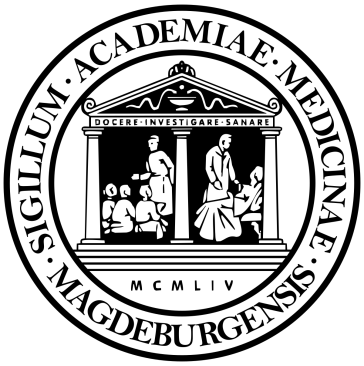

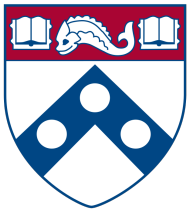

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The Nitrogen and Carbon Metabolism in *Klebsiella pneumoniae*: Implications for Inflammatory Bowel Disease and Hepatic Encephalopathy

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(Dissertation)

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Das Darmmikrobiom spielt eine zentrale Rolle bei der Aufrechterhaltung der Homöostase im Gastrointestinaltrakt. *Klebsiella pneumoniae*, ein opportunistischer gramnegativer Erreger, hat aufgrund der häufiger auftretenden Antibiotikaresistenzen und seiner Rolle bei darmpathogenen Erkrankungen zunehmend an Bedeutung gewonnen. Frühere Arbeiten zeigten, dass Urease, ein bekannter Virulenzfaktor von *K. pneumoniae*, möglicherweise im Kontext der Stickstofflimitierung und im sauren Milieu des Magen-Darm-Traktes eine Rolle spielt. *In-vitro*-Experimente zeigten, dass *K. pneumoniae* Harnstoff als Stickstoffquelle nutzen kann, was unter stickstofflimitierten Bedingungen einen Wachstumsvorteil darstellt. In sauren Umgebungen verschafft Urease jedoch keinen Überlebensvorteil. Unsere *in-vivo*-Studien mit Mäusen ergaben, dass weder Urease noch das Ntr-System, welches weitere Stickstoffquellen zugänglich macht, für eine erfolgreiche Kolonisation des Darms notwendig sind. Dies deutet darauf hin, dass Stickstoff kein limitierender Faktor für die Besiedlung des Darms ist. Im Gegensatz dazu scheinen Kohlenstoffquellen ein limitierender Nährstoff für *Enterobacteriaceae* wie *K. pneumoniae* zu sein. Eine zuvor durchgeführte Ernährungsstudie zeigte, dass Kohlenstoff ein wesentlicher Faktor für die Kolonisierung des Darms ist. Wir stellten fest, dass *K. pneumoniae* einfache Kohlenhydrate gegenüber komplexen, wie sie in Ballaststoffen vorkommen, bevorzugt. Dies könnte bei Erkrankungen wie Dysbiose von Bedeutung sein, da ein übermäßiges Wachstum von *Enterobacteriaceae* oft auftritt und in diesem Fall durch Ernährungsinterventionen eingesetzt werden könnten. Spezielle Kohlenhydrate werden bereits therapeutisch eingesetzt, z.B. Laktulose zur Behandlung von HE. Unsere Untersuchungen zeigten, dass Laktulose die Ammoniakproduktion von *K. pneumoniae* beeinflusst.

Schlüsselwörter

Ammonia - Carbon metabolism - *Escherichia coli* MP1 - Genetic recombineering - Gut microbiome - Hepatic encephalopathy - *In vivo* colonization - Inflammatory bowel disease - *Klebsiella pneumoniae* MGH 78578 - Nitrogen metabolism - Short chain fatty acids - Urea - Urease

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Abbreviations

ABX	antibiotic treatment
ATP	adenosinetriphosphate
AUC	area under the curve
BBB	blood-brain barrier
BCAA	branched chain amino acid
CFU	colony forming units
CR	conventionally raised
CNS	central nervous system
DSI	distal small intestine
DSS	dextran sodium sulfate
E. coli	*Escherichia coli*
EEN	exclusive enteral nutrition
ESBL	extended-spectrum ß-lactamase
FRT	FLP recombination target
GBA	gut-brain axis
GF	germ free
GVB	gut-vascular barrier
HE	Hepatic Encephalopathy
HPLC	(ultra-) high performance liguid chromatography coupled to UV or fluorescence detection
H. pylori	*Helicobacter pylori*
IBD	inflammatory bowel disease
ICU	intensive care unit
K. pneumoniae	*Klebsiella pneumoniae*
LB	Lysogeny Broth
MASLD	metabolic-dysfunction associated steatotic liver disease
MOPS	3-(N-morpholino)propanesulfonic acid (buffering agent, pK_a 7.20)
NAC	nitrogen assimilation control
nac	nitrogen assimilation protein
NADH	Nicotinamide adenine dinucleotide
NMDA	N-methyl-D-aspartate
NMR	proton nuclear magnetic resonance
Ntr	nitrogen regulation
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol

PSI	proximal small intestine
RNS	reactive nitrogen species
rpm	rotations per minute
ROS	reactive oxygen species
SCFA	short chain fatty acid
SPF	specific pathogen-free
Supp. Figure	Supplemental Figure
TAA	thioacetamide
TCA	tricarboxylic acid
TIPS	transjugular intrahepatic portosystemic stent shunt
WT	wild type
w/	with

1. Introduction

1.1 The Intestinal Microbiome

In recent years, the interest in understanding the interactions between the microbiome, physiology, and pathology has grown and several diseases relating to various organ systems have been discovered to be associated with microbial imbalances [**1, 2**].
The colon hosts the greatest number of bacteria in the intestinal tract as it constitutes the most habitable environment. The majority of the colonic microbiota is made up of obligate anaerobes, belonging to the Firmicutes and Bacteroidetes phyla with facultative anaerobes like *Enterobacteriaceae*, such as our species of interest *Klebsiella pneumoniae*, being a minor component [**3, 4**]. The microbiome in the mammalian intestinal tract has evolved to work together with the host in a mutualistic relationship. Nevertheless, the homeostasis between the mammalian host and its resident microbiome depends on the immune system constraining the intestinal microbiota [**5**]. If this balance is disrupted, dysbiosis may develop [**2**]. Dysbiosis is subsequently a driving factor, which may participate in the pathogenesis of various diseases, which we will examine further in this study [**6, 7**].

1.2 The Growing Significance of *K. pneumoniae* in the Clinical Setting

K. pneumoniae is a omnipresent gram-negative opportunistic pathogen that can be found on mucous membranes of mammals and in water or soil [**8, 9**]. It is rapidly spreading geographically and thus becoming a bigger concern to humanity [**8, 10**]. While it poses immediate threat to most healthy individuals who may be asymptomatic carriers of the bacterium in their intestinal tract, nose, or throat it can disseminate systemically and lead to serious infections, especially in hospitalized patients, predominantly causing pneumonia or wound, soft tissue, and urinary tract infections [**8**–**13**]. Several antibiotic multidrug-resistant strains of *K. pneumoniae* have been characterized, with some having the ability to produce extended-spectrum ß-lactamases (ESBLs) and an emerging carbapenem-resistance, leading to treatment failure and prolonged hospital stays. Hypervirulent strains have emerged in recent years due to the growing number of hospital- and community-associated outbreaks [**8, 10**–**17**]. Hypervirulent *K. pneumoniae* increasingly causes infections in immunocompetent individuals and can lead to invasive disease with abscesses in the liver or seeding to the lung, central nervous system (CNS), or musculoskeletal system [**13, 18**]. Even if treatment is carried out with appropriate antibiotics, the mortality and morbidity of severe systemic *K. pneumoniae* infections remain high [**15**]. *K. pneumoniae* is a member of the family of *Enterobacteriaceae* in the phylum of Proteobacteria, which is often found in the gut microbiome of individuals with dysbiosis or inflammatory bowel disease (IBD), respectively [**6, 7**].
The significance of *K. pneumoniae* is ever growing and although many discoveries regarding its virulence and pathogenicity have been made, not all mechanisms involved have been explored to this date [**18, 19**].

1.3 Urease as a Virulence Factor in *K. pneumoniae*

One of the many known virulence factors (capsular polysaccharides, pili, siderophores, biofilm formation, antibiotic-resistance, to name a few) of *K. pneumoniae* is urease, which we will concentrate on in this study [**8, 15**]. Urease can be produced by various bacterial species that can be found in the intestinal or urinary tract of animal hosts, in addition to appearing in other environments like water and soil [**20**–**22**]. Urease-positive organisms include the spectrum from resident flora to pathogenic bacteria and are known to be an essential component in the colonization of hosts and the metabolic maintenance of procaryotes, giving them the ability to access urea as a nitrogen source [**21**–**23**]. The level of urease activity an organism exhibits can vary and is a characteristic and distinguishing feature [**22**]. Ureolytic pathogens include methicillin-resistant *Staphylococcus aureus* and *Staphylococcus saprophyticus*, which are both known to cause urinary tract infections [**24, 25**], as well as *Helicobacter spp.*, all isolates from gastritis were found to be urease-positive [**22**], *Mycobacterium tuberculosis* and *bovis*, *Proteus mirabilis*, *Ureaplasma urealyticum*, *Pseudomonas spp.*, *Corynebacterium spp.* and *Klebsiella spp.*, to name a few [**22**]. Notably, a commonality in all urease-positive bacteria is the ability to cause urinary tract infections [**21**]. Nevertheless, the urinary tract is not the only host environment urease plays an essential role in. A great variety of urease-positive organisms can be found in the gastrointestinal tract, where they are part of the resident microbiome, but can potentially play a role as opportunistic pathogens [**6, 21**].

Previous studies from our research group found a link between urease-positive *E. coli* and the development of dysbiosis [**6**], which is characteristically represented by a proportional increase of Proteobacteria in the gut microbiome [**6, 7**]. This led us to speculate that other urease-positive Proteobacteria, specifically *K. pneumoniae*, may provide another interesting insight into the role urease plays in the development of dysbiosis [**6**].

The reaction catalyzed by the enzyme urease is the hydrolysis of urea, resulting in carbamic acid and ammonia, with the former decomposing into carbonic acid and an additional ammonia molecule (**Figure 1.1**) [**26**]. The ammonia-producing reaction can have multiple effects on the bacteria itself and its environment, for example increasing the environmental pH or making urea available as a nitrogen source [**26, 27**].

Urease is composed of three subunits (UreA, UreB, and UreC), with two nickel ions per active center, located in the cytoplasm of *Klebsiella spp.* [**26**]. To activate urease *in vivo*, accessory genes (UreD, UreE, UreF, and UreG) are necessary to incorporate the nickel-containing metallocenters into the functional urease enzyme [**26**]. In our studied organism, *K. pneumoniae*, the expression of urease is regulated based on the availability of nitrogen through the nitrogen regulatory system (NTR), which is responsible for the activation of the transcription of urease via the positive controller nitrogen assimilation control (NAC) [**26**]. This will be discussed in more detail later in this study.

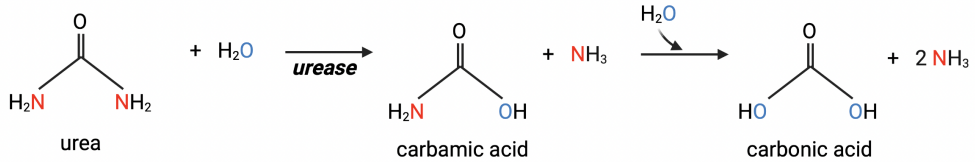

Figure 1.1: Hydrolysis of urea via urease

1.3.1 *Helicobacter pylori* Utilizes Urease for Neutralization of the Acidic Gastric Environment

Urease is an integral part of the colonization of the gastric mucosa by *Helicobacter pylori* [**28**]. *In vitro* experiments have shown that the bacteria is sensitive to acidity in the absence of urea, yet in the presence of urea *H. pylori* can withstand the gastric pH [**26**]. *H. pylori* uses urease to hydrolyze urea, resulting in ammonia (NH_3) and carbonic acid (**Figure 1.1**), with the latter subsequently broken down further into carbon dioxide (CO_2) and water (H_2O) [**26, 27, 29**]. Both ammonia and carbon dioxide subsequently play a role in lowering the gastric pH from 2.5 to a near-neutral micro-environment of pH 6.1 in the periplasmatic space of *H. pylori* [**26, 29**]. This effect is in one part achieved by carbon dioxide being converted into bicarbonate (HCO_3^-) and (H^+), with the base bicarbonate neutralizing the gastric acid [**29**]. The resulting (H^+) is furthermore neutralized by ammonia, which forms the second part of *H. pylori's* acid resistance as ammonium (NH_4^+) [**29**].

1.3.2 SCFAs Affect Growth of *K. pneumoniae* in Acidic Environments

The gastric acid during passage of the stomach is not the only instance where bacteria may encounter acidic environments in the body. Short chain fatty acids (SCFAs), mainly acetate, propionate and butyrate, are organic acids with a short carbon chain of less than 6 carbon atoms, produced in the colon by fermentation of carbohydrates [**30**–**33**]. Other SCFAs, such as formate, valerate, and the branched chain acid isovalerate, which are products of amino acid fermentation, can be found more sparingly in the intestinal tract [**32**]. Dietary compounds used in the carbohydrate fermentation process are mostly complex carbohydrates, such as polysaccharides, oligosaccharides, as well as some disaccharides, which are hydrolyzed by microbiome-produced hydrolases [**4**]. This reaction results in the production of SCFAs, as well as gases, such as carbon dioxide (CO_2), methane (CH_4), and hydrogen (H_2) [**4, 32**]. Acetate is found to a greater extent than propionate and butyrate [**4, 31**]. SCFAs, as weak acids, have a pK_a of around 4.8, so their production in the colon subsequently leads to a decrease in pH [**4**]. The normal range of pH in the human colon of 5.5 - 7.5 [**4**]. *K. pneumoniae* is capable of maintaining a slightly alkaline intracellular pH, even in slightly acidic (pH 5.75) environments. Additionally, it was previously observed that the presence of SCFAs at a neutral pH of 7 does not inhibit the growth of *K. pneumoniae* [**34**]. While neither condition is sufficient to inhibit growth alone, if SCFAs are present in an acidic environment of pH 5 - 6 they may hinder the growth of *K. pneumoniae* [**34**].
Our aim was to test whether *K. pneumoniae* can utilize urease and the resulting production of ammonia to neutralize mildly acidic environments, like the one encountered in the colon due to increased SCFA concentration.

1.4 Essential Resources for Microbial Growth in the Gut

The mammalian intestinal tract is one of the densest microbial communities in the biosphere, with the colon being the most hospitable due to its neutral pH, longer retention time, and greater volume [**3, 35**]. Since many dietary nutrients are absorbed by the host in the small intestine, it is believed that only a limited number of essential nutrients for bacterial growth reach the colon [**3, 36, 37**]. One nutrient that plays an important role in the colonization of the gut is nitrogen. It is hypothesized that nitrogen is one of the limiting factors for the growth of the microbiota in the colon, as it is a limiting reagent for the growth of many other organisms in other environments

[**3, 38**]. Through studies in germ free (GF) mice, it was observed that the microbiota can increase host protein requirements by consuming dietary nitrogen, in addition to utilizing host-derived urea through its hydrolyzation into ammonia, and is therefore thought to be a consumer of dietary nitrogen [**3, 21, 22, 39**]. The main sources of nitrogen in the gut are dietary protein and urea, with the latter being hydrolyzed into ammonia by microbial urease [**21, 22**]. The ammonia present in the colon can be absorbed by the host and added into the urea cycle in the liver, subsequently ending up in the intestinal lumen again [**40**]. Excess ammonia in the colon that is neither absorbed by the host nor utilized by the microbiota is excreted with the feces [**40**].
Besides nitrogen, an important nutrient to consider for the microbial colonization of the intestinal tract is carbon. It is one of the main building blocks of microbial life and is needed for anabolic processes in growth as well as for energy accumulation. Different carbon sources have been linked to a change in the microbiome, with carbon sources contained in the standard Western diet, mainly simple carbohydrates, being associated with dysbiosis and diets containing more dietary fiber in the form of microbiota-digestible carbohydrates favoring a diverse gut microbiome [**41, 42**].
Given the importance of dietary nutrients on the composition of the intestinal microbiome, understanding the effects variations in diet have on the microbiota becomes essential. Previous observations have been made that indicate changes in the microbiome composition following dietary changes [**40–42**]. *K. pneumoniae* as part of the family of *Enterobacteriaceae* in the phylum of Proteobacteria is often found in the intestinal microbiome of individuals with IBD [**43**]. Previously published work by our group, working with fecal samples obtained from the Food and Resulting Microbial Metabolites (FARMM) study [**44**], revealed *K. pneumoniae* to be a major contributor to microbiome reconstitution after antibiotic treatment [**45**]. Healthy volunteers, of whom 20 followed a typical Western diet (furthermore referred to as omnivore diet) and 10 a vegan diet. The omnivore group was divided into groups of 10 patients each, one group continuing the omnivore diet and 10 receiving a fiber-free exclusive enteral nutrition (EEN) diet. All three groups were subsequently given an antibiotic regime to diminish their intestinal microbiome. The vegan group continued their previous vegan diet. When looking at the composition of the EEN diet, it is apparent that it contains a majority of simple carbohydrates and no dietary fiber, as opposed to the omnivore and especially vegan diet, which are made up of fiber from complex carbohydrates. The samples taken from the group revealed to be colonized by a different make-up of bacteria before and after reconstitution of the microbiome, as well as depending on the diet they received. In the group receiving a diet consisting mainly of simple carbohydrates (EEN diet), Proteobacteria was found in a greater quantity after recovery of the microbiome than other groups usually found in a healthy gut microbiome, such as Bacteroidetes or Firmicutes (**Figure 1.2**). An outgrowth of Proteobacteria is associated with dysbiosis leading to an increase of Western diseases such as IBD [**6, 43, 44, 46, 47**].

1.4.1 NtrC is a Critical Activator of Nitrogen Scavenging in Nitrogen Limited Environments

As nitrogen is a scarce but integral nutrient for bacterial growth in many environments, many species are able to metabolize a variety of different compounds as a source of nitrogen [**3, 38**]. If available, ammonia is often the preferred nitrogen source of bacteria, including *K. pneumoniae* [**48**]. In an ammonia-abundant environment, ammonia is converted into glutamine, acting as the primary nitrogen donor for other cellular processes [**48**]. In a nitrogen-depleted environment, low cellular glutamine levels activate the nitrogen regulation (Ntr) stress response and allow *K. pneumoniae*

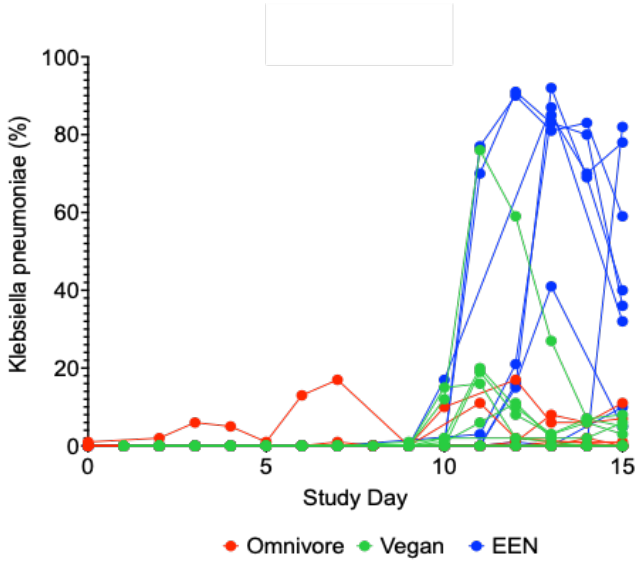

Figure 1.2: Reconstitution of *K. pneumoniae* in the microbiome (in percentage) of patients from the FARMM study [**44**] after antibiotic treatment for three days (day 6 - 8), following either a vegan, omnivore, or EEN diet.

to utilize alternative nitrogen sources [**48**]. The Ntr system consists of two components, namely NtrC and NtrB. This system is activated by the phosphorylation of the transcriptional activator NtrC by NtrB [**48**]. Two phosphorylated NtrC-P dimers interact, which changes the conformation of NtrC-P into its active form [**49**]. The activated NtrC-P dimers bind to two enhancer sites (-155 and -135 in relation to the transcription-start-side), which activates the $\sigma^5$4-dependent RNA polymerase and leads to the transcription of the nitrogen assimilation protein (*nac*)gene [**50**] and the subsequent production of NAC [**48**]. The NAC protein then regulates many different genes with a function in the nitrogen metabolism and assimilation and is responsible for the utilization of nearly all alternative nitrogen sources [**48**]. The produced NAC not only affects the nitrogen metabolism in case of nitrogen limitation but additionally plays a role in the metabolism of carbohydrates as well as in the synthesis of macromolecules and cell division [**48**]. Given that NtrC and subsequently NAC are necessary for the effective utilization of alternative nitrogen sources in nitrogen-limited environments, we hypothesized that the deletion of NtrC in *K. pneumoniae* would lead to insufficient nitrogen accessibility in the case that ammonia is not available and thus limited growth.

1.4.2 Amino Acid Metabolism of *K. pneumoniae*

Glutamine, which is utilized as a nitrogen donor, is only one amino acid neccessary for microbial growth [**48**]. Amino acids are an important part of the bacterial metabolism as they are the building blocks for protein synthesis and the products of protein degradation. The resulting amino acids can be used as a source of carbon as well as nitrogen by further degradation [**51**]. One mechanism for the degradation of amino acids is deamination, where amino groups are split from the amino acid by a deaminase enzyme, resulting in the production of ammonia, which can subsequently be used for nitrogen (**Figure 1.3**).
E. coli and *K. pneumoniae* are able to metabolize amino acids as a source of both carbon and nitrogen [**51**]. While *E. coli* and *K. pneumoniae* share many common metabolic pathways, there are some distinctions between their metabolism of amino acids. To give an example, *K. pneumoniae*

uses L-tryptophan as a sole nitrogen source but fails to use it as a sole carbon source. *E. coli*, however, can use L-tryptophan as a source of carbon but is not able to metabolize it to function as a sole nitrogen source [**52**]. Anaerobic conditions do not exclusively inhibit deamination. In some cases, for example, L-alanine and glycine, deaminase production is decreased, while the presence of L-glutamate under anaerobic conditions increases the formation of deaminase [**53**]. However, there remains a large knowledge gap in the metabolism of amino acids as nitrogen and carbon sources of *K. pneumoniae* under mixed amino acid conditions and in an anaerobic environment, which we will investiagte in this study.

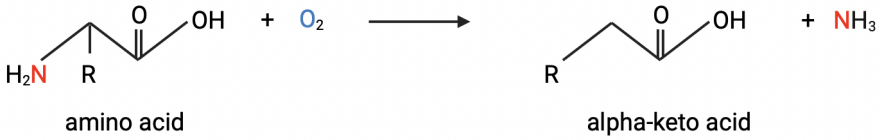

Figure 1.3: Deamination reaction of amino acids

1.5 The Oxygen Availability in the Intestinal Environment Decreases Along its Radial and Longitudinal Axis

Availability of nutrients is not the only factor impacting the colonization of the gut with varying bacterial species. The healthy resident microbiome of the colon, as the largest bacterial habitat of the intestinal tract, is mainly made up of obligate anaerobes like Bacteroidetes, as well as Firmicutes such as *Clostridia spp.* [**4, 54**]. Many different bacterial species, for example, *Enterobacteriaceae*, can grow and survive under both aerobic and anaerobic conditions as facultative anaerobes, due to the branched respiratory chain, equipped to generate energy under different aerogenic conditions by using varying electron acceptors [**55, 56**]. Under aerobic conditions, the terminal electron acceptor is oxygen, supplied with electrons from the respiratory chain via oxidoreductases, while under anaerobic conditions different electron acceptors come into play, such as nitrate, nitrite, dimethyl sulfoxide, trimethylamine N-oxide, and fumarate, with their corresponding reductases [**55**].
In the case of inflammation, this gains the bacteria a fitness advantage in the case of the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) by the host [**54**]. These are employed as a defense mechanism but can be used by *Enterobacteriaceae* as additional electron acceptors, the most potent one being nitrate, favoring the facultative pathogenic bacteria over the healthy microbiota [**54**].
The oxygen concentration decreases following the radial axis from closest to the submucosa leading into the lumen of the gut [**57**]. This implies that the environmental oxygen availability can differ in the same intestinal segment depending on the distance into the lumen. The oxygen concentration changes similarly on a longitudinal axis from the proximal small intestine (PSI), with its relatively greater abundance of oxygen, to the colon, which is virtually anaerobic [**58**] (**Figure 1.4**). This leads to differences in the oxygen environment between the different compartments of the intestinal tract. This is mirrored in the distribution of the resident microbiota. The PSI exhibits larger numbers of facultative anaerobes, with *K. pneumoniae* and *E. coli* as Proteobacteria being part of this group, while the distal small intestine (DSI) and colon are mostly colonized by obligate

anaerobes [**2, 58**]. This distinction can additionally be seen on the radial axis, as greater numbers of Proteobacteria are colonizing the DSI and colon closer towards the edge of the lumen at the epithelium, where the oxygen content is higher [**58**].
Originally, this phenomenon of decreasing oxygen availability along the course of the gut was thought to be due to the oxygen consumption by the microbiota along the longitudinal axis of the gut [**2, 58**]. However, previous studies from our lab found it to be present in GF mice as well, showing that the effect of the microbiome on the oxygen content in the intestinal lumen is marginal and rather mainly governed by the epithelium of the intestinal tract itself [**58**]. It was observed that one main aspect coming into play is the surface area of the epithelium, with the greater surface area in the PSI due to increasing numbers of villi, resulting in a higher rate of oxygen diffusion from the epithelium into the gut lumen [**58**].
Given the dependence of the microbiome composition on the distribution of oxygen, it is not surprising that changes in oxygen availability influence changes in the microbiota and *vice versa*. This can be seen in dysbiosis and the associated outgrowth of facultative anaerobes like Proteobacteria. Many conditions that are associated with dysbiosis subsequently cause an increase in available oxygen in the colon, for example, Western diets low in fiber but high in simple sugars and fat, antibiotic treatment or IBD and cancer, and therefore lead to an increase of facultative anaerobes in the colon [**2**]. One example of this phenomenon previously studied is the production of ROS during inflammation in *Salmonella* infection, leading to an outgrowth of the pathogen and inhibition of the resident microbiota, due to *Salmonella* being able to access additional electron acceptors via a reaction with ROS [**59**].

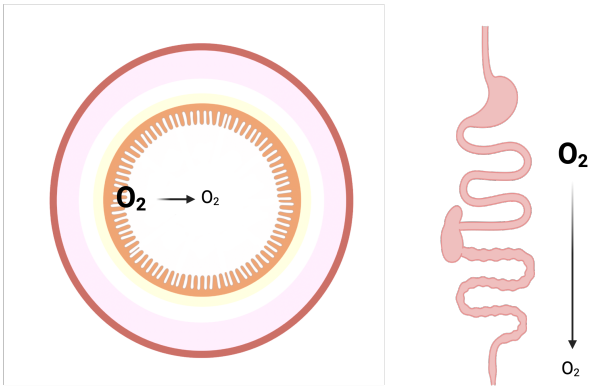

Figure 1.4: Distribution of oxygen in the intestinal tract on the radial and longitudinal axis. The oxygen concentration decreases from the submucosal layers to the lumen and from the proximal to the distal segments.

1.6 Dissemination and Intestinal Inflammation

One major complication of disruptions of the homeostasis of the gut microbiome is dissemination from the intestinal microbiota into the systemic circulation, which is a possible cause of sepsis and systemic infections like urinary tract infections or cholangitis, especially in immunosuppressed and otherwise susceptible patients [**9, 60**]. Under normal circumstances, the gut exhibits a barrier function keeping its microbiome from crossing over into the systemic circulation. This so-called gut-vascular barrier (GVB) is composed similarly to the blood-brain barrier (BBB) and is, likewise, prone to infection-induced changes in its permeability [**60**]. A second critical host defense

is the mucus layer, which protects the epithelial cells in the intestinal tract from the gut flora. It is divided into an inner layer, close to the epithelium and made up of tightly packed mucins that allow for a low density of bacteria and an outer layer, towards the lumen, that hosts a higher density of bacteria. This effectively protects the epithelial cells from the gut microbiota by interposing the highly colonized lumen with the mucus layer low in bacterial colonization [**2, 61**]. However, if the mucus-inhabiting bacteria are devoid of effective nutrition such as dietary fiber, some species, specifically gut commensal *Bacteroidetes*, can turn to metabolize the mucins making up the intestinal mucus layer. Destruction of this mucus layer can in turn increase susceptibility to systemic dissemination of the microbiome [**2, 62**]. Pathogenic bacteria are known to cross over the GVB even in healthy individuals, one example being hypervirulent *K. pneumoniae* [**9, 13, 60**]. However, in the context of inflammation, opportunistic pathogenic bacteria are able to disseminate into the systemic circulation and seed in downstream organs, such as the liver or spleen. Therefore, diet influences the susceptibility of patients for the dissemination of bacteria in dysbiosis.
In addition to diet, another critical factor contributing to dysbiosis is antibiotic treatment. We previously discussed that the host reaction to inflammation favors the outgrowth of *Enterobacteriaceae*, due to their ability to utilize additional electron acceptors produced in the presence of ROS and RNS [**54**]. This phenomenon was not only observed in severe inflammation during colitis or infections but additionally during antibiotic treatment, which can result in a fitness advantage of *Enterobacteriaceae* over the healthy microbiome of *Bacteroidetes* and *Firmicutes* [**54**].

1.7 Systemic Interactions of the Intestinal Microbiome

1.7.1 The Gut-Brain Axis

Dissemination is not the only systemic complication of a disrupted gut homestasis. The connection between the microbiome in the intestinal tract and the host nervous system has long been established [**63, 64**]. Various modes of communication have previously been explored, such as the gut interacting with the CNS via the immune system, metabolites like SCFAs or branched-chain amino acids, the vagus nerve and the enteric nervous system, in addition to other mediators like hormones, neurotransmitters, and neuropeptides [**63, 65**]. This interaction allows the nervous system to influence the microbiome [**65, 66**]. The gut-brain axis (GBA) is associated with multiple disorders including anxiety, obesity, cardiovascular disease, neuropsychiatric disorders, and degenerative diseases such as Alzheimer's disease or Parkinson's disease [**64**]. One mode of action is the disruption of the GVB in the case of dysbiosis, as mentioned previously. Dissemination can lead to systemic inflammation and the release of inflammatory cytokines, subsequently leading to a disruption of the BBB with subsequent neuroinflammation [**60, 64, 66**].

1.7.2 Hepatic Encephalopathy as a Complication of Liver Cirrhosis

Liver cirrhosis, the end stage of chronic liver disease, is most often the result of chronic and excessive alcohol consumption or hepatitis C [**67, 68**]. Recently, with the rise of excessive food consumption and a predomination of the Western diet leading to obesity, the prevalence of non-alcoholic fatty liver disease (also known as metabolic-dysfunction associated steatotic liver disease (MASLD)) has increased [**67, 69**]. In addition, there are a few etiologies found infrequently, like hepatitis B or D, as they are treatable in developed nations, or autoimmune diseases, like primary biliary cirrhosis and autoimmune hepatitis [**67, 68, 70, 71**]. The defining features of cirrhosis include fibrosis of the

liver, changes in the lobular structure and nodule formation, as well as vascular remodeling, leading to shunts between the portal and systemic circulation, bypassing intrahepatic metabolization of metabolites that were absorbed in the gut [**67, 72**].
As a neuropsychiatric complication of cirrhosis, the symptoms of Hepatic Encephalopathy (HE) range from changes in personality and sleep habits to some dementia-like symptoms, such as a shortened attention span and memory deficits, as well as muscular symptoms like asterixis, before the disease finally compounds in stupor and coma [**73**]. HE develops in around 30 - 45% of cirrhosis patients and is usually accompanied by a poor prognosis, as it is difficult to treat the disease definitely without liver transplantation and is accompanied by an approximate mortality rate of 60% in the first year after diagnosis [**72, 74**]. Several factors interlock, ultimately resulting in the development of HE. On one hand, an inflammation-favoring environment in combination with dysbiosis leads to an increasing release of proinflammatory cytokines (interleukin-1 and -6, as well as tumor necrosis factor). On the other hand, hyperammonemia, which in conjunction with the aforementioned, can result in cerebral edema [**73, 75**].

1.7.2.1 *K. pneumoniae* and Ammonia in the Context of Cirrhosis

Hyperammonemia, in particular, is thought to play a substantial part in the development of HE [**76**]. In a healthy liver, ammonia is metabolized into urea and subsequently excreted by the kidneys. In cirrhosis, however, the decreased liver function is unable the metabolize all accruing ammonia, leading to its accumulation [**75,77**]. Blood ammonia levels are attributable to **i)** production in the human body, part of it through amino acid transamination and the purine-nucleotide cycle by the skeletal muscle, and **ii)** production by the microbiome in the colon and small intestine via glutamate degradation or protein and urea metabolism [**73, 78**]. If the ammonia concentration in the blood increases considerably, it can lead to the development of hyperammonemia, a condition that can present with neurological symptoms due to the innate neurotoxic character of ammonia [**78**]. Additionally, if hyperammonemia is not diagnosed and treated in time, it can lead to serious complications like cerebral edema and, subsequently, brain herniation [**78, 79**]. Multiple pathomechanisms of the neurological damage caused by hyperammonemia have been proposed but the exact reason is not yet established. One hypothesis is that ammonia can disrupt the neurotransmitter system of the CNS by increasing extracellular glutamate, which activates NMDA receptors that subsequently activate the Na/K-ATPase through decreased phosphorylation of protein kinase C [**77**–**79**]. This leads to ATP depletion, which may be causative for the neurotoxicity of ammonia that results in seizures in acute hyperammonemia [**78**]. In turn, the increased extracellular glutamate concentration leads to the downregulation of glutamate receptors in chronic hyperammonemia and the disrupted glutamate-nitric oxide-cGMP pathway leads to impaired transmission in the NMDA receptors and, therefore, cognitive dysfunction [**77, 78**]. Another hypothesis to explain the cerebral edema found in hyperammonemia is the swelling of astrocytes, the only cells able to detoxify ammonia in the brain [**73**]. However, cerebral edema is not a direct cause of increased ammonia levels but rather a result of the increased metabolism of ammonia in astrocytes. In the cell, it is metabolized by glutamate-synthetase, resulting in the production of glutamine, which is osmotically active and leads to intracellular swelling due to its changes in the osmotic gradient [**75**]. Following this are changes in water and K^+ metabolism, activation of tumor suppressor protein p53, increased uptake of compounds like pyruvate, lactate, and glutamine, and decreased uptake of ketone bodies, glutamate, and free glucose [**78**].
In addition to liver cirrhosis, reasons for hyperammonemia are manifold. One example is metabolic

abnormalities that lead to inadequate metabolization of ammonia in the liver, such as disorders that lead to redirection of the portal blood to the systemic circulation, bypassing the liver, or increased production by the gastrointestinal microbiota, which can be observed in the treatment of portal hypertension via transjugular intrahepatic portosystemic stent shunt (TIPS) [**73, 78**]. The presenting symptoms, irritability, headaches, vomiting, ataxia, and gait abnormalities, and in severe cases, seizures, encephalopathy, coma, and death are not always a clear indicator of hyperammonemia. Therefore, other possible etiologies like sepsis, meningitis, or other neurological diseases need to be ruled out for diagnosis [**78**].
Liver cirrhosis was found to be associated with an altered microbiome and an increase of *Enterobacteriaceae*, such as *K. pneumoniae*, especially in patients who had developed HE [**80**].

1.7.2.2 Lactulose, a Treatment Option for Hepatic Encephalopathy, Reduces Intestinal Ammonia Production

Lactulose has been a popular treatment option for HE for several years, as it is widely available and has little to no serious side effects [**32, 81, 82**]. It is a non-absorbable synthetic disaccharide, made up of galactose and fructose. As no specific enzyme, present in the human small intestine, is able to hydrolyze the disaccharide, it arrives in the colon unchanged, where it is fermented by the microbiome, leading to the production of the SCFAs acetate and lactate, as well as H_2 and CO_2 [**32, 83**].
Lactulose is hypothesized to affect the intestinal ammonia production in multiple ways, **i)** the acidic pH may inhibit urease-producing bacteria that can produce ammonia, **ii)** lactulose is thought to promote bacterial growth and, therefore, uptake of ammonia by bacteria can be used for protein synthesis, **iii)** its metabolism can have a laxative effect and reduces colonic retention time, **iv)** the microbiome-metabolized disaccharide may inhibit the uptake of glutamine by the intestinal mucosa and therefore its metabolization into ammonia and, lastly, **v)** acidification of the gut lumen can convert ammonia to ammonium leading to decreased intestinal absorption [**32, 79, 83**]. The exact mechanism by which lactulose interacts with the microbiome and reduces the intestinal ammonia concentration is still under investigation to this date [**84, 85**].

1.8 Objectives

This thesis aims to explore the nitrogen and carbon metabolism of *K. pneumoniae* in regard to clinical diseases such as IBD, and HE. The specific details we will focus on in this study are, **i)** investigate the role of the virulence factor urease in *K. pneumoniae* in different nitrogen environments and its impact on gut colonization, dysbiosis, and IBD, **ii)** assess the utilization of a range of different nitrogen sources in *K. pneumoniae*, the constraint that deficiencies in the bacterial systems used to access additional nitrogen sources produce, and how the colonization with *K. pneumoniae vice versa* impacts the nitrogen composition of the gut, **iii)** explore the preferences in carbon sources of *K. pneumoniae* and how changes in the available carbon sources from the host's diet influence colonization with *K. pneumoniae*, dysbiosis, and resulting diseases such as IBD, **iv)** determine the interactions between the intestinal colonization and ammonia production, hyperammonemia, the development of HE, and lactulose to further the understanding of this treatment choice for HE. Overall, our objectives are to deepen the understanding of the metabolism of *K. pneumoniae* and its role in gut-related disease, possibly leading to new insights that could be the basis for new therapeutic strategies.

2. Materials and Methods

2.1 *In Vitro* Studies

2.1.1 General Growth Conditions, Bacteria, Antibiotics, Defined Media, Quantification

Bacterial strains. Strains used in this study were *Klebsiella pneumoniae* MGH 78578 and *Escherichia coli* MP1, as specified (Table 2.2).

Bacterial cultures. Cultures were grown at 37°C (unless otherwise specified) with or without aeration in Lysogeny Broth (LB) Miller medium, low-salt LB or minimal M9 media. LB consisted of 10 g of tryptone, 5 g of yeast, and 10 g of NaCl (5 g of NaCl for low-salt conditions) in 1 liter autoclaved water, adjusted to a pH of 7-7.4. Minimal M9 contained 6.8 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 100 μM $CaCl_2$, and 2 mM $MgSO_4$ per liter with 1 g NH_4Cl and 2 g dextrose added according to protocol. For the growth of *E. coli* strains, 100 μM of Iron-(III)-sulfate were added. Stock solutions of $CaCl_2$, $MgSO_4$, $Fe_2(SO_4)^3$, NH_4Cl and dextrose were filter-sterilized through syringe driven 0.20 μm membranes and subsequently added individually to autoclaved solutions containing Na_2HPO_4, KH_2PO_4 and NaCl.

Defined minimal media. Minimal M9 media with added ammonium chloride (hence forth referred to as "ammonia") (0 mM, 1.8 mM or 18 mM) was used as defined minimal media and supplemented with urea (0 mM, 7.5 mM or 33.3 mM) according to protocol.

Amino acid model. The MOPS EZ rich defined media kit M2105 (SKU: LM0194; Teknova) was used as media by adding our own amino acid solution. The final media contained: 40 mM MOPS, 4 mM tricene, 0.01 mM iron sulfate, 9.5 mM ammonium chloride, 0.276 mM potassium sulfate, 0.5 μM calcium chloride, 0.525 mM magnesium chloride, 50 mM sodium chloride, 2.92×10^{-7} mM ammonium molybdate, 4.00×10^{-5} mM boric acid, 3.02×10^{-6} mM cobalt chloride, 9.62×10^{-7} mM cupric sulfate, 8.08×10^{-6} mM manganese chloride, 9.74×10^{-7} mM zinc sulfate, 1.32 mM potassium phosphate dibasic (K_2HPO_4) and each of the 20 proteinogenic amino acids (glycine, L-alanine, L-arginine, L-aspartate, L-aspartic acid, L-cysteine, L-glutamine, L-glutamic acid, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine) in an equal molar concentration of 1 mM. Glucose (0 mM, 10 mM, 100 mM) and lactulose (0 mM, 100 mM) were added according to protocol. Stock solutions of each of the amino acids, lactulose and dextrose were filter-sterilized through syringe driven 0.45μm PTFE filter membranes and subsequently added individually to the purchased MOPS media. Fresh media was prepared the previous day and left overnight on the bench at room temperature or in the anaerobic chamber at 37°C. *K. pneumoniae* was added in a 1:100 dilution from an overnight culture grown aerobically in LB at 37°C. About 11 mL of media were incubated at 37°C, either aerobic or anaerobic, and 1.6 mL were taken at specific time points (start = 0 hours, 2 hours, 3.5 hours, 5 hours, overnight = 23 - 24 hours), when OD_{600} was measured. Additionally, we froze samples at -80°C to test urea and ammonia concentrations after all time points were reached and colony forming units (CFU) was calculated by serial dilution on LB-ampicillin plates. The samples taken at 5 and 24 hours were diluted 1:10 for the aerobic and 1:2 for the anaerobic set-up before measuring OD_{600}, to allow the manual photometry apparatus to measure the very high densities. This was subsequently factored into the calculation to reflect the OD_{600} without dilution.

Antibiotics. Ampicillin (100 μg/mL), apramycin (100 μg/mL) and hygromycin (50 μg/mL) were

used in our experiments, the latter exclusively in conjunction with low-salt LB.

Overnight cultures. *K. pneumoniae* overnight cultures were grown in M9 minimal media with 18 mM of ammonia at 37°C from a single colony, picked from an LB-ampicillin plate. *E. coli* was grown with the addition of 100 μM iron as an essential element. Bacteria was washed in a 1:10 dilution in minimal media containing no ammonia and diluted into different media conditions, as specified for the experiment, in a 1:10 ratio. The dilution was spun down at 8,000 * g for 3 minutes at room temperature and resuspended in 1 mL of fresh media. The resuspended culture was diluted 1:100, resulting in a total dilution of 1:1000 and incubated at 37°C.

Growth curves. Manual growth curves were produced via inoculation from bacterial cultures in autoclaved glass bacterial culture tubes on a roller drum at 200 rpm. Machine growth curves were produced by using a 96-well-plate in a BioTek Epoch 2 plate reader, measuring the OD_{600} every 15 minutes for a 20- to 24-hour period under continuous shaking at 37°C. Anaerobic growth curves were created using the same model of plate reader and settings inside of an anaerobic chamber. Overnight cultures for inoculation of anaerobic growth curves were grown in an anaerobic chamber. The media used was incubated overnight in the anaerobic chamber.

Quantitative measurement of bacterial growth. Optical density at 600 nm (OD_{600}), as well as colony forming units per defined quantity (CFU/mL or CFU/g) were used to quantify growth. Both cuvettes, to manually monitor growth over a defined period of time, and 96-well-microplates, to measure the growth continuously over a 24-hour period, were utilized to assess the OD_{600}. CFU were calculated by plating a serial dilution on LB-ampicillin plates to isolate *K. pneumoniae*, as well as no antibiotics for the quantification of *E. coli*. Plates were incubated overnight at 37°C.

2.1.2 Survival Studies of *K. pneumoniae* and *E. coli*

Acidic shock of *K. pneumoniae* and *E. coli*. Bacterial cultures were grown at 37°C overnight in LB (pH 7), diluted 1:100 into fresh LB, either pH 5 or 7, and grown until late exponential phase (OD_{600} of 0.7 - 0.9). 25 μL of the subcultures were transferred into 1 mL of pre-warmed LB (pH 2.5) and incubated at 37°C, standing. Another 25 μL were transferred into 1 mL of phosphate-buffered saline (PBS) and a serial dilution was plated on an LB plate as a control. After incubating for one hour, the cultures shocked in acidic LB were serially diluted as well and plated on LB. Both plates were incubated overnight at 37°C and CFU/mL was calculated the following day.

Growth of *K. pneumoniae* in high SCFA concentrations. An experimental set-up, modeled after experiments previously conducted by our group using *E. coli*, was created [**86**]. LB media was corrected to pH 6 or 5 and autoclaved. Overnight cultures of *K. pneumoniae* were normalized to an OD_{600} of 3 and diluted 1:1000 into media containing 0 mM or 100 mM of acetate, with or without the addition of 10 mM urea. The cultures were grown in 96-well-plates and continuously monitored in a plate reader for 24 hours. The experiment was repeated in the anaerobic chamber. The media was placed in the chamber overnight to remove oxygen prior to conducting the experiment. The media was acidified using HCl and autoclaved afterwards. The acetate stock was filter sterilized.

2.2 Strain Construction

Recombineering. Previously published protocols were utilized [**87**]. Both the urease operon and the ntrC gene were excised from the *K. pneumoniae* WT genome using apramycin-resistance cassettes flanked by FLP recombination target (FRT) sites. First, λ-red system expressing cells were constructed by electroporation of the pACBSR-hyg plasmid into electrocompetent WT MGH 78578

cells. The FRT/apramycin cassette was PCR-amplified from a pMDIAI plasmid and integrated into MGH 78578-pACBSR-hyg, correct insertion was confirmed by PCR. Loss of the pACBSR-hyg plasmid was achieved via serial passage on LB-apramycin plates until loss of hygromycin-resistance was detected. To construct the final strains, pFLP-hyg plasmids were electroporated into competent *K. pneumoniae*-FRT/apramycin cells and heat-treated overnight at 43°C to induce the FLP recombinase. Colonies were screened for loss of apramycin-resistance and the pFLP-hygromycin plasmid was cured via serial passage. Correct deletion was subsequently confirmed via oligonucleotide sequencing. Electrocompetent cells were again prepared as previously described [**87**].

Resistance marking. Strains were constructed to distinguish different *K. pneumoniae* strains in competition. The pACBSR-hygromycin or the pMDIAI-apramycin (**Table 2.7**) plasmids containing antibiotic resistance against hygromycin or apramycin were integrated into the bacteria using electrocompetent cells. Correct insertion was subsequently confirmed via polymerase chain reaction (PCR).

Competition of resistance-marked *K. pneumoniae* strains was constructed *in vitro* by inoculating multiple overnight cultures into defined minimal M9 media, with or without the addition of urea, or *in vivo* by inoculating mice with multiple overnight cultures. Self-competition between the apramycin- and hygromycin-marked strains was tested to exclude differences produced by the resistance-marker and quantified by CFU.

2.3 *In Vivo* Studies

2.3.1 Animal Studies

All animal experiments were conducted in accordance to protocols approved by the Institutional Animal Care and Use Committee and Biosafety Committees of the University of Pennsylvania. Female specific pathogen-free (SPF) C57BL/6 J mice, 8 weeks of age, with a native microbiota from the Jackson laboratory were used for all manipulative experiments. Mice were kept in a conventional laboratory animal facility at the University of Pennsylvania (housed under standard lighting cycle conditions (12 hours on/12 hours off) and provided with acidified water). Sample size for experiments was estimated based on prior animal modeling studies utilized within the laboratory and no animals were excluded from analysis. As the human data used in this study combined both male and female participants, without apparent significant differences in the groups, sex was not expected to be a confounding variable. Murine studies were therefore conducted solely on female mice. Investigators were not blinded for these studies.

Colonization of conventionally raised mice with *K. pneumoniae*. conventionally raised (CR) mice were pre-treated for 72 hours with vancomycin (2.5 g/L), neomycin (5 g/L) and aspartame (25 g/L) in their drinking water, given *ad libitum*, with the addition of polyethylene glycol (PEG) 10 % (w/v) for the last 12 hours [**6**]. Mice were switched back to regular drinking water on the day of orogastric gavage with $1 * 10^8$ CFU of *K. pneumoniae* in 100 µL of PBS, resuspended from an aerobically grown overnight culture using PBS instead of minimal media [**88**].

Germ free colonization model. Female GF C57BL/6 mice about 8 weeks of age were used for all manipulative experiments. Mice were breed in-house and kept in a GF laboratory at the Gnotobiotic Facility of the University of Pennsylvania Veterinary School.

Fecal pellets were collected and tested for CFU to ensure no native intestinal microbiota was present prior to the start of the experiment. GF mice underwent orogastric gavage with one of the *K. pneumoniae* strains and were kept in isolators as to not cross-contaminate the different

strains. One group (n = 9) received the wild type (WT) strain and the other (n = 9) the ΔUrease stain. Engraftment was tested via CFU from fecal pellets the following day. One week after gastric gavage, half of each group (n = 4, for each the WT and the ΔUrease strain) was switched to water containing 3% lactulose given *ad libitum*, the control group continued receiving standard autoclaved water. One week later (day 14 after gavage), mice were euthanized and samples for further testing of metabolomics and CFU were collected.

IBD mouse model. IBD in mice was modelled with the use of dextran sodium sulfate (DSS) [**89, 90**]. Mice received drinking water with 5% of DSS and were subsequently monitored for preestablished criteria (**Table 2.9**). Euthanization was performed at time points indicated in the results or if moribund was in accord with established criteria by the Animal Use Protocol.

Sample collection and preparation. Fresh fecal pellets were collected, weighed and resuspended in 1.5 mL of PBS. Serial dilutions were plated on LB agar, containing ampicillin, apramycin, or hygromycin, as indicated in results. Plates were inoculated overnight at 37°C, after which CFU/mg of stool was calculated. Fecal samples collected for metabolomics analysis were flash frozen on dry ice and stored at -80°C.

Mice were euthanized at time points indicated in the results or if moribund was in accord with established criteria by the Animal Use Protocol. Euthanization was performed in compliance with the Animal Use Protocol. Fecal pellets and blood samples (jugular vein puncture) were collected before euthanization. After confirmed death, mice were dissected and tissue of interest was collected. The spleen and left liver lobe were placed in 1.5 mL of PBS to be homogenized for 10 minutes and centrifuged at 10,000 x g for 10 minutes twice. Intestinal samples were collected by dividing the gut into sections of colon, PSI and DSI and luminal mucous contents were collected from 10 cm of PSI as well as DSI and colon. Length of the colon was measured at the time of euthanasia. All samples were serially diluted and spot plated for CFU on LB with kanamycin and vancomycin. CFU were calculated per weight or per organ (liver, spleen, bile), as indicated.

2.3.2 Analysis of Human Colonization Data

Stocked fecal samples from the previously reported human study called Food and Resulting Microbial Metabolites (FARMM) were tested. All research subjects had given written informed consent. All protocols were approved by the University of Pennsylvania Institutional Review Board (IRB), which considered it exempt from clinical trial registration requirement [**44**]. Healthy adult volunteers were at least 18 years of age. Both female and male volunteers were included in the study. In total, the study consisted of 30 healthy human subjects, divided into three cohorts. 10 practiced a vegan diet at baseline and the remaining 20 ate a standard Western (omnivore) diet. Vegan subjects maintained an outpatient vegan diet during the course of the study. The omnivore group was randomized to follow a standardized omnivore diet or exclusive enteral nutrition (EEN) diet, consisting of Modulen® IBD, after completion of the antibiotic regimen. The omnivore standardized diet was designed to have similar composition as the EEN diet, except for the lack of dietary fiber. All subjects used in the present study were treated with a bowel-cleansing protocol consisting of vancomycin (500 mg, every 6 hours) and neomycin (1,000 mg, every 6 hours) daily for 3 days with a PEG-purge (GoLytely®) (4 liters) on day 2. Afterwards, the two omnivore cohorts stayed inpatient on the ward, while the patients following a vegan diet continued their vegan diet on their own accords (**Figure 2.1**).

Fecal samples were collected before, during and after the antibiotic clearing and reconstitution of the gut microbiome and subsequently tested for ammonia and urea, as well as the percentage of

K. pneumoniae in the intestinal microbiome.

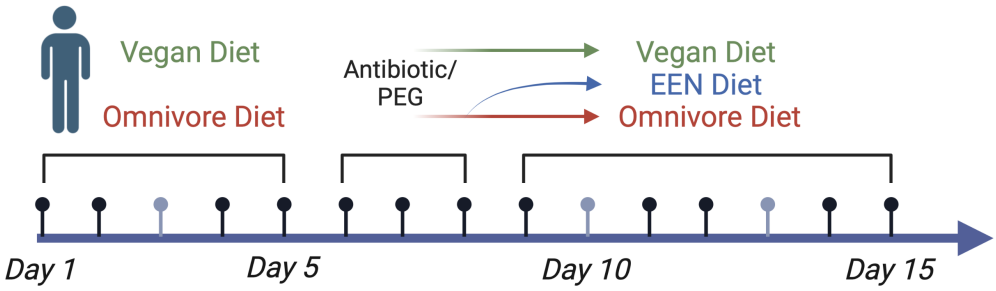

Figure 2.1: Experimental set-up of the FARMM study (created with BioRender.com)

2.4 Quantification and Statistical Analysis

Nitrogen and carbon source analysis. Biolog Phenotype microarray 96-well plates were used to test utilization of different compounds as a sole nitrogen or carbon source. Overnight cultures of *K. pneumoniae* in M9 minimal media with ammonia (carbon testing) or glucose (nitrogen testing), diluted 1:10 and washed with M9 minimal media without ammonia (nitrogen testing) or glucose (carbon testing) to eliminate any residual nitrogen or carbon sources left from the overnight growth medium were used. The washed bacteria was diluted 1:100 once more afterwards. 100 µL of the suspension were pipetted into each well of the 96-well plate and incubated in a BioTek Epoch2 plate reader at 37°C for 20 hours while being shaken continuously. Biolog Phenotype microarray 96-well plates PM1 (**Supp. Figure A.1**), PM2A (**Supp. Figure A.2**) were used for carbon testing, PM3B (**Supp. Figure A.3**) for nitrogen testing. For anaerobic conditions, the media was prepared the night before and incubated anaerobically, in conjunction with the overnight culture of *K. pneumoniae*, after which proceedings were as described above. Background absorbance was subtracted and resulting area under the curve (AUC) was calculated using Prism software Version 9.5.1.

Quantitative Assays. Culture samples were prepared by centrifuging and then dividing the supernatant from the pellet into different microcentrifuge tubes. Metabolites were quantified in fecal samples obtained from mice or human patients (FARMM study). Samples were homogenized with 10 µL of sterile water per mg of fecal material and spun down to remove solid parts. All samples were stored at -80°C. **Ammonia** was quantified using the SIGMA-ALDRICH® Ammonia Assay Kit (MAK310-1KT). **Glucose** was quantified using the Abnova Glucose Assay Kit (KA1648) Kit. **Urea** was quantified using the QuantiChrom™ Urea assay Kit (76237-110 (EA)). The protocol was modified for samples with a suspected low concentration of urea. 25 μL of the sample were mixed with 100 μL of the working reagent and incubated at room temperature for 50 minutes. The optical density (OD) was read at 520 nm and the concentration calculated by using the equation for low urea samples provided in the kit's protocol. Fecal samples were homogenized in 10 μL of ddH_2O per mg of stool. The resuspension was centrifugation at 2500 x g for 10 minutes to remove solid particles. The supernatant was for urea quantification as describes above. **Urease** was quantified using the SIGMA-ALDRICH® Urease Activity Assay Kit (St Louis, MI).

Amino acids were quantified using a Waters Acquity uPLC System with an AccQ-Tag Ultra C18 1.7 μm 2.1 × 100 mm column and a Photodiode Detector Array as previously published [**6**]. Fecal samples were homogenized in methanol (5 μL/mg stool) and centrifuged for 5 minutes at 13,000 *

g twice. Amino acids in the supernatant were derivatized using the Waters AccQ-Tag Ultra Amino Acid Derivatization Kit (Waters Corporation, Milford, MA) and analyzed using the UPLC AAA H-Class Application Kit (Waters Corporation, Milford, MA) following manufacturer's instructions. All chemicals and reagents used were mass spectrometry grade.

Metabolites were quantified utilizing ^1H proton nuclear magnetic resonance (NMR)-based metabolomics on fecal or cecal samples. Stool samples were weighted, approximately 50 mg were resuspended and extracted using phosphate-buffered solution containing 50% D_2O with 0.29 mM trimethylsilylpropanoic acid (TMSP) as an internal standard, following homogenization by vertexing. ^1H NMR spectra were acquired at 298 K on a Bruker Avance NEO 600-MHz spectrometer equipped with a SampleJet sample changer (Bruker Biospin; Billerica, MA). Metabolites were assigned based on known spectral peaks and confirmed with a series of 2D NMR spectra. The *noesygppr1d* pulse sequence was used for recording ^1H 1D NMR experiments with presaturation water suppression during relaxation and mixing time. All ^1H NMR spectra were processed automatically via Chenomx NMR Suite (Chenomx Inc., version 9.05; Edmonton, Canada). Each spectrum was checked and adjusted manually for phase and baseline. Metabolites were identified and spectral fit using an in-house metabolite library. Metabolite concentrations were calculated according to an internal standard for further statistical analysis.

General statistics. GraphPad Prism software (GraphPad, Inc.; version 9.5.1 and version 10.2.3; Boston, MA) was used to perform all statistical analyses. Two-way ANOVA with Šídák correction for multiple comparisons and a 95% confidence interval was used for comparison of growth on commercially available Biolog Phenotype microarray 96-well plates, as well as to compare colonization and dissemination of intestinal sections after euthanization of GF mice. One-way ANOVA with multiple comparisons using the Kruskal-Wallis test and a 95% confidence interval was used to compare ammonia concentrations in subjects from the FARMM study. Multiple unpaired, 2-tailed t-tests were used to compare stool urea in GF mice, with Bonferroni's multiple corrections to compare colonization with *K. pneumoniae* and stool ammonia. Mann-Whitney's U-tests with multiple comparison two-stage step-up method of Benjamini, Krieger and Yekultieli were used to compare concentrations of amino acids and other metabolites in GF mice. One-way Brown-Forsythe and Welch ANOVA test with Dunnett T3 correction for multiple comparisons and a 95% confidence interval was used to compare stool urea after euthanization of GF mice. Presentation of p values is noted in figure legends, p values less than 0.05 were considered statistically significant.

2.5 Protocols

Liquid Chromatography/Mass Spectrometry. Bile acids were quantified using a Waters Acquity uPLC System with a Cortecs UPLC C-18+ 1.6 μm 2.1 x 50 mm column and a QDa single quadrupole mass detector. Briefly, fecal samples were suspended in methanol (5 μL/mg stool), vortexed for 1 minute, and centrifuged twice at 13,000 * g for 5 minutes. Intestinal flushes were vortexed for 1 minute, and centrifuged twice at 13,000 * g for 5 minutes. The supernatant was transferred to a new tube, sealed, and stored at 4°C until analysis. The flow rate was 0.8 mL/min, the injection volume was 4 μL, the column temperature was 30°C, the sample temperature was 4°C, and the run time was 4 min per sample. Eluent A was 0.1% formic acid in water, eluent B was 0.1% formic acid in acetonitrile, the weak needle wash was 0.1% formic acid in water, the strong needle wash was 0.1% formic acid in acetonitrile, and the seal wash was 10% acetonitrile in water. The gradient was: initial flow 70% eluent A, linear gradient to 100% eluent B over 2.5 minutes, hold at 100% eluent B for 0.6 minutes, and linear gradient to 70% eluent A over 0.9 min-

utes. The mass detection channels were: +357.35 for chenodeoxycholic acid and deoxycholic acid; +359.25 for lithocholic acid; -407.5 for cholic, alphamuricholic, betamuricholic, gammamuricholic, and omegamuricholic acids; -432.5 for glycolithocholic acid; -448.5 for glycochenodeoxycholic and glycodeoxycholic acids; -464.5 for glycocholic acid; -482.5 for taurolithocholic acid; -498.5 for taurochenodeoxycholic and taurodeoxycholic acids; and -514.4 for taurocholic acid. Samples were quantified against standard curves of at least five points run in triplicate (chemicals obtained from Santa Cruz Biotechnology, Dallas, TX, and Steraloids Inc., Newport, RI). Standard curves were run at the beginning and end of each metabolomics run. Blanks and standards were run every eight samples. Amino acids were quantified using a Waters Acquity uPLC System with an AccQ-Tag Ultra C18 1.7 μm 2.1x100 mm column and a Photodiode Detector Array. Fecal samples were homogenized in methanol (5 μL/mg stool) and centrifuged twice at 13,000g for 5 minutes. Intestinal flushes were vortexed for 1 minute, and centrifuged twice at 13,000 * g for 5 minutes. Amino acids in the supernatant were derivatized using the Waters AccQ-Tag Ultra Amino Acid Derivatization Kit (Waters Corporation, Milford, MA) and analyzed using the UPLC AAA H-Class Application Kit (Waters Corporation, Milford, MA) according to manufacturer's instructions. All chemicals and reagents used were mass spectrometry grade. Short chain fatty acids were quantified using a Waters Acquity uPLC System with a HSS T3 1.8 μm 2.1x150 mm column and a Photodiode Detector Array. Fecal samples were homogenized in volatile free fatty acid mix (5 μL/mg stool, Supelco, Bellefonte, PA) and centrifuged twice (13,000 * g for 5 minutes). The supernatant was filtered using 1.2, 0.65, and 0.22 μm filter plates (Millipore, Billerica, MA) and the filtrate was loaded into a total recovery vial (Waters, Milford, MA). Intestinal flushes were vortexed for 1 minute, and centrifuged twice at 13,000 * g for 5 minutes. The flow rate was 0.25 mL/min, the injection volume was 5 μL, the column temperature was 40°C, the sample temperature was 4°C, and the run time was 25 minutes per sample. Eluent A was 100 mM sodium phosphate monobasic, pH 2.5, eluent B was methanol, the weak needle wash was 0.1% formic acid in water, the strong needle wash was 0.1% formic acid in acetonitrile, and the seal wash was 10% acetonitrile in water. The gradient was 100% eluent A for 5 min, gradient to 70% eluent B from 5-22 min, and then 100% eluent A for 3 min. The photodiode array was set to read absorbance at 215 nm with 4.8 nm resolution. Samples were quantified against standard curves of at least five points run in triplicate. Standard curves were run at the beginning and end of each metabolomics run. Blanks and standards were run every eight samples.

Notes: The following publications have been published using these methods for amino acid [1, 2], bile acid [2-5], and short chain fatty acid [2, 6]:

1. Ni, J., et al., A role for bacterial urease in gut dysbiosis and Crohn's disease. *Science Translational Medicine*, 2017. 9(416): p. eaah6888.
2. Ramsteijn, A.S., et al., Antidepressant treatment with fluoxetine during pregnancy and lactation modulates the gut microbiome and metabolome in a rat model relevant to depression. *Gut Microbes*, 2020. 11(4): p. 735-753.
3. Friedman, E.S., et al., FXR-Dependent Modulation of the Human Small Intestinal Microbiome by the Bile Acid Derivative Obeticholic Acid. *Gastroenterology*, 2018. 155(6): p. 1741-1752 e5.
4. Wang, S., et al., Diet-induced remission in chronic enteropathy is associated with altered microbial community structure and synthesis of secondary bile acids. *Microbiome*, 2019. 7(1): p. 126.
5. Grau, K.R., et al., The intestinal regionalization of acute norovirus infection is regulated by the microbiota via bile acid-mediated priming of type III interferon. *Nature Microbiology*, 2020. 5(1):

p. 84-92.
6. Uribe-Herranz, M., et al., Gut microbiota modulate dendritic cell antigen presentation and radiotherapy-induced antitumor immune response. *The Journal of Clinical Investigation*, 2020. 130(1): p. 466-479.

2.6 Materials

Table 2.1: Antibiotics

Antibiotic	**Manufacturer**	**Identifier**
Ampicillin	RPI Research Products International	CAS:69-52-3; Lot:155806-160204
Apramycine Sulfate	Alfa Aesar	REF:J66616
Hygromycine B	CORNING	REF:30-240-CR; Lot:30240134
Kanamycin Sulfate	Fisher	BP906
Neomycine Trisulfate Salt Hydrate	Sigma	REF:N6386
Vancomycin Hydrochloride	Fresenius Kabi	NDC 63323-314-61

Table 2.2: Bacterial Strains

Strain	**Description**	**Source**
Klebsiella pneumoniae MGH 78578	Isolated from a pneumonia patient sputum (1994) (https://www.genome.jp/entry/gn:T00566). Parental (wild type) strain	ATCC
ΔUrease	In-frame deletion of Urease operon	This study
ΔNtrC	In-frame deletion of NtrC gene	This study
E.coli MP1	Isolated from the murine intestinal tract [**91**]	Department of Microbiology, (Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA)

Table 2.3: Laboratory Animals

Laboratory animal	**Specification**	**Origin**
Conventional mice	SPF C57BL/6J	Jackson Laboratory, (University of Pennsylvania, Philadelphia, PA)
Germ-free mice	C57BL/6J	Bred in-house in Hill Laboratory, (University of Pennsylvania Veterinary School, Philadelphia, PA)

Table 2.4: Commercial Assays

Assay	**Manufacturer**	**Identifier**
Ammonia Assay Kit	SIGMA-ALDRICH®, (St Louis, MI)	MAK310-1KT
AccQ-Tag Ultra Amino Acid Derivatization Kit	Waters Corporation, (Milford, MA)	
Carbon Utilization Assay	Biolog, (Hayward, CA)	Microbial Phenotype Microplate PM1, PM2A
Glucose Assay Kit	Abnova, (New Providence, NJ)	KA1648
Nitrogen utilization Assay	Biolog, (Hayward, CA)	Microbial Phenotype Microplate PM3B
Quantichrom™ Urea Assay Kit	BioAssay Systems, (Hayward, CA)	DIUR-100
UPLC AAA H-Class Application Kit	Waters Corporation, (Milford, MA)	
Urease Activity Assay Kit	SIGMA-ALDRICH®, (St Louis, MI)	MAK120

Table 2.5: Chemicals

Chemical	**Manufacturer**	**Lot**	**CAS**
Dextran Sulfate	Fisher	NA	NA
Glycine			
L-alanine	fisher scientific education	0GKX26B011	56-41-7
L-arginine	Sigma-Aldrich	082K1450	74-79-3
L-aspartate	cell5		
L-Asparagine	Sigma-Aldrich	51K0079	5794-13-8
L-aspartic acid	Sigma-Aldrich	98H01651	56-84-8
L-cysteine	Sigma-Aldrich	020M1561	56-89-3
L-glutamine	Sigma-Aldrich	50K0237	56-85-9
L-glutamic acid	Sigma-Aldrich	19H0642	19473-49-5
L-histidine	Sigma-Aldrich	76H00885	7048-02-4
L-isoleucine	ACROS Organics	A0420335	73-32-5
L-leucine	Sigma-Aldrich	31K0911	61-90-5
L-lysine	TCI Tokyo Chemical Industry Co., Ltd.	T7WCH-FX	657-27-2
L-methionine	Sigma-Aldrich	25H08295	63-68-3
L-phenylalanine	Sigma-Aldrich	125H07391	63-91-2
L-proline	Sigma-Aldrich	072K0183	147-85-3
L-serine	Sigma-Aldrich	042K0881	56-45-1
L-threonine	Sigma-Aldrich	34H1319	72-19-5
L-tryptophan	Sigma-Aldrich	56H04215	73-22-3
L-tyrosine	Sigma-Aldrich	122K0741	60-18-4
L-valine	Sigma-Aldrich	77H1808	72-18-4
Lactulose	Thermo Scientific	Q25I006	4618-18-2
Miller's LB Agar Powder	dot scientific inc.	6040-6813	9002-18-0
Miller's LB broth powder	fisher bioreagents	213129	
Polyethylene Glycol 3350	Miralax	NA	NA
Sodium Salt	Fisher		
TAA	Sigma-Aldrich	BCBJ4361V	62-55-5
Urea	fisher scientific	105609	57-13-6

Table 2.6: Oligonucleotide Primers

Primer	**Resulting construct or purpose**	**Sequence**
Kp-NtrC-LRed$_F$	*ntrC* deletion Recombineering	GCCGGGTCATACCGAATT TTCGGTATACCTGCCTAT TCGGAAGTAGAGGT-GTTT atgCAAgggatccgtcgacctgca gttc
Kp-NtrC-LRed$_R$	*ntrC* deletion Recombineering	ACAAAATAGCAGCACTTT GCGCCGATCGGCTATT TTTCATCATGCTGTT GAACCctaCTCatgtgtaggctg gagctgcttc
Kp-UreaseOp-LRed-F	Urease operon deletion Recombineering	TACGGATGACATAAGCGTTT CGTATGACCGGGATAAA CTCCCGCCGATCAATACT CATTGattccggggatccgtcgacc
Kp-UreaseOp-LRed-R	Urease operon deletion Recombineering	AGAGAGAGCAGAGGCTGC ACCATCCGGACGCGCTT GCGCCCGGCTGGTGCAA CAGGCCTAatgtgtaggctggag ctgcttc
Kp-NtrC-Check-F	*ntrC* deletion test	GCACCGCTTTCCAGCTGACGC
Kp-NtrC-Check-R	*ntrC* deletion test	CCCGAATGCAGCAGTTCT CACGGG
Kp-UreaseOp-Check-F2	Urease operon deletion Test	CAATACGTTAGCAGCATG GAAAGGCAAAAGTTGC
Kp-UreaseOp-Check-R	Urease operon deletion Test	GCGGGGCGTAACGTAAGG TGTAATCT

Table 2.7: Plasmids

Plasmid	**Relevant features**	**Source**
pACBSR	arabinose inducable λ-Red recombinase ; hygromycin resistance marker	Huang,T et al.
pFLP-hyg	heat-shock inducable FLP recombinase; hygromycin resistance marker	Huang,T et al. [**87**]
pMDIAI	template for amplification of the FTR/apramycin-resistance cassette	Addgene (51655)

Table 2.8: Special Equipment

Equipment	**Manufacturer**	**Specifications**
centrifuge	Kendro Laboratory Products	SORVALL® Biofuge pico
homogenizer	pro scientific	PRO scientific 01-01200 Bio-Gen PRO200 homogenizer
gyratory shaker 2	B. Braun Biotech International	CERTOMAT® RM at 200 rpm
gyratory shaker 2	New Brunswick Scientific Co., Inc.	G10 GYROTORY SHAKER at 200rpm
micro pulser	Bio Rad	
plate reader	BioTek	EPOCH 2 microplate reader
roller drum	New Brunswick Scientific Co., Inc.	model TC-7 / Serial No. 790960431
Software and algorhythms	GraphPad	Prism version 9.5.1 and 10.2.3

Table 2.9: DSS Model - Disease Activity Index

Score	**Weight Loss [%]**	**Stool Consistency**	**Rectal Bleeding**
0	< 1	normal	none
1	1 - 5	normal	none
2	5 - 10	loose stool	hemoccult positive
3	10 - 20	loose stool	hemoccult positive
4	> 20	diarrhea	gross bleeding

3. Results

3.1 The Competitive Advantage of Urease

3.1.1 Urea Provides a Growth Advantage under Nitrogen Limitation

The expression of urease in *K. pneumoniae* is dependent on the availability of nitrogen in the environment [**26**]. Similarly, as in other urease-positive organisms, it is thought that urease in *K. pneumoniae* makes urea available as an additional nitrogen source, aiding in survival during nitrogen-limitation and increasing colonization fitness [**26, 27**]. We hypothesize that urease in *K. pneumoniae* supports growth in the presence of urea, with this effect being increased when alternative nitrogen sources are not accessible. ΔUrease *K. pneumoniae*, which is unable to express urease, was used as a negative control, to confirm that the observed results are solely due to the effects of urease.

K. pneumoniae WT and ΔUrease were grown overnight in minimal media with varying concentrations of ammonium chloride (furthermore referred to as "ammonia", as previously described in materials and methods) as a nitrogen source in the presence or absence of urea and OD_{600} was continuously monitored. Ammonia concentrations of 0%, 0.01%, and 0.1% imitated environments with no, limited, or abundant nitrogen availability. Urea was added to the media as specified in the figure legend. The growth of the WT without the addition of urea, as well as of ΔUrease with and without urea was limited when no available nitrogen source was present (**Figure 3.1 A**).

The WT can grow in the presence of urea, as it is able to utilize urea as a nitrogen source through the urease-catalyzed hydrolyzation. When the ammonia concentration increased, we no longer observed the same differences (**Figure 3.1 B and C**), as all strains grew at similar rates. In addition to recording growth curves, we tested the concentration of ammonia and urea at different time points during the growth of *K. pneumoniae*. We saw that the concentration of ammonia decreased over time in the ΔUrease, as well as the WT without the addition of urea (**Figure 3.1 D**). Only the WT in the presence of urea experienced an increase in the ammonia concentration. As expected, we saw the concentration of urea decrease in the presence of the WT, while it was unchanged by ΔUrease (**Figure 3.1 E**).

The sum of our findings supports our hypothesis. The urease-positive *K. pneumoniae* WT can utilize urea as an additional nitrogen source, increasing growth in nitrogen-limited media, while ΔUrease is unable to express urease and therefore displays a growth defect in nitrogen-limited media in comparison. Urea is being consumed by the WT, which in turn increases the ammonia concentration in the media. The striking ammonia increase in media due to the hydrolyzation of urea by the WT leads us to believe that the reaction produces more ammonia than is needed for growth and excess ammonia is released into the environment.

3.1.2 Urease Does Not Provide an Advantage in the Survival of the Gastric Acidic Environment in *K. pneumoniae* or *E. coli*

Accessing additional nitrogen sources is only one of many mechanisms by which urease may be beneficial for *K. pneumoniae*. Given the rationale that *H. pylori* is able to neutralize the acidic environment created by gastric acid in the stomach through the use of urease [**26, 27, 29**], we hypothesized that urease produced by *K. pneumoniae* would exhibit the same buffering capacity

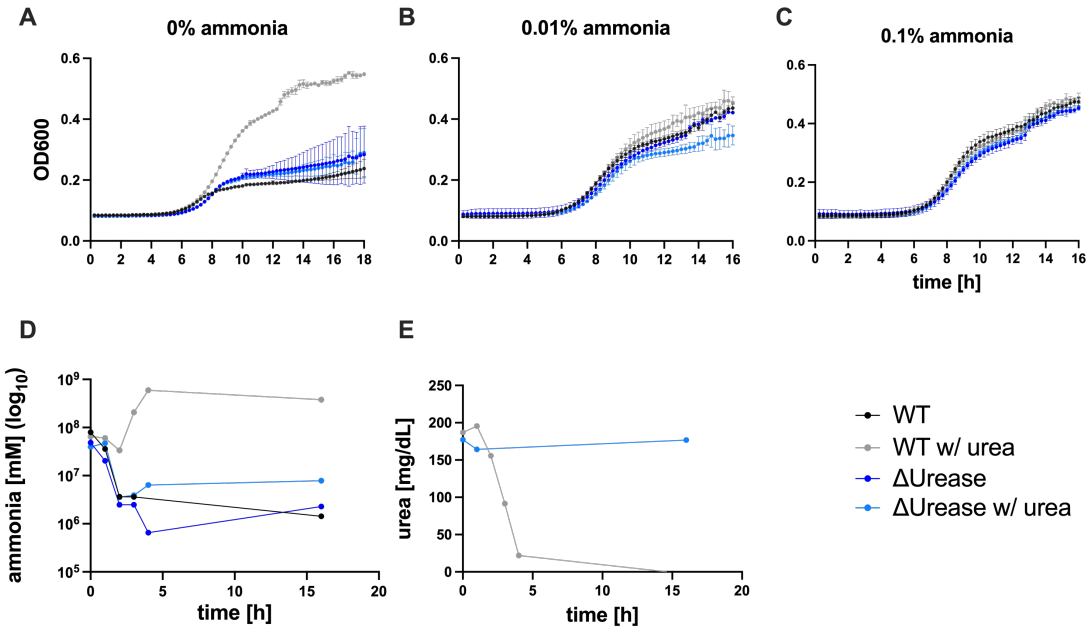

Figure 3.1: (**A - C**) Overnight growth curves of *K. pneumoniae* WT and ΔUrease in minimal media with or without the addition and different concentrations of ammonia ((**A**) 0%, (**B**) 0.01%, (**C**) 0.1%) as a nitrogen source, urea was added as specified in the legend; (**D, E**) media was manually tested for (**D**) ammonia ($log_(10)$ transformed), and (**E**) urea at start (0h) and the time points 1h, 2h, 3h, 4h, and 16h.

and subsequently increase survival of the harsh conditions encountered in the passage of the stomach. We imitated the physiology of the stomach by having bacteria (*K. pneumoniae* and *E. coli*) undergo an acid shock in media with a pH of 2.5 for an hour in the presence of urea, as well as without urea, before being washed and grown in neutral media to test for survival by calculating the CFU. Urease-positive strains as well as strains without the ability to express urea were used for both *K. pneumoniae* (WT and ΔUrease) and *E. coli* (MP1 and MP1 + Urease).

We observed *K. pneumoniae* growing to the same level in the control group, regardless of the ability to produce urease or the presence of urea (**Figure 3.2 A**). The same was observed in the group experiencing the acid shock, as the same CFU/mL was reached, regardless of the ability to produce urease. The bacteria shocked in acid grew to a lower CFU/mL overall (10^4 compared to 10^8 in the control). The experiment was repeated using *E. coli*. We observed a similar effect, as the ability to produce urease in the originally urease-negative MP1 did not increase survival in the presence of urea after being shocked at a pH of 2.5 (**Figure 3.2 B**).

This led us to reject our hypothesis of urease increasing survival after acid shock in *K. pneumoniae*, as the deletion of the urease-operon and therefore the inability to utilize urease in ΔUrease did not result in a disadvantage during acid shock. Our additional test using *E. coli* aimed at controlling for confounders affecting results in *K. pneumoniae* WT and ΔUrease by adding the gene to produce urease to the urease-negative MP1 strain, which produced the same results, further supporting us in rejecting the hypothesis.

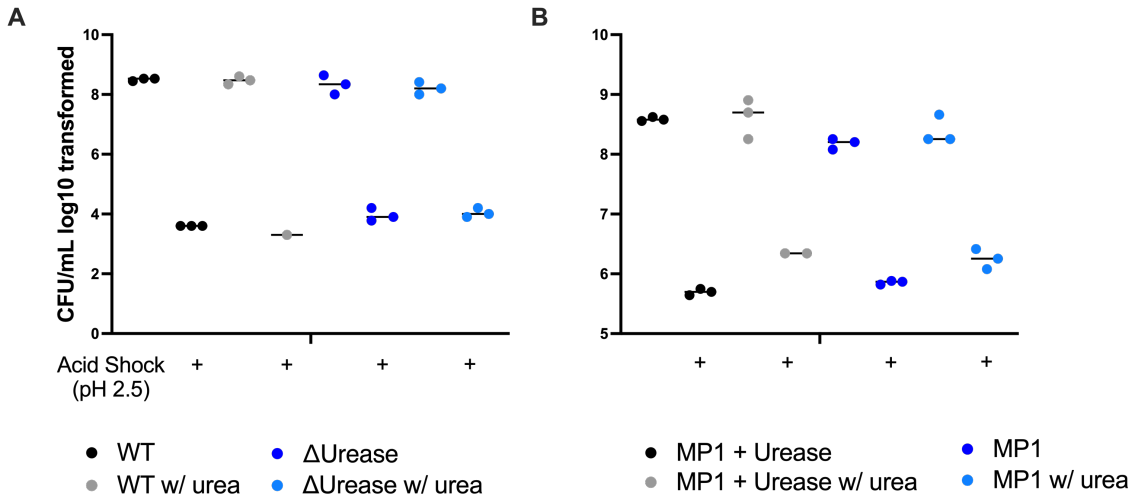

Figure 3.2: Bacteria experienced an acid shock at pH 2.5 as specified in the legend (control at pH 7) after overnight growth in LB (pH 5) with or without the addition of urea; (**A**) *K. pneumoniae* WT or ΔUrease; (**B**) *E. coli* MP1 or urease-competent MP1+Urease.

3.1.3 Urease Does Not Provide an Advantage in the Survival of the Colonic Acidic Environment in *K. pneumoniae*

Pamer *et al.* previously demonstrated that the growth of *K. pneumoniae* is stunted by the presence of SCFAs in an acidic environment [**34**]. Similar results were additionally found by our group in previous experiments examining the effects of acetate on the growth of *E. coli*. These results showed that increasing concentrations of acetate, an SCFA, stunted growth [**86**]. These circumstances are often experienced by bacteria in the colon, where SCFAs are produced through the fermentation of complex carbohydrates, resulting in a decrease in pH and the normal range of the human colonic pH of 5.5 to 7.5 [**4, 31**]. We hypothesized that the presence of acetate as an SCFA in acidic media would inhibit the growth of *K. pneumoniae*. ΔUrease was utilized as a control for the relevance of urease under these circumstances, as in the previous experiments.

We recreated the aforementioned conditions of the colon by adding 100 mM acetate into acidic media (pH 5-6) and recording the growth overnight. We tested the growth of either the *K. pneumoniae* WT or ΔUrease, with or without the addition of acetate and urea (**Figure 3.3 A - F**). Urea was used to investigate the effects that deletion of the urease-operon would have on the ability to withstand the environmental presence of acetate. The WT was additionally tested in an anaerobic environment, as the gut exhibits both aerobic and anaerobic conditions (**Figure 3.3 C, D**). The addition of acetate stunted the growth of the WT regardless of the pH of the media, or the presence of urea. We observed a comparable effect in ΔUrease (**Figure 3.3 E and F**). However, in the urease-negative strain, we observed a difference in the effect acetate has on growth of *K. pneumoniae* in different pH environments. Addition of acetate stunted growth to a greater extent in media with a pH of 6, than it did in a pH of 5.

These results provide support for our hypothesis, as the growth of *K. pneumoniae* was inhibited by the addition of acetate. Deletion of the urease-operon did not have a clear effect on the ability of *K. pneumoniae* to withstand acetate. We are unable to say with certainty whether the observed differences between the *K. pneumoniae* WT and ΔUrease are a product of the expression of urease, as the observed effects differed depending on the specific pH of the medium. Our results lead us

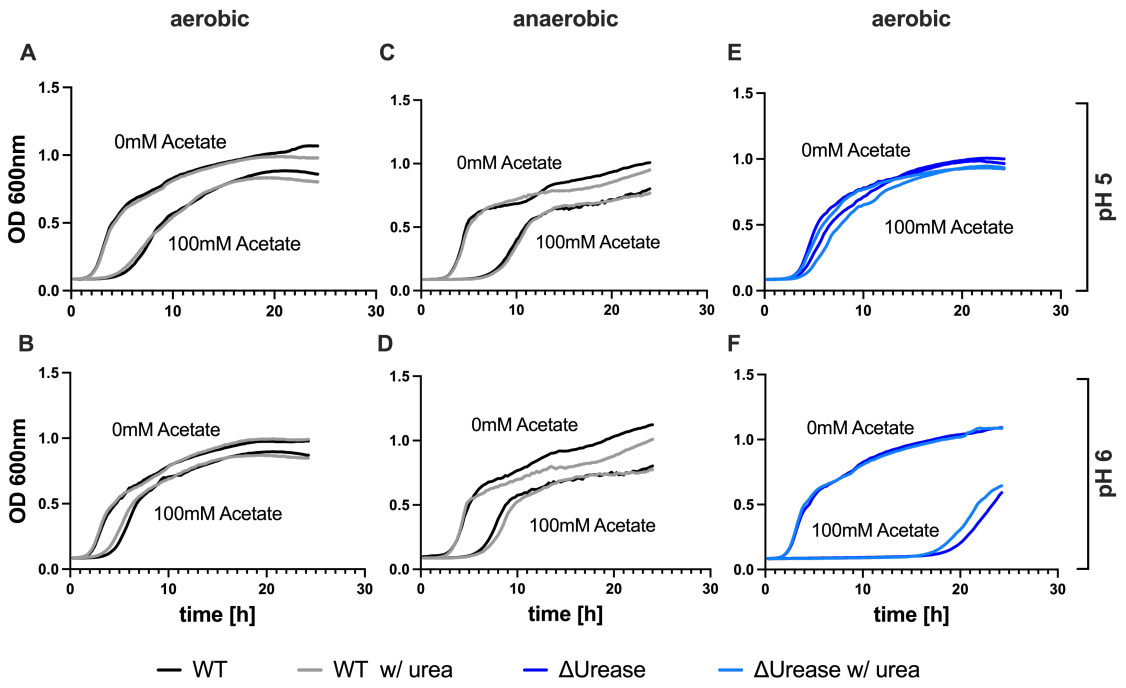

Figure 3.3: Growth of *K. pneumoniae* in LB media with the addition of 0 or 100 mM acetate, with or without the addition of urea inn different media conditions; (**A**) WT in pH 5, aerobic; (**B**) WT in pH 6, aerobic; (**C**) WT in pH 5, anaerobic; (**D**) WT in pH 6, anaerobic; (**E**) ΔUrease mutant in pH 5, aerobic; (**F**) ΔUrease mutant in pH 6, aerobic.

to believe that urease may play a role in the survival of acidic environments with a pH of 6 and the presence of acetate, yet we could not see the same effect in more acidic environments with a pH of 5. Further studies will be needed to examine the relevance of the environmental pH on the ability of urease-positive organisms to survive increased SCFA concentrations and the impact acidity plays regarding the function of urease.

3.1.4 Urease Does Not Provide an Advantage During *In Vivo* Colonization

Nitrogen limitation is believed to be one of the key factors controlling bacterial colonization in the mammalian intestinal tract. Given the growth advantage that the urease enzyme provided in a nitrogen-limited environment *in vitro* (**Figure 3.1 A-C**), we hypothesized that urease can provide an advantage during colonization of the mouse gut.

To determine this, we colonized antibiotic pre-treated mice with *K. pneumoniae* WT and ΔUrease and monitored the level of colonization in the feces over a month. When introduced separately, we did not observe an advantage of the WT over ΔUrease during the colonization of the murine intestinal tract (**Figure 3.4 A**). We additionally introduced the WT and ΔUrease in direct competition into the mouse gut. The CFU of the innoculation was tested to exclude a quantitive advantge of one of the strains (**Figure 3.5 C**). In this instance, we observed that contrary to our prediction, ΔUrease exhibited an advantage over the WT (**Figures 3.4 B and C**). Antibiotic resistance markers were alternated to control for possible interference. To account for the competition results possibly being skewed by the antibiotic markers themselves, we competed each of the resistance-marked strains with the contrastingly marked one (**Figure 3.5**). We saw

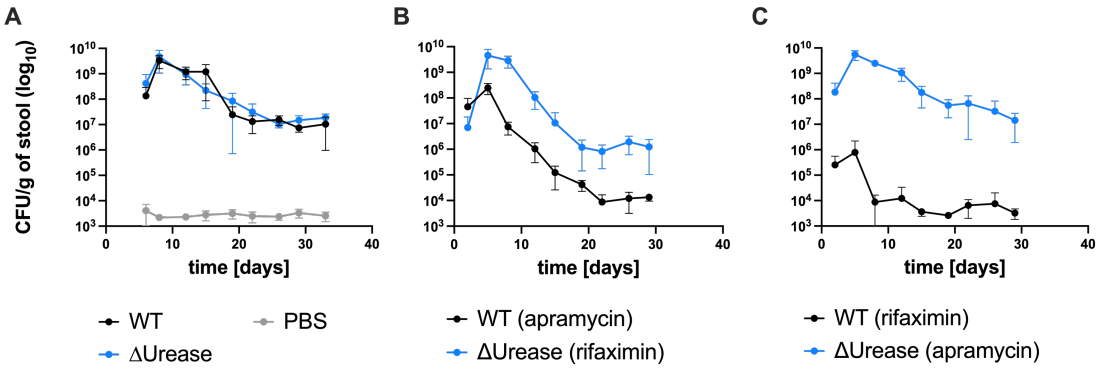

Figure 3.4: Competition of *K. pneumoniae* WT and ΔUrease mutant in the mouse gastrointestinal tract in (**A**) monoculture (including PBS control, $log_(10)$ transformed), (**B**) in competition (WT apramycin marked and Δurease mutant rifaximin marked, $log_(10)$ transformed) and (**C**) in competition (WT rifaximin-marked and Δurease mutant apramycin-marked, $log_(10)$ transformed).

that both times, when the apramycin- and rifaximin-marked strains competed in culture that the apramycin-marked strain showed a slight growth advantage (**Figure 3.5 A and B**). Given the fact that ΔUrease outperformed the WT regardless of the resistance marker, we are reinforced in our assumption that the ΔUrease mutant might have an advantage in direct competition when colonizing the intestinal tract.

Overall, our results suggest that urease is not needed for successful colonization of the mouse intestinal tract.

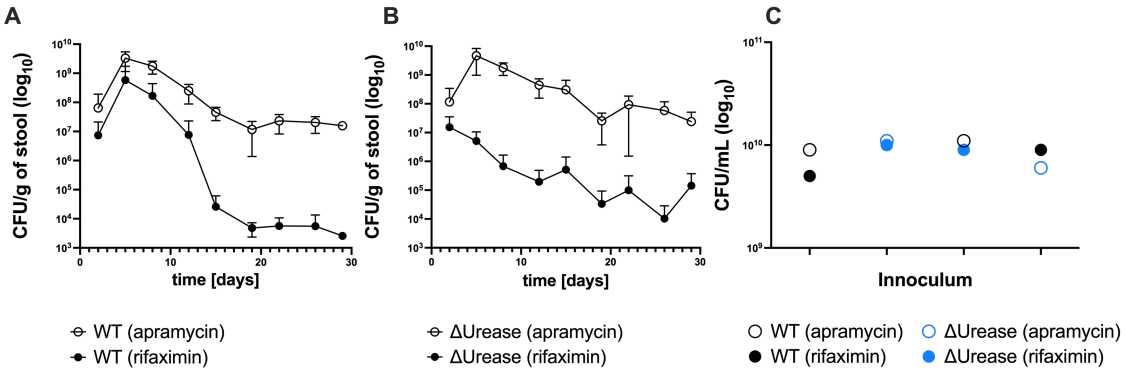

Figure 3.5: Competition of *K. pneumoniae* WT and ΔUrease mutant *in vivo*, (**A**) apramycin-marked WT vs. rifaximin-marked WT, (**B**) apramycin-marked ΔUrease vs. rifaximin-marked ΔUrease. (**C**) Inoculum for the *in vivo* colonization competition of *K. pneumoniae* WT and ΔUrease.

3.2 Nitrogen and Carbon as Essential Resources of Bacterial Colonization in the Intestinal Tract

3.2.1 *K. pneumoniae* Colonization Alters the Nitrogen Composition of the Intestinal Tract

We were previously able to show that the ability of *K. pneumoniae* to hydrolyze urea leads to an increase in ammonia concentration (**Figure 3.1 D and E**). This leads us to hypothesize that colonization with *K. pneumoniae* will alter fecal ammonia levels by metabolizing urea in the gut. To test the effect *K. pneumoniae* had on the concentration of ammonia and urea, we investigated individuals whose gut microbiome was predominant colonized by *K. pneumoniae* (*K. pneumoniae*-high) and in those whose microbiome was made up of mostly other microbes (*K. pneumoniae*-low), using fecal samples from the FARMM study [**44**]. We compared the ammonia and urea levels in fecal samples of *K. pneumoniae*-high or -low individuals from the FARMM study, before and after antibiotic treatment, as well as after the recovery of the microbiome. Ammonia levels decreased significantly after antibiotic treatment in both groups (**Figure 3.6 A and B**). After recovery, only the *K. pneumoniae*-high group experienced a significant increase in ammonia levels (**Figure 3.6 B**), while the ammonia levels in the *K. pneumoniae*-low group did not change significantly after recovery of the intestinal microbiota (**Figure 3.6 A**). Quantification of the urea concentration revealed no significant change in the urea levels from before to directly after antibiotic treatment (**Figure 3.6 C and D**). However, after reconstitution of the microbiome, the *K. pneumoniae*-high group showed a significant decline in urea levels (**Figure 3.6 D**), the *K. pneumoniae*-low group on the other hand only had a slight non-significant decrease in urea levels (**Figure 3.6 C**). This supports our hypothesis that colonization with *K. pneumoniae* increases ammonia production by urea-consumption.

Our data showed that *K. pneumoniae* metabolizes urea as a nitrogen source (**Figure 3.1 A-E**). Other relevant nitrogen sources in the gut are amino acids from dietary proteins. We hypothesized that *K. pneumoniae* colonization would alter the amino acid metabolization in the gut microbiome by accessing alternative nitrogen sources such as urea. To test this, we evaluated the level of all 20 proteinogenic amino acids in stool samples from the FARMM study (**Figure 3.6 C**). Before antibiotic treatment, all amino acids were low in concentration in all three dietary groups. After antibiotic treatment, when the numbers of bacteria in the intestinal tract were reduced, the concentration of amino acids increased, supporting the notion that the gut microbiome consumes intestinal amino acids. After the return of the microbiota, these levels returned to baseline in the omnivore and vegan groups. The EEN group, which is mainly colonized with *K. pneumoniae*, however, did not return to their prior amino acid levels but instead remained elevated. Combined with our previous data of *K. pneumoniae* decreasing stool urea and increasing stool ammonia, this finding of increased amino acid levels in highly *K. pneumoniae*-colonized subjects, supports our hypothesis that *K. pneumoniae* colonization alters the nitrogen composition of the gut.

3.2.2 The Nitrogen Preferences of *K. pneumoniae*

To investigate the specific compounds *K. pneumoniae* can metabolize, a commercially available array of nitrogen-containing compounds was tested. We observed that K. pneumoniae is able to

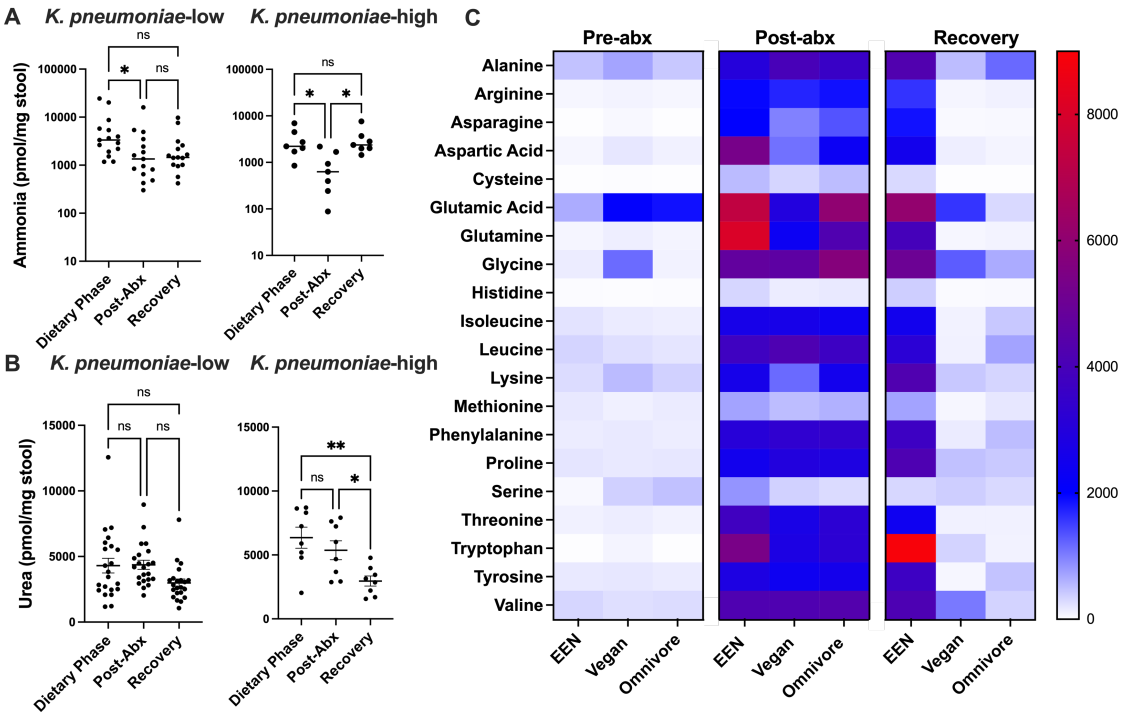

Figure 3.6: Association of ammonia, urea, and amino acid concentration with antibiotic use and *K. pneumoniae* colonization in fecal samples from the FARMM study (before antibiotic treatment (pre-abx), after antibiotic treatment (post-abx), and after recovery of the microbiome) [**44**]: (**A**) ammonia quantification in *K. pneumoniae*-low and -high subjects in each phase of the FARMM study; (**B**) urea quantification in *K. pneumoniae*-low and -high subjects in each phase of the FARMM study; (**C**) heat map showing the NMR quantification of the 20 proteinogenic amino acids in stool samples in each phase of the FARMM study; (**A, B**) one-way ANOVA with multiple comparisons using the Kruskal-Wallis test, 95% confidence interval, n.s. not significant, *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001.

utilize a wide array of single nitrogen sources (**Figure 3.7**).
To gain insight into the nitrogen preferences used under conditions more similar to the gut, we additionally tested this under anaerobic conditions. We observed that the sole nitrogen sources best suited for growth are amino acids as well as dipeptides containing glycine, asparagine, and glutamine, in addition to ammonia and urea. We identified a significant difference in many compounds that led to increased growth of *K. pneumoniae* aerobically, compared to the anaerobic conditions. This difference was apparent in most amino acids and dipeptides, as well as ammonia and urea, with the exception of the amino acids L-cysteine and L-serine.
The Ntr stress response is important for the utilization of alternative nitrogen sources when ammonia is present at low concentrations [**48**]. To determine the impact the deletion of the *ntrC* gene has on the utilization of single nitrogen sources, we tested a Δ*ntrC* mutant under the same conditions. We found that, as anticipated, growth on ammonia is unaffected by the mutation, while the growth on urea as a sole nitrogen source is significantly delayed. When comparing the WT with the Δ*ntrC* mutant in both aerobic and anaerobic environments, as anticipated, we find the Δ*ntrC* mutant is unable to utilize most nitrogen sources to the same extent as the WT. However, growth on several nitrogen sources was unaffected by the deletion of the *ntrC* gene, namely ammonia, L-glutamine, and the L-glutamine-containing dipeptides. This is likely due to the abundance of glutamine, making the Ntr stress response obsolete under these circumstances.

3.2.3 Nitrogen is Not a Limiting Factor for *K. pneumoniae* Colonization of the Gut

3.2.3.1 Microbiome-Produced Ammonia is Not Essential for Growth of *K. pneumoniae* in the Gut

Previously, we demonstrated that urease, and therefore the ability to utilize urea as an additional nitrogen source, is not needed for successful colonization of the intestinal tract of CR mice (**Figure 3.4**).
It is possible that cross-feeding from the microbiota produces enough ammonia to provide a sufficient nitrogen source for colonization with the urease-negative *K. pneumoniae* strain. We saw in previous experiments that *K. pneumoniae* produces a considerable amount of ammonia, more than is needed to sustain its growth, which is subsequently released into the environment (**Figure 3.1 E**). To exclude this possibility, we inoculated GF mice, that have no resident microbiome, with either WT or ΔUrease *K. pneumoniae* and monitored the *in vivo* growth via CFU. Additionally, the concentration of ammonia was measured in fecal samples over the course of colonization, as well as urea on day 8, after colonization with *K. pneumoniae* had occurred. Both strains were able to colonize the intestinal tract without a disadvantage of ΔUrease (**Figure 3.8 A**). Before introduction of bacteria into the GF mice, the ammonia concentration in the fecal samples was notably low, subsequently increasing with colonization in both the WT, approximately 10-fold, and ΔUrease, approximately 4-fold, inoculated groups (**Figure 3.8 B**). The increase in ammonia concentration with ΔUrease is significantly lower than the WT but, nevertheless, remarkable compared to the starting point at day 0. The fecal concentration of urea after 8 days coincides with our previous results in CR mice, showing a decrease in the WT compared to colonization with ΔUrease (**Figure 3.8 C**). When comparing these findings in GF mice to our previous data, testing the colonization of the WT and ΔUrease mutant in CR mice after antibiotic clearance of the microbiome (**Figure**

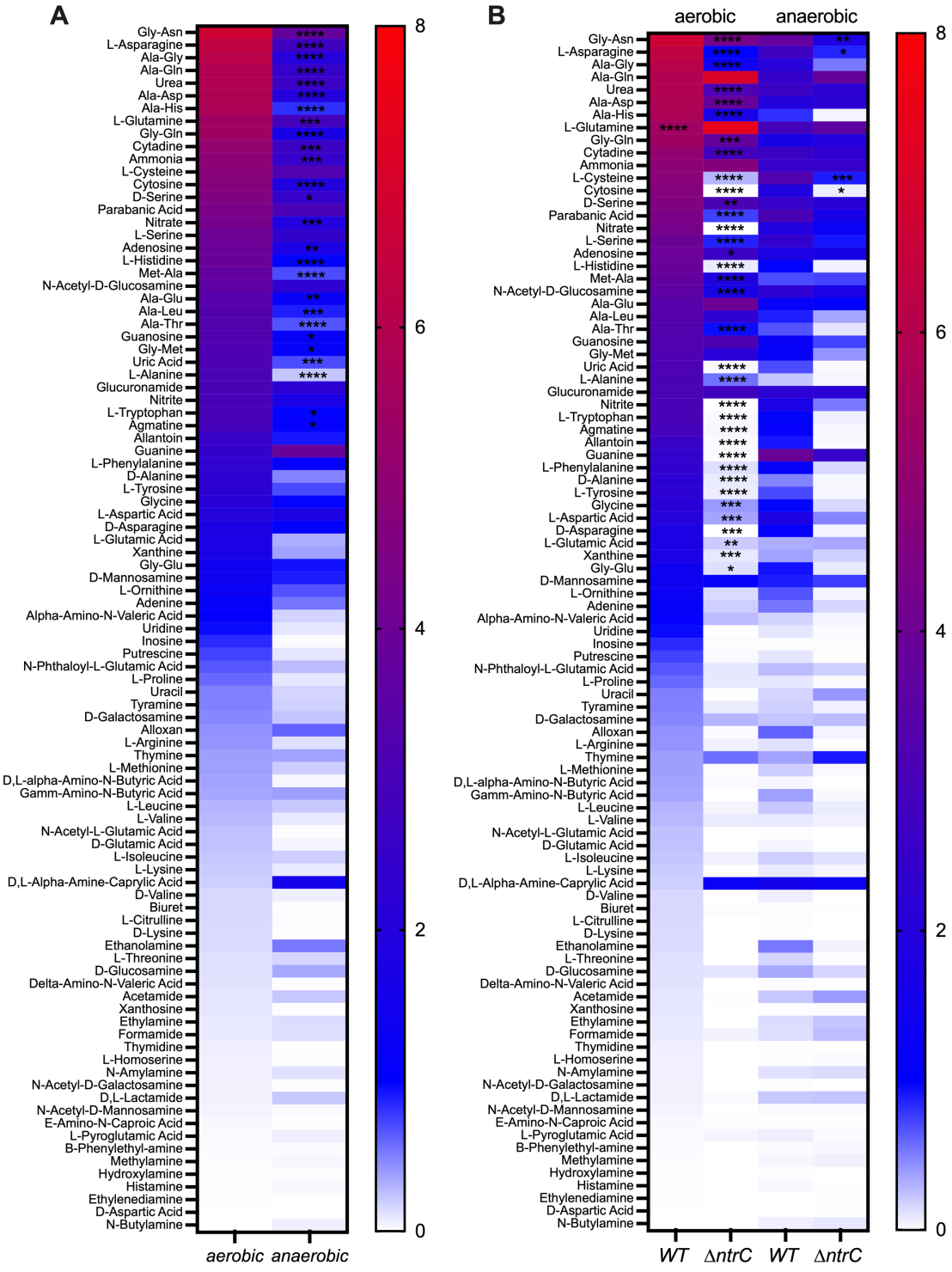

Figure 3.7: Growth of *K. pneumoniae* on Biolog (PM3B) Nitrogen plates; (**A**) heat map of the AUC of the *K. pneumoniae* WT growing overnight on different sole nitrogen sources; (**B**) heat map comparing the growth of *K. pneumoniae* WT and Δ*ntrC* on different sole nitrogen sources under aerobic or anaerobic conditions; results of two-way ANOVA with Šídák correction for multiple comparisons, 95% confidence interval, n.s. not significant, *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001.

3.4), we observe that urease has minimal impact on colonization regardless of microbiota.

3.2.3.2 Intestinal Colonization Does Not Require Nitrogen Scavenging

In addition to the present microbiome, another factor impacting the colonization of the intestinal tract by *K. pneumoniae* is the available nutrition. Previously published work has shown that the gut microbiome is a consumer of dietary nitrogen in addition to urea [**3, 21, 22**]. We, therefore, hypothesize that changes in dietary nitrogen availability will impact the necessity of urea metabolization as a nitrogen source, favoring urease-positive bacteria. We additionally included Δ*ntrC*, unable to access most nitrogen sources with the exception of ammonia and L-glutamine, to address the necessity of alternative nitrogen sources other than urea that can aid in survival and be of an advantage during colonization of nitrogen-limited environments. We repeated our experiment of testing the *in vivo* colonization of mice by gavaging *K. pneumoniae* WT, ΔUrease, and Δ*ntrC*, respectively, into CR mice receiving either a standard diet or a low protein diet, aimed at reducing dietary nitrogen availability. We did not observe a significant difference in the CFU quantification of the different bacterial strains during colonization of mice, receiving either a standard or low protein diet (**Figure 3.9 A - D**). The limitation of dietary nitrogen through a low protein diet did not affect the ability of *K. pneumoniae* WT, or ΔUrease and Δ*ntrC*, either deficient in a nitrogen scavenging mechanism, to colonize the murine intestine (**Figure 3.9 C and D**).
Ammonia and urea concentrations in the stool were quantified before and after antibiotic treatment, as well as after recovery of the microbiome 3 days after gavage. Ammonia decreased after antibiotic treatment, while urea increased, both in the samples gavaged with WT and ΔUrease, due to the decimation of the microbiota, leading to a decrease in urea hydrolyzation into ammonia (**Figure 3.9 E and F**). After the reconstitution of the microbiome and growth of either *K. pneumoniae* WT or ΔUrease, we observed that the ammonia concentration increased in the WT-colonized samples, while ΔUrease did not lead to an increase in ammonia (**Figure 3.9 E**). We saw the contrary effect in urea concentrations, where colonization with the WT led to a significant decrease compared to ΔUrease (**Figure 3.9 F**).
During colonization of the intestinal tract under normal circumstances, the bacteria will have to compete with other members of the microbiome to gain access to limited nutrients, possibly nitrogen. Under these circumstances, the accessibility of additional nitrogen sources through the Ntr system may grant an advantage. We, therefore, hypothesize that deletion of the *ntrC* gene will lead to a growth disadvantage in competition with the Ntr-competent *K. pneumoniae* WT. To test this, we observed the colonization of the murine intestinal tract by the WT as well as Δ*ntrC* strain, introduced individually, as well as inoculated in competition. Both the WT and Δ*ntrC* strains colonized the murine gut individually without an apparent growth defect of Δ*ntrC* (**Figure 3.10 A**). When inoculated concomitantly, despite growing in competition, we, likewise, did not see a disadvantage of Δ*ntrC* (**Figure 3.10 B**). This led us to reject our hypothesis, as the results suggest that Ntr is not needed for successful colonization in competition with other bacteria.
Our results show that neither the hydrolyzation of urea through urease, cross-feeding of ammonia, nor additional nitrogen sources accessed via Ntr are necessary for successful colonization of the murine intestinal tract, suggesting that the murine gut is not a nitrogen-limited environment for the growth of *Enterobacteriaceae* such as *K. pneumonia*.

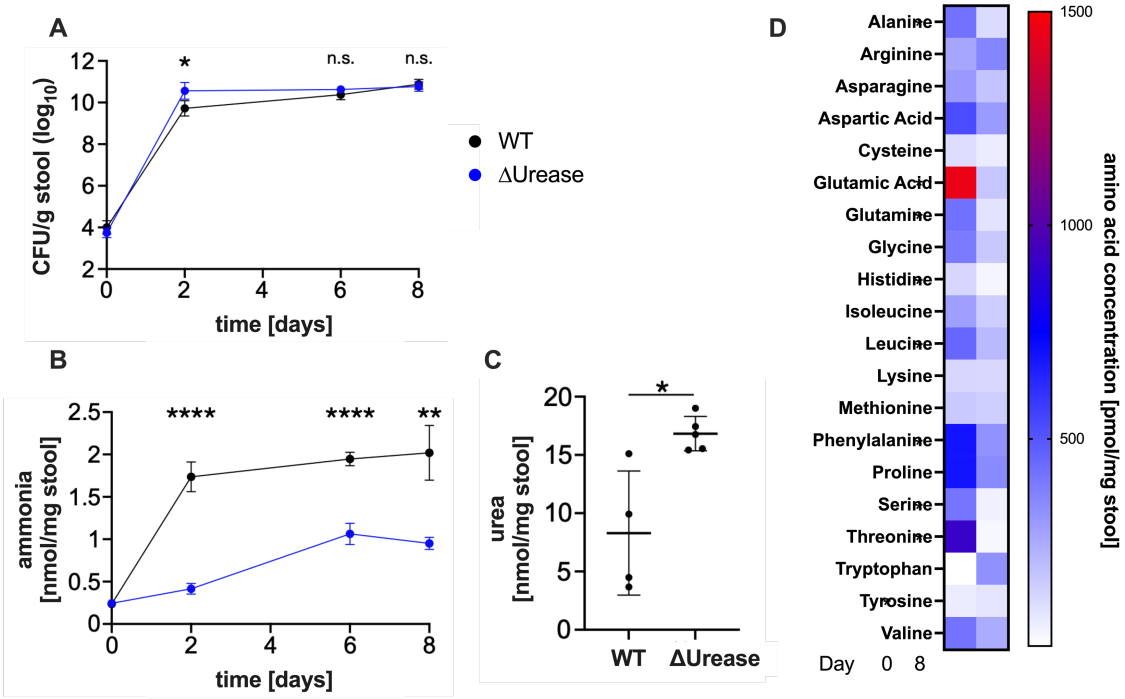

Figure 3.8: Colonization of GF mice with *K. pneumoniae* WT or ΔUrease; serial stool collections were performed to quantify (**A**) CFU and (**B**) stool ammonia; (**C**) stool urea was tested on day 8; (**D**) levels of stool amino acids tested before (day 0) and after (8 days) colonization of *K. pneumoniae*; Data represent combined results from 2 independent experiments; (**A, B**) *p<0.05, **p<0.01, and ****p<0.001, by multiple unpaired, 2-tailed t-tests with Bonferroni's multiple corrections or (**C**) by unpaired, 2-tailed t-test; (**D**) results of Mann-Whitney's U-tests with multiple comparison two-stage step-up method of Benjamini, Krieger and Yekultieli, not significant q>0.01,* q<0.01.

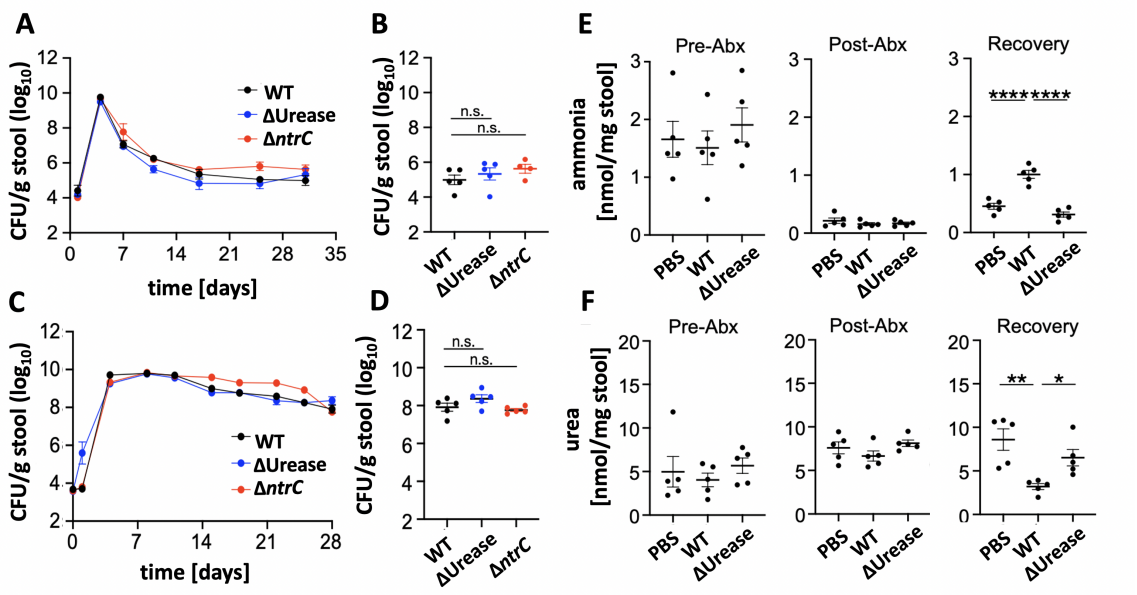

Figure 3.9: (**A-D**) Mice were provided a standard chow diet (**A** and **B**), or a low protein diet (**C** and **D**) and gavaged with WT, Δ*ntrC*, or ΔUrease *K. pneumoniae* after antibiotic (abx) pre-treatment and fecal CFU was monitored for the subsequent 4 weeks (**A**, **C**); CFU quantification 4 weeks after gavage for each respective diet (**B**, **D**); (**E**) fecal ammonia was quantified in mice colonized with WT, or ΔUrease *K. pneumoniae* before abx, after abx, and 3 days after gavage; **(F)** fecal urea was quantified in mice colonized with WT, or ΔUrease *K. pneumoniae* before abx, after abx, and 3 days after gavage.

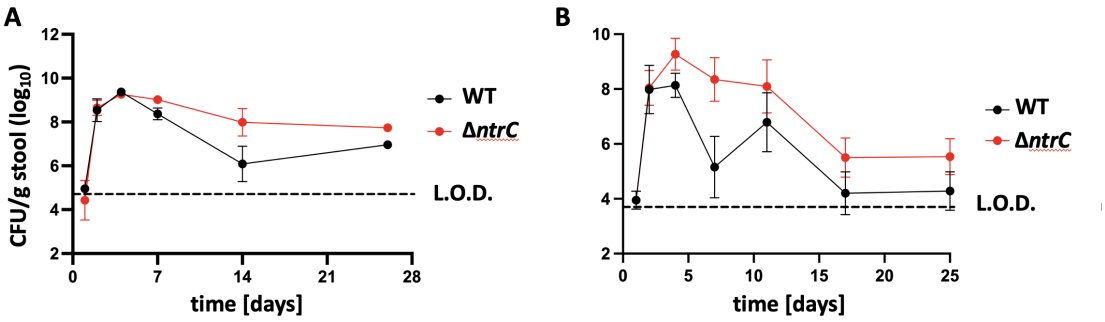

Figure 3.10: (**A**) Monocolonization of *K. pneumoniae* WT or Δ*ntrC* mutant in mice after antibiotic clearing of the intestinal flora; (**B**) competitive colonization of the WT and Δ*ntrC* in mice after antibiotic clearing of the intestinal flora; L.O.D: Limit of detection.

3.2.4 *K. pneumoniae* Prefers Simple Over Complex Carbohydrates

Nitrogen availability alone does not regulate bacterial growth in the intestinal tract, as carbon is another essential nutrient relevant for colonization of the gut.
The FARMM study, previously conducted by our group on healthy volunteers, assessed the impact different diets had on intestinal colonization [**44**]. Healthy volunteers were divided into three groups, receiving different diets after antibiotic eradication of the inherent microbiome. In the group receiving the EEN diet, *K. pneumoniae* comprised a great portion of the bacteria colonizing the gut to shape the newly formed microbiome. Meanwhile, the omnivore or vegan group did not experience a bloom of *K. pneumoniae* to the same extent (**Figure 1.2**) [**45**].
Based on this observation, we hypothesized that the outgrowth of *K. pneumoniae* in the EEN group is due to the preference for simple carbohydrates as a carbon source instead of complex carbohydrates found in dietary fiber. To investigate our hypothesis, we tested the growth of *K. pneumoniae* with a commercially available panel of carbon sources (**Figure 3.11**). We tested the growth under aerobic and anaerobic conditions, to mimic both ends of the aerogenic spectrum in the intestinal lumen [**57**]. We observed that *K. pneumoniae* thrives on mono- and disaccharides, which are sugars such as glucose, fructose, lactose, maltose, or sucrose often found in processed foods. Considering this, it is foreseeable that *K. pneumoniae* flourishes in the EEN group, as the Modulen® IBD used for the EEN diet mainly contains glucose and sucrose as carbohydrate sources. *K. pneumoniae* does not grow well on complex carbohydrates that mainly make up dietary fiber such as glycogen, gelatin, amygdalin, chondroitin sulfate, or N-acetylneuraminic acid, to name a few (**Figure 3.11**). With few exceptions (e.g., mannitol, D-gluconic acid, D-raffinose, and stachyose), the utilization of carbon sources does not differ significantly between aerobic and anaerobic environments (**Figure 3.11**).

3.2.5 Amino Acids are Accessible as Additional Carbon Sources

We previously conducted research on the utilization of different carbon sources and were able to show that *K. pneumoniae* has a preference for simple carbohydrates in the form of mono- and disaccharides (**Figure 3.11**). In addition to sugars, we observed that *K. pneumoniae* can utilize amino acids as a carbon source (**Figure 3.11 and 3.14 B**). To understand the impact of easily accessible carbon sources in the form of glucose on the growth of *K. pneumoniae* and the metabolization of amino acids as either a carbon or nitrogen source, we conducted a test using a specifically made amino acid mix in MOPS media, testing the overnight growth with different concentrations of glucose. Urea was introduced as an additional nitrogen source, to evaluate the impact of other available nitrogen sources on the utilization of amino acids as a carbon source.
We observed that *K. pneumoniae* grew to a similar CFU on glucose concentrations ranging from 0 mM to 100 mM (**Figure 3.13 A**). When we observed the growth via the OD_{600}, we saw that a higher concentration of glucose (100 mM) led to greater growth of *K. pneumoniae* (**Figure 3.13 B**). Here, we additionally observed that the addition of urea led to an even greater increase in the growth of *K. pneumoniae* than the high concentration of glucose alone. Addition of urea to media with a lower concentration of glucose did not have the same effect and did not increase growth. This led us to suspect that carbon became the limiting factor under these circumstances, prior to nitrogen. *K. pneumoniae* was able to grow in media without any glucose present as a carbon source, suggesting that amino acids are able to be utilized as a carbon source under these circum-

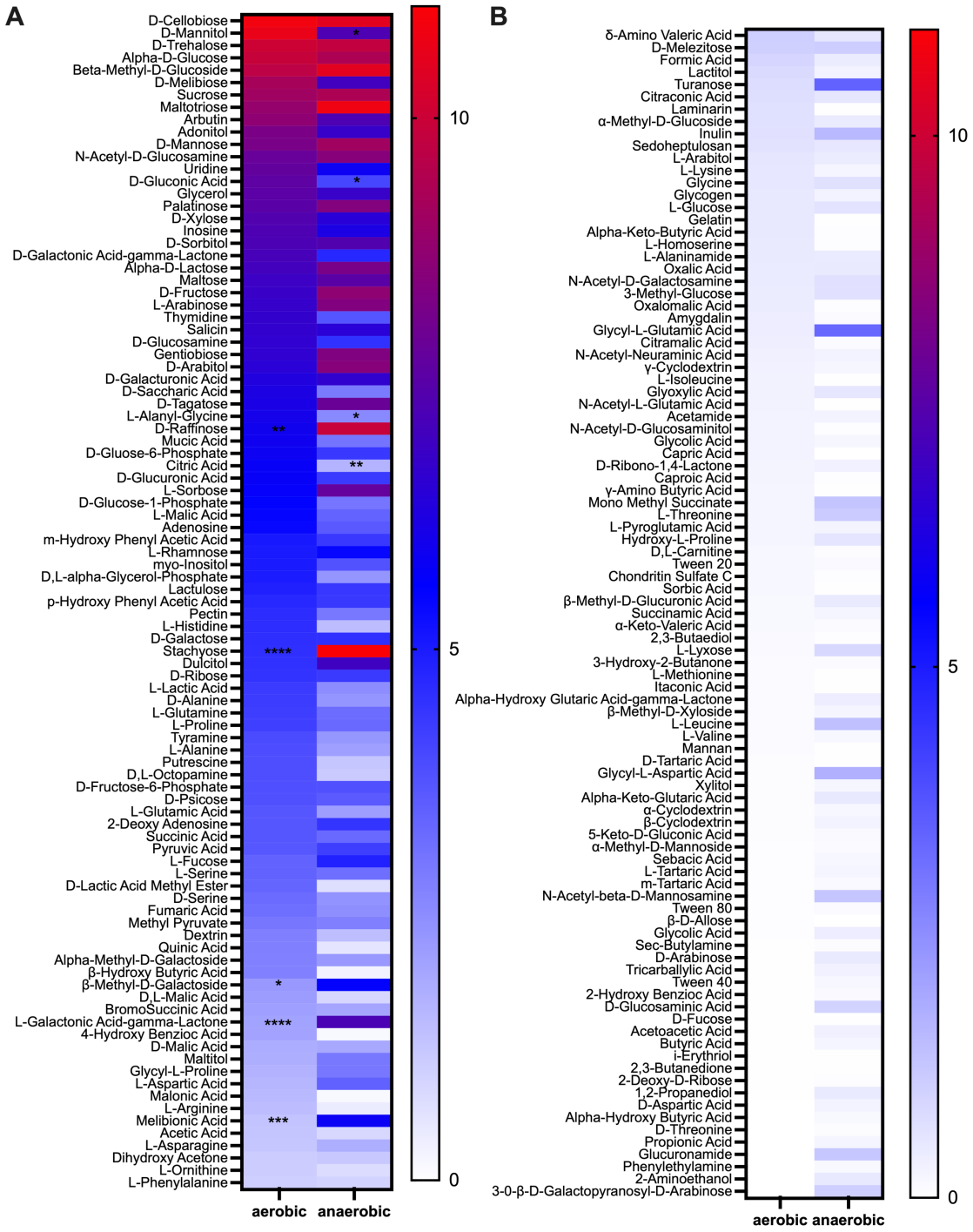

Figure 3.11: Heat map showing the area under the curve analysis of growth of *K. pneumoniae* WT on Biolog plates with different sole carbon sources (PM1 and PM2A), combined and arranged from most to least growth aerobically continuing from top to bottom and left (**A**) to right (**B**); Results of two-way ANOVA with Šídák correction for multiple comparisons, 95% confidence interval, n.s. not significant, *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001.

stances. To evaluate whether *K. pneumoniae* prefers glucose or amino acids as a carbon source, we tested the glucose concentration at multiple time points during the growth. We observed that the glucose concentration decreased in all glucose-containing media (**Figure 3.13 E**). When 10 mM of glucose was supplied, the carbon source was exhausted in the first 6 hours, suggesting that after this time point, amino acids are the sole carbon supplier. This was mirrored in the consumption of amino acids, as more amino acids were metabolized in media containing 10 mM of glucose than 100 mM of glucose (**Figure 3.12 A**).
To gain insight into the nitrogen preferences used under conditions more similar to the gut, we additionally tested this under anaerobic conditions. Anaerobically, we saw a similar impact of the glucose concentration on the growth of *K. pneumoniae* as we observed aerobically (**Figure 3.13 B, D, and F**). The main difference seen, was *K. pneumoniae* not growing in media with a glucose concentration of 0 mM (**Figure 3.13 B and D**), suggesting that amino acids are not able to sustain the bacterial growth as a sole carbon source under anaerobic conditions. We believe that this is a product of the metabolic pathways, that result in the accessibility of amino acids as carbon sources and rely mainly on the availability of oxygen. We will discuss the metabolic pathways of amino acid utilization in more detail later in this study.

Our results showed the utilization of amino acids as a carbon source *in vitro*. To evaluate our findings in an *in vivo* context, we revisited a previous experiment, in which GF mice were colonized with *K. pneumoniae* and tested the concentration of amino acids in fecal samples (**Figure 3.8 D**). The concentration of the targeted amino acids were quantified before (day 0) and after (day 8) colonization with *K. pneumoniae* in the stool via NMR. After colonization with *K. pneumoniae*, the analysis of the fecal amino acid concentration revealed a significant decrease, suggesting consumption by *K. pneumoniae* (**Figure 3.8 D**). The simultaneous release of ammonia (**Figure 3.8 B**), measurable in ΔUrease although no urea was consumed (**Figure 3.8 C**), is consistent with the notion that amino acids are being deamidated as they are consumed as a carbon source by *K. pneumoniae*.

3.2.6 Amino Acid Utilization of *K. pneumoniae*

We previously showed *K. pneumoniae* to be able to utilize a great variety of different compounds as a sole carbon or nitrogen sources (Figures 3.7 and 3.11). We assessed the compounds available in the panels and noted the different amino acids used as carbon and nitrogen sources, respectively (Figures 3.14).
The amino acids *K. pneumoniae* is able to use as a single nitrogen source are, in descending order of maximum growth and under aerobic conditions, L-asparagine, L-glutamine, L-cysteine, L-serine, L-histidine, L-alanine, and L-tryptophane, while anaerobic conditions lead to limited growth on any of the aforementioned amino acids (**Figure 3.14 B**). This distribution changed, when we investigated the amino acids that can be utilized as a carbon source. Under aerobic conditions, in descending order, these are L-histidine, L-glutamine, L-proline, L-alanine, L-glutamate, and L-serine. Anaerobically, some amino acids could be utilized for carbon including L-aspartate, L-serine, L-proline, and L-glutamine (**Figure 3.11**). The amino acids used as both a nitrogen and a carbon source are L-histidine, L-glutamate, L-glutamine, L-alanine, L-aspartate, and L-serine. This suggests that amino acid utilization is highly selective and dependent on the metabolic needs of the organism. We wanted to determine the amino acids that are preferably used in a mixed-

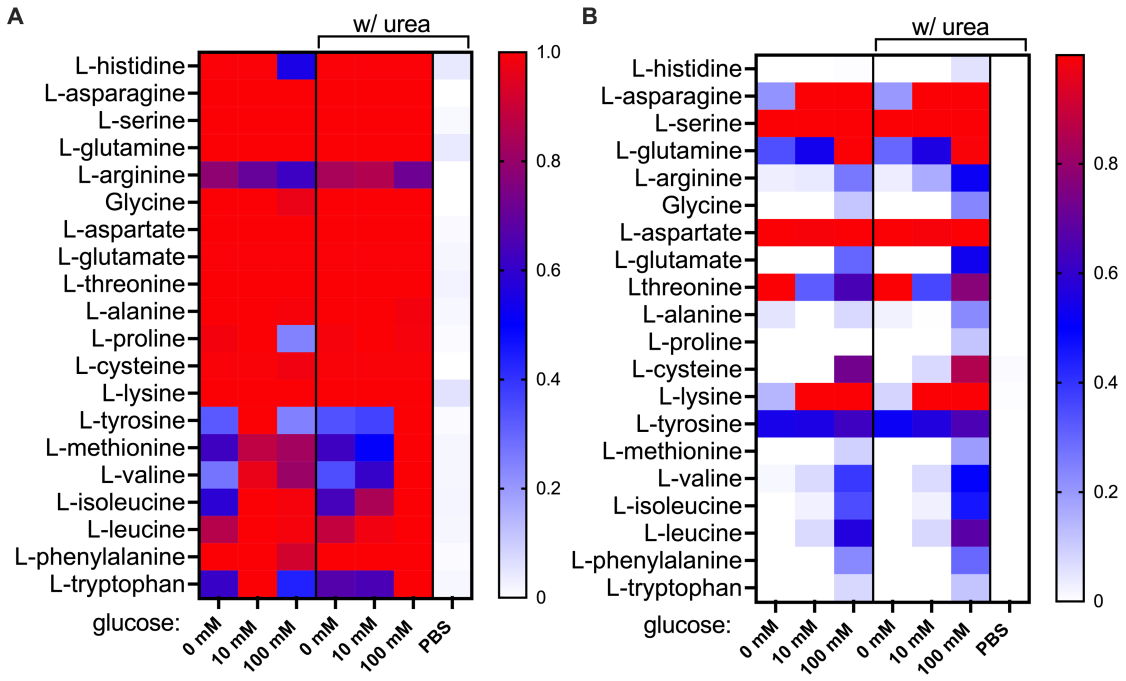

Figure 3.12: Percentage of amino acids used over a 24 hour growth period in MOPS media with all amino acids at equal concentration, different amounts of glucose added (0 - 100 mM), with or without added urea, PBS control with 100 mM of glucose; (**A**) aerobic; (**B**) anaerobic.

amino acid environment and when the bacteria is encountering changing availabilities of nitrogen and carbon, as is the case in the intestinal tract due to changes in diet as well as absorption of nutrients by the host.
To investigate this preference, we revisited a previous experimental set-up, where we utilized a defined media and added a mixture of all proteinogenic amino acids in equal-molar concentration. We monitored the growth over 24 hours and measured the concentration of amino acids and other metabolites. We additionally tested the impact of increasing quantities of free carbon and nitrogen sources in the form of glucose and urea to determine the impact of nutrient limitation on amino acid consumption (**Figure 3.12**).

L-proline was the only amino acid shown to only be utilized as a carbon source in our previous assays (**Figure 3.14 A and C**). In our experiment testing amino acid consumption, L-proline was consumed with all glucose concentrations, but utilization decreased at the highest glucose concentration (**Figure 3.12**). This may be due to the fact that nitrogen becomes limiting under those circumstances, thus limiting the need for proline consumption. When urea was added, supplying more nitrogen, all of the L-proline contained in the media was metabolized. L-proline is barely consumed anaerobically. Previous work shows that the degradation of L-proline results in the formation of L-glutamate [**92**]. This pathway results in the production of energy in the form of Nicotinamide adenine dinucleotide (NADH). As a result, L-proline can be metabolized as a source of carbon and subsequently energy [**93**]. However, even though L-proline is degraded into L-glutamate, it is unable to sustain substantial growth as a sole nitrogen source (**Figure 3.14 B**).

L-asparagine, L-cysteine, and L-tryptophan only sustain the growth of *K. pneumoniae* as a nitrogen source. We observed that they are exhausted in all media conditions, suggesting that under

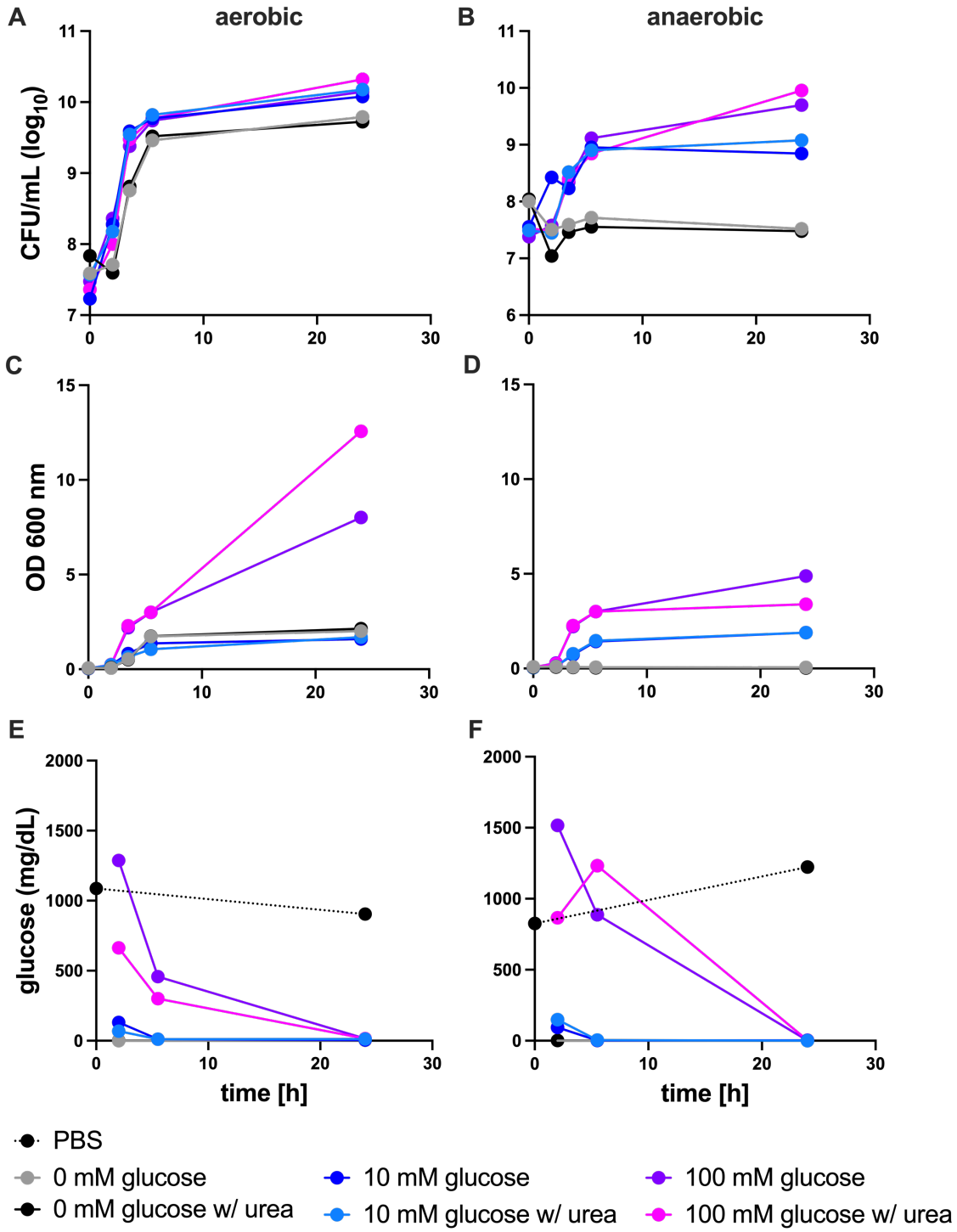

Figure 3.13: Quantification of growth of the *K. pneumoniae* WT over 24 hours, urea added as specified; measured **(A, B)** via CFU/mL; **(C, D)** via OD_{600nm}; **(E, F)** concentration of glucose over the time of growth; PBS control with urea as well as 100 mM of glucose; **(A, C, E)** aerobic; **(B, D, F)** anaerobic.

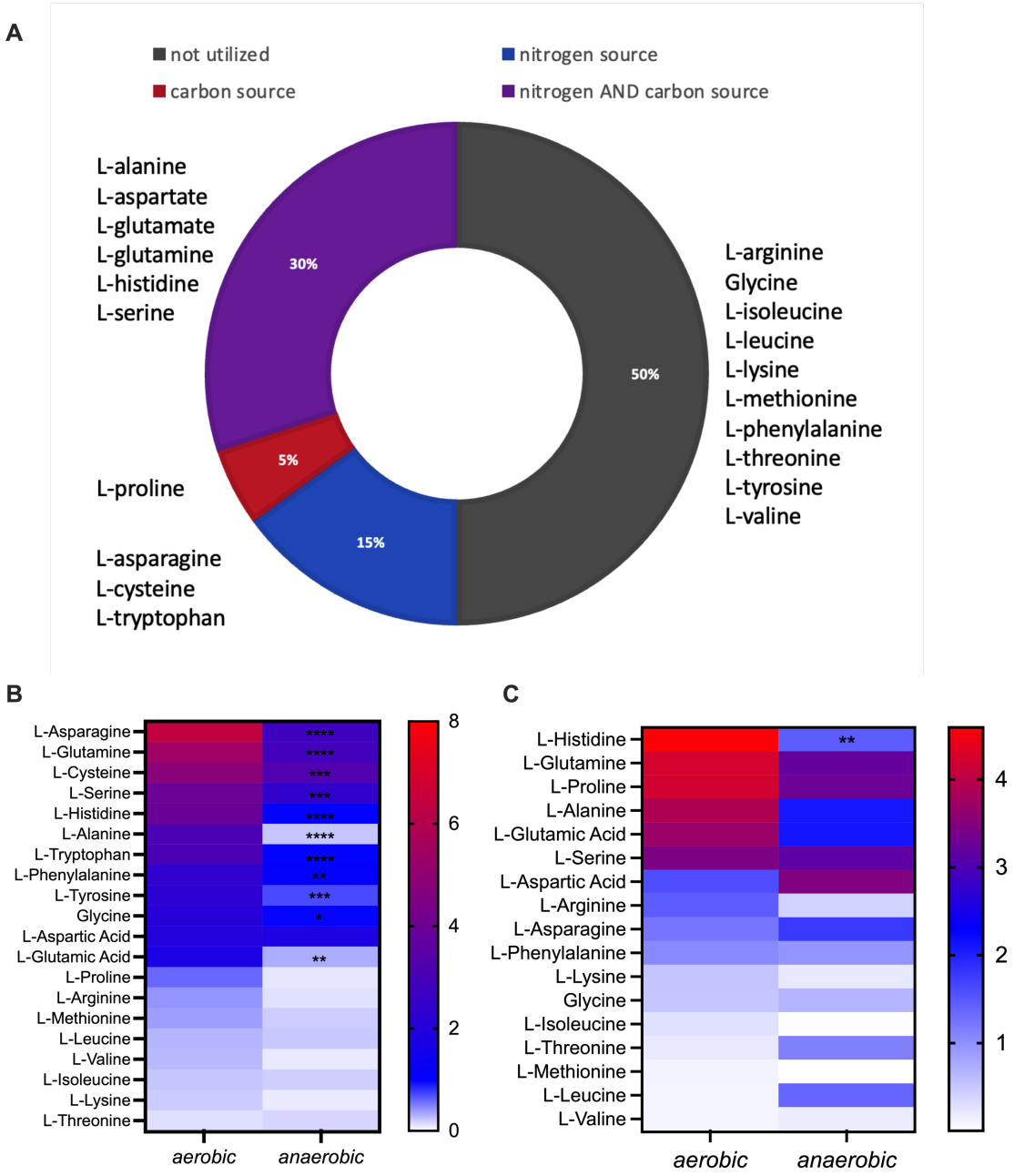

Figure 3.14: **(A)** Graph showing the percentages of amino acids being utilized for either nitrogen or carbon, as well as for both or not being utilized for either; **(B)** heat map showing the growth of the K. pneumoniae WT on proteinogenic amino acids as a single nitrogen source, arranged from most to least growth aerobically; (**C**) heat map showing the growth of the *K. pneumoniae* WT on amino acids as a single carbon source, arranged from most to least growth aerobically (left panel) with the corresponding anaerobic values (right panel); two-way ANOVA with Šídák correction for multiple comparisons, 95% confidence interval, n.s. not significant, *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001.

these circumstances, with increasing glucose concentrations, nitrogen has become limiting prior to carbon (**Figure 3.12**). Tryptophan is not always consumed to its full extent. This, however, may be due to the low solubility of tryptophane, which led to difficulty normalizing the starting concentration and may have interfered with measurements. Under anaerobic conditions, only L-asparagine is utilized with all glucose concentrations, yet it is only consumed partially in 10 mM glucose media, suggesting carbon availability is limiting growth before L-asparagine becomes depleted. L-cysteine and L-tryptophane are only metabolized by the bacteria in 100 mM glucose media. This suggests that these amino acids are only utilized when high quantities of nitrogen are needed as they may be harder to metabolize for nitrogen than L-asparagine or other amino acids. The degradation of L-asparagine involves the hydrolyzation via the enzyme asparaginase into L-aspartate and ammonium [**94**].
The degradation of L-cysteine in bacteria results in the formation of hydrogen sulfide. This pathway has not yet specifically been shown in *Enterobacteriaceae*, yet it is deduced from similar gene sequences found responsible for the mammalian pathway of L-cysteine degradation. Another product of this pathway is pyruvate, which as the end product of glycolysis can supply energy and function as a carbon source [**95**]. We observed the consumption of amino acids, specifically the ones solely used as a nitrogen source, to be dependent on the need for nitrogen in situations of carbon limitation. Our results led us to assume that *K. pneumoniae* may have preferences in amino acids, observed here under carbon limitation, that direct utilization.

L-alanine, L-aspartate, L-glutamate, L-glutamine, L-histidine, and L-serine can be metabolized as both a carbon and nitrogen source by *K. pneumoniae*. They are completely consumed by *K. pneumoniae*, both with and without added urea and in all glucose concentrations. Only L-histidine showed a lower utilization with 100 mM glucose and without added urea. This suggests that L-histidine may serve as a strong source of carbon, as it is not limiting in this media, but a poorer source of nitrogen, which is in congruence with our previous findings (**Figure 3.14 B and C**). Anaerobically, L-histidine and L-alanine are not consumed, while L-glutamine is utilized mainly in media with a higher glucose concentration (**Figure 3.12 B**). Considering it is one of the easily available nitrogen sources for *K. pneumoniae*, it is likely that it will serve as a nitrogen source in high-carbon environments. L-serine is depleted to its full extent with any glucose concentration, both aerobically and anaerobically.
The degradation of L-serine is catalyzed by an initial enzymatic reaction, which results in an unstable intermediate that immediately tautomerizes, building a structural isomer that is converted into pyruvate and ammonium via non-enzymatic hydrolyzation [**96**]. Pyruvate as the end product of glycolysis can subsequently be utilized for energy production. The pathway of degradation is consistent with our findings of L-serine being able to be metabolized as a sole nitrogen and carbon source (**Figure 3.14**). The deaminase responsible for the hydrolyzation into ammonium and pyruvate is also known in *E. coli*, where its production is correlated to the absence of glucose and its oxygen sensitivity suggests it mainly functions under anaerobic conditions [**96**]. However, we did not see the same outcomes in *K. pneumoniae*. Here, L-serine was consumed as a nitrogen and carbon source both under aerobic and anaerobic conditions, although to a varying extent (**Figure 3.14 B and C**), additionally we saw L-serine being consumed in both high and low concentrations of glucose present (**Figure 3.12**).
The degradation of L-histidine begins with the formation of ammonium and uronate via the enzyme histidase, which is then degraded further via multiple steps, subsequently resulting in the formation of L-glutamate and formamide. The nitrogen-containing compound formamide is not

metabolized further [**97**], which is apparent in formamide resulting in virtually no growth of *K. pneumoniae* (**Figure 3.7 A**). L-glutamate, however, can be metabolized further, resulting in the availability of L-histidine as a nitrogen and carbon source (**Figure 3.14 A**).
The first step of the L-glutamate degradation results in the formation of L-aspartate and is therefore also part of the synthesis of aspartate [**98**]. In a second step, the enzyme aspartase splits L-aspartate into ammonium and fumarate, which is subsequently used in energy production via the tricarboxylic acid (TCA) cycle [**98**]. Via the connecting degradational pathways, L-histidine, L-glutamate, and L-aspartate are all able to be utilized as a source for nitrogen and carbon, with L-histidine resulting in the formation of two ammonium molecules, L-glutamate and L-aspartate in one ammonium molecule and all subsequently entering the TCA cycle as fumarate. It is interesting to note, that the degradation of all three amino acids results in the same carbon-containing compound, but only L-histidine leads to substantial growth as a sole carbon source, while the growth on L-glutamate, L-aspartate, and the compound itself, fumarate, as the sole carbon source lags behind (**Figure 3.11 and 3.14 C**). Given the fact that L-glutamate is needed to produce L-glutamine in the presence of ammonia and serves as a storage for nitrogen, we suspect that it is mainly utilized in that process and not metabolized as a source of nitrogen or carbon itself. This would explain why we saw very minimal growth on L-glutamate as a single nitrogen or carbon source, yet the compound is completely exhausted in our mixed amino acid experiment (**Figure 3.12**).
L-glutamine is one of the most important nitrogen sources, as it is produced from L-glutamate and ammonia in order to store and supply ammonia to cellular processes and, therefore, serves as the main source of nitrogen, not affected by the nitrogen scavenging system [**48, 99**]. *K. pneumoniae* is able to grow on L-glutamine as a sole nitrogen or carbon source, respectively. The degradation of L-glutamine results in the formation of ammonia and L-glutamate [**99**].
E. coli can utilize L-alanine as a sole source of either carbon or nitrogen. As this is consistent with our data on *K. pneumoniae*, we deduce that the same deamination reaction is responsible for the metabolism of L-alanine in *K. pneumoniae* as is in *E. coli* [**100**].

In addition to the amino acids shown to facilitate growth in our previous commercial assays (**Figure 3.14 B and C**), glycine, L-aspartate, L-threonine, L-lysine, L-leucine, and L-phenylalanine are also consumed completely in the defined media containing all proteinogenic amino acids (**Figure 3.12**). Some additional amino acids are utilized marginally too. This suggests that they may be able to be utilized in conjunction with each other or are needed for other processes.
We, additionally, measured the concentration of urea and ammonia in samples from the aforementioned experiment in defined media containing all proteinogenic amino acids. We observed, as in our previous *in vitro* tests that, during the growth of *K. pneumoniae*, urea was hydrolyzed and its concentration decreased (**Figure 3.15 A**). Meanwhile, ammonia was produced by the hydrolyzation of urea and therefore the concentration increased (**Figure 3.16 A**). We have to note that this was mainly the case when glucose was present. In media with 0 mM glucose, urea was barely consumed, suggesting that under these circumstances carbon was the limiting nutrient. We observed that more ammonia was produced in the 10 mM glucose media, possibly due to the limited reuptake of ammonia by the bacteria as the growth became carbon-limited. This is supported by the fact that we did not see the same effect with 100 mM of glucose. We saw a similar trend under anaerobic conditions, however, the carbon limitation appeared to be more pressing, as urea was only depleted in 100 mM glucose media (**Figure 3.15 B**). It is interesting to note that, anaerobically, less ammonia is released into the media when glucose is present than otherwise. That may

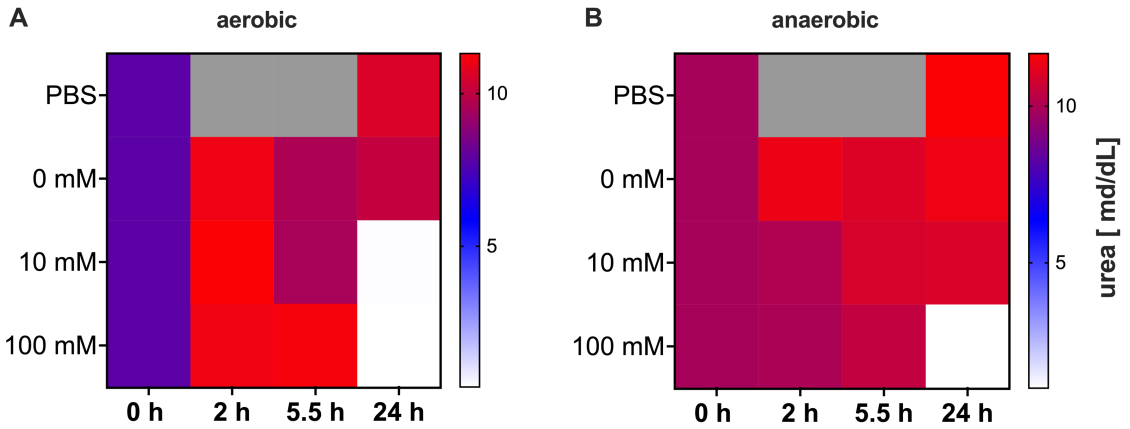

Figure 3.15: Concentration of urea over a 24 hour growth period in MOPS media with all amino acids at equal concentration, different amounts of glucose added (0 - 100 mM) and with our without Urea, PBS control with Urea as well as 100 mM of glucose; (**A**) aerobic; (**B**) anaerobic.

be due to the minimal growth without glucose that does not allow for the production of ammonia to the same extent (**Figure 3.16 B**).

3.2.7 *K. pneumoniae* Can Systemically Disseminate During Intestinal Inflammation

Under normal circumstances and in healthy individuals, the intestinal microbiome is not able to cross from the gut into the systemic circulation and adjacent organs, as the GVB inhibits the dissemination of bacteria [**60**]. Outgrowth of *K. pneumoniae*, as part of Proteobacteria, is often found in dysbiosis and IBD, which can increase suseptibility to dissemination of bacteria from the gut microbiome [**6, 7**]. We aimed to evaluate the effect of inflammation on the colonization and possible dissemination of *K. pneumoniae*. Our tested strain, *K. pneumoniae* MGH 78578, unlike other hypervirulent strains of *K. pneumoniae*, is not known to disseminate systemically, crossing the barrier between the gut and the systemic circulation, without host predisposition [**9,13**]. Since the disruption of the intestinal barrier function by intestinal inflammation, such as observed in IBD, reduces the ability to inhibit translocation of the intestinal bacteria to other organs, we examined the liver, spleen, and bile for bacterial growth.
Before euthanization, mice were treated with DSS and PEG. Modeling IBD with the use of DSS is a widely used approach in mouse models as it induces acute intestinal inflammation and its accompanying features of weight loss, diarrhea, and possible rectal bleeding [**89, 90**]. We observed dissemination of bacteria into all evaluated organs, namely liver, spleen, and bile (**Figure 3.17**). We additionally tested, whether urease, as a virulence factor of *K. pneumoniae*, had an impact on the dissemination of the bacteria. However, we were unable to show a significant effect of urease on the dissemination of *K. pneumoniae* (**Figure 3.17**).
Our data suggests that *K. pneumoniae* can disseminate systemically in the case of intestinal inflammation, possibly leading to subsequent infection. It additionally suggests that urease is not needed in the process of dissemination and does not grant an advantage in the colonization of compartments outside of the intestinal tract.

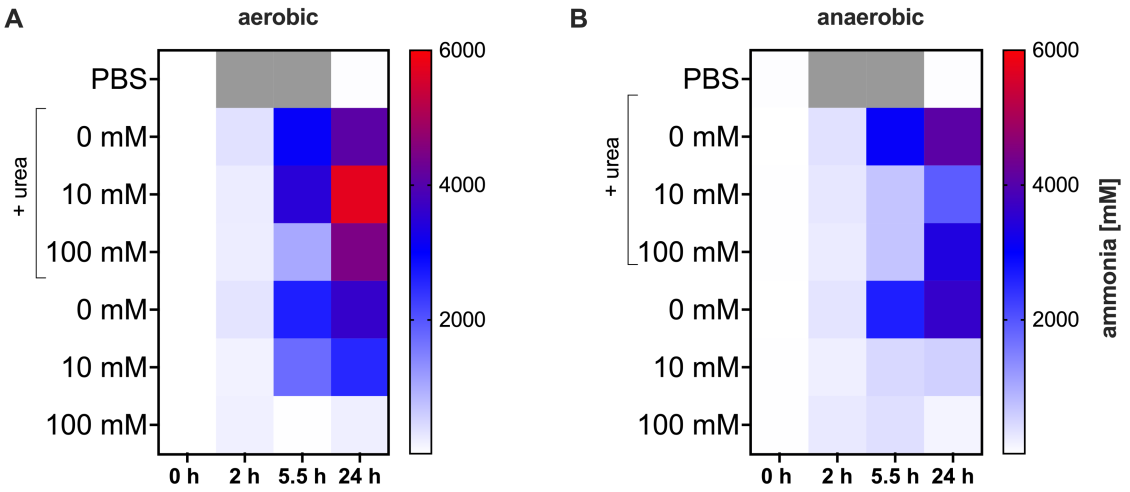

Figure 3.16: Concentration of ammonia over a 24 hour growth period in MOPS media with all amino acids at equal concentration, different amounts of glucose added (0 - 100 mM) and with our without Urea, PBS control with Urea as well as 100 mM of glucose; (**A**) aerobic; (**B**) anaerobic.

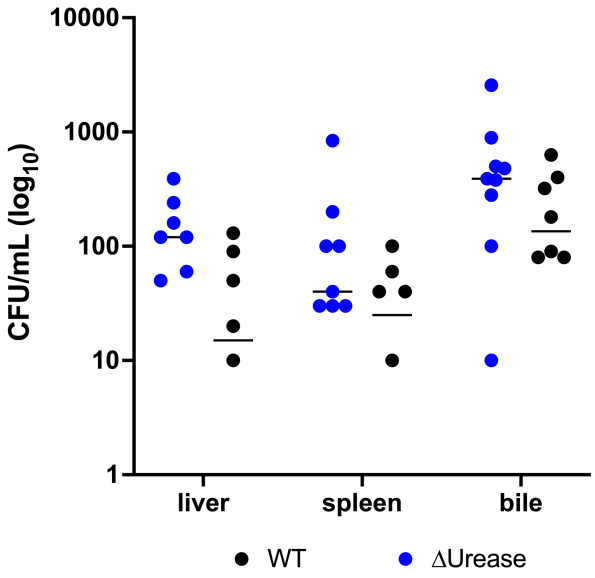

Figure 3.17: Dissemination of bacteria (CFU/mL) after euthanization; Results of two-way ANOVA with Šídák correction for multiple comparisons, 95% confidence interval, n.s. not significant, *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001.

3.3 Lactulose as an Additional Carbon Source in the Treatment of Hepatic Encephalopathy

3.3.1 Lactulose Does Not Inhibit the Growth of Urease-Positive *K. pneumoniae*

K. pneumoniae, as *Enterobacteriaceae*, is often increased in the intestinal microbiome of patients developing HE in the context of liver cirrhosis [**80**]. Lactulose is part of the treatment regimen used in patients with HE but the exact mechanism of action of the drug is not yet completely understood.
One hypothesized mechanism of action of the lactulose treatment is the inhibition of urease-positive organisms, such as *K. pneumoniae*, in the intestinal tract [**32, 83**]. This would subsequently lead to a decrease in ammonia production in the gut via hydrolyzation of urea and, therefore, decrease the risk for the development of HE through hyperammonemia. We hypothesized that treatment with lactulose will decrease the growth of *K. pneumoniae* and, therefore, increase the intestinal concentration of urea, as it is not being consumed.
To test this hypothesis, we conducted an experiment on the colonization by *K. pneumoniae* of the murine intestinal tract. To determine whether the observed result was due to urease specifically or rather a result of a different aspect present in urease-positive organisms, we additionally tested the artifically-made urease-negative *K. pneumoniae* ΔUrease. GF mice were gavaged with the bacteria and the colonization was monitored. During this initial part of the experiment, mice received regular drinking water *ad libitum*. We colonized one group of mice with the urease-competent *K. pneumoniae* WT (n=9) and a second group with the urease-deficient ΔUrease (n=9), adding lactulose to the drinking water of half of each group after 7 days. Therefore, half of the subjects were switched to a lactulose treatment and received water, containing 3% of lactulose, *ad libitum*. The remaining mice continued receiving regular drinking water. After 14 days, mice were euthanized and mucus was collected, as well as fecal samples from the intestinal tract. The gut was divided into sections and CFU was determined. We did not observe a significant difference in the colonization of the gut of the subjects receiving the lactulose treatment (**Figure 3.18 A**).
We additionally tested the urea concentration in the aforementioned samples, to observe possible interactions between to functionality of urease with the introduced lactulose. [**32, 79, 83**]. We did not see a significant difference in the concentration of urea between the groups receiving the lactulose treatment and the control groups (**Figure 3.18 B**).
Contrary to our hypothesis, we did not see a difference in the colonization of the murine intestinal tract in mice receiving lactulose, compared to the control group receiving regular drinking water. Our results suggest that lactulose does not inhibit the growth of *K. pneumoniae*, a urease-positive organism, in the gut of GF mice.

3.3.2 Lactulose Can Be Consumed as an Alternative Carbon Source by the Microbiome, Leading to a Decrease in Ammonia Production

Another hypothesis is that lactulose, as an available carbon source for the microbiome, increases bacterial growth and therefore the utilization of ammonia in anabolic processes rather than excretion into the lumen and subsequent absorption by the host [**84, 85**].

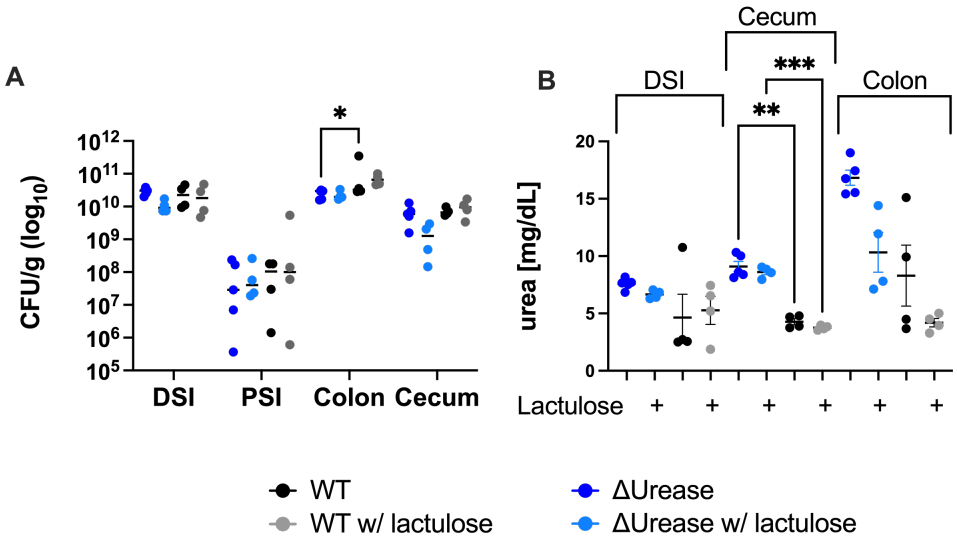

Figure 3.18: GF mice were inoculated with *K. pneumoniae*, after 7 days half the cohort was given lactulose (3%) via drinking water, and euthanization after 14 days. (**A**) Colonization of the intestinal tract (CFU/g of stool) after euthanization; (**B**) Concentration of urea in the intestinal tract after euthanization, (**A**) results of two-way ANOVA with Šídák correction for multiple comparisons, 95% confidence interval, n.s. not significant, *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001, (**B**) results of one-way Brown-Forsythe and Welch ANOVA test with Dunnett T3 correction for multiple comparisons, 95% confidence interval, n.s. not significant, *p<0.0332, **p<0.0021, ***p<0.0002, ****p<0.0001.

We first reviewed the growth of *K. pneumoniae* WT and our previously established strain, Δ*ntrC*, on lactulose as a sole carbon source using a commercially available array, by extracting the data from a previous experiment (**Figure 3.11**). Δ*ntrC* was included in this investigation, as the strain was needed in the following experiment, to determine whether changes in the utilization of amino acids as nitrogen sources play a role in the function of lactulose. Our results show that lactulose can serve as a sole carbon source to support the growth of both tested *K. pneumoniae* strains (**Figure 3.19**). Additionally, we did observe Δ*ntrC* to have a growth advantage over the WT on lactulose as a sole carbon source. Lactulose was able to sustain the growth of the *K. pneumoniae* WT under aerobic as well as anaerobic conditions. Knowing that *K. pneumoniae* is able to consume lactulose as a carbon source to sustain growth, we hypothesized that the addition of lactulose into media would lead to increased growth of the bacteria, since we previously established that carbon-limitation is more pressing during colonization and will lead to metabolization of available amino acids as a carbon source (**Figure 3.8**). The deamination process involved in the utilization of amino acids as carbon sources releases additional ammonia. We, therefore, additionally hypoth-

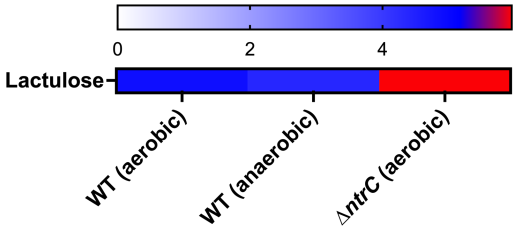

Figure 3.19: AUC of the growth of the WT and Δ*ntrC* mutant on lactulose in aerobic or anaerobic environments on a commercially available assay.

esized that the addition of another carbon source, in this case, lactulose, will lead to decreased deamination of amino acids and therefore to a decrease in ammonia concentration.
As a first step, we monitored the growth of *K. pneumoniae* (WT, Δ*ntrC*, and ΔUrease) in MOPS media, containing all 20 proteinogenic amino acids, under the addition of lactulose. Δ*ntrC* and ΔUrease were included to investigate possible differenes in the growth on lactulose when the utilization of amino acids as a nitrogen source was limited, leading to more available amino acids, or the hydrolization of urea as a nitrogen source was restricted, leading to a greater consumption of amino acids as a nitrogen source. We only saw a marginal increase in the growth of the strains receiving additional lactulose compared to the ones having only amino acids available as a carbon source (**Figure 3.20**). This effect was not only visible in the WT but also in Δ*ntrC* and ΔUrease. The advantage of lactulose as an additional carbon source seemed greater in Δ*ntrC* and ΔUrease, especially under anaerobic conditions (**Figure 3.20 B**). We additionally tested the concentration of ammonia in our samples to evaluate the effect of the lactulose treatment on the production of ammonia. The absolute concentration of ammonia in the media increased in the first 6 hours of all samples. Following that, only the bacteria in the media without lactulose continued to produce ammonia (**Figure 3.20 A**). The absolute ammonia concentration in the samples with added lactulose decreased substantially. Since we saw different rates at which the bacteria grew, depending on whether lactulose was added, we calculated the concentration of ammonia per CFU in our culture, to receive a more comparable value. We were able to observe a similar effect, as the addition of lactulose to the media led to a decrease in ammonia concentration per CFU (**Figure 3.20 C**). By combining the aforementioned data, we revealed that the addition of lactulose led to increased growth of *K. pneumoniae*, while simultaneously decreasing the amount of ammonia released into the environment. As oxygen is not available in all of the intestinal tract, we repeated the experiment under anaerobic conditions. The absolute ammonia concentration did not rise to the same levels as aerobically (**Figure 3.20 B**). After an initial increase, the ammonia concentrations remained stagnant, except for in the media that contained both lactulose and urea. The ammonia concentration per CFU decreased in the lactulose-containing cultures (**Figure 3.20 D**), yet it remained at a higher level than aerobically, suggesting that the difference in metabolism under anaerobic conditions, previously established (**Figure 3.12**), may lead to a higher ammonia production.
As discussed, it is hypothesized that the increased biomass due to the addition of lactulose as a carbon source will lead to greater incorporation of ammonia into anabolic processes, removing it from the environment. We saw the ammonia concentration in the media decrease as the *K. pneumoniae* colonization grew. Biomass increased, while the ammonia concentration in the environment decreased, which woud support the hypothesis. However, if this was solely due to an increase in biomass and incorporation of available ammonia, the ammonia concentration produced per CFU should remain the same, as the metabolism of the bacteria itself would not change. Nevertheless, when we calculated the amount of ammonia produced per CFU of *K. pneumoniae*, we saw that lactulose addition led to a decrease. This is likely due to a decrease in deamination of amino acids, as lactulose acts as a carbon source, making them available as nitrogen sources, decreasing ammonia production of the bacterial metabolism. Therefore, the overall decrease in ammonia concentration is not only ascribable to the increase in biomass but additionally to the change in metabolism of amino acids.

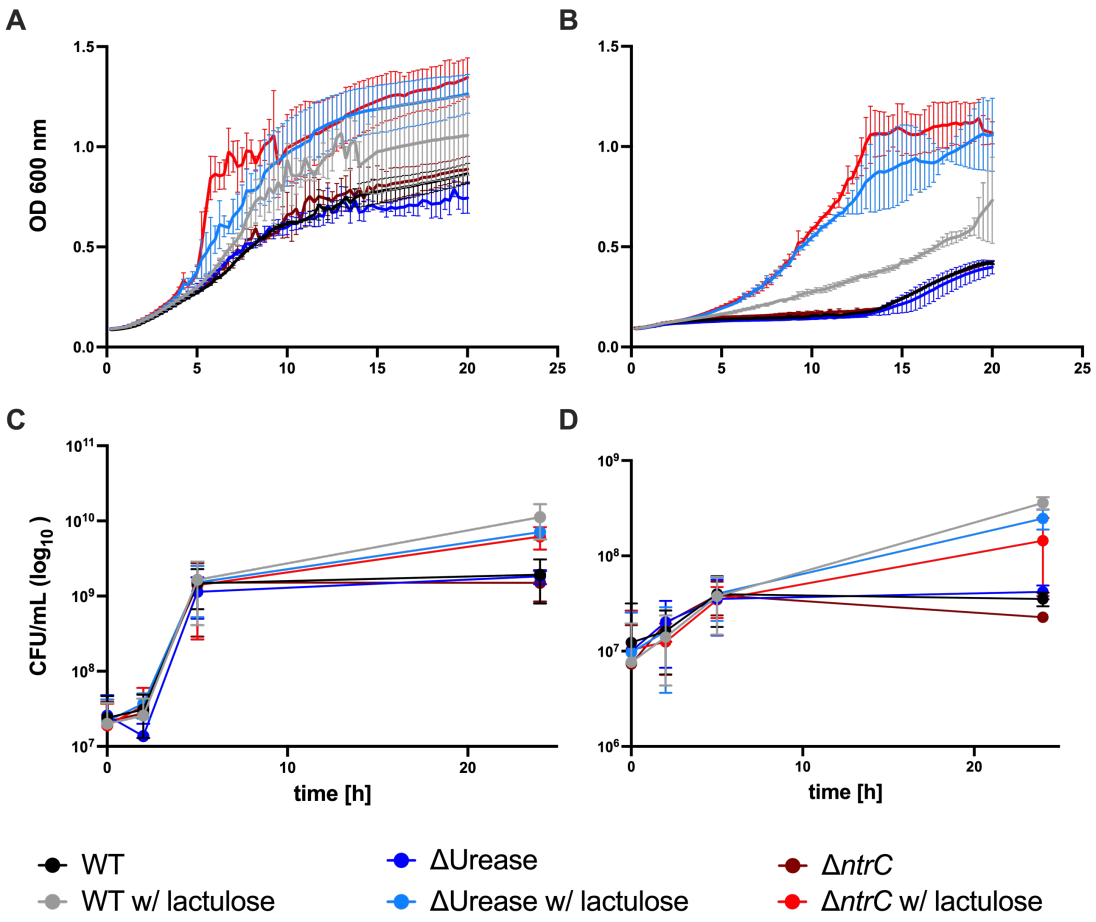

Figure 3.20: Growth of *K. pneumoniae* over 24 hours in MOPS media containing all 20 proteinogenic amino acids; (**A**) aerobically and (**B**) anaerobically, measured continuously via OD at 600nm; (**C**) aerobically and (**D**) anaerobically, measured via CFU/mL.

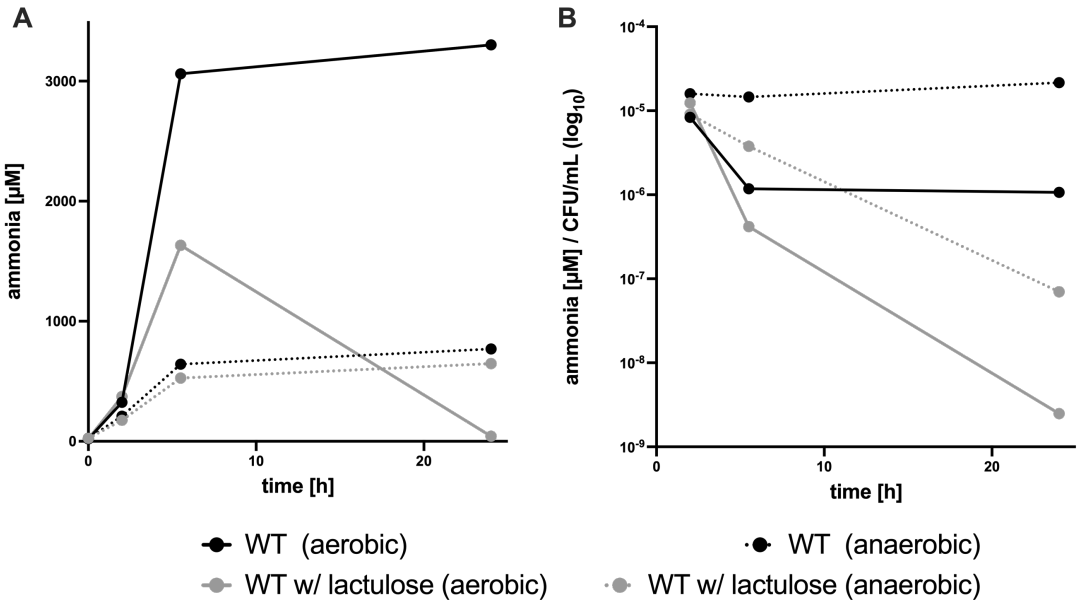

Figure 3.21: Growth of *K. pneumoniae* over 24 hours in MOPS media containing all 20 proteinogenic amino acids; (**A**) production of ammonia (in μM) over time, aerobically and anaerobically, with and without the addition of lactulose; (**B**) production of ammonia (in μM) over time, calculated per CFU/mL, aerobically and anaerobically, with and without the addition of lactulose.

3.3.3 Metabolic Analysis of the Effects of the Lactulose Treatment *In Vivo*

Our results *in vitro* suggested that treatment with lactulose influences the production of ammonia by the microbiota (**Figure 3.21**). We hypothesized this to be a result of changes to the amino acid metabolism, subsequently decreasing the amount of ammonia that is released into the environment. To verify this hypothesis, we evaluated the concentration of amino acids in the gut *in vivo*, after lactulose treatment.

GF mice, colonized with *K. pneumoniae* WT, were given regular drinking water for 7 days, after which they were switched to water containing 3% lactulose, *ad libitum*, for a week. Amino acid concentrations in cecal samples were tested via HPLC, after euthanization, and compared to a control group of mice that continued to receive regular drinking water. The concentration of amino acids in the fecal samples stayed consistent regardless of the added lactulose (**Figure 3.22 A**). Ammonia was tested in the same samples from cecal material and, likewise, showed no significant difference between the group receiving the lactulose treatment and the group continuing on regular water (**Figure 3.22 A**). These results stand contrary our hypothesis, suggesting that the effects of lactulose we saw *in vitro*, resulting in a decreased ammonia production in *K. pneumoniae*, do not maintain in an *in vivo* environment, as we did not see a decrease in ammonia concentration in the group treated with lactulose.

Another hypothesis states that lactulose inhibits the absorption of glutamine and its metabolization into ammonia by the intestinal mucosa [**84, 85**]. We did not find a change in the glutamine concentration in the group receiving the lactulose treatment, contrary to the increased glutamine concentration we were to expect if its absorption and metabolization by the host was inhibited (**Figure 3.22 A**). Overall, our results led us to believe that lactulose does not interact with the

amino acid metabolism by *K. pneumoniae* in the intestinal tract nor that it leads to changes in the absorption of amino acids by the intestinal epithelium.
Furthermore, the concentration of a variety of metabolites was tested, including the SCFAs acetate, formate, fumarate, propionate, and the branched chain acid 3-hydroxyisovalerate, as well as sucrose, and derivates of glycolysis and the TCA cycle, such as succinate and pyruvate, in fecal samples, obtained from GF mice colonized with *K. pneumoniae* before (day 8) and after (day 15) the initiation of the lactulose treatment. We saw a non-significant change that occurred after the lactulose treatment in acetate, which decreased in concentration after the addition of lactulose (**Figure 3.22 B**). Other metabolites, such as succinate and lactate, saw a slight, however, non-significant decrease after treatment with lactulose, while the concentrations of the remaining SCFAs stayed consistent (**Figure 3.22 B**).
Lastly, it is hypothesized that acidification of the gut lumen and the subsequent increase of ammonium ions relative to ammonia may lead to decreased intestinal absorption of ammonia [**32**]. To quantify the concentration of ammonia, we tested both the ammonium ion (NH_4^+) and ammonia (NH_3), in conjunction, subsequently labeled ammonia. We neither saw an increase in the concentration of ammonia in our *in vitro* test (**Figure 3.21**), nor did we see a change in the concentration of ammonia *in vivo* (**Figure 3.22 B**). As we saw an overall decrease in the concentration of ammonia in our media study, but observed an unchanged ammonia concentration in the murine model, it is possible that an increased ratio of ammonium ion led to its retention in the gut lumen and, therefore, a seemingly unchanged ammonia concentration *in vivo*. However, more tests will be needed to investigate this possible relationship between lactulose and the intestinal ammonia absorption further.

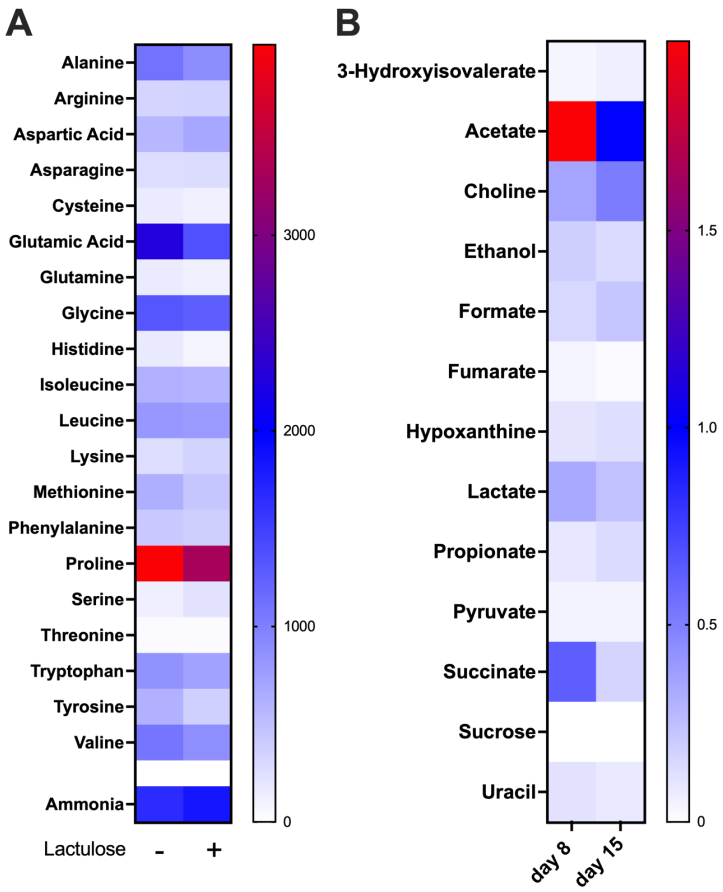

Figure 3.22: Levels of stool (**A**) amino acids and ammonia, measured via UPLC testing in cecal samples from *K. pneumoniae*-colonized GF mice and (**B**) metabolites, measured via NMR testing before (day 8) and after (day 15) start of lactulose treatment in *K. pneumoniae*-colonized GF mice; results of Mann-Whitney's *U*-tests with multiple comparison two-stage step-up method of Benjamini, Krieger and Yekultieli, not significant $q>0.01$, $^*q<0.01$.

4. Discussion

In this section, we present the main conclusions of our research and discuss how they can be interpreted in light of the current literature. Additionally, we explore how our results may change the current understanding and close research gaps. Ultimately, the limitations of this study are identified and proposals for future research are made.

Urease is a well-described virulence factor in a variety of gastrointestinal organisms [**22, 26, 27**]. However, the specific role of urease during the intestinal colonization of *K. pneumoniae* is not completely understood. We hypothesized that urease would increase the colonization fitness of *K. pneumoniae* by making urea available as an additional nitrogen source as the gut is believed to be a nitrogen-limited environment, due to the absorption of dietary protein by the host before it reaches the colon.
Our results demonstrated that urease provided a growth advantage in *in vitro* during nitrogen limitation (**Figure 3.1 A-C**). Under these circumstances, the ability to hydrolyze urea and access it as an additional nitrogen source increased the bacteria's colonization fitness and led to greater growth. *In vivo* inoculation, however, appeared to not require urease for successful colonization. We observed that ΔUrease colonized the murine intestinal tract in mono-colonization without a growth disadvantage compared to the WT (**Figure 3.4 A**). Additionally, contrary to our prediction, the urease-deficient ΔUrease outperformed the urease-positive WT in direct competition (**Figure 3.4 B and C**). These results led us to believe that urease may not be as essential in the process of colonizing the mammalian gastrointestinal tract as previously believed and the enzyme may rather be necessary for other functions in this species.
Another prediction of the function of urease as a virulence factor was derived from other organisms such as *H. pylori*. We hypothesized that urease in *K. pneumoniae* would similarly increase survival when encountering acidity by neutralizing the pH through the production of ammonia. However, our results revealed that urease was not advantageous for *K. pneumoniae*in the survival of an acidic environment. We hypothesized that conditions of increased SCFA concentration, as is often the case in the colon, would negatively impact the growth of *K. pneumoniae* and wanted to investigate the impact of urease. The effects of urease on the survival of presence of increased SCFAs concentrations, leading to an acidic environment, did not have a uniform appearance, as growth in a pH of 6 (**Figure 3.3 B and F**) and the addition of acetate of the urease-negative strain ΔUrease was weaker compared to the colonization of the WT under identical conditions. However, in media with a pH of 5 (**Figure 3.3 A and E**) we were able to observe ΔUrease outperform the WT in the presence of acetate. Given these results, it is possible that the neutralizing effect of urease in the presence of SCFAs is pH-dependent and mainly plays a role in lower pH environments. Our data alone is unable to reveal the exact mechanism of how urease interacts with its surrounding pH environment. Nevertheless, by modeling obstacles *in vitro* of the colonization of the gastrointestinal tract (namely acidity of the gastric and colonic environment), we showed that urease does not pose an advantage during the passage of the gastric acidic environment or during encounters of increased SCFA concentrations in the colon.
Our experiments revealed that urease is not needed for successful colonization of the murine intestinal tract by *K. pneumoniae*. We interpret this result as an indication for there to not be a need for the utilization of urea as a nitrogen source. Future research will be needed to determine the specific role of urease in *K. pneumoniae* that is responsible for its virulence, for example the

relationship between urease and its effects on pH in *K. pneumoniae*.

We were able to show that nutrition and diet can have an effect on the colonization of the intestinal tract with *Enterobacteriaceae* like *K. pneumoniae* in *in vitro*, as well as in *in vivo* models, such as the FARMM study. This may be partly due to alterations of the available nutrition in the intestinal tract, supplied through the host's diet but other factors may play an addtional role in this interaction that were not specifically examined in this study and will need further evaluation. One key element interacting with the microbiota may be the fiber content of the host's diet. Further studies by our group have evaluated the effects of fiber on the microbiota, suggesting that prebiotic interventions may have implications for future treatment options of diseases such as IBD [**45**].
Similar to other *Enterobacteriaceae*, *K. pneumoniae* is considered to play a crucial part in IBD and other critical diseases, including sepsis, urinary tract infections, or cholangitis, that can be a result of dissemination of bacteria from the gastrointestinal tract [**8, 9, 11, 13**]. We uncovered specific dietary factors that can increase the colonization of *K. pneumoniae* in the gut and, therefore, favor dysbiosis and increase the likelihood of IBD. This knowledge can guide us in understanding how diet can increase the susceptibility of patients for IBD or systemic disease and how we can intervene before changes in the microbiome increase the risk of serious illness. We determined the importance of nutritional factors regulating the microbiome, specifically nitrogen- or carbon-containing nutrients, and their impact on the colonization of the murine and human gastrointestinal tract.
One dietary factor that plays a crucial role in the constitution of the microbiome is carbon and, therefore, carbohydrates. We found *K. pneumoniae* to thrive on simple carbohydrates as a source of carbon. On the contrary, the growth of *K. pneumoniae* was suppressed on complex carbohydrates, such as inulin or dextrin, which can be found in dietary fiber [**45**]. Disproportionate dietary simple carbohydrates can overload the absorption in the small intestine and increase the availability in the colon, leading to an outgrowth of dysbiosis-associated bacteria like *K. pneumoniae* [**2**]. In addition to dietary choices, simple carbohydrates are also occasionally used as a treatment option. One example of this is the EEN diet, predominantly used to treat IBD in pediatric patient groups [**101**] and as a source of nutrition in intensive care unit (ICU) patients [**102,103**]. We revealed the EEN diet to favor the outgrowth of *Enterobacteriaceae*, in our case *K. pneumoniae*. *Enterobacteriaceae* are often involved in dysbiosis, which is an important feature of IBD. This would suggest that the EEN diet, often given as treatment for IBD, may favor the growth of organisms like *K. pneumoniae* that are found in dysbiosis and can lead to or exacerbate IBD. The EEN diet has previously been proven to induce remission in IBD patients [**104**], yet we found it to support the outgrowth of IBD-supporting microbiota. We presume additional factors, next to the modification of the gut microbiome, play a role in the effectiveness of the EEN diet as a treatment intervention for IBD [**105**]. Nevertheless, it is possible that the antibiotic intervention, implemented in the FARMM trial [**44**], had an impact on the effect of the EEN diet and we would see different results if tested in patients with IBD, who did not receive antibiotic treatment previous of the dietary intervention. To determine this, additional future studies will be necessary.
Dysbiosis is not the only complication of *K. pneumoniae* outgrowth. We showed previously (**Figure 3.17**), that *K. pneumoniae* is able to disseminate into other abdominal organs in the case of a disruption of the GVB, by employing DSS to induce colitis. While *K. pneumoniae* relies on simple carbohydrates as a source of carbon, other inhabitants of the colonic microbiome, namely *Bacteroides*, can utilize complex carbohydrates as a source of carbon and can metabolize the intestinal mucus layer in the case of insufficient dietary fiber as a nutritional source and an oversupply of simple carbohydrates [**2, 62**]. The overconsumption of simple sugars and subsequent excess

supply in the intestinal tract, in conjunction with the lack of dietary fiber in a diet high in simple carbohydrates, would favor the outgrowth of *Enterobacteriaceae*, like *K. pneumoniae*, while potentially leading to the degradation of the protective mucus layer by bacteria, such as *Bacteroides*. Together, this creates an advantageous environment for dysbiosis and translocation of the intestinal bacteria through the weakened mucus layer and subsequent systemic dissemination. In critically ill ICU patients, the application of an EEN diet without the supplementation of fiber may favor the outgrowth of *Enterobacteriaceae* and, therefore, predispose the patient to dysbiosis, subsequent dissemination, and systemic disease. This creates an opportunity for future pre- and probiotic treatment options via dietary interventions, employing an increased ratio of dietary fiber.

Another dietary component with a great impact on the intestinal microbiome is nitrogen. It was long believed that nitrogen is one of the biggest limiting factors of bacterial growth in the gut microbiome, as it was hypothesized that the intestinal tract is a nitrogen-limited environment, given that most dietary nitrogen is absorbed by the host in the small intestine and thus unavailable to sustain the intestinal microbiome [**3, 40**]. Contrary to this, we observed nitrogen to not be a constraining factor for the growth of *K. pneumoniae* in the gut. Urea, a nitrous waste product expelled into the gut by the host, can function as a nitrogen source for urease-producing organisms. In our human data from the FARMM study [**44**], we suspected urease to be of an advantage, utilized by *K. pneumoniae* during the reconstitution of the microbiome. However, after testing the virulence factor extensively *in vitro* as well as *in vivo*, we determined urease to not play an essential advantageous part in the colonization of the gut. Additionally, we found the Ntr system, which makes alternative nitrogen sources available in the case of nitrogen starvation, to not be a necessary factor in the colonization of the intestinal tract. It can be suspected that ammonia cross-feeding from urease-potent organisms can potentially sustain urease-negative species in the gut, as we saw in our experiments that *K. pneumoniae* produced a greater amount of ammonia than needed for its growth. In *K. pneumoniae*, however, this can be refuted, as the urease-deficient strain did not show a growth defect in GF mice, where no inherent microbiome was present to supply ammonia. On the contrary, we saw that the urease-deficient strain was able to sustain itself, likely in part through the availability of ammonia through the deamination of dietary amino acids. We conclude that the intestinal tract is not nitrogen-limited. Next to urea and ammonia, other nitrogen-suppliers in the intestinal tract are amino acids. *K. pneumoniae* is able to utilize many of them as a nitrogen, as well as a carbon source. As this mainly involves deamination, ammonia is not only produced by the hydrolyzation of urea but also by metabolizing amino acids.
We additionally observed the ability of *K. pneumoniae* to metabolize amino acids as both nitrogen and carbon sources, if needed, in human subjects, looking at samples from the FARMM study [**44**], previously conducted by members of our group. *K. pneumoniae*-high colonized patients showed increased amino acid concentrations compared to patients colonized with lower levels of *K. pneumoniae*. This shows that *K. pneumoniae* is able to alter the balance of the nitrogen composition of the intestinal tract, possibly favoring outgrowth of other *Enterobacteriaceae*, which may add to the vicious cycle of dysbiosis.
We found that not only is the nitrogen availability impacting the composition of the microbiome but *vice versa* the microbiome and colonization with different bacteria have an impact on the nitrogen composition of the gut.

HE is a complication of liver cirrhosis, in which the nitrogen composition of the gut can play a pivotal role. The absorption of intestinal ammonia has been linked to an increased risk of hyper-

ammonemia, which is believed to be a substantial contributor to the pathomechanisms underlying the development of the disease [**76, 78**]. The disaccharide lactulose can be used as a therapeutic agent to treat and reduce the risk of HE, acting through a number of potential pathways. One hypothesis is that lactulose inhibits urease-producing bacteria in the intestinal tract, one of those being *K. pneumoniae*, which would lead to a decrease in ammonia production through urea hydrolyzation [**32, 79, 83**]. This most likely happens via production of SCFAs through fermentation by the resident microbiome, for example *Firmicutes spp.* or *Bacteroidetes spp.*, which subsequently leads to acidification of the intestinal lumen [**32, 33, 83**]. Our results showed that the addition of lactulose did not inhibit the growth of *K. pneumoniae* but rather promoted its growth, by acting as an available carbon source (**Figure 3.20 and 3.19)**. As we utilized GF mice in our model, it is possible that the absence of the resident microbiome influenced these results through reduced concentrations of intestinal SCFAs, normally produced by commensal bacteria in the gut. Our group subsequently repeated this experiment in CR mice, which resulted in increased growth of *K. pneumoniae* in the murine gut [**45**]. This not only directly contradicts our original hypothesis, but additionally raised the question of the impact of the resident microbiome on the efficacy of lactulose as a treatment choice in HE. As commensal microbes are present under these circumstances, we are unable to say with certainty whether the results we see in our study conducted on GF mice are impacted by lower SCFA levels in the intestinal tract or whether additional mechanisms need to be considered.

Another aspect thought to explain the mechanism of action in the treatment of HE with lactulose is the fact that, as a carbon source, it can increase bacterial biomass in the gut, which, in turn, decreases available ammonia, being consumed as a nitrogen source in anabolic processes [**84, 85**]. We showed that lactulose increased biomass of the intestinal microbiome by acting as an additional carbon source (**Figure 3.20**). However, our results suggest that this is not the reason for the decrease in ammonia concentration (**Figure 3.21 A**), as we found lactulose to impact the ammonia production per unit of bacteria (**Figure 3.21 B**). This suggests that lactulose has an effect on the production of ammonia by the bacteria independent of solely increasing overall growth.

An alternative hypothesis is that lactulose impedes the absorption of glutamine and its conversion into ammonia by the intestinal mucosa [**84, 85**]. However, our findings indicate that the intestinal glutamine concentration stayed unchanged from its initial state prior to the initiation of the lactulose treatment, suggesting that lactulose did not interact with the amino acid uptake and metabolism in the host epithelium. Finally, increased concentrations of ammonium ions relative to ammonia, as a consequence of the acidification of the gut lumen, may subsequently result in a reduction in the ammonia concentration absorbed by the host [**32**]. We observed a decreased ammonia concentration (ammonium ion and ammonia) *in vitro*. This data, in conjunction with the *in vivo* observation of an unchanged ammonia concentration, may indicate that alterations in the ratio of ammonium ions to ammonia may result in changes in ammonia absorption. Further studies will be necessary to distinguish between the presence of ammonium ions and ammonia in the gut lumen and possible implications for the ammonia homeostasis of the host metabolism.

In conclusion, we were able to show that lactulose can decrease the ammonia concentration *in vitro* by changing the amount of ammonia produced by *K. pneumoniae*. Even though, we did not find a change in the ammonia concentration after lactulose treatment in murine cecal samples, it remains possible that the alterations in ammonia production by the bacteria can influence its concentration in the colon. A reduced production would lead to a lessened ammonia absorption in the intestinal tract and, therefore, decrease its effect on the CNS and subsequently the symptomatic burden of HE in patients with liver cirrhosis. Further investigations are needed to prove this and determine

the specific pathomechanisms involved *in vivo* and the implications this will have on the understanding of lactulose as a treatment in patients with HE. Comparing our results with additional studies done by our group raises the question of the impact the commensal microbiome has on the effectiveness of lactulose as a treatment. Future studies will be needed to determine the implications changes in the intestinal microbiome have on these effects and whether the composition of the gut microbiome, specifically changing percentages of *Enterobacteriaceae* and commensal microbes, may play a key role in understanding lactulose in regards to HE.

This study presents notable strengths that offer valuable insight into the metabolic processes of *K. pneumoniae*, thereby furthering the understanding of their implications for gut-related diseases.
Key metabolic pathways of nitrogen and carbon metabolization in *K. pneumoniae* are addressed, offering insight into the influence of available nutrients on the bacteria's ability to colonize the intestinal tract and impact disease states in the process. The focus on urease, which has been demonstrated to potentially play a role in the virulence of dysbiosis-associated organisms, revealed how this enzyme facilitates the growth of *K. pneumoniae* under nitrogen-limiting conditions and how it may contribute to the progression of diseases such as IBD and HE.
Our study effectively integrates *in vitro* experiments with *in vivo* tests on murine and human subjects, providing an comprehensive exploration of the processes involved in *K. pneumoniae* colonization and advantageous factors thereof. Observations from a study on human volunteers, previously conducted by members of our laboratory, were tested under controlled conditions *in vitro* and subsequently validated *in vivo*, employing mouse models. By following this progression, we were able to explore specific metabolic processes under controlled conditions in a laboratory setting. Furthermore, the utilization of murine models introduced a new layer of biological relevance by mimicking more complex physiological conditions. The assessment of samples obtained during the course of the aforementioned human study, provided insight into the extent to which the findings would be applicable to human physiology. This approach serves to reinforce the conclusions of this study, ensuring that the findings are reproducible across multiple experimental settings.
Investigating the implactions of *K. pneumoniae* colonization and the subsequent involvement in conditions such as IBD and HE is highly relevent to clinical medicine. A deeper understanding of how nitrogen and carbon sources are utilized within the intestinal tract, and how the ways in which particular dietary changes can be advantageous for growth of the opportunistic pathogen may facilitate the development of novel therapeutic interventions. One example of this is the potential of employing pre- and probiotic dietary interventions consisting of increased dietary fiber for the treatment or prevention of dysbiosis.
Sophisticated methodologies were employed, including quantitative assays, NMR-based metabolomics for the measurement of metabolite production, and recombinant DNA technology for the construction of mutant strains utilized to test specific aspects of the bacterial metabolism. These advanced techniques offer a more profound comprehension of the underlying processes and metabolic interactions occurring in both *in vitro* cultures and *in vivo* host environments.
Conventional murine models utilizing CR mice were enhanced by additionally conducting tests employing GF mice. As GF mice are specifically bred to have no inherent microbiome, they provide a unique opportunity to assess effects of colonization with selected organisms without possible interference or cross-reaction with the commensal microbiome. Tests involving GF mice were conducted in specialized laboratories at the University of Pennsylvania Veterinary school by, and under the guidance of, trained professionals. Strict protocolls were employed to minimize the risk of contamination and mice were kept in seperate cages to control for cross-contamination between

inoculated strains.
The objective of our investigation was to ascertain the role of *K. pneumoniae* in the reconstitution of the microbiome after antibiotic treatment. Our results shed light on the interconnections between nutrient availability, dysbiosis, and the host's intestinal microbiome.
The research contributes to a more comprehensive understanding of the interactions between the microbiome and the host, particularly in the context of antibiotic- or diet-induced dysbiosis and its long-term effects on gut health. Additionally, it investigates the involvement of the microbiome in the pathophysiology of HE and the subsequent interaction of ammonia-producing bacteria with treatment efforts. By integrating the underlying metabolic processes observed *in vitro* with their implications for diseases affecting the gut *in vivo*, the research provides a translational aspect that bridges basic science and clinical application. This may potentially inform future treatment stategies for IBD and offer insight into the mechanisms responsible for efficacy of lactulose in the treatment of HE.

Figures were designed to enhance comprehension while adhering to the physical constraints of this work and sized to optimize readability without impeding comprehension. Data sets were arranged to include results from connected experiments unless otherwise preferred to facilitate comprehension of the presented results. Graph descriptions were removed if it prevented redundancy of the figure.
To guide the reader along concepts discussed in this study and aid the connection of investigated hypotheses, conclusions were already included in part in the results section. However, conclusions discussed previously were additionally included in the discussion section.
To interpret the conclusions drawn from the results of this study, it is necessary to consider the limitations of the research and the methodologies utilized.
This study focused primarily on the enzyme urease, which can be regarded as an important virulence factor. However, it is not the sole component of virulence and other virulence mechanisms, such as biofilm formation, antibiotic resistance, and eversion of the host immune system could also play a significant role and influence the ability of *K. pneumoniae* to colonize the intestinal tract. Similarly, lactulose is merely one component of the therapeutic regimen employed to treat HE. The potential impact of interactions between lactulose and other elements of the therapeutic strategy, such as rifaximine or ornithine aspartate, on the results has not been investigated.
The study was conducted with a specific focus on sources of nitrogen and carbon. However, the diversity of available nutrients in the diet is greater than the range of compounds tested in the presented study. Furthermore, the fluctuating concentrations of these compounds in the intestinal lumen may influence their metabolization by *K. pneumoniae*, which was not assessed in this study.
A significant portion of our study was conducted under controlled conditions *in vitro*, which does not fully replicate the complexity of the gut environment in its natural state. *In vivo*, additional factors such as interactions with the commensal microbiome (comprising a variety of different microorganisms), host immune responses, and variations in nutrient and oxygen availability can affect the outcome is ways that are different from those observed in a laboratory setting. Further studies are required to investigate whether the results obtained from our *in vitro* models are fully reproducible *in vivo*.
Murine models are frequently employed to investigate pathophysiology prior to transfering the aquired data to studies involving human subjects. Nevertheless, modeling human physiology using mice is not without limitations, particularly when studying complex conditions such as IBD and HE, which are characterized by intricate interactions between systems in the human body.

Consequently, although murine models provide valuable insight into potential disease processes, the findings must be carefully reevaluated and approached with caution when translating them into human clinical conditions. Although human subjects were included in the investigation of this study, the sample size was relatively small and, therefore, individual variability in the gut microbiome and response to interventions were not able to be fully adressed.
The sample sizes employed in the investigations were relatively restricted and should be augmented for a more comprehensive investigation. Nevertheless, the use of a limited number of subjects allowed for the exploration of concepts and hypotheses, as well as the acquisition of insight into underlying processes, which can subsequently be subjected to further investigation. Murine models were solely conducted on female mice. Although, we did not observe a difference in results regarding sex in previous results from the human intervention study, it is possible that differences in physiology between the sexes may have an influence on our findings. This could be addressed in future studies by including both male and female mice in the tested cohorts.
Antibiotic treatment regimens were employed to induce microbiome depletion and prepare the intestinal tract of our murine and human subjects for colonization with the introduced organisms. It is possible that the treatment with antibiotics may have influenced the observed results and, therefore, may not fully represent the investigated clinical conditions.
In summary, while the study's methods are sound for investigating specific metabolic processes, they fall short in replicating the complexities of real-world conditions, both in the environment of the intestinal tract and in interactions with host's systems. The aforementioned limitations highlight the need for further research to validate and expand upon our findings, particularly in more complex and encompassing models of the gut microbiome interactions.

Ultimately, there are a number of conclusions we can draw from our study.
Urease provides an advantage in bacterial growth in nitrogen-limited environments by providing access to urea as an additional nitrogen source. Nevertheless, is not a prerequisite for the successful colonization of the mammalian intestinal tract. Given that alternative nitrogen scavenging mechanisms are equally ineffective in this context, we can infer that the mammalian intestinal tract is not a nitrogen-limited environment. Attempts to reproduce the mechanisms by which urease exerts its virulence in other urease-positive organisms were unsuccessful. Future research is required to assess the precise mechanisms through which urease functions as a virulence factor in *K. pneumoniae*.
Nutritional factors influence the compositions of the intestinal microbiome, which, *vice versa*, affects the nutritional profile of the gut lumen. *K. pneumoniae* has a preferrence for simple carbohydrates as a carbon source. Exessive ingestion of these, as is the case in the typical Western diet, favors the growth of *Enterobacteriaceae* such as *K. pneumoniae*. The relative lack of dietary fiber in the form of complex carbohydrates further contributes to the outgrowth of the dysbiosis-associated organisms. Subsequently, dysbiosis increases the likelihood of developing conditions such as IBD. Dietary interventions increasing the intake of fiber may prove beneficial for patients experiencing dysbiosis or suffering from IBD.
In vitro observations indicated the potential for lactulose to affect the metabolism of *K. pneumoniae*, reducing the amount of ammonia produced by the bacterium. However, these results were not reproducible *in vivo*, with cecal samples obtained from mice colonized with *K. pneumoniae* and receiving treatment with lactulose. Future research is necessary to elucidate the mode of action of lactulose *in vivo* and aid in understanding the commonly given treatment for HE.

5. Summary

In this thesis we explored the role of dietary nutrients, particularly carbon and nitrogen, on the colonization of the gut by *K. pneumoniae* and the subsequent implications for disease processes. Prior research has indicated that the intestinal tract is a nitrogen-limited environment. Our findings revealed that urease, a previously established virulence factor and associated with dysbiosis, facilitates the conversion of urea into an additional nitrogen source in nitrogen-limited environments, conferring a growth advantage to the bacteria under these circumstances. However, this function of urease was not observed to translate from the *in vitro* studies to the *in vivo* colonization of the murine intestinal tract. On the contrary, our findings indicate that supplementary nitrogen sources, made accessible by urease or nitrogen scavenging via the Ntr system, were not of assistance during the colonization of the gut. This suggests that, contrary to previous assumptions, the intestinal microbiome is not constrained by nitrogen limitation. Furthermore, our results revealed that the colonization of the gut by *K. pneumoniae* resulted in alterations of the nitrogen composition of the gut and change of the amino acid signature. It may therefore be assumed that the outgrowth of *Enterobacteriaceae* exerts an influence on the commensal microbiome. Amino acids present in the intestinal tract can be utilized as a nitrogen source, but additionally supply carbon, accessible via deamination. This sustains bacterial growth if carbohydrates are not present. We differentiate complex from simple carbohydrates, with the latter being associated with a decrease in fiber intake, particularly prevalent in a Western diet, and subsequent dysbiosis and the proliferation of *Enterobacteriaceae* in the gut. Carbon was identified as the limiting nutrient in the intestinal microbiome, specifically related to the growth of *Enterobacteriaceae* such as *K. pneumoniae*, with the introduction of additional carbon sources resulting in increased colonization. Fecal samples from a previous human dietary intervention, which revealed that *K. pneumoniae* prefers simple over complex carbohydrates, were analyzed using commercially available nutrient assays. It is important to consider that diets high in simple carbohydrates are predominantly consumed by patients with IBD. Dietary interventions that increase fiber intake may prevent the outgrowth of *Enterobacteriaceae* and subsequently protect against dysbiosis, IBD. One carbohydrate of particular interest in the context of this study was lactulose, which is commonly employed as a treatment for HE, a complication of liver cirrhosis. The precise mechanism of action of this host-indigestible disaccharide remains uncertain. Our *in vivo* and *in vitro* studies investigated the pathomechanisms and demonstrated that lactulose was capable of decreasing the ammonia production of *K. pneumoniae*, even though it increased the bacterial biomass as an additional carbon source. This suggests that lactulose may have an impact on the metabolism of the bacteria itself.
This summary encapsulates the main points from this study, providing a comprehensive overview of the research, methodologies, results, and implications discussed in the thesis.

6. Zusammenfassung

In dieser Arbeit wurde die Rolle von Nährstoffen aus der Nahrung, insbesondere Kohlenstoff und Stickstoff, auf Kolonisation des Gastrointestinaltraktes mit *K. pneumoniae* und deren Auswirkungen auf verschiedene Krankheitsprozesse untersucht.
Frühere Forschungsergebnisse deuten darauf hin, dass der Darm ein stickstoffarmes Milieu ist. Unsere Ergebnisse zeigten, dass Urease, ein bekannter Virulenzfaktor, in stickstoffarmen Umgebungen die Umwandlung von Harnstoff in eine zusätzliche Stickstoffquelle erleichtert und so den Bakterien unter diesen Umständen einen Wachstumsvorteil verschafft. Diese *in-vitro* beobachtete Funktion von Urease ließ sich jedoch nicht auf *in-vivo*-Studien zur Kolonisation des Darmes übertragen. Im Gegenteil, unsere Ergebnisse deuteten darauf hin, dass zusätzliche Stickstoffquellen, die durch Urease oder den Stickstoffabbau über das Ntr-System zugänglich gemacht wurden, bei der Besiedlung des Darms keinen Vorteil bringen. Dies deutet darauf hin, dass das Darmmikrobiom nicht, wie angenommen, durch Stickstofflimitierung eingeschränkt wird. Darüber hinaus zeigten unsere Ergebnisse, dass die Besiedlung mit *K. pneumoniae* die Stickstoffzusammensetzung und die Aminosäuresignatur des Darmes verändert. Ein übermäßiges Wachstum von *Enterobacteriaceae* könnte daher einen Einfluss auf das kommensale Mikrobiom ausüben. Die im Darm vorhandenen Aminosäuren können als Stickstoffquelle genutzt werden, liefern aber auch Kohlenstoff, der durch Desaminierung zugänglich ist. Dies unterstützt das bakterielle Wachstum, sollten keine Kohlenhydrate vorhanden sind. Es werden komplexe von einfachen Kohlenhydraten unterschieden, letztere werden in Verbindung mit einer geringeren Aufnahme von Ballaststoffen, wie sie vor allem in der westlichen Ernährung vorkommen, sowie mit Dysbiose und der Vermehrung von *Enterobacteriaceae* im Darm gebracht. Kohlenstoff wurde als der limitierende Nährstoff im Darmmikrobiom identifiziert, insbesondere für das Wachstum von *Enterobacteriaceae* wie *K. pneumoniae*, wobei die Einführung zusätzlicher Kohlenstoffquellen zu einer verstärkten Kolonisierung führt. Fäkalproben aus einer vorhergehenden Ernährungsstudie beim Menschen, bei der sich herausstellte, dass *K. pneumoniae* einfache gegenüber komplexen Kohlenhydraten bevorzugt, wurden mit handelsüblichen Nährstofftests analysiert. Es ist wichtig zu beachten, dass Patienten mit chronisch entzündlichen Darmerkrankungen überwiegend einfache Kohlenhydrate konsumieren. Diätetische Maßnahmen, die die Aufnahme von Ballaststoffen erhöhen, könnten das Wachstum von Enterobakterien verhindern und somit vor Dysbiose und chronisch entzündlichen Darmerkrankungen schützen. Ein Kohlenhydrat, das im Rahmen dieser Studie von besonderem Interesse war, war Laktulose, welches üblicherweise zur Behandlung von hepatischer Enzephalopathie, einer Komplikation der Leberzirrhose, eingesetzt wird. Der genaue Wirkmechanismus dieses für den Wirt unverdaulichen Disaccharids ist noch unklar. Unsere *in-vivo*- und *in-vitro*-Studien zeigten, dass Laktulose die Ammoniakproduktion von *K. pneumoniae* verringert, obwohl die zusätzliche Kohlenstoffquelle das Bakterienwachstum erhöht. Dies deutet darauf hin, dass Laktulose möglicherweise den Stoffwechsel des Bakteriums beeinflusst.
Diese Zusammenfassung fasst die wichtigsten Punkte dieser Studie zusammen und bietet einen umfassenden Überblick über die Forschung, die Methoden, die Ergebnisse und die daraus gefolgerten Auswirkungen, die in dieser Arbeit diskutiert wurden.

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9. Sworn Declaration

I hereby declare that I did produce the doctoral dissertation submitted to the Faculty of Medicine of the Otto-von-Guericke University Magdeburg entitled

The Nitrogen and Carbon Metabolism in *Klebsiella pneumoniae*: Implications for Inflammatory Bowel Disease and Hepatic Encephalopathy

in the Clinic for General, Abdominal, Transplant and Visceral surgery
with support from Professor Frank Meyer

and in the Division of Gastroenterology at the Hospital of the University of Pennsylvania, as well as the Department of Biology at the University of Pennsylvania
with support from Aaron Hecht MD, PhD, Gary Wu MD, PhD and Mark Goulian PhD

without any other assistance and that I did not use any other resources in the writing of the dissertation than those listed here.

No rights of third parties were infringed in the writing of this dissertation.

I have not previously submitted this dissertation to any domestic or foreign university. I hereby confer upon the School of Medicine the right to produce and distribute additional copies of my dissertation.

Magdeburg, May 15, 2025

Lisa Harling

10. Educational Background

Education

05/2024	Certificate of Medical Examination (1.8)
10/2016–05/2024	Medical School, Otto-von-Guericke-University Magdeburg, Germany
09/2008–06/2016	Secondary School ("Gymnasium"), Martino-Katharineum in Braunschweig Grade "Abitur": 1.5

Research Experience

09/2021–09/2022	Research Exchange, Department of Gastroenterology, Prof. Dr. Gary Wu, in collaboration with the Division of Biology, Prof. Mark Goulian, University of Pennsylvania

Scholarships and Certificates

04/2020 - 10/2023	Germany scholarship "Deutschlandstipendium" of the Otto-von-Guericke-University
09/2021 - 05/2021	Peer-Reviewed Research Funding, Biomedical Exchange Program (DAAD funded)
07/2023	USMLE Step 2 (pass)
08/2022	USMLE Step 1 (pass)
03/2022 - 06/2022	Pediatric Cardiology Virtual Internship (Save a Child's Heart)

Related Professional Experience

03/2024 - Present	**Blood Donation Supervision:** German Red Cross, Braunschweig, Germany
10/2024 - 03/2025	**Ringside Medical Assistance:** The Cage MAA Magdeburg, Germany
10/2022 - 03/2023	**Student Assistant in Telemetry:** Department of Cardiology, University Clinic Magdeburg, Germany
07/2019 - 08/2021	**Student Assistant in Telemetry:** Department of Cardiology, University Clinic Magdeburg, Germany
04/2020 -07/2020	**Emergency 24-Triage during the COVID-19 Pandemic:** University Clinic Magdeburg, Germany
05/2023 -04/2024	**Sub-Internships (Praktisches Jahr)**: Thoracic Surgery, Yale-New Haven Health, USA; Vascular & Visceral Surgery, Angiology & Pneumology, "Harzklinikum" Wernigerode, Germany; Cardiac Anesthesiology, OB-Gyn & Pediatric Anesthesiology, University Clinic Magdeburg, Germany
08/2017 -07/2021	**Clinical Electives (Famulaturen und Hospitationen)**: Cardiothoracic Surgery, University Clinic Magdeburg, Germany; Cardiac Surgery, Jilin Heart Hospital, Changchun, China; Hepatobiliary Surgery, FAW General Hospital, Changchun, China; Maxillofacial Surgery, "Stadtklinikum" Braunschweig and Practice Dr.med. Dr.dent. Bartels, Braunschweig, Germany; Plastic Surgery, University Clinic Magdeburg, Germany; Hematology and Oncology, Practice "Onkologische Schwerpunktpraxis" Braunschweig, Germany; Pediatrics, Practice "Kinderarztpraxis am Dom" Magdeburg, Germany; Internal Medicine and Family Medicine, Practice "Hausarztpraxis Sellmann und Schwarz", Magdeburg, Germany
12/2024 -02/2025	**Observerships (Hospitationen)**: Cardiac Surgery, New-York Presbytarian Hospital, Columbia University, USA

Volunteering

2023 - Present	"Young Forum" of the DGTHG (German Society for Thoracic, Cardiac and Vascular Surgery), elected council member (2025 - present)
2018 - Present	Student club "Kiste" of the medical faculty of the Otto-von-Guericke University, elected council member (2019 - 2024)
2018 - 2021	Student council of the medical faculty of the Otto-von-Guericke University
2019 - 2020	Volunteer organizer at "TeddyKlinik" Initiative of the Otto-von-Guericke University

Magdeburg, May 15, 2025

Lisa Harling

A. Appendix

A.1 Supplemental Figures

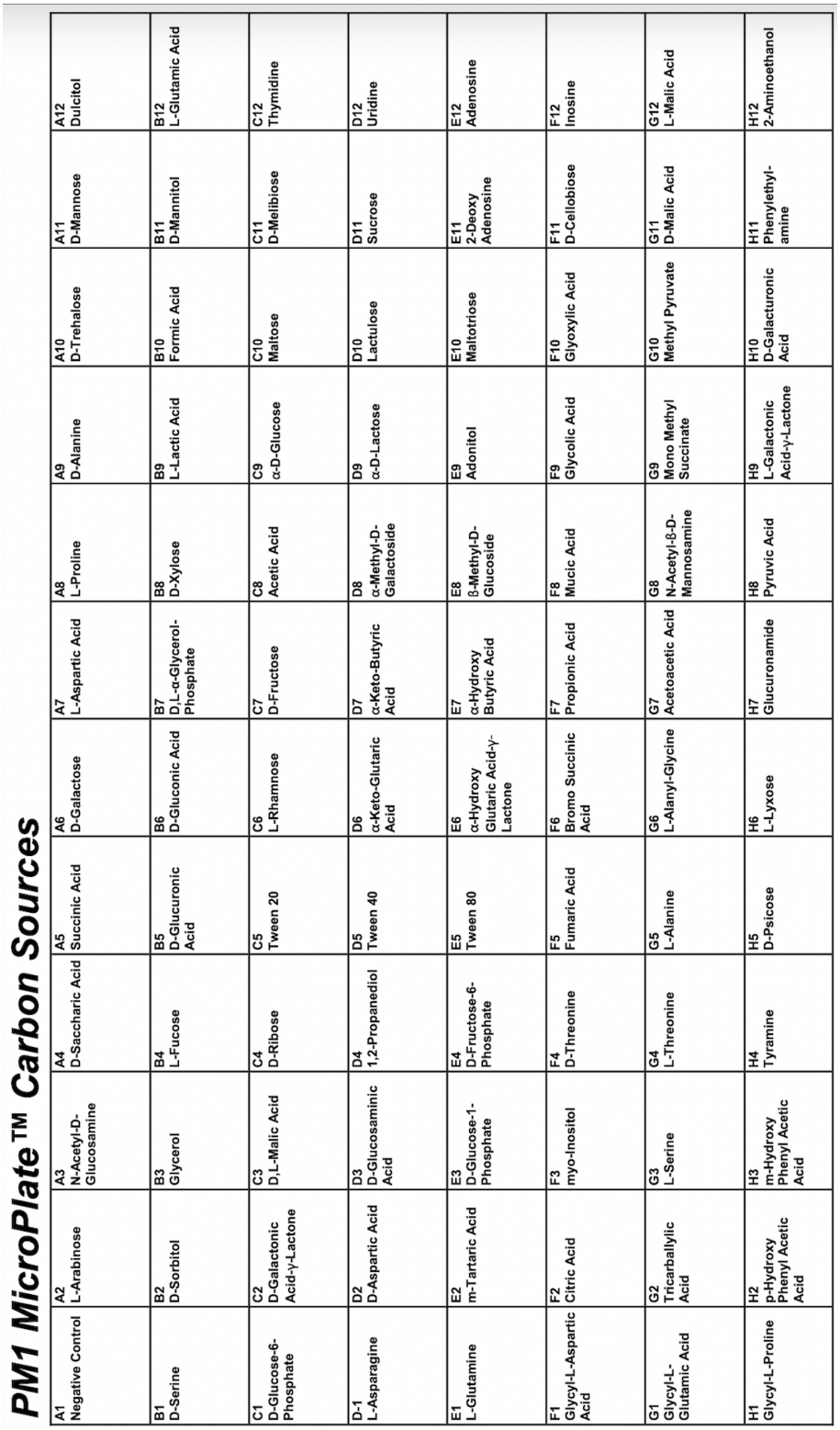

Figure A.1: The PM1 MicroPlate™ was used to test the ability of *K. pneumoniae* to use different carbon-containing compounds as a single carbon source in defined minimal media.

PM2A MicroPlate™ Carbon Sources

A1 Negative Control	A2 Chondroitin Sulfate C	A3 α-Cyclodextrin	A4 β-Cyclodextrin	A5 γ-Cyclodextrin	A6 Dextrin	A7 Gelatin	A8 Glycogen	A9 Inulin	A10 Laminarin	A11 Mannan	A12 Pectin
B1 N-Acetyl-D-Galactosamine	B2 N-Acetyl-Neuraminic Acid	B3 β-D-Allose	B4 Amygdalin	B5 D-Arabinose	B6 D-Arabitol	B7 L-Arabitol	B8 Arbutin	B9 2-Deoxy-D-Ribose	B10 i-Erythritol	B11 D-Fucose	B12 3-O-β-D-Galactopyranosyl-D-Arabinose
C1 Gentiobiose	C2 L-Glucose	C3 Lactitol	C4 D-Melezitose	C5 Maltitol	C6 α-Methyl-D-Glucoside	C7 β-Methyl-D-Galactoside	C8 3-Methyl Glucose	C9 β-Methyl-D-Glucuronic Acid	C10 α-Methyl-D-Mannoside	C11 β-Methyl-D-Xyloside	C12 Palatinose
D1 D-Raffinose	D2 Salicin	D3 Sedoheptulosan	D4 L-Sorbose	D5 Stachyose	D6 D-Tagatose	D7 Turanose	D8 Xylitol	D9 N-Acetyl-D-Glucosaminitol	D10 γ-Amino Butyric Acid	D11 δ-Amino Valeric Acid	D12 Butyric Acid
E1 Capric Acid	E2 Caproic Acid	E3 Citraconic Acid	E4 Citramalic Acid	E5 D-Glucosamine	E6 2-Hydroxy Benzoic Acid	E7 4-Hydroxy Benzoic Acid	E8 β-Hydroxy Butyric Acid	E9 Glycolic Acid	E10 α-Keto-Valeric Acid	E11 Itaconic Acid	E12 5-Keto-D-Gluconic Acid
F1 D-Lactic Acid Methyl Ester	F2 Malonic Acid	F3 Melibionic Acid	F4 Oxalic Acid	F5 Oxalomalic Acid	F6 Quinic Acid	F7 D-Ribono-1,4-Lactone	F8 Sebacic Acid	F9 Sorbic Acid	F10 Succinamic Acid	F11 D-Tartaric Acid	F12 L-Tartaric Acid
G1 Acetamide	G2 L-Alaninamide	G3 N-Acetyl-L-Glutamic Acid	G4 L-Arginine	G5 Glycine	G6 L-Histidine	G7 L-Homoserine	G8 Hydroxy-LProline	G9 L-Isoleucine	G10 L-Leucine	G11 L-Lysine	G12 L-Methionine
H1 L-Ornithine	H2 L-Phenylalanine	H3 L-Pyroglutamic Acid	H4 L-Valine	H5 D,L-Carnitine	H6 Sec-Butylamine	H7 D,L-Octopamine	H8 Putrescine	H9 Dihydroxy Acetone	H10 2,3-Butanediol	H11 2,3-Butanedione	H12 3-Hydroxy-2-Butanone

Figure A.2: The PM2A MicroPlate™ was used to test the ability of *K. pneumoniae* to use different carbon-containing compounds as a single carbon source in defined minimal media.

PM3B MicroPlate™ Nitrogen Sources

A1 Negative Control	A2 Ammonia	A3 Nitrite	A4 Nitrate	A5 Urea	A6 Biuret	A7 L-Alanine	A8 L-Arginine	A9 L-Asparagine	A10 L-Aspartic Acid	A11 L-Cysteine	A12 L-Glutamic Acid
B1 L-Glutamine	B2 Glycine	B3 L-Histidine	B4 L-Isoleucine	B5 L-Leucine	B6 L-Lysine	B7 L-Methionine	B8 L-Phenylalanine	B9 L-Proline	B10 L-Serine	B11 L-Threonine	B12 L-Tryptophan
C1 L-Tyrosine	C2 L-Valine	C3 D-Alanine	C4 D-Asparagine	C5 D-Aspartic Acid	C6 D-Glutamic Acid	C7 D-Lysine	C8 D-Serine	C9 D-Valine	C10 L-Citrulline	C11 L-Homoserine	C12 L-Ornithine
D1 N-Acetyl-L-Glutamic Acid	D2 N-Phthaloyl-L-Glutamic Acid	D3 L-Pyroglutamic Acid	D4 Hydroxylamine	D5 Methylamine	D6 N-Amylamine	D7 N-Butylamine	D8 Ethylamine	D9 Ethanolamine	D10 Ethylenediamine	D11 Putrescine	D12 Agmatine
E1 Histamine	E2 β-Phenylethyl-amine	E3 Tyramine	E4 Acetamide	E5 Formamide	E6 Glucuronamide	E7 D,L-Lactamide	E8 D-Glucosamine	E9 D-Galactosamine	E10 D-Mannosamine	E11 N-Acetyl-D-Glucosamine	E12 N-Acetyl-D-Galactosamine
F1 N-Acetyl-D-Mannosamine	F2 Adenine	F3 Adenosine	F4 Cytidine	F5 Cytosine	F6 Guanine	F7 Guanosine	F8 Thymine	F9 Thymidine	F10 Uracil	F11 Uridine	F12 Inosine
G1 Xanthine	G2 Xanthosine	G3 Uric Acid	G4 Alloxan	G5 Allantoin	G6 Parabanic Acid	G7 D,L-α-Amino-N-Butyric Acid	G8 γ-Amino-N-Butyric Acid	G9 ε-Amino-N-Caproic Acid	G10 D,L-α-Amino-Caprylic Acid	G11 δ-Amino-N-Valeric Acid	G12 α-Amino-N-Valeric Acid
H1 Ala-Asp	H2 Ala-Gln	H3 Ala-Glu	H4 Ala-Gly	H5 Ala-His	H6 Ala-Leu	H7 Ala-Thr	H8 Gly-Asn	H9 Gly-Gln	H10 Gly-Glu	H11 Gly-Met	H12 Met-Ala

Figure A.3: The PM3B MicroPlate™ was used to test the ability of *K. pneumoniae* to use different nitrogen-containing compounds as a single nitrogen source in defined minimal media.

A.2 Peer-Reviewed Publications

Harling L, Zafar M, Ziganshin B, Elefteriades JA. Gene Commonality in Arterial Circuits Throughout the Body. *AORTA*, accepted for publication.

Hecht AL, **Harling L**, Friedman ES, Tanes C, Lee J, Firrman J, Tu V, Liu L, Bittinger K, Goulian M, Wu GD. Colonization and Dissemination of Klebsiella pneumoniae is Dependent on Dietary Carbohydrates. *Journal of Clinical Investigation.* 2024 Mar 21 (DOI 10.1172/JCI174726).

Lemons JMS, Conrad M, Tanes C, Chen J, Friedman ES, Roggiani M, Curry D, Chau L, Hecht AL, **Harling L**, Vales J, Kachelries KE, Baldassano RN, Goulian M, Bittinger K, Master SR, Liu L, Wu GD. Enterobacteriaceae growth promotion by intestinal acylcarnitines, a biomarker of dysbiosis in Inflammatory Bowel Disease. *Cell Mol Gastroenterol Hepatol.* 2023 Sep 20 (DOI: 10.1016/j.jcmgh.2023.09.005).

Harling L, Peglow S, Eger K, March C, Croner RS, Meyer F. Acute epiploic appendagitis - a rare differential diagnosis of acute abdomen. *Z Gstroenterol* 1-6, 2021, (DOI: 10.1055/a-1727-9842).

A.3 Conference Presentations

Harling L, Wacker M, Ruhparwar A, Schmeisser A, Wippermann J. Case Report - Biventricular Heart Failure due to Giant Cell Myocarditis in an 18-year-old patient with new-onset Systemic Lupus Erythematosus. *Annual German Cardiology Congress*, Mannheim, Germany, April, 2024.

Harling L, Aswandi IP, Croner RS, Meyer F. Uncomplicated Diverticulitis of the Sigmoid Colon in Ongoing Immunosuppressive and Anti-inflammatory Treatment of a Female Patient with Rheumatoid Arthritis. *Annual German Surgery Congress*, Leipzig, Germany, April, 2024.

Harling L, Aswandi LP, Croner RS and Meyer F. Uncomplicated diverticulitis of the sigmoid colon in ongoing immunosuppressive and anti-inflammatory treatment of a female patient with rheumatoid arthritis (RA) (A-377). (Poster) TEACHING CASE REPORT. *Symposium "Junge Chirurgen - next generation"*. March 08, 2024, Chemnitz (Germany).

Harling L, Peglow S, Eger K, March C, Croner RS and Meyer F. Acute epiploic appendagitis - a rare differential diagnosis of acute abdomen. *Symposium "Junge Chirurgen - next generation"*. March 08, 2024, Chemnitz (Germany).

Harling L, Hecht A, Lee J, Goulian M, Wu G. The mammalian intestinal tract is not nitrogen limited. *Annual German Surgery Congress*, Munich, Germany, April, 2023.

Hecht A, **Harling L**, Friedman E, Shen D, Tanes C, Tu V, Bittinger K, Goulian M, Wu G. Intestinal Colonization of K. pneumoniae Alters the Nitrogen Composition of the Gut in a Urease-Dependent Manner. (Poster) Philadelphia, PA, USA, August, 2022.

Harling L, Aswandi LP, Croner RS and Meyer F. Uncomplicated diverticulitis of the sigmoid colon in ongoing immunosuppressive and anti-inflammatory treatment of a female patient with rheumatoid arthritis (RA) (A-377). *Annual Meeting of the German Society of Pathology*, Munster, Germany, June, 2022.

Harling L, Peglow S, Eger K, March C, Croner RS and Meyer F. Acute epiploic appendagitis - a rare differential diagnosis of acute abdomen. *Annual Surgical Research Congress*, Leibzig, Germany, September, 2021.

Harling L, Peglow S, Eger K, March C, Croner RS and Meyer F. Acute epiploic appendagitis - a rare differential diagnosis of acute abdomen. *Annual Meeting of the German Society of Gastroenterology*, Digestive and Metabolic Diseases, September, 2021.