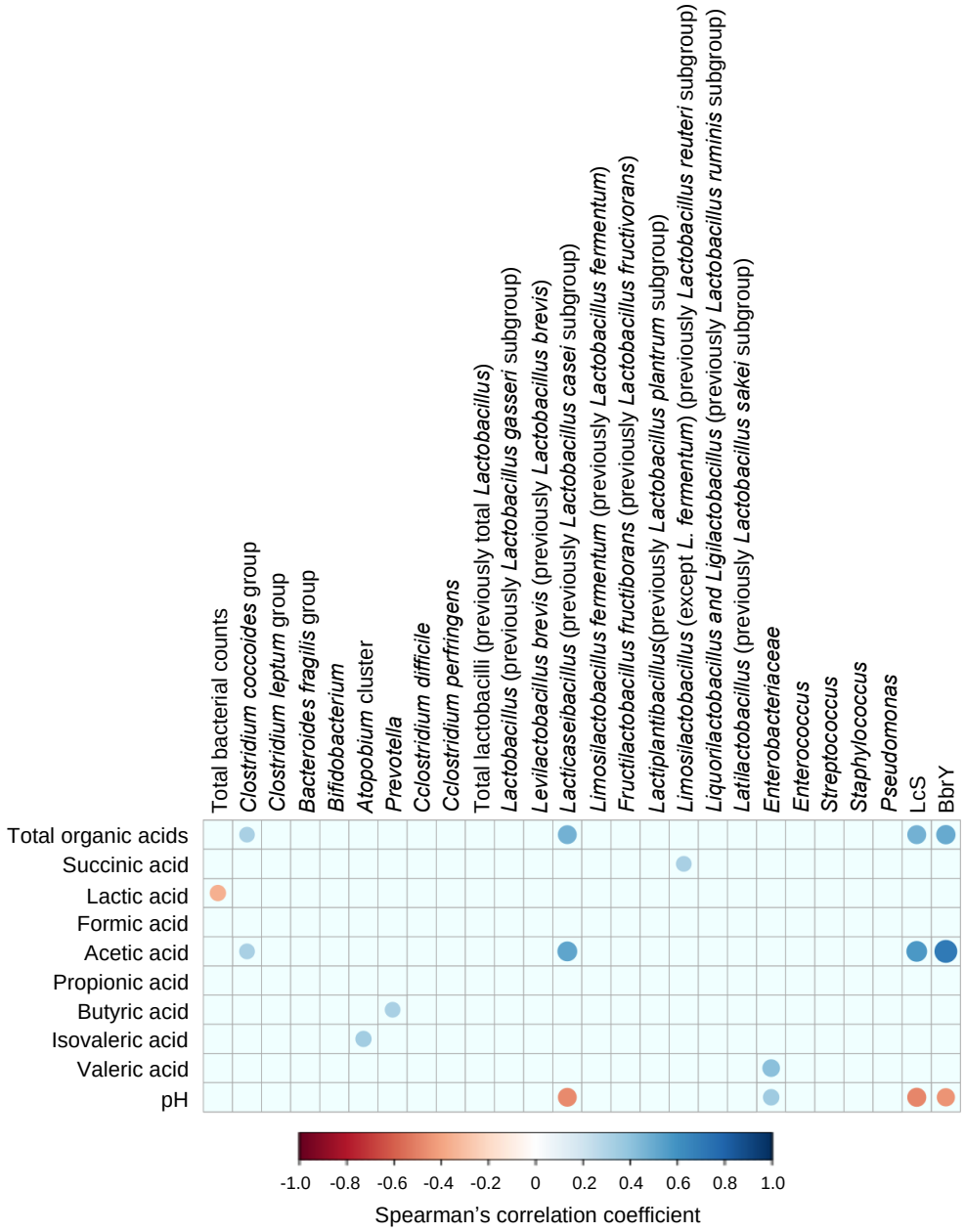

Figure 8.

Spearman's correlation coefficients between fecal microbiota and fecal organic acid concentrations or pH after the second cycle of chemotherapy.

Statistically significant correlations ($p < 0.05$) are shown as colored circles.

Discussion

Neoadjuvant chemotherapy is now a standard protocol for resectable advanced esophageal cancer. Cisplatin and 5-fluorouracil are the most commonly used neoadjuvant drugs for this purpose. The major adverse events resulting from use of these drugs are intestinal mucosal damage and immune suppression. Therefore, bacterial translocation may be induced by these drugs. However, to the best of our knowledge, the occurrence of bacterial translocation induced by chemotherapy and the preventive effects of synbiotics in reducing bacterial translocation has never been elucidated before.

Comparison with other studies

There is only one randomized controlled study that simply examined the effect of synbiotics during neoadjuvant chemotherapy on adverse events in esophageal cancer patients.[22] The primary endpoint of this study was the incidence of chemotherapy-related adverse events. However, no analyses for blood and mesenteric lymph node (sampled during surgery) samples, which are related to the occurrence of bacterial translocation, were performed. Therefore, the role of synbiotics in preventing bacterial translocation during neoadjuvant chemotherapy was unclear. As shown in this study, administration of synbiotics effectively reduced the incidence of bacteremia detected with RT-qPCR during chemotherapy. Presence of bacteria in the MLN sampled during surgery was reduced. Synbiotics also reduced the incidence of side effects induced by neoadjuvant chemotherapy, which is consistent with the previous randomized controlled study.[22] Moreover, both PNI and NLR, which are recognized as predictive factors for a good response in patients undergoing neoadjuvant chemotherapy for esophageal cancer,[18, 23, 24] showed favorable trends in the synbiotics group compared to the control group.

In the control group, the detection rate of bacteria in the blood samples peaked around the second cycle of chemotherapy, and it was rather reduced immediately before surgery. Damage to the intestinal mucosa and immune suppression induced by cisplatin and 5-fluorouracil peak around 2 weeks after administration and gradually subside thereafter. In this study, a 4- to 5-week interval was present between the final administration of cisplatin and 5-fluorouracil and surgery. Therefore, we speculate that damage to the intestinal mucosa and immune suppression may have partly recovered during the waiting period for surgery, and this recovery may have led to a lower detection rate of bacteria in the blood before surgery.

This study first demonstrated that bacteremia occurs at high frequency during neoadjuvant chemotherapy in patients with esophageal cancer. We speculated that this bacteremia is caused by bacterial translocation because most of the detected bacteria were intestinal microorganisms. However, the detection of bacteria may not be possible using conventional culture methods because the number of bacteria in the blood sample was small. The small number of circulating bacteria that is not detectable with the conventional culture method but is detectable with RT-qPCR is called “occult-bacteremia”, meaning that the presence of bacteria is only proven with the highly sensitive detection system. This small number of bacteria that has invaded the blood stream may silently affect the functions of organs including the liver and kidney. The higher rate of side effects including liver dysfunction in the control group may be partly explained by this hypothesis because animal and human studies[25, 26] have shown that liver functions are damaged by septicemia.

The impact of synbiotics treatment in preventing bacterial translocation in this study is rather outstanding compared to previous studies.[3, 4] The reason for this evident difference is unknown but may be partly explained by the long-term administration of synbiotics. In this

study, at least 8 weeks were needed for completion of two cycles of neoadjuvant chemotherapy and the subsequent waiting period. During this period, patients in the synbiotics group had been continuously taking synbiotics, whereas those in the control group were prohibited from taking food or beverages that contained synbiotics or probiotics. This protocol is clearly different from that in previous studies in which patients were asked to take synbiotics for only 2 weeks before surgery.[3, 4] The longer the period of synbiotics treatment, the more the intestinal microenvironment may become favorable and prevent bacterial translocation during chemotherapy and subsequent surgery. Further investigation is necessary to determine the optimal duration of synbiotics treatment before surgery.

As shown in previous studies, synbiotics treatment clearly changed the intestinal microenvironment.[3, 4, 9, 13] Fecal concentrations of pathogenic bacteria such as *Enterobacteriaceae* and *Staphylococcus* were maintained at low levels in the synbiotics group. In addition, the fecal concentration of acetic acid, which is the most predominant organic acid in the feces, was higher in the synbiotics group compared to the control group. Interestingly, the fecal concentrations of *Bifidobacterium breve* strain Yakult and *Lacticaseibacillus paracasei* strain Shirota showed a strong correlation with the fecal acetic acid concentration. Fecal acetic acid improves intestinal barrier function and prevents bacterial translocation.[27] According to previous studies, the major metabolic product of *Bifidobacterium breve* strain Yakult is acetic acid[28] and that of *Lacticaseibacillus paracasei* strain Shirota is lactic acid.[1] Lactic acid is further metabolized to acetic acid by obligate anaerobes.[1] Acetic acid has strict bactericidal effects on pathogenic bacteria[29-31] and upregulates tight junction-related genes such as Claudin1, Occludin, and ZO-1 in the intestinal epithelium.[28] These results indicated that administration of synbiotics possibly improves the intestinal microenvironment by increasing fecal acetic acid, and this change may

lead to prevention of bacterial translocation during chemotherapy. In fact, in this study, fecal concentration of acetic acid was lower in patients with bacteremia compared to others.

Limitations of this study

This study has several limitations. The number of study patients was small. However, the beneficial effect of synbiotics in preventing bacterial translocation was evident. Therefore, it is ethically unacceptable to perform the same study with larger number of patients in the future. The definition of bacterial translocation in this study was the presence of bacteria in the blood or MLN samples when detected using the highly sensitive bacterial detection system (RT-qPCR). Whether the small number of bacteria detected with RT-qPCR is responsible for the clinical symptoms that emerged during chemotherapy is still unclear. Seven patients in the control group and four patients in the synbiotics group received radiotherapy during the term of chemotherapy. Although we did not find any difference between patients with and without radiotherapy in this study, a further large-scale randomized controlled trial with or without radiotherapy is necessary to clarify the impact of radiotherapy on the bacterial translocation.

Conclusions

In conclusion, this study clearly showed the beneficial effects of synbiotics treatment for preventing bacterial translocation during neoadjuvant chemotherapy and subsequent esophagectomy in patients with esophageal cancer. The incidence of gastrointestinal toxicity during chemotherapy was lower in the synbiotics group compared to the control group. Long-term administration of synbiotics may lead to a favorable intestinal microenvironment and allow patients to tolerate neoadjuvant chemotherapy and subsequent highly invasive surgery.

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Obtained from all of the included patients.

Ethics approval:

Approved by the Human Research Review Committee of Nagoya University Hospital (approved number 2011-1337-2). All patients gave informed consent.

Data sharing statement:

All data pertaining to this work are stored in the Nagoya University Graduate School of Medicine. Yukihiro Yokoyama (corresponding author) had full access to all the data in the study and had the final responsibility for the decision to submit the study for publication.

Transparency:

The corresponding author (YY) affirms that the manuscript is an honest, accurate, and transparent account of the study being reported, and follows the CONSORT guidelines for the reporting of clinical trials. No important aspects have been omitted, and any discrepancies from the study as planned have been explained.

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