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Author(s)	Hino, Akihisa; Kusakabe, Shinsuke; Oka, Kentaro et al.
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RESEARCH LETTER

Enterococcus proliferates via the phosphotransferase system in the disrupted gut microbiota after allogeneic haematopoietic stem cell transplantation

To the Editor,

High-throughput approaches, metagenomics and metabolomics have advanced our understanding of the relationship between the microbiota and the host. It is widely recognized that the gut microbiota undergoes alterations after allogeneic haematopoietic stem cell transplantation (allo-HSCT).^{1,2} Importantly, the modulation of the gut microbiota is a novel strategy to reduce post-transplant complications such as acute graft-versus-host disease (aGVHD). Various treatments targeting the gut microbiota, including probiotics and faecal microbiota transplantation, are currently under investigation for patients with allo-HSCT.^{3–5} Several studies have suggested that an increased *Enterococcus* population worsens post-transplant complications.^{4,6,7} Notably, strategies to suppress *Enterococcus* proliferation have been proposed, including phage-derived enzymes, endolysins and lactose restriction.^{8,9} However, it is not yet clear which substrates and metabolic pathways are used by *Enterococcus* for growth and colonization after allo-HSCT. Here, we analysed metabolic pathways in the gut microbiota after allo-HSCT.

This study is a post hoc analysis of our retrospective study evaluating the effects of prophylactic probiotic administration on the gut microbiota after HSCT.³ We analysed the gut microbiota of 40 patients who underwent allo-HSCT at Osaka University and Tokai University Hospital between May 2006 and 2017. Three patients with insufficient faecal samples were excluded from the study. The study was approved by the ethics committees of both institutions and conducted in accordance with the Declaration of Helsinki. All patients provided informed consent for enrolment. Baseline faecal samples were collected before initiation of the conditioning regimens. Post-treatment samples were collected weekly from the start of the conditioning regimen until 35 days after allo-HSCT. Patients placed faecal samples into disposable sterile collection tubes (76 × 20 mm; Sarstedt, Nümbrecht, Germany), and the samples were preserved in RNAlater™ stabilization solution (Thermo Fisher Scientific, Waltham, MA, USA) at –80°C. A total of 196 faecal samples were collected from 37 patients. The methods for sample processing and 16S rRNA gene sequencing are described in detail in our previous study.³ All samples were sequenced

for the 16S rRNA V3–V4 region using an Illumina MiSeq platform. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) (version 2.4.1) was used to predict Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs from the taxonomic composition inferred from amplicon sequencing. All statistical analyses were performed in R (v4.1.1). Alpha diversity was quantified using the Shannon index. Differences in Shannon index between time points were assessed with the Wilcoxon signed-rank test, comparing each post-transplant time point with Day –7 for the same patients; *p*-values were adjusted across the six comparisons using the Benjamini–Hochberg procedure to control the false discovery rate (FDR, *q* < 0.05). Community composition (β -diversity) was visualized by principal coordinates analysis (PCoA) on the Bray–Curtis distance matrix computed from all samples, and group differences were tested by permutational analysis of variance (PERMANOVA) with 9999 permutations using the same distance matrix. Spearman's rank correlations between bacterial taxa and metabolic pathways were computed, and *p*-values across all tested taxa (*m* = 356) were adjusted using the Benjamini–Hochberg FDR procedure (*q* < 0.05).

The study population comprised 15 female and 22 male patients with a mean age of 44 years (range: 20–64). Twenty-one patients developed mild aGVHD (grades 0–1), while 16 patients developed severe aGVHD (grades 2–4). [Table S1](#) lists the patient characteristics. The median time to aGVHD onset was 37 days after transplantation. Twelve patients with severe aGVHD developed lower gastrointestinal graft-versus-host disease (lower GI-GVHD). All patients received oral levofloxacin as prophylactic antibacterial therapy. Fourth-generation cephalosporins and carbapenems were administered to 15 and 33 patients respectively.

[Figure S1](#) shows the changes in the diversity of the gut microbiota before and after transplantation. Reduced alpha diversity was observed from Day 14 to Day 35 after transplantation. We used PCoA to compare metabolic pathway structures over time between patients with mild aGVHD and those with severe aGVHD ([Figure S2A](#)). A significant difference was observed in the metabolic pathway structures between the two groups on Day 14 after

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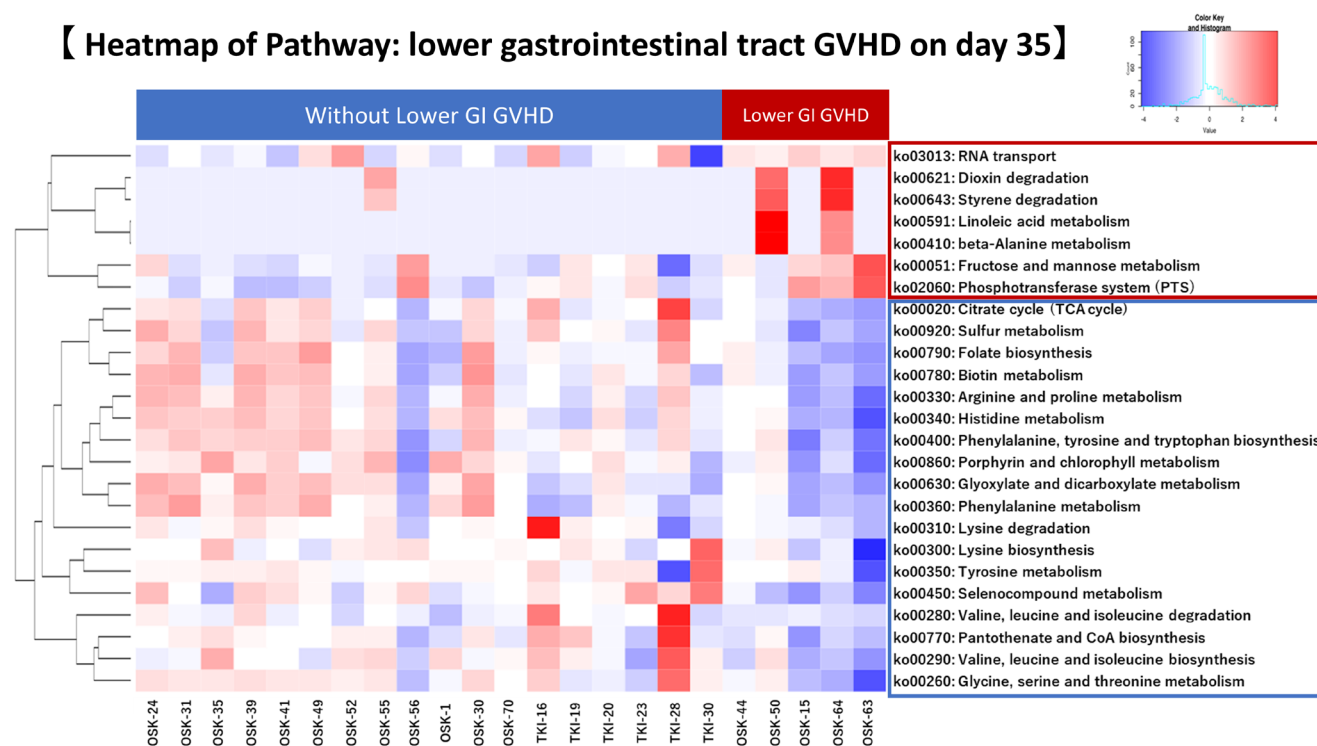
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HSCT ($p=0.0120$). A comparison of patients with and without lower GI-GVHD revealed a significant difference ($p=0.0370$) in metabolic pathways at 35 days after HSCT (Figure S2B). We conducted a detailed comparison of the metabolic pathways at these two time points (14 and 35 days after HSCT).

Metagenomic prediction identified 170 metabolic pathways on Day 14 in patients with mild and severe aGVHD. Significant upregulation was observed in 32 of these pathways between the two groups. Figure S3 shows a heat map of these 32 pathways. The functional pathways exhibiting high expression levels in severe aGVHD included the

(A)

【Heatmap of Pathway: lower gastrointestinal tract GVHD on day 35】



(B)

【Spearman correlation vs PTS】

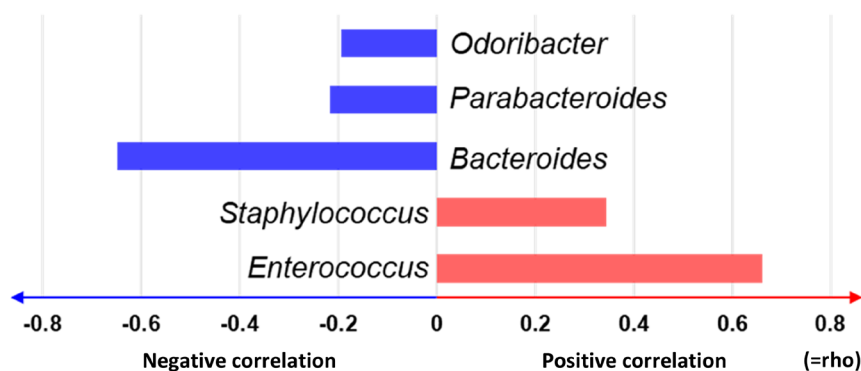
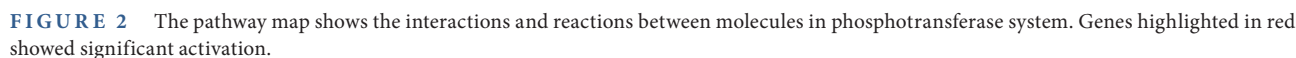


FIGURE 1 Metabolic pathways activated by lower gastrointestinal tract graft-versus-host disease (lower GI GVHD) on Day 35. (A) The heat map shows the comparison of metabolic pathways with and without lower GI GVHD on Day 35. The metabolic pathways surrounded by red squares were activated in cases that developed lower GI GVHD. In contrast, the pathways surrounded by blue squares were active in the group that did not develop lower GI GVHD. (B) Bacteria strongly associated with the phosphotransferase system (PTS) are shown using Spearman correlation. *Enterococcus* and *Staphylococcus* showed a strong positive correlation with PTS.

activation of these metabolic pathways supported previous reports.¹¹ Although we did not measure metabolite levels directly, these data raise the possibility that microbially derived TCA intermediates could influence the local mucosal immune cells.



Furthermore, we compared metabolic pathways in the gut microbiota of patients who developed lower GI-GVHD at 35 days (Figure 1A). Significant differences were found in the activation of 25 pathways in patients with lower GI-GVHD. The phosphotransferase system (PTS) was among the pathways activated in these patients. The PTS is utilized by bacteria such as *Escherichia coli* and *Enterococcus* to take up extracellular sugars. We conducted further analyses to identify the bacteria that correlated with PTS activation.

We conducted a correlation analysis to investigate the relationship between the gut microbiota and PTS in patients with lower GI-GVHD. As expected, the relative abundance of *Enterococcus* showed a strong positive correlation with PTS in the gut microbiota after allo-HSCT ($\rho=0.66$) (Figure 1B). The map of PTS enzyme genes extracted from the KEGG metabolic system is shown in Figure 2. Genes that were significantly activated in the PTS are shown in red, with no genes displaying a significant decrease in activity. All PTS-related metabolic genes correlated positively with the relative abundance of *Enterococcus*. Notably, many of these genes were particularly active in cellobiose and mannose metabolism. The relative abundance of *Enterococcus* was high (25%–75%) in cases where the PTS was activated. These results suggest that *Enterococcus* may utilize the PTS to establish colonization in the intestinal tract after transplantation.

Gut microbial domination of *Enterococcus* after allo-HSCT leads to high rates of GVHD-related mortality. In mouse GVHD models, *Enterococcus* promotes donor T-cell infiltration, increasing CD4⁺ T-cell activation and recruiting TH17 cells in the mucosal lamina propria.⁹ In the human body, enterococci can also induce autoimmune disease by activating T helper 17 immune responses.¹² These effects may be involved in the development of GVHD. Therefore, the modulation of *Enterococcus* outgrowth may improve transplant outcomes.

We discovered that *Enterococcus* utilizes the PTS in the intestinal tract after transplantation. It has been reported that pro-inflammatory enterococci use the PTS to colonize and proliferate.¹³ We suggest that the same mechanism may exacerbate colitis after allo-HSCT. Therefore, regulating the PTS could represent a therapeutic strategy to control *Enterococcus*, requiring the identification of specific metabolic pathways involved. In this study, we observed increased expression of functional genes related to mannose and cellobiose metabolism. All these genes correlated with *Enterococcus*, and increased *Enterococcus* abundance was confirmed in cases with an activated PTS pathway. This finding suggests that these metabolic pathways are involved in the growth and maintenance of *Enterococcus*. Our findings provide a foundation for developing novel nutritional strategies to control *Enterococcus*. This study also demonstrated a negative correlation between *Enterococcus* and *Bacteroides*. A decrease in *Bacteroides ovatus* abundance causes exacerbation of GVHD.¹⁴ Regulating the PTS may not only control *Enterococcus* but also help maintain protective bacteria against GVHD.

Fan et al. used a mouse model of chronic colitis to demonstrate that glucosamine metabolism via the PTS can worsen the inflammation caused by *Enterococcus*.¹⁵ However, we

did not observe any significant differences in the genes involved in glucosamine metabolism in patients with lower GI-GVHD. Differences in the composition and function of the intestinal microbiota and immune responses between humans and mice may account for the observed discrepancies in results. Moreover, we confirmed the metabolic pathways of the gut microbiota over time and found that PTS activation became significant 35 days after transplantation. These results indicate that initiating nutritional restriction before Day 35 may be optimal for managing *Enterococcus* overgrowth and subsequent intestinal inflammation.

One limitation of this study was its reliance on a small number of cases, which may limit the generalizability of our findings. Additionally, the observed activation of these metabolic pathways may represent a response unique to certain individuals or clinical contexts. Another limitation is our use of PICRUSt2, an indirect method, to infer bacterial functional metabolic pathways. Direct evaluation of metabolites is required to confirm their presence and concentration.

Despite these limitations, we identified the metabolic pathways involved in the growth of *Enterococcus* following allo-HSCT. These findings may form the basis for a new nutritional strategy aimed at controlling *Enterococcus*. This strategy has the potential to be a cost-effective approach to minimize the inappropriate use of antibiotics and prevent aGVHD.

KEYWORDS

acute GVHD, allogeneic haematopoietic stem cell transplantation, gut microbiota, metabolites, the phosphotransferase system

AUTHOR CONTRIBUTIONS

A. Hino, K.O. and H.K. designed the study; A. Hino and K.F. performed the statistical analyses; A. Hino, K.O., H.K. and K.F. contributed to manuscript preparation; K.O., H.K., T.A., A. Hayashi and M.T. performed the metagenomic analyses; A. Hino, S.K. and K.F. managed the clinical data; and S.K., K.F. and M.O. critically revised the manuscript; K.T. and M.M. supervised the study. All authors approved the submitted and final versions of the manuscript.

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CONFLICT OF INTEREST STATEMENT

O.K., H.K., A. Hayashi, T.A. and M.T. report employment for Miyarisan Pharmaceutical Co. Ltd. M.M. reports a consulting/advisory role for Miyarisan Pharmaceutical Co. Ltd. K.F. and M.O. received research funding from Miyarisan Pharmaceutical Co. Ltd. The other authors declare no conflicts of interest associated with this study.

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ETHICS STATEMENT

The Institutional Review Board of The University of Osaka Hospital (Osaka, Japan) approved this study (approval number 21244).

PATIENT CONSENT STATEMENT

This study was conducted in compliance with the Declaration of Helsinki (2013 and 2024). All patients provided written informed consent for sample collection.

DATA AVAILABILITY STATEMENT

For original data, please contact kfukushi@bldon.med.osaka-u.ac.jp.

Akihisa Hino¹ 
 Shinsuke Kusakabe¹ 
 Kentaro Oka²
 Hayami Kudo²
 Tadashi Ariyoshi²
 Atsushi Hayashi²
 Motomichi Takahashi²
 Makoto Onizuka³
 Kiyoshi Takeda^{4,5,6}
 Masaki Mori⁷
 Naoki Hosen^{1,5,6}
 Kentaro Fukushima¹ 

¹Department of Haematology and Oncology, The University of Osaka Graduate School of Medicine, Suita, Japan

²R&D Division, Central Research Institute, Miyarisan Pharmaceutical Co. Ltd., Saitama, Japan

³Department of Hematology/Oncology, Tokai University School of Medicine, Isehara, Japan

⁴Laboratory of Immune Regulation, Department of Microbiology and Immunology, Graduate School of Medicine, The University of Osaka, Suita, Japan

⁵World Premier International Immunology Frontier Research Centre, The University of Osaka, Suita, Japan

⁶Integrated Frontier Research for Medical Science Division, Institute for Open and Transdisciplinary Research Initiatives (OTRI), The University of Osaka, Suita, Japan

⁷Faculty of Medicine, Tokai University School of Medicine, Isehara, Japan

Correspondence

Kentaro Fukushima, Department of Haematology and Oncology, The University of Osaka Graduate School of Medicine, Suita, Osaka 565-0871, Japan.
 Email: kfukushi@bldon.med.osaka-u.ac.jp

ORCID

Akihisa Hino  <https://orcid.org/0000-0002-7331-2713>
 Shinsuke Kusakabe  <https://orcid.org/0000-0002-9814-7611>
 Kentaro Fukushima  <https://orcid.org/0000-0002-8003-2584>

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SUPPORTING INFORMATION

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