Spatioregional assessment of the gut microbiota in experimental necrotizing pancreatitis

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Abstract

Background: Infectious complications following experimental pancreatitis involve major disruptions in the gut microbiota. The aim of this study was to characterize this disruption by examining the spatioregional distribution in microbial community structure and function following experimental pancreatitis associated with pancreatic infection.

Methods: Mice were subjected to infusion of the pancreatic duct with either taurocholate to induce necrotizing pancreatitis or normal saline (control group). The spatial (lumen versus mucosa) and regional composition and function of the microbiota from the duodenum, ileum, caecum, colon, pancreas and blood were evaluated using 16S rRNA gene amplicon sequencing.

Results: Mice that developed necrotizing pancreatitis demonstrated a decrease in microbial richness and significantly altered microbiota in distal parts of the gastrointestinal tract, compared with controls. Among the most differentially increased taxa were the mucus-degrading Akkermansia muciniphila, and there was a decrease of butyrate-producing bacteria following pancreatitis. Application of the SourceTracker tool to the generated metadata indicated that the duodenum was the most probable source of bacteria that subsequently infected pancreatic tissue in this model. The functional prediction annotation using pathway analyses indicated a diminished capacity of the caecal microbiota to metabolize carbohydrate, and fatty and amino acids.

Discussion: The distal gut microbiota was significantly impacted in this model of experimental necrotizing pancreatitis. Data suggest that the duodenal microbiota might also play a role in bacterial translation and secondary infections.

Surgical Relevance

Intestinal bacteria are key disease modifiers in severe acute pancreatitis, especially when it comes to secondary infections such as pneumonia, bacteraemia and infected (peri-)pancreatic necrosis. Efficient and safe targeting of gut microbes as a way of prophylaxis warrants a deeper understanding of which bacteria in which intestinal compartment contribute to disease progression. This study investigated whether induction of necrotizing pancreatitis impacted the composition and function of the spatioregional gut microbiota in mice.

Necrotizing pancreatitis had a major effect on the microbiota distribution of the distal gastrointestinal tract with a reduction of butyrate-producing bacteria. The duodenum was identified as the main source of pancreatic infection in the model. These data suggest that microbial communities of both the upper and lower gastrointestinal tract might represent future targets for prophylaxis in patients with severe acute pancreatitis.

Introduction

Infection-related complications remain the most important determinant of the course, outcome and healthcare burden following severe acute pancreatitis. Although the intestinal tract is recognized to be the main site of origin of pathogens that cause infections during acute pancreatitis, the precise site and species that drive disease progression have been incompletely investigated. Manipulations of the microbiome may fail to recognize off-target effects, such as when probiotics are used to replace lost microbiota or when broad-spectrum antibiotics are applied to eliminate emerging pathogens. Empirical treatment with systemic meropenem, for example, prior to induction of experimental pancreatitis in mice, results in acceleration of mortality and increased infections, predominantly caused by gut-derived Enterococcus gallinarum. Such findings may indicate that while certain pathogenic bacteria might require targeted elimination, others may need to be preserved in order to prevent loss of colonization resistance afforded by the normal microbiota.

Evaluation of the gut microbiota following experimental pancreatitis has the potential to uncover which members of the microbiota are beneficial (such as butyrate-producers) and which are not, and, importantly, where such alterations occur.

It was hypothesized that region-specific changes in the gut microbiota could be defined that would predict subsequent
pancreatic infection in this model. The aims of this study were therefore to define the spatial (luminal versus mucosal) and regional (duodenum, ileum, caecum and colon) differences in microbial community structure and function using 16S rRNA gene amplicon sequencing in mice subjected to experimental pancreatitis and to assess how these changes related to pancreatic infection.

Methods

Ethical approval

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Chicago (ACUP 72540).

Animal experiments

Six-week-old C57BL/6 mice were purchased (Charles River Laboratories International Inc., Wilmington, Massachusetts, USA) and housed under standard conditions (12 hours dark/light cycle) for 6 weeks before any study procedures were performed. Animals had ad libitum access to tap water and standard chow diet. Mice were anaesthetized with intraperitoneal ketamine (100 mg/kg) and xylazine (0.1 mg/kg) and meloxicam (1 mg/kg) was administered before surgery, an injection of bupinorphine (0.1 mg/kg) and meloxicam (1 mg/kg) was administered postoperatively by intraperitoneal injections of bupinorphine every 12 hours for at least 48 hours. All procedures were performed under sterile conditions according to local guidelines and policies.

Murine model of acute biliary necrotizing pancreatitis

Necrotizing pancreatitis was induced by infusion of the pancreatic duct with taurocholate acid, as previously described12. This involved midline laparotomy, exposure of the posterior side of the duodenum and identification of the papilla of Vater followed by cannulation of the common bile duct for 2 mm and connection with PE-10 tubing (Fisher ScientificTM, Waltham, Massachusetts, USA) attached to a syringe pump (Harvard apparatus, Holliston, Massachusetts, USA). The cannula was temporarily fixed with an 8/0 prolene suture, and a micro-clamp placed at the proximal hepatic duct. The pancreas was infused for 10 minutes with either 50μl saline (controls, n = 7) or an equal volume of 4 per cent sodium taurocholate hydrate (pancreatitis, n = 7) with 1 per cent methyl-blue (Sigma-Aldrich, Saint-Louis, Missouri, USA). The cannula, micro-clamp and ligature were removed and the duodenal puncture closed, followed by routine wound closure. A group of mice that did not undergo surgery (untreated, n = 5) acted as a further comparator group.

All animals were sacrificed by carbon dioxide suffocation at either 24 or 72 hours following surgery. Sterile blood was collected by cardiac puncture. Pancreas and intestinal contents were aseptically removed and stored in saline with 10 per cent glycerol as cryopreservative at -80°C until analysis. Histology of pancreatic tissue was performed as previously described13. No technical replicates were used.

16S rRNA gene amplicon sequencing

Microbial DNA was extracted from pancreatic tissue and intestinal (duodenum, ileum, caecum, colon) tissues and contents using the MagAttract® PowerMicrobiome DNA/RNA KF kit and from blood using the DNeasy® Blood & Tissue kit (Qiagen, Germantown, Maryland, USA). Amplicons of the V4 region of the 16S rRNA gene were constructed using 515F/806R primer pair, according to the Earth Microbiome Project protocols (EMP, http://www.earthmicrobiome.org/emp-standard-protocols/16s/). Gene amplicon sequencing was done on a MiSeqTM platform (Illumina, San Diego, California, USA) at the Argonne Sequencing Facility, generating 150bp pair-end reads14,15. Decontaminating exact sequence variants (ESV) were removed using water control samples and the R package Decontam using the prevalence method with a threshold of 0.516. Statistical analysis and visualization were done using R packages phyloseq and ggplot217,18. A panel of butyrate-producers based on genus taxonomy was constructed as described previously13,19. The Inverse Simpson index was used as measure for alpha diversity which takes abundance into account. For beta diversity, the UniFrac distances were used to compare two communities based solely on the presence or absence of bacterial taxa (unweighted), or taking abundance into account (weighted). Differential abundance and microbial predictors were determined using R packages Deseq2 and Randomforest20,21. Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) 2 tool that predicts functional aspects from 16s rRNA amplicon data using Kyoto Encyclopedia of Genes and Genomes (KEGG) and MetaCyc metabolic databases were used with QIME2. Significant differences between predicted functional pathways were calculated using the R package Aldex22,23. Blast was used for reads to determine a match at the species level24. The SourceTracker tool was designed to use 16S rRNA gene sequencing data to predict from which source a sample is determined24. Pancreatic and blood samples were used as ‘sink’, and the luminal and mucosal gut samples as ‘source’ using standard settings.

Untargeted gas chromatography–mass spectrometry metabolomics analysis

Following sacrifice at 24 hours following surgery, caecal content was collected, snap frozen and stored at -80°C until analysis. Ice-cold high-performance liquid chromatography (HPLC) grade water (100 μl) was added, homogenized and centrifuged at 16,000 rpm for 20 minutes at 4°C. Supernatant was collected and 450 μl ice cold HPLC-grade methanol added, homogenized and centrifuged. The supernatants were combined and filtered through a 0.22 μm filter. Then 5 μl of 3 mg/ml myristic acid d27 (Sigma Aldrich, Saint-Louis, Missouri, USA) was added as internal control. The samples were evaporated in a Vacufuge® (Eppendorf, Hamburg, Germany) and the residue stored at -80°C.

Samples were derived by adding 50 μl of 20 mg/ml methoxyamine (Sigma) in pyridine. Samples were incubated at 37°C for 90 minutes in a shaking incubator. Next, 100 μl of MSTFA + 1 per cent TMC (Sigma) was added and incubated at 37°C for 30 minutes before analysis.

Samples were analysed using a high-resolution accurate mass 7200B QTOF gas chromatography–mass spectrometer (Agilent, Santa Clara, California) with a mass range 35–800, acquisition rate 6 spectra/s. Samples were randomized in batches of five, and injected at a 25 : 1 split ratio. A temperature programme of 70°C for 5 minutes was used, ramped at 5°C/min until 320°C, and held for 3 minutes. A blank sample containing solvent (pyridine) was run between each sample to clean the column of any residue. The chromatograms were processed (peak picking, definition of pseudospectra and annotation) using R (R Core Team, 2014) with the ‘runGC’ function of the metaMS package25. The resulting pseudospectra were annotated using the commercial Fiehn Gas Chromatography–Mass Spectrometry (GC/MS) Metabolomics RTL Library26. Features with matching factors 60 per cent or greater were positively identified.
Statistical analysis

Data were analysed using GraphPad Prism 8 (Graphpad Software, San Diego, California, USA) or R (R Core Team, 2014). Unless stated otherwise, results were expressed as mean(s.d.). Non-parametric Mann–Whitney or student t test was used to test statistical significance, depending on the distribution of normality. ANOVA was used to test significant differences between multiple groups. Benjamini–Hochberg correction was used for multiple comparison of groups. The Metaanalyst 4.0 webserver module was used for the metabolome pathway analysis using normalized data and default settings with an FDR cut-off of 0.227.

Results

Histology of the pancreatic tissue of pancreatitis mice confirmed the presence of focal necrosis (Fig. S1a,b), in comparison with control mice that only showed mild oedema (Fig. S1c,d) and untreated mice (Fig. S1e,f). No mortality was observed prior to sacrifice.

The microbiota in all gut compartments in both mucus (Fig. 1a) and lumen (Fig. 1b) were affected by acute necrotizing pancreatitis (ANP) as seen by the distribution of ESVs. Overall, there was a relative loss of taxa in the pancreatitis group, as demonstrated by a decrease of richness in caecal (P = 0.044) and colonic mucosa (P = 0.044), but not in luminal samples (Fig. 1c). However, alpha diversity did not differ between groups (Fig. 1d) suggesting that mainly low-abundance taxa were affected.

Variance analysis of unweighted beta diversity showed that the centroids of the experimental groups significantly differed in the mucosal ileum (R² = 0.162, P = 0.032), mucosal and luminal caecum (R² = 0.199, P = 0.024 and R² = 0.16, P = 0.043) and mucosal and luminal colon (R² = 0.146, P = 0.008 and R² = 0.189, P = 0.008 by PERMANOVA), but not in the duodenum (Fig. 1e). Weighted beta diversity did not differ significantly (Fig. 1f), again indicating that mostly low-abundance microbes were impacted. These differences were observed among both the luminal and mucosal microbiota. When comparing alpha and beta diversity of untreated and control mice, no major differences were observed (Fig. S2).

In comparing gut segments (Fig. S3) using the unweighted UniFrac measure, mucosal duodenal microbiota in animals with ANP closely resembled mucosal ileal microbiota (P = 0.056). In the control group mucosal duodenal and ileal microbiota differed markedly (P = 0.001) (Fig. S3).

To investigate possible involved bacterial pathways in the disease course of necrotizing pancreatitis, the authors next applied functional prediction on the microbiome data set (Fig. 2a). Prediction analysis demonstrated that multiple Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways involved in carbohydrate, nucleotide and fatty acid metabolism were significantly downregulated in the caecal lumen following pancreatitis. Conversely, non-homologous end-joining was upregulated. MetaCyc pathway analysis additionally demonstrated upregulation of glucose, aromatic biogenic amine and 1,4-dihydropyrazole-2-naphthoate metabolism. In the luminal colon, only degradation of purine nucleobases was decreased. There were no identifiable changes in regulatory pathways in the mucosal segments, nor in the duodenal or ileal luminal microbiota.

The metabolic profile of caecal luminal content in necrotizing pancreatitis and controls was further assessed with untargeted GC-MS metabolomics. Differential abundance analysis (Deseq2) demonstrated that two short-chain fatty acids, 2-hydroxybutyric acid and 5-aminovaleric acid, were decreased in pancreatitis (Fig. 2b). Amongst the increased metabolites were monosaccharides (D-mannose, D-lyxosylamine), phenolic acid (3-hydroxyphenylacetic acid), amino acid (L-threonine) and bile salt (cholic acid).

Analysis of differential abundant taxa of integrated luminal and mucosal segments (duodenum, ileum, caecum and colon) between the pancreatitis and control groups (Fig. S4) identified Verrucomicrobia, with its representative species Akkermansia muciniphila, as the sole phylum (Fig. 3a, P < 0.050) that differed between the groups, with an increase in the pancreatitis group. The phylum Firmicutes demonstrated an increase in the Erysipelotrichaceae family, but a decrease in Lachnospiraceae in the pancreatitis group (P < 0.050). Although there was no difference in Proteobacteria, the phylum known to contain the majority of pathogens, bacteria from the genus Escherichia/Shigella were increased with pancreatitis (P < 0.050). Furthermore, there was both a decrease (mostly from the families Lachnospiraceae and Rumminococcaceae) and increase (genera Butyricimonas and Ruminococcus) of known butyrate-producing taxa (P < 0.050).

Investigation of the separate gut segments indicated no major shift in relative abundances of the four major phyla (Fig. S5a–d). Notably, Firmicutes: Bacteroidetes ratio demonstrated an approximately 50-fold decrease in the colonic mucosal (Fig. 3b), but not the luminal microbiota (Fig. 5e). Overall, there was a decrease of relative abundance of a panel of butyrate-producers in caecal mucosa (Fig. 3c). Akkermansia muciniphila, a mucus-degrading bacteria, was enriched in both the lumen and mucosa of caecum and colon (Fig. 3d and Fig. S5f).

Deseq2 analysis for differential abundance and a random forest classifier identified the genera that contributed the most in the prediction of the experimental groups. This analysis identified 43 decreased ESVs and 13 increased ESVs when combining gut segments of the groups (P < 0.050) (Fig. 4), with the majority overlapping in lumen and mucosa. In accordance with the alpha and beta diversity measures, the number of differential genera gradually increases along the gastrointestinal tract, with the majority found in the colon (47 genera), followed by caecum (29 genera), ileum (15 genera) and duodenum (15 genera) (Fig. S6a–d, P < 0.050). Akkermansia muciniphila was among the most increased abundant taxa in mucosa and lumen with both Deseq2 (Fig. 4) and random forest classifier (Fig. 57). Escherichia/Shigella was increased in the luminal compartment and mucosa of the caecum (Fig. S6c).

Sequencing of the 16S rRNA gene of pancreatic tissue after pancreatitis induction identified pathogens that translocate from the gastrointestinal tract to distant tissues. At the phylum and genus level, there were no major differences in relative abundances between the experimental and control group (Fig. 5a and Fig. S8). There were only two bacterial genera that were significantly overrepresented in pancreatic tissue based on Deseq2: Proteus and Lachnospiraceae_FCS02_group (Fig. 5b). The Proteus ESV in these samples had a 100 per cent identical match with Proteus mabii. Routine aerobic culture of pancreatic tissue following pancreatitis in this model revealed no Proteus species. Intestinal abundance of Proteus and Lachnospiraceae_FCS02_group were not significantly increased in the pancreatitis group. However, when comparing relative abundances between gut segments it was noted that Proteus abundance was highest in the duodenum, especially in mucosa (Fig. S9).

SourceTracker analysis indicated that the duodenum contributed 57 per cent to the disseminated bacteria, of which the majority were derived from the luminal compartment (Fig. S5c). In the control group 86 per cent could not be attributed to the
Fig. 1 Compositional analysis (16S rRNA gene analysis) of segmental lumen- and mucosa-associated microbiomes in acute necrotizing pancreatitis

Microbiota analysis of gut segments from mice with retrograde infusion of the pancreatic duct (pancreatitis) and saline-infused (control) mice (n = 7 per group). a, b Venn diagrams of exact sequence variants (ESVs) of gut segments from mucosa a and lumen b. c Richness (observed taxa) of luminal and mucosal gut segments. Richness was significantly decreased in caecum mucosa (P = 0.044, paired t-test with Benjamini-Hochberg correction) and colon mucosa (P = 0.044) of pancreatitis mice. d Alpha diversity of luminal and mucosal gut segments. No significant differences were found. e, f Beta diversity of luminal and mucosal gut segments measured by unweighted e and weighted f UniFrac. There were significant differences in ileum mucosa, caecum lumen and mucosa and colon lumen and mucosa for weighted UniFrac e, and in colon lumen and mucosa for unweighted UniFrac f. Beta diversity P and R² values based on PERMANOVA test with Benjamini-Hochberg correction. *P < 0.050, †P < 0.010
Discussion

Although bacterial overgrowth, impaired intestinal permeability and bacterial translocation to peripheral organs are widely recognized as hallmarks of disease progression in severe acute pancreatitis, the species involved in this process and their site of origin remain poorly understood. The present study investigated the biogeographical changes in microbial community composition, membership and function between different regions of the gastrointestinal tract. In blood samples, only about 3 per cent of bacterial reads were predicted to have been derived from the gut segments in both control and pancreatitis animals (Fig. 5d). Although the relative abundance of *P. mirabilis* was highest in the duodenum compared with other gut segments, there was no difference in relative abundance of duodenal *P. mirabilis* between the pancreatitis and control group.

The present study demonstrated that necrotizing pancreatitis induced a shift within the lumen to mucosal-associated microbiota in some regions, with the mucosal microbiota appearing to display greater alterations in alpha (for mucosal caecum and colon) and beta diversity (for luminal ileum, caecum and colon). These differences appeared to be most pronounced in the colon. Although the mechanisms that govern this response remain to be identified, alterations in perfusion and oxygen tension as a result of the profound physiological perturbation that occurs during pancreatitis could be relevant. A more complete description of the local physicochemical cues at precise sites within the spatial and regional contexts where microbiota reside during necrotizing pancreatitis is needed to elucidate these potential mechanisms fully.

The identification of *A. muciniphila*, a commensal mucus-degrading bacterium of the phylum Verrucomicrobia, in this model may be important, given increasing understanding into its role in intestinal health and gastrointestinal disorders. *A. muciniphila* produces short-chain fatty acids, propionate and acetate, and works in synergy with downstream metabolizing bacteria that produce butyrate. Experimental and clinical studies have shown that supplementation of live pasteurized strains of *A. muciniphila* and even purified membrane proteins have a beneficial effect on obesity and diabetes. The role of *A. muciniphila*, which demonstrated a 20-fold increase in caecal mucosal
samples and a 100-fold increase in the colonic mucosa at 72 hours after induction of necrotizing pancreatitis, remains to be clarified. It is possible that a bloom of mucus-degrading bacteria, such as *A. muciniphila*, may play a part in the known mucosal damage and altered intestinal integrity that develops in clinical and experimental pancreatitis. In line with previous work, an increase of the *Escherichia/Shigella* genus in some gut compartments was noticed, implying a potential pathogenic role in this model of necrotizing pancreatitis. The majority of pathogens cultured in infected pancreatic collections (among

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**Fig. 3** Taxonomic tree and relative abundances in integrated gut segments of pancreatitis and control mice

- **a** Taxonomic tree indicating significantly increased and decreased relative abundance of bacteria at different taxonomic levels of integrated luminal and mucosal segments (duodenum, ileum, caecum and colon) in pancreatitis group compared with controls (*P* < 0.05 by Kruskall–Wallis test with FDR correction).
- **b** Firmicutes/Bacteroidetes (F/B) ratio of gut segments. F/B ratio is decreased in colon mucosa of pancreatitis mice (*P* = 0.018).
- **c** Relative abundance of butyrate-producers in caecum mucosa is decreased in the pancreatitis group (*P* = 0.004).
- **d** Relative abundance of *Akkermansia muciniphila* demonstrates an increase in mucosal caecum (*P* = 0.003) and colon (*P* = 0.010) in the pancreatitis group compared with the control group. *P* values based on Mann–Whitney test. *P* < 0.05, †*P* < 0.01.
others Escherichia coli and P. mirabilis) are known gut inhabitants that are thought to translocate and cause (extra-) pancreatic infections. There were no major differences in the pancreatic microbiome between pancreatitis and control mice, indicating that the majority of these taxa were either representations of a native pancreatic microbiome (if one exists), the results of intrinsic contamination of the DNA isolation or sequencing methods (despite computational decontamination) or as a result of the surgical procedure. Pancreatic over-representation of Proteus mirabilis, a facultative anaerobic Gram-negative pathogen frequently cultured in experimental and human infected necrotizing pancreatitis, might be the result of bacterial translation. One explanation why P. mirabilis was not cultured is that it was unable to survive and proliferate in the pancreatic tissue in this model. The high abundance of Proteus in the duodenum compared with levels in the distal gastrointestinal tract adds support to this.

A major finding in the present study was the implication that the pancreatic duct may be a route of infection from the duodenum which acts as a reservoir of potential invading pathogens. In contrast to other studies, the use of the SourceTracker tool pointed towards the duodenum as the most likely origin of pancreatic infection in this model. Other studies have demonstrated that bacteria can migrate to the pancreas of healthy mice within 30 minutes following their administration, suggesting that infection occurs proximally, perhaps directly via the pancreatic duct. In this specific model, it is accepted that surgical trauma (laparotomy and cannulation) followed by inflammation (taurocholate

**Fig. 4 Microbiome differential abundance analysis**

Differential abundance of genera in mucosa and lumen of combined gut segments is tested using Deseq2. Colours indicate phylum. Wald-test is used for statistical significance (P < 0.050).
infusion) could lead to more contamination compared with trauma without subsequent inflammation (saline-infused control group).

There was discrepancy in terms of the gut regions where microbiota changes were most pronounced (caecum and colon), and the likely source of pancreatic infection in the present model (duodenum). This might reflect duodenal overgrowth with downstream microbes due to decreased intestinal motility. The timing of microbiota sampling might also be relevant. Duodenal microbiota changes that preceded pancreatic infections might have occurred before 72 hours.

In general, results from the present study were complementary to similar studies in this area and parallel findings in patients with acute pancreatitis, and the likely source of pancreatic infection in the present model (duodenum). This might reflect duodenal overgrowth with downstream microbes due to decreased intestinal motility. The timing of microbiota sampling might also be relevant. Duodenal microbiota changes that preceded pancreatic infections might have occurred before 72 hours.

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Supplementary material
Supplementary material is available at BJS Open online.

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