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The Impact of Physiological Tolerances on the Colonization of Gut Bacteria in Preterm Infants

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ADAR	arginine-dependent acid stress resistance 3 system
AgD	agmatine deiminase
AMiGA	Analysis of Microbial Growth Assays (software)
AMP	antimicrobial peptide
AR	acid resistance
AR1	acid stress resistance system 1
ASC	antibody-secreting cell
ASP	acid shock protein
ASR	acid stress response
asr	acid shock RNA gene
AUC	area under the curve
BR	biological replicate
C-section	Cesarean section
cAMP	cyclic adenosine monophosphate
CE	capillary electrophoresis
CFA	cyclopropane fatty acid
CHO	complex carbohydrates
CRP	cyclic adenosine monophosphate (cAMP) receptor protein
CV	coefficient of variation
DoME	Department of Microbiology and Ecosystem Science
GBA	gut-brain axis
GDAR	glutamate-dependent acid stress resistance 2 system
GIT	gastrointestinal tract
GMEs	guanidino-group modifying enzymes
GMH-IVH	germinal matrix hemorrhage- intraventricular hemorrhage
HBM	human breast milk
HCl	hydrochloric acid
HCO_3^-	bicarbonate, hydrogen carbonate
HMO	human milk oligosaccharides
HPLC	high-performance liquid chromatography

IgA	immunoglobulin A
IL-2	Interleukin 2
LAB	lactic acid bacteria
JMF	Joint Microbiome Facility
LDAR	lysine-dependent acid stress resistance 4 system
LOS	late onset sepsis
MQW	MilliQ® water
NaOH	sodium hydroxide
NC	negative control
NEC	necrotizing enterocolitis
NH_3	ammonia
NH_4^+	ammonium
NICU	neonatal intensive care unit
$OD_{(min/max)}$	minimum optical density/ maximum optical density
OTC	ornithine carbamoyltransferase/ ornithine transcarbamylase
ODAR	ornithine-dependent acid stress resistance 5 system
PBS	phosphate buffered saline
pH_{ex}	extracellular pH
pH_{in}	intracellular pH
pK_a	acid dissociation constant
PLP	pyridoxal-5'-phosphate
PMF	proton motive force
PVL	paraventricular leukomalacia
PWMI	perinatal white matter injury
RCT	randomized controlled trial
RpoS	alternative sigma factor σ^S
ROS	reactive oxygen species
SCFA	short chain fatty acid
sIgA	secretory immunoglobulin A
TR	technical replicate
TUM	Technische Universität München
ΔpH	pH gradient
ΔΨ	transmembrane potential

ABSTRACT

Preterm infants face unique challenges due to their immature immune system, increasing their risk for conditions such as necrotizing enterocolitis (NEC) and late-onset sepsis (LOS). A critical factor influencing these outcomes is gut microbiota composition, which in preterm infants is often imbalanced, with an overgrowth of potentially pathogenic *Enterobacteriaceae* (e.g., *Klebsiella pneumoniae*, *Escherichia coli*) and a reduction in beneficial *Bifidobacteria* and *Lactobacilli*. This dysbiosis has been linked to poor health outcomes, emphasizing the importance of investigating the physiological mechanisms that influence microbial growth.

This study examines the growth of 23 bacterial isolates representative of the preterm gut microbiome under varying pH- and short-chain fatty acid (SCFA)- conditions, and with alternating oxygen availability. Facultative anaerobes, including *Enterobacteriaceae*, exhibit altered growth patterns in response to environmental factors, thriving at acidic pH in the presence of oxygen. Additionally, SCFAs, particularly acetate, drastically inhibit the growth of pathogenic bacteria, an effect enhanced under acidic conditions. In contrast, *Bifidobacteria* and *Lactobacilli* demonstrate acid stress resistance, allowing them to persist despite SCFA exposure, potentially contributing to colonization resistance against opportunistic pathogens.

These findings highlight the complex interplay between pH, oxygen levels, and metabolic by-products in shaping the preterm gut microbiota. By investigating the physiological tolerances of key bacterial species, this study provides insight into potential microbiome-targeted interventions aimed at promoting gastrointestinal health in preterm infants.

ZUSAMMENFASSUNG

Frühgeborene sind aufgrund ihres unreifen Immunsystems besonders anfällig für Erkrankungen wie nekrotisierende Enterokolitis (NEC) und Neugeborenensepsis (LOS). Eine zentrale Rolle spielt das Darmmikrobiom, das bei Frühgeborenen häufig durch eine verstärkte Vermehrung pathogener Enterobakterien (z. B. *Klebsiella pneumoniae*, *Escherichia coli*) und eine verminderte Zahl nützlicher *Bifidobakterien* und *Lactobacillen* gekennzeichnet ist. Diese Dysbiose wird mit ungünstigen Gesundheitsverläufen assoziiert, weshalb ein besseres Verständnis der mikrobiellen Wachstumsmechanismen essentiell ist.

Diese Studie untersucht das Wachstum von 23 bakteriellen Isolaten, die typisch für das Frühgeborenen-Mikrobiom sind, unter verschiedenen Umweltbedingungen (pH-Wert, kurzkettige Fettsäuren [SCFAs], Sauerstoffverfügbarkeit). Unsere Ergebnisse zeigen, dass fakultativ anaerobe Enterobakterien ihr Wachstum anpassen und besonders bei saurem pH in Anwesenheit von Sauerstoff gedeihen. Gleichzeitig hemmen SCFAs – insbesondere Acetat – das Wachstum pathogener Bakterien stark, wobei dieser Effekt unter sauren Bedingungen verstärkt wird. Im Gegensatz dazu sind *Bifidobakterien* und *Lactobacillen* resistenter gegenüber Säurestress, was ihnen einen Vorteil bei der Darmbesiedlung verschaffen und die Vermehrung opportunistischer Pathogene begrenzen könnte.

Diese Ergebnisse verdeutlichen die komplexe Wechselwirkung zwischen pH-Wert, Sauerstoffverfügbarkeit und Stoffwechselprodukten in der Gestaltung des Frühgeborenen-Mikrobioms. Durch die Untersuchung physiologischer Toleranzen liefert diese Studie wertvolle Erkenntnisse für Mikrobiom-basierte Interventionen zur Förderung der Darmgesundheit bei Frühgeborenen.

INTRODUCTION

I. Preterm Birth

The term “preterm birth” refers to children being born alive before a complete 37 weeks of gestation and can further be subdivided into extremely preterm births (< 28 weeks), very preterm births (28 to < 32 weeks) and moderate to late preterm births (32 to < 37 weeks). The complications of being born prematurely lead to it being the most frequent cause of death in neonates (within the first 28 days after birth), in infants (within a year after birth), and in children under the age of 5. Preterm birth often goes hand in hand with poor health outcomes in these young patients with diseases such as NEC or LOS arising, as well as life-long neurodevelopmental impairment occurring (Abebaw et al., 2021; Beghetti et al., 2023; Cao et al., 2022; Seki et al., 2021). For the further development of the brain, the third trimester of pregnancy, starting at week 27, is of special importance, contributing to its refinement and maturation needed for later cognitive functions by increasing synaptic complexity and dendritic branching (Matthews et al., 2018; Seki et al., 2021).

The human gut microbiota has been the focus of research in the past few decades, and it has been generally established that disturbances in the composition of the microbiota can result in various diseases not only in the GIT. This so-called “dysbiosis” refers to the imbalance of the microbial species inhabiting the gut which can lead to the onset of different human illnesses such as Inflammatory Bowel Disease (IBD), autoimmune diseases (e.g., Multiple Sclerosis) or neurological disorders such as anxiety, depression or schizophrenia (Mitrea et al., 2022; Weiss & Hennet, 2017). Regarding preterm born infants, it has been observed that aberrations in the gut microbiome can have detrimental effects on their development by interfering with the integrity of the gut-brain axis (GBA). The GBA is of high importance as it is the foundation of several bidirectional signaling pathways, including hormonal, immunological and neural signaling. Apart from that, various microbial metabolites can also use the axis to influence their target areas, e.g., short-chain fatty acids (SCFAs) responsible for early neurodevelopment and immune regulation. Due to several factors (delivery mode, hospital environment, administration of antibiotics, etc.) preterm infants frequently must battle with structural

and immunological immaturity of the gut microbiota and the consequences thereof. This immaturity predisposes the infants to negative outcomes such as NEC which heavily impairs the function of the GBA and is thought to be an underlying factor for neurodevelopmental impairment in these children (Beghetti et al., 2023; Seki et al., 2021).

As mentioned above, the third trimester of pregnancy (starting at week 27) is of critical importance for the development of the human brain and its complexity. Especially extremely preterm infants represent a significantly vulnerable group with an increased risk of perinatal white matter injuries (PWMI) encompassing germinal matrix hemorrhage- intraventricular hemorrhage (GMH-IVH), paraventricular leukomalacia (PVL) and diffuse white matter injury (Lee, 2017). Here, the production of metabolites by early gut colonizers plays a very important role. It has been observed that gut microbes and their metabolites are involved in immune inflammatory responses and affect processes of perinatal brain development including neurogenesis, myelination and blood-brain barrier development. Hence, aberrations in the gut microbial composition and consecutive deficiency of the metabolites may facilitate PWMI (Y. Wang, Zhu, et al., 2023). Recent research has shown that the composition of the gastrointestinal microbiota in preterm infants differs significantly from that in term born infants. Whilst term newborns are usually colonized with mostly *Streptococcus*, *Enterobacteriaceae* and *Bifidobacteria* spp., the preterm infants' gut microbial landscape paints a different picture entirely. It has been discovered that preterm infants have a lower bacterial diversity compared to healthy term infants, leading to species such as *Escherichia coli*, *Clostridium*, *Staphylococcus* and *Streptococcus* being the most prevalent (Y. Chen et al., 2020; Y. Wang, Zhu, et al., 2023).

The pattern of colonization of the GIT with different bacterial species in preterm infants is influenced by several factors one of which is the mode of delivery. When a child is vaginally delivered, overall gut bacterial richness and diversity remains at a higher level when compared to children born by "unnatural" Cesarean section (C-section), with elective Cesarean sections reducing richness and diversity even more than spontaneous/emergency C-sections (Azad et al., 2013; Mitchell et al., 2020) It has been observed that the intestinal microbiome of the infants delivered by C-section resembles

the microbiota of the mother's skin showing high amounts of *Corynebacterium*, *Acinetobacter, Propionibacterium* and *Staphylococcus*, but at the same time having significantly lower prevalence for *Escherichia* and *Shigella* and lacking *Bacteroides* and *Bifidobacterium* (Mitchell et al., 2020; Ximenez & Torres, 2017). Vaginally delivered infants, however, displayed colonization with bacterial species that resemble the vaginal flora such as *Lactobacillus, Prevotella* and higher amounts of *Bacteroides* and *Bifidobacteria* (Ximenez & Torres, 2017). Another important factor influencing the intestinal microbiota is breast milk. Breast milk contains complex oligosaccharides called "human milk oligosaccharides" (HMOs) which can only be utilized by specific bacterial species. It has been observed that *Bifidobacterium* spp. are enriched in the gut microbiota of breast-fed children and it has been shown that HMOs are able to promote growth of these bacterial species (Azad et al., 2013; Neu & Rushing, 2011; Ximenez & Torres, 2017; Yamada et al., 2017). The HMOs found in human breast milk serve as source for specific beneficial health-promoting gut bacteria (especially the *Bifidobacterium* genus) due to their ability to escape host digestion. It has been observed that the gastrointestinal microbiota of breast-fed infants is composed mainly of *Bifidobacterium,* and *Ruminococcus*. In comparison to formula-fed infants, breast-fed infants have lower numbers of *Escherichia coli, Bacteroides fragilis, Clostridium difficile* and *Lactobacilli* present in their gut and, interestingly, also a lower bacterial complexity, richness and diversity. The microbiome of formula-fed infants on the other hand is composed mainly of members of the *Enterobacteriaceae* family, *Clostridium, Bacteroides* and *Streptococcus* as well as *Bifidobacteria* and *Atopobium* (Lordan et al., 2024; Power et al., 2014). Being born prematurely can, as mentioned before, lead to severe disturbances in the further development of the infant. One of the consequences of a child being born too early is the uncompleted maturation of the immune system leading to the newborn being more susceptible to pathogens and infections. Because of that, preterm infants are often treated in NICUs right after their birth. However, it has been observed that a prolonged stay in NICUs and the accompanying clinical interventions can lead to an abnormal development of the neonatal gut microbiota and nosocomial infections. The perinatal exposure to antibiotics, which are often administered due to a higher risk of onset of LOS and NEC, has been shown to lead to altered maturation, composition and perturbations in the functions of the gut microbiota, all of which taken together is also called a "microbiome shift". Furthermore, it has been

observed that the use of antibiotics and the associated shifts support the colonization of the preterm gut microbiota with pathogenic bacteria such as *Enterococcus faecalis, Escherichia coli* and *Klebsiella pneumoniae*, which often carry antibiotic-resistance genes, while at the same time decreasing beneficial bacterial taxa including the SCFA-producing *Bifidobacteria* and *Veillonella* (Thänert et al., 2024; Ximenez & Torres, 2017; Xu et al., 2022). All these factors taken together are clearly influencing the composition of the gut microbiota of prematurely born infants and can therefore have long-lasting effects on the development and health of these children. One of the main goals, therefore, is to diminish the negative health outcomes in preterm infants. Recent research has hence been focusing on the modulation of the intestinal microbiota by the administration of e.g., probiotics, prebiotics or compounds such as HMOs and lactoferrin, thereby changing the ratio of bacteria in favor of beneficial gut inhabitants and consequently relieving the burden off these infants (Y. Wang, Florez, et al., 2023).

II. The importance of pH tolerance in the colonization of the gut microbiota

The human gastrointestinal tract is a complex heterogenous landscape, influenced by multiple parameters such as pH, mucus type, nutrient and oxygen availability, presence of antimicrobial peptides (AMPs) and peristaltic rates. The different regions of the healthy adult human GIT harbor different amounts and types of bacterial species depending on their environmental conditions, the two most important conditions being the availability of oxygen as well as the environmental pH. Under normal circumstances, the pH level ranges from pH ~ 2.0 – 3.0 in the stomach where conditions are still somewhat aerobic to pH ~ 7.0 in the colon where no oxygen is present anymore (McCallum & Tropini, 2024). In preterm infants, however, aberrations in the healthy and normal pH levels are not uncommon due to several factors influencing the composition of the gut microbiota such as the mode of delivery, the administration of antibiotics or the lack of breastfeeding as mentioned in the previous chapter. It has been observed that over the past one hundred years the gastrointestinal pH levels of preterm infants experienced a sharp increase in the median pH of almost two log units, climbing from a median of 4.88 in 1926 to one of 6.5 nowadays (Duar et al., 2020). Since the presence or absence of bacterial species and communities is closely related to specific pH ranges (e.g., *K. pneumoniae* has its growth

optimum between pH 6 to 8; *Bifidobacteria* mostly grow the best at pH 6.5 to 7 with a few exceptions having their growth optimum at more acidic pH levels (e.g., *Bifidobacterium bifidum*)), deviations herein can lead to problems in the development of a healthy and stable gut microbiota and compromise the neonates' overall well-being (J. Chen et al., 2021; Kawasaki et al., 2006; Mitrea & Vodnar, 2019).

II.I The fight against acidity – acid stress resistance mechanisms in bacteria

The environmental pH and the associated redox potential do not only influence growth and metabolism of the microbes inhabiting the GIT, but they also determine nutrient availability and enzyme activity rates. By doing so, the bacterial landscape is being shaped in a unique way that should ultimately benefit the host (Jin & Kirk, 2018).

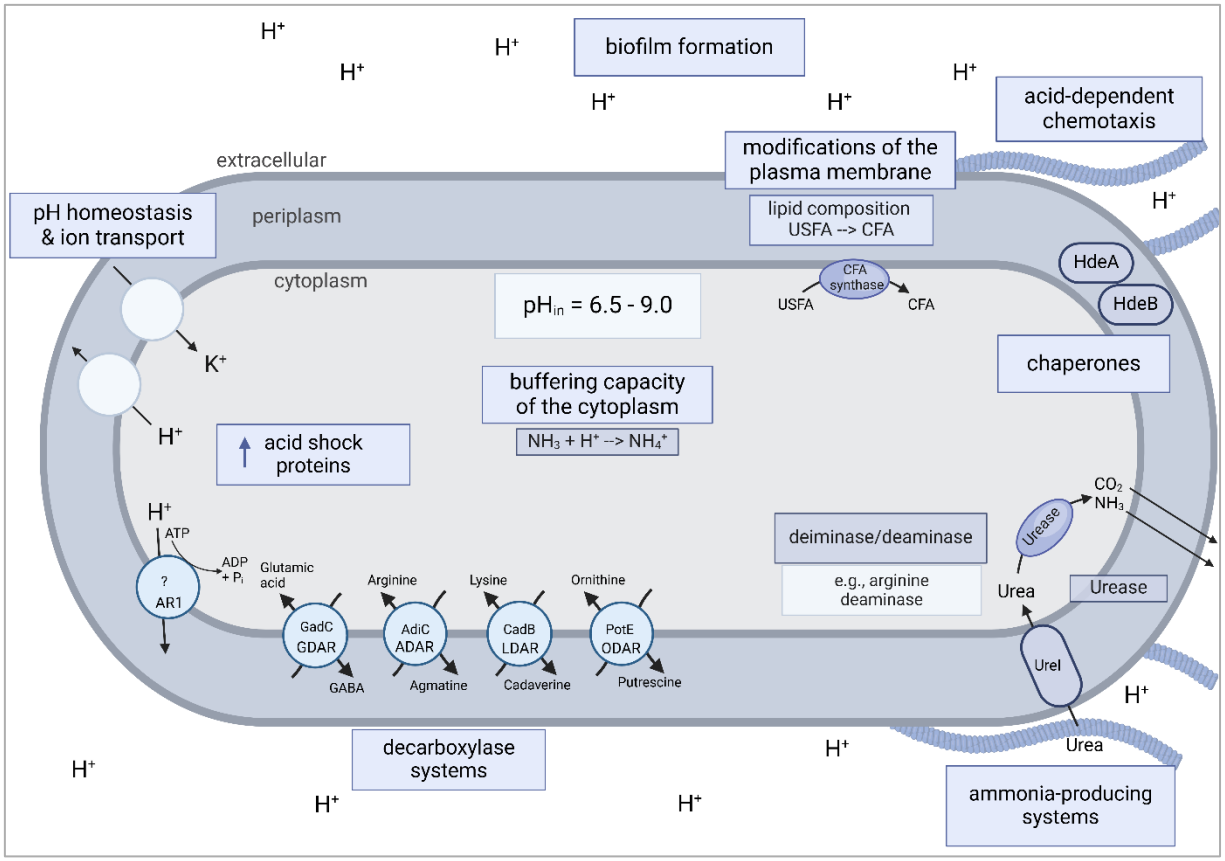

Figure 1: ***Overview of mechanisms in bacteria to counteract acid stress****. Adapted from Schwarz et al., 2022. Created with BioRender.com.*

One essential concept that has a major impact on the gastrointestinal microbial composition is that of the so-called acid stress response (ASR), also referred to as acid resistance (AR). ASR involves resistance mechanisms through which the bacterial cells that are exposed to acidic environmental pH levels can tolerate these harsh conditions by regulating their cellular functions in order to help them survive. Depending on the bacterial species, different mechanisms are employed, all aiming to induce tolerance (Guan & Liu, 2020; Schwarz et al., 2022).

pH homeostasis and ion transport

pH homeostasis is essential for growth and metabolism functions of the bacterial cell, it influences the absorption and utilization of nutrients as well as the synthesis of proteins and nucleic acids and the degradation of substrates (Guan & Liu, 2020). The difference between intracellular pH (pH_{in}) and extracellular pH (pH_{ex}) is called the pH gradient (ΔpH). There are three different groups of microorganisms each having a different optimal growth pH: the acidophiles grow optimally at pH < 5.0, the neutralophiles grow best between pH 5.0 to 9.0 and the alkaliphiles which have their growth optimum at pH > 9.0. All these microorganisms need to be able to keep their pH_{in} relatively constant, even when pH_{ex} sharply decreases. Acidophiles stabilize their pH_{in} at 6.5 to 7.0, neutralophiles between pH 7.5 to 8.0 and alkaliphiles retain a pH_{in} of 8.4 to 9.0. Since acidophiles have a pH growth optimum at very low pH levels, they must maintain a ΔpH of several log units across the cell membrane at all times (Baker-Austin & Dopson, 2007; Schwarz et al., 2022). One important player in keeping a constant intracellular pH is the intrinsic buffering capacity of the cytoplasm where intracellular protons present as a surplus are sequestered by pH-titratable components of the cell (e.g., inorganic phosphates, polyamines, amino acid side chains, etc.) thereby leading to a stabilization of pH_{in} (Schwarz et al., 2022). Furthermore, another important mechanism is the so-called proton motive force (PMF). The PMF measures the energy state of the cell membrane which is generated due to a charge separation between the cytoplasm and the extracellular milieu created by the transmembrane potential (ΔΨ; describes the electrical potential difference across the plasma membrane) and ΔpH across the membrane (Baker-Austin & Dopson, 2007; Chrysafides et al., 2024). It helps to enhance the activity of proton pumps which are essential for promoting proton efflux (= H^+ efflux) such as the

H^+-ATPase leading to a relief of acid stress. Additionally, acidophilic bacteria have the ability to reverse ΔΨ, which is usually negative on the inside of the cell. Upon acid stress, ΔΨ is reversed leading to a positive intracellular milieu that inhibits further entry of protons due to charge repulsion (Baker-Austin & Dopson, 2007; Guan & Liu, 2020; Schwarz et al., 2022). Furthermore, the active import of monovalent cations, such as potassium (K^+), helps in restoring a normal pH_{in}. This is due to a net exchange with a proton as soon as K^+ is imported into the bacterial cell leading to an alkalization of the intracellular pH levels (Booth, 1985; Schwarz et al., 2022).

Enzyme-based H^+-consuming acid stress resistance system

Acid stress resistance can, however, not only be brought on by the active regulation of the transport of H^+ but can also be achieved through the consumption of the excess of these protons by using the enzyme system of the bacterial cells. In total, there have been five different enzyme-based resistance systems identified in *E. coli*- the AR1 system which is activated by the alternative sigma factor σS (RpoS) and the cyclic adenosine monophosphate (cAMP) receptor protein (CRP), the glutamate-dependent AR2 system (GDAR), the arginine-dependent AR3 system (ADAR), the lysine-dependent AR4 system (LDAR) and the ornithine-dependent AR5 system (ODAR). The mechanism behind each of the latter four systems involves a cytoplasmic amino acid decarboxylase together with its respective corresponding antiporter which together perform an H^+-consuming reaction. In this reaction, the pyridoxal-5'-phosphate (PLP)-dependent decarboxylase converts a specific amino acid (i.e., glutamate, arginine, lysine, ornithine) into a more alkaline product (i.e., an amino acid derivative), leading to its expulsion through the respective antiporter (GadC in the GDAR system, AdiC in the ADAR system, CadB in the LDAR system and PotE in the ODAR system). Thereby, it is exchanged for the respective amino acid derivative which further causes an increase in the intracellular and extracellular pH levels. AR1, however, employs a different mechanism. The F_0F_1-ATP-synthase (also referred to as F_0F_1-ATPase) hydrolyzes ATP in order to pump protons out of the cell to increase the pH_{in} independent of the presence of an external amino acid. Note that the exact mechanism of AR1 has not yet been completely unveiled. Furthermore, the AR2-AR5 systems require different pH ranges (all under pH 6) since each decarboxylase has its own optimal working pH, whereas the AR1 system is activated only

under extremely acidic conditions around pH 2.5. Interestingly, while all five resistance systems protect bacterial cells from acidity during their stationary phase, only the AR2 and AR3 system were discovered to also do so during the exponential phase. Equally important to note is that out of the five systems AR2 is undoubtedly the most effective one (Schwarz et al., 2022; Xu et al., 2020).

Enzyme-based ammonia-producing acid resistance systems

There are two different ammonia-producing systems found in bacteria which are utilized to neutralize acidic stress within the cell – the deiminase/deaminase system and the urease system. The deiminase/deaminase system is employed by both gram-negative and gram-positive bacteria. The mechanism underlying this system contains two steps: first, excess protons are being used to produce ammonia (NH_3) through these two enzymes and second, the conversion of NH_3 into ammonium (NH_4^+) ions. Naturally, various deiminase and deaminase systems have been established in different bacterial groups. The enzymes involved in the biochemical reactions are part of the guanidino-group modifying enzyme (GMEs) superfamily which comprises many different metabolic enzymes targeting different pathways in the bacterial cell. For instance, the agmatine deiminase (AgD) carries out the hydrolyzation of agmatine to N-carbamoylputrescine and NH_3 in bacteria such as *Heliobacter pylori* or *Pseudomonas aeruginosa* to counteract acid stress within the cell (Schwarz et al., 2022; Shirai et al., 2006).

Another GME present in e.g., *Bacillus, Pseudomonas* or lactic acid bacteria (LAB) is the arginine deiminase system which comprises three different enzymes with specific tasks responsible for inducing acid tolerance: the arginine deiminase which degrades arginine into citrulline and NH_3, the ornithine carbamoyltransferase (OTC) which is cleaving citrulline into carbamoyl phosphate and ornithine and lastly, the carbamate kinase which dephosphorylates the carbamoyl phosphate and thereby generates ATP, NH_3 and CO_2. All of these three enzymes are most active at a pH level of 3.1 or even lower and by producing NH_3 and ATP they contribute to the re-establishment of the pH homeostasis through the F_0F_1-ATPase (Casiano-Colón & Marquis, 1988; Schwarz et al., 2022).

In *E. coli* ammonia is produced using on the one hand the GDAR system where the glutaminase GlsA (also known as YbaS) is activated under acidic stress conditions (i.e., at pH levels of 6.0 and lower, with the highest activity at pH 4.0) and converts L-glutamine into L-glutamate and concomitant release of NH_3 (Lu et al., 2013; Schwarz et al., 2022).

On the other hand, NH_3 can also be produced by relying on the deamination of adenosine. This reaction causes the intracellular pH to rise and acidic stress being evaded (Schwarz et al., 2022).

The second system producing ammonia that is used to protect against acidic stress is the urease system which is found in a lot of different bacterial species including *Heliobacter pylori, Klebsiella pneumoniae, Clostridium perfringens, Salmonella spp., Bifidobacterium longum* subsp. *infantis, Staphylococcus* and *Proteus* (Mora & Arioli, 2014; Schwarz et al., 2022). In this specific system, the enzyme urease hydrolyzes urea to NH_3 and carbamate. Carbamate in turn subsequently decomposes to another molecule of NH_3 and CO_2. The generated ammonia molecules help in buffering the cytoplasmic and periplasmic pH levels by using protons in order to produce NH_4^+, whereas CO_2 is converted to bicarbonate (also known as hydrogen carbonate; HCO_3^-) with the help of the alpha-carbonic anhydrase (Schwarz et al., 2022).

Cellular remodeling to protect against acidic stress

Aside from maintaining the pH homeostasis and enzyme-based resistance mechanisms, bacterial cells also have the ability to undergo physiological changes in order to protect themselves against acidity-induced stress. Various processes are involved in this such as i) the synthesis of chaperones, ii) the alteration of the cytoplasmic membrane, iii) the synthesis of acid-shock proteins, iv) the acid-dependent chemotaxis and v) biofilm synthesis (Guan & Liu, 2020; Schwarz et al., 2022).

i) A decrease in the pH levels is very often followed by processes within the cell that lead to negative outcomes such as protein unfolding, protein denaturation and protein damage due to amino acids becoming fully protonated. These can further cause damage to the structures of the DNA. In such cases, the synthesis of chaperones is a major key player in protecting and repairing damaged cellular structures. In gram-negative bacteria, especially, acid stress can have more detrimental ramifications on enzymes, transmembrane antiporters and transporters located in the periplasm since they lack the inner membrane. Therefore, in *E. coli*, for instance, HdeA and HdeB- two periplasmic chaperones- act as protectors against damage through gastric juice, while HdeA is also able to help bacteria to tolerate acid stress arising due to the accumulation of organic

acid. It has been observed that HdeA functions optimally at very acidic pH levels (pH 2.0 to 3.0) whereas HdeB has its optimum at a pH of 4.0. These two chaperones act in an ATP-independent manner to minimize protein aggregation through high proton and chloride concentrations by binding to the unfolded proteins during acid stress, as well as also taking part in refolding substrates after acid stress relief. (Guan & Liu, 2020; Schwarz et al., 2022).

ii) The outer membrane of a bacterial cell is, naturally, the part of the cell that is exposed to environmental stress conditions the most. Therefore, it is especially important for bacteria, also during acid stress, to be able to regulate the integrity, permeability, fluidity and lipid composition of the cytoplasmic membrane. Acidophilic bacteria in general possess a rather impermeable cell membrane for protons in order to maintain pH homeostasis. For bacteria that are not acidophilic, modulating the lipid composition of the cytoplasmic membrane is essential. Here, the ratios of saturated to unsaturated, branched to unbranched and *cis*- to *trans*-unsaturated fatty acids can be modified. Of special importance is the increase in the ratio of saturated to unsaturated fatty acids, i.e., when more saturated fatty acids are present relative to unsaturated fatty acids under acidic conditions. This is thought to lead to a reduced membrane fluidity which possibly impedes the entry of protons into the cell. Additionally, unsaturated fatty acids can be converted into cyclopropane fatty acids (CFAs) through the addition of a methyl-group from S-adenosyl-methionine to a double bond which are then subsequently incorporated into the cytoplasmic membrane leading to reduced membrane fluidity. All of this taken together seems to be very important to maintain membrane integrity during acid stress (Baker-Austin & Dopson, 2007; Schwarz et al., 2022; Yang et al., 2014).

iii) Extremely acidic conditions put a lot of pressure on bacteria to survive. An important part in tolerating these harsh conditions is the induction of the synthesis of the so-called acid shock proteins (ASPs). It is believed that these proteins play an essential role in the acid resistance system of bacteria, contributing to biochemical reactions which neutralize extracellular acidic pH, repair damaged DNA, synthesize membranes and adjust the cellular metabolism according to the environmental circumstances (Šeputienė et al., 2003). One example of an ASP is the periplasmic protein Asr which is encoded by the *asr* (acid

shock RNA) gene in *E. coli*. It has been shown that Asr is required for maintaining growth under moderate acidic conditions with a pH of 4.0 to 4.5. The exact mechanism underlying this mechanism, however, remains unclear and further research needs to be done (Schwarz et al., 2022; Šeputienė et al., 2003).

iv) Bacterial chemotaxis has been a well-known method used by bacteria to respond to changes in their surroundings and it describes the active movement towards or away from an environment (Wadhams & Armitage, 2004). This behavior can also be seen when the pH levels change toward a more acidic milieu. When confronted with low pH, bacterial cells tend to move away from the more acidic environment toward a more welcoming one, i.e., they exhibit negative chemotaxis. On top of that, it has been observed that the presence of acids results in an interference in the flagellar motor causing prolonged tumbling of the bacteria. This is a scenario that can have negative effects on the cells which is why they move towards more favorable conditions (Maurer et al., 2005; Schwarz et al., 2022).

v) The formation of biofilms in general is a useful tool for bacteria to enhance their survival under harsh conditions. This behavior is very interesting as it involves a lot of cell-to-cell communication. It results in communities wrapped in a surface-attached biofilm with the bacterial cells finding themselves in the innermost part, thereby defending them against extracellular acid shock. Even though, biofilms have been shown to be effective in protecting against decreasing pH_{ex}, they are less potent in shielding bacteria from internal acid shock resulting from the presence of acidic by-products of metabolism (Cotter & Hill, 2003; Guan & Liu, 2020; Y. Liu et al., 2015).

III. The importance of short chain fatty acids in the preterm gut

Short chain fatty acids (SCFAs) are saturated aliphatic organic acids consisting of one to six carbon atoms and are produced through anaerobic fermentation of indigestible complex carbohydrates (CHO) by different members of the microorganisms living in the colon. The three principal SCFAs in the adult gut are acetate (C2), propionate (C3) and butyrate (C4), with acetate making up approximately 60% while the other two account for ~ 20% each. Overall molar concentrations can be influenced by the diet of the host, however on average the amounts present in healthy adults are thought to be between 70-

140 mM in the proximal colon, between 20-70 mM in the distal colon and 20-40 mM in the distal ileum. Aside from the three conventional SCFAs, lactate isomers, valerate, caproate and branched SCFAs such as iso-valerate and iso-butyrate are often also included when mentioning SCFAs. In general, short chain fatty acids have been observed to play an important role in lipid metabolism, mucosal integrity and maintenance, glucose homeostasis and immune function, therefore being essential for host health (Cifuentes et al., 2024; den Besten et al., 2013; Fusco et al., 2023; X. Wang et al., 2021). Each of the main SCFAs (here, also including lactate, since it is part of the study described later) possesses specific functions in the host that will be described in the following section:

i. Acetate: acetate is the main fermentative metabolite of the genera *Bifidobacterium*, *Lactobacillus*, *Bacteroides* and *Prevotella,* generated via the acetyl-CoA or Wood-Ljungdahl pathway. This SCFA is involved in immune, endocrine and metabolic mechanisms within the human body as well as in the protection of the intestinal mucosa and eliciting anti-inflammatory effects (Cifuentes et al., 2024; X.-C. Liu et al., 2022).

ii. Propionate: *Bacteroides, Propionibacterium* and *Veillonella* are the main producers of propionate with different pathways being used to generate this fatty acid. While *Bacteroides* and *Veillonella* prefer using the succinate-pathway to produce propionate out of succinate, *Propionibacterium* uses either the acrylate- or the propylene glycol-pathway to generate propionate from lactate. In humans, this SCFA is involved in glucose homeostasis and in satiety and it acts as a precursor for hepatic gluconeogenesis (Cifuentes et al., 2024; X.-C. Liu et al., 2022).

iii. Butyrate: butyrate is produced by *Clostridium* and *Lactobacillus* as well as members of the Firmicutes and Bacteroidetes phyla (e.g., *Faecalibacterium prausnitzii, Butyricicoccus pullicaecorum*) from acetyl-CoA and butyryl-CoA via the “classical pathway” or via the lactate-pathway using acetate and lactate as substrates. Butyrate has been shown to not only be the main energy rich substrate for colonocytes, but also to be involved in inhibiting pro-inflammatory responses by reducing the expression of cytokines (i.e., modulating immune functions) as well as exerting anti-carcinogenic effects and promoting intestinal

gluconeogenesis (Cifuentes et al., 2024; den Besten et al., 2013; X.-C. Liu et al., 2022).

iv. Lactate: while lactate is technically not a "real" SCFA it still plays an important role in the host. It is produced via fermentation by so-called lactic-acid bacteria comprising, among others, *Lactobacillus* and *Bifidobacterium*. It has been shown that HMOs and specific *Bifidobacterium* species such as *B. breve, B. bifidum* and *B. longum* subsp. *infantis* are involved in the generation of aromatic lactic acids. Moreover, lactate has been observed to inhibit growth of potentially pathogenic bacteria, including *E. coli* (Cifuentes et al., 2024; S. P. Wang et al., 2020).

Pathogenic bacteria from the *Enterobacteriaceae* family, such as *Klebsiella, Escherichia, Enterobacter, Shigella* and *Salmonella* as well as members of the *Bacillota* family (e.g., *Clostridium*) are often enriched in infants born prematurely due to antibiotic interventions coupled with an immature immune system. In this case, beneficial bacterial species, especially *Bifidobacterium* and *Lactobacillus*, are often used as probiotic supplements, defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (Reid, 2016). Thereby, their abundance is increased and long-term colonization is supported in the GIT of preterm infants (Alsharairi, 2023). In a randomized controlled trial (RCT) Plummer et al. have investigated the impact of probiotic supplements containing *Bifidobacterium longum* subsp. *infantis, Bifidobacterium animalis* subsp. *lactis* and *Streptococcus thermophilus* on the composition of the gut microbiome in preterm infants. Results showed that during the supplementation, all three of the administered species were present at increased levels. However, after supplementation had been terminated, only *B. longum* subsp. *infantis* and *B. animalis* subsp. *lactis* displayed higher detection levels, whereas *S. thermophilus* showed no difference in detection levels when comparing the probiotic and the placebo group (Plummer et al., 2021). In 2020, a retrospective observational study by Robertson et al. was published that concerned the impact of the administration of probiotics on the incidence of NEC over a ten year period. The probiotics used were initially Infloran® (containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum*) and later Labinic™ Drops (containing *L. acidophilus, B. bifidum*, and *B. longum* subsp. *infantis*). It was shown that NEC rates were more than halved (from 7.5% to 3.1%) in a five-year period following the administration of probiotics. Moreover, also LOS rates

decreased during the study period, falling from 22.6% to 11.5% (Robertson et al., 2020). In another observational study Alcon-Giner et al. compared two different groups of preterm infants – one group receiving oral supplementation of probiotic *Bifidobacterium*- and *Lactobacillus*-species (again Infloran® was used) whereas the other group did not receive any probiotic administration. They found that the gut microbiota composition in preterm babies who received Infloran® closely resembled that of healthy full-term breast-fed infants. Interestingly, this study also showed a decrease in the abundance of pathogenic bacteria such as *Klebsiella* in the group receiving *Bifidobacterium* and *Lactobacillus* (Alcon-Giner et al., 2020).

As already mentioned, aberrations in the microbial composition, among other factors like infection, sepsis, low birth weight or premature rupture of membranes, have been linked to the onset of NEC. Another influential factor is breast feeding. Human breast milk (HBM) contains HMOs in concentrations of 12-24 g/L and they act as a substrate for beneficial bacteria such as LAB including *Bifidobacterium* spp. However, HMB is also a great source of secretory immunoglobulin A (sIgA) and IgA-producing antibody-secreting cells (ASCs), both of which are needed for the proper maturation of the gut microbiome. They also enhance the immune system in preterm infants by binding to SCFA-producing *Bifidobacterium* and *Lactobacillus* which further leads to a decreased chance of NEC onset (Alsharairi, 2023; Cifuentes et al., 2024; Yamada et al., 2017).

The SCFAs acetate, propionate and butyrate are mainly produced by the members of the gram-positive Firmicutes phylum including *Ruminococcus, Clostridium, Enterococcus* and *Roseburia* (Alsharairi, 2023). Additionally, *Bifidobacterium* spp. are also producers of acetate via the so-called "*Bifido* Shunt" pathway. Since *Bifidobacteria* lack the enzymes aldolase and glucose-6-phosphate $NADP^+$ oxidoreductase, they cannot metabolize glucose via glycolysis or the hexose monophosphate pathway. Instead, they yield 1.5 moles of acetate and 1 mole of lactate as well as 2.5 moles of ATP per mole of glucose (Palframan et al., 2003). However, lactate, which is often produced as intermediate and then further metabolized into butyrate, is not only a product of *Bifidobacterium* but also of LAB such as *Lactobacillus* spp. (Alsharairi, 2023). As mentioned in the previous chapter, premature birth very often leads to dysbiosis of gut microbiota with decreased abundances of *Bifidobacterium* and *Bacteroides* and increased amounts of *Enterobacteriaceae, Streptococcus, Prevotella* and *Staphylococcus*. One of the tasks of SCFA-producing bacteria in the preterm gut is to

inhibit members of the *Enterobacteriaceae* family, such as *Klebsiella, Escherichia, Enterobacter, Shigella* and *Salmonella* as well as members of the *Bacillota* family (e.g., *Clostridium*). These bacteria have very often developed antibiotic-resistance and can therefore endure medical interventions so that the inhibition by SCFAs is a crucial factor. Since it has been observed that both *Lactobacillus* spp. and *Bifidobacterium* spp. can not only secrete antimicrobial SCFAs and peptides to confer colonization resistance, but they are also able to carry out immunomodulatory functions which may help to enhance the host's immune response to pathogens, their importance as potential probiotic supplements increases strongly (Buffie & Pamer, 2013). Sorbara et al. have been able to show that the production of SCFAs together with the acidification of the pH is imperative in order to inhibit growth of *Enterobacteriaceae*. In their study, they observed that acidic pH levels are essential for the growth inhibition of *K. pneumoniae*, however it is not sufficient in preventing it on its own. Additionally, the presence of SCFAs, more specifically, a mixture of acetate, propionate and butyrate, is required. Interestingly, *K. pneumoniae* as well as *E. coli* were only significantly inhibited when the pH was acidic and when physiological concentrations of SCFAs (approximately 30-70 mM in this study) were present. This became obvious when they were measured at two different pH levels, acidic (5.75) and neutral (7), and managed to grow well at pH 5.75 without SCFAs while not being inhibited at pH 7 with SCFAs being present (Sorbara et al., 2018). Similarly to these findings Chang et al. also observed that acidic pH alone is not sufficient in inhibiting *Enterobacteriaceae* species. Three different concentrations (10 mM, 50 mM and 100 mM) of each acetate, propionate and butyrate individually were used to see the most inhibitory effect on pathogenic bacterial species. Under aerobic conditions, at the maximum concentration of 100 mM at pH 5.75, all three of the SCFAs were successfully preventing growth of all tested species (i.e., two *E. coli* strains, one *K. pneumoniae, S. enterica* and *E. cloacae*), however, at lower concentrations there were strain-specific variations visible. Butyrate as well as propionate appear to be more inhibitory than acetate leading to reduced growth of the tested strains at the lower concentration of 50 mM (Chang et al., 2024). Both studies were also able to emphasize the role oxygen plays in the inhibition, showing that under anaerobic conditions even the lower amounts of SCFAs (i.e., 50 mM) at an acidic pH severely inhibit the tested species when compared to the aerobic inhibition (Chang et al., 2024; Sorbara et al., 2018). Chang et al. even observed slight growth retention at neutral pH 7 when oxygen was absent. SCFAs are

uncharged weak acids with similar pK_a (acid dissociation constant which is an equilibrium constant showing how strong an acid is in a solvent (Kütt et al., 2018)). They diffuse through the cell membrane in their neutral, protonated form (i.e., undissociated; HA) and then dissociate into anions and protons inside the cell. At more acidic environmental pH values, a higher extent of the protonated HA form is present which can enter the bacterial cell (Chang et al., 2024). However, especially in regions with higher pH levels (e.g., the colon) another major route for SCFA entry into the cell is the one using active transporters such as proton-coupled transporters or sodium-coupled transporters (Sivaprakasam et al., 2017). As mentioned in the previous chapter II.I, the entry of protons into the intracellular space is coupled with a decrease in pH_{in} leading to internal acidification also causing the disruption of the PMF by affecting ΔΨ and ΔpH. Chang et al. observed that the combination of an acidic environmental pH (5.75) and SCFAs appears to negatively impact the maintenance of ΔΨ in the tested bacterial species. However, only small differences were found between individual SCFAs regarding the reduction of ΔΨ. Sorbara et al. investigated the changes in ΔpH upon administration of physiological concentrations (50 mM and 100 mM) of acetate and were able to see that intracellular acidification occurred resulting in a greater ΔpH. When compared to the effect of propionate and butyrate in similar concentrations, it becomes visible that acetate is likely the most effective SCFA out of the three in decreasing pH_{in} (Chang et al., 2024; Sorbara et al., 2018).

All of this taken together paints a good picture of why the presence of beneficial bacteria in the gut has the potential to confer colonization resistance against pathogens such as *Enterobacteriaceae* which have been proven to negatively affect the development of the brain as a result of inflammation. Since these young patients may experience severe medical interventions, often coupled with often life-long complications, it is imperative to enhance the abundance of beneficial species e.g., *Bifidobacterium* or *Lactobacillus,* in order to prevent severe health issues such as NEC or LOS.

MATERIALS

Bacterial species

The species used (as shown in Table 1) for the experiments below were chosen based on two studies from Seki et al. (2021, 2024) in order to resemble the preterm gut microbiota as closely as possible. Most of the species were isolated from a cohort of extremely preterm infants investigated in the PreMiBraIn study of Seki et al. (2012). A few species were kindly provided by Lindsay Hall from the Technische Universität München (TUM). Before using the isolates, their purity was confirmed by Sanger sequencing or the 16S rRNA- or *dnaJ* gene in case of *Enterobacteriaceae*. Also, the genomes of some members of the collection have been sequenced (Oxford Nanopore Technologies-WGS) by the Joint Microbiome Facility (JMF; University of Vienna and Medical University of Vienna) and their taxonomic classification was confirmed. The remaining isolates have been submitted for whole-genome sequencing.

Table 1: Isolates used for experimental set up. Isolates marked with (ti) have been isolated from feces of term infants. "LH" indicates isolates provided by Lindsay Hall. The Veillonella atypica isolate marked with an asterisk (*) was isolated from a single preterm infant which was not part of the cohort. The combination of numbers and letters on the left of each species represents a unique isolate number.

obligate anaerobic species		**facultative anaerobic species**	
Bifidobacterium bifidum (ti; LH)	WS111	*Enterobacter hormaechei*	11.2A
Bifidobacterium breve (ti; LH)	WS23	*Enterobacter hormaechei*	11.2C
Clostridium perfringens	12.6B	*Enterobacter hormaechei* (LH)	LH_NM_074
Clostridium sporogenes	8.8A	*Enterococcus durans*	12.4A
Lactobacillus fermentum	10.2E	*Enterococcus faecalis*	5.5A
Lactobacillus plantarum	37.6A	*Escherichia coli*	13.3C
*Veillonella atypica**	3.11	*Escherichia coli*	14.3B

	Klebsiella michiganensis	8.3A
	Klebsiella michiganensis	8.4A
	Klebsiella pneumoniae	2.1A
	Klebsiella pneumoniae	2.1E
	Klebsiella pneumoniae	2.3A
	Klebsiella pneumoniae	2.7B
	Klebsiella pneumoniae	40.9B
	Staphylococcus epidermis	8.2A
	Staphylococcus epidermis (LH)	LH_NM_637

Media and Solutions

All general media and solutions used were prepared according to Table 2 to

Table 6, then autoclaved before usage and stored at either 4 °C (BHI media) or room temperature (PBS, glycerol) until further use.

Table 2: Brain Heart Infusion (BHI) medium (undiluted, 100 mL)

Chemical	**Company & catalog number (CN)**	**Amount**
BHI broth	Oxoid; CN: CM1135	3.70 g
MilliQ® Water	-	100 mL

Table 3: Brain Heart Infusion (BHI) medium (1:10 diluted, 100 mL)

Chemical	Company & catalog number (CN)	Amount
BHI broth	Oxoid; CN: CM1135	0.37 g
MilliQ® Water	-	100 mL

Table 4: 10x Phosphate Buffered Saline (PBS, 1000 mL)

Chemical	Company & catalog number (CN)	Amount
NaCl	Carl Roth GmbH + Co. KG; CN: 3957.1/3957.3	80.00 g
KCl	Sigma-Aldrich; CN: 1.04936	2.00 g
$Na_2HPO_4*H_2O$	Carl Roth GmbH + Co. KG; CN: 4984,1	17.80 g
KH_2PO_4	Carl Roth GmbH + Co. KG; CN: 3904,2	2.40 g

Table 5: 1x Phosphate Buffered Saline (PBS, 100 mL)

Chemical	Company & catalog number (CN)	Amount
10x PBS	-	10 mL
MilliQ® Water	-	90 mL

Table 6: 60% Glycerol Solution (100 mL)

Chemical	Company & catalog number (CN)	Amount
Glycerol	Carl Roth GmbH + Co. KG; CN: 3783,1	60 mL
MilliQ® Water	-	40 mL

L-cysteine stock solution

The L-cysteine (10% w/v) stock solution was prepared according to Table 7 and filtered (0.22 µm Sartorius Minisart® non-pyrogenic filter) afterwards. It was aliquoted and stored at -20°C until further use.

Table 7: L-cysteine (5 mL)

Chemical	Company & catalog number (CN)	Amount
L-cysteine	Carl Roth GmbH + Co. KG; CN: 3468.2	0.50 g
MilliQ® Water	-	5 mL

Experiment-specific pH media

Two different sets of pH media were prepared - one with undiluted BHI medium and one with 1:10 diluted BHI medium. For this, seven different buffer solutions of 1 M K_2HPO_4 and 1 M KH_2PO_4 were prepared by mixing them according to Table 8.

Table 8: pH media (100 mL)

pH	1 M K_2HPO_4 (mL)	1 M KH_2PO_4 (mL)
5	0.75	49.25
5.5	2.50	47.50
6	4.25	45.75
6.5	13.90	36.10
7	30.75	19.25
7.5	43.30	6.70
8	47.00	3.00

pH-specific media were prepared with undiluted and 1:10 diluted BHI in the same manner. 90 mL of each medium were prepared as described above (Table 8) and filled up with 10 mL of the respective pH buffer to reach 0.1 M final concentration of medium.

Subsequently, the pH levels were measured using a pH electrode and were adjusted by adding either 5 M hydrochloric acid (HCl) or 5 M sodium hydroxide (NaOH).

Experiment-specific SCFA media

First, 5 mM stock solutions of acetate and lactate were prepared (see Table 9 and Table 10).

Table 9: 5 mM Stock of Acetate Solution (5 mL)

Chemical	Company & catalog number (CN)	Amount
Sodium Acetate anhydrous	Sigma-Aldrich; CN: S8750	2.05 g
MilliQ® Water	-	5 mL

Table 10: 5 mM Stock of Lactate Solution (5 mL)

Chemical	Company & catalog number (CN)	Amount
Sodium L-Lactate	Thermo Scientific; CN: L14500.14	2.80 g
MilliQ® Water	-	5 mL

Further on, the respective SCFA stock solutions were diluted in BHI media (see Table 2) with a pH of either 5.75 or 7 to reach a final concentration of 10 or 50 mM respectively (see Table 11).

Table 11: SCFA-BHI media with different concentrations and pH values.

Medium	SCFA	Concentration	pH
BHI	Acetate	10 mM	5.75
BHI	Acetate	50 mM	5.75
BHI	Acetate	10 mM	7
BHI	Acetate	50 mM	7
BHI	Lactate	10 mM	5.75

BHI	Lactate	50 mM	5.75
BHI	Lactate	10 mM	7
BHI	Lactate	50 mM	7

METHODS

Determination of growth under aerobic and anaerobic conditions

In order to gain more insight into the physiological growth capabilities of our bacterial collection under various conditions, we performed growth assays with essentially the same workflow in oxic and anoxic environments (as for the SCFA experiments only under anaerobic conditions). For the pH experiments, we established a range from pH 5 to 8, increasing in steps of 0.50, to assess the ability of the bacterial isolates to grow across a broad pH spectrum and to ensure the coverage of the pH range relevant to preterm infants. For the SCFA experiments we used eight SCFA-containing media with different concentrations and different pH levels (see Table 11), to evaluate whether pH impacts the susceptibility of premature infant microbiota to SCFAs in terms of growth capabilities.

Starting of cultures

Bacterial overnight cultures were started in the laminar flow using either frozen glycerol stocks or by picking colonies from agar plates and inoculating them in 5 mL of undiluted BHI. Cultures used for the anaerobic workflow were incubated for ~ 24 hours at 37 °C in the controlled environment of the anaerobic chamber (85% N_2, 10% CO_2, 5% H_2). 50 µL of L-cysteine stock solution were added to cultures of *Bifidobacterium bifidum* WS111 and *Bifidobacterium breve* WS23 since they are auxotrophic for this amino acid. The aerobically used cultures were mostly incubated at room temperature for the same amount of time. Exceptions here were slow growers such as the two *Staphylococcus epidermidis* isolates 8.2A and LH_NM_637, *Enterococcus durans* 12.4A and *Enterococcus faecalis* 5.5A, which were incubated at 37 °C.

Passaging of cultures

50 µL of all species were passaged in the laminar flow after approximately 24 hours into separate glass tubes containing 5 mL of fresh medium to condition the isolates for the medium of the growth assay resulting in a 1:100 dilution. For the aerobic and anaerobic

pH experiments, the medium used to prepare them for the growth conditions was a 1:10 dilution of BHI, except for the two *S. epidermidis* isolates, which were transferred into undiluted BHI since they were subjected to growth in undiluted BHI-pH media subsequently. For the SCFA experiments, all species were passaged into undiluted BHI. Again, 50 µL of L-cysteine were added to *B. bifidum* and *B. breve* to ensure proper growth. Anaerobic cultures grew at 37 °C for approximately 24 hours, whereas aerobic cultures (with the same exceptions as mentioned above) grew at room temperature for the same amount of time.

Washing of cultures

Prior to inoculation, bacterial cultures were washed following a series of steps:

- 1 mL of each culture was centrifuged for 60 seconds at 13000 rpm to collect the cells. The supernatant was removed carefully.
- The pellet was washed twice with 1 mL of 1x PBS.
- Centrifugation was performed as before.
- 1 mL of 1x PBS is added to each Eppendorf tube and the pellet was resuspended.
- Finally, a 1:100 dilution of each isolate in 1x PBS was prepared and vortexed for 2-3 seconds.

Inoculation of 96-well plates

Pre-prepared 96-well plates containing 200 µL of medium with different pH levels or different SCFA media in each well were used. The wells containing no pH medium were filled with 200 µL of MilliQ® water (MQW). 2 µL of cell culture were pipetted into the respective wells. The overall dilution of bacterial cells was therefore 1:10,000 to ensure enough cell divisions prior to the exponential phase in order to gain a better reproducibility. Before adding the cell cultures, the Eppendorf tubes were vortexed very shortly to ensure a proper resuspension. After finishing, the plates were sealed with the Breathe-Easy® sealing membrane.

Cell growth determined in the plate reader

Aerobic bacterial growth was measured in 96-well plates in the TECAN® plate reader at 37°C and at OD_{600} for approximately 24 hours. For the anaerobic bacteria and thanks to a new technical setup which was originally developed by Müller et al., it was possible to measure the growth of bacteria in up to 20 microtiter plates in one experimental run within 24 hours. For this, the plate stacking device "Biostack 3" and the plate reader "Epoch2 multi-plate reader" were installed into the anaerobic tent and used for obtaining our results (Müller et al., 2024).

Preparation of 96-well plates

VWR® Tissue Culture 96-well plates used for the determination of both aerobic and anaerobic growth were prepared prior to the experiments in a pre-cleaned laminar flow, sealed with parafilm and stored at 4°C until further use. All experiments were conducted with 200 µL per well. Each bacterial isolate was tested in three biological replicates (BR), each of which was conducted on a different day. For use under anoxic conditions, the plates were introduced into the anaerobic chamber approximately 24 hours before the start of the experiment in order to ensure the complete removal of oxygen.

Data analysis of growth curves

The output data from the plate reader was generated in an Excel format. The growth curves were then submitted to AMiGA (Analysis of Microbial Growth Assays) software, which is a tool written in Python 3 and applies, among others, a Gaussian process regression to streamline the analysis of growth curves without any assumption about their shapes (Midani et al., 2021). The pH growth curves used for the analyses were all (aerobic and anaerobic) cut off at cycle 52 (after 17 hours and 20 minutes), the SCFA growth curves were cut off at cycle 64 (after 21 hours and 20 minutes). Next, AMiGA was used to fit the growth curves and afterwards predict growth metrics (AUC, carrying capacity), all performed with "Default" settings. The output generated was further imported into RStudio (version 2024.12.0+467). The code used to visualize growth curves of the aerobic pH assay (exports of the TECAN® plate reader) were provided by David Berry and further adapted by Anna-Celine Danklmaier. To visualize growth curves

generated with the high-throughput set-up (exports of Agilent plate reader), the neckaR package, version 0.2.0 (Müller et al., 2024) was implemented. To analyze the output of AMiGA, R-scripts were created by Anna-Celine Danklmaier to perform outlier deletion, normalization and plotting. The curves were visually inspected to identify biological and technical outliers (i.e., random growth aberrations vs. pipetting error). Certain growth curves were excluded from the analysis due to several reasons (e.g., machine-based errors, measurement errors). For normalization, z-score normalization was applied to each biological replicate of individual isolates to minimize batch effects. This normalization was performed across all pH levels within the respective groups (pH 5 to 8 jointly normalized). Subsequently, the mean was calculated for each individual pH level and isolate. Finally, the obtained mean values were scaled between 0 and 1 to enhance visual representation and comparability. In general, growth of each bacterial isolate was measured three times as a triplicate, however, certain isolates were measured four times. *E. hormaechei* 11.2A, *E. hormaechei* LH_NM_074, *C. perfringens* 12.6B and *L. fermentum* 10.2E were tested four times during the anaerobic pH experiments, whereas *K. michiganensis* 8.3A, *K. michiganensis* 8.4A, *E. hormaechei* LH_NM_074 and *C. sporogenes* 8.8A were tested four times during the SCFA experiments.

RESULTS

Both the pH growth assay, as well as the SCFA growth assay allow us to gain deeper insight into the physiological tolerances of bacteria from different genera under varying environmental conditions. This is especially important to better understand the niche colonization abilities of microbes in the preterm gut and how they are influenced by varying environmental conditions.

I. Growth of bacterial species under anaerobic and aerobic conditions at different pH values

Different pH levels were used to test growth of the respective bacterial isolates under anoxic (anaerobic) and oxic (aerobic) conditions in order to define their growth optima.
To illustrate the effects of the pH levels, the heatmaps below (Figure 2 and Figure 3) show two different growth metrics, namely the normalized area under the curve (AUC; the integrated area of the growth curve) and the normalized carrying capacity (K). The difference between these two is that the AUC represents the total accumulated growth over a specific period of time, whereas the carrying capacity describes the maximum population density a culture can reach in a defined amount of time. While the AUC is influenced by both growth rate and growth duration (i.e., how quickly do the bacteria grow and how long can they sustain growth), the carrying capacity is the plateau of bacterial growth within a culture. The carrying capacity represents the difference between the maximum optical density (OD_{max}) and the minimum optical density (OD_{min}).
In Figure 2, one can see the results normalized based on the AUC, which takes on values between 0.00 and 1.00 and, again, describes the total growth of the respective bacterial species over a specific amount of time (in this case 17 hours and 20 minutes). The value 0.00 represents the minimum AUC, whereas the value 1.00 accounts for the maximum AUC.

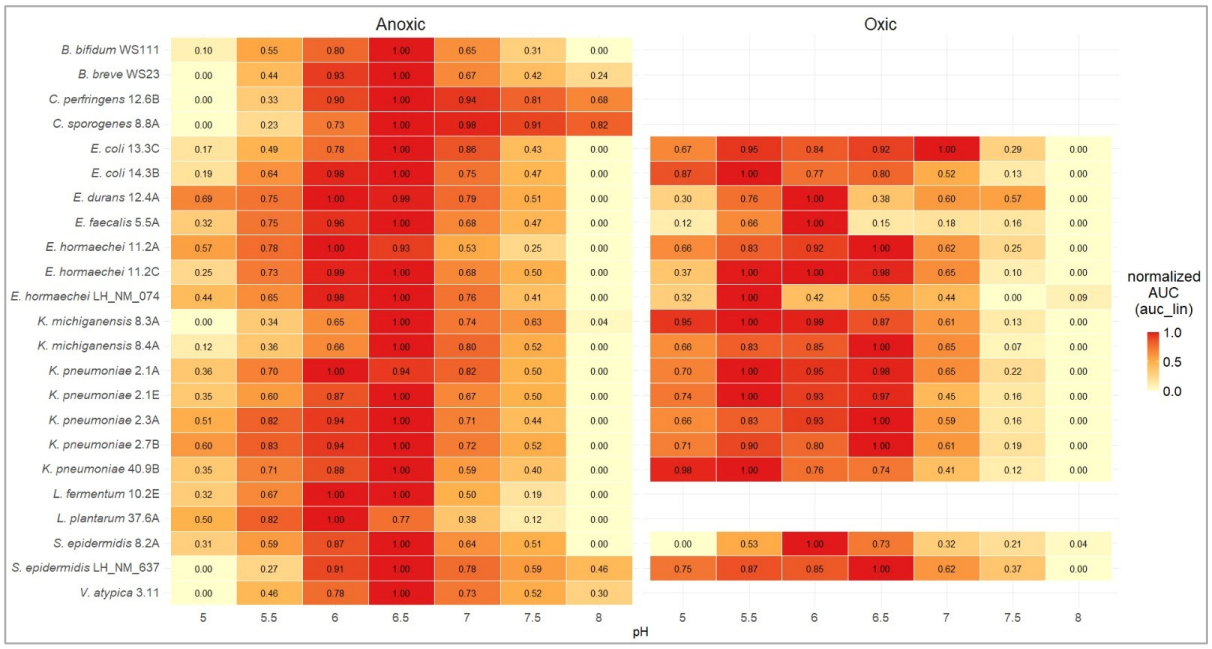

Figure 2: ***Heatmap of the normalized AUC under anoxic (left) and oxic (right) conditions.*** *The AUC was normalized for each biological replicate group of a strain individually over the range of all pH levels (pH 5 to 8) to compare growth optima trends.*

When comparing the bacteria under anaerobic conditions (left), it is clearly visible that there is a certain trend underlying their growth. Almost all tested bacterial species reach the highest AUC at pH 6.5 except *Enterococcus durans* 12.4A, *Enterobacter hormaechei* 11.2A, *Klebsiella pneumoniae* 2.1A and *Lactobacillus plantarum* 37.6A which have their optimal growth at pH 6. Interestingly, *Clostridium perfringens* 12.6B and *Clostridium sporogenes* 8.8A also manage to grow well under conditions with pH values of 7, 7.5 and 8, although *C. sporogenes* growth exceeds that of *C. perfringens* slightly. Opposed to this, it becomes clear that members of the *Enterobacteriaceae* (*E. coli, K. pneumoniae, Klebsiella michiganensis, E. hormaechei*) and the *Enterococcaceae* family (*Enterococcus durans, Enterococcus faecalis*) reach minimum AUCs at pHs higher than 7.5. The same pattern can be seen in *L. fermentum, L. plantarum* and *Staphylococcus epidermidis* LH_NM_637, all of which display minimum growth at pH 8. When looking at the two tested *Bifidobacteria* species (*Bifidobacterium bifidum, Bifidobacterium breve*) one can see minor differences in their growth capabilities over the pH range, except when looking at pH 6 and pH 8. Here, *B. breve* WS23 exceeds *B. bifidum* WS111 and manages to grow better. When focusing on the more acidic pH values (i.e., pH 5 and pH 5.5) it is apparent that *L. plantarum*, *E. hormaechei* 11.2A, *E. durans* and two *K. pneumoniae* isolates 2.3A and 2.7B obtain the highest AUCs in comparison to other isolates. Surprisingly, when looking at the two

Bifidobacteria it becomes clear that both reach minimal AUCs at pH 5 and grow moderately at pH 5.5.

Under aerobic conditions (right), a similar trend to the one under anaerobic conditions can be observed, showing overall minimum AUCs between pH 7.5 to 8. Growth optima again lie mostly around pH 6 and pH 6.5, however, the presence of oxygen leads to a shift of growth optima represented by the AUC towards more acidic pH levels in most isolates. Here, especially, the two *E. coli* isolates 13.3C and 14.3B, *K. michiganensis* 8.3A and the five *K. pneumoniae* isolates, but especially 40.9B grew better at more acidic pH values when compared to the anaerobic conditions. The exceptions here are *E. faecalis* 5.5A, the *E. hormaechei* isolate 11.2C and LH_NM_074 and *S. epidermidis* LH_NM_637 whose growth capabilities at pH 5 are similar to the ones without oxygen or declined under oxygen presence. These trends indicate that most bacterial isolates grow optimally at pH 6 to 6.5 under anoxic conditions. Under oxic conditions, especially members of the *Enterobacteriaceae* family manage to grow more efficiently at more acidic pH level as well.

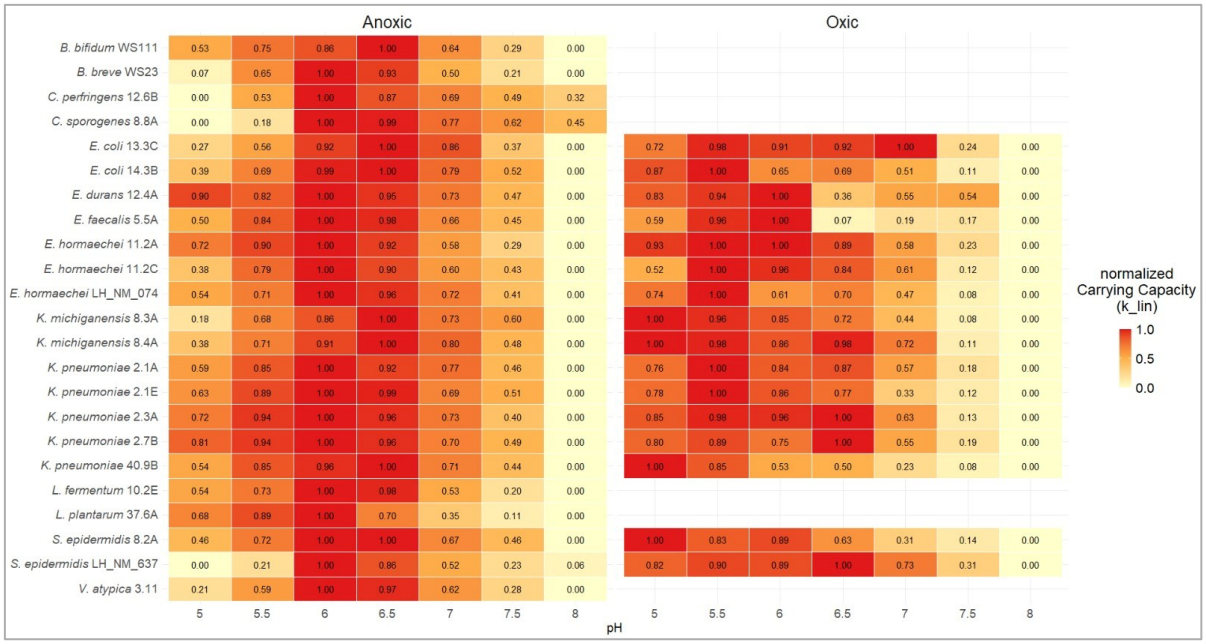

Figure 3: ***Heatmap of the normalized carrying capacity under anoxic (left) and oxic (right) conditions.*** *The carrying capacity was normalized for each biological replicate group of a strain individually over the range of all pH levels (pH 5 to 8) to compare growth optima trends.*

In Figure 3, the carrying capacity, such as the AUC above, takes on values between 0.0 to 1.0, as a minimum and maximum, respectively, and indicates the growth capabilities of the bacterial isolates. Similarly to Figure 2, most bacterial species have their maximum

carrying capacity under anaerobic conditions (left) at pH 6 or pH 6.5 while not managing to grow very well at higher pHs such as pH 8. However, since the carrying capacity is based on subtracting the minimum OD from the maximum OD it does not encompass all phases of the bacterial growth as the AUC does (i.e., lag phase, exponential phase, stationary phase), which might lead to some differences in the observed growth optima. An example of this are the members of the *Clostridiaceae* family, *C. perfringens* 12.6B and *C. sporogenes* 8.8A. At pH 7.5 as well as at pH 8, both species show reduced growth in terms of carrying capacity when compared to the AUC, and their growth optima shift towards pH 6. Another example is *B. bifidum* WS111, which reaches its growth optimum around pH 6 and pH 6.5 in terms of carrying capacity, however, displays maximum AUC at pH 5 and 5.5.

Under aerobic environmental conditions (right) there are only very minor differences when comparing the carrying capacity to the AUC. Here, the trend of bacteria showing minimal carrying capacity at pH 7.5 and pH 8 is visible, as well as all of the *K. pneumoniae* isolates (particularly *K. pneumoniae* 40.9B), both *K. michiganensis* isolates 8.3A and 8.4A and *S. epidermidis* LH_NM_637 managing to grow well at the acidic pH 5 and 5.5. Taken together, similar to the heatmap based on the AUC, the bacterial species obtain higher carrying capacity at more acidic pH levels (pH 5.5 and lower) in the presence of oxygen. Supporting the trends seen in the AUC heatmap, these trends indicate that most bacterial isolates grow optimally at pH 6 to 6.5 under anoxic conditions. Under oxic conditions, especially members of the *Enterobacteriaceae* family manage to grow more efficiently at more acidic pH level as well.

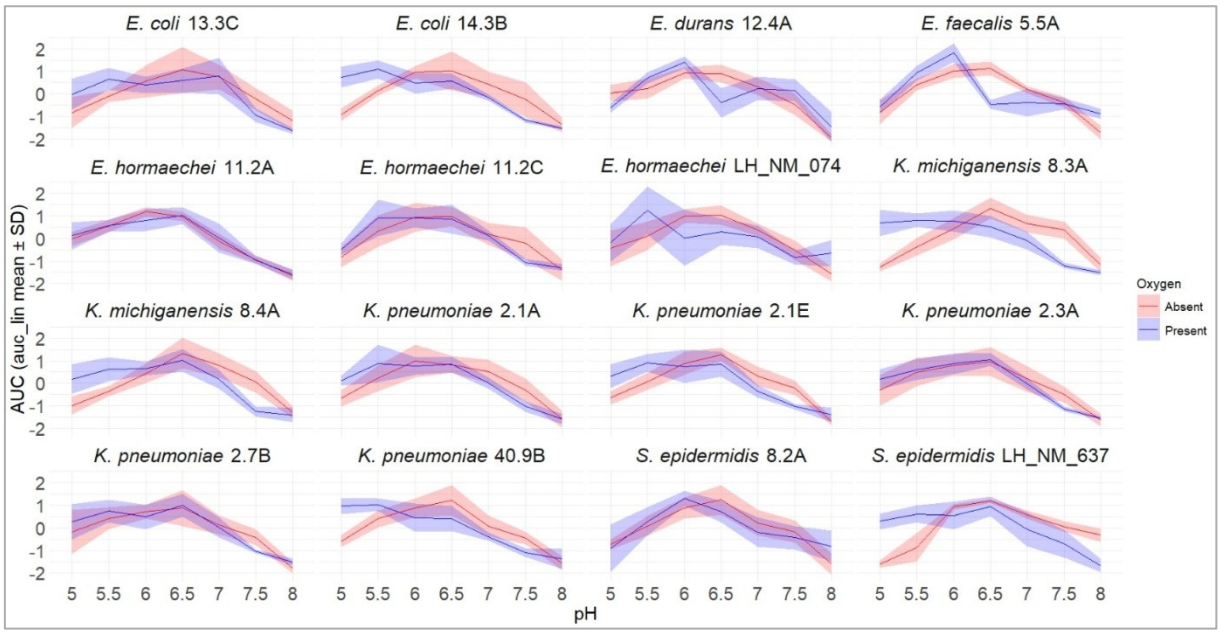

Figure 4: ***Direct comparison of growth under conditions with and without oxygen.*** *Each of the bacterial species is plotted separately. On the x-axis are the different pH values, whereas on the y-axis the z-score normalized AUC without scaling between 0 and 1 is shown. Blue curves show growth under conditions with oxygen, red curves show growth under conditions without oxygen.*

In Figure 4 are depicted only the AUCs of the facultative anaerobic bacterial species in order to show in detail the difference between growth under aerobic or anaerobic conditions of these isolate. In some cases (e.g., *E. hormaechei* 11.2A, *K. pneumoniae* 2.3A and 2.7B) the trend of minimal to maximum growth between the two conditions is very similar over the whole pH range. However, there it is visible that almost all of the species grow less efficiently under anaerobic conditions especially at acidic pH levels (pH 5.5 and lower). Very distinct examples for that are *E. coli* 14.3B, both *K. michiganensis* isolates although the difference is more visible in 8.3A than in 8.4A, *K. pneumoniae* 40.9B and *S. epidermidis* LH_NM_637. Two very intriguing growth patterns are displayed by the two members of the *Enterococcaceae* family, *E. faecalis* 5.5A and *E. durans* 12.4A. Not only do they grow better at acidic pHs (5 and 5.5) in the absence of oxygen, but both of the isolates experience a sharp decrease in growth at pH 6.5 under aerobic conditions. Whilst *E. durans* manages to somewhat recover at more the basic pHs of 7 and 7.5, *E. faecalis*' growth remains at a minimum. Taken together, it becomes clear that, especially members of the *Enterobacteriaceae* family as well as one of the two *Staphylococci*, display less growth at acidic pH levels in the absence of oxygen compared to when oxygen is present. This goes to show that the combination of oxygen and pH may have growth retention effects at more acidic pH levels. At the same time, it is visible that the overall trend of growth decreasing towards

more basic pHs (pH 7.5 and pH 8), which was also seen in the two heatmaps above, is seemingly confirmed with Figure 4.

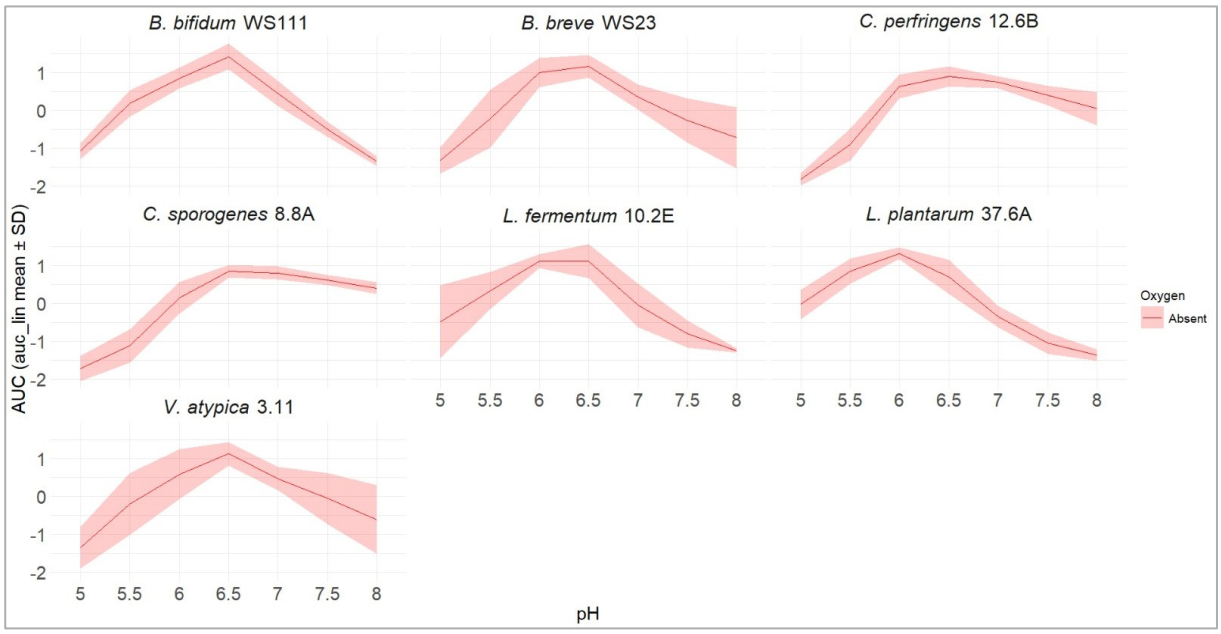

Figure 5: ***Growth patterns of strict anaerobic bacterial isolates.*** *On the x-axis are the different pH values, whereas on the y-axis the z-score normalized AUC without scaling between 0 and 1 is shown. Each of the bacterial species is plotted separately. Red curves show growth under conditions without oxygen.*

Additionally to the heatmaps (Figure 2 and Figure 3) and to give another perspective on the results of the strict anarobic bacterial species, Figure 5 shows the trends of growth capabilities of each of the anaerobic species seperately. Consistent with the results of the two heatmaps, the optimal growth for the two *Bifidobacteria* as well as for the two *Lactobacilli* and the *Veillonella* isolate is between pH 6 and pH 6.5 with slight interindividual differences. Furthermore, growth patterns of *C. sporogenes* 8.8A and *C. perfringens* 12.6B are also compliant with the heatmap results showing that they are the only ones managing to grow well at pH values of 7.5 and 8.

II. Inhibition of potentially pathogenic bacteria in the presence of SCFAs

In the following plots (Figure 6 to Figure 8), one can see the results obtained from the SCFA experiments. Figure 6 gives an overview of the 23 tested bacterial isolates and their growth capabilities in terms of AUC in the absence or presence of 50 mM SCFAs at either acidic pH 5.75 or neutral pH 7 (the growth capabilities at 10 mM SCFAs can be found in Figure S1 in "Supplemental Materials"). The concentrations were chosen to resemble the lower range of the overall concentration of SCFAs in a healthy GIT. Each of the isolates is plotted separately to allow for a direct comparison between the tested bacteria. The overall trend for the *Enterobacteriaceae* (i.e., *E. coli, E. hormaechei, K. michiganensis* and *K. pneumoniae*) shows that the inhibition effect of acetate at acidic pH is greater than that of lactate. Especially, the five *K. pneumoniae* isolates as well as the two *E. coli* isolates and the three tested isolates of *E. hormaechei* show a great reduction in growth when compared to the Control condition. Note that 11.2A, 11.2C, 2.1E and 2.7B display a bigger variance in comparison to the other *Enterobacteriaceae* family members, which is possibly due to biological differences in growth depending on the day (Figure S2 and Figure S3 in "Supplemental Materials"). Interestingly, the inhibition effect seems to be weaker in case of the two *Enterococcaceae* as well as at the two isolates of *S. epidermidis*. Both tested isolates of *Enterococci* and of *Staphylococci* did not experience a large reduction in growth in the presence of either of the SCFAs or either of the pH levels. As for the strict anaerobic bacteria, the addition of acetate and lactate as well as the difference in the pH mostly lead to a slight growth reduction but only minor differences between the respective conditions can be observed. Summarizing, the overall trend, especially in the facultative anaerobic bacteria, shows a greater inhibition effect of acetate when compared to lactate but only at the acidic pH 5.75. Regarding the obligate anaerobic species, there is little to no difference between the respective SCFAs and the two different concentrations.

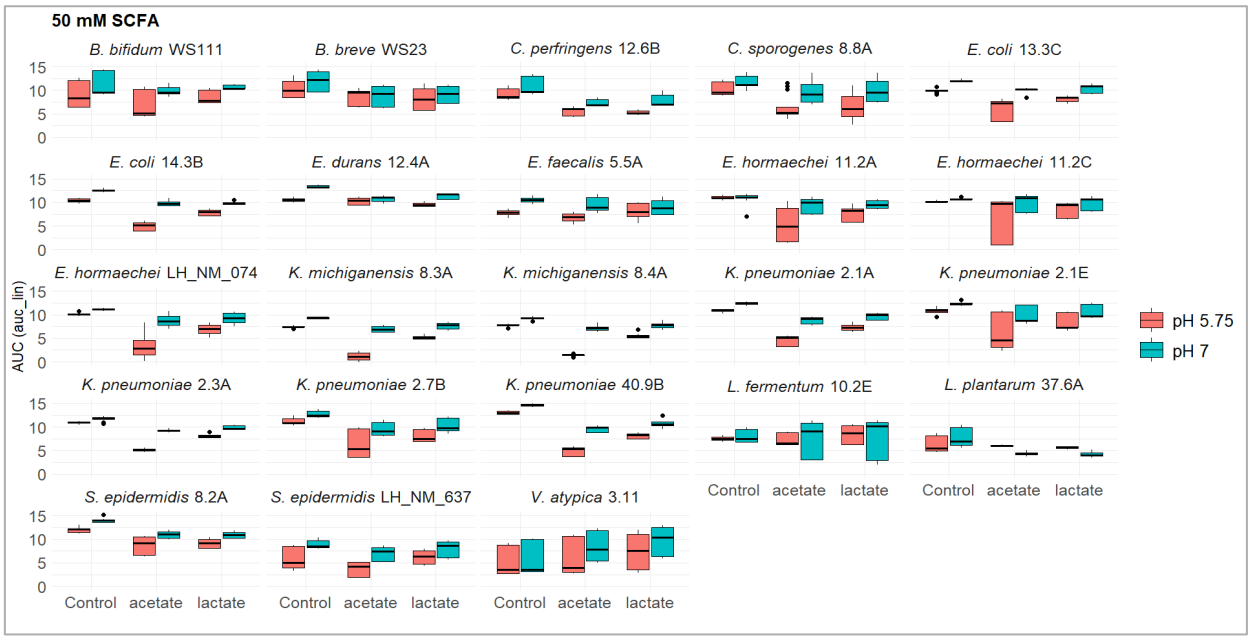

Figure 6: ***Overview of the inhibition effect of acetate and lactate at 50 mM at pH 5.75 (red) and pH 7 (blue) in comparison to the Control.*** *The two respective SCFAs as well as the Control are depicted on the x-axis and the AUC is depicted on the y-axis*

In order to get a more detailed insight and a direct comparison between the effects of 10 mM versus 50 mM of the respective SCFA, the following bar plots show the AUC of selected members of the *Enterobacteriaceae* family (Figure 7) as well as of the strict anaerobic bacteria (Figure 8). Each bacterium is depicted separately to allow for easier comparison between the isolates. Figure 7 distinctively shows that members of the *Enterobacteriaceae* family are inhibited the most in the presence of 50 mM of acetate at the acidic pH level, with the *K. pneumoniae* isolates displaying the greatest inhibition. It is apparent that at pH 5.75, 50 mM acetate restricts growth more than lactate does at the same concentration, while such a stark difference is not observed at 10 mM. Also, it becomes visible that the inhibitory effect of 10 mM of either SCFA respectively at neutral pH is similar to the one at pH 5.75. However, focusing only on pH 7, the differences in growth inhibition are less pronounced between acetate and lactate at both concentrations.

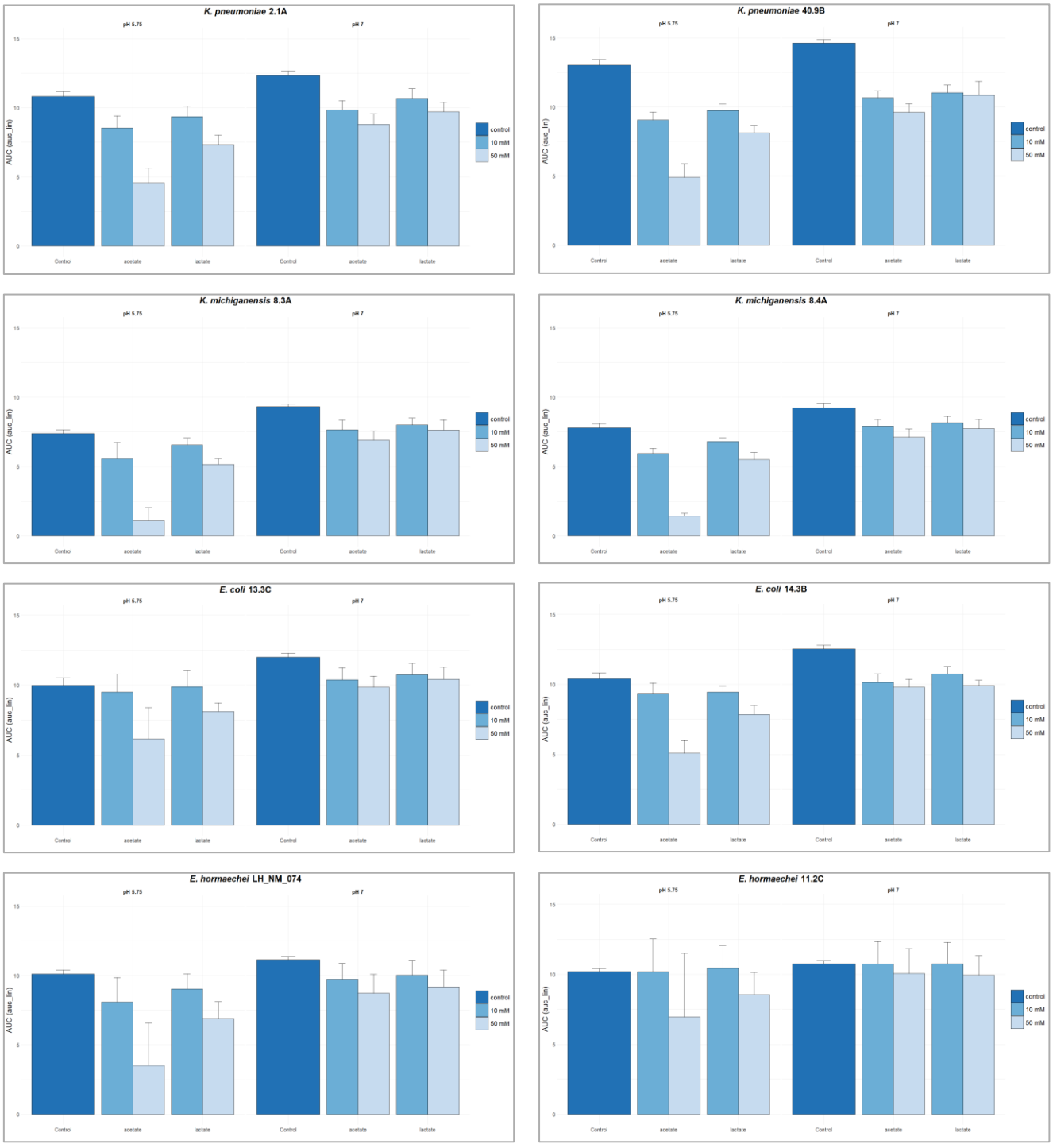

Figure 7: ***Growth inhibition of representatives of the Enterobacteriaceae family in the presence of different SCFAs compared to the Control.*** *Each isolate is depicted in a separate plot. On the left side of the individual plot is always the acidic pH condition that was tested whereas the neutral pH is depicted on the right side. The darkest blue color shows the growth under "Control" conditions (i.e., when no SCFA was added). While the medium blue color shows the lower of the two concentrations (10 mM), the light blue color represents the higher concentration (50mM) of the respective SCFA ("acetate" or "lactate").*

Figure 8 depicts the AUC of obligate anerobic species at different pH levels and with different SCFA concentrations. In contrast to the *Enterobacteriaceae* described above, the tested anaerobic isolates do not show a consistent trend. Also, there are differences within one bacterial family visible. First, concerning the two *Bifidobacteria* species, *B. bifidum* is slightly inhibited in the presence of either of the SCFAs at pH 7 and astonishingly managed to grow better at pH 5.75 with both acetate and lactate at 10 mM. However, at acidic pH and 50 mM of either SCFA, *B. bifidum* growth is decreased, although acetate is slightly more effective in doing so. *B. breve* on the other hand is inhibited more at the neutral pH than at the acidic pH, albeit there is no real difference between the SCFA source and the concentrations. At the same time, at pH 5.75 there is no growth inhibition in the presence of both acetate and lactate at 10 mM, and only a slight decrease in AUC at 50 mM of the SCFAs. Secondly, there are also interspecies differences between the two *Lactobacilli* that were tested. Whilst *L. fermentum* growth is either improving or remaining similar in the presence of SCFAs (no matter the pH and the concentration), *L. plantarum* is not inhibited at the acidic pH but its growth is decreased at the neutral pH similarly with both acetate and lactate and at 10 mM as well as at 50 mM. As for *C. perfringens* and *C. sporogenes*, the trend of growth inhibition is the same for both. At pH 5.75, acetate as well as lactate manages to decrease growth compared to the control, both being similarly effective at 10 mM and 50 mM. At pH 7, growth is also inhibited consistently under all four SCFA conditions, although *C. perfringens* is affected more strongly than *C. sporogenes*. Opposed to this, growth of the *V. atypica* 3.11 isolate is not inhibited at all. At each of the environmental pH levels and in the presence of both acetate and lactate at both concentrations, *V. atypica* growth increased compared to the control. Under acidic conditions, 10 mM of acetate and lactate respectively improve growth more than when 50 mM of each were added. At the neutral pH of 7, there was seemingly no or just a slight difference between the concentration and also between the two SCFAs.

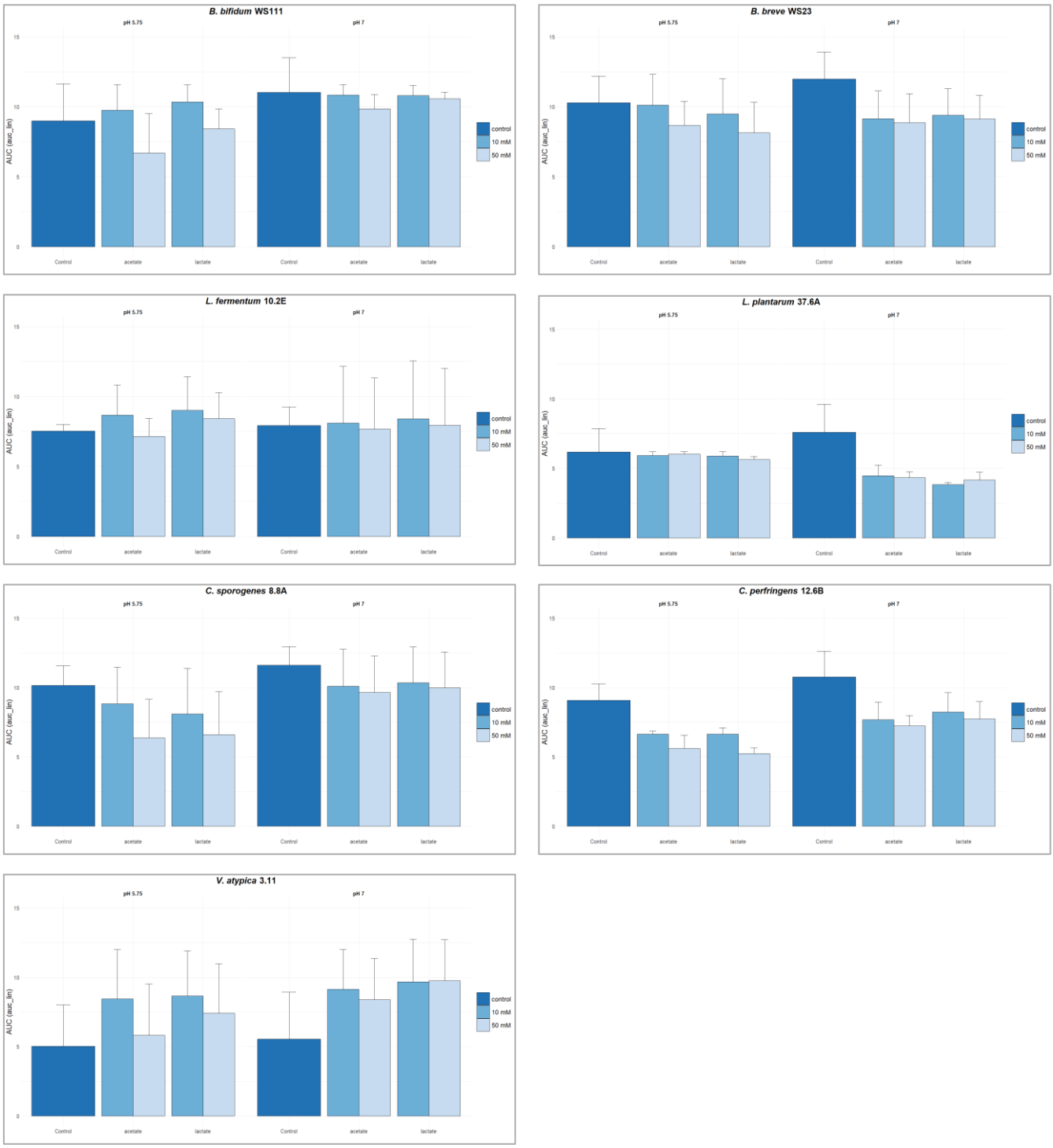

Figure 8: ***Growth inhibition of obligate anaerobic bacteria in the presence of different SCFAs compared to the Control.*** *Each isolate is depicted in a separate plot. On the left side of the individual plot is always the acidic pH condition that was tested whereas the neutral pH is depicted on the right side. The darkest blue color shows the growth under "Control" conditions (i.e., when no SCFA was added). While the medium blue color shows the lower of the two concentrations (10 mM), the light blue color represents the higher concentration (50mM) of the respective SCFA ("acetate" or "lactate").*

It is important to note that some of the isolates measured (e.g., *K. pneumoniae* 2.1E, *E. hormaechei* LH_NM_074, *K. michiganensis* 8.3A, *V. atypica* 3.11 or *C. sporogenes* 8.8A) in the SCFA experiments display a relatively high coefficient of variation (CV). This might be because the control conditions were not performed together with its biological replicate of each isolate due to time reasons. CV is considered as “high” when it is > 50%, “moderate” between 10 to 50% and “low” when it is < 10%. This means that, for example, *V. atypica* 3.11 has a moderate to high variation in the presence of either SCFA depending on the concentration and pH. An exact overview of the CV for each isolate and condition can be found in Figure S2 and Figure S3) in “Supplemental Materials”.

DISCUSSION

Preterm birth accounts for approximately 10% of all births worldwide and still remains the leading cause of death in neonates (Ahmed et al., 2024; Ohuma et al., 2023). Over the past few decades, it has been established that a healthy gastrointestinal microbiome in neonates supports the proper neurological and immunological development as well as it infers colonization resistance to various enteric pathogens (Sanidad & Zeng, 2020). However, it has been observed that the gut microbiota of preterm infants differs significantly from that of term infants showing higher numbers of potentially pathogenic and opportunistic bacteria such as *Proteobacteria, Enterobacteriaceae* and *Staphylococci* and at the same time displaying reduced abundances of beneficial bacteria such as *Bifidobacterium* (Beghetti et al., 2023; Korpela et al., 2018). These aberrations often have detrimental effects on neurodevelopment and overall infant health (Seki et al., 2021).

Because of these challenging circumstances, this study aims to shed some light on the basic physiological behaviors of members of the preterm gut microbiota and thereby give deeper insight into how growth of beneficial and potentially pathogenic bacteria can be influenced depending on environmental conditions. Therefore, 23 different bacterial isolates resembling the preterm gut microbiota were grown under different pH conditions alone, as well as at acidic and neutral pH in combination with two SCFAs, acetate and lactate. The pH experiments were carried out in both aerobic and anaerobic conditions, since the initial establishment of the gastrointestinal microbiota of preterm infants and neonates in general is, amongst others, influenced by the presence of oxygen. Right after birth the intestine of the neonate is an aerobic environment which favors the expansion of facultative anaerobes such as *Enterobacteriaceae* or *Enterococcus*. In healthy infants (i.e., term infants), the switch from an anaerobic to an anaerobic environment happens within a matter of a few days, promoting growth of obligate anaerobic bacteria such as *Bifidobacterium* and *Clostridiaceae* (Arrieta et al., 2014). Based on a study by Arboleya et al., it appears that the change from an oxic to an anoxic condition in the GIT in preterm infants happens at a later timepoint compared to term infants, consistently showing lower abundances of strict anaerobes over a 90-day period after birth and higher amounts of *Enterobacteriaceae* over a 30-day period (Arboleya et al., 2012). It is, however, not clear yet whether the difference in the oxygen levels between term and

preterm infants is due to natural causes, or if e.g., specific medical practices in NICUs (i.e., use of continuous positive airway pressure, use of antibiotics) cause an increase in oxygen concentrations (Chong et al., 2018).

With our experiments, we were able to show that the growth of facultative anaerobic gut bacteria of preterm infants at various pH levels indeed differs depending on the presence of oxygen. Whilst the growth optimum of members of the *Enterobacteriaceae* is at pH 6 to 7 under anoxic conditions, it shifts towards more acidic optima for each tested species (pH 5.5 to pH 6.5; based on AUC as a growth metric). A similar trend becomes apparent for one of the two *S. epidermidis* isolates (i.e., *S. epidermidis* LH_NM_637) which also seems to be growing slightly better at acidic pH levels in the presence of oxygen. Furthermore, the results for growth of the two *Enterococci*, namely *E. durans* and *E. faecalis*, are especially intriguing and lead to additional assumptions that oxygen plays a very important role concerning the optimal growth pattern of bacteria. In comparison to their growth capability across different pH levels under anoxic conditions, the two *Enterococcus* spp. show a sharp decrease in growth under oxic conditions at pH > 6. For *E. faecalis* this decline even extends over a broader range (pH 6.5 to 8) when compared to *E. durans*, which manages to recover at pH7 and 7.5. This might be due to enzymatic systems being sensitive to both pH and oxygen presence. For instance, the pyruvate formate lyase (PFL) present in *E. faecalis* is active at pH levels higher than 6.0 but at the same time very sensitive to oxygen. Since the PFL system is used to produce acetyl-CoA under anaerobic conditions and is known as an enyzme of the anaerobic metabolism, its functions are prevented in the presence of oxygen, thereby potentially causing a decrease in energy production and growth (Doi & Ikegami, 2014; Sarantinopoulos et al., 2001). Another interesting finding of our study is the growth behavior of *Enterobacteriaceae*, here especially the five different *K. pneumoniae* isolates, at neutral and basic pH levels (i.e., > pH 7). According to literature, optimal growth of this bacterium is typically observed around pH 6 to pH 8 under anaerobic conditions (Mitrea & Vodnar, 2019). However, our results suggest slight differences. Under anoxic conditions all tested *Enterobacteriaceae* family members grow moderately well at pH 7 and pH 7.5 and growth only declines at pH 8. On the other hand, in the presence of oxygen, *Klebsiella* spp., *E. hormaechei* and *E. coli* are subject to drastic growth inhibition at pH > 7. This is congruent with our finding of these bacteria having their growth optima shifted to a more

acidic pH level under oxic conditions. Concerning beneficial bacteria – *Bifidobacteria* spp. and *Lactobacilli* spp. which are often administered to preterm infants as probiotics to mitigate the dysbiosis inducing effects of antibiotics and improve infant health (Alsharairi, 2023) – our findings indicate a few differences in the behavior of our isolates to that in literature. The *B. bifidum* and *B. breve* from our collection both have their growth optimum at pH 6.5 followed by pH 6 based on the AUC as a growth metric. They grow equally well at pH 5.5 and pH 7. This is somewhat overlapping with the findings from J. Chen et al. and Kawasaki et al. who state that optimal *Bifidobacterium* growth occurs around pH 6.5 to pH 7 with some exception of e.g., *B. bifidum* that showed its optima in the acidic region (pH 5). *Lactobacilli* spp. have been reported to have their growth optimum between pH 4.5 and pH 6.5 (Śliżewska & Chlebicz-Wójcik, 2020). Since our pH range started at pH 5, we cannot fully prove or refute these findings, however, we saw interspecies differences in growth between the two *Lactobacilli* isolates. Whilst *L. fermentum* grew best at pH 6 and pH 6.5 but did not do so well at more acidic pHs, *L. plantarum* did manage to show growth at pH 5 and pH 5.5, although its optimum was at pH 6 as well.

The second goal of this study was to show the influence of two individual SCFAs, acetate and lactate, on the growth of different gut bacteria at either environmental pH 5.75 or pH 7 and whether they can inhibit potentially pathogenic bacteria. Acetate and lactate were chosen since they are, among others, produced by *Bifidobacterium* spp. and *Lactobacillus* spp. to show the effect they have on our bacterial collection. To highlight the significance of SCFAs, one must consider their relationship with pH. SCFAs are weak acids, with similar pK_a values, which can diffuse into the bacterial cell in their protonated form. This happens more frequently at acidic environmental pH levels since the ΔpH between the cytoplasm and the surroundings outside of the cell becomes larger, which leads to more of the SCFAs being protonated and therefore more of them being able to transverse the cell membrane (Salmond et al., 1984; Sorbara et al., 2018). Once inside the cell, they dissociate into protons and anions and can thereby acidify the cytoplasm and further inhibit growth of various bacterial species. However, the amount of protonated SCFAs depends not only on the ΔpH but also on the pK_a. Acetate has a pK_a of 4.76, whereas the one of lactate is 3.86 (Ng et al., 2024). According to the Henderson-Hasselbalch equation, 50% of the weak acid is protonated (for acetate: CH_3COOH) and

50% is present in a deprotonated form (CH_3COO^-) when pH = pK_a. Consistently, there is a logarithmic decrease at pH < pK_a, so when the environment is acidic, meaning that the protonated form of the acid is more prevalent than the deprotonated (i.e., ionized) form. Coincidentally, at pH > pK_a (i.e., basic environment) a logarithmic increase leads to more of the weak acid being deprotonated and less being present in a protonated state (Kundukad et al., 2017). The magnitude of the difference between pK_a and pH determines the amount of the SCFA existing in a protonated vs. deprotonated form. As mentioned before, when the pH is higher than the pK_a, more of the acid is deprotonated, meaning that the amount diffusing through the membrane is lower. Even though in our experiments both SCFAs have smaller pK_a values than the pH levels tested, the difference between acetate's pK_a and the two pHs chosen (pH 5.75 and pH 7) is lower than the one between lactate's pK_a and the pHs. Therefore, connecting this information to our study design, one would assume that acetate would be more efficient when compared to lactate. Indeed, we were able to see the inhibition of potentially pathogenic bacteria belonging to the *Enterobacteriaceae* family with both SCFAs, although acetate was more effective in doing so. Still, the presence of lactate led to a higher inhibition than initially expected due to its pK_a of 3.86, which would allow only approximately 1% of lactic acid to diffuse into the cell at pH 5.75 (in contrast, in the case of acetate approximately 10% of acetic acid could diffuse into the cell). These findings are congruent to the underlying mechanism described above and have also been observed in similar studies. In 2024, Chang et al. tested growth of various potentially pathogenic bacteria (i.e., *E. coli*, *K. pneumoniae, Enterobacter cloacae* and *Salmonella enterica Typhimurium*) in the presence of 10, 50 and 100 mM of acetate, propionate and butyrate respectively at pH 5.75 and 7.0. Due to relevancy for our study, only the observed effect of acetate will be elucidated here. Similar to our discoveries, they also observed the highest inhibition at concentrations ≥ 50 mM of acetate but only in combination with an acidic pH of 5.75 (Chang et al., 2024). On top of that, Sorbara et al. also detected a reduction of *E. coli* under acidic pH conditions in the presence of SCFAs, however, they used a mixture of acetic acid, butyric acid and propionic acid with concentrations of 50 mM, 25 mM and 6 mM respectively (Sorbara et al., 2018). Accordingly, Ng et al. tested the effect of 20 structurally different SCFAs at various concentrations, including acetate and lactate, on the growth of *S. enterica* at an acidic pH (pH 4.5 and 5.5) and at a near-neutral pH (6.5). They observed a similar pattern of inhibition with 50 mM of acetate

and lactate (and other SCFAs, which will not be mentioned here), showing growth of *S. enterica* reduced by 50% or more at pH 4.5 and 5.5 but not at pH 6.5 (Ng et al., 2024). Another very intriguing finding of our study showed enhanced growth of *V. atypica* in the presence of both SCFAs. *V. atypica* and other members of the *Veillonella* genus are strict anaerobes and are commonly found in the human oral cavity microbiome and can also be found as a commensal in the preterm gut microbiota (Seki et al., 2024; Zhou et al., 2021). Since they lack the gluco- and fructokinase, they are unable to metabolize glucose, fructose and disaccharides. Instead, their primarily consumed carbon source is lactate which they ferment into propionate, acetate, CO_2 and H_2. Besides that, *Veillonella* spp. have been found to be often co-localized with *Lactobacilli* spp. which suggests a potential relationship between these two genera (Zhang et al., 2024; Zhou et al., 2021). This leads to the assumption that the *V. atypica* isolate from our collection potentially displayed increased growth in the presence of the two SCFAs because of its ability to metabolize lactate. As for the growth enhancing effect of acetate, there have not been studies to support this finding. However, even though acetate is not used as a primary energy source, it is still an important by-product in the fermentation of lactate and might serve to maintain the redox balance of the biochemical reaction at hand. By doing so, one can argue that it indirectly supports *Veillonella* growth, and we therefore saw an increase in our experiments.

A key finding of our study is that *Enterobacteriaceae* grow better at acidic pH in the presence of oxygen than under anaerobic conditions. Since members of this family are usually classified as “potentially pathogenic”, understanding the mechanisms that regulate their growth in the preterm gut is crucial. Typically, the human GIT is hypoxic with an oxygen gradient that decreases from the stomach to the colon. This decrease in the oxygen level influences the composition of the gut microbiome, favoring the expansion of oxygen-sensitive anaerobic species (e.g., *Bifidobacteria*) (McCallum & Tropini, 2024; Moreira de Gouveia et al., 2024). However, preterm infants are often subject to prolonged aerobic conditions within the GIT compared to term infants (Arboleya et al., 2012). Factors such as antibiotic treatments and inflammation – both of which preterm infants are frequently confronted with – have been known to increase epithelial oxygenation and elevate subsequent diffusion of oxygen into the gut lumen. This altered oxygen landscape provides a competitive advantage for *Enterobacteriaceae* over strict anaerobic bacteria

(Moreira de Gouveia et al., 2024; Rivera-Chávez et al., 2016). Unlike strict anaerobes, *Enterobacteriaceae* can utilize oxygen, nitrate and other substrates as terminal electron acceptors for respiration, enabling them to generate more energy than just via the fermentation pathway (Sorbara et al., 2018). In contrast, oxygen-sensitive bacteria such as *Bifidobacteria* experience significant stress when exposed to oxygen which can lead to the accumulation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2). ROS can be lethal to these bacteria, since they lead to protein misfolding and aggregation and cause DNA damage as well as lipid peroxidation (Averina et al., 2023). At the same time, acid stress plays a crucial role in shaping microbial populations. Most bacteria possess robust acid resistance mechanisms, including proton pumps, amino acid decarboxylation systems, and membrane modifications that help them maintain intracellular pH homeostasis, as discussed thoroughly in Chapter II.I. This is also mostly the case for beneficial bacteria such as *Bifidobacteria* and *Lactobacilli,* who can also protect themselves against acid stress in similar ways (i.e., membrane modifications, use of proton pumps for pH homeostasis, formation of biofilms). Also, these bacteria produce short-chain fatty acids, which acidify the gut environment and exert inhibitory effects on *Enterobacteriaceae* (Schöpping et al., 2022; Sorbara et al., 2018). Since *Enterobacteriaceae* usually grow best under slightly acidic to slightly basic conditions (pH 6 to 8), acidification of the gut lumen and of their cytoplasm in the presence of SCFAs limits their expansion. Apparently, this particularly happens under anaerobic conditions where their acid stress response mechanisms may be less effective, since our findings suggest more resilience to acid stress under oxic conditions. This could indicate that oxygen availability facilitates activation of acid stress resistance mechanisms that allow for survival at lower pH levels. Summarizing, the interplay between oxygen availability, SCFA production and acid stress resistance are of the utmost importance in shaping the microbial landscape and are essential parameters that need to be considered when investigating the preterm gut microbiota.

CONCLUSION AND OUTLOOK

This study highlights the crucial role of environmental factors – such as pH, oxygen availability, and the presence of SCFAs – in shaping the microbial composition of the preterm infant gut. We observed that facultative anaerobic bacteria, including members of the *Enterobacteriaceae* family, exhibit enhanced growth in acidic conditions when oxygen is present. Moreover, our findings suggest that SCFAs, particularly acetate, strongly inhibit the growth of potentially pathogenic bacteria, with this effect being amplified at more acidic pH levels. In contrast, we found that beneficial bacteria such as *Bifidobacteria* and *Lactobacilli* grow best at slightly acidic to neutral pH levels and display acid stress resistance mechanisms since they are not inhibited noticeably in the presence of SCFAs. These results provide further insights into how metabolic by-products influence microbial dynamics and contribute to colonization resistance against opportunistic pathogens.

Looking ahead, several important questions remain open and will need to be addressed in future research. One important aspect is intracellular acidification, which could be examined using reporter assays to determine how bacterial cells respond to acidic stress at a molecular level. Additionally, co-culture experiments provide insights into interactions between beneficial bacteria and potential pathogens like *Klebsiella* in terms of inhibition by SCFAs. Therefore, analytical techniques such as high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) could help to quantify SCFA production under different environmental conditions. Gaining deeper insight into the production of SCFA is essential, especially in the presence of other substrates such as HMOs which can influence bacterial community dynamics.

Another important question to address is how oxygen availability affects SCFA production of facultative anaerobes like *Enterobacteriaceae*. Measuring SCFA concentrations under aerobic conditions, as well as monitoring pH changes after microbial growth in SCFA-rich environments, could elucidate the interplay between oxygen, fermentation processes, and gut acidification.

Regarding the SCFA experiments, it is necessary to repeat at least some of the bacterial isolates (i.e., the ones with a high CV) in order to get more distinctive results. As mentioned before, the controls were not tested on the same day as the SCFA conditions. Due to time reasons, it was not possible for this thesis to repeat the

experiments. Therefore, the resulting data has not been normalized, however, a calculation of the CV was performed to review on the comparability of the results without normalization.

Furthermore, the levels of oxygen in the preterm gut remain an open question, and whether they are comparable to those used in our aerobic growth conditions is difficult to determine due to ethical constraints. However, understanding the realistic oxygen concentrations and their impact on microbial behavior would be critical for refining experimental conditions and improving the robustness of our findings. Given the delayed transition to an anaerobic environment in preterm infants, interventions that promote acid-tolerant beneficial bacteria, while simultaneously limiting *Enterobacteriaceae* overgrowth could be a promising strategy to support gut health and reduce the risk of dysbiosis-associated complications such as NEC.

Additionally, other environmental conditions (e.g., presence or absence of antibiotics, urea or iron) need to be tested to gain further insight into the colonization abilities of microbes in the preterm gut and to better understand the negative effects pathogens such as *Klebsiella* can have.

Ultimately, these additional investigations could potentially contribute to the establishment of microbiome-targeted interventions aimed to promote gastrointestinal health, thereby possibly reducing the risk of severe complications in preterm infants.

SUPPLEMENTAL MATERIALS

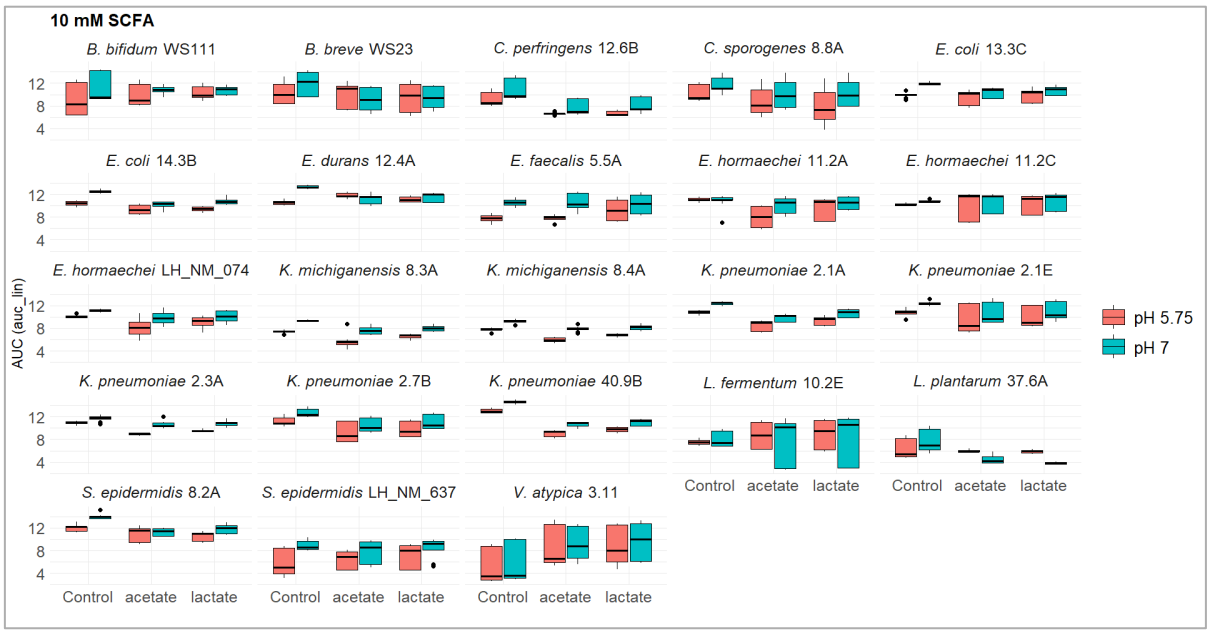

Figure S1: ***Overview of the inhibition effect of acetate and lactate at 50 mM at pH 5.75 (red) and pH 7 (blue) in comparison to the Control.*** *The two respective SCFAs as well as the Control are depicted on the x-axis and the AUC is depicted on the y-axis*

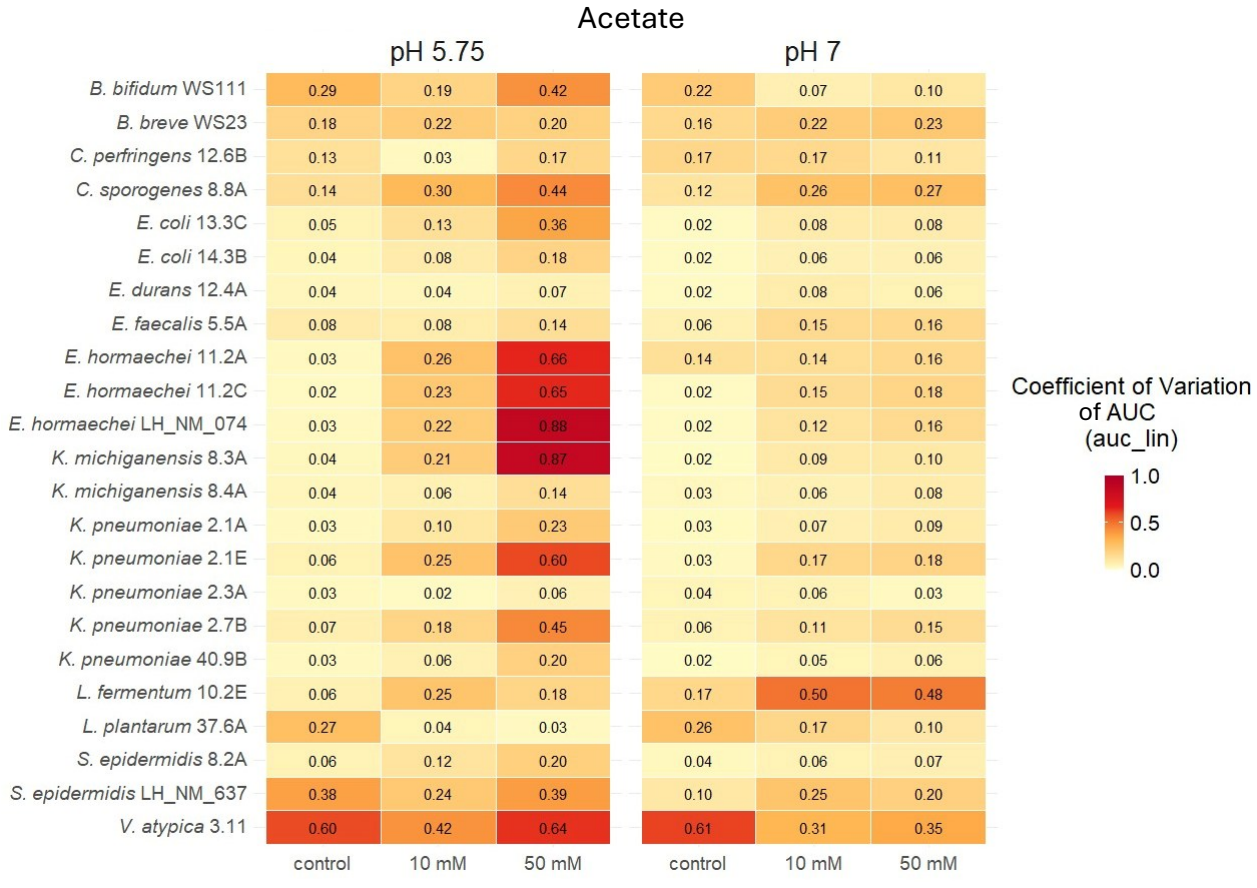

Figure S2: ***Coefficient of Variation (CV) for each individual bacterial isolate under anaerobic conditions in the presence of acetate.*** *Depicted on the x-axis are the different conditions (control,*

10 mM and 50 mM), whereas on the y-axis one can see the individual bacterial isolates. The CV was calculated based on the AUCs of the individual isolates and can have values between 0.0 (0%) and 1.0 (100%). CVs of > 50% are regarded as "high" CVs, whereas CVs between 10-50% are regarded as "moderate" and the ones < 10% are classified as "low" CVs.

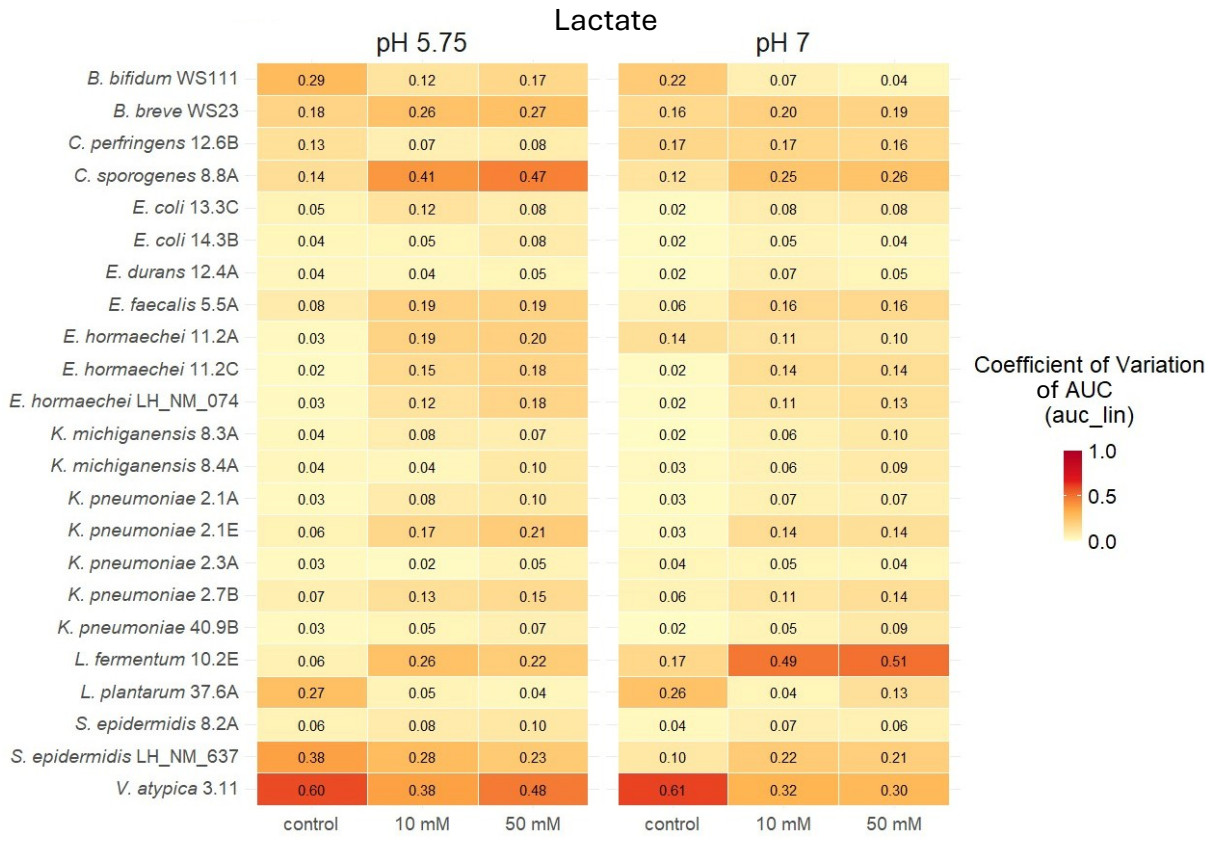

Figure S3: ***Coefficient of Variation (CV) for each individual bacterial isolate under anaerobic conditions in the presence of lactate.*** *Depicted on the x-axis are the different conditions (control, 10 mM and 50 mM), whereas on the y-axis one can see the individual bacterial isolates. The CV was calculated based on the AUCs of the individual isolates and can have values between 0.0 (0%) and 1.0 (100%). CVs of > 50% are regarded as "high" CVs, whereas CVs between 10-50% are regarded as "moderate" and the ones < 10% are classified as "low" CVs.*

DISCLAIMER

During the writing process of this work, I have used OpenAI ChatGPT-4 to occasionally translate and/or harmonize text passages and as support when using R Studio (e.g., for writing code correctly). After using this AI tool, I have reviewed and edited the content as needed and I therefore take full responsibility for the content of this publication.

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REFERENCES

Abebaw, E., Reta, A., Kibret, G. D., & Wagnew, F. (2021). Incidence and Predictors of Mortality among Preterm Neonates Admitted to the Neonatal Intensive Care Unit at Debre Markos Referral Hospital, Northwest Ethiopia. *Ethiopian Journal of Health Sciences*, *31*(5), 937–946. https://doi.org/10.4314/ejhs.v31i5.4

Ahmed, A. M., Grandi, S. M., Pullenayegum, E., McDonald, S. D., Beltempo, M., Premji, S. S., Pole, J. D., Bacchini, F., Shah, P. S., & Pechlivanoglou, P. (2024). Short-Term and Long-Term Mortality Risk After Preterm Birth. *JAMA Network Open*, *7*(11), e2445871. https://doi.org/10.1001/jamanetworkopen.2024.45871

Alcon-Giner, C., Dalby, M. J., Caim, S., Ketskemety, J., Shaw, A., Sim, K., Lawson, M. A. E., Kiu, R., Leclaire, C., Chalklen, L., Kujawska, M., Mitra, S., Fardus-Reid, F., Belteki, G., McColl, K., Swann, J. R., Kroll, J. S., Clarke, P., & Hall, L. J. (2020). Microbiota Supplementation with Bifidobacterium and Lactobacillus Modifies the Preterm Infant Gut Microbiota and Metabolome: An Observational Study. *Cell Reports Medicine*, *1*(5), 100077. https://doi.org/10.1016/j.xcrm.2020.100077

Alsharairi, N. A. (2023). Therapeutic Potential of Gut Microbiota and Its Metabolite Short-Chain Fatty Acids in Neonatal Necrotizing Enterocolitis. *Life*, *13*(2), Article 2. https://doi.org/10.3390/life13020561

Arboleya, S., Binetti, A., Salazar, N., Fernández, N., Solís, G., Hernández-Barranco, A., Margolles, A., de los Reyes-Gavilán, C. G., & Gueimonde, M. (2012). Establishment and development of intestinal microbiota in preterm neonates. *FEMS Microbiology Ecology*, *79*(3), 763–772. https://doi.org/10.1111/j.1574-6941.2011.01261.x

Arrieta, M.-C., Stiemsma, L. T., Amenyogbe, N., Brown, E. M., & Finlay, B. (2014). The Intestinal Microbiome in Early Life: Health and Disease. *Frontiers in Immunology*, *5*, 427. https://doi.org/10.3389/fimmu.2014.00427

Averina, O. V., Kovtun, A. S., Mavletova, D. A., Ziganshin, R. H., Danilenko, V. N., Mihaylova, D., Blazheva, D., Slavchev, A., Brazkova, M., Ibrahim, S. A., & Krastanov, A. (2023). Oxidative Stress Response of Probiotic Strain Bifidobacterium longum subsp. Longum GT15. *Foods*, *12*(18), Article 18. https://doi.org/10.3390/foods12183356

Azad, M. B., Konya, T., Maughan, H., Guttman, D. S., Field, C. J., Chari, R. S., Sears, M. R., Becker, A. B., Scott, J. A., & Kozyrskyj, A. L. (2013). Gut microbiota of healthy Canadian infants: Profiles by mode of delivery and infant diet at 4 months. *CMAJ*, *185*(5), 385–394. https://doi.org/10.1503/cmaj.121189

Baker-Austin, C., & Dopson, M. (2007). Life in acid: pH homeostasis in acidophiles. *Trends in Microbiology*, *15*(4), 165–171. https://doi.org/10.1016/j.tim.2007.02.005

Beghetti, I., Barone, M., Brigidi, P., Sansavini, A., Corvaglia, L., Aceti, A., & Turroni, S. (2023). Early-life gut microbiota and neurodevelopment in preterm infants: A narrative review. *Frontiers in Nutrition*, *10*, 1241303. https://doi.org/10.3389/fnut.2023.1241303

Booth, I. R. (1985). Regulation of cytoplasmic pH in bacteria. *Microbiological Reviews*, *49*(4), 359–378.

Buffie, C. G., & Pamer, E. G. (2013). Microbiota-mediated colonization resistance against intestinal pathogens. *Nature Reviews. Immunology*, *13*(11), 790–801. https://doi.org/10.1038/nri3535

Cao, G., Liu, J., & Liu, M. (2022). Global, Regional, and National Incidence and Mortality of Neonatal Preterm Birth, 1990-2019. *JAMA Pediatrics*, *176*(8), 787–796. https://doi.org/10.1001/jamapediatrics.2022.1622

Casiano-Colón, A., & Marquis, R. E. (1988). Role of the arginine deiminase system in protecting oral bacteria and an enzymatic basis for acid tolerance. *Applied and Environmental Microbiology*, *54*(6), 1318–1324.

Chang, K. C., Nagarajan, N., & Gan, Y.-H. (2024). Short-chain fatty acids of various lengths differentially inhibit Klebsiella pneumoniae and Enterobacteriaceae species. *mSphere*, *9*(2), e00781-23. https://doi.org/10.1128/msphere.00781-23

Chen, J., Chen, X., & Ho, C. L. (2021). Recent Development of Probiotic Bifidobacteria for Treating Human Diseases. *Frontiers in Bioengineering and Biotechnology*, *9*, 770248. https://doi.org/10.3389/fbioe.2021.770248

Chen, Y., Brook, T. C., Soe, C. Z., O'Neill, I., Alcon-Giner, C., Leelastwattanagul, O., Phillips, S., Caim, S., Clarke, P., Hall, L. J., & Hoyles, L. (2020). Preterm infants harbour diverse Klebsiella populations, including atypical species that encode and produce an array of antimicrobial resistance- and virulence-associated factors. *Microbial Genomics*, *6*(6), e000377. https://doi.org/10.1099/mgen.0.000377

Chong, C. Y. L., Bloomfield, F. H., & O'Sullivan, J. M. (2018). Factors Affecting Gastrointestinal Microbiome Development in Neonates. *Nutrients*, *10*(3), 274. https://doi.org/10.3390/nu10030274

Chrysafides, S. M., Bordes, S. J., & Sharma, S. (2024). Physiology, Resting Potential. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK538338/

Cifuentes, M. P., Chapman, J. A., & Stewart, C. J. (2024). Gut microbiome derived short chain fatty acids: Promising strategies in necrotising enterocolitis. *Current Research in Microbial Sciences*, *6*, 100219. https://doi.org/10.1016/j.crmicr.2024.100219

Cotter, P. D., & Hill, C. (2003). Surviving the Acid Test: Responses of Gram-Positive Bacteria to Low pH. *Microbiology and Molecular Biology Reviews*, *67*(3), 429–453. https://doi.org/10.1128/MMBR.67.3.429-453.2003

den Besten, G., van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D.-J., & Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research*, *54*(9), 2325–2340. https://doi.org/10.1194/jlr.R036012

Doi, Y., & Ikegami, Y. (2014). Pyruvate Formate-Lyase Is Essential for Fumarate-Independent Anaerobic Glycerol Utilization in the Enterococcus faecalis Strain W11. *Journal of Bacteriology*, *196*(13), 2472–2480. https://doi.org/10.1128/JB.01512-14

Duar, R. M., Kyle, D., & Casaburi, G. (2020). Colonization Resistance in the Infant Gut: The Role of B. infantis in Reducing pH and Preventing Pathogen Growth. *High-Throughput*, *9*(2), 7. https://doi.org/10.3390/ht9020007

Fusco, W., Lorenzo, M. B., Cintoni, M., Porcari, S., Rinninella, E., Kaitsas, F., Lener, E., Mele, M. C., Gasbarrini, A., Collado, M. C., Cammarota, G., & Ianiro, G. (2023). Short-Chain Fatty-Acid-Producing Bacteria: Key Components of the Human Gut Microbiota. *Nutrients*, *15*(9), 2211. https://doi.org/10.3390/nu15092211

Guan, N., & Liu, L. (2020). Microbial response to acid stress: Mechanisms and applications. *Applied Microbiology and Biotechnology*, *104*(1), 51–65. https://doi.org/10.1007/s00253-019-10226-1

Jin, Q., & Kirk, M. F. (2018). pH as a Primary Control in Environmental Microbiology: 1. Thermodynamic Perspective. *Frontiers in Environmental Science*, *6*. https://doi.org/10.3389/fenvs.2018.00021

Kawasaki, S., Mimura, T., Satoh, T., Takeda, K., & Niimura, Y. (2006). Response of the Microaerophilic Bifidobacterium Species, B. boum and B. thermophilum, to Oxygen. *Applied and Environmental Microbiology*, *72*(10), 6854. https://doi.org/10.1128/AEM.01216-06

Korpela, K., Blakstad, E. W., Moltu, S. J., Strømmen, K., Nakstad, B., Rønnestad, A. E., Brække, K., Iversen, P. O., Drevon, C. A., & de Vos, W. (2018). Intestinal microbiota development and gestational age in preterm neonates. *Scientific Reports*, *8*, 2453. https://doi.org/10.1038/s41598-018-20827-x

Kundukad, B., Schussman, M., Yang, K., Seviour, T., Yang, L., Rice, S. A., Kjelleberg, S., & Doyle, P. S. (2017). Mechanistic action of weak acid drugs on biofilms. *Scientific Reports*, *7*(1), 4783. https://doi.org/10.1038/s41598-017-05178-3

Kütt, A., Selberg, S., Kaljurand, I., Tshepelevitsh, S., Heering, A., Darnell, A., Kaupmees, K., Piirsalu, M., & Leito, I. (2018). P*K*a values in organic chemistry – Making maximum use of the available data. *Tetrahedron Letters*, *59*(42), 3738–3748. https://doi.org/10.1016/j.tetlet.2018.08.054

Lee, Y. A. (2017). White Matter Injury of Prematurity: Its Mechanisms and Clinical Features. *Journal of Pathology and Translational Medicine*, *51*(5), 449–455. https://doi.org/10.4132/jptm.2017.07.25

Liu, X.-C., Du, T.-T., Gao, X., Zhao, W.-J., Wang, Z.-L., He, Y., Bao, L., & Li, L.-Q. (2022). Gut microbiota and short-chain fatty acids may be new biomarkers for predicting neonatal necrotizing enterocolitis: A pilot study. *Frontiers in Microbiology*, *13*, 969656. https://doi.org/10.3389/fmicb.2022.969656

Liu, Y., Tang, H., Lin, Z., & Xu, P. (2015). Mechanisms of acid tolerance in bacteria and prospects in biotechnology and bioremediation. *Biotechnology Advances*, *33*(7), 1484–1492. https://doi.org/10.1016/j.biotechadv.2015.06.001

Lordan, C., Roche, A. K., Delsing, D., Nauta, A., Groeneveld, A., MacSharry, J., Cotter, P. D., & van Sinderen, D. (2024). Linking human milk oligosaccharide metabolism and early life gut microbiota: Bifidobacteria and beyond. *Microbiology and Molecular Biology Reviews: MMBR*, *88*(1), e0009423. https://doi.org/10.1128/mmbr.00094-23

Lu, P., Ma, D., Chen, Y., Guo, Y., Chen, G.-Q., Deng, H., & Shi, Y. (2013). L-glutamine provides acid resistance for Escherichia coli through enzymatic release of ammonia. *Cell Research*, *23*(5), 635–644. https://doi.org/10.1038/cr.2013.13

Matthews, L. G., Walsh, B. H., Knutsen, C., Neil, J. J., Smyser, C. D., Rogers, C. E., & Inder, T. E. (2018). Brain Growth in the NICU – Critical Periods of Tissue-Specific Expansion. *Pediatric Research*, *83*(5), 976–981. https://doi.org/10.1038/pr.2018.4

Maurer, L. M., Yohannes, E., Bondurant, S. S., Radmacher, M., & Slonczewski, J. L. (2005). pH Regulates Genes for Flagellar Motility, Catabolism, and Oxidative Stress in Escherichia coli K-12. *Journal of Bacteriology*, *187*(1), 304–319. https://doi.org/10.1128/JB.187.1.304-319.2005

McCallum, G., & Tropini, C. (2024). The gut microbiota and its biogeography. *Nature Reviews Microbiology*, *22*(2), 105–118. https://doi.org/10.1038/s41579-023-00969-0

Midani, F. S., Collins, J., & Britton, R. A. (2021). AMiGA: Software for Automated Analysis of Microbial Growth Assays. *mSystems*, *6*(4), 10.1128/msystems.00508-21. https://doi.org/10.1128/msystems.00508-21

Mitchell, C. M., Mazzoni, C., Hogstrom, L., Bryant, A., Bergerat, A., Cher, A., Pochan, S., Herman, P., Carrigan, M., Sharp, K., Huttenhower, C., Lander, E. S., Vlamakis, H., Xavier, R. J., & Yassour, M. (2020). Delivery Mode Affects Stability of Early Infant Gut Microbiota. *Cell Reports Medicine*, *1*(9), 100156. https://doi.org/10.1016/j.xcrm.2020.100156

Mitrea, L., Nemeş, S.-A., Szabo, K., Teleky, B.-E., & Vodnar, D.-C. (2022). Guts Imbalance Imbalances the Brain: A Review of Gut Microbiota Association With Neurological and Psychiatric Disorders. *Frontiers in Medicine*, *9*, 813204. https://doi.org/10.3389/fmed.2022.813204

Mitrea, L., & Vodnar, D. C. (2019). Klebsiella pneumoniae—A Useful Pathogenic Strain for Biotechnological Purposes: Diols Biosynthesis under Controlled and Uncontrolled pH Levels. *Pathogens*, *8*(4), 293. https://doi.org/10.3390/pathogens8040293

Mora, D., & Arioli, S. (2014). Microbial Urease in Health and Disease. *PLoS Pathogens*, *10*(12), e1004472. https://doi.org/10.1371/journal.ppat.1004472

Moreira de Gouveia, M. I., Bernalier-Donadille, A., & Jubelin, G. (2024). Enterobacteriaceae in the Human Gut: Dynamics and Ecological Roles in Health and Disease. *Biology*, *13*(3), 142. https://doi.org/10.3390/biology13030142

Müller, P., de la Cuesta-Zuluaga, J., Kuhn, M., Baghai Arassi, M., Treis, T., Blasche, S., Zimmermann, M., Bork, P., Patil, K. R., Typas, A., Garcia-Santamarina, S., & Maier, L. (2024). High-throughput anaerobic screening for identifying compounds acting against gut bacteria in monocultures or communities. *Nature Protocols*, *19*(3), 668–699. https://doi.org/10.1038/s41596-023-00926-4

Neu, J., & Rushing, J. (2011). Cesarean versus Vaginal Delivery: Long term infant outcomes and the Hygiene Hypothesis. *Clinics in Perinatology*, *38*(2), 321–331. https://doi.org/10.1016/j.clp.2011.03.008

Ng, K.-S., Bambace, M. F., Andersen, E. B., Meyer, R. L., & Schwab, C. (2024). Environmental pH and compound structure affect the activity of short-chain carboxylic acids against planktonic growth, biofilm formation, and eradication of the food pathogen Salmonella enterica. *Microbiology Spectrum*, *12*(11), e01658-24. https://doi.org/10.1128/spectrum.01658-24

Ohuma, E. O., Moller, A.-B., Bradley, E., Chakwera, S., Hussain-Alkhateeb, L., Lewin, A., Okwaraji, Y. B., Mahanani, W. R., Johansson, E. W., Lavin, T., Fernandez, D. E., Domínguez, G. G., Costa, A. de, Cresswell, J. A., Krasevec, J., Lawn, J. E., Blencowe, H., Requejo, J., & Moran, A. C. (2023). National, regional, and global estimates of preterm birth in 2020, with trends from 2010: A systematic analysis. *The Lancet*, *402*(10409), 1261–1271. https://doi.org/10.1016/S0140-6736(23)00878-4

Palframan, R. J., Gibson, G. R., & Rastall, R. A. (2003). Carbohydrate preferences of Bifidobacterium species isolated from the human gut. *Current Issues in Intestinal Microbiology*, *4*(2), 71–75.

Plummer, E. L., Danielewski, J. A., Garland, S. M., Su, J., Jacobs, S. E., & Murray, G. L. (2021). The effect of probiotic supplementation on the gut microbiota of preterm infants. *Journal of Medical Microbiology*, *70*(8), 001403. https://doi.org/10.1099/jmm.0.001403

Power, S. E., O'Toole, P. W., Stanton, C., Ross, R. P., & Fitzgerald, G. F. (2014). Intestinal microbiota, diet and health. *British Journal of Nutrition*, *111*(3), 387–402. https://doi.org/10.1017/S0007114513002560

Reid, G. (2016). Probiotics: Definition, scope and mechanisms of action. *Best Practice & Research Clinical Gastroenterology*, *30*(1), 17–25. https://doi.org/10.1016/j.bpg.2015.12.001

Rivera-Chávez, F., Zhang, L. F., Faber, F., Lopez, C. A., Byndloss, M. X., Olsan, E. E., Xu, G., Velazquez, E. M., Lebrilla, C. B., Winter, S. E., & Bäumler, A. J. (2016). Depletion of butyrate-producing Clostridia from the gut microbiota drives an aerobic luminal expansion of Salmonella. *Cell Host & Microbe*, *19*(4), 443–454. https://doi.org/10.1016/j.chom.2016.03.004

Robertson, C., Savva, G. M., Clapuci, R., Jones, J., Maimouni, H., Brown, E., Minocha, A., Hall, L. J., & Clarke, P. (2020). Incidence of necrotising enterocolitis before and after introducing routine prophylactic Lactobacillus and Bifidobacterium probiotics. *Archives of Disease in Childhood. Fetal and Neonatal Edition*, *105*(4), 380–386. https://doi.org/10.1136/archdischild-2019-317346

Salmond, C. V., Kroll, R. G., & Booth, I. R. (1984). The Effect of Food Preservatives on pH Homeostasis in Escherichia coli. *Microbiology*, *130*(11), 2845–2850. https://doi.org/10.1099/00221287-130-11-2845

Sanidad, K. Z., & Zeng, M. Y. (2020). Neonatal gut microbiome and immunity. *Current Opinion in Microbiology*, *56*, 30–37. https://doi.org/10.1016/j.mib.2020.05.011

Sarantinopoulos, P., Kalantzopoulos, G., & Tsakalidou, E. (2001). Citrate Metabolism by Enterococcus faecalis FAIR-E 229. *Applied and Environmental Microbiology*, *67*(12), 5482–5487. https://doi.org/10.1128/AEM.67.12.5482-5487.2001

Schöpping, M., Zeidan, A. A., & Franzén, C. J. (2022). Stress Response in Bifidobacteria. *Microbiology and Molecular Biology Reviews*, *86*(4), e00170-21. https://doi.org/10.1128/mmbr.00170-21

Schwarz, J., Schumacher, K., Brameyer, S., & Jung, K. (2022). Bacterial battle against acidity. *FEMS Microbiology Reviews*, *46*(6), fuac037. https://doi.org/10.1093/femsre/fuac037

Seki, D., Kirkegaard, R., Osvatic, J., Hausmann, B., Séneca, J., Pjevac, P., Berger, A., J Hall, L., Wisgrill, L., & Berry, D. (2024). Gut microbiota genome features associated with brain injury in extremely premature infants. *Gut Microbes*, *16*(1), 2410479. https://doi.org/10.1080/19490976.2024.2410479

Seki, D., Mayer, M., Hausmann, B., Pjevac, P., Giordano, V., Goeral, K., Unterasinger, L., Klebermaß-Schrehof, K., De Paepe, K., Van de Wiele, T., Spittler, A., Kasprian, G.,

Warth, B., Berger, A., Berry, D., & Wisgrill, L. (2021). Aberrant gut-microbiota-immune-brain axis development in premature neonates with brain damage. *Cell Host & Microbe*, *29*(10), 1558-1572.e6. https://doi.org/10.1016/j.chom.2021.08.004

Šeputienė, V., Motiejūnas, D., Sužiedėlis, K., Tomenius, H., Normark, S., Melefors, Ö., & Sužiedėlienė, E. (2003). Molecular Characterization of the Acid-Inducible asr Gene of Escherichia coli and Its Role in Acid Stress Response. *Journal of Bacteriology*, *185*(8), 2475–2484. https://doi.org/10.1128/JB.185.8.2475-2484.2003

Shirai, H., Mokrab, Y., & Mizuguchi, K. (2006). The guanidino-group modifying enzymes: Structural basis for their diversity and commonality. *Proteins: Structure, Function, and Bioinformatics*, *64*(4), 1010–1023. https://doi.org/10.1002/prot.20863

Sivaprakasam, S., Bhutia, Y. D., Yang, S., & Ganapathy, V. (2017). Short-Chain Fatty Acid Transporters: Role in Colonic Homeostasis. *Comprehensive Physiology*, *8*(1), 299. https://doi.org/10.1002/cphy.c170014

Śliżewska, K., & Chlebicz-Wójcik, A. (2020). Growth Kinetics of Probiotic Lactobacillus Strains in the Alternative, Cost-Efficient Semi-Solid Fermentation Medium. *Biology*, *9*(12), 423. https://doi.org/10.3390/biology9120423

Sorbara, M. T., Dubin, K., Littmann, E. R., Moody, T. U., Fontana, E., Seok, R., Leiner, I. M., Taur, Y., Peled, J. U., van den Brink, M. R. M., Litvak, Y., Bäumler, A. J., Chaubard, J.-L., Pickard, A. J., Cross, J. R., & Pamer, E. G. (2018). Inhibiting antibiotic-resistant Enterobacteriaceae by microbiota-mediated intracellular acidification. *Journal of Experimental Medicine*, *216*(1), 84–98. https://doi.org/10.1084/jem.20181639

Thänert, R., Schwartz, D. J., Keen, E. C., Hall-Moore, C., Wang, B., Shaikh, N., Ning, J., Rouggly-Nickless, L. C., Thänert, A., Ferreiro, A., Fishbein, S. R. S., Sullivan, J. E., Radmacher, P., Escobedo, M., Warner, B. B., Tarr, P. I., & Dantas, G. (2024). Clinical sequelae of gut microbiome development and disruption in hospitalized preterm infants. *Cell Host & Microbe*, *0*(0). https://doi.org/10.1016/j.chom.2024.07.027

Wadhams, G. H., & Armitage, J. P. (2004). Making sense of it all: Bacterial chemotaxis. *Nature Reviews Molecular Cell Biology*, *5*(12), 1024–1037. https://doi.org/10.1038/nrm1524

Wang, S. P., Rubio, L. A., Duncan, S. H., Donachie, G. E., Holtrop, G., Lo, G., Farquharson, F. M., Wagner, J., Parkhill, J., Louis, P., Walker, A. W., & Flint, H. J. (2020). Pivotal

Roles for pH, Lactate, and Lactate-Utilizing Bacteria in the Stability of a Human Colonic Microbial Ecosystem. *mSystems*, *5*(5), 10.1128/msystems.00645-20. https://doi.org/10.1128/msystems.00645-20

Wang, X., Li, J., Li, N., Guan, K., Yin, D., Zhang, H., Ding, G., & Hu, Y. (2021). Evolution of Intestinal Gases and Fecal Short-Chain Fatty Acids Produced in vitro by Preterm Infant Gut Microbiota During the First 4 Weeks of Life. *Frontiers in Pediatrics*, *9*. https://doi.org/10.3389/fped.2021.726193

Wang, Y., Florez, I. D., Morgan, R. L., Foroutan, F., Chang, Y., Crandon, H. N., Zeraatkar, D., Bala, M. M., Mao, R. Q., Tao, B., Shahid, S., Wang, X., Beyene, J., Offringa, M., Sherman, P. M., El Gouhary, E., Guyatt, G. H., & Sadeghirad, B. (2023). Probiotics, Prebiotics, Lactoferrin, and Combination Products for Prevention of Mortality and Morbidity in Preterm Infants. *JAMA Pediatrics*, *177*(11), 1158–1167. https://doi.org/10.1001/jamapediatrics.2023.3849

Wang, Y., Zhu, J., Zou, N., Zhang, L., Wang, Y., Zhang, M., Wang, C., & Yang, L. (2023). Pathogenesis from the microbial-gut-brain axis in white matter injury in preterm infants: A review. *Frontiers in Integrative Neuroscience*, *17*. https://doi.org/10.3389/fnint.2023.1051689

Weiss, G. A., & Hennet, T. (2017). Mechanisms and consequences of intestinal dysbiosis. *Cellular and Molecular Life Sciences*, *74*(16), 2959–2977. https://doi.org/10.1007/s00018-017-2509-x

Ximenez, C., & Torres, J. (2017). Development of Microbiota in Infants and its Role in Maturation of Gut Mucosa and Immune System. *Archives of Medical Research*, *48*(8), 666–680. https://doi.org/10.1016/j.arcmed.2017.11.007

Xu, Y., Milburn, O., Beiersdorfer, T., Du, L., Akinbi, H., & Haslam, D. B. (2022). Antibiotic exposure prevents acquisition of beneficial metabolic functions in the preterm infant gut microbiome. *Microbiome*, *10*, 103. https://doi.org/10.1186/s40168-022-01300-4

Xu, Y., Zhao, Z., Tong, W., Ding, Y., Liu, B., Shi, Y., Wang, J., Sun, S., Liu, M., Wang, Y., Qi, Q., Xian, M., & Zhao, G. (2020). An acid-tolerance response system protecting exponentially growing Escherichia coli. *Nature Communications*, *11*, 1496. https://doi.org/10.1038/s41467-020-15350-5

Yamada, C., Gotoh, A., Sakanaka, M., Hattie, M., Stubbs, K. A., Katayama-Ikegami, A., Hirose, J., Kurihara, S., Arakawa, T., Kitaoka, M., Okuda, S., Katayama, T., &

Fushinobu, S. (2017). Molecular Insight into Evolution of Symbiosis between Breast-Fed Infants and a Member of the Human Gut Microbiome *Bifidobacterium longum*. *Cell Chemical Biology*, *24*(4), 515-524.e5. https://doi.org/10.1016/j.chembiol.2017.03.012

Yang, Y., Kadim, M. I., Khoo, W. J., Zheng, Q., Setyawati, M. I., Shin, Y.-J., Lee, S.-C., & Yuk, H.-G. (2014). Membrane lipid composition and stress/virulence related gene expression of *Salmonella* Enteritidis cells adapted to lactic acid and trisodium phosphate and their resistance to lethal heat and acid stress. *International Journal of Food Microbiology*, *191*, 24–31. https://doi.org/10.1016/j.ijfoodmicro.2014.08.034

Zhang, S., Hung, J., Yen, T. N., & Huang, S. (2024). Mutualistic interactions of lactate-producing lactobacilli and lactate-utilizing Veillonella dispar: Lactate and glutamate cross-feeding for the enhanced growth and short-chain fatty acid production. *Microbial Biotechnology*, *17*(5), e14484. https://doi.org/10.1111/1751-7915.14484

Zhou, P., Manoil, D., Belibasakis, G. N., & Kotsakis, G. A. (2021). Veillonellae: Beyond Bridging Species in Oral Biofilm Ecology. *Frontiers in Oral Health*, *2*, 774115. https://doi.org/10.3389/froh.2021.774115