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Research article

Microbial and host factors contribute to bloodstream infection in a pediatric acute lymphocytic leukemia mouse model

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ABSTRACT

Background: Hematological malignancies are the most common cancers in the pediatric population, and T-cell acute lymphocytic leukemia (T-ALL) is the most common hematological malignancy in children. Bloodstream infection (BSI) is a commonly occurring complication in leukemia due to underlying conditions and therapy-induced neutropenia. Several studies identified the gut microbiome as a major source of BSI due to bacterial translocation. This study aimed to investigate changes in the intestinal and fecal microbiome, and their roles in the pathophysiology of BSI in a pediatric T-ALL mouse model using high-throughput shotgun metagenomics sequencing, and metabolomics.

Results: Our results show that BSI in ALL is characterized by an increase of a mucin degrading bacterium (*Akkermansia muciniphila)* and a decrease of butyrate producer *Clostridia spp.*, along with a decrease in short-chain fatty acid (SCFA) concentrations and differential expression of tight junction proteins in the small intestine. Functional analysis of the small intestinal microbiome indicated a reduced capability of SCFA synthesis, while SCFA supplementation ameliorated the development of BSI in ALL.

Conclusions: Our data indicates that changes in the microbiome, and the resulting changes in levels of SCFAs contribute significantly to the pathogenesis of bloodstream infection in ALL. Our study provides tailored preventive or therapeutic approaches to reduce BSI-associated mortality in ALL.

1. Introduction

Leukemia is a group of cancers affecting the hematopoietic system. Over 400,000 people are diagnosed with leukemia worldwide, and approximately 309,000 people die of this disease annually [1]. Hematological malignancies are the most common cancers during childhood, and leukemia comprises 30% of all pediatric cancers. Ninety-seven percent of childhood leukemias are acute, and acute lymphocytic leukemia (ALL) is the most frequently occurring pediatric leukemia, comprising 80% of childhood leukemias, with the incidence highest between 2-5 years of age [2].

Bloodstream infection (BSI) is a severe, often fatal complication in leukemia due to underlying conditions and therapy-induced neutropenia. BSIs are also the most concerning complications in the treatment of leukemia, as they lead to increased mortality rates, extended hospital stays and delays or dose reductions in chemotherapy [3, 4]. Despite the recognized importance and severity of infectious complications, little is known about the pathophysiology of endogenous infections. The intestinal microbiome has been recognized as a major source of BSI, as ALL [5] and therapy-induced neutropenia leads to increased intestinal permeability and subsequent translocation of bacteria [6, 7].

Several studies describe microbiome changes [8, 9, 10] in feces from ALL patients. However, chemotherapy and antibiotics treatments, as part of the standard protocol for most ALL patients, heavily influence microbiome composition. That is one of the possible reasons why findings in microbiome compositions differ significantly between studies [11]. In addition, clinical studies only analyze feces, which may not reflect the complex development of the microbiome in the small intestine and the colon [12]. We hypothesized that the compositions and metabolites of microbiome in both intestine and feces contribute together to the pathophysiology of gut bacterial translocation and subsequent BSI in pediatric patients with ALL. In this study, we applied shotgun metagenomics sequencing to a pediatric T-ALL mouse model to investigate the changes in composition, microbial metabolites and metabolic processes of the bacterial, fungal and viral microbiome by analyzing intestinal contents and longitudinal investigation of feces.

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2. Methods

2.1. Mice

Three-week-old female Nod/Scid mice were used in this study (Jackson Laboratories, Bar Harbor, ME, USA). All experimental procedures were approved by the University of Illinois College of Medicine at Peoria Institutional Animal Care and Use Committee review board (protocol number #1643433) and all methods and procedures were performed in accordance with the relevant guidelines and regulations. Leukemia was induced (n = 8 leukemic and n = 8 controls) as previously described [13] using 10^6 CCL-119 cells (lymphoblasts from a pediatric acute lymphocytic leukemia patient, from ATCC) in 50 μl PBS (pH = 7.84) and equal volume of PBS without cells for controls per mice. The average weight of the mice were 17.5 ± 1 gr for controls, and 17.1 ± 2.4 g for leukemic mice ($p = 0.6$). Mice were housed in a barrier room with a 12-hr dark–light cycle, in cages (four mice/cage) with UV-treated shred bedding. Water and regular chow were provided ad libitum. Fecal and blood samples were freshly collected weekly from arrival (week 1) until the end of the experiment (week 5) with the exception of the xenograft (week 2). Blood samples were collected using tail nicking. Fecal DNA was extracted from 50 mg fecal material using the QIAmp DNA Stool mini kit, DNA from blood was extracted using the QIAmp DNA Blood mini kit. Mice were sacrificed at the end of the 5th week, small intestines (n = 8 in the control group, n = 6 in the leukemic group) and colons (n = 8 in the control group, n = 6 in the leukemic group) were removed, and intestinal contents were thoroughly washed with 1ml sterile PBS (pH = 7.84). DNA from intestinal content was extracted using the QIAmp DNA Stool mini kit, while DNA from intestinal tissue was extracted using the DNeasy Blood and Tissue kit (all from Qiagen). One small intestinal content from a leukemic mouse did not have sufficient DNA for sequencing following extraction. RNA was extracted from intestinal tissue using the Zymo Quick RNA miniprep kit. Euthanasia was performed with cervical dislocation in anesthesia induced by 5% Isothesia in 1.0 L/min oxygen in compliance with the ARRIVE guidelines. The following humane endpoints were used for immediate euthanasia: severe weight loss (>15% from starting weight), body condition score 2, ascites, moribund condition, and dehydration. The mouse model was validated on mouse blood and intestinal samples using TPOX [14].

2.2. Sequencing

Intestinal contents and feces were subjected to shotgun metagenomics, performed on an Illumina HiSeq 4000 instrument using 150 base pair reads with an average read number of over 13M reads per sample. No template control (sterile water) did not result in a library. Reads shorter than 30 base pair or with quality score (≥10% of bases in a read) below 30 were discarded. RTG Core 3.4 was used to filter reads against the mouse genome (GRCm38-p6) and to map against bacterial, fungal and viral genomes by using the composition-meta-pipeline command with default settings [15]. The assay sensitivity and specificity were verified using ZymoBIOMICS Microbial Community Standard II with minimum sensitivity detected at relative abundance of 0.00089 (Suppl Figure 1).

MEGAHIT was used for genome assembly [16], with d = 2 minimum multiplicity for filtering, and minimum contig length was set to 200. Genes were predicted and annotated using Prokka [17], with locus tag counter increment set to 1 using gff3 Genbank-compliant output (–gffver 3, –compliant). Prokka GenBank outputs were converted to KEGMapper for pathway analysis [18] using Prokka2KEGG (https://github.com/SilentGene/Bio-py/tree/master/prokka2kegg).

2.3. RT-qPCR and qPCR

Real-time qPCR to quantify microbial DNA and RT-qPCR to quantify expression changes in intestinal tissues was performed using a Corbett RotorGene 6000 instrument. The PowerUp SYBR Green qPCR and RT-qPCR master mix was used for setting up qPCR and RT-qPCR reactions with 200 nM primer concentrations. Bacteria were quantified using the 530F-806R 16S primers [19], and fungi were quantified using the ITS3-ITS4 primers on DNA extracted from 10 μl blood. Occludin, zonulin and claudin genes were quantified as described by Ohtsuki et al. [20] and MUC2 gene was detected using Qiagen Quantitect Mm_MUC2_2_SG primers with 50 ng of intestinal RNA as a starting material. All PCR reactions were subjected to 2 min denaturation, then cycled at 95 °C for 20'', 50 °C for 30'' and 72 °C for 30''. Mouse GAPDH was used for relative quantification of MUC2, claudin, zonulin and occludin genes, and fold change was determined using the ΔΔCt method [21]. Standard curves were created using *Staphylococcus aureus* and *Saccharomyces cerevisiae* genomic DNA (Sigma, St. Louis, MI, USA) with known concentrations. Samples for short-chain fatty acid (SCFA) concentrations used 50 mg feces prepared in 50 μl 1xPBS and 8% acetic acid and centrifuged at 14,000 RPM for 30 min, and measured in LC-MS (University of Illinois Biotechnology Center, Urbana-Champaign, IL, USA) using the Sciex LC/MS—6500 + Triple Quadrupole Mass Spectrometer. pH was measured using an Accumet AE150 instrument (Fisher Scientific, Pittsburgh, PA, USA). The presence of *A. muciniphila* was confirmed in fecal and intestinal samples using 5'-CAGCACGTGAAGGTGGGGAC-3' forward, and 5'-CCTTGCGGTTGGCTTCAGAT-3' reverse primers as described [22].

2.4. Statistical analysis

Statistical analysis was performed using Student's *t*-test or ANOVA, with significance set to 0.05. Phylogeny was performed using Mega 7 using UPGMA default settings, and PCA graphs were created using STAMP [23]. Linear regression analysis by using the least squares method was used for independent variables, with ANOVA to determine significance with significance level set to 0.05. PERMANOVA was used with Bray-Curtis similarity index in Primer7 (v0.21) with 999 permutations.

2.5. SCFA treatment

An additional 12 NOD/Scid mice were used for SCFA experiments (4 controls, 4 leukemic, 4 leukemic + SCFA treated). A mixture of sodium salts of 100mM acetate, propionate and butyrate dissolved in 100 μl water was administered via oral gavage to leukemic mice (n = 4) every second day following xenograft until the end of the experiment. Untreated leukemic mice (n = 4) and untreated control mice (n = 4) received sterile water via oral gavage.

3. Results

3.1. Bacterial microbiome

3.1.1. Akkermansia muciniphila *abundance increased in ALL feces*

Fecal compositions have been investigated from week 3 to week 5. At week 3, there was no significant difference in the abundance of *A. muciniphila* between control and ALL groups (Figure 1). The relative abundance of *A. muciniphila* significantly increased (Figure 2) from 0.33 (week 3) to 0.71 by week 5 ($p < 0.01$) in ALL. In the control group, there was no significant change in the relative abundance of *A. muciniphila* between weeks (week 3: 0.43, week 5: 0.44, $p = 0.47$). Differences between ALL and control fecal microbiome were investigated with principal component analysis showing clustering of ALL and control groups in week 5 (PERMANOVA $p < 0.05$, Suppl. Figure 2). In order to identify microbial biomarker signatures in feces in week 5, we used the linear discriminant analysis (LDA) effect size method [24]. *A. muciniphila* and *Bacteroides oleiciplenus* were identified as overrepresented in ALL feces, while *Alistipes* and *Clostridium* species were dominant in the control group (Figure 3).

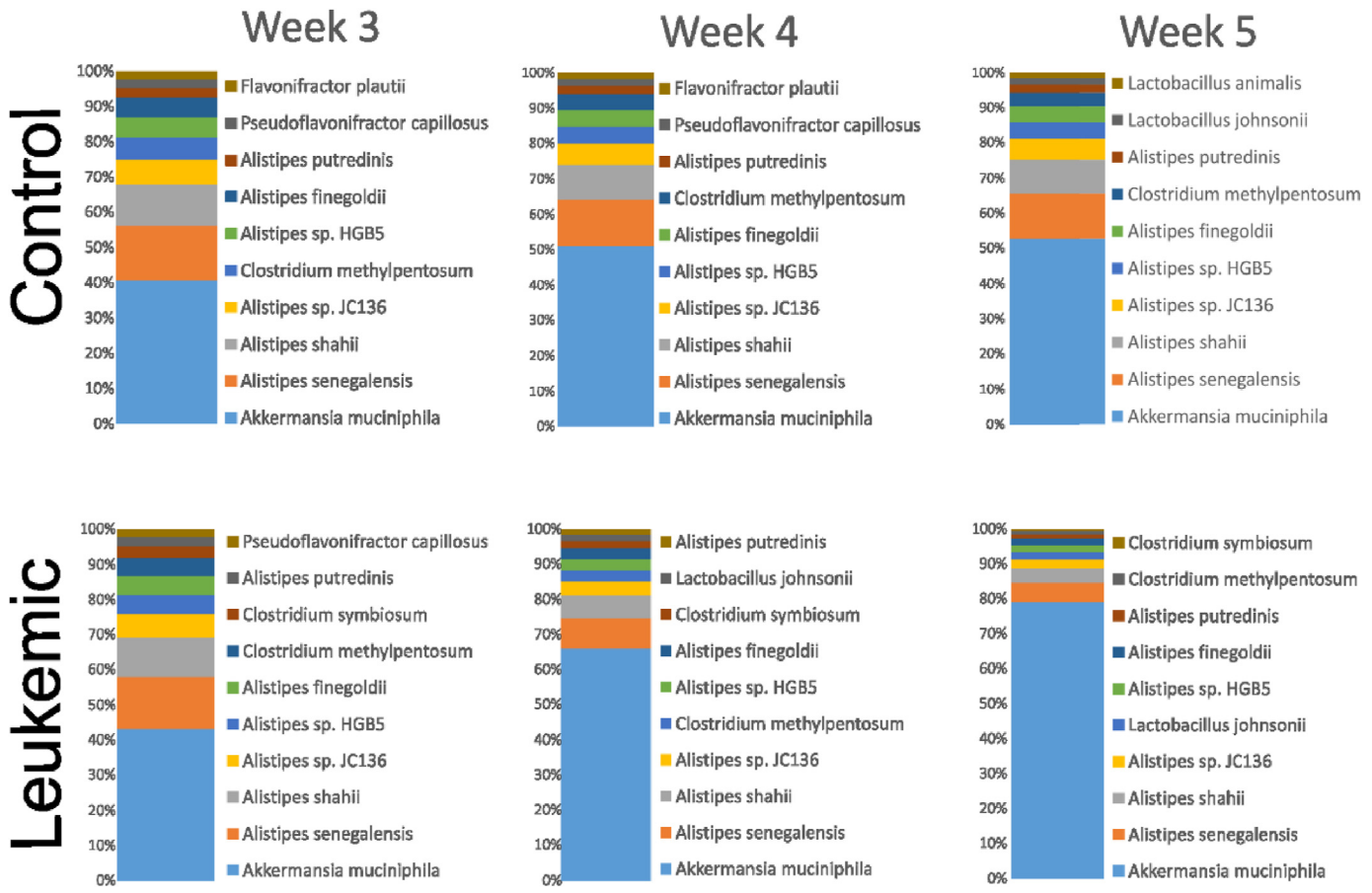

Figure 1. Bacterial compositions of control and ALL feces from week 3 to week 5 show a significant increase of *Akkermansia muciniphila* in ALL. Taxa with the 10 highest abundances are shown.

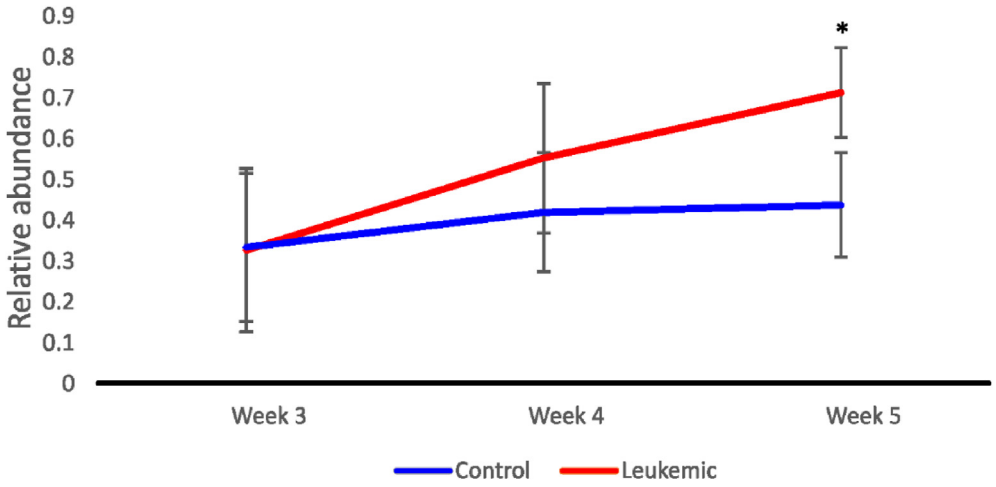

Figure 2. *A. muciniphila* abundances are shown in control and leukemic groups throughout the study. Asterisk represent statistically significant difference and error bars represent standard deviation.

Whole genome sequences were assembled by mapping reads to *A. muciniphila* ATCC BAA-835 (GCF_000020225.1) as reference resulting in an average > 99% coverage, with average coverage depth >15. Phylogenetic tree constructed using the whole genome sequences of *A. muciniphila* showed a clustering of ALL samples from weeks 4 and 5, indicating changes in the species sequence with disease progression (Suppl. Figure 3).

To analyze these sequence differences, we conducted a Pfam domain search [25] to identify potential protein domains that were acquired throughout the disease development (from week 3 to weeks 4 and 5). The most common protein domain which *A. muciniphila* genomes leukemic mice gained was alpha/beta hydrolase (5 samples out of 8), along with AMP-binding enzyme (2/8), and NADH dehydrogenase (1/8).

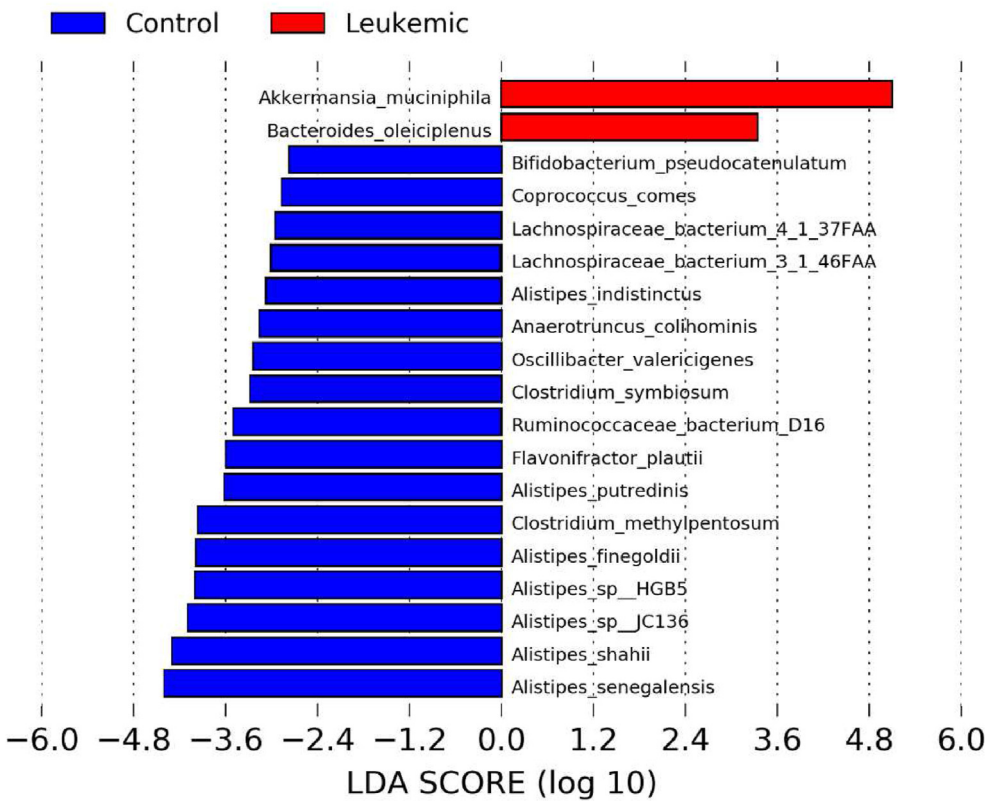

Figure 3. LefSe [21] was used to identify taxa with statistically different abundance between control and leukemic groups in feces.

3.1.2. Compositional changes in the bacterial microbiome in the small intestinal and colonic content

Intestinal compositions have been investigated at the end of the experiment (week 5). A compositional graph of the small intestinal content with the 10 highest abundance taxa is shown in Figure 4A as determined by LefSe [24]. An increase in *Clostridia spp.* were identified as differentially abundant species in the control group (*C. scindens, C. methylpenthosum, C. symbiosum*, Figure 4B).

A compositional graph of the colonic content in ALL and control groups with the 10 highest abundance taxa is shown in Figure 5A. Differentially abundant taxa showed an increase in the relative abundance of *Edwardsiella ictaluri* in ALL, and *Clostridia* and *Alistipes* spp. among others in the control group (Figure 5B). Principal component analyses showed partial clustering between leukemic and control samples both in the colon (PERMANOVA $p < 0.05$, Suppl. Figure 4) but not in the small intestine (PERMANOVA $p > 0.05$).

3.1.3. Differences in alpha diversity of the bacterial microbiome

Alpha diversity was measured by the Shannon index. There was no significant difference between ALL (1.14 ± 0.2) and controls (1.43 ± 0.4) in the small intestinal contents ($p = 0.08$). However, a significant decrease was identified in the colonic contents of ALL mice compared with controls (1.43 ± 0.6 in ALL, 2.27 ± 0.5 in controls, $p = 0.01$). A significant decrease was also measured in ALL in feces in week 5 (1.87 ± 0.4 in controls, 1.05 ± 0.4 in ALL, $p < 0.01$), but not in weeks 3–4.

3.1.4. SCFA levels decrease in the small intestine in ALL

Acetic acid, formic acid, propanoic acid and butanoic acid were measured in feces (from week 3 to week 5) and in colonic and small intestinal contents. All SCFA levels were significantly reduced in ALL compared with the control group in the small intestine (Figure 6), while no significant changes were found in the colonic contents or feces. Acetic acid levels were 3.02 ± 0.88 mM (mean ± SD) in the control group, and 1.28 ± 1.25 mM ($p = 0.02$) in the ALL group. Formic acid levels were 1.71 ± 0.52 mM in controls, and 0.75 ± 0.26 mM in the ALL group ($p = 0.01$). Propanoic acid levels were 0.42 ± 0.17 mM in controls, and 0.15 ± 0.18 mM in the ALL group ($p = 0.03$). Butanoic acid levels were 0.13 ± 0.04 mM in the control group, and 0.09 ± 0.01 mM in the ALL group ($p = 0.05$). pH values were measured of small intestinal and colonic contents dissolved in PBS (pH = 7.84). There was a significant increase in pH values in the small intestinal contents in ALL mice (pH = 8.33) compared with the control group (pH = 8.12, $p = 0.001$). In the colonic contents, there was no difference in pH values (control: 8.16; ALL: 8.12, $p = 0.112$).

3.1.5. Correlation between SCFA levels and the intestinal and fecal microbiome

A. muciniphila is a major producer of acetic acid [26], and its abundance was correlated with acetic acid levels in feces in ALL mice throughout weeks 3–5 ($n = 18$, $R2 = 0.22$, $p = 0.05$), but not in controls ($n = 20$, $R2 = 0$, $p = 0.73$) or in small intestinal or colonic content. *A. muciniphila* abundance was also correlated with propionate levels in feces in ALL ($R2 = 0.32$, $p = 0.02$), but not in controls, or small intestine and colonic content.

Butanoic acid plays an important role in gut barrier homeostasis and is an important source of nutrition for gut epithelial cells [27]. Regression analysis showed a significant correlation between *C. methylpenthosum* and butanoic acid levels ($R2 = 0.55$, $p = 0.05$) in the small intestinal contents. *C. methylpenthosum*, *C. scindens* and *C. symbiosum* abundances significantly decreased in the small intestinal contents in ALL (Figure 4), and the sum of the 3 *Clostridia spp.* abundances were also correlated with increased butanoic acid level ($R2 = 0.59$, $p = 0.04$). However, *Clostridia spp.* abundances were not correlated with other SCFA levels.

3.1.6. Functional analysis of small intestinal contents

In order to characterize their ability to synthetize SCFAs, we performed a functional analysis on the microbiomes of small intestinal contents. Our analysis shows that the microbiome from small intestines

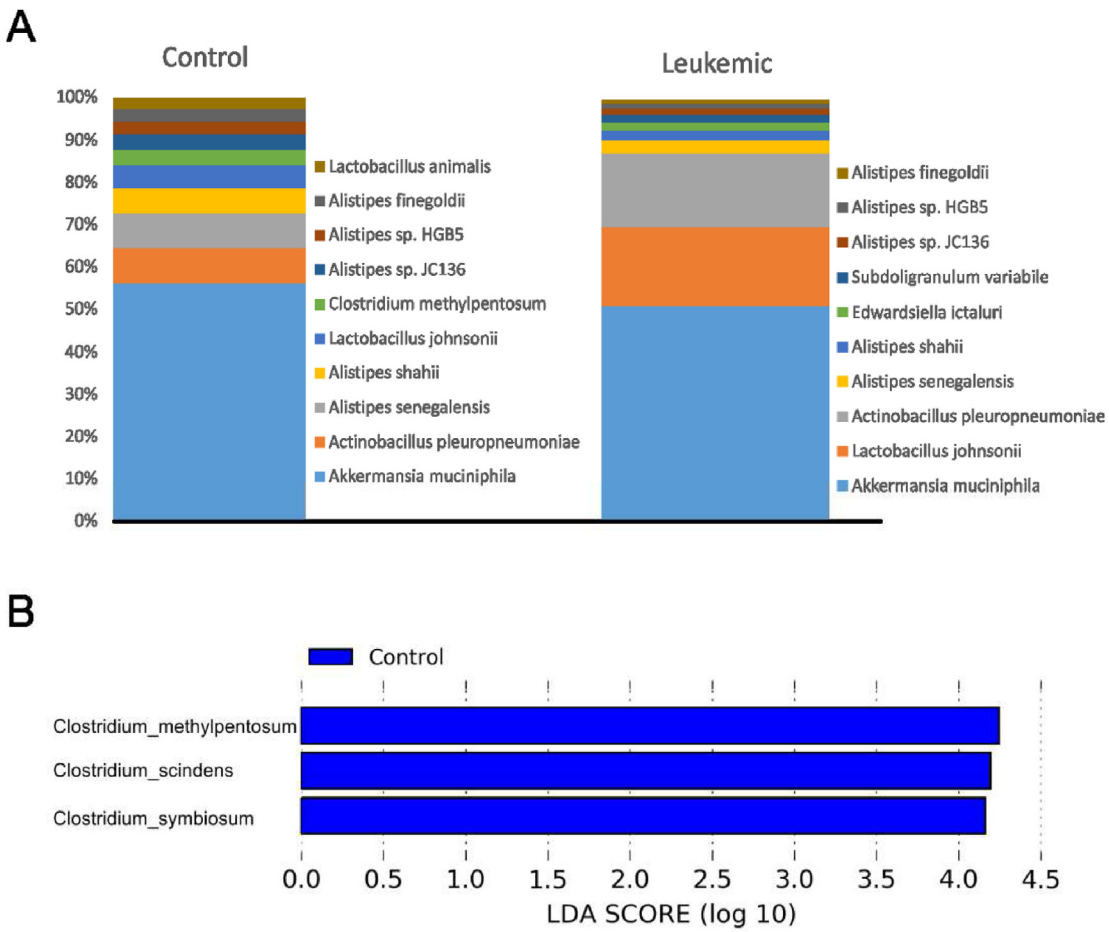

Figure 4. Small intestine content compositions in control and leukemic groups (A) and differentially abundant taxa (B). Taxa with the 10 highest abundances are shown.

of ALL mice lack key enzymes for de novo fatty acid biosynthesis (Suppl. Figure 5). Only 20% of ALL samples expressed 3-hydroxyacil-dehydratase (63% of controls), an enzyme that catalyzes the synthesis of fatty acids from acetyl-CoA. Sixty percent of ALL samples lacked 3-oxoacyl-reductase (present in 88% of controls), another key enzyme in the biosynthesis of fatty acids from acetyl-CoA as it reduces beta-ketoacyl-ACP substrates to beta-hydroxyacyl-ACP. S-malonyltransferase, an enzyme participating in fatty acid synthesis by transferring malonate from malonyl-CoA to acyl-carrier protein was present in 40% of ALL, and 100% of control samples (Suppl. Figure 5).

3.2. Bacterial translocation

We evaluated bacterial levels in the intestine via 16S qPCR of small intestine and colon tissue samples from ALL and control groups (Figure 7A). There was no significant change in the small intestine in ALL compared with controls in bacterial levels measured by 16S qPCR ($25,699.8 \pm 2,961$ in control group vs $52,767.3 \pm 36,447.7$ in ALL group, $p = 0.2$) or in the colon ($58,635 \pm 26,128.4$ in controls and $57,826.3 \pm 38,413.8$ in ALL, $p = 0.49$).

We further evaluated translocation of bacteria to the bloodstream by 16S qPCR of blood samples (Figure 7B). In ALL mice, bacterial copy numbers significantly increased from week 3 throughout the experiment (531.5 ± 133.1, mean $\pm$ SEM) compared with controls (167.5 ± 26.9, $p < 0.01$), as a probable result of bacterial translocation in ALL as shown earlier [5]. Moreover, 16S copy number showed a positive correlation with fecal *A. muciniphila* abundances ($R2 = 0.34$, $p = 0.02$) from week 4.

3.2.1. Host factors in bacterial translocation

As parts of the intestinal barrier, the mucus layer and the epithelial tissue protect the gut microbiome from translocation to sterile body sites [6]. MUC2 is a major mucin component secreted by goblet cells [28], while occludin, zonulin and claudin are core components of tight junctions in epithelial tissues [29]. We measured MUC2, claudin, zonulin and occludin levels in small intestine and colon tissues from control and ALL mice using RT-qPCR. Mouse GAPDH was used to measure fold change.

There was no significant change in MUC2 and claudin expression levels in small intestine or colonic tissues ($p > 0.05$). There was a significant increase of zonulin expression levels in the small intestinal tissue in the ALL group ($ddCt = -0.77$ between ALL and control groups, $p < 0.01$), indicating a 1.7-fold change [21]. Occludin expression levels showed a >600-fold decrease in the ALL group in the small intestine ($ddCt = 9.3$, $p = 0.02$). There was no significant difference of zonulin and occludin expression levels in the colonic tissue.

3.3. SCFA treatment ameliorates bacterial translocation

SCFAs play an important role in maintaining gut barrier function to prevent bacterial translocation [27, 28, 29], while our results indicated a depletion of SCFAs in the small intestine in ALL (Figure 6). We tested the hypothesis that SCFA supplementation may prevent bacterial translocation and differential expression of occludin in ALL by using 4 SCFA treated leukemic (LS), 4 leukemic (L), and 4 control (C) mice. Confirming our previous result of bacterial translocation, 16S qPCR of blood samples on weeks 4–5 indicated bacteremia ($p < 0.05$) in L mice, but was not significantly different in LS mice (Figure 7C). Occludin expression levels

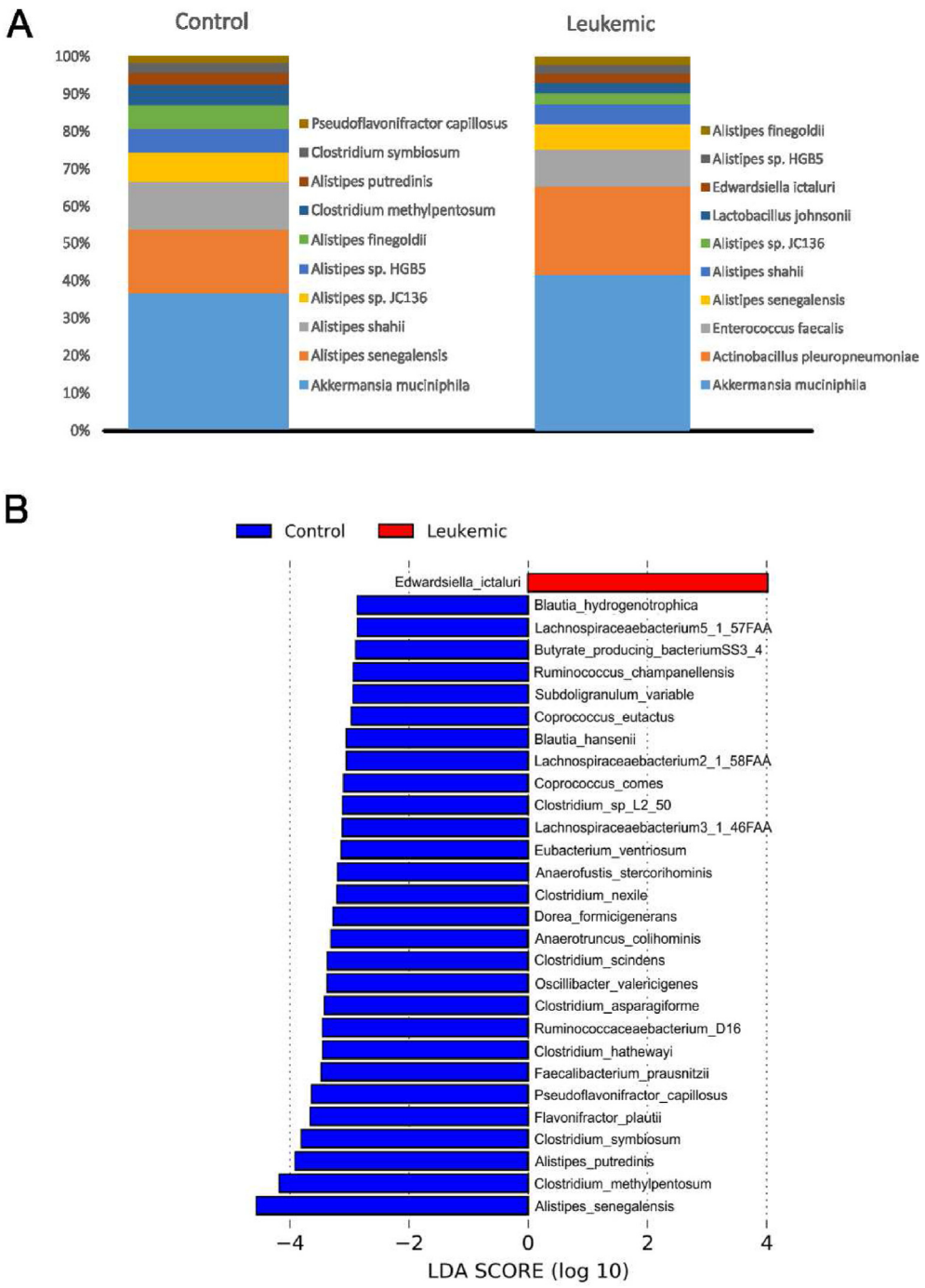

Figure 5. Colonic content compositions in control and leukemic groups (A) and differentially abundant taxa (B). Taxa with the 10 highest abundances are shown.

decreased in leukemic mice ($\Delta\Delta Ct = 7.4$) similarly to our previous findings, and slightly improved in LS mice ($\Delta\Delta Ct = 5.2$, $p > 0.05$).

3.4. Fungal microbiome

Even though >99% of the sequencing reads belonged to bacteria, shotgun sequencing identified fungal species in intestinal contents and feces.

In the small intestinal contents, *Aspergillus flavus* (in 3 samples out of 5) and *Malassezia globosa* (2/5) were only present in ALL mice. *Botryotinia fuckeliana* were present both in controls (4/5) and in ALL mice (5/5).

Similarly to the small intestine, *A. flavus* (3/5) and *M. globosa* (2/5) were only present in ALL mice, and *Botryotinia fuckeliana* were present both in controls (2/5) and in ALL mice (5/5) in the colonic contents. In feces, no fungal species was present in more than a single sample.

Aspergillus spp were shown to increase gut permeability [30], therefore we investigated the correlation between the abundance of *A. muciniphila* and the presence of *A. flavus* (Suppl. Figure 6). In the small intestine, where *A. flavus* was present, the abundance of *A. muciniphila* was significantly higher (0.64 ± 0.22) than in samples without *A. flavus* (0.12 ± 0.25, $p = 0.04$). There was no correlation between *Aspergillus* and *Akkermansia* in the colon. In comparison, we investigated the correlation between *M. globosa* and *A. muciniphila*, but there was no significant correlation in *A. muciniphila* abundance between samples with or without *M. globosa* ($p = 0.15$ in small intestinal content, and $p = 0.3$ in colonic content) in ALL mice.

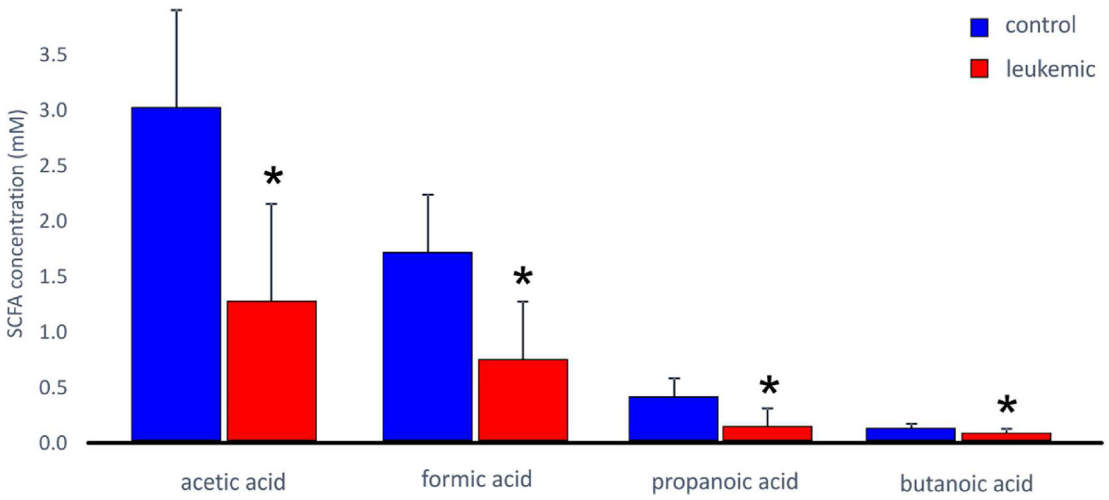

Figure 6. SCFA levels are shown from the small intestine content of control and leukemic mice. Asterisk represent statistically significant difference and error bars represent standard deviation.

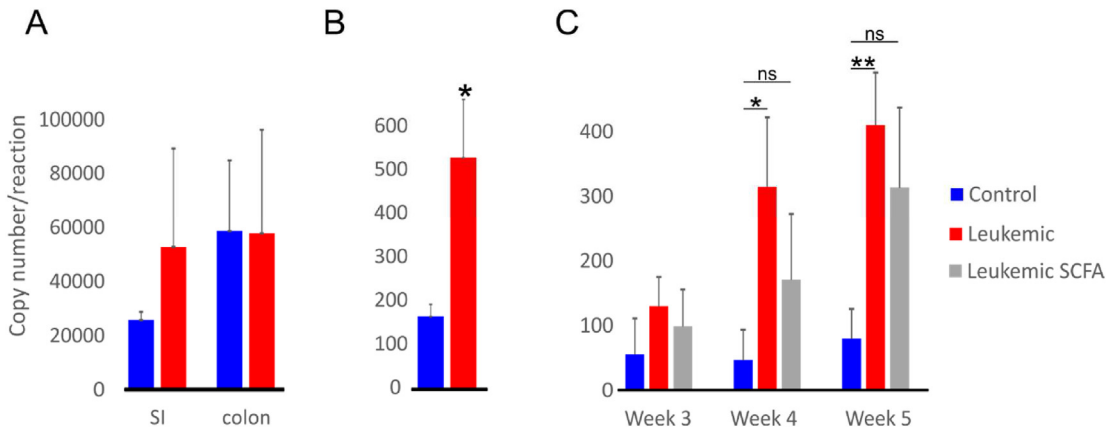

Figure 7. Bacterial levels using 16S qPCR were measured in small intestine and colon tissues in control and leukemic mice (A). Standard curves using *S. aureus* reference genomic DNA were used to measure copy numbers, with R2 > 0.99. Bacterial levels using 16S qPCR were measured in whole blood of control and leukemic mice on week 5 (B). Standard curves using *S. aureus* reference genomic DNA were used to measure copy numbers, with R2 = 0.99. Bacterial levels using 16S qPCR were measured in whole blood of control and leukemic mice, and in leukemic mice treated with SCFAs (C). Standard curves using *S. aureus* reference genomic DNA were used to measure copy numbers, with R2 = 0.99. * = $p < 0.05$, ** = $p < 0.01$, ns = non-significant. Error bars represent SEM.

3.4.1. Fungal translocation

We investigated the possibility of fungal translocation from the intestinal contents to the intestinal tissue using universal fungal (ITS 3–4) primers. No fungi have been detected in any of the samples from the small intestine and colon tissues.

3.5. Viral microbiome

Encephalomyocarditis virus (EMCV) is a member of the Picornaviridae family, and it has been isolated from over 30 animal species and can be spread with feces [31]. EMCV was detected in both control (n = 4) and ALL (n = 4) groups in the small intestinal contents, and it was also detected in colon in control (n = 2) and ALL (n = 5) mice. In the feces, no viral species were detected in more than one animal.

4. Discussion

BSI is a significant complication and a major source of mortality in ALL due to underlying immunosuppression. The gut microbiome has been identified as a source of BSI via bacterial translocation as a consequence of an increased intestinal permeability. Understanding the role of the microbiome in the development of BSI in ALL is crucial for the discovery of preventive and therapeutic treatments. Our study highlights the complex intertwining mechanisms between the host and the microbiome contributing to the pathophysiology of BSI in ALL. We have previously shown that the damage of the gut-associated lymphoid tissue is one of the elements of BSI pathogenesis in ALL [5]. In this work, we further investigated alterations in the compositions and microbial metabolites in intestinal and fecal microbiome and gut barrier functions, which may play a role in the pathophysiology of BSI in ALL. The current study determined that changes in the bacterial and fungal microbiome, in SCFA levels and in the intestinal barrier may potentially contribute to bacterial translocation and subsequent BSI.

4.1. Changes in the intestinal and fecal microbiome

Microbiome changes were characterized by a decrease in *Clostridia spp.* in the leukemic group in the small intestine, colon, and feces (Figures 3, 4, and 5), and an increase of *A. muciniphila* in feces (Figure 2).

Clostridia spp. are commonly present in the gut microbiome [11], and have been reported to increase [8] and decrease [9, 10] in ALL. We found that *C. methylpentosum* has decreased in both intestinal contents and feces in the ALL group compared with control mice (Figures 3, 4, and 5). *C. methylpentosum* is part of the normal microbiome, and can utilize

pentoses and methylpentoses as fermentable substrates to produce SCFAs [32]. *C. symbiosum*, a bacterium which produces butyrate in the intestine in physiological conditions [33] also decreased in the ALL group in all sites (Figure 6). Butyrate improves gut barrier function by facilitating tight junction assemblies [34], and through regulating nuclear NF-κB and inhibition of histone deacetylase [28]. We detected a decrease in butyrate levels in the small intestinal contents of the ALL group, and the relative abundance of *Clostridia spp.* were correlated with the decrease in butyrate level ($R2 = 0.59$, $p = 0.04$). In agreement with this finding, it has been previously shown that butyrate regulates tight junction protein production in epithelial cells [29, 34, 35], and upregulation of zonulin [36, 37] and downregulation of occludin [38, 39] leads to an increase in intestinal permeability. Therefore, our results combined with other sources suggest that the ALL microbiome led to alterations in expression levels of tight junction proteins occludin and zonulin via butyrate, and possibly other factors.

Functional analysis of the microbiome indicated that the reduced SCFA levels in the small intestine of leukemic mice are a result of an incomplete pathway of fatty acid synthesis (Suppl. Figure 5). Our preliminary data suggest that gut barrier integrity and bacterial translocation in ALL can be ameliorated, but not reversed by SCFA supplementation. Optimization of composition, dosage and frequency of administration may further enhance the protective effect of SCFAs on gut barrier function [40].

A. muciniphila is part of the normal microbiome, and can contribute to an increase in gut permeability by degradation of the mucus layer [41]. However, it has also been shown to be beneficial in obesity [42] as a probiotic. *A. muciniphila* produces acetic acid from mucin to supply nutrition to goblet cells [26]. This was supported in this work as *A. muciniphila* abundance showed a linear association with acetic acid levels in feces. Our work also shows that *A. muciniphila* gained protein domains throughout ALL development, such as hydrolase and dehydrogenase which may also contribute to mucin degradation [34] and gut barrier disruption. Therefore, this study suggests that the gut microbiome changes in ALL, which weakens the intestinal barrier via decreased butyrate production by *Clostridia spp.* [35, 43], increased abundance of *A. muciniphila,* and mucus degradation [26].

In accordance with this finding, linear regression showed a strong correlation between bacterial copy numbers in the blood and fecal *A. muciniphila* abundance. In addition, our results show that the abundance of *A. muciniphila* in feces was positively correlated with the presence or absence of *A. flavus* in small intestinal content. *A. flavus* also negatively impacts the gut barrier integrity via mycotoxins [31], and this effect may be enhanced when both microbes are present.

4.2. Bacterial translocation

The intestinal barrier prevents the gut microbiome from exiting the lumen and invading sterile body sites. The intestinal barrier is composed of a mucus layer containing antimicrobial peptides, a monolayer columnar epithelium with tight junctions, and the gut-associated lymphocytic tissue (GALT). Tight junction proteins are key components of the intestinal barrier as they form a penetrable layer between epithelial cells. SCFAs produced by the gut microbiome provide nutrition to epithelial cells and help maintaining gut barrier integrity. Failure in the intestinal barrier function is crucial in the pathogenesis of BSI in ALL [5, 6, 44].

It has been demonstrated previously that acute leukemia can result in leaky blood brain barrier due to impaired tight junctions [45]. Our results show that ALL resulted in bacterial translocation, dominantly localized in the small intestine (Figure 7A), which led to bacteremia (Figure 7B). We showed expression differences in 2 key tight junction proteins, zonulin and occludin in the small intestine, which may have contributed to bacterial translocation [46, 47]. The small intestine is a primary location for nutrient absorption, as well as for bacterial translocation through a disruption in the gastrointestinal equilibrium [6, 48]. The pH in the gastrointestinal tract increases from the stomach to the colon [49], while metabolic processes change from saccharolytic to proteolytic fermentation [12]. We detected an increase in pH in the small intestine in ALL, which may have contributed to the change in the microbiome (and vice versa) and subsequent alterations in the intestinal barrier.

Microbial diversity has been associated with a healthy microbial ecosystem, while a decrease in diversity is commonly associated with pathological conditions [12]. A decrease in diversity leads to reduced colonization resistance, and may allow perturbations in the microbiome characterized by an increase of opportunistic pathogens [11, 26]. We found that alpha diversity decreases in colonic content and feces, but not in the small intestine in ALL. In accordance with this finding, reports describing the fecal microbiome in leukemia are consistent that microbial diversity decreases both in clinical samples and in animal models of leukemia [9, 50, 51, 52, 53, 54].

This study has several limitations. We used a limited number of mice (4 per group for SCFA supplementation and 8 per group for metagenomics and transcriptomics experiments) – however, this number is comparable to or higher than similar studies [55, 56, 57]. pH values of intestinal contents have been recorded in PBS buffer, which was necessary to remove and wash intestinal tissues without cell damage.

5. Conclusions

Our findings show a change of the microbiome in the small intestine in ALL, characterized by a decrease in *Clostridia spp.*, and a subsequent reduction in SCFA levels. This change likely contributed to an imbalance in gut barrier function, expression differences of tight junction proteins [58] and the resulting bacterial translocation in the small intestine in ALL. In addition, we also detected an increase of *A. muciniphila*, a mucin degrading bacterium and acetic acid producer in leukemic feces, which may have helped the degradation of the mucus layer and further weaken the intestinal barrier in ALL. In summary, this work suggests that both microbial and host factors contribute to translocation of bacteria and subsequent BSI in ALL.

Declarations

Author contribution statement

Yajing Song, Katherine Perlman: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Peter Gyarmati: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data associated with this study has been deposited to the Metagenomics analysis server under the accession number MGP93427.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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