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Alterations in the gut microbiome in patients with esophageal carcinoma in response to esophagectomy and neoadjuvant treatment

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Author contributions

All authors contributed to the study's conception and design. Hirofumi Hasuda: collected data and samples and wrote the first draft of the manuscript; Yutaka Makizaki, Haruka Yokota, Yoshiki Tanaka, and Hiroshi Ohno: analyzed the gut microbiome data; Mototsugu Shimokawa: contributed intellectual inputs, mainly for statistics; Hiroya Matsuoka: collected data and samples; Yasue Kimura: contributed intellectual inputs, mainly for treatment; Tetsuo Ikeda, Eiji Oki, and Tomoharu Yoshizumi: supervised the entire study. All authors participated in writing the manuscript and/or critically revising the content. All authors approved the final manuscript.

Abstract

Purpose: Analyzing the gut microbiome is essential for planning treatment strategies to manage esophageal squamous cell carcinoma. This study aimed to characterize the gut microbiome of patients with esophageal squamous cell carcinoma and to identify alterations in its composition during treatment.

Methods: We observed alterations in the gut microbiome in 21 consecutive patients with esophageal squamous cell carcinoma at five different time points, from neoadjuvant treatment to postoperative surgery. Ten healthy individuals were used as a non-cancer control group. Fecal samples were collected and analyzed using 16S ribosomal ribonucleic acid sequencing.

Results: Before treatment, participants with esophageal squamous cell carcinoma had different alpha and beta diversity in comparison to healthy controls. The number of *Streptococcus*, a facultative anaerobic bacterium, was significantly higher, whereas that of *Faecalibacterium*, an obligate anaerobic bacterium, was significantly lower. Both alpha and beta diversity remained unchanged during neoadjuvant treatment, but the alterations were pronounced after surgery. The increase in the relative abundance of *Streptococcus* and the decrease in that of *Faecalibacterium* also tended to be more pronounced after surgery.

Conclusions: The gut microbiome in patients with esophageal squamous cell carcinoma is altered with surgical intervention.

Keywords: gut microbiome, esophageal squamous cell carcinoma, esophagectomy, neoadjuvant treatment, chemotherapy

25 **Introduction**

26 Squamous cell carcinoma accounts for 90% of esophageal cancer cases in East
27 Asian countries [1]. Lifestyle habits, including smoking and alcohol consumption, as
28 well as physical characteristics like the flushing response, influence the carcinogenesis
29 of esophageal squamous cell carcinoma (ESCC) [2, 3]. Thus, the major risk factors for
30 ESCC are heavy smoking and excessive drinking.

31 The composition and diversity of the gut microbiome are associated with some
32 malignant diseases [4, 5], and they serve as sensitivity modulators to immune
33 checkpoint inhibitors (ICIs) in melanoma [6] or prognostic factors for colorectal cancer
34 [7]. Probiotic therapy has been found to significantly prolong progression-free survival
35 and overall survival in lung cancer patients treated with ICIs [8]. Yamamura et al.
36 reported that the tissue microbiome is associated with cancer development and the
37 progression of ESCC [9]. Furthermore, they reported that the intratumoral levels of
38 *Fusobacterium nucleatum* could help predict the therapeutic response to neoadjuvant
39 chemotherapy (NAC) in patients with ESCC [10]. Therefore, the antitumor efficacy of
40 chemotherapy can be promoted by modulating the microbiome diversity. Moreover, the
41 administration of synbiotics during NAC to patients with esophageal cancer reduces the
42 occurrence of adverse events [11]. ICI therapy is considered a standard adjuvant
43 treatment in ESCC [12]. Since differences in microbial composition are associated with
44 the efficacy of ICI therapy [6], changes in the gut microbiome during treatment should
45 be identified.

46 Previous studies on the gut microbiome in patients with ESCC primarily
47 focused on preoperative cases [13]. This study aimed to characterize the gut

- 48 microbiome of patients with ESCC and report alterations in its composition during
- 49 neoadjuvant treatment and thoracoscopic subtotal esophagectomy.

50 **Materials and Methods**

51 *Patients*

52 This study included consecutive patients with ESCC who received NAC or
53 neoadjuvant chemoradiotherapy (NACRT) and who underwent thoracoscopic subtotal
54 esophagectomy at Kyushu University between June 2018 and March 2020. Initially,
55 forty patients were recruited; however, 19 were excluded. Therefore, 21 patients
56 participated in the study (Online Resource 1). Participants were asked to collect fecal
57 samples at five different time points: (1) before treatment; (2) on the fifth day of
58 NAC/NACRT; (3) after NAC/NACRT; (4) two weeks after surgery; and (5) three
59 months after surgery. Additionally, physical examinations and blood tests were
60 performed at five different time points. Smoking and alcohol consumption data were
61 obtained using questionnaires, and the alcohol intake was converted to ethanol
62 consumption [3]. Ten healthy individuals without any serious medical history were
63 recruited as a healthy control (HC) group, regardless of their smoking and drinking
64 habits. Physical examination data and fecal samples were collected only once from the
65 participants in the HC group. The study was approved by the Ethics Review Board of
66 Kyushu University, and written informed consent was obtained from all participants
67 (permission number: 2021-188). This study was registered in the UMIN Clinical Trials
68 Registry System (UMIN000044878).

69

70 *Neoadjuvant treatment, surgical procedure, and perioperative management*

71 NAC/NACRT was performed according to the Japanese esophageal cancer
72 guidelines [14, 15]. For NAC, either 5-fluorouracil plus cisplatin (FP) or docetaxel plus
73 cisplatin and 5-fluorouracil (DCF) were administered. For NACRT, FP plus radiation

was administered. At least two courses of both FP and DCF therapy were administered every four weeks. The thoracoscopic subtotal esophagectomy was scheduled within two weeks after the administration of NAC/NACRT. Esophagectomy was performed with standard two- or three-field lymphadenectomy. Gastric tube reconstruction was performed by laparoscopic-assisted surgery. Enteral feeding was initiated the day after surgery using a jejunostomy tube. For cases without postoperative complications, oral intake was resumed from postoperative day 6–10. Cefazolin was used as a routine perioperative antimicrobial agent from the day of surgery until postoperative day 2 or 3. Broad-spectrum antimicrobial agents were used to treat postoperative complications. Proton pump inhibitors (PPIs) were regularly administered after surgery.

Microbiome analysis

Next-generation 16S ribosomal ribonucleic acid sequencing was performed. Fecal samples were immediately stored at -80°C. Deoxyribonucleic acid was extracted from the fecal samples using the beads-phenol method [16]. 16S ribosomal ribonucleic acid sequencing was performed using the V3-V4 region of the 16S ribosomal ribonucleic acid on the Illumina MiSeq platform (San Diego, CA) [17]. The collected data were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (<http://qiime.org/>) [18], as previously described [19]. An alpha diversity analysis was performed to examine the richness (using observed operational taxonomic units [OTUs], Chao1, and abundance-based coverage estimator [ACE]) and evenness (Shannon index) according to the QIIME pipeline. The unweighted UniFrac distance was calculated for each sample using QIIME. Finally, a beta diversity principal

coordinate analysis was performed using R (R Foundation for Statistical Computing, Vienna, Austria; <https://www.R-project.org/>) with the `vegdist` function.

Statistical analysis

Categorical and numerical variables are presented as the median (range) and were compared using the Mann–Whitney U test, Wilcoxon signed-rank test, and Fisher’s exact test. The mean (\pm standard error) of both the microbiome and alpha diversity data were analyzed and appropriately compared using Welch’s *t*-test or a paired *t*-test. The unweighted UniFrac distances were analyzed using a permutational multivariate analysis of variance. Spearman’s rank correlation analysis was performed to evaluate the association between the clinical variables and microbiome parameters. Two-sided *P* values of <0.05 were considered statistically significant. As this was an exploratory study, multiple corrections were not performed. All statistical analyses were completed using the JMP Pro software program (version 15.1.0, SAS Institute, Cary, NC) and the R package “vegan” (version 3.1.3).

Results

Characteristics of the HCs and patients with ESCC

Table 1 shows the clinical characteristics of the HCs and patients with ESCC. The median age of the HCs and patients with ESCC was 51.5 and 69 years, respectively ($P < 0.001$). Patients with ESCC consumed significantly higher amounts of tobacco ($P < 0.001$) and alcohol ($P = 0.002$) in comparison to the HCs. Fecal samples were collected at five different time points from the 21 patients with ESCC. In six patients, fecal samples were not collected on the fifth day of NAC/NACRT due to constipation. Therefore, a total of 99 fecal samples were obtained from the patients with ESCC. Table 1 shows the clinicopathological factors of patients with ESCC. NAC was administered to 19 patients with ESCC. Thoracoscopic subtotal esophagectomy was performed for 21 patients, and robot-assisted surgery was used in 12 of these patients. Eight patients experienced postoperative complications. Grade 2 or 3 pathological therapeutic effects were observed in seven patients [20]. The clinical data are summarized in Online Resource 2, and their alterations are shown in Online Resource 3.

Diversity of the gut microbiome in HCs and patients with ESCC

The alpha diversity in patients with ESCC was significantly lower in comparison to the HCs (Fig. 1a–d). Further, a principal coordinate analysis was performed to confirm the beta diversity (Fig. 1e). The analysis showed that the data of patients with ESCC formed a dispersed cluster in a different location from that of the HCs ($P = 0.011$).

Comparison of the gut microbiome at the phylum and genus levels between HCs and patients with ESCC

The composition of the phyla and major genera is shown in Online Resource 4. The top five genera were as follows: *Blautia*, *Bacteroides*, *Faecalibacterium*, *Bifidobacterium*, and *Eubacterium* in HC; conversely, *Blautia*, *Bacteroides*, *Bifidobacterium*, *Streptococcus*, and *Faecalibacterium* were observed in patients with ESCC (Fig. 1f). In patients with ESCC, the relative abundance of *Streptococcus* was significantly higher ($P=0.009$), and that of *Faecalibacterium* was significantly lower ($P=0.009$) in comparison to the HCs.

Relationship between the gut microbiome and nutritional index before treatment

The correlation between the gut microbiota data and each nutritional parameter was analyzed using the pre-treatment data to examine the relationship between the nutritional status and the microbiome of patients with ESCC (Fig. 2a). An accurate numerical value was assigned when a moderate positive correlation of ≥ 0.30 or a negative correlation of ≤ -0.30 was observed on the heat map [21]. The abundance of *Streptococcus* showed a moderate negative correlation with hemoglobin, albumin, and total cholesterol on the heat map. Moreover, the abundance of *Streptococcus* was associated with prognosis-related nutritional scores, including moderate positive correlations with the controlling nutrition status score, Glasgow prognostic score, C-reactive protein-albumin ratio, and platelet-lymphocyte ratio. An inverse correlation was noted between the abundance of *Streptococcus* and the prognostic nutritional index. The abundance of *Faecalibacterium* was negatively correlated with aspartate aminotransferase and γ -glutamyl transpeptidase levels.

Influence of tobacco and alcohol consumption on the abundance of Streptococcus and Faecalibacterium

In patients with ESCC, the abundance of *Streptococcus* was significantly higher, and that of *Faecalibacterium* was significantly lower in comparison to the HCs. Therefore, further examinations were performed on these two genera. PPIs reportedly increase the abundance of *Streptococcus* and reduce the abundance of *Faecalibacterium* [22]. Thus, to negate the effect of PPIs, three patients with ESCC who had taken PPIs were excluded (the HCs had not received PPIs), and the abundance of *Streptococcus* and *Faecalibacterium* was re-examined in both groups. In the 18 patients with ESCC, the abundance of *Streptococcus* was significantly higher ($P=0.032$), and that of *Faecalibacterium* was significantly lower ($P=0.017$) in comparison to the HCs (Fig. 2b). Furthermore, the influence of tobacco and alcohol consumption on the abundance of these two genera was analyzed by integrating the data from the HCs and patients with ESCC. Smokers who consumed ≥ 40 packs/year [3] had a significantly higher relative abundance of *Streptococcus* than smokers who consumed < 40 packs/year or non-smokers ($P=0.040$) (Fig. 2c). There were no significant differences in the abundance of *Streptococcus* between current and former smokers (Fig. 2d). Additionally, alcohol users who consumed > 70 g of ethanol/week [23] had a significantly lower relative abundance of *Faecalibacterium* ($P=0.030$) than participants who consumed ≤ 70 g of ethanol/week (Fig. 2e).

Alterations in diversity during the treatment of patients with ESCC

The alpha diversity at five different time points is shown in Figure 3a–d. All alpha diversity factors, including observed OTUs ($P=0.008$), Chao1 ($P=0.010$), ACE

($P=0.016$), and Shannon index ($P=0.031$), were significantly decreased at two weeks after surgery in comparison to after NAC/NACRT. The observed OTUs ($P=0.048$), Chao1 ($P=0.021$), and Shannon index ($P=0.038$) significantly increased at three months after surgery in comparison to two weeks after surgery. However, no significant alterations were observed between the data before and after the administration of NAC/NACRT. The beta diversity at each of the five-time points is shown in Figure 3e. No significant alterations were observed two weeks ($P=0.153$) and three months ($P=0.053$) after surgery in comparison to after NAC/NACRT. However, different clusters were detected two weeks ($P=0.042$) and three months ($P=0.036$) after surgery versus before NAC/NACRT.

Gut microbiome alterations at the phylum and genus levels

Alterations in the microbiome at the phylum level are shown in Online Resource 5. Alterations at the genus level are shown in Figure 4a. The abundance of facultative anaerobes increased, whereas that of obligate anaerobes decreased after surgery (Fig. 4b). The abundance of *Streptococcus*, a facultative anaerobe, increased significantly at three months after surgery in comparison to after NAC/NACRT ($P=0.001$). The abundance of *Faecalibacterium*, an obligate anaerobe, was low until two weeks after surgery and then significantly decreased at three months after surgery in comparison to the 2-week levels ($P=0.033$). The abundance of *Enterococcus* at three months after surgery was significantly increased in patients with postoperative complications in comparison to patients without postoperative complications ($P=0.042$) (Online Resource 6). The abundance of *Blautia* in patients with high pathological therapeutic effects (Grade 2/3) was significantly lower in comparison to patients with low

208 pathological therapeutic effects (Grade 0/1) before treatment ($P < 0.001$), on day 5 of
209 NAC/NACRT ($P = 0.003$), and after NAC/NACRT ($P = 0.007$) (Online Resource 6).

210

211 Discussion

212 To the best of our knowledge, this is the first study identifying alterations to
 213 the gut microbiome in patients with ESCC treated with NAC/NACRT followed by
 214 esophagectomy. Patients with ESCC, had high and low relative abundance of
 215 *Streptococcus* and *Faecalibacterium*, respectively. The relative abundance of
 216 *Streptococcus* and *Faecalibacterium* remained unchanged until two weeks after surgery.
 217 However, the relative abundance of *Streptococcus* was increased, while that of
 218 *Faecalibacterium* was decreased at three months after surgery.

219 *Streptococcus*, a facultative anaerobe, is an essential bacterium of the oral
 220 microbiome [24]. In our study, the relative abundance of *Streptococcus* before treatment
 221 was higher in patients with ESCC than in the HCs. Deng et al. reported that the
 222 abundance of *Streptococcus* in patients with esophageal cancer was higher than that in
 223 healthy individuals [13], consistent with our findings. Additionally, the abundance of
 224 *Streptococcus* was significantly increased at three months after surgery in comparison
 225 to after NAC/NACRT, indicating that surgery alters the relative abundance of
 226 *Streptococcus*. Moreover, *Klebsiella* and *Enterococcus*, which are facultative anaerobes,
 227 showed a remarkable increase after surgery in comparison to the pre-surgery levels. An
 228 increased abundance of facultative gut anaerobes, including *Streptococcus* spp.,
 229 *Klebsiella pneumoniae*, and *Enterococcus faecalis*, after Roux-en-Y bypass surgery in
 230 obese patients has been reported [25]. Increases in the abundance of these facultative
 231 anaerobes may be caused by the accumulation of oxygen in the distal parts of the gut
 232 after surgery [25], supporting a shift to an aerobic environment following
 233 esophagectomy. Moreover, surgical procedures involving the stomach reduce gastric
 234 acid secretion, which weakens the barrier against the settlement of oral *Streptococcus*

[25]. Furthermore, sleeve gastrectomy in obese patients increases the abundance of *Streptococcaceae* after surgery [26]. Reconstruction using a gastric tube or sleeve gastrectomy can similarly reduce gastric acid secretion and gastric transit time [26-28], increasing the abundance of *Streptococcus*.

In this study, the abundance of *Streptococcus* in heavy smokers (including former smokers) was significantly higher than that in non-smokers. However, a direct relationship between smoking and the abundance of *Streptococcus* could not be established due to the limited number of cases, and a multivariate analysis was not performed. Although the difference between former and current smokers was non-significant, current smokers tended to have a higher abundance of *Streptococcus* than former smokers. Thus, quitting smoking may reduce the levels of *Streptococcus* in the gut. Smoking increases glycolysis and other oxygen-independent carbohydrate metabolism pathways that create a favorable environment for facultative anaerobe growth, leading to increased levels of *Streptococcus* in the oral cavity [29]. Moreover, the abundance of *Streptococcus* was correlated with nutritional parameters and prognosis-related nutritional scores. Therefore, the abundance of *Streptococcus* indicates the prognosis in patients with ESCC.

Faecalibacterium, represented by *Faecalibacterium prausnitzii*, is an obligate anaerobe and an essential component of the gut microbiome; *Faecalibacterium* accounts for >5% of the entire bacterial population in healthy adult individuals, and it enhances the immune system functioning [30]. In this study, the HCs showed a high relative abundance of *Faecalibacterium*, whereas patients with ESCC exhibited a low abundance before treatment. Moreover, alcohol consumers showed a lower abundance of *Faecalibacterium* in comparison to non-consumers. Since alcohol consumption

decreases the level of *Faecalibacterium* [31], the low abundance of *Faecalibacterium* in patients with ESCC may be due to alcohol consumption. In this study, the abundance of remained low until two weeks after surgery and further decreased at three months after surgery. The decrease in the abundance of *Faecalibacterium* may be due to a shift in the aerobic environment. Palleja et al. [25] reported that Roux-en-Y bypass in obese patients decreased the abundance of *Faecalibacterium* at three months after surgery, which is consistent with our results. Chaput et al. [32] reported that high baseline levels of *Faecalibacterium* prolonged progression-free survival and overall survival in patients with melanoma treated with ICIs. The effectiveness of ICI treatment as an adjuvant therapy has recently been observed in ESCC [12]. Future studies on the relationship between postoperative *Faecalibacterium* baseline levels and the therapeutic effects of ICIs may improve treatment outcomes. Furthermore, Hibberd et al. reported potential therapeutic benefits in patients with colorectal cancer who received probiotics preoperatively [33]. These patients showed an increased abundance of butyrate-producing bacteria (including *Faecalibacterium*) but a decreased abundance of colorectal cancer-associated genera. The administration of probiotics may be helpful for improving the treatment results of patients with ESCC with low levels of *Faecalibacterium*.

Alpha and beta diversity factors showed remarkable alterations after surgery in this study. The high preoperative abundance of oral bacteria and facultative anaerobes in the intestine may be associated with these alterations. In addition to *Streptococcus* and *Faecalibacterium*, a relationship was observed between several microbiomes and clinical outcomes. Cases with a high chemotherapeutic response had a significantly lower abundance of *Blautia* throughout the first three time points. Although it has been

reported that after ICI treatment for metastatic colorectal cancer and non-small cell lung cancer, patients with *Blautia* SR1/5-positive fecal samples showed significantly better progression-free survival in comparison to patients with negative fecal samples [34]. The relative abundance of lower *Blautia* was associated with a lower response to chemotherapy in our study. The difference in the evaluation of the role of *Blautia* in therapeutic sensitivity might be due to ethnic difference in the gut microbiome, as mentioned above (i.e., *Blautia* species are dominant in the Japanese gut microbiome [35]. Therefore, by changing the gut microbiome environment (dysbiosis or low diversity), a change in the abundance of *Blautia* was seen and could be highlighted more clearly in comparison to other genera. Hence, more comprehensive studies are required to further clarify the association between the abundance of *Blautia* and chemosensitivity in Japanese patients with ESCC. Despite insufficient reports on the association between the therapeutic effects of chemotherapy and *Blautia*, *Blautia* may serve as a biomarker for chemosensitivity. Since the abundance of *Enterococcus* was high in cases with postoperative complications, the impact of broad-spectrum antibiotics should be considered [36]. Shi et al. [37] reported the effects of neoadjuvant chemoradiotherapy on the gut microbiota in patients with rectal cancer. Although some gut microbiome alteration was observed before and after treatment, the diversity remained unchanged. The fact that diversity was not altered by neoadjuvant treatment was consistent with the findings of our study.

One of the limitations of this study is that the microbiome data of HCs and patients with ESCC should be cautiously interpreted because of differences in the participants' backgrounds. In this study, the sample size was reduced to characterize the intestinal flora of ESCC patients; moreover, Japanese patients with ESCC who had

characteristics such as a lifestyle of heavy smoking and heavy drinking, and who had a flushing reaction were compared with an average middle-aged healthy Japanese population. Patients with ESCC were characterized by low diversity before treatment, a high abundance of facultative anaerobes, and a low abundance of obligate anaerobes, which were more pronounced after surgery. After surgery, the intestinal environment of patients with ESCC is more prone to dysbiosis in comparison to before surgery, indicating that treatment does not improve the dysbiosis. Since ICI treatment was not covered in our study, we believe that future studies to investigate the alterations in the gut microbiome during ICI treatment are essential.

The composition, diversity, and alterations in the intestinal microbiome that occur in response to surgery and neoadjuvant treatment for gastrointestinal malignancies should be investigated further. We believe that the analysis of the gut microbiome should be performed before NAC/NACRT, and about three months after surgery, when the patients have recovered from surgical invasion. The administration of probiotics should be started before treatment and continued over the long term after surgery; at the same time, precautions to suppress the migration of oral bacteria (e.g., oral care and avoiding the administration of antacids) are essential.

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330

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447 associated with therapeutic responses and toxicities of neoadjuvant
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450

Figure legends:

Fig. 1 Comparison of the gut microbiome diversity and gene levels in HCs and patients with ESCC.

(a) Observed OTUs, (b) Chao1, (c) ACE, (d) Shannon index, and (e) PCoA of the microbiome in HCs and patients with ESCC. Blue marks represent HCs, and orange marks represent patients with ESCC. (f) At the genus level, the relative abundance of *Streptococcus* was significantly higher, and that of *Faecalibacterium* was significantly lower in patients with ESCC in comparison to HCs. HC, healthy control; ESCC, esophageal squamous cell carcinoma; OTUs, operational taxonomic units; ACE, abundance-based coverage estimator; PCoA, principal coordinate analysis.

Fig. 2 Relationship between the microbiome and clinical data.

(a) Heat map of the correlation between the gut microbiome and nutritional index based on the data before treatment. A numerical value was assigned for a positive correlation of ≥ 0.30 or a negative correlation of ≤ -0.30 . (b) The relative abundance of *Streptococcus* and *Faecalibacterium* in HCs and patients with ESCC (three patients who received PPIs were excluded). (c) The influence of tobacco consumption on the abundance of *Streptococcus*. (d) Comparison of the relative abundance of *Streptococcus* between former and current smokers. (e) Influence of alcohol consumption on the abundance of *Faecalibacterium*. HC, healthy control; ESCC, esophageal squamous cell carcinoma; BMI, body mass index; TLC, total leukocyte count; Hb, hemoglobin; Alb, albumin; T.Chol, total cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ -GTP, γ -glutamyl transpeptidase; CONUT score, controlling nutrition status score; GPS, Glasgow prognostic score; CAR,

C-reactive protein-albumin ratio; NLR, neutrophil-lymphocyte ratio; PLR, platelet-lymphocyte ratio; PNI, prognostic nutritional index; OTUs, operational taxonomic units; ACE, abundance-based coverage estimator; Cor, correlation coefficient; PPI, proton pump inhibitor.

Fig. 3 Alterations in alpha and beta diversity during treatment of patients with ESCC. Alpha diversity included: (a) observed OTUs, (b) Chao1, (c) ACE, and (d) Shannon index. (e) PCoA (unweighted UniFrac distances) of the microbiome during treatment of patients with ESCC. Point 1: before treatment. Point 2: on the fifth day of NAC/NACRT. Point 3: after NAC/NACRT. Point 4: two weeks after surgery. Point 5: three months after surgery. ESCC, esophageal squamous cell carcinoma; OTUs, operational taxonomic units; ACE, abundance-based coverage estimator; PCoA, principal coordinate analysis; NAC, neoadjuvant chemotherapy; NACRT, neoadjuvant chemoradiotherapy.

Fig. 4 Alterations in the relative abundance of the microbiome at the genus level in patients with ESCC. (a) Alterations in the microbial composition at the representative genera. (b) Alterations in the relative abundance of facultative and obligate anaerobes. . Point 1: before treatment. Point 2: on the fifth day of NAC/NACRT. Point 3: after NAC/NACRT. Point 4: two weeks after surgery. Point 5: three months after surgery. ESCC, esophageal squamous cell carcinoma; NAC, neoadjuvant chemotherapy; NACRT, neoadjuvant chemoradiotherapy.

499 **List of supporting information:**

500 Online Resource 1. Study design.

501 Online Resource 2. Blood test data and prognosis-related nutritional score in patients
502 with ESCC before treatment.

503 Online Resource 3. Alterations of body mass index and blood test data in patients with
504 ESCC.

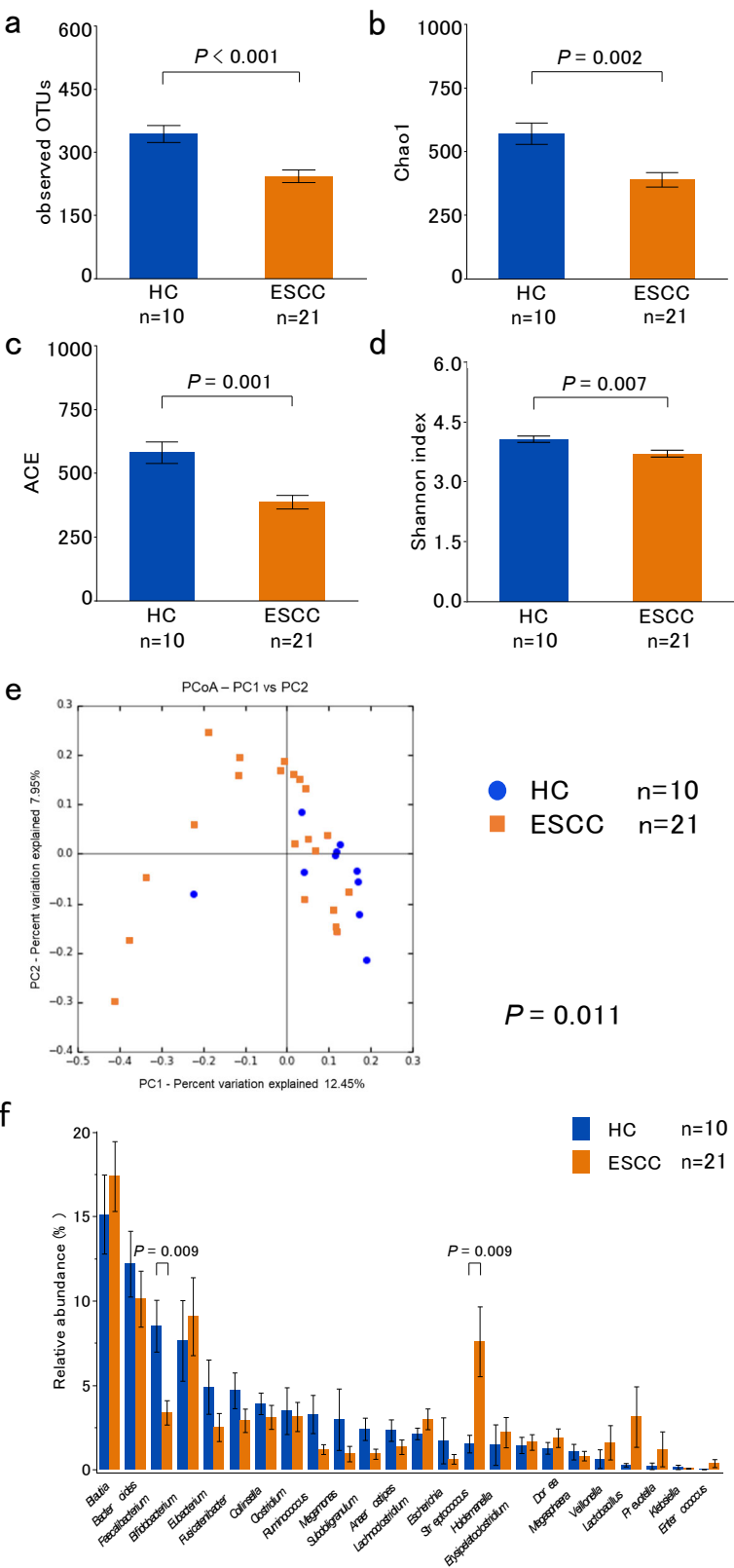
505 Online Resource 4. Comparison of the gut microbiome at the phylum and genus levels
506 between HCs and patients with ESCC.

507 Online Resource 5. Alterations in the relative abundance of the microbiome at the
508 phylum level in patients with ESCC.

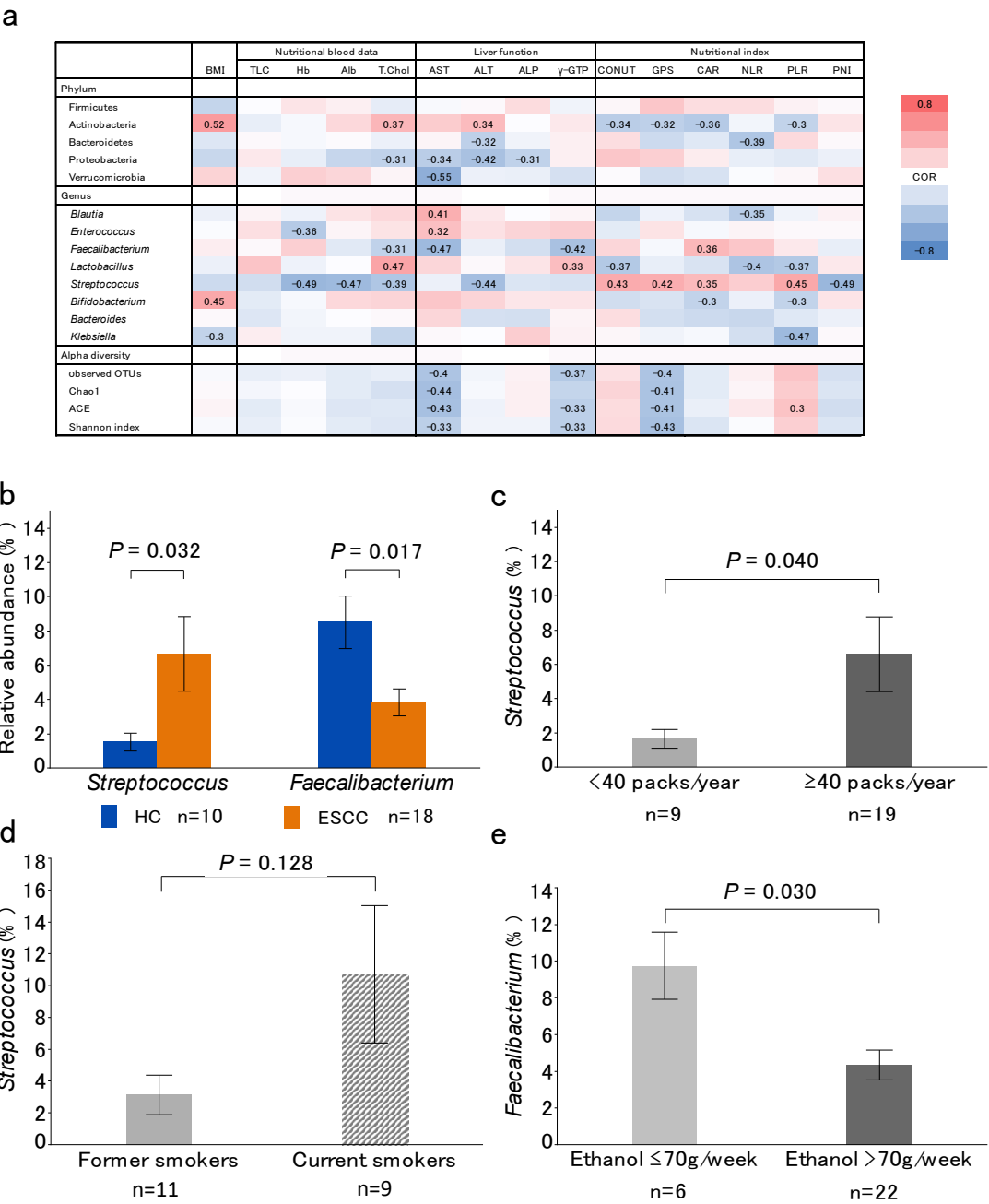
509 Online Resource 6. Relationship between the microbiome and clinical outcomes in
510 patients with ESCC.

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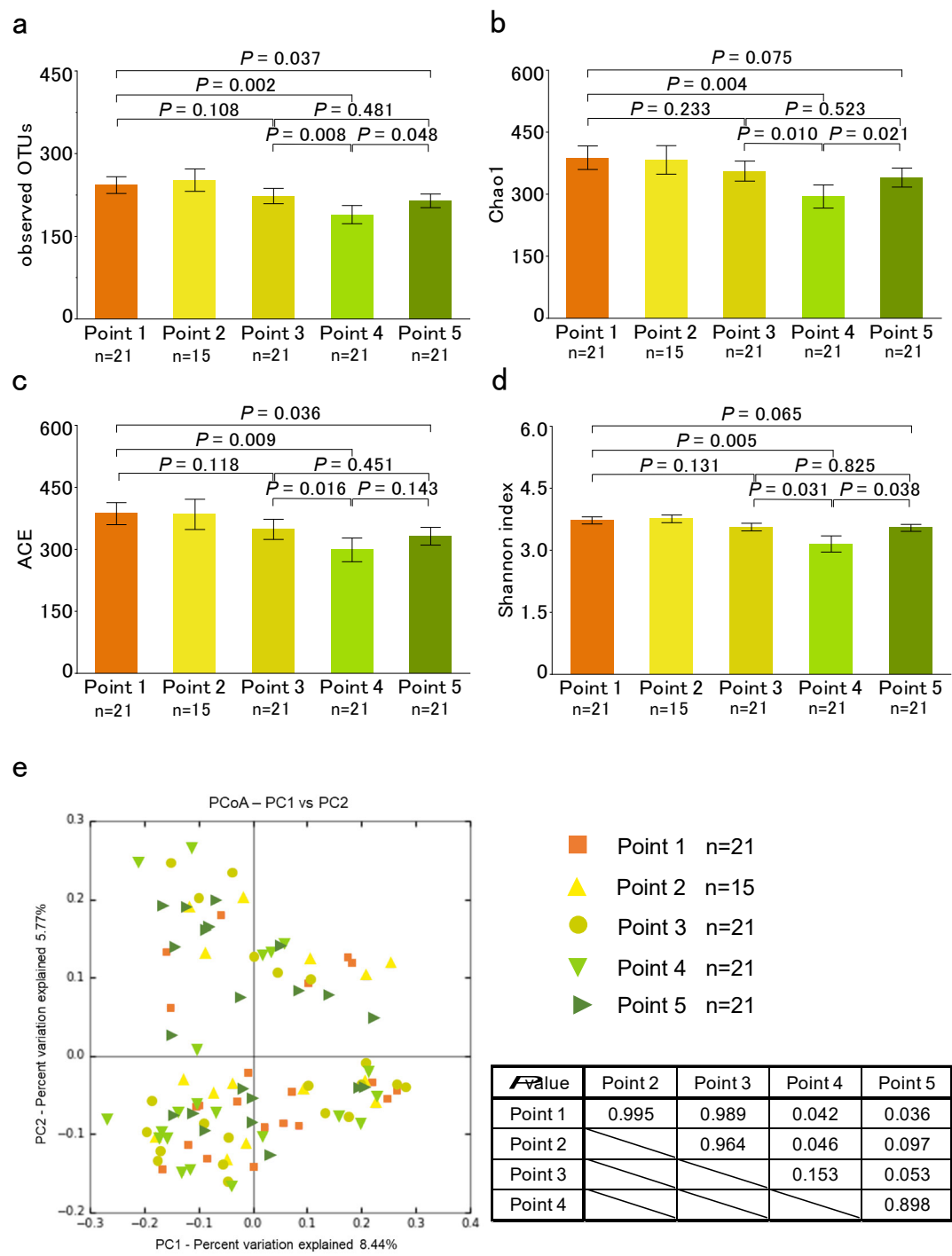
512 Fig. 1



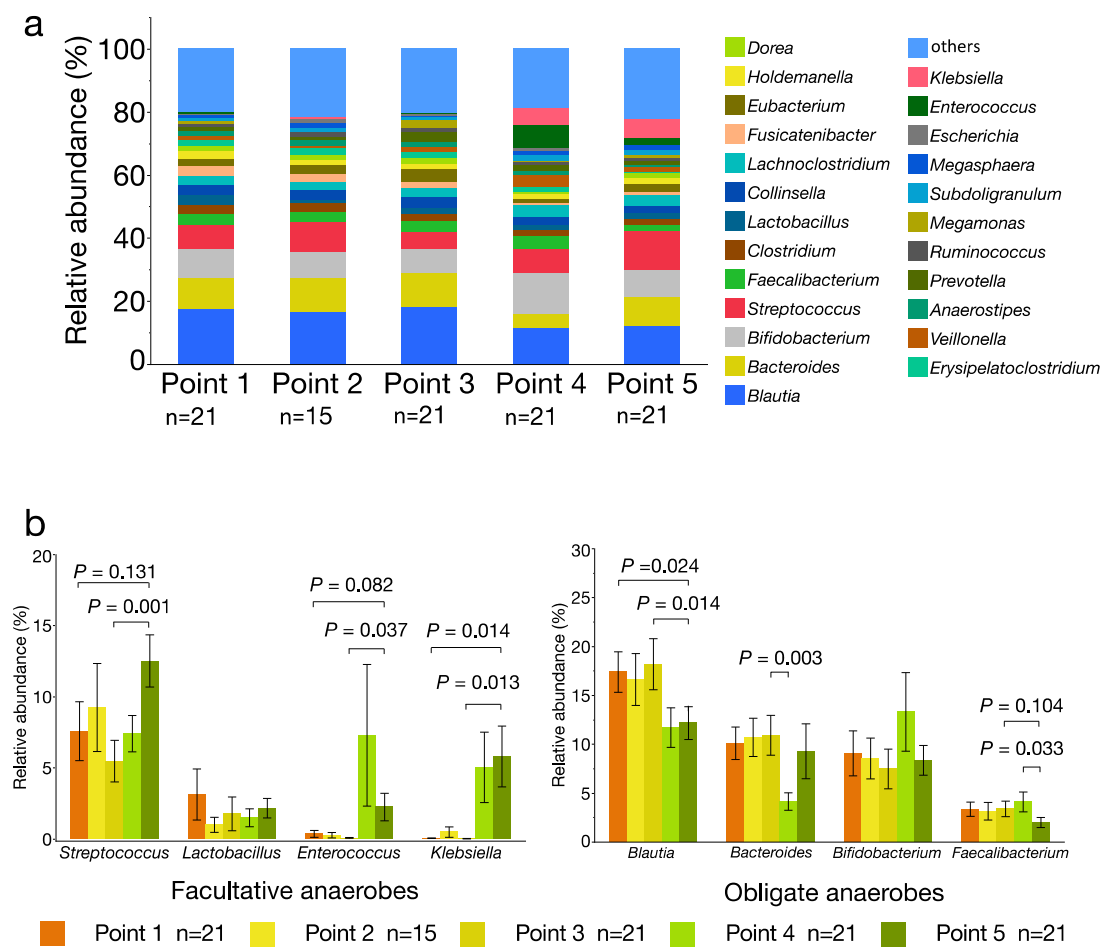
514 Fig. 2



517 Fig. 3



519 Fig. 4



521 Table 1. Characteristics of HCs and patients with ESCC

Factor	HCs	Patients with ESCC	P-value
	n=10 (%)	n=21 (%)	
Age, years			
Median, range	51.5 (50–61)	69 (55–79)	< 0.001
Sex			
Male	8 (80)	14 (67)	0.677
Female	2 (20)	7 (33)	
Body mass index, kg/m ²			
Median, range	22.9 (17.8–26.1)	22.1 (15.7–25.8)	0.352
Tobacco consumption			
≥40 packs/year	2 (20)	17 (81)	< 0.001
<40 packs/year	8 (80)	4 (19)	
Alcohol consumption			
>70 g of ethanol/week	4 (40)	20 (95)	0.002
≤70 g of ethanol/week	6 (60)	1 (5)	
Alcohol flushing response			
Negative	5 (50)	5 (24)	0.29
Positive	5 (50)	14 (67)	
Unknown	0 (0)	2 (9)	
Tumor location			
Cervical esophagus		1 (5)	
Upper thoracic esophagus		1 (5)	

Middle thoracic esophagus	11 (52)
Lower thoracic esophagus	8 (38)
Depth of tumor invasion (TNM 7th)	
cT1	3 (14)
cT2	5 (24)
cT3	12 (57)
cT4	1 (5)
Clinical <i>N</i> factor	
cN (-)	10 (48)
cN (+)	11 (52)
Neoadjuvant treatment	
FP therapy	13 (62)
DCF therapy	6 (29)
FP plus radiation therapy	2 (9)
Surgical procedure	
Minimally invasive surgery†	20 (95)
Minimally invasive surgery plus	1 (5)
TPLE	
Postoperative complications	
Anastomotic leakage	1 (5)
Pneumonia	3 (14)
Ileus	1 (5)
Others	3 (14)

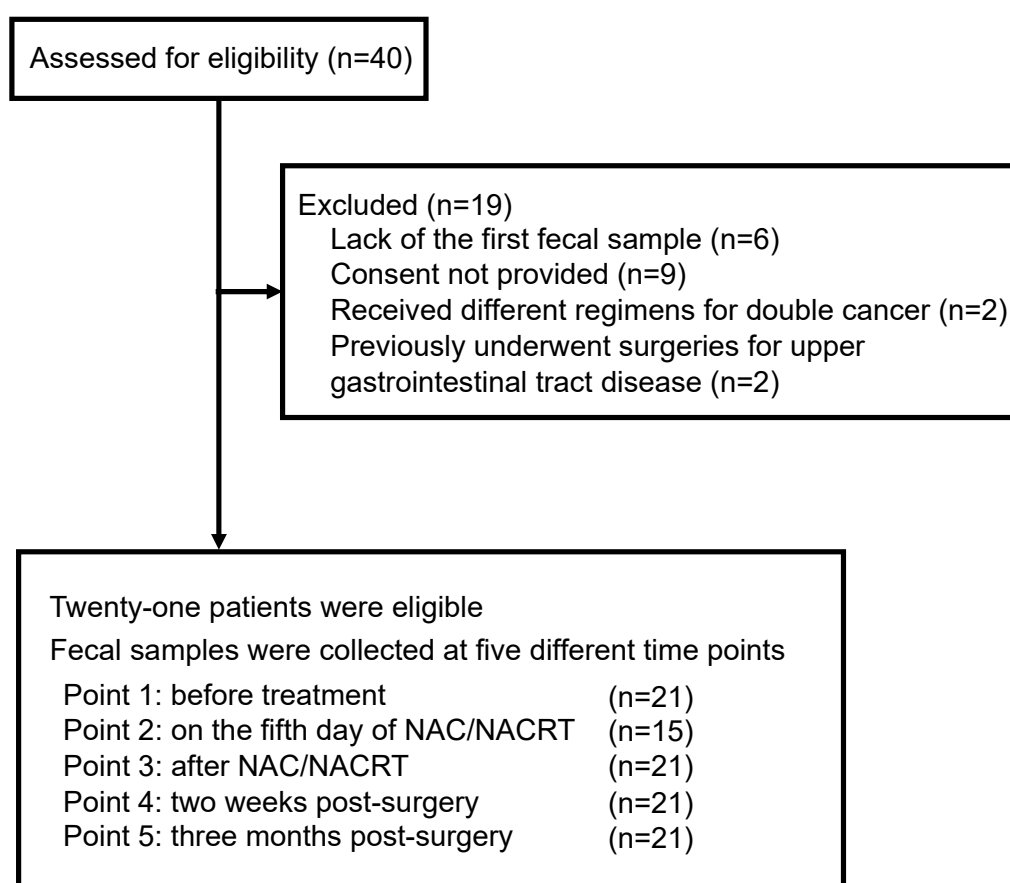
None	13 (62)
Pathological therapeutic effects	
Grade 2 or 3	7 (33)
Grade 0 or 1	14 (67)

522

523 HC: healthy controls, ESCC: esophageal squamous cell carcinoma, FP: 5-fluorouracil plus
524 cisplatin, DCF: docetaxel plus cisplatin, and 5-fluorouracil, TPLE: total
525 pharyngolaryngoesophagectomy, †Minimally invasive surgery includes robot-assisted and
526 thoracoscopic subtotal esophagectomy.

527 Grade 3: markedly effective, Grade 2: moderately effective, Grade 1: slightly effective, Grade 0:
528 ineffective

529



Online Resource 1. Study design.

NAC: neoadjuvant chemotherapy, NACRT: neoadjuvant chemoradiotherapy

Online Resource 2

Blood test data and prognosis-related nutritional scores in patients with ESCC before treatment

Factor	Value
WBC, 10 ³ /μL	6.4 (3.6–17.6)
TNC, 10 ³ /μL	3.8 (1.7–14.2)
TLC, 10 ³ /μL	1.5 (0.9–2.4)
Hb, g/dL	13.2 (8.2–16.2)
PLT, 10 ³ /μL	248 (161–534)
Alb, g/dL	4.1 (3.2–4.7)
T.Chol, mg/dL	176 (144–257)
AST, U/L	20 (14–46)
ALT, U/L	12 (6–28)
ALP, U/L	189 (67–370)
γ-GTP, U/L	24 (13–353)
CRP, mg/dL	0.08 (0.02–5.47)
CONUT score, (n)	
0–1	13
2–8	8
GPS, (n)	
0	16
1–2	5
CAR	0.012 (0.004–1.709)
NLR	2.84 (1.35–6.91)
PLR	158 (110–356)
PNI	48.1 (37.3–58.8)

ESCC: esophageal squamous cell carcinoma, WBC: white blood cell, TNC: total neutrophil count, TLC: total leukocyte count, Hb: hemoglobin, PLT: platelet, Alb: albumin, T.Chol: total cholesterol, AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, γ-GTP: γ-glutamyl transpeptidase, CRP: C-reactive protein, CONUT: controlling nutritional status, GPS: Glasgow prognostic score, CAR: C-reactive protein-albumin ratio, NLR: neutrophil-lymphocyte ratio, PLR: platelet-lymphocyte ratio, PNI: prognostic nutritional index

547 Online Resource 3.

548 Alterations of body mass index and blood test data in patients with ESCC

549

550

Factor	Point 1	Point 2	Point 3	Point 4	Point 5
BMI, kg/m ²	22.1 (15.7–25.8)	21.3 (16.8–27.2)	21.9 (16.6–26.0)	20.7 (15.2–27.4)*	19.3 (16.6–22.9)*
WBC, 10 ³ /μL	6.4 (3.6–17.6)	6.4 (3.2–16.6)	5.1 (3.1–8.1)*	6.5 (4.3–10.5)	4.6 (2.4–9.1)*
TNC, 10 ³ /μL	3.8 (1.7–14.2)	4.9 (1.7–15.4)	3.2(1.4–5.1)*	4.4 (2.3–8.2)	2.5 (0.7–7.6)*
TLC, 10 ³ /μL	1.5 (0.9–2.4)	1.0 (0.4–3.0)	1.5 (0.5–2.5)	1.0 (0.5–2.0)*	1.1 (0.7–2.3)*
Hb, g/dL	13.2 (8.2–16.2)	12.4 (8.4–16.2)*	11.7 (8.7–14.2)*	10.2 (7.6–13.2)*	11.4 (8.7–13.9)*
PLT, 10 ³ /μL	248 (161–534)	210 (104–433)*	219 (130–317)*	434 (13.7–737)*	205 (132–411)*
Alb, g/dL	4.1 (3.2–4.7)	3.6 (2.9–4.2)*	4.0 (2.9–4.7)	3.2 (2.4–4.5)*	3.8 (1.8–4.3)*
AST, U/L	20 (14–46)	21 (16–45)	19 (11–25)*	18 (10–29)	20 (13–41)
ALT, U/L	12 (6–28)	19 (11–141)*	11 (6–20)	17 (9–54)*	15 (6–44)

551

552 ESCC: esophageal squamous cell carcinoma, Point 1: before treatment, Point 2: on the

553 fifth day of NAC/NACRT, Point 3: after NAC/NACRT, Point 4: two weeks post-

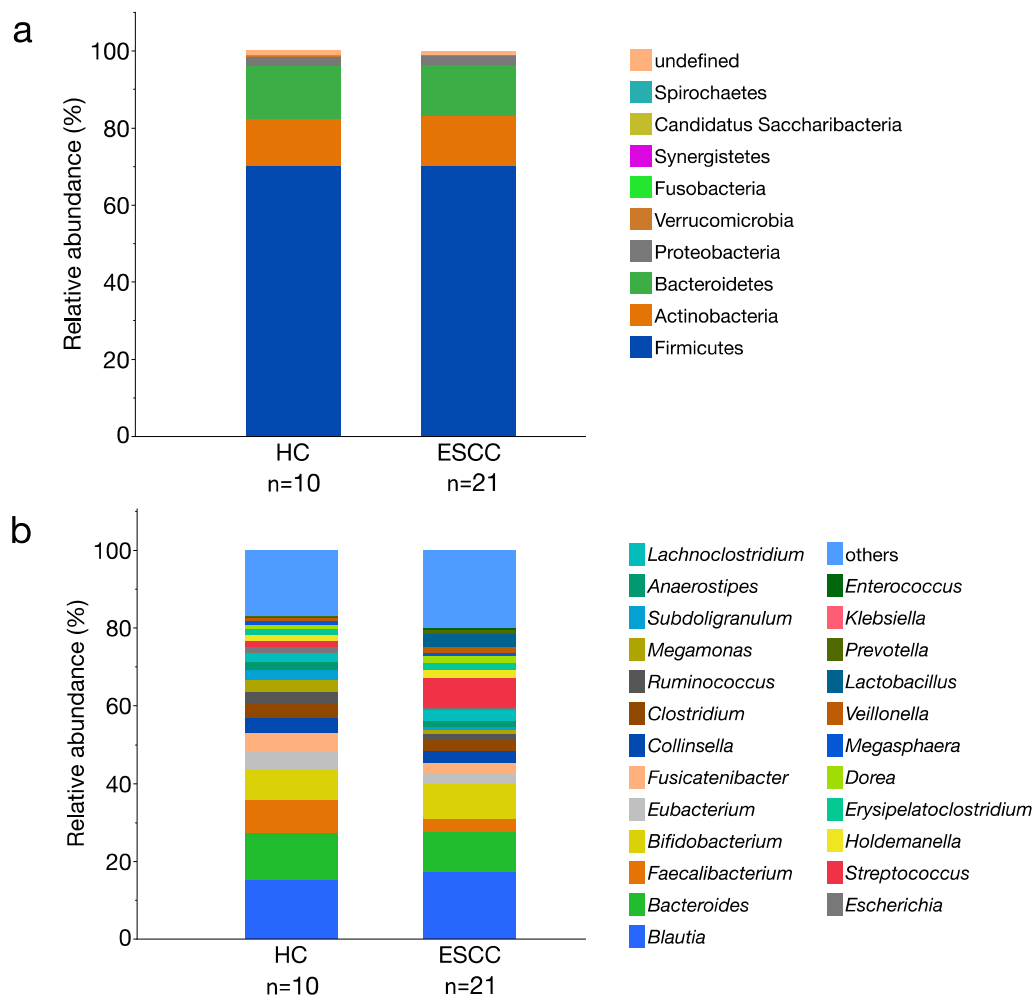
554 surgery, and Point 5: three months post-surgery. BMI: body mass index, WBC: white

555 blood cell, TNC: total neutrophil count, TLC: total leukocyte count, Hb: haemoglobin,

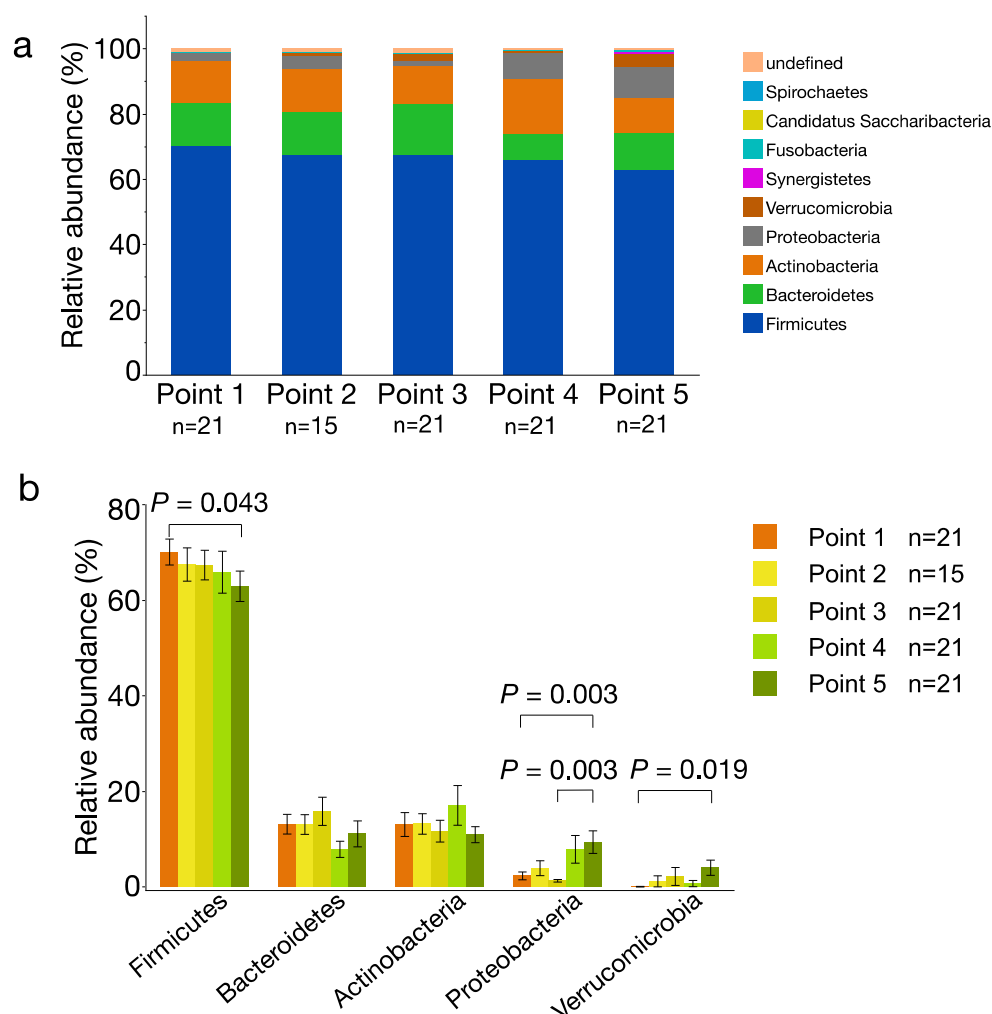
556 PLT: platelet, Alb: albumin, AST: aspartate aminotransferase, ALT: alanine

557 aminotransferase. * $P < 0.050$ versus Point 1

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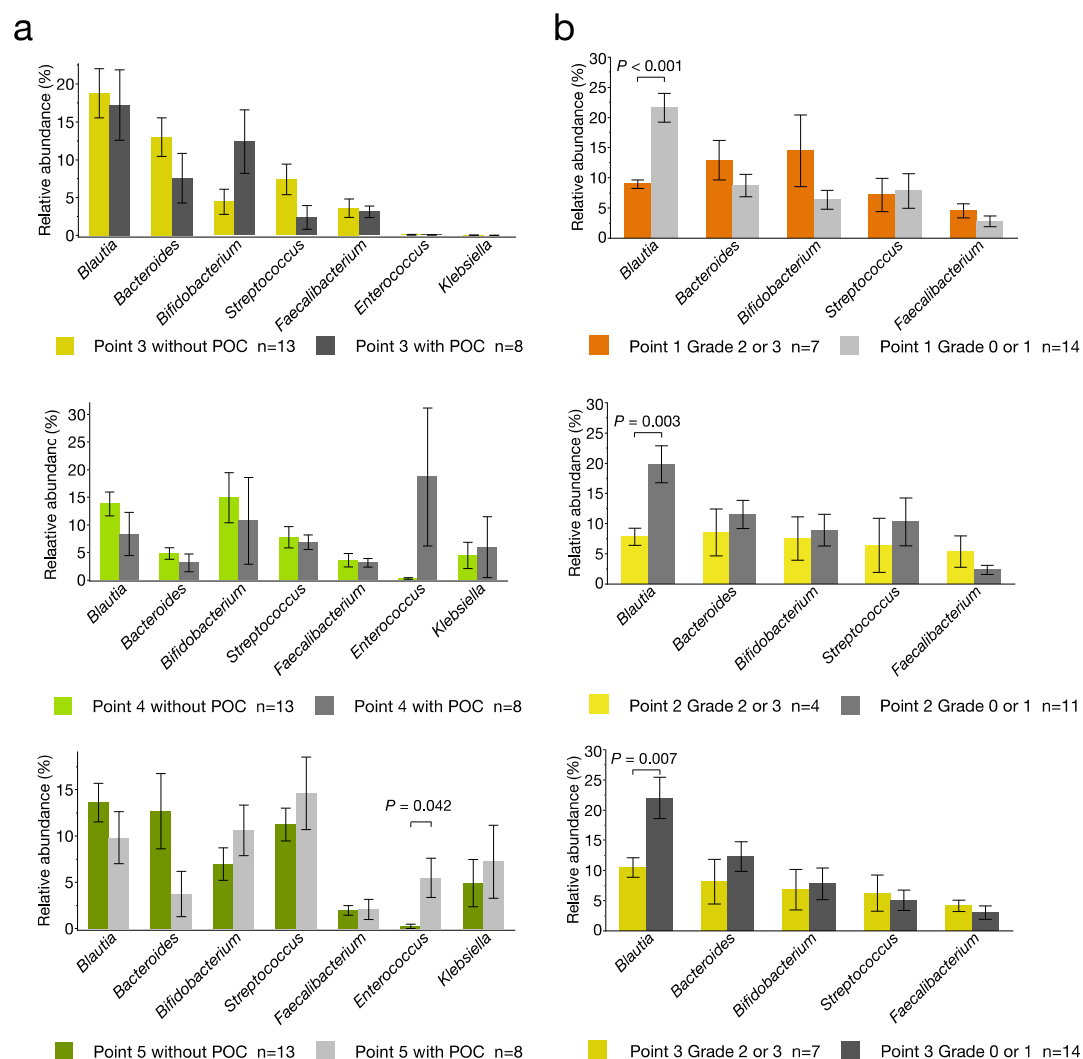


Online Resource 4. Comparison of the gut microbiome at the phylum and genus levels between HCs and patients with ESCC. (a) The composition of the phylum level. (b) The composition of the major genera. HC: healthy control, ESCC: esophageal squamous cell carcinoma.



Online Resource 5. Alterations in the relative abundance of the microbiome at the phylum level in patients with ESCC.

(a) Alterations in the microbial composition at the phylum level during treatment. (b) Alterations of the major phyla. Point 1: before treatment, Point 2: on the fifth day of NAC/NACRT, Point 3: after NAC/NACRT, Point 4: two weeks post-surgery, and Point 5: three months post-surgery. ESCC: esophageal squamous cell carcinoma, NAC: neoadjuvant chemotherapy, NACRT: neoadjuvant chemoradiotherapy.



Online Resource 6. Relationship between the microbiome and clinical outcomes in patients with ESCC.

(a) Comparison of the relative abundance of the gut microbiome according to postoperative complications. (b) Comparison of high (Grade 2 or 3) and low (Grade 0 or 1) pathological therapeutic effects groups. Point 1: before treatment, Point 2: on the fifth day of NAC/NACRT, Point 3: after NAC/NACRT, Point 4: two weeks post-surgery, and Point 5: three months post-surgery. ESCC: esophageal squamous cell carcinoma, NAC: neoadjuvant chemotherapy, NACRT: neoadjuvant chemoradiotherapy, POC: postoperative complication.