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Influence of intestinal microbiota modulations on the severity level of acute pancreatitis

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I hereby declare that I independently prepared the preset thesis at the Technical University of Munich, suggested and guided by Dr. Christoph Stein-Thöringer and Dr. Raquel Mejías-Luque. This work has been done without external help using only the listed literature references.

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Abstract

In this dissertation we show that nearly complete eradication of the gut microbiota has the potential to ameliorate the severity of the acute pancreatitis (AP). The mechanisms remain to be further elucidated, but our data presents a strong case for the communication between the gut and the pancreas in which absence of microbiota leads to a milder immune response upon AP induction. Additionally, we were able to show that certain bacterial strains translocated to the pancreas, and that they provoke an immune reaction in the pancreas.

Zusammenfassung

In dieser Arbeit zeigen wir, dass die fast vollständige Ausrottung der Darm Mikrobiota das Potenzial hat, den Schweregrad der akuten Pankreatitis zu verbessern. Die Mechanismen werden weiterhin verstanden, aber unsere Daten zeigen, dass die Abwesenheit von Mikrobiota bei der AP-Induktion zu einer milden Immunantwort führt. Darüber hinaus konnten wir zeigen, dass bestimmte Bakterienstämme in die Bauchspeicheldrüse verlagert wurden und eine Immunreaktion in der Bauchspeicheldrüse hervorrufen.

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Acronyms

AP	Acute pancreatitis
APCs	Antigen presenting cells
ATP	Adenosine triphosphate
BB	Brucella broth
BHI	Brain heart infusion
CCK	Cholecystokinin
CFU	Colony-forming unit
CT	Computed tomography
DAMPs	Damage associated molecular patterns
dNTP	Deoxynucleoside triphosphate
dsDNA	Double-strand DNA
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FISH	Fluorescence *in situ* hybridization
GF	Germ-free
HBSS	Hank's Balanced Salt Solution
HE	Haematoxylin and eosin
HF	High-fat
HMGB 1	High-mobility group box protein 1
HPF	Histopathological field
HSP70	Heat shock protein 70
IL	Interleukin
INF-γ	Interferon γ
IPMN	Intraductal papillary mucinous neoplasm
LEfSe	Linear discriminant analysis of effect size
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell sorting
MAMPs	Microbe-associated molecular patterns
MCP-1	Monocyte chemoattractant protein 1
MDR	Multidrug-resistant

Acronyms

mLNs	Mesenteric lymph nodes
MOF	Multiple organ failure
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
NOD1	Nucleotide-binding oligomerization domain-containing protein 1
OD	Optical density
OTUs	Operational taxonomic units
PRRs	Pattern recognition receptors
RPM	Rounds per minute
RT	Room temperature
SCFA	Short-chain-fatty acid
SIRS	Systemic inflammatory response syndrome
SPF	Specific pathogen free
SWA	Serum work area
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor α
t-SNE	t-Distributed Stochastic Neighbor Embedding
UFA	Unsaturated free fatty acid
WOPN	Walled-off pancreatic necrosis

1 Introduction

1.1 Epidemiology of acute pancreatitis

The global incidence of acute pancreatitis (AP) is 34 affected individuals per 100,000 person, per year, and it has been increasing worldwide [1]. The burden of the disease on the healthcare resource consumption is expected to increase in the near future [2]–[5]. The global mortality rate is 5% to 17% in severe acute pancreatitis, and 1.5% mild acute pancreatitis [2], [4], [6], while necrotizing pancreatitis occurs in 5–10% of patients and has a mortality rate of 43% [7]. Even though acute pancreatitis is characterized by significant morbidity and mortality, thanks to the improvements in the treatment of critically ill patients, and timely and accurate diagnoses in the last ten years, the mortality has decreased from 1.6% to 0.8% [8]. In spite of this, morbidity and long-term consequences still remain considerable [9]–[11]. For example, after their first episode of acute pancreatitis, a quarter of all patients develop exocrine pancreatic insufficiency, and nearly half of patients develop prediabetes or diabetes [12], [13].

The largest number of cases of acute pancreatitis are alcohol related, gallstone related, or idiopathic [2], [10], [14], [15]. Gallstone related pathology was likely to account for 28%-38% of the cases while alcohol related causes were 19%- 41% of the cases [10], [14], [16]. With respect to gender, females are more likely to have gallstone related pancreatitis [5], [17] and the rise in frequency of acute pancreatitis has been observed in women and men older than 35 years [2].

The global increase in obesity is also suspected to contribute to the rising worldwide incidence of acute pancreatitis [18]. Several studies associate most common health problems experienced by obese people, such as gallstones, hypertriglyceridaemia and diabetes, with acute pancreatitis [19], [20]. Aetiologies of acute pancreatitis are shown in Table 1.1.

Aetilogy	Examples	Suggestive clinical data
Gallstones	NA	Choledocholithiasis; alanine aminotransferase over three times the upper limit of normal; cholelithiasis when other causes have been ruled out.
Alcohol	NA	Drinking history described as more than 35 standard drinks per week, for more than 5 years.
Smoking	NA	Long-term smoking habit.
Trauma	• Post-operative trauma. • Gunshot or stab wounds.	Onset of pancreatitis following trauma.
Metabolic	• Hypertriglyceridaemia • Hypercalcaemia • Diabetes	• Elevated triglyceride levels. • Elevated calcium levels. • Diabetes diagnosis.
Genetic	Risk genes: *PRSS1*, *SPINK1*, *CFTR*, *CASR*, *CTRC*	Family history of pancreatitis or pancreatic cancer; pancreatitis onset in people under 30 years of age.
Pre-malignant and malignant conditions	• Intraductal papillary mucinous neoplasm • Ductal adenocarcinoma • Metastatic carcinoma to the pancreas	Pancreatic cancer diagnosis or detection of metastases to the pancreas.
Autoimmune Pancreatitis (AIP)	• Type 1 AIP - IgG4-related pancreatitis. • Type 2 AIP - idiopathic duct-centric pancreatitis.	Published diagnostic criteria [22].
Animal toxins	• Scorpion venom (*Tityus trinitatis).* • Snake venom (*Vipera berus*).	The sting of the scorpion, or a snakebite.
Drugs	Losartan, didanosine, pentamidine, valproic acid, pentavalent antimonials.	Administration of drugs known to induce AP.
Infections	• Viruses (mumps, Coxsackie B, hepatitis). • Bacteria (*Mycoplasma pneumoniae*, *Legionella pneumophila, Leptospira sp. Salmonela sp.*). • Parasites (Ascaris lumbricoides, Fasciola hepatica, and hydatid disease).	Onset of pancreatitis coinciding with the infection.
Idiopathic	NA	When all other causes have been ruled out.

Table 1.1: Summary of acute pancreatitis aetiologies. Updated and modified from Lee and Papachristou [21]. NA: not applicable.

1.2 Pathophysiology of acute pancreatitis

At the cellular level, events that lead to the pathogenesis of acute pancreatitis include dysregulated calcium signaling [23]–[26], premature activation of pancreatic digestive enzymes within the acinar cells [27]–[29], endoplasmic reticulum stress and impaired unfolded protein response [30]–[33], mitochondrial dysfunction [30], [34], [35], as well as impaired autophagy [30], [36]. The main culprits behind the initial induction of pancreatitis are usually the established acinar cell toxins, such as alcohol, nicotine and bile acids [23]. Other causes, some of them listed in Table 1.1, can be infections, drugs or malignancies. Inflammatory response is initiated and propagated by the interaction between acinar cells and the immune system [66]–[68].

1.2.1 Premature trypsin activation

While still in the acinar cells and the draining ducts, pancreatic digestive enzymes are maintained in an inactive form (i.e. zymogens such as trypsinogen). Upon entry into the gut lumen, they are cleaved by duodenal enterokinase or trypsin itself to produce the active enzyme trypsin [40]–[43]. This is the reason why factors, such as cerulein hyperstimulation or ethanol consumption that influence premature activation of the digestive enzymes within acinar cells, have the capacity to cause auto-digestion of acinar cells that starts the cascade of inflammatory events.

As summarized in Figure 1.1, the proposed mechanism suggests that supramaximal dose of cerulein causes activation of acinar cells via low-affinity cholecystokinin (CCK) receptors (CCKRs). This induces intracellular cytoskeletal changes that result in inhibition of acinar cell secretion [42], which leads to the rapid accumulation of trypsinogen-carrying vesicles within acinar cells and their fusion with the lysosome. After this event, cathepsin B, a key lysosomal enzyme, catalyzes cleavage of trypsinogen into trypsin [44]. It is still a matter of debate how trypsin and cathepsin B are released from the lysosomes. Some publications suggest that enzyme accumulation in the lysosomes might lead to their rupture, while other studies have suggested that trypsin causes lysosomal membrane leakiness [45].

Presence of trypsin and other pancreatic digestive enzymes in the cytosol of acinar cells causes their autodigestion. Cathepsin B aids this process by inducing a spillage of cellular contents or necroptosis, a regulated form of necrosis [45], [46]. Altogether, these events lead to the generation of many components of dying autodigested cells, also known as damage associated molecular patterns (DAMPs), that trigger the immune response (Figure 1.1).

1.2 Pathophysiology of acute pancreatitis

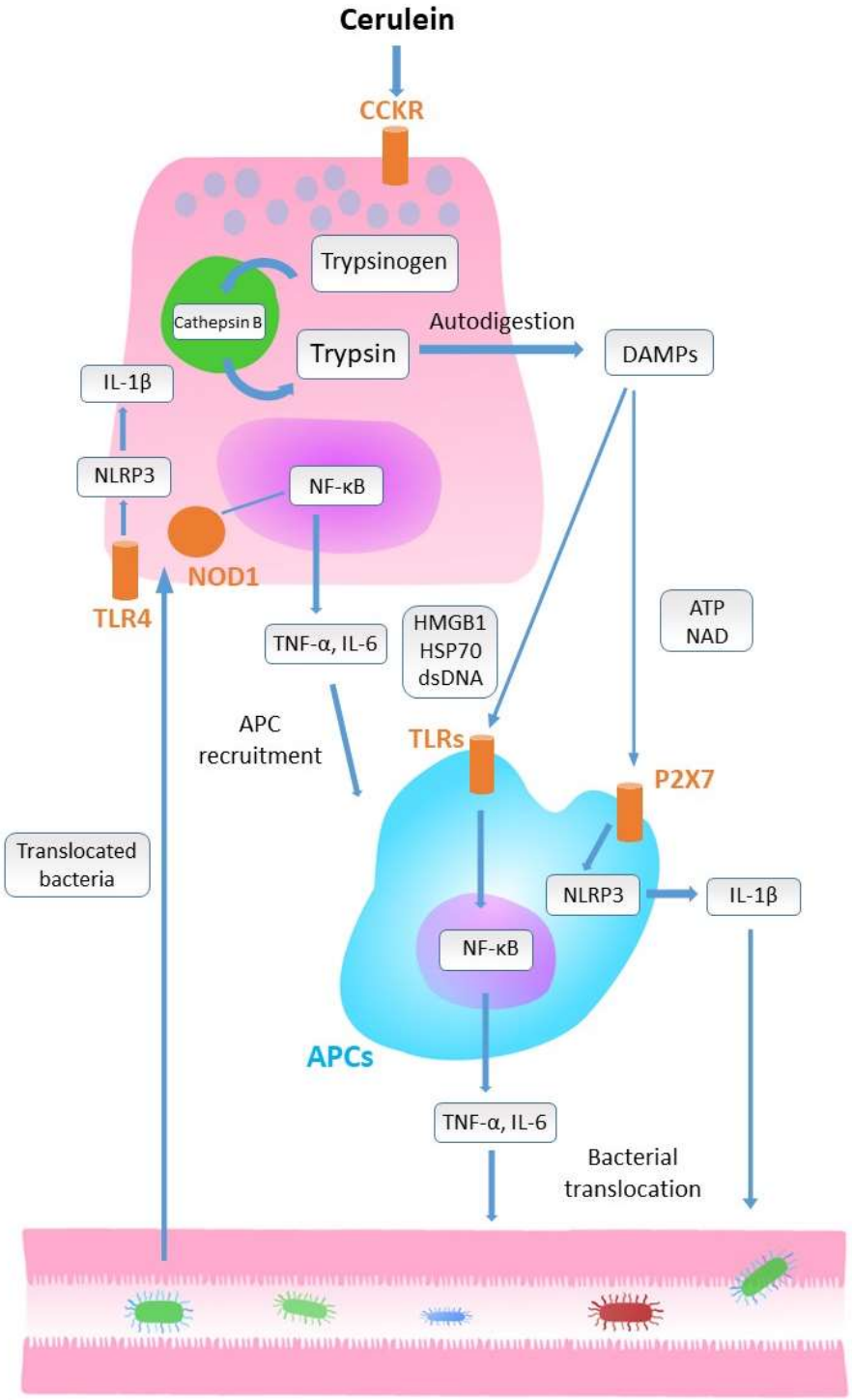


Figure 1.1: Initial events leading to the induction of pancreatitis. In the cerulein mouse model of pancreatitis, cholecystokinin receptors (CCKR) hyper-stimulation leads to generation of trypsin from trypsinogen. This causes acinar cell autodigestion and release of damage associated molecular patterns (DAMPs). Some DAMPs such as

double-strand DNA (dsDNA) released from autodigested cells, high-mobility group box protein 1 (HMGB1) and heat-shock protein 70 (HSP70) act via toll-like receptors (TLRs) to induce nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, which leads to generation of pro-inflammatory cytokines such as TNF-a and interleukin 6 (IL-6). Some DAMPs, such as adenosine triphosphate (ATP) or nicotinamide adenine dinucleotide (NAD) act via purinergic receptor P2X7 to activate inflammasome and lead to production of interleukin 1β (IL-1β). Release of these cytokines leads to changes in intestinal permeability and the translocation of gut commensal flora into the circulation. Translocated bacteria stimulate acinar cells via Toll-like receptor 4 (TLR4) and nucleotide-binding oligomerization domain-containing protein 1 (NOD1). TLR4 signaling leads to the activation of the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome and the production of IL-1β, while NOD1 signaling leads to production of pro-inflammatory cytokines which leads to recruitment of antigen-presenting cells (APCs) and exacerbates the bacterial translocation.

1.2.2 Role of immune system response and cytokine release in pathophysiology of acute pancreatitis

It is a widely considered fact that the uninflamed pancreas is a sterile organ [47]. Therefore, it is assumed that the initial cellular injury, triggered by pathological trypsinogen activation in a model of cerulein-induced pancreatitis, occurs in an aseptic environment. Many studies strongly suggest that the injury is likely to result from the release of DAMPs from autodigested pancreatic acinar cells [48].

As described in Figure 1.1, various kinds of DAMPs, also known as pancreatitis-associated ‘‘danger signals’’, including high-mobility group box protein 1 (HMGB1), adenosine triphosphate (ATP), released cellular DNA, and nucleosomes mediate their effects by binding to different receptors on the immune cells [49]–[52] of the innate immune system and trigger the inflammation [48]. For example, released cellular DNA, HMGB1, and heat-shock protein 70 (HSP70) signal through Toll-like receptors (TLRs) leading to activation of the nuclear

factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway [53]. NF-κB is a prominent transcription factor that mediates the gene expression of pro-inflammatory cytokines, chemokines and adhesion molecules [54].

In cerulein-induced pancreatitis, Hoque *et al.* have proposed a molecular mechanism mediated by DAMPs interaction with pattern recognition receptors (PRRs) [47]. This study showed that activation of TLR9 on pancreatic macrophages by the DNA released from the autodigested acinar cells is one of the crucial events in the early phase of cerulein-induced pancreatitis [48], [52], [55]. At the same time, released ATP induces the activation of purinergic P2X7 receptors, which then together with TLR9 activate NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome leading to the conversion of the pro-IL-1β to mature interleukin 1β (IL-1β) [56].

IL-1β is a potent neutrophil chemotactic factor, and its activation points to the possibility that the early neutrophil infiltration into the pancreas is one of the main characteristics of the acute pancreatitis. In addition to their role in killing the invading organisms, neutrophils also produce extracellular traps, which are adhesive nets largely made of DNA and granular proteins. Their role is to activate pro-inflammatory signals, cause ductal obstruction, and also prematurely activate trypsinogen [38], [57].

Along with neutrophils, macrophages are the first immune cells to be recruited into the pancreas in the initial phases of pancreatitis [56]. In addition to their recruitment, macrophages at distant organs are also activated, and this activation leads to worsening of the systemic inflammation and distant organ injury, although the exact mechanisms of the resulting injury have not been fully explained [58].

The cellular contents released from autodigested and dying cells further activate infiltrated immune cells, leading the positive-feedback loop that exacerbates the inflammation [59], [60].

It has also been shown that the initial onset of cerulein-induced acute pancreatitis is dependent on the activation of nucleotide-binding oligomerization domain-containing protein 1 (NOD1) in acinar cells by commensal bacteria translocated from the gut, which causes activation of NF-κB; and also on the ongoing pro-inflammatory cytokine release by antigen

presenting cells (APCs) [48], [55], [61]. The inflammation is thereby aggravated by the exposure of acinar cells to organisms originating in the gut [62].

Additionally, there is evidence that a precursor form of IL-1β, may have a role in the induction of gut permeability to commensal organisms [52].

A comprehensive study of bacterial invasion in pancreatitis by Li *et al.* [63] found that bacterial DNA can be detected in the circulation of about 70% of patients with pancreatitis and this percentage increased with disease severity. Most of the organisms were similar to those found in the gastrointestinal tract and probably originated in the bowel lumen. In addition, pathogens were also found which account for the more severe complications of bacterial invasion in pancreatitis, such as pancreatic necrosis.

1.2.3 Pro-inflammatory cytokine activity in acute pancreatitis

In summary, pathogenesis in cerulein-induced model of pancreatitis starts with initiation of acinar cell damage by dysregulated trypsinogen activation. This leads to the release of DAMPs that act on PRRs on antigen-presenting cells to cause the generation of cytokines [64]. That is why one of the hallmarks of acute pancreatitis is elevation of serum levels of pro-inflammatory cytokines such as IL-6, IL-1β, and tumor necrosis factor α (TNF-α) [65]–[67] and various chemokines such as IL-8, monocyte chemoattractant protein 1 (MCP-1), which are secreted from acinar cells and recruited immune cells [67]–[69].

1.2.4 Role of gut barrier failure in acute pancreatitis

After the onset of pancreatitis and initiation of inflammation, the inflammation rapidly leads to changes in the gut permeability that facilitate bacterial translocation, a process of migration of intraluminal bacteria and bacterial fragments from intestine into circulation [70]. This process which may promote secondary infections has been shown to occur very early in animal models of acute pancreatitis as enteric bacteria have been found in mesenteric lymph nodes, liver, spleen, lungs, as well as pancreas [59], [71]–[73]. In fact, infection of necrotic

pancreatic tissue is one of the most important causes of mortality in acute pancreatitis [74], [75].

Clinical studies are showing that severe pancreatitis can lead to pancreatic necrosis and systemic inflammatory response syndrome (SIRS) with multiple organ failure (MOF) and death being the end result [62], [76]–[78]. These local and extra-pancreatic complications occur mainly due to invasion of pancreas by members of gut microbiota and their subsequent dissemination to other organs [62].

There are several explanations how pancreatic damage increases intestinal permeability and causes ischemia and bacterial overgrowth in the gut, as summarized in Figure 1.2 [79]. One of them postulates that the decreased gut motility, caused by disturbances in the secretion gastrointestinal peptides and hormones during acute pancreatitis, is one of the leading factors contributing to the bacterial overgrowth and translocation [80]. Another important cause is shown to be the reduced oxygen delivery by the impaired blood supply to the gut [79], as well as the massive distortion of the local and systemic microvasculature, which results in damage to the tight junctions and the enteric epithelium [81].

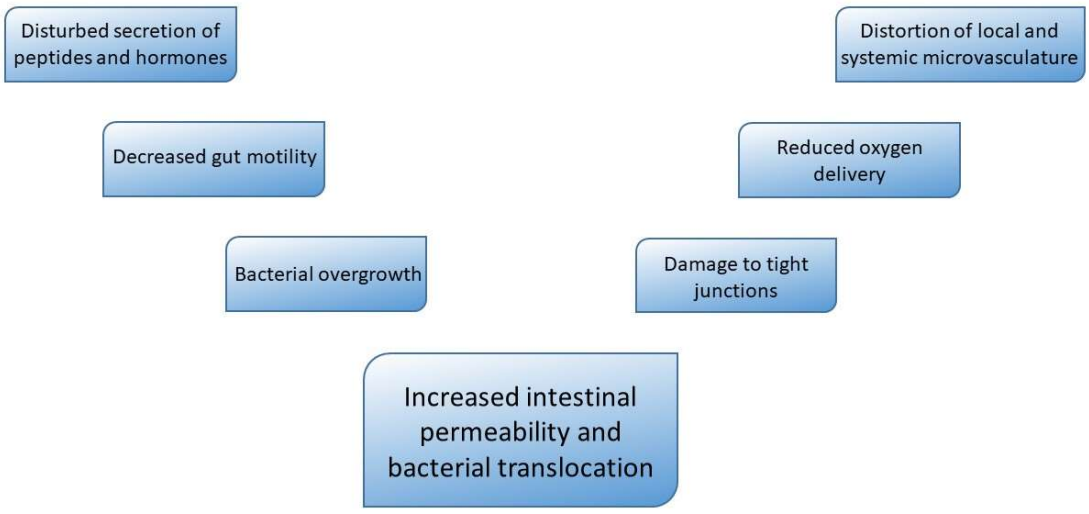


Figure 1.2: Schematic representation of causes leading to increased intestinal permeability and bacterial translocation.

In summary, bacterial overgrowth, damaged gut epithelium, together with decreased motility due to the gastric peptide and hormone de-regulation, represent the main reasons for bacterial translocation that leads to continuous pancreatic contamination in acute pancreatitis. This is hypothesized to be the major driving force of pancreatic inflammation after its initiation.

1.3 Clinical characteristics and diagnosis of acute pancreatitis

In order to diagnose the acute pancreatitis two of the following three criteria need to be met: abdominal pain localized to the upper-to-middle abdomen; elevated levels of pancreatic enzymes more than three times the normal upper limit; and imaging findings characteristic for acute pancreatitis. In addition to this, other clinical characteristics of acute pancreatitis are jaundice, diarrhea, vomiting, flank pain, fever, back pain, abdominal distension, hematemesis and nausea [82].

1.3.1 Measurement of serum levels of digestive enzymes

The most common serum-based biomarkers used for the diagnosis of acute pancreatitis are amylase, and lipase [83]. In acute pancreatitis, the level of amylase (glycoside hydrolase) rapidly increases in 4 to 6 hours after the onset of the disease. It remains high for 3 to 4 days in humans and gradually decreases afterwards [84]–[86]. When compared to levels of amylase, levels of lipase are found to be higher during the onset of acute pancreatitis. This marker is also more specific and sensitive than amylase for detecting acute pancreatitis because its serum levels remain elevated for about two weeks in patients before they return to the normal level [85], [87]. Because of the use of different laboratory methods for measuring these enzymes, there is no standardized reference range for levels of serum amylase and lipase. It is important to mention the limitations of serum amylase and lipase as diagnostic tests for acute pancreatitis. Amylase levels can be increased by other causes unrelated to acute pancreatitis like bowel obstruction, perforation and infraction, as well as appendicitis. Conversely, amylase levels can be normal in patients with alcoholic or

hypertriglyceridaemic pancreatitis [88], [89]. Lipase can be elevated as a side effect of drugs such as psychopharmacological agents, or in different intestinal pathologies, such as biliary obstruction, cholecystitis and duodenal ulceration [89]. Since diagnosis might be challenging in those patients it is necessary to complement diagnostic work with different imaging techniques.

1.3.2 Direct visual assessment of pancreas via computed tomography scan

The most commonly used imaging technique in diagnostics of acute pancreatitis is abdominal computed tomography scan (CT) [90]. Findings can include gland edema, lack of contrast enhancement in the parenchyma (signifying necrotizing pancreatitis) and peripancreatic fluid collections [91]. It is also important to mention peripancreatic fat stranding which manifests itself as blurred interface between the pancreatic parenchyma and surrounding fat on a CT scan and is an indicator of interstitial pancreatitis [90].

1.4 Assessment of severity of acute pancreatitis in patients

1.4.1 Classifications of acute pancreatitis

The revised Atlanta and Determinant-based classification of acute pancreatitis were developed with aim to establish patient severity classification with prognostic significance, and reflect important developments in the understanding of the main factors contributing to morbidity and mortality in acute pancreatitis. These two categorizations have been extensively compared and validated by multiple clinical centers [92]–[94], as summarized in Table 1.2.

Classification	Mild AP	Moderately severe AP	Severe AP	Critical AP
Revised Atlanta classification	No organ failure and no local complications.	Organ failure that resolves within 48 hours (transient organ failure). Local or systemic complications without persistent organ failure.	Persistent organ failure (lasting longer than 48 hours).	NA
Determinant-based classification	No organ failure and no local complications (no pancreatic or peripacreatic necrosis).	Sterile pancreatic and peripancreatic necrosis and/or transient organ failure.	Persistent organ failure or infected pancreatic or peripancreatic necrosis.	Persistent organ failure and infected pancreatic or peripancreatic necrosis.

Table 1.2: Comparison between revised Atlanta classification and Determinant-based classification. Modified from Lee and Papachristou [21].

1.4.2 Prediction of severity

Because of the significant mortality rate of severe acute pancreatitis, many prognostic tools have been developed with the aim of predicting it early in the disease course [21]. They include laboratory markers [95]–[97], biomarkers [98]–[101] and clinical scoring systems [95], [98], [102]–[105].

Despite many available prognostic modalities, the ability to predict severe disease early in acute pancreatitis with accuracy of about 80% is still modest [105]–[107]. That is the reason why doctors rely upon simple and accurate clinical predictors of severity. Those include hematocrit [108], serum urea nitrogen elevation [95], [97] and persistent SIRS with elevation of IL-6 [109]. The strongest advantages of these scores over other complex scoring systems is that they are freely available and can be easily followed.

1.5 Clinical management of acute pancreatitis

Once the diagnosis of acute pancreatitis is made, patients are classified on the basis of predicted severity. Of all the prognostic factors, the presence SIRS is highly associated with severity and mortality of acute pancreatitis [109], [110]. The presence of SIRS can be easily calculated based on body temperature, respiratory rate, heart rate, and white blood cell count.

The main factors for early management of acute pancreatitis include fluid resuscitation, analgesia, nutritional support, as well as identification of etiology.

1.5.1 Intravenous fluid resuscitation

Established guidelines for pancreatitis management agree that early fluid administration is the foundation of management in acute pancreatitis [89], [92], [111], [112]. A fluid of choice in management of acute pancreatitis is lactated Ringer's solution, which is currently preferred over other crystalloids. The prevalence of use of lactated Ringer's solution is based on a pilot randomized clinical trial [113] supported by mechanistic evidence [114], which showed that its administration is associated with a significant reduction in SIRS by 84% compared to saline.

1.5.2 Nutritional support

It has been shown that early and aggressive approaches to feeding have an impact on the reduction of the length of hospital stay in patients with mild or moderately severe pancreatitis. Early introduction of solid, low-fat diet is supported by evidence for patients with mild or moderately severe pancreatitis [115]–[117]. For patients who tolerate oral diet, an initial low-fat solid diet is preferred whereas enteral tube feeding is considered for patients who do not tolerate oral feeding [115], [117].

1.5.3 Analgesia

Effects of analgesia on the course of acute pancreatitis are still largely unknown. Regarding the use of opioids, one animal study showed that morphine administration was associated with increase in acute pancreatitis severity and the inhibition of subsequent pancreatic regeneration [118]. Even though one study shows that opioid analgesia, frequently used in the USA, seems to be effective [119], opioid medication might not be an ideal choice because of the high risk of development of the addiction in patients. Therefore, non-steroidal anti-inflammatory agents, such as metamizol are being explored as options in pain management of acute pancreatitis [120]. In a large study with patients from intensive care units, epidural analgesia, based on non-narcotic anaesthetics such as bupivacaine, was associated with significant reduction in mortality [121]. It is shown that benefits of epidural analgesia stem from improved splanchnic and pancreatic blood flow and anti-inflammatory effects [122], [123].

1.5.4 Identification of aetiology and its management

Many studies show that promotion of awareness of the damage induced by regular smoking and alcohol consumption significantly reduce acute pancreatitis recurrence and readmission rates [124]–[126]. Additionally, emerging evidence suggests that even mild increase in triglyceride blood levels might contribute to acute pancreatitis severity [127]. This highlights the importance of regular check-ups of serum triglyceride levels and treatment when above 1000 mg/dL. In the absence of prior history of pancreatitis or chronic pancreatitis, main-duct intraductal papillary mucinous neoplasm (IPMN) should also be considered as an aetiology because of their high malignant potential. Pancreatic cancer is also a cause of acute pancreatitis that accounts for approximately 1% of all cases [128].

1.5.5 Antibiotic prophylaxis

The use of prophylactic antibiotics in patients with either severe pancreatitis or necrotizing pancreatitis is still a matter of heated debate. Presently, the guidelines recommend no routine

antibiotic prophylaxis in acute pancreatitis [89], [92], [112]. This issue will be discussed in more detail in the following chapter.

1.6 Complications during the course of acute pancreatitis

1.6.1 Local complications

Local complications mainly refer to pancreatic fluid collections that form in and around the pancreas. There are two types of collections: acute fluid collections, that consist of fluid with minimal or no solid debris, and acute necrotic collections that contain necrotic debris from pancreatic or peripancreatic necrosis. When these collections become persistent, organized and encapsulated, they are called pseudocyst and walled-off pancreatic necrosis (WOPN), each having different management guidelines [92]. The aim of naming local complications is to simplify and standardize the definitions and consequently, the patient treatment.

1.6.2 Disease progression to chronic pancreatitis

After an initial episode of acute pancreatitis, 18% of patients develop recurrent acute pancreatitis which results in reduced quality of life [10], [11], [129], [130]. Additionally, these patients are at substantially increased risk of chronic pancreatitis [129], [131]. This is especially problematic since many cohort studies have suggested that chronic pancreatitis might be an important risk factor for pancreatic cancer [128], [132]–[134]. The strongest risk factors for progression to recurrent acute pancreatitis and chronic pancreatitis are active alcohol consumption and smoking, but it can also be idiopathic [129], [131], [135].

1.6.3 Endocrine and exocrine complications

The most common endocrine and exocrine complications of acute pancreatitis are diabetes and exocrine pancreatic insufficiency. Even though the mechanisms and risk factors need to be further explained, it has been established that approximately one-third of acute pancreatitis patients will develop prediabetes or diabetes within 5 years of the initial AP

occurrence [12], [136]. Even more common is exocrine pancreatic insufficiency which occurs in 24–40% of patients who suffered from acute pancreatitis [9], [13], [136], [137]. Described risk factors for exocrine pancreatic insufficiency that occurs after acute pancreatitis include alcohol-related aetiology of acute pancreatitis, pancreatic necrosis, and severe acute pancreatitis [13], [136].

1.7 Antibiotic prophylaxis in acute pancreatitis

A large number of conditions such as diabetes, rheumatoid arthritis, inflammatory bowel disease, Parkinson's disease have been associated with gut dysbiosis [138]. Although it is hard to determine if dysbiosis is the cause or consequence of inflammation, many studies suggest that the use of antibiotics can cause dysbiosis [139]–[142]. The dysbiosis leads to the dissemination of multidrug-resistant (MDR) bacteria and increased mortality in patients suffering from inflammatory diseases [143]–[145]. For example, in a study based on dextran sodium sulfate mouse model of colitis it has been shown that an antibiotic treatment leads to the death of mice due to the translocation and systemic dissemination of a multi-drug resistant *Escherichia coli* strain, [143]. Conversely, a recent study showed that broad-spectrum antibiotic treatment of patients who underwent hematopoietic stem cell transplantation leads to the development of intestinal graft versus host disease due to loss of microbial diversity [146]. These two examples indicate that certain antibiotics have a harmful impact on intestinal inflammation in both mice and humans.

However, the use and efficacy of prophylactic antibiotic therapy in acute pancreatitis has long been a point of controversy. Although the pathogenesis of secondary bacterial pancreatic infection is still debated, bacteria found in necrotic pancreatic lesions are identified as gut-derived bacteria [147]. Therefore, it is considered that gut-derived bacteria are responsible for up to 70% of infections of pancreatic necrotic lesions [148]. These bacteria translocate due to a number of reasons, the main ones being disruption of the intestinal flora, bacterial overgrowth and damage to the bowel mucosa. Additionally, Gram-positive bacteria (*Staphylococcus aureus, Streptococcus faecalis, Enterococcus sp*.), anaerobic bacteria and, sporadically, fungi have also been identified in the lesions [149]–[151].

1.7 Antibiotic prophylaxis in acute pancreatitis

The route to the pancreas is supposed to be by migrating from the duodenum via the main pancreatic duct [152]. This occurs either through transmural colonic migration or directly from the bowel since gut mucosal defenses against bacterial translocation become impaired in severe acute pancreatitis [153], [154]. Pathogens can also reach the pancreas via the blood stream, biliary system, or over intertwined lymphatic vessels [152]. It has long been established that pancreatic necrosis can set in as early as the time of hospital admission, when it can be observed by CT scan [155]. However, the dangerous necrotic infection can later become apparent when it comes to multiple organ failure. This points to the existence of a window of opportunity of around 1-2 weeks after disease onset when administering antibacterial therapy may prevent the infection [156]–[159]. Downside of this approach is that the administration of potent antibacterial therapy for 2 weeks or more could potentially increase the risks of antibacterial resistance and facilitate opportunistic fungal infection [160].

In the 1990s a prophylactic antibiotic treatment has been first introduced with the goal of preventing the onset of infected pancreatic necrotic lesions [161]. The most commonly used antibiotics in the prophylaxis of acute pancreatitis are the broad-spectrum carbapenems, such as imipenem, because of their higher penetration of pancreatic tissue compared to other antibiotics [162]–[166]. Even though current guidelines for the management of acute pancreatitis do not recommend antibiotic prophylaxis [92], antibiotic prophylaxis is still sometimes applied [167]–[171]. The reason for this discrepancy is that the benefits of antibiotic prophylaxis for patients with severe acute pancreatitis are still debated. For instance, Røkke *et al.* [172] showed that a prophylactic treatment with imipenem, largely eliminated infected complications, although without having an effect on patient mortality rates. In contrast, prophylactic treatment with a related drug, meropenem did not eliminate pancreatic or peripancreatic infectious complications, and therefore did not affect mortality and the need for surgical intervention [173]. A meta-analysis by Lim *et al.* [174] (details displayed in in Table 1.3) found that antibiotic prophylaxis did not significantly change the rate of incidence of infected pancreatic necrosis. These findings are contradictory and far from conclusive, however, there is still an open question whether antibiotics influence the acute pancreatitis severity and the spread of gut bacteria. Comprehensive summary of meta-analyses showing benefits of antibiotic treatment in acute pancreatitis is given in Table 1.3.

Study	Year	Study type	Studies (n)	Patients	Pancreatitis	Significant reduction with antibiotics	
						All-cause mortality	Infection/pancreatic necrosis
Moggia *et al.*	2017	RCT	17	1058	NP	No	No
Ukai *et al.*	2015	RCT	6	397	NP	Yes	Yes
Lim *et al.*	2015	RCT	11	864	NP	Yes	No
Jiang *et al.*	2012	RCT	11	621	SAP	Yes	No data
Wittau *et al.*	2011	RCT	14	841	SAP	No	No
Bai *et al.*	2010	RCT	9	519	NP	No	No
Yao *et al.*	2010	RCT	9	564	NP	No	Yes
Villatoro *et al.*	2010	RCT	7	404	NP	No	No
Jafri *et al.*	2009	RCT	8	502	SAP	No	No
Hart *et al.*	2008	RCT	7	429	NP	No	No
Bai *et al.*	2008	RCT	7	467	NP	No	No
Xu *et al.*	2008	RCT	8	540	NP	No	Yes
Dambrauskas *et al.*	2007	RCT	10	1279	NP	Yes	Yes
De Vries *et al.*	2007	RCT	6	397	SAP	No	No
Mazaki *et al.*	2006	RCT	6	329	NP	No	No
Xiong *et al.*	2006	RCT	6	338	SAP	No	No
Villatoro *et al.*	2006	RCT	5	294	NP	Yes	No
Sharma *et al.*	2002	RCT	3	160	NP	Yes	No
Golub *et al.*	1998	RCT	8	514	SAP	Yes	No data

NP: necrotising pancreatitis; RCT: randomised controlled trial; SAP: severe acute pancreatitis

Table 1.3: List of meta-analyses showing the outcome of antibiotic treatment in acute pancreatitis. Updated and modified from Mourad *et al*. [171].

1.7.1 Role of bowel decontamination in prophylaxis of acute pancreatitis

There are several ways by which antibiotics can intercept bacteria, even though they may not effectively enter the necrotic areas, despite high pancreatic tissue penetrability of certain antibiotics [175]–[180]. Antibiotics in blood-circulation can prevent infection via bloodstream and lymphatic routes [157], also near-total bowel decontamination has emerged as a potential therapy [149], [181].

As it has been explained in more detail, microbe-associated molecular patterns (MAMPs) originating from the intestinal microbiota activate the host innate immune system via pattern recognition receptors such as Toll-like receptors (TLRs) and nucleotide-binding domain and leucine-rich repeat containing molecules (NLRs) [61], [182]. In addition, NF-κB, a downstream transcription factor of the TLR and NLR signaling pathways [55], [61], plays a critical role in the development of acute pancreatitis [74], [183], [184]. This points to the fact that activation of TLRs and NLRs by translocated gut bacteria is one of the events that lead to the development of severe acute pancreatitis.

In accordance to this, Tsuji *et al.* [185] showed that induction of pancreatitis with consecutive, high doses of cerulein, requires the presence of gut commensal organisms acting through NOD1 for the development of pancreatic inflammation (Figure 1.1), and that bowel sterilization by broad-spectrum antibiotics attenuates pancreatitis induced with high doses of cerulein.

Here we can conclude that bacterial translocation from the gut to the pancreas and the subsequent induction of acinar cell expression of inflammatory cytokines are also necessary elements of acute pancreatitis induction, in addition to the established mechanism of acinar cell autodigestion by premature activation of pancreatic enzymes [53], [186]. This offers a new

approach to the treatment of acute pancreatitis through development of therapeutic measures preventing bacterial overgrowth and translocation, such as total bowel decontamination.

Another important controlled clinical trial by Luiten *et al.* [149], pointed to the importance of selective decontamination for the treatment of severe acute pancreatitis. This clinical trial showed that selective decontamination that led to the near-total elimination of Gram-negative bacteria in the gut, prevented the onset of pancreatic infection, and significantly reduced mortality in patients with severe acute necrotizing pancreatitis.

Gianotti *et al.* [187] argued for the application of selective bowel decontamination with polymyxin B, amikacin, amphotericin B. In this mouse study, the group concluded that Gram-negative and Gram-positive bacteria and fungi play a prominent role in morbidity and mortality in acute pancreatitis, since the application of selective bowel decontamination improved the survival rates of mice. It also reduced the incidence of infections of necrotic lesions with gut bacteria.

However, in contrast to these studies, Villatoro *et al.* analyzed data from 7 trials involving 404 patients randomly allocated to receive antibiotics or placebo (Table 1.3). They concluded that there was no statistically significant effect on reduction of mortality with antibiotic therapy [188].

1.8 Animal models of acute pancreatitis

The main obstacles in elucidating the pathophysiology of acute pancreatitis are the high variability of the disease severity and the problems in accessing the pancreas in a clinical study [189]. Since exact etiological, environmental, and genetic factors that have an impact on the severity level of acute pancreatitis are still unclear, using animal models is crucial to increase the understanding of this pathology. First use of animals in acute pancreatitis modelling was by Claude Bernard in 1856 [190]. Since then different animal models have been developed [191]. Rodents are the most widely used animals due to the ability to adequately model the

disease, in addition to high reproducibility and low cost [192]. Advances in genetic engineering further increased the capacities of the use of mice in modelling different conditions [192]. As the first step when using mouse models of acute pancreatitis, the elevation of pancreatic enzymes should be assessed in order to confirm the induction of acute pancreatitis. In addition, interstitial edema, parenchymal loss, inflammatory cell infiltration, acinar cell necrosis, hemorrhage, and the evaluation of local or systemic complications, are used in order to assess severity of pancreatitis [193].

Acute pancreatitis classification	Animals	Models
Mild acute pancreatitis	Rats and mice	Cerulein-induced model
Moderate acute pancreatitis	No model	No model
Severe acute pancreatitis	Mice	Cerulein-induced model – higher number of doses.
	Mice and Rats	Closed duodenal loop model
	Mice	Alcohol-induced model
	Mice and Rats	Nutrient-induced model
	Mice and Rats	Biliopancreatic duct injection model
	Mice and Rats	Vascular-induced model
	Mice and Rats	Ischemia/Reperfusion model
	Mice and Rats	Duct ligation model

Table 1.4: Animal models for acute pancreatitis according to the severity degree. Modified from Silva-Vaz *et al*. [194].

The severity of acute pancreatitis can be divided into three degrees: mild, moderately severe, and severe [7]. This classification is based on the absence or presence of multiple organ failure and local and systemic complications. The early prediction of severity is of great importance in the management of acute pancreatitis, since in the severe form there is significant

pancreatic necrosis and systemic inflammation, which may lead to multiple organ failure and death.

Determining severity of acute pancreatitis in murine models is a challenging task. However, it can still be predicted how will the mice react to certain treatments and if they will develop mild or severe form of pancreatitis (Table 1.4).

1.8.1 Cerulein-induced acute pancreatitis model

Cerulein-induced acute pancreatitis is the most widely used acute and chronic pancreatitis model. It is based on the use of cerulein, a cholecystokinin analogue that stimulates pancreatic secretion [195]. Cerulein was first isolated and characterized by Anastasi *et al.* from extracts of the skin of the Australian tree frog *Hyla caerulea* [196]. Later, it was isolated from the skin of several other species including *Xenopus laevis* and *Leptodactylus pendactylus* [197]. Authors of this study hypothesized that cerulein may have a role in the regulation of secretory processes of the skin and in the exchange of water and electrolytes through the skin.

In this model, acute pancreatitis results from administration of supra-physiological doses of cerulein, which leads to overproduction of pancreatic digestive enzymes. This model histologically simulates the early phase of acute pancreatitis in humans, and is a highly reproducible, rapid, non-invasive and economical model in rats and mice [34], [198], [199]. Table 1.5 summarizes clinical relevance of cerulein model, the different routes of administration, as well as the most commonly used doses. The model of cerulein-induced pancreatitis in rats was developed by Lampel *et al.* [200], and is a model of mild, interstitial, and edematous pancreatitis. Almost a decade later, this model has been adapted by Niederau *et al.* [201] to induce severe acute pancreatitis with necrosis of acinar cells in mice. The supra-physiological dosage of cerulein can be achieved intravenously [200], [202], [203], subcutaneously [204], [205], or intraperitoneally [206]. Despite intravenous mode of administration being the best way of achieving the right dosage, it is not a standard practice due to the tedious procedure and the requirement of anesthesia. Therefore, this method was modified for intraperitoneal administration in the lower left or right quadrant of the abdomen.

1.8 Animal models of acute pancreatitis

As explained in the chapter 1.2, the mechanism of cerulein-induced pancreatitis starts with overstimulation of CCK receptor, which leads to initiation of premature trypsinogen activation within acinar cells. This causes infiltration of immune cells into the pancreas, pancreatic edema, and acinar cells vacuolization that are comparable to acute pancreatitis in humans. On histological level, interstitial edema develops one hour after the last cerulein injection, reaching a peak after 12 h [200]. Evaluation of the acute pancreatitis is done by analyzing the pathophysiology [207], [208], severity [209], [210], course of the disease and mortality [31], and associated lung [211], [212] and heart muscle [213] injuries. Interestingly, it is also a useful model for studying the pathophysiology of scorpion venom and other animal toxins-induced acute pancreatitis in humans [214], [215].

Acute pancreatitis model	Animals	Protocols		References	Clinical relevance
		Administration route	Doses		
Cerulein	Mice	Intravenous	6 h continuous infusion of 100µg/kg/h	[203]	- Relevant to understanding the early acute pancreatitis mechanisms. - Pulmonary injury mimics the respiratory involvement in humans - Structural changes of acinar cells are similar to human acute pancreatitis. - Preserves acinar physiology throughout the experimental disease course. - Mimics the pathophysiology of acute pancreatitis caused by scorpion venom and cholinergic toxins in humans.
		Subcutaneous	7 h of injections at 50 µg/kg	[205]	
		Intraperitoneal	8 h of injections of 10 µg/mL, 0.2 mL/mouse) over two consecutive days	[206]	
			7 h of injections at 50 µg/kg	[216],[209]	
			50 µg/kg every two hours for five rounds	[208]	
			10 h of injections at 50 µg/kg	[207],[217]	

Table 1.5: Protocols of the cerulein-induced model of acute pancreatitis in mice. Excerpt from Silva-Vaz et al. [194].

1.9 Aims of the study

It has been postulated that commensal gut microbiota plays a role in the onset of pancreatic inflammation. The current hypothesis explains that after the initiation of pancreatitis, the inflammation rapidly leads to changes in the gut permeability that facilitate bacterial translocation. However, the precise mechanisms remain to be further explored. Furthermore, infection of pancreatic tissue which leads to necrotic lesions is one of the most important causes of mortality in acute pancreatitis.

The main objective of this study is to investigate if the change in composition of gut bacteria, whether subtle or drastic as with total bowel decontamination, has the potential to ameliorate acute pancreatitis. Therefore, we wanted to answer the following questions:

Does antibiotic-induced microbiota modulation influence the severity of acute pancreatitis? If so, are there specific bacterial strains responsible for this effect?

Can diet modification ameliorate the severity of acute pancreatitis?

We believe that answering these questions will help to elucidate the connection between the gut microbiota and the pancreas, and by doing so, improve our understanding of the involvement of gut microbiota composition in induction and the course of innate immune reactions.

2 Materials and methods

2.1 Materials

2.1.1 Equipment

BX61VS slide scanner microscope	Olympus
C1000 Touch™ Thermal cycler	Biorad
C1000 Touch™ Thermal cycler CFX384™ Real-Time System	Biorad
Cytoflex S™ flow cytometer	Beckman coulter
NanoDrop®1000 Spectrophotometer	Thermo Scientific®

2.1.2 Software

CFX Manager™	Biorad
FlowJo v10 (Flow cytometry analysis software)	TreeStar
GraphPad Prism V 5.0a (Biostatistics software)	GraphPad Software
Nanodrop®1000 V 3.7.0	Kisker
OlyVIA (Image viewer software)	Olympus

2.1.3 Kit systems

DNA-free™ DNA Removal Kit	Invitrogen
GenElute™ Mammalian Total RNA miniprep kit	Sigma
GoTaq® qPCR Master Mix	Promega
MACS® Cell separation	Miltenyi Biotec

2.1.4 Reagents

0% fiber diet: AIN-93G without fiber or starch	Testdiet®
2.3% fiber diet AIN-93G w/ 2.3% fiber from cellulose and guar gum	Testdiet®
40% fiber diet: AIN-93G w/ 40% fiber from cellulose and guar gum	Testdiet®
60% high-fat diet: DIO Rodent Purified Diet w/60% Energy From Fat	Testdiet®
Agencourt AMPure XP	Beckman Coulter
Ammonium chloride (NH_4Cl)	Roth®
Ampicillin	Chevita GMBH
Brain heart infusion (BHI)	MP Biomedicals
CD16/CD32 Monoclonal Antibody	eBioscience™
Cerulein	Sigma
Collagenase (from *Clostridium histolyticum*)	Sigma
Deoxynucleoside triphosphates (dNTPs)	Promega

2.1 Materials

Dexamethasone	Sigma
Dulbecco's PBS	Gibco™
Eosin 1%	Morphisto
EpCAM CD336 MicroBeads	Miltenyi Biotec
Ethanol	Fischar
Ethanol absolut for molecular iology use	AppliChem
Fetal bovine serum (FBS)	Pan-Biotech
Fluconazol Claris	Pharmore
Formaldehyde 4%	Fischar
Glucose 40%	Braun
Hank's Balanced Salt Solution HBSS	Gibco™
LPS	Sigma
M-MLV RT 5x Buffer	Promega
Mayer's Haematoxylin	Roth®
Metronidazol	Braun
MLV RT RNase	Promega
Nuclease-free water	Promega
Paraformaldehyde (PFA)	Sigma
Penicillin-Streptomycin (5,000 U/mL)	Gibco™

Random primers	Promega
Roti®-mount	Roth®
Roticlear®	Roth®
Sodium chloride (NaCl)	AppliChem
Sodium lauryl sulphate (SDS)	Sigma
Tris(hydroxymethyl)aminomethane (Tris)	AppliChem
Trypsin inhibitor Type I-S: from Soybean	Sigma
Vancomycin	Hikma Pharma GBH
Vectashield antifade mounting medium	Vector laboratories Inc
Waymouth medium	Gibco™
Zombie Aqua™ Fixable viability kit	BioLegend

2.1.5 Buffers and solutions

All buffers and solutions were prepared with Millipore Q distillated water.

2.1.5.1 FISH staining buffers

FISH hybridisation buffer:	20 mM	Tris-HCl
.	0.9 M	NaCl
FISH washing buffer:	20 mM	Tris-HCl

.	0.9 M	NaCl
.	0.1%	SDS

2.1.5.2 Buffers and solutions for FACS

FACS buffer	1x	PBS
.	2%	FCS
ACT buffer for erythrocyte lysis	17 mM	Tris
.	160 mM	NH_4Cl
.	pH 7.2	.

2.1.5.3 Solutions for bacterial lysis

MD solution:	0.1M	$MgCl_2$
.	100 µg/mL	DNase

2.1.5.4 Buffers for isolation and maintenance of acinar suspension

5% FBS:	2 mL	FBS
.	38 mL	HBSS

30% FBS:	6 mL	FBS
.	14 mL	HBSS
Collagenase solution:	20 mg	Collagenase
.	50 mL	HBSS

Growth medium:

.	17.6 mL	Waymouth medium
.	2 mL	FBS
.	200 µL	Penicillin/Streptomycin
.	100 µg/mL	Soybean trypsin inhibitor
.	1 µg/mL	Dexamethasone

2.1.5.5 Probe for the FISH staining

Pan-bacterial probe EUB338: 5'-GCTGCCTCCCGTAGGAGT-3' conjugated with Cy3 fluorophore.

2.1.6 Primer sequences

Name	**Forward**	**Reverse**
GAPDH	GCCTTCTCCATGGTGGTGAA	GCACAGTCAAGGCCGAGAAT
TNF-α	CGATGGGTTGTACCTTGTC	CGGACTCCGCAAAGTCTAAG
IL-6	AGTTGCCTTCTTGGGACTGA	CAGAATTGCCATTGCACAAC
IL-1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA

Table 2.1: Primers used for qPCR

2.1.7 Antibodies

α-mouse CD11c PE-Cy7	BioLegend
α-mouse CD19 PE eFluor 610	eBiosciences
α-mouse CD3 PE Cy7	eBiosciences
α-mouse CD4 eFluor 450	eBiosciences
α-mouse CD45 APC	eBiosciences
α-mouse CD45 APC eFluor 780	eBiosciences
α-mouse CD8a PE	BioLegend
α-mouse F4/80 APC	BioLegend
α-mouse Ly6G BV421	BioLegend
α-mouse MHCII (A-A/A-I) PE	eBiosciences

Table 2.2: Antibodies used for FACS.

2.2 Methods

2.2.1 Animals

2.2.1.1 Mouse strains and housing

C57BL/6 mice were housed in the animal facility of the Institute for Medical Microbiology, Immunology and Hygiene (MIH), Technical University Munich, Munich, or at the animal facility (RARC) of Memorial Sloan Kettering Cancer Center (MSKCC), NYC. Mice were purchased from Envigo in Munich or from Jackson Laboratories in NYC, and were maintained under specific pathogen free (SPF) conditions. Only male, 8-10 week old mice were used, and the experiments were performed in accordance with local government regulations.

Germ-free (GF) mice on a C57BL/6J background were bred and housed in the MSKCC gnotobiotic facility with weekly microbiological monitoring of germ-free status. Experiments with germ-free mice were performed in individual gnotobiotic isocages (SentrySPP, Allentown).

2.2.1.2 Cerulein-induced acute pancreatitis model

Our cerulein model is based on protocols by Zhang *et al.* and Gao *et al.* [209], [216].

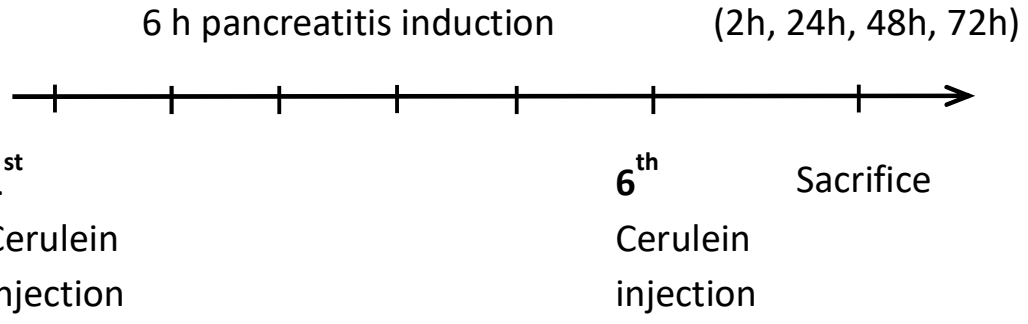


Figure 2.1: Schematic representation of the timeline of injections and sacrifice in cerulein model of acute pancreatitis.

As summarized in Figure 2.1, mice were given 6 intraperitoneal injections of cerulein at 50 µL/kg, diluted in PBS, with 200 µL PBS as a sham control in a one-hour interval. Mice were sacrificed at different time points by cervical dislocation in order to examine the course of pancreatitis and to determine the acute phase of pancreatitis.

2.2.1.3 Colonization of GF mice with specific bacterial strains

GF mice were inoculated once by oral gavage with 200 µL of over-night bacterial culture of *Escherichia coli, Burkholderia cepacia* and *Lactobacillus johnsonii*, two weeks prior to acute pancreatitis induction. Bacteria were cultured in the following conditions: *Escherichia coli*: liquid culture in BB medium, aerobic conditions, at 37°C; *Burkholderia cepacia*: Columbia agar plate with 5% sheep blood, aerobic conditions, at 37°C; *Lactobacillus johnsonii*: Columbia agar plate with 5% sheep blood, anaerobic conditions, at 37°C.

2.2.1.4 Colonization of GF mice with cecal content-derived microbes of mice fed with high-fat diet

GF mice were inoculated once by oral gavage with 200 µL of cecal content of mice fed with high-fat diet or with 200 µL of cecal content of regular chow-fed SPF mice two weeks prior to acute pancreatitis induction. Cecal content was collected after euthanasia from mice that were fed with high-fat diet for two weeks and diluted in sterile PBS under anaerobic conditions.

2.2.1.5 Diet modulation of gut microbiota

Mice were fed with 0%, 2.8% or 40% fiber diet (Testdiet®) for two weeks, prior to acute pancreatitis induction. 2.8% fiber is considered to be normal amount of fiber in mouse chow, and served as a control.

Mice were fed with high-fat diet (Testdiet®), containing 60% fat, for two weeks, prior to acute pancreatitis induction.

2.2.1.6 Antibiotic treatment

The antibiotic cocktail was loaded into mouse water bottles and provided to mice *ad libitum*. In order to prepare antibiotic cocktail, the following antibiotics were diluted in autoclaved water: Ampicillin (1 g/L), Vancomycin (0.5 g/L) and Metronidazole (1 g/L). Additionally, Fluconazole (2 mg/100 mL), an antifungal medicine, was added in order to prevent the growth of opportunistic fungi. Glucose (5 mL/100 mL) was also added in order to neutralise the bitter taste of Metronidazole that sometimes prevents mice from drinking water. The treatment lasted for two weeks.

2.2.2 Biochemical analysis

In order to measure amylase and lipase serum levels, blood was collected in gel tubes with clotting activator (Sarstedt) and centrifuged at 3000 RPM for 10 minutes. The serum was collected and stored at -20°C. For analysis, serum was diluted in 1:10 ratio in the double-distilled water (total 100 µL) and sent to the Clinical chemistry department at Klinikum rechts der Isar. The analysis was performed using Roche COBAS 8000 high-throughput modular analyzer. It is a scalable, module-based serum work area (SWA) solution for a wide range of in vitro diagnostics testing of clinical chemistry and immunochemistry designed for high throughput laboratories.

2.2.3 Histological methods

2.2.3.1 Tissue preparation for histological staining

A piece duodenal portion of pancreas from each individual mouse was placed in histological cassette and fixed for 1 day in 4% formaldehyde at 4°C. Tissue was dehydrated using the Shandon Excelsior ES tissue processor and embedded in paraffin using the TB 588 paraffin embedding system. RM 2245 automatic rotary microtome was used for cutting 4 µm sections that were fixed on glass slides and used for staining.

2.2.3.2 Haematoxylin and eosin histological staining

In order to perform the assessment of the tissue damage induced by acute pancreatitis, pancreatic tissue was stained with haematoxylin and eosin histological staining (HE).

Reagent	**Incubation time**
Roticlear®	10 min
Ethanol 96%	2 min
Ethanol 70%	2 min
Ethanol 50%	2 min
Aqua bidest.	2 min
Mayer's haematoxylin	5 min
Tap water	Rinsing until water becomes clear
1% (v/v) Eosin	5 min
Tap water	Rinsing until water becomes clear
Ethanol 80%	1 min
Ethanol 96%	4 min

Roticlear®	10 min
Roti®-Mount	-

Table 2.3: Haematoxylin and eosin staining protocol.

2.2.3.3 Histopathological scoring

Images of HE stained pancreatic tissue were taken with Slide scanner microscope, 20x magnification. Histopathological scoring was performed on one section per pancreas, in a blinded fashion, according to Schmidt *et al.* [218] as shown in Table 2.4. Histopathologic scores of 10 analyzed fields per mouse were added to form a sum score for each variable. Variable scores were then added to form a total score.

Edema	
0	absent
0.5	focal expansion of interlobar septae
1	diffuse expansion of interlobar septae
1.5	same as 1 + focal expansion of interlobular septae
2	same as 1 + diffuse expansion of interlobular septae
2.5	same as 2 + focal expansion of interacinar septae
3	same as 2 + diffuse expansion of interacinar septae
3.5	same as 3 + focal expansion of intercellular spaces
4	same as 3 + diffuse expansion of intercellular spaces

Acinar necrosis	
0	absent
0.5	focal occurrence of 1-4 necrotic cells/HPF
1	diffuse occurrence of 1-4 necrotic cells/HPF
1.5	same as 1 + focal occurrence of 5-10 necrotic cells/HPF
2	diffuse occurrence of 5-10 necrotic cells/HPF
2.5	same as 2 + focal occurrence of 11-16 necrotic cells/HPF
3	diffuse occurrence of 11-16 necrotic cells/HPF (foci of confluent necrosis)
3.5	same as 3 + focal occurrence of >16 necrotic cells/HPF
4	>16 necrotic cells/HPF (extensive confluent necrosis)
Inflammation and perivascular infiltrate	
0	0-1 intralobular or perivascular leukocytes/HPF
0.5	2-5 intralobular or perivascular leukocytes/HPF
1	6-10 intralobular or perivascular leukocytes/HPF
1.5	11-15 intralobular or perivascular leukocytes/HPF
2	16-20 intralobular or perivascular leukocytes/HPF
2.5	21-25 intralobular or perivascular leukocytes/HPF
3	26-30 intralobular or perivascular leukocytes/HPF

3.5	more than 30 leukocytes/HPF or focal microabscesses
4	more than 35 leukocytes/HPF or confluent microabscesses

Table 2.4: Histopathological scoring HPF: histopathological field.

2.2.4 FISH (Fluorescence *in situ* hybridization) staining

FISH staining was performed on paraffinized pancreatic tissue sections, 4 µm thickness, according to the protocol from Johansson and Hansson [219].

Slides were dewaxed by incubation in the oven for 10 minutes at 60°C and subsequent incubation in Roticlear®, first 10 minutes in prewarmed Roticlear®, second 10 minutes at room temperature. Sections were dehydrated by incubation in absolute ethanol for 5 minutes. After drying, FISH EuB probe conjugated with Cy3 fluorescent fluorophore was applied, diluted in hybridization buffer. Incubation was performed in the dark humid chamber, overnight, at 50°C. After incubation, sections were washed in FISH washing buffer (10 minutes, RT), followed by washing in PBS (3 x 10 minutes, RT). After drying, sections were covered in Vectashield mounting media for fluorescence and left to dry. Slides were stored at 4°C.

2.2.5 Molecular biological methods

2.2.5.1 RNA isolation

RNA isolation from pancreatic tissue, or pancreatic suspension pellet was performed according to instructions from the GenElute™ Mammalian Total RNA miniprep kit (Sigma).

Briefly, tissue was homogenised in lysis buffer with β-Mercaptoethanol (10 µL per 1 mL lysis buffer). Homogenate was loaded on filtration columns and centrifuged (2 minutes, 130000 RPM, RT). Filtrate was then loaded onto the binding column and centrifuged (15 seconds, 13000 RPM, RT). Column was washed with washing buffers 1 and 2 provided in the kit.

Afterwards, column was eluted with application of elution buffer and centrifugation (1 minute, 13000 RPM, RT). The yield and purity of the eluted RNA was determined using NanoDrop 1000 (Kisker). RNA samples were then stored at -80°C for use in qPCR.

2.2.5.2 DNAse digestion

Since the previously described RNA isolation method provides mixed yield with DNA molecules, it is important to perform DNA digestion, in order to get the pure RNA yield.

The DNase digestion was done according to instruction from the Invitrogen DNase kit and incubated at 37°C for 20-30 minutes. DNase inactivation reagent was added and incubated for 2 minutes at RT. Samples were centrifuged (2 minutes, 13000 RPM, RT), and RNA was transferred to a fresh tube.

2.2.5.3 Reverse transcriptase PCR (RT-PCR)

1 µg of RNA was mixed with 1 µL (150 ng/µL) of random primers, and nuclease-free water was added to the total volume of 14 µL. Samples were incubated at 70°C for 5 minutes, followed with incubation on ice (or 4°C in Thermal cycler) for 5 minutes.

Master mix prepared from the following ingredients was added to the samples.

cDNA	**Negative control**
5 µL buffer (5X)	5 µL buffer (5X)
1.25 µL dNTP min (10 mM)	1.25 µL dNTP min (10 mM)
1 µL Enzyme	----------
3.75 Nuclease-free water	4.75 Nuclease-free water

Table 2.5: Master mix ingredients for RT-PCR.

Samples were first incubated for 10 minutes at RT, and then in Thermal cycler for 50 minutes at 50°C, followed with 10 minutes at 70°C.

Resulting cDNA was stored either short-term at 4°C, or long-term at -20°C.

2.2.5.4 qPCR

qPCR was performed with cDNA from the previous section. The qPCR reaction mix was prepared as follows:

qPCR mix component	**Volume**
SYBR Green	5 µL
Forward primer	0.5 µL
Reverse primer	0.5 µL
cDNA sample	6 µL

Table 2.6: qPCR reaction mix.

The reaction mix was loaded on 384-well plate and the PCR was run on the C1000 Touch™ Thermal Cycler (BioRad) with the following settings:

95°C 5 min

(95°C 0.10 min; 59°C 0.30 min; measure fluorescence) x 40

60°C 0.31 min

(60°C 0.05 min +0.5°C/cycle (ramp 0.5°C/s); measure fluorescence) x 2

The results were analysed using the BioRad CFX Manager™ software.

2.2.5.5 16S rRNA amplicon sequencing

Sample preparation and 16S rRNA amplicon sequencing were performed at MSKCC, van den Brink laboratory, according to their established protocol.

DNA was extracted from mouse samples using a phenol-chloroform protocol, and the genomic 16S ribosomal-RNA gene V4-V5 variable region was amplified and sequenced on the Illumina MiSeq platform according to the previously published protocols [220]–[222]. PCR products were purified using the Agencourt AMPure PCR amplicon purification system (Beckman coulter) following the manufacturers' instructions.

2.2.6 Flow cytometry

2.2.6.1 Isolation of pancreatic cells

Resected pancreata were put on a 100 µm cell strainer and grinded with the syringe plunger. The strainer was washed with the FACS buffer, and the flow-through was collected into the 50 mL tube. The flow-through was passed through the 40 µm cell strainer, and centrifuged (25 minutes, 300xg, 4°C). Supernatant was decanted, and pellet was resuspended in PBS.

2.2.6.2 Isolation of splenocytes

Resected spleen was transferred onto the 100 µm cell strainer and grinded with the syringe plunger. The strainer was washed with PBS, and the flow-through was collected into the 50 mL tube. The flow-through was centrifuged (5 minutes, 300xg, 4°C). Erythrocytes from the spleen were lysed by adding 5mL of Erythrocytes lysis buffer for 5 minutes at RT. Lysis was stopped by adding 7 mL of PBS. The mix was passed through the 40 µm cell strainer to remove the tissue clumps and centrifuged (5 minutes, 300xg, 4°C). Supernatant was decanted and pellet was resuspended in PBS.

2.2.6.3 Multicolor surface staining

Resuspended pellet was loaded onto a plate and centrifuged again (5 minutes, 300xg, 4°C). PBS was removed and cells were resuspended in 100 µL FACS buffer with Zombie Aqua™ (1:1000) which was added as a marker that discriminates between live and dead cells, and Fc-block (1:500) for 30 minutes in the dark, at 4°C. After washing in FACS buffer, cells were resuspended in 50 µL FACS buffer with different antibodies (listed below) and incubated for 30 minutes in the dark at 4°C. After washing in FACS buffer cells were fixed in 200 µL FACS/0.5% PFA for the measurement on the next day in Cytoflex S (Beckman Coulter).

T cell panel:	**Other immune cells:**
CD45 APC	CD45 APC 780
CD3 PE Cy7	CD19 PE EF 610
CD8 PE	F4.80 APC
CD4 EF450	CD11b FITC
	CD11c PECy7
	Ly6G BV 420
	MHCII PE

Table 2.7: List of immune cell markers used in construction of FACS panels.

2.2.7 Bacterial culture

Resected pancreata were homogenised in 1 mL BHI medium and plated on Columbia agar 5% sheep blood plates. Every sample was plated onto two plates; one plate would be incubated in aerobic conditions in humified incubator at 37°C, and the other in anaerobic condition, at 37°C.

Plates were incubated for 7 days before being sent to the Diagnostics department of MIH to be analysed by MALDI Biotyper.

2.2.8 Preparation of protein lysate from bacterial culture

Bacteria from liquid overnight culture were pelleted by centrifugation (10,000 RPM, 10 minutes, RT). After washing in PBS bacteria were resuspended in sterile PBS (1 mL); and 250 µL of MD solution was added per 1 mL bacterial suspension. Bacterial suspension was transferred to 2 mL tubes filled with 0.1 mm zirconium beads and disrupted by using a Bead Beater. Tubes were left on ice for 5 minutes to cool down before being centrifuged (7500xg, 5 minutes, 4°C). Supernatant was sterilized by using a 0.2 µm syringe filter. Protein concentration was determined by Bradford assay and lysate was stored in aliquots at -80°C.

2.2.9 Preparation and treatment of acinar culture

2.2.9.1 Harvesting adult pancreas

Pancreas was resected and placed in Hanks BSS (HBSS) (without phenol red) on ice. Pancreas was swiftly minced into small pieces using sterile scissors. Pieces were transferred into the 50 mL tube and centrifuged (720xg, 2 minutes, 4°C).

2.2.9.2 Isolation of adult exocrine pancreas epithelium

Supernatant was removed and 5 mL of collagenase (20 mg in 50 mL HBSS) was added. The mix was transferred into the small petri dish and incubated at 37°C for 10 minutes with occasional shaking. The reaction was stopped by adding 5 mL of 5% FBS and content was spinned at 720xg for 2 minutes at 4°C. Supernatant was removed and pellet resuspended with 10 mL of 5% FBS with trituration. After another centrifuge step (420xg, 2 minutes at 4°C) the supernatant was removed and the pellet was resuspended with trituration. Centrifuging and trituration were once again repeated and the resuspended pellet was passed through 100 µm cell strainer. Some tissue pieces left on the cell strainer were grinded with syringe plunger.

Collected suspension was slowly pipetted on top of the 30% FBS in HBSS. The tube was spinned (180xg, 2 minutes, at 4°C) in order to remove erythrocytes. Supernatant was removed and pellet resuspended in growth medium and plated.

Acinar suspension was treated for 4 hours with: LPS (10 µg/mL), cerulein (10 nM, 20 nM, 50 nM), protein lysate (15 mg/mL). Live bacteria were added to acinar culture: *Lactobacillus johnsonni* 2x10^6 CFU (OD 0.5=10^8 CFU/mL); *Burkholderia cepacia* 4x10^6, 10^7, 10^8 CFU (OD 0.5=10^8 CFU/mL).

After the treatment suspension as collected in 15 mL tubes and centrifuged at 4000 RPM, for 4 minutes. Pellet was resuspended in lysis buffer for RNA isolation and stored at -80°C.

2.2.9.3 MACS sorting of acinar suspension

In order to remove the immune cells from the acinar suspension and determine of the remaining epithelial cells can also trigger the immune response, acinar suspension underwent the magnetic-activated cell sorting (MACS) separation with the use of EpCAM326 magnetic beads (Miltenyi Biotec). The separation was performed according to the manufacturer's instructions. Briefly, acinar suspension was incubated with the EpCAM326 magnetic beads, and passed through LD columns attached to the MACS Separator magnet. The flow-through containing non-labelled cells was discarded. The columns were removed from the magnet and washed again, collecting the labelled cells.

2.2.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism software. In data sets with 3 or more test groups, data were analysed using One-way ANOVA multiple comparison test. For normally distributed data, Newman-Keuls multiple comparison post-hoc test was used, and for not normally distributed data, Dunn's multiple comparison post-hoc test. In data sets with 2 test groups, for normally distributed data, Student's t test was used, and for the not normally distributed data, Mann-Whitney test. Statistical significance was determined based on p-values <0.05.

Data obtained by 16S rRNA amplicon sequencing were analysed at MSKCC, according to a locally established bioinformatics pipeline [223]. Briefly, operational taxonomic units (OTUs)

were classified to the species level against the Greengenes database [224], with gaps in taxonomic annotation filled in by classification against the NCBI 16S ribosomal RNA sequence database [225]. To map sequences on green genes database, the qiime [226] function assign_taxonomy.py and the assignment_method mothur [227] were used. Lastly, blastn on the 16S NCBI database was used to supplement genus and species annotation in sequences that had no genus/species assigned and a 97% match identity.

In order to statistically analyze the taxonomic differences between groups, the linear discriminant analysis of effect size (LEfSe) bioinformatics tool was used. LEfSe is an algorithm for high-dimensional biomarker discovery and explanation that allows for the identification of taxonomic composition differences between two or more treatment conditions [228].

t-Distributed Stochastic Neighbor Embedding (t-SNE) is a technique for dimensionality reduction that is used for the visualization of high-dimensional datasets, data by giving each datapoint a location in a two or three-dimensional map [229]. t-SNE analysis was implemented in the R package Rt-SNE with generalized UniFrac distances as the distance measure as described previously [230].

3 Results

3.1 Establishment of the cerulein-induced model of acute pancreatitis

3.1.1 Identification of the acute phase in our model of acute pancreatitis.

In order to establish a model of acute pancreatitis, we injected mice with six hourly doses of cerulein and sacrificed them at different time points (2h, 24h, 72h). Assessment of the severity of acute pancreatitis was performed by measuring levels of amylase and lipase in serum as biochemical parameters, by determining histological severity scores and by measuring expression of pancreatic pro-inflammatory cytokines in pancreatic tissue.

Amylase and lipase are digestive enzymes, normally secreted into the pancreatic duct and released into the duodenum. However, due to the hyperstimulation of the pancreas by cerulein, they are released into the bloodstream, and can therefore serve as markers for the severity of the inflammation. As shown in Figure 3.1, amylase and lipase levels peak 2 hours after the last cerulein injection.

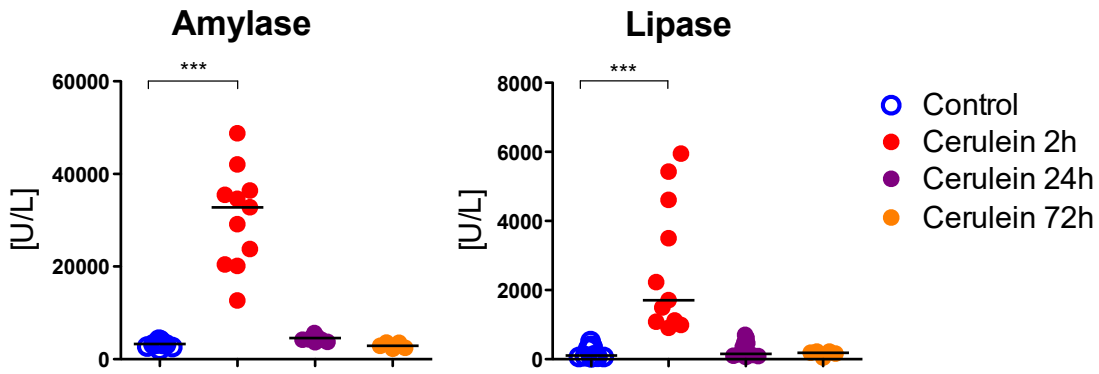
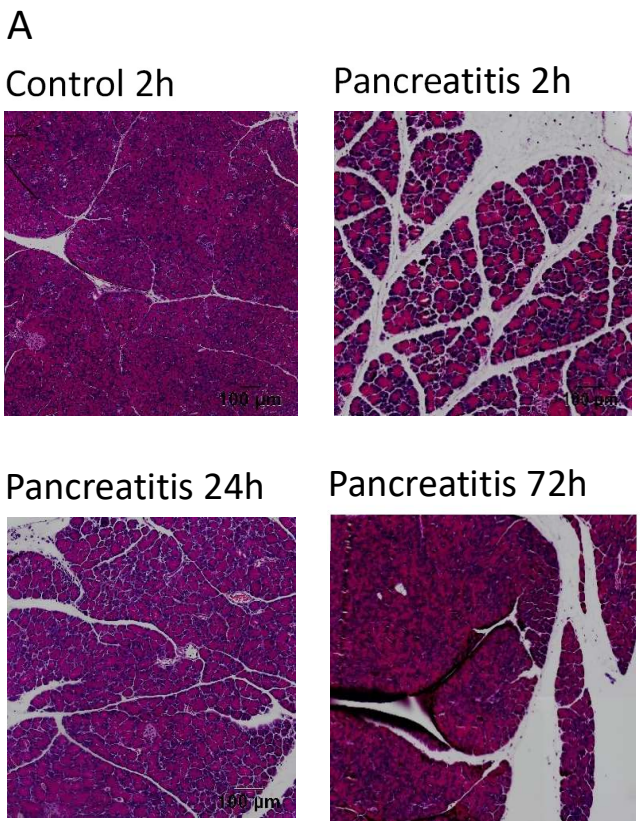


Figure 3.1: Amylase and lipase serum levels at different time points. Serum levels of amylase and lipase measured at 2h, 24h, and 72h after the last cerulein injection by Cobas 8000 Roche diagnostic modular analyser. U/L units per liter. Horizontal lines depict medians. Statistical significance was calculated using one-way ANOVA, with Newman-Keuls post-hoc test. (* p< 0.05, ** p< 0.01, *** p< 0.001).

Histological scoring was done in order to assess the damage induced to pancreatic tissue at different time points (Figure 3.2). Tissue samples were scored according to presence of edema, level of immune cell infiltration and presence of apoptotic cells, according to Schmidt *et al.* [218]. 2h after the last cerulein injection, pancreatic tissue shows considerable damage with the most prominent feature being edema and infiltration of immune cells. After 24h tissue is showing signs of recovery with reduction of both edema and levels of immune cell infiltration. At the 72h time point the severity of inflammation has been further reduced, with absence of infiltrating immune cells, and almost complete absence of edema.



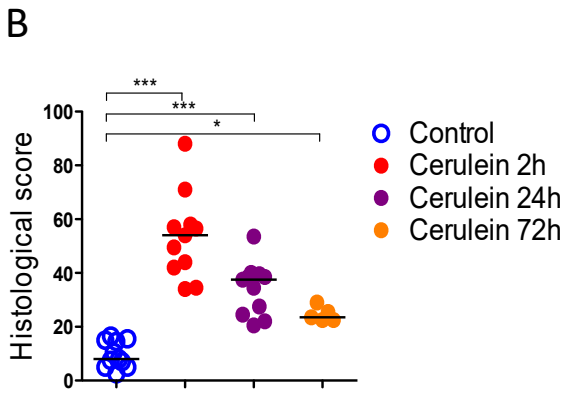


Figure 3.2: Histological scoring at different time points of acute pancreatitis. (A) Representative images of mouse pancreata stained with HE histological staining. Pancreata were harvested from mice sacrificed at different time points (2h, 24h and 72h) after the last cerulein injection. (B) Histological score of mice pancreata. Histological scoring was established based on Schmidt *et al.* [218]. Horizontal lines depict medians. Statistical significance was calculated using one-way ANOVA, with Dunnett's multiple comparison post-hoc test. (p value: * < 0.05, ** < 0.01, *** < 0.001).

AP is characterized by the presence of cytokines released by innate immune cells, such as TNFα and IL-6. Therefore, as a next parameter of AP severity, the expression of these cytokines was analyzed in pancreatic tissue (Figure 3.3). 2h after pancreatitis induction the expression levels of both cytokines are significantly upregulated in comparison to later time points.

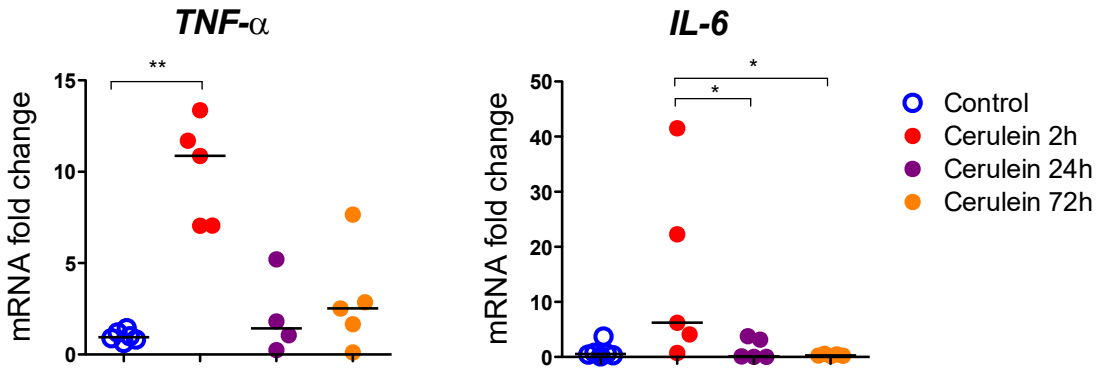


Figure 3.3 mRNA fold change of pro-inflammatory cytokines at different time points of acute pancreatitis. Gene expression is normalized to expression of the housekeeping gene *gapdh*. Horizontal lines depict medians. Statistical significance was calculated using one-way ANOVA, with Dunn's multiple comparison post-hoc test. (p value: * < 0.05, ** < 0.01, *** < 0.001).

This data led us to the conclusion that that acute pancreatitis in mice occurs 2 hours after cerulein injection. Therefore, this time was selected to examine the effects of microbiota manipulations on the severity of the acute pancreas inflammation.

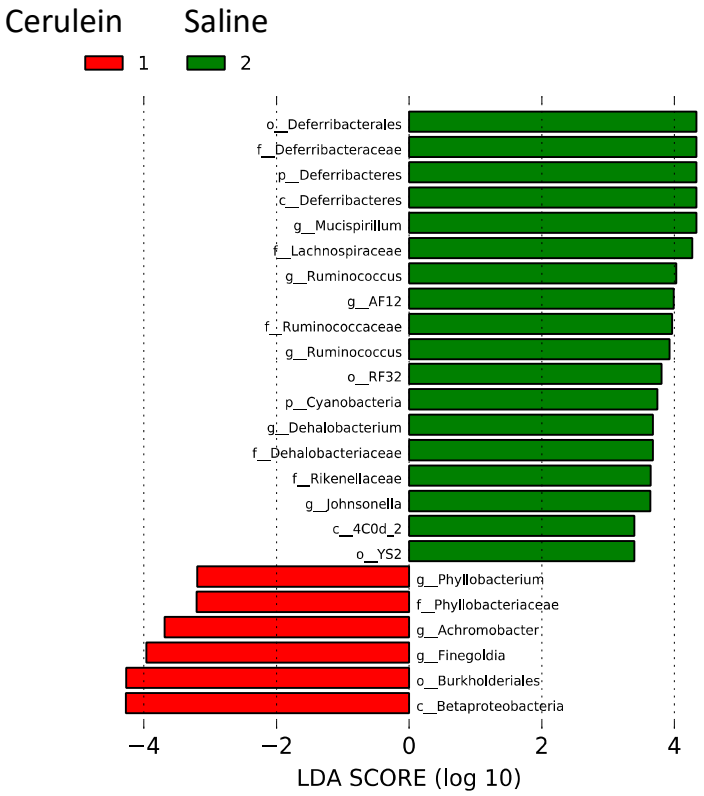
3.1.2 Cerulein-induced pancreatitis changes pancreatic microbial composition

Several publications point to the influence of pancreatitis on the gut barrier function, and suggest translocation of gut bacteria to the pancreas, which are hypothesized to aggravate AP symptoms. Therefore, we wanted to determine if the induction of pancreatitis per se changes the pancreatic microbiota. There are conflicting reports regarding the question of pancreatic sterility, thus, this was the first question we addressed. Our 16S sequencing results showed that pancreas is not a sterile organ (Figure 3.4 A), confirming previously published findings from Pushalkar *et al*. [231]. Moreover, we observed differences in microbial

populations in cerulein-treated mice in comparison to the control group as determined by LefSe analysis (Figure 3.4 A). For example, the order *Burkholderiales* and the *Phyllobacterium* genus are some of the most abundant bacteria of the inflamed pancreas, in comparison to the genus *Mucispirillum* (belonging to family *Deferribacteriaceae*) and the family *Lachnospiraceae* that have prevalence in the uninflamed pancreas.

Sequencing of other tissues, specifically mLNs, ileal contents and fecal samples showed that they harbor a distinctive microbial composition, as represented in the t-SNE plots (Figure 3.4 B). Cerulein treatment also caused a change in microbial communities, which further points to effects of inflammation on bacterial composition (Figure 3.4 C).

A



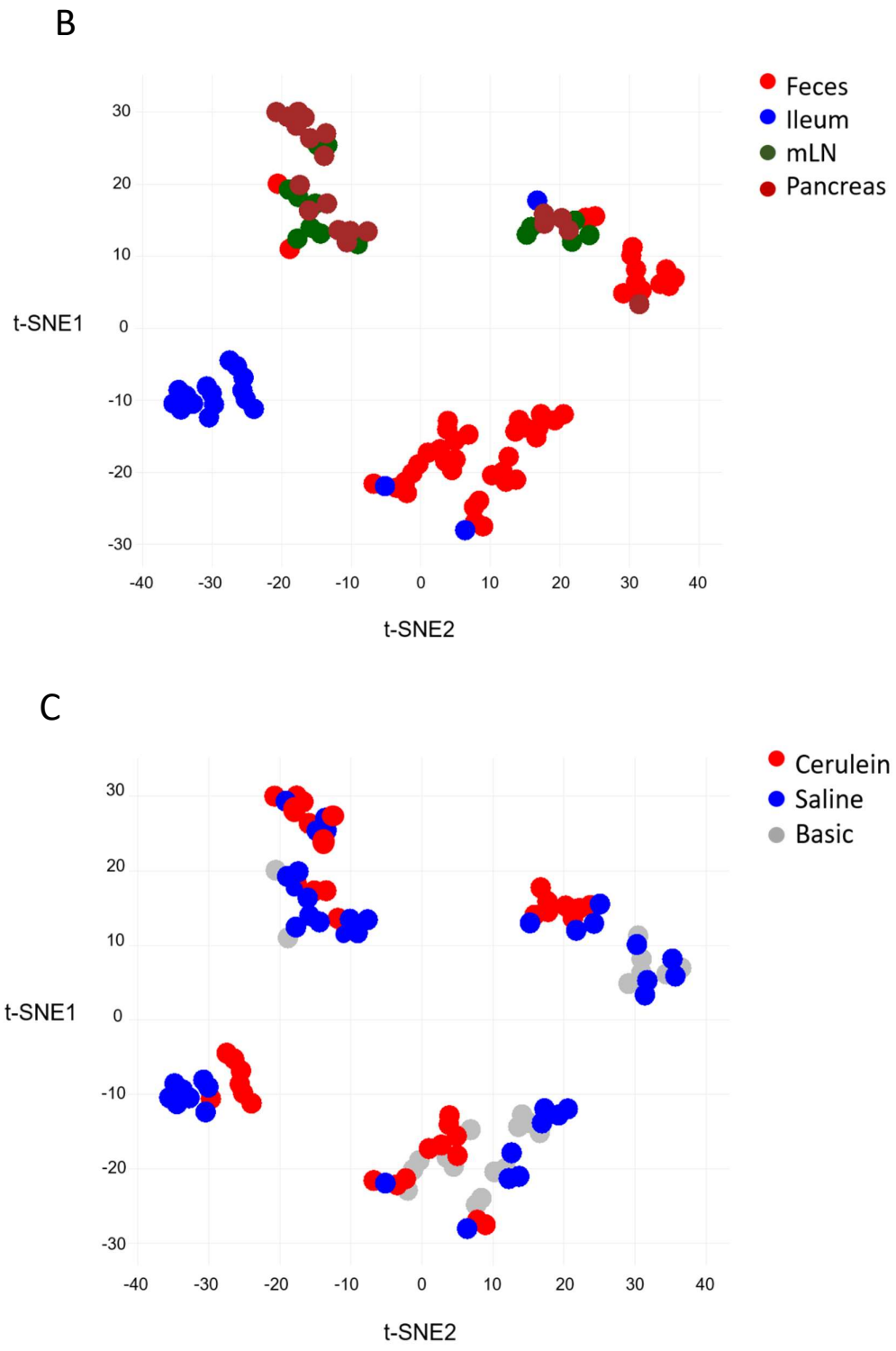


Figure 3.4: Different bacterial composition is observed in acute pancreatitis. (A) Analysis of treatment differences on individual operational taxonomic unit (OTU) differences using LefSe analysis. Microbiota from pancreata of cerulein-treated mice shows

different composition in comparison to the saline-treated control mice. (B) t-SNE plot of UniFrac distances. A two-dimensional representation of unweighted UniFrac distances was generated using t-distributed stochastic neighbor embedding (t-SNE). Samples are colored to indicate different tissues. (C) t-SNE plot of UniFrac distances. Samples are colored to indicate different treatment (cerulein: cerulein-treated samples; saline: saline-treated control samples; basic: untreated samples).

To further demonstrate the presence of bacteria in the pancreas, we performed FISH staining using the pan-bacterial probe EuB. Results confirmed the presence of bacteria in inflamed pancreata (Figure 3.5).

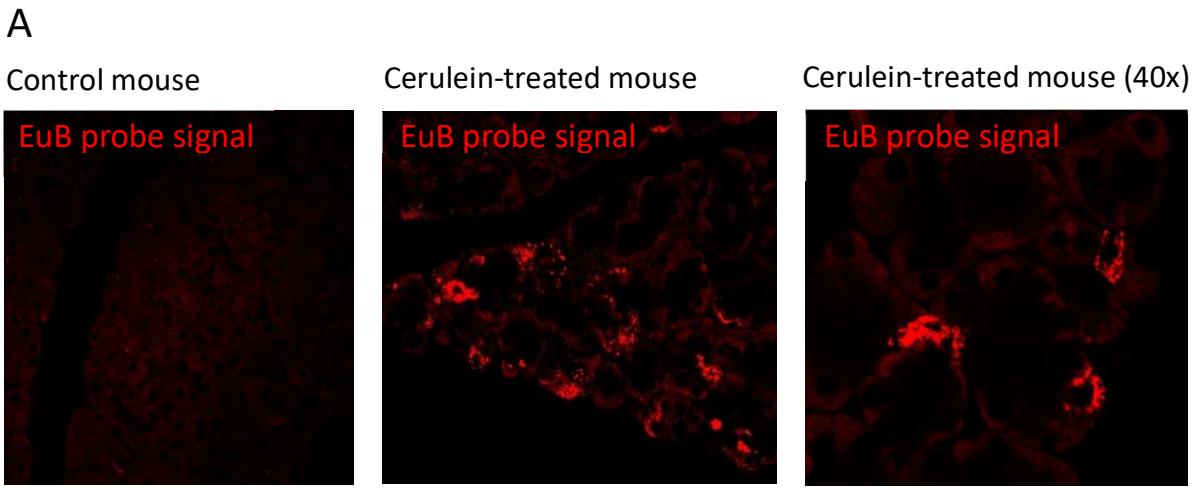


Figure 3.5: Bacterial presence in the inflamed pancreata detected by FISH staining. Bacteria (red) are present as single puncta and clusters in pancreata of cerulein-treated mice.

Presence of bacteria in the pancreas was further confirmed by homogenizing and plating pancreata from control and AP mice. Samples were cultured in aerobic and anaerobic conditions on non-selective media. Besides detecting viable bacteria, this experiment also served to compare the bacterial populations between control and cerulein-treated, inflamed pancreata. As a result, in AP samples different bacterial strains were identified, as compared to controls. Predominantly *Escherichia coli* and *Burkholderia cepacia* were observed. Colonies

of *Lactobacillus reuteri, Lactobacillus johnsonii* and *Corynebacterium amycolatum* were detected in homogenized pancreata from control mice.

Plates	**Control**	**Acute pancreatitis**
Aerobic 1	-	*Escherichia coli*
Aerobic 2	-	*Escherichia coli*
Aerobic 3	*Corynebacterium amycolatum*	*Burkholderia cepacia, Escherichia coli*
Aerobic 4	-	*Burkholderia cepacia*
Aerobic 5	-	*Escherichia coli*
Anaerobic 1	*Lactobacillus johnsonii*	*Lactobacillus reuteri, Lactobacillus johnsonii*
Anaerobic 2	-	*Escherichia coli, Lactobacillus reuteri*
Anaerobic 3	*Lactobacillus reuteri*	*Lactobacillus murinus, Lactobacillus johnsonii*
Anaerobic 4	-	*Corynebacterium thomssenii, Lactobacillus johnsonii*
Anaerobic 5	-	*Lactobacillus reuteri*

Table 3.1: Different bacteria were found in samples from control and cerulein-treated mice. Results obtained from MALDI analysis of colonies grown from homogenized pancreata cultures in aerobic or anaerobic conditions.

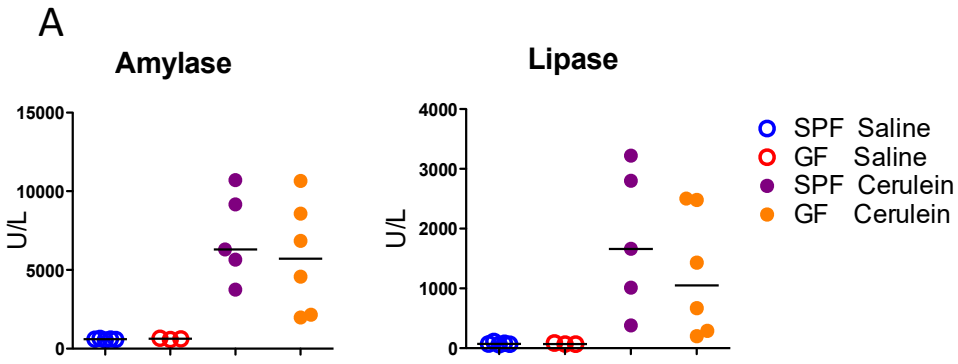
3.2 Influence of microbiota manipulations on the severity of acute pancreatitis

There are still conflicting reports regarding the use of antibiotics in the clinical treatment of pancreatitis. One study showed that reduction of Gram-negative bacteria significantly reduced morbidity and mortality in patients with severe acute pancreatitis [149]. Another suggested that bowel sterilization by broad-spectrum antibiotics attenuates cerulein-induced pancreatitis in mice [185]. In contrast, a meta-study performed in 2010 showed that there

was no statistically significant effect on reduction of mortality with antibiotic therapy [188]. To thoroughly explore the influence of antibiotics on acute pancreatitis, we used the previously established mouse model of acute pancreatitis under different experimental conditions.

3.2.1 Induction of pancreatitis in germ-free mice results in reduction of the severity of acute pancreatitis

To determine whether bowel decontamination would have an impact on the course of acute pancreatitis, we used the GF mice as a paradigm of complete eradication of the gut microbiota. Therefore, we induced pancreatitis in GF and SPF mice and compared the results. No significant differences in the levels of amylase or lipase were detected between SPF and GF mice treated with cerulein (Figure 3.6 A), although GF mice showed a tendency to lower levels of these biochemical parameters. Despite no difference in biochemical parameters, we found a significant reduction in histological scores of the AP of GF compared to SPF mice, predominantly in scores for edema and immune cell infiltration (Figure 3.6 B). This suggests that the presence of gut microbiota influences the severity of the AP, and points to a link between gut microbiota and pancreatitis.



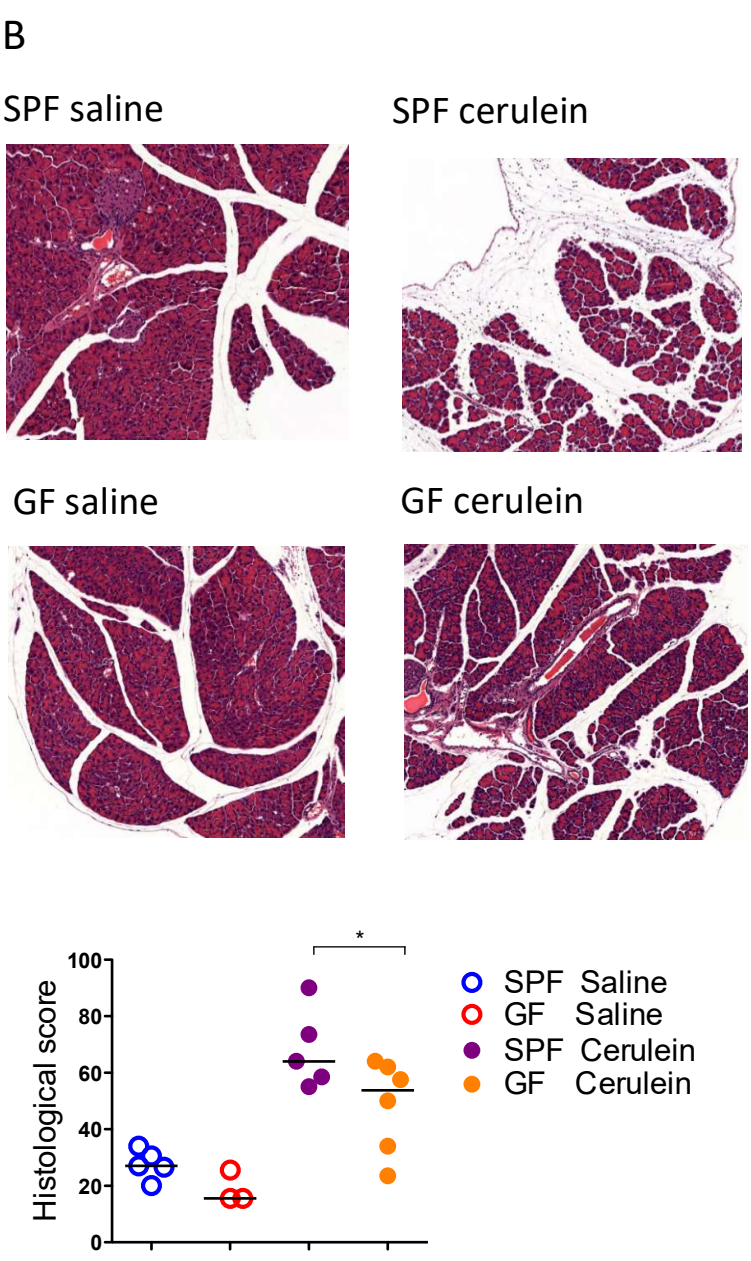


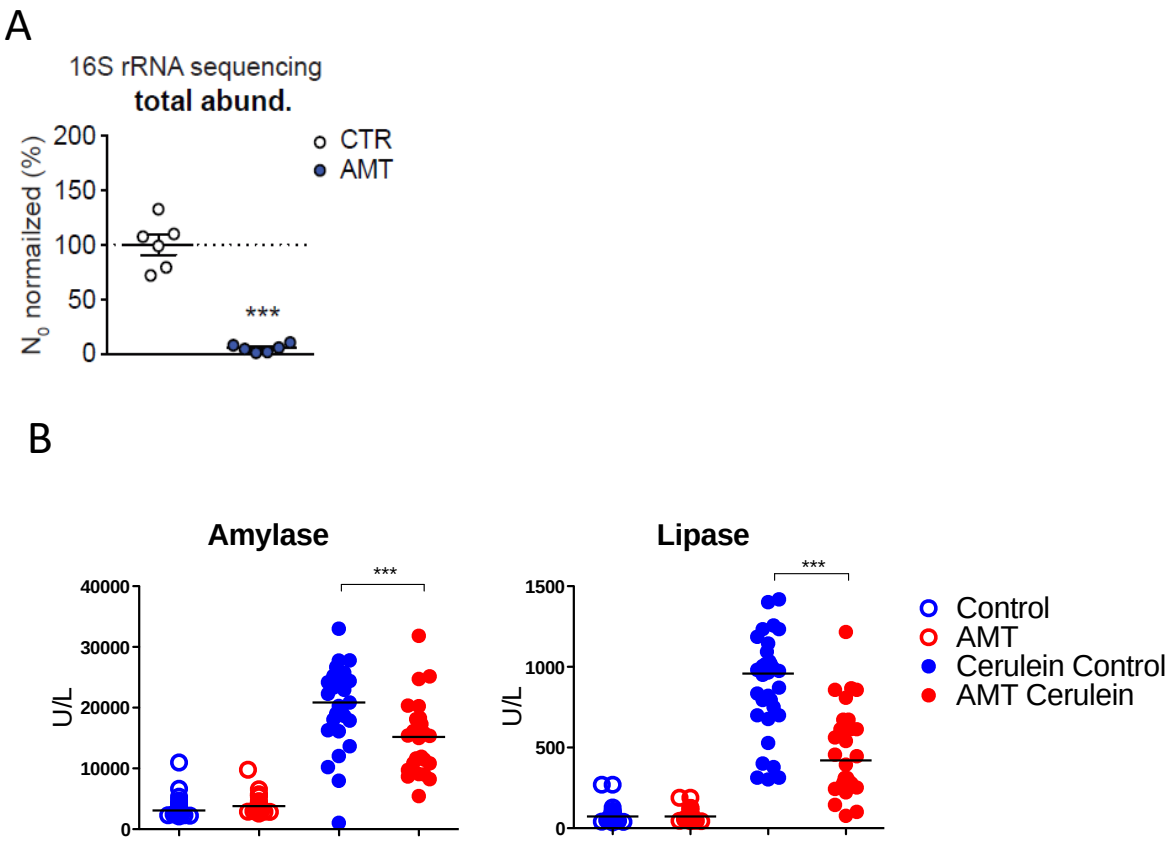
Figure 3.6: Acute pancreatitis is reduced in GF mice. (A) Analysis of biochemical parameters of pancreatitis severity. U/L units per liter. (B) Histological analysis of the tissue of GF and SPF mice. Representative images of HE staining of the pancreas of GF and SPF mice. Histological scoring was established based on Schmidt *et al.* [218]. Horizontal lines depict medians. Statistical significance was calculated using one-way ANOVA, with Dunn's multiple comparison post-hoc test. (p value: * < 0.05, ** < 0.01, *** < 0.001).

3.2.2 Broad-spectrum antibiotic treatment results in decrease of acute pancreatitis severity

In addition to experiments with GF mice, we pretreated mice with a cocktail of antibiotics (ampicillin, vancomycin, metronidazole) called AMT, to induce broad-spectrum microbial decontamination. As shown in Figure 3.7 A, application of AMT cocktail caused a significant drop in bacterial load in the gut.

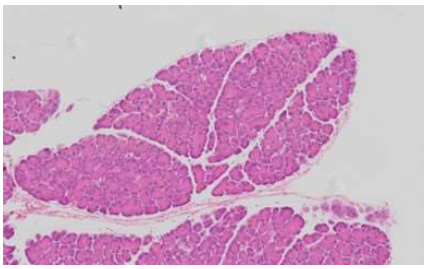
After antibiotic treatment and induction of AP, mice showed a significant reduction in amylase and lipase serum levels (Figure 3.7 B). In addition, antibiotic treated mice (or AMT mice) showed reduced edema, necrosis, and notably, less infiltration of immune cells (Figure 3.7 C). In addition, pro-inflammatory cytokine levels were significantly decreased in AMT-pretreated mice (Figure 3.7 D).

Taken together, our results indicate that AMT pretreatment attenuates the severity of AP.

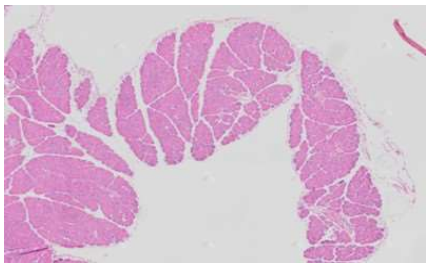


C

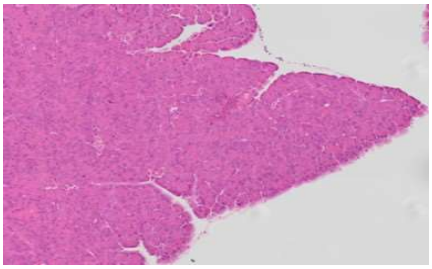
Cerulein control



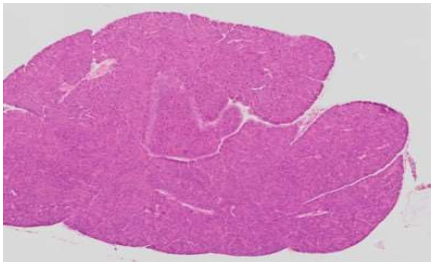
AMT cerulein

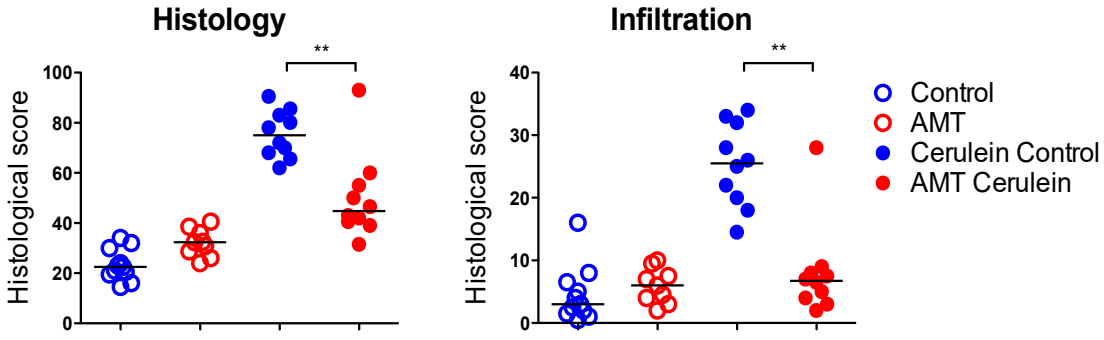


Saline control



AMT control





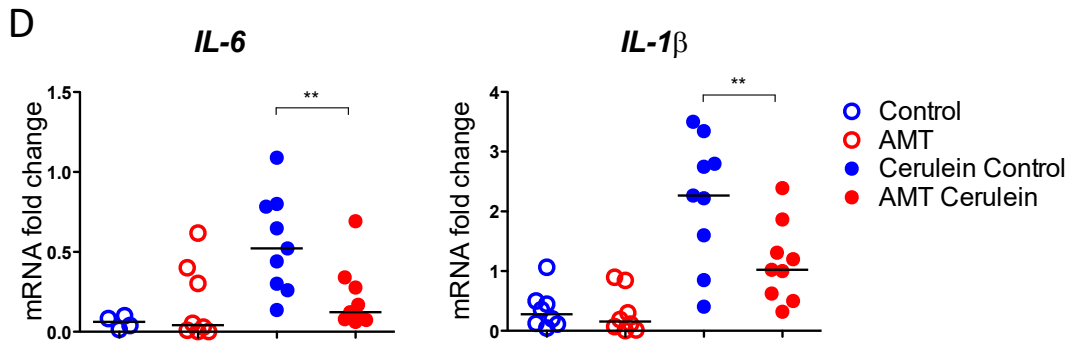


Figure 3.7: AMT treatment attenuates acute pancreatitis. (A) Total abundance of bacteria in fecal samples from control and AMT-pretreated mice. (B) Analysis of biochemical parameters of pancreatitis severity. U/L units per liter. (C) Histological analysis of the pancreatic tissue and representative images of HE staining of the pancreas. Histological scoring was established based on Schmidt *et al.* [218]. (D) mRNA fold change of pro-inflammatory cytokines between different treatment groups. Gene expression is normalized to expression of the housekeeping gene *gapdh*. Horizontal lines depict medians. Statistical significance was calculated using one-way ANOVA, with Dunn's multiple comparison post-hoc test. (p value: * < 0.05, ** < 0.01, *** < 0.001).

3.2.3 Innate immune response is the main driver of inflammation in acute pancreatitis

To further investigate the nature of the immune response in our model of AP and to identify infiltrating cell subsets differentially recruited upon AMT treatment, we performed FACS analysis of pancreata and spleens resected 2 hours after induction of pancreatitis with or without 2-week antibiotic pretreatment. A higher proportion of CD4+ cells was detected in the pancreas of mice undergoing AMT treatment, while the frequency of CD8+ cells was similar in both groups (Figure 3.8 A). In spleen, antibiotic treatment alone induced increased frequency of CD8+ cells, while no differences between control and antibiotic treated mice were detected upon pancreatitis induction. (Figure 3.19 B).

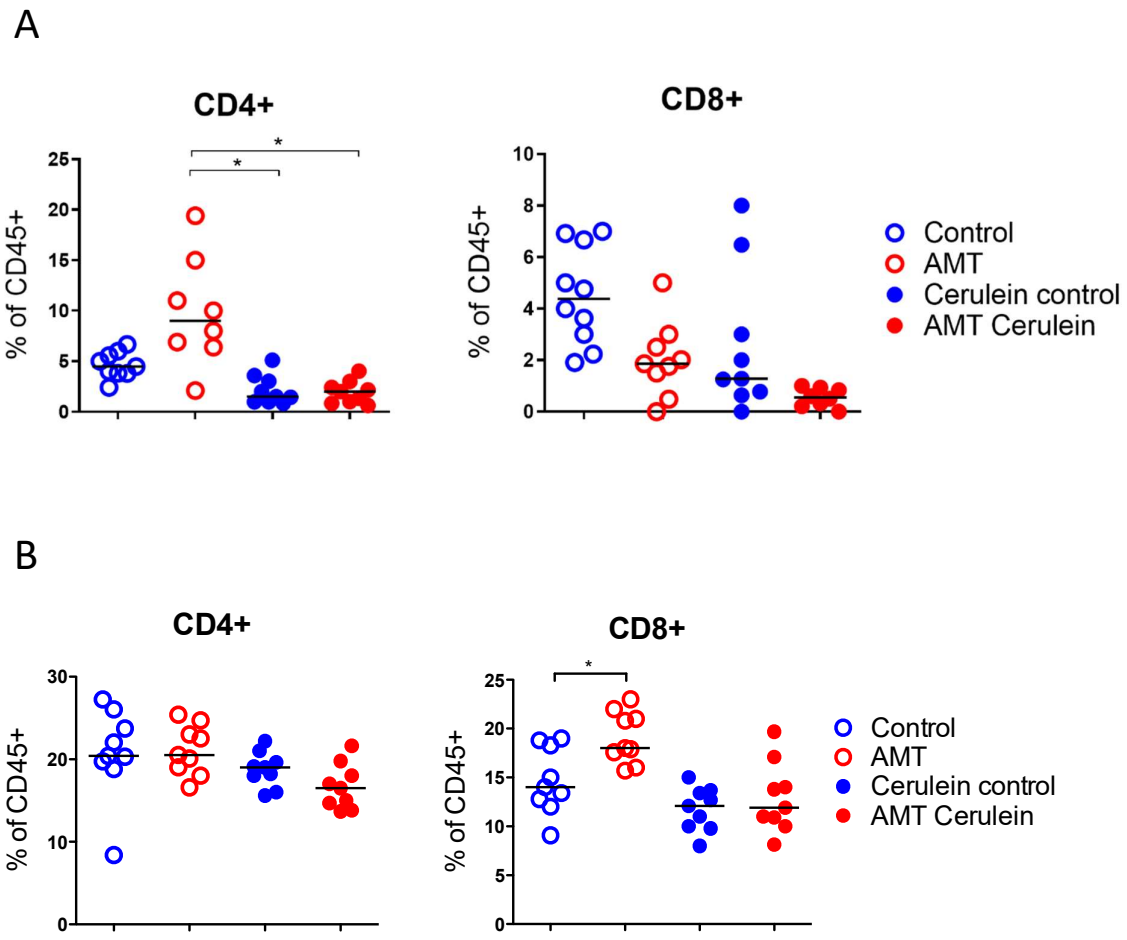
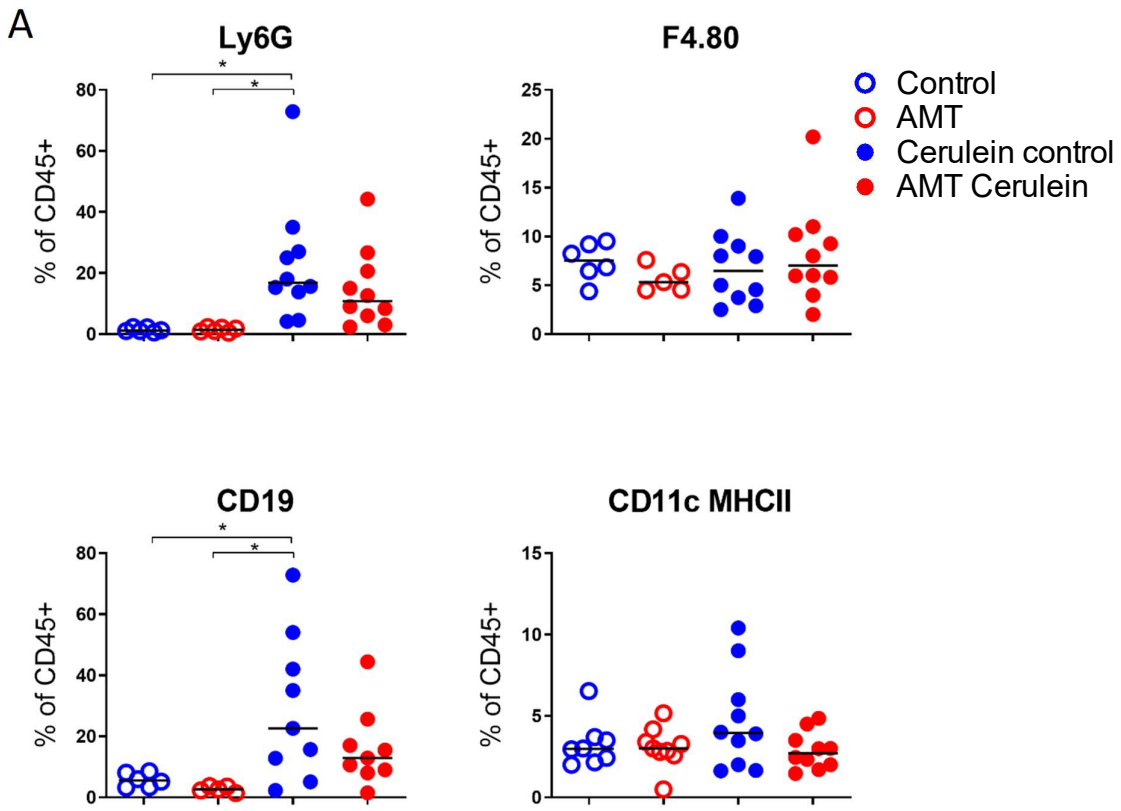


Figure 3.8: T cells are not affected by pancreatitis. Percentage of CD4 (helper T lymphocytes) and CD8 (cytotoxic T lymphocytes) positive cells of total CD45 positive cells in the pancreas (A) and in the spleen (B) of control and antibiotic treated (AMT) mice during AP. Each data point represents data from one mouse. Horizontal lines depict medians. Statistical significance was calculated using One-way ANOVA with Newman-Keuls post-hoc test (p value: * < 0.05, ** < 0.01, *** < 0.001).

As indicated before, the innate immune response has been established to be the main driver of the immune reaction occurring during AP [74], [114], [232]. [74], [114], [232]. Therefore, we also examined the presence of innate immune cells in pancreas and spleen upon antibiotic treatment. As a result, we observed an increase in the proportion of neutrophils and B cells

in the inflamed pancreata, while AMT pretreatment only led to a trend in reduction of these cells in inflamed pancreata (Figure 3.20 A). The inflammation observed in the pancreas upon cerulein treatment can therefore be attributed to the increased infiltration of neutrophils and B cells. Macrophages and CD11c+/MHCII+ antigen-presenting cells seem unaffected by inflammation, AMT treatment, or the combination of both (Figure 3.20 A). In spleen, there is a notable upregulation of neutrophils during inflammation. However, AMT treatment reduces the observed increase in percentage of neutrophils during inflammation.

Therefore, when mice receive antibiotic treatment, on the systemic level, there is a reduction of neutrophils, which may account for the observed trend in decrease of neutrophils in AMT-pretreated pancreata, and the amelioration of acute pancreatitis symptoms.



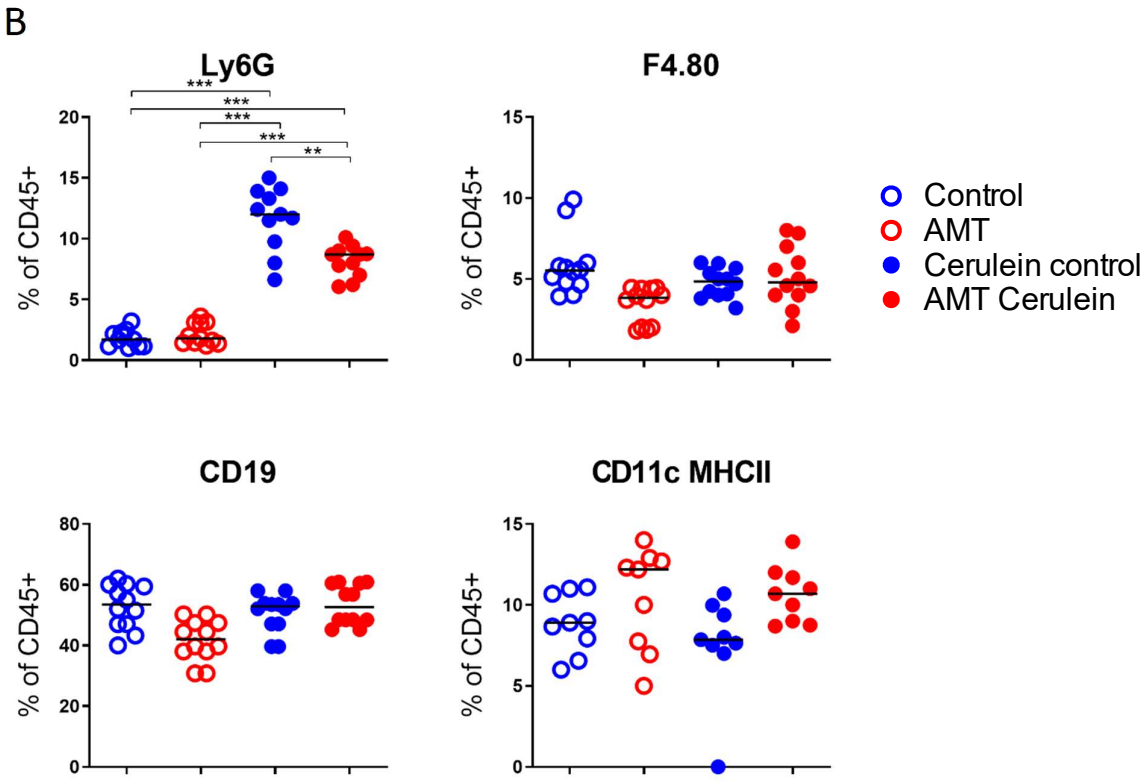


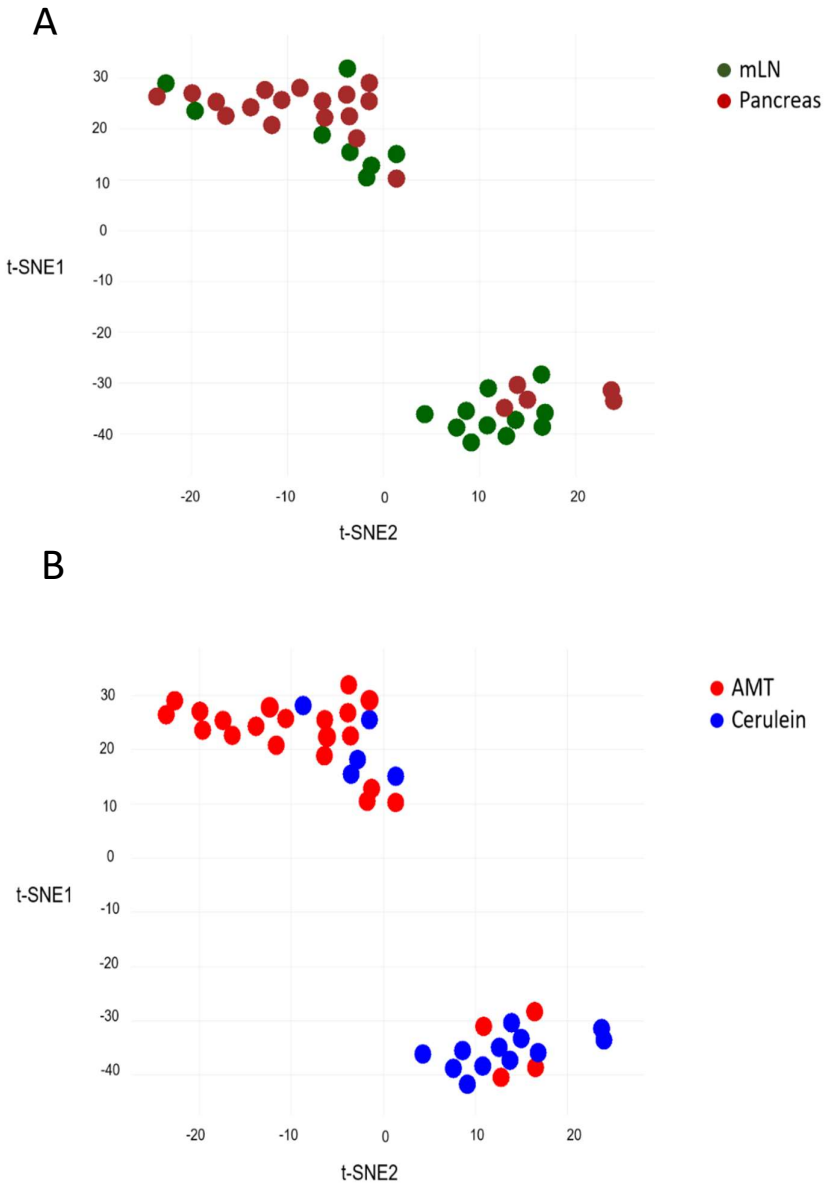
Figure 3.9: Neutrophils and B cells are the main drivers of acute pancreatitis. (A) Percentage of Ly6G (neutrophils), F4.80 (macrophages), CD19 (B cells) and CD11cMHCII (dendritic cells) positive cells of total CD45 positive cells in the pancreas (A) and in the spleen (B) of control and antibiotic treated (AMT) mice during AP. Each data point represents data from one mouse. Statistical significance was calculated using One-way ANOVA with Newman-Keuls post-hoc test (p value: * < 0.05, ** < 0.01, *** < 0.001).

3.2.4 Antibiotic pretreatment influences microbial composition of pancreas and mLNs in acute pancreatitis

In order to examine the influence of pancreatitis induction as well as antibiotic pretreatment on microbial composition of the pancreas, we performed 16S rRNA sequencing of pancreata and mLNs. When grouping OTUs by the tissue of origin, we observed a certain overlap

between pancreas and mLNs (Figure 3.10 A). This could indicate that translocation of gut bacteria to the pancreas occurs via mLNs.

No clear separation of OTUs in clusters was detected when analyzing Beta diversity of pancreatitis and control mice (Figure 3.10 C), however, simulation according to the pretreatment, showed a prominent separation of OTUs (Figure 3.10 B). This is suggesting that the effect of broad-spectrum antibiotic pretreatment on the microbial composition of the pancreas and mLNs is more prominent than the induction of pancreatitis itself.



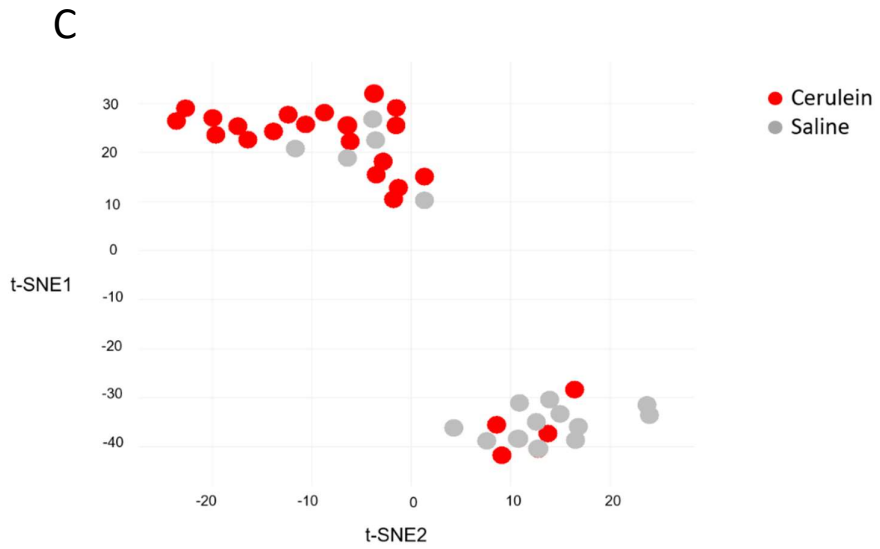


Figure 3.10 t-SNE visualization of the beta-diversity shows influence of antibiotic pretreatment on microbial composition in acute pancreatitis. (A) Color-coding according to tissue. (B) Color-coding according to pretreatment. (C) Color-coding according to cerulein treatment vs. saline-administration. t-SNE as ordination method based on weighted normalized Unifrac distances.

Our 16S rRNA sequencing results show that microbial compositions in the mouse pancreata were impacted by the different treatments (Figure 3.11). First, we observed that cerulein treatment altered the microbial composition observed in control mice. Inflamed pancreata are dominated by the genus *Propionibacterium*, belonging to the phylum *Actinobacteria*, in contrast to control pancreata, which are dominated by bacteria from the phylum Firmicutes, and the family *Lachnospiraceae*. As a reminder I will mention that family *Lachnospiraceae* was previously detected in sequencing of control pancreata in the experiment with different time points (Figure 3.4, section 3.1.2). Regarding the AMT treatment, sequencing results show that the AMT treatment alone favors the *Shewanella*, *Afipia* and *Moraxella* genera, all containing antibiotic resistant species. Interestingly, pancreatitis induction in AMT pretreated mice allowed the expansion of genus *Burkholderia*, that became the most dominant genus. Note that *Burkholderia* order was also identified in sequencing of inflamed pancreata in the previous experiment (Figure 4, section 3.1.2), and *Burkholderia cepacia* was cultured in samples of homogenized inflamed pancreata (Table 3.1).

This represents another line of evidence that inflammation, both with and without AMT treatment, is making an impact on the pancreatic microbiota.

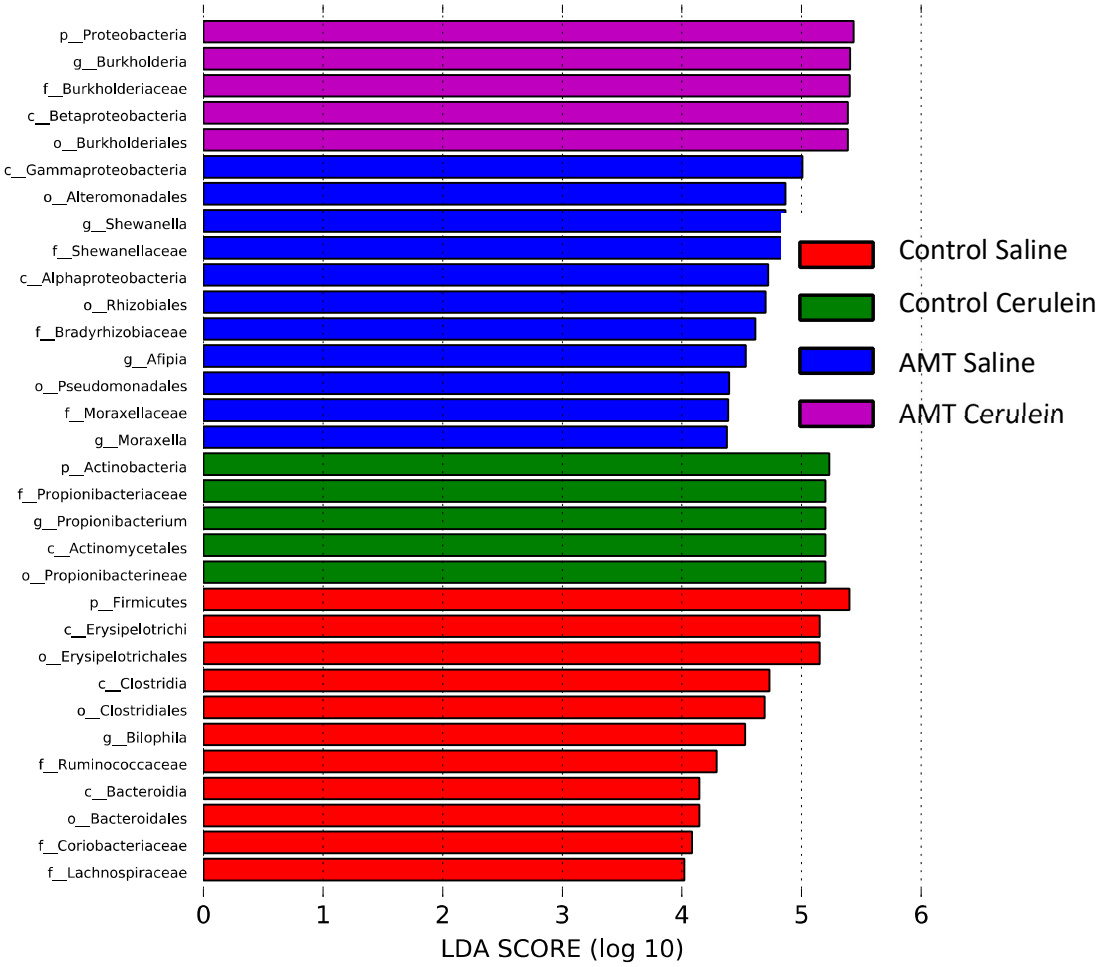


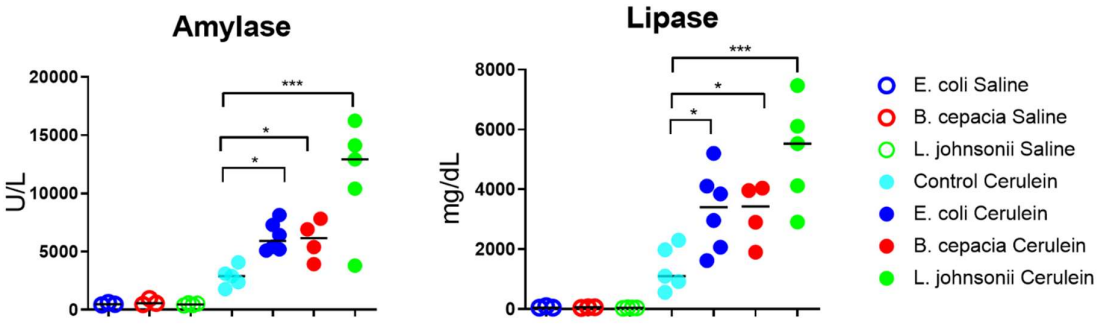
Figure 3.11: Different treatments influence the microbial composition of the pancreas. Statistical analyses of taxa overrepresentation in the four treatment conditions using LefSe.

3.2.5 Inoculation of GF mice with bacterial species previously identified in inflamed pancreata increases the severity of acute pancreatitis

Bacterial species that have been identified by culturing bacteria from homogenized mouse pancreata as shown in Table 3.1, or by 16S sequencing as shown in Figure 3.11, were used for inoculation of GF mice. Three strains that were the most represented (*Escherichia coli, Lactobacillus johnsonii* and *Burkholderia cepacia)* were chosen. Mice were gavaged with bacteria two weeks prior to AP induction. This was done to determine the effects of single bacterial strains on pancreatitis severity in a gnotobiotic setting.

These results suggest that mono-colonization of GF mice with certain bacterial species worsen the inflammation in AP, since the AP parameters (biochemical and histological) are higher in mono-colonized mice than in ceruluein-treated control mice (Figure 3.12). Also, as Figure 3.12 shows, *Lactobacillus johnsonii* has the most prominent effect on the severity of AP, followed by *Burkholderia cepacia*, and *Escherichia coli*.

A



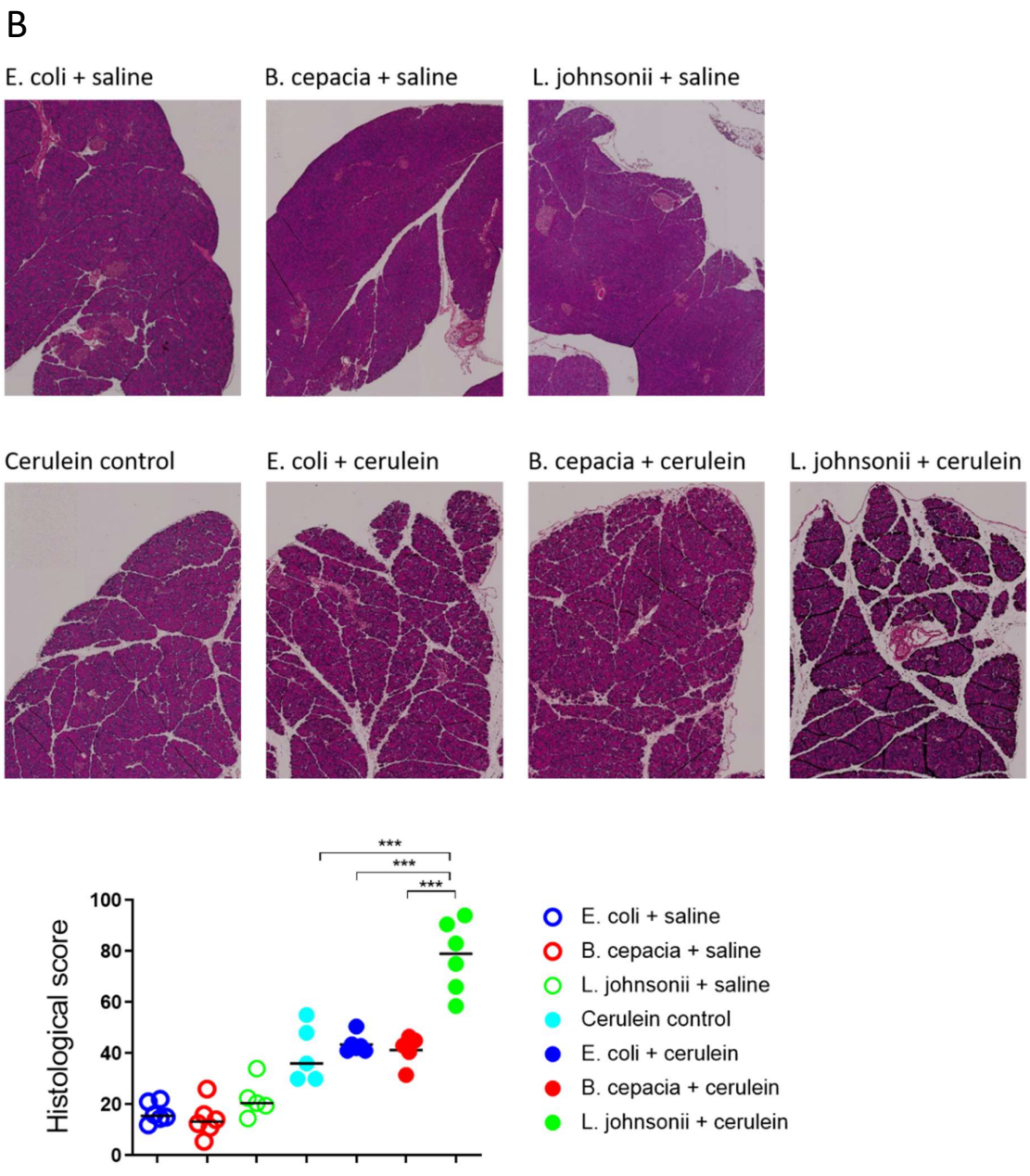


Figure 3.12: Pancreatitis severity in GF mono-colonized mice is higher than in cerulein-treated control mice. (A) Analysis of biochemical parameters in GF mice inoculated with different bacterial strains. U/L units per liter. (B) Histological analysis of the pancreatic tissue and representative images of HE staining of the pancreas. Histological scoring was established based on Schmidt *et al.* [218]. Horizontal lines depict medians. Statistical significance was calculated using One-

way ANOVA, with Dunn's multiple comparison post-hoc test. (p value: * < 0.05, ** < 0.01, *** < 0.001).

3.2.6 Single antibiotics pretreatment has no significant influence on acute pancreatitis severity

In order to show if the absence of a particular microbial population is responsible for the observed effect of bowel decontamination, we pretreated mice with single antibiotics and observed the effect on the severity of pancreatitis. For this purpose, ampicillin, streptomycin and vancomycin were used. The reason these antibiotics were chosen is that they target different bacterial populations. Streptomycin targets aerobic, Gram-negative bacteria; vancomycin targets Gram-positive bacteria; and ampicillin is a broad-spectrum antibiotic. Biochemical parameters did not show any significant difference in AP severity and this led us to the conclusion that use of single antibiotics with limited effect on the gut microbiota was not an effective way of counteracting AP.

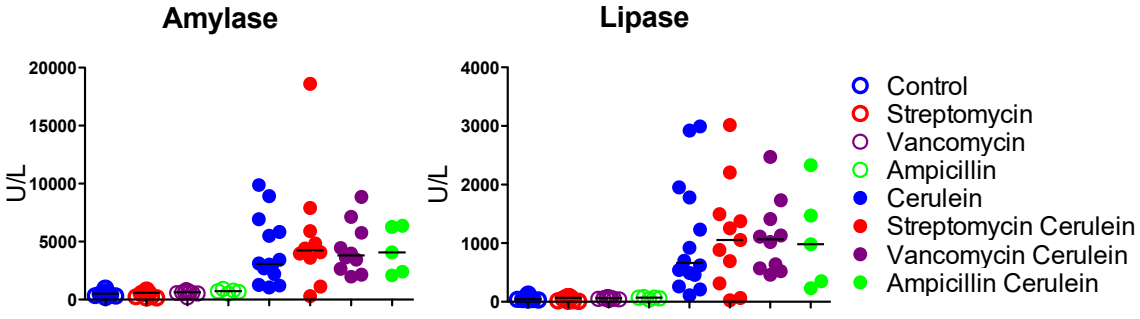


Figure 3.13: Pretreatment with single antibiotics has no influence on biochemical parameters of pancreatitis severity. Analysis of biochemical parameters of pancreatitis severity. U/L units per liter. Statistical significance was calculated using one-way ANOVA, with Newman-Keuls post-hoc test.

3.3 Diet modulation and acute pancreatitis

3.3.1 Diet with different fiber content does not have an effect on acute pancreatitis severity

Another way to modulate the gut microbiota and thereby interfere with the severity of AP is by administration of different diets to mice. Mice were fed either low or high fiber diet in order to examine if the anti-inflammatory properties of short chain fatty acids produced by increased fiber digestion [233]–[235] will reduce AP severity. Our data shows that there is an increase in amylase and lipase markers of pancreatitis in mice fed with low- and high-fiber diet as compared to normal-fiber diet. This data points to an interesting avenue for future research, suggesting that variation in fiber content of the diet can have an effect on the severity of AP (Figure 3.14).

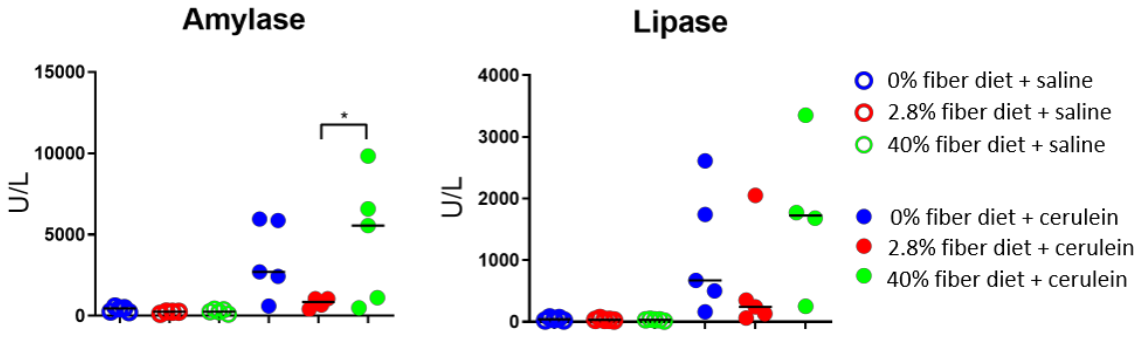
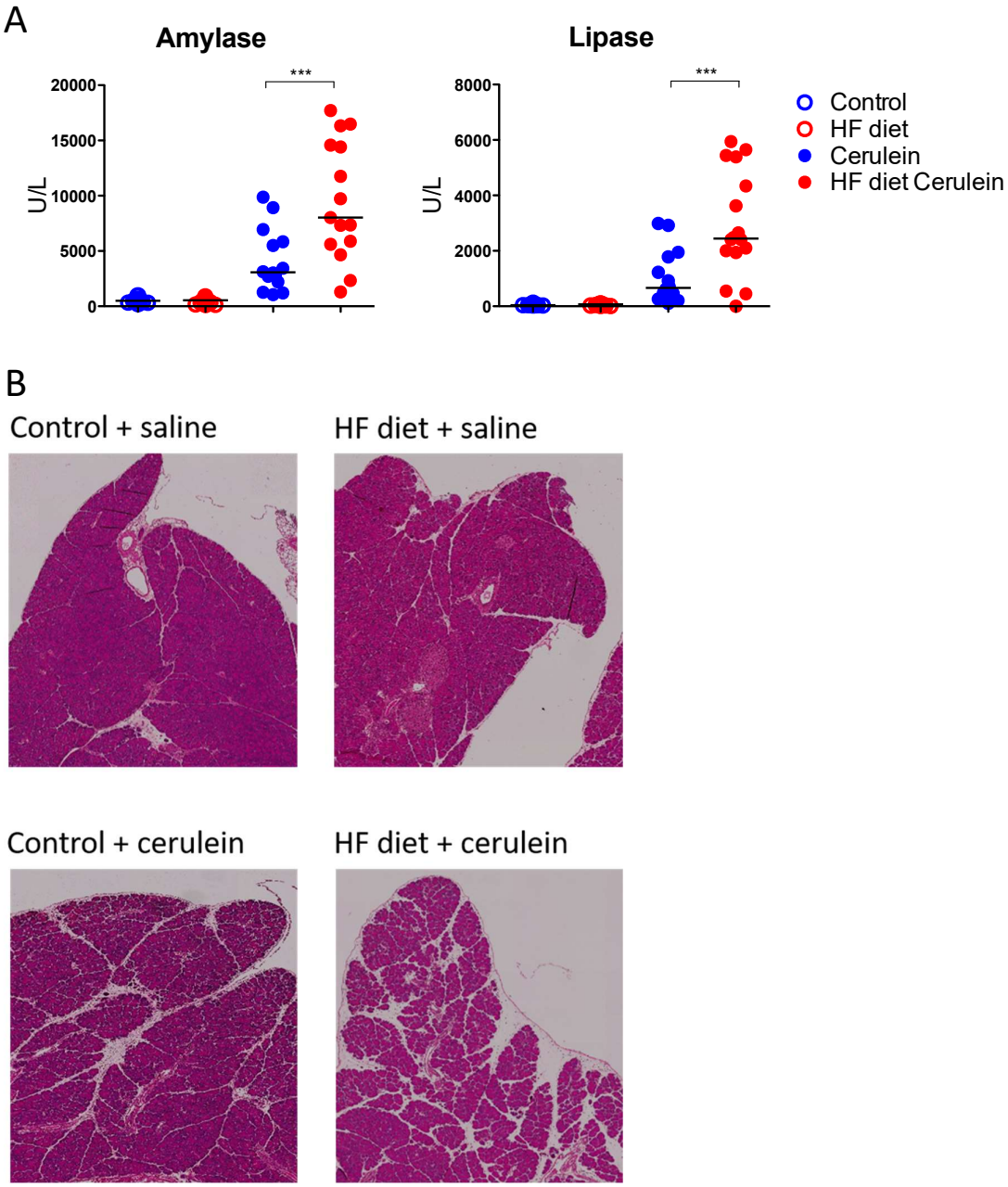


Figure 3.14: Low and high fiber diet does not influence pancreatitis severity. Analysis of biochemical parameters of pancreatitis severity. U/L units per liter. Horizontal lines depict medians. Statistical significance was calculated using One-way ANOVA with Newman-Keuls post-hoc test (p value: * < 0.05, ** < 0.01, *** < 0.001).

3.3.2 High-fat diet results in increased severity of acute pancreatitis

Based on accumulating evidence of pro-inflammatory effects of the high-fat (HF) diet [236], [237], we wanted to test if the gut microbiota modulation caused by the HF diet worsens AP, and therefore further prove that there is causal interaction and communication between the gut and the pancreas. Our results show worsening of the pancreatitis phenotype in mice fed with HF diet, based on the observed biochemical parameters and histological scoring (Figure 3.15).



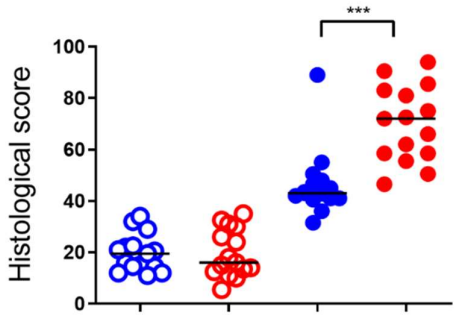


Figure 3.15: High-fat diet increases the severity of acute pancreatitis. (A) Analysis of biochemical parameters of pancreatitis severity. U/L units per liter. (B) Histological analysis of the pancreatic tissue and representative images of HE staining of the pancreas. Histological scoring was established based on Schmidt *et al.* [218]. Horizontal lines depict medians. Statistical significance was calculated using one-way ANOVA, with Newman-Keuls post-hoc test (p value: * < 0.05, ** < 0.01, *** < 0.001).

3.3.3 High-fat diet influences acute pancreatitis severity by means different from microbial composition modifications

Next, we wanted to see whether worsening of pancreatitis is associated with microbiota changes associated with HF diet or other effects of this diet on inflammatory processes within the pancreas. For this reason, GF mice were inoculated with cecal content of mice fed with either normal chow or HF diet. As Figure 3.16 shows, there was no difference in the severity of pancreatitis between mice inoculated with cecal contents from regular chow or HF diet-fed mice. This suggests that HF diet influences AP by mechanisms different from microbiota alterations.

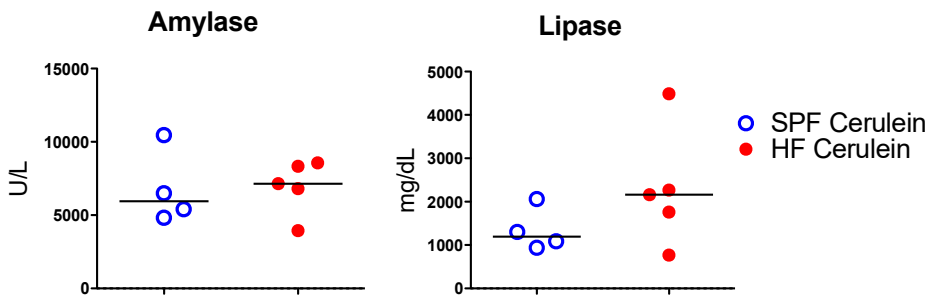


Figure 3.16: Inoculation of GF mice with cecal content of HF diet-fed mice does not result in a change of pancreatitis severity. Analysis of biochemical parameters of pancreatitis severity. U/L units per liter. Horizontal lines depict medians. Statistical significance was calculated using Mann-Whitney Test for nonparametric distributed data.

3.4 Establishment of an *in vitro* model of pancreatitis

Given the importance of microbiota for the severity of pancreatitis, there was a need for an *in vitro* model where the effects of microbiota could be analyzed in more detail. For this reason, we established an *in vitro* model in which the effects of different bacteria, bacterial protein lysates or cerulein treatment could be analysed.

Because of rapid loss of functional differentiation that regularly occurs *in vitro*, there was no instance of pancreatitis induction in long-term culture of pancreatic acinar cells. However, since we were interested into the initial phases of acute pancreatitis, the short time-window in which acinar cells still kept their identity did not represent a problem. Also, there was no case in the literature that acinar suspension or acinar explant culture was used to model pancreatitis. The reason for this could be the aforementioned problem of fast trans-differentiation, or that no research group expressed interest in *in vitro* modelling of the early phases of acute pancreatitis.

First, we had to establish whether acinar suspension or acinar explant culture could give a readout that could be used for assessing the severity of pancreatitis. The chosen readout was measurement of pro-inflammatory cytokine levels by qPCR. Since recognition of bacterial lipopolysaccharide (LPS), a component of the outer cell wall of Gram-negative bacteria, by the

innate immune system elicits strong pro-inflammatory responses, we have compared acinar suspension and acinar explants by stimulating them with LPS and performing subsequent measurements of the cytokine levels. Acini isolated in both ways responded to LPS in comparably high levels of cytokine transcripts (Figure 3.17 B). Since there was no difference in responsiveness between acinar suspension and acinar explants, we have opted for acinar suspension as an *in vitro* model, because the isolation was less time consuming and because a simple acinar suspension meets the demands of the planned experiments. Figure 3.12 A represents an image of acini in the acinar suspension culture.

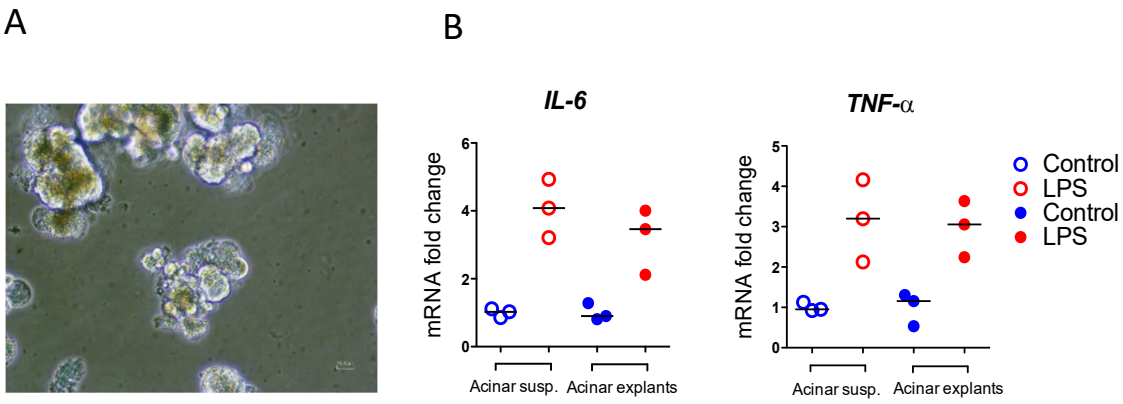


Figure 3.17: Establishment of an *in vitro* model of pancreatitis. (A) Representative image of acini in acinar suspension. (B) Comparison of responsiveness to LPS of acinar suspension and acinar explants. Expression levels of *IL-6* and *TNFα* cytokines were assessed by qPCR. Each data point represents a technical triplicate from one mouse. Horizontal lines depict medians. Statistical significance was performed using Mann-Whitney Test for nonparametric distributed data.

We chose a 4h time point because without the addition of growth factors, acinar cells transdifferentiate into ductal cells within 24h, and lose responsiveness to LPS (Figure 3.18).

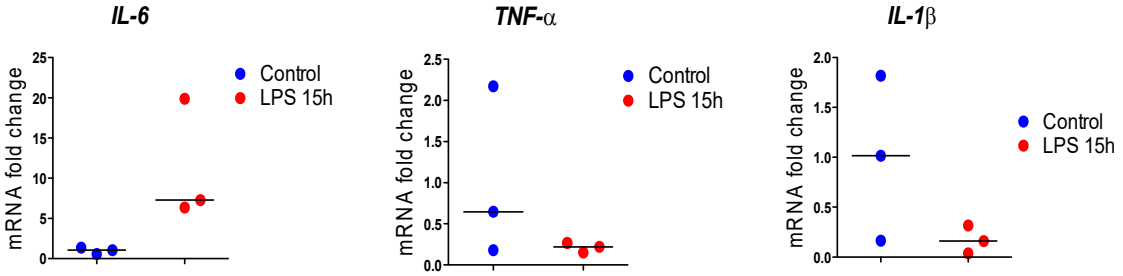


Figure 3.18: Acinar cells lose responsiveness to the LPS treatment at the later time point. Acinar suspension was treated with 10 µg/mL LPS for 15h. Expression levels of *IL-6* and *TNFα* cytokines was assessed by qPCR. Each data point represents a technical triplicate from one mouse. Horizontal lines depict medians. Statistical significance was calculated using Mann-Whitney Test for nonparametric distributed data.

3.4.1 Co-stimulation of acinar suspension with cerulein and alive bacteria

After choosing the model and observing the readout after LPS stimulation, we wanted to try to model pancreatitis *in vitro*. Since cerulein was used in our *in vivo* model, several doses of cerulein were applied to the acinar suspension, which induced the expression of pro-inflammatory cytokines (Figure 3.19). This shows that treating isolated acini with supraphysiological doses of cerulein triggers early pathologic responses of acute pancreatitis (trypsinogen activation, dysregulated secretion of digestive enzymes, vacuole accumulation) and can be considered an *ex vivo* disease model. 10 nM concentration was chosen because of the induction of several tested pro-inflammatory cytokines.

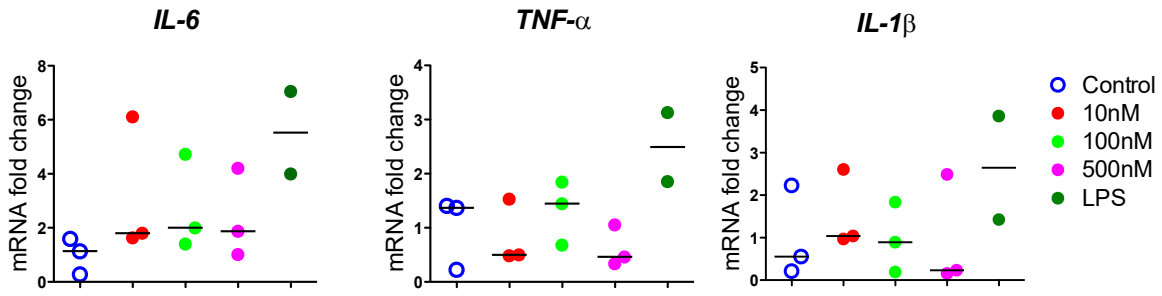


Figure 3.19: Cerulein dose of 10 nM causes mRNA fold increase for the three most prominent AP cytokines. Acinar suspension was treated either with different doses of cerulein or with 10 µg/mL LPS for 4h. Expression levels of *IL-6*, *TNFα* and *IL-1β* cytokines was assessed by qPCR. Each data point represents a technical triplicate from one mouse. Horizontal lines depict medians. Statistical significance was calculated using one-way ANOVA with Newman-Keuls post-hoc test.

Next, we wanted to simulate the *in vivo* situation with presence of certain bacterial strains in an already inflamed pancreas. For this reason, the co-stimulation of acinar suspension with cerulein and $2x10^6$ CFU of *Lactobacillus johnsonii* was performed (Figure 3.20). *TNFα* showed a strong upregulation during the co-stimulation, while *IL-6* and *IL-1β* showed only a modest increase in co-stimulated samples, compared to bacteria-only treated, or cerulein-only stimulated samples.

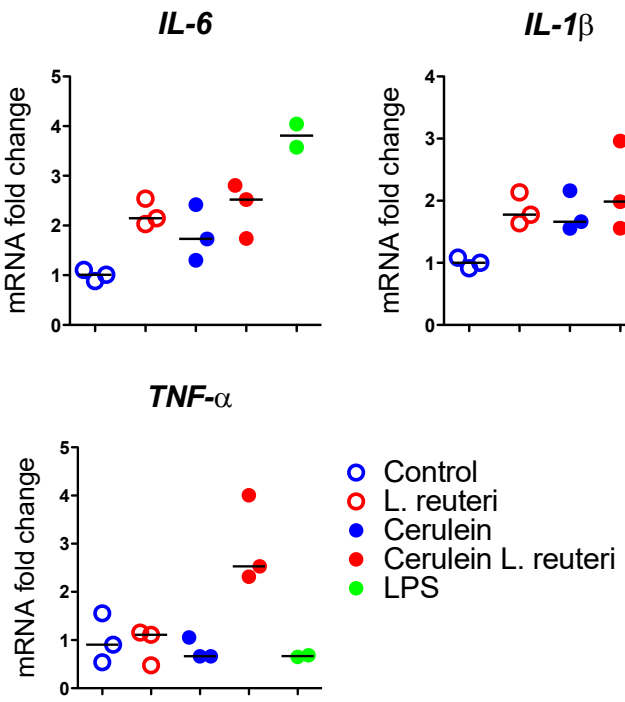


Figure 3.20: Co-stimulation of acinar suspension with cerulein and alive bacteria results in mRNA fold increase of pro-inflammatory cytokines. Treatment with $2x10^6$ CFU *Lactobacillus* johnsonii; OD 0.5=10^8 CFU/mL; cerulein 10 nM and LPS 10 µg/mL. Acinar suspension was treated with bacteria, cerulein or LPS for 4h. Expression levels of *IL-6* and *TNFα* cytokines were assessed by qPCR. Each data point represents a technical triplicate from one mouse. Horizontal lines depict medians. Statistical significance was calculated using one-way ANOVA with Newman-Keuls post-hoc test.

3.4.2 Acinar suspension responds to the treatment with bacterial protein lysates

The aim of this experiment was to define a direct influence of bacterial presence on acinar suspension by determining its responsiveness to proteins present on bacteria. Therefore, acinar suspension was treated with bacterial protein lysates, in order to eliminate the consequences of live bacteria culturing, such as metabolite production and change of pH.

This experiment shows that acinar suspension is also responsive to bacterial proteins only, regardless of the metabolite production and pH change (Figure 3.21). Interestingly, the discrepancy in *L. johnsonii* effect on *TNF-α* mRNA fold change between alive bacteria treatment (Figure 3.20) and protein lysate treatment (Figure 3.21) can suggest that metabolites or pH change in the presence of alive bacteria may lead to the reduced expression of this cytokine.

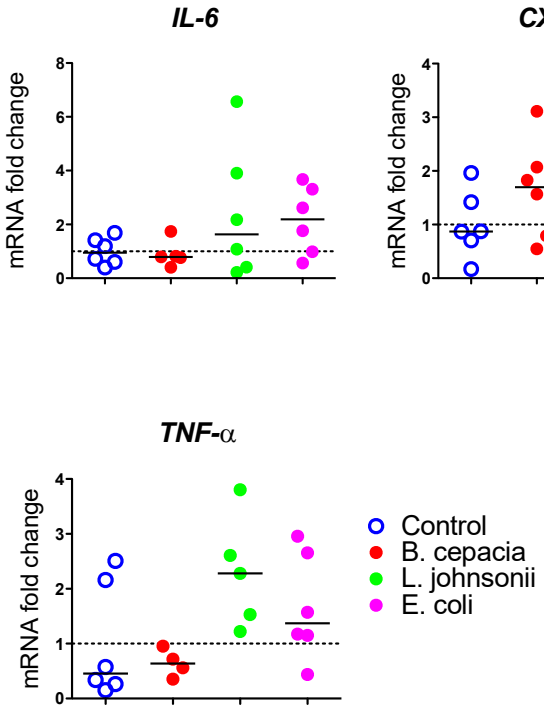


Figure 3.21: Acinar suspension responds to the treatment with bacterial protein lysates by changing the transcription levels of pro-inflammatory cytokines. Treatment with 15 g/mL of protein lysates from different bacterial strains for 4 h. Expression levels of *IL-6*, *CXC1* and *TNF-α* cytokines was assessed by qPCR. Each data point represents a technical triplicate from one mouse. Horizontal lines depict medians. Statistical significance was calculated using One-way ANOVA with Newman-Keuls post-hoc test.

3.4.3 Acinar cells do not respond to stimulation with bacterial protein lysates

As the last step, we wanted to investigate if the acinar cells are also contributing to the immune response observed in our in vitro model of AP, or is the immune response solely coming from pancreas-resident immune cells. The rationale for this is coming from the fact that acinar cells are equipped with innate immune receptors such as TLR4 and NOD1 that can recognize bacteria [47], [56], [238]. Acinar suspension underwent MACS sorting for EpCAM, so that only acinar cells were filtered and collected. After treatment of pure acinar cell suspension with bacterial protein lysates, there was no upregulation of pro-inflammatory cytokines. On the contrary, their levels were even lower than those of the untreated controls (Figure 3.22). In conclusion, by excluding pancreas-resident immune cells and treating the acinar cells suspension with different bacterial protein lysates, we prevent the onset of the immune response. Therefore, we can conclude that the immune response to the bacterial presence in acute pancreatitis, as seen in Figures 20 and 21 is generated by the resident immune cells.

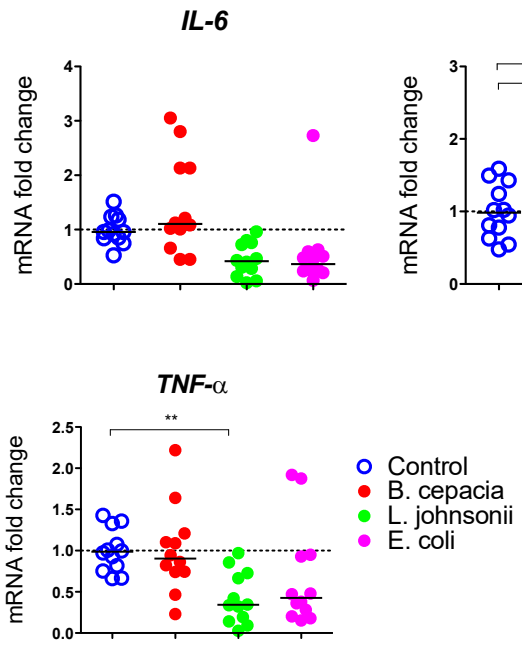


Figure 3.22: Acinar cells are irresponsive to the bacterial protein stimulation. Acinar suspensions were sorted for EpCAM antibody for epithelial surface antigen using a MACS sorting system. Treatment with 15 µg/mL of protein lysates from different

bacterial strains for 4 h. Expression levels of *IL-6*, *CXC1* and *TNFα* cytokines was assessed by qPCR. Each data point represents a technical triplicate from one mouse. Data was shown as group median. Statistical evaluation was calculated using One-way ANOVA with Newman-Keuls post-hoc test (p value: * <0.05, ** < 0.01, *** < 0.001).

4 Discussion

4.1 Establishment of a cerulein-induced model of acute pancreatitis

In establishing cerulein-induced pancreatitis model, we have used several parameters in order to assess the successful induction of acute pancreatitis as well as the severity level. Supra-physiological doses of the secretagogue cerulein lead to a high secretion of pancreatic digestive enzymes into the serum, most notably amylase and lipase. Cerulein treatment also caused infiltration of inflammatory cells into the pancreas, caused pancreatic edema and acinar cells vacuolization. These features have been described before in mice [239], [240] and are comparable to acute pancreatitis feature in humans [21], [241]. This model histologically simulates the early phase of AP in humans [194] and enables the analysis of early, intracellular events in an early phase of pancreatitis.

In the present thesis, we used the analysis of serum levels of amylase and lipase, as main surrogate parameters of successful induction of acute pancreatitis as this has been shown to be a useful parameter in numerous publications [24], [53], [204], [210], [240], including ours [242]. In acute pancreatitis patients, the level of amylase rapidly increases with 4 to 6 hours after the onset of the disease. It remains high for 3 to 4 days and is gradually decreasing afterwards [84]–[86]. Even though amylase levels can be normal in patients with alcoholic or hypertriglyceridemic pancreatitis, in our study, for obvious reasons, this was not considered as a limitation of these two enzymes in diagnosis of acute pancreatitis. Some authors suggest that even though elevated serum levels of amylase and lipase are reliable indicators of successful induction of acute pancreatitis, they cannot be used to monitor or predict the severity of acute pancreatitis in patients [243], [244]. This notion was established in the study by Lankisch *et al.* [245] where patients with only a small increase in amylase and lipase levels also had or developed severe acute pancreatitis. However, instead of predicting the severity and clinical course of the disease, we used these biochemical serum parameters to assess the severity of the inflammatory process in the pancreas at a given time point. Being able to do

this is important in order to investigate how different antibiotic pretreatments or dietary modulation impact the severity of acute pancreatitis. Still, having all the limitations of amylase and lipase measurements in mind, these parameters are considered to be surrogate parameters of acute pancreatitis in patients and mouse models and were complemented with histological and cytokine analysis.

Main histological parameters of acute pancreatitis are presence of edema, level of immune cell infiltration and presence of apoptotic cells, according to Schmidt *et al.* [240]. These authors described that acute pancreatitis is morphologically characterized by edema, hemorrhages, parenchymal necrosis with fat necrosis and infiltration of leukocytes. With regard to absence or presence of necrosis, acute pancreatitis can be classified into edematous or hemorrhagic-necrotizing pancreatitis [239]. Since the most prominent histological feature of cerulein-induced model of pancreatitis is edema, this model is considered to be a model of edematous pancreatitis which barely develops tissue necrosis [194]. Therefore, our evaluation is made by giving scores to different types of morphological alterations in histological samples as described before [240].

Furthermore, levels of mRNA of pro-inflammatory cytokines in the pancreas were measured. The cytokines known to be most prominently upregulated in acute pancreatitis are IL-6, TNF-α and IL-1β [65], [101], [246], [247]. When compared directly with TNF-α and IL-1β, IL-6 was superior with an overall accuracy of 88% in detecting acute pancreatitis [247].

In our experiment to validate the time course of the inflammatory process, these parameters were measured 2, 24 and 72 hours after the last cerulein injection. All parameters were significantly elevated at a 2-hour time point, and were gradually decreasing as the time progressed. In addition to a successful induction of acute pancreatitis, this assessment allowed us to identify the time point of an acute inflammatory phase, when inflammation is reaching its maximum. This time point is important for the examination of biological events occurring in the early phase of acute pancreatitis.

4.2 Identification of pancreatic microbiota and its changes induced by acute pancreatitis

There is still an ongoing debate regarding the question of pancreatic sterility. It has been a widely held notion that the pancreas is a sterile organ. However, several recent findings have shown that bacteria are residing in the pancreas of mice and men. Pushalkar *et al*. [231] showed by FISH and qPCR analysis the presence of bacteria in pancreas. Additionally, they oral gavaged wild-type mice with fluorescently labelled *Escherichia coli* and *Enterococcus faecalis* and observed the presence of these bacteria in the pancreas as quickly as half an hour after the gavage, therefore showing that translocation of bacteria from the gut to the pancreas via intestine-pancreatic route is possible, probably via the primary pancreatic duct. In line with this, microbiota sequencing results of pancreata showed that the most abundant genera in wild-type mouse pancreata are *Agrobacterium* and *Rhizobium*, which are associated with mouse chow diet [231], further suggesting a gut origin of pancreatic bacteria. We confirmed presence of bacteria in the pancreas by different means. Our 16S rRNA gene sequencing results showed that pancreas is not a sterile organ. Moreover, experiments with homogenized pancreata, that were plated and incubated in different conditions, also showed that there are several bacterial genera that reside in uninflamed pancreas, the most prominent being Lactobacilli. A problem with these findings could be a possible contamination of samples with bacteria from other gastrointestinal sites during organ extraction.

The question of pancreatic sterility has been brought up mostly by the studies researching the important role of pancreatic microbiota in pancreatic cancer, as summarized in a review by Ertz-Archambault *et al.* [248]. However, changes introduced to the pancreatic microbiota, in terms of total bacterial abundance or compositional changes, induced by the acute pancreatitis have not yet been explored in detail. Our data show that induction of acute pancreatitis in mice is reflected by compositional changes of the intrapancreatic microbiota. FISH staining with pan-bacterial EuB probe also shows increased total abundance of bacteria in pancreatic tissue samples from cerulein-treated mice compared to non-pancretitis control animals. In line with this finding, experiments where pancreata were homogenized, plated

and incubated in different conditions in order to support the growth of bacteria showed more diverse bacterial colonies in plates with pancreata originating from cerulein-treated mice.

Another confirmatory result comes from beta-diversity analyses using t-SNE visualization, where we observed that pancreatic, as well as mesenteric lymph node, ileal and fecal microbial communities are affected by the onset of cerulein-induced pancreatitis.

It is established that pathogenesis of acute pancreatitis has its origin in the gut, where microbial dysbiosis contributes to the intestinal barrier dysfunction. It has been further confirmed in a meta-study that the majority of patients with acute pancreatitis also have gut barrier dysfunction [249]. What is not known so far, is how gut dysbiosis affects the pancreas. We hypothesized that the dysbiosis, that occurs due to multiple reasons, affects the onset and severity of acute pancreatitis, by utilizing the dysfunctional intestinal barrier to translocate bacteria to pancreas and exacerbate the inflammation. We tried to confirm this hypothesis by introducing different modulations to the gut microbiota and observing the repercussions on the pancreatic microbial population as well as on the severity of inflammation.

4.3 Effect of broad-spectrum antibiotic treatment on the severity of acute pancreatitis

The link between gut microbiota dysbiosis and human inflammatory diseases is very well documented [250]–[253]. Many studies show that intestinal microbiota is associated with inflammation and is involved in the progression of acute pancreatitis [254]–[257].

Conversely, patients with acute pancreatitis are more prone to a gut dysbiosis which manifests as higher relative abundances of *Enterobacteriaceae* and *Enterococcus* spp., and lower *Bifidobacteria* in fecal biospecimens [254]. All the studies, both preclinical, and human, that point to the role of intestinal dysbiosis in the pathogenesis and level of severity of acute pancreatitis, raised the question of modulation of the gut microbiome to counteract the AP [254], [255], [257], [258]. Therefore, it has been postulated that alterations of acute

pancreatitis-associated gut microbiota by broad-spectrum antibiotics treatment might alleviate the severity of pancreatitis [257].

Before applying the bowel decontamination with the use of broad-spectrum antibiotics, we first used germ-free mice to study the overall contribution of commensal bacteria on pancreatitis. Mice treated with antibiotics have significant reductions in bacterial load that are associated with changes in signaling pathways, or inflammatory response, with results often being similar to what is seen in germ-free mice [259]. Here, we found that germ-free mice exhibited alleviated pancreatic injury after AP induction by measuring serum levels of amylase and lipase and performing the histological analysis, which was recently described in a similar preclinical study by Zhu *et al*. [255]. Our histological analysis of pancreatic tissue further revealed that the GF mice exhibited reduced pancreatic injury after cerulein treatment, compared to SPF mice.

As a conclusion, this experiment identified the gut microbiota as an important mediator during AP and that its absence is associated with a decrease in AP severity, which suggests its role as potential therapeutic target.

In our study of the application of broad-spectrum antibiotic pretreatment in pancreatitis, mice were treated with a cocktail of antibiotics for two weeks, before the induction of acute pancreatitis. 2 hours after the last cerulein injection, mice were sacrificed and the pancreatitis severity was assessed with standard parameters. The histopathology score showed that antibiotics treatment markedly decreased morphological damage in the form of edema and inflammatory infiltration in AP mice. The serum level of amylase and lipase was lower in AP mice treated with antibiotics than controls, and levels of pro-inflammatory cytokines were significantly decreased in antibiotic treated AP group. Similar results were previously published in a study by Zhu *et al*. [255] where they treated C57BL/6 mice, in which cerulein model of pancreatitis was previously induced, with antibiotic cocktail (ampicillin, neomycin, metronidazole and vancomycin). Histological evaluation of pancreas as well as serum amylase and lipase levels revealed that antibiotic-treated mice had alleviated symptoms of acute pancreatitis compared to mice without antibiotics. Numerous clinical studies also assessed

the application of bowel decontamination in prevention of severe necrotizing pancreatitis, showing mixed results.

The explanation for the reduction in all of the observed parameters is probably due to the change of the microbial composition that reflects on the change of the immune response. As it has been explained before that pathogenesis of acute pancreatitis originates in the gut, where microbial dysbiosis contributes to the intestinal barrier dysfunction, our results speak in favor of bowel decontamination as a mean to mitigate the influence of the dysbiotic gut microbiota.

To investigate the microbiota signatures associated with antibiotic treatment in AP, we have 16S sequenced samples of pancreata and mLNs, and performed flow-cytometric analysis of pancreas and spleen to delineate the effects on immunophenotypes in the pancreas.

4.3.1 Impact of broad-spectrum antibiotic pretreatment on microbial composition of the pancreas and mLNs

Our 16S sequencing results showed several important effects: First, we saw an overlap of the microbiota between mLNs and the pancreas. This is pointing to the origin of bacteria being in the gut lumen and their path of translocation leading, at least in part, over mLNs. This is supported by the evidence from numerous studies showing that a usual route of gut bacteria towards liver or other organs leads over mLNs [260]–[262].

t-SNE visualization of Beta-diversity by treatment showed that antibiotic pretreatment had an impact on bacterial communities, even more so than presence or absence of pancreatitis. Together with findings from 16S sequencing of antibiotic treatment having an overall influence on bacterial populations, this suggests a correlation between diminished parameters of inflammation, and changed bacterial communities. In line with this, Zhu *et al.* [255] showed that the antibiotic-treated mice with a depleted gut microbiota had alleviated pancreatic injury compared with controls after the induction of acute pancreatitis.

4.3.2 Local and systemic immune response in acute pancreatitis and antibiotic pretreatment

In order to explain how induction of acute pancreatitis and broad-spectrum antibiotic treatment influence local pancreatic and systemic immune responses, we performed FACS analysis of the mouse pancreas and spleen.

The effects of the gut microbiota on T cell induction and development have been widely documented [263]–[267]. Mouse studies exploring the effect of broad-spectrum antibiotic application and effects on immune cell populations in different tissues showed that upon antibiotic treatment number of CD4+ T cells is reduced in Peyer's patches, small intestines, the colon, mesenteric lymph nodes, spleen and the blood [142], [268]–[270].

Similar to CD4+ T cells, number of cytotoxic CD8+ T cells generally decreased in the intestine after antibiotic treatment. Results at other sites vary, showing CD8+ decrease in colon and blood, similar or higher numbers in mesenteric lymph nodes, or increase in spleen and Peyer's patches [270]–[273].

The severity of pancreatitis is also reduced *in vivo* by CD4+ (but not CD8+) T-cell depletion, showing that CD8+ T-cells do not influence the course of acute pancreatitis [274]. CD4+ T cells are very important in the inflammatory response in acute pancreatitis because activated neutrophils potentially recruit and activate CD4+ T cells [275], which then release IL-8 [276], resulting in recruitment of more neutrophils and additional CD4+ T cells to the inflamed pancreas. Our results showed a slight increase in percentage of CD4+ T cells in the pancreas after antibiotic treatment. Cerulein-induced pancreatitis, or pancreatitis after antibiotic pretreatment did not cause any changes in CD4+ T cell presence in the pancreas. This could be due to the fact that the time point at which we did the measurement was too early for the CD4+ T cells to be recruited to the pancreas.

Regarding systemic effects of antibiotic treatment on immune cells, our results are showing only slight decrease in percentage of CD4+ T cells in the spleen, but no overall difference of immune cell subtypes after cerulein induction compared to control mice. CD8+ T cells were increased in antibiotic treated mice, which is in contrast to other publications that show either

decrease, or no change in CD8+ T cell counts in spleen after antibiotic treatment [272]. In pancreatitis, however, regardless of presence or absence of antibiotic pretreatment, we did not observe any change of CD8+ T cell counts.

Together, these results show that CD4+ and CD8+ T cells do not play a role in the early phase of acute pancreatitis inflammatory response, and that consequently, their levels in pancreatitis are not influenced by the antibiotic pretreatment.

There are varied reports of B cell shifts after antibiotic treatment. Some studies showed a decrease in small intestine, colon and Peyer's patches [269], while some showed not difference, or a slight reduction in B cells in spleen and blood [277]. Some observe no antibiotic effect on B cell counts in mesenteric lymph nodes or the liver [272], [273]. Our results are in line with this, showing no difference in presence of B cells in pancreas after antibiotic treatment, and a slight reduction in spleen.

It is important to note that no previous study researched the role of B cells in cerulein-induced acute pancreatitis. Our results show that in pancreatitis-only, as well as in pretreated mice, there is an increase of percentage of B cells in the pancreas, while no difference is observed in the spleen. This indicates that B cells could be the drivers of early immune response in our model of acute pancreatitis, and that antibiotic pretreatment can potentially reduce the presence of B cells in this condition.

One recent study concluded that high levels of CD4+ T and CD19+ B lymphocytes in patients' blood during the early phase of AP can predict organ failure [278]. Therefore, it is important to work towards optimization of the antibiotic pretreatment that would reduce the number of these cells.

Next we looked at the cells of myeloid lineage labelled with markers Ly6G (monocytes, neutrophils and granulocytes) and F4/80 (macrophages).

Reports regarding monocytes, neutrophils and granulocytes show that upon antibiotic treatment they are predominantly decreasing in spleen, liver and small intestine [279]–[281]. Our results show that levels of Ly6G+ cells in both pancreas and spleen are going up after the cerulein treatment, and that antibiotic pretreatment has an effect on reducing their levels.

This is important because neutrophil infiltration of the pancreas following pancreatitis induction is an early phenomenon that results in trypsinogen activation [232], [282]. Neutrophil recruitment and activation is central to acinar cell necrosis in the cerulein model, since in the absence of neutrophils only apoptosis occurs, and not necrosis [283]. Being able to reduce the level of neutrophils in the pancreas at the early stage of pancreatitis and therefore prevent cellular necrosis and further worsening of the inflammation is one of our very important findings.

Macrophages mostly go down in antibiotic treatment, in small intestine, colon, spleen, liver, or are at similar levels in Peyer's patches, mesenteric lymph nodes, kidneys and lungs [269], [284]–[286].

Folias *et al*. [287] found F4/80+ macrophages were the predominant immune cells present in the uninflamed pancreas, and that is what we also observe in our data set. In accordance with these findings, our data shows that out of all the observed immune cells, macrophages were the most prominent ones in uninflamed pancreas. Antibiotic treatment alone, has indeed reduced the levels of macrophages in both pancreas and spleen, just as it has been shown to be the case in other examined organs.

In the cerulein model, macrophages are known to progressively infiltrate the pancreas and are a major source of TNF-α [288], which potentially both attracts and activates neutrophils [289]. Activated macrophages also secrete IL-12, which stimulates CD4+ T cell to secrete more INF-γ, promoting more macrophage activation [290], the appearance and activation of neutrophils, and ultimately more acinar cell necrosis.

Our data, however, does no support these findings, since there was no increase in macrophage percentage in pancreas, nor systemically. Consequently, antibiotic pretreatment did not change the levels of macrophages in pancreatitis or systemically. The possible explanation for this is that our time point is very early when macrophages are still not recruited into the inflamed pancreas.

Here, we looked for pancreas-specific dendritic cells double positive for MHCII and CD11c. A study by Bedrosian *et al*. [291] showed that levels of these dendritic cells are increased in

pancreas after induction of acute pancreatitis with cerulein or L-arginine in mice. These increased levels of dendritic cells contributed to faster regeneration after the pancreatitis and increased survival of mice.

The inflammation in acute pancreatitis is in part sustained by translocation of commensal microbiota and their consequent stimulation of innate immune responses in acinar cells, which leads to necrosis and production of cellular necrotic debris. Interestingly, a study by Bedrosian *et al.* [291] is showing that dendritic cells might put a stop to this due to their capacity to clear the necrotic debris. This study is showing that depletion of CD11c+/MHCII+ dendritic cells results in exacerbation of acute pancreatitis and increased acinar cell death [291].

Our data shows that levels of CD11c+/MHCII+ dendritic cells are increased in the pancreas, after the cerulein treatment, while there is no increase on the systemic level, in the spleen.

Regarding antibiotic treatment, dendritic cells follow the trend of other immune cell types and are decreased, or similar to normal in many analysed tissues, spleen, small intestine, colon and mesenteric lymph nodes [272], [273], [279]. In our data, dendritic cells in the pancreas correlate with findings from the previous studies, and do not show any increase due to the antibiotic treatment. However, in the spleen, there is an increase in percentage of these dendritic cells following the antibiotic treatment, regardless of cerulein treatment. It would be important to see whether those dendritic cells are recruited to the pancreas at later time points, where they contribute to the debris clearance and improve the tissue regeneration.

Overall, our data obtained with FACS analysis is showing that at our early time point, the inflammation is mainly driven by Ly6G+ cells and B cells in the pancreas. These seem to be the first immune cells to infiltrate the pancreas, and that is why it is promising to see that our antibiotic pretreatment managed to reduce the levels of these cells in the pancreas. The mechanism behind this is still unclear, but with the help of the following data sets we will try to give answer to this question.

4.4 Influence of diet modulations on the severity of the acute pancreatitis

4.4.1 Influence of fiber-rich diet

Short chain fatty acids are produced in the gut by degradation of dietary fiber by the commensal bacteria, with most prominent producers being *Clostridia* cluster XIVa [141], [292]. Two recent studies showed an effect of short-chain-fatty acid (SCFA) butyrate in ameliorating cerulein-induced acute pancreatitis and associated intestinal injury [293], [294]**.** The proposed mechanism is by directly interfering with histone acetylation, and subsequent suppression of inflammasome activation [293]. A study by Zhu *et al*. [255] also showed that the early dysbiosis of gut microbiota leading to the depletion of SCFA-producing bacteria is associated with the impaired gut barrier that leads to the progression of acute pancreatitis.

In our experiment, mice were fed with low-, normal- and high- fiber diet in order to examine the influence of dietary fiber degradation and increased or decreased short-chain-fatty acid production in our model of acute pancreatitis. Our results are not showing a change in the severity of acute pancreatitis. Possible explanation is that acute pancreatitis induction overrides the anti-inflammatory effect of the high-fiber diet, or that the effect can only be observed in the later stage of pancreatitis, where it possibly prevents the onset of organ failure, or decreases the overall severity of the disease.

4.4.2 Influence of high-fat diet

Obesity and high triglyceride levels in the blood are known risk factors for acute pancreatitis [19], [295]. The reason why these conditions are a risk factors for acute pancreatitis is that acinar cell necrosis causes release of digestive enzymes to areas of the pancreas otherwise protected from contact with digestive enzymes [295]. For example, lipase hydrolyses circulating triglycerides in the blood-stream and those stored in the intrapancreatic and peripancreatic adipocytes into saturated and unsaturated free fatty acids (UFAs) [295]. UFAs such as linoleic, oleic and linolenic acids are causing the increase in inflammatory response by increasing the levels of TNF-α and other pro-inflammatory cytokines [19], [296]–[298]. In

clinical studies, patients with acute pancreatitis who have increased visceral fats and hypertriglyceridemia were found to be at increased risk of multisystem organ failure and pancreatic necrosis [299]–[302]. Moreover, clinical and preclinical studies demonstrated that HF diet drastically changes the gut microbiota composition [236], [303] leading to increase in gut permeability which attenuates intestinal barrier function, resulting in leaky gut [237].

Our results from mice that were fed with a HF diet before induction of acute pancreatitis, confirmed these findings. In HF diet-fed mice serum level of amylase and lipase are significantly higher than in mice fed a normal chow. Next, we wanted to determine if gut microbiota was also a contributing factor in the severity of pancreatitis. To explore this question, we inoculated germ-free mice with cecal contents of mice either fed with HF diet or with normal chow. Mice inoculated with cecal content of mice fed with high-fat diet did not show an exacerbated acute pancreatitis. Therefore, we can conclude that the gut microbiota of mice fed with HF diet does no predispose these mice to more severe acute pancreatitis. The observed effect was most likely due to the increased presence of free fatty acids in blood stream, as well as increase in visceral fat.

4.5 Modelling of acute pancreatitis *in vitro*

The establishment of the in vitro model of pancreatitis meant that the isolated pancreas suspension was responsive to LPS and cerulein treatments, with the readout being upregulation of pro-inflammatory cytokines, such as IL-6 or TNF-α. In the literature, there are only few examples of application of cerulein in vitro, for instance, its application on rat acinar AR42J cell lines [210], and on rat acinar suspension [304]. After the optimization of LPS and cerulein treatments of acinar suspension, that made it a suitable in vitro model of acute pancreatitis, other treatments were applied with the goal of delineating the underlying mechanisms of microbiota-immune interactions.

4.5.1 Co-stimulation of acinar suspension with cerulein and bacteria leads to upregulation of pro-inflammatory cytokines

In order to see if the bacteria detected in culturing experiments as well with 16S sequencing had an effect on acinar cells, we incubated acinar suspensions with alive bacteria, or protein lysates of bacteria that were collected in culturing experiments. Treatment with the alive bacterial species *Lactobacillus johnsonii* and *Burkholderia cepacia* induced an upregulation of the expression of IL-6, TNF-α and IL-1 β pro-inflammatory cytokines.

In order to complement these findings with the context of acute pancreatitis, acinar suspensions were treated with both cerulein and alive *Lactobacillus johnsonii*. In this case, combination of cerulein treatment and exposure of acinar cells to bacteria induced a higher upregulation of pro-inflammatory cytokines, especially TNF-α. The possible explanation for this could be lowering of the pH by live bacteria, that sensitizes the pancreatic acinar cells to secretagogue-induced zymogen activation. This explanation is given in the study by Bhoomagoud *et al*. [304], where they reduced the pH of acinar culture while exposing it to cerulein. The result was increase in zymogen activation and tissue injury (in *in vivo* experiment), which implies to the increased risk for the development of severe acute pancreatitis.

Altogether, these findings implicate that bacteria can exacerbate cerulein-induced pancreatitis. Whether it is due to the reduction or pH, or presence of bacteria being sensed by acinar cells' TLRs and resident immune cells, we try to answer by treating acinar suspension with bacterial protein lysates.

Our data demonstrate that bacterial protein lysates are able to induce the upregulation or pro-inflammatory cytokines. Interestingly, lysates from different bacteria upregulated different cytokines: *Burkholderia cepacia* upregulated CXCL1, *Lactobacillus johnsonii* TNF-α, while *Escherichia coli* upregulated IL-6 and TNF-α. This is probably due to recognition of different bacterial molecular patterns by different TLR receptors, which leads to different innate immune responses.

The last thing we explored in our in vitro pancreatitis model was whether acinar cells alone were able to trigger the immune response in presence of bacteria. For this reason, we eliminated immune cells from our acinar suspension by magnetic cell sorting, producing a pure acinar cell suspension. Here we observed that acinar cells alone cannot trigger the upregulation of pro-inflammatory cytokines. This raises the importance of pancreas-resident immune cells, as well as the immune cell recruitment into the pancreas upon pancreatitis induction.

4.6 Conclusions

Reliable and reproducible models of acute pancreatitis *in vivo* and *in vitro* were established. These models allowed us to study the course of acute pancreatitis and the influence of gut microbiota on the severity of the inflammation.

The results indicate that the gut microbiota is an important mediator of the onset of acute pancreatitis. Absence of gut microbiota is associated with a decrease in AP severity, suggesting its role as potential therapeutic target. This might direct the new approach to the treatment of AP through development of therapeutic measures aimed at preventing bacterial overgrowth and translocation, or modulating the microbiota to eliminate bacterial strains known to exacerbate the acute pancreatitis.

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A Publications

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