FACTORS GOVERNING GASTROINTESTINAL COLONIZATION OF *CANDIDA ALBICANS*

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DEDICATION

To my parents and my family

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I would not have been able to complete this journey without the support of so many amazing people.

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FACTORS GOVERNING GASTROINTESTINAL COLONIZATION OF *CANDIDA ALBICANS*

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Candida albicans can colonize the human gastrointestinal tract (GI) and cause disseminated infections in immunocompromised hosts. Depletion of specific gut commensal microbiota is associated with or results in increased *C. albicans* burden in the gut and increased likelihood of dissemination in human patients and mice, respectively. The exact mechanisms by which gut microbiota mediate *C. albicans* colonization resistance in the gut, however, are unknown. Here, we show that gut microbiota-derived short chain fatty acids (SCFA) directly inhibit *C. albicans* growth *in vitro*. SCFA inhibit *C. albicans* hexose uptake and induce intracellular acidification. In contrast, SCFA promote *C. albicans* GI colonization resistance *in vivo* but only when an intact gut microbiome is present. SCFA induce gut microbiota composition changes that promote *C. albicans* colonization resistance. Commensal gut microbiota unable to produce SCFA have a diminished capacity to reduce *C. albicans* GI colonization. Prebiotic therapy results in increased GI SCFA levels which enhance *C. albicans* GI clearance. This work also describes two *C. albicans* isolates 529L and CHN1 that can stably colonize the murine GI tract without the use of antibiotics. These clinical isolates have a higher resistance to antimicrobial peptide CRAMP compared to the most commonly studied *C. albicans*

laboratory strain SC5314. Thus, the work sheds light on mechanisms that might be critical in governing *C. albicans* gastrointestinal colonization levels. It provides mechanistic insights into the importance of gut microbiota-derived metabolites in maintaining *C. albicans* colonization resistance and may have therapeutic implications for modulating *C. albicans* gastrointestinal colonization levels in order to prevent invasive candidiasis in immunocompromised patients. Further, *C. albicans* strain-specific difference in colonization ability appears to depend on the sensitivity to these host immune effectors. The described isolates can further serve as valuable tools to probe the mechanisms of *C. albicans* gastrointestinal colonization without the intervention of any antibiotics.

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***Candida albicans* Isolates 529L and CHN1 Exhibit Stable Colonization of the Murine Gastrointestinal Tract**
McDonough L[#], Mishra AA[#], Tosini N[#], Kakade P, Penumutchu S, Liang S, Maufrais C, Zhai B, Taur Y, Belenky P, Bennett RJ, Hohl TM, Koh AY, Ene IV. In review at mBio.
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The microbial and host factors that govern Candida gastrointestinal colonization and dissemination.
Mishra AA, Koh AY. Current Opinion in Microbiology, 2021

Adaptation of *Candida albicans* during gastrointestinal tract colonization.
Mishra AA, Koh AY.
Current Clinical Microbiology Reports, 2018

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LIST OF ABBREVIATIONS

GI – gastrointestinal

CA – *Candida albicans*

B. theta – Bacteroides thetaiotaomicron

B. producta – Blautia producta

CFU – Colony forming units

SCFA – Short Chain fatty acids

BA – Butyric acid

PA – Propionic acid

AA – Acetic acid

CRAMP – cathelicidin-related antimicrobial peptide

HIF1α – Hypoxia inducible factor 1- alpha

FFAR2 – Free fatty acid receptor 2

FFAR3 – Free fatty acid receptor 3

PPARγ – Peroxisome proliferator- activated receptor gamma

GC-MS – Gas chromatography-mass spectrometry

YPD – Yeast Peptone Dextrose

YNB – Yeast Nitrogen Base

TSA – Trypticase Soy Agar

YVG – Yeast peptone dextrose agar vancomycin gentamycin

PBS – Phosphate Buffer Saline

NS – Normal Saline

gDNA – genomic DNA

rRNA – ribosomal RNA

2-DG – 2- deoxyglucose

MTBSTFA – N-(tert-Butyldimethylsilyl)-N-methyltrifluoroacetamide

QIIME – Quantitative Insights Into Microbial Ecology

LDA – Linear discriminant analysis

LefSe – Linear discriminate analysis coupled with effect size measurements

CHAPTER ONE
Literature Review

Infectious diseases are the leading cause of death worldwide and third leading cause of death in the United States, with majority of the infections transpiring through mucosal surfaces such as the linings of respiratory, gastrointestinal and genitourinary tracts [1]. The human gastrointestinal (GI) tract harbors numerous microbial species in the GI tract termed collectively as the microbiota, and can outnumber human cells by a factor of 10 to 1 [2]. A small subset of the microbiota (up to 0.1% of the total microbiota) is made of fungal species, termed the mycobiota. Although the proportion of fungal species is small, fungal species can be up to a 100 times larger than bacterial species and are recognized as key players in maintaining host homeostasis [3-9].

C. albicans (CA) is one of the most prominent members of the human mycobiota and is present in almost 70% of individuals [10-12]. The carriage of CA is found to be higher in populations living in Western societies compared to individual livings in indigenous populations, such as inhabitants of the Amazon rainforest, suggesting a role of lifestyle choices governing the colonization of CA [13,14].

CA is a pathobiont, which resides as a commensal in a healthy host but can become invasive in immunocompromised setting [15,16]. CA can translocate from the GI tract into bloodstream leading to bloodstream infection and mortality in patients undergoing allogenic hematopoietic cell transplant or cancer chemotherapy [17,18]. A modest decrease in the GI

levels of CA has been shown to decrease the probability of invasive bloodstream infection and subsequent mortality [18,19]. Apart from bloodstream infections, CA carriage and burden appears to be associated with ulcerative colitis and Crohn's disease and severity [20]. The magnitude of CA GI colonization seems to depend on three critical factors, 1) CA genetic determinants, 2) host immunity and 3) gut microbiota.

Genetic determinants of CA and GI colonization

CA is a dimorphic organism and can exist in either the spheroid yeast form or as filamentous hyphae. The ability of CA to switch from yeast to hyphae has been implicated as important for its GI colonization potential [17,21]. Current dogma suggests that the yeast form is favorable for *Candida* when colonizing the GI tract whereas the hyphae form is associated with a higher degree of invasiveness and thus associated with increased virulence [22,23]. The filament locked form of CA, however, has been shown to be less virulent than the wildtype strain, as it has reduced ability to colonize the GI tract [17]. Also, strains with reduced filamentation ability induce lower mortality from disseminated disease, despite colonizing the GI tract to the same levels as wildtype counterparts [17]. Thus, both the carriage of CA in the GI tract and its ability to filament determines the overall virulence and its invasiveness to cause bloodstream infection.

CA can readily colonize germ-free mice as opposed to conventional mice, thought to be due to the absence of gut microbiota [21,24]. In germ-free mice, *Candida* has been shown to exist primarily in its yeast form as opposed to the hyphae form. Also, mutants with increased ability to filament (such as ZCF8, ZFU, and TRY4) are defective in colonizing the

germ-free murine GI tract [21]. One of possible rationale for this observed defect is attributed to the observation that the yeast form results in lower secretory levels of the host immune effector molecule granulocyte-colony stimulating factor compared to the hyphae form [21]. Thus, a milder host immune response against the yeast form could be facilitating improved colonization ability.

The absence of Ume6, a central regulator of hyphal genes, results in increased CA colonization ability [25-27]. Interestingly, this superior colonization ability in the GI tract was not found to be dependent on the morphological status of the mutant, as the yeast to hyphae ratio were similar in both the wildtype and mutant strain. The differential colonization ability was rather shown to be associated with proteins that elicit a stronger host immune response, such as secreted aspartyl protease 6 (SAP6). SAP6 has been recognized as pro-inflammatory molecule and plays a role in CA invasiveness [28-31]. *Sap6* mutant strains also display a higher colonization potential in the murine GI tract similar to the *ume6* mutant. Thus, the differences observed in the colonization ability of yeast and hyphae form may not be due solely to physical properties such as adhesion to host mucosal surfaces but also due to variability in inducing host immune effectors. This difference in immune modulation could play a critical role in the GI carriage of CA -- with strains that induce higher innate immune response exhibiting lower GI colonization levels.

Recently other CA morphologies have been identified as potential factors in modulating GI colonization. One such reported morphology is GUT (gastrointestinally induced transition) morphology which is induced by the passage of CA through the GI tract [32,33] and is dependent on the *wor1* gene, which is an essential regulator of CA white-

opaque switching in mating [34,35]. Overexpression of *wor1* leads to a competitive fitness advantage over wildtype strains in the GI tract whereas deletion of *wor1* leads to significant GI disadvantage compared to wildtype counterparts.

Underscoring the importance of morphology and colonization potential, a recent study from the Bennett lab, highlighted the role of *efg1* [36]. The *efg1*+/- strain can adopt a gray morphology that has previously been reported to rely on epigenetic switching [37]. This study, however, showed that a switch to gray morphology was dependent on the loss of *efg1* through *de novo* mutations. When *efg1*+/- strains of CA passed through the GI tract the recovered colonies from the fecal pellets displayed a rapid loss of *efg1* and conversion to gray morphology. *efg1*+/- strain was also able to outcompete *efg1*+/+ strain, highlighting the significance to be able to lose *efg1* and adopt gray morphology. Collectively, these studies highlight the ability of CA to adopt different morphologies which facilitates and determines its ability to colonize different host niches in conjunction with specific environmental cues.

As mentioned earlier, CA is dimorphic, with its hyphal form associated with higher virulence and invasiveness. There are CA derived factors that facilitate invasion into host tissues. CA secrete adhesins (Als3) [38] and secreted aspartyl proteases (SAP6) [39] that facilitate its epithelial invasion. Recently, the first CA effector molecule, candidalysin, essential for its invasive ability was discovered [40]. Candidalysin is a 31 amino acid long cytolytic peptide toxin that is generated from precursor protein enhancement of chain elongation 1 (ECE1) [41]. In the oral candidiasis model, CA strains lacking candidalysin had lower colonization potential and decreased invasive capacity [40]. In contrast, no such colonization defect was observed in a vulvovaginitis model, though the presence of

candidalysin did exacerbate the disease [42]. With regards to GI colonization, the role candidalysin is not clear, although it is shown to be important for intestinal epithelial translocation *in vitro* [43].

In summary, the genetic factors of CA that governs its morphology, invasiveness, immune evasion and epithelial damage are critical in defining its life as a pathobiont. Comprehensive knowledge of these factors and mechanisms will be critical in illuminating the biological intricacies that prime CA for a successful lifestyle within the host.

Host immune system

The host immune system is critical in defending against CA dissemination and bloodstream infections. Epithelial damage and neutropenia in the host increase the probability of CA dissemination and bloodstream infection [17,44]. One of the major pattern recognition receptor for CA is Dectin-1, a C-type lectin receptor. Dectin-1 has been shown to be essential in preventing GI dissemination of CA [45-47]. Interestingly, TLR-2 and TLR-4 have been shown to impact the GI fungal load even though they do not directly recognize CA [48-50], findings which likely emphasize the importance of bacterial gut microbiota and their ability to induce host immune effectors (e.g. antimicrobial peptides) that promote CA colonization resistance. In fact, intestinal epithelial secreted antimicrobial peptides such as CRAMP (cathelicidin related antimicrobial peptide) have been shown to be important for maintaining GI colonization resistance against CA [19,51,52]. Our group has shown that pharmacologic induction of intestinal CRAMP can result in decreased levels of CA in GI

tract and subsequently inhibit dissemination. Similarly, a loss of CRAMP increases the colonization ability of CA in the GI tract

Th17 has been well established to be critical for protection against oral candidiasis. Multiple seminal studies have interrogated the relationship between CA and Th17 response [53-55]. In one such study, intestinal CA was shown to be important to generate anti-CA Th17 to provide protection against invasive infection from CA [3]. Interestingly, this Th17 augmentation also provided cross-protection against *Staphylococcus aureus* infection. This cross-reactivity is not objectively beneficial as it can exacerbate airway inflammation when challenged with dust mites. In a separate study, circulating Th17 cells against intestinal CA were generated that could also cross-react with other fungal species and expand in peripheral spaces such as lungs, again highlighting the capacity to invoke cross-protective responses [6]. In the setting of intestinal inflammation, these cross-reactive Th17 cells can also lead to an augmented response in these extra intestinal sites, exacerbating the pathogenesis of pulmonary aspergillosis in this instance.

CX3CR1+ gut resident mononuclear phagocytes (MNP) are important for generating an effective Th17 response against intestinal CA as well [56]. The CX3CR1+ MNP function was critically dependent on Spleen Tyrosine kinase (Syk), and loss of CX3CR1+ MNP led to ulcerative colitis, that could be reversed by the administration of anti-fungal compounds.

An unexpected and intriguing potential mechanism of protecting the host epithelium against the fungal metabolites and toxins by macrophages focuses on colonic macrophages forming balloon-like protrusions that sample the fluid absorbed for fungal toxins and metabolites [57]. These balloon-like protrusions then regulate the rate of fluid absorption and

prevent epithelial damage. In the absence of these macrophages, there is increased epithelial damage that can be prevented by the administration of anti-fungal compounds. Our group and others have shown that damage of intestinal epithelial integrity is associated with CA invasion and dissemination, thus this novel attribute of colonic macrophages in sensing fungal toxins and promoting epithelial integrity could contribute to reducing CA dissemination and preventing bloodstream infection.

While most studies have focused on cellular immunity's role in defending against CA infections, recent evidence suggests that the humoral component may be important as well. A recent study showed that intestinal CA can generate systemic anti- CA IgG antibodies in the serum [5]. The immunogenic potential of CA was compared with other food and skin associated fungal species. CA was found to be significantly more immunogenic and provided protection against disseminated infection. The generation of these circulating antibodies was also shown to be critically dependent on the presence of Card9, a well-known as a regulator of anti-fungal immunity [58,59]. Deleterious mutations resulting in full deficiency of Card9 are associated with invasive candidiasis [60], and polymorphisms in Card9 gene are associated with colitis and Crohn's disease [58,61].

Overall, there are numerous facets involved in immunity against CA, which include well-accepted mechanisms of protection provided by neutrophils, antimicrobial peptides and Th17 response. In the past several years, there have also been critical advances in unraveling novel mechanisms of systemic immunity and cross-reactivity arising from GI colonization of CA. Further investigation will tease out these mechanisms at play and paint a clearer picture of host immune effector mechanisms and its interaction with *C. albicans*.

Gut Microbiome and *Candida*

The gut microbiome is a critical host factor that modulates GI colonization of CA [18,19,62]. Decreases in the levels of specific gut microbiota facilitate the colonization of *C. albicans*. For example, mice with intact microbiota cannot be colonized with numerous isolates of CA, including the most commonly used laboratory isolate SC5314. Thus, to achieve high levels of CA colonization in the murine GI tract, mice are administered antibiotics to decrease the levels of gut microbiota [17,63,64]. Specific gut microbiota are known to induce the production of antimicrobial peptide such as CRAMP from myeloid and epithelial cells [65]. CRAMP production from intestinal epithelial cells limits the colonization and dissemination ability of CA [19]. This could be one of several mechanisms through which the presence of gut microbiota might be contributing to colonization resistance against CA. Presence of specific commensal anaerobes is shown to be critical in promoting this colonization resistance. Specifically, loss of commensal anaerobes belonging to the *Bacteroidetes* and *Firmicutes* phylum by the administration antibiotics leads to the maximal loss of colonization resistance. Supplementation of these phyla, such as *Bacteroides thetaiotaomicron* (Phylum, *Bacteroidetes*, Family *Bacteroideaceae*) and *Blautia producta* (Phylum, *Firmicutes*, Family, *Lachnospiraceae*) have been shown to promote colonization resistance against CA in germ free and conventional mice models of CA colonization [19]. A study conducted in adult stem cells transplant patients that developed Candidemia further corroborated these findings. High resolution microbiota and mycobiota analyses of patients who did or did not develop *Candida* spp. bloodstream infections (BSI) revealed a significant

correlation between abundance of Lachnospiraceae and Bacteroidetes and protection from *Candida* spp. bloodstream infections [18]. *Candida* spp. BSIs were preceded by an expansion of *Candida* spp. in the GI tract. *Candida* spp. BSI was inversely associated with the abundance of *Bacteroidetes* and *Lachnospiraceae*. Interestingly, the use of anti-anaerobes positively correlated with the incidence of BSI. Both these studies highlight the importance of commensal microbiota in maintaining *Candida* colonization resistance and modulating GI fungal load to prevent bloodstream infections.

There have been other studies as well implicating direct interaction between gut microbiota and *Candida* spp. A study showed that an effector molecule, EntV from *Enterococcus faecalis* inhibits the *C. albicans* potential to form biofilms, although no fungicidal activity was observed by this effector [66]. Type VI secretion apparatus present in certain bacteria have been known to secrete anti-eukaryotic effector molecules. *S. marcescens* has been shown to secrete anti-CA effector molecule, thus presence of such bacteria could also serve to directly inhibit the colonization of *C. albicans* by engaging their fungicidal effectors [67].

The interaction between CA and the bacterial gut microbiota is not unidirectional, CA can also affect the members of the bacterial gut microbiota. *Candida* spp. can modulate the recovery and reconstitution of the gut bacteria after administration of antibiotics. Cefoperazone inhibits *Lactobacillus* spp. colonization in mice and promotes the growth of Enterococcus after cessation of antibiotic [68,69]. Our lab has also previously shown that CA can inhibit *Pseudomonas aeruginosa* virulence without directly inhibiting its growth *in vitro* or colonization levels in mice [70]. Finally, the efficacy of fecal transplants to treat

Clostridium difficile infections by fecal transplants has been shown to depend on the composition of the mycobiota, with a dysbiotic mycobiota reducing the probability of success [71].

The crosstalk between *C. albicans,* microbiota and the host is important for the development of all three stakeholders. A recent study investigated the evolution of CA in the absence or presence of microbiota. CA when evolved in antibiotic treated mice with reduced bacterial microbiota lost its hyphal generating ability through *de novo* mutations, leading to CA better able to colonize the gut but less virulent [72]. Interestingly, the evolved CA also induced host protection against other microbes through innate immune dependent cross-reactivity.

Gut microbiota secretes numerous metabolites that can also interact and impact the colonization ability of CA. The gut metabolome depends not only on the composition of the microbiota but it on lifestyle and environmental factors, such as diet, antibiotic treatment, etc. Previously, it has been shown that mice fed 'purified' diets consisting of cornstarch, soybean oil and sucrose could colonize the GI tract of mice without any antibiotic treatment, although the level of colonization is significantly lower compared to the antibiotic treated mice or germ free mice [73,74]. Further studies are required to tease out the mechanisms that might contribute to enhanced colonization. The emergent phenotype is likely a result of the combinatorial effect of the microbiome, metabolome, host factors and *C. albicans*. Further studies will allow us to delineate the mechanisms and further define the degree of cross talk involved in these interactions.

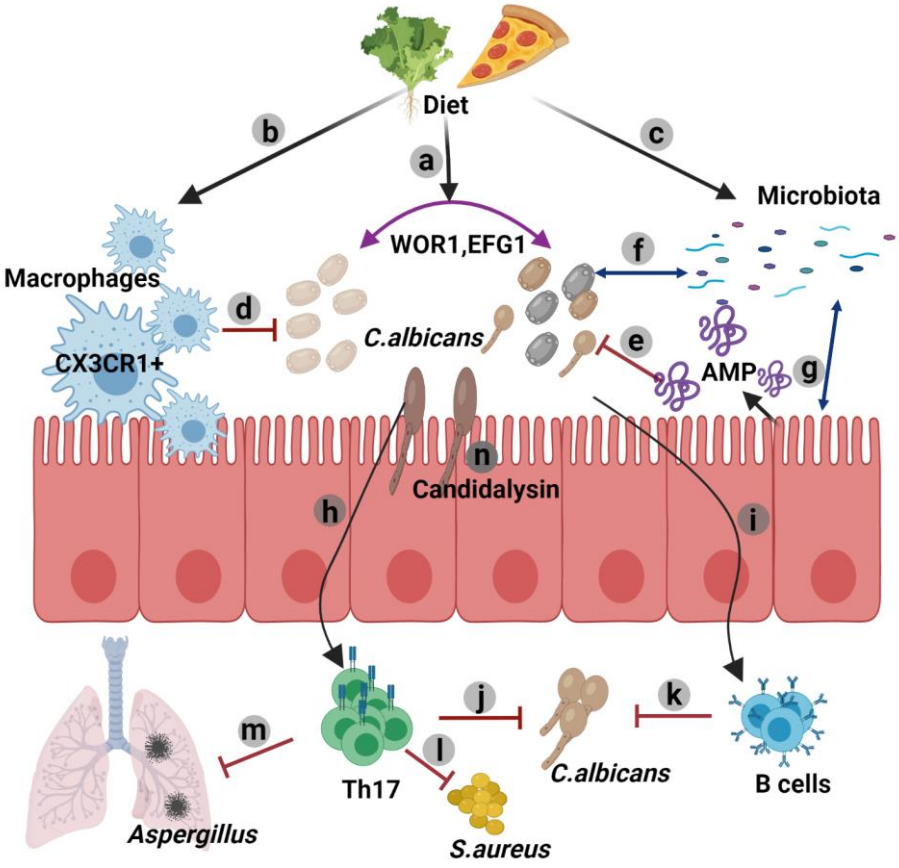

Figure 1. The microbial and host factors that govern *Candida* gastrointestinal colonization and dissemination. 1) CA genetic determinants. CA can adopt different morphologies based on its niche by modulating the expression of genes such as *wor1* and *efg1* to suit its lifestyle (a). Effector molecule Candidalysin helps CA to invade host tissue and disseminate (n). 2) Host Immune Effectors. CXRCR1^{+} macrophages (b, d) and antimicrobial peptides reduces fungal load in GI tract (e, g). Intestinal CA also induces anti-CA Th17 T (h, j) cells and anti-CA IgG (i, k) response that provide protection against systemic CA infection. Anti-CA Th17 T cells are cross-reactive either providing protection against pathogens such *as S.aureus* (l) or exacerbating pathophysiology in case of aspergillosis (m). 3) Gut Microbiota. The crosstalk between CA and microbiota affects colonization of CA and composition of microbiota (c, f). Finally, environmental factors (such as diet) have the potential to influence all three factors (CA, the host immune system and microbiota) which subsequently dictates CA status as a pathobiont (a, b, c).

Murine Models of *Candida albicans* GI colonization and dissemination

C. albicans does not have any environmental reservoir and is only found associated with human or mammalian host. Interestingly, the unperturbed conventional adult mice GI tract is not amenable to CA colonization. Thus, several different murine models have been devised to study host and CA interactions.

Antibiotic-treated adult murine models. To achieve high, sustained levels of CA GI colonization in the mouse, antibiotics are widely used [17,63,64]. Administration of antibiotics depletes the microbiota, which decreases colonization resistance and allows for CA GI colonization. The level of colonization depends on the choice of antibiotics, with the administration of penicillin inducing substantially higher levels of CA colonization than other commonly used antibiotics such as clindamycin or metronidazole. Of note, penicillin markedly reduces the levels of commensal anaerobes in the GI tract which have been shown to be protective against CA GI colonization [17,19].

Neonatal murine model. In contrast to the adult murine GI tract, CA can colonize the GI tract of infant mice without the administration of antibiotics [24]. This disparity is likely attributable to differences in the microbiota of infant and adult mice. Infant mice have a higher abundance of *Proteobacteria,* and a paucity of *Bacteroidetes and Firmicutes* which dominates the microbiota of the adult mice and is protective against CA GI colonization [75,76]. The neonatal mice used in this model are generally 5-7 days old, and the colonization levels achieved is between 10^3 to 10^5 CFU/g of intestinal tissue [77,78], which is less than what is achieved in adult mice after administration of antibiotics (penicillin). *Candida* spp. is also the second leading cause of deaths related to infectious disease in

premature infants [79]. This model might be beneficial in understanding the factors affecting CA colonization that might be uniquely important in neonates.

Germ-free mice. Finally, germ free mice can be utilized to study the factors contributing to CA GI colonization. Germ free mice do not have any gut microbiota, allowing CA to readily colonize their GI tract. The levels of colonization achieved is as high as 10^8 CFU/g of feces [80-82]. The germ free system can help facilitate ascertaining causality and mechanistic insights, as the role of specific bacterial species and its interaction with CA can be studied in a system with fewer confounding variables.

Overall, these are the widely used murine models used to study CA GI colonization and dissemination and each is able to achieve substantial CA GI colonization due to defect or depletion of the microbiota, specifically commensal anaerobes.

Microbiota produced short chain fatty acids

Short chain fatty acids (SCFA) are microbial products generated by gut microbiota in the large intestine from the unabsorbed dietary components (e.g. indigestible fibers found in ingested plants and vegetables) which cannot be absorbed by the small intestine [83]. Short chain fatty acids are characterized as branched or unbranched carboxylic acids containing less than six carbon atoms. The major short chain fatty acids produced in the GI tract are acetate, propionate and butyrate. SCFA are primarily generated from carbohydrates that are broken down by the microbiota. Acetic acid although can be generated by acetogenic bacteria directly by Wood-Ljungdahl pathway from hydrogen and carbon dioxide or formic acid [84].

Acetic acid is also the most abundant SCFA and accounts for more than 50% of the total SCFA [85].

Propionate can be generated by three pathways: 1) succinate pathway, 2) acrylate pathway, and 3) propanodiol pathway [86]. The most commonly utilized pathway is the succinate pathway [87]. Succinate generated from the breakdown of carbohydrates, is converted into methylmalonyl-CoA. Decarboxylation of methylmalonyl-CoA then generates propionyl-CoA and finally a CoA transferase generates propionate [86]. This pathway is present in bacteria belonging to the *Bacteroidetes* family which includes *Bacteroides thetaiotamicron* and also in members of *Firmicutes* phylum belonging to *Negativicutes* class. Deletion of propionyl decarboxylase in *Bacteroides thetaiotaomicron* loses its ability to synthesize propionate [88]. The acrylate pathway utilizes lactate to convert it into propionyl-CoA and finally into propionate [89]. This pathway is present in bacteria belonging to *Negativicutes* class. Finally, the propanodiol pathway utilizes deoxysugar rhamnose and fucose to synthesize propionate. This pathway is present in bacteria belonging to *Lachnospiraceae* family including *Blautia spp* [86].

Production of butyrate utilizes two molecules of acetyl-CoA to produce acetoacetyl-CoA which is reduced to butyryl-CoA. Butyryl-CoA can then be reduced to butyrate by two separate pathways. The more common pathway utilizes butyryl-CoA:acetate CoA-transferase, and is present in genera such as *Faecalibacterium, Eubacterium, Roseburia* [90]. The less common pathway utilizes phosphotransbutyrylase and butyrate kinase and is found in *Coprococcus* spp.[91] Thus members of gut microbiota utilize different pathways to produce SCFA.

Gut microbiota produced SCFA especially propionate and butyrate have now been shown to have myriad effects ranging from their anti-inflammatory effect on ulcerative colitis [92], potential infectious disease preventing role (by enhancing intestinal barrier integrate) [93], and finally, to bolstering efficacy of radiation therapy for cancer [94].

Recently, there have been seminal studies studying the effect of SCFA on colonization of pathogenic microbes. Study from Eric Pamer's lab showed that the presence of SCFA suppresses the growth of clinical isolates of *Klebsiella pneumonia*, *Proteus mirabilis,* and *Escherichia coli* [95]. The study showed that the suppression observed in all the three bacterial isolates was present only under acidic conditions, and SCFA induced inhibition was dependent on increased intracellular acidification observed for these isolates. The study also showed negative correlation between the level of SCFA and the expansion of *E.coli* in an allogenic hematopoietic stem cell transplant patient.

Propionate was shown in another study to inhibit the growth of *Salmonella typhimurium* [88]. The study also showed that an increase in the levels of propionate was able to reduce the colonization levels *S.typhimurium* in the murine gut. Interestingly, in this study the inhibition was observed only with propionate but not with acetate or butyrate. The inhibition observed in this study as well was shown to be dependent on increased intracellular acidification of *S.typhimurium* in presence of propionate

There have been other studies where butyrate has inhibited the expansion of *Enterobacteriaceae* and colonization of *S.typhimurium* in murine GI tract but this was not due to the direct impact of butyrate on these microbes. The mechanism involved altered

metabolism in the host epithelium resulting in increased concentration of oxygen and nitrate that can serve as electron acceptors boosting the growth of these microbes in the gut [96,97].

Apart from this, host sensing of SCFA can also impact the production of epithelial derived antimicrobial peptide such as β-defensins and CRAMP [98], which as mentioned previously, effect CA colonization. The two major surface receptors that can sense SCFA are FFAR2 and FFAR3. There exists differential ability for these receptors to sense SCFA. FFAR2 is more sensitive to acetate compared to acetate and it is vice versa for FFAR3. Both the receptors are most sensitive for propionate [99]. Apart from these two PPARγ is an intracellular sensor that can sense butyrate and control the cell metabolism [96,97,100]. Overall, microbiota produced SCFA are metabolites of great interest that can be sensed and impact host homeostasis, as well as have antimicrobial effect.

CHAPTER TWO
Overall Objectives and Synopsis

C. albicans (CA) is a major cause of nosocomial infections. The source of CA associated bloodstream infections is the gastrointestinal (GI) tract, a natural reservoir for CA in humans [10]. Diminished CA colonization in the GI tract decreases the probability of bloodstream infections [18,19]. Still, there is not much known about the factors that impact CA GI colonization. This study tries to elucidate the role of microbiota derived metabolite short chain fatty acids on GI colonization of CA. The work also highlights strain-specific differences in GI colonization by CA clinical isolates. It is known that the bacterial gut microbiota is essential for providing colonization resistance against CA. Our lab has previously shown that *Bactoeroides thetaiotaomicron* and *Blautia producta* antagonize colonization of CA in the murine GI tract in a HIF1α and CRAMP dependent manner. We further wanted to identify if there are specific metabolites produced by these microbes that might be contributing to the colonization resistance against CA. One such metabolite is short chain fatty acids produced by the members of microbiota including *Bactoeroides thetaiotaomicron* and *Blautia producta*. Antibiotics (penicillin/streptomycin) which massively deplete *B. theta* and *B. producta* facilitate CA GI colonization. As such, we studied the effect of SCFA on CA growth *in vitro* in the presence of SCFA under different environmental settings. In all the settings, SCFA was found to inhibit the growth of *C. albicans*. Transcription profiling studies of CA exposed to SCFAs lead to the subsequent

experimental observations that SCFA-induced growth inhibition is dependent on reductions in glucose uptake and decreases in intracellular pH. Interestingly, *in vivo*, butyric acid and propionic acid but not acetic acid was found to be protective against CA GI colonization. The colonization ability of CA is regulated by gut microbiota and host immune effectors, both of which can be impacted by SCFA. Thus, we also studied the effect of SCFA on gut microbiota reconstitution after antibiotic cessation and its importance in mediating colonization resistance. Gut microbiota was found to be essential for protective effect of SCFA. We also analyzed the role host SCFA sensing in mediating colonization resistance against CA. SCFAs were found to still be protective against CA in single gene SCFA receptor knockout mice. Finally, we genetically modified the ability of microbiota to synthesize propionic acid specifically. Microbiota with higher ability to synthesize propionic acid was able to confer better colonization resistance against CA.

Another impeding factor in generating holistic and physiological knowledge about the factors contributing to GI colonization of CA is the lack of murine models that can be stably colonized with CA without the use of antibiotics. We analyzed the ability of two clinical isolates of CA, namely CHN1 and 529L, to colonize the murine GI tract without the use of antibiotics. CHN1 and 529L were able to colonize the murine GI tract of different mice strains stably and at higher levels compared to the common lab strain SC5314. We further studied these strains to tease out the mechanism conferring this better colonization ability of these strains. Both CHN1 and 529L displayed increased resistance to CRAMP compared to SC5314 and this better tolerance might be contributing to its GI colonization ability.

Overall, this study shows the importance of microbiota-derived metabolites in governing CA GI colonization. The study sequentially analyzes the critical factors such as intrinsic CA pathways, gut microbiota and host sensing of the metabolite for their contribution towards the observed phenotype. This study might further paves the way for future studies involving prebiotic and probiotic strategies for the development of therapeutic interventions. The study also describes two isolates with superior potential to colonize the murine GI tract without any antibiotic supplementation. These isolates can serve as valuable tools to further elucidate importance of novel factors by studying the CA GI colonization in an unperturbed system.

CHAPTER THREE
Material and Methods

Growth of *C. albicans* isolates

Unless otherwise specified, isolates were cultured overnight in 30ml YPD (10g/l yeast extract, 20g/l peptone, 2% dextrose) at 30°C. Cultures were then harvested, washed twice with sterile PBS and resuspended in 10 ml PBS. The concentration of CA was then calculated using a hemocytometer. Required amount of cells were then used based for the assay in question. YVG plates (20g/l agar, 30mg/l vancomycin, 30mg/l gentamicin per liter of YPD) are used to grow and quantify CA isolates from *in vitro* cultures and *in vivo* tissue homogenates. Vancomycin and gentamicin are used to prevent gram-positive and gram-negative bacterial growth, respectively.

Aerobic growth curve of *C.albicans* with short-chain fatty acids (SCFA)

CA strain SC5314 isolate was grown overnight in YPD and processed as described above. For aerobic growth curve experiments, isolates were seeded in YPD in 96-well plates at a starting OD_{600} of 0.1. The wells had varying concentrations of SCFA (butyric acid, Sigma-Aldrich, B10350; propionic acid, Sigma Aldrich, P1086; and acetic acid, Amresco, 0714) or HCl (Fischer, 414- in YPD). Cells were then grown at 37°C with orbital shaking in plate reader (Biotek Synergy HT) for 12 h. OD_{600} reading was taken every hour to monitor growth in each well. Experiments were performed independently three times, with each

experiment having three technical replicates. Growth curves were compared using the linear mixed effect model for significance.

Anaerobic growth curve of *C. albicans* with SCFA

SC5314 was grown overnight in YPD and processed as above. For anaerobic growth curve experiments, isolates were seeded in YPD in 96-well plates at a starting OD_{600} of 0.1. Individual wells had varying concentrations of SCFA or HCl in YPD. The cells were then grown at 37°C without shaking for 96 h in an anaerobic chamber (Coy). OD_{600} reading was taken every 24 h to monitor growth in each well. Experiments were performed independently three times, with each experiment having three technical replicates. The curves were compared using the linear mixed effect model for significance.

Measuring anaerobic growth of *C.albicans* using dilution plating with SCFA

SC5314, Can092 or CHN1 strains were grown overnight in YPD and processed as above. Isolates were seeded in YPD in 12-well plates at a starting concentration of 10^5 cells/ml. Each well contained 25 mM of SCFA at pH 5.0 or YPD adjusted to pH 5.0 with HCl. Cells were then grown at 37°C without shaking for 4 h in an anaerobic chamber at 37°C. Cultures from each well was serially diluted and plated on YVG plates which were then incubated overnight at 30°C. Colonies were counted to evaluate the growth. Experiments were independently performed two times, with each experiment having three technical replicates. Differences in growth were compared using unpaired parametric t-tests.

C. albicans growth with HT-29 and SCFA

HT-29 cells (human colon adenocarcinoma) from ATCC were seeded in 12-well plates. Cells were grown in DMEM with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine. SC5314, Can092 or CHN1 strains were grown overnight in YPD and processed above. Spent media was removed. Cells were washed twice with PBS. Fresh media (with either 25 mM SCFA at pH 5.0 or media adjusted to pH 5.0 with HCl) was added to all wells. Isolates were then incubated with HT-29 cells at a concentration of 10^5 cells/ml for 4 h at 37°C with 5% CO_2. The media was then removed and plated on YVG plates at different dilutions. Experiments were independently performed two times, with each experiment having three technical replicates. The plates were incubated overnight at 30°C and the colonies were counted to evaluate the growth. Growth was compared using unpaired parametric t-tests. Each experiment was performed independently twice, with each experiment having three technical replicates.

Growth of *C. albicans* with cathelicidin-related antimicrobial peptide (CRAMP) *in vitro*

C. albicans isolates were grown overnight in Synthetic Complete Medium (SC) at 30°C under aerobic conditions. Cells were inoculated in 3 ml of liquid SC at OD_{600} 0.25, grown at 30°C until OD_{600} of 1, harvested by centrifugation and washed twice with 10 mM sodium phosphate buffer pH 7.4 (NaPB). Cells were then resuspended in 3 ml of NaPB. 10 µl of cell resuspension was added to 140 µl YPD media with or without the desired concentration of CRAMP (Anaspec, AS-61305) and incubated for 1h at 37°C with shaking. 40 µl of each culture was then added to individual wells of 96-well plate containing 60 µl

YPD with the desired concentration of CRAMP. The plate was then incubated in a plate reader (Biotek Synergy HT) at 37°C with orbital shaking for 16 h. Growth was assessed by taking OD_{600} readings every hour. Aerobic experiments were performed independently three times, with three technical replicates per experiment. For anaerobic growth in the presence of CRAMP, the 96- well plate was incubated at 37°C in an anaerobic chamber without shaking. Growth was evaluated by measuring the final biomass (OD_{600}) at the end of the 16 h incubation period. Anaerobic experiments were performed independently three times, with three technical replicates per experiment.

***in vitro Candida albicans* RNASeq Experiment**

C. albicans strain SC5314 was grown in YPD with or without 50 mM acetic acid, 50 mM butyric acid, or 50 mM propionic acid. pH was adjusted in the untreated control sample to be equivalent to the SCFA samples. Cultures were grown in aerobic conditions at 37C for 90 minutes. CA cells were harvested, washed with sterile PBS x 3, and flash frozen. Total RNA was extracted (Trizol reagent). Crude RNA were treated with DNaseI (Qiagen) and column purified (RNEasy Kit, Qiagen). RNA concentrations were quantified by spectrophotometry (Nanodrop). The quality of the resultant RNA was determined using an Agilent Bioanalyzer. For samples with RNA Integrity Numbers of greater than 7.5, the RiboMinus Kit (Life Technologies) was used to deplete rRNA from the total RNA samples. 500 ng of mRNA was used to create cDNA libraries (UTSW Microarray Core). Paired-end libraries for Illumina sequencing were prepared from purified cDNA (TruSeq RNA Sample preparation kit, Illumina). Sequencing was performed on an Illumina Hi-Seq (PE-150). Reads

were aligned to CA SC5314 genome using CLC-Biosystems RNA-Seq module. For each ORF the number of reads per kilobase of gene model per million mapped reads (RPKM) was calculated [101]. The raw read counts for each gene was processed using DESeq software (utilizing negative binomial distribution analysis), and differentially expressed genes were identified using adjusted p-values < 0.05 and fold-change > 2 (Benjamini-Hochberg procedure) [102].

2-deoxyglucose uptake assay

Glucose uptake assay kit from Abcam was used (Product # 136956). SC5314 was grown and harvested overnight. SC5314 cells were then resuspended to a concentration of 10^8 cells/ml, washed three times with sterile PBS, resuspended in Krebs-Ringer-Phosphate-Hepes buffer (20 mM Hepes, 5 mM KH2PO4, 1 mM MgSO4, 1 mM CaCl2, 136 mM NaCl, 4.7 mM KCl, pH 7.4) at same concentration; and incubated for 120 minute at 37°C. 100 µl of cells were then aliquoted for each technical replicate in each group. The cells were spun down and resuspended in 100 µl YP (10 g/l yeast extract and 20 g/l peptone). The cells then received varying concentrations of SCFA and were incubated for 20 minutes. After 20 minutes, 10 µl of 10 mM 2-DG was added to each replicate and incubated for another 20 minutes. 10 µl of each sample was plated to quantify the viability in each sample. The cells were then washed three times with sterile PBS, and 90 µl of extraction buffer was added. Samples were flash frozen, thawed, and incubated at 85°C for 40 minutes. The cells were then cooled and 10 µl of neutralization buffer was added. The contents were mixed thoroughly and centrifuged for 2 minutes at 500g at room temperature. 50 µl of supernatant

were transferred in black 96-well plate with clear bottom. 50 µl of reaction mix was added to all samples, and incubated at 37°C for 40 minutes. The plate was then measured (excitation wavelength of 537 nm, emission at 585 nm) in a plate reader (Synergy HT). The readings were then converted into 2-DG concentration based on the standard curve generated from the standards of known concentrations. The concentration was then normalized by the cell viability of each sample to quantify the 2-DG uptake. Unpaired parametric t-tests were used to analyze the significance of the difference in 2-DG uptake between different groups.

Intracellular pH measurement in *C.albicans*

To evaluate the intracellular pH in *C.albicans*, strain JKC1559 (which has the phluorin gene encoded in its genome and was graciously provided by Julie Kohler, MD, Harvard Medical School) was utilized. Phluorin is a genetically engineered version of GFP that undergoes a change in its emission spectra based on pH. To evaluate the effect of SCFA on CA intracellular pH, a calibration curve was generated. JKC1559, was grown overnight in YPD for 19 h. The cells were then resuspended in 30 ml normal saline (NS) and mixed thoroughly. Cells were centrifuged at 2000 x g, and the supernatant was discarded. Cells were then resuspended in NS and transferred to 2 ml microfuge tubes. The cells were centrifuged again; the supernatant was discarded; cells were weighed; and cells finally resuspended in NS at a concentration of 0.5 g/ml. 100 µl of cells were then inoculated into 10 ml of the YNB LO FLO (Formedium, CYN6202) medium and incubated at 30°C for 4 h. After 4 h, cells were collected in 15 ml centrifuge tubes at 2000 x g for 1 min. Cells were washed with the culturing medium (5ml) once, and centrifuged. Supernatant was discarded.

Cells were weighed and resuspended to a final concentration of 0.5 g/ml cells. 20 µl of cell suspension was added to each pH calibration tube (2ml) (pH 5, pH 5.5, pH 6, pH 6.5, p H7, pH 7.5, pH 8). Monesin (15 mM stock) and nigericin (2 mM stock) were added to final concentrations of 110 µM monensin and 15 µM nigericin, respectively. The tubes were then incubated at 30°C for one hour with gentle mixing. 300 µl of each sample was then transferred in 96-well plate and fluorescence was measured using a plate reader (Synergy HT) at excitation wavelength of 405 nM and 485 nM with emission wavelength at 515 nM. Ratio of emission intensity at 405 nM and 485 nM was calculated for all the samples and plotted against the pH of each of calibration standard to generate the calibration curve. This curve was used to evaluate the pH of the experimental samples. For experimental samples, cells were grown (exactly as noted previously to generate the calibration curve) to the final density of 0.5 g/ml. This final culture was then used to inoculate 297 µl YNB LO-FLO media per well with varying concentrations of short chain fatty acid in a 96-well plate. 3 µl of the culture was used to inoculate 297 µl of the media in 96-well plate. The media was mixed thoroughly and the fluorescence (405 nM and 485 nM excitation; 515 nM emission) was taken 10 minutes after resuspending cells in the experimental condition. The ratio of emission intensity at 405 nM and 485 nM excitation wavelength was calculated. The calibration curve was then used to convert the ratio into corresponding intracellular pH value. Unpaired parametric t-tests were used to analyze the significance of the difference in intracellular pH between different groups.

Short chain fatty acid quantification

Mouse cecal contents were transferred into pre-weighed tubes, weighed, and resuspended in 1ml PBS. Samples were vortexed and then centrifuged at 6000x g for 15 minutes at 4°C. The supernatant was collected. 95µl of the supernatant was mixed with 5 µl of 100 µM d-butyrate. The samples were completely dried (Eppendorf Vacufuge0. Dried samples were then resuspended in 100 µL of pyridine. All samples were then sonicated (water bath sonicator, 50% amplitude) for 1 minute. The samples were then incubated for 20 minutes at 80°C. The samples were cooled and derivatized with MTBSTFA. 100µL of MTBSTFA was added to all the samples and mixed thoroughly. The samples were then incubated at 80°C for one hour. After one hour, samples were centrifuged at 13,000 x g for 1 minute. Following centrifugation at 13,000 x g, the derivatized samples were moved to autosampler vials for gas chromatography-mass spectrometry (GC-MS) analysis (Shimadzu, TQ8040). The injection temperature was 250°C and the injection split ratio was set to 1:100 with an injection volume of 1 µl. The oven temperature was set at 50°C for 2 min, increasing to 100°C at 20°C per min and to 330°C at 40°C per min with a final hold at 330°C for 3 min. The flow rate of the helium carrier gas (99.9999% purity) was kept constant at a linear velocity of 50 cm/s. We used a 30 m × 0.25 mm × 0.25 µm Rtx-5Sil MS (Shimadzu) column. The interface temperature was 300°C. The electron impact ion source temperature was 200°C, with 70 V ionization voltage and 150 µA current. Acetate (m/z of 117 and 159), propionate (m/z of 131, 132, and 75), butyrate (m/z of 145, 146, and 75) and deuterated butyrate (m/z of 152, 153, and 76) were quantitated in single ion monitoring mode. The target (quantitation) ion is underlined, all other fragments were used as reference (qualifier)

ions. Concentrations were calculated based on an external standard curve and deuterated butyrate as the internal standard.

Synthesizing Inulin propionate ester

Inulin propionate ester was synthesized following a previously published procedure for inulin esterification [88]. Inulin (20 g, 0.123 mole), propionic anhydride (18.3 g, .014 mol), 1-methyl imidazole (10.1 g, 0.123 mol) was combined in 60 ml DMSO and stirred at room temperature for 4 h. The mixture was diluted with 120 ml water. The reaction mixture was dialyzed against water for 2 days at 4°C. Following dialysis, samples were freeze-dried. Product was tested using ^{1H}NMR, a peak in 1-2 ppm region validated the successful synthesis of inulin propionate ester.

Mouse strains used in this study

Wildtype C57BL/6J mice (Jackson, female, 6-8 weeks, Stock 000664, Room RB12) and C3H/HeN mice (Envigo, female, 6-8 weeks, Stock 040) were used for studying the effect of SCFA on CA GI colonization. Heterozygous breeding pairs for *Ffar2* KO mice (MMRRC, stock 047690-UCD) were bred to generate *Ffar2* KO mice. Heterozygous breeding pair for *Ffar3* KO (RIKEN,stock RBRC06327) were bred to generate *Ffar3* KO mice. *Camp* KO mice (Jakcson, Stock 017799) were bred in our animal facility. *Hif1a*$^{fl/fl}$ *vil-Cre*$^{+}$ were bred in house, which was originally created from B6.129-Hif1a^{tm3Rsjo}/J, C57/BL background, Jackson Laboratories. *Pparγ*$^{fl/fl}$ *vil-Cre*$^{+}$ were propagated in house from the breeding pair received from the laboratory of Dr. Sebastian Winter. Germ free C57Bl/6 mice were provided by the Gnotobiotic Animal Core at the University of Texas Southwestern

Medical Center. All the procedures performed in the experiment were done in accordance to the IACUC approved protocols.

Effect of oral administration of SCFA on *C. albicans* SC5314 GI colonization in mice

Adult mice (female, 6-8 weeks old) were given 2 mg/ml of streptomycin and 1500 U/ml of penicillin in drinking water to deplete endogenous bacterial gut microbiota. After 7 days, bacterial microbiota clearance was checked by plating fecal pellets from mice on BHI-blood plates (anaerobic incubation, 37°C) and MacConkey and TSA plates (aerobic incubation, 37°C). Clearance was confirmed by absence of growth from these media. Mice were then gavaged with 200 µl of $1x10^9$ CFU/ml of SC5314 suspended in sterile PBS. Antibiotic treatment was stopped. Mice were started either on sterile water at pH 5.0 or water with 150 mM SCFA at pH 5.0 *ad libitum.* Mice were fed Teklad Global 16% Protein Rodent Diet chow (Teklad 2916, irradiated). Mouse cages were changed once weekly. In order to quantify CA distal GI tract colonization levels, mouse fecal pellets were collected, weighed, homogenized in PBS, serially diluted, plated on YVG plates and incubated at 30°C. Colonies were counted and normalized to weight of the fecal pellets to quantify CA colonization levels. CA colonization levels were assessed weekly for four weeks. To determine CA colonization in different GI segments, mice were euthanized on day 7 and day 28 after SCFA water initiation. Luminal contents from duodenum, ileum, cecum and colon were processed and plated as described above.

To determine the impact of microbiota recovery on the protective effect of SCFA, experimental schema described above was amended so that antibiotic treatment continued throughout the duration of the experiment.

In germ-free mouse experiments, no antibiotic treatment was given prior to colonizing the mice with CA. Fecal pellets were sampled weekly for CA levels as described above after the mice were started on SCFA water.

Differences in colonization levels were checked for statistical significance using Mann-Whitney tests for all experiments.

The effect of *B. theta* mutant unable to produce propionate on *C. albicans* GI colonization

Germ-free mice were gavaged with 200 µl of 1 x 10^9 CFU/ml of CA strain SC5314. The following day fecal pellets were collected prior to quantify SC5314 colonization levels. Mice were then gavaged with 200µl of 2x10^9 CFU/ml wildtype B.theta (Δtdk strain) or B. theta Δ1686-89 mutant strain (graciously provided by Denise Monack, PhD, University of California at San Francisco). Fecal pellets were collected every third and seventh day of the week for four weeks. Fecal pellets were processed as described above to quantify colonization levels of SC5314 in mice. Differences in colonization levels were checked for statistical significance using Mann-Whitney tests. Mice were euthanized at the end of the experiment, and cecal contents were collected to measure propionate levels.

Quantifying the impact of inulin propionate ester on *C. albicans* GI colonization

Microbiota of C57BL/6J mice were depleted with antibiotics and colonized with SC5314 as described above. After colonization with SC5314 was confirmed, mice were put on sterile water without antibiotics and were gavaged with 200µl of $2x10^9$ CFU/ml wildtype *B.theta* (VPI-5482 strain). Mice were subsequently orally gavaged with 200 µL 1% inulin or 1% inulin propionate ester daily. Fecal pellets were collected every two days, over a period of four weeks. Fecal pellets were processed as described previously to quantify the colonization levels of SC5314 in mice. Differences in colonization levels were assessed using Mann-Whitney tests. Mice were euthanized at the end of the experiment, and cecal contents were collected to measure propionate levels.

16S rRNA gene PCR amplification and sequencing

Bacterial gDNA was extracted from fecal samples using the MagAttract Power Microbiome DNA/RNA KF kit (Qiagen) on the Kingfisher Flex machine (Thermo Fisher Scientific). 16S rRNA genes (variable region 4, V4) were amplified from each sample in 96-well plates using a composite forward primer and a reverse primer containing a unique 8-base barcode that was used to tag PCR products from respective samples [103]. We used the reverse primer 926R, 5'-*CAAGCAGAAGACGGCATACGAGAT*-NNNNNNNN-**AGTCAGTCAG-CC-GGACTACHVGGGTWTCTAAT**-3": the italicized sequence is the reverse MiSeq primer i7; NNNNNNNN designates the unique 8-base barcode used to tag each PCR product; the bold sequence is the broad-range 16S bacterial primer containing the pad-link-16SR. The forward primer used was 515F, 5'-*AATGATACGGCGACCACCGAGATCTACAC*-NNNNNNNN-**TATGGTAATT-GT**-

GTGCCAGCMGCCGCGGTAA-3': the italicized sequence is MiSeq Primer i5; the NNNNNNNN designates the unique 8-base barcode used to tag each PCR product; and the bold sequence is the broad range 16S bacterial primer containing the pad-link-16SF. PCR reactions consisted of 17ul Accuprime Pfx Supermix, 1000 nM of each primer, and 20ng of template. Reaction conditions were 2 min at 95°C, followed by 30 cycles of 20 s at 95°C, 15 s at 55°C, 5 min at 72°C, then 10 min at 72°C, and a hold at 4°C on an Eppendorf Mastercycler. Products were verified on a 1% agarose gel, and all samples were cleaned and normalized using the AmPure Normalization plate protocol using the KingFisher Flex platform. Each plate was then pooled into a single tube, and the PCR product size and library quality of each individual pooled plate was checked using Agilent Technologies D1000 ScreenTape electrophoresis. Additionally, KAPA Biosystems PCR Library Quantification kit was used to quantify each pooled plate. Illumina spike-in (PhiX) was included at 4 pM at 10%, and the pooled sample library was included at 4pM at 90% yielding a final library concentration of 3.6 pM and PhiX concentration of 0.4 pM. Pooled samples were then sequenced with Illumina MiSeq (PE-250) at the Microbiome Research Laboratory at UTSW Medical center.

16S rRNA gene sequence analysis

Raw sequences generated from Illumina MiSeq for 16S rRNA gene PCR amplicons were quality filtered. Sequences shorter than 200 nucleotides or longer than 1000 nucleotides were removed. Sequences containing ambiguous bases, primer mismatches, homopolymer runs in excess of 6 bases and uncorrectable barcodes were also removed. Sequences that

passed the quality filtration were denoised and analyzed using the open source software package Quantitative Insights Into Microbial Ecology (QIIME2). 16S rRNA gene sequences were then classified taxonomically using the classifier Silva database. Differential taxonomic abundance between different phenotypic groups was analyzed by linear discriminate analysis (LDA) coupled with effect size measurements (LEfSe). Only taxa noted to have >2 log fold increase in LDA score and $p< 0.05$, Kruskal-Wallis test were identified as significantly enriched or depleted.

Strain specific differences in *C. albicans* GI colonization

For the GI colonization experiments with single strain infection, C3H/HeN mice (female, 6-8 weeks old, Envigo) were used. *C. albicans* strains SC5314, CHN1 and 529L were grown overnight in YPD at 30°C with shaking under aerobic conditions. Cells were harvested, washed twice with PBS and resuspended in PBS at a concentration of 1×10^9 CFU/ml. C3H female mice were gavaged with 200 µl of cell suspension containing a total of 2×10^8 *Candida* cells. To determine fungal burdens, fecal pellets were collected every 7 days for 35 days, homogenated and plated on YPD agar supplemented with antibiotics (30 µg/ml of vancomycin and 30 µg/ml of gentamicin). For competition experiments, *C. albicans* isolates SC5314 (containing the *SAT1* gene, *SAT1*+), CHN1 and 529L were grown overnight in YPD at 30°C with shaking under aerobic conditions. Cells were harvested, washed twice with PBS and resuspended in PBS at a concentration of 1×10^9 CFU/ml. Equal cell numbers of SC5314 (*SAT1*+) and CHN1 or 529L were mixed together. 6-8 weeks old C57BL/6J (Jackson Laboratories, room RB12) or *Camp* KO (Jackson Laboratories, Stock number

017799) female mice were gavaged with 200 µl of cell suspension containing a total of 2 x 10^8 *Candida* cells. Equal strain ratios were confirmed by plating the initial inoculum. Fecal pellets were collected every two days for 19 days, homogenated and plated on YPD agar supplemented with nourseothricin (200 µg/ml) and antibiotics (30 µg/ml of vancomycin and 30 µg/ml of gentamicin).

CHAPTER FOUR
Effect of short chain fatty acids on *C. albicans* gastrointestinal colonization

Introduction

Infectious diseases are the third leading cause of death in the United States and the leading cause worldwide [1]. Most infectious diseases invade the human host through mucosal surfaces to cause disease. Thus, understanding the underlying mechanisms by which microbes colonize mucosal surfaces and further disseminate to other organs is of great interest. *Candida albicans* (CA) exists as a pathobiont – acting as a commensal, colonizing the gastrointestinal and geneto-urinary tract in normal healthy host, without causing disease, but in immunocompromised hosts, it can disseminate and cause invasive infections. Therefore, understanding the mechanisms that allow CA to effectively colonize gastrointestinal tract remains extremely relevant to human health and may lead to developing better interventions to decrease invasive and life-threatening fungal infections.

Our lab has previously developed a mouse model to study the mechanisms of gastrointestinal colonization of CA [17]. Our lab identified commensal species, *Bacteroides thetaiotamicron* and *Blautia producta* promote colonization resistance against CA [19]. This resistance in part was shown to be dependent on HIF1α and the anti-microbial peptide CRAMP. In another study from the lab of Tobias Hohl, showed in human specimen that *Candida albicans* fungal load was inversely correlated with the abundance of *Bacteroidetes* family and *Lachnospiraceae* family [18]. Thus it is a relevant problem to further understand

the mechanisms that might be allowing these bacterial species to mediate colonization resistance against CA.

One of the common characteristics of these two bacterial species, *Bacteroides thetaiotamicron* and *Blautia producta* is that both these species produce short chain fatty acids [104]. There is also a drastic decrease in the levels of short chain fatty acids (SCFA) in the antibiotic treated mice that can be colonized by CA strain SC5314 compared to the mice with intact microbiota that is resistant to CA strain SC5314. Short chain fatty acids are known to be immunomodulatory molecules that can impact the production of anti-microbial peptides as well including CRAMP [98]. Not only that, now there are studies, demonstrating a direct anti-microbial properties of short chain fatty acids on *enterobacteriaceae* family and *salmonella* [88,95]. Thus we decided to probe the impact of short chain fatty acids on factors governing colonization levels of CA.

There are three pathways that SCFA can modulate to impact the colonization potential of *C.albicans*, [105] 1) directly interacting with CA and impacting its genetic pathways to decrease the ability of GI colonization 2) interacting with the host and impacting the defense mechanism such as the secretion of anti-microbial peptide that can promote colonization resistance against CA and 3) modulating the composition of the microbiome such as increase in the levels of *Bacteroidetes* or *Firmicutes* that can then either directly or indirectly lead to colonization resistance against CA.

Based on these three different possibilities the study investigates the impact of short chain fatty acids on CA GI colonization and underlying mechanism that might be involved. To study the impact of SCFA on CA, growth under different *in vitro* settings were quantified.

After characterizing the impact of SCFA on CA, the genetic pathways of CA that gets impacted by SCFA through RNAseq was analyzed. The analysis of RNAseq allowed us to further investigate the pathways that were crucial in inhibiting the growth of CA in the presence of short chain fatty acids. This led to elucidating the role of glucose uptake pathway and intracellular pH in mediating the effect of short chain fatty acids on CA.

Further the impact of SCFA on GI colonization in *in vivo* models of CA, demonstrating the protective effect of SCFA against CA GI colonization was analyzed. This also allowed investigating the importance of microbiota in mediating the protective effect and further to study the specific differences in the recovery of the microbiota depending on the treatment regimen that the mice received.

To elucidate the importance of immunomodulatory mechanisms that might be involved in this overall protection, numerous genetic knockout mice to study the effect of SCFA on CA colonization were utilized. We analyzed the protection provided by SCFA in different single gene knockout mouse model. We initially measured the protection in *Hif1α*$^{fl/fl}$ *vil-Cre*$^{+}$ and *Camp* KO mouse models, which have been previously shown to be important for CA colonization resistance. We also studied the protective effect SCFA on CA colonization in genetic knockout of SCFA sensors in mouse, namely *Ffar2* KO mice, *Ffar3* KO mice and *Pparγ* $^{fl/fl}$ *vil-Cre*$^{+}$.

Finally, the effect of change in endogenous SCFA concentration on CA GI colonization was studied. The microbiota produced propionate levels were modulated, either by utilizing a *B.theta* strain lacking the potential to produce propionate or by providing prebiotic that could be processed by the microbiota to increase the level of propionate.

In summary, this study demonstrates that the microbiota produced SCFA has a protective effect on host by reducing the colonization levels of CA in GI tract. It establishes the mechanisms through which SCFA can directly impact CA genetic pathways to provide this protection. It also elucidates the importance of the resident microbiota, and how it could be engineered to provide protection. The study furthers our understanding of GI colonization by CA as well as provides evidence and mechanisms to develop SCFA as a therapeutic intervention to decrease the gastrointestinal level of CA, subsequently decreasing the probability of dissemination and blood stream infections.

Results

SCFA inhibit *C.albicans* growth *in vitro*

C.albicans (CA) strain SC5314 is the most commonly used isolate in CA research. SC5314 cannot colonize the gastrointestinal tract of mice with an intact gut microbiota [24]. Thus, the murine model developed by our lab utilizes penicillin and streptomycin administered in drinking water *ad libitum* for one week to deplete endogenous mouse gut bacterial microbiota prior to colonizing mouse with CA [17]. This antibiotic treatment significantly decreases the levels of anaerobes belonging to the *Bacteroidetes* and *Firmicutes* phylum which have been shown by our group and others to provide colonization resistance against *C.albicans* [19]. A common feature for certain members of phyla is that they produce short chain fatty acids (SCFA) [104]. Therefore, we first sought to measure distal gut short chain fatty acids levels in mice with intact microbiota compared to mice receiving penicillin and streptomycin with depleted microbiota. Indeed, mice that received penicillin and streptomycin were significantly reduced for all the three SCFA (acetic acid, propionic acid and butyric acid) (Figure 2A-C).

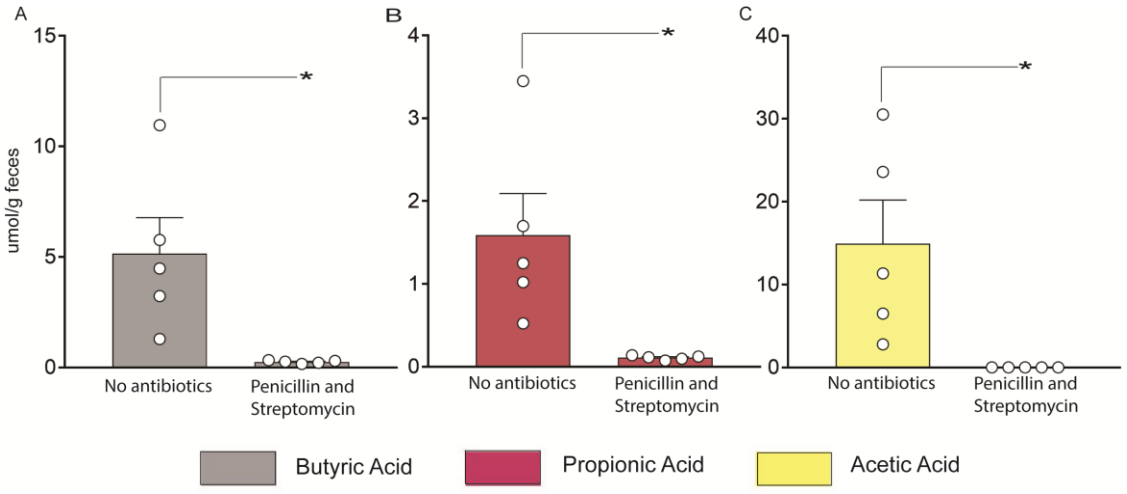

Figure 2. Penicillin and streptomycin treatment decreases levels of SCFA in C57BL/6J mice. (A) Butyric acid. (B) Propionic acid. (C) Acetic acid. Graphs show mean + SEM value of SCFA measured from the cecal contents of the mice using GC-MS. Statistical significance of comparisons is calculated using unpaired parametric t-tests. * $P < 0.05$.

We then investigated the effect of SCFA on CA growth *in vitro*. We grew CA strain SC5314 in the presence of varying concentrations (0-50 mM) of acetic, butyric, and propionic acid in YPD media for 12 h at 37°C under aerobic conditions. The SCFA concentrations tested are physiologically relevant for the mammalian GI tract: acetic acid 60 mM-100 mM, propionic acid 10-20 mM and butyric acid 20-30 mM [106]. The growth of CA strain SC5314 was significantly reduced in the presence of increasing concentrations of SCFA in the media. The minimum inhibitory concentration observed for both butyric acid and propionic acid was 25 mM (Figure 3A-B). Acetic acid provided significant growth inhibition as well, but the minimum inhibitory concentration was 50 mM of acetic acid (Figure 3C). Since SCFA acidifies media, we sought to understand whether the growth inhibition was attributable to reduction in pH alone. Thus, we conducted growth experiments in YPD media pH 3-6, in which HCl was added to change the pH. CA growth was not impeded by changes in pH alone (Figure 3D). This data suggest that SCFA-induced growth inhibition of CA is not dependent on the acidification of the growth media.

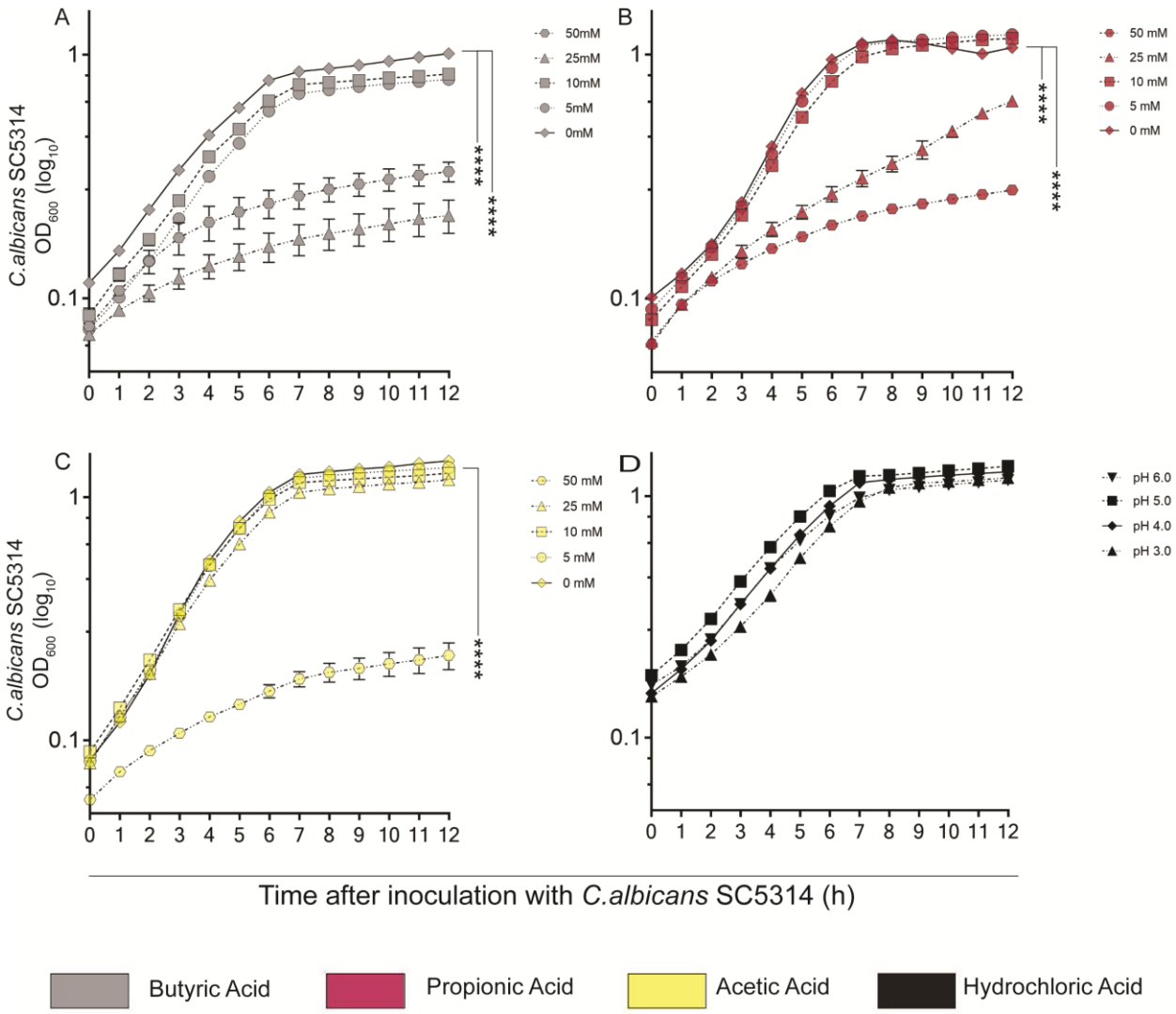

Figure 3. *in vitro* inhibition of *C. albicans* isolate SC5314 aerobic growth at 37°C under different SCFA concentration. (A) butyric acid, (B) propionic acid, (C) acetic acid, (D) HCl. Plots show means ± SEM growth levels over 12 h from three independent experiments. Statistical significance of comparisons is calculated using linear mixed effect model. **** $P < 0.0001$.

Oxygen concentrations are scarce (microaerophilic) or absent in the gastrointestinal tract, particularly the distal gut [96,107]. As such, we assessed the effect of SCFA on

SC5314 growth under anaerobic conditions. We tested the same range of SCFA concentrations (0-50 mm) and also tested the effect of acidification alone (pH 3.0, HCl). All three SCFA were able to inhibit the growth of SC5314 under anaerobic condition (Figure 4 A-C). In contrast to the aerobic growth experiments, SCFA induced CA growth inhibition at lower minimal inhibitory concentrations: butyric acid 5 mM (versus 25 mM aerobic); propionic acid 10 mM (25 mM aerobic); and acetic acid 25 mM (50 mM aerobic). In summary, all three SCFA were able to inhibit the growth of SC5314 under anaerobic condition, and the minimal inhibitory concentration was lower in anaerobic conditions compared to aerobic conditions.

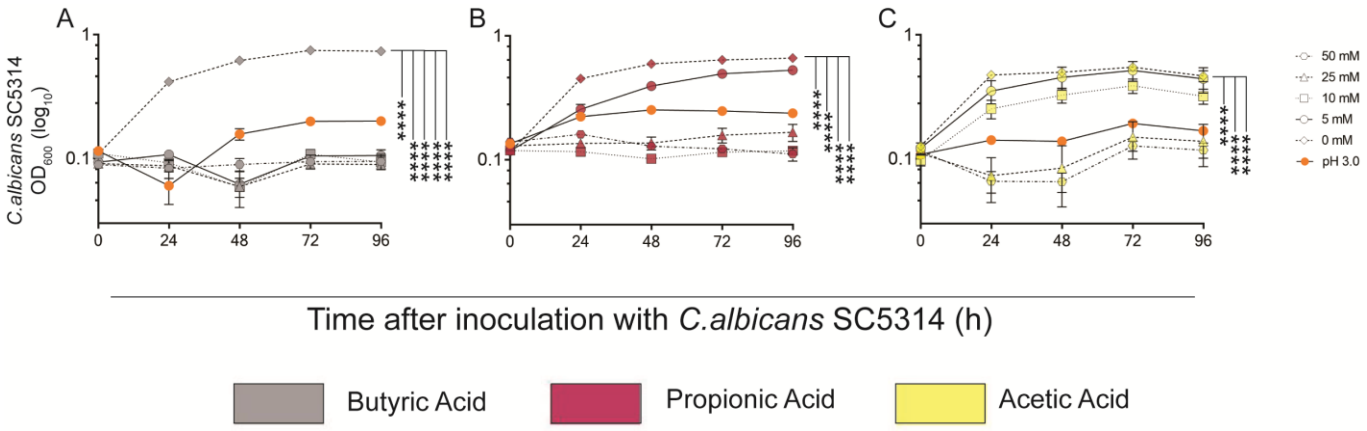

Figure 4. *in vitro* inhibition of *C. albicans* isolate SC5314 anaerobic growth at 37°C under different SCFA concentration. (A) Butyric acid. (B) Propionic acid. (C) Acetic acid. Plots show means ± SEM growth levels over 96 h from three independent experiments. Statistical significance of comparisons is calculated using linear mixed effect model. **** $P < 0.0001$.

CA is a dimorphic organism that can exist either in a spherical yeast form or as an elongated hyphae and pseudohyphae form. Since measurement of growth using spectrophotometric methods is influenced by the cell morphology and does not differentiate between live and dead cells, we decided to further quantify the growth inhibition by SCFA by cultured enumeration of CA grown in presence or absence of SCFA under anaerobic conditions. Consistent with our growth curve experiments, the number of viable CA (as determined by enumeration of cultured CA on selective media) was significantly lower when grown in the presence in butyric and propionic acid compared to CA grown without any SCFA (Figure 5 A). In contrast, acetic acid had no inhibitory effect at 25 mM (Figure 5 A). Interestingly, all three SCFAs (mixed in proportions physiologically relevant to the mammalian GI tract; 13 mM acetic acid, 5 mM propionic acid, 7 mM butyric acid) were able to inhibit the growth of SC5314 but not to the extent of butyric acid and propionic acid alone (Figure 5A).

Microbial strain-specific variations in phenotype is a commonly observed phenomenon. In order to determine if our observations were generalizable to other CA strains, we tested two clinical isolates of *C.albicans* Can092 and CHN1. We grew both these isolates in the presence or absence of 25 mM SCFA (individual acids or the combination of three equal to 25 mM). Both strains exhibited growth inhibition with the addition of SCFA to the media anaerobically at 37°C (5 B-C). As observed with CA strain SC5314, butyric and propionic acid were more effective in inhibiting CA growth as compared to acetic acid. These data suggest that SCFA-induced CA growth inhibition is not a strain-specific phenomenon.

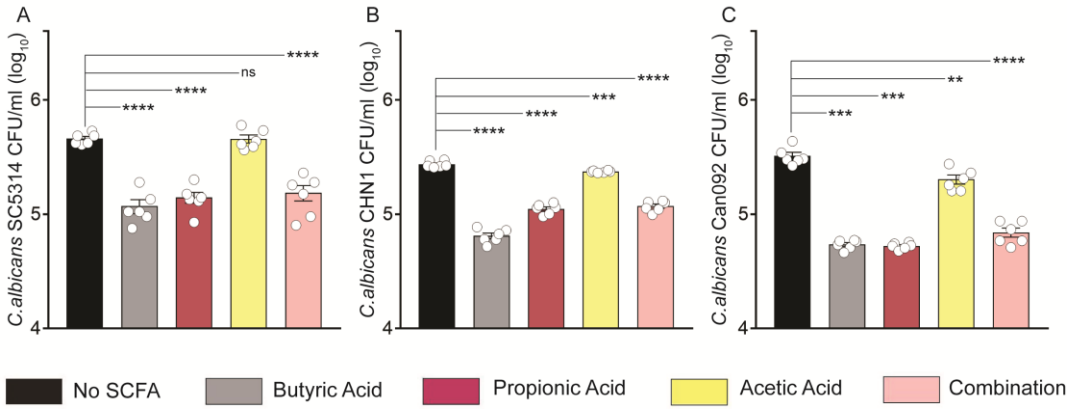

Figure 5. *in vitro* inhibition of *C. albicans* isolate SC514, Can092 and CHN1 anaerobic growth at 37°C under 25 mM butyric acid, propionic acid, acetic acid, or combination (7 mM butyric acid, 5 mM propionic acid, 13 mM acetic acid). (A) SC5314. (B) Can092. (C) CHN1. Growth was measured by CFU enumeration by diluting plating on YVG plates after 4 hours. Plots show means ± SEM CFU recovered from cultures from two independent experiments. Statistical significance of comparisons is calculated using unpaired parametric tests. ** $P < 0.1$, *** $P < 0.01$, **** $P < 0.0001$, ns, not significant.

Finally, we sought to understand the impact of host mucosal immune effectors in the context of both CA and SCFAs. Therefore, we utilized a well-established cell culture protocol utilizing human colonic cells, HT-29. All the three strains of CA SC5314, CHN1 and Can092 were co-cultured with colonic HT-29 cells. HT-29 and *C.albicans* strains were grown in DMEM media with or without SCFA. CA growth/levels were determined by culturing cell culture media (after 4 h of co-incubation) and enumeration CA. Consistent with the experiments described above, inhibition was observed with butyric acid and propionic acid (Figure 6A-C). Acetic acid, however, only inhibited CHN1 strain growth. (Figure 6C).

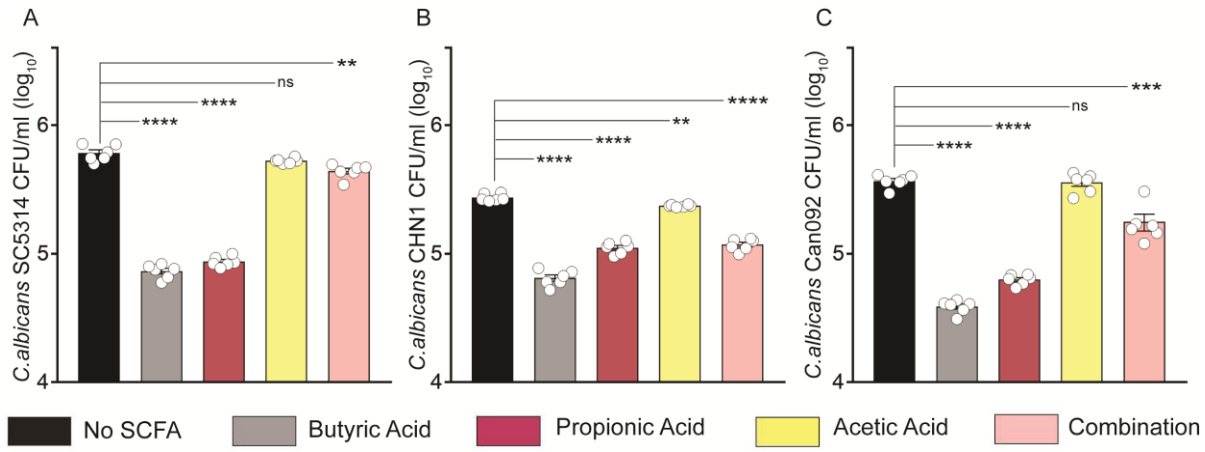

Figure 6. *in vitro* inhibition of *C. albicans* isolate SC514, Can092 and CHN1 in presence of HT-29 colonocytes at 37°C aerobically with 5% CO_2 under 25 mM butyric acid, propionic acid, acetic acid, or combination (7 mM butyric acid, 5 mM propionic acid, 13 mM acetic acid). (A) SC5314, (B) Can092 (C), CHN1. Growth was measured by CFU enumeration by diluting plating on YVG plates after 4 hours. Plots show means ± SEM CFU recovered from cultures from two independent experiments. Statistical significance of comparisons is calculated using unpaired parametric tests. ** $P < 0.1$, *** $P < 0.01$, **** $P < 0.0001$, ns, not significant.

Overall, these data suggest that SCFA directly inhibit CA growth. Butyric acid and propionic acid exhibit stronger growth inhibitory effects than acetic acid. And these phenomena are not strain-specific and may be generalizable to all CA.

SCFA inhibit glucose uptake pathways in *C.albicans*

To gain further mechanistic insight, we explored the effect of SCFA on the CA transcriptome. To this effect, SC5314 was grown in YPD or YPD supplemented with 50mM of one of the three SCFA for 90 minutes at 37°C. Total RNA was extracted from CA cells, and RNAseq was performed. Focusing on genes that were significantly (> 2 fold change, p<

0.05) downregulated in the presence of all three SCFA, we identified genes that were integral for hexose uptake and carbohydrate metabolism (Figure 7). These data suggest that one potential mechanisms by which SCFA inhibit CA growth could be secondary to downregulation of hexose uptake and carbohydrate metabolism pathways that would ultimately lead to CA starvation and growth inhibition.

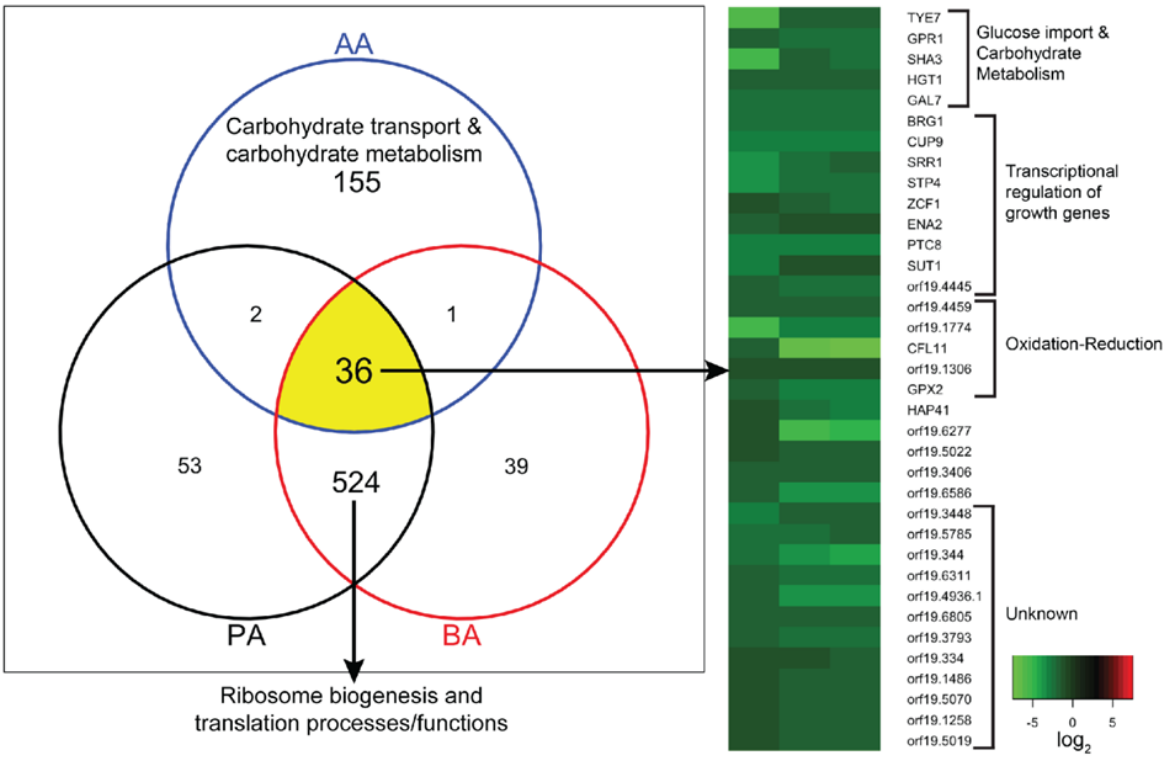

Figure 7. List of common set of genes downregulated (> 2 fold change, p< 0.05) downregulated in the presence of all three SCFA using RNA-seq

To further test this hypothesis, we utilized an assay to quantify glucose uptake by CA. This assay utilizes a glucose analog 2-deoxyglucose. SC5314 can uptake 2-DG but cannot metabolize it. Thus, intracellular levels of 2-DG can serve as a marker for the rate of glucose

uptake under different conditions. CA cells were first starved of glucose before providing them with 2-DG. Strikingly, CA 2-DG levels decreased with exposure to SCFAs (0 mM to 25 mM) in a dose-dependent fashion (Figure 8A-C). As observed with the previous growth inhibition assays, butyric acid had the most potent effect, with significant inhibition of glucose uptake at 1 mM (Figure 8A). Propionic acid was able to inhibit 2-DG uptake at 1 mM in more than 50% of our samples but not in all the samples (Figure 8B). In contrast, acetic acid had the least inhibitory effect on glucose uptake at 1 mM (Figure 8C). The trends observed with the *in vitro* growth curve experiments was also noted with these experiments: higher concentrations of acetic acid are required to induce the phenotype when compared to butyric and propionic acid. These 2-DG uptake data support the RNASeq findings and thus provide one potential mechanism by which SCFA directly inhibit CA growth.

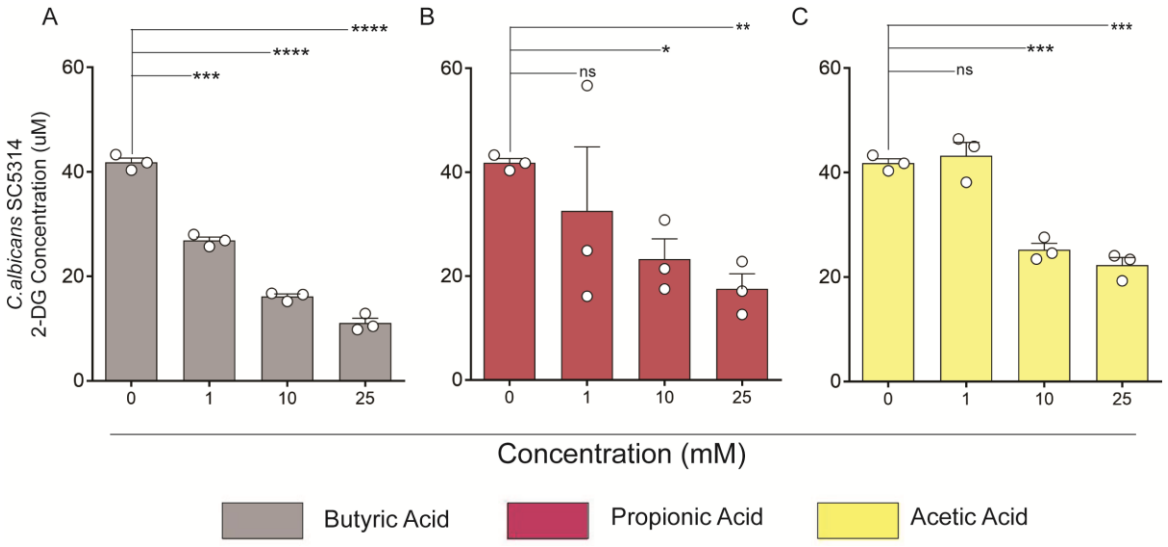

Figure 8. Inhibition of 2-DG uptake in *C. albicans* isolate SC5314 under different SCFA concentration. (A) Butyric acid, (B) propionic acid (C) acetic acid. Plots show mean + SEM value of 2-DG uptake from three independent experiments. Statistical significance of comparisons is calculated using unpaired parametric t-tests. ** $P < 0.1$, *** $P < 0.01$, **** $P < 0.0001$, ns, not significant.

SCFA decreases intracellular pH of *C.albicans*

SCFA have been shown to inhibit the growth of other microbial species (*Salmonella* spp. and *Enterobacteriaceae*) [88,95], and the purported mechanism is via SCFA-induced intracellular acidification of the bacteria. Interestingly, in CA and other yeast species, such as *S.cerevisiae,* pH, glucose sensing and glucose uptake processes have been shown to be intimately linked to each other [108,109]. For example, intracellular pH is proposed to serve as a second messenger of glucose availability in *S.cerevisiae*. When extracellular glucose levels are low, the intracellular pH of *S.cerevisiae* decreases (pH 6.2 to 6.4). As glucose availability increases in the media, intracellular pH increases. Thus, we investigated whether SCFA induces CA intracellular acidification well. We utilized a *C.albicans* strain JKC1559, an engineered strain of that encodes for Phluorin [110]. Phluorin is a variant of GFP whose fluorescence spectra changes with change in surrounding pH. Briefly, Phluorin can be excited at wavelengths, 405 nM and 485 nM with the emission wavelength for both at 515 nM. As the pH of the medium decreases the emission peak for the excitation wavelength 485nM increases whereas that for 405 nM decreases. A calibration curve at different pHs and the emission ratio for both the wavelength is then used to convert the ratio of samples to respective intracellular pH. Intracellular pH of JKC1559 in presence of SCFA at a concentration of 10 mM or 25 mM was measured. Both butyric and propionic acid were able to reduce the intracellular pH of JKC1559 to 6.2, while untreated JK1559 cells had significantly higher pH values (Figure 9A). Acetic acid at 10 mM concentration was not able to significantly reduce the intracellular pH of JKC1559 (Figure 9A). JKC1559 cells grown with 25mM of SCFA showed a comparable reduction in pH (Figure 9B). At 25mM, acetic

acid was able to significantly reduce the intracellular pH but not to the degree of BA, PA or the combination of all three SCFAs (Figure 9B). These data further confirm that higher concentration of acetic acid appear be required to exert its effect on CA.

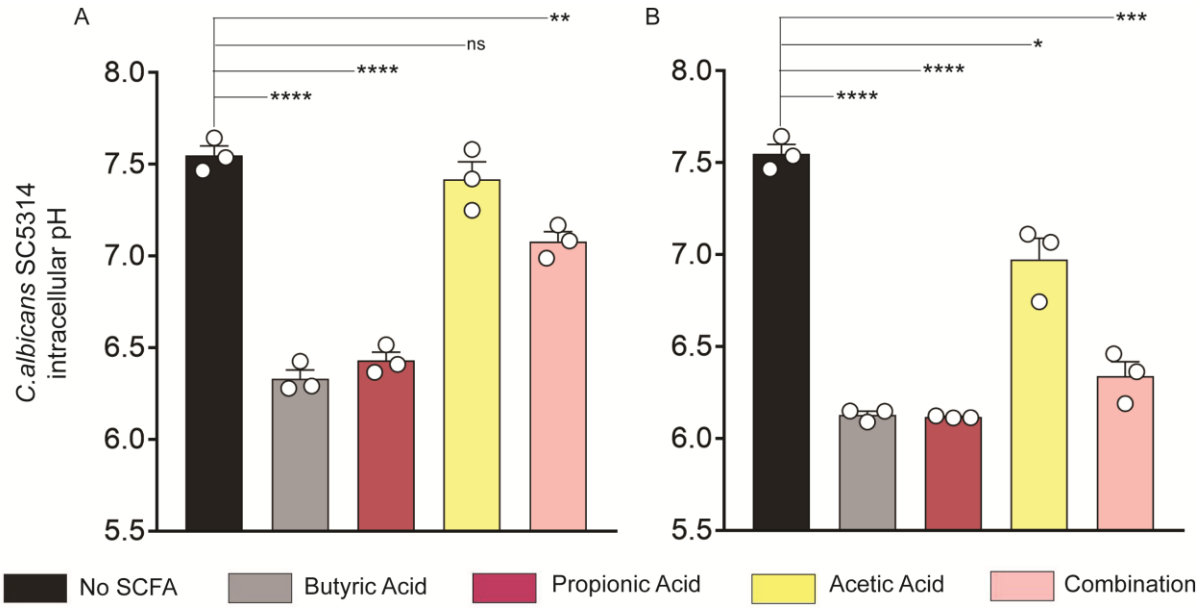

Figure 9. Decrease in cytosolic pH of *C. albicans* isolate SC5314 in presence of SCFA. (A) 10 mM of butyric acid, propionic acid, acetic acid or combination (3 mM butyric acid, 2 mM propionic acid, 5 mM acetic acid). (B) 25 mM of butyric acid, propionic acid, acetic acid or combination (7 mM butyric acid, 5 mM propionic acid, 13 mM acetic acid). Plots show means + SEM value of cytosolic pH from three independent experiments. Statistical significance of comparisons is calculated using unpaired parametric t-tests. ** $P < 0.1$, *** $P < 0.01$, **** $P < 0.0001$, ns, not significant.

In summary, SCFAs significantly downregulate CA genes involved in hexose uptake pathway and carbohydrate metabolism. As intracellular pH can serve as a second messenger of glucose availability, a decrease in the intracellular pH can signal unavailability of glucose

leading to decrease in the levels of genes involved in glucose uptake pathway. These events would culminate in CA being driven into a starvation state, thus inhibiting the overall growth of CA.

SCFA provides colonization resistance against *C.albicans* in mice GI tract

To determine if our *in vitro* observations hold true *in vivo,* we utilized a murine model of gastrointestinal colonization by *C.albicans* used in our lab. Mice with intact microbiota cannot be stably colonized with CA strain SC5314. Thus, we administer antibiotics (penicillin and streptomycin) in drinking water to mice for 7 days before establishing colonization with SC5314.

We used C57BL/6J mice (female, 6-8 weeks old, Jackson) to study the effect of SCFA on SC5314 gastrointestinal colonization. After mice were colonized by SC5314, antibiotic water was discontinued and transitioned to sterile water at pH 5.0, HCl or water with 150 mM of one of the three SCFA at pH 5.0. One group of mice received all the three SCFA with combined concentration equal to 150 mM at pH 5.0. To quantify GI CA load, fecal pellets were collected from individual mice from all the groups weekly. The fecal pellets were homogenized in PBS, serially diluted, plated on YVG, and enumerated (normalized to fecal pellet weight) to determine the CA colonization levels. Mice treated with butyric acid or propionic acid exhibited significantly lower CA GI colonization levels compared to mice receiving sterile water, as soon as day 7 after SCFA treatment was initiated (Figure 10A-B). Mice that received acetic acid or the combination treatment did not show any significant difference in the fungal load compared to the mice receiving sterile water on

day 7 (Figure 10C-D). CA SC5314 GI colonization levels were followed weekly for four weeks. Mice treated with butyric acid and propionic acid had the lowest CA GI colonization levels compared to all other treatment groups, throughout the duration of the experiment. (Figure 10A-B). The propionic acid treated group had the lowest fungal burden, while mice treated with acetic acid or combination SCFA did not show any significant differences in CA GI levels except day 21 (Figure 10C-D). These data are consistent with our *in vitro* findings, in which acetic acid had the least inhibitory effect on the growth of *C.albicans* species.

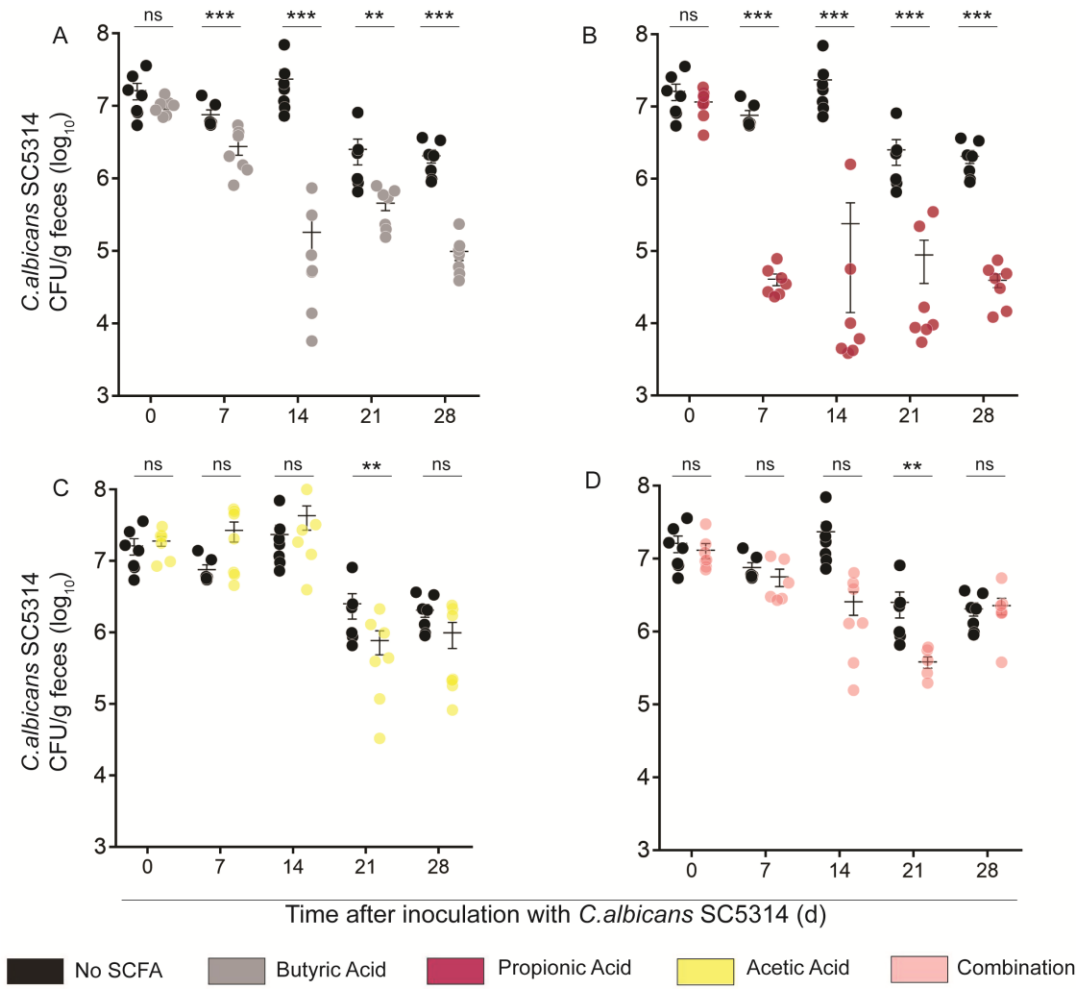

Figure 10. Effect of 150 mM SCFA in drinking water on *C. albicans* isolate SC5314 gastrointestinal colonization in C57BL/6J mice after penicillin and streptomycin cessation. (A) 150 mM butyric acid at pH 5.0 (n=7) vs sterile water at pH 5.0 (n=7). (B) 150 mM propionic acid at pH 5.0 (n=7) vs sterile water at pH 5.0 (n=7). (C) 150 mM acetic acid at pH 5.0 (n=7) vs sterile water at pH 5.0 (n=7). (D) 150 mM combination (42 mM butyric acid, 24 mM propionic acid, 81 acetic acid) (n=7) at pH 5.0 vs sterile water at pH 5.0 (n=7). Graphs represent mean ± SEM fecal colonization load (CFU/g) over time. Statistical significance of comparisons is calculated using Mann-Whitney tests. ** $P < 0.1$, *** $P < 0.01$, ns, not significant.

Inbred mouse strains have distinct gut microbiota, even among the same strain from different vendors (Jackson C57BL vs Taconic C57BL). Thus to exclude mouse strain-specific effects, we repeated the experiment using C3H/HeN mice (female, 6-8 weeks, Envigo). Consistent with our results in Jackson C57BL/6 mice, both butyric acid and propionic acid induced colonization resistance against SC5314 but no such protection was conferred by acetic acid or combination SCFA. Again, propionic acid was most effect in enhancing CA GI colonization reduction in C3H/HeN mice (Figure 11A-D).

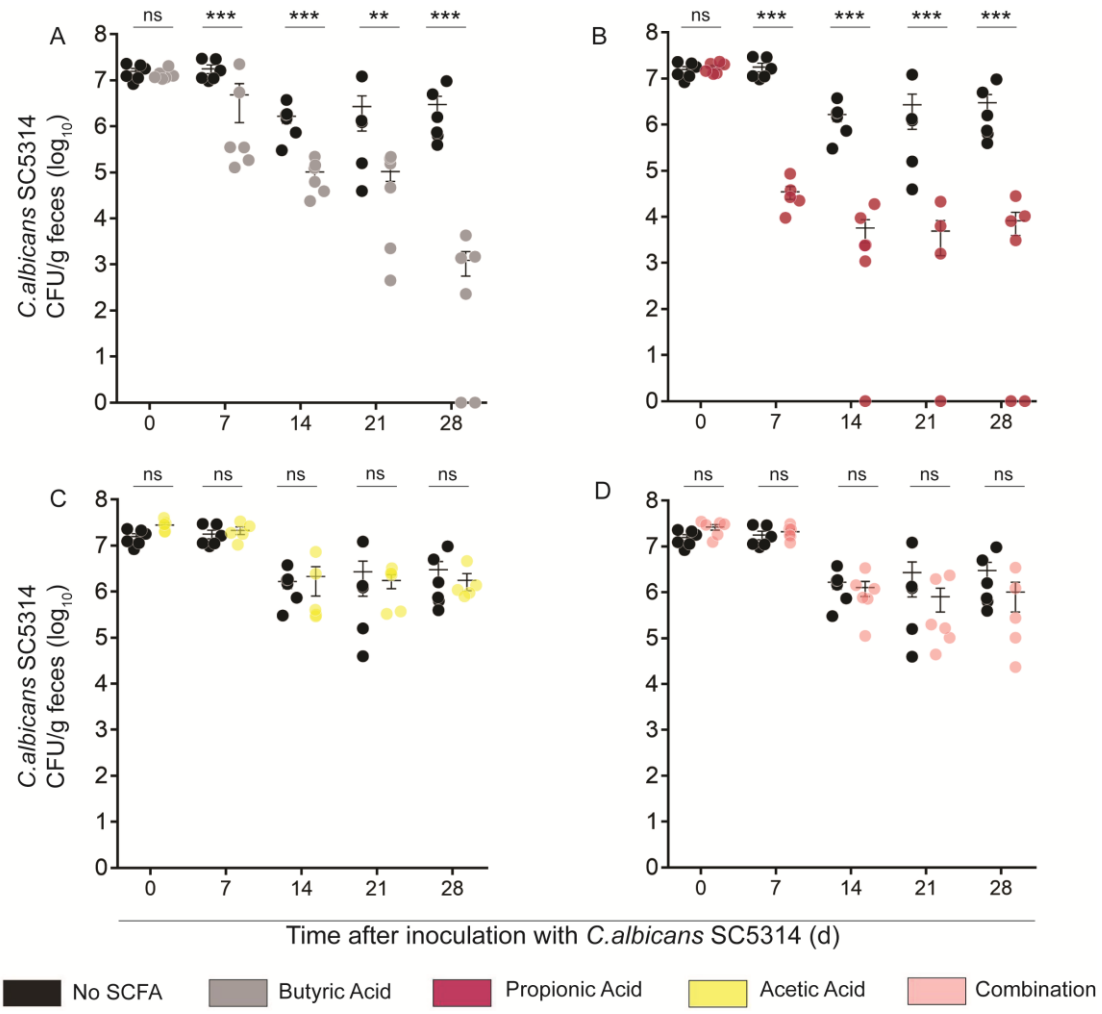

Figure 11. Effect of 150 mM SCFA in drinking water on *C. albicans* isolate SC5314 GI colonization in C3H/HeN mice after penicillin and streptomycin cessation. (A) 150 mM butyric acid at pH 5.0 (n=6) vs sterile water at pH 5.0 (n=6). (B) 150 mM propionic acid at pH 5.0 (n=6) vs sterile water at pH 5.0 (n=6). (C) 150 mM acetic acid at pH 5.0 (n=6) vs sterile water at pH 5.0 (n=6). (D) 150 mM combination (42 mM butyric acid, 24 mM propionic acid, 81 acetic acid) (n=6) at pH 5.0 vs sterile water at pH 5.0 (n=6). Graphs represent mean ± SEM fecal colonization load (CFU/g) over time. Statistical significance of comparisons is calculated using Mann-Whitney tests. ** $P < 0.1$, *** $P < 0.01$, ns, not significant.

We had previously shown that there is heterogeneity in CA fungal burden in different GI segments [19]. As such, we quantified SC5314 burden in different GI segments day 7 and day 28 by sacrificing mice and culturing intestinal luminal contents for CA. Mice treated with butyric acid or propionic acid had significantly lower CA burden in the duodenum, ileum, cecum and colon compared to mice treated with sterile water (Figure 12A-B).

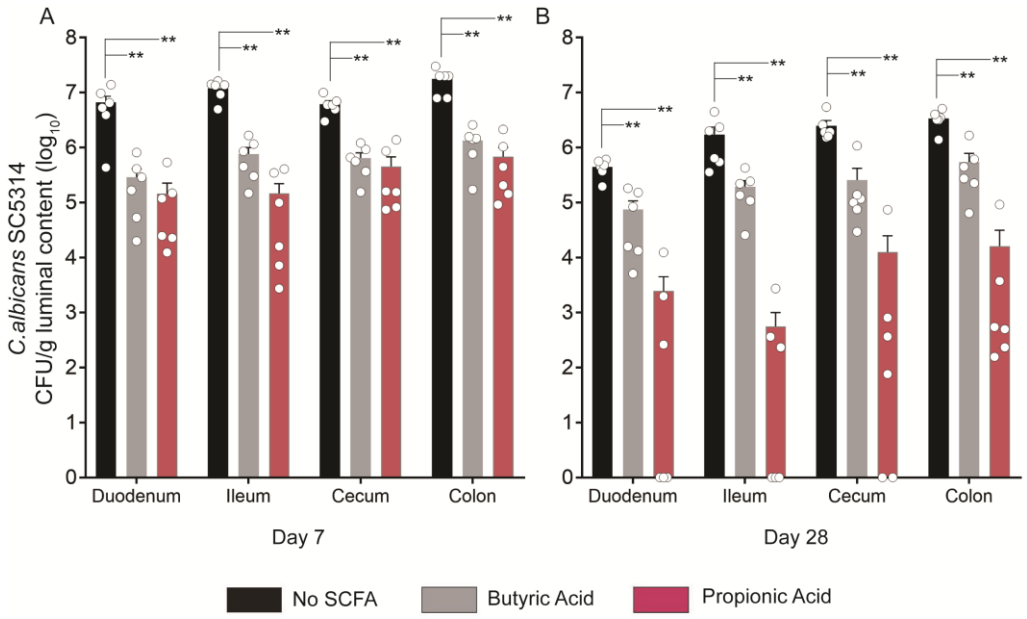

Figure 12. Effect of 150 mM SCFA in drinking water on *C. albicans* isolate SC5314 colonization in duodenum, ileum, cecum and colon of C57BL/6J mice after penicillin and streptomycin cessation on different days (A) 150 mM butyric acid at pH 5.0 (n=6) or 150 mM propionic acid at pH 5.0 (n=6) vs sterile water at pH 5.0 (n=6) on day 7 post SCFA treatment. (B) 150 mM butyric acid at pH 5.0 (n=6) or 150 mM propionic acid at pH 5.0 (n=6) vs sterile water at pH 5.0 (n=6) on day 28 post SCFA treatment. Graphs represent mean ± SEM luminal colonization load (CFU/g) in the GI segment. Statistical significance of comparisons is calculated using Mann-Whitney tests. ** $P < 0.1$.

Overall, consistent with our *in vitro* experiments, there appears to be protective effect against CA GI colonization provided by SCFA *in vivo* as well. Again, there is differential capacity of individual SCFAs to induce CA colonization reduction: propionic > butyric >>> acetic acid.

Gut microbiota is essential in mediating protective effect of SCFA

Gut microbiota is one of the critical host factors that can modulate gastrointestinal colonization of *C.albicans*. As noted before, antibiotic-depletion of bacterial gut microbiota is the most common methods for establishing stable CA colonization in the murine gut. Once antibiotics are discontinued in our preclinical model, the microbiota recovers or reconstitutes. Thus, one critical question is whether SCFAs affect either the rate or quality of gut microbiota recovery and whether these changes enhance or diminish CA colonization resistance. The rationale for this line of thought is that SCFA can also interact with bacterial species and has been shown to decrease Enterobacteriaceae colonization [95]. Thus there may be similar mechanisms relevant to CA colonization resistance.

To test whether the recovery of the microbiota is integral to SCFA-dependent inhibition of CA colonization, we inhibited the recovery of microbiota by continuing antibiotic (penicillin/streptomycin) treatment in the drinking water for all mice treated with sterile water, propionic acid, or butyric acid. Since, butyric acid and propionic acid had the most prominent protective effect in reducing SC5314 colonization, we chose to focus on these two treatment group. Surprisingly, the CA protective effect of both butyric acid and propionic acid was no longer observed: no significant difference differences in CA GI

colonization levels were observed at any time throughout the duration of the experiment (Figure 13A-B).

We also tested the effect of SCFA on CA GI colonization in germ-free mice. Indeed, there were no significant differences in SC5314 GI levels of SC5314 in germ-free mice treated with butyric acid or propionic acid when compared to counterparts receiving sterile water (Figure 14A-B).

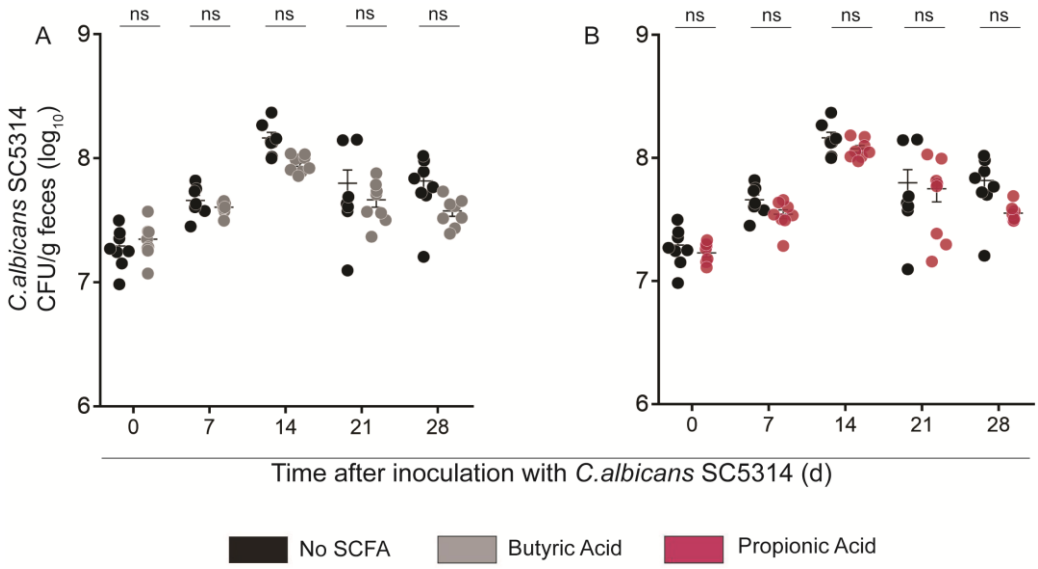

Figure 13. Effect of 150 mM SCFA in drinking water on *C. albicans* isolate SC5314 gastrointestinal colonization in C57BL/6J mice in presence of penicillin and streptomycin. (A) 150 mM butyric acid at pH 5.0 (n=8) vs sterile water at pH 5.0 (n=8). (B) 150 mM propionic acid at pH 5.0 (n=8) vs sterile water at pH 5.0 (n=8). Graphs represent mean ± SEM fecal colonization load (CFU/g) over time. Statistical significance of comparisons is calculated using Mann-Whitney tests. ns, not significant.

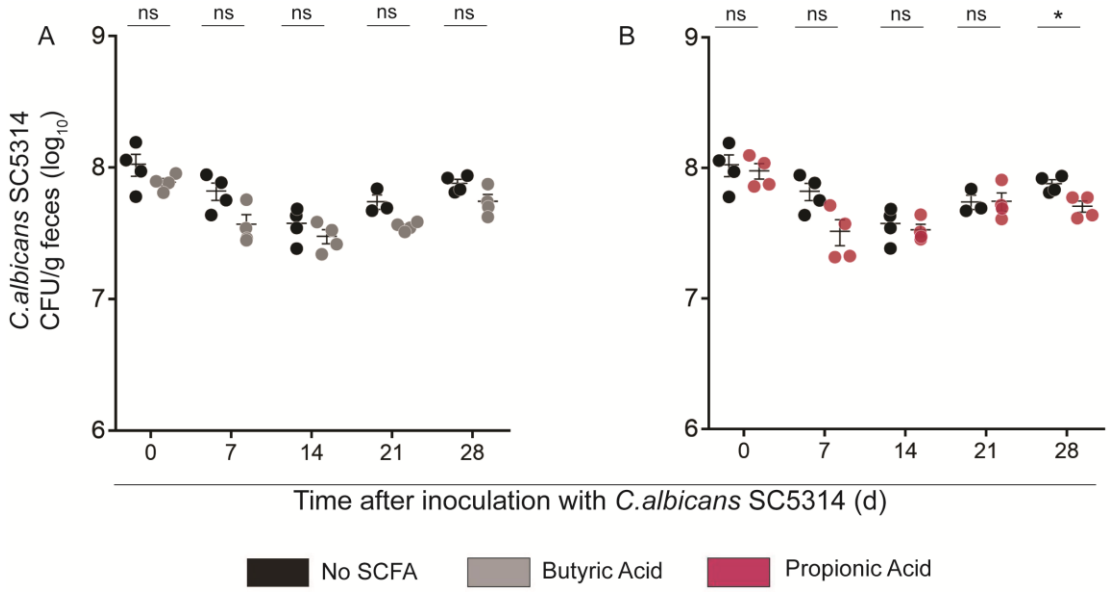

Figure 14. . Effect of 150 mM SCFA in drinking water on *C. albicans* isolate SC5314 gastrointestinal colonization in germ-free mice. A) 150 mM butyric acid at pH 5.0 (n=4) vs sterile water at pH 5.0 (n=4). (B) 150 mM propionic acid at pH 5.0 (n=4) vs sterile water at pH 5.0 (n=4). Graphs represents mean ± SEM fecal colonization load (CFU/g) over time. Statistical significance of comparisons is calculated using Mann-Whitney tests. * $P < 0.05$, ns, not significant.

Collectively, these data suggest that the presence of microbiota is essential in mediating the protective effect of butyric acid and propionic acid on CA GI colonization. In contrast to our *in vitro* experiments, SCFAs alone are not sufficient for reducing SC5314 from the GI tract. As mentioned above, SCFA can impact the reconstitution of the microbiota, which could then modulate colonization resistance against CA. Thus, during this recovery/reconstitution period, we decided to analyze the differences in gut microbiota composition between groups receiving sterile water or SCFA water. Thus, we performed gut microbiome profiling on genomic DNA extracted from fecal pellets (16S rRNA sequencing, V4 region). In order to identify statistically significant differences in gut microbiome

composition, we used a well-established statistical approach to quantitate differential taxonomic abundance (linear discriminate analysis coupled with effect size measurements, LEfSe). An enrichment in the Phylum *Bacteroidetes* (p= 0.006323 for butyric acid and p= 0.011719 for propionic acid, LEfSe, Kruskal-Wallis test) was observed groups treated with butyric acid and propionic acid compared to mice on sterile water on day 28 post SCFA treatment (Figure 15F-G). As mentioned previously, our group and others have previously shown *Bacteroidetes* be inversely correlated with *C.albicans* gastrointestinal colonization levels [18,19]. Thus, an enrichment of *Bacteroidetes* could very well be contributing to further bolstering CA colonization resistance. This *Bacteroidetes* enrichment was also observed in the group that received acetic acid or the combination of the three acids (Figure 15F-I).

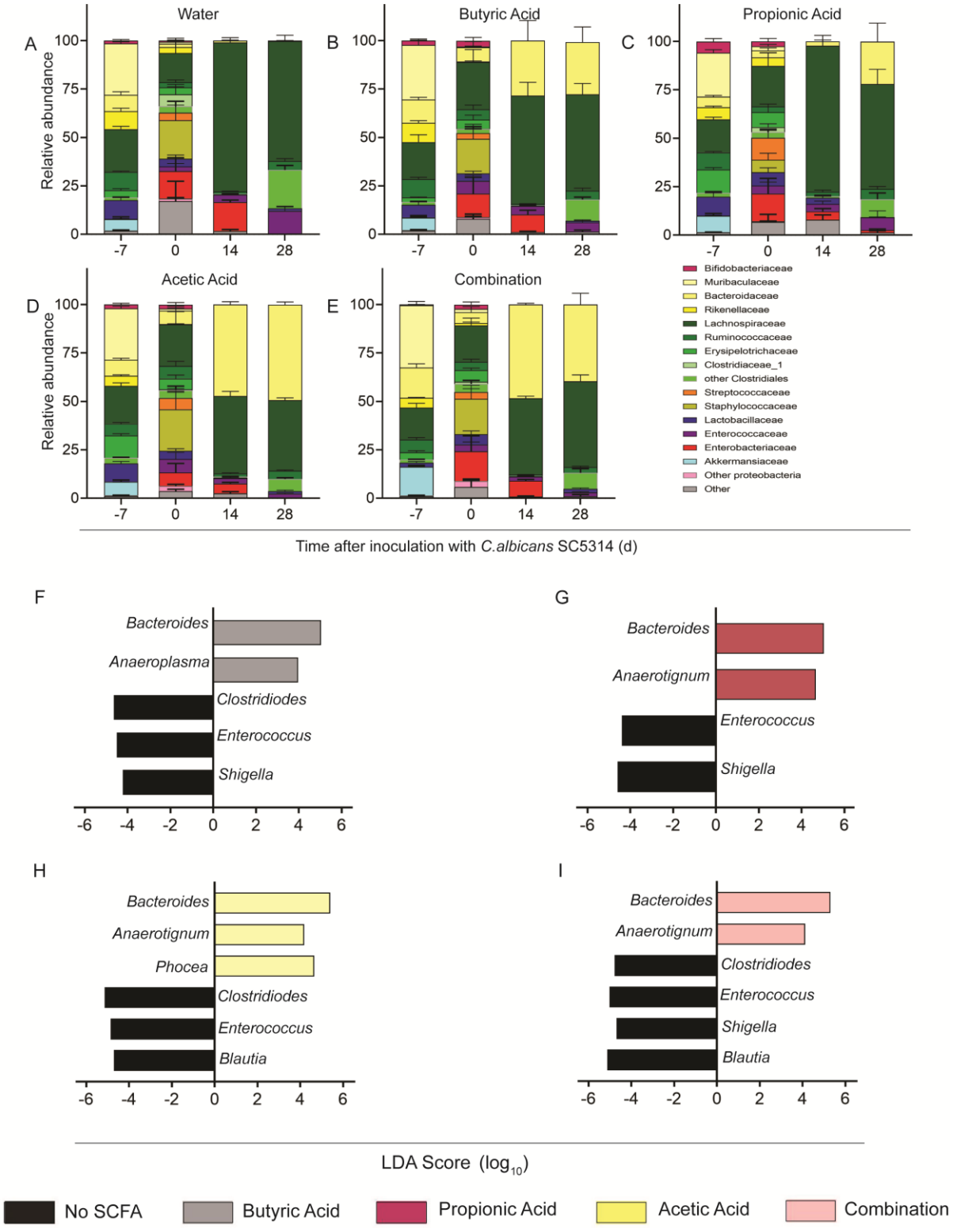
A
Water
B
Butyric Acid
C
Propionic Acid
D
Acetic Acid
E
Combination
Relative abundance
100
75
50
25
0
-7
0
14
28
Bifidobacteriaceae
Muribaculaceae
Bacteroidaceae
Rikenellaceae
Lachnospiraceae
Ruminococcaceae
Erysipelotrichaceae
Clostridiaceae_1
other Clostridiales
Streptococcaceae
Staphylococcaceae
Lactobacillaceae
Enterococcaceae
Enterobacteriaceae
Akkermansiaceae
Other proteobacteria
Other
Time after inoculation with C.albicans SC5314 (d)
F
G
H
I
Bacteroides
Anaeroplasma
Clostridiodes
Enterococcus
Shigella
Anaerotignum
Phocea
Blautia
-6
-4
-2
0
2
4
6
LDA Score (log10)
No SCFA
Butyric Acid
Propionic Acid
Acetic Acid
Combination

Figure 15. Relative abundance of bacterial families in the microbiota before and after penicillin and streptomycin in different treatment groups over days (A) Water at pH 5.0 (n=8). (B) Butyric acid at pH 5.0 (n=8). (C) Propionic acid at pH 5.0 (n=8) (D) Acetic acid at pH 5.0 (n=8) (E) Combination (42 mM butyric acid, 24 mM propionic acid, 81 acetic acid) at pH 5.0 (n=8). LefSe analysis to identify significantly different bacterial genus in water vs SCFA groups was performed. (F) 150 mM butyric acid at pH 5.0 (n=8) vs sterile water at pH 5.0 (n=8). (G) 150 mM propionic acid at pH 5.0 (n=6) vs sterile water at pH 5.0 (n=8). (H) 150 mM acetic acid at pH 5.0 (n=8) vs sterile water at pH 5.0 (n=8). (I) 150 mM combination () (n=8) at pH 5.0 vs sterile water at pH 5.0 (n=8).

SCFA retains protective effect in single SCFA receptor knockout mice

SCFA can act as an immunomodulatory molecule, as they are sensed by receptors present on epithelial cells. SCFA can induce the secretion of antimicrobial peptides, such as CRAMP that are effective in killing CA. HIF1α regulates CRAMP as well. Our group had previously shown that pharmacologic HIF1α agonist could augment CRAMP production in the gut and ultimately lead to decreased *C. albicans* GI colonization levels [19]. Thus, we first studied the effect of butyric acid or propionic acid on CA GI colonization in both *Camp* and Hif1α conditional (intestinal epithelial cell) knockout mice. In the *Camp* knockout mice, butyric and propionic acid treatment still resulted in significant reductions in CA colonization levels throughout the time course of the experiment (Figure 16A-B). A similar phenotype was also observed in *Hif1α* conditional knockout mice (Figure 17A-B). These data suggest that CRAMP or HIF1α are not essential for SCFA-dependent reduction in CA GI colonization.

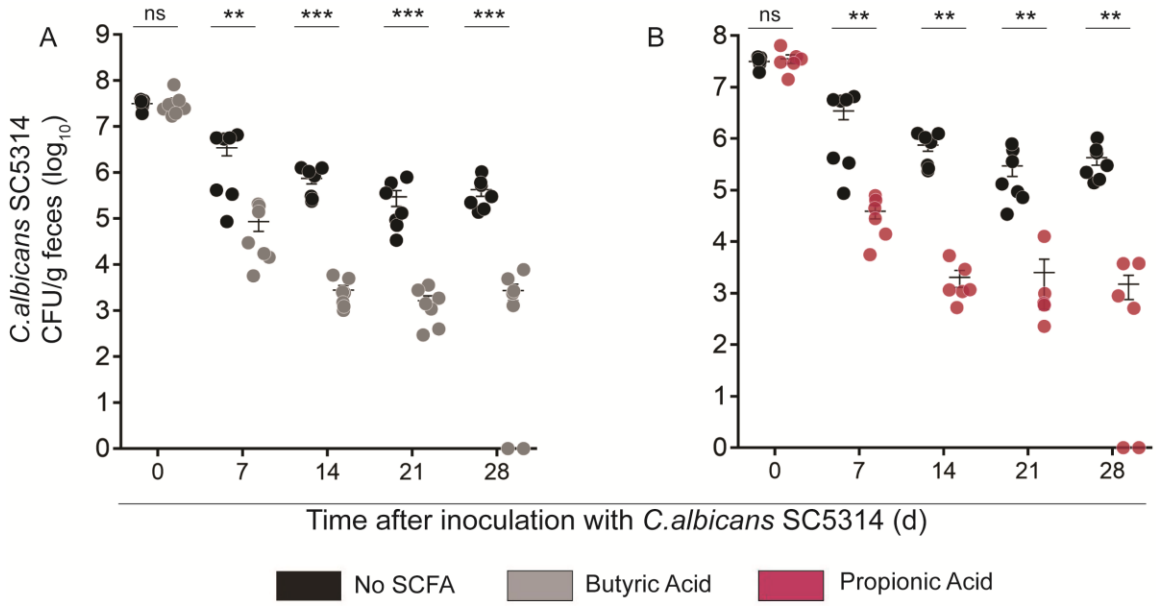

Figure 16. Effect of 150 mM SCFA in drinking water on *C. albicans* isolate SC5314 GI colonization in *Camp* KO mice after penicillin and streptomycin cessation. (A) 150 mM butyric acid at pH 5.0 (n=7) vs sterile water at pH 5.0 (n=7). (B) 150 mM propionic acid at pH 5.0 (n=6) vs sterile water at pH 5.0 (n=7). Graphs represent mean ± SEM fecal colonization load (CFU/g) over time. Statistical significance of comparisons is calculated using Mann-Whitney tests. ** $P < 0.1$, *** $P < 0.01$, ns, not significant.

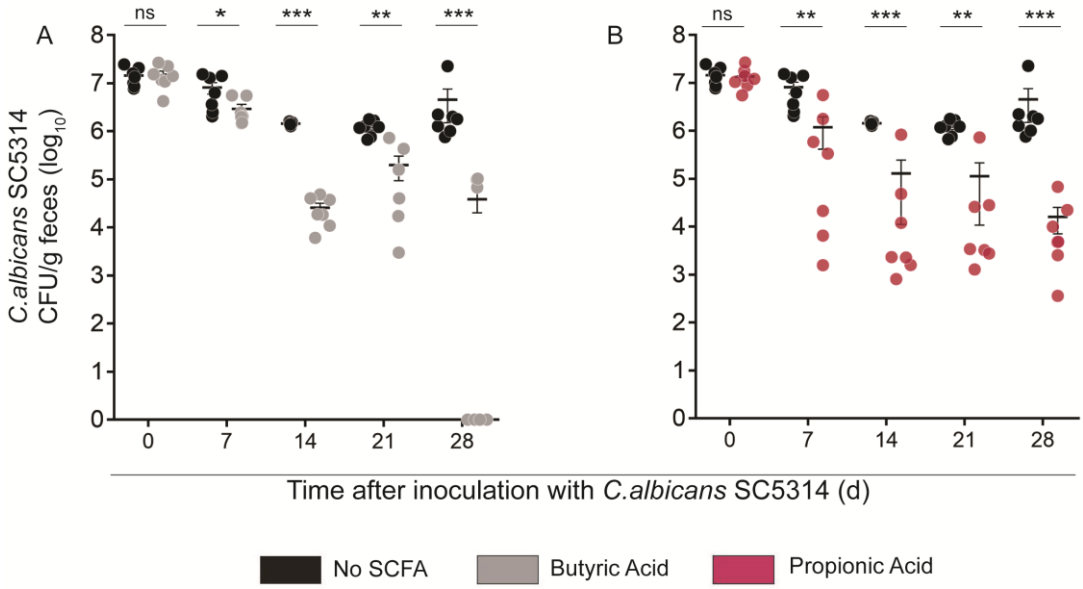

Figure 17. Effect of 150 mM SCFA in drinking water on *C. albicans* isolate SC5314 GI colonization in *Hif1a*$^{fl/fl}$ *vil-Cre*$^{+}$ mice after penicillin and streptomycin cessation. (A) 150 mM butyric acid at pH 5.0 (n=7) vs sterile water at pH 5.0 (n=7). (B) 150 mM propionic acid at pH 5.0 (n=6) vs sterile water at pH 5.0 (n=6). Graphs represent mean ± SEM fecal colonization load (CFU/g) over time. Statistical significance of comparisons is calculated using Mann-Whitney tests. ** $P < 0.1$, *** $P < 0.01$, ns, not significant.

We next sought to assess the effect of SCFA on CA GI colonization in SCFA receptor knockout mice. There are two major surface receptors for SCFA: FFAR2 and FFAR3. These receptors have differential sensitivity towards each SCFA with propionic acid being the most sensitive for both the receptors [99]. Sensing of SCFA by these receptors in immunomodulatory effecting the levels of antimicrobial peptide as well [98]. There is also an intracellular receptor for butyric acid that is PPARγ. PPARγ is known to alter epithelial cell metabolism that can also increase luminal oxygen concentration [96,97,100]. Therefore, we studied the effect of butyric acid and propionic acid in mice lacking each of these SCFA receptors individually (single knockout mice). Propionic and butyric acid still induced significant reduction in CA GI colonization in each FFAR2, FFAR3, and PPARγ conditional KO mice (intestinal epithelial cell) (Figure 18A-B, 19A-B, 20A-B). As there is likely functional redundancy in SCFA detection pathways, it is possible that the loss of a single SCFA receptor would not be sufficient to result in a significant phenotype. We did not perform experiments in double or triple SCFA receptor knockout mice, in which a stronger phenotype may be observed.

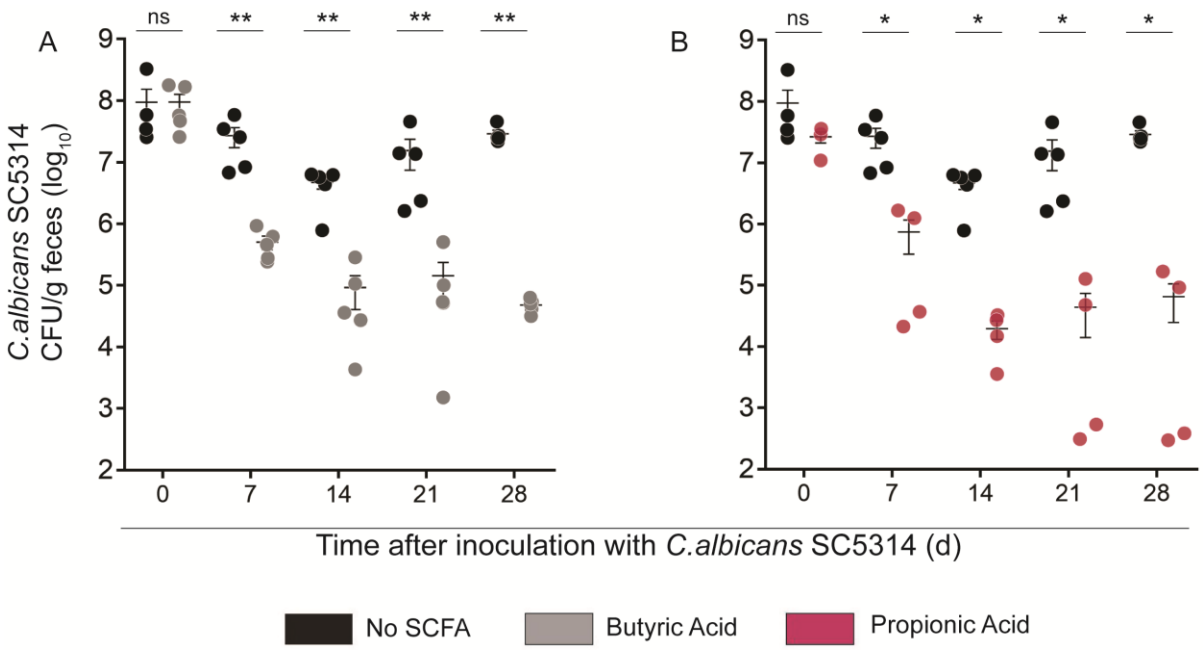

Figure 18. Effect of 150 mM SCFA in drinking water on *C. albicans* isolate SC5314 GI colonization in *Ffar2* KO mice after penicillin and streptomycin cessation. (A) 150 mM butyric acid at pH 5.0 (n=5) vs sterile water at pH 5.0 (n=5). (B) 150 mM propionic acid at pH 5.0 (n=4) vs sterile water at pH 5.0 (n=5). Graphs represent mean ± SEM fecal colonization load (CFU/g) over time. Statistical significance of comparisons is calculated using Mann-Whitney tests. * $P < 0.05$, ** $P < 0.01$, ns, not significant.

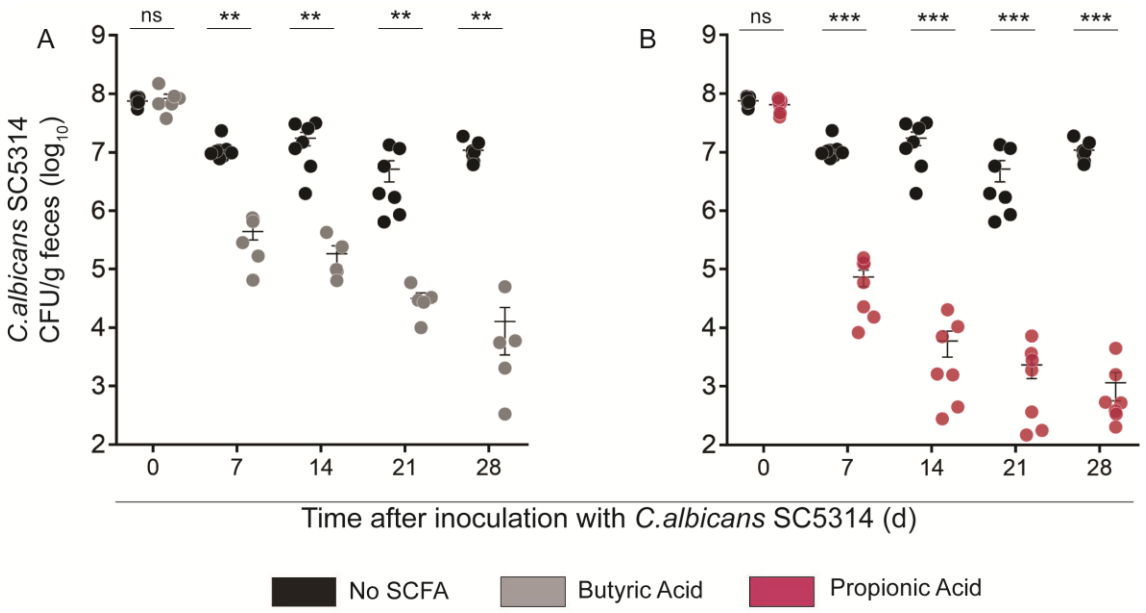

Figure 19. Effect of 150 mM SCFA in drinking water on *C. albicans* isolate SC5314 GI colonization in *Ffar3* KO mice after penicillin and streptomycin cessation. (A) 150 mM butyric acid at pH 5.0 (n=5) vs sterile water at pH 5.0 (n=7). (B) 150 mM propionic acid at pH 5.0 (n=7) vs sterile water at pH 5.0 (n=7). Graphs represent mean ± SEM fecal colonization load (CFU/g) over time. Statistical significance of comparisons is calculated using Mann-Whitney tests. ** $P < 0.1$, *** $P < 0.01$, ns, not significant.

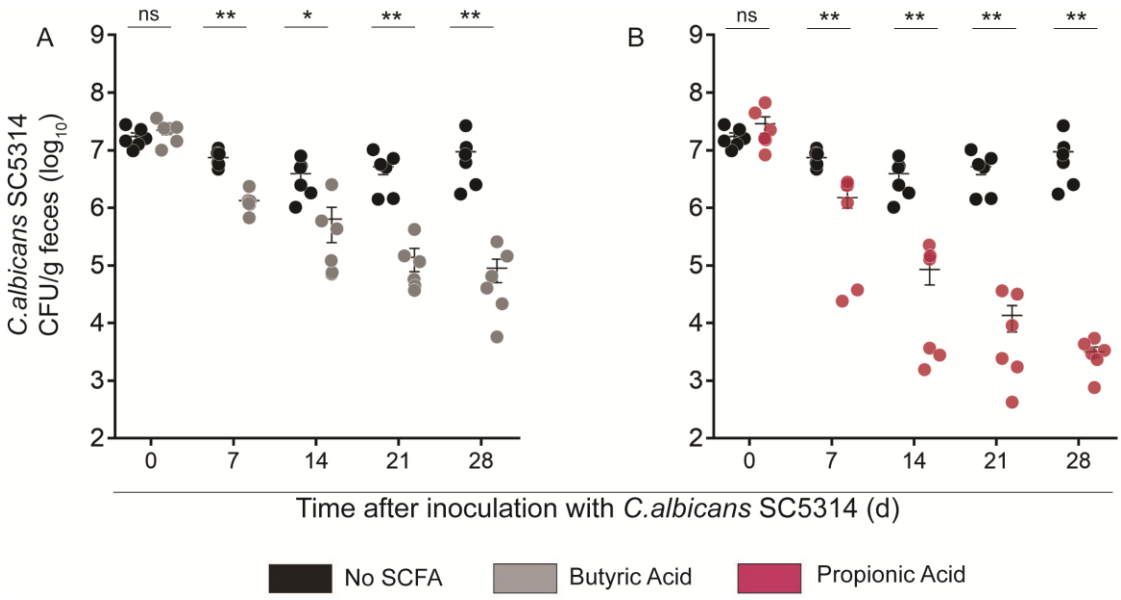

Figure 20. Effect of 150 mM SCFA in drinking water on *C. albicans* isolate SC5314 GI colonization in *Pparγ*$^{fl/fl}$ *vil-Cre*$^{+}$ mice after penicillin and streptomycin cessation. (A) 150 mM butyric acid at pH 5.0 (n=5) vs sterile water at pH 5.0 (n=7). (B) 150 mM propionic acid at pH 5.0 (n=7) vs sterile water at pH 5.0 (n=7). Graphs represent mean ± SEM fecal colonization load (CFU/g) over time. Statistical significance of comparisons is calculated using Mann-Whitney tests. * $P < 0.05$, ** $P < 0.01$, ns, not significant.

Endogenous modulation of propionic acid levels in the gut promotes colonization resistance against *C.albicans*

SCFA is a microbial-derived metabolite produced by specific gut microbiota, including *Bacteroides thetaiotamicron* (*B.theta)*. As *B. theta* genetics are well-understood and tools for engineering genetic mutants are validated, we sought to create a *B.thetaiotamicron* mutant that lacks the capacity to synthesize SCFA and then assess the B. theta SCFA-deficient mutant's ability to promote CA GI colonization resistance [88]. Towards this effort, we utilized the *B.thetaiotamicron* mutant *Δprop*, which lacks the ability to synthesize propionic acid. Germ-free mice were first colonized with CA strain SC5314. Mice were then colonized with either wildtype *B.theta* (*Δtdk* strain) or the *B. theta Δprop* mutant. Indeed, mice co-colonized with CA and the wildtype *B. theta* had significantly lower CA levels than those mice co-colonized with CA and the B. theta *Δprop* mutant (Figure 21A). Propionic acid levels in the gut (as measured in the cecal contents) were significantly lower in mice co-colonized with the mutant strain compared to the wildtype B. theta group (Figure 21B).

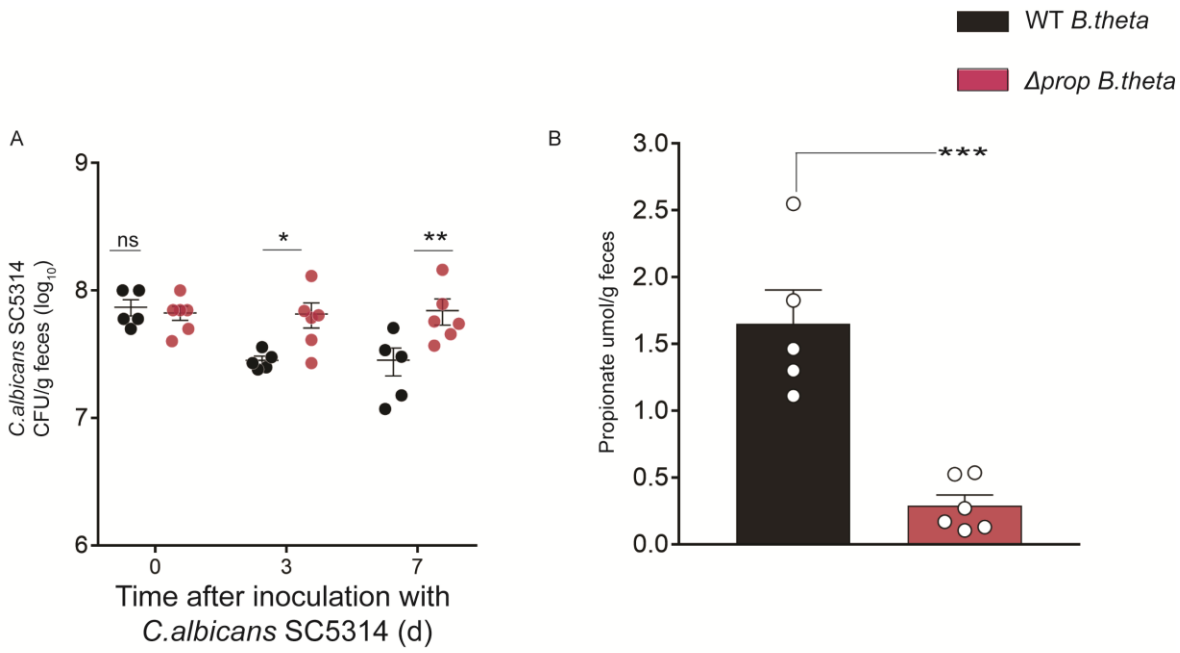

Figure 21. Effect of propionate deficient B.theta mutant *Δprop* on *C. albicans* isolate SC5314 GI colonization in germ-free mice. (A) Colonization level of *C. albicans* isolate SC5314 in germfree mice after colonization with WT *B. theta (Δtdk)* (n=5) or *B.theta* mutant *Δprop* (n=6). Graphs represent mean ± SEM fecal colonization load (CFU/g) over time. Statistical significance of comparisons is calculated using Mann-Whitney tests. * $P < 0.05$, ** $P < 0.01$. (B) Propionate levels in germ free mice colonized with WT *B. theta (Δtdk)* or *B.theta* mutant *Δprop*. Graph shows means + SEM value of propionate measured from the cecal contents of the mice using GC-MS. Statistical significance of comparisons is calculated using unpaired parametric t-tests. *** $P < 0.01$.

The levels of endogenously produced SCFA can also be augmented by providing a prebiotic that can be utilized by the resident microbiota as a substrate to produce SCFA. For example, indigestible starches, such as inulin or fructo-oligosaccharides, have been used to increase endogenous gut SCFA production [104]. Thus, we hypothesized that a prebiotic inulin propionate ester could be utilized for these efforts [88]. The inulin propionate ester is

synthesized by attaching propionate moiety on the complex carbohydrate inulin through an esterification reaction between inulin and propionic anhydride. Both inulin and inulin propionate ester can be digested by the anaerobic members of the microbiota including *B.theta*. When inulin propionate ester is digested, the attached propionate moieties are released, directly increasing the levels of propionic acid in the distal GI tract. Further, inulin can then be utilized by SCFA-producing gut microbiota to produce additional propionate as well. Thus, we pre-treated C57BL/6J mice with antibiotics and subsequently established CA colonization, as described above. Wildtype *B.theta* (strain VPI-5482) was then administered orally, and mice were then treated with either 1% inulin or inulin propionate ester every day (via oral gavage) for 14 days. Mice receiving inulin propionate ester displayed lower fungal loads, (Figure 22A) and exhibited higher propionic acid levels (in cecal contents) in the gut (Figure 22B), as compared to inulin only treated groups.

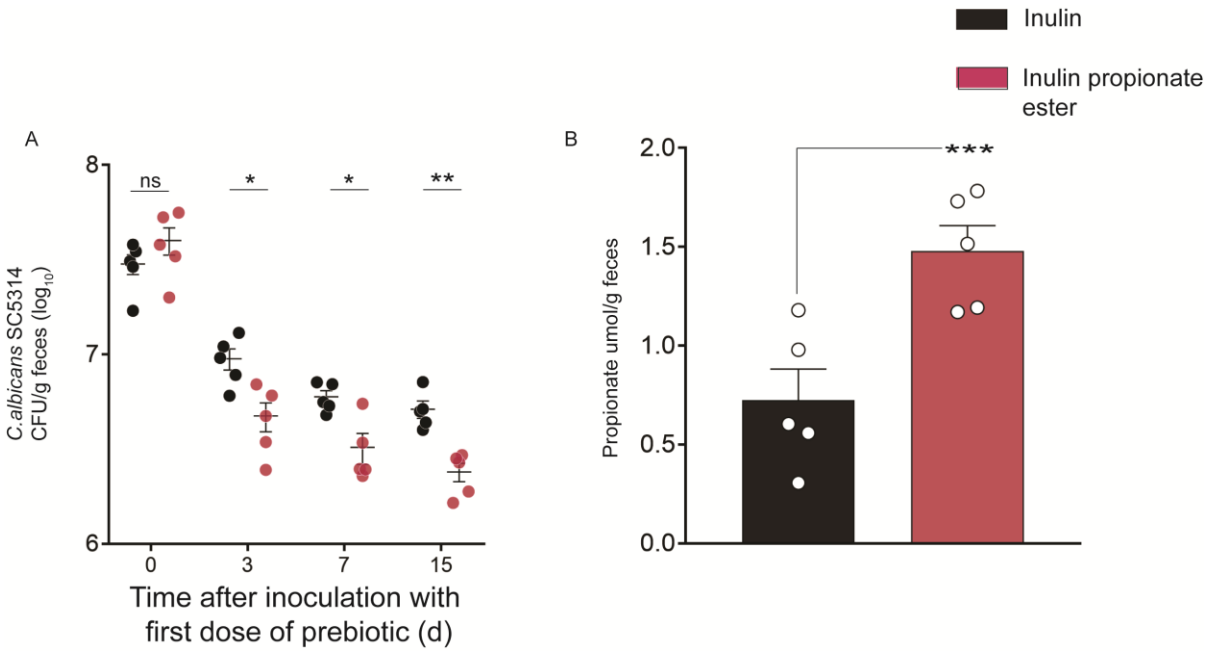

Figure 22. Effect of prebiotic inulin propionate ester on *C. albicans* isolate SC5314 GI colonization in C57BL/6J mice. A) Colonization level of *C. albicans* isolate SC5314 in C57BL/6J mice receiving inulin or inulin propionate ester. Graphs represent mean ± SEM fecal colonization load (CFU/g) over time. Statistical significance of comparisons is calculated using Mann-Whitney tests. * $P < 0.05$, ** $P < 0.01$. (B) Propionate levels in C57BL/6J mice receiving inulin or inulin propionate ester. Graph shows mean + SEM value of propionate measured from the cecal contents of the mice using GC-MS. Statistical significance of comparisons is calculated using unpaired parametric tests. *** $P < 0.01$.

Overall, these experiments confirmed that modulating the capacity of gut microbiota to produce propionic acid is a viable strategy for reducing fungal burden in the murine GI tract. These observations lead to the enticing prospect of manipulating the microbiome with probiotics and/or prebiotics to increase SCFA levels in human patients to reduce the GI carriage of *C.albicans* and thus reducing the likelihood of disseminated infection.

Overall, our study validates the inhibitory effect of SCFA on CA and delineates specific mechanisms that mediate this effect. Both *in vitro* and *in vivo* approaches highlight unique and complimentary observations that aim to provide insight into the very complex interactions and impact that SCFAs have on the host and/or CA. Ultimately, the conclusions derived from this project may lay the foundation for manipulating the ability of endogenous gut microbiota by prebiotic or probiotic to modulate both SCFA and gut microbiota taxa levels with the final goal of promoting CA GI colonization resistance.

Discussion

C. albicans (CA) is the third leading cause of nosocomial infections in the United States. *C. albicans* colonization levels in the GI tract dictate risk of developing CA bloodstream infections in immunocompromised hosts [18,19]. Previous studies, including those from our laboratory, have shown the protective effect of specific members of the gut microbiota (e.g. *Bacteroidetes* and *Firmicutes* phylum) against CA GI colonization [19]. Here, we show that the microbiota-derived metabolites short chain fatty acids, which can be produced by *Bacteroidetes* member *Bacteroides thetaiotaomicron* and *Firmicutes Blautia producta,* are protective against CA GI colonization. Both these species have previously been shown by our lab to promote colonization resistance against CA in mice. Previously, there have been few studies that documented the effect of SCFA on CA physiology and growth *in vitro* [111,112]. None of these studies identified any specific molecular mechanisms underlying these observed phenotypes or extensively studied the role of SCFA *in vivo*. Recently, other studies have shown a direct inhibition of bacterial growth in the presence SCFAs. For example, SCFA inhibited growth of clinical isolates of *Klebsiella pneumonia, Escherichia coli* and *Proteus mirabilis* [95]. In another study, propionate (but not the other two SCFA) inhibited the growth of *Salmonella typhimurium* [88]. In this current study, we also noted a differential effect of specific SCFAs on CA growth inhibition and GI colonization. In all the *in vitro* experiments, acetate was found to be least effective in inhibiting CA growth. Commensurate with the *in vitro* phenotype, acetate supplementation did not induce CA GI colonization reduction, whereas propionate and butyrate treatment significantly reduce CA gut burden.

Nutrient uptake and utilization is essential for the growth in the GI tract. CA can uptake and utilize different carbon sources at the same time by use of hexose transporters. In contrast, *S. cerevisiae*, utilizes glucose as a preferred carbon source [113]. Here, we showed that the presence of SCFA represses genes involved in hexose uptake and carbohydrate metabolism. One of the repressed genes, Tye7, has been shown to be essential for CA growth *in vitro* under oxygen limiting condition as it regulates the glycolytic pathway [114]. Absence of Tye7 in CA results in decreased intestinal colonization in a mouse model [115]. Since the GI tract ranges from microaerophilic to completely anaerobic, downregulation of Tye7 and the subsequent glycolytic pathway could contribute to GI colonization defect as well. Mechanisms for sensing glucose availability and regulation of intracellular pH are closely linked in both *C. albicans* and *S. cerevisiae* [108,109]. The presence of glucose increases the level of cytosolic pH in both species. In *S. cerevisiae,* cytosolic pH is thought to act as a second messenger of glucose availability. SCFA mediated growth inhibition in bacterial species has been shown to depend on increased intracellular acidification [88,95]. Our study also identified that SCFA decrease the cytosolic pH of *C. albicans*. Interestingly, the SCFA-induced changes in pH (~ 1-1.5 pH units) is similar to that observed in glucose absent conditions [108,109]. Thus, it is possible that SCFA may be inducing an aberrant signal by decreasing the intracellular pH thereby indicating unavailability of glucose and thus pushing CA into starvation mode.

SCFA can be sensed by the host and results in physiologic changes. One such example, is butyrate as a histone-deacetylase inhibitor which can selectively alter gene transcription [116]. SCFA has also been implicated in modulating numerous host immune

effectors, including intestinal derived antimicrobial peptide such CRAMP and β-defensins [98]. These antimicrobial peptides have been shown to exhibit fungicidal effects on CA and thus modulate CA GI colonization level [19]. Therefore, we tested whether SCFA could still promote CA GI colonization resistance in the absence of intestinal epithelial CRAMP and found the elimination of CRAMP was not sufficient to abrogate the protective effects of SCFA. Further, we found that SCFA were still to induce CA colonization reduction in mice lacking a single SCFA receptor (FFAR2, FFAR3, or PPARγ). These data suggest that loss of a single immune effector or pathway does not appear to be sufficient to mitigate the protective effect of SCFA on CA GI colonization. This is likely due to the functional redundancy of host immune effectors (e.g. multiple antimicrobial peptides effective in killing CA) and pathways (e.g. multiple SCFA signaling pathways) that are critical for maintaining CA colonization resistance. Therefore, the role of the host factors requires further interrogation in a system where multiple host factors can be removed to study their contribution more definitively.

SCFA are produced by the members of the gut microbiota, including *Bacteroides thetaiotaomicron* [83]. We were able to modulate the propionate producing capacity of the gut microbiome (by engineering gut microbiota unable to produce SCFA or by using dietary supplementation to augment overall SCFA production) to study its impact on CA GI colonization. Indeed, increasing levels of propionate in the GI tract is protective against *C. albicans* GI colonization. In our study, we only modulated the microbiota capacity to produce propionate, but an even more effective approach may be to augment levels of multiple SCFA in the gut (e.g. administer inulin butyrate ester with inulin propionate ester).

Cross-feeding, where one gut microbiota taxa supplies a nutrient to another taxa, is well established in gut microbiota communities [117,118]. Therefore, leveraging this knowledge and implementing combinatorial dietary treatment approach might be synergistic and allow for greater effect in promoting pathogen colonization resistance

CHAPTER FIVE
Strain specific differences in *C. albicans* ability to colonize murine gastrointestinal tract

Introduction

C. albicans (CA) is a prominent member of the human gastrointestinal mycobiota colonizing approximately 70% of the population [10]. CA colonization in normal healthy hosts is generally harmless and rarely causes disease. But in immunocompromised hosts, CA colonization can results in serious infections [18]. *C. albicans* carriage has been shown to have immunomodulatory effects as well. *C.albicans* in GI tract of mice and humans can drive systemic Th17 responses, which can provide protection against systemic fungal and microbial infections but when overexuberant can lead to autoimmune phenomena, including airway inflammation [3,6].

Given the importance in driving/activating host immune responses and the potential to cause infections, it is critical to gain insight regarding the mechanisms by which CA achieves and maintains GI colonization. Interestingly, the inbred mouse strains are refractory to CA GI colonization: by the most commonly studied laboratory strain SC5314, but also by WO-1, Can098, 3153A, ATCC 18804, and OH1 isolates [17,19,63,64]. These CA strains cannot colonize the murine GI tract with intact microbiota, and thus antibiotic pretreatment is required to establish sustained CA GI colonization [24]. Other *Candida* species such as *Candida glabrata* ATCC15126, a *Candida parapsilosis* clinical isolate and *Candida tropicalis* ATCC 66029 also cannot colonize murine GI tract without the use of antibiotics.

Very little is known about the mechanisms that regulate GI colonization of CA. Most models rely on antibiotic depletion of the microbiota or germ-free mice that allow for CA colonization of the murine GI tract. These models have shown the importance of commensal bacterial microbiota species in modulating CA colonization resistance in the gut. Our group has identified specific host factors (HIF1α and antimicrobial peptide CRAMP) that work in concert with bacterial gut microbiota in regulating fungal burden in GI tract [19].

As mentioned above, CA strain SC5314 is the most commonly studied isolate used research studies. Yet, microbial strain-specific differences have been identified which influence microbial virulence and/or the capacity to induce host immune responses [119-122]. Therefore, in this study, we identified two *C.albicans* clinical isolates CHN1 and 529L that were able to colonize the murine GI tract without any antibiotic treatment. We worked with two other research groups (The Bennet Lab, Brown University, and Hohl Lab, Memorial Sloan Kettering Cancer Center) who also arrived at these results independently. We confirmed that CHN1 and 529L were able to establish murine GI colonization in three distinct inbred mouse strains: C57BL/6 (Jackson), BALB/c (Charles River), and C3H/HeN (Envigo).

All three groups worked to identify potential mechanisms by which CHN1 and 529L exhibited such a distinct GI colonization phenotype compared to SC5314. Our group focused on the potential differential sensitivity of these strains to host immune effectors, specifically antimicrobial peptides. Thus, we evaluated the effect of antimicrobial peptide CRAMP, which our group and others have shown to have potent anti-Candida activity, on these two isolates as compared to SC5314 *in vitro*. Growth (in media with varying concentrations of

CRAMP) was analyzed for all three isolates under aerobic and anaerobic conditions. CHN1 and 529L exhibited significantly greater growth compared to SC5314 in the presence of CRAMP

To determine whether increased resistance to CRAMP was a feasible mechanism in vitro, we utilized genetically engineered mice lacking CRAMP. The rationale was that in the absence of CRAMP, the differences in GI colonization phenotype between CHN1 and 529L versus SC5314 would be attenuated. Therefore, competition assays using CHN1 or 529L versus SC5314 were conducted in wildtype C57BL/6J mice and CRAMP knockout mice. While SC5314 exhibited inferior GI colonization fitness when compared to CHN1 or 529L in wildtype mice, SC5314 displayed improved GI colonization fitness in CRAMP KO mice. Overall, this study highlights that different *C.albicans* strains can exhibit differential colonization potential in the murine GI tract which may depend, in part, on their strain-specific sensitivity to specific host immune factors such as CRAMP. This study also provides an additional tool to study host-fungal interactions in a commensal niche without having to perturb the microbiota which has greater physiological relevance to the human condition.

Results

Numerous *C.albicans* strains, including the widely used laboratory isolate SC5314 which was originally isolated from a human bloodstream infection, cannot colonize the murine GI tract without antibiotic treatment. Here, SC5314 and two other CA clinical isolates -- 529L, isolated from the human oral cavity [123], and CHN1, isolated from a human lung [68]-- were tested for their ability to colonize the murine GI tract without any antibiotic pre-treatment. We utilized C3H/HeN mice (female, 6-8 weeks, Envigo) for these experiments. As we have previously shown. SC5314 was not able to stably colonize the GI tract of C3H/HeN and was rapidly cleared (1-2 log-fold drop in the first 7-14 days and falling below the limit of detection after 14 days). In contrast, both CHN1 and 529L had 1-2 log-fold higher fungal loads than SC5314 (as determined by culturing fecal homogenates and determining CA CFU/g feces). Both CHN1 and 529L persisted in the GI tract (~ 10^3 CFU/g through day 35 when experiment was stopped) (Figure 23A).

As microbial GI colonization phenotypes can vary widely based on mouse strain choice and breeding/experimental facility, CHN1 and 529L isolates were also tested for their GI colonization phenotype by the laboratories of Tobias Hohl, MD, PhD, at Memorial Sloan Kettering Medical Center, New York, and lab of Richard Bennett, PhD, at Brown University, Rhodes Island. CHN1 and 529L strains were tested in C57BL/6J (New York and Rhode Island) and BALB/c mice (Rhode Island) at these facilities. Both CHN1 and 529L mice had a superior GI colonizing ability compared to SC5314 in all mice tested. C57BL/6J mice exhibited a similar GI colonization phenotype to C3H/HeN mice and exhibited stable GI

colonization by CHN1 and 529L until the end of experiments (Figure 23B-C), whereas BALB/c mice eventually cleared the two strains by day 28 (Figure 23D). Overall, these experiments showed that CHN1 and 529L are superior colonizers of murine GI tract and can stably colonize the murine GI tract for extended period of time without the use of any antibiotics.

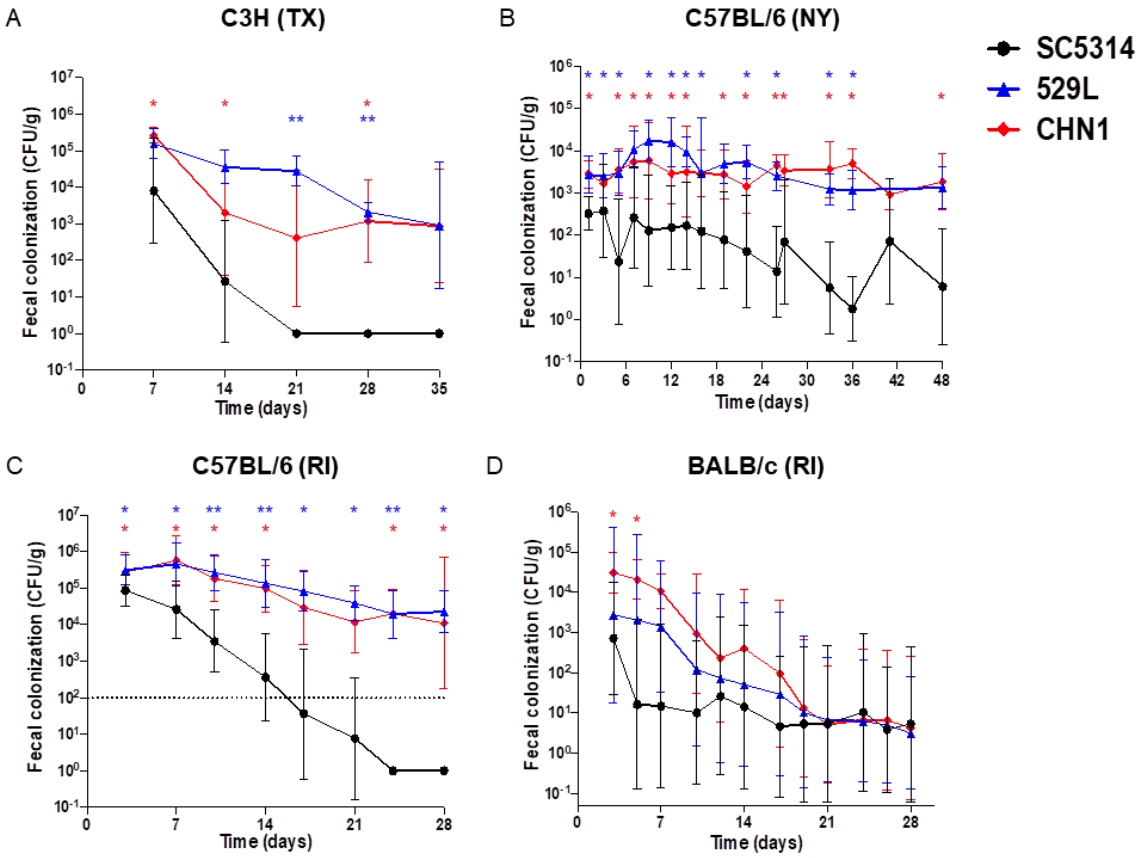

Figure 23. *C. albicans* isolates 529L and CHN1 can stably colonize the gastrointestinal tract of mice without antibiotic treatment. (A) C3H/HeN, Koh lab, TX, (n = 8). (B) C57BL/6J, Hohl lab NY, (n = 10-18 mice). (C) C57BL/6J, Bennett lab,RI, (n = 8 mice). (D) Bennett lab,RI, (n = 8 mice). Panels show fecal colonization levels (CFUs/g) over time. *, $P < 0.05$, **, $P < 0.01$ by *t* test.

CA GI colonization ability has been previously shown to be inhibited by intestinal epithelial-derived antimicrobial peptide CRAMP [19]. Therefore, we hypothesized that one potential mechanism which might explain the ability of CHN1 and 529L to exhibit improved GI colonization ability is these strains are more resistant to CRAMP compared to strain SC5314. To test this, SC5314, 529L and CHN1 were grown both aerobically and anaerobically at 37^{o}C with various concentrations of CRAMP. Under aerobic conditions, the growth was monitored continuously. SC5314 displayed higher sensitivity and was inhibited by CRAMP at 5 µM concentration and showed no growth at 10 µM (Figure 24A). In contrast, CHN1 and 529L were able to grow in the presence of 5 µM CRAMP, although the kinetics of growth were slower in presence of 5 µM CRAMP compared to without CRAMP (Figure 24 B-C). A similar phenotypic difference was observed between the isolates when experiments were repeated under anaerobic conditions. SC5314 growth was reduced by ~70% at 10uM CRAMP where CHN1 and 529L showed ~42% and 25% growth inhibition, respectively at the same concentration of CRAMP (Figure 25). Differential sensitivity between the isolates was also observed at higher concentrations - with 529L being the most resistant to CRAMP (Figure 25).

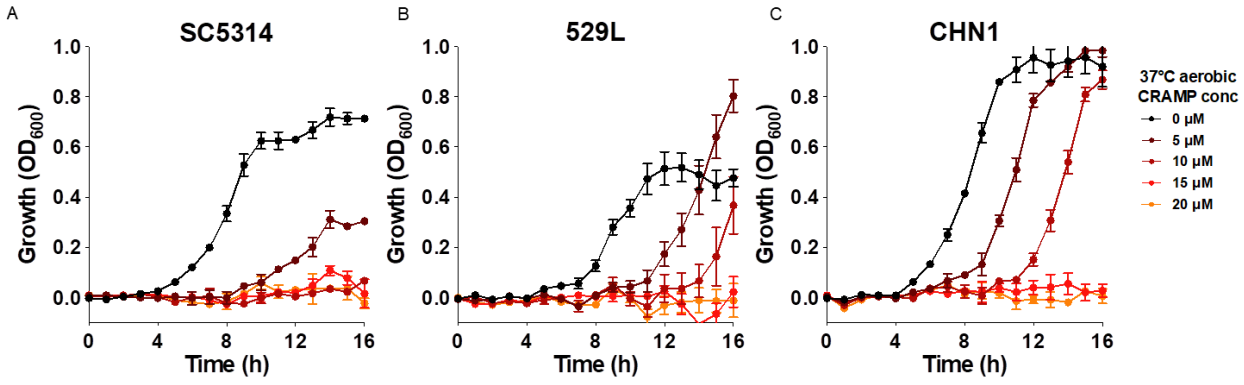

Figure 24. Effect of different concentrations of CRAMP on *C. albicans* growth under aerobic conditions at 37°C. (A) SC5314. (B) CHN1. (C) 529L. to different CRAMP concentrations under aerobic growth at 37°C. Plots show mean ± SEM growth levels over 16 h from three independent experiments.

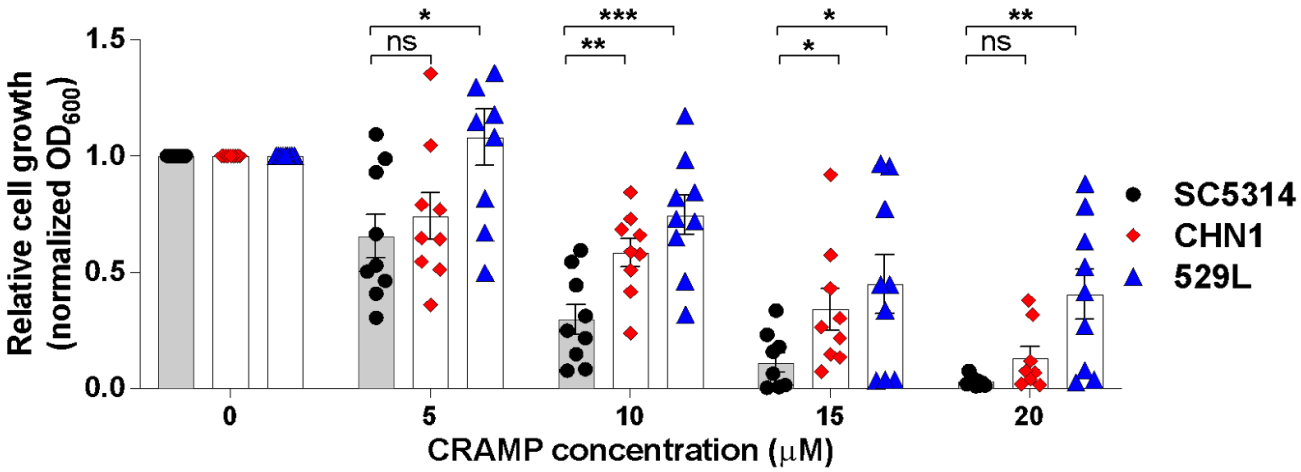

Figure 25. *in vitro* susceptibility of *C. albicans* isolates to different concentrations CRAMP at 37°C under anaerobic conditions. Histograms show mean relative fungal growth ± SEM values from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** P<0.001 based on comparison between SC5314 and CHN1 or 529L using unpaired parametric t-tests.

To test whether these *in vitro* findings held true *in vivo*, we performed competition experiment between SC5314 and CHN1 or 529L, in wildtype C57BL/6J mice and *Camp* knockout mice. SC5314 showed a GI colonization defect in both the wildtype and *Camp* knockout mice compared to CHN1 or 529L, although the proportion of SC5314 in *Camp* knockout mice was higher at specific time points early on in the experiment (Figure 26 A-B). The phenotypic difference was modest, which is not surprising as there are other immune effectors (e.g. antimicrobial peptides, such as β-defensins, and humoral factors IgA) which

likely play a critical role in maintaining CA colonization resistance in the gut. As such, differential susceptibility to CRAMP alone would not be expected to be sufficient to completely explain the observed phenotype.

The study highlights CA strain-specific differences in their ability to colonize murine GI tract. These findings also provide a valuable tool to further study the mechanisms of GI colonization by CA as these isolates can stably colonize murine GI tract without the use of antibiotics.

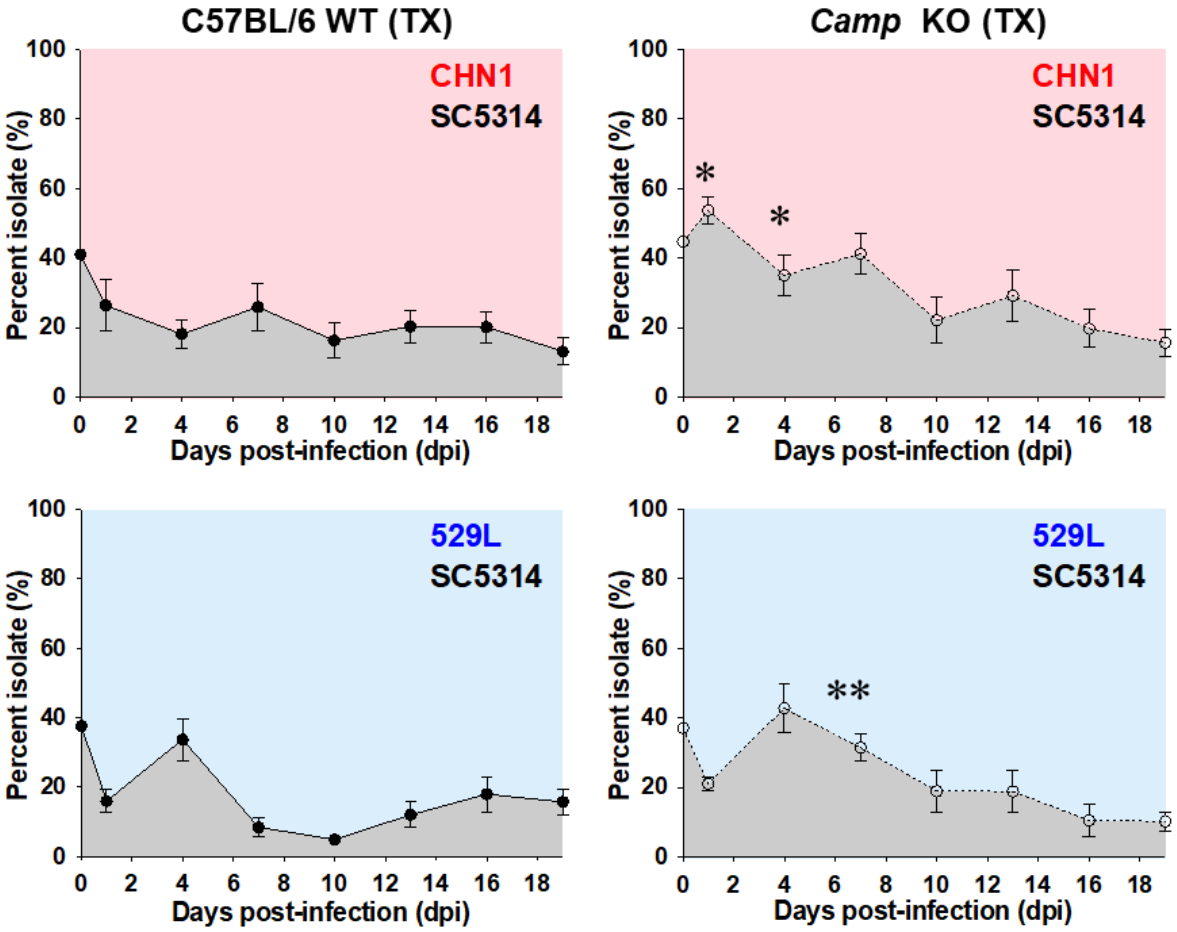

Figure 26. Direct competitions between SC5314 and 529L or CHN1 in the GI of C57BL/6J wild type (WT) and *Camp* KO mice (TX). Isolates were co-inoculated 50:50 and analyzed using nourseothricin selection upon

recovery from fecal pellets. Plots show mean values ± SEM from 8 mice per group, * $P < 0.05$, ** $P < 0.01$ based on comparisons of individual time points between WT and *Cramp* KO mice using Mann-Whitney tests.

Discussion

C. albicans is one of the most prominent members of the human mycobiota. Yet current dogma suggests that CA cannot stably colonize the murine GI tract [17,24]. Here, we identified two clinical CA isolates CHN1 and 529L that can stably colonize the murine GI tract without the use of antibiotic pre-treatment. This CA GI colonization phenotype was confirmed in different mice strains and in different facilities.

These results highlight the importance of intra-strain differences that might affect the natural history and/or disease causing potential of these pathobionts. Strain-specific differences have previously been implicated as determining changes in CA virulence. For example, the most well-studied, most commonly used lab isolate SC5314 has been shown to be more invasive than other isolates in oropharyngeal model of candidiasis as well has higher propensity to filament [122,124]. Filamentation is often associated with reduced commensalism and higher invasiveness [17,21]. Colonization differences between SC5314 and 529L had been reported previously in murine oral candidiasis models. While 529L can stably colonize the murine oral cavity, SC5314 is eliminated for the mouse oral cavity[123]. This difference was attributed to 529L inducing a weaker host inflammatory response [119], which has been shown to be an important factor governing GI colonization as well. In another study, CHN1 and SC5314 were shown to have similar impact on the reconstitution of the microbiota after cessation of the antibiotic Cefoperazone [68].

Relevant to this study is the fact that neither CHN1 nor 529L is considered a GI isolate. 529L was originally isolated from the human oral cavity, whereas CHN1 was isolated from lung. Thus, CA strains from disparate host niches may be more effective in colonizing new host tissues. This underscores the ability of CA to exist in different morphological states that effectively allows it to colonize numerous environmental niches [105].

One of the shortcomings of the murine model of CA colonization has been the use of antibiotics for stable colonization in adult mice. While this highlights the importance of microbiota in conferring colonization resistance against CA, this also handicaps studying the CA colonization mechanisms relevant to more relevant human physiologic settings (e.g. CA commensalism in a normal human host). Neonatal mice do not require antibiotics for colonization, likely because of immature bacterial gut microbiota [24]. As such, the neonatal CA GI colonization model does not accurately reflect the adult GI environment and conditions. Thus, the potential of these isolates to stably colonize the murine GI tract without antibiotic intervention opens the avenue to study colonization mechanisms employed by CA and its interaction with the microbiota and host in an unperturbed setting.

Finally, we show that CHN1 and 529L are more resistant to the antimicrobial peptide CRAMP compared to SC5314. CRAMP is related to the human antimicrobial cathelicidin, LL37, that has both antibacterial and antifungal properties [51,52,125]. CRAMP has been shown to kill CA *in vitro*. Our lab has shown in a previous study that it is critical in maintaining the GI colonization levels of CA as well. We tested the effect of loss of CRAMP on differential colonization between the isolates by performing competition experiments between *Camp* knockout mice that do not produce CRAMP peptide and wildtype mice. The

loss of CRAMP in these mice resulted in a modest phenotype, which is not surprising as myriad factors contribute to CA GI colonization including other intestinal-derived antimicrobial peptide such as β-defensins [126,127]. Previously, resistance to endogenously produced antimicrobial peptides has been invoked as potential mechanism for how specific gut microbiota taxa establish dominance in the gut: *Bacteroidetes* are more resistant to intestinal-derived antimicrobial peptides compared to members of the *Enterobactericeae* family (e.g. *E. coli*), thereby promoting *Bacteroidetes* abundance over *Enterobacteriaceae* [128]. Further investigation into the role that other antimicrobial peptide such as β-defensins, as well as pattern recognition receptors such as Dectin-1, play in maintaining CA GI colonization resistance may elucidate the intricacies contributing to this observed differential GI colonization.

CHAPTER SIX
Discussion and Future Directions

The mammalian GI tract is a unique milieu of host cells, host-derived factors, microbial cells, and microbial-derived signaling molecules. The ability for both host and microbe to sense and adapt to these signals accordingly are essential for establishing commensalism. *C. albicans* (CA) does not have an environmental reservoir thus has evolved strategies to colonize the mammalian GI tract [24]. CA can undergo morphological and genetic changes as it passes through the mammalian GI tract [25,33,36]. One such change is the CA adoption of the GUT morphology [33], which is dependent on overexpression of Wor1 or gray morphology that is dependent on the loss of EFG1 [36]. These genetic changes in the GI tract repress CA hyphal growth and promote the yeast form. It is hypothesized that the CA yeast form results in reduced host innate immune activation (e.g. decreased inflammation) and thus facilitates CA GI colonization [21,25]. Relevant to this study, CA also upregulates its hexose uptake and carbohydrate metabolism pathways while traversing through the GI tract to efficiently utilize any available carbon source [113]. Tye7, an important regulator of the glycolytic pathway has been shown to be important for its growth under hypoxic condition *in vitro* [114], and during GI colonization [115]. These factors, and multitude of others, are critical for promoting commensalism within a healthy host but also contribute to disease pathogenesis, particularly in immunocompromised hosts where sundry host and microbial signals have changed. Thus, a deeper understanding of the microbial and

host factors that govern commensalism and transition to pathogenic states is needed to ultimately develop novel approaches to reducing CA colonization and subsequent dissemination.

In this study, we studied CA GI colonization with the intention of ascertaining the contributions of 1) bacterial gut microbiota, 2) host immune system and 3) CA in governing this phenotype. First, we showed that microbiota-derived SCFA inhibit the growth of CA *in vitro* and *in vivo*. This growth and colonization inhibition appears to depend on SCFA-dependent inhibition of hexose uptake and carbohydrate metabolism pathways, such as GAL7 and TYE7 known to be important for GI colonization. Since CA morphogenesis pathways and metabolism pathways appear to be intertwined, it would be interesting to see if restriction of CA morphology (e.g. using yeast locked mutants with higher GI colonization ability) attenuates or modulates the protective effect of SCFA. This question is clinically relevant as the propensity of EFG1 hemizygous clinical isolates undergo loss of EFG1 through *de novo* mutation to be better colonizers [36].

Interestingly, inhibiting the reconstitution of bacterial gut microbiota abolished the protective effect of SCFA. These results may be reflective of the fact that depletion of endogenous anaerobic bacterial gut microbiota increases the nutrient availability in the GI tract as well as promoting greater oxygen availability in the gut. Both these changes could compensate for SCFA-induced decreased hexose uptake and carbohydrate metabolism pathway in CA.

We did not observe loss in protection from SCFA in *Camp* KO mice or other single SCFA sensor KO mice in this study. We attribute this to the vast degree of functional

redundancy and compensatory mechanisms found within the gut -- due to multiple sensing pathways and multiple immune effectors. Nonetheless, we do show the contribution of CRAMP in the differential GI colonization potential of SC5314, CHN1, and 529L underscoring the importance of host immunity in modulating GI levels of CA.

The concept of manipulating the gut microbiome to induce a phenotype of interest (e.g. reduction in pathobiont colonization) is of great interest for a novel therapeutic platform. In this study, as a proof of concept experiment, we used a prebiotic inulin propionate ester to increase the levels of propionate which had a protective effect against CA GI colonization. A similar approach of utilizing an inulin butyrate ester or inulin acetate ester could afford potential clinical benefit as well. These prebiotic therapies can be utilized by numerous members of gut microbiota to increase concentrations of the SCFA. The effect of the cocktail might be synergistic as increasing levels of all three SCFA would promote cross-feeding mechanisms prevalent in microbiota [117,118].

Finally, our study describes two clinical isolates of *C. albicans* 529L and CHN1 able to colonize the murine GI tract without the use of antibiotics. This could serve as a valuable tool for studying *C. albicans* GI colonization in an unperturbed system, perhaps emulating commensalism in humans better. It will also allow comparing and contrasting the factors that might regulate the GI colonization in antibiotic treated vs non-antibiotic treated hosts. The genes in SC5314 considered to be important for effective GI colonization could be tested in antibiotic and non-antibiotic treated hosts to determine if the colonization phenotype is context dependent (e.g. presence of gut microbiota) or universal regardless of environmental or host conditions. We have shown in this study that CHN1 is inhibited by SCFA *in vitro*. A

natural follow-up study would be to assess effect of SCFA in a non-antibiotic treated mice stably colonized by CHN1 and 529L in the murine GI tract. These strains could be the basis of novel preclinical models. One such idea could be targeted depletion of select species in the microbiota, and its effect on the expansion of these isolates stably colonizing the mice before the depletion. This would be closer to the sequence of events observed in humans where CA exists as a pathobiont. These strains opens up the possibility to develop several such models to more effectively study such interactions and answer relevant questions.

Overall, this study has attempted to explore the pathophysiologic process of CA GI colonization in a comprehensive fashion. The study has utilized both *in vitro* and *in vivo* approaches to delineate the contributions of the gut microbiota, the host immune system and CA itself in determining the natural history and course of colonizing the mammalian GI tract. The results of this could lay the foundation for conducting future experiments that are more physiologically relevant to the human condition. A deeper understanding of the mechanisms which govern pathobiont GI colonization resistance could allow for the development of novel and more effective therapeutic interventions to reduce the clinical burden of *C. albicans* associated blood stream infections and mortality.

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